Towards the inventory of pathogenicity effectors on *Plasmopara halstedii*, the obligate oomycete responsible for Downy Mildew in sunflower *Helianthus annuus*

Quentin GASCUEL [1], Falah AS SADI [1], Marie-Claude BONIFACE [1], Charles ROSENBERG [1], Laurence GODIARD [1], Patrick VINCOURT[1,2]
[1] INRA, CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, F-31326 Castanet-Tolosan, France.
2 Corresponding author: patrick.vincourt@toulouse.inra.fr

ABSTRACT:

- Downy mildew in sunflowers (*Helianthus annuus* L.) is caused by the oomycete *Plasmopara halstedii* (Farl.*)* Berlese et de Toni. Novel sequence data of this oomycete obligate parasite have been recently provided to the community (As sadi *et al.,* 2011, http://www.heliagene.org/HP). Putative RXLR and CRN pathogenicity effectors, typical of other oomycetes, have been found expressed during the interaction of sunflower with downy mildew. The aim of this work was to acquire knowledge on the role of these effectors in the pathogenicity of downy mildew.

- The RXLR and CRN putative effectors were selected to have their sequences with (i) their expression during plant-pathogen interaction, (ii) the RxLR or LxLFLAK conserved translocation signal after signal peptide, (ii) a good level of similarity with effectors of the well studied oomycete *P. infestans*, and (iv) various C-terminal domains. They were amplified from cDNA of *P. halstedii* race 710, and further cloned in pSK vector and sequenced. Golden gate (Engler *et al.,* 2009) cloning was realized to integrate effector sequences under the control of the 35S promoter, and fused to the 3-HA tag within a binary expression vector pBi discount 35S. The *A. tumefaciens* strain LBA4404 was transformed with pBi discount 35S containing different constructs of putative *P.halstedii* CRN effectors. Leaves of the sunflower genotypes XRQ, PSC8 and CF33 were transformed *in planta* according to Manavella *et al*. (2009) with some modifications.

- While 14% of the 14 infiltrated areas showed necrosis on the control, PhCRN07, PhCRN11 and PhCRN20 CRN effectors under the control of 35S promoter showed respectively 88%, 40% and 77% of necrosis on XRQ for 18, 10 and 22 total infiltrated areas (khi 2: 8.1, significant at 2%) , suggesting that their *in planta* effects are not identical. In CF33 cultivar, necrosis was only induced by expression of PhCRN11 (40% of necrosis for 10 total infiltrated areas), and PhCRN20 induced a slight discoloration in infiltrated areas. No significant difference between control and others tested constructions have been observed.

- We hypothesize that, depending on the genetic pattern of the sunflower host, the *P. halstedii* biotrophic pathogen is inducing different phenotypes according to its own pattern of effectors, which able or not to manipulate host cell in keeping it alive to facilitate infection and to draw its nutrients from host. According to Zig-Zag-Zig model our results suggest that plant can recognize some of these effectors activating plant responses that could determined the outcome of the interaction.

- These results could provide a background to decipher some components of the interaction between *P.halstedii* races and *H.annuus* differential lines.

Keywords: Effectors, Helianthus annuus, oomycete, Plasmopara halstedii, sunflower,

INTRODUCTION:

H. annuus and its obligate parasite *P. halstedii*, the oomycete being the causal agent of downy mildew (Berl. & De Toni 1888), are both originated from North America where a co-evolution took place before the sunflower domestication. Many oomycetes are known as plant pathogens (60%) drawing their nutrients from the host, and using for that purpose infection structures named haustoria to release effectors into the host cells (Morgan and Kamoun,2007). Effectors are secreted in the apoplast and some of them could be translocated into plant cell cytoplasm to suppress defenses activated by the recognition of pathogen through pathogen associated molecular patterns (PAMPs). This plant defenses suppression induced susceptibility and compatibility of the interaction named Effector-Triggered-Susceptibility (ETS) (Morgan and Kamoun,2007). Alternatively, plants can recognize effectors as non-self (avirulence protein) leading to the activation of its defenses and to the incompatibility of the interaction named Effector Triggered Immunity (ETI). These mechanisms were described in the Zig-Zag-Zig model (Jones and Dangl,2006, Hein et al.,2009). In oomycetes, two classes of cytoplamic effectors have been characterized up to now: (i) the RxLR family of effectors is defined by the RxLR-dEER motif necessary and sufficient to translocate into host cells (Birch et al., 2009); (ii) LxLFLAK motif provides the same functions in CRN (Crinkling and Necrosis) effectors (Schornack et al., 2010). The development of new genomic resources of *P.halstedii* (As sadi et al., 2011) allowed the identification of putative RxLR and CRN sequences. Starting in this work with a subset of CRN effectors, we aimed to understand their role in the pathogenicity of *P.halstedii*, with the ultimate goal to decipher the pattern of the downy mildew races * sunflower differential lines interaction.

MATERIAL AND METHODS:

Genetic material:

Sunflower inbred lines XRQ, carrying Pl5 and QTL involved in quantitative resistance to downy mildew (Vear et al., 2008) and PSC8, susceptible to race 710 of downy mildew, and the cultivar CF33,susceptible to race 710 of downy mildew,were used for transient expression experiment. CF33 was previously used by Manavela and Chan (2009) to perform transient transformation of sunflower leaf discs via an *Agrobacterium*-mediated method. Every genotype was grown under the same conditions as further. The day before agroinfection, the plants were recovered with a plastic cover to keep plant in high humidity environment. In this way, plants keep their stomata open. Agroinfection was realized on 3 week old plants. 4-weeks old *Arabidopsis thaliana* Col-0 and *Nicotiana benthamiana* were used in preliminary agroinfection experiments in heterologous system.

Selection and cloning of putative effectors:

The effector sequences are originated from 454 sequencing and assembly of cDNA obtained from infected sunflower and spores of *P. halstedii* and were selected from those available at http://www.heliagene.org/HP (see also As sadi et al., 2011, supplementary data) according to (i) their expression during plant-pathogen interaction, (ii) if possible, the presence of a signal peptide followed by a pattern RxLR or LxLFLAK, (iii) a good level of similarity with effectors previously characterized in other oomycetes pathogens (mainly *Phytophthora infestans*), (iv) for putative CRN, various C-terminal domains.

Agroinfection:

Tests were realized using two strains of A. tumefaciens (AGL1 & LBA 4404) transformed by electroporation with an expression vector carrying the GUS gene without a promoter or with an intron (pBI101 and pBi121Gi, used as negative control), or fused to the 35 S promoter (pBi 121). Single strand cDNA from P. halstedii race 710 was used as matrix to amplify putative effectors, with Finnzymes'Phusion High-Fidelity Taq DNA polymerase. Primers were designed for later use of the cloning method "golden gate" (Engler et al., 2008). PCR products were purified on agarose gel with Wizard SV Gel and PCR Clean-Up System (Promega; 2800 Woods Hollow Road, Madison, WI 53711 USA) and cloned in pSK Bluescript (Stratagene) (carries a gene for resistance to Kanamycin) using SmaI restriction enzyme. All recombinant plasmids were purified with Promega Wizard Plus SV Mini-Prep, checked on agarose gel after restriction analysis and used to transform Escherichia Coli DH5α (bacteria without antibiotic resistance) was transformed with these plasmids have integrated putative effectors (pSK::putative effectors). E. Coli DH5α are grown 16h at 37°c on selective medium containing Kanamycin, to extract plasmids in large amount.

The pSK plasmids containing the putative effectors were then fused in frame to a peptidic tag (3-HA) and cloned in one step in an expression vector called pBi discount 35S using the cloning method "golden gate" (Engler et al, 2008). This method is based on the use of type IIs restriction enzymes which cut outside of their non-palindromic recognition sequence. After each cloning step, plasmid constructions were sequenced using T3and T7 primer for pSK inserts, and pro35S-F and Ter35S-R primers for pBi discount inserts. *Agrobacterium tumefaciens* (LBA 4404) strain was transformed by electroporation with pBi discount 35S carrying the putative effectors. Culture methods and plant transient assays are derived from Manavella and Chan (2009). The agrobacteria washed and resuspended at an OD 600nm of 1 in infiltration buffer medium [30] were infiltrated by pressure at the lower leaf part in 1 cm2 leaf sectors on 2 to 3 leaves per plant using a 1ml syringe without a needle. These solutions were infiltrated in *A. thaliana* and *N. benthamiana* using the same protocol.

RESULTS:

- *In planta* transient expression into sunflower leaves has not been routinely applied because of the lack of efficient protocol and difficulties with infiltration, especially due to the structure of the leaf and the thickness of the epidermis. A method of transient transformation of sunflower leaf discs with *A. tumefaciens* has been indeed proposed by Manavella and Chan (2009) in order to overcome structural leaf problems and low bacterial transformation efficiencies. According to this protocol, we tested vacuum infiltration of detached leaf discs on XRQ, PSC8 and CF33 sunflower genotypes to transiently express the reporter gene GUS in leaf cells, using two strains of *A. tumefaciens*, LBA4404 and AGL1. *A. tumefaciens* strain LBA 4404 was found more efficient than AGL1 to transiently transformed sunflower (XRQ, PSC8 and Advanta) and *N. benthamiana*. In parallel we developed a protocol allowing us to express transiently *in planta* putative effectors in undetached leaf sectors to analyze possible phenotypes on genotypes XRQ, PSC8 and CF33. We infiltrated leaf sectors of whole plants with the same solution of *A. tumefaciens* used in vacuum infiltration of detached leaf discs. As well as in leaf cell discs agroinfiltration, *Agrobacterium* strain LBA4404 was more efficient to express transiently the reporter gene GUS.
- 6 CRN putative effectors out the 19 selected effectors (15 CRN and 4 RxLR, Table 1) were successfully cloned by ligation in pSK vector, sequenced and further integrated within the expression vector pBi discount 35S. Then the *Agrobacterium* strain LBA4404 was transformed with expression vectors with or not putative effectors.
- Infiltration of LBA4404 control (pBi disc 35S without any effector) showed only slight necrosis localized at the wound inflected during the infiltration in XRQ and CF33 genotypes, as expected for the control vector, but induced clear necroses in PSC8 may be due to excessive concentration of agrobacteria used. There was no difference between necrosis induced by control and other constructions in PSC8 preventing the interpretation for this line. Expression in XRQ leaves of PhCRN07, PhCRN11 and PhCRN20 CRN effectors under the control of 35S promoter induced necrosis in infiltrated areas. They showed respectively 88%, 40% and 77% of necrosis on XRQ for 18, 10 and 22 total infiltrated areas suggesting that their *in planta* effects are not identical (Figure 1). In CF33 plants, necrosis was only induced by expression of PhCRN11 (40% of necrosis for 10 total infiltrated areas), and PhCRN20 induced a slight discoloration in infiltrated areas (Figure 1). For the phenotype "induction of necrosis", no difference between control and others tested constructions have been observed.

				Homology with known effector (NCBI-blastP)				
gene name	**Signal Peptide**	**Translocation motif**	**CRN domain**	**score**	**E value**	**oomycete**	**effector name**	**domain**
PhCRN01	**no**	LRLFLAK	DO	141	1,00E-33	Phytophthora infestans	Crinkler (CRN) family protein	
PhCRN02	yes	LRLSLAK	SN6	131	1,00E-31	Phytophthora infestans	crinkler (CRN) family protein	Coil
PhCRN03	**no**	LELSLAK	DN17	301	4,00E-82	Phytophthora infestans	crinkler (CRN) family protein	
PhCRN04	**no**	LELSLAK	DBF	1873	0	Phytophthora infestans	Crinkler (CRN) family protein	Aminoglycoside phosphotransferase
PhCRN05	yes	LQLFLAK	no	166	1,00E-41	Phytophthora infestans	crinkler (CRN) family protein	
PhCRN06	**no**	IELFLSKK	DBF	1873	0	Phytophthora infestans	Crinkler (CRN) family protein	Aminoglycoside phosphotransferase
PhCRN07	yes	LKLSLAKK	DN17	571	1,00E-163	Phytophthora infestans	crinkler (CRN) family protein	WD40 repeat
PhCRN09	**no**	LELSLAK	no	311	1,00E-85	Phytophthora infestans	crinkler (CRN) family protein	
PhCRN11	yes	LELSLAK	DBF	179	1,00E-43	Phytophthora infestans	Crinkler (CRN) family protein	
PhCRN16	**no**	LTLYLAK	DXZ 100%	407	1,00E-111	Phytophthora infestans	CRN-like CRN11	
PhCRN17	yes	LELFLGK	DN17	331	2,00E-83	Phytophthora infestans	CRN-like CRN5	
PhCRN18	yes	LELFLAK	newDWL	152	3,00E-35	Phytophthora infestans	Crinkler (CRN) family protein	
PhCRN19	**no**	LQLFLAK	DBF	114	3,00E-24	Phytophthora infestans	Crinkler (CRN) family protein	Aminoglycoside phosphotransferase
PhCRN20	**no**	LELFLAK	DO	1041	0,00E+00	Phytophthora infestans	Crinkler (CRN) family protein	
PhCRN21	yes	LQLFLAK	DBF 57 %	129	7,00E-28	Phytophthora infestans	Crinkler (CRN) family protein	
PhRXLR06	yes	DEER		44,3	0,005	Phytophthora infestans	secreted RxLR effector peptide protein, putative	IQ motif, EF-hand binding site
PhRXLR07	yes	RxLR		88,2	3,00E-16	Phytophthora infestans	secreted RxLR effector peptide protein, putative	
PhRXLR08	yes	DEER		133	7,00E-30	Phytophthora infestans	conserved hypothetical protein	
PhRXLR09	yes	RxLR + DEER						

Table 1: 19 putative CRN and RXLR effectors of *P.halstedii* seems to be diversified and closed to *P.infestans* effectors.

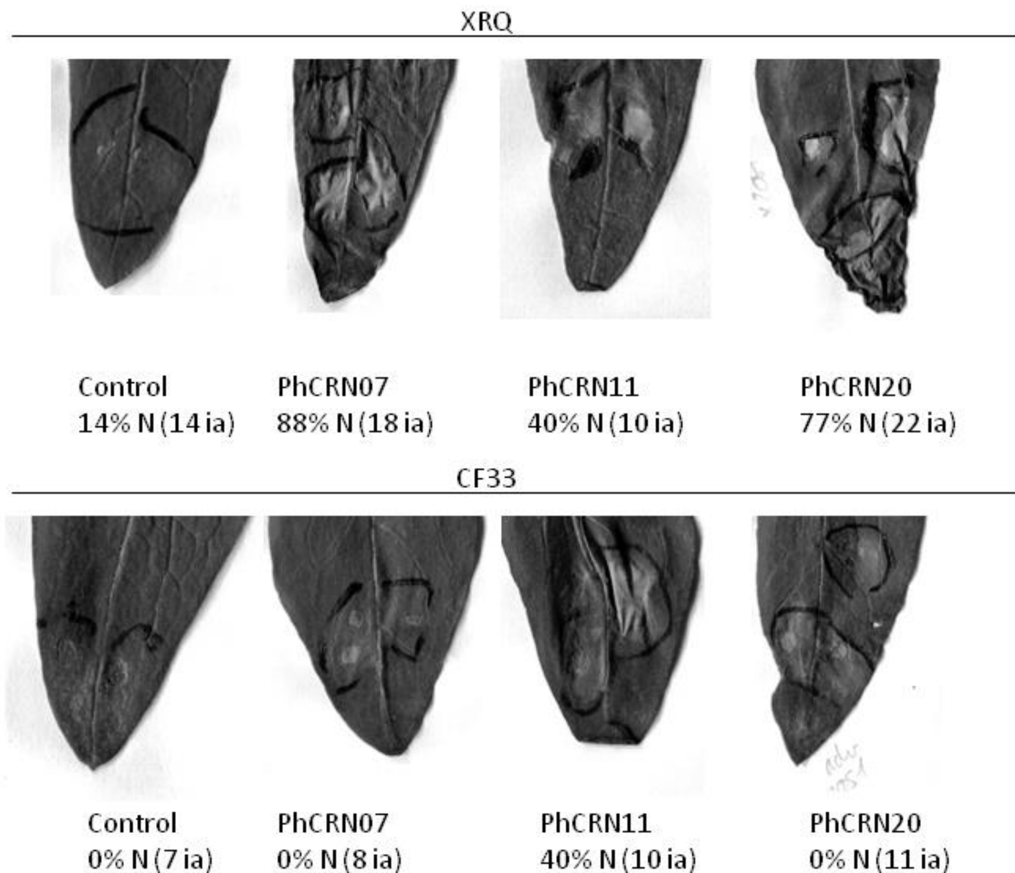

Figure 1: Phenotypes observed of sunflower leaves 5 days after agroinfection with PhCRN effectors. Induction of necrotic symptoms on XRQ (resistant to the race 710 of P.halstedii) and on CF33 (susceptible to the race 710 of P.halstedii). Percentages of necrosis (N) and number of infiltrated leaf areas (ia) are indicated.

DISCUSSION:

Bioanalyses of sequences present in HP basis showed that *P. halstedii*, as most of oomycete obligate biotrophs, have cytoplasmic CRN-like and RxLR-like effectors which may be involved in host cell interactions. Indeed, presence of signal peptide and conserved motives of translocation (RxLR and LxLFLAK) in N-terminal part of encoded proteins, suggest they can act into host cells. Moreover they present a good similarity with effectors (putative or not) of *P. infestans*, another biotrophic oomycete that attacks potato. This similarity is not confined to 5' sequences, and effector sequences (C-terminal domain) supposed to play a role in host cells also looks like some C-terminal domains of *P. infestans* (1)). In the first experiment PhCRN11 which expression induces necrosis in sunflower, has a C-terminal domain similar to necrotic domain DBF of *P. infestans* effector proteins. We hypothesize that *P. halstedii* biotrophic pathogen inject in its host only effectors, which manipulate host cell in keeping it alive to facilitate infection and to draw its nutrients from host. According to Zig-Zag-Zig model our results suggest that plant can recognize some of these effectors activating HR response leading to incompatible interaction. These results seem to be confirmed by the specificity race genotype of these interactions. For example, PhCRN07 induce necrosis in XRQ (resistant to *P. halstedii* race 710), not in Advanta sunflower (susceptible to *P. halstedii* race 710) and PhCRN20 induce necrosis and discoloration respectively in XRQ and Advanta. These results showed that some C-terminal domains, such as of PhCRN07 DN17 and PhCRN20 DO, not necrotic in the case of *P.infestans* and *N.benthamiana*, could be necrotic at least in sunflower particularly in incompatible interaction, probably due to plant recognition.

Although preliminary, these first results are consistent and give some information on the possible role of effector molecule in inducing necrosis depending on the sunflower genotype. This should help to a better

understanding of interactions between *H. annuus* and *P. halstedii.* Nevertheless they have to be confirmed by complementary experiments refining the developed protocol. Future experiments, such as of sub-cellular localization, should be done to better characterize these molecules functionally. Co-agroinfection with different putative effectors could help us to place them on Zig-Zag-Zig model. All these experiments could be done for all putative available effectors which number should increase thanks to the soon availability of the genomic sequence of race 710 of *P. halstedii*. Selection of robust *P. halstedii* effectors could be used in the future to identify the plant targets with which they interact *in planta*. By this way, we should be able to identify molecular determinants of plant involved in susceptibility or resistance whose expression does not change during the interaction.

ACKNOWLEDGEMENTS:

We are grateful to ADVANTA Semillas for kindly providing us with seeds of the CF33 sunflower variety, which was previously used by Manavella and Chan (2009) in their transient transformation experiment.

REFERENCES:

As sadi F., Carrere S., Gascuel Q., Hourlier T., Rengel D., Le Paslier M.C., Bordat A., Boniface M.C., Brunel D., Gouzy J., Godiard L., Vincourt P. 2011. Transcriptomic analysis of the interaction between Helianthus annuus and its obligate parasite Plasmopara halstedii reveals single nucleotide polymorphisms in CRN sequences. BMC Genomics 2011;12(498)

Birch P.R.J., Armstrong M., Bos B., Boevink P., Gilroy E.M., Taylor R.M., Wawra S., Pritchard L., Conti L., Ewan R.Whisson S.C.Van West P., Sadanandom A., Kamoun S. 2009. Towards understanding the virulence functions of RXLR effectors of the oomycete plant pathogen *Phytophthora infestans*. Journal of experimental botany, 60: 1133-40.

Engler C., Kandzia R., Marillonnet S. 2008. A one pot, one step, precision cloning method with high throughput capability.PloS one, 3, e3647

Hein I., Gilroy E.M., Armstrong M.R., Birch P.R.J. 2009. The zig-zag-zig in oomycete–plant interactions, Molecular plant pathology, 10: 547–562.

Huitema E. 2010. Ancient class of translocated oomycete effectors targets the host nucleus. Proceedings of the National Academy of Sciences of the United States of America, 2-7
Jones J.D.G., Dangl J.L. 2006. The plant immune system.,*Nature*, 444: 323-329

Manavella P.A., Chan R.L. 2009 Transient transformation of sunflower leaf discs via an Agrobacterium-mediated method: applications for gene expression and silencing studies.Nature protocols, 4: 699-707.

Morgan W. , Kamoun S. 2007. RXLR effectors of plant pathogenic oomycetes.,” *Current opinion in microbiology* 10:332-338.

Schornack S., Van Damme S., Bozkurt T.O., Cano L.M., Smoker M., Thines M., Gaulin E., Kamoun S.,

Vear F., Serre F., Jouan-Dufournel I., Bert P.F., Roche S., Walser P., Tourvieille de Labrouhe D., Vincourt P. Inheritance of quantitative resistance to downy mildew (Plasmopara halstedii) in sunflower (Helianthus annuus L.) Euphytica 164 (2) :561-570,