LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

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

ANNEX 8

DELIVERABLE ACTION B4

Demonstration of Kilo-scale biotechnological synthesis of the anti-virulence peptides by recombinant technology

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1. INTRODUCTION

In human and veterinary medicine, bacterial infections are increasingly becoming difficult to be treated for the development of resistance to antibiotics at an alarming rate. Antibiotic resistance is recognised as one of the most serious dangers to human health worldwide in the 21st century, because bacteria have already become resistant also to those antibiotics, such as colistin, considered the last resort in the antibiotics armoury (Prim *et al.*, 2016).

However, antibiotic resistance should be considered a natural phenomenon, because the same antibiotics are naturally present in almost every ecosystem as weapons in the fight for survival. Therefore, many microorganisms have indigenous genetic determinants encoding resistance to some antibiotics which they naturally produce themselves or which they commonly found in the environment where they live as produced by other bacterial or fungal competitors. The genes coding for antibiotic resistance are generally associated with mobile genetic elements, called plasmids, with are capable to be quite easily transferred to other bacteria *via* "Horizontal Gene Transfer" (HGT) events, thus causing antibiotic resistance spread. Moreover, during bacterial evolution, genes for the resistance to different antibiotics frequently clustered together onto the same conjugative plasmids thus originating the so called "multi-drug resistant" (MDR) bacteria now observed globally (Ventola, 2015).

Furthermore, antibiotic resistance has traditionally considered as a clinical-originated problem, while recently non-clinical environments have been highlighted as an important factor in the dissemination of antibiotic resistance genes (Nesme and Simonet, 2015). In particular, in natural environments high levels of antibiotics usually select for resistant bacteria directly, while sub-inhibitory concentrations seem to facilitate HGT (Sengupta *et al.*, 2013). Therefore, the decrease of antibiotic does prevent the spread and maintenance of antibiotic resistance genes in both clinical and natural environments. Moreover, many evidence have accumulated about the exchanges of antibiotic resistance genes between pathogenic and environmental bacteria (Forsberg *et al*., 2012).

Moreover, the spread of antibiotic resistance in the environment is also triggered and/or favored by several anthropogenic pollutants, which promote a co-selection process that indirectly selects also for the resistance to antibiotics (Seiler and Berendonk, 2012). Among these pollutants, heavy metal contaminations are widely spread into the water and the soil. As far as copper (Cu) is concerned, its high levels intro agro-soils were reported to drive co-selection for antibiotic resistance genes (Berg *et al*., 2010; Knapp *et al*., 2011). Despite the extraordinary value of copper in plant protection, its continuous and repeated use endangers also its durable effectiveness, because of the resistance

mechanisms which bacteria develop to evade copper antimicrobial activity (Becerra-Castro *et al.*., 2015).

This phenomenon is dramatically increasing, because the periodic application of copper-based bactericides on crops to control bacterial diseases increases the selection pressure for the development of epiphytic bacterial populations resistant to copper and streptomycin, elevating the risks for transfer of these resistance genes within the plant pathogenic bacterial population and also to other bacteria belonging to the resident microflora. Over the last years, phytopathogenic strains that are resistant to copper treatments have been detected worldwide and are threatening the efficacy of this strategy. Many different strategies have been introduced to fight this phenomenon, such as the use of health seed, the biological control by antagonistic microorganisms, the soil solarization, and the use of natural-occurring antibacterial compounds, in a frame of a sustainable agriculture where the use of chemicals is gradually decreasing. In this frame, wide space has been given to the development of several defense strategies, including the production of natural AMP (Zasloff, 2002). In general, antimicrobial peptides are characterized by a net positive charge and an amphipathic three-dimensional structure that gives the peptides an electrostatic affinity to the outer leaflet of the microbial membrane, mediated by lipid molecules on bacterial surfaces (Powers, Robert E.W. Hancock, 2003). This affinity leads to binding, disruption of the membrane and microbial cell death. The advantage of using antimicrobial peptides as antibacterial agents is that bacteria are less likely to become resistant to these compounds in comparison to antibiotics. However, the peptide availability is one of the major factors that determine the feasibility of their widespread usage as antibiotics and a number of fundamental issues such as mechanisms, efficacy and safety must be addressed. Answering these questions requires extensive functional and structural studies, the progress of which partially relies on the availability of pure peptides. Here, we present a scale up of biotechnological synthesis of the anti-virulence peptides by recombinant technology. Chemical synthesis, although very efficient, is a complex and costly process (Andersson *et al.*., 2000), in comparison to recombinant DNA technology that provides an economical means for protein manufacture. Among the systems available for recombinant protein production, *Escherichia coli* has been the most widely used host (Ingham *et al*, 2007; Chen, 2008). Expression of antimicrobial peptides in *E. coli* faces two challenges. First, the peptides' antibacterial nature makes them potentially fatal to the producing host. Secondly, the peptides' small size and high cationic property makes them highly susceptible to proteolytic degradation. In this era of molecular farming, an ideal system for the production of recombinant proteins would be the one which produces the safest biologically active proteins with ease and at minimum cost. In this frame, the use of plants as biofactories for AMPs might represent an economical and safe alternative. In fact, plants are easy to

transform and recombinant proteins can be produced cost effectively (Giddings *et al.*, 2000; Twyman *et al.*, 2003) and the technology for processing and harvesting plant products already exists (Daniell *et al.*, 2001). Furthermore, mature seeds are in a state of physiological quiescence, with significantly low water content (Parmenter *et al*., 1995; Nykiforuk and Johnson-Flahagan, 1998) accordingly hydrolytic activity is very low and protein degradation is minimal. The recombinant proteins expressed in seeds can be stored for longer periods than those obtained from other sources. For example, GUS enzyme has been reported to remain active for more than one year when expressed as oleosin fusion in seeds (Van Rooijen and Moloney, 1995). Moreover, oleosin gene from a particular source can be expressed in various systems, which includes diverse plants, yeast and even *E. coli*. Oleosin gene from maize has been expressed and oleosin so formed was correctly targeted in Brassica (Lee *et al.*, 1991) and yeast (Ting *et al.*, 1997), from Brassica in tobacco (Batchelder *et al.*, 1994), from soybean in Brassica (Sarmiento *et al.*, 1997), from sunflower in yeast (Beaudoin and Napier, 2002) and from sesame in *E. coli* (Peng *et al.*, 2004). The expression of oleosin gene can occur under the promoter of its own or equivalent seed storage protein even from other plant species (Lee *et al.*, 1991). The heterologous protein can be fused to either N or C terminal (Chiang *et al.*, 2005). The presence of heterologous protein fused with oleosin does not alter the correct targeting of oleosin into the oil bodies (Liu *et al.*, 1997; Nykiforuk *et al.*, 2005). For example, the production of several recombinant proteins and peptides has been successfully accomplished in transgenic rice seeds, including vaccines, hormones, antibodies, and other pharmaceutical peptides. In conclusion, there is no doubt that the production of recombinant proteins in microbial systems has revolutionized biochemistry. In fact, the ability to express and purify the desired recombinant protein in a large quantity allows for its biochemical characterization, its use in industrial processes and the development of commercial goods. However, we also have to take into consideration the production of peptides in bacteria by recombinant DNA technology has been impeded by the antimicrobial activity of these peptides and their susceptibility to proteolytic degradation, while subsequent purification of recombinant peptides often requires multiple steps and has not been cost-effective. For references concerning the costs of recombinant peptides production we refer to at the following links:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4271771/

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3813893/

2. DELIVERABLE ACTION B4

2.1 Experimental procedures and results

2.1.1 Scale-up peptides synthesis by recombinant DNA-based technology

The biotechnological synthesis at kilo-scale of AFTER CU anti-infective peptides and of its target protein HrpA was performed using the methodological approach reported in Action B2. The bacterial culture for scale-up peptides synthesis by recombinant DNA-based technology were performed in 2-10L.

2.1.2 Anti-infective peptides scale-up synthesis by recombinant technology

According to the previous results obtained for the peptides production at laboratory scale by recombinant technology, first of all we have developed the procedure on HrpA protein and subsequently on the After-Cu peptides. The HrpA protein and AP17, Li27 peptides obtained by recombinant technology from 2-10L of bacterial culture have been analyzed by SDS-PAGE and showed below. The HrpA protein and peptides obtained have showed a not very high yield around at 80-100mg.

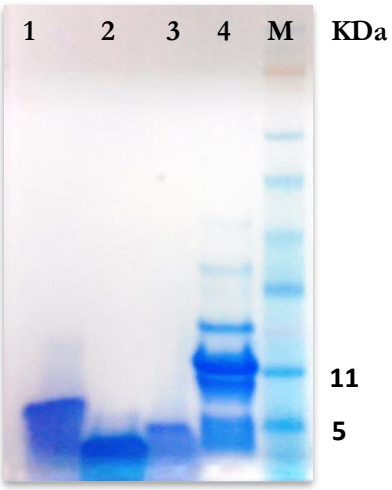

Fig. 1 Final result of scale-up recombinant production of Li27(1), Ap17 (2-3) and HrpA protein (4).

In particular, the fraction corresponding to HrpA protein have showed contaminants in the background and for this reason a subsequently purified procedure by size-exclusion (Superdex™ 75pg, GE Healthcare) was performed and the results was showed below. The recovered fraction corresponding to HrpA protein was quantified, as reported in Action B2, with a yield around at 80mg.

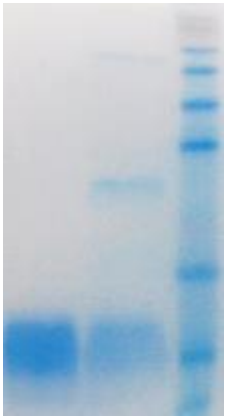

Fig. 2 SDS-PAGE of recombinant HrpA after size-exclusion on the left and on the right before size-exclusion.

2.1.3 Polyclonal antibody against *P. savastanoi* pv. *nerii* HrpA and AP17 and Li27

The obtained HrpA protein was then used for the production of polyclonal antibodies necessary for subsequent tests and analysis. Polyclonal primary antibodies against HrpA protein of *Psn23* were obtained from Primm srl (Milano-Italy) following immunization of two rabbit with recombinant protein HrpA. The antibody performance was checked and verified by Western blot analysis.

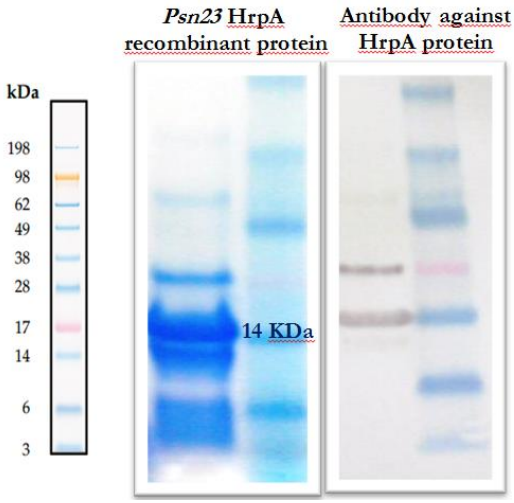

Fig. 3 SDS-PAGE analysis of HrpA recombinant protein of *Psn23* (left). Western blot with polyclonal antibody against *Psn23* HrpA protein.

Finally, the HrpA antibody was used in order to perform ELISA assay to quantify the peptides obtained by recombinant technology, given their common primary structure.

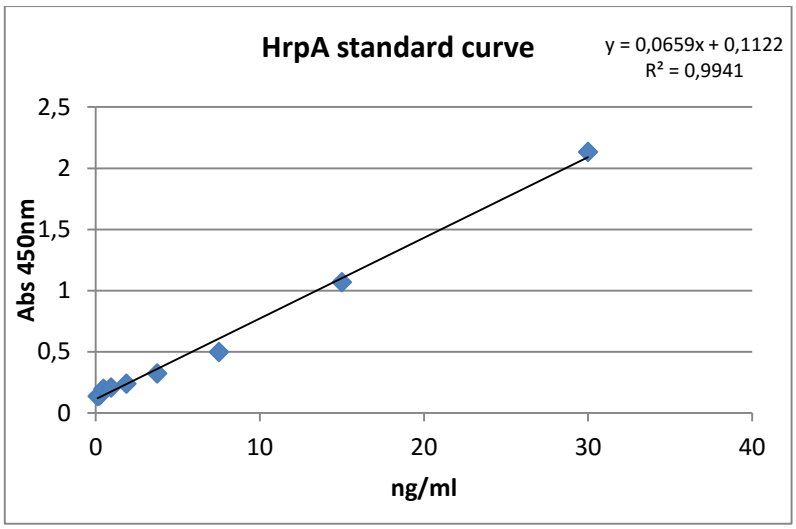

Fig. 4 Standard curve performed with serial dilution of HrpA recombinant protein.

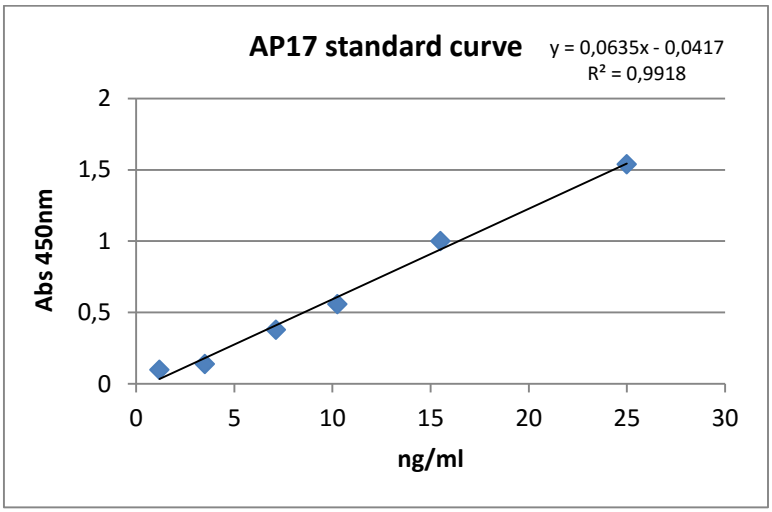

Fig. 5 Standard curve performed with serial dilution of AP17 recombinant peptide.

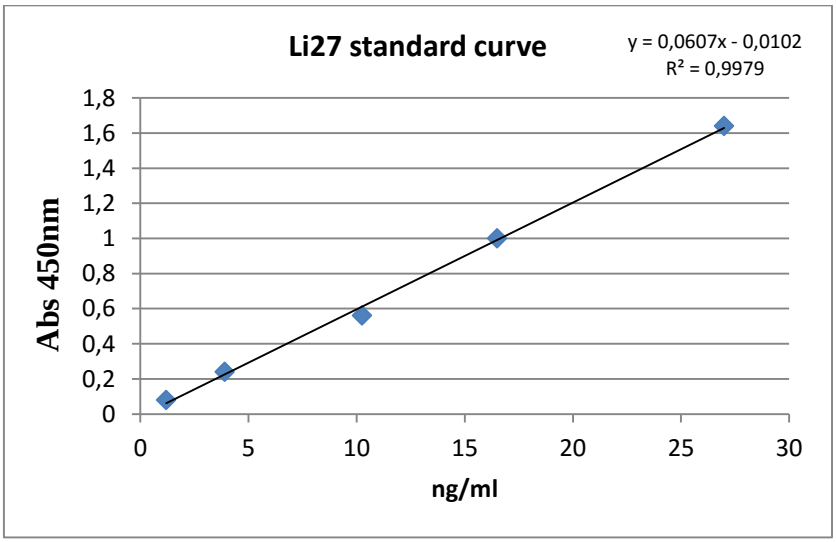

Fig. 6 Standard curve performed with serial dilution of Li27 recombinant peptide.

3. CONCLUSIONS

The scale up production of AFTER Cu peptides by DNA recombinant technology has produced a not very high yield. The quantity obtained is not proportional and affordable in comparison to chemical synthesis and concerning time and employed costs. Nevertheless, the recombinant strategy still remains the most preferable procedure, also in view of an eco-friendly production for the AFTER Cu peptides. Therefore, an alternative strategy for the more effective production of recombinant peptides on industrial scale should be further evaluated, working in close relationship with biotech companies involved in this sector and interested in the AFTER Cu technology.

4. REFERENCES

- Ingham A.B., Moore R.J. (2007) Recombinant production of antimicrobial peptides in heterologous microbial systems. *Biotechnol. Appl. Biochem*. 47: 1–9.
- Beaudoin F, Napier JA. (2002) Targeting and membrane-insertion of a sunflower oleosin in vitro and in *Saccharomyces cerecisiae*: the central hydrophobic domain contains more than one signal sequence, and directs oleosin insertion into the endoplasmic reticulum membrane using a signal anchor sequence mechanism. *Planta* 215:293-303.
- Becerra-Castro C, Machado RA, Vaz-Moreira I, Manaia CM. (2015) Assessment of copper and zinc salts as selectors of antibiotic resistance in Gram-negative bacteria. *Sci Total Environ*. 15:530-531.
- Berg J, Thorsen MK, Holm PE, Jensen J, Nybroe O, Brandt KK. (2010) Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environ Sci Technol.* 44:8724-8728.
- Batchelder C., Ross JHE, Murphy DJ (1994) Synthesis and targeting of *Brassica napus* olesin in transgenic tobacco. *Plant Science* 104 39-47.
- Chi-Chung Peng, Jeff C.F. Chen, Douglas J.H. Shyu, Ming-Jinn Chen, Jason T.C. Tzen. A System for purification of recombinant preoteins in Escherichia coli via artificialo il bodies constituted with their oleosin-fused polypeptifes. *Journal of Biotechnology* (2004) 51–57.
- Chiang CJ, Chen HC, Chao YP, Jason TC, Tzen (2005) Efficient System of Artificial Oil Bodies for Functional Expression and Purification of Recombinant Nattokinase in *Escherichia coli*. *J. Agric. Food Chem*. 53:4799−4804.
- Daniell H, Lee SB, Panchal T, Wiebe PO (2001) Expression of the native cholera toxin b subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *Journal of Molecular Biology* 311:1001-1009.
- Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337: 1107-1111.
- Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. *Nature Biotechnology* 18:1151-1155.
- Liu JH, Selinger LB, Cheng KJ, Beauchemin KA, Moloney MM (1997) Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus *Neocallimastix patriciarum*. *Molecular Breeding* 3: 463-470.

- Powers JPS, Hancock REW (2003) The relationship between peptide structure and antibacaterial activity. *Peptides* 24:1681-1691.
- Knapp CW, McCluskey SM, Singh BK, Campbell CD, Hudson G, Graham DW (2011) Antibiotic Resistance Gene Abundances Correlate with Metal and Geochemical Conditions in Archived Scottish Soils. *PLoS ONE* 6(11): e27300.
- Andersson L, Blomberg L, Flegel M, Lepsa L, Nilsson B, Verlander M (2000) Largescale synthesis of peptides, *Biopolymers* 55:227–250.
- Lee WS, Tzen JTC, Kridl JC, Radke SE, Huang AHC (1991). Maize oleosin is correctly targeted to seed oil bodies in *Brassica napus* transformed with the maize oleosin gene. *Proc. Natl. Acad. Sci.* USA 88:6181–6185.
- Nesme J, Simonet P (2015) The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. *Environ Microbiol*. 17:913-30.
- Nykiforuk CL, and Johnson-Flanagan AM (1998). Low temperature emergence in crop plants: Biochemical and molecular aspects of germination and early seedling growth. *J. Crop Production* 1:249-289.
- Nykiforuk CL, Boothe JG, Murray RG, Keon HJ, Goren NA, Markley MM, Moloney (2005) Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. *Plant Biotechnol. J.* 4:77-85.
- Parmenter DL, Boothe JG, van Rooijen GJ, Yeung EC, Moloney M.M. (1995) Production of biologically active hirudin in plant seeds using oleosin partitioning. *Plant Mol Biol.* 29:1167-1180.
- Sarmiento C, Ross JH, Herman E, Murphy DJ. (1997) Expression and subcellular targeting of a soybean oleosin in transgenic rapeseed. Implications fot the mechanism of oil-body formation in seeds. *Plant J.* 11:783-796.
- Seiler C, Berendonk TU (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology* 3:399. doi:10.3389/fmicb.2012.00399.
- Sengupta S, Chattopadhyay MK, Grossart HP (2013) The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology* 4:47. doi:10.3389/fmicb.2013.00047.
- Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003). Molecular farming in plants: host systems and expression technology. *Trends in Biotechnology* 21:570-578.
- van Rooijen GJ, Moloney MM. (1995) Plant seed oil-bodies as carriers for foreign proteins. *Biotechnology* 13:72-77.

- Ventola CL (2015) The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics* 40:277-283.
- Li Y, Chen Z (2008) RAPD: a database of recombinantly-produced antimicrobial peptides, *FEMS Microbiol. Lett*. 289:126–129.
- Zasloff M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415:389-395.