Enhancing disease resistance against microbial pathogens by expression of Et-AMPs in Arabidopsis plants

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List of Abbreviations

alfAFP	alfalfa antifungal peptide
AMPs	Antimicrobial peptides
AsA	ascorbic acid
Avr	Avirulence
Bgh	Blumeria graminis f. sp. hordei
bp	base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
C.F.U	colony-forming unit
CHI2	chitinase gene
CRPs	cysteine-rich peptides
CSαβ	Cystine stabilized αβ motif
cv.	Cultivar
Cys	cystine
DHA	dehydroascorbate
DNA	Desoxyribonucleic acid
dai	day(s) after inoculation
EDTA	ethylenediaminetetraacetic acid
endo-PGs	endopolygalacturonases
ER	endoplasmic reticulum
Et	*Eristalis tenax*
et al.	and others
FHB	Fusarium head blight
Fig.	Figure
FRR	Fusarium root rot
GNBP	Gram-negative binding proteins
GP	Golden Promise
HEWL	hen egg white lysozyme
His	Histidine
IMD	Immune deficiency pathway
IWF	intercellular washing fluid
IPAZ	Institute of Phytopathology and Applied Zoology
IPTG	Isopropyl-β-D-thiogalactopyranoside
JA	Jasmonic acid
kDa	Kilo Dalton
L	Liter
LB	Luria-Bertani medium
M	Molar
MF3	microbial factor 3 gene
Min	Minute (s)
Mtk	Metchnikowin

List of Abbreviations

Ni-NTA	nickel-nitrilotriacetic acid affinity chromatography
ORF	Open reading frame
PAGE	Polyacrylamid gelelektrophorese
PCD	programmed cell death
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	potato dextrose broth
PGRP	peptidoglycan recognition proteins
pPGIP	polygalacturonase inhibitor protein
PR	Pathogenesis related
Pst	*Pseudomonas syringae* pv. *tomato* strain DC3000
RCC2	rice chitinase gene
RNA	Ribonucleic acid
rpm	rounds per minute
ROS	reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
SP	signal peptide
Tab	Table
TBE	Tris-Borate-EDTA
Tris	Tris-(hydroxymethyl)-aminomethan
Wt	Wild type

1. Introduction

Plants are vulnerable to attack by pathogenic organisms and environmental stresses. To ensure a long-lasting existence under these conditions, they need fast defense responses to inhibit pathogen spread after primary infection and hence to limit disease. Among these defense responses are a rapid oxidative burst, local transcriptional activation of defense-related genes, and the generation of yet unknown systemic signals that trigger and induce a state of systemic immunity (Ali and Reddy, 2000).

Living organisms exude a broad range of antimicrobial peptides-as the first line of defense in plants and animals-produced through ribosomal (defensins and small bacteriocins) or non-ribosomal synthesis (peptaibols, cyclopeptides and pseudopeptides, Montesinos, 2007).

Genetic engineering has emerged as a new strategy for ameliorating disease resistance through cellular and molecular tools (Beers and McDowell, 2001).

Animal and plant produce antimicrobial peptides by ribosomal synthesis, thus supplying tools for improving transgenic plants expressing genes coding for the synthesis of these compounds granting partial or total resistance to plant pathogens (Montesinos, 2007).

1.1 Defensins

In the 90s of the last century, this term has emerged to be given to a class of plant peptides (small basic proteins) with structural and functional properties gathering those of insect and mammalian defensins (Terras *et al.,* 1995), except that insect defensins lack the domain corresponding to the amino-terminal b-strand of plant defensins (Portieles *et al.,* 2006).

Hitherto, there is a comprehensive collection of 363 defensin records each containing sequence, structure and activity information defensins knowledge base (http://defensins.bii.a-star.edu.sg/).

The fast biotechnological development has contributed to the application of defensins in plant improvement, increased pest resistance; in addition crop production was increased.

Moreover, defensins are also being exploited in the pharmaceutical field for developing new antibiotics and fungicides active against pathogenic microorganisms (Murad *et al.,* 2007).

Comparatively small preproteins produce the defensins which are targeted to the endomembrane system, the apoplast, and in some conditions the vacuole, by short amino-terminal and/or carboxy-terminal signal motifs (Lay and Anderson, 2005).

Portieles *et al.* (2006) pointed out that the defensins, thionins and lipid transfer proteins (LTP) comprise the major groups of antimicrobial peptides in plants.

Defensins, found widely in plants, insects and mammals, are a family of endogenous cationic antimicrobial peptides with broad-spectrum bactericidal activity and low susceptibility to acquired bacterial resistance (Ganz, 2005). They can be found throughout the plant kingdom, they are highly basic cysteine-rich peptides (CRPs) belonging to the large defensin family, and contribute to the ancestral non-specific innate immune defense system. Furthermore, defensins are involved in mediating plant responses to pathogens (Huffaker *et al.,* 2006).

Collila *et al*. (1990) ; Mendez *et al.* (1990) ; Terras *et al.* (1993) and Sjahril *et al.* (2006) reported about isolation of plant defensins from wheat, barley (as the first two isolated plant defensins), sorghum, pepper, mustard, radishes and other species of Brassicaceae. An analysis of amino acid sequence of this class (defensins) revealed that it was probably a new family of thionins, with a and b-thionins, so it named a gamma-thionins (Collila *et al.,* 1990) because the number of their disulfide bridges (four) and size (5kDa) is similar to alpha-thionins and beta-thionins. Afterward, studies have proved that gamma-thionins are structurally unrelated to alpha-thionins and beta-thionins (Terras *et al.,* 1992; Bruix *et al.,* 1993; Bohlmann, 1994).

Other studies trended to consider the g-thionins as a super family of antimicrobial peptides not evolutionarily related to b-thionins, with representatives in vertebrates, invertebrates and plants, renamed them as plant defensins (Broekaert *et al.,* 1995).

Plant defensins contribute as a part of the innate immunity arsenal and are omnipresent in plants. Small multigene families encode plant defensins and are expressed in various plant tissues with best characterization in seeds. Typically, they are produced as preproteins, although a small subset is produced as larger precursors with C-terminal prodomains (Lay and Anderson, 2005).

Genes encoding defensins in plants are developmentally adjusted, with a prevalent expression in outer cell layers (Moreno *et al.,* 1994; Terras *et al.,* 1995); these genes can be stimulated as result to pathogen infection and other stresses above basal levels (Chiang and Hadwiger, 1991; Terras *et al.,* 1995; Penninckx *et al.,* 1996; Epple *et al.,* 1997). Furthermore, some defensin genes are down-regulated by some pathogens (Moreno *et al.,* 1994). According to that, patterns of Gene expression of defensins are compatible with a hypothetical role in plant defense (Broekaert *et al.,* 1995; Broekaert *et al.,* 1997).

1.1.1 Source of plant defensins

Defense peptides are characterized in diverse eukaryotic organisms, including mammals (Ganz and Lehrer, 1994), plants (Broekaert *et al.,* 1995), birds (Djik *et al.,* 2008), teleost fish (Zou *et al.,* 2007), mollusks (Charlet *et al.,* 1996), insects (Bulet *et al.,* 1999; Fehlbaum *et al.,* 1994) aracnidae (Cociancich *et al.*, 1993), crustaceans (Saito *et al.,* 1995) and fungi (Mygind *et al.,* 2005).

1.1.2 Structure of plant defensins

Defensins are one class among the several types of Cys-rich antimicrobial peptides that vary in bonds, folding patterns, length and number of cystine (Boman, 1995).

Plant defensins are a super family of similarly folded antimicrobial peptides found in vertebrates, invertebrates, mollusks, and plants. They are very stable to temperature and pH variations (Terras *et al.,* 1995; Selitrennikoff, 2001) and consist of 45 to 54 amino acids residues long. Thus they form a small molecule with a molecular mass between 5 and 7 kDa – as opposed to up to 10 kDa mentioned in some reviews (Olli and Kirt, 2006; Olli *et al.,* 2007) – with a net positive charge and clear, relatively limited sequence conservation that contains eight strictly conserved Cys residues which are involved in four intrachain disulfide bridges being responsible for the stabilization of the three-dimensional structure. In addition to some other amino acid positions, that three-dimensional structure of plant defensins, being small and globular, resembles that of insect, mollusks and mammalian defensins (Bruix *et al.,* 1992; Carvalho and Gomes, 2009; Yang *et al.,* 2009).

They have three anti-parallel β-sheets and one α-helix that is stabilized by a structural motif composed of disulfide bridges. That motif is found in other peptides with biological activity and called the Cys stabilized αβ motif (CSαβ). The CSαβ defensins are critical

effector elements for innate immunity defense against microbial infection (Bulet and Stocklin, 2005).

The encoded polypeptide by the mature copy of the majority of plant defensins consists of two parts (Carvalho and Gomes, 2009).

Part1: It is the amino-signal peptide that targets the peptide to the extracellular space.

Part2: It is the mature peptide domain.

Furthermore, some flowering plant defensins also have a carboxyl-terminal domain.

The defensins display significant variations in their amino acid sequences, possibly based on selective pressures to enable them to challenge with a wide range of microbial agents (Oppenheim *et al.,* 2003). A number of antimicrobial peptides such as defensins have multiple functions in host defense; they activate not only innate but also adaptive immune responses (Oppenheim *et al.,* 2003).

It has been obvious that plant defensins are complex and sophisticated peptides with functions that exceed their role in defense of plants against microbial infection relying on the knowledge on defensin structure, gene expression and regulation, and also their *in vitro* biological activity.

Using several representative members of the defensins, for instance the antifungal plant defensin from *Raphanus sativus*, antibacterial arthropod defensins from *Ornithodoros moubata*, *Pyrrhocoris apterus*, *Tenebrio molitor* and mussel defensin from *Mytilus galloprovincialis*, for studying the structure–function relationships of the defensins revealed their functional region (Ahn *et al.,* 2006; De Samblanx *et al.,* 1997; Lee *et al.,* 1998; Romestand *et al.,* 2003; Varkey *et al.,* 2006), principally located in the carboxy-terminal β-sheet domain, named the 'γ-core motif' by Yount and Yeaman (2004).

It is noteworthy that peptides with principally antifungal activity, like several of those isolated from plants, tend to be relatively rich in polar neutral amino acids, suggesting a unique structure-activity relationship (Hancock and Chapple, 1999).

An amphipathic construction in the γ-core, distinguished by positively charged and hydrophobic residues structurally clustered in two detached domains, is an essential demand for antimicrobial activity of these peptides, which seems to be unconnected to the charge property of the entire molecule (Lai *et al.,* 2004).

The three-dimensional structure for insect α-amylases (SIα1) by nuclear magnetic resonance ^{1}H NMR were established by Bloch *et al.* (l998), who discovered the topologic

similarity to charybdotoxin (2CRD), a small basic protein (37 residues with 3 disulfide bridges) that was isolated from the scorpion (*Leiurus quinquestriatus*), that links specifically to potassium channels, blocking them. There are some studies about the structure of defensins saying that they are flat triple-stranded molecules assembled together by three intramolecular disulfide bonds. Furthermore, the lack of alpha helix in α-defensins, α- and ß-defensins contrast in their cysteine amino acids interval, disulfide bond linkage and gene organization (Selsted and Ouellette, 2005; Ganz, 2005; Lehrer, 2007). Gamma-defensins are small cyclic peptides, originated from α-defensins (Tang *et al.,* 1999). Additionally, ß-defensins are to some extent larger, and some of them have modified termini (Boman, 2003). They consist of two distinct genes producing two separate defensins and are eventually linked covalently (Boman, 2003).

Due to the plant defensins having a triple stranded, antiparallel β-sheet and a single α-helix standing in parallel with the β-sheet, they have a prevalent three-dimensional structure (Bruix *et al.,* 1993). In spite of that, Bert *et al.* (2003) mentioned that the defensin of *Petunia hybrida*, PhD1 has five disulfide bonds.

Consequently, the fifth disulfide bond, that is neighboring to the hydrogen bond between the conserved threonine and glutamic acid residues of the other plant defensins, efficiently fixes the protein to compose part of the hydrogen bond network dispensable, thus playing a complicated role in the stabilization of the protein in a manner that is harmonizing to those of other plant defensins. The conserved hydrogen bond is significant for the function of plant defensins in respect of the fact that mutation of Thr10 (residue B) to glycine, or mutation of Leu28 or Phe49, each of which is next to Glu27 (residue A), to an arginine results in a remarkable loss of activity in Rs-AFP2 (De Samblanx *et al.,* 1997). Subsequently, the hydrogen bond between the threonine and the glutamic acid established on electrostatic considerations are likely to disrupt due to all these mutations.

Some plant defensins exhibit antifungal activity, although it is not known whether they have a common mode of action (Broekaert *et al.,* 1997).

All plant defensins are part of a common three-dimensional folding pattern, endured by eight disulphide linked cysteines (Broekaert *et al.,* 1995 and 1997). They are also structurally correlated to antibacterial insect defensins (Cociancich *et al.,* 1993).

Finally, the majority of plant defensins are a serine at position 7, while an aromatic residue at position 10, with two glycines at positions 12 and 32, and a glutamic acid at position 27 (numbering relative to NaD1, Broekaert *et al.,* 1995; Lay *et al.,* 2003).

1.1.3 Classification of defensins

We can divide the plant defensins into two major classes in view of the structure of the precursor proteins predicted from cDNA clones (Lay *et al.,* 2003):

1- This class is the largest, in which the precursor protein is composed of an endoplasmic reticulum (ER) signal sequence and a mature defensin domain. These proteins enter the secretory pathway and have no obvious signals for post-translational modification or subcellular targeting.

2- In the second class, defensins are produced as larger precursors with C-terminal prodomains of about 33 amino acids. These defensins have only been found in solanaceous species where they are expressed constitutively in floral tissues (Lay *et al.,* 2003; Gu *et al.,* 1992; Milligan and Gasser, 1995; Brandstadter *et al.,* 1996). In addition, defensin expression can also be induced by salt stress in the leaves of some Nicotiana species (Yamada *et al.,* 1997; Komori *et al.,* 1997).

Depending on the annotation information of protein sequences, Zuo and Li (2009),after omitting the redundant sequences, had 286 non-redundant defensin proteins and classified them into four families:

1- The first one comprises 48 plant defensins.

2- While the second family included 37 insect defensins.

3- The largest one included 190 vertebrate defensins.

4- The last family included 11other defensins.

Then they selected three main vertebrate subfamilies: Alphatype (60 sequences), Beta-type (126 sequences) and Theta-type (4 sequences).

After that, they explained the distribution of diverse sequence identities for each defensin family shown in Fig. 1.

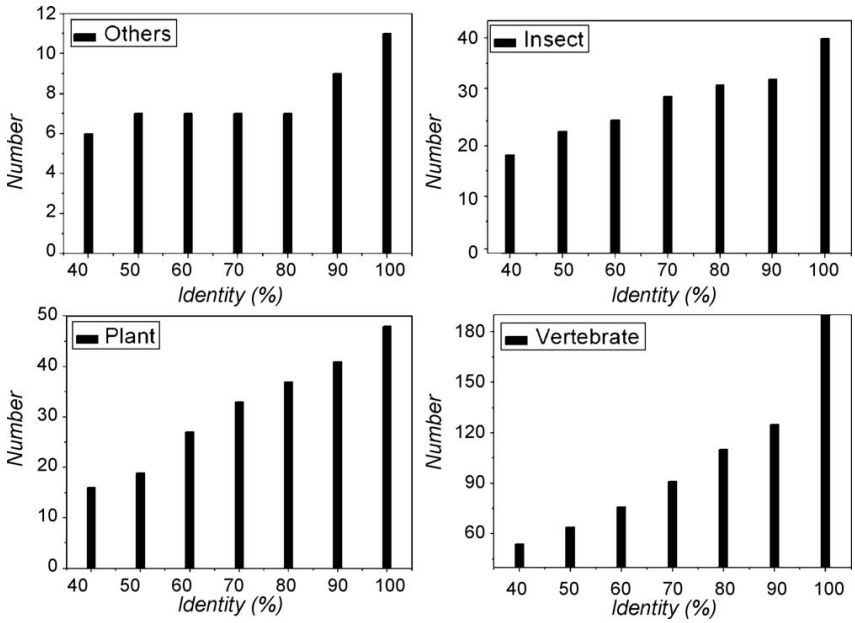

Fig. 1: The distribution of sequence identity of defensin family dataset by using CD-HIT program (Zuo, 2009).

Zhu (2008) pointed out the computational identification and clearly assigning of six families of fungal defensin-like peptides which contain three known defensin types (antibacterial ancient invertebrate-type defensins, antibacterial classical insect-type defensins, and antifungal plant/insect-type defensins.

Several studies devide defensins into vertebrate, invertebrate and plant defensins (Lehrer and Ganz, 2002; Froy and Gurevitz (2003). As well as, vertebrate defensins are extra classified into three sub-families: α, β and θ theta defensins (Klotman and Chang, 2006)

Whereas, according to the morphogenic effects caused on treated fungal hyphae, antifungal plant defensins can be devided into two groups (Broekaert *et al.,* 1995):

1- Morphogenic plant defensins: cause reduced hyphal elongation that is accompanied by increasing hyphal branching. This group comprises plant defensins of some plant families such as Brassicaceae and Saxifragaceae.

2- Nonmorphogenic plant defensins: cause slow down of hyphal extension but do not induce marked morphological distortions. This group comprises plant defensins of Asteraceae, Fabaceae and Hippocastanaceae. Morphogenic and nonmorphogenic plant defensins also differ in their antifungal spectrum.

Another study carried out by Thevissen *et al.* (1999) divides the plant defensin family into two main groups (A and B) with 25% similarity to each other (Harrison *et al.,* 1997). Also, they subdivide group A into four subfamilies (A1, A2, A3, and A4) with at least 50% similarity within each subfamily. The plant defensins of subfamily A2, comprising Dm-AMP1 of *Dahlia merckii*, reduce hyphal elongation without affecting fungal morphology previously termed nonmorphogenic plant defensins (Broekaert *et al.,* 1995). On the other hand, the members of subfamilies A3 and A4 comprising Rs-AFP2 of *Raphanus sativus* and Hs-AFP1of *Heuchera sanguinea*, stimulate tip ballooning and branch formation on susceptible fungi (Osborn *et al.,* 1995). Therefore, they termed morphogenic plant defensins (Broekaert *et al.,* 1995).

As to *Arabidopsis thaliana*, Thomma and Broekaert (1998) recognized 13 putative plant defensins and classified them into two main groups; the first one contains seven proteins which exhibit a high degree of conservation, while the second one is more different at the amino acid level. Whilst, Thomma *et al.* (2002) subdivided the *A. thaliana* defensin family into two subfamilies named AtPDF1 and AtPDF2. Whereas, Penninckx *et al.* (1996) concluded that Arabidopsis comprises two genes that encode highly homologous plant defensins with totally different expression patterns. PDF1.1 is expressed prevalently in seed and is accounted to be the analog of the radish Rs-AFPI and Rs-AFP2 genes, while PDF1.2 is expressed in leaves upon pathogen-induced stress and can be accounted the analog to the Rs-AFP3 and RS-AFP4 genes of radish (Terras *et al.,* 1995). They noted the accumulation of plant defensin with antifungal activity in pathogen-stressed Arabidopsis leaves (Penninckx *et al.,* 1996). They also noted hyphal hyperbranching and tip swelling as evidence to growth inhibition that is similar to what was noted for Rs-AFP1 and correlated plant defensins to other Brassicaceae, including PDF1.1 (At-AFPI) to Arabidopsis seed (Terras *et al.,* 1993). They concluded from their experiments (Penninckx *et al.,* 1996) that the induction of plant defensin genes upon pathogen challenge synchronizes with an increase in endogenous jasmonic acid (JA) levels, both in inoculated and in noninoculated (with *Alternaria brassicicola*) systemic leaves. Furthermore, three Arabidopsis genes, PR-1, PR-2 (they encode a α-1, 3-glucanase), and PR-5 which encodes an osmotin-like protein, were previously discovered. These genes are coordinately and systemically induced when infected with a pathogen (Dempsey *et al.,* 1993; Mauch-Mani and Slusarenko, 1994; Uknes *et al.,* 1993).

Silverstein *et al.* (2007) compared cysteine-rich peptides sequences in both of Arabidopsis and rice genomes; stating that this point is very effectively. For instance, while defensin-like sequences are the most plentiful CRP sequences in the Arabidopsis genome, with 323 members, it is only 93 defensin-like genes in rice. In contrast, the rice genome has 13 CRPs sequences for Bowman-Birk protease inhibitor, whilst these sequences are missing from the Arabidopsis genome.

Plant defensins are structurally similar to insect ones. Insect defensin A represents a typical sample of the insect defensin family; it is a 40 amino acid antibacterial protein that is produced in the fat body and accumulates in the hemolymph of bacterially challenged larvae of the black blowfly, *Phormia terranovae* (Lambert *et al.,* 1989).

Insect defensins consist of 34-43 residues with three disulfide bridges, like cecropins, which are produced by the insect fat body and secreted in the hemolymph by pathogen inducible manner (Hoffmann and Hétru, 1992).

The majority of insect defensins have three disulfide bonds, whereas a number of related proteins such as drosomycin of *Drosophila melanogaster* have four disulfides (Fehlbaum *et al.,* 1994; Landon *et al.,* 1997) created by six cysteine residues, with three characteristic domains: a N-terminal flexible loop, followed by an a-helix and two C-terminal anti-parallel b-sheets (Bulet *et al.,* 1999; Dassanayake *et al.,* 2007; Thevissen *et al.,* 2004).

Until 1999, forty insect defensins were characterized from different species (Bulet *et al.,* 1999), to be increased afterwards (Lopez et al., 2003). Also, insect defensin-like peptides have been found in molluscs, scorpions and ticks (Froy and Gurevitz, 2003). Moreover, mammals produce defensins but there is no similarity to insect defensins, so they are considered to be evolutionarily unrelated (Gurevitz, 2003).

Insect defensins have a broad-spectrum antibacterial activity (Dimarcq *et al.,* 1998) and may also show antifungal activity (Lambert *et al.,* 1989; Lamberty *et al.,* 1999).

An illustration of the three-dimensional structures of numerous insect defensins has been done by Cornet *et al.,* 1995 and Da Silva *et al.,* 2003. Their global fold, as typified by insect defensin A, feature an a-helix, a double-stranded antiparallel b-sheet and a long N-terminal loop (equivalent to the third b-strand in plant defensins), that are stabilized by three disulfide bonds centred on the CSab motif (Bonmatin *et al.,* 1992; Broekaert *et al.,* 1995; Cornet *et al.,* 1995). Drosomycin replaces the N-terminal loop of the insect

defensins and has a fourth disulfide bond and a third b-strand that is analogous to that in plant defensins (Fehlbaum *et al.,* 1994; Landon *et al.,* 1997; Landon *et al.,* 2000).
Drosomycin was discovered in 1982 (Fehlbaum *et al.,* 1994); it shares about 30% amino acid sequence identity with plant defensins and thus comprises a fungus- specific defensin family with similar structural and functional features (Yuan *et al.,* 2007).

1.1.4 Role of plant defensins in plants

Defensins have been isolated from many parts of plants:
1- seeds: the best characterized defensins have been isolated from seeds (Osborn *et al.,* 1995; Broekaert *et al.,* 1997). A single seed can release at least 1 µg of plant defensin that is sufficient to form an inhibitory zone around the seed to protect the germinating seedling against soil-borne fungal pathogens. Whereas the defensins comprise about 30% of the proteins released by mechanical wounding of radish seeds, it represents only 0.5% of the total seed proteins (Terras *et al.,* 1995).
2-vegetative tissues: in addition, the vegetative tissues also release plant defensins and accumulate them in the endosperms, hypocotyls, tubers and floral structures and peripheral cell layers of cotyledons (Lay *et al.,* 2003; Moreno *et al.,* 1994; Gu *et al.,* 1992; Thomma *et al.,* 1998; Penninckx *et al.,* 1996). Due to their accumulation in these sites, plant defensins play a role in a first line of defense against potential pathogens.
Plant defensins consist of a family of highly stable basic proteins (Broekaert *et al.,* 1995). These defensins are characterized in numerous plants with the DNA and protein level from various plant tissues, comprising leaves, pods, tubers, roots, fruits and flowers (Broekaert *et al.,* 1997; Lay *et al.,* 2003; Thomma and Broekaert, 1998). The common thing that gathers all plant defensins is a three-dimensional folding pattern, stabilized by four disulfide bridges (Broekaert *et al.,* 1995).
It is clear that defensins play a significant role for defense in plants, since they are inducible by biotic and abiotic stresses. Furthermore, some studies referred to the accumulation of defensins in leaves, stomatal cells and in the cell walls that line the substomatal cavities in sugar beet. This is interesting because as stomata are potential entry points for fungal pathogens (Kragh *et al.,* 1995). For example, *Nicotiana alata* accumulate floral defensin (NaD1) in the epidermal cells of the sepals and petals and in the cortical cells of the style and the connective cells of the anther (Lay *et al.,* 2003). This creates

compatibility with the role of protecting the valuable germ cells against damage by potential pathogens due to the accumulation in these outermost layers (Lay *et al.,* 2003). This is analogous to the expression of mammalian defensins in reproductive tissues (Li *et al.,* Yamada *et al.,* 1997; Chiang and Hadwiger, 1991; Maitra and Cushman, 1998; Koike *et al.,* 2002). On the other hand, they are able to confer increased protection against pathogens in vegetative tissues of transgenic plants (Gao *et al.,* 2000; Elfstrand *et al.,* 2001; Park *et al.,* 2002).

It is possible that the plant defensins play a key role as first-line defense for vulnerable tissues. Therefore, they are vital components of the defense system in plants in addition to several organs (peripheral cells of leaves, flowers, seeds and tubers) producing these antimicrobial peptides (http://www.copewithcytokines.de)

There is asubclass of defensins that is named alpha-amylase inhibitor including some plant defensins which inhibit alpha-amylases from insects and humans (Bloch and Richardson, 1991; Osborn *et al.,* 1995). On the other hand, plant defensins show weak activity against fungi (Osborn *et al.,* 1995), and they possibly play a role in the protection against herbivores. It has been proven that expression of alpha-amylase inhibitors in seeds has been contributed to protection against some seed-feeding insects (Shade *et al.,* 1994).

1.1.5 Mode of action

Plant defensins are potent antimicrobial peptides (Thevissen *et al.,* 2007). They display activity by various mechanisms:

1- Activity against a-amylases of insects: like g-thionins from sorghum (*Sorghum bicolor*) as first plant defensin described with α-amylase inhibitory activity (Bloch and Richardson, 1991). There are six different α-amylase inhibitor classes that can be used in pest control: Kunitz-like, lectin-like, γ-purothionin-like, cerealtype, thaumatin-like and knottin-like (Murad *et al.,* 2007). They are also able to inhibit proteinases from insects and sometimes display an ability to restrain both trypsin and chymotrypsin (Pelegrini and Franco, 2005).

2- Inhibition protein synthesis: For instance, g-hordothionin of barley (Mendez *et al.,* 1990).

3- Reversible blocking of sodium ion channels in tumor cell: g-zeathionins of corn (Kushmerick *et al.,* 1998).

4- Inhibition of calcium ion channels: for example, medicago sativa defensin 1 of *Medicago sativa* (Spelbrink *et al.,* 2004). One suggested hypothesis for the mechanism of action is related to the fundamentality of the Ca^{2+} ion for some insect α-amylase activity (Pelegrini *et al.,* 2006), whereas another hypothesis supposes that defensins are able to chelate calcium also, destabilizing the enzyme and extra causing its inhibition (Castro and Vernon, 2003; Pelegrini and Franco, 2005).

5- Membrane permeabilization: It takes place by interacting with binding sites on fungal cells, particularly to fungal shingolipids and glucosylceramides (Thevissen *et al.,* 2004).

In *Neurospora cra*ssa, the membrane permeabilization induction by plant defensins is biphasic, according to the plant defensin dose. With low defensin levels (0.1 to 1 mM), a weak permeabilization is detected with a more cation-resistant permeabilization correlating with the inhibition of fungal growth, whereas at high defensin levels (10 to 40 mM), strong permeabilization is detected which can be suppressed by cations in the medium. The effect of divalent cations, especially Ca^{2+} on the reduction of the antimicrobial activity has also been observed for mammalian and insect defensins (De Samblanx *et al.,* 1997).

Thevissen *et al.* (1996) pointed out that the plant defensins induce ion fluxes across the plasma membranes of living fungal hyphae. Plant defensins neither form ion permeable pores in artificial membranes nor change the electrical properties of artificial lipid bilayers, in contrast to insect (Cociancich *et al.,* 1993) and mammalian defensins (Kagan *et al.,* 1990).

The ion fluxes may result from:

1- The interaction of the plant defensins with a binding site that transduces a signal to endogenous ion channels in the membrane

2- binding-site-mediated insertion of the plant defensins into the membrane with subsequent ion channel formation.

According to the previous judgment there is a direct interaction with lipid components of the plasma membrane, a mechanism proposed to explain the anti (Cociancich *et al.,* 1993; Kagan *et al.,* 1990) is improbable for plant defensins.

Some studies with radiolabeled plant defensins have identified specific, high-affinity binding sites for plant defensins on fungal cells and microsomal membranes (Thevissen *et al.,* 1997).

Thevissen *et al.* (2000) reported that the binding site is essential for the antifungal activity of Dm-AMP1 plant defensin: He noted that the capacity of mutant yeast *Saccharomyces cerevisiae* (called DM1) to bind Dm-AMP1 to its plasma membrane is more than 10-fold less than that of the wild type; therefore mutant yeast is not sensitive to the plant defensin Dm-AMP1. Thevissen (2000; 2004) revealed the mode of antifungal action for Rs-AFP2 and Dm-AMP1 plant defensins isolated from radish and dahlia (*Dahlia merckii*) respectively. Both peptides bound on distinct sphingolipids (glucosylceramide and manosyl diinositol phosphorylceramide for Rs-AFP2 and Dm-AMP1, respectively) in fungal membranes consequently showed a different specificity against fungal and yeasts species, including the human pathogen *Candida albicans*.

A mode of action of plant defensins was suggested by Thevissen *et al.* (2000) referring to the membrane patches including mannosyl diinositol phosphoryl ceramide M (IP) 2C that constitute binding sites for DmAMP1 or, alternatively, are required for fixing of membrane or cell-wall-associated proteins, which themselves interact with DmAMP1. Furthermore, structurally homologous antifungal peptides present in species from different eukaryotes interact with the same target in the fungal plasma membrane, namely glucosylceramides (Thevissen *et al.,* 2004).

Plant defensins are extremely diverse in their primary amino acid sequences with only eight structure stabilizing Cys residues in general. Due to this diversion in the different primary sequences there are different biological activities (http://www.plantphysiol.org/cgi/content/full/135/4/2038), for instance, antibacterial activity (Segura *et al.,* 1998), antifungal activity (Terras *et al.,* 1995), amylase inhibitory activity (Bloch and Richardsonet, 1991) and proteinase activity (Wijaya *et al.,* 2000).

Furthermore, in a lot of situations, we can expect the specificity of the role of defense with small differences in amino acid sequence (Garcia-Olmedo *et al.,* 1998).

The killing action of the defensin consists of various activities in which membrane damage plays a significant role; however, it may also comprise activities at specific sites, for instance, on the cell surface (Sassa *et al.,* 2008). While they refer to that it is not clear how the defensin could mechanistically cause inhibition of cell wall biosynthesis.

1.1.6 *In vivo* activity of plant defensins

Plant defensins are vital components of host defense. Therefore they can be exploited to produce transgenic crops with enhanced pathogen resistance. Consequently the constitutive expression of plant defensins in potato tubers (Moreno *et al.,* 1994), flower organs in tobacco (Gu *et al.,* 1992), peripheral cells of radish seeds (Terras *et al.,* 1992) and pea leaves (Broekaert and Terras, 1995) is congruous with the first-line defense function of vulnerable tissues. Hence enhanced resistance has been achieved in transgenic tomatos, chinese cabbage and oil rapeseed plants by constitutive plant defensin expression (Park *et al.,* 2002).

It has become clear that g-hordothionins inhibits *in vitro* protein synthesis (Mendez *et al.,* 1990). Other studies dealing with the isolation of three isoinhibitors of insect a-amilases (SIa1, SIa2 and SIa3) discovered a high sequential similarity to g-purothionins (Bloch *et al,* 1991). Some of plant defensins present a potent fungicide action, referring that they can act as defense proteins (Terras *et al.,* 1992; Thevissen, *et al.,* 1999).

Defensins carry out some critical functions in the innate immunity of their hosts by objecting the assaulting pathogens. The distribution of defensins in life is very huge; they are ancient molecules with a joint precursor belonging to an era of more than one billion years ago (Aerts *et al.,* 2008).

Most of the discovered insect defensins exhibit antibacterial activities, and yeasts are less distressed by insect defensins (Bulet and Stocklin, 2005). So it is difficult to observe insect antifungal defensins until now: termicin from the termite *Pseudacanthotermes spiniger* (Lamberty *et al.,* 2001) or defensin-like peptide drosomycin from the fruit fly *Drosophila melanogaster* (Fehlbaum *et al.,* 1994) and gallerimycin from greater wax moth *Galleria mellonella*.

Genes encoding defensins in plants are evolutionary adjusted, with a prevalent expression in outer cell layers according to Moreno *et al.,* 1994; Terras *et al.,* 1995; this expression may be stimulated above basal levels as response to pathogen infection and other stresses (Penninckx *et al.,* 1996; Epple *et al.,* 1997).

In some cases, plant defensins has antifungal activity, but it is not known if they have a common mode of action (Broekaert *et al.,* 1997). In this context Thevissen *et al.* (1996) mentioned Rs-AFP2 that was isolated from radish seeds which act principally on the cell membrane. He also mentioned that the Rs-AFP2 stimulate rapid Ca^{2+} uptake and K^{+} flux

from *Neurospora crassa* hyphae and hence may inhibit the growth of filamentous fungi by disrupting cytosolic Ca^{2+} gradients essential for hyphal tip growth. Using plant defensins as resistance traits in transgenic plants will create improvement of protection against pathogen attack (Montesinos, 2007).

Hyperbranching and the growth of fungal hyphae were suppressed by both MsDef1 and the Ca^{2+} channel blocker, EGTA (Spelbrink *et al.,* 2004) indicating that defensins delay hyphal growth by affecting a Ca^{2+} transport. Furthermore, a three-dimensional structure will be formed by correlating MsDef1 and RsAFP2 to each other. The structure is distinct in the primary amino acid sequence (Fant *et al.,* 1998) referring to dissimilar modes of action for these two structurally related defensins.

Other biological activities by the plant defensins are for example proteinase (Melo *et al.,* 2002), inhibition of protein translation (Mendez *et al.,* 1996) and α-amylase inhibitory activity (Zhang *et al.,* 1997) that perhaps contribute to their function in defense. Therefore, the transgenic expression of plant defensins contributes to the protection of vegetative tissues against pathogen attack. Paramount expression of a radish defensin evidently enhanced resistance of tomatoes to *Alternaria solan*i (Parashina *et al.,* 2000), and likewise in tobacco plants to the fungal leaf pathogen *Alternaria longipes* (Terras *et al.,* 1995).

Wang *et al.* (1999) reported an enhanced resistance against blackleg (*Leptosphaeria maculans*) disease by Canola (*Brassica napus*) constitutively expressing a pea defensin. Whereas Gao *et al.* (2000) mentioned a vigorous resistance against the agronomically important fungus under field conditions was achieved in situation of constitutive expression of an alfalfa defensin in potatoes.

Zhu (2008) proposed that invertebrates and fungi participate in a similar immune defense mechanism to control infection of gram-positive bacteria which is evidently different from plants.

A broad range of mechanisms was developed by insects for defense against pathogens and parasites. The organisms that are able to invade the insect through the insect cuticle or the gut are recognized by specific receptors that stimulate multiple cellular and humoral immune reactions. The latter demand Toll and Imd (Immune deficiency) signaling pathways (Fig. 2) for production of antimicrobial peptides, which are synthesized principally by the insect fat body and released into the haemolymph (Gillespie *et al .,* 1997; Hoffmann, 2003). As pointed out by Lemaitre and Hoffmann (2007), there are two

major members for the activation of a prophenoloxidase cascade and of Toll/IMD pathways resulting in the massive production of immune effector molecules in insects; they are the Gram-negative binding proteins (GNBP) and peptidoglycan recognition proteins (PGRP).

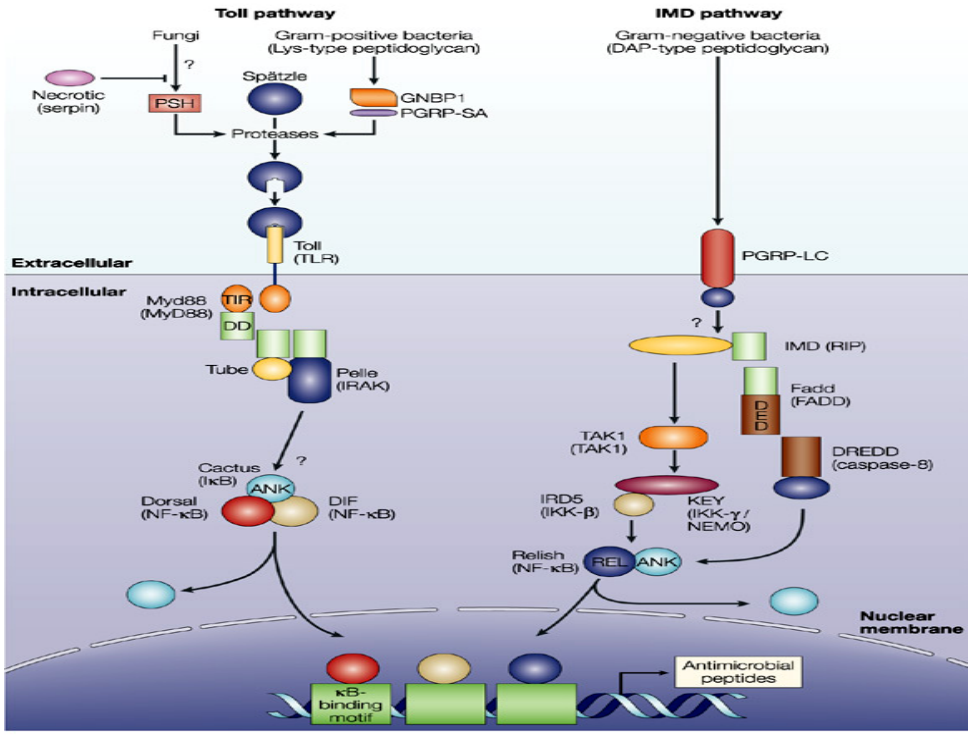

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Fig. 2: Explains Toll/IMD pathways.

The genes that encode antimicrobial peptides are regulated by a balance between two signaling pathways: the Toll pathway which is activated mainly by fungi and Gram-positive bacteria, and the Immune deficiency (IMD) pathway which is activated mainly by Gram-negative bacteria. In spite of the majority of antibacterial peptides attacking Gram-negative bacteria, defensins act mainly against Gram positive bacteria. The negatively charged bacterial cytoplasmic membrane is attacked by a positively charged defensin by electrostatic and hydrophobic interactions resulting in the disruption of the bacterial membrane (Froy and Gurevitz, 2003).

There are many works referring to plants acquiring fungal pathogen protectio by expressing a plant defensin peptide, for example the potato plant (Gao *et al.,* 2000). They

mentioned that alfalfa antifungal peptide (alfAFP) shows strong activity against the agronomically important fungal pathogen *Verticillum dahlia* and provides strong resistance in the green house to transgenic potato plants. El-Awady *et al.* (2008) succeeded in transforming the gene kate to canola plants. The gene causes defense against the fungi *Peronospora parasitica* and *Erysiphe polygoni* responsible for downy mildew and powdery mildew. Subsequently the production of resistant canola plants enhanced.

Plant defensin protein families play a defensive role against plant pathogenic bacteria (Bohlmann, 1994) and fungi (Thevissen *et al.,* 1996). Some studies have been carried out about integration and over-expression of defensin genes in monocotyledonous plants such as rice, wheat (Kanzaki *et al.,* 2002; Koike *et al,* . 2002; Liang *et al.,* 1998), wasabi (*Wasabia japonica*, Sjahril *et al.,* 2006) and bananas (Chakrabarti *et al.,* 2003), but also in transgenic dicotyledonous plants, for instance potato, tobacco, arabidopsis, mung bean and canola (Eppel *et al.*, 1997; Gao *et al.,* 2000; Saitoh *et al.,* 2001; Kiba *et al.,* 2003).

It is believed that the antimicrobial activities of some plant defensin such as cysteinerich antimicrobial proteins, hevein-like protein and thionine are controlled by specific structures formed by inter-cysteine disulfide bonds (Broekaert *et al.,* 1995). It is also believed that these proteins invade membrane structures of microorganisms. These proteins are thought to target membrane structures of microorganisms, in view of the fact that these kinds of antimicrobial activity are quite sensitive to the presence of cations (*Kiba et al.,* 2003; Osborn *et al.,* 1995).

Defensins show a broad-spectrum antimicrobial activity that is typically but variably inhibited by increasing physiological salt concentrations (Boman, 1998; Ganz and Lehrer, 2001; Scott and Hancock, 2002).

Plant defensins do not generally affect bacteria, with some exceptions such as *Pseudomonas solanacearum* and *Clavibacter michigansis*, which are susceptible to Pth-Stl, a plant defensin isolated from potato tubers (Moreno *et al.,* 1994), and *Bacillus subtilis*, which is inhibited by Ct-AMPl (Osborn *et al.,* 1995).

As pointed out by Terras *et al.* (1992) and Osborn *et al.* (1995) increasing the ionic potency of the fungal growth assay medium reduces the antifungal activity of plant defensins, morphogenic or nonmorphogenic. Thus the antagonistic effect of ions is strongly reliant on the fungus and consequently on the conformation of the putative target

site. As a general rule, the antifungal activity of plant defensins is slightly more reduced by Ca^{2+} than by Mg^{2+} (Osborn *et al.,* 1995).

Regarding the effect of a presence of inorganic salts on plant defensin, the inorganic salts antagonize the antifungal activity of plant defensins, particularly divalent cations. The sensivity of cation also differed with the defensin and the fungus used (Terras *et al.,* 1993).

Defensins may also play an important role in plant growth and development. For instance, in *A. thaliana* the constitutive expression of AtPep1 induced the expression of PDF1.2 which resulted in better root development referring that plant defensins may regulate root development (Huffaker *et al.,* 2006).

The most important point is that plant defensins are nontoxic to both mammalian and plant cells. A lot of plant defensins can inhibit the growth of a broad range of fungi at micromolar concentrations (Moreno *et al.,* 1994; Osborn *et al.,* 1995), and in some plant tissues, the expression of defensin genes is stimulated as response to fungal infection (Broekaert *et al.,* 1995 and 1997; Thevissen *et al.,* 2004; Tavares *et al.,* 2008).

The expression of defensin genes takes place in response to fungal infection in some plant tissues (Penninckx *et al.,* 1996), in spite of the expression being constitutively in other tissues (Thomma *et al.,* 1998).

1.1.7 *In vitro* activity of plant defensins

Carvalho and Gomes (2009) mentioned that the biological activity of plant defensins is determined *in vitro* as follows:

1- As protein translation inhibitors: The first report about the biological activity of plant defensins was done by Mendez *et al.* (1990). They pointed out that plant defensins can inhibit protein translation in a cell-free system. In plant systems, many plant defensins have been verified to inhibit translation, for instance VaD1 which is derived from wheat germ inhibited protein translation (Chen *et al.,* 2005), and also plant protein translation (Chen *et al.,* 2004; *et al.,* 2002).

2- As enzyme inhibitors:

a) a-Amylase inhibitors: Bloch and Richardson (1991) referred to inhibition of the insect a-amylase as a result to activity of plant defensins, Thus it was suggested that plant defensins play a role in plant defense against insects.

b) As protease inhibitors: 5459 and 5144 are peptides isolated from the seeds of *Cassia fistula*, with homology to plant defensins, Since they have anti-insect properties, it has been supposed that this activity may contribute to plant defense against insects by a mechanism relating to insect digestion (Molosov *et al.,* 2008).

3- As microbial inhibitors

In the early 1990s, Terras *et al.* (1992) discovered the antimicrobial activity of plant defensins. The activity was primarily observed against fungi. Some Gram positive groups are also inhibited, as well as a few Gram-negative bacteria (Carvalho and Gomes, 2009).

4- As zinc tolerance mediator in plants:

In the last century, heavy metals like copper, lead, zinc and cadmium polluted several regions (Pilon-Smits *et al.,* 2005). Marque`s *et al.* (2004) demonstrated that a functional manner to metal tolerance is achievable at a cellular level. Mirouze *et al.* (2006) expressed a cDNA library of the zinc hyper-accumulated plant *Arabidopsis halleri* in the yeast *Saccharomyces cerevisiae*, and then defined the tolerant strains by incubation on a medium containing a toxic concentration of the metal. The sequences of cDNAs revealed that they encode four peptides with striking homology to plant defensins.

5- As enzymatic activity involved in ascorbic acid redox state:

This function was imputed to the SPD1 defensin which has the ability to regenerate dehydroascorbate (DHA) to ascorbic acid (AsA) in the presence of glutathione, subsequently regulating the AsA redox state.

6- As ion channel blockers.

The best observed *in vitro* activity of plant defensins is the ability to inhibit the growth of a broad range of filamentous fungi and yeasts. For example, a broad-spectrum antifungal activity of numerous plant defensins derived from Brassicaceae species, comprising radish, was detected against several filamentous fungi including *B. cinerea*, *A. brassicicola* and *F. culmorum* (Terras *et al.,* 1993; 1995).

There are two defensins isolated from maize kernels called g1-zeathionin and g2-zeathionin that can reversibly inhibit the sodium currents at an IC_{50} of 62 and 33 mM, respectively, probably by influencing the conductance activity of the channel by reducing the number of the open channels or decreasing the number of channels that open during the voltage step (Kushmerick *et al.,* 1998).

1.1.8 Biotechnological applications

The family of plant defensins contributes to the innate immune system of insects and plants (Yang and Lyu, 2008). Since the plant defensins can contribute to biocontrol as insecticidal, antimicrobial and anti-parasitic agents, more interest should be put in using them in protein engineering and transgenic production of agronomically important plants which are resistant to pathogens and pests, as well as using the R gene mediated transformation in plants (Carvalho and Gomes, 2009). In addition to that plant defensins thogether with another antimicrobial compounds contribute to increasing the resistance (Chen *et al.,* 2006; Oh *et al.,* 2005). Furthermore, a lot of reviews mentioned the contributing of defensins to abiotic stress adaptation with their the major role in antimicrobial activity (Carvalho *et al.,* 2006; Do *et al.,* 2004; Mirouze *et al.,* 2006).

According to Giuliani *et al.,* 2007 and Koike *et al.,* 2002 plant defensins have some interesting important features as follows:

1- Cooperation with other antimicrobial peptides

2- Broad-spectrum activities against positive and Gram negative bacteria, fungi, protozoa and virus.

3- In general, they have the very important feature of a low toxicity to mammalian cells.

4- Rapid murder of microorganisms with low doses.

5- Some of them have the capability to modulate the innate immune response in mammals, creating a new therapeutic opportunities.

6- On account of that, they principally attack plasma membrane; the emerging of resistance is unbelievable.

7- They can avoid septic shock in view of the ability to suppress endotoxins.

Antimicrobial peptides, comprising defensins, are main components of innate immunity which have the ability for protection against gram- positive and negative bacteria, fungi, viruses and protozoa parasites (Chrudimska *et al.,* 2010). The innate immunity relies on pathogen detection that is essentially mediated by host proteins called pattern recognition receptors (Altincicek and Vilcinskas, 2007).

A lot of plant defensins are expressed plentifully, for example in seed (Terras *et al.,* 1995) but others are developmentally regulated (Vanoosthuyse *et al.,* 2001) or stimulated by various biotic and abiotic stress factors, comprising heavy metals (Mirouze *et al.,* 2006),

potassium starvation (Armengaud *et al.,* 2004), cold (Koike *et al.,* 2002), drought or salt stresses (Do *et al.,* 2004; Maitra and Cushman, 1998; Yamada *et al.,* 1997).

Plant defensins play a role in the plant defense response, which is protruded by enhanced disease resistance phenotypes found in various plant species heterologously over-expressing plant defensin genes (Sels *et al.,* 2008). Therefore the over-expression of an alfalfa plant defensin gene increased the resistance of transgenic potatoes to *Verticillium dahlia* (Gao *et al.,* 2000). Over-expressing the Rs-AFP2 gene in transgenic tobacco enhanced resistance to the fungal pathogen *Alternaria longipes* (Terras *et al.,* 1995). Kang *et al.* (2006) reported that an RsGluR over-expression resulted in growth inhibition of the necrotic fungal pathogen *Botrytis cinerea*, probably as a consequence of up-regulation of the defensins. However, Sels *et al.* (2008) mentioned that there is no enhanced resistance by over-expressing of a plant defensin gene in the same plant from which it derived.

1.2 Objectives of the study

Microbial diseases cause crop losses worldwide; they are currently estimated $720B (US) annually, whereas additional $20B (US) are spent on countervailing pesticides. The losses are untenable considering the increase of human populations accompanied by the decrease of available arable cropland and irrigation (Borlaug, 2000). Fungal and bacterial diseases are one of the main factors effecting crop production.

Antimicrobial peptides from insects have been used for transgenic expression as promising tool to produce crops resistant to biotic stress (Vilcinskas and Gross, 2005; Yevtushenko *et al.,* 2005; Coca *et al.,* 2006).

In this study, we aimed at the generation of transgenic *Arabidopsis thaliana* expressing Et-AMP1 and Et-AMP2 antimicrobial peptides which we expect to offer promising resistance against fungal and bacterial pathogens and be antifungal agents in disease resistance.

2. Materials and Methods

2.1 Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was used to produce the *Eristalis tenax* AMPs (Et-AMP1 and Et-AMP2) expressing transgenic plants.

Seeds of all transgenic Arabidopsis and wild types were first surface-sterilized with Sodium hypochlorite 20% (NaClO) for 20 min at room temperature. They were then washed 3 times with sterilized-distilled water and germinated in Petri dishes on half-strength MS-medium (Murashige and Skoog, 1962) supplemented with 0.5% sucrose, 0.4% Gelrite (Roth, Germany) and with or without 30 mg L^{-1} hygromycin (Roche, Mannheim, Germany). To achieve synchronized germination, Petri dishes with seed were incubated firstly at 4 ºC for 2 days then placed in a growth chamber (Percival scientific, Boone, Iowa, USA) under photoperiodic conditions of 16 h light (180 µmol m^{-2} s^{-1} Photon flux density), 22 °C/18 °C day/night temperatures for 2 weeks. The plants were then transplanted into pots containing a soil mixture of 1:1 sand: soil Type ED 73 (Einheitserde und Humuswerke Gebr. Patzer GmbH+ Co.KG, Sinntal-Jossa, Germany) and cultured at the same condition as mentioned above with 60% relative humidity. Five weeks later, plants of uniform size were selected for pathogenicity studies.

2.2 Fungal and bacterial strains

In this study, *Fusarium culmorum* (strain KF 350, obtained from Prof. Chelkowski, Institute of Plant Genetics, Poznan, Polen) were used for *in vitro* antifungal assays. For *in vivo* assays, the fungal pathogens grey mold *Botytis cinerea* strain B05.10 (provided by Prof. M. Hahn, Kaiserslautern, Germany) in addition to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 were used.

For antifungal assays, growth and harvesting of spores from the fungus *F. culmorum* was carried out as described by Broekaert *et al*., 1990. Fungus was grown on Potato Dextrose

Agar medium (PDA) containing 15 g L^{-1} agar, Roth, Germany) for 10 days at room temperature (RT).

2.3 Preparation of fungal spore suspension

Fungal spore suspensions were prepared by flooding plates with 5 ml sterile double distilled water and gently scraping them with a sterile loop. The resulting crude suspension was filtered through a layer of sterile cheesecloth to remove mycelial fragments. Inoculum concentration was determined by using a Fuchs-Rosenthal counting chamber (Roth, Germany) and then adjusted to 4×10^4 conidia mL^{-1} for *F. culmorum*.

B. cinerea was grown on HA-Agar medium (1% Malt extract, 0.4% Glucose, and 0.4% Yeast extract) for 10 days at RT. Spore suspension (2.5×10^4 conidiospores mL^{-1}) was prepared in 12 g L^{-1} potato dextrose broth (PDB) medium.

2.4 ET-AMP1 and ET-AMP2

ET-AMP1 (GenBANK: AM706434); Et-AMP2 (GenBank: AM706429, Altincicek and Vilcinskas, 2007) are two new antifungal peptides derived from insect *Eristalis tenax* (Fig. 3).

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Syrphidae
Genus	*Eristalis*
Species	*E. tenax*

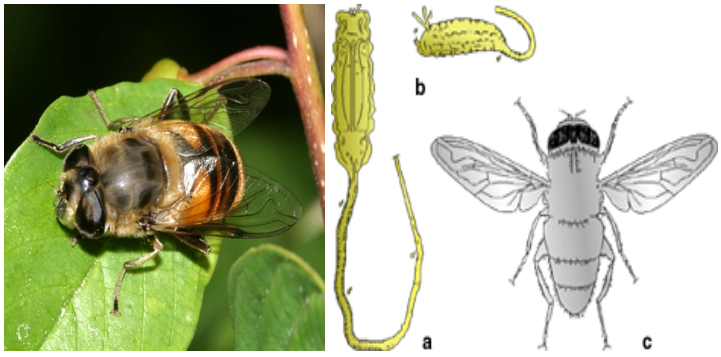

Fig. 3: Classification and life stages of *Eristalis tenax*: (a) larva; (b) pupa; and (c) adult fly (The Medical Journal of Australia eMJA, 2000, http://www.mja.com.au/public/issues/173_11_041200/whishwilson/whishwilson.html).

2.5 Production of Et-AMP1 recombinant protein using pEXp5-CT/Topo expression vector

PCR product encoding the mature active peptide of Et-AMP1 (that is presented in pGEM-T vector, Altincicek and Vilcinskas, 2007) was done by Et-AMP1 specific primers (see

Table 1), and then cloned into a pEXp5-CT/Topo expression vector (containing His-tag at C-terminal, Invitrogen, Germany, Fig. 4 and 5). The plasmid were transferred into *E. coli* BL21 (DE3, Stratagene, La Jolla, USA) cells for protein expression; after Nucleotide sequence (AGOWA, Berlin, Germany).

Transformed cells were grown at 37 °C in Luria-Bertani (LB) medium (1.0% sodium chloride, 1.0% tryptone, and 0.5% yeast extract) until they reached an OD_{600} of 0.8. Expression was then induced by the addition of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4h of induction, cells were harvested by centrifugation at 2830 rpm for 20 min. The pellet was resuspended in lysis buffer (50 mM sodium dihydrogen phosphate, 300 mM sodium chloride, 10% glycine, and 1 mg mL^{-1} lysozyme, adjusted to pH 8.0 using NaOH) and disrupted using a French® press cell press at a pressure of 8000 lb in^{-2}. Purification was achieved by using nickel-nitrilotriacetic acid affinity chromatography (Ni-NTA; Qiagen, Germany), following the manufacturer's instructions. The fractions were collected and applied to 15% Tricine-PAGE for pursuing recombinant protein at different times after induction with IPTG and in different fractions (Schägger and Jagow, 1987).

After that, the bacteria was grown, then miniprep and electroporation into *E. coli* RIL; BL21 (DE3) contained expression vector and induction of target protein (10 kDa) using Isopropyl β-D-1-thiogalactopyranoside (IPTG); then production and purification of protein by Histidine column under native and denaturing conditions, and after that precipitation of protein.

Finally, the *in vivo* activity of peptide (the MW of the recombinant protein is 7025g/mol) was tested against the germination of *Fusarium culmorum* conidia.

2.6 Purification of fusion protein (6His-Et-AMP1)

The bacterial pellet was dissolved in 30 mL lysis buffer (see section 2.5**)** and cell disruption by French press was performed two times at a pressure of 8000 lb in^{-2}. Subsequently, the lysate was mixed with 30 mL binding buffer (8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 8.0) and incubated under shaking for 3 h at RT. Thereafter, the cell debris was precipitated from the lysate solution by centrifugation for 15 min in a Beckman coulter centrifuge (15000 rpm). The supernatant containing soluble protein was collected and stored at 4 °C. To purify the fusion protein, tagged with 6×His at the N-terminus, supernatant was applied to a Ni^{2+}-chelating column packed with

1 mL of Ni-NTA resin (Qiagen, Hilden, Germany) that had been previously equilibrated with binding buffer. The column was washed three times with 4 mL washing buffer (8 M urea, 25 mM imidazole, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 6.3). Finally, the column was eluted three times with elution buffer (8 M urea, 500 mM imidazole, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 4.5). The fractions were collected and applied to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). After electrophoresis, gel was fixed by fixation solution (one part glacial acetic acid, 3 parts isopropanol and 6 parts water) for 30 min. Eventually, the gel was visualized with colloidal Coomassie blue (Roth, Germany). Staining solution was prepared (20 mL coomassie brilliant blue stock solution, 20 mL methanol and 60 mL water) and added to the gel till the bands were clearly seen. Destaining was performed with destaining solution (40% methanol, 10% glacial acid, 50% water).

Purity of Et-AMP1 recombinant protein was determined by separating protein aliquots using SDS-PAGE (Fig. 4).

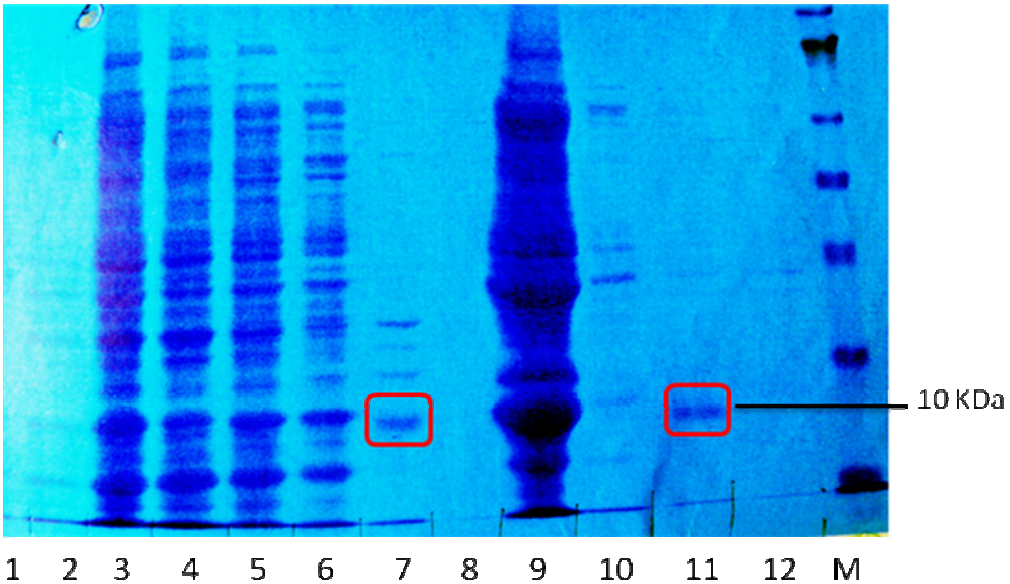

Fig. 4: SDS-PAGE showing expression and Ni-NTA purification of recombinant PEXP5-Et-AMP1 under denaturating conditions. Lane M, BenchMark^{TM} prestained protein marker (Invitrogen); lane 1 represents uninduced *E. coli* BL21/ PEXP5- Et-AMP1; lanes 2 and 3 represent total protein from induced BL21 cells containing recombinant PEXP5- Et-AMP1 after 2 and 4 h of IPTG induction; lane 4 represents flow through; lanes 5 and 6 (also 9 and 10) represent washing steps; lanes 7 and 8 (also, 11 and 12) represent elution steps. Square refers to the purified recombinant PEXP5- Et-AMP1 of 10 kDa.

2.7 Generation of transgenic plant

Two different cDNA Et-AMP1 and Et-AMP2, isolated from *Eristalis tenax* larvae were transformed into five-week-old *A. thaliana* ecotype Col-0 using *Agrobacterium tumefaciens* to confer resistance against fungal and bacterial plant pathogens.

2.7.1 Cloning strategy for Et-AMP1 and Et-AMP2

We used the same strategy for both of AMPs for DNA cloning and production of transgenic plants.

The complete open reading frame of Et-AMP2, including its predicted signal peptide (SP) and pro-sequence, was provided by B. Altincicek (Justus-Liebig University, Giessen, Germany, Fig 5) cloned in pGEM-T easy vector (Promega, Germany). A 350 base pairs (bp) fragment containing the complete coding region of the SP- Et-AMP2 was amplified by a PCR assay using the specific primers (Et-AMP2_BglII-start and Et-AMP2_stop_HindIII, Table 1).

Table 1: Gene-specific primers and universal primers used in this study.
At= Annealing temperature

Primers	Sequence 5`- 3`	AT
Et-AMP1_BamHI	GCGCGGATCCATGGCGATCAAACTTATCAT	60°C
Et-AMP1_stop_HindIII	GCGCAAGCTTCTAGAAGTTGTATCTCAAG	
Et-AMP2_BglII	GCGCAGATCTATGATTTCAAAAGTATT	50°C
Et-AMP2_stop_HindIII	GCGCAAGCTTACTTTGGGCATCTGACAA	
Et-AMP1_ for3	GCATGTTCGAAGGAAACTT	55°C
Et-AMP1_ rev2	GAAGTTGTATCTCAAGCCAACGTGTC	
pGY1 fwd2	CGTTCCAACCACGTCTTCAA	50°C
T7- Term-rev	ATCCGCATATAGTTCCTCCTTTC	
BamHI-Et-AMP1_ fwd	GGATCCGATCCAGCTACATGTGATCTGCT	50°C
HindIII-Et-AMP1_ rev	AAGCTTCTAACGCCGGCAATTGCAGACT	

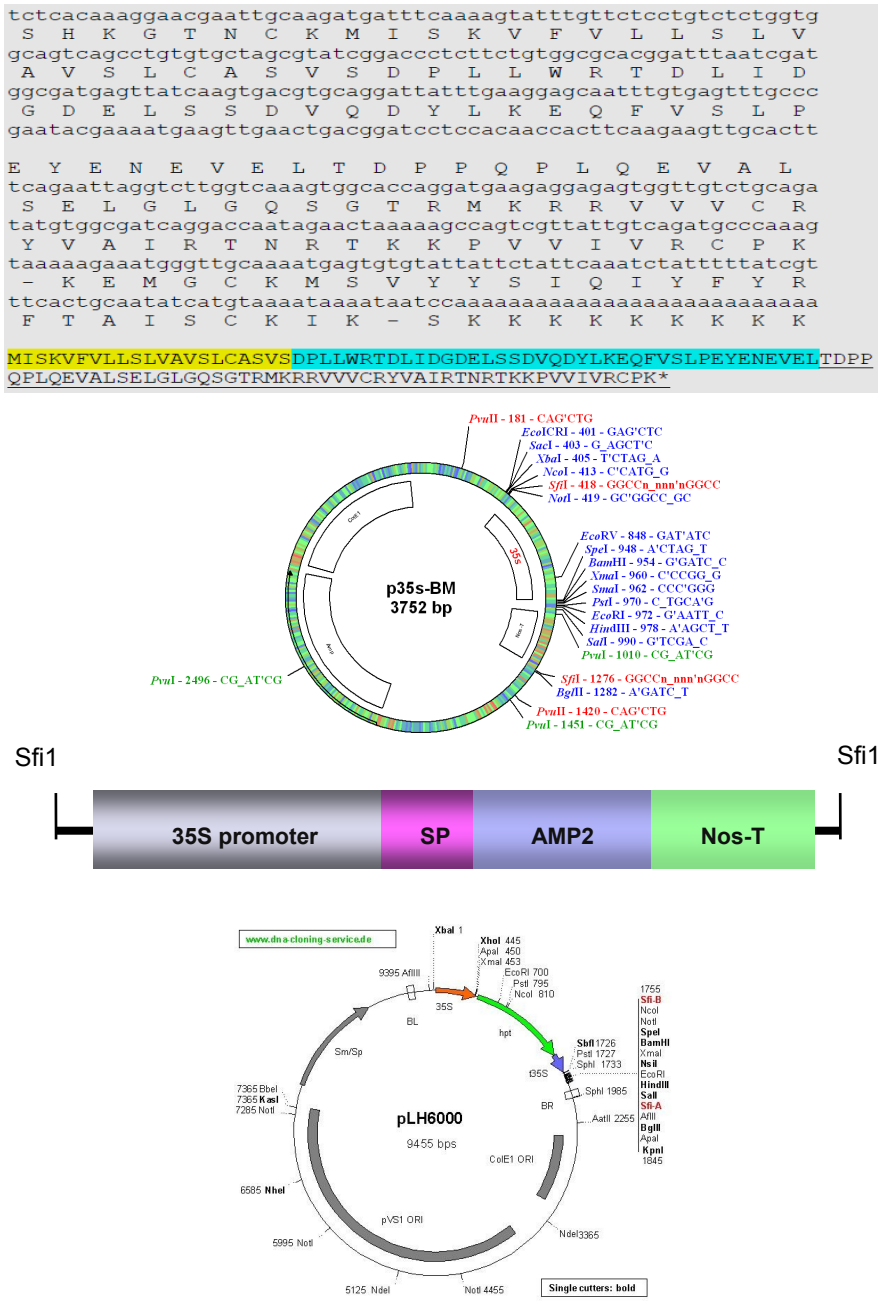

Fig. 5: Schematic representation of the inserted fragment into intermediary p-35s-BM and T-DNA region of transformation vector pLH6000. (a) Nucleotide sequence of Et_AMP2 (signal peptide, propeptide, and mature peptide) with the whole amino acid sequence, with signal peptide (yellow), propeptide (turquoise) and mature peptide (underlined). (b) 350 bp fragment containing the complete coding region of the SP- Et_AMP2 were first inserted into p-35s-BM. (c) Afterward the whole fragment together with 35s promoter and Nopaline synthase terminator (d) were inserted into transformation binary vector (pLH6000) with appropriate enzyme SfiI.

2.7.2 Transformation of cloned vector into *Agrobacterium tumefaciens*

Subsequently, the later constructs were transformed into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.,* 1991) through electroporation (*E. coli* Pulser, Biorad, USA) according to manufacturer`s instruction.

The transformed cells were platted on YEP (Table 2) agar medium containing 25 mg L^{-1} carbenicillin, 25 mg L^{-1} rifampicin and 50 mg L^{-1} spectinomycin on a rotary shaker at 150 rpm and 28 °C in dark for 2 days. Growing, antibiotica-resistant colonies of Agrobacterium were subcultured in liquid medium and then screened by PCR amplification using pGY1for2 and Et-AMP1_stop_HindIII for Et-AMP1 or PGY1for2 and Et-AMP2_stop_HindIII primers for Et-AMP2 (Table 1).

2.7. 3 Transformation of *Arabidopsis thaliana* plants

The transformed Agrobacterium Culture (section 2.7.2) was inoculated into 250 mL YEP medium containing the same antibiotics and grown at 28 °C for 6 h to the relative density of OD_{600}= 1.8. Agrobacterium cells were centrifuged for 10 min at 2700xg and resuspended in a transformation medium consisting of 5% sucrose, 0.4% ½MS-salts, 1xB5 vitamin, 10 µl L^{-1} BAP, and 0.01% Silwet-L77, pH 5.8 to a final OD_{600} of 1.1 – 1.3. According to Bechtold *et al*. (1993) the inflorescences of 6 week old Arabidopsis plants were dipped into bacteria solution and infiltrated by the (530 HPa for 5 min, see Fig. 6). The transformed plants were then grown in a climatic chamber for seed maturation. Afterward the T_1 generation seeds were harvested, surface sterilized and selected on ½ MS-medium containing hygromycin (30 mg L^{-1}) and ticarcillin (150 mg L^{-1}); after that the transgenic plants were transferred into a preserving jar containing ½MS-medium without antibiotics. Finally, when the plants were growing well, they were transferred into pots containing a soil mixture of 1:1 sand: soil Type ED 73 (Einheitserde- und Humuswerke Gebr. Patzer GmbH + Co.KG, Sinntal-Jossa, Germany). For acclimatization the transformants were covered for one week with transparent cover and grown in a growth chamber under controlled environmental conditions (see section 2.1) to raise the T_1 plants.

Fig. 6: Vacuum infiltration method: plants with flowering stage are ready for transformation (a); plants were immersed into a bacteria suspension in a vacuum chamber (b). Figures were provided by Dr. Imani

Hygromycin-resistant transformants (T_1) were self-pollinated, and harvested seeds of each T_2 line were checked for inheritance of foreign gene by selection of plants on medium with hygromycin (30 mg L^{-1}). Homozygous lines for the transgene were then selected by allowing hygromycin-resistant T_2 progeny to self-pollinate and by screening for plants whose seeds were 100% hygromycin-resistant. Homozygous lines for each gene were used for experiments in addition to the wild type as control plants**.**

Table 2: Composition of YEB (Yeast Extract Broth) medium (1L)

Beef extract	5g
Yeast extract	1g
Peptone	5g
Sucrose	5g

Adjust pH to 7.2 with 0.5 M NaOH
After autoclaving and cooling down, add 2 ml filter sterilized 1M $MgCl_2$.

2.8 Molecular characterization of transgenic lines

2.8.1 Extraction of genomic plant DNA

DNA was extracted from leaf of both transgenic and wild type plants by (REDExtract-N-Amp^{TM} Plant PCR Kits Sigma, Germany). A 0.5 cm disk of leaf tissue was punched and

immediately transferred into a 2 ml collection tube, then adding 100 µl of extraction solution to the collection tube and mixing it. After that the sample was incubated at 95°C for 10 minutes. After adding 100 µl of dilution solution the sample mix was stored at 4 °C.

2.8.2 Polymerase chain reaction (PCR) analysis

PCR amplifications were carried out on Biometra® PCR System thermocycler (Applied Biosystems). Primers Et-AMP1_BamHI-for and Et-AMP1_stop_HindIII-rev (Table 1) were used for the amplification of the fragment of SP-Et-AMP1 and primers Et-AMP2_BglII-for2 and Et-AMP2_stop_HindIII-rev for the amplification of SP- Et-AMP2. The reaction for both genes was carried out in 10 µL reaction mixture containing 5–10 ng of extracted plant DNA, PCR Master Mix (Extract-N-Amp Kit, Sigma, Germany), and 10 pmol of each primer. The PCR program profile for both genes was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30s at 94 °C, 30s at 50 °C and 40s at 72 °C. Finally, an additional elongation step was performed for 5 min at 72 °C. The amplification products were analyzed on 1.5% (w/v) agarose gel in Tris–Borate–EDTA (TBE) buffer containing 90 mM Tris–HCl (pH 7.5), 90 mM Boric acid, and 1 mM EDTA (pH 8) and visualized by staining with ethidium bromide (0.5µg/ml) as final concentration. 1KB Plus DNA Ladder (Invitrogen, Germany) was used as size marker. The gel was then visualized using a UV transilluminator (Fröbel-Labortechnic) at 312 nm wavelength. The stained bands were digitalized using digiStore software (INTAS, Gottingen) on a personal computer connected to thermo pointer.

2.9 *In vitro* antifungal assays

2.9.1 *In vitro* antifungal activity of Et-AMP1 peptide

In vitro antifungal activity of Et-AMP1 was evaluated against fungal pathogen *F. culmorum* by determining the number of germinated spores in the presence of the peptide.

2.9.2 Spore germination assay

To evaluate spore germination, spore suspension ($4x10^4$ conidia mL^{-1}) of *F. culmorum* was prepared as described in section 2.3 Spore suspensions were incubated with different

protein concentrations of Et-AMP1 at RT. The number of germinating spores was counted and the percentage inhibition was calculated for each concentration.

2.10 Plant resistance bioassays

To assess resistance, T3 homozygous of both Et-AMP1 and Et-AMP2 expressing transgenic plants and non-transformed Arabidopsis Col-0 were used. Antifungal resistance of Et-AMP1 and Et-AMP2 transformants was evaluated by inoculation with the necrotic fungal pathogen *B. cinerea* causing grey mold. For antibacterial resistance assays, 5 transgenic plants with four week old ones from each line were inoculated with *P. syringae* strain.

2.10.1 Inoculation with plant pathogen grey mold (*B. cinerea*)

Botrytis inoculation was done using the detached-leaf assay (modified after Ferrari *et al.,* 2003). 15 rosette leaves from 5 transgenic plants as well as wild type plants (5-week-old) were detached and placed in Petri dishes containing 0.5% agar, with the petiole embedded in the medium. Inoculation with *B. cinerea* was performed by placing 5 µL droplet of a spore suspension of 2×10^4 conidiospores mL^{-1} in 12 g L^{-1} potato dextrose broth (PDB) on the middle vein. The Petri dishes were sealed by parafilm in order to maintain a high humidity. The plates were then incubated in a growth chamber with 16 h photoperiod and 22/18 °C day/night temperatures. 4 days after inoculation, pictures of the infected leaves were taken. For assessing the progression of disease symptom of *B. cinerea*, the lesion size (diameter of the lesion area, in mm) was measured from the digital images using the free software Image J programme (http://rsb.info.nih.gov/ij/index.html).

2.10.2 Antibacterial resistance in transgenic Arabidopsis plants

Et-AMP1 and Et-AMP2 transgenic Arabidopsis (5-week-old, soil-grown) plants were infected with *P. syringae* strain DC3000. For plant treatment, bacteria were cultured at 28 °C on King's medium (20 g Bacto protease peptone, 5 g Dipotassium phosphate (K2HPO4),15g Magnesium Sulfate heptahydrate $MgSO_4$-$7H_2O$, 0.8 % glycerol, 15 g Difco Bacto agar per litter) and 50 mg L-1 rifampicin was added. After 2 days, bacterial culture was collected by scraping the culture from the plates and washing it twice with

sterile 10 mM $MgCl_2$. The bacterial concentration was brought to OD_{600}=0.04; then pressure was infiltrated into the abaxial side of the leaves using a syringe without a needle (Swanson *et al.,* 1988).

Inoculated plants were incubated in a growth chamber under conditions similar to those of pre-inoculation. Four days after inoculation, levels of bacterial growth in the leaves were determined as described (Whalen *et al.,* 1991). Leaf disks (0.5 cm diameter) were punched from the infiltrated area with a cork borer and grounded in 1 mL 10 mM $MgCl_2$. Bacterial populations were measured by the standard plate-dilution method, using King's medium B amended with rifampicin (50 mg L^{-1}, Whalen *et al.,* 1991).

2.11 Statistical analysis

All data sets were analyzed using one-way-ANOVA of the SPSS for windows statistical data analysis package (SPSS Inc., release 16, Chicago, IL, USA) to determine if significant differences of antimicrobial activity between transgenic and non-transgenic plants were presented with a rejection limit of $P \leq 0.05$.

3. Results

Transgenic expression of antimicrobial peptides from insects has been emerged as a promising tool to render crops resistant to a wide range of fungal and bacterial pathogens (Vilcinskas and Gross, 2005). In this study, we aimed to generate transgenic *Arabidopsis thaliana* lines expressing *Erestalis tenax* antimicrobial peptide (Et-AMP1 and Et-AMP2) which expected to be promising candidate conferring plant resistance against fungal and bacterial pathogens.

3.1 Generation of transgenic *Arabidopsis thaliana* plant

3.1.1 Et-AMP2

We prepared Et-AMP2 for insertion into *Arabidopsis thaliana* ecotype Columbia plants as described in Fig. 5.

For selection of transgene the harvested seed were cultivated on ½ MS medium containing antibiotic Hygromycin. The transgenic seed could be able grow onto whole plant (Fig. 7).

Fig. 7: Selection on half MS medium containing hygromycin, transgenic plants: vigorously growth of root and leaves (arrow) comparing nontransgenic one on half-strength MS-medium with 30 mg L^{-1} hygromycin.

3.1.1.1 Molecular analysis of transgene

Also, the integration of gene into *Arabidopsis thaliana* genome was confirmed by Molecular analysis (PCR). Fragment with 350 bp (Et-AMP2_BglII/ Et-AMP2_stop_HindIII primers) including the complete coding region of the SP-Et-AMP2 was detected in several transgenic lines (Fig. 8).

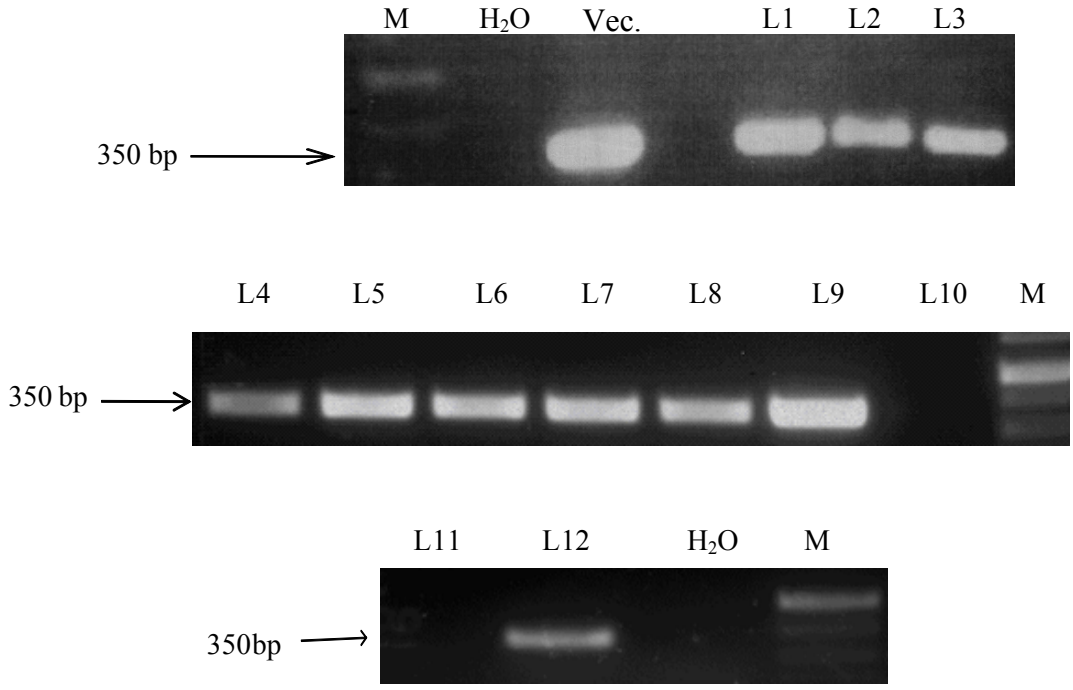

Fig. 8: PCR confirms the integration of Et-AMP2 in most of putative transgenic Arabidopsis lines. A 350 base pairs (bp) fragments were amplified with gene specific primer (Et-AMP2_BglII/ Et-AMP2_stop_HindIII).

Ln= transgenic line number; Vec: Plasmid as positive control; M= DNA marker, H_2O: water control

3.1.2 Et-AMP1

We applied the same strategy for preparing the Et-AMP1 for insertion into *A. thaliana* ecotype Columbia plants.

3.1.2.1 Molecular analysis of transgen

The integration of transgene in *A. thaliana* was confirmed by Molecular analysis (PCR). Fragment with 375 bp (Et-AMP1_BamHI-start and Et-AMP1_stop_HindIII primers, Table

1) including the complete coding region of the SP-Et-AMP1 was detected in several transgenic lines (Fig. 9).

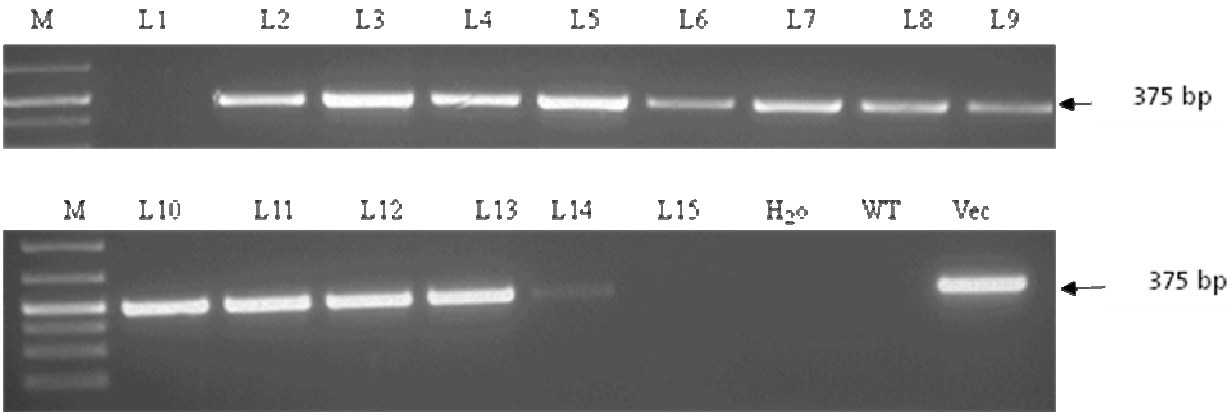

Fig. 9: Conformation of gene integration in genome of *A. thaliana* plant by mean of PCR. The 375 bp fragments of introduced gene were detectable with Et-AMP1 specific primers (Et-AMP1_BamHI/ Et-AMP1_stop_HindIII).

M: DNA Marker; L1-L15 putative transgenic lines; Vec: Plasmid as positive control; WT: Wild type non transgenic plant; H_2O: water control.

3.1.2.2 Production of recombinant Et-AMP1 protein

One of the major concerns regarding the application of synthetic AMPs is their high production costs. Thus, it was attempted in this study to establish a method to permit the production of recombinant ET-AMP1 confers protein in *E. coli* in large quantities with low costs. To achieve that, the expression vector pEXp5/CT (containing His-tag and V5 epitope at C-terminal) was used to produce the recombinant ET-AMP1 protein in the *E. coli* BL21 (DE3) expression system. The obtained target recombinant pEXp5-ET-AMP1 protein was purified using Ni-NTA column (see section 2.6). Tricin-SDS-PAGE analysis (Fig. 10) showed that the target protein was successfully expressed in insoluble form after 0.3 and 1 mM IPTG induction.

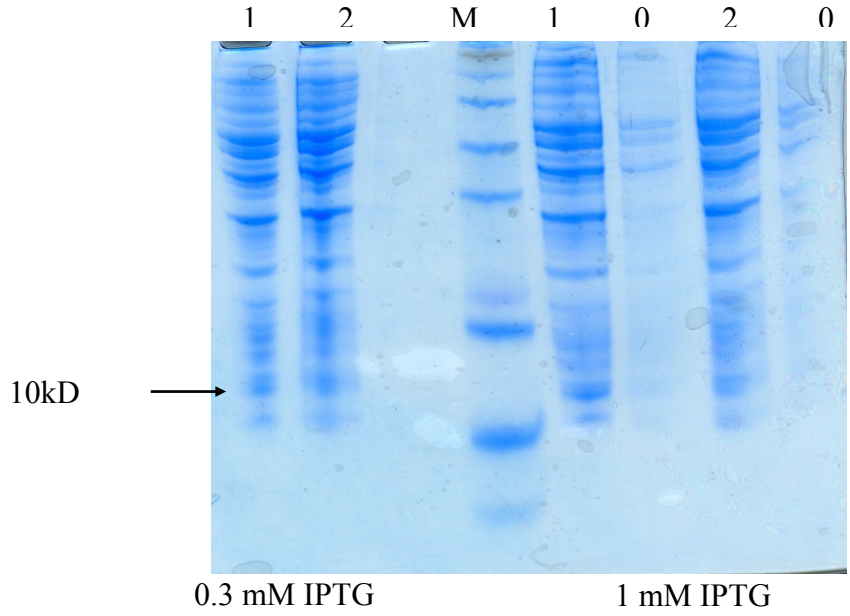

Fig. 10: Tricin-SDS-PAGE showing expression of *E.coli* BL21 (DE3) that was induced by IPTG, and Ni-NTA purification of recombinant pEXP5-Et-AMP1 under denaturating conditions.
Lane M, BenchMark™ prestained protein marker (Invitrogen); lane 0, represents uninduced *E. coli* BL21/ PEXP5-Et-AMP1; lane 1 and 2 represent a total protein from induced BL21 cells containing recombinant PEXP5-Et-AMP1 after 2 and 4 h of IPTG induction; lane 0 represents noninduced BL21 cells.

3.1.2.3 *In vitro* activity test

We tested the recombinant protein against *Fusarium culmorum* conidia germination and hyphal growth (Table 3, Fig. 11). There were effects on the conidia germination (10.5 % inhibition) and hyphal growth (stunting).

Table 3: Effect of recombinant protein (Et-AMP1) on germination of *Fusarium culmorum* conidia with different concentration.

Conc. of Recom. Protein (µM)	0.9		0.45		0.3		0.09		0	
No. of spores	Germi-nated	Non	Germi-nated	Non	Germi-nated	Non	Germi-nated	Non	Germi-nated	Non
	87	11	43	5	71	8	88	7	89	1
	62	8	57	3	45	2	74	3	128	1
	81	8	85	12	71	2	62	5	99	1
Total	230	27	185	20	187	12	224	15	227	3
% germination	89.5		90.3		93		93.7		98.7	

We can observe the higher inhibition percentage of recombinant protein against *Fusarium culmorum* conidia germination did not exceed 10.5% with the higher purified concentration (0.9 µM), whilst it was decreased to 6.3% with 0.09 µM.

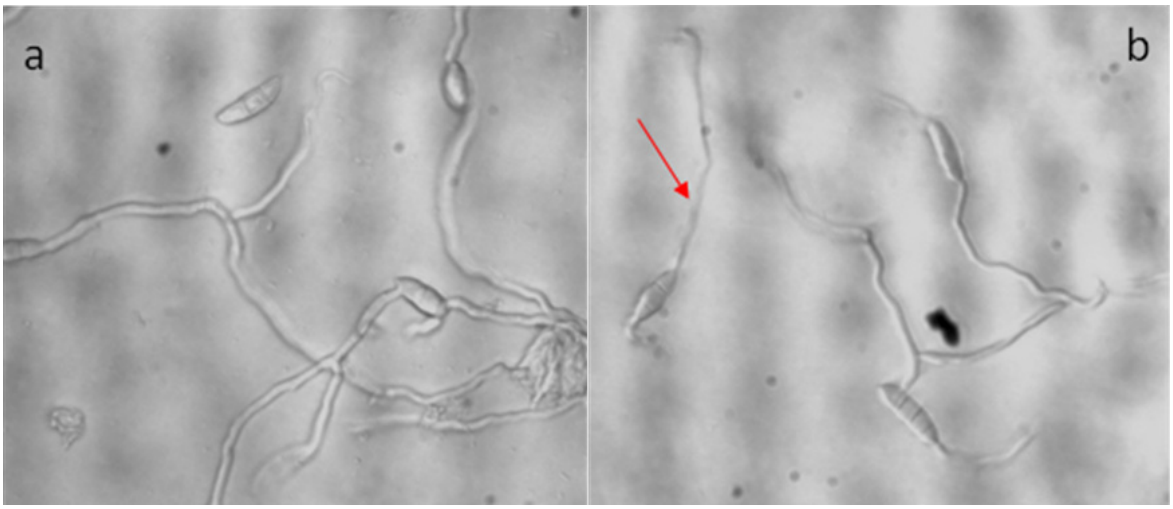

Fig. 11: Effect of the recombinant protein Et-AMP1 on the growth of fungal hypha. The hypha growth (b) in presence of the recombinant protein (0.9 µM, see red arrow) was less than (a) control (phosphate buffer 10 mM) indicating the effect of recombinant protein on hypha growth.

3.2 Pathogen assay

3.2.1 Pathogen assay with ET-AMP2

3.2.1.1 *In planta* resistance against *B. cinerea*

Botrytis cinerea strain B05.10 was grown on HA-Agar medium for 10 days at room temperature (RT). Spore suspension concentration (2.5×10^4 conidiospores mL^{-1}) in 12 g L^{-1} potato dextrose broth (PDB) was used for inoculation of both wild type and transgenic *Arabidopsis thaliana* plants (Fig. 12).

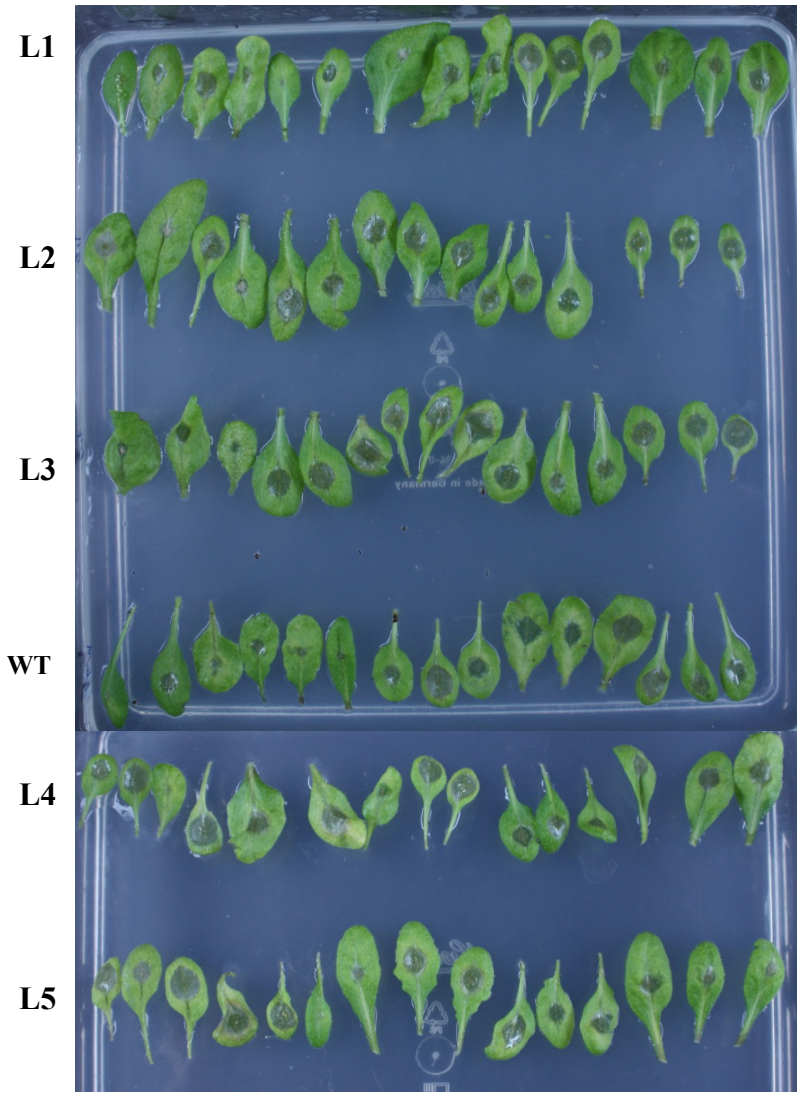

Fig. 12: Disease symptoms of *B. cinerea* evaluated 4 days after the inoculation on fifteen leaves representing five plants for both non-transgenic control Arabidopsis Col-0 (WT), representative transgenic lines expressing ET-AMP2 (lines 1, 2, 3, 4 and 5), note necrotic lesion with different diameter on the different lines. There is different in severity of infection among non-transgenic control and transgenic lines.

As can be seen in Figure 13, the transgenic expression of ET-AMP2 in Arabidopsis plants result to reduce the spot diameter occurred on the leaves in lines 1, 4 and 5, whilst did not significantly affect the diameter on the leaves in lines 2 and 3.

We can note that there are significant difference between wild type and lines number 1, 4 and 5. Also, there is difference between wild type and lines number 2 and 3. The most resistant lines to *Botrytis cinerea* were lines number 1, 5 and 4, respectively. Importantly, line 1 exhibited significantly ($P \leq 0.05$) the lowest leaf diameter with reduction of about 32.5% relative to wild type.

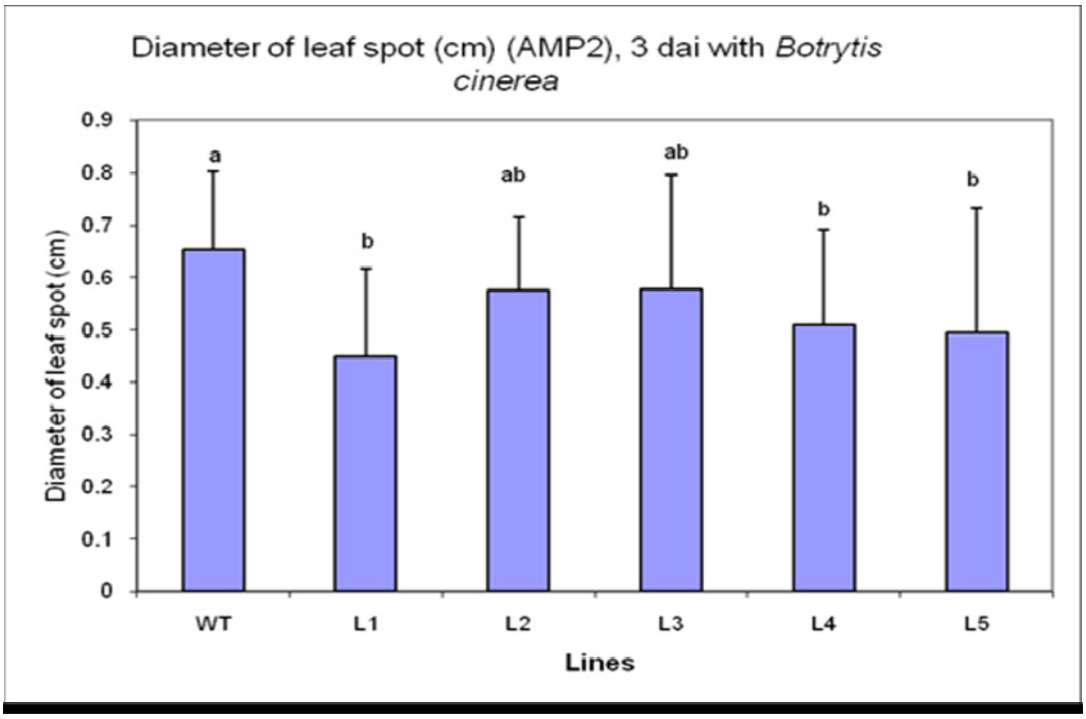

Fig. 13: *In planta* antifungal activity of ET-AMP2 against *B. cinerea* inoculation. Mean necrotic lesions on the leaves of different Arabidopsis transgenic lines and wild type. This chart explains that significant differentiations are found between wild type and some transgenic lines indicating to resistance of these lines. Columns with the same letter are not significantly ($P \leq 0.05$).

The results are from one representative of two experiments and are averages of 15 leaves from 5 plants (replicates) per line. The same tend was observed in the second experiment (season) therefore we took the first experiment as representative sample.

3.2.1.2 *In planta* resistance against *P. syringae* pv. *tomato*

To determine whether the constitutive expression of ET-AMP2 could confer resistance against *P. syringae* in Arabidopsis, five independent T3 progeny homozygous ET-AMP2 expressing transgenic lines (1, 2, 3, 4 and 5, Fig. 14) were evaluated, as well, non-transgenic Col-0 (WT) plants were used as control. Inoculation of Transgenic Arabidopsis plants with *Pseudomonas syringae* pv. *tomato* DC3000 with an inoculum concentration OD_{600}=0.01 by syringe infiltration.

Small necrotic lesions areas of inoculated leaves were noted on the leaves of non-transgenic Col-0 (WT) plants, transgenic plants 4 days after infection, but the symptoms were very slight and did not accompany by chlorosis.

Fig. 14: Arabidopsis plants inoculated with *Pseudomonas syringae*. 4 dai, Wild type and ET-AMP2 lines. We can note that the symptoms were very slight and the spots were not accompanied by chlorosis, inoculated leaves infiltrated with black marker, arrows refer to some inoculated leaves.

To calculate the number of colony forming units/mL:

C.F.U/mL original sample = C.F.U/plate x (1/mL aliquot plated) x dilution factor

Where: C.F.U/plate: number of colonies growing on the plate.

Aliquot plated: amount of bacterial (crushed leaf discs in Mg So4) suspension which added to the plate.

Dilution factor: No. of dilution times of original bacterial suspension.

To calculate C.F.U for Line 1: 13x (1/0.01ml) $x10^6$=$13x0^8$

As can be seen in Figure 15, the transgenic expression of ET-AMP2 in Arabidopsis plants result to reduce the C.F.U of the *Pseudomonas syringae* in lines 1, 2, 3, 4 and 5 whilst result to increase the C.F.U in line 4.

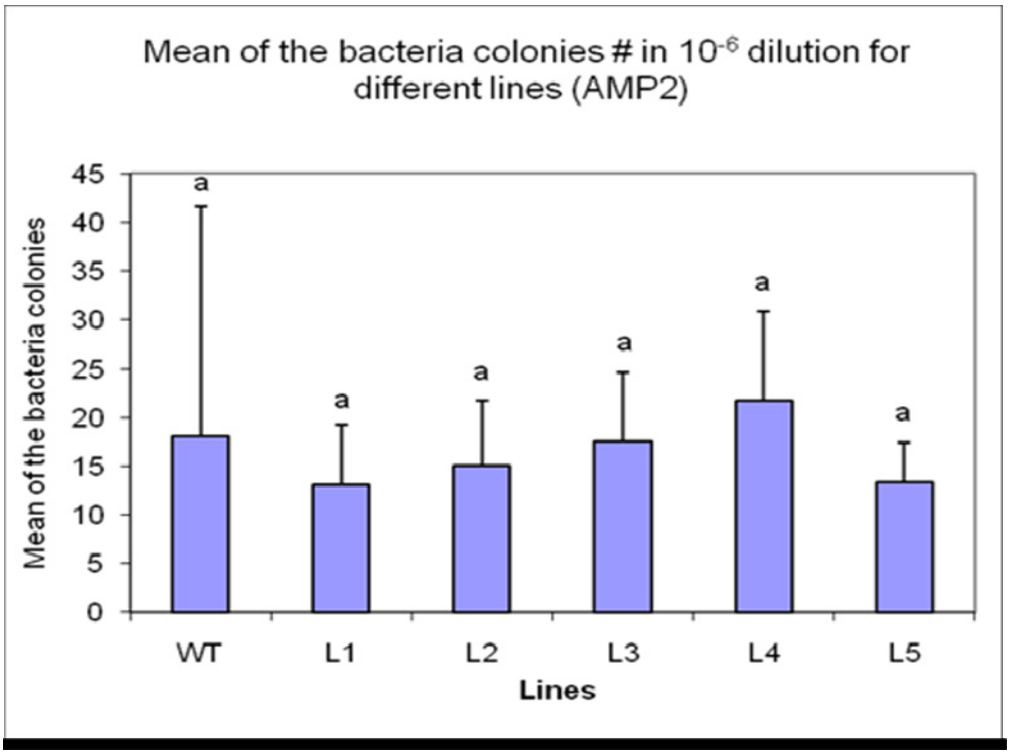

Fig. 15: Mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution for different ET-AMP2 and wild type lines. In spite of, the line number 1, 2 and 5 exhibit resistance against bacterial infection but there is no significant differentiations between wild type and transgenic lines. The results are from one experiment and are averages of 5 plants (replicates) per line.

As can be seen in Figure 15, the transgenic expression of ET-AMP2 in Arabidopsis plants result to reduce the C.F.U of the *Pseudomonas syringae* in lines 1, 2, 3 and 5 whilst, result to increase the C.F.U in line 4.

We can note that there are no significant difference between wild type and all of lines. But Lines number 1and 5 seems more resistant than others.

Repeating the previous experiment

We repeated last the experiment (3.2.1.2) but with increasing of the *Pseudomonas syringae* inoculum concentration to OD_{600}=0.04, Fig. 16). The symptoms were restricted on necrotic lesions as watered spots in areas of inoculation on the leaves of non-transgenic Col-0 (WT) plants, transgenic plants and did not accompany by chlorosis.

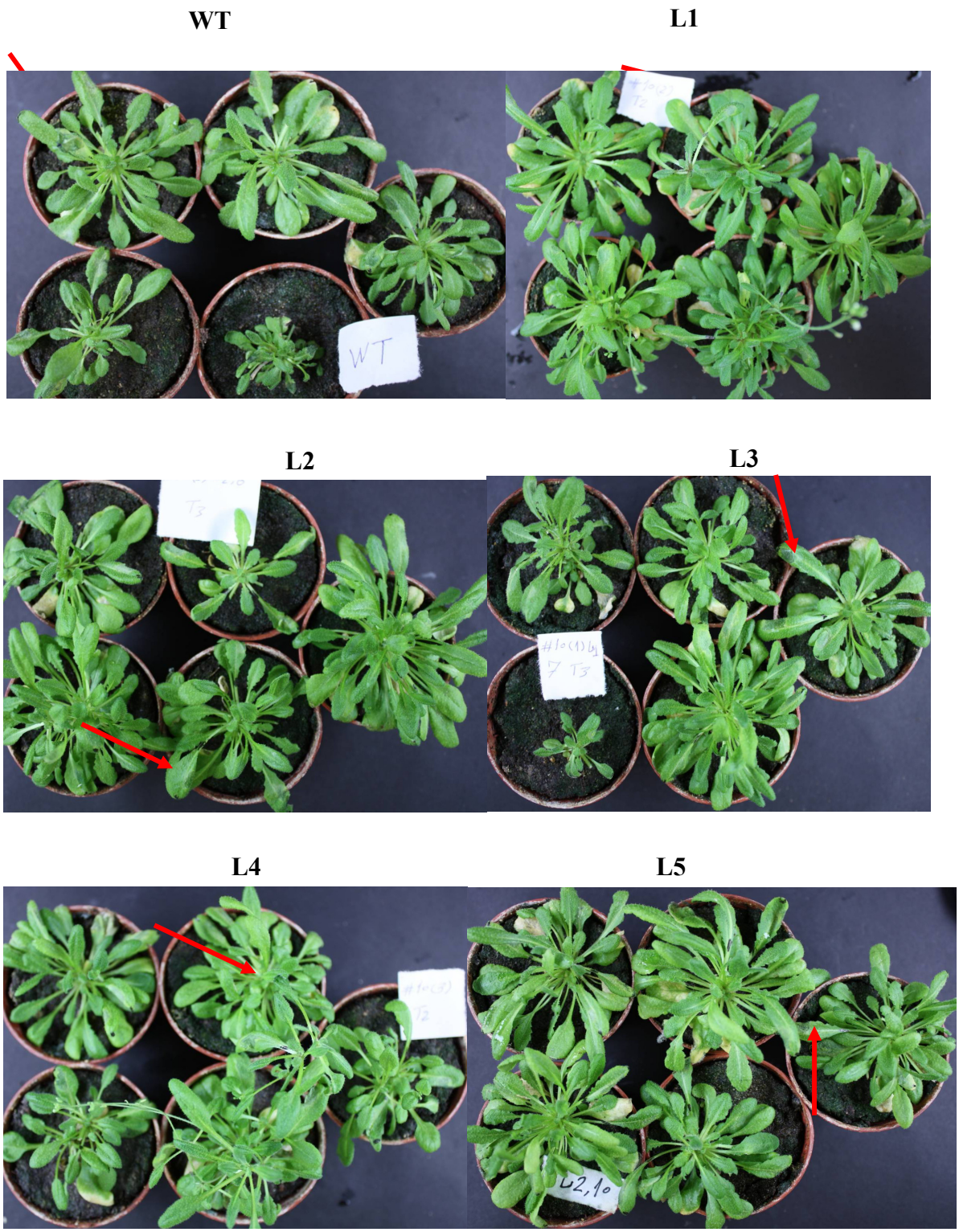

Fig. 16: Arabidopsis plants inoculated with *Pseudomonas syringae*. 4 dai, Wild type and ET-AMP2 lines. We can note that the symptoms were slight and the spots did not accompany by chlorosis, except some leaves (some of them labeled with red arrow).

As can be seen in Figure 17, the transgenic expression of ET-AMP2 in Arabidopsis plants inoculated with *Pseudomonas syringae* result to reduce the C.F.U in lines 1, 2, 3, 4 and 5. We can note that there is significant difference between wild type and all of transgenic lines indicating that ET-AMP2 gene confers resistant in transgenic Arabidopsis plants against *Pseudomonas syringae*, But the Lines number 1 and 2 seems to be more resistant than others.

The same tend was observed in the second experiment (season) therefore we took the first experiment as representative sample.

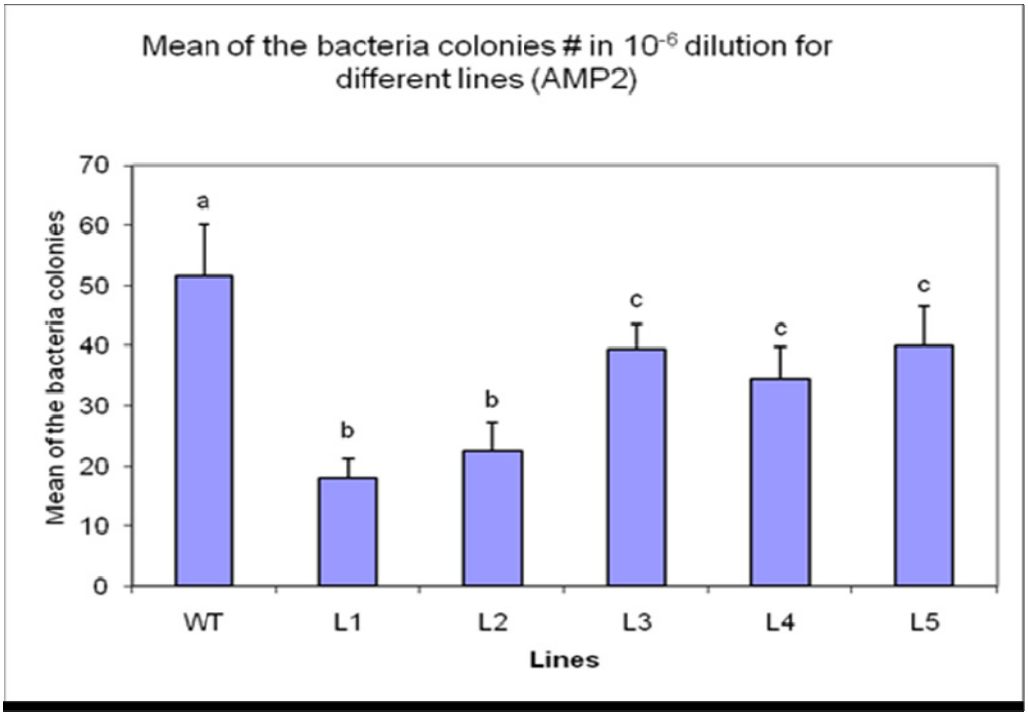

Fig. 17: Mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution for different ET-AMP2 and wild type lines. We can observe that line number 1 and 2 exhibit resistance against bacterial infection. Also, there are significant differentiations between wild type and some transgenic lines whereas, there is no significant differentiations with others. The results are from one representative of two experiments and are averages of 5 plants (replicates) per line.

We can observe in Figure 18 that the expression of ET-AMP2 in transgenic Arabidopsis plants inoculated with *Pseudomonas syringae* result to reduce the C.F.U in lines 1, 2, 4, 3 and 5 respectively.

Also, we can note that there are significant difference between wild type and all of transgenic lines indicating to ET-AMP2 confers resistant in transgenic Arabidopsis plants against *Pseudomonas syringae*, But the Lines number 1and 2 seem to be more resistant than others.

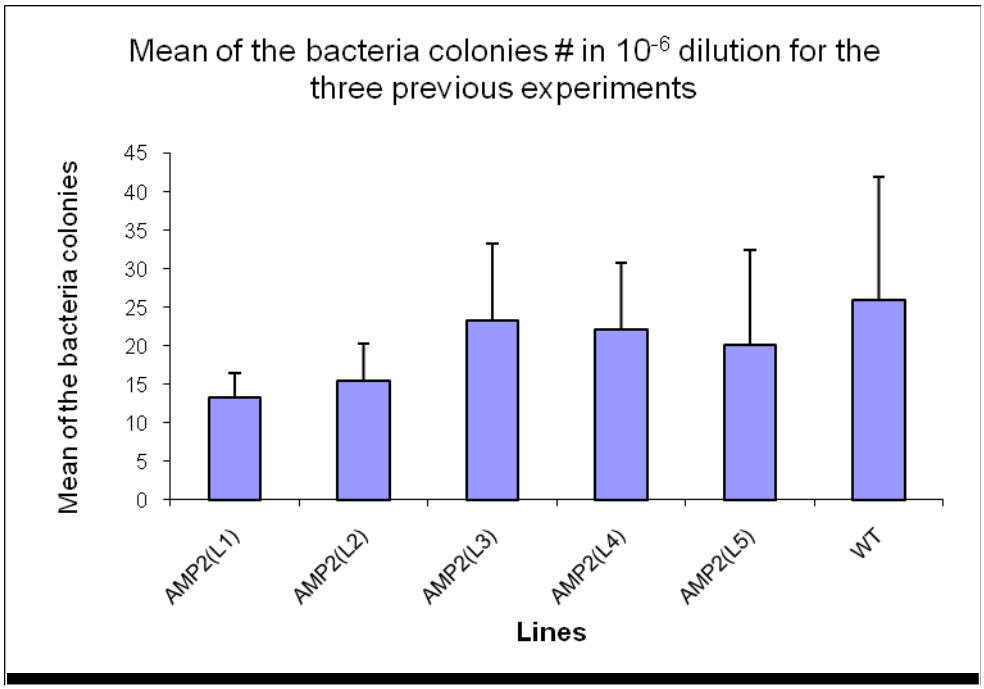

Fig. 18: Mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution for wild type and different transgenic lines (ET-AMP2) for the three experiments. The chart explains that line number 1 and 2 exhibit resistance against bacterial infection. Also, there is significant differentiations between wild type and some transgenic lines indicating to resistance whereas, there is no significant differentiations with others indicating to no resistance.

3.2.2 Pathogen assay with Et-AMP1

3.2.2.1 *In planta* resistance against *B. cinerea*

Inoculation with *Botrytis cinerea* spores (inoculum concentration was $2.5x10^5$ spores $mL^{-1,}$ Fig. 19). Spore suspension concentration (2.5×10^4 conidiospores mL^{-1}) in 12 g L^{-1} potato dextrose broth (PDB) was used for inoculation of *Arabidopsis thaliana* plants.

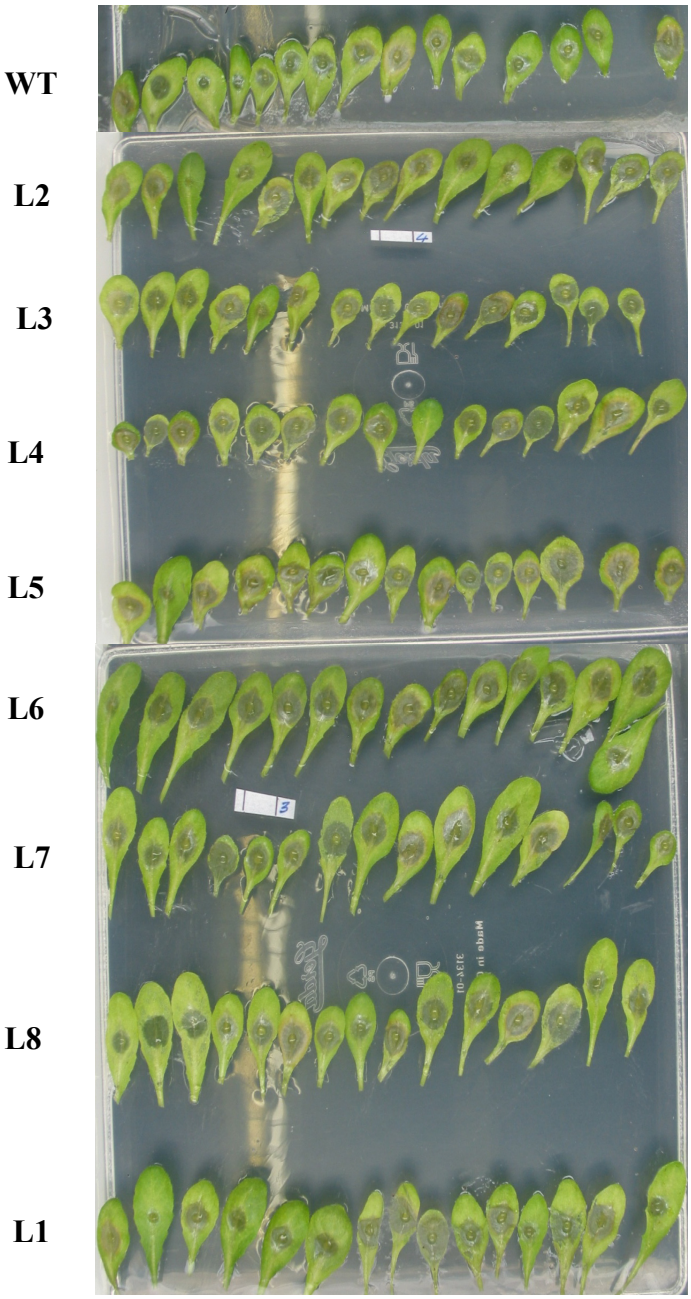

Fig. 19: Disease symptoms of *B. cinerea* evaluated 4 days after the inoculation on fifteen leaves represent five plants for both non-transgenic control Arabidopsis Col-0 (WT), representative transgenic lines expressing ET-AMP1 (lines 1, 2, 3, 4, 5 6, 7 and 8). We can recognize necrotic lesion with different diameter on the different lines accompanied by chlorosis, but it seems there is no different in severity of infection among non-transgenic control and transgenic lines.

As can be seen in Figure 20, the expression of ET-AMP1 in Arabidopsis plants did not result in reduce of the spot diameter in the all lines.

We can note that there are no significant difference between wild type and all of lines indicating that the expression of ET-AMP1 in Arabidopsis plants did not result in reduce the necrotic lesions and did not increase the resistance to *Botrytis cinerea*.
The same tend was observed in the second experiment (season) therefore we took the first experiment as representative sample.

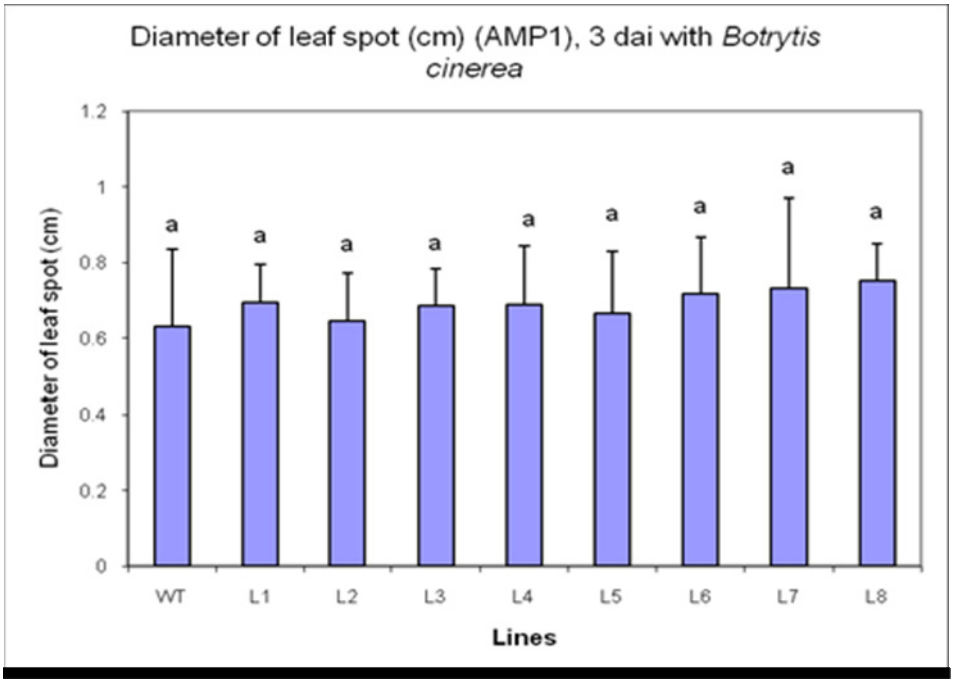

Fig. 20: *In planta* antifungal activity of ET-AMP1 against *B. cinerea* inoculation. Mean necrotic lesions on the leaves of different ET-AMP1 Arabidopsis transgenic lines and wild type, the peptide has no activity against the pathogen. The results are from one representative of two experiments and are averages of 15 leaves from 5 plants (replicates) per line.

3.2.2.2 *In planta* resistance against *P. syringae* pv. *tomato*

Inoculation of transgenic Arabidopsis plants with *Pseudomonas syringae* pv. *tomato* DC3000 with inoculum concentration OD_{600}=0.04 (Fig. 21). Typical necrotic lesions with chlorosis spreading out from lesions in areas of inoculation were noted on the leaves of non-transgenic Col-0 (WT) plants, transgenic plants.

WT

L1

L2

L3

L4

L5

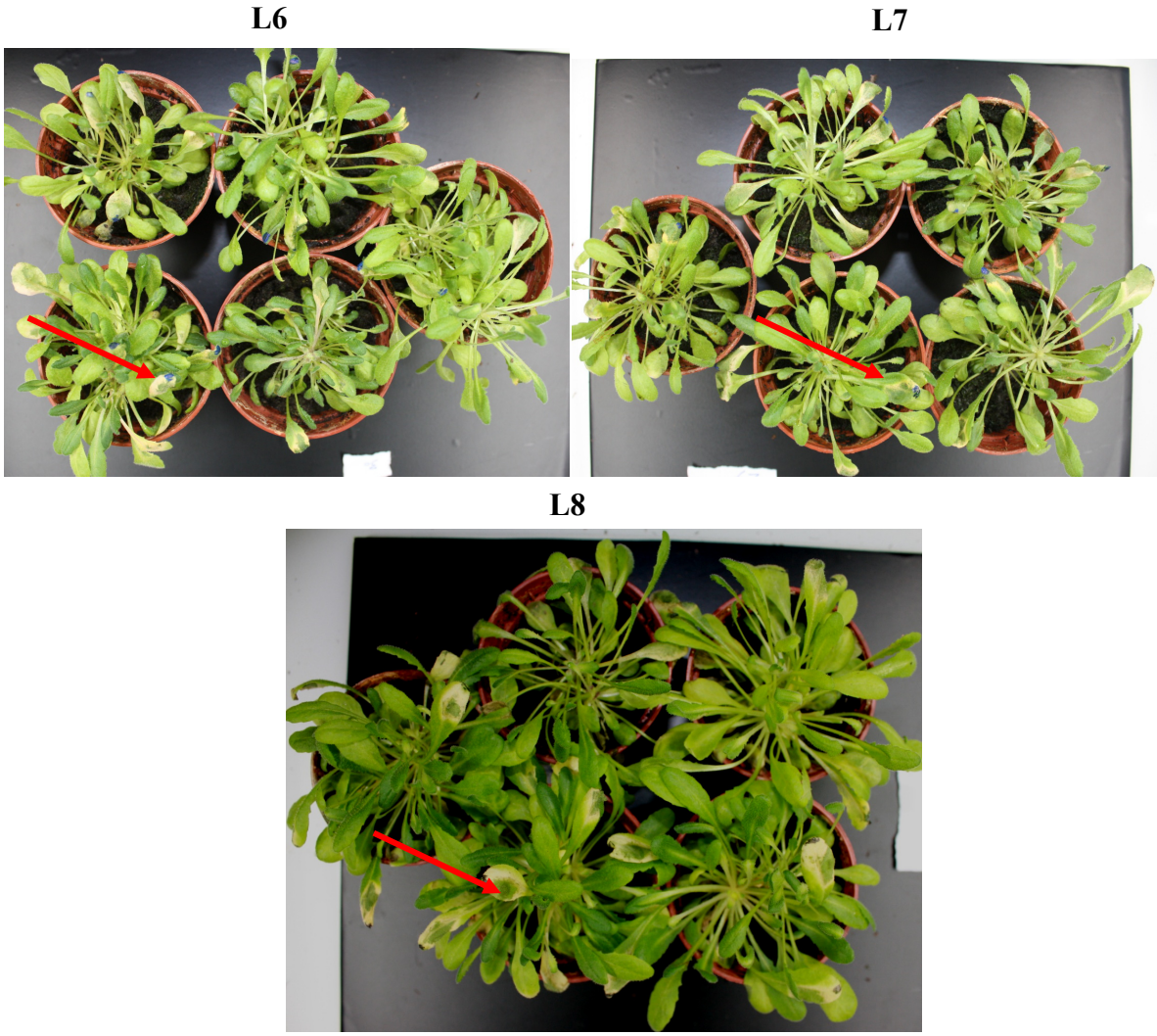

Fig. 21: Arabidopsis plants inoculated with *Pseudomonas syringae*. 4 dai, Wild type and ET-AMP1 Lines. The pathogen caused lesions with yellow halos as well, overall yellowish in the plants, see red arows.

We can observe in Figure 22 for the mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution that: the expression of ET-AMP1 in transgenic Arabidopsis plants inoculated with *P. syringae* result to reduce the C.F.U in lines 1, 2, 3, 4, 5, 7 and 8 lines. In contrast, the expression of ET-AMP1 in transgenic line 6 increased the C.F.U.

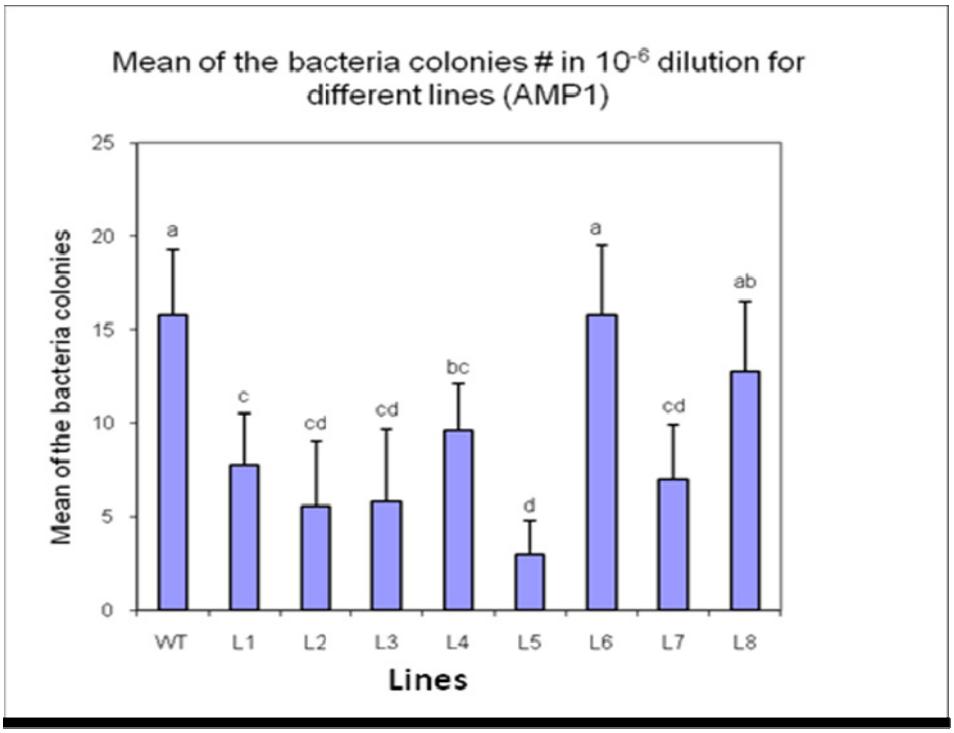

Fig. 22: Arabidopsis plants inoculated with *Pseudomonas syringae*. 4 dai, Wild type and ET-AMP1 Lines. Significant differences are found between the most of transgenic lines with wild type and among some of transgenic lines themselves. The results are from one representative of two experiments and are averages of 5 plants (replicates) per line.

Also, we can note that: there are a significant difference between wild type and all of transgenic lines (except line number 6) indicating to ET-AMP1confers resistance in transgenic Arabidopsis plants against *P. syringae*, but the Lines number 5, 2, 3, 7 and 1 respectively seem to be more resistant.

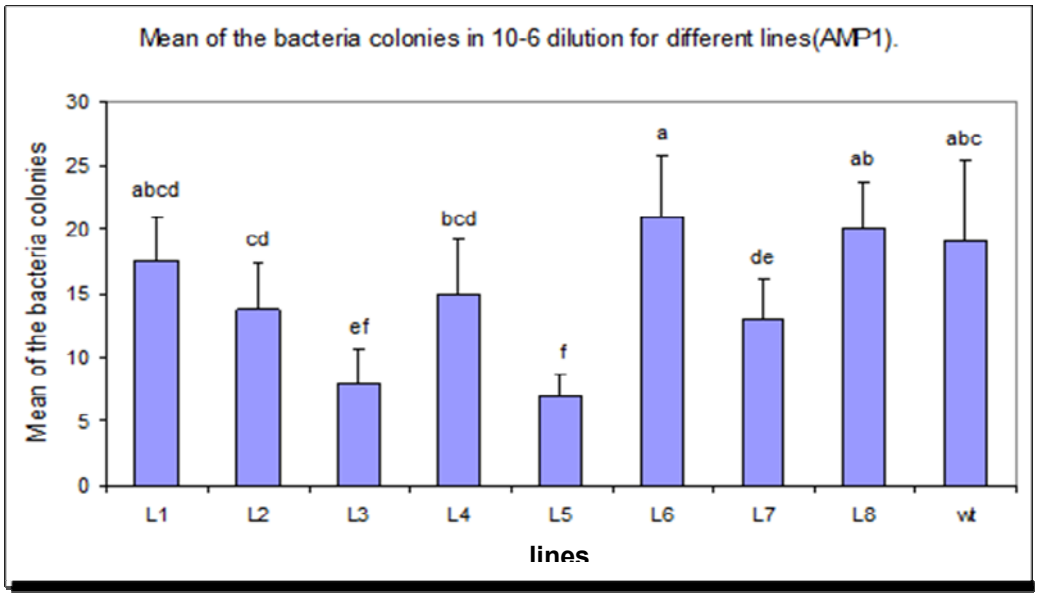

Fig. 23: Mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution for wild type and different transgenic lines (ET-AMP1) for the two experiments. Significant differences are detected between the majority of transgenic lines with wild type and among some of transgenic lines themselves. As a Mean of the *P. syringae* colonies number in 10^{-6} dilution for wild type and different transgenic lines (ET-AMP1).

We can observe in Figure 23 for the Mean of the *Pseudomonas syringae* colonies number in 10^{-6} dilution that: the expression of ET-AMP1 in transgenic Arabidopsis plants inoculated with *P. syringae* result to reduce the C.F.U in lines 1, 2, 3, 4, 5 and 7 lines. In contrast, the expression of ET-AMP1 in transgenic lines 6 and 8 increaased the C.F.U.

We can observe that: there are significant differences between wild type and all of transgenic lines indicating to ET-AMP1 confers resistant in transgenic Arabidopsis plants against *P. syringae*, the higher resistant was found in lines number 5, 3, 7, 2 and 1 respectively. Whereas**,** the Lines number 8 and 6 are susceptible to *P. syringae* inoculation.

4. Discussion

Plants are always attacked by pathogens, pests and exposed to environmental stresses. Therefore, they have developed sophisticated and complex defense mechanisms throughout their evolution. To reduce pathogen infections, plants synthesize numerous compounds, like for example defensins which reportedly contribute to the plant innate host defense (Fujimura *et al.,* 2004; Pelegrini and Franco, 2005; Franco *et al.,* 2006).
In the last few years, a mixture of biotechnological tools has been developed to diminish the resistance of hurtful pests and pathogens that react in plants towards chemical products administrated against them. One of these wonderful techniques consists in the possibility of inserting, silencing or over-expressing different genes in plants, creating an organism with improved resistance to pathogens, and improving crop production (Bhargava et al. 2007; Sesmero et al. 2007).

4.1 Et-AMP1 and Et-AMP2 mode of action

The common feature of most AMPs is penetration of microbial membranes, disturbing an array of intracellular target molecules (Shai, 1999; Brogden, 2005; Marcos, 2008). Regarding Et-AMP1 and Et-AMP2, the mode of action is not yet clarified and still needs to be studied. It is supposed that the initial association of AMPs with the bacterial membrane occurs generally through electrostatic interactions between the cationic AMPs and the outer membrane of bacteria (Vaara, 1992; Otvos, 2000).

Additionally, the use of signal sequences from different origins for targeting of AMPs into the intercellular spaces (where the proteolytic degradation is expected to be minimum) is supposed to prevent the cellular degradation of AMPs, and avoid the possible harmful effects on the plant cells (Sharma *et al.,* 2000).
As well, extracellular targeting of AMPs allow the plant to produce peptides to be secreted into the battleground between pathogens and host, providing direct access to the pathogen target and hence effectively improving plant resistance against invading pathogens.

4.2 Production and *in vitro* activity of recombinant Et-AMP1 protein

Due to the high cost of producing large amounts of synthetic antimicrobial peptides, their direct utilization in phytopathogen control, *in vitro* screenings and mode of action studies are highly restricted. Therefore, it was sought to establish a method for the production of recombinant Et-AMP1 in the *E. coli* expression system to obtain sufficient quantities required for detailed biological *in vitro* and *in vivo* assays on its activity spectrum. For this reason, preliminary antifungal assays were performed *in vitro* with Et-AMP1 on the spore germination of the ascomycetes (*F. culmorum*). The inhibition percentage was low (Table 3, Fig. 12) because of the low concentration of recombinant protein, whereas the higher concentration of the Et-AMP1 recombinant protein was 0.9 µM, which may be attributed to the antibacterial activity of Et-AMP1 against *E. coli* or the susceptibility of peptide to proteolytic degradation or both together (Piers *et al.,* 1993; Makrides, 1996; Zhou *et al.,* 2009).

E. coli BL21 (DE3) was used as expression host for the production of recombinant Et-AMP1 protein, in view of the fact that this strain encodes the T7 RNA polymerase and can be utilized for protein expression under the control of a T7 promoter. We have primarily cloned Et-AMP1 mature peptide with an added C-terminal part with V5-epitope and His-tag in the pEXp5 expression vector. After the purification using Ni-NTA, the recombinant target protein (pEXp5- Et-AMP1) with an expected molecular mass of 10 kDa was detected, though only in a small amount, using Tricin-SDS-PAGE (Fig. 4 and 10).
In vitro antifungal activity of this recombinant protein against conidial germination of *F. culmorum* (Table 3, Fig. 11) revealed a slight effect which did not exceed 10.5 % inhibition of conidia germination at the highest peptide concentration (0.9 µM).
In comparison, Langen *et al.* (2006) studied the antifungal activity of gallerimycin, a novel antifungal peptide from the greater wax moth *Galleria mellonella*, against conidial germination of the biotrophic tobacco powdery mildew fungus *Erysiphe cichoracearum*. When conidia incubated in intercellular washing fluid (IWF) from transgenic plants, their germination was strictly reduced and appressorium-like structures were rarely observed in the germinated conidia. Depending on the transgenic line from which the IWF was prepared, germination of powdery mildew conidia was reduced by 45–66%. In contrast,

conidia incubated in the IWF from non-transgenic tobacco could germinate and form an appressorium-like structure at the tip of the germ tube within approximately 24 hours.
Rahnamaeian *et al.* (2009) reported that the Metchnikowin (Mtk) proline-rich peptide synthesized in the fat body of *Drosophila melanogaster* inhibited the germination of conidia of the plant pathogenic fungus *Fusarium graminearum* with IC_{50} value of 1 μM.
Also, Mtk enhanced resistance to powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Bgh), where the number of germinated conidia that penetrated successfully into epidermal cells of the transgenic lines was reduced significantly. Less than 10% of germinated conidia penetrated an epidermal cell, formed a functional haustorium, and elongated secondary hyphae. By contrast, more than 50% of germinated conidia produced functional haustoria in wild-type (barley cv. Golden Promise, GP) plants.

4.3 *Arabidopsis thaliana* transformation

Et-AMP1 and Et-AMP2 encoding sequences consist of three parts, i.e., signal peptide, propeptide and mature peptide. Precisely, propeptide mediates transport of protein from the endoplasmic reticulum into the trans-Golgi network by protecting molecules from degradation in order to facilitate packaging of protein into vesicles (Stoller and Shields, 1989).

The signal peptide is a precursor with an amino-terminal extension of 20 to 25 amino acid residues for synthesizing most secretory proteins in both prokaryotic and eukaryotic cells. This signal peptide is removed during translocation of the secretory proteins across the membrane (Coleman *et al.,* 1985).
In this study, Et-AMP1 and Et-AMP2 including their putative signal peptide, propeptide and mature peptide were cloned downstream of the constitutive CaMV 35S promoter into a binary victor to target the mature peptide into the plant apoplastic space to engineer transgenic Arabidopsis plants via Agrobacterium-mediated transformation by the vacuum infiltration method (Bechtold *et al.,* 1993). This strategy has been successfully employed to confer resistance against fungal pathogens in tobacco by transgenic expression of the antifungal peptide gallerimycin from *Galleria mellonella* (Langen *et al.,* 2006).
A number of independent transgenic Arabidopsis lines constitutively expressing either Et-AMP1 or Et-AMP2 genes were successfully obtained.

Notably, all transgenic plants were healthy, fertile and did not show any morphological or developmental abnormalities compared with the wild type. This suggests that the constitutive expression of these peptides targeted to apoplast does not seem to influence plant physiology.

The integration of both genes has been confirmed by PCR. Fig. 8 and 9 confirm the integration of Et-AMP1 and Et-AMP2 in most of putative transgenic Arabidopsis lines.

4.4 Et-AMP1 and Et-AMP2 expression, biotrophic pathogenic interaction of Pseudomonas-Arabidopsis and necrotrophic pathogenic interaction of Botrytis-Arabidopsis

There is no previous evaluation of Et-AMP1 and Et-AMP2 influences on Pseudomonas bacteria and Botrytis fungus during its interaction with Et-AMP1 and Et-AMP2 Arabidopsis.

In this work, we studied the ability of two new antimicrobial peptides (Et-AMP1 and Et-AMP2) from *Eristalis tenax* (Altincicek and Vilcinskas, 2007) to engineer Arabidopsis plants for disease resistance against *Pseudomonas syringae* bacteria and *Botrytis cinerea* fungus.

4.4.1 Use of transgenic plants as a tool to enhance plant resistance against fungi

The losses in cultivated and stored crops due to phytopathogens are estimated to be $30-$50 billion annually (Baker *et al.,* 1997).

Applications of pesticides as countervailing approaches not only significantly increase production costs and are regarded as serious environmental hazards, but also contribute to the increase in antimicrobial-resistant species (Khan et al., 2006).

Gray mold (*Botrytis cinerea*) is one of the major destructive diseases throughout the world which causes serious losses in many crops. The disease symptoms are observed under humid conditions and characterized commonly by gray, fuzzy sporulating lesions (Sutton, 1995; Jayaraj and Punja, 2007). Using fungicides to control gray mold is common, however became less accepted because of increasing the potential for the build-up of resistance in *B. cinerea* against fungicides.

Biological control strategies which have advantages over fungicides arose as effective method, although its efficacy differs according to the timing and the environmental conditions. Among the used strategies are transformation of technologies to improve crop resistance against *B. cinerea* (Punja and Raharjo, 1996; Jayaraj and Punja, 2007).

In the present study, the antimicrobial activity of Et-AMP1 and Et-AMP2 antimicrobial peptides against the fungal pathogen *Botrytis cinerea* was evaluated. Under favorable climatic and physiological conditions, it is capable of growing on all species of dicotyledons.

Transgenic Arabidopsis plants carrying Et-AMP1 and Et-AMP2 genes were obtained, thus indicating that transformation with plant defense genes can be an approach to protect crops against *B. cinerea* infection. The disease symptoms of *B. cinerea* (necrotic lesions and leaf yellowing to different degrees) started to appear three days after the inoculation. The lesion diameter was recorded 4 days after the inoculation. Expectedly, non-transgenic Col-0 (WT) plants showed the typical symptoms on all inoculated leaves (Fig. 12, 19). All Et-AMP1 and Et-AMP2 transgenic lines showed these typical symptoms except for the transgenic Et-AMP2 lines 1, 4 and 5, where the diameters of necrotic lesions were significantly ($P \leq 0.05$) lowest (Fig. 13). When *B. cinerea* grow on tissues of transgenic Arabidopsis plants expressing Et-AMP2 they develop fewer gray mold symptoms than those on inoculated control plant tissues (Fig.12 and 13). Interestingly, the Et-AMP2 transgenic lines 1, 5 and 4 were able to significantly reduce the necrotic lesion size caused by *B. cinerea*. This reduction of disease symptoms implies that Et-AMP2 can alter fungal pathogen maceration of plant tissues; in contrast to Et-AMP1 (Fig. 19 and 20), where no lines exhibited resistance against *B. cinerea*. This can be attributed to the short persistence of Et-AMP1 in transgenic plants because of the post-translational degradation by proteinases present in the intercellular fluid (Mills *et al.,* 1994; Owens and Heutte, 1997). Several previous studies aiming to improve plant disease resistance using AMPs from insects have shown that the transgenic plants failed to show enhanced resistance expected from *in vitro* assays. For example, initial experiments to express cecropin in tobacco to enhance resistance against *P. syringae* pv. *tabaci* were little successful (Hightower *et al.,* 1994).

As a necrotrophic pathogen *B. cinerea* is known to induce a lesion response in the infected plant tissues, promoting host cell death at early stages of infection (Elad, 1997; Prins *et al.,* 2000; Govrin and Levine, 2002). Cell death caused by *B. cinerea* is largely attributed to the accumulation of reactive oxygen species (ROS) (Govrin and Levin, 2000; Colmenares *et al.,* 2002). Increasing ROS within the plant cells upon infection would result into oxidative destruction of these antimicrobial peptides or modifying them to inactive forms (Florack *et al.,* 1995), which in turn, lead to reduce their levels in the plant tissues.

In the same context, Langen *et al.* (2006) tested antifungal activities of gallerimycin *in planta*, for resistance against the fungal pathogens. Transgenic plants (tobacco cv. Xanthi-nc) developed necrotic lesions 3 days after inoculation in response to *Sclerotinia minor*. The transgenic plants showed a reduction of the lesion area by 73%. Gallerimycin-expressing tobacco was also more resistant to *E. cichoracearum*. By 8 days post inoculation, powdery mildew pustules were only rarely observed on leaves of 5-week-old plants of transgenic line 21L5, while non-transgenic plants were covered by fungal mycelium.

Furthermore, the effect of Mtk peptide in barley plants on necrotrophic Fusarium species was evaluated by Rahnamaeian *et al.* (2009), Mtk plants were infected with *F. graminearum* that causes root rot (FRR) and head blight (FHB). Some of the transgenic lines showed only a 10% cutback in biomass compared with 50% reduction in the wild-type (barley cv. Golden Promise, GP) plants. The seed remnant and root bases of infected GP displayed discoloration, a typical symptom of *F. graminearum* root rot, whereas the inoculated Mtk plants developed a non-symptomatic root system. In addition, they evaluated the effect of Mtk in barley plants on all Mtk-containing lines that showed a strong reduction (60% on average) in the frequency of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Bgh) colony as well as in colony size compared with GP, which shows the enhanced resistance of Mtk plants to powdery mildew.

Actually, transgenic plants expressing AMPs from different families demonstrated and improved resistance against *B. cinerea*. Reportedly*, Arabidopsis thaliana* plants expressing Human beta-defensin-2 (hBD-2) were more resistant against the broad-spectrum fungal pathogen *Botrytis cinerea* as compared to untransformed *A. thaliana* plant (Aerts *et al.,* 2007).

Schestibratov and Dolgov (2005) pointed out that the strawberry (*Fragaria ananassa*) engineered by thaumatin II was resistant against gray mold (*Botrytis cinerea*) by infection with a conidial suspension. Also, high levels of resistance against gray mold disease (*Botrytis cinerea*) were observed in transformed strawberry plants expressing the ch5B gene which encodes a chitinase from *Phaseolus vulgaris* (Vellicce *et al.,* 2006). As well, transgenic Chrysanthemum with a rice chitinase gene (RCC2) showed enhanced resistance against *Botrytis cinerea* (Takatsu *et al.,* 1999). Whereas, the pear fruit expressing polygalacturonase inhibitor protein (pPGIP) in tomato plants revealed that this inhibitor of fungal pathogen endopolygalacturonases (endo-PGs) affects disease development due to the accumulation of abundant heterologous protein in all tissues of transgenic pPGIP plants which was active in vegetative and fruit tissues as an inhibitor of endo-PGs from *Botrytis cinerea*. The growth of *B. cinerea* on ripe tomato fruit expressing pPGIP was minified, and tissue collapse was scaled down by as much as 15%, compared with nontransgenic fruit. Whilst, the lesions of macerated tissue in transgenic leaves decreased approximately by 25% due to the expression of pPGIP (Powell *et al.,* 2000).

Increased resistance against *Botrytis cinerea* was observed by the overexpression of a gene encoding cysteine-rich antimicrobial protein (Ace-Amp-1) from onion in geranium (*Geranium cinereum*, Bi *et al.,* 1999). Also, the expression of a hen egg white lysozyme (HEWL) gene in transgenic potato and tobacco plants exhibited antimicrobial activity towards several bacteria and chitin containing fungi such as *Botrytis cinerea* (Trudel *et al.,*1995). While Khan *et al.* (2006) used detached leaves from non-transformed control and transgenic potato plants (*Solanum tuberosum* L.) harboring the wasabi defensin gene (isolated from *Wasabia japonica* L.) for antifungal assay, Khan *et al.* (2006) showed that transgenic plants were partially resistant to the fungal pathogen, *B. cinerea*. Furthermore, an overexpression of a stilbene synthesize gene from grape in transgenic tobacco plants exhibits resistance against *Botrytis cinerea* (Hain *et al.,* 1993; Fischer and Hain, 1994). Additionally, the expression of the rice chitinase gene in cucumber boosted the resistance against *B.cinerea* (Yin *et al.,* 2005). Indeed, overexpression of the rice chitinase cDNA (RCC2) gene in the cucumber-T1 progeny improved the resistance against gray mold (*B.cinerea*, Tabei *et al.,* 1998).

Punja and Raharjo (1996) showed that upon the inoculation of cucumber cv. Endeavor with *Alternaria cucumerina*, *Botrytis cinerea*, *Sclerotium rolfsii*, and *Rhizoctonia solani*, the rate and final extent of lesion development after 7 days were significantly lower in the transgenic plants expressing the tobacco (basic) chitinase gene. Besides, the symptoms of *Botrytis cinerea* were reduced in transgenic cucumber plants (CC2) expressing an endogenous class III chitinase gene (CHI2) and the class I chitinase cDNA (RCC2) of rice (Kishimoto *et al.,* 2002, 2004).

Moreover, transgenic carrot plants engineered with the microbial factor 3 (MF3) gene from a plant-growth promoting rhizobacteria *Pseudomonas fluorescence* showed significantly enhanced resistance against *Alternaria dauci*, *Alternaria radicina* and *Botrytis cinerea*, on average by 20–40%, in comparison to the non-transformed control plants (Baranski *et al.,* 2007).

Heliomicin and drosomycin expressed in transgenic tobacco conferred a minor but statistically significant enhanced resistance against the fungal pathogen *Cercospora nicotianae* (Banzet *et al.,* 2002).

4.4.2 Use of transgenic plants as a tool to enhance plant resistance against bacteria

As to bacterial pathogens, the study of genes, mechanisms of pathogenesis, natural or induced plant resistance, and parallel work with antibacterial proteins from various sources, have supplied a foundation for implementing a range of molecular strategies to introduce novel forms of transgenic resistance in plants. These approaches fall in three basic categories according to Panopoulos *et al.* (1996):

1- Incorporation of pathogen-derived genes for resistance against bacterial phytotoxins.

2- Introduction of bacterial avirulence genes.

3- Expression of antibacterial proteins from plants, insects, or bacteriophages as bactericidal or bacteriolytic agents.

Transformation of crop plants with a specific gene has been a general approach for obtaining transgenic plants with disease resistance against pathogens (Beachy *et al.,* 1990; Broglie *et al.,* 1991; Meeusen *et al.,* 1989).

Anzai *et al.* (1989) developed the first engineered bacterial resistance of tobacco against *P. syringae* pv. *tabaci.* Since then, several transgenic approaches have been developed that display either partial or complete resistance against plant pathogenic Erwinia, Pseudomonas, Ralstonia, or Xanthomonas spp. (Mourgues *et al.,* 1998).

In our study, transgenic Arabidopsis plants that carried Et-AMP1 and Et-AMP2 were infected with highly virulent Gram-negative bacteria *Pseudomonas syringae* pv. *tomato* DC3000. The disease symptoms of *P.syringae* were small necrotic lesions in areas of inoculation. These lesions were shown on the leaves of non-transgenic Col-0 (WT) plants, transgenic plants 4 days after infection, but the symptoms were very slight and were not accompanied by chlorosis with the transgenic Et-AMP2 lines, especially lines number 1 and 2. The transgenic Et-AMP1 lines exhibited typical necrotic lesions with chlorosis spreading out from lesions in the areas of inoculation on the leaves of non-transgenic Col-0 (WT) plants and transgenic plants, however, the symptoms were less on the leaves of lines 5, 2, 3 and 7. The transgenic Arabidopsis plants expressing Et-AMP1, especially lines number 5, 3, 7 and 2, were resistant, where the expression of ET-AMP1 in transgenic Arabidopsis plants resulted into a significant reduction in the C.F.U. In contrast, the expression of ET-AMP1 in transgenic lines number 6 and 8 increased the C.F.U (Fig. 21, 22 and 23). Also, the transgenic Arabidopsis plants expressing Et-AMP2, especially lines number 1, 2 and 5 (Fig. 16, 17 and 18), were more resistant compared to nontransgenic control plant. Therefore, Et-AMP1 and Et-AMP2 can contribute to a resistance against *P. syringae* infection in Arabidopsis plants.

Plant resistance against *P. syringae* pv. *tabaci* infection was enhanced by constitutive expression of GbERF in transgenic tobacco because GbERF mediates the expression of a wide array of pathogen-related (PR) and ethylene-responsive genes and plays an important role in the plant's response to biotic stress (Qin *et al.,* 2006).

It has been reported by Huang *et al.* (1997) that a significantly higher density of necrotic lesions was observed on leaves of control plants as compared with MB39-tobacco transgenic plants infected with *P. syringae* pv. *tabaci*. Furthermore, the transgenic tobacco plants overexpressing Sarcotoxin IA (bactericidal peptide) exhibited high resistance against several phytopathogens such as the bacterial soft rot disease (*Erwinia carotovora* subsp. *carotovora*), and the wild fire disease (*P. syringae* pv. *tabaci*) was observed in transgenic tobacco plants which overexpress the Sarcotoxin IA (Ohshima *et al.,* 1999).

Furthermore, Norelli *et al.* (1994) and Jaynes *et al.* (1993) succeeded in improving the resistance of transgenic tobacco plants overexpressing Sarcotoxin IA against bacterial soft rot disease (*Erwinia carotovora* subsp. *carotovora*) and wild fire disease (*P. syringae* pv. *tabaci*) by exploiting the wound inducible promoter for protease inhibitor II (PI-II) instead of the CaMV35S promoter to overproduce attacin E, an antibacterial peptide derived from the cecropia moth, and Shiva-1, which is an analog of cecropin B altered by protein engineering, respectively.

Tobacco leaf-disc explant tissue was resistant to *P. syringae* pv. *tabaci* when having been transformed with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid pARK21, which contains NPTII gene and tabtoxin resistance gene (*ttr*, Batchvarova *et al.,* 1998).

Transgenic tobacco plants expressing a rice OsLOL2 gene, orthologous to LSD1 of Arabidopsis (LSD1-related proteins of Arabidopsis with LSD1-like zinc finger domains regulating disease resistance and programmed cell death (PCD) exhibited enhanced disease resistance against a virulent bacterium *P. syringae* pv. *tabaci* (Pst, Bhatti *et al.,* 2008).

On the other hand, several studies failed to ameliorate plant disease resistance in the transgenic plants using AMPs from insects. For example, the expression of cecropin in tobacco to enhance resistance against *P. syringae* pv. *tabaci* were hardly successful (Hightower *et al.,* 1994). That has been attributed to the short persistence of cecropin in transgenic plants because of post-translational degradation by proteinases in the intracellular fluid (Mills *et al.,* 1994; Owens and Heutte, 1997).

4.5 Antimicrobial peptides expression in transgenic plants

Antimicrobial peptides are increasingly highlighted as effective inhibitors of plant microbial pathogens for enhancement of plants resistance against pathogens, especially insect origin peptides which have a sparkling history of sheltering their hosts sturdily against different pathogens. Accessibility of synthetic analogues of cecropins and other antimicrobial peptides facilitated the AMPs ectopic expression in plants to develop their fitness in biotic stress circumstances (Jaynes *et al.,* 1987).

A massive amount of gene construction with AMPs coding sequences transgenically expressed *in planta* resulted into various degrees of protection against fungal and bacterial pathogens (Mills *et al.,* 1994; Owens and Heutte, 1997).

Some reports revealed the resistance degree is not dependent on the AMP production level in transgenic plants (Balconi *et al.,* 2007), whereas others observed that resistance granted by AMPs to plants is associated with the level of active peptide produced in transgenic individuals (Chen *et al.,* 2006; Patkar *et al.,* 2006; Aerts *et al.,* 2007). Moreover, Hoffmann (1995) and McCafferty *et al.* (1999) pointed out the significance of synergism. Defensins and linear peptides work in synergy depending on *in vivo* data on functions of different AMPs (Ong *et al.,* 2002).

4.6 AMPs from insects

Insects produce a set of bactericidal or bacteriostatic peptides or polypeptides as response to bacterial injury or challenge, that show a broad of activity against gram negative and/or gram positive bacteria (Boman *et al.,* 1991; Hoffmann and Hetru, 1992).

We tested the Et-AMP1 recombinant protein against *Fusarium culmorum* conidia germination and hyphal growth (as in table 3 and fig.11), but its influence was limeted because the purified protein concentration was low (0.9 µM), so the growth inhibition ratio was about 10.5 % indicating a weak effect on fungi as we found out later with *in vivo* experiments.

Insect defensins have been expressed in plenty of plants. Expression of attacin from lepidoptera contributed to resistance against fire blight on apples (Ko *et al.,* 2000) and pears (Reynoird *et al.,* 1999). Cecropin A-derived peptides granted the tobacco and tomato plants protection against *P. infestans* (Cavallarin *et al.,* 1998). The insect defensins drosomycin and heliomicin expressed in tobacco displayed tolerance against *B. cinerea* and *Saccharomyces cerevisiae* (Banzet *et al.,* 2002). Expression of *Gallerimycin* from *Galleria mellonella* sheltered tobacco plants from *Erysiphe cichoracearum* and *Sclerotinia minor* (Langen *et al.,* 2006). Arabidopsis plants possessing Cecropin *B* showed higher resistance against *P. syringae* pv. *tomato* (Oard and Enright, 2006). Howerever, the

production of transgenic resistant plants by insertion of genes encoding of AMPs to plants faces some restrictions, such as:

1- Leakage of this type of resistance after a while due to of resiliency of disease-causing microbes (Li *et al.,* 2006).

2- Modest enhanced resistance (Balconi *et al.,* 2007).

3- Incitation of infertility (Coca *et al.,* 2006).

4- Species or race-specificity of the peptides (Schlaich *et al.,* 2006).

Scientist suggested many approaches to diminish these imperfections in addition to heighten the antimicrobial potency of existing peptides.

Among these proposed methods:

1- Using AMPs with paralyzing effect on a broad spectrum of microorganisms and/or co-expression of a mixture of AMPs with diverse modes of mechanism that demotes the opportunity of resistance to drain attributable to the escape of microorganisms

2- Using a chloroplast genome as substitute nuclear genome for engineering crop plants genetically for disease resistance against reach high levels of expression and to prevent pollen-mediated escape of transgenes (DeGray *et al.,* 2001).

3- Diminishing potential infertility in transgenic plants by targeting the AMPs into endoplasmic reticulum as alternative to apoplastic space (Coca *et al.,* 2006).

4- Synthesizing or manipulating the peptides for base substitution/deletion and chimeric hybridization, subsequently enhancement of induced resistance in plants (Osusky *et al.,* 2005; DeGray *et al.,* 2001; Rajasakeran *et al.,* 2005; Yevtushenko *et al.,* 2005, 2007; Oard and Enright, 2006).

In spite of that, a group of low-molecular-mass inhibitors against microbial proteases accompanied by antimicrobial peptides are synthesized by insects (Vilcinskas and Götz, 1999), that have the ability to suppress the digestive action of proteolytic enzymes secreted form plant pathogenic fungi.

Accordingly, concurrent transmission/expression of insect antimicrobial peptides and their inhibitors of microbial proteases may avoid the selection of pathogens that disintegrate the defense peptides in transgenic plants.

In our study, the bioassays for disease resistance revealed that pathogens *B. cinerea* and *P. syringae* were relatively suppressed by Et-AMP2, Thus, some transgenic lines generated in the present study might provide a promising source for breeding programs aimed at

improving the resistance to diseases caused by *B. cinerea* and *P. syringae.* Whereas, the transgenic lines expressing Et-AMP1 did not suppress the pathogen *B. cinerea*, they could relatively suppress the pathogen *P. syringae*. Thus, they may provide a promising source for breeding programs aimed at improving the resistance to diseases caused by *P. syringae*, but not to *B. cinerea*.

Subsequently, we can suggest the following points to improve the ET-AMP1 and ET-AMP2:

Synthesizing of synthetic analogues and hybrids for these antimicrobial peptides to prevent degradation of them in transgenic plants. As well as increasing the activity of ET-AMP1 and ET-AMP2 by modification of the structure and charge by replacement or adding some amino acids to our peptides, especially Arginine and lysine (they possess positively charged R groups) and hydrophobic (Nonpolar) residues (such as Valine, Isoleucine and Leucine), which is essential for increasing the activity of ET-AMP1 and ET-AMP2 against bacterial membranes (Chan *et al.,* 2007). One of the key properties of AMPs is their ability to differentiate between foreign and host cells, which is defined by the peptide structure and target membrane properties (Papo and Shai, 2003). Importantly, eukaryotic and bacterial membranes have very different lipid compositions. The membrane of host cells comprises mainly phosphatidylcholine, sphingomyelin and cholesterol, whereas bacterial cells expose negatively charged phospholipids, phosphatidylglycerol, cardiolipin and lipopolysaccharides. The host cells has zwitterionic lipid, whereas bacterial cells have anionic lipids that are negatively charged (Sood and Domanov, 2007). Zorko *et al.* (2009) found that the peptide binds to the anionic but not to zwitterionic lipid vesicles and that the type of lipid mimetics significantly affects the peptide structure and may account for its antibacterial activity.

Antimicrobial peptides are under consideration as new substitutes for conventional pesticides and antibiotic, and there are many cases of successful use of antimicrobial peptides in agriculture and food industry that indicate a promising future for extensive application of these peptides (Keymanesh *et al.,* 2009). In addition, AMPs are less susceptible to the development of bacterial resistance because they act on the bacterial membrane causing multiple stresses through several targets (Pag *et al.,* 2008). Consequently, antimicrobial peptides and proteins have been proposed for potential use as

a strategy in agriculture (Casteels *et al.,* 1989; Jaynes *et al.,*1987 ; Montesinos, 2007 ; Rao, 1995 ; Van der Biezen, 2001).

Finally, some of the transgenic lines in our study seem to deserve further investigations to evaluate the potential of Et-AMP1 and Et-AMP2 *in planta* against other phytopathogens. In addition, further information about physiological and molecular characterization, in order to uncover the antimicrobial mode of action of these peptides is required.

5. Summary

The aim of the study was to detect the potency of the novel insect antimicrobial peptides Et-AMP1 and Et-AMP2 derived from drone fly *Eristalis tenax* to engineer disease resistance in the model plant *Arabidopsis thaliana* against the fungal pathogen grey mold *Botrytis cinerea* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000. For the first antimicrobial peptide (Et-AMP1), a protocol for the production of recombinant protein in *E. coli* expression system was established by inserting the sequence for mature Et-AMP1 peptide in frame downstream of the pEXp5-CT/Topo expression vector. The recombinant protein was evaluated *in vitro* as to its effects on the devastating phytopathogen *Fusarium culmorum* by using spore germination inhibition assays. Results of this assay exhibited a weak effect with an inhibition percentage about 10.5% against this plant pathogenic fungus with the maximum concentration of produced recombinant protein (0.9 µM).

Both antimicrobial peptides (Et-AMP1 and Et-AMP2) were introduced into Arabidopsis plants via Agrobacterium-mediated transformation. After the production of transgenic Arabidopsis plants, *in vitro* antifungal assays were carried out by using T3 plants for evaluating their resistance against the fungal pathogens *B. cinerea* and the bacterial pathogen *P. syringae*. Our results show that Et-AMP1 transgenic plants did not exhibit resistance against *B. cinerea*. In contrast, these plants were more resistant to *P. syringae,* especially lines 5, 3, 7 and 2. On the other hand, the Et-AMP2 transgenic plants were resistant both to *B. cinerea* and *P. syringae*. Lines 1, 5 and 4 reduced significantly the necrotic lesion size caused by *B. cinerea* pathogen. Lines 1, 2 and 5 were more resistant to *P. syringae* infection. Subsequently, further research is required to identify the potential of Et-AMP1 and Et-AMP2 for plant protection approaches.

5. Zusammenfassung

Diese Studie soll die Potenziale der neuartigen antimikrobiellen Peptide Et-AMP1 und Et-AMP2 von der Mistbiene *Eristalis tenax* untersuchen, um Resistenz gegen das pilzliche Pathogen *Botrytis cinerea* (Grauschimmel) und das bakterielle Pathogen *Pseudomonas syringae* pv. *tomato* Stamm DC3000 in der Modellpflanze *Arabidopsis thaliana* zu erzeugen. Zuerst wurde für das antimikrobielle Peptid Et-AMP1 ein Protokoll für die Produktion von rekombinanten Protein in dem *E. coli* Expressionssystem durch Insertion der Et-AMP1-Sequenz in den Expressionsvektor pEXp5-CT/Topo erstellt. Der Effekt dieses rekombinanten Proteins wurde *in vitro* gegen den phytopathogenen Pilz *Fusarium culmorum* in einem Sporenkeimungs-Hemmungstest evaluiert.

Die Ergebnisse dieses Tests zeigen eine schwache Hemmung (ca 10,5%) der Sporenkeimung durch 0,9 µM dieses Proteins.

Arabidopsis-Pflanzen wurden mit den Sequenzen beider antimikrobielle Peptide (Et-AMP1 und Et-AMP2) mittels *Agrobacterium tumefaciens* transformiert. Danach wurde mit den transgenen Pflanzen (T3-Generation) *in vivo*-Assays zur Ermittlung ihrer Resistenz gegen das Pilzpathogen *B. cinerea* und den bakteriellen Erreger *P. syringae* durchgeführt.

Et-AMP1 transgenen Pflanzen zeigten keine erhöhte Resistenz gegen *B. cinerea*, im Gegensatz dazu wurde eine deutliche Resistenzsteigerung gegen *P. syringae* vor allem in den transgenen Linien 5, 3, 7 und 2 festgestellt. Die Et-AMP2 transgenen Pflanzen waren resistenter gegen *B. cinerea* und *P. syringae* im Vergleich zu den Wildtyp-Pflanzen. Die transgenen Linien 1, 5 und 4 zeigten eine signifikante Verringerung der nekrotischen Läsionen, die von *B. cinerea* verursacht werden. Die Linien 1, 2 und 5 waren außerdem resistenter gegen *P. syringae*.

Weitere Untersuchungen sind erforderlich, um das Potenzial der Peptide Et-AMP1 und Et-AMP2 für Pflanzenschutzansätze zu ermitteln.

7. References

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Declaration

Hiermit erkläre ich, dass diese Arbeit selbstständig und ohne Benutzung anderer als der abgegebenen Quellen und Hilfsmittel verfasst habe. Alle Stellen der Arbeit, die wörtlich oder sinngemäß aus Veröffentlichungen oder aus anderen fremden Mitteilungen entnommen wurden, habe ich einzeln kenntlich gemacht.
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