LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

"Anti-infective environmental friendly molecules
against plant pathogenic bacteria for reducing Cu"

ANNEX 4

DELIVERABLE ACTION B3

Demonstration of the chemical and biological stability of anti-virulence peptides produced by conventional chemical and biotechnological synthesis

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1. INTRODUCTION

As previously described sustainable protection of plants against bacterial diseases has to rely on a more rational and environmental use of pesticides, starting from a deep revision of copper application. More generally there is a strong and urgent need to reduce the number of active ingredients, that are asked to be more selective, less toxic and with a lower negative environmental profile. In this frame antimicrobial peptides (AMPs) have had a large attention in the next years (Lay & Gallo, 2009). These compound can be found in nature, produced in low amounts by a wide range of organisms and microorganisms for their natural defenses against biotic stresses, often having a wide range of action spectrum. For these features AMPs have been often indicated with terms such as "host defense peptides", "alarmins", and even "defensins" (Wimley, 2010). Over the past two decades, more than 1,200 AMPs have been identified or predicted from various organisms. For a partial list of these compounds, the Antimicrobial Peptide Database was set up (http://aps.unmc.edu/AP/main.php). Generally, AMPs consist of 10–50 amino-acid residues, and lack any specific consensus amino-acid sequences that are associated. Most of them have got some common features, such as a to contain a positive charge, and a relatively hydrophobic and amphipathic structure. On the basis of their amino-acid composition, size, and conformational structures, AMPs can be divided into several categories, such as peptides with a-helix structures, peptides with b-sheet structures stabilized by disulfide bridges, or peptides with extended or loop structures (Lai & Gallo, 2009). Classic AMPs, such as LL-37 and human b-defensins (hBDs), are amphipathic molecules that possess clusters of positively charged and hydrophobic charged amino-acid chains. This amphipathic feature is thought to allow them to interact with negatively charged phospholipid head groups and hydrophobic fatty acid chains of microbial membranes, resulting in pore formation on the microbial membrane and release of cytosol components (Glaser *et al.*, 2005; Wimley, 2010). It is the membrane-active nature of the AMPs that seems to control their function. And it is the membrane-active nature of the AMPs that likely controls their function. It is important to point out that the difference in prokaryotic and eukaryotic membrane structure gives a certain selectivity to AMPs for bacteria and/or fungi, thus reducing toxic side effects against cells of higher organisms (Alan & Earle, 2002; Montesinos, 2007; Montesinos & Bardaji, 2008).

Some of them have been proved to be toxic, some others are low active when purified or even unstable. Very often complex and costly procedures are required for their extraction and purification starting from the producing organisms/microorganisms. One of the main limitation of the antimicrobial peptide technology for its application in plant protection is due to the production costs. Therefore,

synthetic AMPs, that are about the same molecules but produced *in vitro* or chemically synthesized, can offer a good alternative for their industrial production in the view of their use for replacing conventional pesticides.

The AFTER CU peptides production by a low-cost recombinant biotechnological approach is fully described in the Annex Deliverable B2. These peptides harbor different structural and chemical properties in comparison to traditional AMPs and their target is the so called bacterial Type Three Secretion System (T3SS), essential for the pathogenicity of a broad spectrum of Gram-negative bacteria, that infect both plant and mammalian hosts including humans (He *et al.*, 2004; Mota & Cornelis 2005). It is a syringe-like apparatus, which has been found and deeply studied in over two dozen of Gram-negative phytopathogenic bacteria, where it was shown to be very well conserved both structurally and functionally (Tegli *et al*., 2011). Basically, T3SS is essential to cause disease into host plants by injecting pathogenicity and virulence effector proteins (named "Type Three Effectors", T3Es) directly into the cytosol of host cells. In other words, without a properly working T3SS, pathogenic bacteria are unable to cause disease. This makes T3SS an attractive and ideal target for novel antimicrobial drugs, to replace or reduce the use of copper in agriculture, and of antibiotics for animal and human health (Clatworthy *et al*., 2007; Keyser *et al*., 2008; Baron, 2010; Kline *et al*., 2011; Miles *et al*., 2012; Lun *et al*., 2013). Up to now several classes of synthetic compounds have been identified, as well as natural products, as active T3SS inhibitors in a wide range of Gram-negative bacterial pathogens for animals and humans, such as *Escherichia coli*, *Salmonella*, *Yersinia*, *Shigella* and *Chlamydia* (Kauppi *et al*., 2003; Oh & Beer, 2005; Oh *et al*., 2005; Gauthier *et al*., 2005; Muschiol *et al*., 2009). In particular, and as fully reported in the Annex Deliverable B2, the AFTER CU peptides are targeting T3SS HrpA protein of the phytopathogenic bacteria here used as a model [*Pseudomonas savastanoi* pv. *savastanoi* (Psv), *P. syringae* pv. *syringae* (*Psy*) and *P. syringae* pv. *actinidiae* (*Psa*)].

2. DELIVERABLE ACTION B3

2.1 Experimental design and results

The AFTER CU Action B3 has to demonstrate one of the most challenging aspect of the design and the development of novel compounds for plant disease control, including those against phytopathogenic bacteria, that is to demonstrate their chemical and biological stability on the plant surfaces and/or into the plant apoplast.

To this purpose, pathogenicity trials were carried out on *in vitro* Oleander plants with *Psv* strain Psn23, that is one of the phytopathogenic bacteria here used as a model, in presence or not of an aqueous solution of peptides AP17 and Li27, chemically synthesized. Several experiments were carried out to establish the right peptides' concentration to be used, and then a 30 µM concentration was selected. *In vitro* Oleander plants were simultaneously inoculated into a wound caused by cutting a leaf (at the second internode level, starting from the upper part of the plant) with *Psv* strain Psn23 and 5 µL/inoculum of a 30 µM aqueous solution of peptides AP17 and Li27.

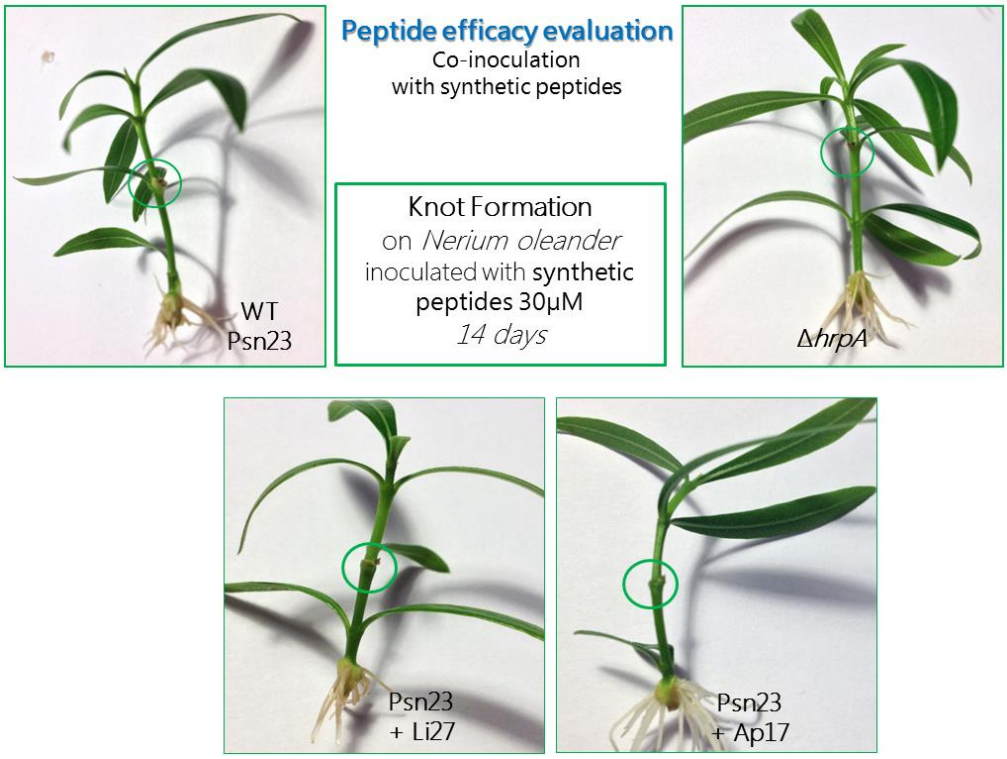

Fig.1. Inoculation trials on *in vitro* Oleander plants challenged with *Psv* wild type Psn23 and its non pathogenic mutants ΔhrpA (unable to produce HrpA protein. Psn23 was also co-inoculated with peptide AP17 and Li27, in 30 µM aqueous solution. Pictures taken 14 days after inoculation and peptides' treatment.

The plants were then incubated into a growth chamber at 24±1 °C, 1500 lux and 16 h light photoperiod. Plants were daily observed and periodically (7, 14 and 21 days post-inoculation) the disease severity and incidence evaluated. The results obtained are shown in Figure 1. As it can be directly observed the results strongly suggest a quite good level of chemical and biological *in planta* stability for the AP17 and Li27 peptides. In fact, 14 days after *Psv* inoculation no typical hyperplastic symptoms were visible on peptides' treated plants, which thus appeared to be "protected" by the AFTER CU anti-virulence peptides by the *Psv* attack (Figure 1). As a positive control the *ΔhrpA* mutant of Psn23 was also used: because of its impaired T3SS, this mutant was unable to cause disease, and its comparison with AP17 and Li27 treated plants further confirmed the AFTR CU anti-virulence peptides' efficacy and stability.

Moreover, the stability of the anti-virulence features of peptides AP17 and Li27 were also demonstrated by examining Psn23 *in planta* multiplication. At 7, 14 and 21 days post-inoculation, the plants were macerated in sterile physiological solution, then using the supernatant for plating on KB medium. After 48 h of incubation at 26°C, the number of colonies formed was evaluated. The results obtained are shown in Figure 2, where the *in planta* inhibitory effect of AP17 and Li27 on the growth of Psn23 is clearly evident.

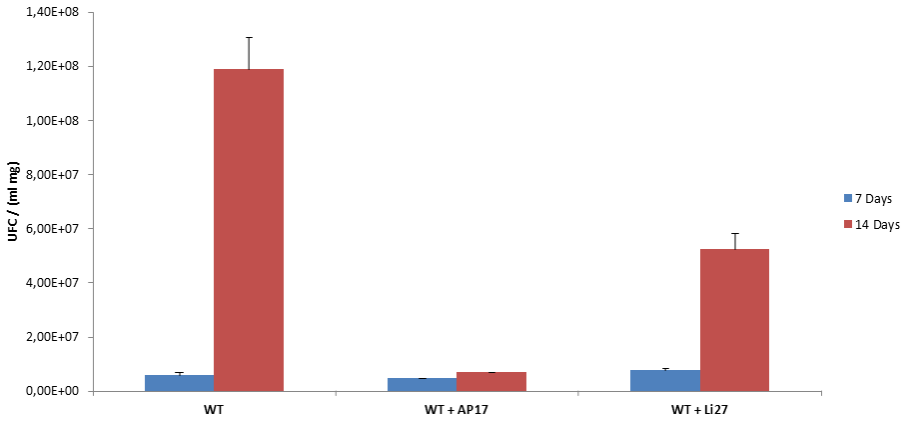

Fig. 2 *In planta* inhibition of Psn23 growth by AP17 and Li27, at 7 and 14 days post-inoculation. *In vitro* Oleander plants were challenged with *Psv* wild type Psn23, co-inoculated with peptide AP17 and Li27, in 30 µM aqueous solution. Pictures taken 14 days after inoculation and peptides' treatment.

In order to be able to experimentally and directly demonstrate that the AFTER CU anti-infective peptides are not readily degraded into biologically inactive products when *in planta*, allowing the maintenance of an acceptable anti-virulence effect, it is also essential to set up and develop some tools, such as antibodies specific for anti-infective peptides and their target protein HrpA. To this aim HrpA

and peptides produced by recombinant synthesis (according to Actions B2) will be really useful to detect and maybe quantify anti-infective peptides and their target HrpA. Conversely, in the present Action B3 a tool was developed, which was firstly useful as positive control in its demonstration activities, but also very supporting for the activities of Actions B2, B4, C2, C3, and C4 as well. Briefly, using the binary vector pCambia1305.2, carrying a signal peptide determining apoplastic localization of any recombinant protein coded by this plasmid, as here monitored *in planta* by using a GUS fusion (Figure 3).

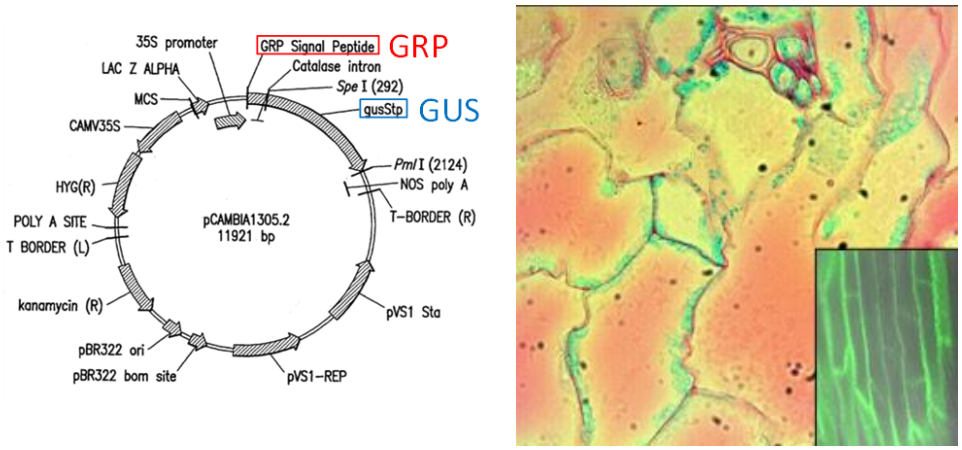

Fig. 3 Map of pCambia1305.2 vector and apoplastic expression of GUS reporter in transiently transformed Tobacco plants.

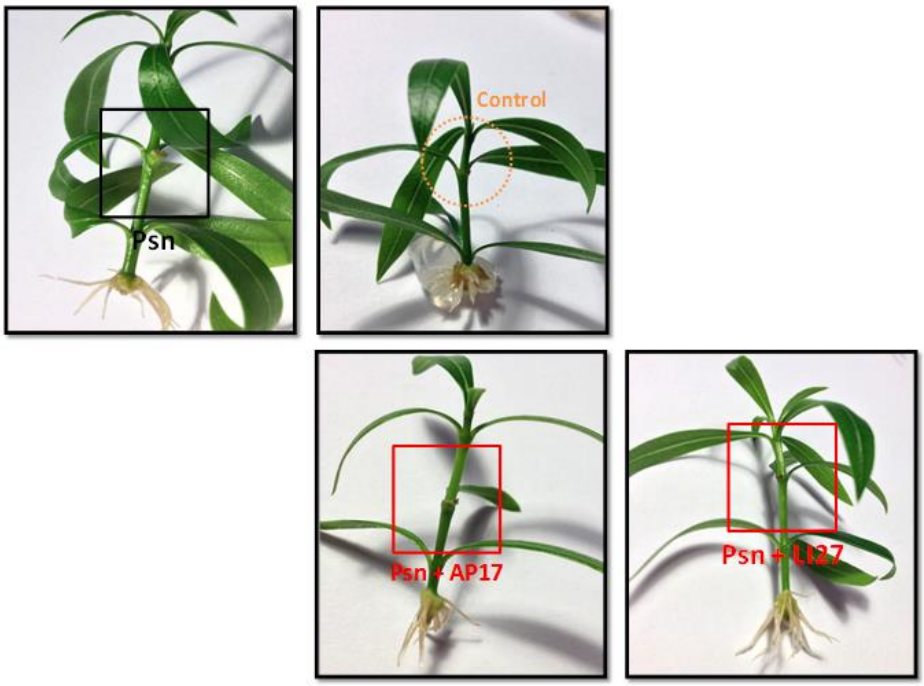

Fig. 4 Inoculation trials on *in vitro* Oleander plants challenged with Psv wild type (Psn23) alone or with *A. tumefaciens* carrying pCambia1305.2-LI27 and pCambia1305.2-AP17 recombinant plasmids, inducing transient expression of each of these peptides by the plant. Pictures taken 14 days after bacterial inoculation.

Two recombinant plasmids were obtained, by fusing to the pCambia1305.2 vector the coding sequences for AP17 and Li27 peptides, named pCambia1305.2-LI27 and pCambia1305.2-AP17 (Figure 4). These plasmids, coding for the LI27 and the AP17 anti-infective peptide, respectively, were electroporated into *Agrobacterium tumefaciens* EHA105 strain, and used the transient expression of these peptides *in planta*, using both *in vitro* Oleander and Tobacco plants, challenged with *Psv* strain Psn23 (Figure 4 and Figure 5).

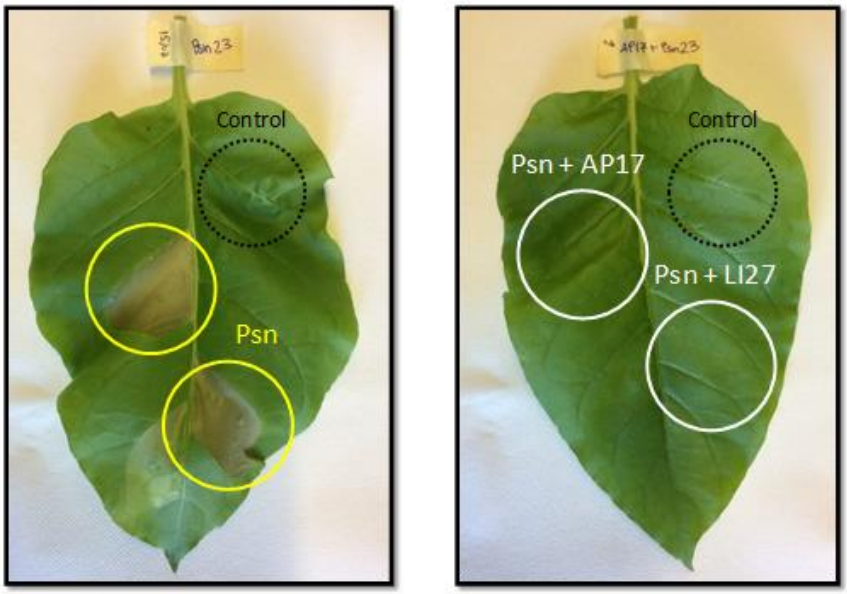

Fig. 5 Inoculation trials on Tobacco leaves challenged with Psv wild type (Psn23) alone or with *A. tumefaciens* carrying pCambia1305.2-LI27 and pCambia1305.2-AP17 recombinant plasmids, inducing transient expression of each of these peptides by the plant. Pictures taken 48 h post-inoculation.

In Figure 4 and Figure 5 it is clearly evident that the *in planta* transient expression of AP17 and Li27 stops Psn23 attack, avoiding the onset of symptoms and of the hypersensitive reaction (HR) on Oleander and Tobacco plants, respectively.

Further confirmation of these data may be obtained in after-LIFE period, when obtaining fully-grown transgenic plants (Tobacco, Oleander and kiwifruit) expressing peptides LI27 and AP17 will be inoculated with *Pseudomonas* spp. These activities are in progress, testing the ability of the different plants species to regenerate *in vitro* after *A. tumefaciens* transformation (Figure 6).

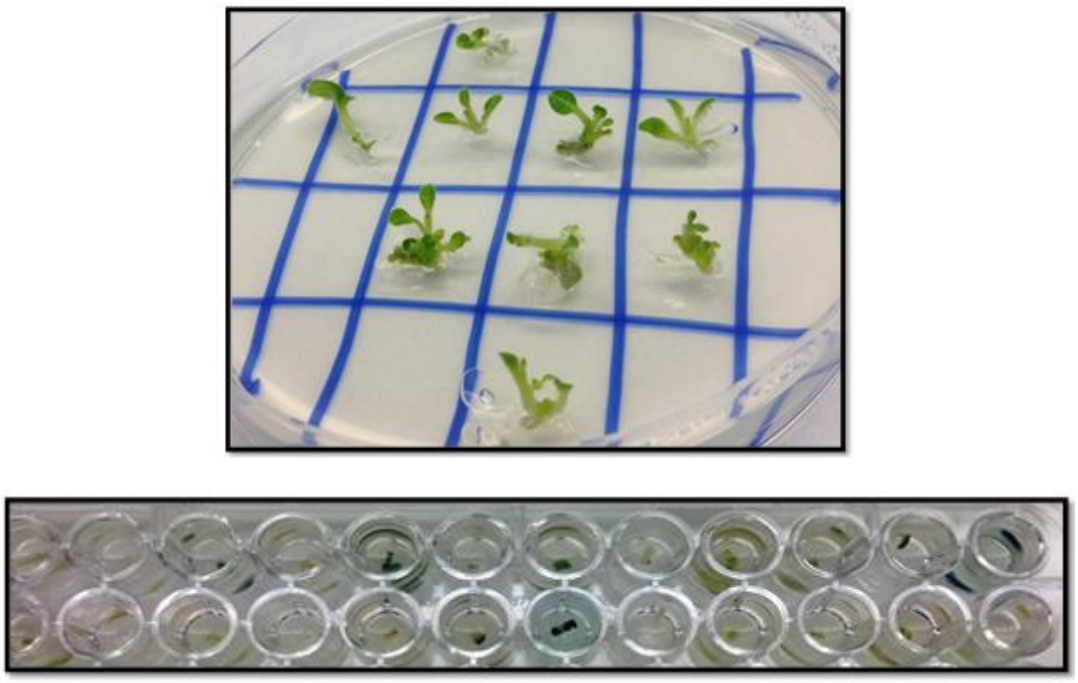

Fig. 6 Regenerating Tobacco shoots transformed with *A. tumefaciens* carrying pCambia1305.2 recombinant plasmids.

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3. CONCLUSIONS

The chemical and biological stability of the AFTER CU anti-virulence peptides was demonstrated using various approaches.

Firstly a 30 µM aqueous solution of peptides AP17 or Li27 was assessed able to block the onset of symptoms on *in vitro* Oleander plants inoculated with *Psv* strain Psn23. In fact, 14 days after *Psv* inoculation no typical hyperplastic symptoms were visible on peptides' treated plants.

Furthermore *in planta* transient expression of AP17 or Li27 peptides *via* agroinfiltration proved these peptides to be efficient competitors of T3SS, preventing the onset of bacterial disease symptoms on host plant and of HR on Tobacco non-host plant.

4. REFERENCES

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