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Summary

The oomycete *Aphanomyces euteiches* is an important pathogen infecting roots of legumes (pea, alfalfa...) and the model legume *Medicago truncatula*. Oomycetes and other microbial eukaryotic pathogens secrete and deliver effector molecules into host intracellular compartments (intracellular/cytoplasmic effectors) to manipulate plant functions and promote infection. CRN (Crinkling and Necrosis) proteins are a wide class of intracellular, nuclear-localized effectors commonly found in oomycetes and recently described in true fungi whose host targets, virulence roles, secretion and host-delivery mechanisms are poorly understood. We addressed the functional characterization of CRN proteins AeCRN5 and AeCRN13 of *A. euteiches* and AeCRN13's homolog of the chytrid fungal pathogen of amphibians *Batrachochytrium dendrobatidis*, BdCRN13. Gene and protein expression studies showed that AeCRN5 and AeCRN13 are expressed during infection of *M. truncatula's* roots. Preliminary immunolocalization studies on AeCRN13 in infected roots indicated that the protein is secreted and translocated into root cells, depicting for the first time CRN secretion and translocation into the host during infection. The heterologous ectopic expression of AeCRNs and BdCRN13 in plant and amphibian cells indicated that these proteins target host nuclei and lead to the perturbation of host physiology. By developing an *in vivo* FRET-FLIM-based assay, we revealed that these CRNs target host nucleic acids: AeCRN5 targets plant RNA while AeCRN13 and BdCRN13 target DNA. Both CRN13 exhibit a HNH-like motif commonly found in endonucleases and we further demonstrated that both CRN13 display a nuclease activity *in vivo* inducing double-stranded DNA cleavage. This work reveals a new mode of action of intracellular eukaryotic effectors and brings new aspects for the comprehension of CRN's activities not only in oomycetes but, for the first time, also in true fungi.

Keywords: CRN, oomycetes, nucleus, effector, secretion, FRET-FLIM, A*phanomyces euteiches*

Résumé

L'oomycète *Aphanomyces euteiches* est un pathogène racinaire de légumineuses cultivées (pois, luzerne …) et de la plante modèle *Medicago truncatula*. Les oomycètes, comme d'autres microorganismes pathogènes eucaryotes, secrètent et transloquent des molécules à l'intérieur des cellules de l'hôte (effecteurs intracellulaires/cytoplasmiques) dans le but de manipuler les fonctions de la plante et de faciliter l'infection. Les protéines CRN (Crinkling and Necrosis) constituent une famille d'effecteurs nucléaires largement répandue chez les oomycètes et récemment décrites chez des espèces fongiques. Leurs cibles et rôle dans la virulence ainsi que leurs mécanismes de sécrétion et de translocation sont encore mal compris. Nous avons entrepris la caractérisation fonctionnelle des protéines AeCRN5 et AeCRN13 d'*A.euteiches* ainsi que de l'homologue d'AeCRN13 du champignon pathogène d'amphibien *Batrachochytrium dendrobatidis*, BdCRN13. Les analyses d'expression génique et protéique ont permis de montrer que AeCRN5 et AeCRN13 sont exprimés durant l'infection des racines de *M. truncatula*. Des résultats préliminaires d'immuno-localisation d'AeCRN13 ont révélé, pour la première fois, la sécrétion et translocation d'un CRN durant l'infection. Leur expression hétérologue, à la fois dans les cellules de plantes et d'amphibiens, a montré que ces protéines se localisent dans les noyaux où leurs activités conduisent à la perturbation de la physiologie de l'hôte. En développant un système *in vivo* basé sur la technique de FRET-FLIM, nous avons démontré que ces CRN ciblent les acides nucléiques: AeCRN5 cible l'ARN des plantes tandis qu'AeCRN13 et BdCRN13 lient l'ADN. Ces deux effecteurs CRN13 exhibent un motif de type HNH, lequel est typiquement retrouvé dans des endonucleases. Nous avons démontré que les CRN13 présentent une activité nuclease *in vivo* conduisant à la génération de coupures double brin de l'ADN. Ce travail a permis de mettre en évidence un nouvel mécanisme d'action des effecteurs de microorganismes eucaryotes et apporte des nouveaux aspects pour la compréhension de l'activité des protéines CRN d'oomycète mais aussi, pour la première fois, de champignon.

Mots clefs : CRN, oomycète, noyau, effecteur, sécrétion, FRET-FLIM, *Aphanomyces euteiches*

List of abbreviations

Ae	*Aphanomyces euteiches*
AM	Arbuscular Mycorrihiza
AT	Adenosine Tyrosine
Avr	Avirulence
Bd	*Batrachochytrium dendrobatidis*
BFA	Brefeldin A
BIC	Biotrophic Interfacial Complex
CBEL	Cellulose Binding ELicitor
CD	Cell-Death
CN	Cyst Nematode
CRN	Crinkling and Necrosis
CSEPs	Candidate Secreted Effector Proteins
CWDEs	Cell Wall Degrading Enzymes
DBD	DNA Binding Domain
EIHM	ExtraInvasive Hyphal Membrane
ER	Endoplasmic Reticulum
ESTs	Expressed Sequence Tags
ETI	Effector-Triggered Immunity
FLIM	Fluorescence Life Image Measure
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
GWAS	Genome Wide Association Study
HATs	Histone AcetylTransferases
HDACs	Histone DeAcetylases
HIGS	Host-Induced Gene Silencing
HMTs	Histone MethylTranferases

JA	Jasmoninc Acid
MAMP	Microbe-Associated Molecular Pattern
MTFs	MAD-box Transcription Factors
NE	Nuclear Envelop
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NPCs	Nuclear Pore Complex
NTR	Nuclear Transport Receptor
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PCW	Plant Cell Wall
PI(3)P	PhosphoInositol-3-Phosphate
PR	Pathogenesis Related
PTI	PAMP-Triggered Immunity
PVX	Potato Virus X
QTL	Quantitative Trait Loci
R	Resistance
RBPs	RNA Binding Protein
rDNA	ribosomal DNA
RKNs	Root Knot Nematodes
RLKs	Receptor Like Kinases
RLPs	Receptor Like Proteins
ROS	Reactive Oxygen Species
RRM	RNA Recognition Motif
RZE	Repeated Zoospore Emergence
SA	Salicylic Acid
SDS	Sodium Dodecyl Sulfate

SIX	Secreted In Xylem
SNPs	Single Nucleotide Polymorphism
SylA	SyringoLin A
T2SS	Type 2 Secretion System
T3E	Type 3 Effector
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
TALE	Transcription Activator-Like Effector
TBS	Tris-Buffered Saline
TE	Transposable Element
TFs	Transcription Factors
TIR	Toll Interleukin Receptor NB-LRR
TMV	Tomato Mosaic Virus
Tris	Tris (hydroxymethyl)-aminomethane
Ub	Ubiquitin
UBCEPs	Ubiquitin Cterminal Extension Proteins
WGA	Wheat Germ Agglutinin
Y2H	Yeast-2-Hybrid

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General introduction

General introduction

The emergence of agriculture took place 10,000 years ago in eastern China, Mesopotamia, and MesoAmerica (Purugganan and Fuller, 2009) and was a crucial step for human survey and the blowing of civilizations. The cultivation of plants, rather than their gathering from nature, permitted humans to control when and how much food plants were grown and by this way ensure their food supply. This practice resulted in the domestication of wild plant species and conducted progressively to the appearance of plants presenting advantageous traits for humans (as rapid growth, bigger fruits…) and to modern crops. First agro-systems initiated, thus, with the beginning of plant domestication and, ever since their emergence, infectious plant diseases have manifested as a threat for food production and other human activities that depend on it.

Plant-pathogen microorganism associations are as old as 315 Ma, as evidenced in plant fossil records documenting the first pathogenic oomycetes in vascular plants (Strullu-Derrien et al., 2011). Human awareness of plant diseases date back to 3500 BC in Greek civilization and first descriptive reports were brought by Théophraste (370-286 BC). Plant diseases caused by microorganisms posed problems in ancient crop systems: japanese literature of the 600 century AC makes allusion to infectious diseases (blast disease on rice) that resulted in serious famines back in the days (Akai, 1974). But one can only speculate about their causative agents since it was only till principles in biology were cemented that plant pathology and etiology of plant diseases established their biotic origin.

From the beginning of our modern times, serious episodes of plant infectious diseases have taken place on various important crops. An emblematic example of how plant diseases affect humans is the potato blight disease caused by the oomycete *P. infestans*, responsible for the “Great Irish Famine” in the 1840s that led to important demographic and cultural changes in Ireland. Another oomycete, *P. viticola,* caused considerable losses for the European grape industry in the beginnings of the 20th century, reaching up to 70% loss in 1915 in France (Gessler et al., 2011).

Today the problem persists. Actually, it has been estimated that only infectious diseases, are responsible for 10% of all crop losses (Strange and Scott, 2005) and recent studies have raised the alarm on novel infectious diseases that are emerging and spreading, putting at risk plant health and thereby food security (Fisher et al., 2012). For instance, the black stem of wheat caused by the strain Ug99 of the fungus *P. gramini sf. tritici*, which has being threating wheat cultures since 1998, is having a calamitous impact in the Middle East

and West Asia and other wheat-growing countries (Flood, 2010; Pennisi, 2010). Another example is the fungus *Magnaporthe oryzae* (the rice blast disease agent) aggressively infecting rice as well as other grass species including wheat. Only on rice, estimations indicate that harvest losses per year could feed 60 million people (Pennisi, 2010). Hence, it is urgent to provide means to avoid or to better manage these infections. To address this challenge it is crucial to better comprehend plant-pathogen interactions at the molecular level.

Among all microorganisms responsible for the contemporary menaces, the filamentous eukaryotic fungi and oomycetes stand as the most serious (Fisher et al., 2012; Flood, 2010; Pennisi, 2010). Indeed, oomycetes stand as notorious plant pathogens causing dramatic losses on crops, estimated at $ 6.7 billion for *P. infestans* on tomato and potato (Haas et al., 2009) and from $1 to $2 billion for *P. sojae* on soybean (Tyler, 2007). In addition to crop systems, oomycetes can severely affect semi-natural and natural ecosystems, like in the case of *P.ramorum*, responsible for the sudden-oak-death disease (Grünwald et al., 2012). Despite the relevance of these diseases, means to avoid and control oomycete are scarce and often unsuccessful. Oomycete control recommendations include continuous spraying of complex fungicidal mixtures and prophylactic measures (Blum et al., 2010; Hobbelen et al., 2011). Fungicides extensively used for the control of fungal diseases often become ineffective because of the rapid appearance of insensitivity and long standing resistance (Judelson and Senthil, 2006; Pang et al., 2013). In addition, these can be inefficient because the metabolic pathways and key components that they target in fungi can be absent in oomycetes. Studies on the molecular basis governing pathogenicity and plant susceptibility can provide answers of why plants are susceptible to certain pathogens and identify the mechanisms through which pathogens infect successfully their hosts.

The general introduction of the manuscript is divided in different sections. First section is devoted to a general presentation of the current knowledge of the biology of oomycetes with a particular focus on the legume root rot pathogen agent *Aphanomyces euteiches*. Next section presents main concepts of molecular plant-microbe interactions and the emerging role of the plant nucleus in this context, followed by a description of microbial effectors that target this organelle. Lastly, the objectives of the PhD work are presented.

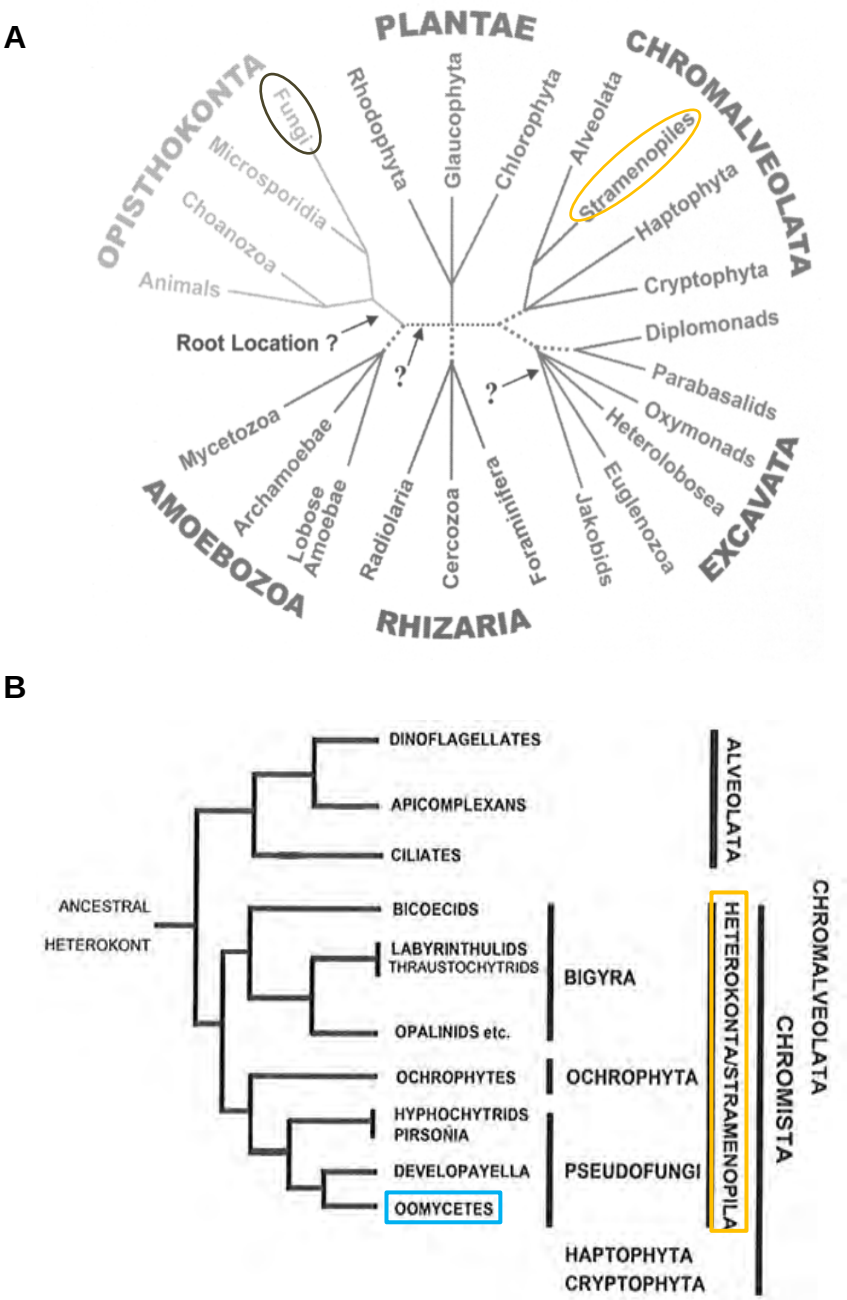

Figure 1. Schematic representations of the likely phylogeny of eukaryotes and the relationships of main phyla and classes of the Chromalveolata ensemble. **A.** Scheme of the current consensus of eukaryotic phylogeny showing the six super-ensembles: Opisthokonta, Plantae, Chromalveolata, Excavata, Rhizaria and Amoebozoa. Stramenopiles (within Heterokonts) are high-lighted in yellow. Adapted from Brinkmann and Phillippe (2007). **B**. Summary of the phylogenetic relationships of oomycetes (blue squared) within Stramenopiles (yellow squared) and their closest related groups. Adapted from Beakes and Sekimoto (2009).

Oomycetes: plant and animal disease agents.

Oomycetes are distinct from fungi

Oomycetes (commonly referred to as water moulds) are eukaryotic microorganisms regrouped in the Stramenipila kingdom (Brinkmann and Philippe, 2007; Beakes et al., 2012). They are heterotrophic organisms acquiring nutrients by absorption (osmotrophs) thanks to the transport into cells of nutrients produced by secreted depolymerizing enzymes on extracellular complex biological material. They present a hyphal tip-growth development that leads to the formation of complex branched mycelia. Because of these characteristics, oomycetes were thought to be related to fungi. It is known, now, that is not the case and that many traits shared with fungi are the result of convergent evolution. In fact, molecular phylogenetic studies based on the mitochondrial cox2 gene (Thines et al., 2008), SSU and LSU rDNA genes (Voglmayr and Riethmüller, 2006) have replaced oomycetes in the super ensemble Chromalveolata (and within it, in Stramenopiles) far distant from true fungi (Opistokonta) (figure 1 A). Oomycetes appear closely related to brown algae and diatoms and quite close to Apicomplexans (figure 1 B). The relatedness to brown algae together with the fact that the most ancestral oomycetes known today (*Eurychasma dicksonii* and *Haptoglossa spp*) are marine habitants, supports a marine origin of oomycetes (Beakes et al., 2012).

Cytological and biochemical studies corroborate the lack of relatedness to fungi and highlight main differences distinguishing them. Oomycetes present a coenocytic thallus which remains diploid throughout its vegetative stage (only formation of haploid nuclei occurs through meiosis for gamete formation). In contrast, fungal thalli are septate and carry haploid nuclei during vegetative stages. Additionally, their cell-wall polysaccharide composition is different. While chitin, N-acetylglucosamine residus 1,4-linked (1,4-GlcNac), remains largely the primary structural component of fungal cell-walls, oomycetes present a more diverse polymer composition. Cellulose and β-glucans remain the principle structural cell-wall constituents of late-divergent oomycetes (like *P. infestans),* whereas early-divergent species like *Saprolegnia spp* and *A. euteiches* present, in addition to cellulose, different levels of chitin (Badreddine et al., 2008; Guerriero et al., 2010). Recently, the cell-wall analysis of *A. euteiches* evidenced the presence of original polymers found for the first time in eukaryotes: 1,6-linked GlcNac residus in association to β-1,6 glucans (Mélida et al., 2013; Nars et al., 2013). Another major trait resides in the absence of biosynthetic pathways implicated in the synthesis of sterols in a large majority of oomycetes, compounds that oomycetes acquire from hosts by the secretion of sterol-carrier proteins during infection

(Mikes et al., 1998).

Oomycetes: diverse microorganisms and notorious plant pathogens

Between 600 and 1500 species are counted in the oomycetes lineage and, within Stramenopiles, they define a solely branch of highly diverse microorganisms. This is reflected, in a first instance, by the variety of their ecological niches since they occupy fresh-water, marine and terrestrial environments ubiquitously throughout the globe (Thines and Kamoun, 2010). In these environments, they present different life styles: as free-living organisms able to acquire nutrients from complex dead organic matter (saprophytes) and/or in association with other organisms. These symbioses are very often parasitic and ensure the derivation of all or part of their nutrition from the living host. When taking place, they are in a large extent pathogenic as they lead to the establishment of disease and mortality of hosts. It is worth to note that, to date, no mutualistic symbiotic oomycete has been described and that the earliest divergent oomycete, *Eurychasma dicksonii*, is an obligate pathogen of brown sea weeds (Grenville-Briggs et al., 2011). Thus, the capacity of parasitism seems to have appeared early in the history of oomycetes as well as their pathogenic behaviour which is a hallmark of this lineage.

Pathogenic oomycetes are highly diverse themselves, presenting a wide range of hosts which can be animals (like crustaceans, fishes, mammals including humans, nematodes) as well as algae and plants (Thines & Kamoun, 2010). Their impact can be seriously detrimental for natural ecosystems with important ecological consequences. For example, the plant pathogen oomycete *Phytophthora ramorum* (the agent of the sudden oak death disease) is destroying entire native North American forests and leading to a decrease of the woody species diversity as well as to changes on carbon and soil nitrogen cycling (Cobb et al., 2013). Another example is the crayfish plague caused by *Aphanomyces astaci* (listed as a notifiable disease of the World Organisation for Animal Health (http://www.oie.int/en/animal-health-in- causing the disappearance of entire species populations (Filipova et al., 2013; Vrålstad1 et al., 2011). Pathogenic oomycetes also constitute a serious threat to agricultural systems and therefore to food security (Fisher et al., 2012; Phillips et al., 2008). In this regard, phytopathogenic oomycetes merit considerable attention. It is estimated that 60% of oomycete species known today are plant pathogens (Thines & Kamoun, 2010). Among these, several stand as the most deadly pathogens of important cultivated plants. As already mentioned, the "plant destroyer" *Phytophthora infestans*, has been and continues to be a threat to potato and tomato representing an economical and societal problem worldwide (Nowicki et al., 2012). Other cultivated plants

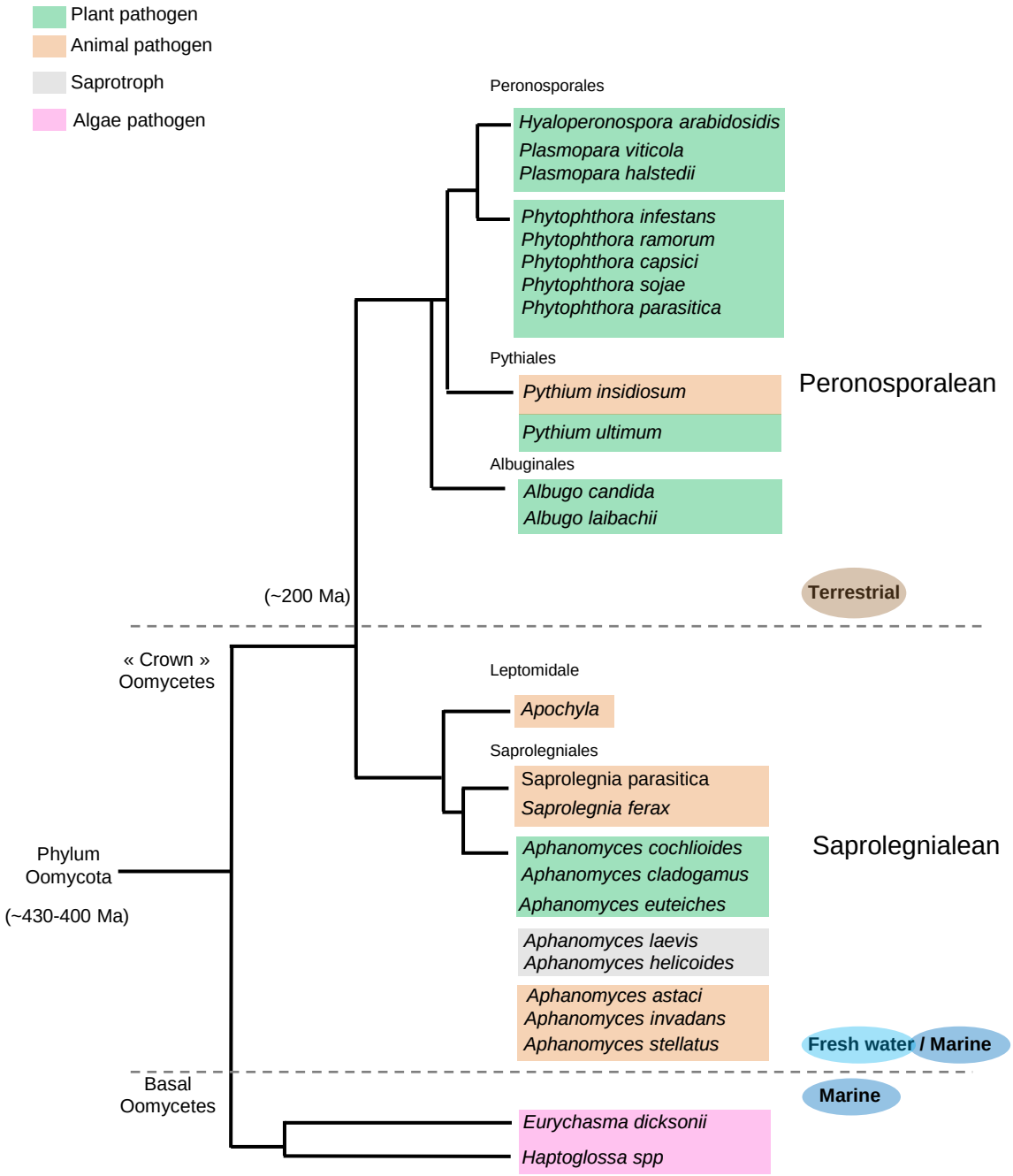

Figure 2. Summary scheme showing the phylogenetic relationships of main taxa groups of oomycetes, their environmental prevalence and lifestyles. Estimations place the origin of oomycete between ~430 and 400 million years ago (Ma). Late divergent « Crown » oomycetes are regrouped into two mayor ensembles: the Peronospolean lineage and the Saprolegnialean lineage, having separated ~200 million years ago (Ma). Representative species and their lifestyles are given. The scheme was generated based on information provided by Beakes and Sekimoto (2012), Dieguez-Uribeondo *et al* (2009) and Matari *et al* (2014).

like soybean, grape, broccoli, lettuce and sugar beet endure important diseases hosting species like *Phytophthora sojae*, *Plasmopara viticola*, *Peronospora parasitica*, *Bremia lactucea* and *Aphanomyces cochlioides*, respectively.

Phylogenetic distribution of oomycetes

Oomycetes appeared 430-400 million years ago (Matari and Blair, 2014) and are grouped in two major taxonomic groups/lineages that encompass the most late-diverging groups of oomycetes or "crown oomycetes" (to exclude early-diverged basal lineages) as termed by Beakes and Sekimoto (2009): the Saprolegnialean and the Peronosporalean lineages (figure 2). The latter groups have been estimated to split 200 million year ago in the history of oomycetes and have, thus, different evolutionary histories which can be evidenced by biochemical features as for example the ability to synthesize sterols, a trait found only in Saprolegniales and in their closest relatives, the brown algae (Gaulin et al., 2010). Another distinctive trait concerns their ecological prevalence. Saprolegniales are predominantly found in aquatic environments (fresh water and estuarine) while Peronosporales occupy mainly terrestrial environments. The water-dependence of Saprolegniales is considered an ancestral trait and the tendency to have a lesser dependence on water is thought to be an evolutionary trend in oomycetes (Beakes and Sekimoto 2009).

Phytopathogenic oomycetes species are found in both lineages (figure 2) with a majority in the Peronosporalean lineage which stands as a phytopathogenic line. Species ascribed to genera *Phytophthora* (over 100 species, Kroon et al., 2012), *Albugo* and *Hyaloperonospora* as well as *Pythium* are phytopathogenic. Main exceptions are restricted to *Pythium* species since among the 150 plant pathogenic species some are animal pathogens like *P. insidiosum* (infecting mammals, Uzuhashi et al., 2010).

In contrast, the Saprolegnialean lineage is more heterogeneous since in addition to a large number of exclusively saprophytic species the group harbors zoopathogenic and phytopathogenic facultative species. Animal pathogens species of the genus *Saprolegnia* (i.e: *S. parasitica)* infect freshwater fishes, insects and amphibians (Sarowar et al., 2013). In *Aphanomyces* genus, species *A. invadans* and *A. astaci* develop on fishes and crayfishes species with elevated economic and ecological impacts (Phillips et al., 2008). Few plant pathogens species have been identified in Saprolegniales and are all included in the genus *Aphanomyces*.

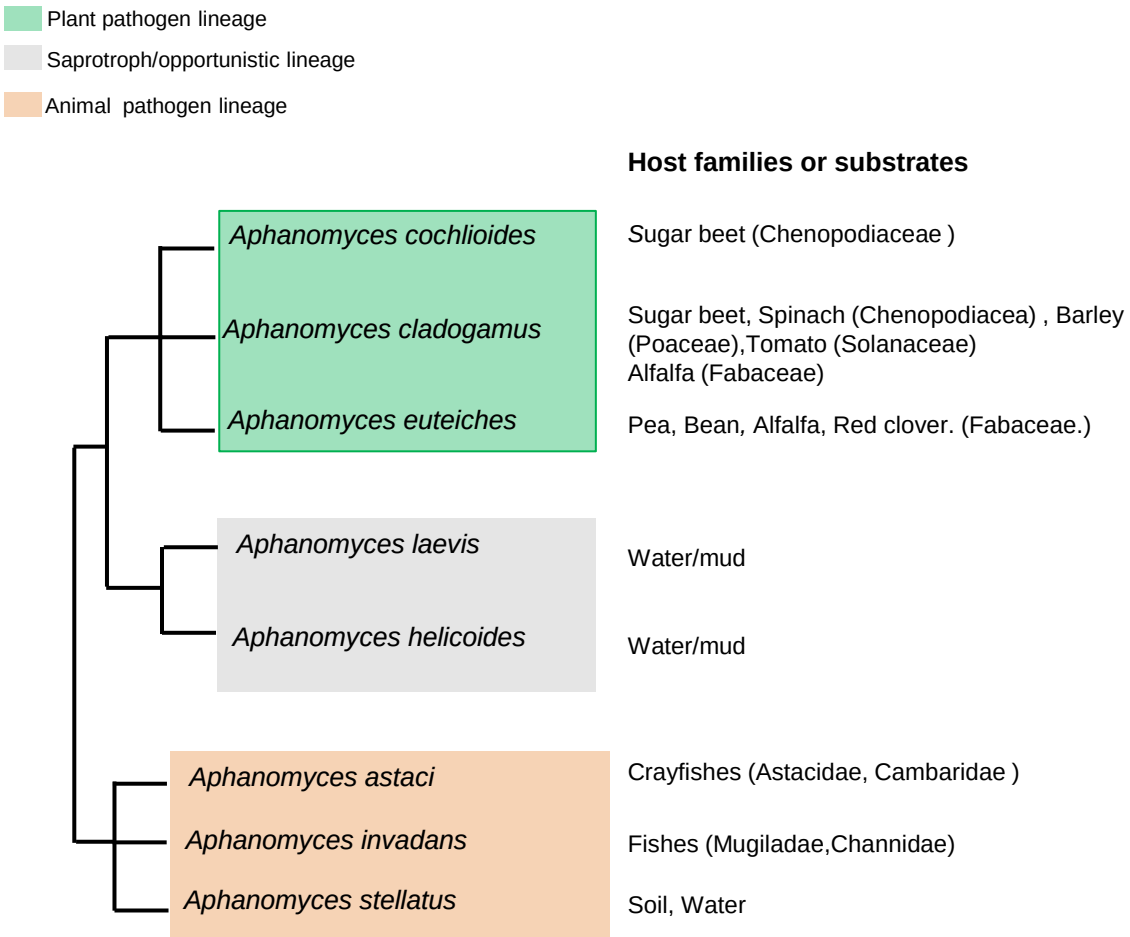

Figure 3. Close up summary scheme on *Aphanomyces* phylogeny, lifestyle and principal hosts or substrates.

The phylogenetic relationships were established by analysis of ITS sequences of nuclear rDNA of the principal *Aphanomyces spp* identified till date. Lifes styles displayed by these species correlates to their phylogenetic regrouping defining three independent lineages: a plant pathogen lineage, a saprophytic/ oportunistic lineage and animal pathogenic lineage. Species *A. laevis* and *A. helicoides* are generally assigned as saprotrophs but isolated studies have documented their development on animals (insects). *A. stellatus* has been found as a free-living species but its ITS sequence analysis assigned it to the zoopathogenic lineage.The scheme was performed based on Dieguez-Uribeondo *et al*., (2009).

Aphanomyces genus

An ancestral genus harboring diverse species

Aphanomyces genus regroups about 40 species (Diéguez-Uribeondo et al., 2009). This is an approximate number because their proper identification is difficult due to the reticence of some species to be isolated and maintained in pure cultures. Hence, its taxonomy is still in progress. Nevertheless, studies converge to the conclusion that *Aphanomyces* is an ancestral Saprolegniale group (Petersen and Rosendahl, 2000).

In an overall view, most species have prevalence for aquatic niches (fresh water and marine, mostly estuarine) with the exception of plant pathogens which occur in terrestrial wet environments. Phylogenetic studies of *Aphanomyces* genus regroup species in three independent lineages globally correlated to their life-styles (figure 3). A first line comprises plant pathogenic species, which in the Saprolegnialean line are only restricted to *Aphanomyces* genus. Within, species like *A. cladogamus* have a broad range of hosts of different families as Fabaceae (*Phaseolus vulgaris*, common bean), Poaceae (*Hordeum vulgare*, barley), Solanaceae (*Lycopersicon esculentum*, tomato) and Chenopodiaceae (*Spinacia oleacera*, spinach). *A. euteiches* displays a quite marked specialization for Fabaceae species while *A. cochlioides* is a host-narrow species reported so far only on sugar beet (*Beta vulgaris*) (Diéguez-Uribeondo et al., 2009). A second lineage harbors species with prevalence for saprophytism as *A. laevis* and *A. helicoides* which can exhibit opportunistic parasitism. Lastly, the zoopathogenic lineage regroups *A. astaci* (infecting freshwater crayfishes, Filipova et al., 2013) or *A. invadans* (responsible for the epizootic ulcerative syndrome of various species of estuarine fishes, (Boys et al., 2012) as well as *A. stellatus*, which, consensualy defined as a saprotroph, has been found to develop on crustaceans (Royo et al., 2004).

The diversity of life styles and hosts of *Aphanomyces* species (animal/ plant pathogens and saprobe species) gives to this genus a special taxonomic position among Saprolegniales and towards Peronosporales.

Aphanomyces life cycle presents sexual and asexual stages. Sexual reproduction leads to the formation of oospores as the result of the fertilization of oogonia (female reproductive structures carrying the female haploid nuclei) by antheridia (male reproductive structures delivering haploid male nuclei). Oospores are long-resting structures which germinate to produce biflagellate motile zoospores (the primary infection entity). Such zoospores can also be produced as the result of asexual reproduction by live mycelium in roots via specialized hyphae (sporangium). As zoospores reach host surfaces, they encyst and

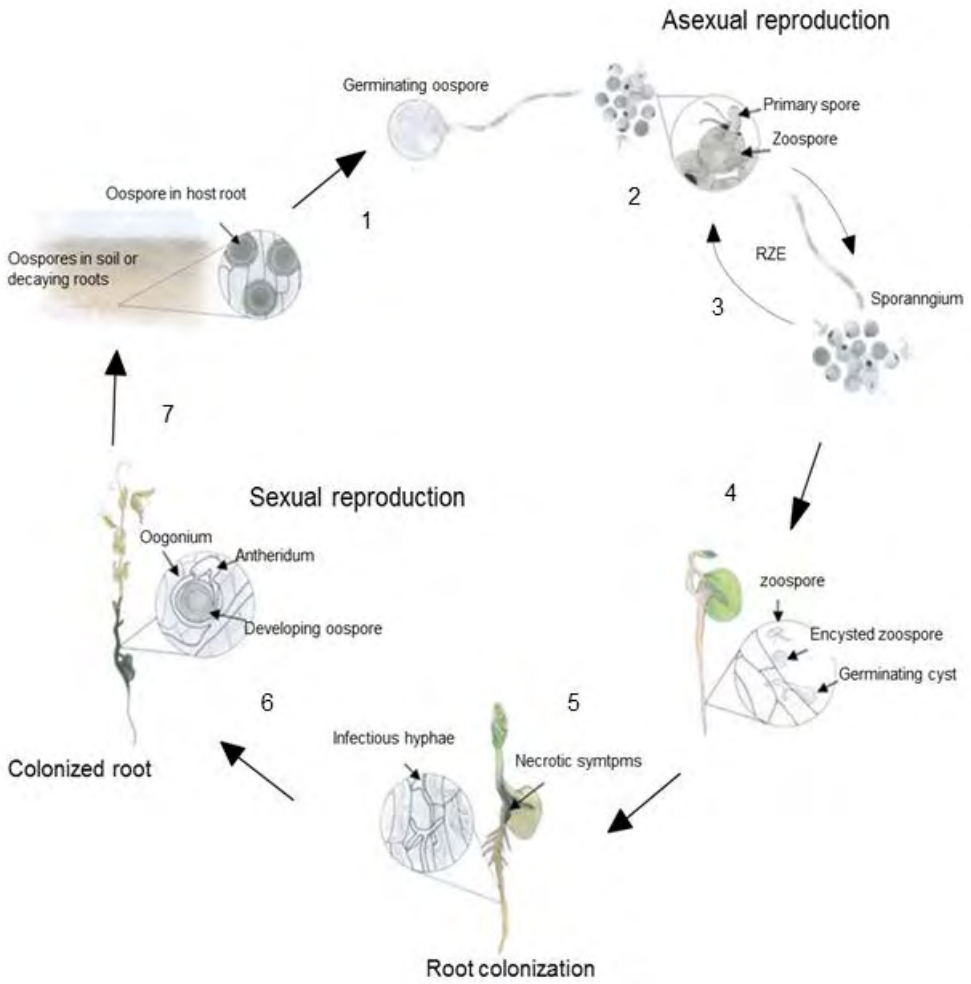

Figure 4. Infection life cycle of *A. euteiches* (Saprolegniales). Oospores present in soil germinate producing a sporangium (1). Primary spores (2N) are formed in the apex of the sporangium and release zoospores (asexual, biflagellate spores) through a pore of their cell wall (2). Zoospores can encyst en germinate to produce a novel sporangium giving rise to a second generation of primary spores and zoospores (3) a phenomenon called Repeated Zoospore Emergence (RZE). Zoospores that reach the host rhizoplane encyst and germinate producing an hyphae that directly penetrates host tissues (4). Infectious hyphae develops intercellularly (5) to completely colonize the root system and subsequently progress to hypocotyls (6). Within roots, sexual reproduction is assured by the differentiation of hyphae into antheridia and oogonia, carrying haploid nuclei (1N) and whose fusion results in the formation of oospores (2N). During the decomposition of dead plant tissue, oospore are then released in soils were they can remain dormant for up to 10 years (7). Adapted from the American Phytopathological Society (APS) https://www.apsnet.org/

germinate to penetrate host surfaces (figure 4). It is worth to note that some physiological and developmental behaviours are linked to the parasitic life style. Parasitic species present the capacity of Repeated Zoospore Emergence (RZE). This refers to the ability of a motile zoospore to encyst and to produce a second generation of zoospore rather than directly infect the host. It is believed that RZE allows to extend the duration of the infective stage and so the chances to come upon a suitable host. Also, plant parasitic species usually present both sexual and asexual stages whereas in animal pathogens the sexual stage is often absent or rare (Diéguez-Uribeondo et al., 2009).

A. euteiches and the root rot disease of legumes

Aphanomyces euteiches was first described by Dreschler in 1925 from infected pea (*Pisum sativum*) in the United States. It is a soil borne pathogen infecting roots of legumes as well as a facultative pathogen which means that it is able to grow as a saprobe outside the host (Papavizas and Davey, 1960). This renders its axenic culture possible. Its developmental cycle includes sexual and asexual stages. The species is homothallic (self-fertile) and presents sexual reproduction typically achieved by the formation of diploid oospores capable to subsist up to 10 years in soils on harsh conditions (Gaulin et al., 2007).

The disease caused by *A. euteiches* is commonly known as the root rot of legumes. Legumes affected include pea (*Pisum sativum*), alfalfa (*Medicago sativa*), snap and red kidney bean (*Phaseolus vulgaris*), faba bean (*Vicia faba*,) red clover (*Trifolium pratense*) and white clover (*Trifolium repens*). Thus, *A. euteiches* has a relative large range of hosts within Fabaceae. Nevertheless its occurrence and degree of pathogenicity can differ from one host to another. Its characterization on pea and alfalfa has defined pathotypes: pea-infecting strains and alfalfa infecting strains from the USA and from France (Malvick and Grau 2001; Moussart et al., 2007; Wicker and Rouxel, 2001).

Infection takes place in roots as zoospores, chemo-attracted by root compounds (Sekizaki et al., 1993), encyst in the rhizoplane and germinate to penetrate root cortex tissues. Total colonization of roots is followed by the spread of the pathogen to stem (hypocotyls, epicotyls). Typically, its development provokes the disintegration of cortex tissues denoted by the appearance of softened and water-soaked areas of roots which become orange-brown or blackish-brown at later times of pathogen development. Symptoms can advance to stems and become evident by the necrosis of epicotyls and hypocotyls, and chlorosis of cotyledons followed by total discoloration (death) or foliage milting. Completion of infection is marked by the formation of oospores that ensure the dissemination. When

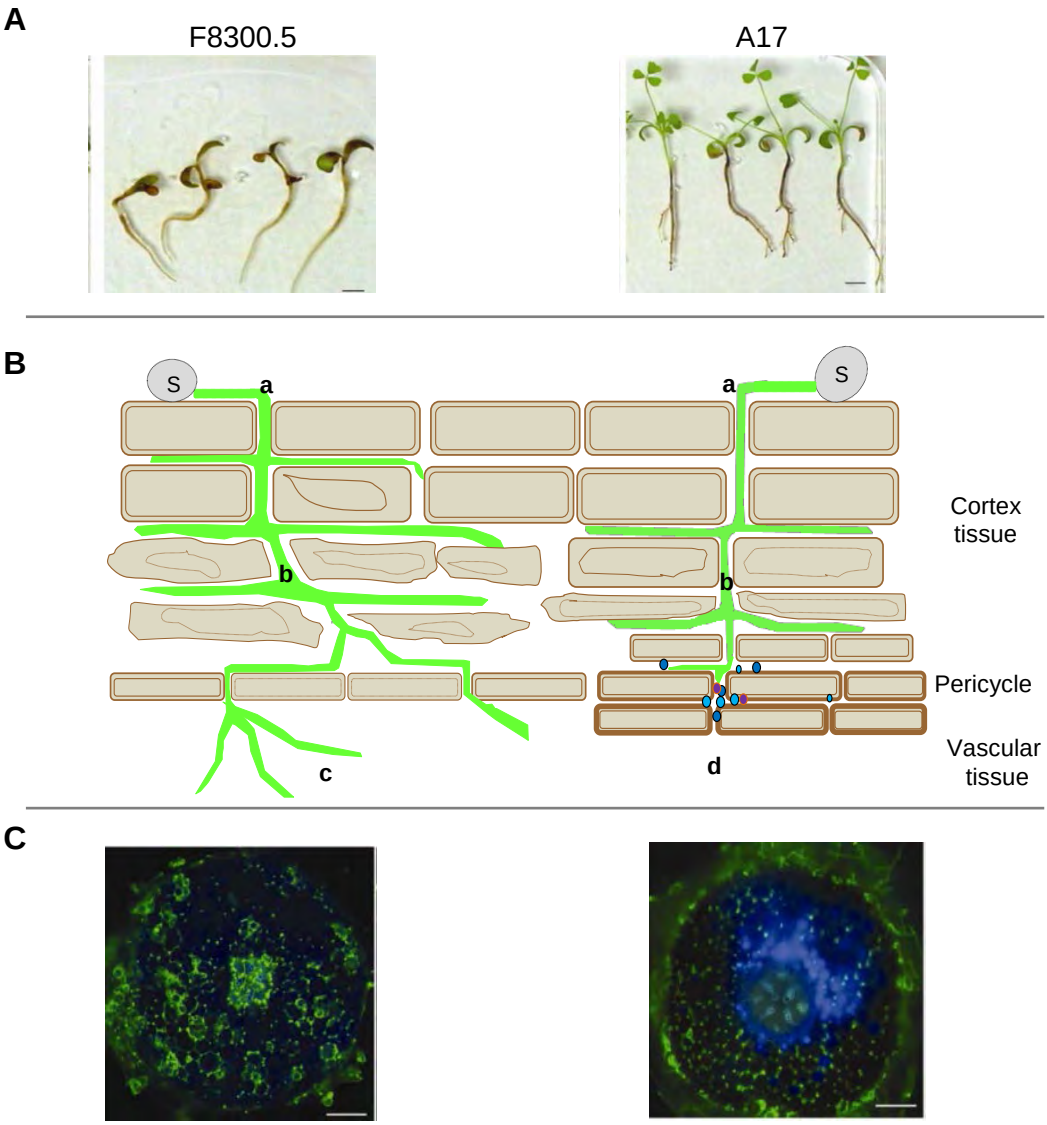

Figure 5. Infection model in the *Medicago truncatula*/ *Aphanomyces euteiches* pathosystem. A. Macroscopic symptoms displayed by *M. truncatula* F83005.5 (highly susceptible) and ecotypes A17 (partially resistant) infected with *A. euteiches in in vitro* conditions (Djébali *et al*.,2009). **B**. Scheme of a transversal section of a root infected by *A. euteiches* (green). On the left side, the scheme describes infection in F83005.5 were an asexual spore (S) has landed on the rhizoplane and germinated to produce a germ tube giving rise to an infectious hyphae that directly penetrates root cortex tissues (a). Hyphae develops between root cells of cortex which becomes completely colonized 6 days post inoculation. Cortical cells died as *A. euteiches* develops leading to root disassembly and water-soaked symptoms typical of root rot disease (b). The pathogen reaches the vascular cylinder before completion of its cycle (not shown) (c). On the right side, the infection is depicted in the tolerant host (A17) were the plant produces supplementary pericyle cell layers with higher levels of lignin in their cell-walls, reinforcing the root stele. In addition to this mechanical barrier, cells produce antimicrobial compounds (d). These cytological responses restrain the advance of the pathogen to the vascular cylinder. **C**. Cross-sections of infected roots showing full invasion (F83005.5) and partial invasion (A17) by *A. euteiches* (green) and the production of antimicrobial compounds by A17 (bleu) in the root stele, 15 days post inoculation (Djébali *et a*l.,2009).

conditions are appropriate, oospores germinate in the vicinity of hosts to produce zoosporangia.

Root rot disease occurs wherever host species are grown. Specially, it is a problem for pea and alfalfa-growing regions in North American and European countries. In France, it affects primary forage pea in northern regions (Gaulin et al., 2007). No effective control exists once *A. euteiches* is installed in soils. In addition, no fully resistant line of pea or alfalfa is available. Only prophylactic measures and crop rotation are preconized to prevent crop losses. Currently, improvement of crop resistance is done via the characterization of broad range resistance and identification of QTLs, which is the only type of resistance towards this pathogen (Djébali et al., 2009; Hamon et al., 2013; Bonhomme et al., 2014).

A.euteiches and *M. truncatula* pathosystem

The legume *Medicago truncatula* is a close relative of the cultivated alfalfa (*M. sativa*) that has become a largely used model plant (Cook, 1999). Protocols suited for laboratory research have been established and a wide collection of mutants and natural genotypes are at disposition for the scientific community. Genomic resources include sequences of over 288 accessions (Stanton-Geddes et al., 2013). More interestingly, it is a host of *A. euteiches* showing great variability of susceptibility to this pathogen (Moussart et al., 2008).

A. euteiches/*M. truncatula* pathosystem has been developed and exploited in our research group to understand the molecular aspects behind the suceptible variability of the plant. The pathosystem makes use of a pea isolate of *A. euteiches (*ATCC 201684) and different genotypes of *M. truncatula* that display different degrees of tolerance to this strain. Two accessions exemplifying the opposite resistance degrees are line A17 (partially resistance) and line F83005.5 (highly susceptible). Genetic approaches and the use of an *in vitro* infection assay coupled to the characterization of infection phenotypes have led to the identification of a QTL (Djébali et al., 2009). Recently, the variability of 176 *M. truncatula* genotypes towards *A. euteiches* has been correlated to gene sequence architecture through a Genome Wide Association Study (GWAS). Single Nucleotide Polymorphisms (SNPs) in the promoter and coding region of an F-box gene have been spotted out and linked to *M. truncatulas's* variability to *A. euteiches* (Bonhomme et al., 2014). Although no expression polymorphism between resistance and susceptible lines could be shown, the identified SNPs are preconized to lead to functional and non-functional forms of the F-box protein which are associated to susceptibility and resistance, respectively. In addition to the F-

box gene, and consistent with the quantitative type of resistance towards *A. euteiches*, other genes have also been identified through the GWAS. These latter are associated to hormone regulation, notably the biosynthesis of cytokinine and transcription factors associated to ethylene response as well as giberrilin and abcissic acid (Bonhomme et al., 2014).

Molecular and cytological approaches carried in lines A17 and F83005.5 have evidenced particular plant mechanisms that might be implicated in the contrasting tolerance to *A. euteiches.* In both lines, upon inoculation of roots with zoospores, *A. euteiches* penetrates and starts to develop between cells of the outer cortex tissue within 1 day. No specialized infectious hyphae structures as appressoria or haustoria have been evidenced. The pathogen presents an intercellular development and invades the whole cortex area within 3 to 6 days. But, while in F83005.5 total invasion of cortical tissues is followed by the progression of the pathogen to vascular system, in A17 roots the pathogen progression is mostly restrained to cortical tissues. This inability to progress into the vascular system is correlated to the capacity of A17 to deploy a whole set of defense mechanisms like the production of phenolic compounds, the reinforcement of cell walls and, more particularly, the formation of supplementary pericycle cell layers that might act as a physical barrier for the invading hyphae (figure 5). Intriguingly, partial resistance of A17 is accompanied by an increase of lateral roots (Djébali et al., 2009).

Molecular interplay between pathogen effectors and plant immunity

Plant pathogen microorganisms have acquired the ability to take profit of the nutrient-rich niche that may be provided by plants to develop and reproduce. Indeed plants represent a well lasting source of carbon, nitrogen and water as well as a physical protection (Zuluaga et al., 2013). This mode of living implies an intimate association with plants in order to exploit goods. Because the development of associated microorganisms affects host plant health, conducting eventually to plant disease and death, these associations are termed as pathogenic.

Plant immunity and effectors

As any other living organism exposed to a multitude of microorganisms, plants sense these external cues to respond in the best-adapted manner. To defend themselves against pathogens, plants possess constitutive defenses consisting in pre-existing physical or

Table 1. Pathogen Molecular Associated Patterns (PAMPs) of bacteria fungi and oomycetes and their cognate receptors in plants.

Organism	PAMP (name)	Plant Receptor	Reference
Bacteria	Flagellin (flg22)	FLS2 (*A.thaliana*)	Félix et al. , 1999 Gomez-Gomez et al., 2001
	Elongtion factor EF-Tu (elf18/26)	ERF (*A.thaliana*)	Kunze et al., 2004
	Cold shock protein (RNP)	Not identified	Félix and Boller, 2003
	Lipopolysaccharide	Not identified	Newman et al., 2005
	Peptidoglycan	Lym1, Lym2 (*A. thaliana*)	Erbs et al., 2008a Willmann et al., 2011
Fungi	chitin	CeBip, (rice) CERK1 (*A.thaliana*)	Kouzai et al., 2014 Petutschnig et al., 2010
	Xylanase (EIX)	EIX (tomato)	Ron and Avni, 2004
Oomycetes	CBEL	Not identified	Gaulin et al.,2006 Larroque et al., 2013
	Pep13	Not identified	Nürnberger et al.,1994 Brunner et al., 2002
	B-hepta glucans	GEBP (putative, soybean)	Umemoto et al., 1997
	INF1	NbLRK1 (*N.benthamiana*)	Kanzaki et al., 2008

biochemical barriers (i.e. hydrophobic cuticles, surface antimicrobial compounds…) that act as a first level of protection to avoid and limit development of potential harmful microorganisms. In addition, they possess an inducible multi-layered immune system that allows them to perceive microorganisms and activate a whole set of modular defenses (figure 6 A) (Dodds and Rathjen, 2010).

The first level of plant immunity is activated by the direct perception of conserved epitopes in commonly occurring molecules of microbes (pathogen or non-pathogen) called "Pathogen/Microbial-Associated Molecular Patterns "P/MAMPs". These molecular signatures can be of different nature (protein, oligosaccharides...), they identify a whole classe of microbes and are absent from plants (table 1). Their recognition involves Pattern Recognition Receptors (PRRs) associated to the plasma membrane that belong to the family of Receptor-Like-Kinases (RLKs) and Receptor-like Proteins (RLP) (Boller and Felix, 2009) (figure 6). Best characterized examples are the 22-peptide present in the bacteria flagellin protein (flg22) and peptides elf18/26 of the bacterial EF-Tu elongation factor protein perceived by the RLKs FLS2 and EFR, respectively (Boller & Felix, 2009). Fungal chitin is perceived in rice by CEBiP (Kouzai et al., 2014) and by CERK1 in *A. thaliana* via their LysM extracellular domains (Petutschnig et al., 2010). The activation of the defense signaling after PAMP perception requires the association of these receptors to a central RLK regulator, named BAK1 (Kim et al., 2013) (figure 6). The importance of BAK1 in mediating PAMP-defense signaling and activation is well known for the above mentioned bacterial and fungal PAMPs and has been recently established for defense against nematodes and insects (Peng and Kaloshian, 2014). Oomycete PAMPs that elicite defense in plants have been identified but their cognate receptors and mechanisms behind their recognition remain for the most unknwon. These include the epitote pep13 of transglutaminases (Brunner et al., 2002), the heptaglucans of *P. sojae* which has been proposed to be perceived by the GEBP (soybean) (Umemoto et al., 1997), the cellulose- binding domain of the cell-wall protein CBEL of *Phytophthora parasitica* (Gaulin et al., 2006) for which its eliciting activity requires BAK1 (Larroque et al., 2013) and finally, the sterol-binding protein elicitin INF1 from *Phytophthora spp* (Kamoun et al., 1998) for which a RLK has been identified (Kanzaki et al., 2008) (table 1).

PAMP/MAMPs recognition triggers a level of immunity referred to as PAMP-Triggered Immunity (PTI) also called basal immunity given the broad sprectrum of occurrence of PAMP/MAMPs, not specific to a given pathogen. PTI includes subsets of early responses like the production of ROS species and ion fluxes. More intermediate events include the activation of signaling cascades involving mitogen-activated protein kinases (MAPKs)

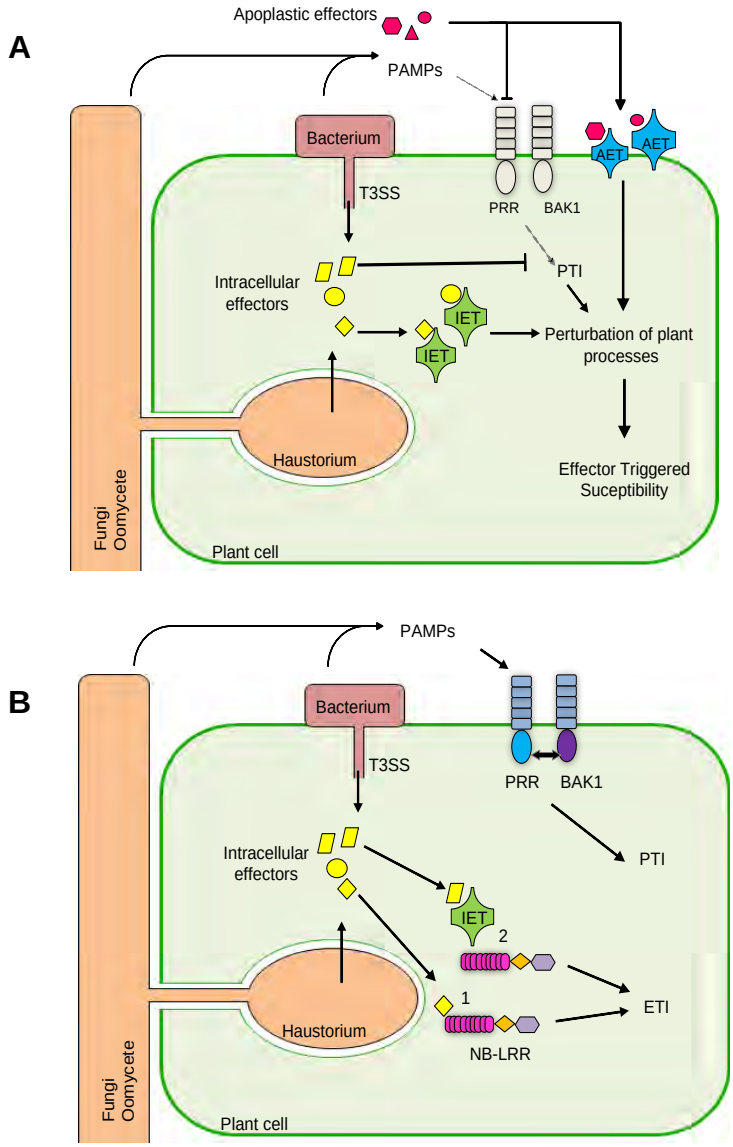

Figure 6. Global concepts of plant susceptibility triggered by microbial effectors (A) and plant immunity (B). **A.** Pathogens such as bacteria, fungi and oomycetes establish a close physical encounter with host cells. They secrete effectors into the apoplasm (apoplastic effectors) of inside plant cells (intracellular effectors). Bacteria deliver them into host cells via the Type 3 secretion system (T3SS) and filamentous pathogens via infectious structures (haustoria). Apoplastic effectors interact with apoplastic effector targets (AET) and intracellular effectors with intracellular effector targets (IET). In susceptible plants (A), their interaction with targets perturb different plant processes that benefit the outcome of infection. Among these processes, PTI is an important target whose suppression is required for successful infection. **B**. Plant immunity activation resides on the perception of pathogens, which can be performed through two modes. A first mode, consists on the perception of PAMPs by PRRs (Patter Recognition Receptors) at the plasma membrane leading to the activation of PTI (PAMP-Triggered Immunity). Initiation of PTI signalling pathway requires the co-receptor BAK1. A second mode, relies on the direct perception of effectors (1) or on the perception of effector activities on plant targets (2) via intracellular nucleotide-binding receptors (NB-LRR), conducting to ETI (Effector-triggered Immunity).
(Modified from Win et al., 2011)

(Pitzschke et al., 2009) that ultimately conduct to the activation of transcription factors (TFs) and expression of pathogenesis-related genes (PR) encoding antimicrobial compounds like chitinases and glucanases which directly degrade cell-wall structural components of pathogens (Dodds & Rathjen, 2010). Phytohormones like salicylic acid (SA) and jasmonic acid (JA) also play key roles in the signal transduction (Tsuda and Katagiri, 2010). All together, these defenses are generally sufficient to arrest pathogen infection and maintain plant health. Nevertheless, adapted pathogens have evolved means to break down or evade such defenses. These pathogens produce secreted molecules termed “effectors” which designates “all proteins and small molecules” produced and secreted by pathogens that “alter host-structure and function” and facilitate infection (Hogenhout et al., 2009). Effectors are secreted into the apoplasm interface (apoplastic effectors) or are addressed inside host cells (intracellular effectors) to interact with plant targets. By doing so, effectors can suppress plant immunity and perturb other plant processes that conduct to host susceptibility or Effector-Triggered Susceptibility, a state benefiting the outcome of the infection (figure 6 A).
Manipulation of PTI can be achieved by avoiding PAMP recognition or by directly suppressing PTI. For instance, the fungal LysM apoplastic effectors bind chitin, preventing its recognition by plants (Kombrink and Thomma, 2013). In oomycetes, several apoplastic-secreted protease inhibitors form *P. infestans* have been shown to directly bind plant apoplastic enzymes, suppressing directly their activities in this host space (Tian et al., 2005, 2007; Song et al., 2009).

Another type of defense relies on the recognition of specific effectors or effectors’s activities and is referred to as Effector-Triggered Immunity (ETI) (figure 6 B). This mechanism for pathogen recognition involves a particular class of receptors called R proteins (Resistance proteins) that are generally cytosolic. Effector recognition by R proteins leads to specific resistance and incompatible interactions, reason why in this context, recognized effectors are called Avirulence (Avr) proteins. This mechanism follows the gene-for-gene model conceptualized by Flor’s work (1971). ETI is similar in nature but is more rapid and strong than PTI (Tao et al., 2003; Tsuda and Katagiri, 2010) and is often accompanied with a cell-death or Hypersensitive Response (HR). Because it relies on the perception of effectors, it is a specific immunity.
R proteins are classified into two classes depending on the type of Nterminal domain: (Toll-like Receptor) TIR-NB-LRR or (coiled-coiled) CC-NB-LRR proteins. Two modes of effector recognition exist: (1) direct physical interaction of R proteins to effectors or (2) indirect interaction of R proteins. In this case, R proteins perceive modifications of plant proteins to which the R protein is associated and monitors (figure 6 B). These modified plant

proteins can be genuine virulence target of the effector (the guard model) or a mimic of one (the decoy model). It has been shown that activated R proteins interact with other proteins forming complexes mediating the signaling that conducts to defense response such signaling requires complexes to relocalize to specific subcellular compartments of plant cells (Dodds & Rathjen, 2010; Engelhardt et al., 2012; Qi and Innes, 2013).

In that instance, studies have recently demonstrated the importance of a nucleo-cytoplasmic trafficking of R proteins and other immune components for the activation of defense responses. The accepted model is that such relocalization is induced by pathogen perception and allows immune receptors to accumulate in the plant nucleus where they activate immune responses through transcriptional reprogramming (Qi and Innes, 2013).

Effector identification

Effector proteins were firstly identified thanks to their avirulence activity. In an ETI context, the effector behaves as an avirulent protein and the resultant incompatible interaction was of great aid to identify avirulence proteins in fungi, bacteria and oomycetes. Their cloning and molecular characterization pointed out the presence of secretion leaders either typical of the Type 3 Secretion System (T3SS, for bacteria Avr proteins) or canonical eukaryotic signal peptides (in oomycetal and fungal Avr proteins) (Armstrong et al., 2005; Staskawicz et al., 1984; van Kan et al., 1991). It was then suggested that such key factors were likely to be secreted by pathogens to reach host structures and compounds.

Effector identification has been possible by the use of transcriptomic, proteomic and genomic approaches coupled to functional screening systems based mostly on the capacity of effectors to modulate plant immunity. Transcriptomic studies have allowed to determine genes differentially expressed during infection and even specifically in particular infection stages and pathogen structures as haustoria (Godfrey et al., 2010; Hahn and Mendgen, 1997; Huang et al., 2004). Expressed Sequenced Tags (ESTs) obtained on cDNA libraries coupled with computational searches for secretion peptides provide putative effector secretomes and thus putative effector repertoires. By this means, 31 hautoria-specific *in planta*-induced genes from the rust fungus *Uromyces faba* were identified (Hahn and Mendgen, 1997) as well as 100 putative secreted proteins expressed predominantly in haustoria of *Blumeria gaminis* (Godfrey et al., 2010). Moreover, *P. infestans* secretome analysis based on ESTs led to the identification of CRN proteins (Torto et al., 2003).

Proteomic approaches and/or biochemical purification of active secreted molecules have

proven to be successful on the characterization of key factors of infection. Mass spectrometry analysis of total proteins extracted from xylem sap of susceptible tomato infected with the vascular pathogen *Fusarium oxysporum* resulted on the identification of SIX (Secreted In Xylem) proteins of *F. oxysporum* specifically present during infection (Houterman et al., 2007). Further characterization of SIX1 and SIX2 proteins demonstrated their requirement for full virulence and as poteins recognized in tomato lines expressing I-3 and I-2 resistance proteins (Rep et al., 2004; Houterman et al., 2009). Proteomic approaches can be also useful when combined with predicted approaches. For instance, mining of *P. infestans's in vivo* secretome has not only corroborated repertoires established *in silico* via gene model prediction on genomic data but has also extended the secretome repertoire (Meijer et al., 2014).

As numerous complete genomes are available, effector repertoires can be established directly on gene models and can be combined to transcriptomic and proteomic data to further support their effector role. *In silico* criteria to determine candidate secreted effector proteins (CSEPs) on genomic data are principally based on the presence of secretion leaders and the absence of transmembrane domains. For such criteria, sequence surveys make use of computational strategies developed to ensure accuracy of predictions (Bendtsen et al., 2004; Torto et al., 2003). Other criteria can be taken into account for the determination of CSEPs and have become widely used and accepted in the scientific community throughout observations made on some experimental data. As very often, the studied effector proteins display small sizes and a high content of cysteine, such caracters are usually taken into account when defining potential candidates. These criteria have led to determine 929 proteins in the oomycete *Albugo candida* (Links et al., 2011) and 491 and 365 proteins from fungi *Blumeria graminis* and *Colletotrichum higginsianum*, respectively, have been proposed as putative effectors (Pedersen et al., 2012b; O'Connell et al., 2012). Genome survey on *Ustilago maydis* revealed 426 proteins potentially secreted (Mueller et al., 2008) and tissue-specific expressed, as revealed by transcriptome profiling (Gao et al., 2013).

Further amino acid sequence inspection of CSEPs repertoires has evidenced the presence of commonly occurring amino residues for some of them. These amino acids define motifs that serve as a basis for protein family classification and as a criterion when inspecting novel pathogen genomes (table 2). This was typically the case of oomycetes, for which sequence comparison of Avr proteins like ATR13 of *H. parasitica*, and Avr3a of *P. infestans* (Allen et al., 2004; Armstrong et al., 2005) led to determine a conserved Nterminal RxLR (Arginine, any amino acid, Leucine, Arginine) motif (Rehmany et al., 2005). Since evidenced as a sequence trait of Avr effectors, this motif has been used on genomic data of

Table 2. Conserved Nterminal motifs identified on Candidate Secreted Effectors Proteins (CSEPs) in fungi anf oomycetes.

Motifs	Protein	Species	Reference
RxLR(dEER)	RxLR effectors	*Phytophthora spp,* *H. arabidopsidis* *A. Laibachii*	Rehmany et al., 2005; Haas et al., 2009; Baxter et al., 2010 Kemen et al., 2011
LSSLR(ILKS)L(KQ)SL	Ac-RXL	*A. candida*	Links et al. ,2011
LxLFLAK	CRN effectors	*Phytophthora spp* *H. arabidopsidis,* *A. euteiches*	Torto et al.,2003 ; Haas et al.,2009 ; Stam et al.,2013 Baxter et al.,2010
	CRN-like	*R. irregularis*	Lin et al., 2014
HVVVxxP	CRN effectors	*A. euteiches*	Gaulin et al.,2008 ; Gaulin et al., in preparation
LxLYLAR /K	CRN effectors	*P.ultimum*	Adhikari et al.,2013
CHxC	CHxC	*A. candida,* *A. laibachii* *P. infestans*	Pais et al., 2013 ; Kemen et al.,2011 ; Links et al., 2011
Y/F/WxC	Y/F/WxC	*B. graminis* *P. graminis* *M. lini*	Godfrey et al., 2010b ; Pedersen et al., 2012
			Saunders et al., 2012
			Duplessis et al. , 2011
RFYR	AvrL567	*M. lini*	Rafiqi et al.,2010
RGFLR, KFLK, RDLA	AvrM	*M. lini*	Rafiqi et al.,2010
RYWT, RTLK	AvrLm6	*L. maculans*	Kale et al. , 2010
RMLH and RIYER	Avr2	*F. oxysporum*	Kale et al., 2010
FYIQYLxNQPV and/or LVAA	CRN-like	*B. dendrobatidis*	Sun et al., 2011

all oomycetes and has led to the identification of a large number of RxLR proteins defining the RxLR class of oomycete effectors (563 coding genes in *P.infestans*, Haas et al., 2009, 134 in *H. parasitica*, and 396 in *P.sojae*, Baxter et al., 2010). The conservation of the Nterminal motif L/Q/FLAK has also allowed the identification and cataloguing of CRN effectors in oomycetes (Baxter et al., 2010; Haas et al., 2009; Stam et al., 2013a). Moreover, a CHXC motif has been recently proposed as a motif characterizing a third large class of effectors in oomycetes (Pais et al., 2013; Kemen et al., 2011; Links et al., 2011). In fungi, RxRL-like motifs (ie, RRLQ RGFLR... Rafiqi et al., 2010; Khang et al., 2010) have been proposed in Nterminal domains of CSEPs. In addition, a Nterminal Y/F/WxC motif, firstly found in the 100 haustoria-expressed CSEPs of *Blumeria graminis* (Godfrey et al., 2010; Pedersen et al., 2012) have also been reported in CSEPs of *Puccinia graminis* and *Melampsora larici-populina* (Duplessis et al., 2011; Saunders et al., 2012).

In addition to these criteria (presence of secretion signals, absence of transmembrane domains, small size, high content of cysteine residues), other sequence signatures like the signs of diversifying selection can pinpoint their potential effector role as this may infer that the protein in question is submitted to a counter selection by hosts. Indeed, this has been demonstrated for the carboxyl terminal domains of RxLR effector secretomes (Win et al., 2007).

With established effector repertoires in a considerable number of species, it is now possible to perform comparative genomics to provide clues explaining pathogen host-adaptation and particular infection strategies. For example, comparison of putative effectors of the closely related smut fungi *U. maydis* and *Sporisorium reilianum* (both infecting maize) have determined specific subsets of effectors in both species. These subsets could be at the basis of their different infection behaviours on maize and may also imply the targeting of distinct maize functions (Schirawski et al., 2010). Another example is the comparison made on seven pathogenic necrotrophic *Pythium* species that revealed the absence of RxLR effectors. Because RxLR proteins are present in the related hemibiotrophs *Phytophthora spp* and the obligate biotroph *H. arabidopsidis*) and display *in planta* functions related to the suppression of host defenses, they have been generaly attributed to the support of biotrophic lifestyles. The lack of RxLR effectors in *Pythium spp* could further support the latter idea as RxLR functions seem unrelevant for *Pythium* necrotophic lifestyle (Adhikari et al., 2013).

Genomic studies aiming to identify putative effectors can be directed on particular genomic environments. Actually, in *Leptosphaeria maculans*, putative effector coding genes are mainly found in AT-rich isochores and are often associated to transposable elements (TE) (Rouxel et al.,2011). These effector-TE associations have also been observed for *P. infestans*

RxLR and CRN genes (Haas et al., 2009). Therefore, identifying such genetic environments in species might be used as a strategy to spot out CSEPs.

Functional characterization of effectors

The different predictive approaches for effector identification can result in important number of candidates. Demonstrating their actual contribution to virulence, studying their effect and biochemical activities in plants as well as identifying their targets can be time consuming because of their number, because reverse genetic approaches may not lead to a phenotype and because, for some pathosystems, genetic manipulation of pathogens is not possible. Given this, rapid and efficient functional methods are required in order to screen and functionally characterize effectors.

For this purpose, and based on the concept that effectors are secreted molecules that act in plant cell interfaces, different systems have been developed to heterogously express effectors *in planta,* in order test directly their cellular localization and any disturbance of plant physiology. Typically, effector proteins tagged with fluorescent proteins are overexpressed with or without their signal peptide (SP) to directly test their cellular localization by plant cell imaging (by means of confocal microscopy) (Caillaud et al., 2012; Stam et al., 2013a/b). Heterologous expression systems are also used to test the "pathogen-independent'" host-delivery of effectors. As the presence of the SP is expected to drive the secretion of effectors into the plant apoplasm, by expressing full length effector proteins (containing their SP), it is inferred that effectors localizing inside cells have re-entered the cytoplasm autonomously via intrinsic translocation signals in the protein. Based on this, it is then inferred that these effectors are likely to localize inside plant cells during infection (Rafiqi et al., 2010; Ribot et al., 2013).

N.benthamiana and *N.tabaccum* (Solanaceae) have become widely used for molecular plant-microbe interactions studies since they display different degrees of susceptibility to a variety of microorganisms as oomycetes (*P.capsici*, *P.infestans*), fungi (*Verticillium dahlia, Colletotrichum orbiculare*), bacteria (*P. syringae*), viruses (TMV, PVX…) and several cDNA libraries and molecular tools like microarrays are available (Goodin et al., 2008). Their foliar tissues are particularly amenable for genetic transformation via the bacteria *Agrobacterium tumefaciens,* compared to other plant model species like *A. thaliana* and crop species like barley. Infection with *A. tumefaciens* strains carrying an effector coding gene allows to, locally and transiently, transform plant cells and constitutes an easy way to express

efficiently an important number of effectors in plant cells. As an example, Caillaud and associates (2012) used this approach to screen the localization and cell-death activity of 49 intracellular RxLR effectors of *H. arabidopsidis*, evidencing the diversity of the subcellular compartments targeted by these proteins. Co-agroinfiltration of *N.benthamiana* leaves is also possible and has been validated, for example, for the study of Avr/R proteins resulting in the expected HR response (Van der Hoorn et al., 2000; Bos et al., 2006). Besides agroinfiltration, *Pseudomonas syringae* and its T3SS provide another strategy for delivering effector proteins directly in the cytosol of plant cells by translationnally fusing T3 secretion leaders to the studied effectors. This strategy was used by Whisson and collegues (2007) to demonstrate that the RxLR effector, Avr3a, is recognized by the R protein R3a in the cytoplasm of plant cells and thus that Avr3a is translocated inside plant cells during infection, proving for the first time that RxLR proteins are intracellular effector proteins (Whisson et al., 2007).

Cellular systems have also been developed for rapid and accurate functional studies. Methods based on protoplasts have been described and improved to transiently express effectors and/or reporter genes on various plant crop species like rice, maize, parsley, tomato...(Chen et al., 2006; Sheen, 2001). Such systems have been used to characterize molecular events linked to MAMP/PAMP perception, signal transduction of defense responses and effector activities (Brunner et al., 2014; Kansaki et al., 2014). Recently, a screen on 33 RxLR effectors using protoplasts of tomato and *A. thaliana* allowed to identify immune suppressive RxLR effectors and to correlate these activities to their cellular localization (Zheng et al., 2014). In addition, screening of 42 *in silico*-identified putative secreted effectors of *M. oryzae* permitted to identify 5 effectors capable of triggering cell-death on rice protoplast (Chen et al., 2013). Finally protoplasts can be also used to screen cDNA libraries for protein-protein interactions, analogously to Yeast-two Hybrid approaches (Y2H). This has been performed to identify plant protein partners of effectors of *A.tumefaciens* (Lee et al., 2012).

All together, these systems have rendered possible to bridge *in silico* identification to rapid validation of effector candidates and even their functional molecular dissection. Still, although providing a rapid way to initiate an effector characterization, experimental systems providing a natural host/microorganism context are required to accurately assess effector's functions.

Effector delivery and localization

Effectors are secreted to exert their functions either on the apoplasm (apoplastic effectors) or inside host cells (intracellular/cytoplasmic effectors). In the case of gram negative bacteria, apoplastic effectors are secreted via the Type 2 Secretion System (T2SS) (Nivaskumar and Francetic, 2014) while apoplastic effectors from filamentous eukaryotic microorganisms are secreted via the classical endomembrane secretion pathway (Giraldo et al., 2013).

To reach host cytoplasm, intracellular effectors require passing across pathogen outer structures, the apoplastic space and outer structural components of host cells (cell-wall and plasma membrane). General concepts of secretion and host-delivery are schematized in figure 7. Delivery systems and mechanisms are well characterized for prokaryotic pathogens, which use the Type 3 and/or Type 4 Secretion System (T3SS/T4SS) to ensure the direct injection of effectors into the host cytosol (Büttner and Bonas, 2006). A total of 52 T3SS effector families have been identified in *Xanthomonas spp*, 58 in *Pseudomona syringae* and 100 *in Ralstonia solanacearum* which display a great variability between strains (Baltrus et al., 2011; Peeters et al., 2013; Ryan et al., 2011). Intracellular effectors of nematodes are delivered into host cytoplasm via a protusible stylet that pierces plant cell-wall and mediates their injection across the host plasma membrane (Mitchum et al., 2013). For filamentous eukaryotic pathogens, secretion and delivery systems are less understood. In the case of fungi, it was very recently shown that intracellular effectors of the rice blast fungus *Magnaporthe oryzae* undergo an alternative secretion pathway different from the classical ER-Golgi secretory endomembrane pathway used for the secretion of apoplastic effectors (Giraldo et al., 2013). Such alternative secretion mechanisms result in the accumulation of effectors in particular zones of the infectious hyphae termed Biotrophic Interfacial Complex (BIC) (Giraldo et al., 2013; Khang et al., 2010).

In oomycetes, it is still not known whether intracellular effectors are secreted by particular secretion routes, but pioneer studies, using GFP-tagged effectors expressed by the pathogen, revealed their accumulation in located zones of haustoria, inferring that these structures ensure effector delivery into host cells. How fungal and oomycete effector proteins are secreted outside the pathogen plasma membrane and transported across the host-cell outer structures is a current topic of considerable interest and controversy.

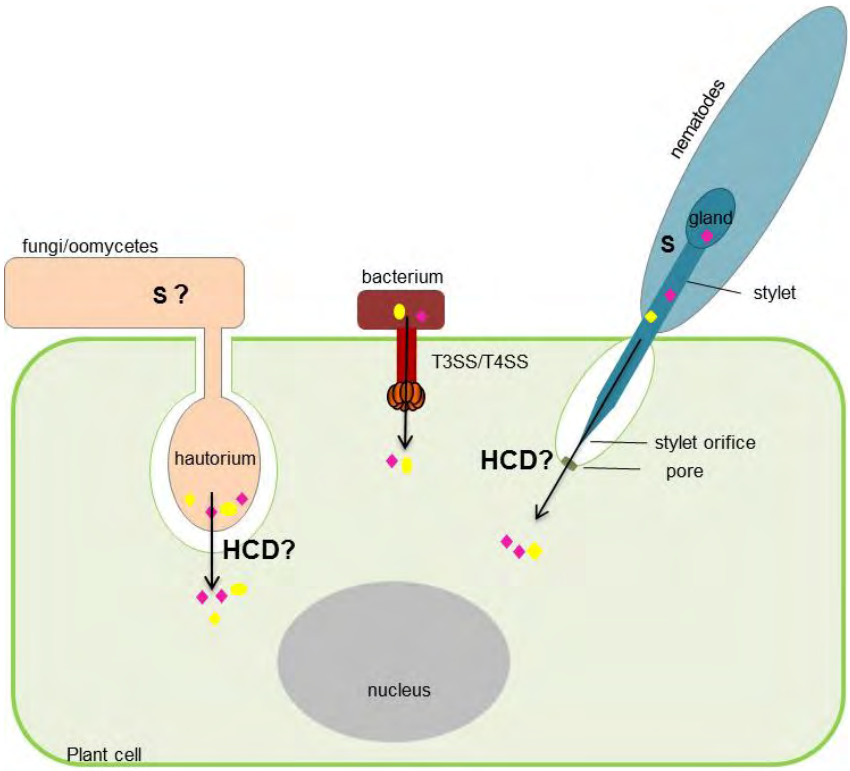

Figure 7. Schematic representation of the general concepts of secretion (S) and host-cell delivery (HCD) of intracellular effectors by phytopathogenic fungi, oomycetes, bacteria and nematodes.

Secretion and translocation of bacterial intracellular effectors to the interior of host cells is ensured by host-cell-contact-dependent secretion molecular systems (T3SS and T4SS).

Fungi and oomycetes intracellular effectors accumulate in infection structures as haustoria from which they translocate inside host cytoplasm. While it has been shown that intracellular effectors undergo ER-Golgi independent secretion pathways in the fungus *M. oryzae* (Giraldo et al., 2103) it is still not clear whether this can be a generality for other fungi and oomycete species.

Nematode intracellular effectors are produced in oesophageal gland cells and secreted via the ER-Golgi secretory network into secretory granules transported along the stylet and subsequently released into host cytoplasm via the stylet orifice. Structural analyses have depicted a small pore in the plasma membrane at the stylet orifice thought to allow direct delivery of effector proteins (Mitchum et al., 2013).

The studies on the translocation of fungal and oomycete effectors have shown that the N-terminal domains might carry the translocation signals. In oomycetes, amino terminal conserved motifs (downstream of their signal peptide sequences) like the RxLR motif (in RxLR effectors) and the L/Q/FLAK (in CRN effectors) characterize these regions and have been shown to act as signals for host delivery (Dou et al., 2008; Kale et al., 2010; Schornack et al., 2010; Whisson et al., 2007). In contrast, fungal amino terminal domains do not present an obvious conservation of residues. For some proteins, algorithm-based sequence surveys have suggested RxLR-like motifs.

Interestingly, the oomycete RxLR motif is highly similar and functionally equivalent to the RxLxE/D/Q (termed ”Pexel”) found in intracellular effectors of the human parasite *Plasmodium falciparum* (phylogenetically related to oomycetes) (Bhattacharjee et al., 2006; Hiller et al., 2004; Marti et al., 2004). The dissection of their export mechanisms has shown that, once addressed into the secretory pathway by secretion signals, these proteins interact with the lipid phosphatidylinositol 3-phosphate PI(3)P present in the endoplasmic reticulum (ER) membrane. In the ER lumen, the RxLxE/ D/Q is cleaved by the aspartic protease Plasmepsin V, a process that is thought to liberate the protein from the ER membrane to allow its subsequence vesicular ER-Golgi export. Once outside the plasma membrane, their actual translocation inside host cells has been proposed to be mediated by a *Plasmodium*-derived protein machinery or "translocon" (Bhattacharjee et al., 2012).

Functional translocation studies directed on the RxLRs motifs of *Phytophthora spp* have been based on recombinant proteins corresponding to tagged-Nterminal halves of RxLR effectors or tagged-full length proteins. First studies showed that the RxLR motif is sufficient to ensure the self-translocation of the fusion protein inside host cells without the need of pathogen-derived machinery, implying that translocation factors are provided by the host (Dou et al., 2008; Whisson et al., 2007). Kale and associates (2010) provided evidence that Nterminal moieties of oomycete RxLR effectors and RxLR-like fungal effectors bind PI(3)P via these motifs and that such binding is necessary for effector entry (Kale et al., 2010). They proposed an internalization model in which binding to PI(3)P present at the surface of the host cell would trigger endocytocis. Effectors would then be in host-derived plasma membrane vesicles and liberated thereafter, localizing freely in host cytoplasm (Kale et al., 2010). But at present, this model has been challenged as the same experiments have not been reproducible by other laboratories (Wawra et al., 2013). Latest studies, indicate that RxLR motifs are not sufficient to such binding and propose also the contribution of Cterminal domains. In addition to the cell entry, Cterminal binding to PI3P would also be required for the stability of the protein and for its virulence activity inside plant cells (Sun et al., 2013;

Yaeno et al., 2011). Such studies have included structural analyses that revealed that, despite the great variability of Cterminal domains between RxLR effectors in the scale of primary sequence, some Ctermini present similar protein folding where positively charged patches may explain their lipid binding properties (Yaeno et al., 2011).

The demonstration of the actual passage of effectors into host cytoplasm during infection has only been brought for fungal effectors like Uf-RTP1p from *U. fabae* and AvrM from *Melampsora lini* thanks to the use of specific antibodies (Kemen et al., 2005; Rafiqi et al., 2010) or by fluorescently tagged effectors expressed by the pathogen like in the case of PWL2, BAS1 and AVR1-CO39 of *M. oryzae* (Khang et al., 2010; Ribot et al., 2013). Deletion analysis of proteins indicates that signals in N-termini are responsible for translocation and that, as for RxLR proteins, the process can also be pathogen-independent (Rafiqi et al., 2010). As mentioned before, well conserved motifs are not apparent, but rather divergent RxLR-like motifs (i.e RRLQ RGFLR...) have been proposed. Structural analyses indicate that, when present, RxLR-like domains are structurally ordered regions which contrasts to the disordered structure of RxLR domains of oomycete effectors. This suggests that they might not be functionally equivalent and/or that both domains use different translocation mechanisms (Ve et al., 2013). Interestingly, in the case of AvrM (proposed as a RxLR-like protein), Nterminal regions necessary for host cell uptake do not bind PI3P (Gan et al., 2010; Ve et al., 2013) thus, it seems that cell uptake does not correlate with lipid binding for fungal effectors.

Once inside the host cell, intracellular effectors can be addressed to neighbor non- infected cells. A cell to cell movement of intracellular effectors has been observed for the PWL2 fungal effector (Khang et al., 2010). Plasmodesmata mediate the viral symplasmic movement (Tilsner et al., 2013) and is also suggested for blast fungal effectors and even the infectious hyphae itself (Kankanala et al., 2007). The trafficking of effectors to adjoining cells is thought to be part of a strategy aimed to prepare host cells for invasion. Various subcellular compartments and structures can be targeted by effectors. Chloroplasts, Golgi and tonoplast endomembranes are targeted by Type 3 effectors of bacteria (Jelenska et al., 2007; Nomura et al., 2011). In addition to these compartments, the plant nucleus is also targeted by intracellular effectors of bacteria, oomycetes (Canonne and Rivas, 2012; Caillaud et al., 2012; Stam et al., 2013b), as well as nematodes (Jaouannet et al., 2012) and fungi (Kemen et al., 2005). These different localizations can be explained by the presence of intrinsic addressing peptide signals (i.e: mitochondrial, plasma membrane, Golgi…). One prominent predictable signal detected in effectors accumulating in plant nuclei is the Nuclear Localization Signal

(NLS), indicating they might co-opt the nuclear trafficking machinery of hosts to access nuclei. This has been actually verified for some since the silencing of plants α-importins uncouples their nuclear accumulation (Kanneganti et al., 2007). The predictability of NLSs during systemic surveys of putative secreted effectors has rendered evident that a considerable number might target the host nucleus. For example, 14 putative effectors of *U. maydis* (Mueller et al., 2008) as well as 15 of the 265 putative effectors of *Heterodera glycines* nematode (Gao et al., 2003) contain NLSs and are, thus, likely to be translocated in nuclei of host cells after secretion. Hence, it seems that manipulation of nuclear functions might be a strategy benefiting pathogen infection.

Function of effectors

Molecular plant-microbe interactions can be seen as interacting networks of biochemical activities. This system biology concept proposed by Pritchard and Birch (2011) postulates that plant networks ensuring a cellular function (i.e, plant defense) are intricate relations of biochemical activities of plant components, each component accomplishing a function in the network. In this context, effectors target plant functions whose alterations lead to a different biochemical behaviour of host's networks and to a beneficial outcome for the pathogen. In such networks, certain functions are important "nodes" or hubs determining the biochemical behaviour of the network. Therefore, it is expected that microbial manipulation strategies converge to the targeting of these hubs.

The plant cell wall (PCW) is one of the first barriers encountered by any microbe tempting to enter plant tissues and represents a source of nutrients given its polysaccharidic nature. It needs to be broken down and/or softened by pathogens in order to establish them in host cells and to progress along host tissues (Cantu et al., 2008; Hématy et al., 2009). A first class of apoplastic effectors is cell-wall degrading enzymes (CWDEs) as pectinases, cellulases, arabinases… (Walton, 1994). Indeed, nematodes, fungi and oomycetes secrete CWDEs in the apoplasm whose activities have been shown to be required for complete virulence (Bohlmann and Sobczak, 2014; Feng et al., 2014; Fu et al., 2013). These enzymes have been shown to act early during fungal infection, accompanying the mechanical pressure exerted by appressoria at penetration sites (Cantu et al., 2008), and intervene also all along the colonization of plant tissues macerating PCW to allow further advance of infectious hyphae. Their importance in the migratory stage of juvenile nematodes in plant roots has also been established (Bohlmann and Sobczak, 2014). Oomycetes and fungi display a large diversity of CWDEs (King et al., 2011; O'Connell et al., 2012; Ospina-Giraldo et al., 2010).

Although CWDEs of oomycetes remain globally poorly functionally characterized compared to fungi CDWEs, their cataloguing and studies point to their requirement during infection (Mingora et al., 2014; Zerillo et al., 2013). Fungal CWDEs have been shown to present a chronologically coordinated *in planta* expression. Their activities correlate to the constitution of host cell walls (King et al., 2011; O'Connell et al., 2012), depicting a strategy of adaptation to a specific host plant. For instance, during late stages of infection, *C. higginissianum* (infecting Brassicaceae species) relies on the activity of pectinases while *C. graminicola* (infecting maize) relies on cellulases and hemicellulases. This is consistent with the cell wall composition of their hosts as dicotyledonous present more pectin than monocotyledonous (containing more hemicellulose) (O'Connell et al., 2012). In addition, the broad host range necrotrophic fungi *Botritis cinerea*, presents a particular endo-arabinase (BcAraA) necessary for its virulence in *A. thaliana* exhibiting a preferential expression in this host rather than in *N. benthamiana* (Nafisi et al., 2014). In the case of phytopathogenic bacteria, which penetrate plant tissues via natural opening (like stomata) or wounds, CWDEs (secreted via the T2SS) mediate the degradation of plant cell-wall carbohydrates and are thought to have, in a general way, a nutrient function (Nivaskumar and Francetic, 2014).

PCW alterations caused by CWDEs and PAMPs, exposed during this close encounter, can be perceived by plants to activate defenses (Hématy et al., 2009). In this context another class of apoplastic effectors is devoted to the manipulation of plant defenses. Best characterized examples in fungi, oomycetes and nematodes, have revealed that manipulation of plant immunity can be based on avoidance or direct suppression of plant defenses (Hewezi and Baum, 2013; Mueller et al., 2013). For instance, avoidance and/or deregulation of plant defenses activated by chitin perception is accomplished by LysM effectors of various fungal species. These effectors are characterized by the presence of LysM carbohydrate-binding domains that bind directly chitin with high affinity, interfering with the binding of chitin to their cognate plant PRR receptors (Kombrink & Thomma, 2013). The LysM Ecp6 from *C. fulvum* masks free chitin-derived fragments preventing their perception by LysM-containing PRR plant receptors. Others, like Avr4 (also from *C. fulvum*) and Mg1LysM from *M. graminicola* have been proposed to protect chitin from degradation by plant chitinases (Kombrink & Thomma, 2013).

Direct suppression of plant defenses can also rely on the inhibition of plant defense factors present in the apoplasm and released upon infection. Several apoplastic effectors display protease inhibitory activities on host apoplastic proteases. For instance, the secreted effector Pit2 from *U. maydis* accumulates in the apoplasm where it interacts with different

host cysteine proteases inhibiting their activities (Mueller et al., 2013). Effectors Avr2 of the fungus *Cladosporium fulvum* and EPIC1 and EPIC2B from the oomycete *P. infestans* interact with and inhibit the cysteine protease Rcr3 of tomato (Song et al., 2009). This plant protease is also targeted by the nematode effector VAP1 from *G. rostochiensis* (Lozano-Torres et al., 2012). Rcr3 is an example of a plant function targeted by different effectors of distinct pathogens protruding, therefore, as an important plant defense factor and illustrating the concept of hub proposed by Pritchard and Birch (2011). In addition to plant proteases other defense-related plant functions have been revealed as direct targets of effectors. The protein Pep1 of *U. maydis* has been demonstrated to inhibit the activity of the host peroxidase POX12 conducting to reduction of H_2O_2 levels, a strategy of crucial importance for *U. maydis* compatibility in maize (Hemetsberger et al., 2012).

Another class of apoplastic effectors seems to contribute in a different manner by displaying a cytotoxic/cell-killing activity. The Nep1-like protein (NLPs) family is widely distributed in bacteria, fungi and particularly in oomycetes (absent in plants and animals). These proteins induce cell-death, ethylene production and wilting in plants (Gijzen and Nürnberger, 2006). Genetic approaches have demonstrated their contribution to virulence and gene expression studies have shown that in hemibiotrophic fungi and oomycetes their pic of expression coincides with necrotrophic infection stages (Feng and Li, 2013; Gijzen & Nürnberger, 2006). Because of this, they are proposed to be secreted toxins facilitating cell-death required for the necrotrophic infection states. Still, their exact biochemical activities and plant targets remain unknown.

Regarding intracellular effectors, the identification and characterization of them in distinct microbial pathogens has shown that they can be addressed to diverse subcellular compartments.

The plant nucleus as a common field for microbial effectors.

The plant nucleus at the center of plant immunity.

Plant immunity resides on the perception of microorganisms either at the surface of cells (i.e, through plasma membrane receptors) or in the cytosol (i.e, via R proteins) and conducts to the activation of a defense program. Upon pathogen perception, robust changes of gene expression occur (Maleck et al., 2000; Tao et al., 2003). The information necessary for

such transcriptional reprogramming needs to be transduced from the cytosol to the nucleus. The signaling convergence towards the nucleus is mediated by defense integrators/activators and nuclear components whose dynamics are required during microbial infection.

A first aspect of these dynamics concerns the nuclear shuttling of activated R proteins and their signaling complexes, which is necessary for the proper induction of disease resistance (Qi and Innes, 2013). In the absence of pathogen, inactivated R proteins can be associated to the plasma membrane and endomembranes (like RPM1 and RPS4 of *A. thaliana*, respectively) or can display a nucleo-cytoplasmic distribution like the barley MLA10, the tobacco N protein and the *Arabidopsis* SNC1 protein (Caplan et al., 2008; Chang et al., 2013). Following recognition of their corresponding cognate effectors, the activated R protein pools can be detected in the nucleus. This has been seen for RPS4 upon activation by AvrRPS4 of *P. syringae* (Heidrich et al., 2011) and it has been shown that the nuclear levels of MLA10 R protein (recognizing Avr10 from the barley pathogen *Blumeria graminis f. sp. horde)* increase during infection (Chang et al., 2013) which is also the case for the tobacco N protein in the presence of the viral effector p50 (Caplan et al., 2008) and Pb1 from rice infected by *M.oryzae* (Inoue et al., 2013). The depletion of nuclear pools of activated R proteins by forced mislocalization to the cytoplasm uncouples full resistance. The nuclear exclusion of MLA10 conducts to the loss of growth arrest of *B. graminis* (Chang et al., 2013) and is also the case for Pb1mediating blast (*M.oryzae*) resistance (Inoue et al., 2013). Interestingly, it has been observed that the nuclear sequestration of activated RPS4 mediates bacterial growth inhibition without HR while its sequestration in cytosol impairs growth inhibition but not the HR (Heidrich et al., 2011). Because of these different outputs of defenses, it has been proposed that each pool of R proteins activate different signaling pathways and that the establishment of a nucleo-cytoplasmic distribution is imperative for the proper resistance (Qi and Innes, 2013).

In addition to R proteins, other immune-regulators proteins require to shuttle between cytoplasm and nucleus. The general immune regulator of *A. thaliana* ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) is one component of various R complexes, able to physically interact with R proteins such as RSP4 and with defense co-regulators PHYTOALEXIN DEFICIENT 4 (PAD4) or Senescence Associated Gene101 (SAG101) to form complexes that localize to the nucleus, necessary for the transcriptional reprogramming of genes and induction of TIR-NB-LRR mediated resistance (García et al., 2010; Heidrich et al., 2011). Another crucial immune component, NPR1 (transcriptional regulator of SA immune signaling) is relocalized from the cytoplasm to the nucleus upon pathogen challenge, where it interacts with TFs of the TGA family (Shearer et al., 2012).

Another aspect of defense transcriptional programs is the involvement of TFs. R proteins can directly bind TFs to mediate gene expression regulations. For instance, MLA10 interacts with TFs of the WARKY (WRK1 and WRK2) and MYB (MYB6) families. Both WRKs are repressors of defense genes while MYB6 was demonstrated to be a positive regulator (Chang et al., 2013). In addition, the rice Pb1 interacts with the WRK45, a positive defense regulator of the SA signaling (Inoue et al., 2013). Moreover, the nuclear-localized N protein of tobacco has recently been shown to directly interact with SPL6, a positive regulator of defense gene expression which has also been suggested to be involved in the nuclear RPS4-mediated defense response (Padmanabhan et al., 2013). As a last example, the TIR-NB-LRR, SNC1 protein delocalizes to the nucleus where it interacts with TPR1 (Zhu et al., 2010). The characterization of one particular R protein, RRS1, has indicated that R proteins may be directly involved in regulation of defense gene expression. RRS1, which participates in AvrRPS4-mediated resistance to *P.syringae* and other pathogens, presents TF properties as it displays, in addition to TIR-NB-LRR domains, a Cterminal WRK domain (Deslandes et al., 2002).

Gene accessibility by the transcriptional machinery is also necessary for the proper induction of disease resistance. Chromatin configuration determines the accessibility of proteins to specific genomic loci. Post-translational modifications (acetylation, deacetylation, methylation ...) of DNA-associated histones proteins, by histone-modifying enzymes (i.e, histone acetyltransferases HAT, histone deacetylases HDA or histone methyltransferase HMT…), mediate chromatin dynamics and are associated to transcription regulation, DNA replication and DNA repair (Kouzarides, 2007). Their involvement in plant developmental processes like seed development and flowering are well documented (Ma et al., 2013) and their implication in plant immunity is beginning to be reported (Berr et al., 2012). For example, the loss of activity of Arabidopsis HDA19 conducts to an increase of SA defense-related genes and enhanced resistance to *P. syringae*. HDA19 interacts with two TFs WRKs that act as transcriptional activators of negative immune-regulator genes. Thus, HDA19 is proposed to regulate negatively plant immunity by impeding the transcriptional activation of negative regulator genes by both WRKs (Berr et al., 2012). In addition, the Arabidopsis HAT, ELP2, influences the expression kinetics of immune regulators EDS1, PAD4, and defense gene PR1 (Wang et al., 2013). Moreover, the HMT SDG8 has been shown to be induced upon infection of necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola* and *sdg8* mutants display enhanced sensitivity to these pathogens which correlates with a decrease expression of hormones ET/JA –responsive genes (Berr et

al., 2012).

Consistent with the importance of the nuclear-cytoplasmic shuttling of these complexes for the correct immune gene activation, components of the nuclear trafficking machinery mediating their nuclear entering and accumulation have been identified. The transport of macromolecules across the Nuclear Envelope (NE) is ensured by Nuclear Pore Complexes (NPCs). NPCs are selective molecular "gates" of the NE formed by nucleoporines proteins (Nups). The passage of cargo molecules is ensured by Nuclear Transport Receptors (NTRs) referred to as importins α/β and exportins. NTRs recognize transport signals present in cargo proteins and direct directionality of the transport: importins α/β recognize Nuclear Localization Signals (NLS) while exportins recognize Nuclear Export Signals (NES) (Wirthmueller et al., 2013). Mutation of the *A. thaliana* nucleoporin MOS7 (also known as Nup88) causes an impairment of immunity responses controlled by SNC1 and EDS1 signaling complexes. EDS1 exhibits predicted NLS and NES sequences although their NTRs partners remain unknown. For SNC1 (carrying one predicted NLS and two predicted NES motifs) importin-α3 has been suggested as mediating its import since its loss impairs SNC1 nuclear accumulation and mediated resistance (Wirthmueller et al., 2013). Thus, an orchestrated nucleo-cytoplasmic traffic accompanying signaling of immune integrators is required to the proper activation of immunity during pathogen infection.

Nuclear localized effectors and their activities

Microbial effectors that target nuclear components and associated processes have been identified. Sequence inspection of effector candidates identified via several approaches (transcriptomics, proteomic and genomics) indicates that an important number of effectors are potentially addressed to host nuclei as evidenced by the presence of predicted NLS sequences or nuclear-related putative functions. In the nematode *M .incognita*, 13.5% of the infection secretome (66 proteins over 486) exhibits NLS and putative DNA/RNA binding domains (Bellafiore et al., 2008) and 10% of CSEPs of *B. graminis* are putative ribonuclease-like proteins (Pedersen et al., 2012). In addition, 14 NLS containing proteins (over 441 putative secreted effectors) of *U. maydis* display similarities to domains typical of transcription factors (Mueller et al., 2008).

Nuclear-associated functions and their dynamics during infection are important to support the plant immunity on set. Owing this, it is likely that nuclear functions related to plant defense or to other physiological processes (cell-growth, nutrition...) controlled in the

nucleus, may be subverted by effectors to allow the proper infection and development of pathogens. The biochemical activities of nuclear effectors and their relevance for virulence is better understood for bacterial effectors (for review refer to Bhattacharjee et al., 2013; Rivas and Deslandes, 2013) and are only beginning to be documented for nematodes, phytoplasma, fungi and oomycetes.

Bacterial effectors

Bacterial proteins often present a modular organization in which each module/domain carries a biochemical activity and a function within the whole protein. The execution *in planta* of each activity promotes the virulence function of the effector and can implicate different host targets. Despite that little is known about the spatio-temporal execution of such activities during infection, the fragmentary information gained through studies has evidenced major host processes that bacteria nuclear effectors are able to manipulate. Among these are host transcription, host mRNA metabolism and host proteasome machinery whose modulation can imply different physiological outcomes for the host.

Direct manipulation of host gene transcription is best exemplified by the Transcription Activator-Like Effectors (TALEs) of *Xanthomas spp* and *Ralstonia spp*. This family of Type 3 secreted effectors can account for up to 28 effectors in one single strain (Scholze and Boch, 2011). TALEs are capable to bind promoters of host genes and to activate their transcription. They present a modular architecture comprising a DNA-binding domain (DBD) of 34/35 amino acids repeats (the number of repeats varying), a NLS, and an acidic activation domain at their C-terminus. Direct binding to host DNA is mediated through their DBD domain and the specificity of the targeted DNA sequence is governed by a DBD amino acid/nucleotide code. While DBD specifically binds DNA, the activation domain at the Cterminus acts as an eukaryotic TF recruiting host transcriptional machinery (Scholze & Boch, 2011). AvrBs3 from *Xanthomonas vesicatoria pv. vesicatoria* was the first TALE identified and is the best characterized. In susceptible pepper it induces the expression of UPA20 gene which codes for a TF that is a key regulator of cell enlargement, leading to hypertrophy of mesophyll cells when expressed in *N. benthamiana,* symptomes that are observed during infection (Kay et al., 2007). The identification of other targeted genes indicates that TALEs manipulate directly the sugar metabolism of hosts. Indeed, in rice, the TALE PthXo1 of *Xanthomonas oryzae pv oryza* activates the transcription of the rice gene

osSWEET11/Xa13 which encodes a plasma membrane sugar transporter. Oncoming results indicate that osSWEET genes are common TALE targets; hence, it is thought that TALEs manipulate plant sugar metabolism and cell growth by inducing the release of sugars as well as larger space niches needed for bacteria growth, proliferation and dispersal (Chen et al., 2010). In addition to the direct binding to promoters, TALEs have also been shown to interact with regulators of RNA polymerase II and III. Such plant regulators control the activities of these RNA polymerases and restrict the assembly of factors implicated in the expression of the targeted gene. Therefore, the interaction of TALEs to these regulators perturbs the synthesis small RNAs, tRNA and the ribosome biogenesis. For instance, the TALE PhtA4 is able to interact with the citrus protein MAF1 (a negative regulator of RNA pol III). The presence of PthA4 in plant cells coincides with the reduction of MAF1 levels and the development of cell hypertrophy and hyperplasia, symptoms typical of canker disease caused by this bacterium (Soprano et al., 2013).

Besides direct DNA-binding, bacteria effectors control host transcription via direct interaction with host TFs and histones. It is the case of XopD, a modular Type 3 effector of *Xanthomonas campestris pv vesicatoria* shown to repress gene transcription, perturbing the onset of defenses and cell-death. XopD presents an N-terminal DNA-binding domain (DBD) characterized by helix-loop-helix motif, two repeated EAR motifs involved in the transcriptional repression, a C-terminal cysteine protease domain involved in the release of SUMO (small ubiquitin-like modifier) and a NLS motif. Consistent with is modularity, XopD presents various activities in plants. First, XopD interacts through its helix-loop-helix domain with AtMYB30, a positive TF regulator of plant defenses to suppress plant immunity and to promote bacteria growth (Canonne et al., 2011). In addition, its SUMO protease Cterminal domain targets sumoylated proteins of plants to deconjugate SUMO from their substrates (Hotson et al., 2003). Because SUMO substrates include histone-modifying enzymes and histone tails, XopD has been suggested to target chromatin regulators via its SUMO protease activity. One particular observation is that XopD displays a “pointed” localization in host nuclei (said to be related to the targeting of nuclear bodies) and its presence is accompanied by the reorganization of DNA material (Canonne et al., 2011). The capacity to interact with host histones has also been proposed for PoP2 effector from *Ralstonia solanacearum,* based on its acetyltransferase activity in plant nucleus (Tasset et al., 2010).

Another strategy resides on the manipulation of host mRNA status. In this context, the Type 3 secreted HopU1 from *P.syringae* interacts with different host RNA- binding proteins (RBPs). RBPs participate in RNA metabolism (ie, splicing, degradation, translation, export…) by directly binding mRNAs via their RNA recognition motifs (RRM) (Keene,

2007). Among HopU1 targets, the RBP GRP7 was reported to be necessary for PTI activation upon perception of PAMPs flg22 (flagellin) and elf18 (elongation factor) (Fu et al., 2007; Nicaise et al., 2013). GRP7 interacts with FLS2 transcripts and HopU1 interaction with GRP7 correlates with a reduction of FLS2 and EFR protein levels. Hence, by manipulating host RNA metabolism of PRR, HopU1 impedes the accumulation of PAMP receptors proteins necessary for the correct PTI input (Nicaise et al., 2013).

Subversion of host ubiquitin-26S proteasome degradation system (UPS) is another virulence strategy of bacteria. Plant UPS is a multisubunit protein complex present in cytoplasm and in the nucleus that selectively degrades proteins conjugated with ubiquitin (Ub). This process regulates several aspects of plant biology including hormone signaling, and immunity (Vierstra, 2009). Addition of Ub to targeted proteins is performed by three enzymes among, which E3-ligase, ensures target specificity of Ub tails. Different type 3 effectors localizing in host cytoplasm exhibit E3-ligase activities leading to the degradation of virulence targets and suppression of plant defense; for review refere to Duplan and Rivas, 2014. A particular polypeptide secreted by *P. syringae*, SyringolinA, necessary for virulence, localizes both in the nucleus and cytoplasm of host cells inhibiting the activity of the proteasome (Kolodziejek et al., 2011).

Nematode effectors

Endoparasitic root-knot nemadotes (RKNs) and cyst nematodes (CNs) are obligate biotroph parasites that enter plant roots and migrate intracellularly to reach host vascular tissues were they become sedentary. In there, they induce tremendous physiological and morphological changes on host root cells that conduct to the formation of feeding cells (FCs): giant cells in the case of RKNs and syncytia in the case of CNs. In both cases, these FCs are physiologically hyperactive as seen by the proliferation of subcellular organelles and secondary vacuoles. FC differentiation and maintenance are triggered by gland-secreted effectors, implying the manipulation of cellular processes like plasma membrane and cell-wall formation (Mitchum et al., 2013). Significant changes occur in host nuclei suggesting important events of plant nuclear biology likely to be triggered by nematodes effectors. These include repeated karyogenesis with aborted cell divisions and successive endoreduplication of DNA material that lead to biogenesis of a multitude of nuclei that can enclose several nucleoli (a sign of intensive transcriptional activity) (Mitchum et al., 2013). Several NLS-containing effectors (66 in RKNs and in 15 CNs) and/or presenting nuclear-

related activities (i.e: DNA/RNA binding, chromatin binding and histone domains) have been predicted (Bellafiore et al., 2008; Elling et al., 2007; Gao et al., 2003) and represent candidate effectors possibly controlling directly host-nuclear biology during infection. Important transcriptional changes have been documented to occur in host cells upon nematode infection and have been long time predicted to be controlled by effectors (Hewezi and Baum, 2013) but the exact effector and targeted genes remain unknown.

A recent report has begun to bring light on this, as a nuclear-localized effector 7H08 of *M. ingonita* was demonstrated to carry a transcriptional activity in plants (Zhang et al., 2014). Two secreted proteins identified via EST mining on the RKNs infecting tomato *Meloidogyne incognita* (Mi) and *Meloidogyne javanica* (Mj) have been demonstrated to be produced in oesophageal cells, secreted and localized in giant cell nuclei during infection (Jaouannet et al., 2012; Lin et al., 2013). MiEFF1 presents a functional NLS and lacks any similarity to other known proteins. Its accumulation outside the nematode is seen in giant cell during the sedentary phase of infection where it accumulates in nuclei (nucleolus excluded). Like MiEFF1, the Mj-NULG1 harbors a NLS, lacks similarity to other proteins and is nuclear-localized in giant feeding cells of roots cells. Mj-NULG1 is required for complete virulence. Consistent with this, its overexpression in plants increases plant susceptibility to Mj (Lin et al., 2013). MiEFF1 and Mj-NULG1 effector proteins appear as RKN-specific as they are absent in free-living and animal nematodes and homologues are found in various *Meloidogyne spp*. It is suggested that they manipulate a host nuclear function specifically leading to giant cells formation. How exactly both proteins participate in this is still unknown as their biochemical activities and host targets remain to be characterized.

16D10 effector of *Meloidogyne incognita* has been shown to stimulate host root proliferation. Although showed to function in the cytoplasm, it manipulates a nuclear-associated function since it interacts with two SCARECROW-like (SCL) transcription factors (Huang et al., 2006) which are known to mediate cell fate in *Arabidopsis* and are members of the GRAS superfamily of TFs largely involved in plant development (Cui et al., 2014; Hirsch and Oldroyd, 2009).

In the case of CNs, *in planta* transient overexpression of several proteins has revealed that specific subnuclear compartments like nucleoli can be targeted as it is the case for 6E07 from *Heteroderea glycines* (Elling et al., 2007) and Hs-UBI1 from *Heterodera schachtii* (Tytgat et al., 2004). 6E07 lacks similarity to known proteins. In contrast, the Nterminal moity of HS- UBI1 identifies to UBiquitin Extension C-terminal Proteins (UBCEPs) while its Cterminal remains particular to this protein. In eukaryotes UBCEPs are hybrid proteins constituted by an N-terminal monomer of ubiquitin followed by a carboxyl-terminal

extension domain. Both are released separately after translational cleavage. Carboxyl-terminal Extension Proteins (CEPs) are constituents of ribosomes and necessary for their biogenesis in the nucleolus and the ubiquitin counterpart acts as a chaperone to mediate this ribosome- related activity (Finley et al., 1989). In the case of plant pathogenic CNs, such type of proteins define a particular class because they are secreted and because their Cterminal do not relate to CEPs (Gao et al., 2003; Tytgat et al., 2004). Because of this latter observation, Hs-UBI1 could intervene in other cellular processes that are regulated in the nucleolus as the cell cycle control (Boisvert et al., 2007). Another ubiquitin extension protein UBCEP12 from the CN *Globodera rostochiensis* has been shown to be cleaved in plant cells; its C-terminal domain is nuclear-cytoplasmic localized and acts as a suppressor of cell-death related to ETI (Chronis et al., 2013).

Phytoplasma effectors

Phytoplasma are obligate phytopathogens bacteria transmitted by insects. In contrast to other phytopathogen bacteria, they present the particularity of colonizing animals (insects from Hemiptera family) and plants. In plant cells, they develop inside the host cytoplasm. About 56 effector candidates have been identified through genome surveys and gene expression profiling indicates they are differentially expressed on hosts, with specific or common repertoires expressed in plants and/or insects (Sugio and Hogenhout, 2012). SAP54 and SAP11 from the Aster Yellow Witches Broom phytoplasma are upregulated during plant infection and are the best functionally characterized. Both effectors display the same mechanism of action by binding to host TFs and triggering their degradation.

SAP11 is a small protein of 90 aa that localizes in nuclei; interacts with and destabilizes class II TCP transcription factors activities. This results in an altered leaf morphogenesis and reduction of hormone levels (Sugio et al., 2014). TCPs are a family of TFs regulating developmental processes including leaf morphogenesis and JA synthesis through the regulation of biosynthetic genes as LOX2. Interestingly, TCPs pop out as common targets of different microbial effectors: the extensive work of Mukhtar and collegues (2011) identified them as direct targets of RxLR oomycete and bacterial type 3 effectors as well (Mukhtar et al., 2011) and further studies on CRNs (see CRN section) confirm the idea that these TFs are important plant hubs whose corruption determines the outcome is important for host infection.

SAP54 targets several MFTs (MAD-domains transcription factors). Its degradation is

mediated by SAP54's capability to interact with RAD23, a proteasome component that mediates polyubiquitination of plant proteins. Thus, SAP54 induces MTFs degradation via the exploitation of host proteasome. In plants, MTFs are involved in flower determination and development and, consistent with their function, ectopic overexpression of SAP54 leads to leaf-like vegetative flowers which is a common symptom of phytoplasma-infected plants. Such plants were shown to be more attractive to insects, suggesting that SAP54 may contribute to dispersal of phytoplasm by increasing chances of transmission via its insect host (MacLean et al., 2011).

Fungal effectors

Despite the evidence of nuclear localized fungal effectors provided by *in silico* prediction of NLS in putative effectors from different fungal species or by their similarity to nuclear-related functions (i.e: ribonuclease-like effectors of *B. graminis* (Pedersen et al., 2012), very few effectors have been functionally characterized.

The best known nuclear effector is Uf-RTP1 from the obligate biotrophic fungi *Uromyces faba, w*hich belongs to a family of cysteine protease inhibitors widely present in related species (Pretsch et al., 2013). Uf-RTP1 is specifically expressed in haustoria and was the first effector protein shown to be translocated inside host cell nuclei during infection (Kemen et al., 2005). Electron microscopy has revealed that, secreted to the extra-hautorial matrix, Uf- RTP1 localizes in protuberances that emerge inside host cells to finally distribute in cytoplasm and nucleoplasm of the invaded cell. Its accumulation inside host cells correlates with the blocking of nucleus and chloroplast cyclosis (Kemen et al., 2013), thus, Uf-RTP1 triggers a steady state in infected host cells. It has been proposed that this could inhibit several physiological responses among which the cell-death involving particularly chloroplasts. Moreover, recombinant Uf-RTP1 proteins showed inhibition of proteolytic activity in *Pichia pastoris*, an activity that might serve as protection from secreted host proteases during and after its translocation into host cells. How this activity contributes exactly to virulence is still not known but RTP1 proteins protrude so far as fungal effectors stabilizing host cells during infection.

In addition to effectors from pathogenic fungi, two proteins of symbiotic fungi have been identified as nuclear-addressed in hosts. The secreted MiSSP7 from the ectomycorrhizal fungus *Laccaria bicolor* corresponds to a small protein of 68 aa with no homology to known proteins. Its gene is the most highly induced during early infection of roots of the tree

Populus trichocarpa and is crucial for the establishment of symbiosis. Binding phopholipid assays indicated that MiSSP7 interacts with PI3P requiring a RxLR-like motif (RALG) present within the protein sequence. Moreover, this motif was also necessary for cell entry. Thus it seems that host cell entry mechanisms of MiSSP7 falls into the model proposed by Kale and associates (2010). Its biochemical activity is still unknown, but transcriptome analyses showed that its presence in nuclei modulates expression of 225 host genes involved in root architecture (auxin- response genes, CLE genes) and cell wall remodelling (Plett et al., 2011).

SP7 is a protein of 270 aa secreted by the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis.* Consistent with the presence of a NLS sequence, the protein enters plant cells and accumulates in nuclei where it interacts with the TF ER19. ER19 is a member of a TF family known to lead to resistance to pathogen attack that correlates with expression of defense- related genes. In *M .truncatula* gene induction of ER19 and defense-related gene was shown to be highly reduced upon expression of SP7. In addition, an enhancement of symbiosis was observed in *M. truncatula* roots over expressing SP7 and by the silencing of root ER19. Results indicate that SP7 contributes to the establishment of symbiosis by attenuating plant defenses, a common infection strategy of pathogen microbes (Kloppholz et al., 2011).

Oomycete effectors

RxLR effectors

RxLR proteins represent one of the two largest families of intracellular protein effectors of oomycetes together with CRNs. 563 RxLR genes have been identified in *P. infestans* (Haas et al., 2009), 396 in *P. sojae,* 374 in *P. ramorum* and 134 genes in the obligate pathogen *H. parasitica* (Hpa) (Baxter et al., 2010). RxLR are modular proteins, comprising conserved Nterminal domains harbouring a “RxLR” amino acid motif and divergent Cterminal domains. It has been generally accepted that, while Ntermini ensure protein translocation inside host cells (Whisson et al., 2007; Kale et al., 2010), Ctermini carry the virulence activity. As reported previously, this functional dichotomy between both domains seems now blurred as recent data indicate that Ctermini might contribute also to the translocation (Yaeno and Shirasu, 2013).

Transcriptional profiles indicate that RxLRs are mostly expressed during early stages

corresponding to biotrophic phases and decrease during the terminal necrotrophic phases of infection (Pais et al., 2013). Functional high-throughputs of RxLR repertoires based on their *in planta* ectopic transient expression point out for defense-suppressive or susceptibility-inducing activities in plants (Fabro et al., 2011; Wang et al., 2011). The exact molecular mechanisms behind these defense-suppressive functions are beginning to be unraveled for studied RxLRs, although, given their number in species RxLR functions and mode of action remain largely elusive.

A substantial number of RxLRs are targeted to the nucleus. Indeed, expression of 49 *Hpa*RxLR effectors in *N. benthamiana* showed that 66% localized in plant nuclei (33% were exclusively nuclear and 33% displayed both cytolasmic and nuclear localization). Among those exclusively accumulating in nuclei, the majority (68%) were nucleolar localized while others presented particular patterns in the nucleoplasm (i.e: fibre-like structures) (Caillaud et al., 2012). Consistent with their localization, the identification of host interactors of HpaRxLR via Y2H screens revealed an overrepresentation of host proteins related to transcriptional regulation (Caillaud et al., 2012). For most of RxLRs studied so far a defense suppression activity has been reported, reason why they stand as a large family of defense suppressor proteins. A recent report on the nuclear localized HpaRxLR44 showed that, by interacting with the nuclear host MED19 protein (a positive regulator of plant immunity) HpaRxLR44 induces MED19 degradation in a proteasome-dependent manner. MED19 regulates negatively JA/ET signaling and its degradation by HpaRxLR44 promotes induction of genes associated with JA/ET signaling pathway (Caillaud et al., 2013). Therefore, *Hpa* attenuates SA-related defense by triggering the antagonistic JA/ET pathway. HpaRxLR44 is an example of RxLR capability to manipulate plant hormone signaling related to defense.

In *P.infestans*, RxLRs that have been studied display other mechanisms of action. Avr3a is a nucleo-cytoplasmic localized effector necessary for virulence and capable to suppress cell death triggered by PAMPs like INF1 in susceptible plants by interacting with the host protein CMPG1 (Bos et al., 2006, 2010). CMPG1 is a E3 ubiquitin ligase whose degradation via the 26S proteasomal complex positively regulates cell-death activated by the perception of PAMPs (Gilroy et al., 2011). Avr3a suppress cell-death by stabilizing CMPG1 which is no longer degraded and as a consequence accumulates in the nucleolus (Bos et al., 2010) Transcriptional induction of Avr3a during infection correlates with CMPG1's at early stages of infection, indicating that Avr3a suppression of PTI via by CMPG1 is important for the proper establishment of biotrophy.

SNE1 RxLR effector also suppresses immune responses but, in contrast to Avr3a, SNE1 suppresses cell-death activated by Avr/R recognition of different pathosystems as well

as cell-death induced by necrosis inducing effectors such as (Nep1)-like proteins toxin-like of *P.infestans* and *P.sojae* associated with necrotrophy (Kelley et al., 2010). Thus, SNE1 seems to suppress a different cell-death pathway than that of Avr3a, but the mechanistics behind this suppressive activity are not known.

PITG_03192 (Pi03192) which is essential for virulence of *P.infestans* has been demonstrated to bind two host NAC transcription factors (NTP1 and NTP2). Both NACs are ER membrane bound and are released from ER endomembrane to become nuclear localized upon pathogen perception. PITG_03192 (Pi03192) impedes their nuclear addressing by interacting directly with them (McLellan et al., 2013).

As a last example, a recent *in planta* functional screen on various RxLR proteins, evidenced SFI1 as a suppressor of immunity activated by the PAMP flagellin. SFI1 was shown to contribute to *P. infestans* and to localize in plant nucleus with prevalence for the nucleolus (Zheng et al., 2014).

CRN effectors.

In oomycetes, a second family of intracellular effectors are Crinklers and Necrosis, CRNs, proteins which protrude as a vast nuclear localized effector class and are the object of this PhD study.

Discovery and distribution

CRN proteins were first identified through a large screen of cDNAs of *P. infestans* obtained during infection and coding for secreted proteins (Torto et al., 2003). The study aimed to identify pathogen-secreted proteins potentially implicated in the manipulation of host processes and, thus, presenting an effect in plants. For this purpose, cDNAs were expressed in *N. benthamiana* and tomato leaves to assess any perturbation of host physiology. Two cDNAs induced Crinkling and Necrosis of leaf tissues and defense gene expression and were named proteins CRN1 and CRN2, upon the obtained macroscopic phenotype. Sequence comparison between them and to others sequences available at the time, including EST databases of *Phytophthora spp* (only type of oomycete genomic resources available at the time) revealed that these proteins corresponded to an ample gene family (Torto et al., 2003). It also evidenced the lack of similarity to any other protein, and so, the impossibility to assign a putative function. As full genome data of various oomycetes have been and are currently being obtained, it has been possible to undertake

Table 3. CRN gene number and consensus CRN Nterminal motifs of oomycetes and true fungi.

Species	Gene number	First Nterminal motif	Infectious life style	Reference
A. euteiches	160	LQLYLALK	Hemibiotroph	Gaulin et al ., in preparation
P. infestans	196	LxLFLAK	Hemibiotroph	Haas et al., 2009
P. capsici	84	LxLFLAK	Hemibiotroph	Stam et al.,2013
P.sojae	100	LxLFLAK	Hemibiotroph	Tyler et al., 2006
P. ramorum	19	LxLFLAK	Hemibiotroph	Tyler et al.,2006
H. arabidopsidis	20		Obligate biotroph	Baxter et al.,2010
P.ultimum	26	LxLYLAR / K	Necrotroph	Lévesque et al., 2010
A.candida	6	LYLAK	Obligate biotroph	Links et al.,2011
A.laibachii	3	ND	Obligate biotroph	Kemen et al.,2011
B.dendrobatidis	84	divergent *	-	Sun et al., 2011
R. irregularis	42	LFLAK	Mutualistic	Lin *et al*., 2014

* Consensus motif for a subset of CRN-like sequences : FYIQYLxNQPV and/orLVAA

analyses to identify CRN repertoires and distribution among oomycete species.

CRN genes in oomycetes stand as plant pathogen–specific as they have been identified in all phytopathogens sequenced so far and are missing in zoopathogens species *Saprolegnia parasitica and Pythium insidiosum* (Jiang et al., 2013; Krajaejun et al., 2011). Their number varies between species, ranging from 196 in *P .infestans*, 100 in *P. sojae*, 84 in *P. capsici* to 26 in *P. ultimum* and only 3 in *Albugo laibachii* (table 3). In our research group, an EST mining of cDNA libraries evidenced the presence of CRN sequences in *A. euteiche*s (Gaulin et al., 2008) and, currently, its genome annotation has revealed 160 CRN gene models (Gaulin et al., *in preparation*). Since *A. euteiches* is an early divergent species among "crown oomycetes", CRN genes protrude as ancient genes acquired early in the oomycete phytopathogen lineage (Schornack et al., 2010).

Outside the oomycete lineage, CRN-like sequences have been observed in the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) and in the fungal symbiont *Rhizophagus irregularis* (Ri) (Sun et al., 2011; Lin et al., 2014). Bd causes chitridiomycosis in a large array of amphibian species and is responsible for the declines of amphibian population worldwide (Fisher et al., 2012). It can infect over 350 amphibian species and has led to the decline of 200 of them with a mortality reaching 100% for some species (Fisher et al., 2009). It has been estimated that 34.5% of all amphibian species are directly threatened by Bd (Kilpatrick et al., 2010) reason why it has been catalogued as an emerging global ecological threat. The pathogen infects and proliferates in keratinized amphibian skin causing its hyperplasia and hyperkeratosis. Its development on skin tissues (which in amphibians is an important organ regulating water uptake, osmotic balance and respiration) leads to the impairment of neurological functions and heart arrest. Little is known about the genetic base of Bd pathogenesis, but in view of its dangerousness, efforts are being made to understand it. Genome of strains JAM81 and JEL423 have been sequenced and surveyed to propose putative virulence factors (Joneson et al., 2011; Sun et al., 2011). Surprisingly, 84 CRN-like sequences presenting up to 46.5 % of similarity to CRNs of *P. infestans* were identified, exhibiting also a modular architecture, a LxFLAK-derived signal and Ctermini resembling oomycete Cterminal organization. Interestingly, these CRN-like are absent in Bd closest relative, the non-pathogen *Homolaphlyctis polyrhiza.* In Bd they appear under positive selection and are transcriptionally induced when the pathogen is cultured on amphibian skin substrate, observations that sustain the idea of their plausible role in virulence (Sun et al., 2011; Rosenblum et al., 2012). In the case of the arbuscular endomycorrhizal (AM) fungus *R. irregularis*, 42 genes models were predicted with high sequence similarity and canonical amino acid motifs of CRNs (Lin et al., 2014). These latest findings pivot CRN

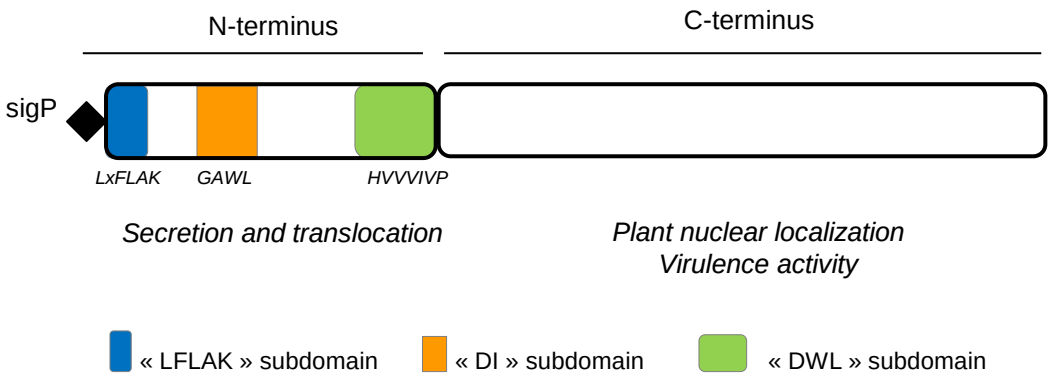

Figure 8 . Sequence and functional architecture of CRN proteins. The typical organization of CRN pre-protein comprises conserved Ntermini were a secretion signal (sigP) is followed the conserved subdomains “LFLAK”, “DI” and “DWL” harboring the conserved amino acid motifs LxFLAK, GAWL and HVVVIVP, respectively. Ntermini mediate secretion and delivery of CRNs to the cytoplasm of host cells. The motif LxFLAK has been shown to be required for this function. Ctermini are responsible for nuclear localization and effects in plant cells. These domains are variable and are composed of the combination of different types of subdomains for which a family categorization has been proposed (figure 8).

conception, which no longer stand as oomycetes-specific proteins nor specific to pathogenic microorganisms.

Protein architecture

At the level of the primary protein sequence, CRN display a modular architecture. A first degree of modularity is reflected by the contrasting degree of conservation between Nterminal and Cterminal moieties. While Nterminal domains are highly conserved among CRN proteins, Cterminal domains are very variable. Both domains are constituted by subdomains characterized by the presence of motifs, defining thus, a second degree of modularity within the protein. In *Phytophthora* CRNs, the conserved Ntermini (< 130aa) comprise the canonical LxFLAK motif (within the first 60aa). Although classical signal peptides (SP) can be predicted in the immature protein sequence, within the first 30 amino acids, around 40% of CRNs do not present a predictable SP for secretion (Haas et al., 2009). The "LxLFLAK" subdomain is followed by a called "DI" domain marked by the presence of conserved GAWL amino acids residues and followed itself by a "DWL" subdomain with a typical HVVVIVP motif denoting the end of the Nterminal moiety (figure 8). Some Ntermini can lack the DI in which case the LxFLAK subdomain is directly followed by the DWL subdomain. The LxFLAK motif (defined for *Phytophtora spp*) can diverge in other species as seen for *P .ultimum* and *A. euteiches* (i,e, LxLALR/K, LQLYLALK respectively) (table 3).

Cterminal domains consist on the juxtaposition of subdomains as well. Their characterization in *P. infestans* by sequence comparison led to propose 36 different conserved subdomains (figure 9) that can assemble in different combinations defining Cterminal subfamilies (Haas et al., 2009). In view of this organization, it has been proposed that the variability of Cterminal domains is the result of recombination events between subdomains. Very recently it was shown that 30 of these subfamilies are present in CRNs of *P.capsici* while 7 new subfamilies (formed by 6 new Cterminal subdomains) appear specific to this species (Stam et al., 2013a). Concerning *A. euteiches,* 160 CRN gene models have been described, among which 12 Cterminal domains are novel subdomains. Hence, *A. euteiches* harbors specific CRN proteins. The presence of CRN specific to certain phythopathogenic oomycete species hints they might manipulate physiological processes/targets specific to hosts and therefore they could define a set of CRNs at the basis of host adaptation. As different *Aphanomyces* species presenting different lifestyles have been sequenced in our research team, other CRN repertoires will be soon available (ANR JCJC APHANO-Effect

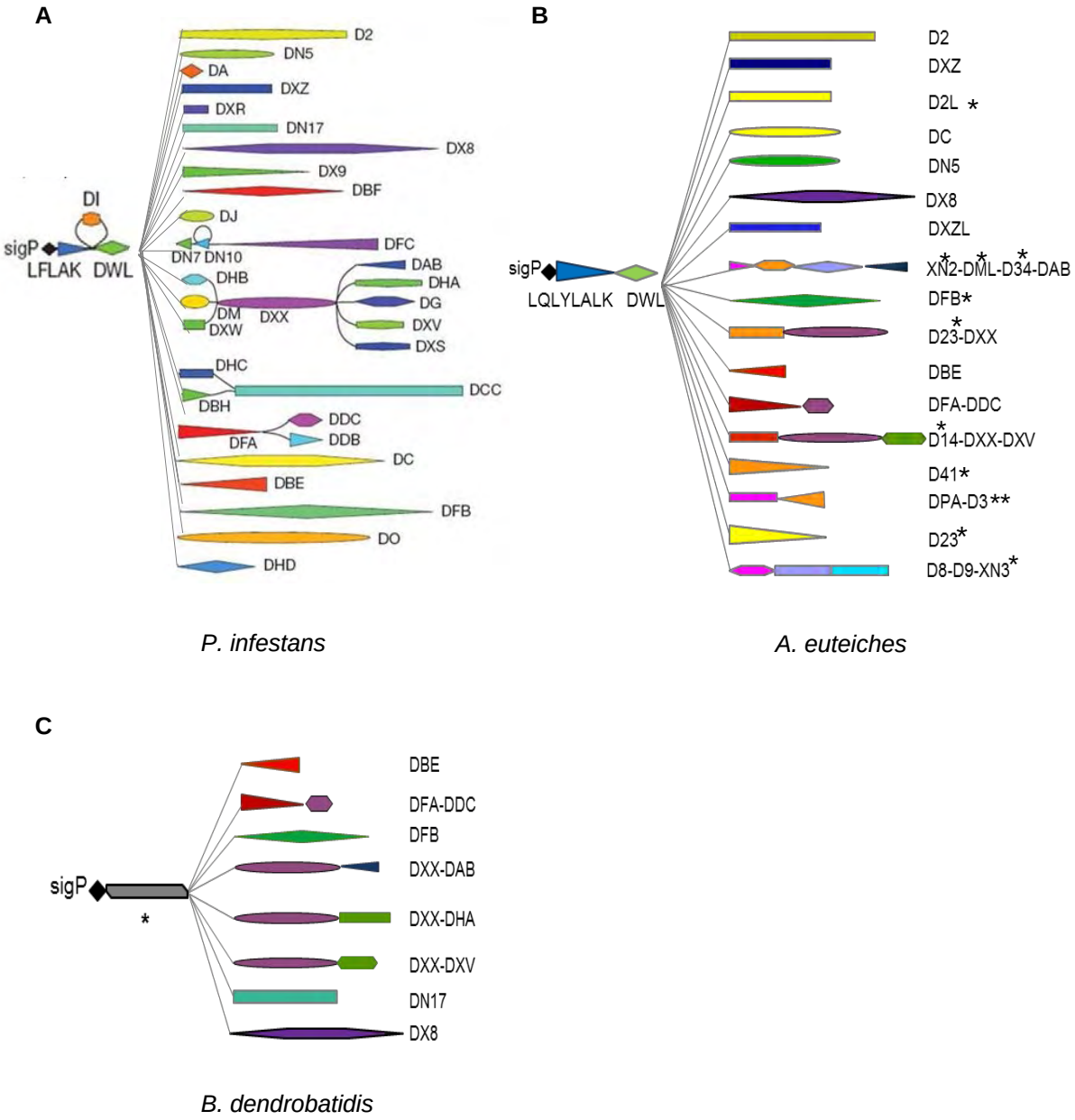

Figure 9. Schemes representing the modular protein architecture of CRNs and Cterminal combinations identified in CRNs of *P. infestans, A. euteiches* and *B. dendrobatidis.*

Scheme representation of CRNs of *P. infestans* (A) and *A. euteiches* (B) *and B. dendrobatidis* (C). Ntermini are highly conserved between both species with light variation of the conserved motifs. **A.** In *P. infestans*, 32 different Cterminal subdomains are present in 36 combinations defining 36 families (Haas *et al*., 2009) **B**. In *A. euteiches*, 27 subdomains have been identified so far, whose combinations define 17 families (Gaulin *et al.*, in preparation). Specific domains to this species are indicated by an asterisk **C**. CRNs of *B. dendrobatidis* present divergent Ntermini compared to oomycete CRNs. Major similarity to oomycete CRNs is displayed in their Ctermini which fall into already identified oomycete CRN families: 12 subdomains ascribing to 8 families (Sun et al., 2010).

2012-2015) and will permit to address CRN occurrence, diversity, evolution-and relevance to lifestyles of Saprolegniale species.

Concerning CRN-like proteins of Bd, their Nterminal domains diverge from the typical Nterminal domains of oomycete CRN. Some present a FLAK motif while others display more divergent (FYIQYLxNQPV or LVAA) motifs (Sun et al., 2011). Actually, major similarities to oomycetes CRNs reside on Ctermini which identify to 8 subfamilies (of the 36 subfamilies of CRN proposed for *P. infestans*) (Figure 9). CRN-like proteins of *R.irregularis* display also the canonical LFLAK motif and identify to 22 Cterminal CRN domains of *P. infestans* (Lin et al., 2014).

Translocation into plant cells

The overall sequence modularity of CRN is a featured shared with RxLR proteins and suggested that amino terminal regions in of CRN proteins could act as translocation signal. This hypothesis was tested via an infection-translocation assay (Schornack et al., 2010), summarized in figure 10.

The system was used to fuse full CRN Ntermini of *P. infestans* (CRN16, CRN2 and CRN8) and *A. euteiches* (AeCRN5) to Avr3a Cterminal domain. As chimera proteins induced depletion of *P. capsici* infection, it was concluded that CRN are cytoplasmic effectors whose Ntermini allow secretion and translocation of CRN proteins into host cells. In addition, mutation of conserved LxFLAK and LQLYLAK showed that, whithin Ntermini, these motifs are signals necessary for translocation function. Importantly, because AeCRN5 and CRN16 lack a predicted signal peptide in the first 30 amino acids, these results demonstrate that a discrete and still unpredictable secretion signal is present in this region and ensure secretion of CRNs in oomycetes (Schornack et al., 2010). Nevertheless, the mechanistics behind the translocation process are not known.

CRN subcellular localization

The use of fluorescent proteins to tag CRNs, followed by microscopy approaches have enabled to study their subcellular localization when ectopically overexpressed in plants, mostly in *N. benthamiana* leaves. Their heterologous expression in this system has shown that all CRN accumulate in nuclei of plant cells (Schornack et al., Stam et al., 2013b). While first localization studies of *P.infestans* CRNs revealed a homogenous nucleoplasm distribution of CRNs that excluded the nucleolus, very recently, CRNs of *P. capsici* were

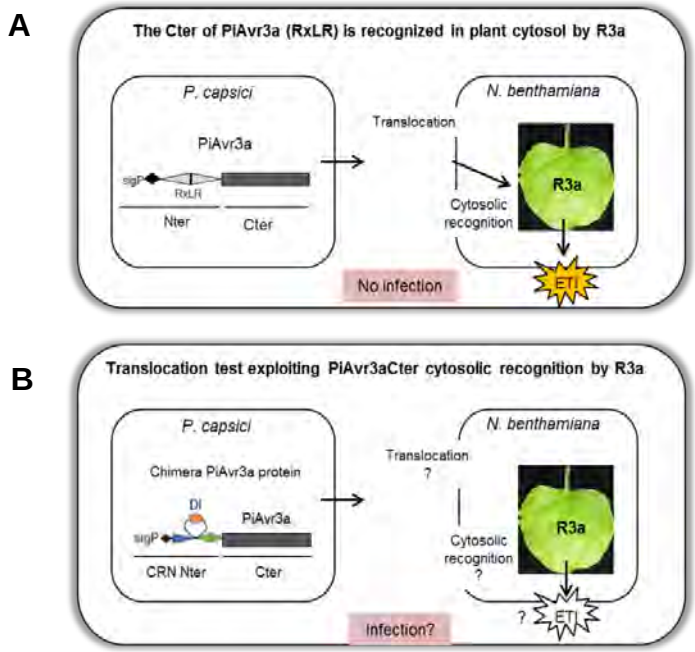

Figure 10. Principle of the Nterminal translocation assay.
The test is based on the recognition of the Cterminal domain of the RxLR Avr3a by the resistance protein R3a which takes place in the cytosol of plant cells and leads to ETI and full depletion of infection. **A.** *P. capsici e*xpressing PiAvr3a is inoculated on *N. benthamiana* leaves expressing R3a. Absence of infection indicates that PiAvr3a has been recognized by R3a in the cytosol and so that PiAvr3a has entered plant cells. **B**. A Nterminal CRN domain is fused to the Cterminus of Avr3a and expressed in *P. capsici.* After inoculation, the absence or presence of infection determines whether the chimera protein has been translocated into plant cytosol. For Nterminal CRN domains tested, *P.capsici* did not develop on leaves, indicating that there was ETI activation and so that the chimera protein was translocated inside plant cells. Mutation of LFLAK and LQLYLALK residues resulted in infection concluding that this motif is necessary for the translocation function of CRN Ntermini (Schornack et al., 2010). sigP: signal peptide

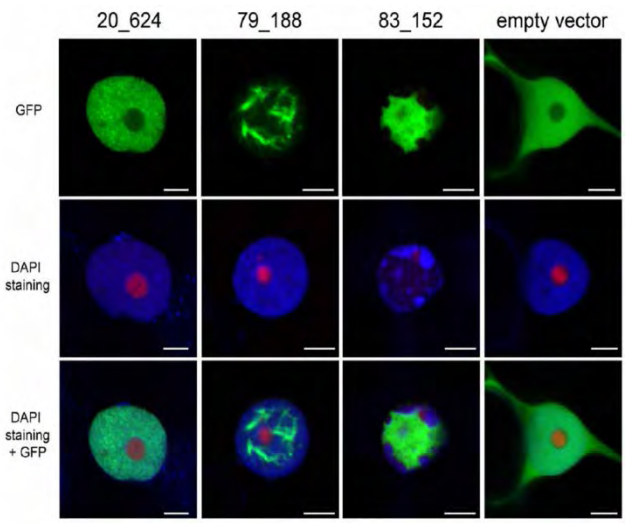

Figure 11. Diverse nuclear localization patterns of three Cterminal domains of CRN of *P. capsici* in epidermal cells of *N. benthamiana.*
Confocal imaging performed on epidermal cells of *N. benthamiana* expressing Cterminal domains of CRN 20_624 (DN17), 79_188 (D2) and 83_152 (DXZ) fused to GFP. GFP alone (empty vector) and CRN localizations are shown in the upper panels and are correlated to DNA by dapi staining (blue) and to the nucleolar marker fibrillin protein (red), in middle and lower pannel.
Adapted from Stam *et al*., 2013a/b

seen also in the nucleolus as well as in yet undetermined regions of nuclei. These localizations are denoted by a constricted fluorescence accumulation in areas of the nucleus depicting particular pattern of fluorescence (i,e filament-like, patchy...) that have, in addition, been suggested to be accompanied of a rearrangement of DNA material (Stam et al., 2013a),(figure 11).

Expression of the full matured protein (lacking the signal peptide) or only the Cterminal domain of CRN of *P. capsici* (CRN20_624, categorized in the DN17 family) results in the accumulation of both protein versions in nuclei, indicating that the nuclear localization is ensured by Ctermini and that Ntermini only mediate translocation inside host cells (Schornack et al., 2010; Stam et al., 2013b). For some CRN proteins, their nuclear localization correlates with the presence of predictable NLS in the primary sequence of carboxyl termini. For this CRN-containing NLS, silencing of α-importin in *N. benthamiana* caused a decrease of CRN accumulation in nuclei (Schornack et al., 2010) proving that, by the presence of NLS, CRNs might exploit host routes of nuclear import.

CRN activities

P. infestans CRN are among the highly expressed genes during plant infection with 50% of CRNs corresponding to 10% of the most highly expressed genes (Haas et al., 2009). CRNs of *P. capsici* show differential expressions in tomato leaves and have been assigned into two classes. First class consists of very early up-regulated genes that are down regulated during biotrophic development and finally increasingly expressed during last stages of infection. A second class comprises CRNs whose expression is less variable showing a progressive increase as infection takes place (Stam et al., 2013a). The expression pattern of the first class suggests that CRNs may be required in very different, almost opposite, infection stages (biotrophic and necrotrophic stages) of *P. capscici* infection. Either CRN function is required on both phases or the same CRN could present more than one activity during infection. As mentioned above, most CRN proteins outstand by their scarcity of similarity to known proteins making difficult to assign putative functions.

Upon overexpression on leaves, some CRNs induce necrosis of epidermal cells while others do not. It is important to emphasize that the necrotic effect is not attributable to a specific subfamily of proteins as *in planta* expression of two CRNs of the same category can conduct or not to necrotic lesions. *P. sojae* PsCRN115 and PsCRN63 differ only by 4 amino acids on their carboxyl terminal domains. While PsCRN63 induces cell-death symptoms in *N. benthamiana,* PsCRN115 does not. In fact, PsCRN115 is able to suppress the

necrosis induced by PsCRN6 itself and by the cell-death inducer PsojNIP (Nep1-like protein). Therefore, these opposite activities are very fine tuned at the protein level. A protein deletion approach allowed to determine that the minimum peptide carrying such activities is contained in Ctermini and that the lack of the Nterminal domains does not abolish them (Liu et al., 2011). An immunity suppressive activity has also been reported for PsCRN70 (Rajput et al., 2014). Because it is able to suppress cell-death elicited by different cell-death pathogen-derived molecules as well as different defense responses as the induction of ROS, expression of PR genes and hormone signaling, PsCRN70 stands as a broad defense suppressor effector.

The only biochemical activity predicted is a phostransferase/kinase activity, based on the presence of serine/threonine kinases (RD kinases) sites for CRNs containing DBF, SN8 and D2 Cterminal domains. CRN8 (D2), from *P. infestans*, displays an autophosphorylation activity *in vitro* and is phophorylated *in planta*, which has been shown as necessary to its cell-death activity, thus, relevant for its function (van Damme et al., 2012).

Finally, the transcription factor TCP14 of tomato has been proposed to be targeted by the *P. capsici* CRN12_997 containing Cterminal domains DHB-DXX-DHA (*Stam Remco, personal communication*). CRN12_997 promotes the degradation of TCP14 preventing its binding to DNA. TCP14 is a member of the TCP family of plant TFs found to be constituents of a plant hub network targeted by bacterial and oomycete effectors (Mukhtar et al., 2011). Because TCP14 seems to be a positive regulator of defenses, CRN12_997 is proposed to contribute to virulence as a suppressor of host gene defense output.

Scope of the thesis

Until today, functional studies have been addressed only on CRNs of *Phytophthora spp* for which elucidation of their function is beginning to be provided. Before the initiation of my PhD work, cDNA libraries of *A. euteiches*, generated in our group, provided first insights into the genetic basis of *A. euteiches* pathogenicity (Gaulin et al., 2008). Mining of ESTs corresponding to 8,000 unigenes from infected and uninfected conditions outlined the absence of RxLR proteins and the presence of CRNs, the first CRN proteins in an oomycete distinct from *Phytophthora spp.* The analysis of AeCRNs ESTs evidenced two major families of AeCRNs with similarities to *P. infestans* CRN13 and CRN5 known at the time (therefore, named AeCRN13 and AeCRN5) and physically represented by full length cDNA clones Ae_9AL5664 and Ae_1AL4462, respectively (Gaulin et al., 2008).

Before the beginning of my work, in September 2010, CRNs were supposed to be intracellular effectors. This was confirmed as the Nterminal domain of AeCRN5 and other *Phythophthora* CRNs were demonstrated to ensure secretion and translocation of these proteins inside plant cells (Schornack et al., 2010). In this context, my work aimed to functionally characterize AeCRN13 and AeCRN5 of the root pathogen *A. euteiches*. During my work, CRN-like proteins were evidenced in the fungal pathogen *B. dendrobatidis*, inferring possible functional commonalities to oomycetal CRNs. This encouraged us to undertake a comparative functional study of AeCRN13 and its putative ortholog BdCRN13, extending CRN functional characterization beyong oomycetes.

The functional study was mainly based on a heterologous ectopic expression of CRNs in order to phenotype the effects and localization of Ctermini expressed directly in host cells. We show that AeCRN5 and AeCRN13 and its ortholog from Bd are host nuclear-localized and cell-death inducing effectors. We demonstrate that AeCRNs target host nucleic acids to perturb host physiology, describing a new mode of action for eukaryotic effectors.

The first chapter of the manuscript presents the characterization of Cterminal activities of AeCRN13 and its ortholog BdCRN13, while the second chapter concerns AeCRN5 characterization.

In the last part of the manuscript, I discuss results that were obtained and contextualize them among latest related findings.

Chapter 1: Functional characterization of AeCRN13 of *A. euteiches* and its ortholog BdCRN13 of *B. dendrobatidis*.

Chapter 1: Functional characterization of AeCRN13 of *A. euteiches* and its ortholog BdCRN13 of *B. dendrobatidis*.

Since the initial identification of CRN proteins by Torto and associates (2003), CRN cataloguing has greatly been favoured by the arrival of several complete genome sequences of Peronosporales (*P. infestans*, *P. ramorum*, *P. sojae*...) and Saprolegniales (*S. parasitica*, Jiang et al., 2013 and *A. euteiches*, Gaulin et al., unpublished). In oomycetes, CRNs protrude as phytopathogen specific outstanding as a large and diversified protein family with more than 150 genes in one species and a total of 43 different subfamilies. All oomycete CRNs studied so far target host nuclei, but their biochemical activities remain poorly characterized. Today, CRN-like proteins have been described in the unrelated mycorrizal fungus *Rhizophagus irregularis* (Lin et al., 2014) and the chytrid pathogen fungus *B. dendrobatidis* (Bd) for which experimental evidence suggests they might contribute to its interaction with amphibians (Sun et al., 2011). Still, no functional studies have been addressed for these CRN-like proteins. Intriguingly, these repertoires harbor Cterminal families also present in oomycetes, suggesting common activities in their respective hosts. Given the different nature of interactions and hosts (symbiotic *vs* pathogenic and animal *vs* plant) it is urgent to understand their mode of action and, by this, their contribution to each type of interaction. The work presented in this chapter ascribes to this problematic by addressing the functional study of AeCRN13 and BdCRN13, two CRN homologs of *A. euteiches* and *B. dendrobatidis*, respectively.

AeCRN13 (Ae_9AL5664) sequence represents one of the two CRN cDNA families identified through a transcriptomic approach on *A. euteiches* during infection of roots (*M. truncatula*) performed in the group (Gaulin et al 2008). Inspection of its protein sequence by comparison to other *Phytophthora* CRNs showed the presence of a canonical Nterminal domain with a LQLYLALK motif and a HLVVVP motif marking its end. Notably, no signal peptide in this region could be predicted, an observation that is consistent with observations made for other CRNs for which secretion and translocation has been shown (Schornack et al., 2010). The Nterminal of AeCRN13 is similar to the tested translocation signal of AeCRN5, suggesting its functionality. We assumed, then, that AeCRN13 may be translocated during the interaction and is a intracellular/cytoplasmic effector. In this context, we focused our work on the Cterminal region of AeCRN13. Since the *in silico* identification of AeCRN13, a protein family classification was proposed for the variable Cterminal domains of CRN of *P. infestans* by Haas and associates (2009). As a first part

of the functional characterization of AeCRN13 Cterminal domain, we compared it to other Ctermini of CRNs and found that it comprises a DFA domain followed by a DDC domain. Thus, AeCRN13 falls into the DFA-DCC CRN family.

AeCRN13 gene expression and protein accumulation during infection of *M. truncatula* roots (ecotype F83005.5) was studied by qRT-PCR and by the use of anti-AeCRN13 antibodies raised in rabbits against a recombinant Cterminal AeCRN13 protein produced in *E. coli*. We found that AeCRN13 gene is expressed during all times of infection tested (between 3, 6 and 9 days after root inoculation, dpi) (figure 1). Western blot analyses confirmed the presence and accumulation of AeCRN13 protein in infected roots (figure 1 C). Altogether, expression data revealed that AeCRN13 is expressed specifically during infection, that the protein is processed and possibly submitted to post-translational modifications. It remains to be elucidated whether these modifications are intrinsic to AeCRN13 regulation (controlled by the pathogen) or an artefact resulting from the action of plant proteases encountering AeCRN13. Nevertheless, because no supplementary products of degradation, we would tend to privilege the possibility of a processing linked to its function.

We tested the contribution of AeCRN13 to virulence. Because genetic transformation of *A. euteiches* is not possible, we made use of a foliar infection assay combined with agroinfiltration of AeCRN13 in *N. benthamiana*, an assay commonly used for the study of *Phytophthora* CRNs (van Damme et al., 2012; Stam et al., 2013a). For this, 24 hours after agroinfiltration of AeCRN13 in leaves, *P. capsici* zoospores were deposited on agroinfiltration zones and infection lesions were measured 3 an 4 days after inoculation.. These lesions were compared to infection lesions on zones agroinfiltrated with GFP. Results revealed greater size lesions for AeCRN13 zones, indicative of the greater colonization of tissues by *P. capsisci*, and led to conclude that AeCRN13 contributes positively to virulence.

CRN-like proteins were identified in *B. dendrobatidis*. We searched for AeCRN13 ortholog (strain JEL423) and found a closest AeCRN13 homolog gene, named BdCRN13 (identified as BDGEG_03200.1) presenting 67 % similarity to AeCRN13. Further sequence inspection of both AeCRN13 and BdCRN13 showed a lack of predictable subcellular localization motifs (including NLS) as well as any similarity to functionally known proteins. Nevertheless, Pfam analysis on one particular region shared between both proteins at the end of the DFA domain, indicated the presence of a HNH-like motif, which is well represented in bacterial endonucleases and DNA binding/cutting factors. Despite the low significance detection via Pfam, we transferred its prediction within the DFA subdomain and hypothesized a possible DNA binding and cutting activities for AeCRN13 and BdCRN13. These putative shared activities encouraged us to undertake a comparative

functional study on both proteins.

The functional characterization of AeCRN13 and BdCRN13 was conducted via the use of heterologous expression systems either on plants (*N. benthamiana* and *M. truncatula*) and/or amphibian (*Xenopus laevis*). To express both proteins in amphibian cells, we used an embryo gene expression system, benefiting of the expertise of the team of Dr. Moreau (Centre de Biologie du Developpment, Toulouse). Both Ctermini were expressed fused to the GFP protein at their Nterminal moity. In plants, both proteins localized in nuclei and inhibited root development of *M. truncatula* and induced necrotic lesions in leaves of *N. benthamiana* (figure 2 and figure 3). In amphibian cells, AeCRN13 and BdCRN13 conducted to bigger cells possively caused by the disruption of cell cycle leading to the absence of cell division and to an abnormal development of embryos (figure 4). In plant and amphibian cells, while GFP:AeCRN13Cter presented an accumulation exclusively in the nucleus, GFP:BdCRN13Cter was nucleo-cytoplasmic localized. Mislocalization of GFP:AeCRN13Cter and GFP:BdCRN13Cter from nuclei was accompanied of the absence of cell-death, demonstrating that the nuclear accumulation of both proteins is necessary to the establishment of necrosis (figure 3). To further dissect the activities of CRN13 Cterminal subdomains DFA and DDC, we generated deleted version of AeCRN13 in *N. benthamiana* and tested their activities and localization (figure 5). Results indicated that the integrity of the protein is required for the activities characterized (cell-death and nuclear accumulation) and infer a synergistic implication of all domains.

The necessity of a nuclear presence to exert a necrotic activity, together with the presence a predicted HNH-like motif, led us to test whether AeCRN13 and BdCRN13 interact with nucleic acids. Because we observed that AeCRN13 and BdCRN13 recombinant proteins were able to bind *in vitro* dsDNA (figure 6), we developed a robust *in vivo* assay to test DNA binding activity based on FRET-FLIM technique in *N. benthamiana* epidermal cells. By this means, we demonstrated that AeCRN13 and BdCRN13 Ctermini bind DNA *in vivo* (table 1). Since the recombinant ΔHNH protein was unable to bind dsDNA *in vitro,* we revealed that this motif is functional in mediating DNA binding (figure 6).

As HNH bacterial endonucleases are known to bind and cleave DNA, we hypothesized that AeCRN13 and BdCRN13 might harbor a DNA cutting activity. We indirectly confirmed a DNA cutting activity for AeCRN13Cter and BdCRN13Cter by showing the accumulation of $_{\gamma}$H2AX (a marker of DNA damage) on epidermal cells of *N. benthamiana* by western blotting (figure 7). Moreover, we also observed the accumulation of $_{\gamma}$H2AX in roots of

M.truncatula infected by *A. euteiches,* which also correlates with the expression of plant DNA damage markers.

These results correspond to the first functional analysis of a CRN protein of *B. dendrobatidis* and of the saprolegniale *A. euteiches.* AeCRN13 and BdCRN13 proteins stand as the first eukaryotic effectors directly targeting host DNA. We showed that both CRN13s (DFA-DDC family) bind DNA and induce its damage, leading to host cell disruption (plant and animal cells). The work shows that sequence similarities support similar activities of homologs CRN proteins, sustaining the idea of shared functions of CRNs in distantly related organisms, and pointing the idea of the targeting of universal cellular functions as likely present in plant and animal cells.

This manuscript was submitted to Plos Pathogens the 10th march 2014. Reviewer's comments were received the 8th may 2014. These concerned (1) the secretion of AeCRN13 during infection: the Nterminal domain of AeCRN13 does not contain a predictable signal peptide for secretion and its function for host-delivery was not tested via the Nterminal translocation domain. Thus, reviewers argued that this most be proven in order for the protein to be considered as an effector and as playing a role during infection (2) the nuclease activity: we hypothesized a nuclease activity that we indirectly showed as happening *in planta* via the evidence of the phosphorylation of H2AX. Reviewers ask to provide the demonstration of a direct cutting activity on DNA. To provide answers to these aspects, at the end of the article manuscript, I present preliminary results concerning the immunolocalization of AeCRN13 in infected roots and results of the *in vitro* nuclease activity on DNA.

CRN13 effectors from plant and animal eukaryotic pathogens are DNA-binding proteins inducing host DNA damage

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CRN13 effectors from plant and animal eukaryotic pathogens are DNA-binding proteins inducing host DNA damage

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Short Title: Genotoxic CRN13 effectors

Abstract

Filamentous plant pathogens deliver effectors proteins inside host cells to facilitate host colonization, but the molecular mechanisms by which eukaryotic effectors promote such effect remain largely unknown. One class of secreted oomycetes effectors comprises the modular CRN (Crinkling and Necrosis) large family of proteins that contain a conserved N-terminal domain specifying translocation into host cells and diverse C-terminal regions harboring effector functions. CRNs were thought to be oomycete-specific until recently identified in the genome of the fungus *Batrachochytrium dendrobaditis* (*Bd*), one of the major contributors to the global amphibian decline. The presence of CRNs genes in phylogenetically distant and unrelated eukaryotic pathogens suggested that eukaryotic effectors might display a conserved mode of action during host infection. In the present study, we functionally characterized the CRN13 from the oomycete pathogen of legume roots *Aphanomyces euteiches* (Ae) and its ortholog from the chytrid pathogen *Bd*. By using model systems in a cross-kingdom approach we detected that both proteins localize in nuclei of plant and amphibian cells triggering cell-death and aberrant cell development. We identify by serial deletion analysis, a conserved HNH-like motif initially found in bacterial endonucleases as essential for this activity. By combining DNA-binding *in vitro* assays with Förster Resonance Energy Transfer (FRET) experiments in tobacco cells we show that both CRN13s interact with DNA. Overexpression of CRN13s induces a phosphorylation of histone H2AX (γH2AX), a marker of DNA double-strand breaks. Accordingly, we show that infection of *M. truncatula* roots by *A. euteiches* induced host DNA damage responses. Overall our results demonstrate for the first time that microbial eukaryotic effectors from phylogenetically distant pathogens, alter host cell physiology through by targeting and cliving DNA.

Author Summary

During infection of their host, microbial pathogens deliver effectors proteins that act on plant or animal cells to promote infection. Some of these effectors are translocated inside host cells where they interact with molecular targets to modify host cell physiology to the benefit of the parasite. Genomic surveys point out the existence of a large repertoire of hundreds of effector genes in oomycetes, which are fungal-like microorganisms comprising devastating plant pathogens. Here we focus on the CRN effector family which was firstly reported in phytopathogenic oomycetes and recently identified in the pathogenic fungus of amphibians *Batrachochytrium dendrobatidis.* We show that a CRN effector from both *Aphanomyces euteiches*, an oomycete infecting plants and from Bd act inside the nucleus of either plant or animal cells where they trigger abnormal cell development. We show that these CRN are able to bind host DNA inducing DNA damage responses. This work shows that oomycete and fungal pathogens infecting plant or animals are able to produce similar translocated effectors which induce DNA damage responses in the host cells.

Introduction

In the pursuit of fungi and fungi-like oomycetes eradication, novels tools are in constant demand due to the emergence of new diseases and resistant-parasites or to the lack of effective molecules [1]. Deciphering the dialog between the host and the parasite at the molecular level is a valid approach to provide reliable knowledge. It is well established that upon microbe perception, thanks to the detection of specific molecular patterns exhibited by the microorganism, plants like animals induce innate immune responses to provide a first type of barrier against the intruder [2]. Adapted filamentous eukaryotic pathogens therefore secrete effectors molecules that modulate host physiology to benefit the microorganism [3]. Upon the last few years ongoing sequencing of fungal and oomycetes genomes enable bioinformatic prediction of whole effector repertoires of pathogens in ever-increasing numbers [4-8] Intriguingly recent cellular studies show that a significant number of effector proteins from prokaryote and eukaryote microorganisms are targeted to the nucleus of host cells [9]. Whereas is has been suggested that effectors may affect nuclear functions through for example a direct activation of host transcription as reported for TAL-bacterial effector [10], the mechanism by which intracellular effectors from filamentous eukaryotic microorganisms interfere with host remains a largely open question.

In the field of phytopathology, eukaryotic intracellular effectors were firstly reported in oomycetes. Oomycetes are fungal-like microorganisms, members of the Stramenopiles and closely related to aquatic organisms such as diatoms and brown algae [11,12]. These filamentous microorganisms are responsible for multi-billion dollar damages in agriculture (e,g late blight of potato, *Phytophthora infestans*), in forestry (e,g sudden oak death, *Phytophthora ramorum*) and aquaculture (e,g crayfish plague, *Aphanomyces astaci*) [13,14]. Two important groups of candidate host-translocated effectors proteins, RxLR and CRN (for crinkling and necrosis) have been revealed by analysis of oomycetes genome sequences. Both

RxLR and CRN effectors proteins present a modular architecture and include conserved N-termini functioning in host delivery and highly diverse C-terminal domains directing the effector activity. While some *P. sojae* RxLRs are able to suppress cell-death triggered by necrosis inducing factors [15], the precise mechanism behind this is still unknown. Similarly, most of the RxLRs from *Hyaloperonospora arabidopsidis* enhance the growth of *Pseudomonas* by suppressing *Arabidopsis* innate immune responses [16]. Subcellular localization of 49 intracellular effectors candidates from *H. arabidopsidis* revealed that one-third is localized strictly to the host nuclei [17]. Among them, the HaRxL44 interact with MED19a, a subunit of the *Arabidopsis* Mediator complex [18]. Although the mechanism by which HaRxLR44 induces the degradation of MED19 to promote host susceptibility is still unclear, these data strongly support the concept that nuclear host components manipulation may be an effector mode of action.

The RxLRs family of effector is predicted for phytopathogenic species including *Phytophthora* (> 350 genes) [4,19] and *Hyaloperonospora* species (>130 genes) [20], whereas it is absent in *Pythium* [21] and *Aphanomyces* [22]. As one main function of the RxLR effector is to suppress host defense responses [23,24], the lack of RxLR effectors in these latter species indicates that modulation of host physiology may rely on other effector types. The CRN family is ubiquitous in plant pathogenic oomycetes including *Aphanomyces* species and their number ranges from 45 genes for *Pythium sp.* [21] to 200 genes for *P. infestans* [4]. All CRNs display a conserved LFLAK N-terminal motif, altered as LYLAK in *Albugo sp.* [25], LxLYLAR/K in *Pythium sp*. [21] and LYLALK in *A. euteiches* [22]. The *Phytophthora* and *Aphanomyces* N-terminal motif has been shown to act as a delivery signal into the cytoplasm of plant cells [26]. Initially reported from *P. infestans* genome mining [4], the CRN C-terminal region is highly diverse and fall into 36 families comprising numerous paralogues. Few new CRNs families have been reported upon complete genome analysis of distinct oomycete species (ie, *Phytophthora capsici, Pythium sp., Aphanomyces euteiches*) [4,27,28]

suggesting that CRNs belong to an ancient effector family that arose early in oomycete evolution [26]. To shed light on the function of CRNs, transient expression assays on plant leaves using C-termini revealed that not all CRN cause cell death suggesting that the perturbation of host processes may rely on different activities [4,26,27,29-31]. Indeed, PsCRN63 and PsCRN115 from *P. sojae* sharing 95% identity at the amino acid level, display contrasted activities since PsCRN63 triggers cell death *in planta* while PsCRN115 is able to suppress this phenotype [30]. Although the subcellular localization of CRNs from *P. sojae* was not investigated, it was proposed that both PsCRNs share the same plant targets required for cell-death response and that their distinct activities depend on their subcellular localization [32]. Subcellular localization studies of unrelated CRN C-termini from two divergent species (CRN15, CRN8, CRN2 from *P. infestans* and CRN5 from *A. euteiches*) revealed the plant nuclear accumulation of CRNs [31,33]. Similarly, CRN localization studies revealed that *P. capsici* CRN C-termini target the plant nucleus and accumulate in specific sub-nuclear compartments [27,29]. Little is known regarding the precise function of CRN, except that the cell-death inducing CRN8 from *P. infestans* displays a functional S/T RD kinase domain at its C-terminus [34] .

Up to recently, CRN proteins were reported exclusively in phytopathogenic oomycetes. By looking for putative effectors in the arbuscular endomycorrhizal fungus *Rhizophagus irregularis* genome, 42 LFLAK-containing proteins with *P. infestans* CRNs C-termini similarity have been recently predicted [35], Thus, it is tempting to speculate that such effectors play important roles in the AM symbiosis. The recent completion of the genome sequence of the pathogenic chytrid *Batrachochytrium dendrobatidis (Bd)* led also to the identification of a set of 84 CRN genes showing similarity with *P. infestans* CRNs [7,36]. *Bd* is responsible of the recent emergence of an infectious disease on amphibians that is causing the declines of hundreds of species worldwide, threatening the amphibian biodiversity [1,37,38]. *Bd* CRNs presented an increased expression when the fungus is grown on host frog

196 tissues [36,39] and are absent in non-pathogenic chytrids suggesting a role in pathogenicity
197 [36]. Nevertheless, the role of CRNs in *Bd* pathogenicity is still unknown, but their presence
198 in the *Bd* genome is certainly intriguing raising the question of their origin and their role in
199 the emergence of chytridiomycosis.

200 In this study we report on the functional characterization of a CRN family detected in
201 oomycetes and in the fungus *Bd*. We focused our work on the CRN13s, from the root legume
202 pathogen *Aphanomyces euteiches* and its ortholog in *Bd*. We demonstrate that both effectors
203 target nuclei of plant and amphibian cells where they exert cytotoxic effect on plant and
204 animal tissue development. We show that both CRN13s target plant nuclear DNA thanks to
205 the presence of an HNH-like domain found in bacterial endonucleases. We combine *in vitro*
206 assays using recombinant proteins with an *in vivo* FRET-FLIM approach on tobacco cells
207 transiently expressing AeCRN13 or BdCNR13 to demonstrate that both CRN13s are able to
208 bind DNA. We finally show that both CRN13 induce plant DNA-damage responses that are
209 also observed in roots of legumes during infection by *A. euteiches.*
210

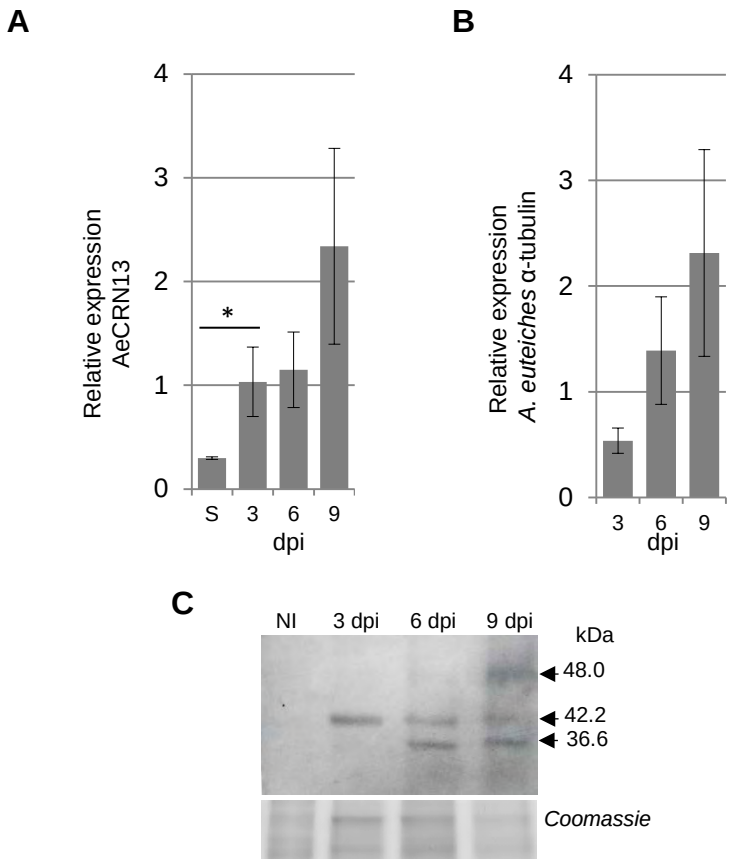

Figure 1. AeCRN13 is expressed during infection of *M. truncatula* roots. (A-B) Graphs show expression level of AeCRN13 (A) and *A. euteiches* α-tubulin (B) measured by qRT-PCR at 3, 6 and 9 days post-inoculation (dpi) in *M. truncatula* roots. S: mycelium grown as saprobe. Error bars are standard deviation errors. Asterisk indicate that the values are significantly different (p-value<0.05, t-test). (C) Immunoblot using anti-CRN13 antibodies showing the proteolytic cleavage of AeCRN13 (42.2 kDa, 36.6 kDa) from 3 to 9 dpi and the presence of non-processed form (48 kDa)of AeCRN13 at 9 dpi. NI: non-infected.

Results

AeCRN13 is expressed and proteolytically processed during infection of *Medicago truncatula* roots

To investigate the expression of CRN13 from *Aphanomyces euteiches* (AeCRN13) during host infection, we performed qRT-PCR analyses on total RNA extracted from growing saprobe hyphae and infected-*Medicago truncatula* roots. AeCRN13 gene expression was detected during saprophytic development and its expression was induced during infection. Its expression level in roots was similar at early stages of the infection (3 to 6 dpi), whereas an induction was detected from 6 to 9 days post infection (dpi) (Fig. 1A). Induction of AeCRN13at 9 dpi corresponds to an infection stage where the entire root cortex is colonized by an actively growing mycelium [40,41] as observed by the expression of α-tubulin (Fig. 1B) at 9 dpi.

To detect protein accumulation during root infection, specific AeCRN13 antibodies were raised against the C-terminal domain of AeCRN13 (AeCRN13Cter) tagged with MBP (maltose-binding protein) and expressed in *Escherichia coli.* The serum was purified by negative adsorption using an unrelated MBP-tagged protein to remove anti-MBP antibodies. At 3 dpi a 42 kDa band was detected while the predicted size of AeCRN13 is 50.1 kDa (Fig. 1C), suggesting a putative cleavage of a 8kDa fragment. At later stages (6 and 9 dpi) a supplementary band of 36.6 kDa corresponding to the predicted size of the C-terminal domain of AeCRN13 was observed, while a 48 kDa form corresponding to the predicted size of AeCRN13 was detected at 9 dpi. These experiments revealed the existence of a putative two-stage proteolytic processing of AeCRN13 during *M. truncatula* infection.

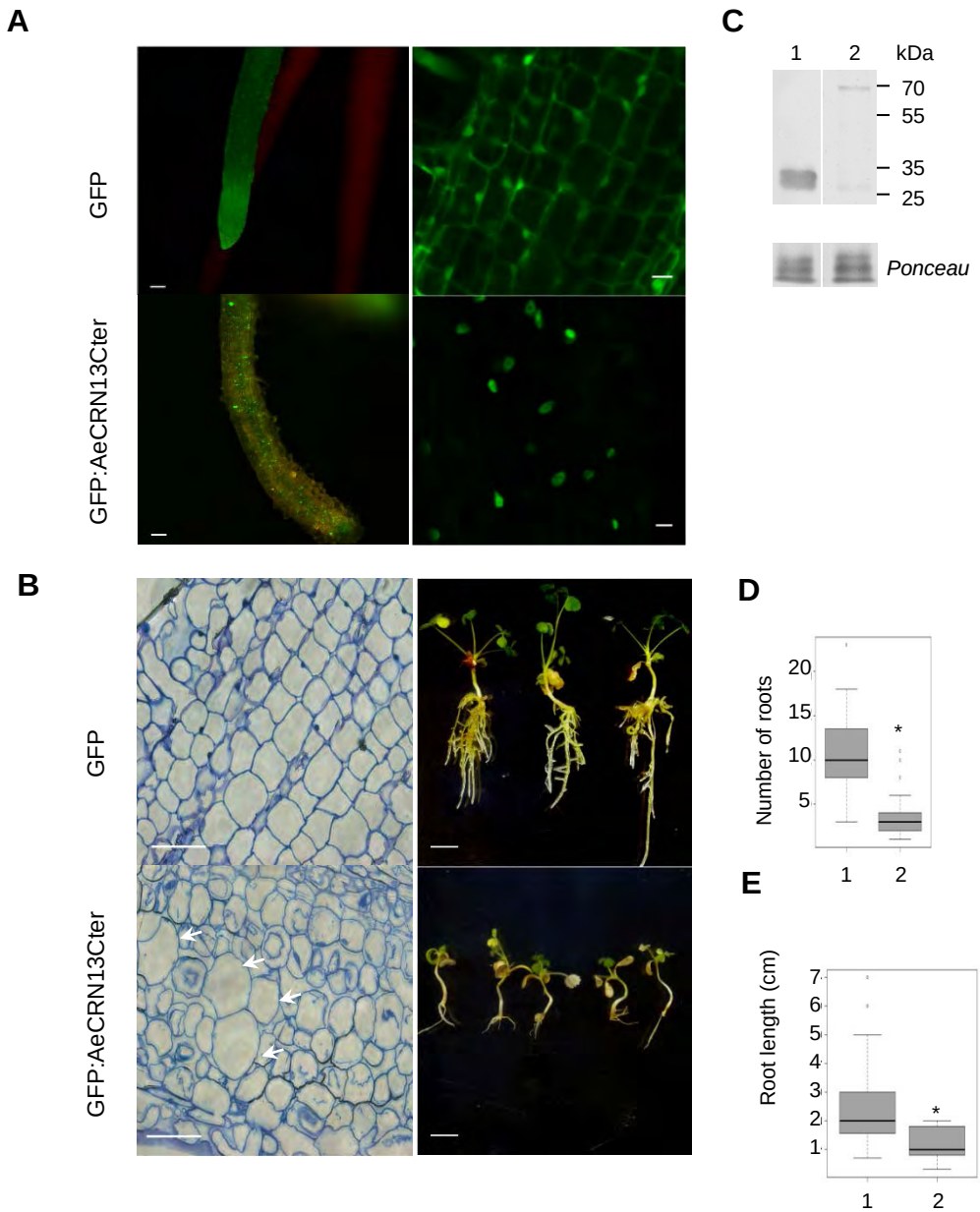

Figure 2. AeCRN13 accumulates in host cell nuclei where it triggers cell size growth and inhibits root development.

(A) *M. truncatula* plantlets were transformed with *A. rhizogenes* to express in roots GFP and GFP:AeCRN13Cter constructs under the control of the cauliflower mosaic virus 35S promoter. At 13 days after *A. rhizogenes* transformation, epifluorescence large-field imaging of transformed roots (Left panel, scale bar: 100µm) and confocal images (Right panel, scale bar: 15 µm) reveal the preferential localization of AeCRN13Cter in host nuclei. (B). Micrographs of *M. truncatula* longitudinal cuts of 13 day-old transformed roots indicate that AeCRN13Cter causes a disorganization of root cells and the formation over-sized cells pointed by white arrows (Left panels, Scale bars: 20µm) as compared to GFP expressing roots. Entire 30 day-old composite plants expressing AeCRN13Cter presented a reduction in root and aerial development (Right panels, Scale bar: 1 cm) (C) Proteins extracts prepared from 30 day-old roots and used for immunoblot (anti-GFP antibody) showed the presence of the GFP (lane 1) and the GFP:AeCRN13Cter (lane 2) proteins as expected size (D-E) Box plot graphics depicting the decrease in the average number of roots per plant (D) and lengths of root systems (E) of 30 day-old composite plants expressing the GFP:AeCRN13Cter construct. 59 GFP-plants (1) and 29 GFP:AeCRN13Cter plants (2) were used for measurements and statistics (* p-value<0.05, t-test).

Nuclear localization of AeCRN13 worries legume and tobacco cells

To substantiate a role for AeCRN13 during host infection we transformed *M. truncatula* roots with the C-terminal domain of the protein consisting of amino acid residues from 106 to 423. *Agrobacterium rhizogenes* was used to express a GFP-tagged *35S:AeCRN13Cter* construct into *M. truncatula* roots. Confocal imaging revealed dots of GFP fluorescence in AeCRN13 expressing roots 13 days after transformation, while a homogeneous fluorescence along the root tip was detected in the GFP-control samples (Fig. 2A). A higher magnification revealed that the AeCRN13Cter protein localized into the nuclei of *M. truncatula* root cells as compared to the GFP-transformed roots in which fluorescence was detected equally in cytoplasm and nuclei. Within two weeks, enlargement of *M. truncatula* cell size was observed upon expression of AeCRN13 (Fig. 2B, left panel) and a large number of the plantlets collapsed without generating new roots, in contrast to control plants. Within three weeks, we noticed a reduction of the development of aerial and root systems (Fig. 2B, right panel). The production of AeCRN13Cter in transformed roots was confirmed by immunoblot and a ~69 kDa band was observed corresponding to the predicted size of the GFP:AeCRN13Cter fusion protein (Fig. 2B). Quantification of the number and length of new generated roots showed that about 10 roots were generated 30 days post-transformation by control plants with a mean length of 2 cm (n=59), whereas less than 1 root with a length lower than 1 cm is statistically observed in *35S:GFP-AeCRN13Cter* composite plants (n=29) (Fig. 2C). Collectively, these results indicate that the C-terminal region of AeCRN13, although devoid of a predicted Nuclear Localization Signal (NLS), is addressed to the nuclei of *Medicago* root cells where it has an inhibitory effect on roots development.

When expressed in leaf tissues of *N. benthamiana*, AeCRN13 induced necrosis at 5 days post inoculation (Fig. 3A). Confocal imaging of infiltrated area at 24 hpi, revealed an exclusive nuclear localization of GFP:AeCRN13Cter. The observed nuclear localization of the protein contrasted with the GFP control, which was detected equally in the cytosol and the

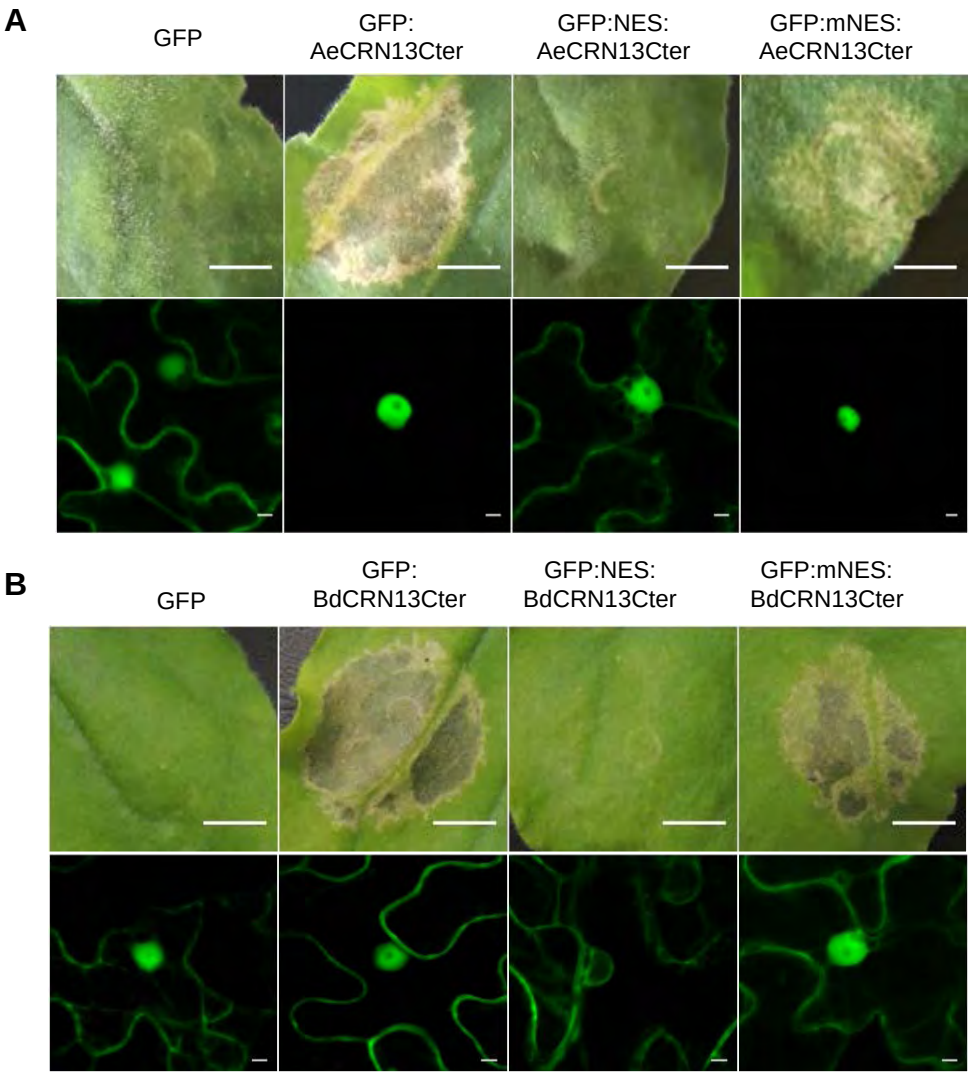

Figure 3. AeCRN13 and BdCRN13 have a cell-death nuclear-dependent activity in *N. benthamiana*

(A). Cell death symptoms are observed 5 days after Agrobacterium-transient expression of AeCRN13Cter in *N. benthamiana* leaves. Confocal imaging 24h after treatment show a nuclear-localization of AeCRN13Cter. The abolishment of necrosis was obtained by the addition of a Nuclear-Export Signal (NES) to the GFP:AeCRN13Cter construct and is correlated to the preferential localization of the fusion protein in the cytoplasm. Necrotic symptoms and nuclear localization of AeCRN13Cter were restored when a non-functional mutated version of NES (mNES) was used. Upper panels, scale bars: 0.5 cm. Lower panels, scale bars: 5µm. (B) Cell death symptoms are also observed 5 days after *Agrobacterium*-transient expression of BdCRN13Cter in *N. benthamiana*. Subcellular localization of the fusion protein at 24h by confocal imaging reveals a preferential nuclear localization of the effector. Macroscopic symptoms on tobacco leaves are abolished when the fusion protein is preferentially addressed to the cytoplasm thanks to the addition of a NES sequence. The GFP:mNES:BdCRN13Cter protein harboring a non-functional NES sequence, is preferentially located in the nucleus and trigger necrotic symptoms on leaves. Upper panels, scale bar: 0.5 cm. Lower panels, scale bar: 5µm.

nucleus (Fig. 3A). To go further and to estimate the importance of nuclear localization in triggering necrosis, we fused the full C-terminal domain of AeCRN13 to a nuclear export signal (NES) or its mutated (mNES) counterpart. The constructs were agroinfiltrated in *N. benthamiana* leaves. No symptoms were observed on treated leaves even at longer time (>10 days) when a NES was fused to the construct. Confocal imaging revealed the enhancement of nuclear export of AeCRN13Cter protein since the GFP signal was recovered mostly in the cytoplasm (Fig. 3A). Immunoblot analysis confirmed the accumulation and stability of the corresponding proteins from 1 to 3 days post inoculation (Fig. S1A). By contrast the addition of a mutated (inactive) NES domain restored the nuclear accumulation of the C-terminus of AeCRN13, as inferred by confocal observations, as well as its cell death effect. Taken together, these results show that nuclear localization is required for AeCRN13 to trigger necrosis in *N. benthamiana*.

BdCRN13 is nuclear-localized and induces necrosis in tobacco cells

Orthologous genes to AeCRN13 were recently reported in the amphibian pathogen *B. dendrobatidis* raising the intriguing question of the origin of these sequences in a distantly related fungus and their role in animal pathogenesis [7]. A *BdCRN13* sequence was amplified from the *Bd* genome (strain JEL 423) and transiently expressed as a GFP fusion protein in *N. benthamiana*. As observed with AeCRN13Cter, cell-death was detected 5 days after agroinfiltration (Fig. 3B), indicating that cell-death inducing activity is conserved among oomycete and chytrid CRN13 C-terminal domains. Subcellular localization assay shows that GFP fluorescence of BdCRN13 is mostly detected in the nuclei of epidermal cells (Fig. 3B). To correlate this preferential nuclear localization with BdCRN13 activity, we fused the full C-terminal domain of the effector to a nuclear export signal (NES) or its mutated counterpart (mNES). No symptoms were observed on agroinfiltrated leaves even at longer times (>10 days) with the NES construct, in contrast to areas treated with the mNES construct (Fig. 3B).

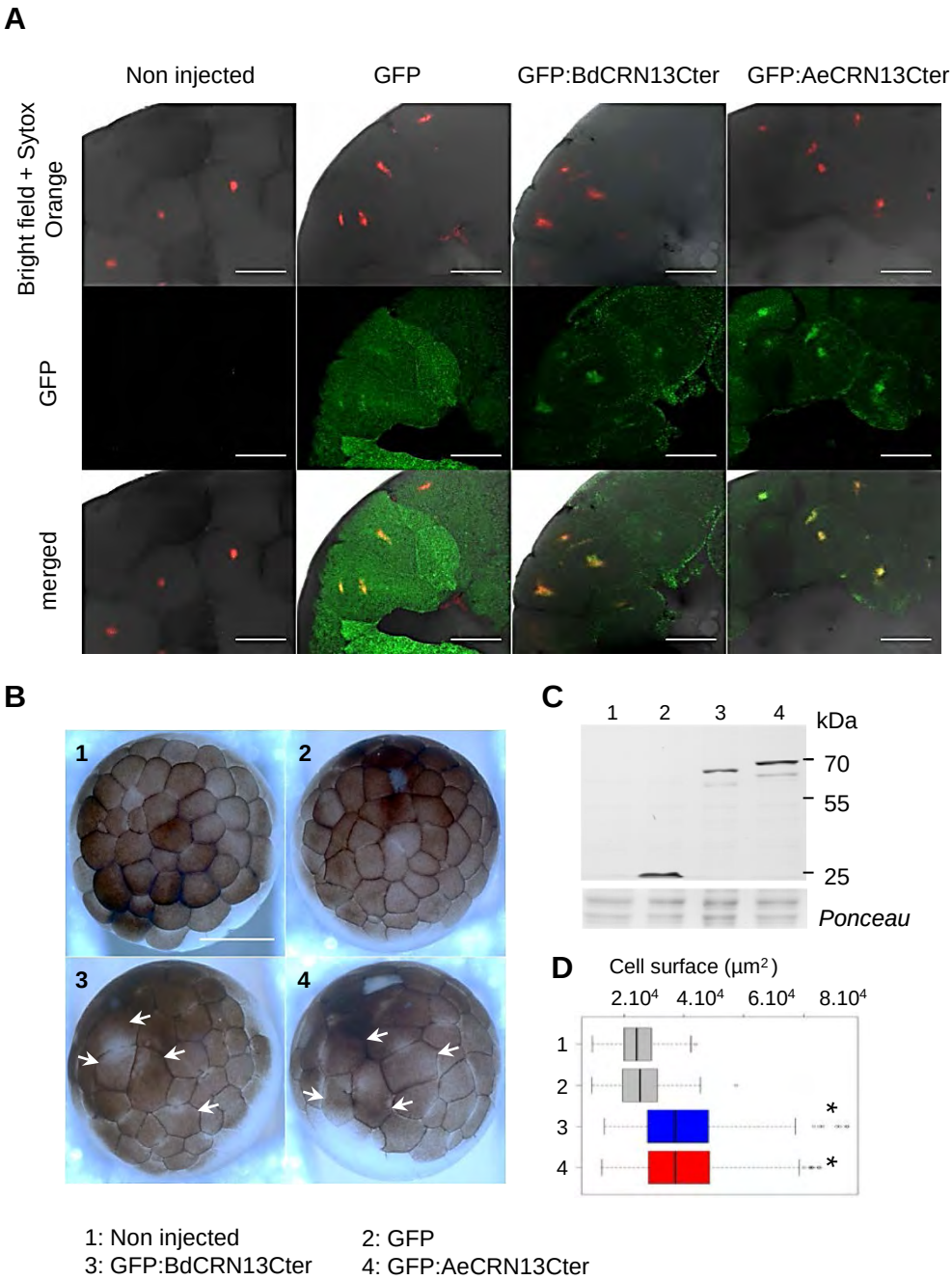

Figure 4. BdCRN13Cter and AeCRN13Cter are nuclear localized in *Xenopus laevis* and affect embryos development.

(A) Confocal images of hemisections of embryos fixed at stage 7 and stained with SytoxOrange, show nuclear localization of GFP:BdCRN13Cter and GFP:AeCRN13Cter fusion proteins. Upper panel: overlay of bright field and SytoxOrange fluorescence, middle panel: GFP fluorescence, lower panel: merged signals. Scale bar: 100µm. (B) Bright field images of stage 7 embryos. Delay in cell division evidenced by larger cell size (arrows) is observed in embryos injected with GFP:BdCRN13Cter or GFP:AeCRN13Cter mRNA. Scale bars: 500µm. (C) Immunoblot performed with anti-GFP antibodies show the stability of the different fusion proteins in Xenopus embryos. (D) Measurements of the cell surface of stage 7 embryo (n=30 embryos/condition). Statistical analyses reveal the larger size of the animal blastomeres from embryos injected with GFP:BdCRN13Cter or GFP:AeCRN13Cter mRNAs (* indicates a statistical difference between cell surface means of Non injected and GFP injected controls and either AeCRN13Cter or BdCRN13Cter injected embryos; p-value<0.05). 1: non injected, 2: GFP, 3: GFP:BdCRN13Cter, 4: GFP:AeCRN13Cter.

Immunoblots confirmed the accumulation of the corresponding fusion protein (Fig. S1B). This suggests that necrotic activity of BdCRN13 is induced by its presence in the plant nucleus, despite not having the same nuclear accumulation pattern as AeCRN13. Together these data strongly support the view that C-termini domain of CRN13s from plant and animal pathogens display a conserved mode of action.

Expression of BdCRN13 and AeCRN13 in *Xenopus laevis* embryo triggers aberrant development

Based on the conserved effect of the C-ter domain of *Ae* and *Bd* CRN13 in plants, we tested whether they both have the same activity in amphibian cells. We benefited from the African clawed frog *Xenopus laevis* embryos expression assay. *Xenopus* embryos undergo time-regulated synchronized cell divisions during the first 3 hours of development and therefore are a useful *in vivo* model system for studying cell division. *GFP:BdCRN13Cter* and *GFP:AeCRN13Cter* constructs were cloned into the pCS2 vector and *in vitro* transcribed. GFP control mRNA was obtained from pCS2-GFP vector. To test the function of both CRNs, one cell of a 2-cell stage embryos were microinjected with either *GFP*, *GFP:BdCRN13Cter* or *GFP:AeCRN13Cter* mRNAs and effect on cell divisions observed at blastula stages. Subcellular localization of the GFP-tagged proteins was conducted on hemisections of injected embryos after staining of nucleic material with Sytox-Orange dye. Cells expressing the GFP alone displayed fluorescence preferentially in the cytoplasm, whereas a green fluorescent signal was also found to co-localized with the red fluorescence of Sytox-Orange for the GFP:BdCRN13Cter or GFP:AeCRN13Cter constructs (Fig. 4A), showing that both proteins were nuclear localized in *Xenopus* embryos. The pattern of cell division of GFP-injected embryos behaved similarly to the uninjected embryos pattern, where blastomeres dividing normally gave smaller blastomeres at each round of division, leading to a typical stage 7 blastula (4 h post fertilization) as described in the normal table of *Xenopus laevis* [42]

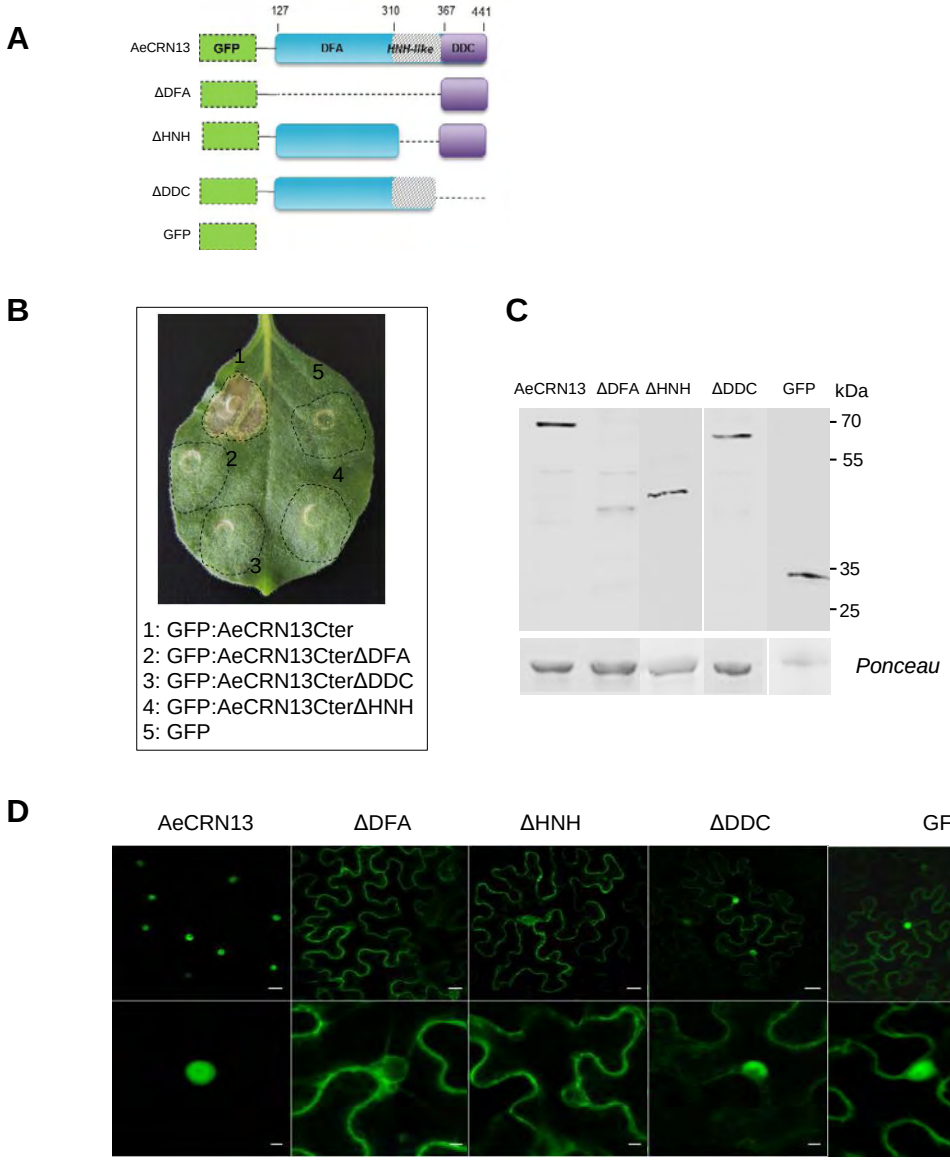

Figure 5. AeCRN13Cter nuclear localization requires a HNH-like motif in association with DFA and DDC subdomains.

(A) Schematic representation of GFP-tagged C-terminal deletions of AeCRN13 under the control of 35S promoter expressed with *A. tumefaciens* in *N. benthamiana* leaves. (B) A typical infiltrated leave observed at 5 days post inoculation shows cell-death symptom only in the area treated with the full length version of AeCRN13Cter. (C) Western blot analysis using anti-GFP antibody reveals the accumulation of GFP:AeCRN13Cter (69,5kDa, lane 1), GFP:AeCRN13CterΔDFA (39,3kDa, lane 2),GFP:AeCRN13CterΔDDC (46,7 kDa, lane 3) and GFP:AeCRN13CterΔHNH (57,7kDa, lane4) fusion proteins 24 hours after agroinfiltration. (D) Confocal images of epidermal cells showing the exclusive nuclear localization of the full length version of AeCRN13Cter, while the DDC-mutated version presented a nucleocytoplasmic localization and the DFA-affected versions are preferentially detected in the cytoplasm (i,e deletion of DFA subdomain or deletion of the HNH-like motif). 1: AeCRN13Cter full length, 2: GFP:AeCRN13CterΔDFA, 3: GFP:AeCRN13CterΔDDC, 4: GFP:AeCRN13CterΔHNH. Upper panels: GFP fluorescence in several cells of a leaf zone. Scale bars: 20µm. Lower panels: focus on one representative cell. Scale bars: 5µm.

(Fig. 4B). In contrast, a cell division delay was observed in *GFP:BdCRN13Cter* or *GFP:AeCRN13Cter* mRNA-injected embryos leading at stage 7 to an accumulation of abnormally large blastomeres (Fig. 4B, lower panel). On later stages, most of these abnormal blastomeres kept developmental delay where cell divisions were not achieved, leading to larger or giant cells (Fig. S2). Evaluation of the average cell surface in injected embryos (n=30 embryos) showed that for *GFP:BdCRN13Cter* or *GFP:AeCRN13Cter* mRNA-injected embryos, cells were statistically larger than the non-injected or GFP-injected embryo cells (Fig. 4D). This result suggests that CRN13 accumulation interfere with cell divisions leading to a progressive accumulation of undivided and larger blastomeres.

A conserved HNH-like motif is required for nuclear localization of AeCRN13

A functional domain search on C-ter of BdCRN13 and AeCRN13using the PFAM database revealed a weak match with a HNH motif (PF13391), hereafter designed as HNH-like motif. Initially found in bacterial proteins, this motif corresponds to a small nucleic acid binding and cleavage module widespread in metal finger endonucleases in all life kingdoms [43]. In CRN13s from *A. euteiches*, *Phytophthora* sp. and *Bd* the HNH-like motif is localized in a highly conserved region at the C-ter of the DFA subdomain (Fig. S3). In order to define whether this motif plays a role in CRN13 function, DFA, DDC and HNH-like deletion mutants of AeCRN13Cter were generated (Fig. 5A) and tested by transient expression in *N. benthamiana* leaves. None of the mutants were able to trigger cell death (10 dpi, Fig. 5B). Specific bands corresponding to the expected protein sizes were detected in all samples indicating the stability of the different fusion proteins, although we noticed a weaker accumulation of the DFA deleted version of AeCRN13Cter (Fig. 5C, lane 2). Removal of the DFA domain encompassing the HNH-like motif led to a GFP signal detected exclusively in the cytoplasm indicating that nuclear import and retention of the protein in the nucleus was impaired. In contrast GFP:AeCRN13CterΔDDC fusion protein , displayed a nucleo-

Table 1. FRET-FLIM measurements showing that AeCRN13Cter and BdCRN13Cter interact with nuclear DNA *in planta*

Donor	Acceptor	τ [(a)]	sem [(b)]	N [(c)]	E [(d)]	[(e)] p-value
GFP	-	2.246	0.036	20	-	-
GFP	Sytox Orange	2.210	0.044	18	1.6	0,52
GFP-H2B	-	2.465	0,017	40	-	-
GFP-H2B	Sytox Orange	1.852	0.047	43	24.8	2,31E-19
GFP-AeCRN13Cter	-	2.154	0.024	36	-	-
GFP-AeCRN13Cter	Sytox Orange	1.655	0.048	35	23	9,58E-14
GFP-AeCRN13Cter	Sytox Orange (RNase treatment)	1.749	0.033	32	19	8,25E-15
GFP-BdCRN13Cter	-	2.196	0.048	37	-	-
GFP-BdCRN13Cter	Sytox Orange	1.915	0.037	30	12.8	3,26E-05
GFP-BdCRN13Cter	Sytox Orange (RNase treatment)	1.963	0.041	32	10.6	5,50E-04

t : mean lifetime in nanoseconds (ns). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with exponential function using a non-linear least-squares estimation procedure. [(b)] s.e.m.: standard error of the mean. [(c)] N: total number of measured nuclei. [(d)] E: FRET efficiency in % : E=1-(tDA/tD). [(e)] p-value (Student's *t* test) of the difference between the donor lifetimes in the presence or absence of acceptor.

cytoplasmic localization similar to the GFP control (Fig. 5D) thus, DFA full domain enters the nucleus but does not maintained in it. The GFP-AeCRN13CterΔHNH was also localized exclusively in the cytoplasm. These results suggest that the HNH-like motif and the DDC domain are likely required for AeCRN13 nuclear localization.

AeCRN13 and BdCRN13 bind DNA *in vitro* and *in planta*

The identification of the HNH-like motif and the requirement of this sequence for CRN13s nuclear localization prompted us to check whether these proteins could interact with DNA. Protein variants were produced in *E. coli* and purified to perform *in vitro* double-strand DNA binding assays. The C-ter domains of AeCRN13 and BdCRN13, but not AeCRN13 deleted of the HNH-like motif, bound to dsDNA-beads (Fig. 6). To confirm that interaction between CRN13 C-ter and dsDNA occurs *in vivo*, we set up a fluorescence resonance energy transfer (FRET) assay coupled with fluorescence lifetime imaging microscopy (FLIM) in *N. benthamiana* cells. GFP-tagged Cter domains of both CRN13s were transiently expressed in *N. benthamiana* epidermal cells by agroinfiltration and plant nucleic acids were stained with SytoxOrange. SytoxOrange in combination with GFP is suitable for FRET-FLIM due to considerable overlap of the acceptor absorption and donor emission spectra [44]. The occurrence of FRET, causing the fluorescence lifetime of the donor GFP to decrease in these conditions, would only be due to the proximity (less than 10 nm) of GFP to SytoxOrange. Energy transfer was detected by fluorescence lifetime imaging microscopy (FLIM). As a positive DNA-binding and FRET control between GFP and Sytox Orange we used *N. benthamiana* leaves expressing a GFP-tagged histone H2B (GFP-H2B) construct. As shown in table 1 the average fluorescence lifetime of GFP and SytoxOrange was 2.20 nsec. In the absence of SytoxOrange the lifetime of GFP:H2B is 2.4651± 0.017 nsec whereas its lifetime decreased to 1.852± 0.047 4 nsec in the presence of the dye, indicating that FRET occurred as expected from the binding of the histone H2B to nucleic acid. Regarding AeCRN13Cter

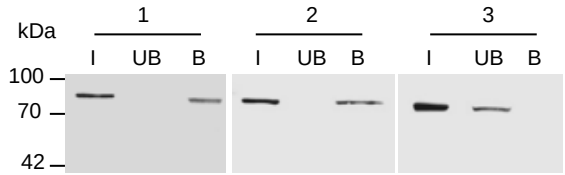

Figure 6. AeCRN13Cter and BdCRN13Cter bind DNA in vitro thanks to a HNH-like motif. Approximately 35 ng of purified recombinant proteins (1: MBP:AeCRN13Cter; 2: MBP:BdCRN13Cter, 3: MBP:AeCRN13CterDHNH) were incubated 20 minutes at room temperature with beads coupled to double strand DNA (dsDNA) before centrifugation. Immunoblot using anti-MBP antibodies showed the presence of AeCRN13Cter and BdCRN13Cter in the fraction in association with dsDNA. In the absence of the HNH-like motif, AeCRN13Cter does not bind to dsDNA. I: input protein fraction, UB: unbound, DNA protein fraction. B: bound-DNA protein fraction.

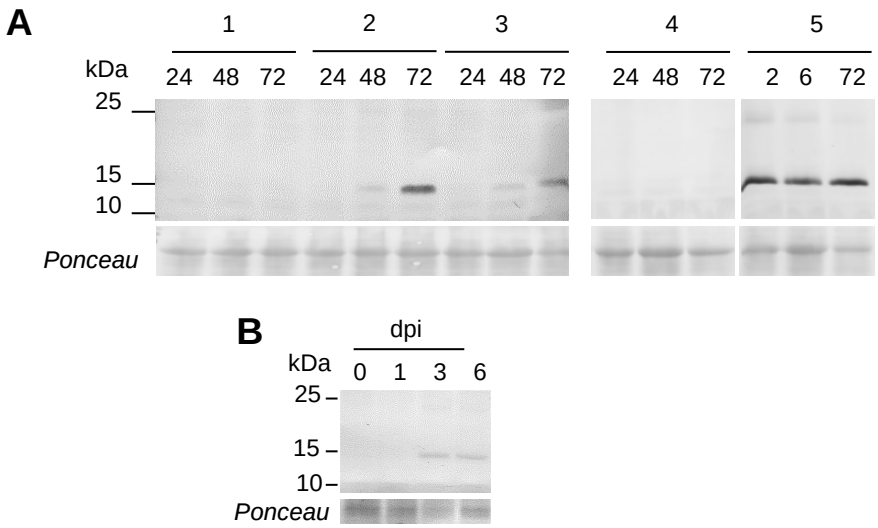

Figure 7. H2AX histone (gH2AX) phosphorylation is induced by AeCRN13Cter and during infection

(A) Tobacco leaves were agroinfiltrated with GFP (1) GFP:AeCRN13Cter (2) GFP:BdCRN13Cter (3) GFP:AeCRN13CterΔDDC (4) constructs and status of histone H2AX phosphorylation was compared using western blotting. Pronounced phosphorylation was observed in leaves cells expressing AeCRN13Cter or BdCRN13Cter two days after treatment with an emphasis at three days, while the deleted DDC version of AeCRN13Cter and the GFP control failed to induce phosphorylation modification. Bleomycin was used to induce DNA damage as a control and cells challenged with the chemical agent display H2AX phosphorylation within 2 hours after treatment. (B) H2AX phosphorylation is detected by western-blot in roots of *M. truncatula* infected by *A. euteiches* from 3 to 6 days post infection (dpi).

construct, the lifetime of GFP:AeCRN13Cter was 2.1542 ±0.024 nsec in the absence of SytoxOrange and decreased significantly to 1.655 ± 0.048 nsec in presence of the dye, a value similar to the one observed with the GFP:H2B positive control. A similar decrease was observed in the GFP life-time in samples expressing the BdCRN13Cter construct. These results indicated an energy transfer and therefore the binding of CRN13s to nucleic acids. While the Sytox Orange dye is not specific for either DNA or RNA [44,45] leaf samples were treated with RNase A to digest RNA before performing the experiment. After RNase treatment the lifetime of CRN13s GFP-tagged proteins remained unchanged as compared to the GFP-H2B control (Table 1). Hence the decrease of the fluorescence lifetime of CRNs-GFP-tagged proteins in the presence of SytoxOrange was only DNA-dependent and indicates that both CRN13s bind nuclear DNA *in vivo*.

CRN13s induce nuclear DNA damage

Because of the observed phenotype on plant and animal cells upon CRN13 Cterminus expression and DNA-binding capacity through a HNH-like motif, the putative DNA-damage inducing activity of CRN13s was tested. A typical marker to examine DNA damage upon genotoxic stress is the phosphorylation of the histone H2AX, namely γH2AX [46]. Using a monoclonal antibody specific for γH2AX, we detected H2AX phosphorylation in agroinfiltrated *N. benthamiana* leaves expressing AeCRN13Cter or BdCNR13Cter 24h after treatment meaning before the apparition of cell-death symptoms (Fig. 7A). In contrast the deleted version of AeCRN13 corresponding to the DFA subdomain and the GFP control are not able to induce H2AX phosphorylation, suggesting that the full length of the C-terminus of AeCRN13is required. When control leaves were treated with the DNA-damaging agent bleomycin (BLM) a strong and early H2AX phosphorylation was detected. Since these results suggest that *A. euteiches* can produce DNA damaging effectors during pathogenesis, the phosphorylation status of H2AX was checked during infection of *M. truncatula* roots upon *A.*

393 *euteiches* infection. As shown by western-blot analysis H2AX phosphorylation is observed at
394 the early stage of *M. truncatula* roots infection (Fig. 7B) strongly suggesting that *A. euteiches*
395 triggers DNA damage during host colonization.
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Discussion

In this study we concentrated on discovering the mode of action of eukaryotic effectors by working on CRN13 intracellular effector from two unrelated filamentous pathogenic microorganisms. Up to now, CRN13s were only predicted in the phytopathogenic oomycetes *Phytophthora* sp. and *A. euteiches* and in the genome of the pathogen of amphibians *B. dendrobatidis (Bd)*. We show that AeCRN13 is expressed during infection of roots of legumes by *A. euteiches*. AeCRN13 C-ter domain and its ortholog from *Bd* triggered tobacco leaf cell death and restricted root development of *M. truncatula*. CRN13s cytotoxic activity in plant cells relied on the nuclear localization of the effectors. Animal cells expressing either AeCRN13 or BdCRN13 at the nuclear level from early *Xenopus* embryos showed aberrant development with an enhancement of cell size. A putative HNH-like motif reported in bacterial endonuclease was predicted in both effectors. This motif was required for AeCRN13 retention in the nucleus of tobacco cells. Both CRN13s exhibited dsDNA-binding affinity *in vitro* thanks to the HNH-like motif. The interaction of effectors with DNA was unambiguously confirmed by FRET/FLIM assays on tobacco cells. Both CRN13s induced phoshorylation of H2AX histone in tobacco leaves, indicating that the effectors trigger plant DNA damage. Infection of host legumes roots by *A. euteiches* was accompanied by phosphorylation of H2AX histone. Altogether these data reveal a new mode of action for intracellular eukaryotic effectors in which host nuclear DNA is the central target. Indeed we provide the first evidence that unrelated filamentous pathogens shared similar effector family able to bind DNA and trigger host DNA damage.

Our first results showed that a CRN13 gene is expressed by A. euteiches during infection of *M. truncatula* roots and the use of CRN13 antibodies revealed a partial processing of the protein suggesting that the translocation signal of CRN is probably cleaved during the translocation process. This result is reminiscent with the proteolytic cleavage occurring during

the translocation of *Plasmodium* effectors. The N-terminus export element (PEXEL; RxLxE/Q/D) of effectors is cleaved at the parasite endoplasmic reticulum by protease plasmepsin V and is required for export V [47]. However, translocation mechanism mediated by the N-terminus targeting signal of CRN is still unknown.

Despite the absence of a predicted Nuclear Localization Signals (NLS), AeCRN13 and BdCRN13 are addressed to the nucleus when heterogously expressed in plant or animal cells. This data indicates that CRN13s C-terminus drives nuclear localization and contains an atypical NLS not detected by available prediction programs, and/or are able to subvert conserved nuclear translocation machinery to promote the efficient import of the effector into nucleus. This result is consistent with previous analyses on CRNs from *P. infestans* and *P. capsici* showing that all CRNs are nuclear localized when expressed in *N. benthamiana* tissues, despite that only some CRNs harbor a predicted NLS [26,29]. The nuclear localization of NLS-containing CRNs required host importin-α factor [26], however its function in the transport of CRNs devoided of a NLS remains to be demonstrated.

CRN13s from *A. euteiches* and *Bd* induced similar phenotypes in plant and animal tissues, and this activity is dependent of their nuclear localization. Overexpression of CRN13s led to an inhibition of root development in *M. truncatula*, to cell-death symptoms on tobacco leaves and to an enhancement of cell size due probably to an arrest of cell division in *Xenopus* embryos. These results corroborate observations made during roots infection of legumes by *A. euteiches*, where susceptible accessions harbor few days after infection a decrease of secondary root development and necrosis of roots [40]. Similarly recent studies show that *Bd* produced compounds that can impair amphibian lymphocyte proliferation to evade host immunity [48]. While some data suggests that these compounds are not proteins, it should be interesting to evaluate the contribution of BdCRNs in this process [48].

AeCRN13 and BdCRN13 show a divergent N-terminal targeting motif but conserved C-terminus organization consisting of two subdomains association (DFA-DDC). *In silico*

analysis lead us to predict a putative HNH motif (PF13391) in the DFA subdomain of both CRN13s. HNH stands for the three most conserved histidine and asparagine residues located within the catalytic site [43]. These residues are found in all the CRN13 sequences analyzed. Less common in eukaryotes, the motif allows DNA-binding and nuclease activities of a large array of prokaryotic enzymes like the colicin E7 toxin from *E. coli* and play important role in many cellular processes including immunity [49-51]. Deletion analysis shows that the HNH-like motif in combination with the DDC domain contribute to CRNs nuclear localization in *Nicotiana.* Thus, CRN13s appear to have a modular organization in which specific subdomains are required for nuclear localization.

We discovered that AeCRN13 and BdCRN13 bind dsDNA *in vitro* and that this capacity is linked to the presence of the HNH-like motif in the DFA subdomain. Although preliminary experiments with recombinant proteins did not allow the detection of a nuclease activity for CRN13 proteins in standard conditions, this possibility cannot be excluded. By setting FRET/FLIM measurements to evaluate protein/DNA interactions in leaves samples, we revealed DNA-binding capacity of CRN13s *in vivo*. These data show unambiguously for the first time that filamentous eukaryotic effectors interact with nuclear DNA. While it is known that the nucleus is a key cellular compartment for expression of immune responses and is a target of many microbial effectors [9] [52], up to now direct interaction between effector and host DNA has been reported only for transcriptional activator-like (TAL) bacterial effectors of *Xanthomonas* sp. and *Ralstonia sp.* [33,53]. Only protein/protein interactions at the nuclear level have been reported for eukaryotic filamentous organisms. In oomycetes, the RxLR Pi03192 effector from *P. infestans* interact with two NAC transcription factors to prevent them from being released from the ER to enter the nucleus where they limit disease progression [16]. The interaction and stabilization of the U-box E3ligase CMPG1, required for cell death, by Avr3a from *P. infestans* occurs in the nucleolus of plant cell [54]. In addition, the RxLR44 from *H. arabidospis* interact with the mediator complex RD19a to

enhance its degradation and *Arabidopsis* susceptibility [18]. Rust transferred protein 1 (RTP1p) from the bean rust fungus *Uromyces fabae* is the only effector from a pathogenic fungus for which a nuclear localization has been described to date but its plant target is still unknown [55]. Here we propose that the CRN-DNA interaction occurs on unspecific nucleic sequences or highly conserved in plant and animals cells, since BdCRN13 binding is effective in *N. benthamiana*. Future work will aim to identify DNA regions targeted by both effectors.

Although nuclease activity of recombinant CRN13s effectors was not detected in standard *in vitro* condition, the presence of a HNH-like motif leads us to hypothesize that binding of CRN13s to host DNA may trigger nucleic damage. This effect was studied by following the phosphorylation of H2AX histone (γH2AX), which is recognized as a solid marker of double strand DNA break (DSB) in plant and animal cells [46]. In *N. benthamiana* cells expressing CRN13s and in *Medicago* roots infected with *A. euteiches*, γH2AX accumulation was observed revealing that CRN13s and *A. euteiches* infection induced DSB. While DNA damage have been well characterized with regard to bacterial toxins upon infection of animal tissues [56,57], this effect has not been reported in the case of plant pathogen interactions. In plants, DSB due to environmental stresses or chemicals agent, have extreme detrimental effects on plant growth with particularly severe effects on actively dividing cells [58,59]. In animal systems, bacterial genotoxins leads to cell cycle arrest and eventually apoptosis [60]. Due to the observed expansion of plant and *Xenopus* cells size and cell-death symptoms upon CRN13 expression, we propose that genotoxic activity of CRNs impacts host cell cycle. Therefore the presence of effectors capable of causing DNA damage may constitute a predisposing factor for host colonization.

Taken together, this work provides first evidences that eukaryotic filamentous pathogens express similar DNA-binding proteins able to trigger host DNA damage. Additionally, our data show that unrelated plant and animal pathogens deployed a common

repertoire of effector with conserved mode of action. This raises the question of the origin and evolution of these genes and their implication on the emergence of new pathogenic species.

Materials and Methods

Plant material, microbial strains, and growth conditions

M. truncatula F83005.5 seeds were scarified, sterilized and *in vitro*-cultured and transformed as previously described [40,61]. Roots infection using *A. euteiches* (ATCC 201684) zoospore inoculum was performed as [41]. *N. benthamiana* plants were grown from seeds in growth chambers at 70% of humidity with a 16h/8h dark at 24/ 20°C regime. All *E.coli* strains (DH5α, DB3.5 and BL21AI), *A. tumefaciens* (GV310::pMP90RK) and *A. rhizogenes* (ArquaI) strains were grown on LB medium with the appropriate antibiotics.

RNA extraction and qRT-PCR

Samples were ground on liquid nitrogen and total RNA extracted using the RNAeasy kit (Qiagen). Reverse transcription was performed on 1µg of total RNA using the AppliedBiosystems kit (Life Technologies-Invitrogen). cDNAs were diluted 50- fold for qPCR reaction. Each qPCR reaction was performed on a final volume of 10µl corresponding to 8 µl of PCR mix (0.5µM of each primer and 5µl SYBRGreen, Applied Biosystems) and 2µl of the diluted cDNA and was conducted on a ABI Prims SDS 7900 HT (Applied Biosystems, Foster City, CA, USA) device using the following conditions: 5min at 95°C, followed by 45 cycles of 15 s at 95°C and 1min at 60°C. Dissociation curves were obtained by applying a 15s 95°C, 15s 95°C and 15s 95°C cycle. Each reaction was conducted on triplicates for cDNAs of four biological replicates. Primers F: 5'-TTATTGCTGTGCCAAATCAG-3' and R: 5'- GATATTGTATCTTGCGGTGAC-3' were used for the detection of AeCRN13 (Ae_9AL5664, http://www.polebio.scsv.ups-

tlse.fr/aphano/, [62]). Primers F: 5'-TGTCGACCCACTCCTTGTTG-3' and R: 5'-TCGTGAGGGACGAGATGACT-3' were used to assess the expression of *A. euteiches*'s α-tubulin coding gene (Ae_22AL7226) and normalize AeCRN13 expression. The histone 3-like of *M. truncatula*, previously described [63], was used to normalize microorganism abundance during infection. Relative expression of AeCRN13 and α-tubulin genes were calculated using the $2^{-\Delta\Delta Cq}$ method described in [64].

Construction of plasmid vectors and *Agrobacterium*-mediated transformation

All primers used for the generation of the CRN constructs are listed in Supplementary Tab. T1. *BdCRN13Cter* was generated by PCR from genomic DNA of *B. dendrobatidis* JAM81 (kindly provided by Jason Stajich, University of California, Riverside, USA) using primers attB1_BdCRN13-F and attB2_BdCRN13-R. Unless noticed otherwise, cDNA clone corresponding to the unigene Ae_9AL5664 (vector pSport_AeCRN13 [62]) was used as template for the generation of C-terminal domain versions of AeCRN13. Gateway technology in combination with pDONR-Zeo (Invitrogen) and pK7WGF2 vectors (http://gateway.psb.ugent.be/) were used for cloning steps. AeCRN13Cter carrying Gateway adaptators was generated by PCR using primers attB1_AeCRN13-F and attB2_AeCRN13-R. AeCRN13CterΔDDC and AeCRN13CterΔDFA carrying the Gateway sequences were amplified with primers set attB1_AeCRN13-F and attB2_DFAend-R and with primers set attB1_AeCRN13_DDC-F and AttB2_AeCRN13-R, respectively. A three-step PCR strategy was used to generate the *GFP:AeCRN13CterΔHNH* construct. Briefly, the amplicon corresponding to the DFA subdomain deleted of the HNH-like motif (106aa-281aa) was mixed with the amplicon corresponding to the DDC subdomain (338aa-423aa) and used as a template for PCR with attB1_AeCRN13-F and attB2_AeCRN13-R primers. Amplicons were BP recombined (Invitrogen) into pDONR-Zeo vector. NES sequence (LQLPPLERLTL) and non-functional mutated NES sequence (mNES: LQAPPAERATL) were added to the

Nterminal moiety of AeCRN13Cter and BdCRN13Cter by PCR [26]. Amplicons were introduced in pENTR/ D-TOPO vector by means of TOPO cloning (Invitrogen) and then transferred to pK7GWF2 vector. Vector *pBI121:H2B:YFP* [65] was used as template to amplify the histone2B from *A.thaliana* and the amplicon was clone into pENTR/ D-TOPO vector and transferred into pK7WGF2 to obtain *GFP:H2B* fusion construct. Generation of *M. truncatula* composite plants was performed as described by [61] using ARQUA-1 *A. rhizogenes* strain. For leaves infiltration, GV3101 *A. tumefaciens* transformed strains were syringe-infiltrated at OD_{600} = 0.3 on 3-4 week-old *N. benthamiana* as described by [66].

MBP-tagged CRN recombinant proteins

AeCRN13Cter, AeCRN13Cter ΔHNH and BdCRN13Cter inserts cloned in pDONR-Zeo entry vector were transferred in pHMWGA [67] expression vector by LR recombination (Invitrogen).

Constructs carrying *the MBP:AeCRN13Cter, MBP:AeCRN13CterΔHNH* and fusions were introduced in *E.coli* BL21-AI (Invitrogen) for protein production as recommended by the provider. Purification of the proteins was performed by affinity chromatography using amylose resin (BioLabs England).

Anti-AeCRN13 antibodies generation and purification

Polyclonal antibodies against the C-terminal domain of AeCRN13 were raised in rabbits (ProteoGenix, France) by injecting the purified recombinant protein MBP:AeCRN13Cter. Sera were purified by negative adsorption against recombinant MBP:AeCRN5Cter protein (Gaulin, data unpublished) adsorbed onto an amylose column resin and used at a dilution of 1:1000 relative to initial sera for western-blots.

584 **Immunoblot analysis**

585 Infected *M. truncatula* roots or roots from *M. truncatula* composite plants were frozen in
586 liquid nitrogen and ground in 50mM NaP, 0.1% (v/v) triton, 10mM β-mercaptoethanol and 1x
587 complete protease inhibitor cocktail (Roche). Supernatants were separated by SDS-PAGE and
588 electroblotted to nitrocellulose membranes (Amersham BioSciences). AeCRN13 was detected
589 using anti-AeCRN13 purified antibodies and anti-rabbit secondary antibodies coupled to
590 alkaline phosphatase (Sigma-Aldrich) and revealed using CDPstar chemioluminescence kit
591 (Roche). *N. benthamiana* protein analyses were performed as described by [6] using a primary
592 rabbit anti-GFP polyclonal antibodies (1:2000, Clontech).
593 For γH2AX experiments, *N.benthamiana* agroinfiltrated leaves and *M. truncatula* infected
594 roots were harvested at different time in liquid nitrogen and grounded in modified Rippa
595 buffer v/v (TrisHCl 50mM, NP-40 1%, NaCl 250mM, SDS 0.1%, EDTA 2mM, DOC 1%,
596 Protease inhibitors 1X (Complete Protease Inhibitors, Roche), orthovanadate 10mM, PMSF
597 1mM (Sigma-aldrich) using 23G needles (0.6mm diameter). Samples were then treated with
598 1µl of Benzonase (Sigma-aldrich, 250U/µl) for 30 min on ice, centrifuged for 20 min at 13000
599 rpm at 4 °C and supernatants were dosed and used for western blot. Approximately 30 µg of
600 total protein extracts were loaded on 12% polyacrylamide gels, transferred on nitrocellulose
601 membrane, blocked in TBS Tween 0.1% BSA 2% for 2 hours and incubated overnight at 4°C
602 with monoclonal antibody γH2AX (ser139) (Cell Signaling Technology) diluted at 1/1000 in
603 TBS-T-BSA2%. After washing steps in TBS Tween 0.05%, blots were incubated with anti-
604 rabbit secondary antibodies coupled to alkaline phosphatase (Sigma-Aldrich) diluted in TBS-
605 T-BSA2% at 1/2000 for 1 hour, washed and then revealed with NBT/BCIP substrate.
606

607 ***In vitro* DNA-binding assays**

608 Beads coupled to calf thymus double-stranded (ds) DNA (Sigma-Aldrich) were suspended in
609 KHN buffer [68] (whose pH was modified and fixed at 7). 35ng of MBP:AeCRN13Cter,

MBP:AeCRN13CterΔHNH and MBP:BdCRN13Cter recombinant proteins were incubated in 400µL of dsDNA beads for 20 min at room temperature (23°C-25°C) under moderate rotation. Beads were centrifuged at 10000xg during 10 min. The first supernatant was retained as the not bound fraction. This fraction (≈ 400µL) was concentrated using Vivaspin 500 column (cut-off 10kDa) for analysis. Beads were washed 4 times with 800µL of KHN buffer. Proteins bound to DNA were eluted by adding 50µl of 2X Laemmli buffer and heating at 95°C for 10 min. Proteins were analysed by immunoblotting using monoclonal anti-MBP antibodies.

Preparation of *N. benthamiana* epidermal leaves for FRET / FLIM experiments

Discs of agroinfiltrated *N. benthamiana* leaves were fixed 24 hours after treatment by vacuum infiltrating a TBS (TRIS 25mM, NaCl 140 mM, KCl 3 mM) 4 % (w/v) paraformaldehyde solution before incubation 20 min at 4°C. Samples were permeabilize 10 min at 37°C using a TBS buffer supplemented with 20µg/ml of proteinase K (Invitrogen). Nucleic acid staining was performed by vaccum-infiltrating a 5µM of Sytox Orange (Invitrogen) solution and before incubating samples 30 min at room temperature. When RNAse treatment was performed, foliar discs were incubated 15 min at room temperature with 0.5µg/ml of RNAse A (Roche) before nucleic acid staining. Foliar discs were washed with and mounted on TBS before observations on an inverted microscope (Eclipse TE2000E, Nikon, Japan).

Confocal microscopy

Scanning was performed on a Leica TCS SP2 DMRXA2 confocal microscope. Excitation wavelengths were 488 nm (GFP) and 543nm (SytoxOrange). Images were acquired with a 40x water immersion lens, or a 20x water immersion lens for embryo *X. laevis* hemisections, and correspond to Z projections of scanned tissues. All confocal images were analyzed and treated using the Image J software.

636

637 **Cytological observations of transformed roots**

638 Roots of composite plants expressing transgene constructs were sampled 30 days after
639 transformation and fixed in 2% (v/v) paraformaldehyde in 50mM sodium cacodylate buffer,
640 pH 7. Fixed roots were dehydrated in a graded ethanol series and embedded in LRW resin
641 (London Resin Company Limited) also in a graded manner. Polymerisation was performed
642 over-night at 60°C. Longitudinal sections (1µm) were performed with an Ultracut microtom
643 (Reichert-Jung), placed on multi-well slide and stained with 0.05% (w/v) toluidine bleu in
644 2.5% (w/v) CaCO3 buffer, pH 11, during 2 minutes at 60°C followed by a water wash.

645

646 **FRET / FLIM measurements**

647 Fluorescence lifetime measurements were performed in time domain using a streak camera
648 [69]. The light source is a mode-locked Ti:sapphire laser (Tsunami, model 3941, Spectra-
649 Physics, USA) pumped by a 10W diode laser (Millennia Pro, Spectra-Physics) and delivering
650 ultrafast femtosecond pulses of light with a fundamental frequency of 80MHz. A pulse picker
651 (model 3980, Spectra-Physics) is used to reduce the repetition rate to 2MHz to satisfy the
652 requirements of the triggering unit (working at 2MHz). The experiments were carried out at λ
653 = 820 nm (multiphoton excitation mode). All images were acquired with a 60x oil immersion
654 lens (plan APO 1.4 N.A., IR) mounted on an inverted microscope (Eclipse TE2000E, Nikon,
655 Japan). The fluorescence emission is directed back into the detection unit through a short pass
656 filter λ<750 nm) and a band pass filter (515/30 nm). The detector is a streak camera
657 (Streakscope C4334, Hamamatsu Photonics, Japan) coupled to a fast and high-sensitivity
658 CCD camera (model C8800-53C, Hamamatsu). For each nucleus, average fluorescence decay
659 profiles were plotted and lifetimes were estimated by fitting data with exponential function
660 using a non-linear least-squares estimation procedure. Fluorescence lifetime of the donor
661 (GFP) was experimentally measured in the presence and absence of the acceptor (Sytox

Orange). FRET efficiency (E) was calculated by comparing the lifetime of the donor in the presence (τ_{DA}) or absence (τ_D) of the acceptor: $E=1-(\tau_{DA}) / (\tau_D)$. Statistical comparisons between control (donor) and assay (donor + acceptor) lifetime values were performed by Student *t-test*. For each experiment, four leaf discs removed from two agroinfiltrated leaves were used to collect data.

Vector constructions and *Xenopus laevis* embryos microinjection

All primers used for cloning are listed in Supplementary Tab. S1. Briefly, GFP C-ter constructs cloned into pK7WGF2 were subcloned into pGEMT (Promega) and inserted into BamHI and XbaI restrictions site of $pCS2^+$ plasmid [70]. *In vitro* transcription was performed using SP6 mMessage mMachine kit (Ambion). Two-stage embryos were obtained by in vitro fertilization [71] and 20 nl of transcripts (6 ng) were pressure-injected in one blastomere at the animal pole in order to target the animal micromeres at later stages. Embryos were allowed to develop at 22 °C and followed by live macro-imaging (Axiozoom). Embryos were fixed in MEMFA buffer (100mM MOPS, pH7.4, 2mM EGTA, 1mM MgSO4, 3.7%(v/v) formaldehyde). Injections and assessment of development was performed on 3 independent experiments where at least 20 embryos were used for each condition. Total proteins of 5 nitrogen frozen embryos were extracted in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP40, 1x complete protease inhibitors (Roche) and analysed by immunoblot using anti-GFP antibodies.

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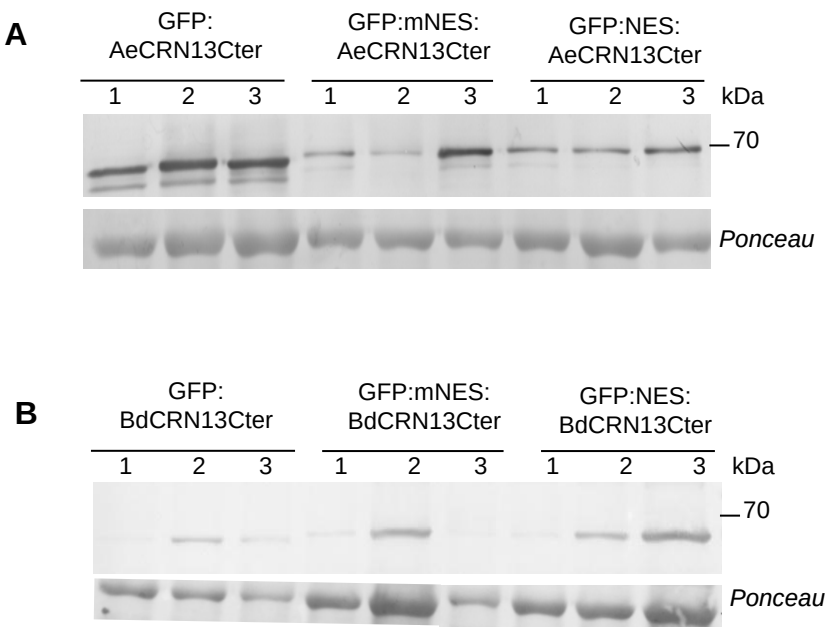
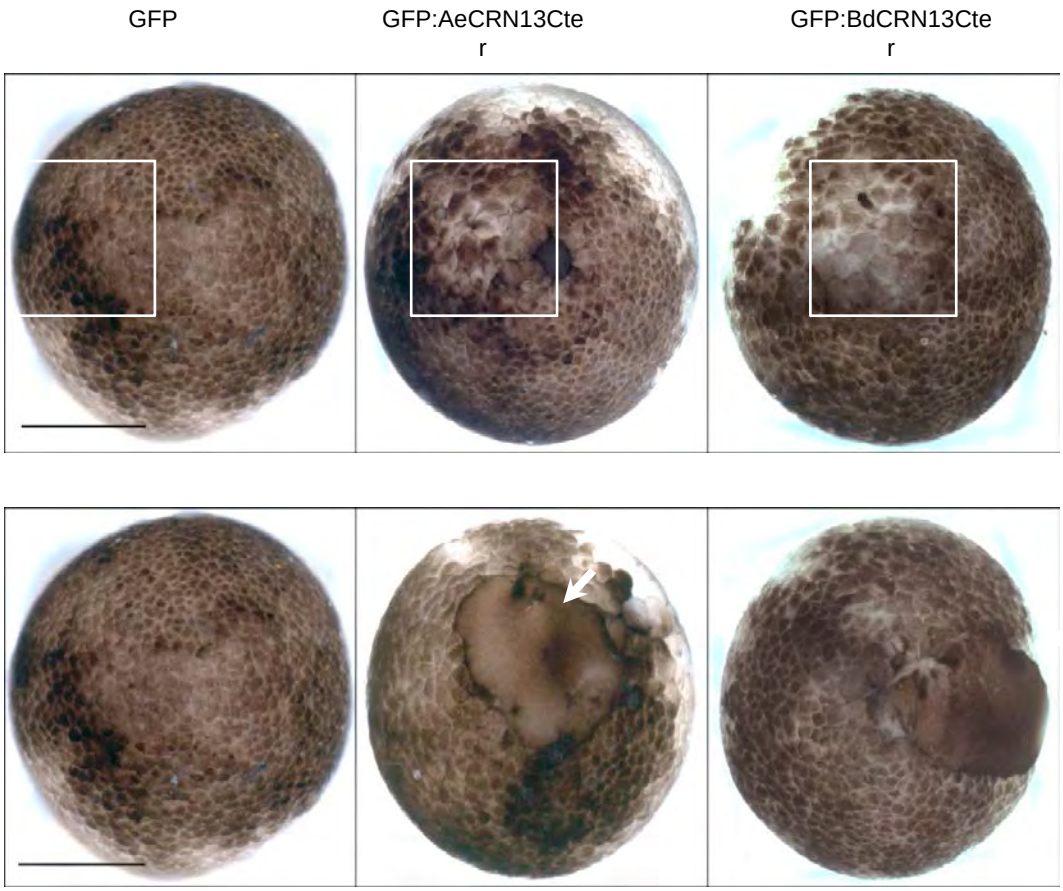
Author Contributions

Conceived and designed the experiments: EG, LC, BD. Performed the experiments: DRG, LC, AJ, YM, BD, IN, CL, MM. Analyzed the data: DRGC, LC, IN, CL, MM, BD, EG. Contributed reagents/materials/analysis tools: DRG, LC, YM, IN, CL. Wrote the paper: DRG, LC, BD, EG. Conceived, directed and coordinated the project: EG.



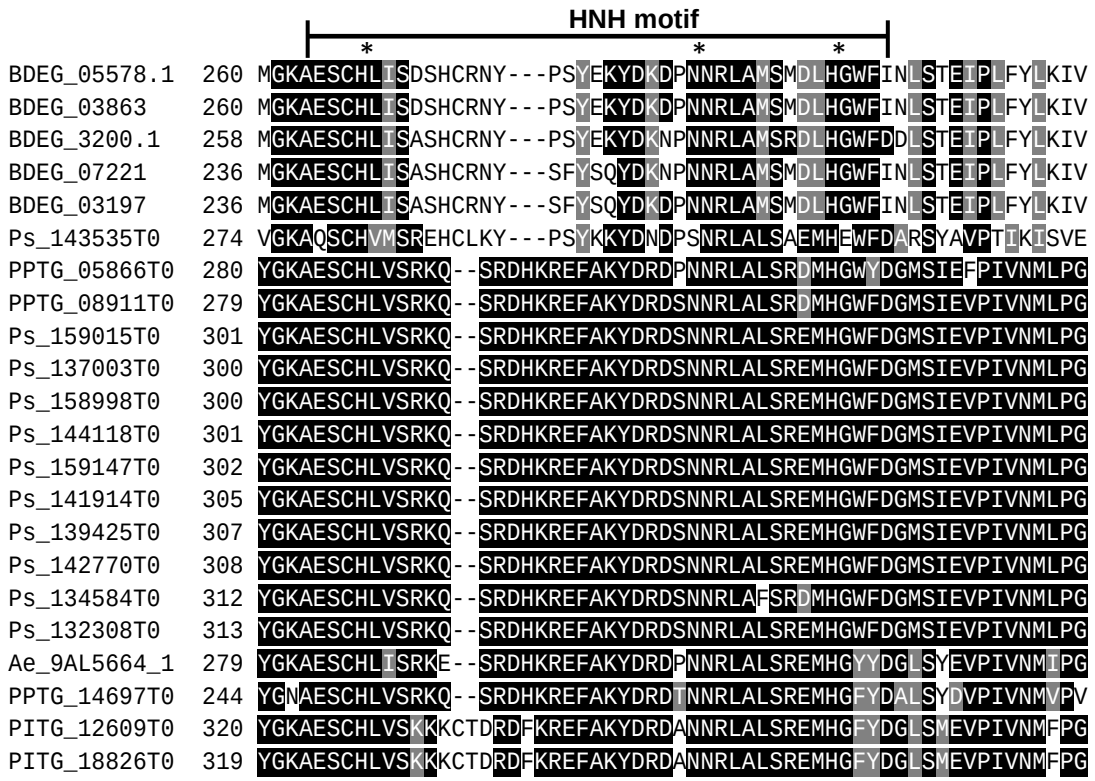
Supplemental Figure S1

Detection of AeCRN13Cter and BdCNR13Cter proteins in *N. benthamiana* leaves upon transient-expression assay. (A) Immunoblot of GFP:AeCRN13Cter, GFP:NES:AeCRN13Cter and GFP:mNES:AeCRN13Cter showing the accumulation of the fusion proteins in agroinfiltrated leaves 1, 2 and 3 days after treatment when probed with a GFP antibody. (B) Immunoblot using a GFP-antibody shows the accumulation of BdCRN13Cter fusion protein and its derivatives in *Nicotiana* leaves from 1 to 3 days post inoculation.



Supplemental Figure S2

AeCRN13 and BdCRN13 cause delay in cell division and aberrant phenotype in *Xenopus laevis*. Embryos injected with *GFP:AeCRN13Cter or GFP:BdCRN13Cter* mRNA presented delay in cell division leading to larger blastomeres (white square). Giant blastomeres (white arrows) are sometimes observed with both CRN13Cter constructs at stage 9 (7 hours post fertilization). Scale bar : 500µm.



Supplemental Figure S3

The DFA subdomain of CRN13 proteins contains a HNH-like motif. The C-terminal part of AeCRN13 (Ae_9AL5664-1) amino acid sequence was used to BlastP *Phytophthora infestans* (PITG), *Phytophthora sojae* (Ps), *Phytophthora parasitica* (PPTG) and *Batrachochytrium dendrobatidis* (BGEG) using the corresponding databases at the Broad Institute. Box shade analysis was done with amino acid region encompassing the HNH-like motif and located at the C-terminus of the DFA subdomain. This motif first identified in bacterial endonucleases is the second most common motif in Type II restriction endonucleases, and is distributed in all life kingdoms. Asterisks indicated the three most conserved (histidine and asparagine) residues standing for the HNH motif (PFAM: PF13391).

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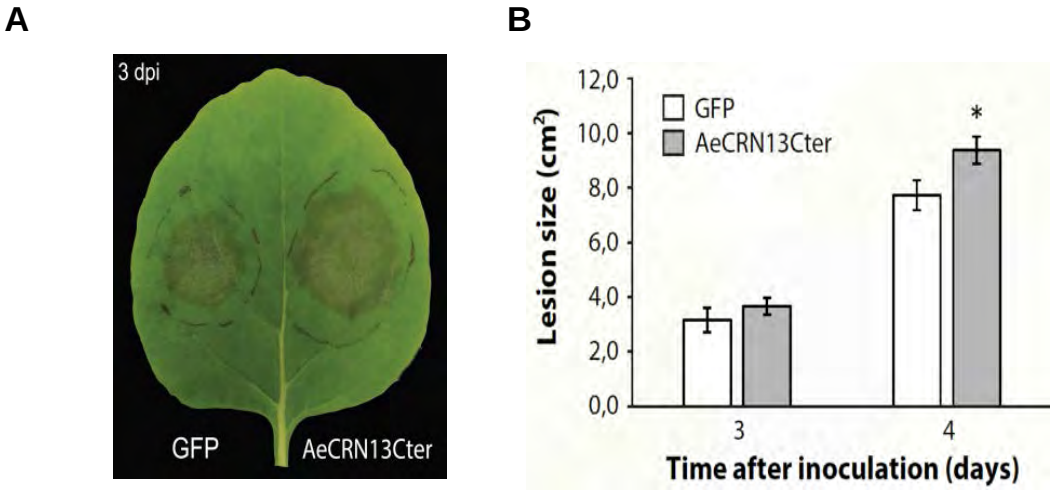
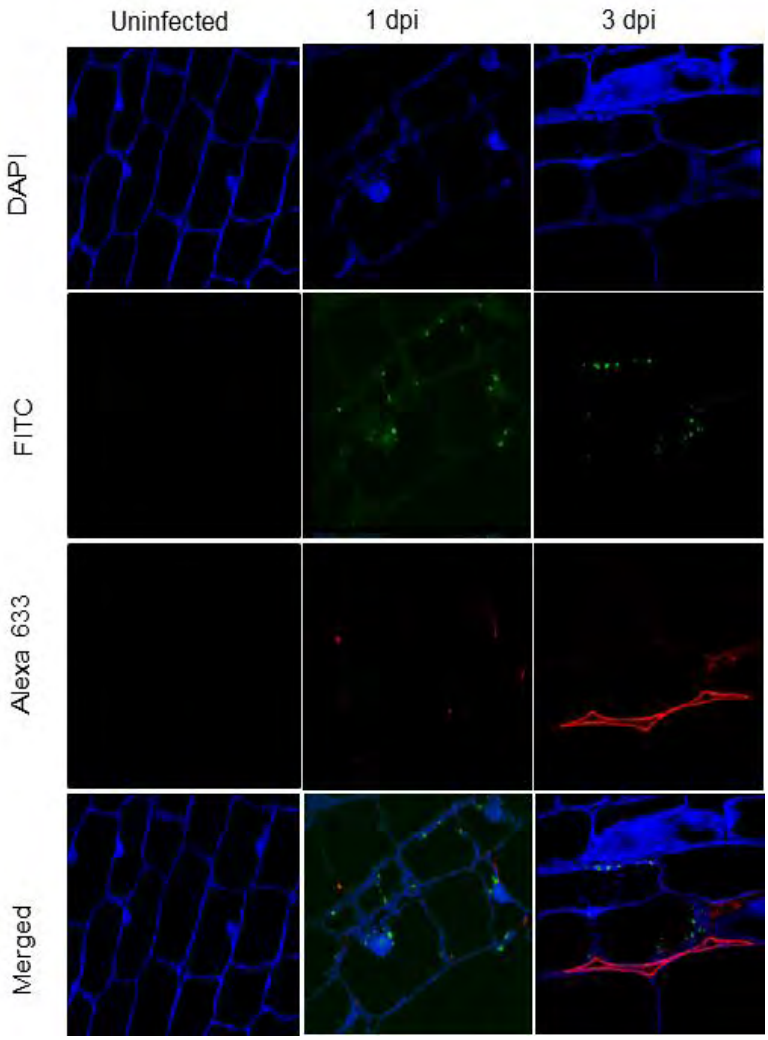
Chapter 1: Complementary results



Complementary figure 1. AeCRN13 presence in plant cells enhances *P. capsici* growth
A. Photograph showing necrotic lesions caused by *P. capsici* 3 days post-inoculation (dpi) on *N. benthamiana* leaves in zones agroinfiltrated with GFP (left side) and GFP:AeCRN13 (right side) constructs. **B**. Graphic showing the average of lesion size (cm2) measured on GFP and AeCRN13 agroinfiltrated zones 3 and 4 dpi.



Complentary figure 2. Secretion and localization of AeCRN13 during infection of roots of *M. truncatula* . Micrographs acquired by confocal microscopy on root sections (1µm thick) of *M. truncatula* infected by *A. euteiches* at 1 and 3 days post-infection (dpi). Anti-AeCRN13 rabbit antibodies were incubated and AeCRN13 signal revealed with anti-rabbit antibodies coupled to FITC (green) while *A. eutiches*'s mycelium was labelled with WGA-Alexa633 (red signal). Roots were treated with DAPI (bleu) to stain nuclei. Images show each signal separately (DAPI, FITC, Alexa633) and merged.

Complementary results

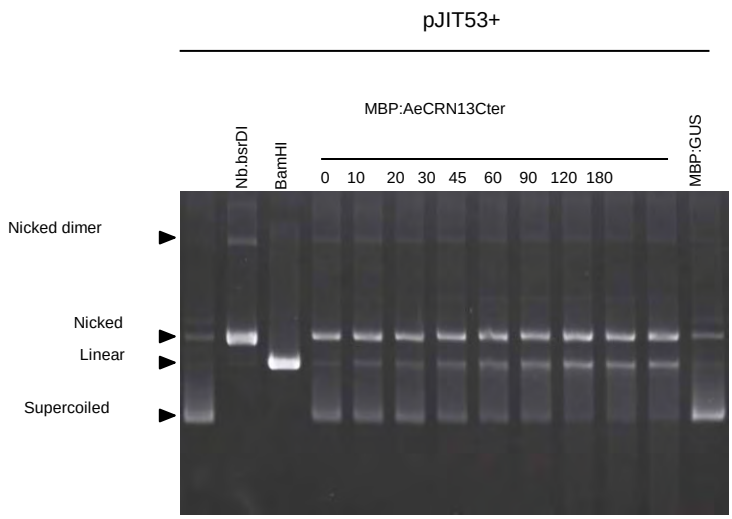
In view of the major points commented by reviewers, we performed complementary experiments to provide answers regarding AeCRN13 contribution to virulence, secretion and localization during infection and nuclease activity.

AeCRN13 enhances *P. capsici in planta* growth

To test the contribution of AeCRN13 to virulence we performed a virulence assay using *P. capsic*i. For this, GFP protein alone and GFP:AeCRN13Cter were transiently expressed side by side on *N. benthamiana* by means of agroinfiltration and detached leaves were challenged on the agroinfiltrated zones by *P.capsici* zoospores 1 day after agroinfiltration. Progression of *P. capsici* on both agroinfiltrated zones was monitored during time and established by the size of necrotic lesions. As shown in complementary figure 1A, 3 days after zoospore inoculation, necrotic lesions were visible on both GFP and AeCRN13Cter agroinfiltrated zones leaves, indicative of *P. capsici* development. Size lesion measures (complementary figure 1B) indicated that lesions were greater for AeCRN13Cter than for GFP zones at 3 dpi and this differential was greater 4 days after. As lesion size depicts *P.capsici's* growth on epidermal tissues, we conclude that the presence of AeCRN13Cer in plant cells contributes positively to the pathogen virulence.

AeCRN13 is secreted during infection into root tissues and addressed to host nuclei.

During our work, we developed anti-AeCRN13 rabbit antibodies raised against the recombinant fusion protein MBP:AeCRN13Cter produced in *E.coli*. We used these antibodies to address the localization of AeCRN13 in roots of *M. truncatula* during infection. Longitudinal sections of infected roots, corresponding to 1 and 3 days of infection were incubated with purified AeCRN13 antibodies and were revealed with secondary anti-rabbit antibodies coupled to FITC. In addition, *A. euteiches* mycelium (cell wall) was labelled using WGA lectin coupled to Alexa 633 fluorochrome and nuclei were stained with DAPI. Same treatments were performed on unifected roots and used as a biological negative control. Internal negative controls (sections not incubated with ACRN13 and/or secondary anti-rabbit antibodies) were used to parameter confocal settings and ensure signal specificity during imaging acquisition. As shown in complementary figure 2, neither FITC nor Alexa 633 signals were detected on uninfected root sections. In infected roots at 1 dpi, *A. euteiches* was



Complementary figure 3. AeCRN13Cter displays a double-stranded nuclease activity *in vitro*

Plasmid DNA (vector pJIT53+) was incubated alone (first lane) during 180 minutes at 37°C with the nicking enzyme Nb.dsrDr, the double stranded cutting enzyme BamHI, with 1µg of recombinant proteins MBP:AeCRN13 and MBP:GUS. The picture corresponds to the electrophoretic analysis of pJIT53+ at the final point reaction (180 min) for the vector alone and controls Nb.bsrDR, BamHI and MBP:GUS and at different time point for MBP:AeCRN13Cter. The different conformations of pJIT53+ DNA molecule are indicated in the left side.

detected intercellularly around root cells. A strong AeCRN13 signal was detected outside *A. euteiches* in circled structures, vesicle-liked, on the apoplasm, and next to host cell nuclei. A faint AeCRN13 signal was detected also inside nuclei. At 3 dpi, these vesicles were still visible and the signal corresponding to *A. euteiches* cell-wall became more apparent confirming with its development in host roots. From these observations, we conclude that AeCRN13 is indeed secreted in roots during infection regardless the presence of a predicted classical signal peptide which suggests that a yet unknwon secretion leader might ensure this function. We evidenced that the release of extracellular vesicles might be at the basis of AeCRN13 secretion. We postulate that AeCRN13 might be released from vesicles neighboring host nuclei as weak signal was recovered also inside nuclei and we explain explained this weak signal as a consequence of AeCRN13 protein diffusion.

While specificity of AeCRN13 antibodies on whole infected root proteins extracts is still under verification, the signal specificity of imaging acquisition revealed for the first time that an oomycetal effector is secreted and addressed into host cells during infection.

AeCRN13Cter displays a double-stranded nuclease activity *in vitro*

AeCRN13 harbors a HNH-like motif withing the end of its DFA Cterminal domain. Because HNH motifs ooccur commonly in proteins with endonuclease activity we hypothesized that AeCRN13 might display such enzymatic activity. We showed that AeCRN13 binds dsDNA *in vitro* and that it induces double-stranded DNA damage *in vivo*. To further sustain is nuclease activity, we set up an *in vitro* assay using the purified recombinant protein MBP:AeCRN13Cter. For this 1 µg of MBP:AeCRN13 were incubated in presence of 1mM of MgCl as a metal ion co-factor and 1µg of pJIT53+ plasmid DNA. The reaction was performed during three hours at 37°C. As a negative control we used the fusion protein MBP:GUS produced and purified using the same conditions as MBP:AeCRN13Cter. To further characterize the different forms of pJIT53+ and a possible activity of AeCRN13Cter on DNA, pJIT53+ was also incubated with commercial enzymes Nb.bsrDr and BamHI, which display a nicking and double stranded cutting activities on DNA, respectively.

As shown in complementary figure 3, pJIT53+ DNA was predominantly in a supercoiled and nicked circular form, presenting low amounts of the linear form before reactions. As expected, control reactions with Nb.bsrDr and BamHI led to the appearance of nicked and linear conformations respectively. Incubation with MBP:AeCRN13Cter resulted in the progressive decrease of the supercoiled form and the increase of the linear form. In latter times (90 min-180 min), a faint smear could be detected, indicative of a total DNA

degradation. No changes of conformation were detected with MBP:GUS protein. Thus, AeCRN13Cter displays a double-stranded nuclease activity on DNA *in vitro*. These results demonstrates that the DNA damage induced *in planta* by agroinfiltration of GFP:AeCRN13Cter is indeed a result of AeCRN13Cter activity on plant DNA.
Having set up this *in vitro* assay, our future experiments will be focused on characterizing AeCRN13 domains implicated in this nuclease activity, notably, the implication of the HNH motif by working with Cterminal deleted versions and point mutated version of the HNH domain.

Materials and methods of complementary experiments.

P. capsici growth assays

GFP:AeCRN13 fusion protein and GFP protein alone were expressed in *N. benthamiana* leaves by agroinfiltration. One day after infiltration, infiltrated leaves were ddetached, placed on plastic dishes and agroinfiltrated zones were drop-inoculated with 5 µL of *P. capsici* (strain LT312) zoospore solution corresponding to 100 zoospores. *P. capsic*i growth was monitored over time. Photographs and leave lesions were assessed 3 and 4 days after inoculation. Lesion areas were measured using Image J software.

Antibody purification.

Polyclonal antibodies against the C-terminal domain of AeCRN13Cter were purified by negative adsorption against proteins from uninfected roots. For this, proteins extracted from uninfected roots of *M.truncatula* were spotted on 25x25 mm nitrocellulose membranes (Protan BA83, GE Healthcare Life Sciences), stained with Ponceau S and washed 3 times with PBS, 0.01% (v/v) Tween20 (PBS-T). Membranes were blocked with PBS-T, 1% (v/v) BSA during 2h at room temperature and incubated with diluted (1:10) sera overnight at 4°C. The obtained sera were used for cytological immunolocalization studies.

Preparation of roots sections and labeling of AeCRN13 and *A.euteiches* cell wall.

Infected and non-infected roots were sampled at 1, 3 and 6 days post inoculation and fixed with 2% (v/v) paraformaldehyde in 50mM of sodium cacodylate buffer, pH 7. Fixed roots were dehydrated in a graded ethanol series and embedded gradually in LR white (LRW) resin (London Resin Company Limited). Resin polymerisation was performed over-

night at 60°C. Roots were sectioned (1µm thick) using an Ultracut microtom (Reichert-Jung) and placed on multi-well slides for immunostaining steps. For this, sections were blocked for 2h30min in PBS, 2 % (v/v) Tween 20, 2 % (w/v) BSA buffer. Sections were then coincubated with purified anti-AeCRN13 rabbit antibodies and with the WGA lectin (Wheat Germ Agglutinin, W21404 Life technologies) conjugated to Alexa633 in blocking solution overnight at 4°C. Slides were washed 3x15 min with PBS, 2% (v/v) Tween 20 and incubated with goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) for 2h at room temperature. Slices were then rinsed and stained with 3µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min and rinsed again before confocal microscopy observation.

Confocal microscopy

Root sections were mounted on oil and scanned with a SP2 AOBS confocal microscope with the following excitation wavelengths: 405nm (DAPI), 488nm (FITC) and 688nm (Alexa688). To avoid signal interference, scanning was performed in a sequential mode. Parameters were set by analyzing all conditions and were the same for all images shown, which were treated using the Image J software.

Nuclease *in vitro* assay activity

1µg of purified proteins MBP: AeCRN13Cter or MBP: GUS (negative control) and restriction enzymes Nb.bsrDI and BamHI (10 units) were incubated in presence of 1µg of plasmid DNA pJIT53+, $MgCl_2$ (10 mM) in a MES (10mM), pH 7 buffer, at 37°C during 180 minutes. The state of DNA was monitored during time by sampling 10µl (≈100 ng) of reaction suspension and adding EDTA (0.2mM) to stop reactions, followed by electrophoresis analysis on an agarose gel (1%) and ethium bromide staining.

Chapter 2: Functional characterization of AeCRN5 of *A. euteiches.*

Chapter 2: Functional characterization of AeCRN5 of *A. euteiches*

Manipulation of plant nuclear biology is emerging as a crucial infection strategy deployed by prokaryotic and eukaryotic microbial pathogens through the secretion of intracellular effectors that target the host nucleus and/or nuclear-related processes (Bhattacharjee et al., 2013). Bacteria nuclear effectors have been shown to suppress plant defenses and manipulate host cellular metabolism by interacting with nuclear host proteins like histones, transcription factors, mRNA regulators as well as host nucleic acids (DNA) (Deslandes & Rivas, 2012). While host-delivery mechanisms, targets and nuclear activities are well characterized for nuclear effectors of bacteria, eukaryotic pathogen effectors are far less characterized.

The study presented in this chapter concerns the characterization of the Cterminal domain of AeCRN5 from the soil borne pathogen *A. euteiches.* AeCRN5 (379 aa) presents Nterminal harboring a LQLYALK (50-55aa) motif ended by a HVVVIVPEVPL (123-130aa) (figure 1 a) that ensures secretion and translocation into plant cells and presents a Cterminus of the DN17 oomycete subfamily containing a nuclear localization signal NLS (149-176aa). Its Cterminus was previously shown to trigger necrosis and to localize in nuclei of epidermal cells of *N. benthamiana* (Schornack et al., 2010). As CRN DN17-like subfamilies have been predicted in fungi *B. dendrobatidis* and *R. irregularis* (Sun et al., 2011; Li et al., 2014), we searched for homolog proteins on proteomes of oomycetes and both fungal species. BlastP resulted on the identification of homolog oomycete CRN ascribed to the DN17 subfamily with which it shares less than 38% identity (supplementary figure 1). The closest CRN sequence was identified in *B. dendrobatidis* with up to 48% identity, while the sequence found in *R. irregularis* only displayed 20% identity.

AeCRN5 was identified in a cDNA library generated from *A. euteiches* grown in close proximity to roots of *M. truncatula*. (Gaulin et al., 2008). The studied of AeCRN5 gene expression during infection showed that AeCRN5 is induced upon interaction with host root with a sustained expression during invasion of cortical root tissues (3-6 dpi) by *A. euteiches* (figure 1b/c). This suggests that AeCRN5 protein is implicated during infection and, thus, likely to be translocated to root cells. To address AeCRN5 effects in *M. truncatula* root cells, we expressed AeCRN5 in roots via *Agrobacterium rhizogenes* -transformation. Composite plants displayed modified root systems with reduced root length and an increased number of roots

(figure 2). Hence, AeCRN5 affects host root architecture, likely by inhibiting root elongation and stimulating root formation.

To rapidly dissect AeCRN5 activity in plant cells, we opted to use *N. benthamiana* ectopic expression system. Exclusion of AeCRN5 from the nucleus prevented its cytotoxic activity (figure 3), indicating that AeCRN5 activity is the result of a perturbation of a plant nuclear-related process.

Localization studies performed on CRNs of *P. capsici* have evidenced distinct nuclear localizations, and since CRN20_624 from *P. capsici*, the only CRN DN17 protein studied so far, was shown to present a punctuated pattern of nuclear localization 2 days after its accumulation in plant cells (Stam et al., 2013), we followed GFP:AeCRN5 nuclear localization over time. We evidenced that AeCRN5 shuttled from the nucleoplasm to unknown nuclear bodies characterized by the absence of DNA material (figure 4). The dynamism of AeCRN5 localization pattern and the absence of DNA led us to hypothesize the possibility of AeCRN5 to be addressed to Speckles, which are highly dynamic nuclear molecular complexes that participate in RNA metabolism and harbor protein factors associated with RNA splicing, RNA capping... . We hypothesize, then, that AeCRN5 could be associated or in close vicinity to RNA.

To first test this, we treated *N. benthamiana* leaves with RNAse and assessed AeCRN5 localization. The treatment resulted in the depletion of the localization to nuclear bodies (figure 4). By FRET-FLIM *in vivo* assay on *N. benthamiana,* we showed that AeCRN5 interacts or is in close vicinity to plant nuclear RNA.

Through this work, we demonstrated that AeCRN5 (DN17) effector interferes with host root development. We showed that it targets plant RNA in the nucleus where it exhibits a dynamic subnuclear localization that depends on RNA. To our knowledge, AeCRN5 is the first eukaryotic effector shown to target RNA. The work provides new insights into the mode of action of eukaryotic effector where the targeting of host RNA seems central.

The corresponding results will be submitted on Molecular Plant Pathology.

AeCRN5 effector from *A. euteiches* targets plant nuclear RNA and worries host root architecture

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AeCRN5 effector from *A. euteiches* targets plant nuclear RNA and worries host root architecture

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Keywords: Effector, Crinkler, Aphanomyces, Medicago, nucleus, RNA, roots

SUMMARY

The Crincklers (or CRN) are a class of translocated effectors produced by oomycete phytopathogens and little is known about their function. Here we report on the function of AeCRN5 from the legume root pathogen *Aphanomyces euteiches*, belonging to a CRN family which is ubiquitous in oomycetes and display orthologs in fungi. AeCRN5 is a modular protein of the CRN effector family containing a functional translocation signal at its N- terminus and a cell-death inducing nuclear C-terminus DN17 domain. The effector expression is induced during colonization of the host root cortex of the legume *Medicago truncatula.* Stable expression of AeCRN5 in *M. truncatula* trigger death of the root system or restricts it drastically. Addition of a nuclear export signal abolished AeCRN5 cell-death inducing activity in *N. benthamiana* leaves. Confocal imaging show that AeCRN5 shuttles in a RNA-dependent manner from plant nuclei to unknown nuclear bodies. FRET-FLIM measurements on *N. benthamiana* leaves demonstrate the close vicinity of AeCRN5 and plant RNA at the nuclear level. These results, combined with the first demonstration that an eukaryotic effector targets plant RNA, reveals a new mode of action of effector where nucleic acid from the host is a central target.

INTRODUCTION

Plant-associated microorganisms rely on the secretion of a particular class of molecules, termed "effectors" to successfully establish infection. These molecules interact with plant targets to modify plant defense responses and to reprogram host physiology, contributing in rendering host niche profitable to sustain growth and spreading of pathogens (Okmen and Doehlemann, 2014). Effectors can be secreted to the apoplastic interfaces (apoplastic effectors) or can be delivered into host cells (intracellular effectors) where they are addressed to different subcellular compartments. A substantial number of microbial effectors are addressed to plant nuclei and their function, assessed mainly through the identification of their plant target, are best characterized in bacteria (Rivas and Genin, 2011). These effectors target different nature of host nuclear factors including proteins, RNA and DNA to perturb plant physiology by, for example, reprogramming host transcription (Deslandes and Rivas, 2012; Bhattacharjee et al., 2013; Canonne and Rivas, 2012). In contrast, the mode of action of nuclear targeted eukaryotic effectors is still elusive.

Oomycetes (Stramenopiles) are eukaryotic filamentous microorganisms comprising several of the most devastating plant pathogens with tremendous impacts on natural and agricultural ecosystems (Thines and Kamoun, 2010). In oomycetes, two main classes of intracellular effectors have been described: the RXLR effectors and Crinklers (CRN), harboring distinct translocation signals. Crinklers (Crinkling and Necrosis, CRN), firstly reported on the potato late blight agent *Phytophthora infestans* (Torto et al., 2003; Haas et al., 2009), are ubiquitous in plant pathogenic oomycetes, with numbers ranging from 45 genes in *Pythium sp* (Lévesque et al., 2010) to 200 genes in *P. infestans* (Haas et al., 2009). All CRNs display a conserved LFLAK N-terminal motif, altered as LYLAK in *Albugo sp* (Kemen et al., 2011), LxLYLAR/K in *Pythium sp* (Lévesque et al., 2010) and LYLALK in *A. euteiches*) (Gaulin et al., 2008) . *Phytophthora* and *Aphanomyces* N-

terminal motifs have been shown to act as host cytoplasm-delivery signals (Schornack et al., 2010). Not all CRNs harbor a predicted signal peptide, although detected by mass spectrometry in culture medium of *P.infestans* (Meijer et al., 2014). CRN Ctermini diversity contrasts to the conservation of Ntermini and is thought be the result of recombinations of different subdomains occurring after a HVLVXXP Nterminal motif that occurs prior to the C-terminus. First reported through a genome mining in *P. infestans*, these subdomains associate, in different combination that define 27 CRN families (Haas et al., 2009), and do not display any significant similarity to known functional domains, except few cases (ie, serine/threonine kinase D2 domain of PiCRN8, (van Damme et al., 2012). Few new CRNs families have been reported upon complete genome analysis of distinct oomycete species (ie, *Phytophthora capsici, Pythium sp., A. euteiches*) suggesting that CRNs belong to an ancient effector family that arose early in oomycete evolution. CRN-like sequences presenting similarities to *Phytophthora* Ctermini were recently evidenced in the genome of the amphibian pathogen fungus *Batrachochytrium dendrobatidis* and the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Sun et al., 2011; Lin et al., 2014).

Oomycetal CRNs Ctermini localize in plant nuclei where they display distinct subcellular localisations including nuclei, nucleoli and unidentified nuclear bodies (Stam et al., 2013a), depicting different nuclear activities and targets. Although initially reported as necrosis-inducing proteins when expressed *in planta*, it has been shown that this is only the case for few CRN as a large number do not cause cell-death (Haas et al., 2009; Shen et al., 2013). *Phytophthora* CRNs have distinct pattern of expression during various life stages and colonization of host plants (Stam et al., 2013c). Several *Phytophthora* CRN can suppress cell death triggered by cell-death inducers or other CRNs (Liu et al., 2011b; Shen et al., 2013), reduce plant defense gene expression or accumulation of reactive oxygen species (ROS) in *N. benthamiana* (Rajput et al., 2014) sustaining the view that CRN might act as suppressors of plant immunity, although not all promote infection (Stam et al.,

2013c). Up to now the precise function of CRN is still elusive, only biochemical activity identified is a kinase activity of CRN8 of *P. infestans* (van Damme et al., 2012).

This work gives first insights into a new mode of action of an eukaryotic effector by deciphering a nuclear activity of AeCRN5 C-terminal region of *A. euteiches*. The soil born pathogen *Aphanomyces euteiches* causes root rot disease on various legumes including alfalfa, clover, snap bean and stands as the most notorious disease agent of pea causing 20 to 100% yield losses and infects the model legume *M. truncatula* (Gaulin et al., 2007). AeCRN5 was firstly identified in a cDNA library from *M. truncatula* roots in contact with *A. euteiches* (Gaulin et al., 2008). It presents a modular architecture with a N-terminal functional LYLALK ensuring host delivery (Schornack et al., 2010) and a DN17 family domain at its Cterminus that triggers cell-death *in planta*. Here we showed that AeCRN5 is induced during the cortex colonization of *Medicago truncatula* roots and that its overexpression in host affects drastically *M. truncatula* root development. Confocal studies on *N. benthamiana* leaves showed that the cell-death inducing activity of AeCRN5 required its nuclear localization. Moreover the observed dynamic relocalization of AeCRN5 from nucleoplasm to unknown nuclear bodies required plant RNA. Finally FRET-FLIM measurements revealed the close vicinity of AeCRN5 Cterminus and RNA at the plant nuclear level. These results indicate that CRN DN17 family function by targeting host plant nucleic acid.

RESULTS

AeCRN5 is a CRN DN17 family protein and is expressed during *M. truncatula* roots infection

Oomycete CRNs proteins display a modular architecture, with conserved N-terminal translocation regions (containing highly conserved LFLAK and HVLVXXP motifs)

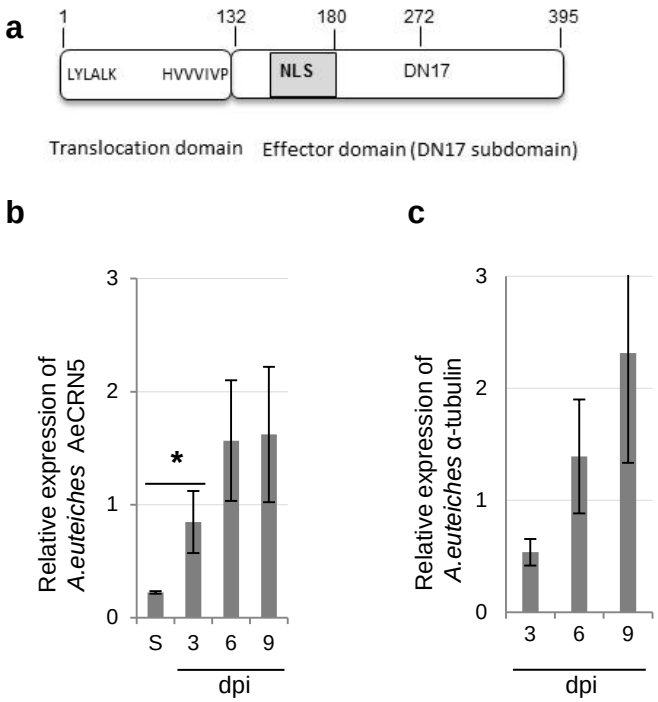

Fig 1. AeCRN5 from the oomycete *Aphanomyces euteiches* is a CRN DN17 effector family expressed during infection of *Medicago truncatula* roots. (a) Schematic representation depicting AeCRN5 protein architecture from the root rot pathogen of legumes *A. euteiches*. At the Nterminus a translocation domain characterized by a LYLALK and HVVVIVP conserved sequence that preceeds the putative DN17_like effector domain (Cterminus) in which a NLS is predicted. Number indicates amino acid positions (b) Graphs show expression level of AeCRN5 (left) and *A. euteiches* α-tubulin (right) measured by qRT-PCR at 3, 6 and 9 days post-inoculation (dpi) in *M. truncatula* roots. S: free-living mycelium. Values are the mean of three independant biological replicates. Error bars are standard deviation errors. Asterisk indicate that the values are significantly different (p-value<0.05, t-test).

followed by variable C-terminal regions organized in subdomain combinations that specify effector function (Haas et al., 2009). The AeCRN5 (Ae_1AL4462, http://www.polebio.scsv.ups- tlse.fr/aphano/) Nterminus (1-132aa) is characterized by a LQLYALK (50-55aa) motif and a HVVVIVPEVPL (123-130aa) motif marking its end (Figure 1a). Although it lacks a predicted signal peptide, the AeCRN5 Nterminus is a functional secretion domain mediating translocation of oomycetal effectors to plant cell (Schornack et al.2010). The Cterminal region shows a sequence identity of 38% with the CRN DN17 family domain of *P. infestans* (Haas et al.2010) and harbors a nuclear localisation signal (NLS 149-176aa) consistent with its plant nuclear localization when expressed in *Nicotiana benthamiana* leaves (Schornack et al., 2010). CRN- like sequences including DN17 family have been recently reported in the fungus *Batrachochytrium dendrobatidis* (Bd), a pathogen of amphibians, and the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (*Ri*) (Sun et al., 2011; Lin et al., 2014). The homology of CRN proteins from the DN17 subfamily is most obvious in the C-terminal region. Sequence comparison showed that AeCRN5 is closest to the *Bd* homolog (45% identity) than to oomycetes CRN (less than 38% identity) (Supplemental Figure 1). The DN17 homolog in *R. irregularis* displayed less than 20% identity with AeCRN5 at its Cterminal region and harbored an extension of about 20 amino acid residues, suggesting distinct function and/or localization for this candidate effector.

AeCRN5 was firstly identified in a cDNA library from *A. euteiches* mycelium grown in close vicinity of *Medicago trunctaula* roots (Gaulin et al., 2007). To characterize its expression during infection, we conducted qRT-PCR analyses on saprophytic mycelium and on infected roots of *M. truncatula*. AeCRN5 is expressed in saprophytic mycelium and induced at the early stage of the infection of *M. trunctaula* (3-6 dpi) (Figure 1b). This expression is correlated to a sustained development of the infectious mycelium as shown by *A. euteiches* quantification in roots (Figure 1c) and corresponds to an infection stage where

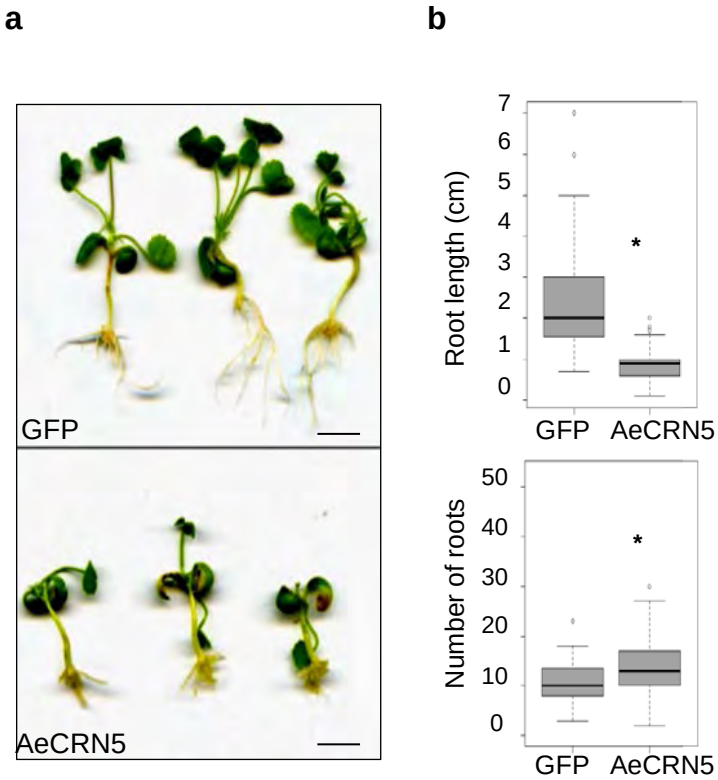

Fig 2. AeCRN5 modifies host root architecture. *M. truncatula* plantlets were transformed with *A. rhizogenes* to express GFP (control) and GFP-tagged AeCRN5 Cterminal domain (AeCRN5) constructs. (a) Photographs of 30 day-old composite plants showing representative phenotypes of composite plants and their effects on overall development and on root system architecture. Scale bars: 1 cm. (b) Box-plot graphics presenting the average of length and root number per root system. Measures and statistical analyses were performed on n=60 (GFP), n=145 (AeCRN5). * (*t- test*, p-value<0.05).

most of the cortical root tissues are colonized (Djébali et al., 2009).

AeCRN5 perturbs root architecture of the host plant *M. truncatula*

AeCRN5 Ctermini (DN17) induces cell death symptoms when overexpress in *Nicotiana benthamiana* leaves (Schornack et al. 2010). To characterize AeCRN5 activity in host cells, we transformed *M. truncatula* roots with a GFP-tagged AeCRN5 Ctermini (130-370) using *Agrobacterium rhizogenes*-mediated transformatiom system (Boisson-Dernier et al., 2001). Within two weeks after transformation, a large number of the plantlets collapsed without generating new roots, in contrast to control plants suggesting a cytotoxic activity for AeCRN5 in *M. truncatula*. Within three weeks, plants that developed presented a reduction of development of aerial and root systems (Figure 2a). Quantification of root length and number (Figure 2b) indicated that AeCRN5 transformed roots presented a decrease of root length and a higher number of roots as compare to GFP- control plants. These observations indicate that AeCRN5 worries the root architecture of the host plant *M. truncatula* by an unknown mechanism.

AeCRN5 cell-death inducing activity requires nuclear localization

To go further we assessed whether the observed cytoxic effect of AeCRN5 Cterminus on *Nicotiana* and *Medicago* plants is the result of a nuclear-related localization. For this purpose a Nuclear Export Signal (NES) or its mutated (mNES) counterpart was fused Nterminally AeCRN5 Cterminus. The resultant fusion proteins were Nterm GFP tagged and the constructs were expressed in *N. benthamiana* leaves by agroinfiltration. Necrotic lesions were observed within 5 days with AeCRN5 construct, whereas no symptoms were detected on leaves treated with NES:AeCRN5, even at longer times (>8

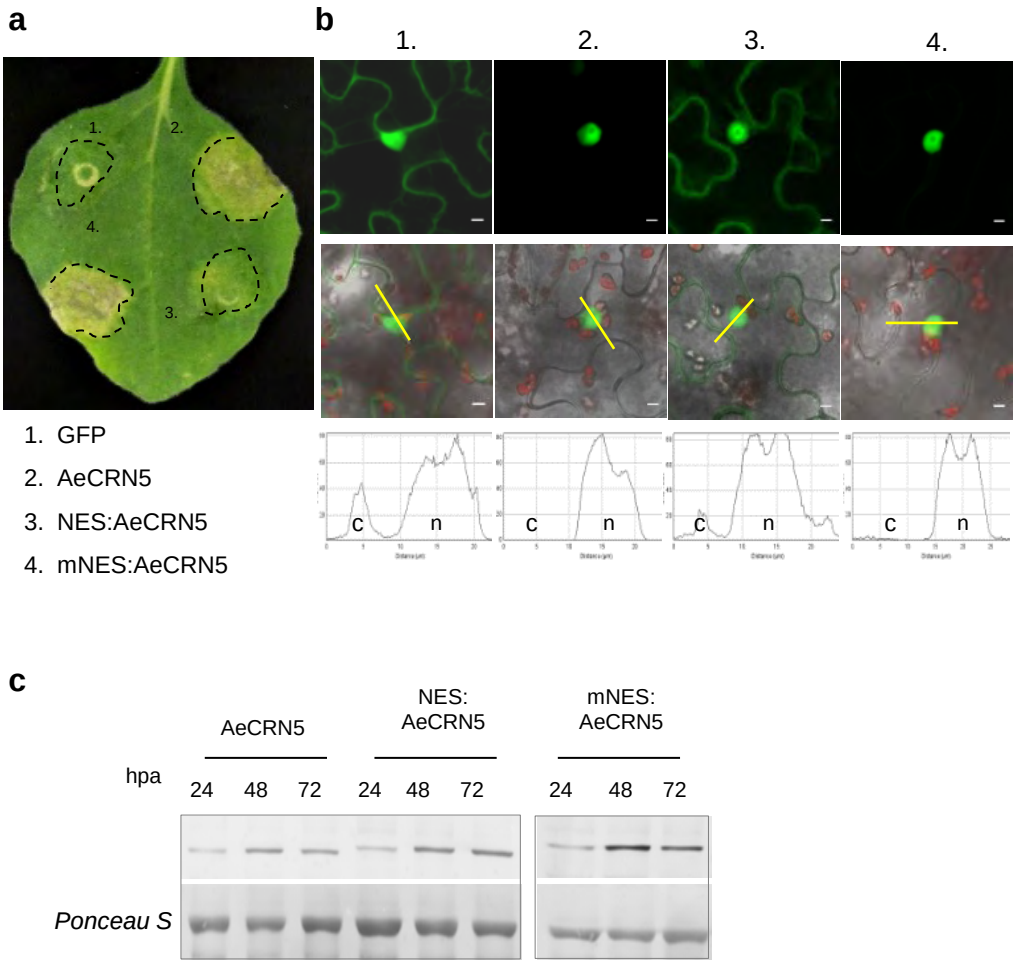

Fig 3. AeCRN5 nuclear localization is required for cell-death inducing activity. (a) Representative symptoms on *N. benthamiana* leaves expressing the indicated constructs (scale bar: 1 cm). Photographs were taken 5 days after agroinfection. (b) Micrographs of epidermal cells acquired 24h after agroifiltration by confocal imaging. Upper panels shown the localization of GFP and GFP-tagged fusion proteins in *N. benthamiana* while middle panels show merged channels in which chloroplasts's autofluorescence appears in red while yellow lines indicate sections measured for GFP signal intensity whoan in lower panels (Scale bars : 5 µm). (c) Western-blot analysis of expression of the indicated GFP-tagged fusion proteins in leaves of *N. benthamiana* 24, 48, and 72 hours post-agroinfiltration (hpa). Blots were probed with GFP antibodies.

days) (Figure 3a). The addition of a mNES, restored the cytotoxic activity of AeCRN5. Confocal microscopy imaging carried 24h after agroinfiltration confirmed that GFP-AeCRN5 fusion protein was restricted to the nucleus (Figure 3b). An enhancement of nuclear export of AeCRN5 protein was detected with NES:AeCRN5 construct, since the GFP signal was recovered also in the cytoplasm. Fluoresence intensity, measured in cells, corroborated NES:AeCRN5 partial mislocalization from the nucleus (Figure 3b, lower panels). A reestablishment of green fluorescence at the nuclear level was obtained for the mNES:AeCRN5 construct. Immunoblot analysis confirmed the accumulation of the fusion proteins from 1 to 3 days after agroinfection (Figure 3c). Altogether the results showed that the cell death phenotype requires AeCRN5 to localize and accumulate in the nucleus.

AeCRN5 accumulates in subnuclear structures and does not co-localize with nuclear DNA

Recent evidences on *P. caspsici* CRNs suggested a link between *N. benthamiana* subnuclear localization and PcCRN activity (Stam et al., 2013b). We thus precised GFP-AeCRN5 Cterminus localization using agrobacterium-mediated transient expression assay on *N. benthamiana* leaves. Two distinct subnuclear localizations of AeCRN5 were observed (Figure 4a-b). As reported above, the fluorescence was either dispersed throughout the nucleoplasm with the exception of the nucleolus or sequestered in patches in unknown nuclear bodies (Figure 4a-c). Both distributions were observed during time. This dynamics and localization pattern in unknown nuclear bodies evokes ‘nuclear speckles pattern. Nuclear speckles are small subnuclear membraneless organelles which appear as irregular and transient punctuate structures at the microscopic level located in interchromatin regions of the nucleoplasm (Lorković et al., 2008; Spector and Lamond, 2011). Speckles act as a reservoir of factors that participate in transcription and pre-mRNA processing. Thereby we evaluated

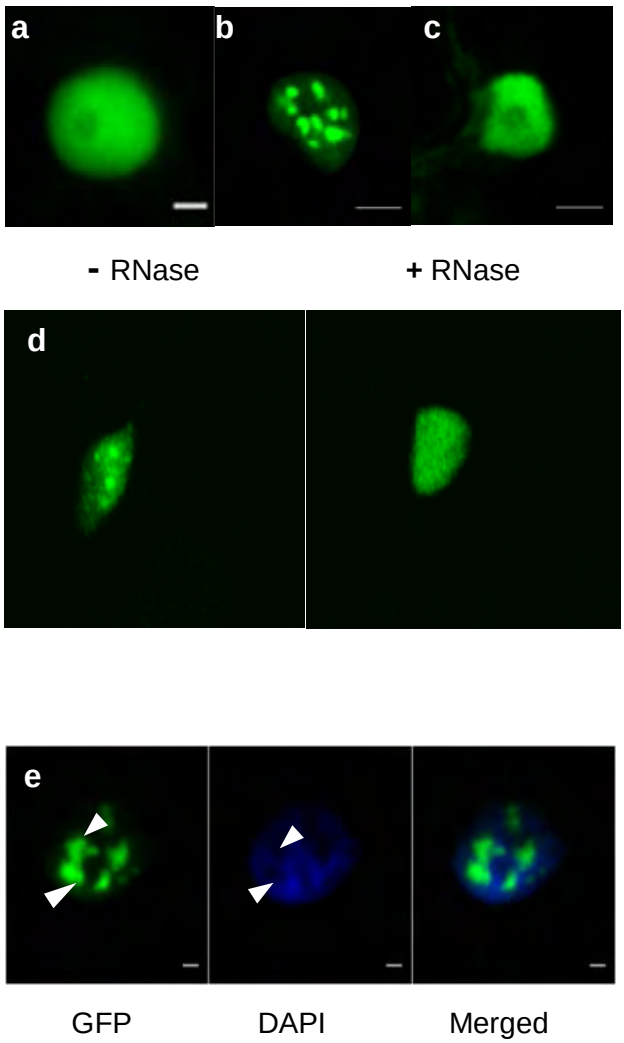

Fig 4. AeCRN5 shuttle from nucleoplasm to unknown nuclear bodies in a RNA-dependent manner Confocal images of *N. benthamiana* leaves expressing GFP-tagged AeCRN5 effector at 1 day post-agroinfiltration. (a-c) Representative micrographs of AeCRN5 subnuclear localisation in which close-up images revealed either an homogeneous (a) or a 'patched' (b) distribution of fluorescence. This pattern is reminiscent with 'nuclear speckle pattern' (white arrows) (Scale bar 5µm). (d) Confocal micrographs depicting AeCRN5 subnuclear localisation after RNAse treatment on cells, where the fluorescence is recovered only in the nucleoplasm with the exception of the nucleolus. (e) Representative images of a "patched" AeCRN5 localization where AeCRN5 distribution (white arrows) is shown in the GFP channel and chromatin distribution is revealed by DAPI labelling (DAPI). Merged signal indicates and absence of DNA material in zones where AeCRN5 localizes (Scale bar 5µm).

whether subcellular localization of AeCRN5 is a RNA-dependent mechanims. As shown on Figure 4d, RNAse treatment on *N. benthamiana* epidermal cells expressing GFP:AeCRN5 abolished the 'speckle pattern' of AeCRN5. Moreover a counterstaining with the nucleic acid stain 4',6-diamidino-2- phenylindole (DAPI), revealed an absence of complementary fluorescence pattern in which nuclear DNA and GFP fluorescence do not colocalized (Figure 4e). These data suggest a dynamic RNA-dependent process for AeCRN5 nuclear localization and therefore activity, where plant DNA might not be a central player.

AeCRN5 targets nuclear RNA *in planta*

We reasoned that AeCRN5 perturbs a plant nuclear-related process leading to cytotoxic activity, probably by targeting plant nuclear components. We firstly assessed the possibility that AeCRN5, like numerous nuclear-targeting bacterial effectors, may be able to bind plant nucleic acids. We thus set up a fluorescence resonance energy transfer (FRET) assay coupled with fluorescence lifetime imaging microscopy (FLIM) in *N. benthamiana* cells. GFP:AeCRN5 and GFP alone were expressed in *N. benthamiana* epidermal cells by agroinfiltration and plant nucleic acids were stained with SytoxOrange. In these conditions, the occurrence of FRET, causing the fluorescence lifetime of the donor GFP to decrease, would only be due to the proximity (less than 10 nm) of GFP to SytoxOrange. Energy transfer was detected by fluorescence lifetime imaging microscopy (FLIM). Additionally to GFP alone, we used cells expressing the DNA-binding protein H2B in fusion to GFP (GFP:H2B).

As reported in Table 1, in presence of Sytox Orange, GFP presented a mean life-time of 2.210 +/- 0.041 ns. Coupled to H2B, its life-time was reduced to 1.852 +/-0.047 nsec attesting that, by interacting to DNA, H2B places GFP close to the a protein energy acceptor. In the case of AeCRN5 construct, the lifetime of GFP:AeCRN5 was 2.128+/-

Table 1. Summary of FRET-FLIM conditions and values obtained on foliar discs of *N. benthamiana* expressing the different fluorescent donor proteins.

Donor	Acceptor	τ (a)	s.e.m (b)	N (c)	E (d)	p-value (e)
GFP	-	2.246	0.036	20	-	-
	Sytox Orange	2.210	0.0417	18	1.6	0.52
GFP:H2B	-	2.465	0,0167	40	-	-
	Sytox Orange	1.852	0,0472	43	24.8	2.31E-19
GFP:AeCRN5	-	2.128	0,0692	27	-	-
	Sytox Orange	1.899	0,0698	10	10.7	5.56E-07
	Sytox Orange (RNAse treatment)	2.228	0,1057	17	0	0.55

t : mean GFP lifetime in nanoseconds (ns). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with exponential function using a non-linear least-squares estimation procedure. (b) s.e.m.: standard error of the mean. (c) N: total number of measured nuclei. (d) E: FRET efficiency in % : E=1- t(DA/ t D). (e) p-value (Student's t test) of the difference between the donor lifetimes in the presence or absence of acceptor

0.069 nsec in the absence of SytoxOrange and decreased significantly to 1.899 +/-0.069 nsec in presence of the dye, indicating an energy transfer and thereby the binding of DN17 domain to nucleic acids. To go further, and since Sytox Orange dye is not specific for either DNA or RNA, samples were treated with RNase A to digest RNA before performing the experiment. After RNase treatment, the 2.228 +/-0.105 nsec lifetime of GFP:AeCRN5 was similar than those observed without Sytox Orange (2.128 2.228 +/-0.069 nsec) (Table 1). Hence in these conditions FRET phenomenon was only RNA-dependent and indicated that DN17 domain of AeCRN5 targets nuclear RNA *in vivo*.

DISCUSSION

To favor the establishment of disease, microorganisms have gained the ability to deliver effector molecules inside host cells. The important number of effectors targeting host nuclei places this organelle, and functions related to it, as important hubs whose perturbations might be of crucial importance for the outcome of infection (Bhattacharjee et al., 2013; Deslandes & Rivas, 2012). CRNs proteins are ubiquitous in plant pathogenic oomycetes and are reported as a wide class of translocated effectors (Schornack et al., 2010). Previously, we showed that the cell-death inducing AeCRN5 effector from the root rot pathogen *A. euteiches* is a plant nuclear localized effector with a functional Nterminus translocation signal (Schornack et al., 2010). In this work we showed that AeCRN5 perturbs root development of the host plant *Medicago truncatula* and targets plant RNA at the nuclear level.

Oomycetal CRN proteins present a modular architecture and include a conserved Ntermini functioning in plant delivery and diverse Cterminal domains thought to direct the activity (Haas et al., 2009). AeCRN5 is a modular CRN DN17 protein family

with orthologous sequences in *Phytophthora* sp. and true fungal species including the chytrid *B. dendrobatidis* and the ectomycorrhiza *R. irregularis*. The functional translocation signal of AeCRN5 is characterized by a LYLALK and a HVVVIP motifs and the absence of an obvious signal peptide (Schornack et al., 2010, Gaulin et al., 2007). The C-terminus corresponds to a CRN DN17 domain family with a NLS (Haas et al., 2009, Schornack et al., 2010) which have no significant similarity to functional domain. The closest ortholog of AeCRN5 is found in the *Bd* fungus rather than *Phytophthora* species, suggesting a conserved function for this ortholgous gene. In contrast the sequences features of DN17 from *R. irregularis* may indicate distinct cellular localization and function for this candidate effector.

Phytophthora CRNs were originally identified as activators of plant cell death upon their *in planta* expression (Torto et al., 2003), although not all CRNs promote infection including the AeCRN5 ortholog from *P. capsici* (Stam et al., 2013b). CRN5 sequences from *A. euteiches* were firstly reported in a cDNA libray from mycelium grown in close vicinity of *M. truncatula* roots (Gaulin et al., 2008) and the corresponding gene models are present in the complete genome sequence of ATTC201684 *A. euteiches* strain (Gaulin et al.,unpublished results). Here we showed by qRT-PCR analysis, that AeCRN5 is expressed during vegetative growth and expression goes up from 3-6 days after infection of roots, a stage where browning of roots is observed in combination to an entire colonization of the root cortex of *M. truncatula*, and the initiation of propagation to vascular tissues (Djébali et al., 2009). Recently two classes of *P. capsici* CRN expression were defined based on contrasted expression pattern during host leaves infection (Stam et al., 2013b) . *P. capsici* DN17 ortholog felt in class 2 with genes expressed in the latest stages of *Solanum lypersicum* infection, suggesting an important role of PcCRN5 at the later stages of colonization. However another similar study on *P.phaseoli* CRN showed that CRNs were mostly repressed in leaves of lima bean (Kunjeti et al., 2012). Thus for conclusive

statements, large gene expression studies of AeCRNs repertoire (> 100 genes models, Gaulin et al., unpublished) are required to precise the putative contribution of AeCRN5 and each AeCRN class during the different stages of *M. truncatula* infection.

We further explored the function of AeCRN5 by using a GFP-DN17 tagged version of the domain. Overexpression of AeCRN5 in *M. truncatula* roots displayed a cytotoxic effect leading in few days to death of transformed plants. The surviving plants were dwarf and harbored reduced root systems with a higher number of roots. These results corroborate observations made during *M. truncatula* roots infection, where susceptible accessions present, within few days after *A. euteiches* infection, a decrease of secondary root development and necrosis of roots (Djébali et al., 2009).

Confocal studies on transiently transformed *N. benthamiana* leaves showed that DN17 cytotoxic effect of AeCRN5 required a plant nuclear accumulation. It is in accordance with the observed reduction of cell death on *N. benthamiana* leaves, upon nuclear exclusion of CRN8 (D2 domain) from *P. infestans* (Schornack et al., 2010). We can therefore suggest that nuclear localization is an important requirement for the cell-death inducing activity of necrotic CRN effectors. Previous study on *P. capsici* DN17 CRN domain has shown its nucleoplasmic localization in *N. benthamiana* leaves upon its expression. Interestingly, we observed here that DN17 shuttles between nucleoplasm and unknown plant nuclear bodies where DNA is excluded. This subnuclear relocation of AeCRN5 is RNA dependent. In addition FRET-FLIM measurements revealed the close vicinity of DN17 with plant RNA at the nuclear level. The dynamics and punctuate localization of DN17 in combination with its proximity to plant RNA, strongly suggest a ‘nuclear speckled pattern’. Nuclear speckles are nuclear granules of variable size and irregular shape, which do not contain DNA and often observed close to highly active transcription site. Nuclear speckles are best known to accumulate spliceosome subunit, splicing factor and many factors involved in mRNA production (Spector and Lamond, 2011). Our results support the

idea that AeCRN5 might be recruited t o speckles and, thus, suggests that AeCRN5 could affect directly or indirectly splicing activities on RNA molecules Future studies will aim to precise the subnuclear localization of AeCRN5 and its effect on host transcriptional activity.

To our knowledge AeCRN5 is the first effector from eukaryotic plant pathogenic microbes known to interfere with nuclear RNA, probably leading to host cytotoxic effect. We are aware that *M. truncatula* root growth inhibition or cell-death inducing symptom s on *N. benthamiana* leaves may be the result of AeCRN5 overexpression. Nonetheless, perturbation of plant development caused by pathogens to facilitate microbial establishment through an undirect manipulation of host RNA machinery have been documented for prokaryotic effector. For instance, TAL effectors (ie, PthA4) from *Xanthomonas citri* target negative regulators of RNA Pol II and Pol III to coordinately increase the transcription activity of host cells thought to enhance bacterial installation (de Souza et al., 2012; Soprano et al., 2013). Microbial manipulation of RNA machinery to subvert host immunity may be another mode of action of effector. Recently two RXLR nuclear-localized effectors from *Phytophthora sojae* were shown to inhibit the biogenesis of small RNA probably by targeting plant DICER-like protein or cofactors, to promote infection (Qiao et al., 2013). In fungi, 72 of the 492 candidate effectors of the fungus *Blumeria graminis* show structural similarity to ribonucleases (Pedersen et al., 2012a). Some of them contribute to infection (BEC1054) and their activity is supposed to be related to host RNA binding (Pliego et al., 2013). Targeting of host RNA has also been proposed for effectors of the nematode *M. incognita* as they present putative RNA binding domains (Bellafiore et al., 2008).

This study shows that the ancestral oomycete *A. euteiches* has evolved plant nuclear CRN effectors that interfere with plant RNA and gives first insights into a new mode of action of eukaryotic effectors. The nature of RNA targeted by DN17 domain as well as interactors that could be involved in such process will provide further understanding on

CRN function and further elucidation of mechanisms by which pathogens may manipulate host RNA machinery and related functions.

EXPERIMENTAL PROCEDURES

Plant material, microbial strains, and growth conditions

M. truncatula F83005.5 seeds were scarified, sterilized, and cultured *in vitro* for root transformation of and infection as previously described (Boisson-Dernier et al.,2001; Djébali et al., 2009). Infection of roots with zoospores of *A. euteiches* (strain ATCC 201684) was performed as Djébali et al., 2009. *N. benthamiana* plants were grown from seeds in growth chambers at 70% of humidity with a 16h/8h dark at 24/ 20°C temperature regime. *A. euteiches* (ATCC 201684) was grown on saprophytic conditions as previously reported (Badreddine et al., 2008). All *E.coli* strains (DH5α, DB3.5, BL21AI). *A. tumefaciens* (GV3101:: pMP90RK) and *A. rhizogenes* (ArquaI) used were grown in LB medium with the appropriate antibiotics.

Sequence analyses

Oomycetal and fungal orthologs of AeCRN5 (Ae_1AL4462, http://www.polebio.scsv.ups- tlse.fr/aphano/) was retrieved by BlastP searches using Broad Institute Database Search tools (http://www.broadinstitute.org) on the corresponding *P. sojae* (Ps_132663) and *P. ramorum* (pRg882635), *P. capsici* (Pc_CRN20_624) and *Batrachochytrium dendrobatidis* (BD_07009.1) genome database. The *Rhizophagus irregularis* ortholog sequence ((Ri, gm1.18625_g) was collected from (Lin et al., 2014); CRN DN17 consensus sequence was retrieved from supplementary data of Haas et al., 2009. All sequences alignments were performed using ClustalW2.

RNA extraction and qRT-PCR

Samples were ground on liquid nitrogen and total RNA extracted using the RNAeasy kit (Qiagen). Reverse transcription was performed on 1µg of total RNA using the AppliedBiosystems kit (Life Technologies-Invitrogen). cDNAs were diluted 50- fold for qPCR reaction. Each qPCR reaction was performed on a final volume of 10µl corresponding to 8 µl of PCR mix (0.5µM of each primer and 5µl SYBRGreen, Applied Biosystems) and 2µl of the diluted cDNA and was conducted on a ABI Prims SDS 7900 HT (AppliedBiosystems, Foster City, CA, USA) device using the following conditions: 5min at 95°C, followed by 45 cycles of 15 s at 95°C and 1min at 60°C. Dissociation curves were obtained by applying a 15s 95°C, 15s 95°C and 15s 95°C cycle. Each reaction was conducted on triplicates for cDNAs of four biological replicates. Primers F:5'- GAAATTCTGCAAGAACTCCA-3' and R:5'- CAATAAAGATGTTGAGAGTGGC-3' were used for the detection of AeCRN5. Primers F: 5'-TGTCGACCCACTCCTTGTTG-3' and R: 5'-TCGTGAGGGACGAGATGACT-3' were used to assess the expression of *A. euteiches*'s α-tubulin gene (Ae_22AL7226) and normalized AeCRN5 expression. Histone 3-like of *M. truncatula*, previously described (Rey et al., 2013) was used to normalize *A. euteiches* abundance during infection. Relative expression of AeCRN5 and α-tubulin genes were calculated using the 2-ΔΔCq method described by (Livak and Schmittgen, 2001).

Construction of plasmid vectors

Sequences and names of primers used in this study are listed in the supplementary Table 1. AeCRN5 Cterminal version carrying Gateway adaptors were generated by PCR on a

template corresponding to the unigene Ae_1AL4462 (vector pSport_Ae_1AL4462). Full length Cterminus AeCRN5 (132aa-370aa) was generated using primer AttB1AeCRN5-F and AttB2AeCRN5-R. Amplicon was BP recombined in pDONR-Zéo vector (Invitrogen) and subsequently introduced in vector pK7GWF2 by means of LR recombination (Invitrogen). *GFP:NES:AeCRN5* and *GFP:mNES:AeCRN5* constructs were generated by adding NES sequence (LQLPPLERLTL) and non-functional mutated NES sequence (mNES: LQAPPAERATL) to the Nterminal moiety of AeCRN5. Amplicons NES:AeCRN5 and mNES:AeCRN5 were obtained using primers NESAeCRN5-F and AeCRN5_end-R and mNES_AeCRN5-F and AeCRN5_end-R respectively and introduced in pENTR/ D-TOPO vector by means of TOPO cloning (Invitrogen) before insertion on vector pK7GWF2. Amplification of the histone 2B of *A. thaliana* was performed on vector pBI121:H2B:YFP REF with primers caccH2B-F and H2B-R. Amplicons were cloned in pENTR/ D-TOPO and subsequently introduced in vector pK7GWF2 to obtain *GFP:H2B* fusion construct. The obtained pK7GWF2 recombined vectors were introduced in *Agrobacterium* strains for *N. benthamiana* agroinfiltration assays and/or *M. truncatula* roots transformation.

Immunoblot analyses

Samples corresponding to agroinfiltrated *N. benthamiana* leaves were frozen in liquid nitrogen. Protein extraction was performed as previously reported (Schornack et al., 2010). Proteins were separated by SDS-PAGE and electroblotted on nitrocellulose membranes (Amersham BioSciences). AeCRN5 and the respective mutant versions were revealed using anti-GFP polyclonal antibodies1:2000, Clontech) and anti-rabbit secondary antibodies coupled to alkaline phosphatase (Sigma-Aldrich).

Agrobacterium-mediated transformation

Generation of *M. truncatula* composite plants was performed as described by Boisson-dernier et al, 2001 using ARQUA-1 (*A. rhizogenes*) strain. Leaf infiltration were performed with *A. tumefaciens* (GV3101:: pMP90RK) as previously reported (Schornack et al., 2010).

Confocal microscopy

Foliar discs (5-8 mm of diameter) of infiltrated leaves of *N. benthamiana* were sampled at different time points after agroinfiltration and fixed in a PBS, 4% (v/v) paraformaldehyde solution and then stained with DAPI (3µg/µL). Scans were performed on a Leica TCS SP2 AOBS device using wavelengths 488nm (GFP) and 350 nm (DAPI) and with a 40x water immersion lens. Acquisitions were done in a sequential mode to avoid overlapping fluorescence signals. Images were treated with Image J software and correspond to Z projections of scanned tissues.

Preparation of *N. benthamiana* epidermal leaves for FRET / FLIM experiments

Discs of agroinfiltrated *N. benthamiana* leaves were fixed 24 hours after treatment by vacuum infiltrating a TBS (TRIS 25mM, NaCl 140 mM, KCl 3 mM) 4 % (w/v) paraformaldehyde solution before incubation 20 min at 4°C. Samples were permeabilized 10 min at 37°C using a TBS buffer supplemented with 20µg/ml of proteinase K (Invitrogen). Nucleic acid staining was performed by vacuum-infiltration of a 5µM of Sytox Orange (Invitrogen) solution, before incubation of the samples 30 min at room temperature. When RNAse treatment was performed, foliar discs were incubated 15 min at room

temperature with 0.5µg/ml of RNAse A (Roche) before acid nucleic staining. Foliar discs were washed with and mounted on TBS before observations on an inverted microscope (Eclipse TE2000E, Nikon, Japan).

FRET/FLIM measurements

Fluorescence lifetime measurements were performed in time domain using a streak camera REF53. The light source is a mode-locked Ti:sapphire laser (Tsunami, model 3941, Spectra- Physics, USA) pumped by a 10W diode laser (Millennia Pro, Spectra-Physics) and delivering ultrafast femtosecond pulses of light with a fundamental frequency of 80MHz. A pulse picker (model 3980, Spectra-Physics) is used to reduce the repetition rate to 2MHz to satisfy the requirements of the triggering unit (working at 2MHz). The experiments were carried out at λ= 820 nm (multiphoton excitation mode). All images were acquired with a 60x oil immersion lens (plan APO 1.4 N.A., IR) mounted on an inverted microscope (Eclipse TE2000E, Nikon, Japan). The fluorescence emission is directed back into the detection unit through a short pass filter λ<750 nm) and a band pass filter (515/30 nm). The detector is a streak camera (Streakscope C4334, Hamamatsu Photonics, Japan) coupled to a fast and high-sensitivity CCD camera (model C8800-53C, Hamamatsu). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with exponential function using a non-linear least-squares estimation procedure. Fluorescence lifetime of the donor (GFP) was experimentally measured in the presence and absence of the acceptor (Sytox Orange). FRET efficiency (E) was calculated by comparing the lifetime of the donor in the presence (τDA) or absence (τD) of the acceptor: E=1-(τDA) / (τD). Statistical comparisons between control (donor) and assay (donor + acceptor) lifetime values were performed by Student t-test. For each experiment, four leaf discs removed from two agroinfiltrated leaves were used to collect data.

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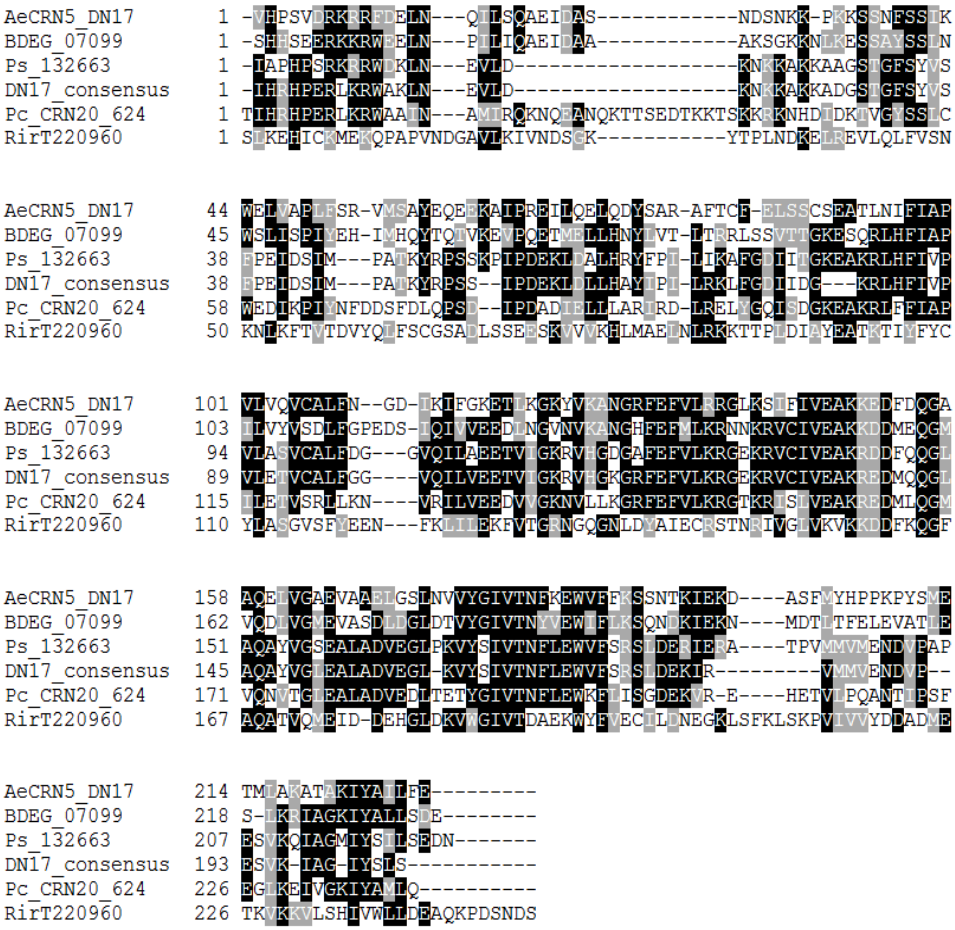
Table 1. Summary of FRET-FLIM conditions and values obtained on foliar discs of *N. benthamiana* expressing the different fluorescent donor proteins.

Donor	Acceptor	τ (a)	s.e.m (b)	N (c)	E (d)	p-value (e)
GFP	-	2.246	0.036	20	-	-
	Sytox Orange	2.210	0.0417	18	1.6	0.52
GFP:H2B	-	2.465	0,0167	40	-	-
	Sytox Orange	1.852	0,0472	43	24.8	2.31E-19
GFP:AeCRN5	-	2.128	0,0692	27	-	-
	Sytox Orange	1.899	0,0698	10	10.7	5.56E-07
	Sytox Orange (RNAse treatment)	2.228	0,1057	17	0	0.55

t : mean GFP lifetime in nanoseconds (ns). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with exponential function using a non-linear least-squares estimation procedure. (b) s.e.m.: standard error of the mean. (c) N: total number of measured nuclei. (d) E: FRET efficiency in % : E=1- t(DA/ t D). (e) p-value (Student's t test) of the difference between the donor lifetimes in the presence or absence of acceptor

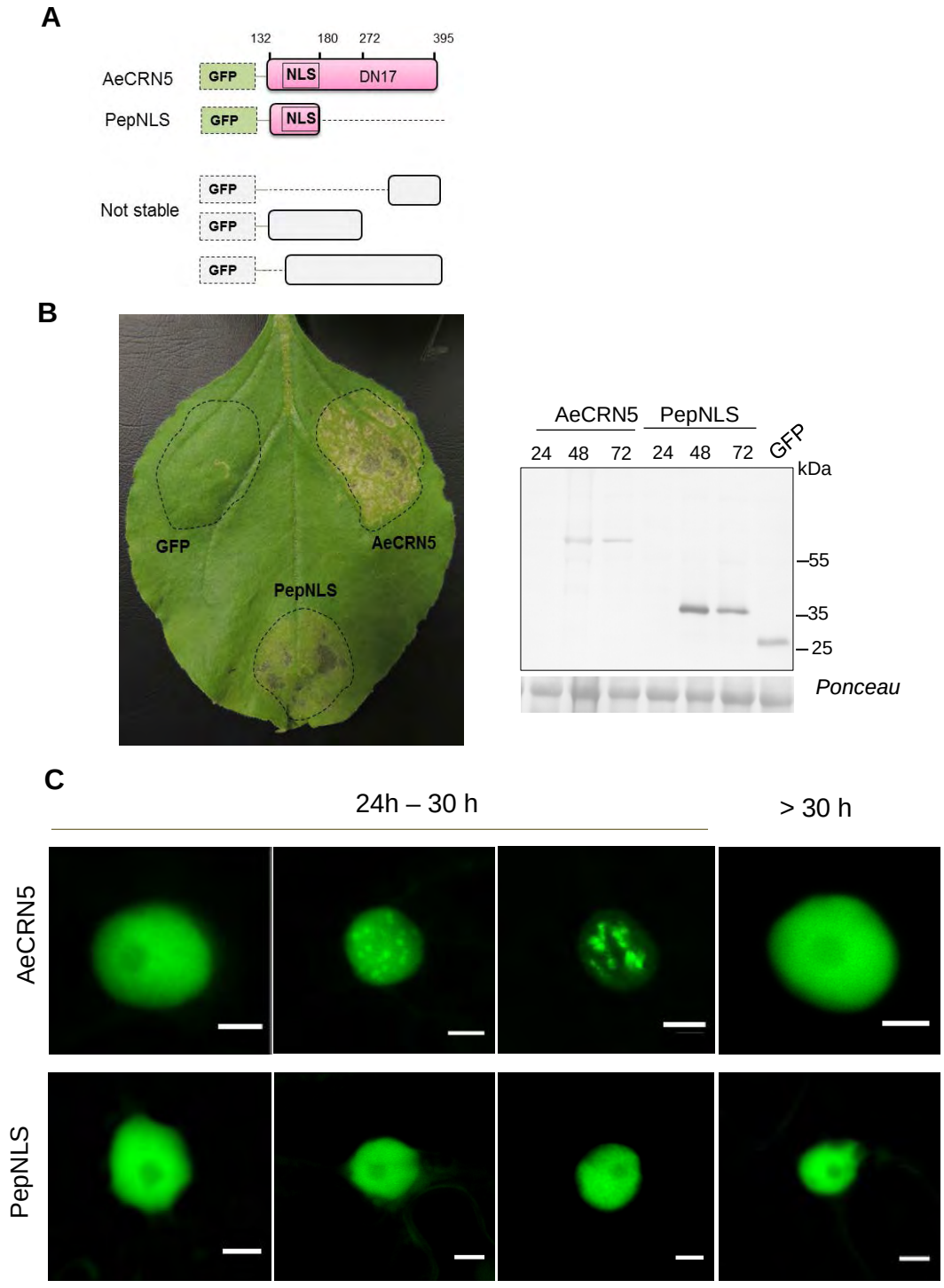
Supplementary Table 1. Primers used for the generation of the different constructs.

Name	Sequence 5'-3'
attB1_AeCRN5-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTGAAGGTG ACCGCTCTAGAACCC
attB2_AeCRN5-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTTATTCA AAAAGTATGGCG
AeCRN5_end-R	TTGTTATTCAAAAAGTATGGCGTAAATTTTGGC
NESAeCRN5-F	CACCCTTCAACTTCCTCCTCTTGAAAGACTTACTCTTTTGA AGGTTGACCGCTCTAGAACCC
mNESAeCRN5-F	CACCCTTCAAGCTCCTCCTGCTGAAAGAGCTACTCTTTTG AAGGTGACCGCTCTAGAACCC
AttB1_NLSDN17-F	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTTATGCC TTCACATATTTCCC
caccH2B-F	CACCATGGCGAAGGCAGATAAGAAACCAGC
H2B-R	TTAAGAACTCGTAAACTTCGTAACCGCC



Supplemental Fig 1: AeCRN5 is a CRN DN17 family**.** Alignment of Cterminus of AeCRN5 with the DN17 consensus sequence of *P. infestans* and its closest orthologs from *P. ramorum* (pRg882635), *P. sojae* (Ps_132663), *P. capsici* (Pc_CRN20_624), the chytrid fungus *Batrachochitrium dendrobatidis* (Bd) (BD_07009.1), the arbuscular mycorrhrizal fungus *Rhizophagus irregularis* (Ri, gm1.18625_g) and the DN17 consensus sequence from *P. infestans*.

Chapter 2: Complementary results



Complementary Figure 1. Identification of a AeCRN5 Cterminal bioactive derivative (PepNLS) in *N. benthamiana.* **A**. Cartoons describe Cteminal AeCRN5 derivatives assayed for cell death activity and nuclear localization on *N. benthamiana* leaves. **B** Photograph of a *N. benthamiana* leaf agroinfiltrated with constructs GFP, AeCRN5 and PepNLS showing representative necrotic symptoms observed 5 days after agroinfiltration and western blot results showing the accumulation of both proteins in plant cells 24, 48, and 72 hours after agroinfiltration. Blots were probed with GFP antibodies. **C.** Panels correspond to micrographs acquired by confocal imaging showing the localization of both GFP fusion proteins AeCRN5 and PepNLS in epidermal cells at 24h-30h and after 30h after infiltration (Scale bars : 5 µm).

Complementary results

Additional experiments were conducted attempting to precise the mechanisms of AeCRN5 nuclear activities.

PepNLS defines a minimal peptide of AeCRN5 presenting necrosis-inducing activity

As previously reported, we showed that AeCRN5 Cterminus domain (DN17) is a cell-death inducing domain which targets plant nuclear RNA with a dynamic localization within the plant nucleus. To identify the regions of DN17 required for these activities, we produced different Cterminal derivatives Nterminally fused to GFP, schematized in complementary figure 1. All constructs were agroinfiltrated in *N. benthamiana* leaves. Only the PepNLS version corresponding to the first 11 aa peptides coupled to the NLS (133aa-179aa) was stably expressed (complementary figure 1 C). Notably, PepNLS accumulation was greater than AeCRN5 and was accompanied of cell-death, similarly to the full DN17 domain (complementary figure 1 C). Confocal microscopy studies revealed a nuclear localization of the GFP:PepNLS fusion protein homogenously distributed in the nucleoplasm over time. Thus, in contrast to the full DN17, it did not display a shuttling to any obvious subnuclear bodies.

PepNLS also displays a RNA binding capacity

We tested whether PepNLS could target plant RNA by FRET-FLIM. Analyses were performed on three independent agroinfiltrations and on a minimum of 10 nuclei per construct and results were compared to GFP and GFP-H2B measurements. For PepNLS, we registered a GFP life- time decrease from 1.995 +/-0.032 to 1.692+/-0.020 in presence of Sytox Orange, indicative of FRET phenomenon (complementary table 1). Since Sytox Orange labels DNA and RNA, to discriminate the nature of nucleic acid targeted by PepNLS, samples were treated with RNAse which resulted in the loss of FRET. Hence, PepNLS targets plant RNA. These preliminary results suggested that this first Nterminal region of DN17 domain may be a minimal peptide ensuring AeCRN RNA binding. Yet, it is not likely to contribute to AeCRN5 localization to nuclear bodies.

Complementary Table 1. Summary of FRET-FLIM conditions and values obtained on foliar discs of *N. benthamiana* expressing the different fluorescent donor proteins.

Donor	Acceptor	t [(a)]	sem [(b)]	N [(c)]	E [(d)]
GFP	-	2.246	0.036	20	-
GFP	Sytox Orange	2.210	0.044	18	1.6
GFP-H2B	-	2.465	0,017	40	-
GFP-H2B	Sytox Orange	1.852	0.047	43	24.8
GFP-AeCRN5	-	2.128	0.069	27	-
GFP-AeCRN5	Sytox Orange	1.899	0.069	10	10.5
GFP-AeCRN5	Sytox Orange + RNAse treatment	2.228	0.105	17	0
GFP-PepNLS	-	1.995	0.032	23	-
GFP-PepNLS	Sytox Orange	1.692	0.020	32	15.2
GFP-PepNLS	Sytox Orange + RNAse treatment	1.969	0.042	31	1.3

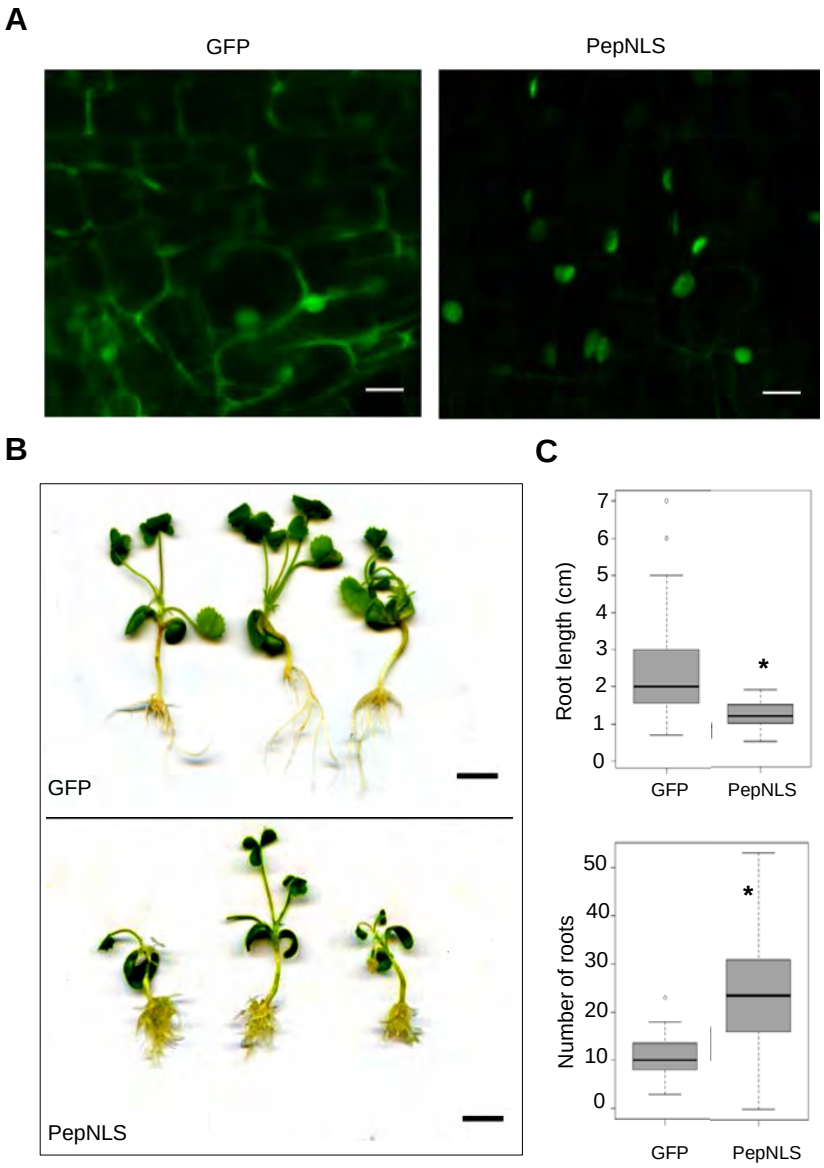
τ : mean GFP lifetime in nanoseconds (ns). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with exponential function using a non-linear least-squares estimation procedure. (b) s.e.m.: standard error of the mean. (c) N: total number of measured nuclei. (d) E: FRET efficiency in % : E=1-(τ DA/ τ D). (e) p-value (Student's t test) of the difference between the donor lifetimes in the presence or absence of acceptor

PepNLS worries host roots similarly to full AeCRN5

We showed that AeCRN5 (complete DN17 domain) affects host root development when overexpressed in *M. truncatula* roots. We further tested whether PepNLS could present similar AeCRN5 in roots of *M. truncatula.* For this, we transiently expressed constructs GFP:PepNLS (PepNLS) and control GFP in roots via a *A. rhizogenes*-mediated transformation. Composite plants were let to develop over time and fluorescent roots were used to study GFP localization by confocal microscopy. GFP corresponding to PepNLS was detected exclusively on nuclei in constrast to GFP alone, which presented its typical nucleo-cytoplasmic localization (complementary figure 3 A). Plants transformed with PepNLS presented an overall size reduction with major changes in root morphology compared to plants transformed with GFP alone (complementary figure 3 B). Quantification of root length and number indicated that roots transformed with PepNLS presented a decrease of root length and a higher number of roots (complementary figure 3 C), similar to effects triggered by AeCRN5. It is worth to note that PepNLS induced stronger effects on root architecture than AeCRN5

As a first approach to further characterize DN17 domain we generated AeCRN5 Ctermical truncated peptide. Only the 11-aa amino terminal peptide coupled to the NLS was stable, accumulating over time in plant cells. This peptide localizes in nuclei, binds RNA, triggers a cell-death but does not transiently localize in nuclear bodies. These results suggest that, within AeCRN5 Cterminal domain, distinct subdomains ensure the nuclear-body localization and that induction of cell-death may be nuclear-body independent. Our preliminary analyses on PepNLS and full DN17 by putative orthologs sequence analysis and 2D modeling did not show any obvious sequence characteristic leading to a particular known activity. In the furure, similar experiments will be perform to generate viable Cterminal derivaties, mainly a DN17 version deleted of its first 11 amino acid to validate the importance of this region for DN17 activities, notably nuclear-body addressing.

In host roots, PepNLS accumulates in nuclei and leads to similarly effects on root morphology that AeCRN5. In contrast to AeCRN5, PepNLS fluorescence accumulated sufficiently to be visualized in host roots. This is consistent with a greater protein accumulation observed for PepNLS in foliar leaves (*N. benthamiana*), and might explain the stronger effects seen for this version compared to those triggered by AeCRN5.



Complementary Figure 2. PepNLS modifies root morphology**.** *M. truncatula* plantlets were transformed with *A. rhizogenes* to express constructs GFP and PepNLS. **A.** Micrographs showing a nucleo-cytoplasmic localization of GFP and a nuclear accumulation of PepNLS. Fluorescent transformed roots were studied by confocal microscopy (scale bar: 20µm). **B.** Photographs of 30 day-old composite plants depict the effects of PepNLS on plant development and on root system morphology (scale bars: 1 cm). **C.** Quantification of length and root number per composite plant is presented in box-plot graphics which represent the average of both parameters and further confirms the impact of PepNLS on root development. Measures and statistical analyses were performed on n=60 (GFP) and n=140 (PepNLS) plants. * (*t- test*, p-value<0.05).

Overall, these preliminary results indicate that whithin the DN17 of AeCRN5 there could be a functional modularity that could explain AeCRN5 localization dynamism within the plant nucleus. Insterstingly this dynamism does not seem to be required for cell-death activity. Why is AeCRN5 addressed to this constricted RNA-containing nuclear zones and how this contributes to its whole protein activity? Whether this fontional modularity supposes different nature of RNA targets and/or interaction to other plant factors needs to be further investigated. Experiments to identify the nature of RNA targets sassociated to PepNLS (nucleocytoplasmic distributed) and to nuclear-bodies are in view to be performed on full DN17 and other protein versions that will be generated.

Materials and methods of complementary experiments

Construction of AeCRN5 Cterminal derivative vectors

Sequences of primers used are listed in complementary table 1. PepNLS, version 2 version 3 and version 4 carrying Gateway adaptors were generated by PCR using the cDNA template Ae_1AL4462 (vector pSport_Ae_1AL4462). PepNLS (132aa-180aa) was amplified using primers AttB1_AeCRN5-F and AttB1_PepNLS-R, version 2 (143aa-370aa) using AttB1_NLSDN17-F and AttB2_AeCRN5-R, version 3 (132aa-272aa) using AttB1_AeCRN5-F and AttB1_DN17dis-R and version 4 (273aa-370aa) using AttB1_Minus-F and AttB1_AeCRN5-R. Amplicons were BP recombined in pDONR-Zéo vector (Invitrogen) and subsequently introduced in vector pK7GWF2 by means of LR recombination (Invitrogen). The obtained pK7GWF2 vectors were introduced in *Agrobacterium* strains for *N. benthamiana* agroinfiltration assays and/or *M. truncatula* roots transformation.

Name	Sequence 5'-3'
attB1_AeCRN5-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTGAAGG TGACCGCTCTAGAACCC
attB2_AeCRN5-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTTATTC AAAAAGTATGGCG
AttB1_PepNLS-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTTATTT CTTTGGCTTCTT GTT
AttB1_NLSDN17-F	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTGTTATGC CTTCACATATTTCCC
AttB1_DN17dis-R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTG TTG TTA TGC CTT CAC ATA TTT CCC
AttB1_Minus-F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC AAT GGT CGT TTT GAA TTT GTA

General discussion and perspectives

General discussion and perspectives

Throughout this work, we performed the functional analysis of two putative cytoplasmic effectors from the root rot pathogen *A. euteiches*, AeCRN5 and AeCRN13. Their corresponding genes were detected in a cDNA library generated from infected roots of *M. truncatula*, before my arrival in the research team. In this section I will discuss the main results obtained during my PhD work and the immediate perspectives of our findings.

CRN proteins, more than just phytopathogenic oomycete proteins.

In the oomycete lineage, CRNs are ancestral proteins described in all phytopathogenic species including the early divergent species *A. euteiches* and latest results of RNA sequencing data indicate that they might be also present in the zoopathogenic species like *A. astaci* (Gaulin et al., in preparation). One characteristic of the CRN protein family is its extensive expansion and diversity, typically found in *Phytophthora* species (*P. infestans, P. ramorum, P. sojae*…) and also in *A. euteiches*. In *Phytophthora* species, this gene number expansion and sequence variability is attributed to gene duplication events and fragment recombination mechanisms between CRN sequences (Haas et al., 2009; Shen et al., 2013) that are thought to give rise to novel CRN proteins and, by this, contribute to host adaption. In *P. sojae* almost 50% of CRNs have been found to issue from gene duplication and are under diversifying selection, denoting likely a neo-functionalization of the resultant CRNs.

At the beginning of this project, CRN proteins were thought to be restricted to the oomycete lineage, but during my PhD work, CRN-like proteins were reported in the chytrid fungus species *B. dendrobatidis*, an amphibian pathogen and in the plant mycorrhizal fungus *R. irregularis.* Thus, CRN are no longer oomycetes specific nor linked to a pathogenic lifestyle but might also contribute to a mutualistic lifestyle. Because CRN-like proteins are limited only to these two fungal species, their occurrence in Bd and Ri is proposed to be the result of Horizontal Gene Transfer (HGT) events from oomycetes (Sun et al., 2011). HGT is very common between bacteria and from bacteria to eukaryotes (Gilbert and Cordaux, 2013) and is becoming evident also between plant parasitic nematodes, oomycetes and fungi for which it has been proposed as a mechanism shaping parasitism (Gardiner, Kazan, & Manners, 2013; Haegeman, Jones, &

Danchin, 2011; Richards et al., 2011). Fixation of new genes supposes then that novel functions somehow fit in functional networks already present in the receiver organism and that these functions add an advantage to the network (Richards et al., 2006). Therefore, the presence in fungi implies that CRN functions fit in the biochemical networks and confer a benefit to the biology of these two organisms.

Several gene transfers have been evidenced between fungi and oomycetes (Richards et al., 2011). 33 fungi-to-*Phytophthora* oomycete species genes have been identified accounting for up to 7.6% of the predicted secretome (*P. ramorum*). Among the transferred genes, some code for secreted proteins with functions in the degradation of cell wall structural polysaccharides specific to plants and therefore putatively involved in the degradation of plant cell wall for tissue colonization and/or nutrient acquisition. Notably, among transferred genes are LysM protein-coding genes which have been demonstrated in phytopathogen fungi as effectors allowing plant defense suppression (Richards et al., 2011).

B. dendrobatidis and *R. irregularis* share a repertoire of oomycete CRNs that include DFA-DDC, DN17, DXX, DX8 Cterminal families with up to 46.5 % of sequence similarity (Sun et al., 2011; Lin et al., 2014). It is expected that fungal CRN could have undergone sequence divergence linked to a specialization and functional adaptation to the recipient's biology since their actual transfer from oomycetes. Nevertheless, this shared core of CRNs could imply similar protein activities and likely similar functions. In the case of *B. dendrobatidis,* the observation that CRN proteins display signs of diversifying selection and are induced upon infection (Rosenblum et al., 2012) supports their possible involvement during host infection. The results obtained on AeCRN13 and BdCRN13 support the idea that sequence similarities can correlate to similar activities and open the question of the exact contribution of similar CRN activities in microorganisms that present different lifestyles and develop in different nature of hosts. If this shared core of CRNs presents indeed similar activities, it would be logic to assume that such activities are necessary for common infectious processes. Then, it is likely that common CRNs target universal functions and/or processes present in both plants and animals. The elucidation of oomycetes and fungal CRN activities together with the identification of targeted processes is of current need to understand the CRN protein family.

AeCRN secretion and translocation

The *in silico* characterization of CRN proteins in oomycetes and fungi has denoted the lack of predictable signal peptides (SPs) in their Nterminal moieties. In fact, 40% of CRNs of *P.infestans* do not contain a SP (Haas et al., 2009) and only 11% of Bd and 12% of Ri CRNs exhibit a SP (Sun et al., 2011; Lin et al., 2014) . The lack of SP is also the case for both AeCRN studied in this work. CRN Ntermini lacking predictable SP, including AeCRN5, were shown to allow the secretion and host-delivery of proteins (Schornack et al., 2010). Hence, it is possible that some CRN possess particular unknown secretion leaders that cannot be accurately predicted by available algorithms (i.e SignalP). Another possibility is that some CRN fall into the category of the so-called leaderless secretory proteins (LSPs) that undergo unconventional protein secretion. The latter idea is sustained by a recent study of *P. infestans*'s *in vivo* proteome that evidenced the presence of proteins devoid of SP in the extracellular space, among which CRNs (Meijer et al., 2014). Unconventional secretion refers to secretion mechanisms other than the classical ER-Golgi trans-membrane route to which canonical SP-containing proteins are addressed, that allow secretion of SP-lacking proteins. Unconventional protein secretion has been described mainly in mammal and fungal systems and in plants (Ding et al., 2012; Oliveira et al., 2013) and its experimental evidence lies on the identification in the extracellular space of proteins that are typically intracellular (cytoplasmic/nuclear) and/or that lack SPs and on the observation that secretion of some proteins is Brefeldin A (BFA) independent. BFA is a potent inhibitor of membrane recruitment of factors that mediate the formation of coated vesicles from Golgi membrane to the plasma membrane. Models for unconventional secretion suppose the direct translocation of the protein across the plasma membrane or a vesicle-dependent secretion, but the exact factors controlling the biogenesis of such vesicles, the shedding and sorting of secreted proteins is not well understood (Nickel and Rabouille, 2009). Studies in various fungal systems, mainly carried in animal pathogenic species (like *Crytococcus neoformans* and *Paracoccidioides brasiliensis*) have described the release of vesicles to the extracellular space during infection. Isolation and analysis of extracellular vesicles or “exosomes” has denoted a variety of cargo molecules (lipids, proteins, polysaccharides…) that have been associated with virulence (Oliveira et al., 2013).

The demonstration that effectors undergo differential secretion pathways has been recently brought thought studies on the phytopathogenic fungus *Magnaporthe oryzae*. Localization studies of effector proteins tagged with fluorescent proteins expressed in this fungus, have shown

that apoplastic effectors are found dispersed and retained in the EIHM compartment (a close-up apoplastic space delimited by the invasive hyphae and the plasma membrane of the invaded host cell) while intracellular effectors accumulate in the BIC (a plant derived structure developed adjacent to the infectious hyphae). Secretion of apoplastic effectors such as Bas4, Bas113and Slp1 was blocked by BFA, indicating that these effectors undergo an ER-to-Golgi secretion. In contrast BFA treatment did not affect the secretion of the intracellular effectors Pwl2, Bas1and Avr-Pita (Giraldo et al., 2013).

Our preliminary immunolocalization results on AeCRN13 during *M. truncatula* root infection provided, for the first time, the visualization of an oomycetal intracellular/cytoplasmic effector secretion and translocation during the natural infection conditions. AeCRN13 was detected in particular cup-shaped structures outside *A. euteiches* infectious mycelium that we denoted as “vesicle-like” or “exosomes”. This localization pattern contrasts to what has been observed in hautoria-forming fungi and oomycetes in which intracellular effectors have been seen to accumulate in located zones of haustoria and other particular biotrophic structures as the above mentioned BIC (Kemen et al., 2005, Whisson et al. 2007, Giraldo et al., 2013). In *A. euteiches* such structures have never been detected and AeCRN13 was not detected in any particular zone of the infectious hyphae.

AeCRN13 signal was detected in vesicle-like structures near host nuclei and a faint AeCRN13 signal was reported in nuclei. We postulate that once in the vicinity of host nuclei, AeCRN13 is released from these structures and addressed into nuclei to exert its function. Future work will be directed to precise the nature of these “vesicles”. Immuno-electron microscopy techniques will be used to precise their size, morphology and the exact localization of AeCRN13 within these structures. To further characterize their molecular composition, these vesicles will be isolated from infected tissues and analysed for protein and lipid composition. Different methods for isolation and analysis have been described in the literature based on ultracentrifugation, filtration and immunoaffinity (Witwer et al., 2013). This latter strategy could be contemplated by using AeCRN13 antibodies. Such studies will be also directed on AeCRN5 and other *Phytophthora* CRNs to see whether secretion is conserved in oomycetes (Saprolegniales and Peronosporales). In addition it will be interesting to direct similar approaches on CRN displaying canonical SP leaders in order to see whether CRN secretion and translocation differ between SP-containing/lacking CRNs.

We followed AeCRN13 protein accumulation during *M. truncatula* infection with AeCRN13 antibodies. The results pointed out the possibility for AeCRN13 to be processed since different specific AeCRN13 signals were detected upon western blot analyses of infected roots. Our interpretation of AeCRN13 processing is based on predicted sizes and on speculations brought by other studies and we are aware of the necessity to sustain our hypotheses with mass spectrometry analyses of the products that were identified to be conclusive.

Nterminal processing of intracellular effectors has been described. For instance, a mass spectrometry analysis of SIX2 (Avr2) protein of *F. oxysporum* isolated from infected tissues revealed that only its Cterminal domain is present in infected xylem tissues (Houtemann et al., 2007: Ma et al., 2013). In addition, it has been demonstrated that the Ntermini of intracellular "Pexel" effectors of *P. falciparum* are processed during its transit on the ER (Osborne et al., 2010) and it it also the case for the Nterminal domain of the RxLR effector Avr3a from *P. infestans* (Warva S, OMGN meeting July 2014).

Post-translational modifications could also constitute a regulation of CRN proteins. So far, phosphorylation has been shown as one type of post-translational decoration of CRN proteins. The recent "phospho-proteome" study on *P. infestans* showed that Cterminal domains belonging to the DBF, DO, DXZ, DXX families are targets of phosphorylation (Resjö et al., 2014) and a previous study indicated that CRN8 (carrying a D2 Cterminal domain) is phosphorylated when expressed *in planta*, a modification necessary to its cell-death activity (van Damme et al., 2012). These observations point to a functionally relevant regulation of CRN at the post-translational level which is in agreement to latest finding on the fungal LysM (Slp1) effector of *M. oryzae* for which its N-glycosylation during its Golgi-dependent secretion is necessary for its chitin binding and virulence activity (Chen et al., 2014).

Nuclear and subnuclear localization of AeCRNs

Nuclear import of proteins larger than 40kDa is an active process mediated by protein complexes associated at the nuclear pores, that solicits import receptors (importins) recognizing NLS motifs in imported cargo proteins (Tamura and Hara-Nishimura, 2014). All CRN proteins studied so far localize in plant nuclei but their nuclear localization not always correlates to the presence of predicted NLS in their Cterminal domains. For instance, only 26% of *P. capsici* CRNs present NLS (Stam et al., 2013). AeCRN13, BdCRN13 and AeCRN5 exemplify this since only AeCRN5 contains a predictable NLS. Their presence in some CRNs infers that CRNs

subvert directly the nuclear transport machinery of host cells by interacting with α/β-importins. This was actually shown for *Phytophthora* NLS-containing CRNs and for AeCRN5 for which silencing of plant α-1- importin partially impairs their nuclear accumulation (Schornack et al.2010). The direct subversion of the host nuclear traffic machinery by bacteria and oomycetal effectors has also been reported (Deslandes and Rivas 2011; Caillaud et al., 2012). How exactly, non-containing NLS CRNs localize in nuclei is still not clear. A possible explanation is that these proteins may harbor discrete NLS, still not characterized, and therefore unpredicted with algorithms at disposition. Other possibility is that they could co-opt host factors that mediate direct interaction with importins as it has been shown for the *Agrobacterium* effector VirE2 that subverts the plant NLS-containing VIP1 protein to mediate its entry (Liu et al., 2010).

AeCRN13 and AeCRN5 localize entirely to nuclei. Confocal imaging performed on GFP-tagged AeCRN5 over time revealed a dynamic subnuclear localization for this protein. AeCRN5, which is a DN17 CRN, first displays an even nucleoplasm distribution then exhibits a punctuated localization that results in its location to uncharacterized nuclear bodies characterized by the lack of DNA material. Another DN17 CRN protein, CRN12_624 (from *P. capsici*) exhibits similar punctuated concentrations of fluorescence all over the nucleus, but the possible dynamism of its localization was not examined.

The dynamics of AeCRN5 localization together with the fact that nuclear bodies do not contain DNA material and required plant RNA integrity to form, suggest that AeCRN5 localization might be linked speckles. Among the different membrane-free nuclear compartments evidenced in plant and animal cells (ie Cajal bodies, nucleoli…), speckles are interchromatin molecular complexes that form throughout the nucleoplasm in regions containing little or no DNA and that exhibit a granular shape. Major components of speckles are proteins involved in pre-mRNA splicing (SR proteins, snRNP protein splicing factors) but transcription factors as well as subunits of RNA poly II and RNA have also been identified (Reddy et al., 2012; Spector and Lamond, 2011). The function of speckles is not clear, but because of their molecular composition together with the fact that their localization often correlates to active transcriptional sites, they have been proposed as splicing centres (spliceosomes) and as domains devoted to the storage and regulation of splicing factors. Speckles are highly dynamics structures, changing in size, shape and number with individual components able to shuttle in and out (Reddy et al., 2012). Future work on AeCNR5 will be devoted to further characterize its subnuclear localization by the use of protein markers of different nuclear compartments.

AeCRNs nuclear targets

An increasing number of experimental data places the nucleus and/or nuclear-related functions as important hubs targeted by microbial effectors. While the nature of nuclear targets are best known for prokaryotic effectors, only few have been identified for eukaryotic effectors which have been shown to be proteins. These include transcription factors of the NAC family (NTP 1 and NTP2) and TCP (TCP14) targeted by RxLR of *P. infestans* and CRN of *P. capsici*, respectively, that have been proposed be positive regulators of plant defense. Other targeted plant defense regulators include the proteasomal protein CMPG1 and MED19 which are targeted by RxLRs (McLellan et al., 2013; Bos et al., 2010; Caillaud et al., 2013; Stam et al., personal communication). This study reveals, for the first time, that filamentous eukaryotic effectors can target nucleic acids. By setting FRET-FLIM measurements *in planta*, we demonstrate the close vicinity of AeCRN13 and AeCRN5 with plant DNA and RNA, respectively. In addition, by working with BdCRN13, we show that this targeting is a conserved CRN activity, suggesting that the targeting host nucleic acids may be a common infection strategy of eukaryotic pathogens. TAL effectors from bacteria illustrate the concept of plant physiology modulation through a direct interaction with nucleic acid. Their binding is DNA sequence specific and has a direct repercussion on host gene transcription.

Next work will aim to decipher the interaction (direct or not) between AeCRNs and plant nucleic acid by developing cutting-edge technology including ChipSeq approach on *M. truncatula*. This approach will also provide answer to a possible specificity of the targeted nucleic sequence. Transcriptionnal modifications caused by CRNs in host plant will be also evaluated to precise their mode of action. Finally, Y2H on infected *M. truncatula* root is underway in the group in order to identify putative co-interactors of AeCRNs.

Cell death activity and contribution to virulence

At the beginning of the work, all *Phytophthor*a CRNs studied were described as cell-death inducing proteins when expressed directly in plant tissues but works have shown that a large number do not carry a cytotoxic activity and/or can even supress cell-death induced by other CRNs and other microbial-derived cell-death inducers (Stam et al., 2013: Shen et al., 2013; Li et al., 2011). As *Phythophthora* species present hemibiotrophic lifestyles, it has been generally

assumed that CRNs with necrotic activities could be required during necrotrophic stages of infection. Nevertheless, gene expression profiles of CRN in *Phythophthora* species indicate that this might not be generally the case as some necrotic CRN are expressed during the biotrophic development of the pathogen (Stam et al., 2013; Li et al., 2011). In addition, CRN proteins of the obligate biotrophic oomycete *Albugo candida* have been shown to trigger cell-death which is in direct contradiction to its infection behaviour (Links et al., 2011). Thus, the cell-death activity observed for some CRNs *in planta* might not be the intrinsic virulence function but rather the consequence of their overexpression and thus "over-activity" in host tissues. Giving the high number of CRN genes, high-through put systems to assess the contribution to virulence of CRN have been based on pathogen challenging of plant tissues expressing ectopically the effectors and measure of pathogen spreading. These studies indicate that not all CRN display an obvious contribution and have suggested that some CRN may present more discrete virulence activities during infection (Stam et al., 2013).

Our work shows that overexpression of both AeCRNs and BdCRN13 has important effects in plant and amphibian cells manifested by tissue necrotic symptoms or host developmental defects, all of which attest for the perturbation of host cellular processes by these AeCRNs. AeCRN13 contributes to *P. capsici* virulence, since AeCRN13 overexpression enhanced *N. benthamiana* leaves colonization. One way to better understand AeCRN13 contribution to infection is to contextualize their expression during infection in regards to the whole CRNs. *A. euteiches* genome contains 160 CRN gene models and currently their expression is being characterized by high-thought put qPCR system (Fluidigm Biomark) on various life stages of the pathogen.

Nuclease activity and DNA damage

During this work we provided first insights into the function of CRN13 by identifying a putative HNH-like endonuclease motif in the Cterminal DFA subdomains of Ae and Bd effectors. *In vitro* assays demonstrated the AeCRN13 binds and cleaves DNA. This is suspected to be mediated by the HNH-like motif. HNH motifs are phylogenetically wide spread functional protein domains associated to DNA-binding factors of prokaryotic and eukaryotic organisms and they occur commonly in homing endonucleases (HNH endonucleases) (Belfort and Roberts, 1997). Studies of bacteria HNH endonucleases place this domain as an important structural domain necessary for the hydrolytic activity on DNA ensuring DNA and cutting activities

(Huang and Yuan, 2007; Shen et al., 2004). Interestingly, some bacterial HNH-containing proteins act like toxins and play ecological roles in bacteria populations. For instance, bacterial colicins have been shown to be secreted and translocated inside other bacteria cells where their nuclease activity leads to cell-death is and is HNH-dependent (Hsia et al., 2004; Pommer et al., 2001 ; Braun and Patzer, 2013) . Future studies will evaluate the functionality of the HNH-like motif of AeCRN13 and its requirement in the cell-death inducing phenotype. For this, different versions of the protein will be made (punctual mutation and HNH-deleted version…) and, for them, DNA-binding affinity as well as nuclease activity will be evaluated.

Lastly, we reported the induction of plant DNA double-strand breaks (DSB) upon overexpression of AeCRN13 in *N. benthamiana* and interestingly during *M. truncatula* infection by *A. euteiches*, using phosphorylated-H2AX detection. This finding identifies CRN effector as inducer of DSB probably through its nuclease activity. Song and Bent (2014) recently demonstrated the occurrence of DSB in plants during infection by phytophatogens *P. syringae* and *P. infestans*. The authors speculate that DSB may be due to a direct effect of microbial effector, toxins or cytotoxic microbial molecules since it was observed only with pathogenic species and in the absence of ROS accumulation (Song and Bent, 2014). In animals and plants, DNA breakage initiates a phosphorylation mediated signal transduction cascade leading to cell cycle arrest, cell-death or repair of DSB. Orthologous genes of the *A. thaliana* or animal signaling cascade have been detected in the *M. truncatula* genomes (i,e . wee1, rad51, brca1…). Future work will aim to evaluate their involvement in the observed mechanism DNA damage.

Concluding remarks

During my PhD work I deciphered a new mode of action of an exciting class of nuclear effectors from the root pathogen *A. euteiches.* I have showed that CRN oomycetal effectors are translocated into the cytoplasm of the host plant during interaction. I identified host nucleic acids as a novel target of AeCRNs that impact plant physiology. Finally I've revealed that eukaryotic effectors induce host DNA damage during infection probably through the secretion of cytoplasmic effector like the CRN DFA-DCC family. Altogether this PhD opens intriguing avenues for future studies.

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Title: Analyses of CRN effectors (Crinkler and Necrosis) of the oomycete *Aphanomyces euteiches*

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Summary: The oomycete *Aphanomyces euteiches* is an important pathogen infecting roots of legumes (pea, alfalfa...) and the model legume *Medicago truncatula*. Oomycetes and other microbial eukaryotic pathogens secrete and deliver effector molecules into host intracellular compartments (intracellular/cytoplasmic effectors) to manipulate plant functions and promote infection. CRN (Crinkling and Necrosis) proteins are a wide class of intracellular, nuclear-localized effectors commonly found in oomycetes and recently described in true fungi whose host targets, virulence roles, secretion and host-delivery mechanisms are poorly understood. We addressed the functional characterization of CRN proteins AeCRN5 and AeCRN13 of *A. euteiches* and AeCRN13's homolog of the chytrid fungal pathogen of amphibians *Batrachochytrium dendrobatidis*, BdCRN13. Gene and protein expression studies showed that AeCRN5 and AeCRN13 are expressed during infection of *M. truncatula's* roots. Preliminary immunolocalization studies on AeCRN13 in infected roots indicated that the protein is secreted and translocated into root cells, depicting for the first time CRN secretion and translocation into the host during infection. The heterologous ectopic expression of AeCRNs and BdCRN13 in plant and amphibian cells indicated that these proteins target host nuclei and lead to the perturbation of host physiology. By developing an *in vivo* FRET-FLIM-based assay, we revealed that these CRNs target host nucleic acids: AeCRN5 targets plant RNA while AeCRN13 and BdCRN13 target DNA. Both CRN13 exhibit a HNH-like motif commonly found in endonucleases and we further demonstrated that both CRN13 display a nuclease activity *in vivo* inducing double-stranded DNA cleavage. This work reveals a new mode of action of intracellular eukaryotic effectors and brings new aspects for the comprehension of CRN's activities not only in oomycetes but, for the first time, also in true fungi.

Keywords: CRN, oomycetes, nucleus, effector, secretion, FRET-FLIM, A*phanomyces euteiches*

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