

Characterization of the membrane-associated HaRxL17 *Hpa* effector candidate

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Abbreviations: *Hpa*, *Hyaloperonospora arabidopsidis*; HaRxLs, *Hpa* RxLR effector candidates; GFP, green fluorescent protein; RFP, Red fluorescent protein; DAI, day after infection; aa, amino acid

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We examined changes to subcellular architecture during the compatible interaction between the biotroph pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) and its host *Arabidopsis*. Live-cell imaging highlighted rearrangements in plant cell membranes upon infection. In particular, the tonoplast appeared close to the extrahaustorial membrane surrounding the haustorium. We investigated the subcellular localization patterns of *Hpa* RxLR effector candidates (HaRxLs) *in planta*. This subcellular localization screening led to the identification of an extrahaustorial membrane-localized effector, HaRxL17 that when stably expressed in *Arabidopsis* increased plant susceptibility to *Hpa* during compatible and incompatible interactions. Here, we report that the N-terminal part of HaRxL17 is sufficient to target the plant cell membranes. We showed that both C- or N-terminal fluorescent-tagged HaRxL17 localizes around *Hpa* haustoria, in early and in late stages of infection. As with *Hpa* infection, GFP-HaRxL17 also localizes around haustoria during infection with *Albugo laibachii*. Thus, HaRxL17 that increases plant susceptibility to *Hpa* during both compatible and incompatible interactions, localizes around oomycete haustoria when stably expressed in *Arabidopsis*.

for studies of the plant immune system.¹ During the compatible interaction (Fig. 1B), *Hpa* maintains a delicate balance between extracting sufficient resources from the plant to complete its life cycle, while avoiding killing its host. The haustorium (Fig. 1C), a projection from the pathogen into the plant cell is surrounded by a membrane from unknown nature and origin, called the extrahaustorial membrane.² In contrast to plasma membrane markers that are excluded from the extrahaustorial membrane, the tonoplast appears to be in close vicinity to the membrane surrounding the haustorium.³ In addition, we observed a close association between the plant cell nucleus and haustoria using live cell imaging.³ Using trypan blue staining in fixed tissues, here we show that whereas the nucleus is free in the cytoplasm in non-infected cells, the plant cell nucleus is positioned close to the haustorium in infected cells along the growing hyphae (Fig. 1C). Using callose staining, we analyzed callose deposition during compatible interactions between *Hpa* Waco9 and *Arabidopsis* Col-0 (Fig. 1D, 1E). At the tip of the *Hpa* hyphae, callose deposition occurs first at the haustorial neck, leading eventually to a thick haustorial encasement in older hyphae⁴ (Fig. 1E). However, the role of callose deposition during *Hpa*-*Arabidopsis* interaction remains unclear.

Introduction

Hyaloperonospora arabidopsidis (*Hpa*), a widely occurring eukaryotic biotroph pathogen of *Arabidopsis* (Fig. 1A), is one of the two most used model pathogens — along with *Pseudomonas syringae* (*Pst*) —

Results

Subcellular localization of the *Hpa* RxLR effector repertoire. In *Hpa*, over one hundred RxLR effector candidates (HaRxLs) are predicted based on the presence of signal peptide and RxLR

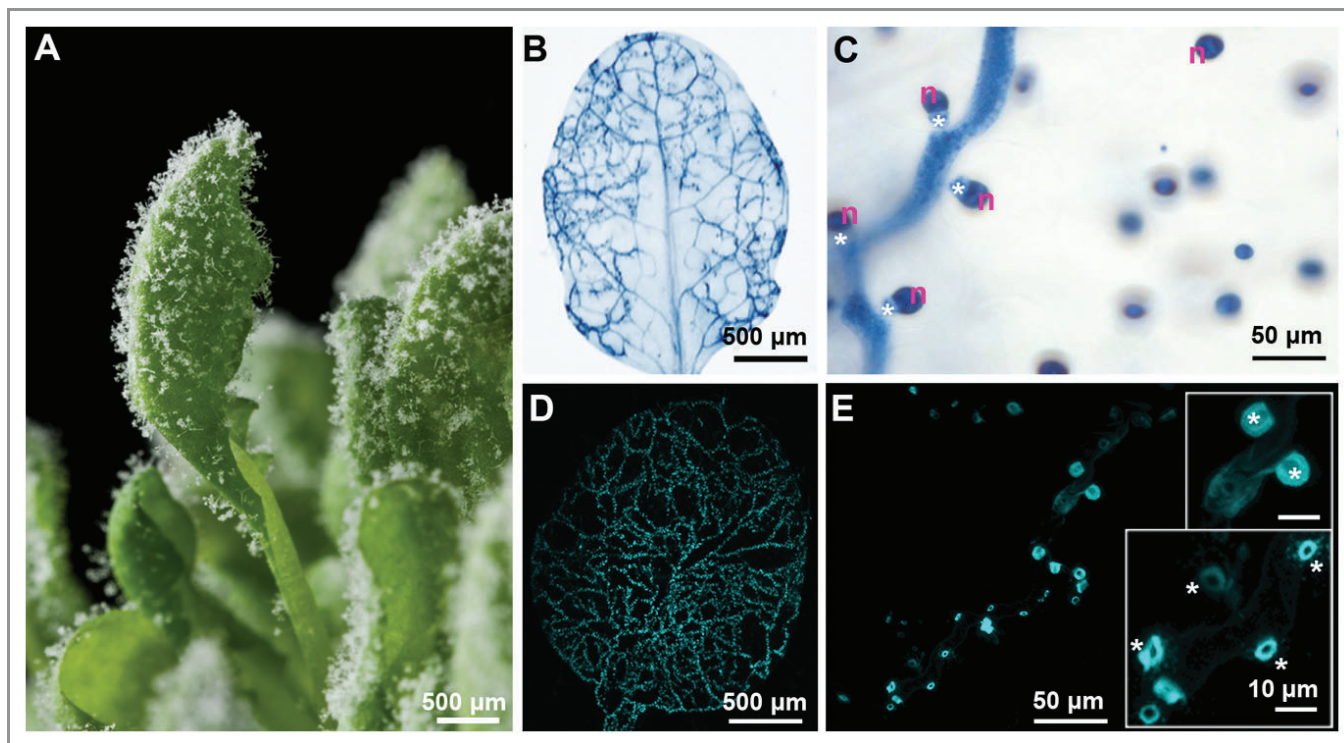


Figure 1. *Hpa* is a model organism in plant pathology. (A) *Hpa* macroscopic symptoms in Arabidopsis infected leaves (B-C) Trypan blue staining *Hpa* Waco9 hyphae in Arabidopsis Col-0 leaves five days after infection (5DAI). (D-E) Aniline blue staining of callose deposition in Arabidopsis Col-0 leaves 5DAI. Asterisk, haustoria; n, nucleus.

motifs.⁵ Recently, the analysis of a collection of over 50 HaRxLs reveals redundant roles of those candidate effectors in suppression of PAMP-Triggered Immune

responses.⁶ We took advantage of this collection to screen for the subcellular localization of the candidate effectors *in planta*.³ By transiently expressing a

fluorescent tagged version of HaRxL candidates, we identified HaRxL17, that localized to the tonoplast.³ We validated the GFP-HaRxL17 localization observed

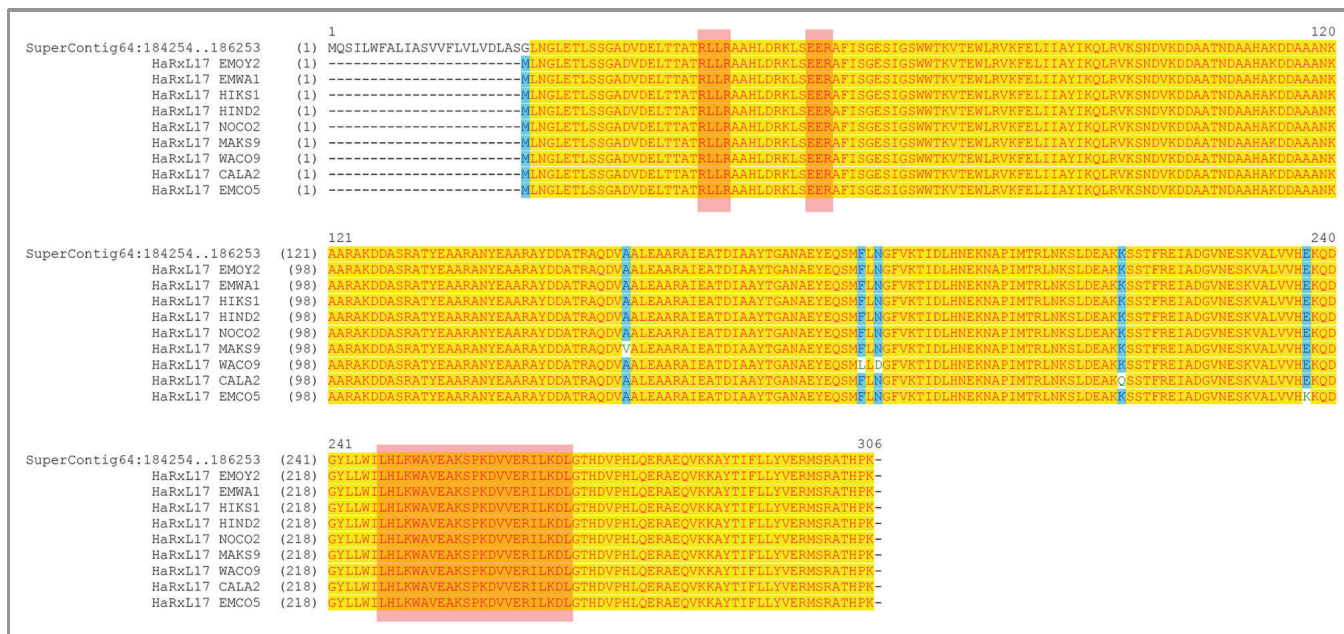


Figure 2. Non-synonymous amino acid polymorphisms in HaRxL17 among the *Hpa* isolates. The motifs in the sequence are indicated in red.

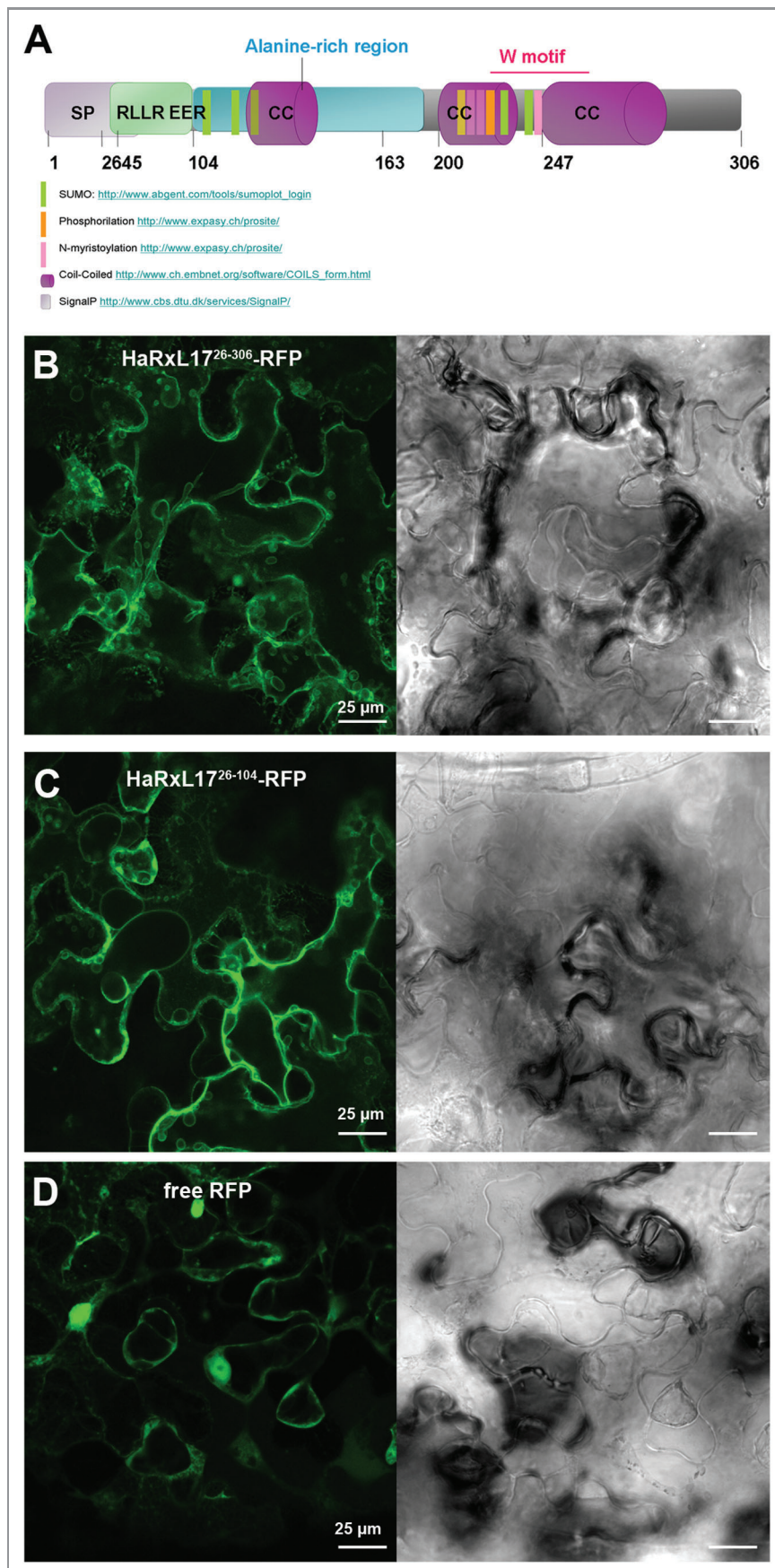


Figure 3. The N-terminal part of HaRxL17 is sufficient to target it to the plant cell membranes. (A) *In silico* prediction of the domain organization in HaRxL17. (B-D) Single-plane confocal images of RFP tagged HaRxL17 truncated version and free RFP in *N. benthamiana* epidermal cells 24 h after infiltration. In order to determine the HaRxL17 truncated version localizations, the cells were plasmolysed by adding sucrose (2M) prior the observations. The Green color corresponds to the RFP signal.

in the screening by generating Arabidopsis transgenic lines.

HaRxL17 confers enhanced plant susceptibility. The role of the membrane-localized HaRxL17 during *Hpa* infection in Arabidopsis was assessed by examining its effects on plant susceptibility, using transgenic lines. We found that independent transgenic lines expressing GFP-HaRxL17 are more susceptible to *Hpa* than the wild type plant during both compatible and incompatible interactions.³

In silico analysis showed that HaRxL17 is a HaRxL of 306 amino acids (aa), which has a predicted signal peptide of 26 aa and RxLR-EER motif (Fig. 3A). The HaRxL17 sequence is predicted to contain an alanine rich region of unknown function and three coiled-coil motifs (Fig. 3A). Notably, HaRxL17 contained a W motif (LHLKWAVEAKS-PKDVVERILKDL, Fig. 3A), a conserved module found in already well characterized *Phytophthora* sp Effectors.⁷

It is known for pathogen effectors that can confer avirulence that polymorphism is a marker of effector evolution.⁸ In order to determine the polymorphism of HaRxL17 among *Hpa* isolates, we performed PCR using specific primer design for *Hpa* Emoy2 isolate, in order to amplify HaRxL17 in the nine *Hpa* isolates (Emoy2, Emwa1, Waco9, Emco5, Noco2, Cala2, Hiks, Hind2, Maks9). Using this approach, we identified five non-synonymous aa polymorphisms in HaRxL17 among the examined *Hpa* isolates (Fig. 2).

Recently, the generation of an interaction network (PPIN1) between *Hpa* (and *Pst*) effectors and Arabidopsis proteins allowed the identification of HaRxLs

putative plant targets.⁹ Out of this screening, perhaps due to the membrane localization of this effector, HaRxL17 interacts only with one protein encoded by At1G14340. At1G14340 encodes a protein that contains a RNA recognition motif, (IPR000504) an Aldo/keto reductase domain (IPR001395) and a Nucleotide-binding domain (IPR012677). In the Arabidopsis Interactome (AI1), At1g14340 encoded protein interacts with proteins implicated in vesicle trafficking and plant immunity.^{9,10} Notably, At1g14340 encoded protein interacts with several prenylated RAB acceptors and the binding partner of acd11 named BPA1.⁹⁻¹¹ Thus, functional analysis of the plant proteins interacting directly or indirectly with HaRxL17 would increase our knowledge on how the pathogen manipulates host membrane trafficking in order to promote virulence.

HaRxL17 localizes to the extrahaustorial membrane when stably expressed in Arabidopsis. We report that in both transient and stably transformed plant cells, C-terminal or N-terminal-tagged HaRxL17 localizes to plant cell membrane³ (Fig. 3B). However, bioinformatic analysis didn't allow us to find any particular motif on the sequence that could explain this localization. We therefore wanted to determine which part of HaRxL17 protein was responsible for its membrane localization. We transiently expressed RFP-tagged truncated versions of HaRxL17 and then performed plant cell plasmolysis in order to determine the precise subcellular localization. Using this approach, we determined that 78 aa from the N-terminal part of HaRxL17 are sufficient to target this effector to the plant cell membrane (Fig. 3C). Recently, Bozkurt et al.,¹² described that the effector AVRblb2 localizes to the cell periphery and accumulates around haustoria in *Phytophthora infestans* infected cells when transiently expressed in *N. benthamiana*.

We next analyzed whether HaRxL17 localization changed during infection with

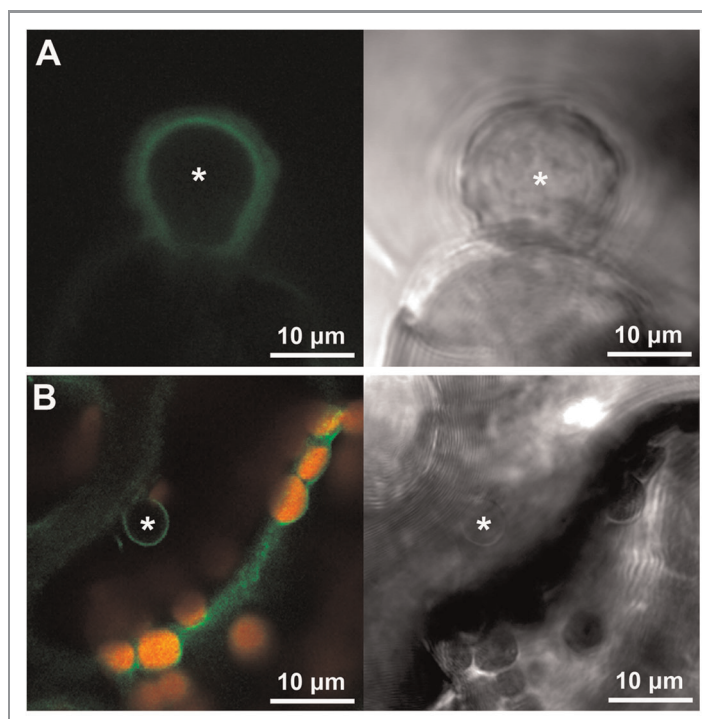


Figure 4. Subcellular localization of fluorescently tagged HaRxL17 in Arabidopsis transgenic line during oomycete infection. In vivo cell imaging in Arabidopsis transgenic lines expressing fluorescently tagged HaRxL17 after infection with biotrophic oomycetes. In the left panel, the green color corresponds to the fluorescence; the red color corresponds to chloroplast auto fluorescence. The right panel represents the corresponding DIC image obtained in bright field. Asterisk, haustoria. (A) HaRxL17-RFP localized around the *Hpa* haustoria 7DAI. (B) GFP-HaRxL17 localized around *Albugo laibachii* haustoria 7DAI.

haustorium-forming pathogens using in vivo cell imaging. As observed in the early stage of infection,³ a strong re-localization of HaRxL17-RFP was observed around the haustoria in late stages of *Hpa* infection (Fig. 4A). In order to determine if HaRxL17 localization was specific to *Hpa* plant-haustorium interface, we next tested Arabidopsis transgenic lines expressing GFP-HaRxL17 with another haustorium-forming Arabidopsis pathogen. Seven days after infection with *Albugo laibachii*, GFP-HaRxL17 localized around haustoria, as we described during *Hpa* infection (Fig. 4A). Thus, HaRxL17 that increases virulence of *Hpa* during both compatible and incompatible interactions, localizes around oomycete

haustoria when stably expressed in Arabidopsis.

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References

1. Jones JD, Dangl JL. The plant immune system. *Nature* 2006; 444:323-9; PMID:17108957; <http://dx.doi.org/10.1038/nature05286>
2. Perfect SE, Green JR. Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. *Mol Plant Pathol* 2001; 2:101-8; PMID:20572997; <http://dx.doi.org/10.1046/j.1364-3703.2001.00055.x>
3. Caillaud MC, Piquerez SJ, Fabro G, Steinbrenner J, Ishaque N, Beynon J, Jones JD. Subcellular localization of the *Hpa* RxLR Effector repertoire identifies the extrahaustorial membrane-localized HaRxL17 that confers enhanced plant susceptibility. *Plant J* 2011. In press. PMID:21914011; <http://dx.doi.org/10.1111/j.1365-313X.2011.04787.x>
4. Donofrio NM, Delaney TP. Abnormal callose response phenotype and hypersusceptibility to *Peronospora parasitica* in defence-compromised arabidopsis nim1-1 and salicylate hydroxylase-expressing plants. *Mol Plant Microbe Interact* 2001; 14:439-50; PMID:11310731; <http://dx.doi.org/10.1094/MPMI.2001.14.4.439>

5. Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A, Kemen E, et al. Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* 2010; 330:1549-51; PMID:21148394; <http://dx.doi.org/10.1126/science.1195203>
6. Fabro G, Steinbrenner J, Coates M, Ishaque N, Baxter L, Studholme DJ, et al. Multiple effectors from the oomycete pathogen *Hyaloperonospora arabidopsidis* suppress host plant immunity. *PLoS Pathog* 2011; 7: e1002348.
7. Jiang RH, Tripathy S, Govers F, Tyler BM. RXLR effector reservoir in two *Phytophthora* species is dominated by a single rapidly evolving superfamily with more than 700 members. *Proc Natl Acad Sci USA* 2008; 105:4874-9; PMID:18344324; <http://dx.doi.org/10.1073/pnas.0709303105>
8. Allen RL, Meitz JC, Baumber RE, Hall SA, Lee SC, Rose LE, et al. Natural variation reveals key amino acids in a downy mildew effector that alters recognition specificity by an *Arabidopsis* resistance gene. *Mol Plant Pathol* 2008; 9:511-23; PMID:18705864; <http://dx.doi.org/10.1111/j.1364-3703.2008.00481.x>
9. Mukhtar MS, Carvunis AR, Dreze M, Epple P, Steinbrenner J, Moore J, et al. Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 2011; 333:596-601; PMID:21798943; <http://dx.doi.org/10.1126/science.1203659>
10. Arabidopsis Interactome Mapping Consortium. Evidence for network evolution in an *Arabidopsis* interactome map. *Science* 2011; 333:601-7; PMID:21798944; <http://dx.doi.org/10.1126/science.1203877>
11. Petersen NH, Joensen J, McKinney LV, Brodersen P, Petersen M, Hofius D, et al. Identification of proteins interacting with *Arabidopsis* ACD11. *J Plant Physiol* 2009; 166:661-6; PMID:18845362; <http://dx.doi.org/10.1016/j.jplph.2008.08.003>
12. Bozkurt TO, Schornack S, Win J, Shindo T, Ilyas M, Oliva R, Cano LM, Jones AM, Huitema E, van der Hoorn RA, Kamoun S. *Phytophthora infestans* effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. *Proceedings of the National Academy of Sciences of the United States of America* 2011; 108:20832-7.