Transgenic expression of antimicrobial peptides from insects as a tool for analysis of compatibility between plants and pathogens

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To my father in spirit whom I always remember, and my dear mother for her love and to my husband who helped me to finish this work and finally to my son Ziad that I wish him a good future.

Contents

List of Abbreviations

Amp	Ampicillin
AMPs	Antimicrobial peptides
Avr	Avirulence
bp	base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
cv.	Cultivar
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
dNTP	Desoxyribonucleosidtriphosphat
dpi	day(s) post inoculation
EDTA	Ethylendiamintetraacetat
ET	Ethylene
et al.	and others
Et-Def	Eristalis defensin
Fig.	Figure
HR	Hypersensitive response
IPAZ	Institute of Phytopathology and Applied Zoology
IPTG	Isopropyl-β-D-thiogalactopyranoside
JA	Jasmonic acid
kDa	Kilo Dalton
L	Liter
M	Molar
MAMP	Microbe-associated molecular pattern
MIC	Minimum inhibition concentration
min	Minute(n)
mRNA	messenger-RNA
ORF	Open reading frame
PAGE	Polyacrylamid gelelektrophorese
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PR	Pathogenesis related
Pst	*Pseudomonas syringae* pv. *Tomato* strain DC3000
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative Real-Time PCR
R-gene	Resistance gene
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR
SA	Salicylic acid
SAR	Systemic acquired resistance

SIR	Systemic induced resistance
Tab.	Table
Taq	Thermus aquaticus
Tris	Tris-(hydroxymethyl)-aminomethan
UV	Ultraviolett
Wt	Wildtyp

1 Introduction

Plants are constantly threatened with a variety of pathogenic microorganisms present in their environments. Worldwide, plant diseases caused by pathogens, including bacteria, fungi, and viruses, contribute to severe loss in crop yield, amounting to 30 – 50 billion dollars annually (Strange and Scott, 2005; Savary *et al*., 2006; Montesinos, 2007). Plant diseases have been the cause of many infamous tragedies in the human history, such as the 1840s Irish potato famine (Agrios, 2005). Consolidated efforts using sustainable agriculture practices, conventional breeding and application of effective microbicidal components are not sufficient or permanently successful in keeping pathogens and pests under control (Moffat, 2001). Although conventional breeding is a major contributor to the production of disease resistant plants, it has some constrains due to interspecific sexual incompatibility, the lack of a desired gene pool in donor species and the time consuming back-crossings due to linkage drag. Meanwhile, the resulting extensively use of agrochemicals in agriculture leads to severe and long-term environmental pollution, since they are toxic, and sometimes even carcinogenic (Daoubi *et al*., 2005). Besides, several pathogens became resistant to many of these chemicals (Russell, 1995; Daoubi *et al.*, 2005). Under these circumstances, tuning of plant defense responses to pathogens for rendering them disease-resistant became an alternative strategy in sustainable agriculture (Kogel and Langen, 2005). In recent years, transgenic expression of genes encoding the so-called antimicrobial peptides (AMPs) could help to enhance resistance against a wide range of phytopathogens (Hancock and Lehrer 1998; Zasloff, 2002; Vilcinskas and Gross, 2005).

1.1 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) have been the object of attention in past years as candidates for plant protection products. AMPs form a heterogeneous class of low molecular weight proteins, being found in the whole living kingdom (Garcia-Olmedo *et al*., 1998; Hancock and Lehrer, 1998; Lehrer and Ganz, 1999). They are multi potent components of the innate defense mechanisms that host organisms have developed to combat assaulting pathogens (Zasloff, 2002; Castro and Fontes, 2005).

Since the discovery of cecropins in the pupae of silkmoth (Steiner *et al*., 1981), a wide repertoire of such molecules were isolated and purified from diverse life forms (Broekaert *et al*., 1997; Schumann *et al*., 2003; Thevissen *et al*., 2007; Aerts *et al*., 2008, Altincicek and Vilcinskas, 2007), and many new ones are being discovered each year. This suggests an important role for these peptides in immunity. Most of these peptides are produced as a prepropeptide consisting of an N-terminal signal sequence (which aids in targeting to endoplasmic reticulum), a pro segment and a C-terminal cationic peptide that demonstrates antimicrobial activity after it is cleaved from the rest of the protein (Bals, 2000). Regardless of their origin, all these molecules are short sequence peptides (usually less than 50 amino acid residues), and polycationic (i.e. contain excess lysine and arginine residues).

Some AMPs exhibit selectivity against different microorganisms, which molecular basis is not completely understood. On the one hand, many AMPs display broad-spectrum activity against Gram-negative, Gram-positive bacteria, and fungi (Miyasaki and Lehrer, 1998). On the other hand, some AMPs, e.g. andropin (Samakovlis *et al*., 1991) and most insect defensins (Meister *et al*., 1997) preferentially eradicate Gram-positive bacteria, while others preferentially kill Gram-negative bacteria, e.g. apidaecin (Casteels and Tempst, 1994), drosocin (Bulet *et al*., 1996), and cecropin (Boman *et al*., 1991). Peptides that preferentially eradicate filamentous fungi (Meister *et al*., 1997; Tailor *et al*., 1997; Langen *et al*., 2003; Rahnamaeian *et al*., 2009), and even protozoa (Arrighi *et al*., 2002).

Considerable attempts have been promoted to express AMPs in plants, with encouraging results on engineering either specific or broad-spectrum disease resistance in tobacco (Jaynes *et al*., 1993; Huang *et al*., 1997; DeGray *et al*., 2001; Langen *et al*., 2006), potato (Gao *et al*., 2000; Osusky *et al*., 2000), rice (Sharma *et al*., 2000; Imamura *et al*., 2009), banana (Chakrabarti *et al*., 2003), hybrid poplar (Mentag *et al*., 2003) and barley (Rahnamaeian *et al*., 2009). Thus, it seems reasonably to predict that genetic engineering using AMPs would represent a powerful tool for developing disease-resistant crop plants (Vilcinskas and Gross, 2005; Coca *et al*., 2006).

1.2 AMPs from insects

With roughly one million characterized species, insects represent the largest class within the animal kingdom. Their enormous colonization success and diversity certainly caused by: (i) their short life spans, (ii) their ability to colonize new niches and to feed on nearly all species of plants and animals and (iii) their capacity to mount a high immune response (Labandeira and Sepkoski, 1993; Bulet and Stöcklin, 2005).

Studying of insect immune defense reactions has attracted great attention during recent decades and revealed alternative antimicrobial strategies. Whereas insect immune defense relies solely on innate immunity (no memory), vertebrates innate immunity coexists with adaptive immunity (clonal) (Hoffmann *et al*., 1999). In insects with complete metamorphosis (holometabolous), AMPs are rapidly and transiently synthesized by the fat body (tissue corresponding to mammalian liver), and by hemolymph cells. When produced by the fat body, AMPs are secreted into the hemolymph, from where they can easily diffuse to act throughout the whole insect (Bulet *et al*., 2003). In contrast, in insects with incomplete metamorphosis (heterometabolous), AMPs are synthesized by hemocytes in the healthy insect and secreted into the hemolymph upon infection (Lamberty *et al*., 2001).

Since the isolation and characterization of the first inducible AMPs in the moth *Hyalophora cecropia*, more than 200 such peptides have been identified in several insect orders (Andreu and Rivas 1998; García-Olmedo *et al*., 1998; Ali and Reddy, 2000; Schumann *et al*., 2003; Altincicek and Vilcinskas, 2007).

Although insect AMPs share common features such as low molecular weight and positive net charge at physiological pH, their primary structure differ markedly. On the basis of their sequence and secondary structural features, insect AMPs are generally classified into three broad categories (Hertu *et al*., 1998; Bulet *et al*., 1999; Bulet and Stöcklin, 2005): (i) peptides usually characterized by abundant cysteine residues, (ii) linear peptides, devoid of cysteine residues and forming α-helices, and (iii) peptides with an overrepresentation in one or two particular amino acids, most frequently proline and / or glycine residues.

The largest and widely-distributed category comprises AMPs with an even number of cysteine residues. Consistent with their secondary structure in aqueous solutions or sequence homology, they can be briefly classified into three main groups: (i) peptides

containing an α -helix and two to four disulphide bonds connecting the helix to ß-strands (e. g., defensins) (Mygind *et al*., 2005; Selsted and Ouellette, 2005; Langen *et al*., 2006). (ii) peptides forming a hairpin-like ß-sheet structure (e. g., thanatin) (Mandard *et al*., 2002; Bulet *et al*., 2003), and (iii) peptides with a triple-stranded antiparallel ß-sheets (Barbault *et al*., 2003).

1.2.1 Insect defensins

Among cysteine-rich peptides, insect defensins constitute a large family of peptides that are widely distributed and account for most antimicrobial activity of hemolymph in several insect orders (Rees *et al.*, 1997; Hertu *et al*., 1998; Bulet *et al.*, 1999). They have been extensively investigated and frequently are at the focus for improvement of plant disease resistance (Thevissen *et al*., 2007).

The first insect defensins were independently isolated from cell cultures of the flesh fly, *Sarcophaga peregrina* (Matsuyama and Nafori, 1988) and from bacteria-challenged larvae of the black brown fly, *Phormia terranovae* (Lambert *et al*., 1989). Since then, more than 60 defensins have been isolated from insects belonging to different phylogenetically orders such as Diptera, Lepidoptera, Coleoptera, Hymenoptera, and Odonata (dragonfly) (Bulet and Stöcklin, 2005; Altincicek and Vilcinskas, 2007).

Generally, insect defensins are tiny small, highly basic, cysteine-rich molecule, mostly consist of 34 – 46 residues, with exception of the 51-residue defensins identified in bees (Dimopoulos *et al*., 1997). Structurally, all insect defensins are triplestranded peptides harbouring a consensus motif of six cysteine residues (Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6) involved in the formation of three disulfide bridges (Thevissen *et al*. 2004). Surprisingly, the three-dimensional structure of different defensin types from insect, plants and vertebrate implicated homology (Fehlbaum *et al*., 1994; Lamberty *et al*., 1999; Schuhmann *et al*., 2003), though sequence similarities were low and restricted to cysteine residues, suggesting that defensins are ancient molecules with a common ancestor that arose more than a billion years ago (Broekaert *et al*. 1995; Thomma *et al*. 2002; Aerts *et al*., 2008).

Apart from the structural homologies between defensins, there also seems to exist functional homology among them. Based on their *in vitro* activity, insect defensins can be classified in two sub-families: antibacterial defensins that preferentially eradicate

bacteria and antifungal defensins that are predominantly effective against filamentous fungi. Whereas defensins with antibacterial activities are extensively reported in the literature (Bulet and Stocklin, 2005), only few antifungal defensins such as defensin-like peptide drosomycin from fruit fly *Drosophila melanogaster* (Fehlbaum *et al*., 1994), heliomycin from Geranium / tobacco budworm *Heliothis virescens* (Lamberty *et al*., 1999), termicin from termite *Pseudocanthotermes spiniger* (Lamberty *et al*., 2001), and gallerimycin from greater wax moth *Galleria mellonella* larvae (Schuhmann *et al*., 2003) have been reported.

It has become evident from several reports that transgenic expression of AMPs from insect origin in higher plants led to an increase in host resistance to bacterial infections, whereas the resistance against fungal infections was less reported. For example, sarcotoxin from fruit fly expressed in tobacco conferred protection against *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* ssp. carotovora (Ohshima *et al*., 1999). The expression of the insect defensins heliomicin and drosomycin in tobacco mediated enhanced resistance against *B. cinerea* (Banzet *et al*., 2002). It was also observed that tobacco plants transformed with gallerimycin, an antifungal peptide from the greater wax moth *G. mellonella*, showed resistance to the fungal pathogens *Golovinomyces cichoracearum* and *Sclerotinia minor* (Langen *et al*., 2006). Recently, overexpression of metchnikowin from *Drosophila melanogaster* into barley plants resulted into enhanced resistance against *Blumeria graminis* and *Fusarium graminearum* (Rahnamaeian *et al*., 2009).

1.2.1.1 Eristalis defensin

Eristalis defensin (*Et*Def) (*syn.* Eristalin) is a novel promising antimicrobial peptide isolated recently from the rat-tailed maggots of the drone fly *Eristalis tenax* during innate immune response (Altincicek and Vilcinskas, 2007). *Et*Def was shown to comprise a predicted signal peptide and pro-sequence and shares sequence similarities to other insect defensins. Phylogenetic analysis using sequences of *Et*Def and other defensin sequences from dipterans indicated that defensins from *E. tenax, S. peregrina,* and *S. calcitrans* were more diverse in sequence (Altincicek and Vilcinskas, 2007). However, information about the antimicrobial activity of *Et*Def and its antimicrobial mode of action is lacking so far and still needs to be investigated.

1.2.2 Thanatin

Thanatin, a hairpin-like ß-sheet peptide, is the smallest (containing only 21 amino acid residues) inducible defence peptide, initially isolated from a hemipteran insect *Podisus maculiventris* (Fehlbaum *et al*., 1996). As has been reported by these authors, thanatin has no particular sequence homology with other insect AMPs, but has noticeable primary and secondary sequence similarities with brevinins, a family of antimicrobial peptides isolated from frog skin secretions. The three-dimensional structure of this peptide has been elucidated by Two-dimensional (2D) H-NMR spectroscopy and molecular modelling (Fehllbaum *et al*., 1996; Mandard *et al*., 1998; Taguchi *et al*., 2000). As has been described, thanatin has a well-defined, two stranded, ß-sheet structure, stabilized by the internal bridging of the two cysteine residues. It includes an N-terminal domain with a large structural variability linked to a well confirmed C-terminal cationic loop (named insect box as opposed to the Rana box). Insect box is delineated by the two cysteine residues and the hydrophilic residues localized at the two opposite sites. The central part is composed of hydrophobic residues that form a kind of belt around the core of the molecule (Fig. 1).

Interestingly, thanatin exhibits the largest antimicrobial spectrum observed so far, since it has potent activity against both Gram-positive and Gram-negative bacteria, filamentous fungi and yeast at physiological concentrations (Fehlbaum *et al*., 1996). Structure-activity relationship studies established that all-D-enantiomer is ineffective against Gram-negative bacteria, but exhibits the same level of activity as the natural L peptide on fungi (Fehlbaum *et al*., 1996). It has been, therefore, suggested that for killing different types of microorganisms, thanatin uses different mechanisms of action, involving a stereospecific interaction with a bacterial target (Fehlbaum *et al*., 1996). In addition, structure-function studies on a series of truncated versions of thanatin show that removing the C-terminal amino acid residue completely abolished the peptide effects against Gram-negative bacteria, as a result of architecture modification of the site that may be involved in the binding with an internal receptor (Mandard *et al*., 1998; 2002). Shin *et al*. (1999) found that a chimeric peptide (T-B1) with the brevinin-1 disulfide loop on the thanatin background elicited higher anti-Gram-positive bacterial activity than thanatin, but showed lower activity against the Gram-negative bacteria.

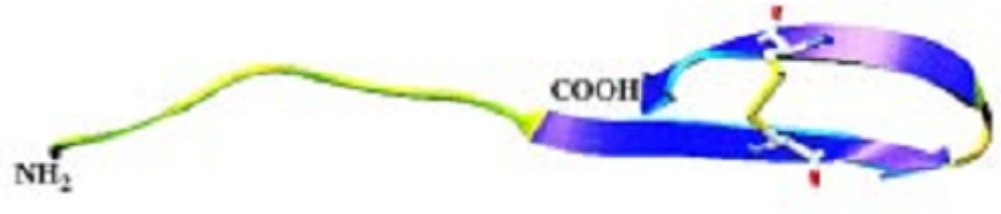


Fig. 1: 3-D structure of thanatin, based on the coordinates from the Brookhaven Protein Data Bank and drawn with Swiss PDB viewer program (Bulet *et al*., 1999).

To investigate the function of disulfide loop, Lee *et al*. (2002) synthesized thanatin with deletion or insertion of amino acid residue(s) between the cysteine residues and characterized the relationships between their structures and antibacterial activities. They found that increasing the number of amino acid(s) using alanine residue led to decrease the antibacterial activity in both Gram- negative and positive bacteria. In addition, thanatin with deletion of threonine at position 15 (Thr15) showed similar antibacterial activity against Gram-negative bacteria, but had higher activity against the Gram-positive bacteria (Lee *et al*., 2002).

The chemically modified thanatin with tertiary-butyl (tBu) group at Cys residues (Cys 11 and Cys 18) exhibited enhanced antimicrobial activity against a Gram-positive bacterium *M. luteus* (Imamura *et al*., 2008). By contrast, tBu-modified thanatin (tBu-Th), which fails to form a disulfide bond, lost its activity against *E. coli* (Imamura *et al*., 2008). Together, these suggest that thanatin has different mode of action depending on the target organisms, and that the disulfide bond is not essential for exhibition of antimicrobial activity against *M. luteus* (Imamura *et al*., 2008). Wu *et al*. (2008) reported that s-thanatin (which synthesized by substituting the amino acid of threonine with serine) exhibited a higher antimicrobial activity and less hemolysis toxicity. Furthermore, s-thanatin was found to display a superior performance on clinical isolates of *Klebsiella pneumoniae,* especially when combined with conventional antibiotics such as cefepime (Wu *et al*., 2009). Finally, Orikasa *et al*. (2009) designed a series of modified thanatins with methyl, ethyl, tBu and octyl groups and examined their

antimicrobial activities. Results of this investigation pointed out to a good correlation between the antimicrobial activity and the hydrophobicity of the side-chain of the cysteine residue.

Owing to its unique spectrum of activity, the expression of thanatin in plants seems to be promising to confer disease protection against a wide range of bacterial and fungal pathogens. Unfortunately, information related to the functional expression of thanatin in plants is still scarcely so far. However, analogues of synthetic thanatin gene have been expressed in rice plants and acquired a sufficient level of resistance against the rice blast fungus, *Magnaporthe oryzae* (Imamura *et al*., 2009).

1.3 Mode of action of AMPs

Although, *in vitro* antimicrobial activities of several AMPs have been characterized, the molecular basis of the mode of their antimicrobial action is still a matter of debate (Otvos, 2002; Shai, 2002; Li *et al*., 2006; Aerts *et al*., 2008).

As previously mentioned, most insect defensins identified to date have antibacterial activity with particular efficacy against Gram-positive bacteria, which are inhibited at low concentrations (1–100 μg mL^{-1}). Gram-negative bacteria, yeast and filamentous fungi are less sensitive to insect defensins (Hoffman, 1995; Hertu *et al*., 1998; Bulet and stocklin, 2005; Aerts *et al*., 2008). This feature of insect defenisns is highly unusual, since all other peptide families are more active against Gram-negative than Gram-positive bacteria (Otvos, 2000).

Numerous studies conducted on defensins from different origin established that these peptides might interact with the plasma membrane of Gram-positive bacteria, leading to membrane permeabilization by either forming pores or blocking Ca^{2+} channels and, thus, mediating lytic effect (Boman *et al*., 1991; Hoffmann and Hetru, 1992; Cociancich *et al*., 1993; Brogden, 2005). Phormia defensin (from *Phormia terranovae*) has been shown to disrupt the permeability barrier of the cytoplasmic membrane of Gram-positive bacteria *Micrococcus luteus in vitro*, resulting into a decrease in cytoplasmic potassium, a partial depolarization of the inner membrane, a reduction in cytoplasmic ATP, and finally an inhibition of the respiration. However, the efficiency is strongly reduced when salt concentration is increased (Cociancich *et al*., 1993). Addition of divalent cations and a decrease in the membrane potential below a threshold of 110 mV

led to reduction in potassium loss. Patch-clamp experiments on giant liposomes supported the hypothesis that Phormia defensin influenced the permeabilization barrier through the formation of channels in the cytoplasmic membrane of *M. luteus* (Cociancich *et al*., 1993).

To date, only few insect antifungal defensins i. e., termicin, drosomycin, heliomicin and gallerimycin have been reported (Fehlbaum *et al*., 1994; Lamberty *et al*., 1999; Lamberty *et al*., 2001; Schuhmann *et al*., 2003). Previous studies revealed that drosomycin at high concentrations (10 µM and above) inhibited completely the spore germination of *Neurospora crassa*, and *Botrytis cinerea*, while low drosomycin concentrations delayed the growth of hyphae, leading thereby to reduction of hyphal elongation with a concomitant increase in hyphal branching (Fehlbaum *et al*., 1994). In addition, exposure of *B. cinerea* to low drosomycin concentrations (1.2 µM) caused a partial lysis of the growing hyphae, resulting into extrusion of cytoplasmic material from the growing hyphae. This effect was, however, much more pronounced in the presence of divalent cations such as Ca^{2+} (Broekaert *et al*., 1997). Lamberty *et al*. (2001) found that termicin at concentration of 100 µM induced several morphologic distortions of *Aspergillus fumigatus* hyphae. At this concentration, termicin led to perforate the hyphal cell wall, with occasionally local leakage of cytosolic material. However, this peptide concentration was not sufficient to inhibit spore germination of this fungus. The exact mechanisms underlying antibacterial and / or antifungal activities exerted by insect defensins are not known, but there is evidence that these peptides strictly function through membrane permeabilization of microorganisms (Broekaert *et al*., 1995; Thevissen *et al*., 1999; Brogden, 2005). While most cationic AMPs are extremely varied regarding their primary and secondary structures, they share two unique features, namely a positive net charge under physiological conditions and they assume amphipathic structures with both a hydrophobic and a hydrophilic domains (Reddy *et al*., 2004; Brogden, 2005). These characteristics underlay the biological activities of AMPs. On one hand, the positively charged domains are proposed to initiate an electrostatical interaction between AMPs and the negatively charged LPS in the outer leaflet of the outer membrane of Gram-negative bacteria. This facilitates the formation of destabilized areas through which the peptide translocates the outer membrane in a process termed self-promoted uptake (Hancock, 1997; Bulet *et al*., 1999;

Otvos, 2000; Jenssen *et al*., 2006). On the other hand, the amphipathic nature enables the AMPs to interact directly with the lipid components of the membrane, and eventually, lead to insertion into the membrane interior (Otvos, 2002; Jenssen *et al*., 2006).

For some plant defensins, it was shown that they could interact with plasma membrane, inducing membrane permeabilization through specific interaction with high affinity binding sites (sphingolipids) on the fungal cells (Thevissen *et al*., 1997; 2000a; 2003; 2004). For example, plant defensin RsAFP2 from *Raphanus sativus*, with sequence similarities to heliomycin was found to interact and bind specifically with glucosylceramide (GlcCer) in *Pichia pastoris* and *Candida albicans*. In adddition, DmAMP1, a defensin from *Dahlia merckii*, could interact and bind specifically with mannosyldiinositolphosphorylceramide in the outer plasma membranes of yeast (Thevissen *et al*., 2000b; 2003; 2004; 2005), leading to a broad-spectrum *in vitro* antifungal activity (Osborn *et al*., 1995; Thomma *et al*., 2002). According to Thevissen *et al*. (2004; 2005), this interaction by itself is not sufficient, though it is necessary to induce fungal growth arrest.

Once AMPs gain an access to the membrane, they either interact with lipid components of the membrane (membrane-disruptive peptides) or translocate into the cytoplasm to act with cytoplasmic targets (non membrane-disruptive peptides) (Bulet *et al*., 2004; Reddy *et al*., 2004; Brogden, 2005; Jenssen *et al*., 2006). Membrane-disruptive peptides are generally reported to be of the α–helical structural class, although several α–helical peptides such as buforin (Park *et al*., 1998), CP10A (Friedrich *et al*., 2001), and pleurocidin analogue (Patrzykat *et al*., 2002) are not membrane-disruptive.

Three prominent models have been proposed to explain membrane disruption and pore-formation, namely: "Barrel-stave", "micellar aggregate", and "carpet model" (Shai, 1999; Bechinger *et al*., 1999; Brogden, 2005). In the barrel-stave model, the peptides reorient perpendicular to the membrane and align in a manner in which the hydrophobic sidechains face outwards into the lipid environment whereas the polar sidechain align inward to form transmembrane pore (Ehrenstein and Lecar, 1977; Yang *et al*., 2001; Brogden, 2005). This model is postulated for alamethicin (North *et al*., 1995). In the alternative micellar aggregate model, it is suggested that peptides reorient and associate in an informal membrane-spanning micellar or aggregate-like arrangement, inducing the

lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups (Matsuzaki *et al*., 1997; Hancock and Chapple, 1999; Brogden, 2005). This pore-forming mechanism is thought to be the mode of action for peotegrins, meltittin, mastoparan X, magainin, and LL-37 (Matsuzaki *et al*., 1996; 1998; Wildman *et al*., 2003). In the so-called carpet model, the peptides align parallel to the bilayer. They are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the membrane surface in a carpet-like manner. At sufficiently high concentration, this would lead to local disturbance in the membrane stability, causing the formation of large cracks, leakage of cytoplasmic components and disruption of the membrane potentials (Bechinger, 1999; Shai, 1999). This pore formation mechanism is symbolized in peptides like PGLa (Bechinger *et al*., 1999), cecropin A (Marassi *et al*., 1999), and ovispirin (Yamaguchi *et al*., 2001). Irrespective of which model is valid, the net result of membrane disruption would be the rapid depolarization of the membrane, leakage of cytoplasmic components and consequently rapid cell death (Friedrich *et al*., 1999; Powers and Hancock, 2003; Boland and Separovic, 2006), although membrane depolarization *per se* is not a lethal event (Powers and Hancock, 2003).

Each of the above mentioned pore-forming models might be correct depending on the experimental conditions and the peptide examined (Hallock *et al*., 2002; Powers and Hancock, 2003; Nomura *et al*., 2004). For example, the pore forming peptide LAH4 was found to operate through the carpet-like and transmembrane orientation at acidic and neutral pH, respectively (Bechinger, 1996). Even under the same experimental conditions, the antimicrobial peptide mastoparan possessed two different pore formation mechanisms simultaneously; 10 % transmembrane and 90 % carpet-like (Hori *et al*., 2001).

Recently, it has been shown that sub-inhibitory concentrations of cecropin A, classified as a lytic peptide, induce transcriptional changes within bacteria (Hong *et al*., 2003). Other studies have indicated that magainin 2 can translocate into the bacterial cytoplasm (Matsuzaki *et al*., 1995). These findings together suggest a role for these peptides in a non-membrane disruptive pathway (Park *et al*., 2000; Powers and Hancock, 2003; Jenssen *et al*., 2006). Several peptides are thought to translocate across the membrane through a process similar to the micellar-aggregate mechanism and accumulate

intracellularly, where they target a variety of essential cellular processes to mediate cell killing (Brogden, 2005; Jenssen *et al*., 2006; van der Weerden *et al*., 2008). Once present into the bacterial cytoplasm, these peptides are thought to target DNA, RNA, and cellular proteins, leading to inhibit the synthesis of these compounds (Lehrer *et al*., 1989; Yonezawa *et al*., 1992;; Futaki *et al*., 2001; Patrzykat *et al*., 2002). Membrane transition has been demonstrated for the frog-derived antimicrobial peptide buforin II. Though, it was found to cause large membrane perturbations in *E. coli*, the disruptions were transient and permeabilization did not occur (Park *et al*., 1998). Similarly, α-helical peptides like pleurocidin from fish, and dermaseptin from frog skin cause inhibition of DNA and RNA synthesis at their MICs without destabilizing the membrane *E. coli* cells (Subbalakshmi and Sitaram, 1998; Patrzykat *et al*., 2002). Several AMPs such as pleurocidin, dermaseptin and PR-39 have been found to inhibit protein synthesis (Bomann *et al*., 1993; Subbalakshmi and Sitaram, 1998; Friedrich *et al*., 2001; Patrzykat *et al*., 2002). Furthermore, specific enzymatic targets have been observed for certain peptides. The proline-rich insect antimicrobial peptide pyrrhocoricin has been shown to bind DnaK (heat shock protein) inhibiting chaperone-assisted protein folding (Otvos, 2002; Kragol *et al*., 2001). Some antimicrobial peptide such as the lantibiotic, mersacidin and nisin, have been found to bind lipid II, leading to the inhibition of peptidoglycan biosynthesis, affecting thereby cell wall synthesis (Brotz *et al*., 1998; Brumfitt *et al*., 2002; Kruszewska *et al*., 2004).

It is worth to mention that loss of viability caused by non-membrane disruptive peptides is much slower compared to membrane-acting peptides, which exert their antimicrobial effects within minutes (Giacomette *et al*., 1998; 1999). For example the ability of pyrrhocoricin to interfere with protein folding in living cells is not observed until 1 h after exposure (Kragol *et al*., 2001) and no observable cell lysis was detected as a result of mersacidin treatment even after 3 h (Brotz *et al*., 1998).

It is valubale to stress that the mechanism of action that individual peptide possesses differ due to the particular bacterial target cell, the concentration at which it is assayed, and the physiological properties of the interacting membrane. Additionally, in context of infection, AMPs may possess several mechanisms to exert their antimicrobial effect (Jenssen *et al*., 2006).

Although much progress has been achieved to unravel the antimicrobial mechanism of action of AMPs recently, reliable information on the putative antimicrobial mode of action of *Et*Def is very scarce in the literature so far.

Similarly, the mode of action of thanatin as antimicrobial peptide is not yet fully understood. However some reports point to a mode of action for thanatin which differs from that of insect defensins. Fehlbaum *et al*. (1996) reported that thanatin is not a pore-forming peptide in contrast to *Phormia* defensin. Additionally, Park *et al*. (1994) reported that unlike brevinins, thanatin don't seem to exert its antibiotic effect through disruption of the permeability of the bacterial membrane. However, a recent study by Pagès *et al*. (2003) on the activity of thanatin against multidrug resistant bacteria isolated from hospitalized patients (*Enterobacter aerogenes* and *Klebsiella pneumoniae*) evidenced that the accessibility of some structurally antibiotics to an internal target of a multidrug-resistant bacteria treated with thanatin is improved when the size of lipopolyssaccharide (LPS) is decreased. This suggests that thanatin may have induced an alteration of the outer membrane structure facilitating the penetration of antibiotics to a periplasmic target of bacteria (Pagès *et al*., 2003). No further information regarding the molecular mode of action of thanatin is currently available.

1.4 Production of recombinant AMPs through bacterial expression systems

AMPs are reported to be promising candidates for therapeutic and industrial application owing to their wide range of activity (Koczulla and Bals 2003; Reddy *et al*. 2004). The low yield of AMPs from their natural origin species and/or the high costs associated with the chemical synthesis of these peptides led to the exploration of an alternative DNA recombinant methods to permit sufficient production of AMPs in microorganisms such as bacterial, yeast or insect cells (Xu *et al*., 2007a; Ingham and Moore, 2007).

Prokaryotic cells of *E. coli* are normally the preferred host for the expression of foreign proteins because they offer (i) inexpensive carbon source requirements for growth, (ii) rapid biomass accumulation, (iii) amenability to high-cell density fermentation, and (iv) simple process scale up (Sahdev *et al*., 2007). *E. coli* has been used for the production of many antimicrobial peptides, e. g. lactoferricin (Kim *et al*., 2006), dermicin (Cipakova *et al*., 2006), defensins (Xu *et al*., 2006) and buforin (Lee *et al*., 1998). This biological expression system is also suitable to obtain uniformly or partially isotopically

enriched peptides, which are required for structural investigations of the ligand–receptor interaction by NMR spectroscopy and provides additional information on molecular dynamics, improvement of the precision of the determined structures and filtered experiments in the complex systems (Majerle *et al*., 2000; Mac *et al*., 2006). However, some technical obstacles encountered in expression of antimicrobial peptides in *E. coli*, such as the intrinsic antibacterial activity to *E. coli* and the susceptibility of peptide to proteolytic degradation (Piers *et al*., 1993; Makrides, 1996). Moreover, lack of post-translational machinery and the production of inactive protein due to the formation of inclusion bodies present a significant challenge in these expression systems. Expression systems with AMPs fused to partner proteins are most efficient due to the decreased toxicity against host cells, improved product stability and facilitated product recovery (Wei *et al*., 2005; Arnau *et al*. 2006; Zhou *et al*., 2009). Usually, such fusion proteins lack antimicrobial activity if they form insoluble products or interact with a carrier protein (Shen *et al*., 2007; Xu *et al*., 2007b). Nevertheless, a number of current protocols are available which describe various strategies for the conversion of inactive protein, expressed as insoluble inclusion bodies, into soluble and active fractions (Forrer and Jaussi 1998; Carrió *et al*,. 2000; Hoffmann *et al*., 2001).

LaVallie *et al.* (1993) reported a fusion expression system of thioredoxin (TrxA), and showed that a number of mammalian cytokines and growth factors, when expressed as C-terminal TrxA fusion proteins, stayed remarkably soluble in the *E. coli* cytoplasm under certain conditions. TrxA is known to be involved in a variety of cellular functions, including the reduction of protein disulfides, sulphate metabolism, as a cofactor for phage T7 DNA polymerase (Adler and Modrich, 1983) and in the assembly of T7 and filamentous phages (Huber *et al*., 1986, Russel and Model, 1986). This protein (TrxA) has been stably expressed at high levels in several expression systems, including the pET system (Invitrogen, Germany) and is extremely soluble in the *E. coli* cytoplasm (Lunn *et al*., 1984). In addition to its solubility, TrxA is small (109 aa; 11.675 kDa), has inherent thermal stability, and is localized onto the cytoplasmic membranes (Bayer, 1968). Apparently, the latter two features may be exploited for rapid purification (LaVallie *et al.*, 1993). Therefore, the use of TrxA as partner protein would, presumably, help to permit production of soluble functional heterologous protein in *E. coli*.

1.5 Plant-Pathogen-interaction

Plant disease resistance and susceptibility are regulated by the combined genotypes of host and pathogen and depend on a complex exchange of signals and responses occurring under given environmental conditions. In response to microbial attack, plants activate a complex series of responses that lead to the local and systemic induction of a broad-spectrum of antimicrobial defenses (Kunkel and Brooks, 2002; Kim and Martin, 2004). While some of these defense mechanisms are preformed to provide physical and chemical barriers (wax layers, rigid cell walls, antimicrobial enzymes, or secondary metabolites), preventing ingress of the pathogen, others are induced only after pathogen attack (i. e., the production of oxidative burst, and antimicrobial compounds) (Hammond-Kosack and Parker, 2003; Park, 2005).

Generally, resistance of an entire plant species to all isolates of a microbial species is referred to as non-host, species resistance or basal disease resistance (Thordal-Christensen, 2003; Mysore and Ryu, 2004; Nürnberger *et al*., 2004; Hückelhoven, 2007). It is believed that the non-host resistance relies on multiple protective mechanisms such as the production of pre-formed and/or inducible barriers against pathogens (Heath, 2000; Kamoun, 2001; Thordal-Christensen, 2003; Nürnberger *et al*., 2004). When a virulent pathogen manages to overcome constitutive defensive layers, it may become subject to recognition at the plasma membrane of plant cells. A huge number of microbe or pathogen-associated molecular patterns (MAMPs/PAMPs) have been shown to trigger receptor-mediated defense responses in non-host plants. MAMPs are structural, highly conserved microbial molecules, which are recognized by plant receptors and activate efficient innate immune responses by distinguishing between self and non-self molecules (Göhre and Robatzek, 2008; Schwessinger and Zipfel, 2008). MAMPs/PAMPs comprise bacterial flagellin, cold-shock proteins (CSPs), lipopolysaccharide (LPS), bacterial elongation factor-Tu (EF-Tu), fungal glucans, chitin, and oomycete elicitor INF1 (Kamoun *et al*., 1997; Nürnberger *et al*., 2004; Chisholm *et al*., 2006). Non host resistance may be attributed to preformed or inducible defense responses, but may also reflect lack of host compatibility or absence of pathogen virulence factors (Heath 2001; Li *et al*., 2005). Three *Arabidopsis* loci, designated *PEN1*, *PEN2* and *PEN3* were identified that are necessary for efficient cell wall penetration resistance against a non-host pathogen (*Blumeria graminis* f.sp. *hordei*)

(Nürnberger and Lipka, 2005, Jones and Dangl, 2006). During evolution, an inappropriate or non-host pathogen must become insensitive to or must suppress or fail to elicit basal defenses in order to cause disease on a new host (Göhre and Robatzek, 2008).

Selective pressure on host plants exerted by virulent pathogens results in the co-evolution of plant resistance (R) genes, which specifically recognize pathogen strain- or race-specific factors, and allow for the establishment of pathogen race/plant cultivar-specific disease resistance (Abramovitch and Martin, 2004; Chang *et al*., 2004; Jones and Takemoto, 2004). Genetically, this type of resistance is determined by complementary pairs of pathogen-encoded avirulence (avr) genes and plant resistance (R) genes, leading to the activation of defenses like the hypersensitive response (HR) (Gabriel and Rolfe, 1990; Prell and Day, 2000; Nimchuk *et al*., 2003; Kamoun, 2006). This gene-for-gene hypothesis was firstly introduced by Flor (1971), and multitude of R-Avr gene combinations have since been characterized (Dangl and Jones, 2001). R-mediated resistance can be activated through the recognition of effectors either by direct physical interaction (ligand-receptor model) between R and Avr proteins or via indirect perception of effectors by R proteins which have been described by the Guard hypothesis (Jia *et al*., 2000; Dangl and Jones, 2001). A recent modification of the Guard model was proposed by van der Hoorn and Kamoun (2008). In this model, known as the Decoy model, the guardee proteins are thought to function as decoy proteins with the exceptional role of mediating perception of the pathogen effector by the R protein. This model recognizes the opposing selective forces that operate on the guardee protein; on the one hand to escape interference by the pathogen effector and maintain its primary function, and on the other to enhance interaction with the effector to trigger effector-triggered immunity (ETI). This form of R-mediated disease resistance is effective against pathogens that can grow only on living host tissue (obligate biotrophs), or hemibiotrophic pathogens, but not against pathogens that kill host tissue during colonization (necrotrophs) (Glazebrook, 2005).

PAMP-induced non-host resistance as well as Avr-induced cultivar-specific resistance should be considered as two complementary elements of plant innate immunity (Espinosa and Alfano, 2004; Nürnberger *et al*., 2004; Jones and Dangl, 2006). According to Jones and Dangl (2006), the plant immune system can be described as a

four phased 'zigzag' model. In this model, plants recognize firstly the pathogen-associated molecular patterns (PAMPs) and as a response to it, PAMP-triggered immunity (PTI) is induced to stop further pathogen invasion. In a second step, well-adopted pathogens promote virulence by delivering effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). In a third step, direct or indirect perception of pathogen effectors by R proteins would lead to disease resistance, known as effector-triggered immunity (ETI). In a fourth step, pathogens exude another set of effector molecules to suppress ETI reestablishing ETS. Ultimately, the plant surveillance system regenerates new R-gene that recognizes these effectors in order to regain ETI.

In addition to basal or R-gene mediated resistance responses that act at the site of pathogen infection, plants are also able to develop a nonspecific systemic resistance that is effective against further pathogen attack. This phenomenon is known as induced resistance, and can be triggered by a variety of biotic and abiotic stimuli (Bostock, 2005). The classic example of an inducible plant defense response is systemic acquired resistance (SAR). It is principally triggered by a localized infection with necrotizing microbes and is manifested on the plant upon secondary challenge by otherwise virulent microbes (Grant and Lamb, 2006). The onset of SAR is characterized in many plants such as tobacco and Arabidopsis by local and systemic increases in endogenously synthesized salicylic acid (SA) and is tightly coupled with the transcriptional reprogramming of a battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Ryals *et al*., 1996; Maleck *et al*., 2000; Durrant and Dong, 2004; Wang *et al*., 2005). Non-expressor of pathogenesis-related genes-1(NPR1) is a key regulator of systemic acquired resistance (SAR) that is crucial for transducing the SA signal to activate pathogenesis-related (PR) gene expression (Vallad and Goodman, 2004). Induced systemic resistance (ISR) is another well known inducible plant defense response, activated by root-associated non-pathogenic bacteria (van Loon, 1997; Pieterse *et al*., 1998; Vallad and Goodman, 2004). Briefly, ISR depends on JA/ET pathways which operate through a SA-independent, but NPR1-dependent system and results consequently into the production of antimicrobial compounds (Pieterse *et al*., 1998; Van Loon *et al*., 1998). Interestingly, plants expressing both types of induced resistance have not shown to raise NPR1-transcript levels, indicating the constitutive

level of NPR1 is sufficient to facilitate expression of SAR and ISR (Pieterse and van Loon, 2004).

1.6 *Arabidopsis thaliana* as a model plant

A. thaliana is a small dicotyledonous species (Family *Brassicaceae*). It has been the focus of intense genetic, biochemical and physiological studies over the last decades because of several traits that make it very desirable for laboratory study. It is easy and cheap to grow and reproduce with relatively short life cycle. Compared to other plants, it is characterized by a small genome, genetically more tractable, high fecundity and ease of mutagenesis. Further, it exhibits the major kinds of defense responses described in other plants. In addition, a large number of virulent and avirulent bacterial, fungal, and viral pathogens of Arabidopsis have been collected. Therefore, it is proving to be an ideal model system to study the host defense responses to pathogen attack (Glazebrook *et al*., 1997; Felix *et al*., 1999; Navarro *et al*., 2006; Robatzek *et al*., 2006; Shen *et al*., 2007b).

1.6.1 Defenses against *Golovinomyces* ssp.

Powdery mildews are Ascomycete fungi (Erysiphales) that are able to colonize about 10,000 distinct plant species (Takamatsu, 2004). They are obligate biotrophic phytopathogens that exclusively feed on living epidermal cells and complete their asexual lifecycle on their host plant leaf surfaces by conidiospore formation. Four powdery mildew species are reportedly known to establish compatible interactions with *A. thaliana*: *Golovinomyces cichoracearum* (Adam and Somerville, 1996) and *G. orontii* (Plotnikova *et al.*, 1998), as well as *Oidium neolycopersici* (Bai *et al.*, 2008; Göllner *et al*., 2008) and *G. cruciferarum* (Koch and Slusarenko, 1990).

Although resistance to powdery mildews is generally conferred by dominantly or semi-dominantly inherited genes which provide race- or isolate specific protection against the fungal parasite, no true race-specific resistance genes against powdery mildew in *A. thaliana* have been yet identified (Göllner *et al*., 2008). This might be due to the fact that Arabidopsis powdery mildew pathosystem have developed relatively recently and didn't have time to mature the classical Avr/R gene pairs (Micali *et al*., 2008). However, the revelation of RPW8-based broad spectrum resistance in Arabidopsis may

have eliminated the evolutionary driving force for the acquisition of prototypic *R* genes conferring race-specific resistance (Xiao *et al*., 2001; Micali *et al*., 2008). The overexpression of *ADR1*, an Arabidopsis R-gene, conferred resistance to *G. cichoracearum*. Additionally, many examples on interactions between the closely related *Blumeria graminis* and barley have been also described (Thordal-Christensen *et al*., 1999; Schulze-Lefert and Vogel, 2000; Hückelhoven and Kogel, 2003). Together, this suggests that gene-for-gene resistance responses does exist in Arabidopsis-powdery mildew interactions and can be effective against these pathogens (Grant *et al*., 2003). Salicylic acid signaling may also play a role in Arabidopsis-powdery mildew interaction. It was shown that Arabidopsis plants bearing *pad4*, *eds5*, or *npr1* mutations displayed enhanced susceptibility to compatible *G. orontii* and *G. cichoracearum* (Reuber *et al*., 1998; Glazebrook, 2005). Clearly, this indicts that SA signaling components are crucial in limiting the growth of powdery mildews on Arabidopsis.

In addition to SA signaling, JA signaling pathway may contribute to powdery mildew resistance. However, this pathway seems not to be important in Arabidopsis, as *jar1* (Reuber *et al*., 1998) and *coi1* (Zimmerli *et al*., 2004) mutations have no effect on susceptibility to *G. orontii* or *G. cichoracearum*, respectively. This may be due to the fact that JA-dependent resistance mechanisms are not induced, rather than that they are ineffective. Indeed, *G. orontii* infection did not induce the JA- and ET-dependent gene *PDF1.2*, suggesting that JA signaling is not activated (Reuber *et al*., 1998).

1.6.2 Defenses against *B. cinerea*

The fungal pathogen *Botrytis cinerea* (necrotroph) is the causative agent of gray mold diseases. It attacks a wide variety of plant crops (more than 200 species), causes serious pre- and post harvest diseases particularly in greenhouse crops and ornamentals, leading to enormous economic losses (Jarvis, 1977; Williamson *et al*., 2007; Tudzynski and Kokkelink, 2009). Disease symptoms are characterized by gray sporulating lesions, commonly observed under humid conditions. These lesions produce masses of conidia which become airborne and are the primary means by which the fungus is spread (Agrios, 2005).

Because it is highly variable (various mode of attack, diverse hosts, and survival as mycelia, conidia or sclerotia), *B. cinerea* can rapidly evolved resistance against

fungicides (Williamson *et al*., 2007). Apparently, due to these reasons, the use of only one control method is unlikely to succeed. A precise understanding of host-pathogen interaction is therefore of particular importance in the control of *B. cinerea*. Though disease control of *B. cinerea* relies frequently on chemicals, consolidated efforts to develop biological control strategies are increasingly successful (Köhl *et al*., 1995; Elad, 1996).

Host defense reaction against *B. cinerea* has been studied in the model plant Arabidopsis. Similar to other necrotrophs, *B. cinerea* infection was found to induce mainly the JA and ET signaling pathways (Thomma *et al*., 2001; Williamson *et al*., 2007). It has been observed that Arabidopsis mutations that block JA signaling pathway such as *coi1* and *jar1* exhibited a partial, sometimes dramatic increase in susceptibility to *B. cinerea* (Thomma *et al*., 1998, 1999; Audenaert *et al*., 2002; Diaz *et al*., 2002; Ferrari *et al*., 2003;). Recent studies showed that the expression of some JA-responsive genes is controlled by the MYC transcription factor *JIN1* (Lorenzo *et al*., 2004), and plants bearing *jin1* mutations were more resistant against *B. cinerea*. Additionally, blocking of ET signaling caused by *ein2* resulted into enhanced susceptibility against *B. cinerea* (Thomma *et al*., 1999; Ferrari *et al*., 2003). Furthermore, overexpression of the transcription factor *ERF1* was found to increase resistance against *B. cinerea* (Berrocal-Lobo *et al*., 2002). It is likely, therefore, that genes play an important role in *B. cinerea* resistance, belong to a group co-regulated by JA and ET, and that *ERF1* activates many of these genes (Glazebrook, 2005).

B. cinerea infection is known to trigger an oxidative burst, both in the plant plasma membrane and in the cell wall of fungal hyphae, promoting thereby plant cell death (Govrin and Levine, 2000; Schouten *et al*., 2002; Tenberge, 2004). Govrin and Levine (2000) proposed that cell death induced by *B. cinerea* is a form of the HR, and that this induction of cell death is an important component of virulence. This is supported by the findings that Arabidopsis mutations that promoted cell death increased susceptibility, whereas those delayed cell death increased resistance against *B. cinerea* (Van Baarlen *et al*., 2007). Furthermore, the growth of *B. cinerea* in Arabidopsis was suppressed in the hypersensitive response defective mutant *dnd1* and was stimulated by hypersensitive response triggered by simultaneous inoculation with an avirulent bacterium (Govrin and Levine, 2000). Together, these indicate that induction of ROI and cell death is an

important determinant in the interaction of *B. cinerea* with its host plants and tolerance to ROI may contribute to resistance.

1.6.3 Defense mechanisms against *Pseudomonas syringae* pv. *tomato*

The bacterial pathogen *P. syringae* pv *tomato* strain DC3000 is often considered as biotroph, occasionally considered as necrotroph (Butt *et al*., 1998), and should probably be a hemi-biotroph (Thaler *et al*., 2004). It infects through wounds and stomata and multiplies in the intercellular spaces. In the early stages of compatible infections, host cell death does not occur, but later stages of infection are usually associated with host tissue chlorosis and necrosis (Buell *et al*., 2003). Many strains, including *Pst* DC3000 are known to cause bacterial speck disease on tomato and Arabidopsis and produce effectors that contribute to pathogenicity (Bender *et al*., 1999; Buell *et al*., 2003). These proteins are called type III effectors and are thought to contribute to virulence, especially in *Arabidopsis* (Alfano and Collmer, 2004; Espinosa and Alfano, 2004).

Reportedly, gene-for-gene resistance is highly effective in Arabidopsis-*P. syringae* interactions (Glazebrook, 2005; Nobuta and Meyers, 2005). It has been observed that the *avrRpt2- RPS2* (Dong *et al*., 1991; Whalen *et al*., 1991; Kunkel *et al*., 1993; Yu *et al*., 1993), *avrB-RPM1* (Bisgrove *et al*., 1994), *avrRpm1-RPM1* (Debener *et al*., 1991), *avrPphB-RPS5* (Simonich and Innes, 1995), and *avrRps4-RPS4* (Hinsch and Staskawicz, 1996) interactions exhibited remarkable reductions of bacterial titers in infected leaves by about 100-fold relative to the isogenic virulent strain *Pst* DC3000. Notably, the oxidative burst generated during gene-for-gene resistance does not seem to play a major role in limiting bacterial growth (Torres *et al*., 2002).

SA-dependent defense responses may be potentially significant in limiting the growth of *P. syringae*. Arabidopsis mutants possess defects in SA signaling, including *eds1* (Aarts *et al*., 1998), *pad4* (Zhou *et al*., 1998), *eds5* (Rogers and Ausubel, 1997), *sid2* (Nawrath and Métraux, 1999), and *npr1* (Glazebrook *et al*., 1996; Shah *et al*., 1997), showed enhanced susceptibility to virulent, and in some cases, avirulent bacterial strains. The observation that *npr1* does not have a defect in resistance to an avirulent *P. syringae* strain whereas *eds5* allows increased bacterial growth provided evidence for SA-dependent, *NPR1*-independent defense mechanisms that are active against *P. syringae* (Clarke *et al*., 2000). Plant treatment with exogenous SA or SA analogs was shown to

inhibit *P. syringae* growth, as did induction of SAR (Cao *et al*., 1994; Lawton *et al*., 1996). In addition, overexpression of WRKY70 increased the plant resistance against *Pst* DC3000 (Glazebrook, 2005).

Besides, recognition of bacterial flagellin mediated by the receptor-like kinase encoded by *FLS2* was found to play an important role in resistance to *Pseudomonas* (Zipfel *et al*., 2004). It activates a MAP kinase cascade that peaks in expression of the transcription factors WRKY22 and WRKY29 (Asai *et al*., 2002). As has been reported, plant treatment with a purified peptide derived from flagellin resulted in activation of a large number of R genes, though the relationships between flagellin-activated signaling, SA signaling, and JA signaling are not fully understood (Navarro *et al*., 2004; Zipfel *et al*., 2004).

1.7 Objectives of the present study

Effective and sustained control of phytopathogens that increasingly account for severe crop losses is one of the most important issues in modern agriculture. Over the last decades, it has become evident that expression of genes encoding AMPs from insects in transgenic plants represents a powerful tool for creating disease-resistant cultivars to a wide range of bacterial and fungal pathogens (Zasloff 2002; Vilcinskas and Gross, 2005; Coca *et al*., 2006). In this context, we reasoned that the expression of the insect antimicrobial peptide thanatin and the new putative peptide *Et*Def may have potentials to provide a broad-spectrum disease-resistance in crop plants. In order to validate this concept, the antimicrobial activities of the synthetic *Et*Def and thanatin peptides against some phytopathogens of agronomic interest such as *Fusarium culmorum*, *Botrytis cinerea* and *Phytophthora parasitica* were firstly *in vitro* assessed. Concurrently, it is attempted here to establish a novel efficient production and purification strategies to permit adequate production level of *Et*Def as recombinant protein in *E. coil* expression system, and to evaluate its *in vitro* activity as a novel antifungal compound. In this study, the questions are addressed whether the *Et*Def and thanatin genes could be functionally expressed in *A. thaliana* and whether expression of these peptides could confer resistant to the economically important fungal pathogens *G. orontii* and *B. cinerea*, and bacterial pathogen *P. syringae* in transgenic *A. thaliana* plants. Thus, transgenic Arabidopsis plants were generated by *Agrobacterium tumefaciens*-mediated

transformation using a construct encoding either *Et*Def or thanatin gene under the regulation of the constitutive CaMV 35S promoter. In order to allow both peptides to enter the secretory pathway of Arabidopsis cells, the coding sequences of complete ORF of both *Et*Def (including its predicted signal peptide) and thanatin peptide (fused to the sequence for the signal peptide of chitinase 26 from *Hordeum vulgare*) were designed for plant transformation. *Et*Def and thanatin transgenic lines were then molecularly characterized and their antimicrobial activities *in vitro* as well as *in planta* were evaluated.

2 Materials and Methods

2.1 Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia 0 (Col-0, N1092, obtained from the European Arabidopsis Stock Centre NASC, University of Nottingham, UK) was used to produce the Eristalis defensin (*Et*Def) and thanatin transgenic plants as well as vector transgenic plant (transgenic control).

Seeds of all transgenic Arabidopsis and wild type were first surface-sterilized with 3 % Sodiumhypochloride (NaClO) for 20 min at room temperature. They were then washed 3 times with sterile d.d water and were germinated on half-strength MS-medium (Murashige and Skoog, 1962) supplemented with 1.5 % sucrose, 0.4 % agar and with or without 30 mg L^{-1} hygromycin (Roche, Mannheim, Germany), respectively. To achieve synchronized germination, seeds were incubated firstly at 4 ºC for 24 h and 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 day / 18 °C night temperatures with 60 % relative humidity for 2 weeks. The plants were then transplanted into pots containing a soil mixture of 1:1 sand: soil Typ ED 73 (Einheitserde- und Humuswerke Gebr. Patzer GmbH+ Co.KG, Sinntal-Jossa, Germany). The plants were kept in a growth chamber under photoperiodic conditions of 8 h light, 22 °C day / 18 °C night temperature with 60 % relative humidity. Three to four weeks later, plants of uniform size were selected for pathogenicity studies.

2.2 Fungal and bacterial strains

In this study, *Botrytis cinerea*, *Fusarium culmorum* and *Phytophthora parasitica* were used for antifungal assays (*in vitro*). For *in vivo* assays, the fungal pathogens grey mold *B. cinerea* and powdery mildew *Golovinomyces orontii* 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* strain KF 350 (obtained from Prof. Chelkowski, Institute of Plant genetics, Poznan, Polen) was carried out as described (Broekaert *et al.* 1990). Fungus was grown on PDA (potato dextrose medium containing 15 g L^{-1} agar, Roth, Germany) for 10 days at room

temperature (RT). Fungal spore suspensions were prepared by flooding plates with 5 ml sterile d.d. water and scraping gently with a sterile loop. The resulting crude suspension was filtered through a layer of sterile cheesecloth to remove mycelial fragments. Inoculum concentration was estimated using a Fuchs-Rosenthal counting chamber (Roth, Germany) and then adjusted to 2×10^4 conidia mL^{-1}.

Phytophthora parasitica (obtained from Institute National de la Recherche Agronomique, France) was cultured on rye agar medium at 25 °C for 7 – 8 days. The sporangia germination bioassay was conducted according to the method of Ali and Reddy (2000). Sporangia were harvested from 4 weeks old cultures by rinsing the plates with 5 mL sterile distilled water. The sporangial suspension was then incubated at 4 °C for 4 h to induce the release of zoospores. The zoospores were 1:50 diluted in RPMI 1640 media (Sigma, Germany) and the concentration was adjusted to 2×10^4 zoospores mL^{-1}.

B. cinerea strain B05.10 (provided by Prof. M. Hahn, Kaiserslauten, Germany) 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).

Powdery mildew *G. orontii* (obtained from Ralph Panstruga, MPI Köln, Germany) was maintained on hyper-susceptible pad 4-1 Arabidopsis plants (Reuber *et al*., 1998) grown under the same conditions as described (see section 2.1).

Pseudomonas syringae pv *tomato* (*Pst*) strain DC3000 (virulent) (obtained from Dr. Schleich, RWTH Aachen, Germany) was grown at 28 °C on King's B medium (King *et al.*, 1954) supplemented with the appropriate antibiotics (50 mg mL^{-1} rifampicin).

2.3 *In vitro* antifungal assays

2.3.1 Synthetic peptides

Amino acid sequence of mature *Et*Def (ATCDLLSFLNVKDAACAAHCLA-KGYRGGYCDGRKVCNCRR) and thanatin (GSKKPVPIIYCNRRTGKCQRM) peptides were synthesized by GL Biochem Ltd (Shanghai, China) with more than 85 % purity. Lyophilized peptides were reconstituted in 1 mM ß-mercaptoethanol (ß-ME) to a stock concentration of 10 mM, and stored as 10 µL aliquots at -20 °C for further use.

2.3.2 *In vitro* antifungal activity of synthetic peptides

In vitro antifungal activity of both synthetic *Et*Def and thanatin was evaluated against fungal pathognes *F. culmorum* and *B. cinerea* by determining the number of germinated spores in the presence of the peptides. Additionallly, the cytotoxic activity of both peptides was determined on the mycelium of *P. parasitica* using MTT- assay according to Meletiadis *et al*. (2000).

2.3.2.1 Spore germination assay

To evaluate spore germination, spore suspension (2×10^4 conidia mL^{-1}) of *F. culmorum and B. cinerea* were prepared as described above in section 2.2. Spore suspensions were incubated with different concentrations of each synthetic peptide at RT. ß-ME control was tested at the same concentrations as in the peptide dilutions except that the peptide was omitted. The number of germinating spores was counted and the percentage inhibition was calculated for each concentration. Germ tube morphology was also examined microscopically using an inverted light microscope (Olympus, Japan) and photographed with a digital camera attached to the photoport of the microscope. The experiment was repeated twice with at least three replications of each concentration.

2.3.2.2 MTT method

To examine the effect of the synthetic peptides on the viability of *P. parasitica* cells, MTT colorimetric assay was conducted. This method based on the reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by reducing enzymes, e.g. mitochondrial dehydrogenases, in metabolically active cells to a blue formazan, which can be measured spectrophotometrically. Zoospores were incubated at RT in microtiter plate at 100 µL of final volume in the presence or absence of the each synthetic peptide (0.5, 1, 2, 5, and 10 µM). After the MICs (minimal inhibitory concentration) were visually determined for each peptide, 20 µL of MTT (Sigma Chemical, St. Louis, USA) at concentration of 5 mg mL^{-1} was added to each well. Incubation was continued at 37 °C for 3 h. The content of each well was removed and 200 µL of isopropanol containing 5 % 1 M HCl was added to extract the dye. After 30 min of incubation at room temperature and gentle agitation, the optical density (OD) was measured with a microtitration plate spectrophotometer (Tecan Deutschland

GmbH, Crailsheim, Germany) at 595 nm. The fungal growth inhibition depending on the percentage of MTT conversion to its formazan for each well was calculated on the following equation:

Growth inhibition % = (A_{595} of the peptid-free well- A_{595} of wells that contained the peptide) / A_{595} of the peptide-free well x 100.

MIC was considered to be the lowest concentrations of synthetic peptide showing 100 % reductions in the OD compared with that of the synthetic peptide free well. All the experiments were run in triplicate and the reading averages, the standard errors and coefficients of variation were calculated. Microscope images were also collected directly from the antifungal assay with an inverted light microscope (Olympus, Japan) at 24 h post treatment. Images were captured with the digital camera (Leica type DFC300FX, Germany).

2.4 *Et*Def recombinant protein

2.4.1 Production of *Et*Def recombinant protein using pCRT7/CT vector

PCR product encoding the mature peptide of *Et*Def (without putative signal peptide and pro-peptide) cloned into a pCRT7/CT vector (containing His-tag and V5 epitope sequences at C-terminal) was obtained from Dr. Altincicek (Justus-Liebig University). Plasmid preparation was performed using the Wizard® Plus SV Miniprep DNA Purification kit (Promega, Germany). Nucleotide sequence was determined by AGOWA Company (Berlin, Germany) using T7-fwd primer (Table 1). *E. coli* BL21 (DE3) (Stratagene, La Jolla, USA) cells for protein expression were transformed with a plasmid with the correctly inserted and error free sequence of the *Et*Def transcript. Transformed cells were grown at 37 °C in Luria-Bertani (LB) (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 4 h of induction, cells were harvested by centrifugation at 2830 rcf 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 at a pressure of 8000 lb in^{-2}. Purification was achieved using nickel-nitrilotriacetic acid affinity chromatography (Ni-NTA; Qiagen, Germany), following the manufacturer's instructions. The fractions were collected and

Table 1: Gene-specific primers and universal primers used in this study. Incorporated restriction enzyme site is shown in bold at the 5'-end of primer. AT: Annealing temperature.

Primers	Sequence 5`- 3`	AT
***Bgl*II-*Et*Def-start**	**GGATCC**CAACGCGAGCGCAGGACAAGC	50 °C
***Hind*III-*Et*Def-stop**	**GTCGAC**GCGGTGACGGTATCTACATG	
***BamH*1-Chi-fwd**	**GGATCC**ATGAGATCGCTCGCGGT	60 °C
***Sal*I-than-stop**	**GTCGAC**TCACATGCGCTGGCACTT	
UBQ5-fwd	CCAAGCCGAAGAAGATCAAG	60 °C
UBQ5-rev	ACTCCTTCCTCAAACGCTGA	
***BamH*I-*Et*Def fwd**	**GGATCC**GATCCAGCTACATGTGATCTGCT	50 °C
***Hind*III-*Et*Def-rev**	**AAGCTT**CTAACGCCGGCAATTGCAGACT	
T7-fwd	TAATACGACTCACTATAGGG	55 °C
T7- Term-rev	ATCCGCATATAGTTCCTCCTTTC	
PGY1-fwd2	CGTTCCAACCACGTCTTCAA	53 °C
Nos-T	ATTGCCAAATGTTTGAACGA	53 °

applied to 15 % Tricine-SDS-PAGE (Schägger and Jagow, 1987). Western-blotting analysis was performed according to the instruction manual. After electrophoresis, the separated proteins were transferred to a nitrocellulose-membrane Protran®BA (Schleicher and Schuell,) using a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad, München). The membrane was blocked with 0.3 % (w/v) bovine serum albumin (BSA) in PBS buffer (containing 0.05 % Tween 20) and incubated with mouse anti-V5 antibody (Invitrogen, Germany) followed by the HRP-conjugated goat anti-mouse IgG (Sigma, Germany). Detection of antigen–antibody complexes was performed with enhanced chemiluminiscence using SuperSignal® West Pico Chemiluminescent Substrate (Pierce protein research products).

1x PBS-buffer

KCl	0.2 g
NaCl	0.8 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g

Complete to 1 L with H_2Odest.

2.4.2 Production of *Et*Def recombinant protein using pET32a(+) vector

In order to obtain large amounts of a soluble, highly purified peptide, we used the plasmid pET32a(+) (Invitrogen, Germany) with thioredoxin (Trx) gene as a fusion partner of the *Et*Def gene. pGEM-T easy/SP-*Et*Def (provided by Dr. B. Altincicek, Justus-Liebig University, Giessen, Germany) served as template to prepare the mature *Et*Def sequence by PCR. A *BamH*I site and codons for Asp-Pro dipeptide were added at 5` end of the forward primer *BamH*I-*Et*Def-fwd for in-frame cloning with the Trx-tag of the vector and a *Sal*I site and a stop codon at 5` end of the reverse primer *Et*Def-*Hind*III (Table 1). Subsequently, *Et*Def fragment was digested with *BamH*I and *Hind*III and then ligated into the *BamH*I-/ *Hind*III- digested and dephosphorylated pET-32a(+), in frame to the Trx-tag, His-tag and S-tag (THS-tag). The resulting plasmid, pET32a-*Et*Def, was transformed into *E. coli* DH5α, and recombinant *E. coli* cells were selected on LB solid medium (1.0 % sodium chloride, 1.0 % tryptone, 0.5 % yeast extract, and 1.5 % agar) containing ampicillin (100 mg L^{-1}) plates and screened by the colony PCR method using *Et*Def specific primers (*BamH*I-*Et*Def-fwd and *Et*Def-*Hind*III-rev) as well as vector primers (T7-fwd and T7 terminal-rev) (Table 1). The resulting plasmid was sequenced to ensure that the coding sequence of pET32a-*Et*Def was correct and in-

frame with the THS-tag. The recombinant plasmid and the empty vector (as a control) were used to transform electrocompetent *E. coli* BL21 (DE3) cells for recombinant protein expression. For large scale protein purification, a single bacterial clone, in which the protein production was highly inducible, was grown in LB- medium overnight at 200 rpm and 37 °C. After inoculation of 1 L medium with the overnight culture, bacteria were allowed to grow until mid log phase (OD_{600} of 0.6 - 0.8) before IPTG was added to a final concentration of 1.0 mM and further incubation for 4-5 hours. Then, bacteria were harvested by centrifugation at 2830 rcf for 20 min at 4 °C.

2.4.3 Purification of fusion protein

The bacterial pellet was dissolved in 30 mL lysis buffer (see section 2.4.1) 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 45 min in a Beckman coulter centrifuge (23700 rcf). 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, Karlsruhe, 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).

2.4.4 Refolding of fusion protein

The purified fusion THS-*Et*Def protein and purified THS-tag from bacterial cell with empty pET32a(+) vector were dialyzed against refolding buffer (10 mM Tris, pH 7.5 and 1 M ß-mercaptoethanol; pH 8.0) containing 6, 4, 2, 1 and 0.5 M urea, respectively to decrease, gradually, the concentration of urea in the protein solutions to 0.5 M, then kept overnight at 4 °C. Finally, the fusion proteins were desalted and concentrated in 10 mM Tri-HCl (pH 7.5) using an ultra-filtrate column (VIVASPIN 6 mL concentrator) with a cut-off at 3 kDa (Vivascience, Lincoln, UK) and stored at -20 °C. Protein concentrations were measured by absorbance at 280 nm using ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Erlangen, Germany). Purity of *Et*Def recombinant protein was determined by separating protein aliquots using SDS-PAGE.

2.4.5 Antifungal activity of recombinant fusion protein (THS-tag-*Et*Def)

Antifungal activity of fusion THS-*Et*Def protein was evaluated on spore germination of *B. cinerea in vitro*. The purified THS-tag from bacterial cells transformed with empty pET32a (+) vector were used as negative controls. *B. cinerea* Spores (2×10^4 spores mL^{-1}) were incubated in the presence of different concentrations of fusion protein THS-*Et*Def as well as purified THS-tags (0.1, 0.5, 1 and 2 µM) on microtiter plate at RT. After 24h of incubation, the percentage of spore germination inhibition was evaluated.

2.5 Construction of expression vectors and transgenic plants

In this study, two different genes *Et*Def and thanatin, isolated from *Eristalis tenax* larvae and *Podisus maculiventris*, respectively, were transformed in *A. thaliana* ecotype Col-0 using *Agrobacterium tumefaciens* to confer resistance against fungal and bacterial plant pathogens.

2.5.1 Construction of plant expression vector for *Et*Def gene

*Et*Def mRNA was identified among immune-related transcripts from *E. tenax* larvae expressed upon injection of microbial elicitors of innate immune responses (Gen-Bank accession number AM706420, Altincicek and Vilcinskas, 2007). The complete open reading frame of *Et*Def, including its predicted signal peptide (SP) and pro-sequence,

was provided by Dr. B. Altincicek (Justus-Liebig University, Giessen, Germany) cloned in pGEM-T easy vector (Promega, Germany). A 309 bp fragment containing the complete coding region of the SP-*Et*Def was amplified by a PCR assay using the SP-*Et*Def specific primers (*Bgl*II-*Et*Def-start and *Hind*III-*Et*Def-stop) (Table 1) harboring the *Bgl*II and *Hind*III restriction sites to facilitate subsequent cloning, ligated into pGEM-T vector and verified by sequencing. For construction of the binary vector, the complete *Et*Def sequence was excised using *Bgl*II and *Hind*III and inserted into the respective restriction sites of the expression vector p35S-BAM (DNA Cloning service, Hamburg, Germany) between the constitutive Cauliflower mosaic virus 35S (CaMV 35S) promoter and the Nopaline synthase terminator (nos-T). This cassette encoding SP-*Et*Def gene was then subcloned into the *Sfi*I restriction site of the pLH6000 binary vector (DNA Cloning Service, Hamburg, Germany), which harbors the hygromycin phosphotrasferase (hpt) resistance cassette giving rise to the final construct pLH6000 35S::SP-*Et*Def::nos.

2.5.2 Construction of the chimeric thanatin gene and plant expression vectors

Immune challenge to the insect *Podisus maculiventris* induces synthesis of a 21-residue peptide, named thanatin (Gen-Bank accession number 6730068, Fehlbaum *et al.*, 1996). In this work, the amino acid sequence of mature thanatin was re-designed to target thanatin to the apoplast. In order to allow the thanatin to enter the secretory pathway of the Arabidopsis cell, the sequence for the signal peptide of chitinase 26 from *Hordeum vulgare* (*Hv*Chi26, Genbank L34210, Jollès and Muzzarelli, 1999) was fused to the mature thanatin gene sequence. DNA encoding thanatin including *Hv*Chi26 signal peptide (SP-thanatin) was chemically synthesized and cloned in pPCR-script vector by the company Sloning Biotechnology GmbH, Puchheim, Germany. A 147 bp PCR product of synthetic SP-thanatin gene was produced using primers *BamH*I-Chi-fwd and than-stop-SalI-rev (Table 1) flanked by *BamH*I/ *Sal*I restriction sites. The newly created chimeric SP-thanatin gene was cloned into the *BamH*I/ *Sal*I sites of p35S-BAM under the control of CaMV 35S promoter and the nos terminator sequences. Finally, the entire cassette for expression of the synthetic SP-thanatin gene was cloned into the *Sfi*I digested pLH6000 binary vector, resulting in plasmid pLH6000 35S::SP-thanatin::nos. The correct insertion and full nucleotide sequence of the promoter and SP-thanatin gene

were confirmed by DNA sequence analysis by AGOWA Company (Berlin, Germany). Standard molecular biology procedures were carried out as described by Sambrook *et al*. (1989). Cloned sequences were analyzed, and multiple sequence analysis was performed using CLUSTALW2 software (Thompson *et al*., 1994).

2.6 Agrobacterium Transformation

The binary vectors designed to express SP-*Et*Def and SP-thanatin genes under the control of a constitutive promoter CaMV 35S were transferred into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) through electroporation (*E. coli* Pulser, Biorad, USA) according to manufacturer`s instruction. As a negative control, a vector containing only the hygromycin phosphotransferase gene conferring hygromycin resistance in the T-DNA region was electroporated into *A. tumefaciens* strain AGL1. The transformed cells were plated on YEP agar medium containing 25 mg L^{-1} carbenicillin, 25 mg L^{-1} rifampicin and 50 mg L^{-1} spectinomycin at 28 °C for 2 days. Growing, antibiotica-resistant colonies of Agrobacteria were subcultured in liquid medium and then screened by PCR amplification using PGY1for2 and nos-T primers (Table 1).

YEB (Yeast Extract Broth)- Medium

0.5% Beef extract
0.1% Yeast extract
0.5% Pepton
0.5% Sucrose
Dilute in 1 L H_2O
Adjust pH to 7.2 with 0.5 M NaOH
After autoclaving and cooling down, add 2 mL filter sterilized 1M $MgCl_2$ per liter.

2.7 *In planta* transformation of *A. thaliana,* selection and propagation of transgenic plants through generations

Five-week-old Arabidopsis plants (ecotype Col-0) were transformed using recombinant *A. tumefaciens* strain AGL1 by the vacuum infiltration method (Bechtold *et al*.,1993). A single colony of Agrobacterium carrying the recombinant vector was inoculated into 50 mL liquid YEP medium (containing 25 mg L^{-1} carbenicillin, 25 mg L^{-1} rifampicin and 50 mg L^{-1} spectinomycin) and grown overnight on a rotary shaker at 200 rpm and 28 °C. The culture 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}= 2.0.

Agrobacterium cells were centrifuged for 10 min at 2700 ×g and resuspended in a transformation suspension consisting of 5 % sucrose, 0.4 % ½ MS-salts, 1x B5 vitamin, 10 µL L^{-1} BAB, and 0.01 % silwet-L77, pH 5.8 to a final OD_{600} of 1.1 – 1.3. The beaker was then placed in a vacuum chamber. Inflorescences were dipped in bacterial suspension and infiltrated under vacuum conditions of 530 HPa for 5 min. The transformed plants were placed into a plastic bag and kept in the dark for 24 h with relative humidity close to 100 %. The plants were then grown in a climatic chamber for seed maturation. T_1 seeds were grown on ½ MS-medium containing hygromycin (30 mg L^{-1}) and ticarcillin (150 mg L^{-1}) for selection. After acclimatization, the transformants were grown in a growth chamber under controlled environmental conditions (see section 2.1) to raise the T_1 plants.

Hygromycin-resistant transformants (T_1) were self-pollinated, and harvested seeds of each T_2 line were checked for inheritance of foreign gene by calculating ratio of the tolerant plants to the non-tolerant plants on selection 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 phenotype characterization and further experiments in addition to the transgenic control plants (empty pLH6000 vector, #14).

2.8 Molecular characterization of transgenic lines

2.8.1 Extraction of plant DNA

Stability of the cloned *Et*Def and thanatin gene integration in Arabidopsis genome was analyzed for two successive generations (T_1, and T_2) using PCR. Genomic DNA was extracted from fresh leaves of transformed and nontransformed (negative controls) plants using the Extract-N-AMP Plant PCR Kit (Sigma, Germany) according to the manufacturer's instructions. One leaf disk (about 0.5 cm^2) from the rosette leaves was collected and extracted in 100 µL of extraction solution. The samples were then vortexed briefly and incubated at 95 °C for 10 min. After that, 100 µL of the dilution solution was added to each sample and vortexed. The samples were stored at 4 °C, and used later as a template for PCR.

2.8.2 Polymerase chain reaction (PCR)

PCR amplifications were carried out on a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems). Primers PGY1-for2 and *Hind*III-*Et*Def-rev (Table 1) were used for the amplification the fragment of SP-*Et*Def and primers PGY1-for2 and *Sal*I-than-rev for the amplification of SP-thanatin. The reaction for both genes was carried out in 10 µL reaction mixture containing 5 – 10 ng of plant DNA, PCR Master Mix (Extract-N-Amp Kit, Sigma, Germany), and 100 ng 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 30 s at 94 °C, 30 s at 50 °C and 40 s at 72 °C. Finally, an additional elongation step was performed for 5 min at 72 °C. The amplification products were mixed with gel loading buffer (0,25 % (w/v) bromphenolblue and 40 % (w/v saccharose) to give a final sample volume of 10 to 20 µL and were then 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 (0.2 µg/ml). 1KB Plus DNA Ladder (Gibco BRL life Technologies GmH, Karlsruhe, 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 thermoprinter.

2.8.3 Detection of gene expression

2.8.3.1 RNA extraction

Total RNA was extracted from 5-week-old transgenic as well as non transgenic Arabidopsis leaves using RNA extraction buffer (Applied Gene technology System, Heidelberg, Germany) according to the manufacturer's instructions. Newly rosette leaves were ground to a fine powder in a mortar with liquid nitrogen and stored at -80 °C. About 150 - 200 mg of the homogenized samples was extracted with 1 mL RNA extraction buffer including guanidiniumthiocyanat and phenol. The samples were then vortexed after the addition of 200 µL chloroform and placed on shaker at RT for 15 min. After centrifugation (20800 rcf for 15 min, at 4 °C), the supernatant was collected and purified with 850 µL chloroform and centrifuged at 20800 rcf for 15 min at 4 °C. An equal volume of 5 M lithium chloride was added to the supernatant, and the mixture

was kept at 4 °C overnight. The RNA was precipitated by centrifugation at 20800 rcf for 20 min at 4 °C. The pellet was washed twice with 70 % ethanol and dissolved in 40 µL H_2O_{DEPC}. The concentration of RNA was determined using NanoDrop ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Erlangen, Germany). The quantity and integrity of mRNA were checked on denaturing 1.5 % agarose-gel containing 5 % formaldehyde. Samples (1 µg RNA) were mixed with loading buffer and separated at 120 V in 1 x MOPS running buffer. The gel was then visualized using a UV transilluminator.

RNA-Extraction Buffer

Phenol in saturated buffer	38 %
Guanidin- Thiocyanat	0.8 M
Amonium- Thiocyanat	0.4 M
Sodiumacetat, pH 5	0.1 M
Glycerol	5 %
H_2O_{DEPC}	

Aqua bidest.DEPC

Aqua bidest. and DEPC (Diethylpyrocarbonat) (0.1 % w/v) were mixed for two hours. The solution was incubated at 37 ºC overnight and finally autoclaved.

10x MOPS

MOPS	200 mM
Sodiumacetat	50 mM
EDTA	10 mM

in autoclaved A. dest $_{DEPC}$. and the pH was adjusted to 7.0 using NaOH (10 M)

2x RNA- Loading puffer

Formamid	720 µL
Formaldehyde (37 %)	260 µL
10x MOPS	160 µL
EtBr (10 mg / ml)	100 µL
Glycerin	80 µL
Bromophenolblue	80 µL
A. bidest.DEPC	100 µL

2.8.3.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Expression of the *Et*Def and thanatin gene in transformed *A. thaliana* plants was tested with RT-PCR. Total RNA was extracted from the leaves of transformed and untransformed, control plants as described above (see section 2.8.3.1), and then treated with RNAse-free DNAseI (Fermentas, Sankt Leon-Rot, Germany) at final concentration of 2 units/µg of total RNA for 30 min at 37 °C. In order to obtain cDNA, mRNA was

reverse transcribed using a One-Step RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 2 µg RNA was used for cDNA synthesis in a final volume of 20 µL according to the manufacturer's instructions. Aliquots were amplified in subsequent PCR reactions using gene-specific primers for *Et*Def (*Bgl*II-*Et*Def-start-Fwd and *Et*Def-*Hind*III-stop-rev) and gene-specific primers for thanatin (*BamH*I-Chi-fwd and than-*Sal*I-stop-rev) (Table 1). In each case, a control PCR with the constitutive Arabidopsis ubiquitin-5 gene (UBQ5) gene was made in parallel with the primers UBQ5-fwd and UBQ5-rev (Table 1). PCR conditions were as follows: a reverse transcription step of 30 min at 50 °C, a denaturation step of 15 min at 94 °C, 30 amplification cycles of 30 s at 94 °C, 30 s at 50 °C (for amplification of *Et*Def and thanatin gene) or at 60 °C (for amplification of Arabidopsis UBQ5), 30 s at 72 °C, and an extension cycle of 10 min at 72 °C. The PCR products were then separated on agarose gel and visualized using a UV transilluminator.

2.8.3.3 Quantitive real-time PCR (qRT-PCR)

Real-time RT-PCR analysis was performed using Mx3000p thermocycler (Stratagene Research, La Jolla, CA, USA). Transcript expression analysis for every gene was performed in *Et*Def- and thanatin- transgenic lines as well as Col-0 (wild type) Arabidopsis plants using the FullVelocity® SYBR® Green QRT-PCR Master Mix kit, 1-Step (Stratagene), according to the manufacturer's protocol. A non-template control was also included for every gene. Each reaction contained 13 µL FullVelocity SYBR Green QRT-PCR master mix, 1µL each of gene specific forward and reverse primers (Table 1), 10 ng of RNA, and 1 µL of RT/RNase block enzyme mixture, which contained dNTPs, Taq polymerase and Reverse transcriptase. Thermocycler conditions were as follows: 50 °C for 30 min; then 95 °C for 7 min; 40 cycles of annealing temperature for 30 s, then 72 °C for 30 s, followed by 95 °C for 1 min. The annealing temperatures are given in Table (1). Dissociation curves were produced to confirm amplicon purity. All reactions were repeated at least triple. From the standard curves, relative expression of each gene was estimated compared to control using Mx3000p MxPro v3.20 software. Cycles of threshold (Ct) values were generated by deducting the raw Ct values of *Et*Def gene and thanatin gene from the respective raw Ct values of the Arabidopsis ubiquitin-5

gene (UBQ5). Within each construct, transcript expression differences were statistically determined using the $2^{-\Delta Ct}$ method.

2.8.4 Antifungal activity of leaf extracts from transgenic Arabidopsis

To evaluate the antifungal activities of *Et*Def and thanatin genes in Arabidopsis plants, T_3 transgenic plants overexpressing *Et*Def and thanatin and transgenic control plants (#14) were used for *in vitro* assays. The antifungal activity of leaf extracts (protein extracts) from 5-week-old Arabidopsis plants was assessed against *B. cinerea*. Protein extracts were pestled with 1 mL precoold extraction buffer (50 mM Tris–HCl pH 7.5), vortexed, and centrifuged (20800 rcf for 10 min at 4 °C). The supernatant was transferred to a clean 1.5 mL microcentrifuge tube and placed on ice (Wang and Constabel, 2004). *B. cinerea* was maintained on HA-agar medium and then agar blocks with fungal mycelium were incubated in leaf extracts at 22 °C for 24 h. Subsequently, agar blocks were transferred to fresh HA-agar plates, and outgrowth of the mycelium was measured 24 h later. Two independent experiments with separate preparations of each plant protein extract, and four replicas for each protein extract were performed.

2.8.5 Antifungal activity of intercellular washing fluids from transgenic Arabidopsis

Intercellular washing fluids (IWFs) were obtained from Arabidopsis transgenic plants as well as non-transgenic Col-0 (5-week-old) by centrifugation according to Lohaus *et al.* (2001). The fully expanded rosette leaves were collected and immersed in a beaker containing extraction buffer (50 mM phosphate buffer and 0.6M NaCl, pH 7.5). The beaker was placed in a vacuum chamber and subjected to six consecutive rounds of vacuum treatment for 2 min followed by abrupt release of vacuum. The infiltrated leaves were dry-blotted and gently placed in a centrifuge tube on a grid separated from the tube bottom. The IWFs were collected from the bottom of the tube after centrifugation of the tubes at 50 rcf for 5 min at 4 °C. The amount of IWF obtained from 1 g of tissue (fresh weight) was 0.2 to 0.3 mL. IWF extracts obtained from transgenic and non-transgenic plants were fractionated on 15 % Tricine-SDS-PAGE (Schägger and Jagow, 1987) and their antifungal activities were evaluated against *B. cinerea* using spore germination assay. Fungal conidia (2×10^4 conidia mL^{-1}) were

incubated in 20 µg IWF from each transgenic lines as well as non-transgenic plants in microtiter plate at RT for 24 h and the percentage spore germination inhibition was then evaluated for each transgenic line.

2.9 Plant resistance bioassays

To assess resistance, T_3 homozygous *Et*Def and thanatin transgenic plants and transgenic control plant (#14) as well as non-transformed Arabidopsis Col-0 were used. Antifungal resistance of *Et*Def and thanatin transformants was evaluated by inoculation with the obligate biotrophic fungal pathogen *G. orontii* causing powdery mildew and the necrotic fungal pathogen *B. cinerea* causing grey mold. For antibacterial resistance assays, transgenic plants were inoculated with *P. syringae* strain DC3000.

2.9.1 Inoculation of powdery mildew

Inoculation with *G. orontii* was performed on 5-week-old soil-grown plants. For inoculum preparation, leaves from heavily infested plants were cut and spores were washed down into 0.02 % Tween solution. A spore suspension with a density of $5x10^5$ conidia mL^{-1} was immediately sprayed on healthy plants. After inoculation, the plants were moved to a growth chamber under the same growth condition as described previously (see section 2.1). A total of 10 plants were used for each treatment, and the experiment was repeated twice.

The growth of *G. orontii* was microscopically evaluated by counting the total number of conidiophores per colony at 5 dpi and the number of new conidia per gram leaf fresh weight of inoculated plant at 10 dpi. To accomplish that, rosette leaves of inoculated plants were cut after 5 dpi and immediately immersed in destaining solution. Afterwards they were stained using acidic blue ink for 60 seconds, mounted on slides and observed with light microscope (Zeiss, Oberkochen, Germany). In each case, pictures of five randomly chosen fields of view per leaf and a minimum of 10 leaves per experiment were used to assess fungal growth. As for conidia number, 3-5 inoculated plants from each treatment were cut, weighed and washed in defined volume of 0,01 % Tween-solution to collect the new grown conidia. Their number were counted then using Fuchs-Rosenthal Counting Chamber. In addition, the visible disease symptoms were photographed at least 10 days after inoculation.

Blue-Ink staining solution
10 % blue ink (v/v) within 25 % acetic acid

Destaining solution
Ethanol (80 %), chloroform (20 %) and 1.5 g L^{-1} trichloroacetic acid.

2.9.2 Inoculation with grey mold *B. cinerea*

Botrytis inoculation was done using the detached-leaf assay (modified after Ferrari *et al*., 2003). 30 rosette leaves from 10 transgenic plants as well as non-transformed 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 ImageJ programme (http://rsb.info.nih.gov/ij/index.html).

2.9.3 Antibacterial resistance in transgenic Arabidopsis plants

*Et*Def and thanatin 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 B (20 g bacto proteose peptone, 15 g K_2HPO_4, 15 g $MgSO_4$-$7H_2O$, 0.8 % glycerol, 15 g agar per liter) containing 50 mg L^{-1} rifampicin. After 2 days, bacterial culture was collected by scraping the culture from the plates and washing twice then with sterile 10 mM $MgCl_2$. The bacterial concentration was brought to OD_{600}=0.2, which corresponds to approximately 1×10^5 cfu/mL and was then pressure 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^2 diameter) were punched from the infiltrated area with a cork borer and ground 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).

2.10 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). Hereabout, the present work aims to introduce genes encoding the novel antimicrobial peptide *Et*Def from *E. tenax* larvae (Altincicek and Vilcinskas, 2007) and thanatin from *P. maculiventris* (Fehlbaum *et al*., 1996) into *Arabidopsis* and to evaluate their *in vitro* as well as *in planta* antimicrobial activities against some agronomically important phytopathogens.

3.1 *In vitro* antifungal activity of synthetic *Et*Def and thanatin

Since available data on the antifungal activity of *Et*Def and thanatin are scarce, the sequences of mature *Et*Def and thanatin peptides were chemically synthesized and their *in vitro* antifungal activities were assessed against different species of phytopathogenic fungi. These include *F. culmorum* and *B. cinerea* (Ascomycetes), and *P. parasitica* (Oomycetes).

Antifungal activity of *Et*Def and thanatin on *F. culmorum* was evaluated using spore germination inhibition assay. Fungal spores were incubated in the presence of various concentrations of *Et*Def and thanatin peptides, with IC_{50} (peptide concentration which leads to reduce the conidial germination by 50 %) and MIC (minimal inhibitory concentration) values being determined after 24 hours of incubation (Table 2). Increasing concentrations of both synthetic *Et*Def and thanatin resulted into a marked reduction in the spore germination and hyphal growth of *F. culmorum* (Fig. 2 and 3). This effect was much obvious for synthetic *Et*Def, with IC_{50} observed at approximately 2 µM. Regarding thanatin, IC_{50} was slightly higher being 2.6 µM (Fig. 3 and Table 2). MICs for both *Et*Def and thanatin were, however, comparable and averaged between 5 – 10 µM (Fig. 3 and Table 2). Light microscopical analyses showed clearly that *Et*Def with its IC_{50} concentration (2 µM) resulted into some abnormalities in the fungal germ tube morphology, such as swelling, shortening, increasing in cell wall thickness (Fig. 2B). Nevertheless, this effect was not observed for thanatin (Fig. 2E).

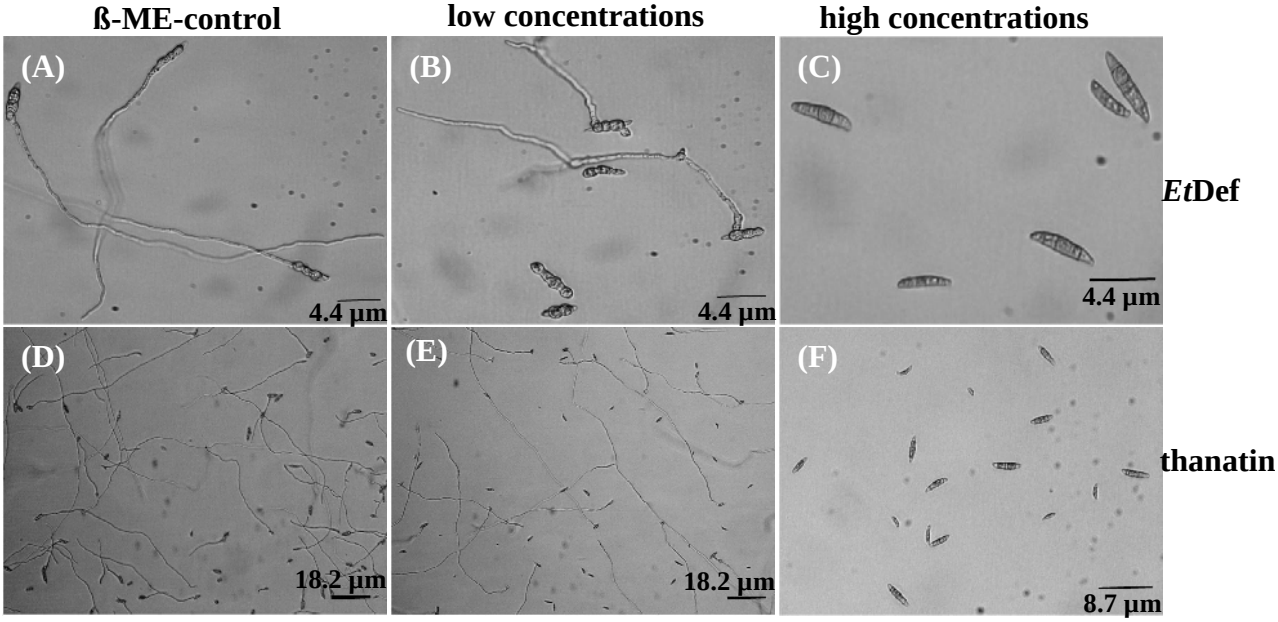


Fig. 2: *In vitro* spore germination of *F. culmorum* in the presence of 10 µM β-ME (as control) (A and D); synthetic *Et*Def at final concentrations of 2 and 10 µM (B and C, respectively); synthetic thanatin at final concentrations of 5 and 10 µM (E and F, respectively) after 24 h incubation. Note the swelling of spores and the growth abnormalities of the germ tube of the germinated spores treated with *Et*Def (B).

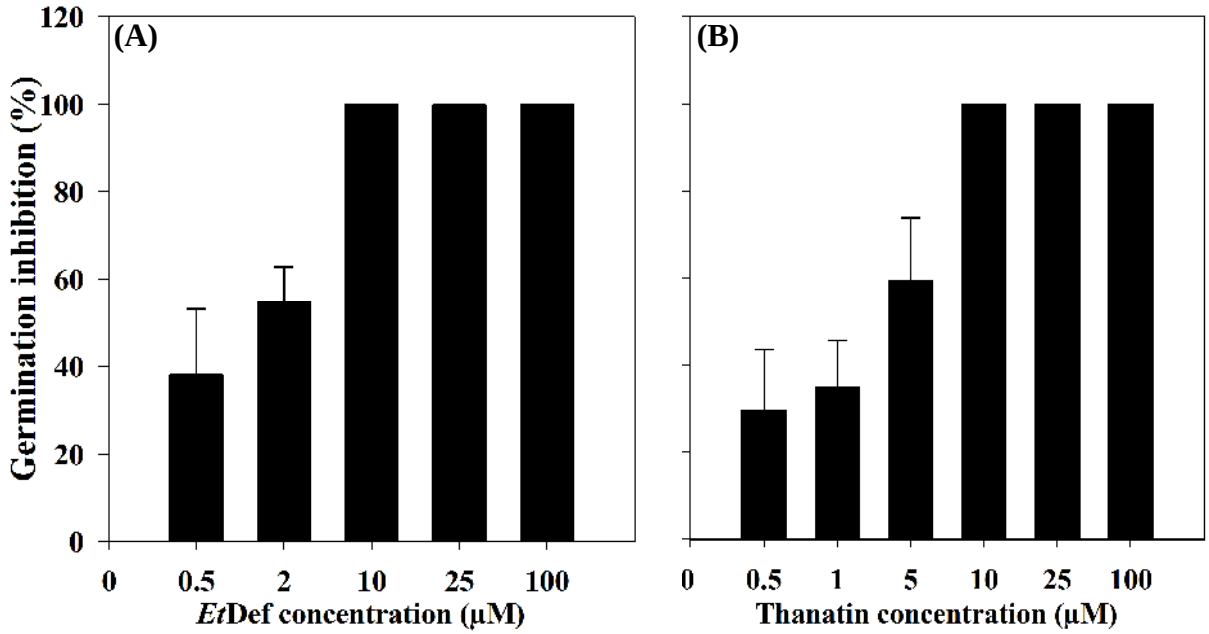


Fig. 3: *In vitro* antifungal activity of synthetic *Et*Def (A) and thanatin (B) on spore germination of *F.culmorum*. The effect of both peptides on spore germination was microscopically investigated and the % spore germination inhibition was evaluated 24 h after incubation. Each value represents the mean of three replicates of two successive experiments ± SE.

High concentrations of both synthetic *Et*Def and thanatin completely abolished the spore germination of *F. culmorum* (Fig. 2C and F, respectively).

The antifungal properties of *Et*Def and thanatin were also studied with respect to *B. cinerea* conidial germination. *B. cinerea* conidia was incubated with either *Et*Def or thanatin at different concentrations and observed microscopically after 24 h of incubation. Irrespective of peptide concentration, spore germination of *B. cinerea* was generally more sensitive to both synthetic *Et*Def and thanatin compared to *F. culmorum*. Microscopic examination of spore germination of *B. cinerea* (Fig. 4) revealed that low *Et*Def and thanatin concentrations significantly reduced the spore germination as well as the growth of germ tube of *B. cinerea* compared with the corresponding controls, whereas high concentrations inhibited completely the spore germination. This inhibitory effect was generally much pronounced for thanatin as compared to *Et*Def. The IC_{50} was 0.5 and 0.1 µM, whereas MICs ranged from 1 to 2 and from 0.5 to 1 µM for *Et*Def and thanatin respectively (Fig. 5 and Table 2).

Because it was difficult to evaluate the *in vitro* antifungal activity of synthetic *Et*Def and thanatin against *P. parasitica* using spore germination inhibition assay, antifungal activity of both peptides was assessed using MTT assay, in which the effect of various concentrations of synthetic *Et*Def and thanatin on the viability of *P. parasitica* cells was colorimetric determined (see section 2.3.2.2). Firstly, the zoospores were incubated with various concentrations of each synthetic peptide for 48 h at RT. Spore germination and hyphal growth were then microscopical investigated. Photomicrographs (Fig. 6) demonstrated that mycelia growth of *P. parasitica* was clearly inhibited as *Et*Def and thanatin concentrations increased compared to the relative controls. Furthermore, thanatin presented comparatively stronger activity than *Et*Def, as moderate concentrations of this peptide (5 µM) inhibited completely the mycelia growth (Fig. 6C). Additionally, no mycelial growth was noted at the highest *Et*Def and thanatin concentrations (10 µM) (Fig. 6). These observations were supported by the results obtained from MTT assay, which showed that mycelial growth and cell viability of *P. parasitica* were distinctly reduced with increasing concentrations of both *Et*Def and thanatin (Fig. 7). IC_{50} was observed at concentration of about 1 µM for *Et*Def and ranged between 1 and 2 µM for thanatin (Fig. 7 and Table 2).

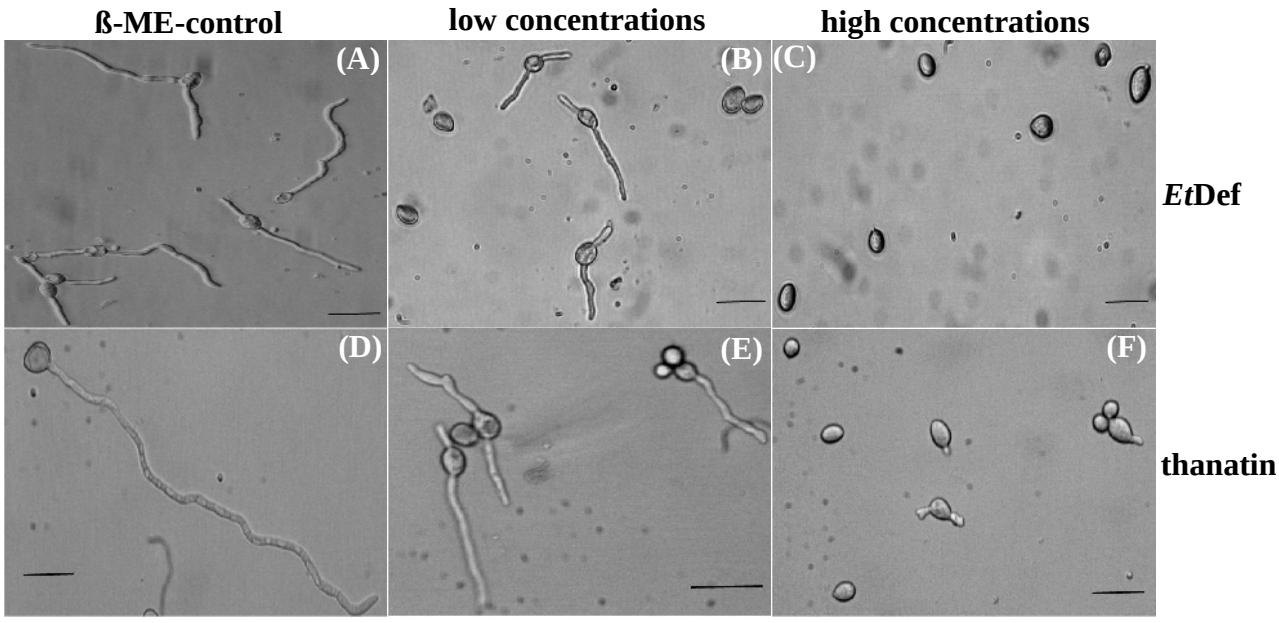


Fig. 4: *In vitro* spore germination of *B. cinerea* in the presence of β-ME (as control) at final concentrations of 2 and 1 µM (A and D, respectively); synthetic *Et*Def at final concentration of 0.5 and 2 µM (B and C respectively), and synthetic thanatin at final concentration of 0.1 and 1 µM (E and F, respectively). Micrographs were taken 24 h after incubation. Bars = 4.4 µm.

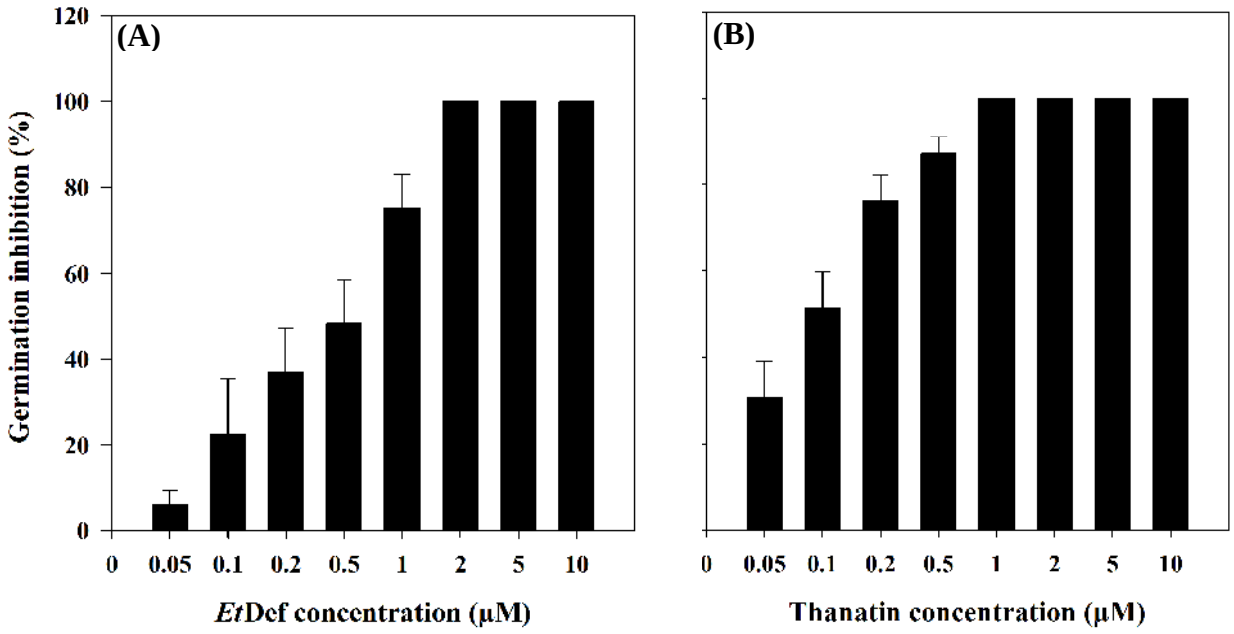


Fig. 5: The effect of synthetic *Et*Def (A) and thanatin (B) on spore germination of *B. cinerea in vitro*. The influence of both peptides on spore germination was microscopically investigated and the % spore germination inhibition was evaluated 24 h after incubation. Each value represents the mean of three replicates of two successive experiments ± SE.

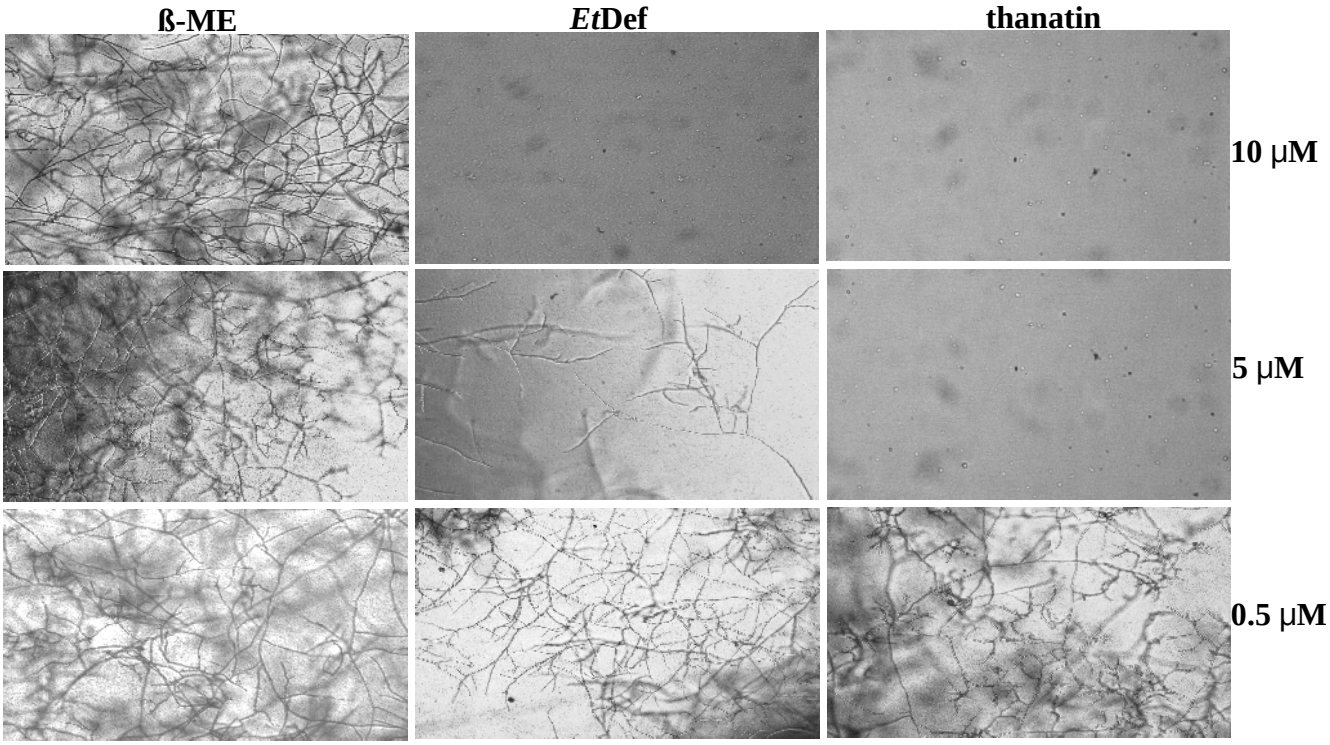


Fig. 6: Hyphal growth inhibition of *P. parasitica* treated with different concentrations of ß-ME (as a control), synthetic *Et*Def, and thanatin. Micrographs were taken 48 h after incubation. Magnification 100 x.

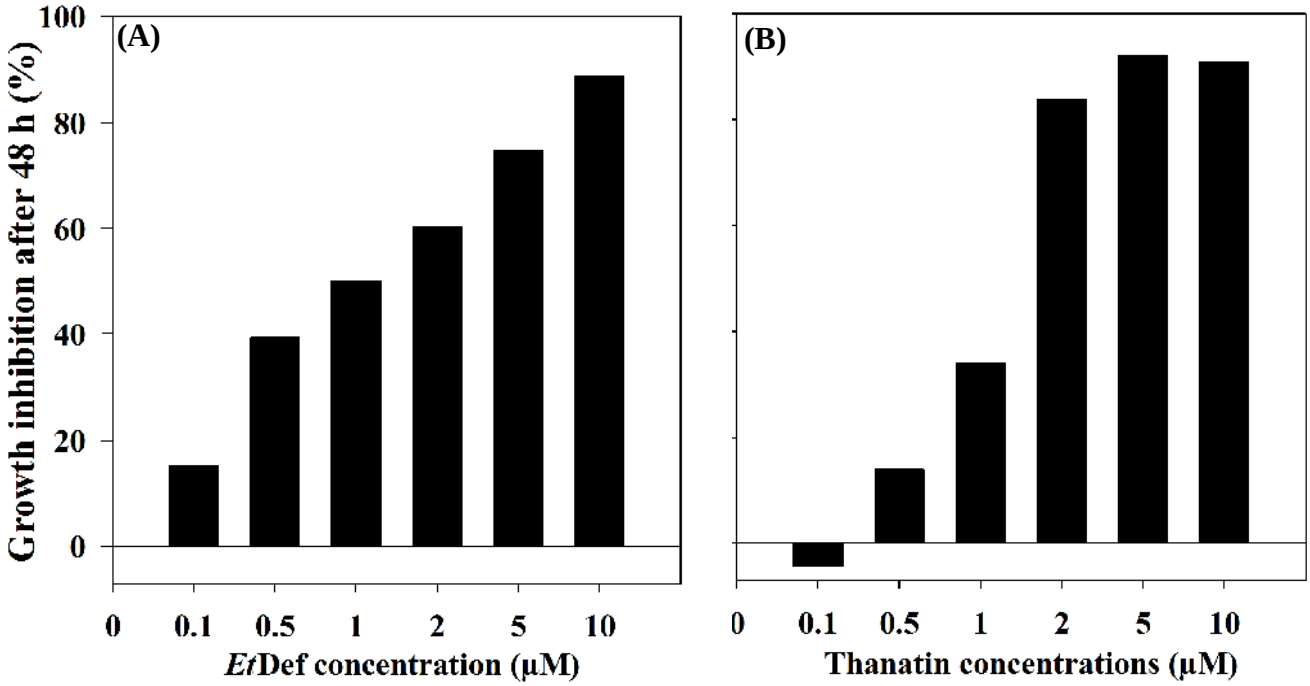


Fig. 7: Effect of various concentrations of synthetic *Et*Def and thanatin on mycelial growth and cell viability of *P. parasitica*. The percentage of growth inhibition of *P. parasitica* after incubation of zoospores with each synthetic peptide for 48 h at RT. Cell viability was determined by MTT-colorimetric assay. The MTT-derived formazan produced during an additional 4 h MTT-incubation was measured. Percentage inhibition was computed from mean absorbance values, as detailed in the material and method (see section 2.3.2.2).

MIC for *Et*Def ranged between 5 – 10 µM, while that of thanatin was attained at comparatively lower concentration (2 – 5 µM) (Fig. 7 and Table 2).

3.2 Expression and purification of recombinant protein *Et*Def

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*Def protein in *E. coli* in large quantities with low costs. To accomplish that, the expression vector pCRT7/CT (containing His-tag and V5 epitope at C-terminal) was used firstly to produce the recombinant *Et*Def protein in the *E. coli* BL21 (DE3) expression system. The obtained target recombinant pCRT7-*Et*Def protein was purified using Ni-NTA column (see section 2.4.3). Tricin-SDS-PAGE analysis (Fig. 8) showed that the target protein was successfully expressed in insoluble form after 1 mM IPTG induction, although, with a little amount. The expression and purity of the target fusion protein were further analyzed by Western-blotting analysis with mouse anti- V5 antibody. As shown in Fig. 9, a band with molecular weight of about 7.6 kDa (corresponding to the expected molecular mass of fusion protein pCRT7-*Et*Def) was detected. Together with the results of Tricin-SDS-PAGE analysis, this confirms that the pCRT7-*Et*Def could be successfully expressed in *E. coli* expression system. Only small amount of peptide could be expressed and purified.

In order to improve the production level, *Et*Def gene was fused with the protein partner Trx-tag under the control of T7 promoter, using pET32a(+) expression vector. All steps of *Et*Def gene synthesis and the recombinant vector pET32a-*Et*Def construction are illustrated in Fig. (10). With the designed primers (P1: *BamH*I-*Et*Def-fwd, and P2: *Hind*III-*Et*Def-rev), DNA fragment with a size of 141 bp (corresponding to the mature peptide coding region of *Et*Def gene) was amplified by PCR from pGEMT-easy/*Et*Def (Fig. 11A). Subsequently, *Et*Def fragment was digested with *BamH*I and *Hind*III and then ligated into pET32a(+) to construct the recombinant expression vector pET32a-*Et*Def. The positive clones were verified by colony PCR using *Et*Def specific primers (P1 and P2) as well as the vector primers P3 (T7-fwd) and P4 (T7-term-rev) (Fig. 11B), and then confirmed by sequencing analysis.

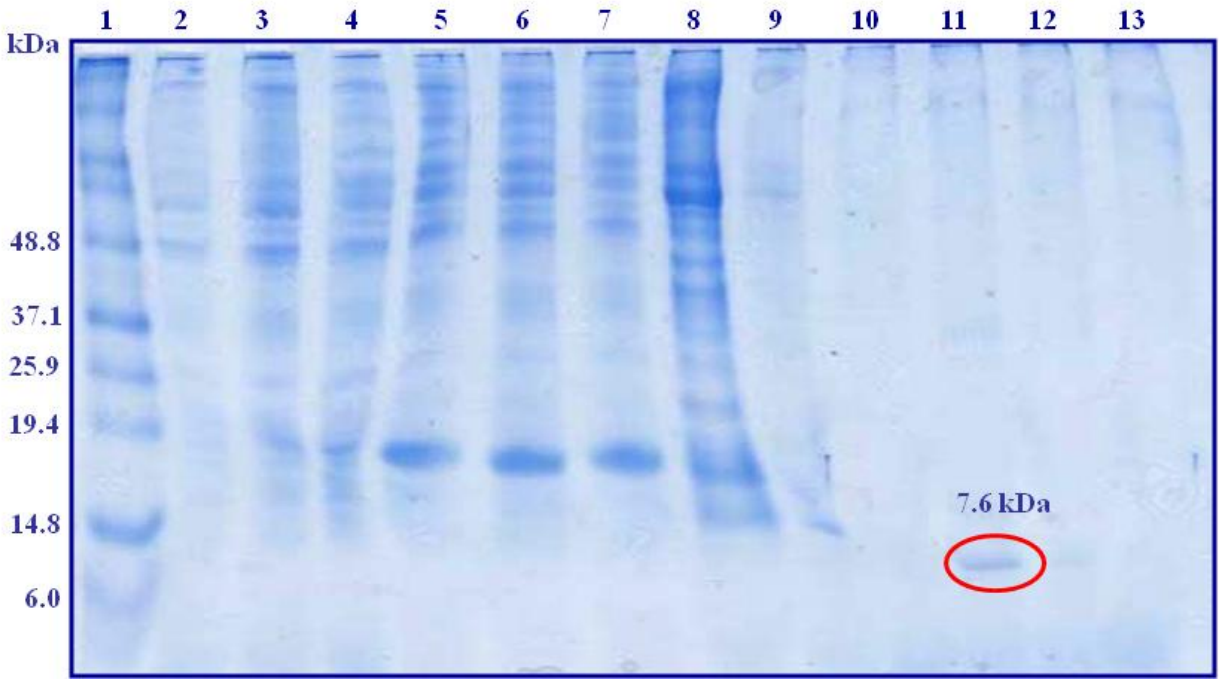


Fig. 8: Tricin-SDS-PAGE (15 %) showing expression and Ni-NTA purification of recombinant PCRT7-*Et*Def under denaturating conditions. Lane 1, BenchMark™ pre-stained protein marker (Invitrogen); lane 2, represents uninduced *E. coli* BL21/ PCRT7-*Et*Def; lanes 3 and 4, represent total protein from induced BL21 cells containing recombinant PCRT7-*Et*Def after 1 and 4 h of IPTG induction; lane 5, represents the inclusion bodies containing fusion PCRT7-*Et*Def after French press; lanes 6 and 7, represent flow through; lanes 8, 9, and 10, represent washing steps; lanes 11, 12, and 13, represent elution steps. Oval refers to the purified recombinant PCRT7-*Et*Def of 7.6 kDa.

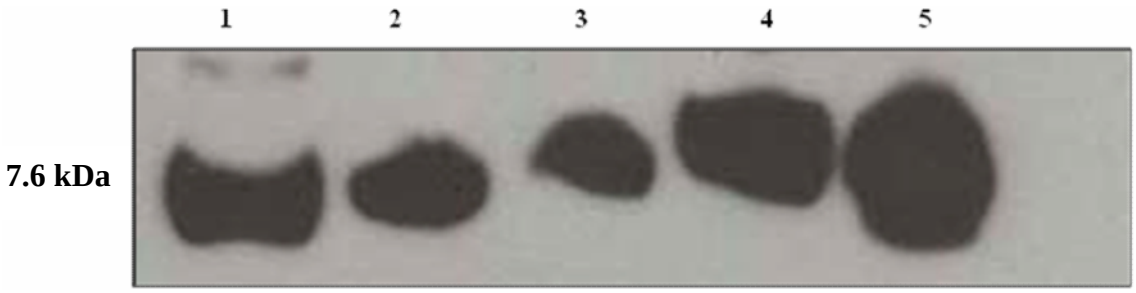


Fig. 9: Western blot analysis of the expression and Ni-NTA purification of recombinant PCRT7-*Et*Def protein using Anti-V5 antibody. Lane 1, shows total protein expression from induced BL21/PCRT7-*Et*Def after 4 h of IPTG induction; lane 2, represents the inclusion bodies containing fusion PCRT7-*Et*Def; lane 3, represents flow through; lane 4, first washing step; lane 5, first elution step.

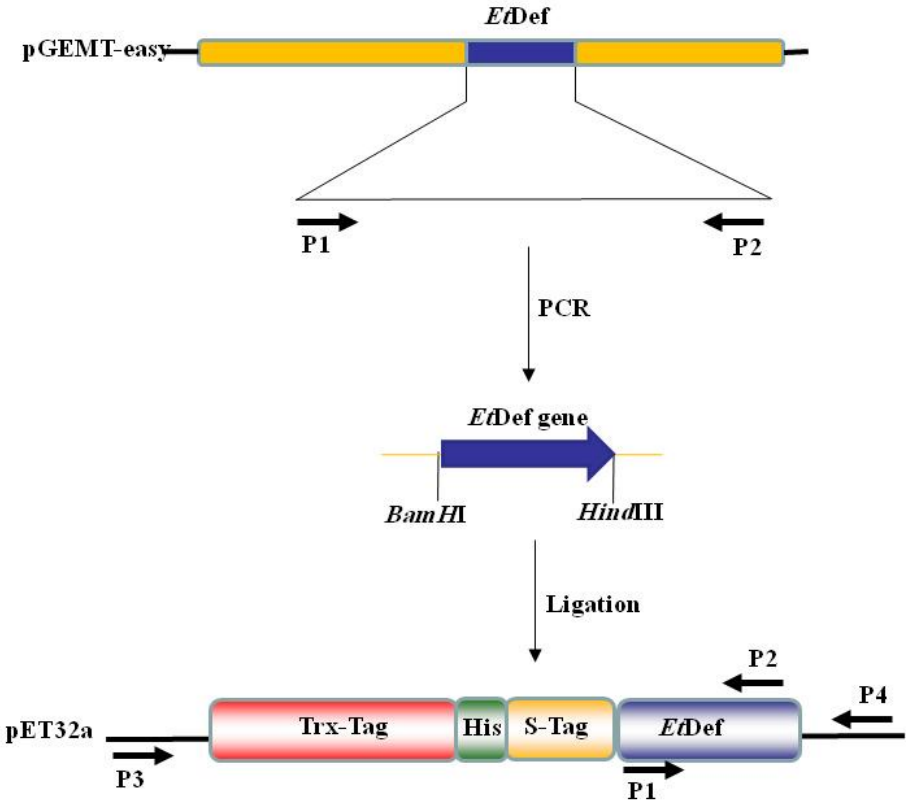


Fig. 10: Schematic representation of the *Et*Def gene synthesis and the construction of expression vector pET32a-*Et*Def with a fusion partner TrxA-His-S-tags. P1, *BamHI-Et*Def fwd; P2, *Hind*III-*Et*Def rev; P3, T7-fwd; P4, T7-term rev.

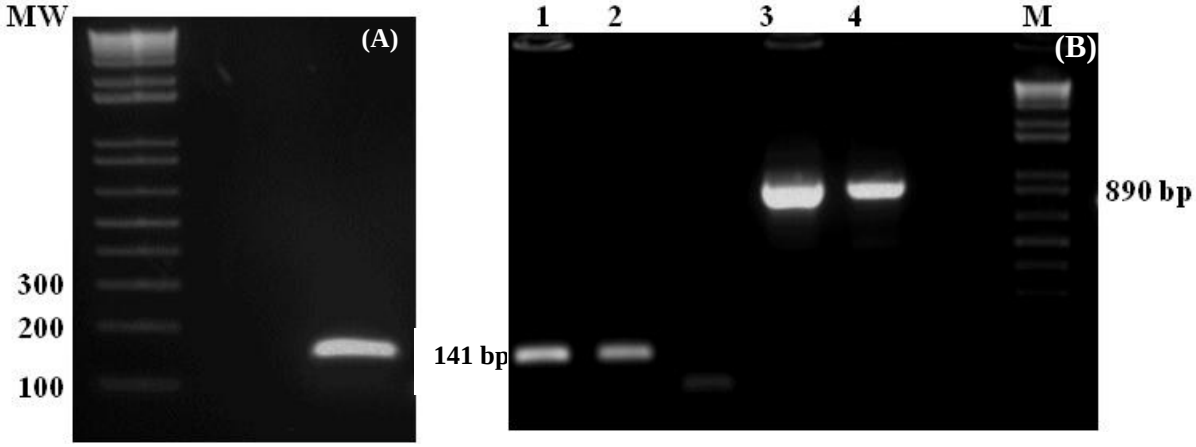


Fig. 11: *Et*Def gene amplified from pGEMT easy/*Et*Def (A), PCR colony check for the recombinant plasmid pET32a/*Et*Def in *E. coli* DH5α (B). Lane 1 and 2 represent PCR products using *Et*Def specific primers; Lane 3 and 4, PCR products using vector primers; M, 1 Kb Plus DNA ladder (Invitrogen).

In a preliminary experiment, *E. coli* BL21 (DE3) containing either empty pET32a(+) or pET32a-*Et*Def was cultured, induced by 0.4 mM IPTG (for 2, 4, and 6 h), and then analyzed by SDS-PAGE (Fig. 12). The maximum induction of THS-tagged-*Et*Def (Trx-His-S-tag- *Et*Def) was observed after 4 h with no considerable variation afterwards. The recombinant protein THS-tagged-*Et*Def was found to have a molecular weight of about 22 kDa which corresponds to the calculated size of *Et*Def (5 kDa) and 17 kDa THS-tags region of pET32a vector (Fig. 12).

The THS-Tagged-*Et*Def was expressed in both the soluble and insoluble (inclusion bodies) fractions of the bacterial lysate. After purification, the purity of protein was much better when purified from the inclusion bodies. Therefore, the pellet fraction of THS-tagged-*Et*Def inclusion bodies was purified by Ni-NTA chromatography under denaturating condition. The purity of the THS-Tagged-*Et*Def protein was analyzed on SDS-PAGE (Fig. 13). After Ni-NTA purification, the target fusion protein THS-*Et*Def was eluted from the column with 250 mM imidazole. Not all fusion peptides could be bound to the matrix (some were removed from the column by the primary salt washing), but large amount of protein was eluted with 250 mM imidazole. The purity of the fusion protein THS-tagged-*Et*Def was around 85 %. Subsequently, the fusion protein THS-tagged-*Et*Def was refolded (see section 2.4.4) and used for further *in vitro* antifungal assays.

3.3 *In vitro* antifungal activity of fusion protein THS- *Et*Def

Antifungal activity of the fusion protein THS-*Et*Def was evaluated against *B. cinerea* using spore germination inhibition assay in a 96 well microtiter plate. The activity of THS-*Et*Def on spore germination was assessed by incubating fungal spores for 24 h in the presence of various peptide concentrations (0.1, 0.5, 1 and 2 µM). Solutions of purified THS-tags from bacterial cells with empty pET32a(+) vector in similar concentrations were used as negative controls.

Microscopic observations (Fig. 14) revealed that low concentrations of the fusion protein THS-*Et*Def caused a marked reduction in the hyphal growth and elongation, with most observed hyphae exhibited various signs of characteristic branching. The grown hyphae appeared jagged, rough, with dense granulated cytoplasmic contents, and thick cell walls compared to well-developed fungal mycelia in empty vector controls

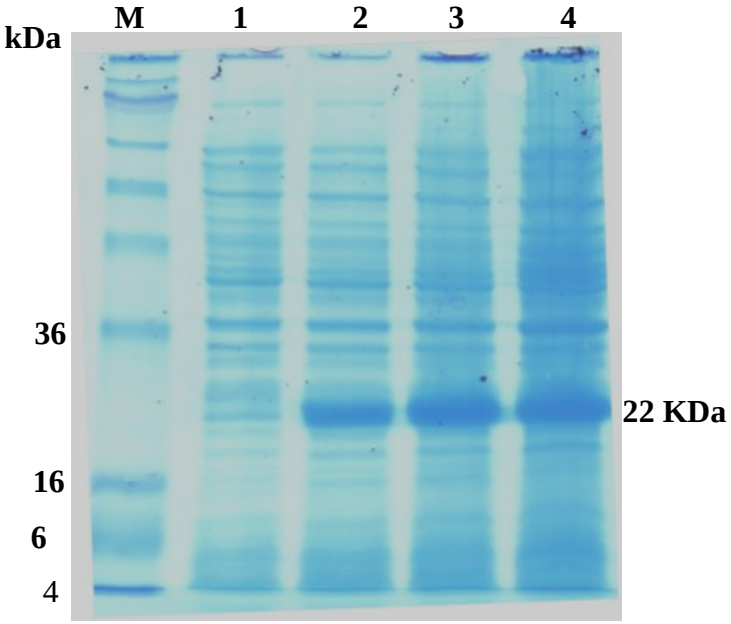


Fig. 12: Coomassie stained SDS-PAGE analysis for the expression of THS-tagged-*Et*Def fusion protein. M, low molecular weight protein marker; lane 1, uninduced *E. coli* BL21/pET32a-*Et*Def; lanes 2, 3 and 4, total protein from induced BL21 cells containing recombinant pET32a-*Et*Def 2, 4, and 6 h after induction with 1 mM IPTG.

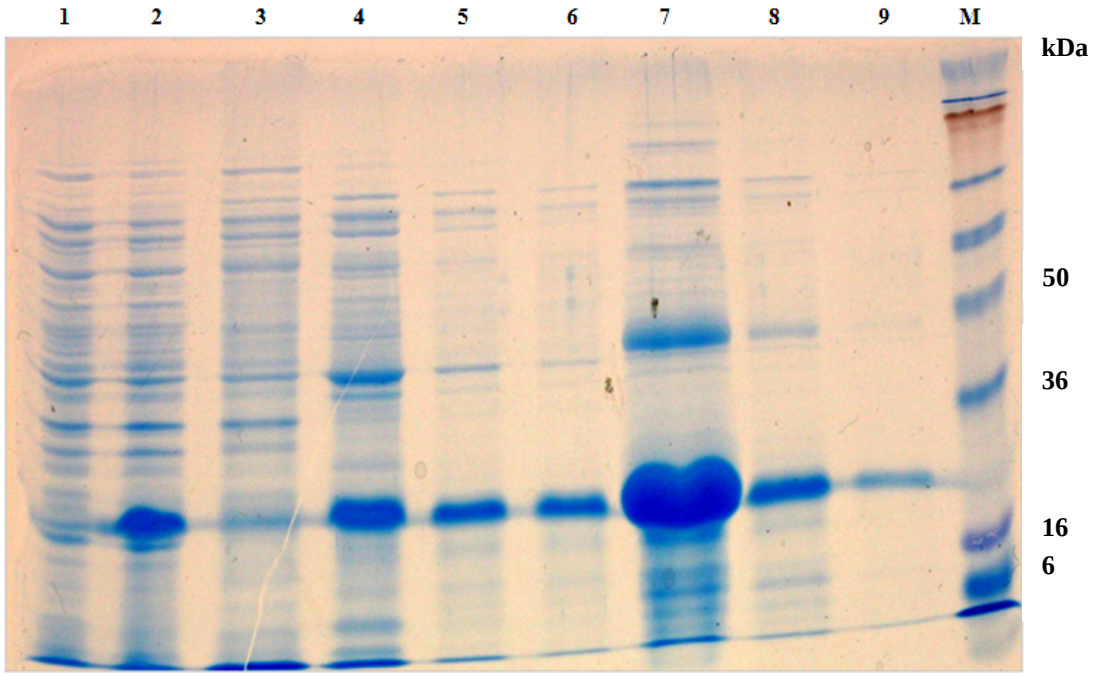


Fig. 13: SDS-PAGE analysis of the expression and Ni-NTA purification of THS-tagged-*Et*Def fusion protein. Lane 1, uninduced BL21/pET32a-*Et*Def; lane 2, total protein expression from induced BL21//pET32a-*Et*Def by IPTG induction (4h); lane 3, flow through; lanes 4, 5 and 6, washing steps; lanes 7, 8 and 9, elution steps; M, low molecular weight protein marker.

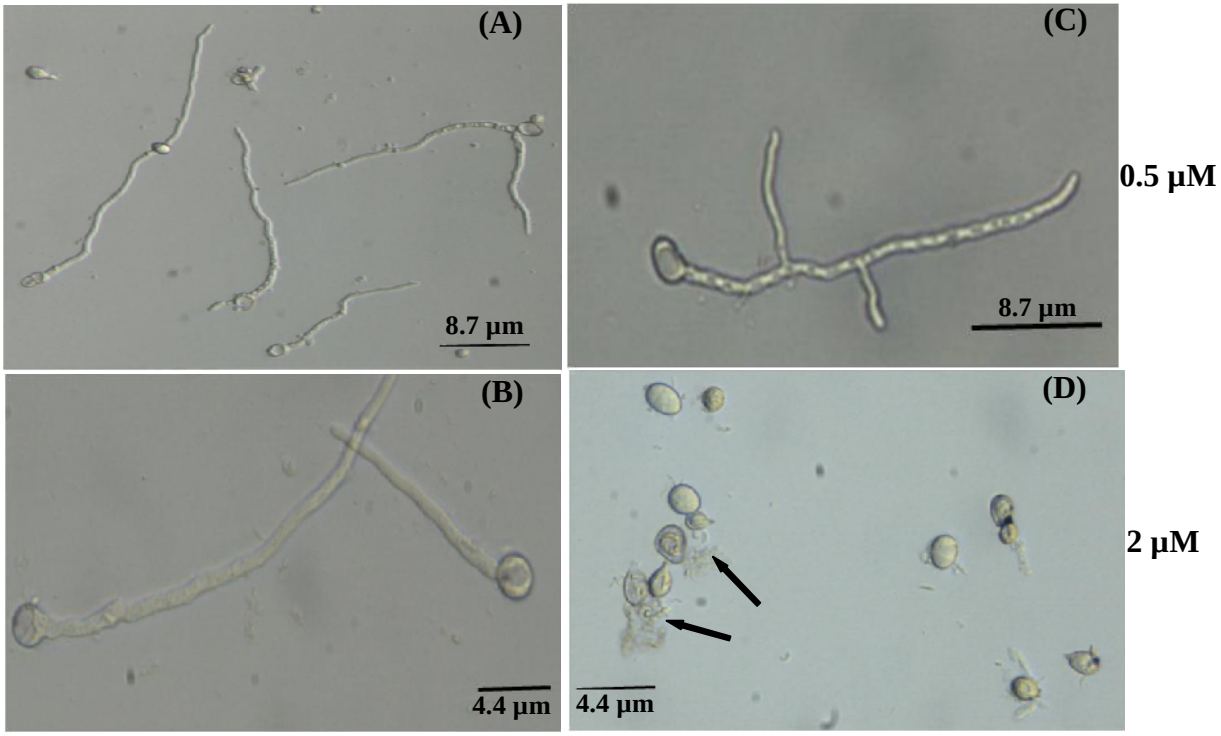


Fig. 14: Effect of various concentrations of THS-tags (A and B), and THS-*Et*Def (C and D) on spore germination of *B. cinerea in vitro*, 24 h after incubation. Arrows indicate extruded cytoplasmic materials surrounding the collapsed spores upon exposure to THS-*Et*Def at a concentration of 2 µM.

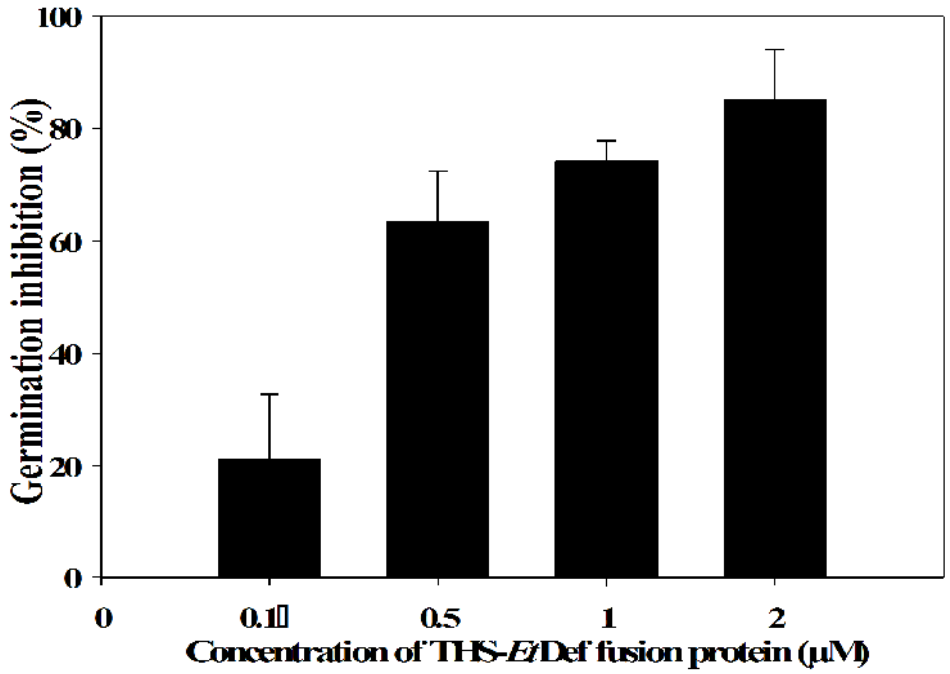


Fig. 15: *In vitro* antifungal activity of various concentrations of fusion protein THS-*Et*Def on the spore germination of *B. cinerea*. Each value represents the mean of three replicates. Bars represent the standard error.

(Fig. 14C). As little as 0.5 µM of the THS-*Et*Def fusion protein was sufficient to inhibit the conidial germination by 50 % (IC_{50}), which is similar to the IC_{50} observed for synthetic *Et*Def peptide. The highest concentration of THS-*Et*Def fusion protein used in this study (2 µM) was able to inhibit about 90 % of spore germination (Fig. 15). Microscopical analysis divulged clearly that these spores appeared swollen, somewhat with different plasmolysis degrees, and release of cytoplasmic materials was observed surrounding the spores (Fig. 14D). Although some conidia (less than 5%) germinated at the highest THS-*Et*Def concentration (2 µM), their germ tubes stopped to grow shortly after the germination.

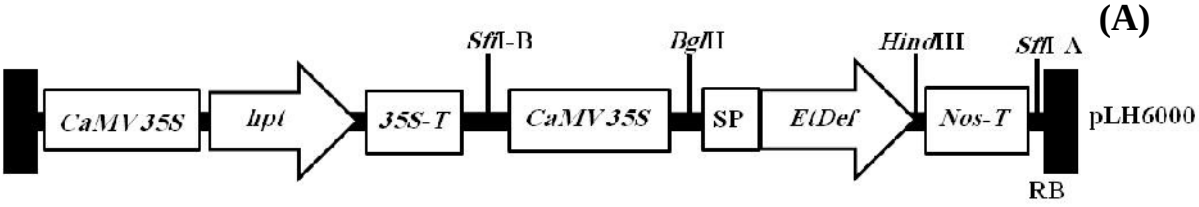
3.4 Transformation of *A. thaliana* with AMP-encoding genes and characterization of transgenic plants

To test whether the insect *Et*Def and thanatin peptides can be functionally expressed in transgenic plants, and targeted to the apoplast, they were transformed into Arabidopsis because of the ease and rapidity with which transgenic plants can be obtained in this species.

The nucleotide sequences encoding the complete ORF of *Et*Def (including its signal peptide) and the chimeric thanatin (including *Hv*Chi26 signal peptide) (Fig. 16 and 17) were inserted into plant expression vector (35S-BM) under the control of enhanced CaMV 35S promoter and nos-terminator. Both expression cassettes were introduced into Arabidopsis via Agrobacterium-mediated transformation using the hygromycin resistance gene as a selectable marker (see section 2.6). The transformation experiments yielded 15 hygromycin-resistant Arabidopsis primary transformants (T_1) with the vector pLH6000 35S::*Et*Def for *Et*Def expression (Lines 391 – 405) and 9 hygromycin-resistant lines with the vector pLH6000 35S::thanatin for thanatin expression (lines 407 – 415). The integration of *Et*Def and thanatin genes into Arabidopsis genome was confirmed by PCR analysis with primers designed to amplify the promoter-transgene region for each construct. Amplicons of 464 bp and 301 bp were observed in the putative transformants overexpressing *Et*Def and thanatin, respectively. No amplification was observed in non-transformed Arabidopsis Col-0 plant.

*Et*Def and thanatin transgenic plants did not show any phenotypic and/or growth behavior differences relative to their wild type plants (Col-0).

(A)



(B)

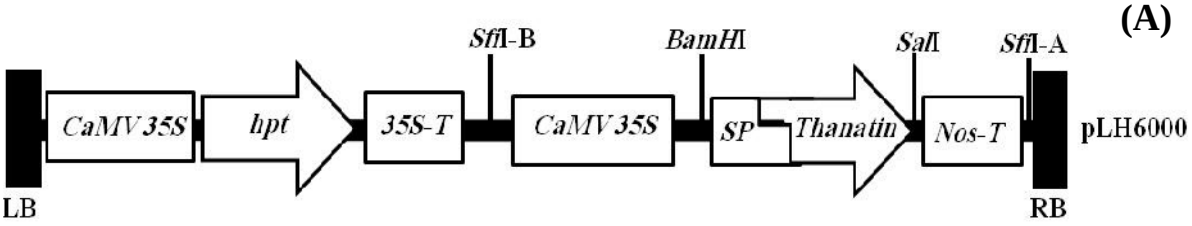
***GGATCC*ATGCGTGCCAGTATCGCAATAACTTTTCTGTTCGGACTCGCCCTGTGCGTTGT**
GTGTTCAGCCGTGCCCGTGGTGGATCCGGAAGCAGCTGCCGAACTCGAATTTGTTGGTG
AGCATGATCTGGAAGCAGTCGCCGACGGAACTGGACTCCGTCAGAAGCGTGCTACATGT
GATCTGCTAAGTTTCTTGAATGTGAAGGATGCTGCTTGTGCTGCTCATTGCTTAGCGAA
GGGATACAGAGGAGGATACTGTGACGGCAGGAAAGTCTGCAATTGCCGGCGTTAA*AAGC*
TT

(C)

MRASIAITFLFGLALCVVCSAVPVVDPEAAAELEFVGEHDLEAVADGTGLRQKRATCDL
LSFLNVKDAACAAHCLAKGYRGGYCDGRKVCNCRR*

Fig. 16: Schematic diagram of the T-DNA construct used for *Et*Def plant transformation. (A), cassette of the pLH6000-*Et*Def vector for *Et*Def expression. (B), nucleotide sequence of *Et*Def ORF (signal peptide, propeptide, and mature peptide) with *Et*Def sequence underlined. Note that the incorporated restriction enzyme sites are shown in italic. (C), whole amino acid sequence of *Et*Def peptide, with signal peptide (yellow), propeptide (turquoise) and mature peptide (underlined). RB, right border; LB, left border; 35S, promoter from Cauliflower Mosaic Virus (CaMV); NOS-T, nopaline synthase terminator; hpt, hygromycin phosphotransferase gene conferring hygromycin resistance. The asterisk sign indicates stop codon.

(A)



(B)

***GGATCC*GGATCCATGAGATCGCTCGCGGTGGTGGTGGCCGTGGTAGCCACGGTGGCCAT**
GGCCATCGGCACGGCGCGCGGCGGCTCCAAGAAGCCGGTCCCGATCATCTACTGCAACC
GCCGCACCGGCAAGTGCCAGCGCATGGTGA*GTCGAC*

MRSLAVVVAVVATVAMAIGTARGGSKKPVPIIYCNRRTGKCQRM* **(C)**

Fig. 17: Schematic diagram of the T-DNA construct used for thanatin plant transformation. (A), cassette of the pLH6000-thanatin vector for thanatin expression. (B), nucleotide sequence of the *Hv*-chitinase signal peptide (*Hv*Chi26)-thanatin open reading frame, with thanatin sequence underlined. Note that the incorporated restriction enzyme sites are shown in italic. (C), whole amino acid sequence of *Hv*Chi26-thanatin, with mature thanatin sequence underlined. RB, right border; LB, left border; 35S, promoter from Cauliflower Mosaic Virus (CaMV); NOS-T, nopaline synthase terminator; hpt, hygromycin phosphotransferase gene conferring hygromycin resistance. The asterisk sign indicates stop codon.

Inheritances of both transgenes were studied by testing the germination of seeds obtained from T_1 self-pollinated plants of each construct on media containing hygromycin. A 3:1 segregation for resistance to hygromycin antibiotic was observed in most of the progenies of each construct indicating a single copy insertion. After self-pollination of the T_2 lines, six *Et*Def transgenic lines (394, 395, 396, 398, 401, and 405), and four thanatin transgenic lines (407, 408, 410, and 411) were selected as homozygous lines from the T_3 generation for further investigations.

3.5 Expression pattern of *Et*Def and thanatin genes in transgenic Arabidopsis plants

Expression analyses of *Et*Def and thanatin were performed by a reverse transcriptase PCR (RT-PCR) using RNA from T_1 hygromicin resistant plants for each construct. Results shown in Fig. 18A and B (upper panels) revealed that *Et*Def and thanatin genes are efficiently transcribed into an mRNA by detecting specific amplicons of the expected sizes (301 and 149 bp, respectively) in their transgenic lines at different levels. Specific ubiquitin (UBiQ5) transcript amplification was detected in all plants as an internal control for cDNA synthesis (Fig. 18A and B, lower panels).

To quantify the level of *Et*Def and thanatin transcripts generated from the CaMV 35S promoter, six *Et*Def and five thanatin transgenic lines (T_1) were analyzed using quantitative real time RT-PCR and used for subsequent bioassays. Compared to the housekeeping gene UBiQ5, three *Et*Def transgenic lines, namely 395, 396 and 405 exhibited noticeably high mRNA level (Fig. 19A). As for thanatin, the highest transcript level was observed in the transgenic line 411 followed by line 410 (Fig. 19B). The same trend of mRNA expression level of both *Et*Def and thanatin was also observed in the T_3 homozygous transgenic lines.

3.6 *In vitro* antifungal activity of leaf extracts and intercellular washing fluids (IWFs) of Arabidopsis transgenic plants

The estimation of spore germination or mycelia growth using crude protein extracts from transgenic plants is commonly used in determining the antifungal activity (Langen *et al*., 2006).

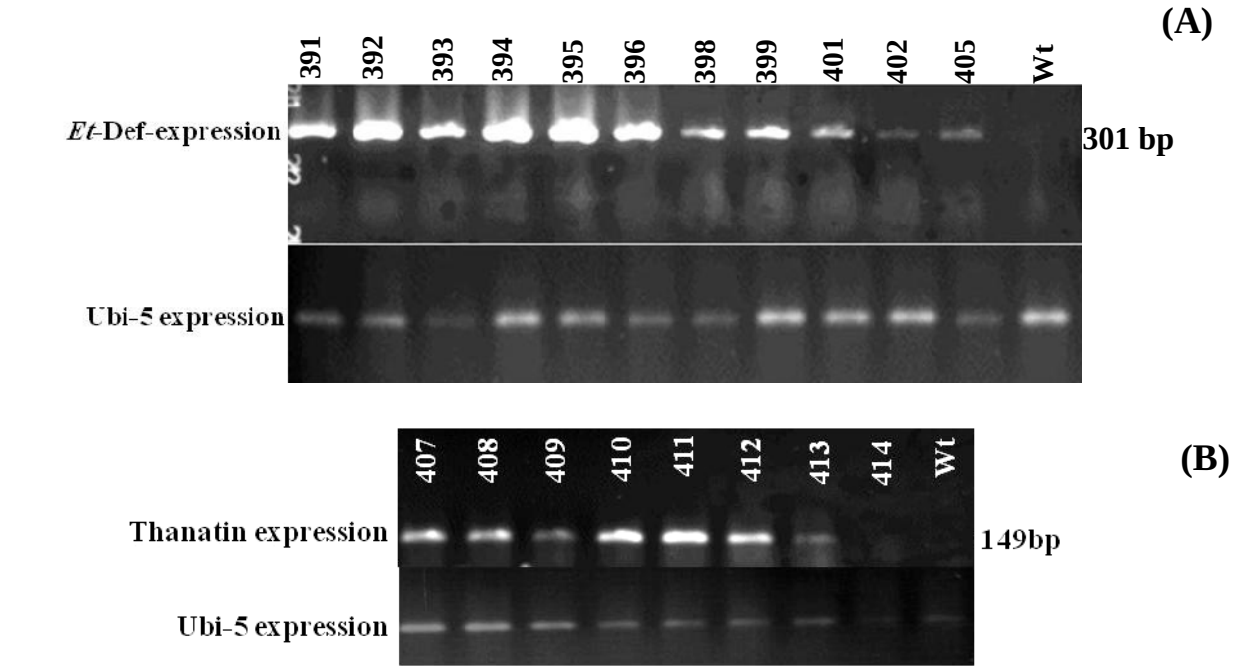


Fig. 18: Expression of *Et*Def and thanatin genes in transgenic Arabidopsis plants. RT-PCR, specific for different transcripts were performed with sets of specific primers (see section 2.8.3.2) from leaf total RNA of T_1 transgenic Arabidopsis plants transformed with (A) *Et*Def and (B) thanatin (upper panel) compared with expression level of housekeeping gene UbiQ5 (lower panel). Wt: Col-0, numbers: respective No. of transgenic lines.

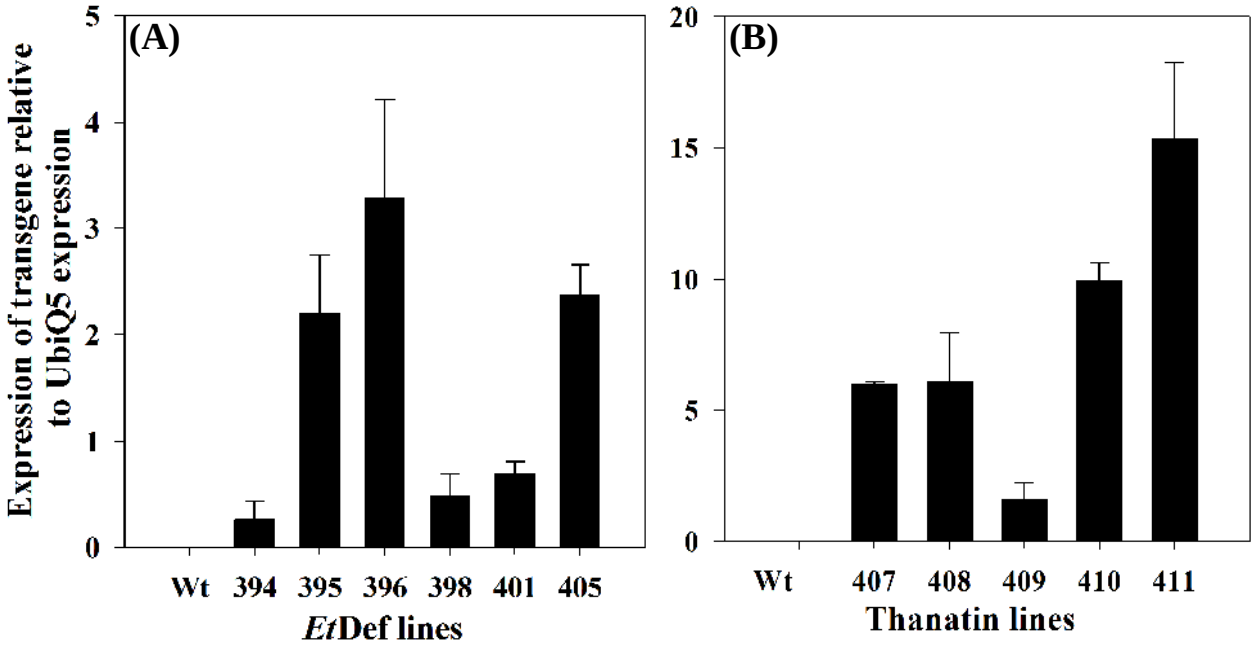


Fig. 19: qRT-PCR analysis of *Et*Def (A), and thanatin (B) of T_1 transgenic Arabidopsis. *Et*Def and thanatin expressions under the control of 35S-CaMV promoter were analyzed in independent transgenic lines. No specific amplification product could be detected in the wild type (Wt) Arabidopsis. Each value represents the mean of three replicates ± SE.

In this study, leaf extracts from 5-week-old transgenic plants (T_1) overexpressing either *Et*Def or thanatin were evaluated against *B. cinerea in vitro*. Fungal mycelia grown on agar blocks were incubated with leaf extracts from each construct for 24 h. Subsequently, the agar blocks were transferred to fresh agar plates and the outgrowth of the mycelium was measured 24 h later. Results of this investigation revealed that leaf extracts from *Et*Def transgenic lines did not show significant reduction in mycelial growth of *B. cinerea* compared to the transgenic control (#14) (Figs. 20, 21). However, leaf extracts from thanatin transgenic lines showed comparatively higher depressive effect on the mycelial growth of *B. cinerea* than *Et*Def, resulting into 45 % reduction in the mycelial growth compared to transgenic control (Figs. 20 and 21).

To verify the secretion of *Et*Def and thanatin into the apoplast of each transgenic line, IWF extracts were prepared from the leaves of transgenic and non-transgenic control plants (see section 2.8.5) and separated by Tricin-SDS-PAGE. After silver staining, an additional band corresponds to the calculated size of thanatin was detected only in the IWF of the transgenic line 410, whereas no signal was found in the IWF of other transgenic and non-transgenic control as well (data not shown).

The antifungal activity of IWFs extracted from homozygous transgenic lines expressing either *Et*Def or thanatin as well as from non-transgenic Arabidopsis Col-0 was assessed against *B. cinerea* using spore germination assay. Microscopical investigations showed that IWFs from both *Et*Def and thanatin transgenic lines led to considerable alterations in the conidial germination of *B. cinerea* as compared with their relative controls (Fig. 22). These alterations include a complete germination inhibition of spores (Figs. 22 D and G), growth abnormalities in the germ tube of the germinated spores (Figs. 22 E and H), and reduced hyphal growth and elongation associated with increasing dichotomous branching (Figs. 22 F and I), compared with thin, well-elongated and extended hyphae treated with IWF from non-transgenic control Col-0 (Fig. 22 A-C). Interestingly, among all transgenic plants, IWFs from lines with highest mRNA expression level of *Et*Def (396, 395, and 405) and thanatin (411, 410, and 407) showed distinctly the strongest inhibitory effect on spore germination of *B. cinerea* (Fig. 23 A and B).

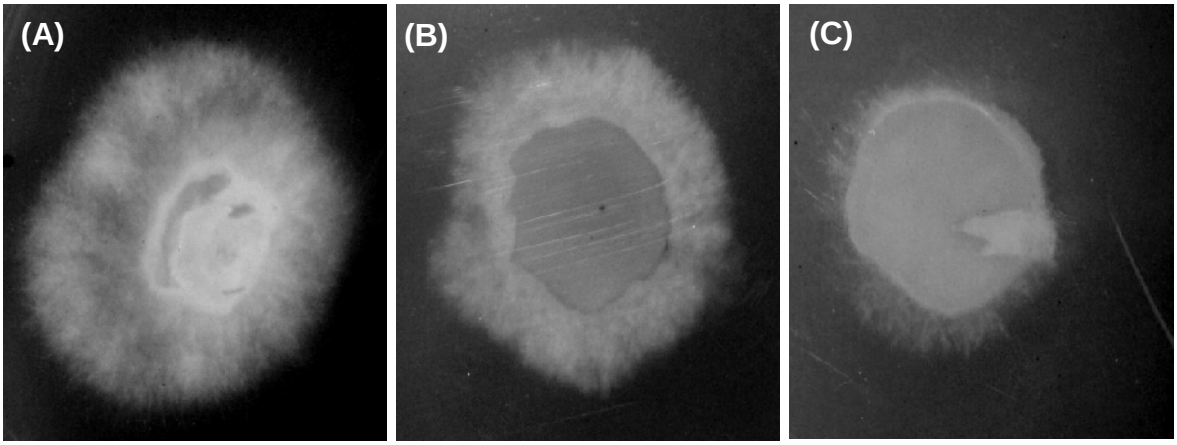


Fig. 20: *In vitro* antifungal activity of leaf extracts from Arabidopsis transgenic control plants #14 (A), *Et*Def transgenic lines (B), and thanatin transgenic lines (C) against *B. cinerea*. Agar blocks with fungal mycelium were incubated for 24 h with leaf sap and transferred afterwards to new agar plates. The hyphal growth retardation was evaluated 24 h later.

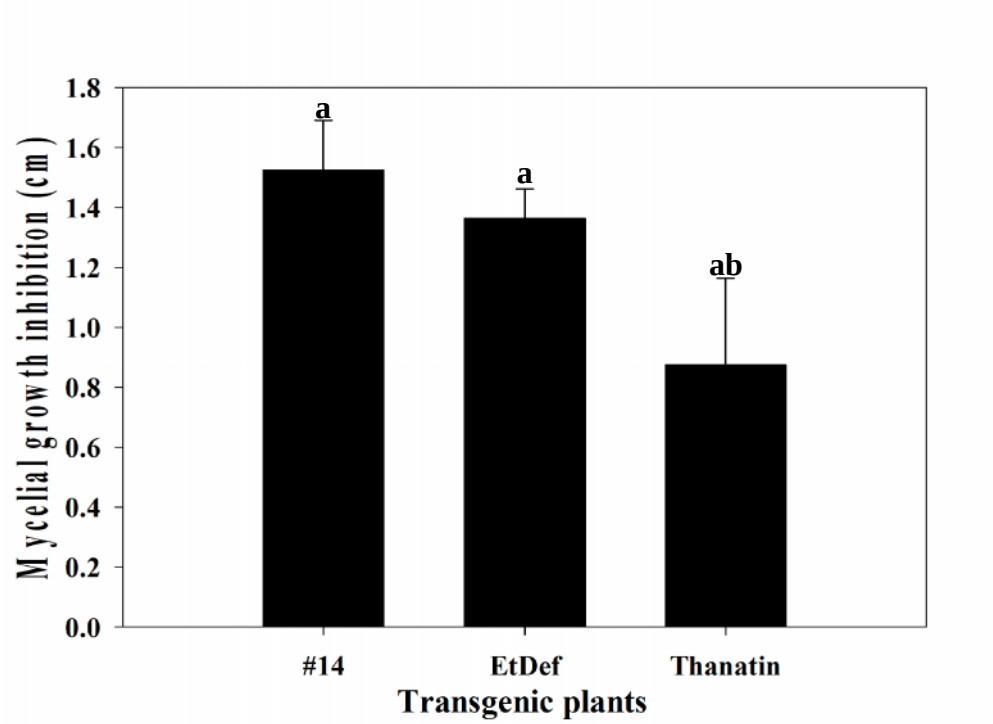


Fig. 21: Growth inhibition of *B. cinerea* mycelia upon treatment with leaf extracts prepared from T_1 *Et*Def and thanatin transgenic lines compared to transgenic control (#14). Two individual transgenic lines were pooled and extracted for each treatment. Values represent the mean and the standard errors of six replicates after 24 h

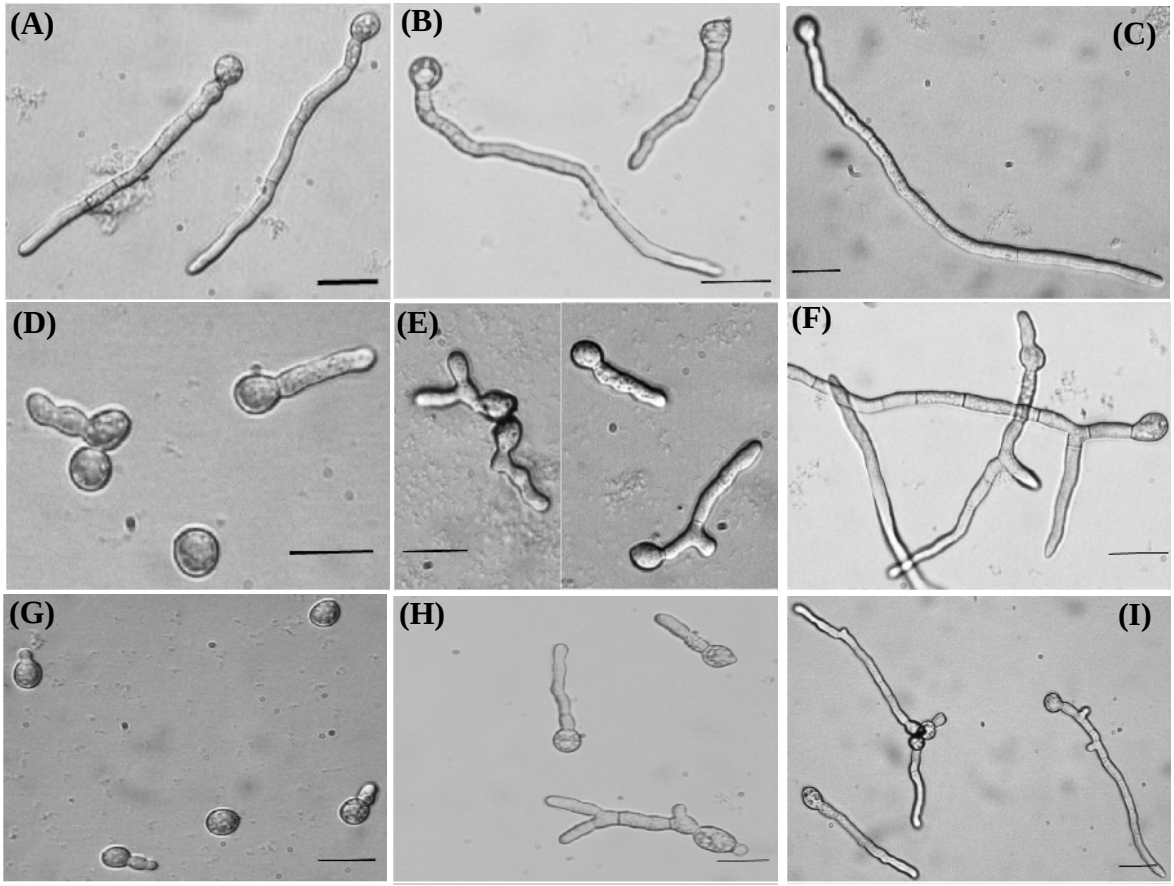


Fig. 22: Representative micrographs of *B. cinerea* conidia after 24 h incubation with IWFs (20 µg/µL) from non-transgenic plants Col-0 (A-C), *Et*Def transgenic plants (D-F), and thanatin transgenic plants (G-I). Bars = 4.4 µm.

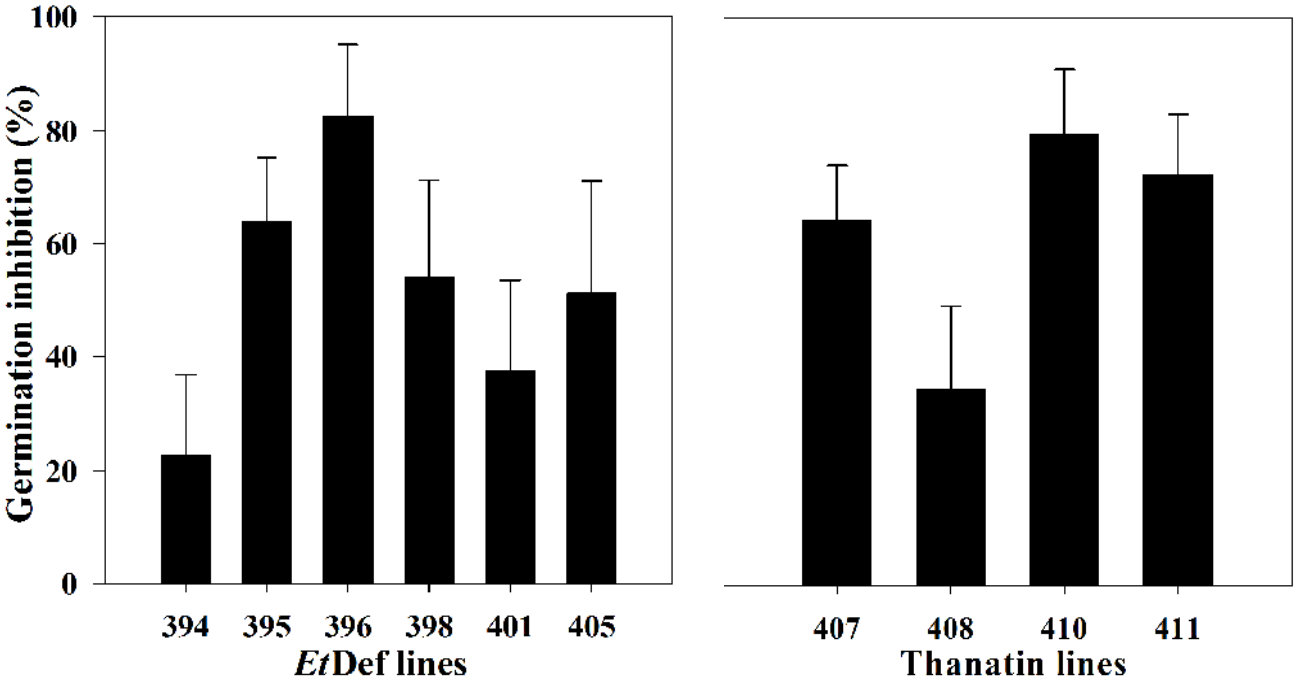


Fig. 23: Percentage of germination inhibition of *B. cinerea* spores after 24 h incubation with intercellular washing fluid (IWFs) (at protein concentration of 20 µg/µL) obtained from leaves of T_3 homozygous transgenic *Et*Def lines (A), and thanatin lines (B) compared to non-transgenic Col-0 (Wt). Values represent the mean of three replicas and the bars represent the standard errors.

3.7 Evaluation of disease resistance in transgenic Arabidopsis plants

To determine whether the constitutive expression of *Et*Def and thanatin could confer resistance against *G. orontii* (biotrophic fungus), *B. cinerea* (necrotrophic fungus), and *P. syringae* in Arabidopsis, six independent T_3 homozygous *Et*Def transgenic lines (394, 395, 396, 398, 401, and 405) and four homozygous thanatin transgenic lines (407, 408, 410, and 411) were evaluated. Plants transformed with the pLH6000 empty vector (#14) as well as non-transgenic plant (Col-0) were used as controls.

3.7.1 *In planta* resistance against *G. orontii*

A total of ten T_3 homozygous 5-week-old soil-grown Arabidopsis plants overexpressing either *Et*Def or thanatin were challenged with a suspension of *G. orontii* conidial spore ($5x10^5$ conidia mL^{-1}) to evaluate the resistance degree mediated by expressing each gene. Ten days after the inoculation, the disease symptoms were recorded. On average, the spread of hyphae as well as the conidial sporulation were remarkably declined on the rosette leaves of all *Et*Def (Fig. 24) and thanatin (Fig. 26) transgenic lines in comparison to the non-transgenic controls (Col-0). The resistance of transgenic plants was further determined by counting the number of conidiophores formed per fungal colony, 5 dpi and by counting spore numbers produced on infected leaves at 10 dpi. Data in Fig. 25A indicate that the number of conidiophores formed per fungal colony was significantly ($P \leq 0.05$) reduced in all *Et*Def lines relative to Col-0 (wild type). This effect was accompanied with a marked reduction in the number of spores formed on the leaves of all *Et*Def transgenic lines compared with the non-transgenic plants at 10 dpi (Fig. 25B).

Regarding thanatin, the transgenic lines 407, 410 and 411 exhibited significantly ($P \leq 0.05$) lower numbers of conidiophores per fungal colony as compared to non-transgenic and transgenic controls (Fig. 27A). Additionally, the number of spores formed was also significantly ($P \leq 0.05$) reduced in these transgenic lines compared with non-transgenic and transgenic controls (Fig. 27B).

Fig. 24: Powdery mildew development on rosette leaves of non-transgenic control Col-0 (Wt), and different *Et*Def transgenic lines 10 dpi with conidial spores of *G. orontii*.

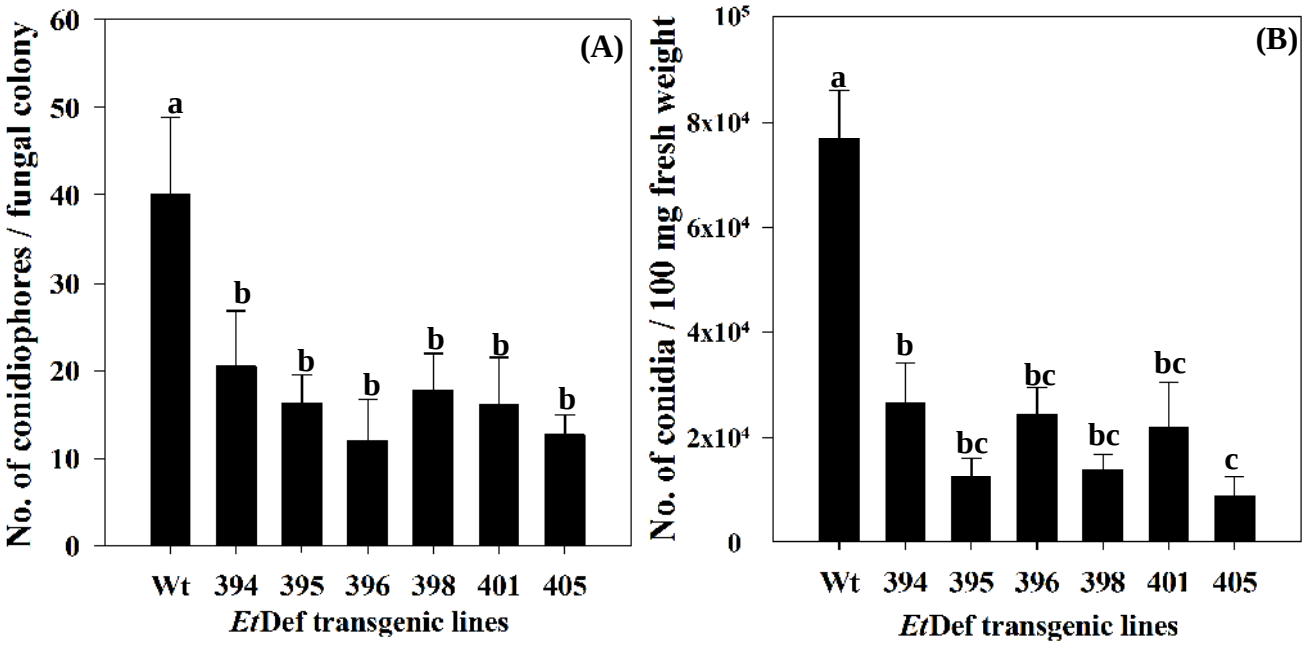


Fig. 25: *In planta* assay of antifungal activity of *Et*Def Arabidopsis transgenic lines against powdery mildew. (A), evaluation of conidiophore numbers after 5 dpi, and (B) conidial numbers after 10 dpi with *G. orontii*. Means are ratings of fungal development on 10 plants. Bars represent the standard error. Different letters indicate data sets significantly different at $P \leq 0.05$.

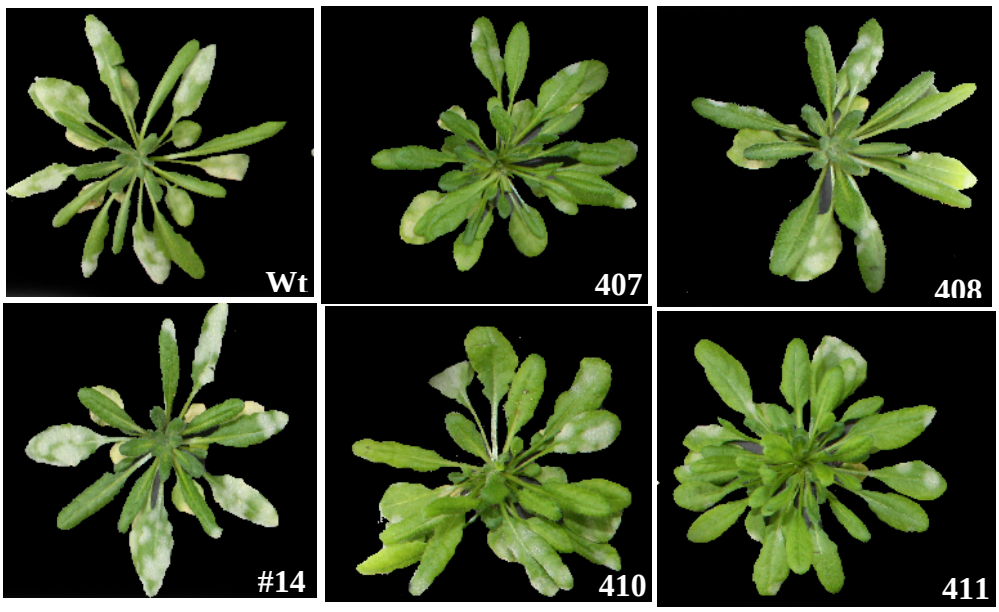


Fig. 26: Powdery mildew development on rosette leaves of non-transgenic control plant (Wt), transgenic control (# 14), and different thanatin transgenic lines 10 dpi with conidial spores of *G. orontii*.

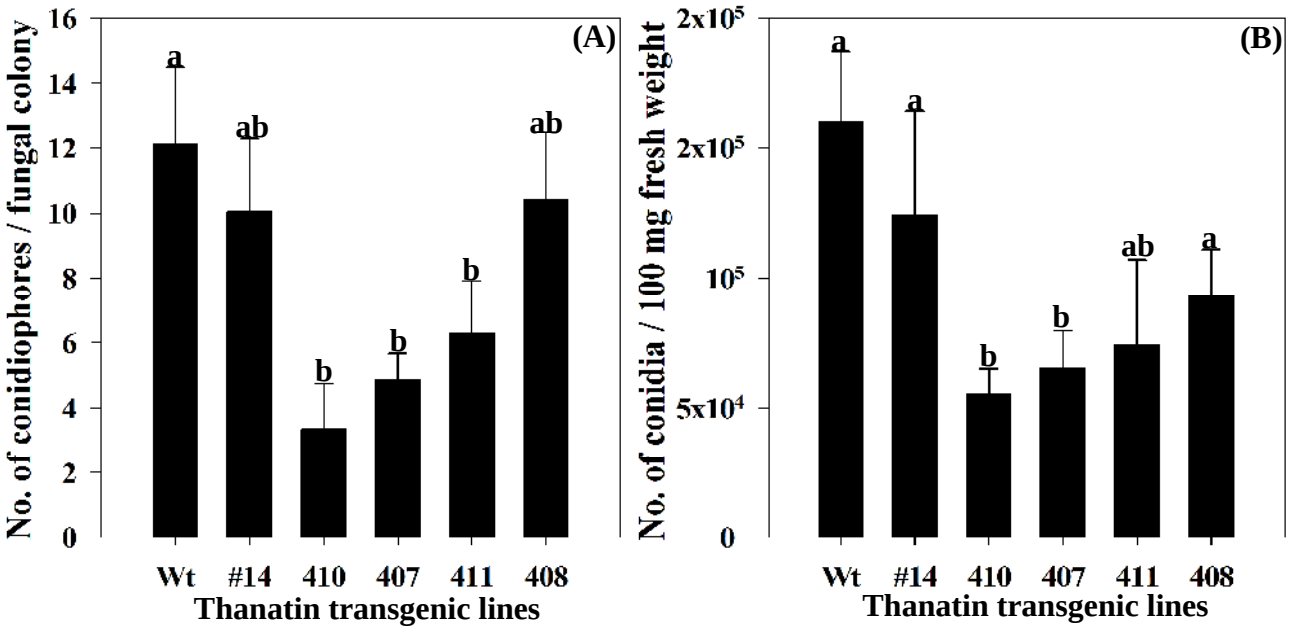


Fig. 27: *In planta* assay of antifungal activity of thanatin Arabidopsis transgenic lines against powdery mildew. (A), evaluation of conidiophore numbers after 5 dpi, and (B) conidial numbers after 10 dpi with *G. orontii*. Means are ratings of fungal development on 10 plants. Bars represent the standard error. Different letters indicate data sets significantly different at $P \leq 0.05$.

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

B. cinerea is the causal agent of grey mold on a broad-spectrum of host plants. To assess whether the expression of *Et*Def and thanatin in homozygous transgenic Arabidopsis could improve resistance against *B. cinerea*, detached leaves from 5-week-old Arabidopsis plants overexpressing either *Et*Def or thanatin as well as from non-transgenic (Col-0) and transgenic control (#14) were inoculated with *B. cinerea* conidia suspension ($2x10^4$ conidiospores mL^{-1}) according to Ferrari *et al*. (2003). The disease symptoms (i.e. necrotic lesions and leaf yellowing to different degrees) started to appear three days after the inoculation. The lesion diameter was recorded 5 days after the inoculation. Expectedly, non-transgenic controls (Col-0) showed the typical symptoms on all inoculated leaves (Fig. 28). Similarly, all *Et*Def transgenic lines showed these typical symptoms except for the transgenic line 405, where the diameters of necrotic lesions were significantly ($P \leq 0.05$) lowest (Figs. 28 and 29A). As for thanatin transgenic lines, typical necrotic lesions were formed on the leaves of all transgenic lines under the study. However, the transgenic lines 410, and 411 exhibited lesions of distinctly smaller size which remained for longer than 5 days without any further increase in their diameters (Figs. 28 and 29B).

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

Resistance of selected homozygous 5-week-old soil-grown Arabidopsis plants expressing either *Et*Def or thanatin against the bacterial pathogen *P. syringae* pv *tomato* DC3000 (*Pst*) was evaluated. All transgenic lines, non-transgenic (Col-0) and transgenic control (# 14) were inoculated by syringe infiltration with bacterial suspension (1×10^5 cfu mL^{-1}). Typical necrotic lesions with chlorosis spreading out from lesions in areas of inoculation were noted on the leaves of non-transgenic Col-0 plants, transgenic control plants (#14), and all *Et*Def transgenic lines 4 days after infection, except for plants of line 405 which showed rarely mild chlorosis. Regarding thanatin, the transgenic lines 407, 410, and 411showed comparatively mild chlorosis. However, no difference in disease symptoms between transgenic plants of line 408 and their relative controls was observed.

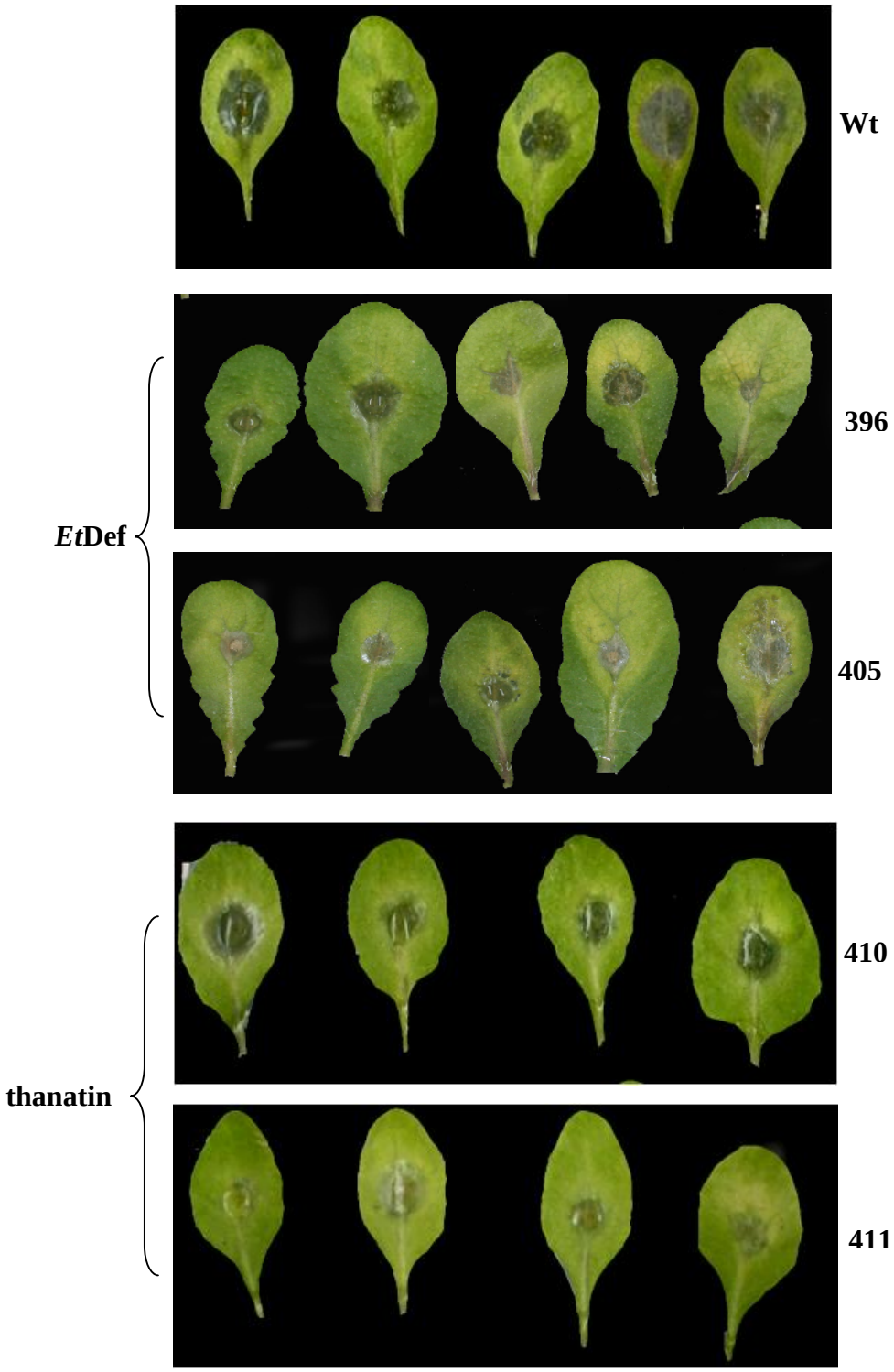


Fig. 28: Disease symptoms of *B. cinerea* evaluated 4 days after the inoculation on non-transgenic control Arabidopsis Col-0 (Wt), representative transgenic lines expressing *Et*Def (lines 396 and 405), and thanatin (lines 410 and 411).

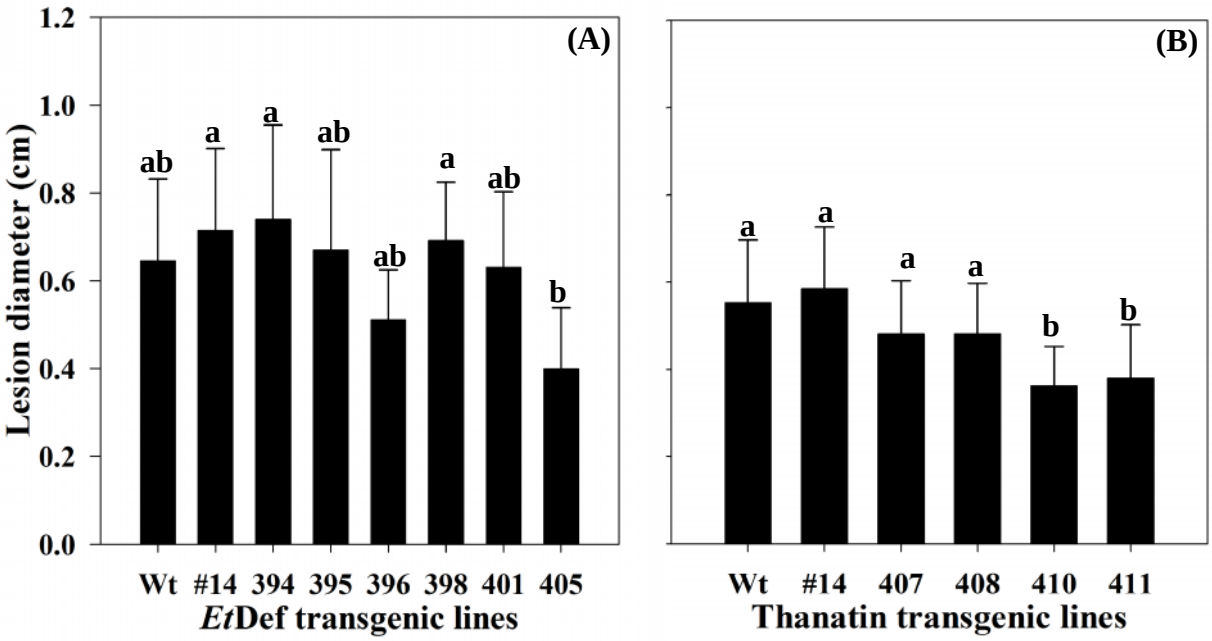


Fig. 29: *In planta* antifungal activity of *Et*Def and thanatin against *B. cinerea* inoculation. Mean necrotic lesions on the leaves of different *Et*Def Arabidopsis transgenic lines (A), and thanatin transgenic lines (B) as compared to non-transgenic Col-0 (Wt) and transgenic control (#14) 5dpi. The results are from one representative of two experiments and are averages of 30 leaves from 10 plants per line. Different letters indicate data sets significantly different at $P \leq 0.05$.

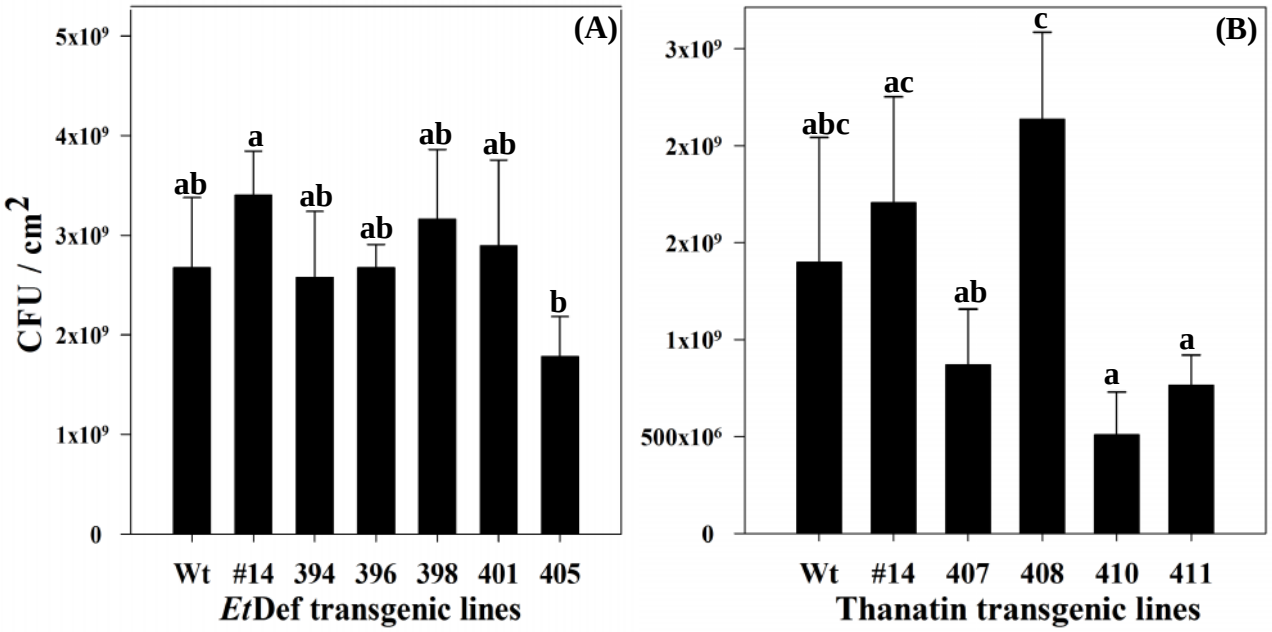


Fig. 30: *In planta* antibacterial activity of *Et*Def and thanatin against *P. syringae* pv *tomato* DC3000. Bacterial cell numbers was measured in leaf tissues of Arabidopsis transgenic lines overexpressing *Et*Def (A) and thanatin (B) compared to non-transgenic Col-0 (Wt) and transgenic control (#14) 4 dpi. Each value represents the mean of five replicates ± SE. Each experiment was repeated twice. Different letters indicate data sets significantly different at $P \leq 0.05$.

The number of bacterial cells in the injected regions was additionally quantified (see section 2.9.3). As shown in Fig. 30, bacterial cell numbers at 4 dpi were not significantly changed in all *Et*Def transgenic lines in respect to both controls, except for the transgenic line 405 where the bacterial cell numbers were 35 % and 48 % lower compared to non-transgenic and transgenic controls respectively (Fig. 30A). Concerning thanatin transgenic lines, the bacterial cell numbers were significantly ($P \leq 0.05$) lowest in line 410 followed by 411 and 407. The transgenic line 410 displayed about 66 % and 72 % reduction in the bacterial population compared with non-transgenic and transgenic controls, respectively (Fig. 30B).

4 Discussion

Modern agriculture is still highly dependent on chemical microbicides to control phytopathogens that continuously threaten agricultural production worldwide. Due to the increasing resistance of plant pathogens to the currently available antimicrobial agents and the emerging need to eliminate toxic chemicals from the agricultural use, developing disease-resistant transgenic plants using genes encoding AMPs could be a potential alternative (Vilcinskas and Gross, 2005; Montesinos, 2007). Research interests on AMPs have drastically increased because of their wide range of activities, and recently there is a huge number of reports in which antimicrobial peptides from plants (Gao *et al*., 2000; Kanzaki *et al*., 2002; Park *et al*., 2002; Li *et al*., 2003), insect (Osusky *et al*., 2000; Vilcinskas and Gross, 2005; Yevtushenko *et al*., 2005; Langen *et al*., 2006; Rahnamaeian *et al*., 2009), frog (DeGray *et al*., 2001; Chakraborti *et al*., 2003; Osusky *et al*., 2004; Osusky *et al*., 2005; Vidal *et al*., 2006; Yevtushenko and Misra, 2007) or mammalian (Zakharchenko *et al*., 2005; Aerts *et al*., 2007) have been used to render the transformed plants more resistant to phytopathogens. Due to their glorious history in protecting their hosts durably against different pathogens, insect AMPs attracted the attention and have been promoted as potent inhibitors of phytopathogens during the last decade (Osusky *et al*., 2000; Vilcinskas and Gross, 2005).

Toward this end, we aimed in the present study to investigate the feasibility of using the novel insect antimicrobial peptide *Et*Def from *Eristalis tenax* larvae (Altincicek and Vilcinskas, 2007) and the well-known, potent AMP thanatin from *Podisus maculiventris* (Fehlbaum *et al*., 1996) to engineer, for the first time, disease-resistance in Arabidopsis against devastating microbial plant pathogens. A prerequisite for the application of these peptides is the precise knowledge about their biological *in vitro* activity and efficacy. Hence, preliminary antifungal assays were performed *in vitro* with the chemically synthesized *Et*Def and thanatin on the spore germination of ascomycetes (*F. culmorum* and *B. cinerea*) and an oomycete (*P. parasitica*).

Figs. 3A, 5A and 7A showed clearly that increasing synthetic *Et*Def concentrations inhibited significantly the spore germination of all studied fungi, with minimal inhibitory concentrations (MICs) varying with the tested fungal pathogen. The MICs

averaged between 5 – 10, 1 – 2, and 5 – 10 µM for *F. culmorum*, *B. cinerea* and *P. parasitica* respectively (Table 2). Antifungal activity of *Et*Def (either *in vitro* or *in vivo*) was tested for the first time in this study, but *in vitro* antifungal activities of other insect defensins against several pathogens have been reported such as drosomycin (Fehlbaum *et al*., 1994), heliomicin (Lamberty *et al*., 1999), termicin (Lamberty *et al*., 2001) and gallerimycin (Schuhmann *et al*., 2003). However, these studies were performed using recombinant defensins.

Similar effects were also found for thanatin on the spore germination of all tested fungi, though, with slightly higher efficacy as compared to *Et*Def (Fig. 3B, 5B and 7B). The minimal inhibitory concentrations were ranged between 5 – 10 µM for *F. culmorum*, 0.5 – 1 µM for *B. cinerea*, and 2 – 5 µM for *P. parasitica* (Table 2). thanatin has been also previously found to be *in vitro* active against *F. culmorum* and *B. cinerea*, however, with slightly lower MIC ranged between 1.2 – 5 for *F. culmorum*, but distinctly higher MIC being 2.5 – 5 µM for *B. cinerea* (Fehlbaum *et al*., 1996). This may be caused by different experimental conditions.

Fungal spore germination and hyphal growth in response to synthetic *Et*Def and thanatin were also monitored microscopically. Photomicrographs showed clearly that spore germination of all fungi was impeded as the peptide concentration rose. At the highest peptide concentrations, spore germination was completely arrested and no hyphae were observed for any of the tested fungi (Fig. 2, 4 and 6). Importantly, low to moderate concentrations of *Et*Def led, unambiguously, to ceased or delay in growth of germ tube, resulting into many morphological abnormalities in their cell walls as compared to the relative controls, particularly in *F. culmorum* (Fig. 2). Abnormal morphological changes have been also reported for fungal hyphae and spores upon exposure to antifungal proteins and are commonly observed in *in vitro* assays (Collinge *et al*., 1993; Lorito *et al*., 1993; Fehlbaum *et al*., 1994; Osborn *et al*., 1995; Terras *et al*., 1995; Cavallarin *et al*., 1998; Ali and Reddy, 2000). Results of the current study implies that *Et*Def may interact with the fungal membrane leading to membrane disruption and destabilization as has been proposed for many other defensins such as plant defensins and defensin-like peptides from insects (Thevissen *et al*., 1996; Hwang and Vogel, 1998; Thevissen *et al*., 1999, 2000, 2004). Nevertheless, these characteristic features were not observed for thanain.

Table 2: *In vitro* antifungal activity spectrum of synthetic *Et*Def and Thanatin on some fungal plant pathogens.

Phytopathogens	IC_{50} (µM)		MIC (µM)	
	*Et*Def	thanatin	*Et*Def	thanatin
F. culmorum *B. cinerea* *P. parasitica*	2 0.5 1	2.6 0.1 1 – 2	5 – 10 1 – 2 5 – 10	5 – 10 0.5 – 1 2 – 5

IC_{50}, peptide concentration that leads to reduce the spore germination by 50%; MIC, minimal inhibitory concentration.

These *in vitro* antifungal assays reflect the potential of both *Et*Def and thanatin for enhancing disease resistance of plants via the transgenic approach. However, the high cost of producing large amounts of synthetic antimicrobial peptides prohibits their direct utilization in phytopathogen control or for large *in vitro* screenings and mode of action studies. Thus, it was attempted to establish, for the first time, a method for the production of recombinant *Et*Def in the *E. coli* expression system to obtain sufficient quantities required for detailed biological *in vitro* and *in vivo* assays on its activity spectrum.

In this study, all expression experiments were conducted using *E. coli* BL21 (DE3) as expression host, since this strain encodes the T_7 RNA polymerase and can be utilized for protein expression under the control of a T_7 promoter. Primarily, we have cloned *Et*Def mature peptide (consisting of 40 amino acids) with an added C-terminal part with V5-epitope and His-tag in the expression vector pCRT7. After Ni-NTA purification, the recombinant target protein (pCRT7-*Et*Def) with an expected molecular mass of 7.6 kDa was detected, though only in a small amount, using both Tricin-SDS-PAGE and western blotting analysis (Fig. 8). This might be largely attributed to the antibacterial activity of *Et*Def against *E. coli* and/or the susceptibility of peptide to proteolytic degradation (Piers *et al*., 1993; Makrides, 1996; Zhou *et al*., 2009). As has been previously reported, fusion expression of the target protein with a partner may diminish the toxic effects of recombinant protein on the host cells and prevent target peptide from proteolytic degradation (Piers *et al*. 1993; LaVallie and McCoy 1995; LaVallie *et al*., 2000; Arnau *et al*. 2006). Therefore, thioredoxin (TrxA) was employed in this study as a fusion partner to alleviate such shortcomings. According to LaVallie and McCoy (1995), LaVallie *et al*. (2000) and Zhou *et al* (2009) TrxA, as a partner protein, could also

accelerate soluble expression of the recombinant target protein. To achieve this, the sequence of mature *Et*Def peptide was inserted in frame downstream of the TrxA gene of pET32a(+) vector which also contains a His-tag for purification and an additional S-tag. After IPTG induction and Ni-NTA purification, the target fusion protein TrxA-His-S-tag-*Et*Def (THS-*Et*Def) with a deduced molecular mass of approximately 22 kDa was successfully detected, apparently, in high quantity (Fig. 12). Unfortunately, again most of the fusion protein was in the insoluble fraction, whereas as expected the THS-tag expressed alone was highly soluble. Therefore, the recombinant peptide was purified under denaturing conditions.

The obtained recombinant THS-*Et*Def was subsequently refolded and its antifungal activity was evaluated *in vitro* against *B. cinerea* using spore germination inhibition assay. Our results showed clearly that recombinant THS-*Et*Def was potent and exerted a similar antifungal activity to the chemically synthesized counterpart. Elevating THS-*Et*Def concentrations caused a considerable reduction in the spore germination (Fig. 14 and 15). This indicates that the presence of the tag, which is bigger than the AMP peptide, didn't much alter the activity of *Et*Def *in vitro*. Therefore, no cleavage of tag from the AMP was attempted in this study. Similar effects were observed for recombinant drosomycin and termicin on the spore germination of *F. culmorum*, *F. oxysporum*, *N. hematococca* and *N. crassa* (Fehlbaum *et al*., 1994, Lamberty *et al*. 2001). Additionally, recombinant heliomicin and drosomycin (at concentrations of 40 µg mL^{-1}) were found to inhibit the spore germination of *F. culmorum* and *B. cinerea*, while antifungal activities of these peptides were weak on the mycelial growth of *B. cinerea* (Banzet *et al*., 2002). Light microscopic investigations demonstrated that low THS-*Et*Def concentrations (0.5 µM) led to reduction in hyphal growth and elongation, but increased their branching compared to the controls. Most hyphae appeared rough with dense and granulated cytoplasmic contents, separated from the cell wall (Fig. 15). Although still some spores (less than 5%) were germinated at the highest tested recombinant *Et*Def concentration (2 µM), their germ tubes apparently stopped to grow and extruded cytoplasmic material was observed surrounding them (Fig. 15). Such lytic effects, frequently reported for AMPs, were also observed for recombinant heliomicin (at concentrations of 40 µg mL^{-1} and higher) on spore germination and hyphal growth of *B. cinerea* (Banzet *et al*., 2002) and recombinant termicin on the mycelia growth of *A.*

fumigatus (Lamberty *et al*., 2001). These observations further confirms that *Et*Def behaves as a "morphogenic" defensin as has been already described for other defensins from plant and insect origins (Osborn *et al*., 1995; Mitsuhara *et al*., 2000, Lamberty *et al*., 2001). Notwithstanding, further studies are needed to assess the activity spectrum of recombinant *Et*Def against microbial pathogens.

Generally, data of *in vitro* antifungal activity indicate that both *Et*Def and thanatin possess the same range of activity, regarding concentration and spectrum of antifungal activities, compared to other known AMPs and consolidate our choice to express them in Arabidopsis plants to enhance their disease-resistance. A relatively large number of gene constructs with insect AMPs coding sequences have been expressed *in planta* and are shown to confer different level of protection against fungal and bacterial pathogens (Osusky *et al*., 2000; DeGray *et al*., 2001; Banzet *et al*. 2002; Chakraborti *et al*., 2003; Osusky *et al*., 2004; Osusky *et al*., 2005; Vilcinskas and Gross, 2005; Yevtushenko *et al*., 2005; Langen *et al*., 2006; Vidal *et al*., 2006; Yevtushenko and Misra, 2007; Rahnamaeian *et al*., 2009). However, data on *in vivo* antimicrobial activity of the novel insect AMP *Et*Def and the relatively well-known AMP thanatin were still missing so far.

Several previous studies aimed to improve plant disease resistance using AMPs from insects have been 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 scarcely successful (Hightower *et al*., 1994). This has been ascribed to the short persistence of cecropin in transgenic plants due to post-translational degradation by proteinases in the intracellular fluid (Mills *et al*., 1994; Owens and Heutte, 1997). Targeting of AMPs using signal sequences from different origins into the intercellular spaces (where proteolytic degradation is expected to be minimal) is assumed to prevent the cellular degradation of AMPs, and avert the possible harmful effects of them on the plant cells (Sharma *et al*., 2000). Extracellular targeting of AMPs would also enable the plant produced peptides to be secreted into the battleground between pathogens and host, providing direct access to the pathogen target and thereby effectively improving plant resistance to invading pathogens. Such a strategy was also successfully employed to acquire resistance to

fungal pathogens in tobacco by transgenic expression of gallerimycin (Langen *et al*., 2006) and in barley by expression of metchnikowin (Rahnamaeian *et al*., 2009).

On this basis, we have attempted to transform *A. thaliana* plants with vector designed to target *Et*Def and thanatin into extracellular spaces. The complete ORF of *Et*Def (including its putative signal peptide, propeptide and mature peptide) and the chemically synthesized gene for chimerical thanatin (including *Hv*Chi26 signal peptide and mature peptide) were cloned in this study downstream of the constitutive CaMV 35S promoter into a binary victor to engineer transgenic Arabidopsis plants via Agrobacterium-mediated transformation by the vacuum infiltration method (Bechtold *et al*., 1993). A large number of independent transgenic Arabidopsis lines constitutively expressing either *Et*Def or thanatin genes were successfully obtained. Importantly, all transgenic plants were healthy, fertile and showed no morphological or developmental abnormalities compared with the wild type, suggesting that the constitutive expression of these peptides targeted to apoplast do not seem to influence plant physiology.

The integration of both genes has been confirmed by PCR. Data in Fig. 18 show clearly that both *Et*Def and thanatin genes are efficiently transcribed into an mRNA, although the levels of expression varied among transformants. Transcripts of transgenes from representative lines expressing either *Et*Def (lines 394 – 405) or thanatin (lines 407 – 411) were then assessed by quantification of *Et*Def and thanatin mRNA using qRT-PCR (Fig. 19). As can be seen in this figure, the expression level was highest for *Et*Def transgenic lines 396, 395 and 405 and thanatin transgenic lines 411 and 410.

In spite of the conspicuous amounts of both *Et*Def and thanatin mRNA which we measured in the generated transgenic lines neither *Et*Def nor thanatin peptide could be unambiguously detected using Tricin-SDS-PAGE, except for a protein band with deduced molecular mass of 3 kDa which could be seen in the IWFs of thanatin transgenic line 410.

The small size of mature *Et*Def and thanatin (4 and 3 kDa, respectively) combined with a limited peptide expression levels might be responsible for the failure in detecting *Et*Def and thanatin peptides in the obtained transgenic Arabidopsis lines. It is likely that expression of such small peptides as a fusion protein would increase their molecular mass which, in turn, may give rise to higher production levels (Okamoto *et al*., 1998). Low peptide expression levels due to either poor translation and/or inefficient post-

translation processing could also be a reason, since *Et*Def and thanatin expression constructs employed in this study were not specifically adapted for optimal codon usage in Arabidopsis, but is rather expected. Such optimization would lead to enhanced production levels of the heterologous protein as was shown for the production of human insulin-like growth factor-1 (hiGF-1) in transgenic rice and tobacco plants (Panahi *et al*., 2004). Alternatively to PAGE and coomassie staining, western blot analysis could have been used to quantify the expression level of both *Et*Def and thanatin, but no specific antibodies were available.

To gain preliminary information on whether *Et*Def and thanatin could be functionally secreted into the apoplast, *in vitro* antifungal activity of intercellular washing fluids (IWFs) from individual homozygous transgenic plants expressing either *Et*Def or thanatin was evaluated against *B. cinerea* using spore germination assay. As can be seen in Fig. 22 and 23, IWF extracts from both *Et*Def and thanatin transgenic plants distinctly inhibited the spore germination and growth of germ tubes of *B. cinerea* to different degrees compared with IWFs from non-transgenic controls. Notably, the inhibitory effect was highly correlated with RNA expression levels of the different transgenic lines tested (Fig. 23). Some morphological abnormalities were also observed, particularly, for spores treated with IWFs from *Et*Def transgenic lines (Fig. 22). These observations support the assumption that *Et*Def may behave as a "morphogenic" defensin, causing membrane destabilization. They also reflect that the expression of both *Et*Def and thanatin peptides was functional and localized to extracellular space in all transgenic lines tested. Similar antifungal activities of IWFs from gallerimycin transgenic tobacco lines on *G. cichoracearum* (Langen *et al*., 2006) and metchnikowin transgenic barley on *F. graminearum* (Rahnamaeian *et al*., 2009) have been also reported.

Using total leaf extracts from *Et*Def transgenic lines, no significant inhibitory effect on the mycelial growth of *B. cinerea* was observed *in vitro*, while those of thanatin transgenic lines strongly retarded the mycelial growth relative to transgenic control plants (Fig. 20 and 21). According to results of similar studies (Everett 1994; Cavallarin *et al*., 1998; Mourgues *et al*., 1998; Ali and Reddy, 2000), this could be explained by an inactivation of *Et*Def peptide due to proteolytic degradation or other inhibitory substances existed in the leaf extracts of *Et*Def transgenic lines, leading to decline the

inhibitory effect of this peptide on the hyphal growth of fungi. Therefore one can speculate that thanatin has the advantage of a better stability towards degradation by plant proteases as compared to *Et*Def.

To assess whether *Et*Def and thanatin transgenic lines acquired enhanced resistance to phytopathogens, Arabidopsis lines transformed with corresponding gene construct were evaluated against the important fungal pathogens *G. orontii* (powdery mildew, biotroph) and *B. cinerea* (necrotroph) and the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*).

Results of the present study showed that Arabidopsis transgenic lines expressing either *Et*Def or thanatin could strikingly suppress the conidial sporulation, hyphal spread and proliferation of *G. orontii* on the rosette leaves relative to the corresponding controls (Fig. 24 and 26), imparting therefore enhanced disease resistance in these plants. Microscopic observations corroborated these findings, and revealed that transgenic *Et*Def and thanatin Arabidopsis lines exhibited comparatively lower conidiophor and conidial numbers than the corresponding controls (Fig. 25 and 27). This pathogenicity assay indicates that transgenic expression of *Et*Def and thanatin could hamper the establishment of biotrophic pathogenic interaction of *G. orontii*, contributing to a significant enhanced resistance against powdery mildew infection in transgenic *A. thaliana*. Reportedly, resistance degree bestowed by expression of AMPs is largely dependent on AMP production level in transgenic plants (Yevtushenko *et al*., 2005; Aerts *et al*., 2007; Yevtushenko and Misra, 2007). As mentioned before, we failed to determine protein expression levels in transgenic lines under the study. However, the suppressive effect conferred by *Et*Def and thanatin against *G. orontii* in Arabidopsis transgenic lines seems to be correlated with observed RNA expression level. Among the tested transgenic lines, *Et*Def transgenic lines 395, 396, and 405 and thanatin transgenic lines 407, 410, and 411 showed high RNA expression levels, and correspondingly high level of resistance to *G. orontii* (Fig 25 and 27). Enhanced resistance to powdery mildew has also been demonstrated by expression of the insect defensin gallerimycin in tobacco plants (Langen *et al*., 2006), the plant defensin Ace-AMP1 in rose plants (Li *et al*., 2003) and metchnikowin in barley plants (Rahnamaeian *et al*., 2009).

To evaluate the resistance degree conferred by expressing *Et*Def and thanatin against necrotrophic fungi, detached leaves from transgenic Arabidopsis plants expressing

either *Et*Def or thanatin were challenged with *B. cinerea* spore suspension. Assessment of resistance against *B. cinerea* revealed generally that *Et*Def and thanatin transgenic lines exhibited varying levels of resistance to *B. cinerea* (Fig. 28). Interestingly, *Et*Def transgenic lines 396 and 405 and thanatin transgenic lines 410 and 411 (with proven high RNA expression level), tended to reduce strongly the necrotic lesion size caused by *B. cinerea* (Fig. 28 and 29), suggesting that these transgenic lines were consistently less susceptible against *B. cinerea*. This contrasts to the results from *in vitro* assays. As a necrotrophic pathogen, *B. cinerea* is known to induce a hypersensitive response in the infected plant tissues, promoting host cell death at very 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) (Makinnon *et al*., 1999; 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. This may explain, at least in part, the limited efficacies of *Et*Def and thanatin in engineered Arabidopsis lines against the broad-spectrum pathogen *B. cinerea* in this study. Presumably, high RNA expression level in *Et*Def transgenic lines 396 and 405 and thanatin transgenic lines 410 and 411 reflects comparable higher remaining peptide concentrations, which might be sufficient to provide significant enhanced resistance against *B. cinerea* in these lines.

Several reports have demonstrated that transgenic expression of defensins from different origins could enhance resistance against several necrotrophic fungal pathogens. For instance, transgenic tobacco plants expressing constitutively heliomicin and drosomycin demonstrated enhanced resistance against *Cercospora nicotianae* (Banzet *et al*., 2002), whereas those overexpressing gallerimycin inducibly showed improved resistance against *Sclerotinia minor* (Langen *et al*., 2006). Constitutive expression of the plant defensin RsAFP2 increased resistance of tobacco plants against *Alternaria longipes* (Terras *et al*., 1995) and tomato plants to *Alternaria solani* (Parashina *et al*., 2000). Overexpression of a pea defensin in canola plants provided robust resistance against *Leptosphaeria maculans* (Wang *et al*., 1999). Transgenic potato plants overexpressing alfalfa defensin exhibited improved resistance against *Verticillium dahlia* (Gao *et al*., 2000). Expression of DmAMP1from dahlia conferred

resistance against *B. cinerea* in eggplant (Turrini *et al*., 2004), and shielded the rice plants from *Magnaporthe oryzae* (Jha *et al*., 2008). Constitutive expression of the human defensin hBD-2 in *A. thaliana* plants could confer protection against *B. cinerea*. This protection was found to be correlated with the levels of transgenically produced hBD-2 (Aerts *et al*., 2007). Indeed, transgenic plants expressing AMPs from different families demonstrated also improved resistance against *B. cinerea*. Expression of maganin2 analogue, MSI-99, in tobacco bestowed protection to *B. cinerea* (Chakrabarti *et al*., 2003). Pathogen-induced expression of the amphibian AMPs *MsrA2* and temporin in tobacco transgenic plants led to increase plant resistance against several necrotrophic fungal pathogens, including *B. cinerea* (Yevtushenko and Misra, 2007). According to these authors, the degree of resistance mediated by transgenic expression of these AMPs was correlated to the protein expression level and the virulence of the tested fungus.

Much less information is available for effect of expression of thanatin in plants. Recent report demonstrated that overexpressing of synthetic thanatin in transgenic rice was shown to enhance resistance against the rice blast fungus *Magnaporthe oryzae* (Imamura *et al*., 2009).

To assess whether disease-resistance displayed by overexpressing *Et*Def or thanatin is extended to bacterial pathogens, Arabidopsis lines transformed with the corresponding gene constructs were evaluated against the highly virulent Gram-negative bacteria *P. syringae* pv. *tomato* DC3000. Inoculation experiments revealed that all transgenic *Et*Def lines were as sensitive as the control plants when challenged with *Pst*, except for the plants of transgenic line 405. This is manifested by minor changes in the numbers of bacterial cell population in the infected leaves of all *Et*Def transgenic lines when compared to non-transgenic and transgenic controls (Fig. 30A). Reliable information about *Et*Def antibacterial activity (*in vitro* or *in vivo*) is generally meager in the literature. Generally, defensins from different origin are reported to selectively kill Gram-positive bacteria, and only few Gram-negative bacteria may be affected by defensins (Bulet and stocklin, 2005; Aerts *et al*., 2008). For example, insect defensin phormia (from *Phormia terranovae*) was found to affect negatively the Gram-positive bacterial *Micrococcus luteus in vitro* by disrupting the permeability barrier of the cytoplasmic membrane (Cociancich *et al*., 1993).

Although no information is yet available concerning the antibacterial mechanism of action of *Et*Def, it is generally proposed 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). In Gram-negative bacteria, an additional outer membrane, composed of a lipid bilayer, some proteins and lipopolysaccharide (LPS), lies above the peptidoglycan layer. As predicted from their positive charge, many antibacterial peptides bind the negatively charged LPS (Vaara, 1992). Examination of the net positive charge/mass ratio of different antibacterial peptide families indicated that this ratio was the smallest for the insect defensins, which, in turn, may explain the general low efficacy of defensins on the permeability of the outer membrane of Gram-negative strains (Otvos, 2000). This may explain, at least in part, the relatively low antibacterial activity observed for *Et*Def Arabidopsis transgenic lines against *Pst* in the present study.

This feature is highly unusual as all other peptide families are more active *in vitro, but* also *in vivo* against Gram-negative than Gram-positive strains. For example, synthetic insect cecropin analogues were reported to improve protection against several pathogenic bacteria such as Gram-negative bacteria *P. syringae* pv. *tomato* DC3000 (Oard *et al*., 2006) and *Erwinia carotovora* ssp. *carotovora* on potato (Arce *et al*., 1999) and *E. amylovora* on Royal Gala apple (Liu *et al*., 2001). Overexpression of insect sarcotoxin in transgenic tobacco improved resistance against *P. syringae* pv. *tabaci* and *E. carotovora* (Ohshima *et al*., 1999; Mitsuhara *et al*., 2000).

Unlike *Et*Def, expression of thanatin in Arabidopsis could provide a higher degree of resistance against *Pst*. Thanatin transgenic lines 410, 411, and 407, which exhibited high RNA expression levels, showed the highest resistance to *Pst*. Plants of these transgenic lines did not show any leaf infection symptoms, except a rarely observed mild chlorosis. Further evidence for enhanced resistance in these lines comes from the significant reduction in bacterial cell numbers compared to controls, particularly, in the transgenic line 410. Plants of this transgenic line caused approximately 66 % and 72% reductions in the bacterial cell numbers as compared with non-transgenic and transgenic controls respectively (Fig. 30B). It is therefore likely that thanatin expression could render higher antibacterial resistance against *Pst* in Arabidopsis as compared to *Et*Def. Reportedly, thanatin is known to possess a wide antimicrobial spectrum (*in vitro*) with

potent activity against both Gram-positive and Gram-negative bacteria, filamentous fungi and yeast at physiological concentrations (Fehlbaum *et al*., 1996). Although the precise mode of action of thanatin is not yet fully understood, several investigations suggested that thanatin don't exert its antimicrobial effect through disruption of the permeability of the bacterial membrane, and considered this peptide as a non pore-forming peptide (Fehlbaum *et al*., 1996; Dimarcq *et al*., 1998; Pagès *et al*., 2003).

Taken together, the results presented here provide experimental evidences that both the novel antimicrobial peptide *Et*Def and thanatin possess at low concentrations a broad spectrum of antifungal activity against the ascomycetes *F. culmorum, B. cinerea* and the oomycete *P. parasitica*. In general, thanatin appears to possess comparatively higher biological activity not only *in vitro*, but also *in vivo* compared to *Et*Def. *In vivo*, both peptides were markedly effective against the biotrophic fungal pathogen *G. orontii* but, clearly, less active against the necrotophic fungi *B.cinerea* and the highly virulent Gram-negative bacteria *P. syringae*. Resistance degree conferred by overexpression of *Et*Def and thanatin varied between individual transgenic lines and is expected to be dependent on the expression level. Although we were not able to quantify the amount of proteins produced in transgenic Arabidopsis plants, a correlation between resistance degree and the level of mRNA expression was observed. Arabidopsis plants of the *Et*Def transgenic lines 395, 396, 398 and 405 and those of thanatin 407, 410 and 411 exhibited distinctly high mRNA levels and were the most resistant. Plants of these transgenic lines seem to be promising to merit further investigations to evaluate the potential of *Et*Def and thanatin *in planta* against other phytopathogens. Further physiological and molecular characterization studies should also be conducted, e. g. detection and quantification of transgenic AMPs *in planta*, and experiments targeted to get an insight into the antimicrobial mode of action of these peptides. Finally, we have to keep in mind that this study is the first step to develop disease-resistant transgenic plants using these peptides. Further prospective investigations are needed to assess the degree of resistance bestowed by transgenically expression of *Et*Def and thanatin either individually or in combination to determine their *in planta* antimicrobial spectrum of activity. In addition, *in planta* efficacy of the transgenically expression of *Et*Def and thanatin under inducible promoter have to be elucidated. Last but not least, transgenic

expression of the novel promising *Et*Def and thanatin should be extended to the economically important crops to render them disease-resistant.

5 Summary

Genetic engineering has proven to be a powerful tool for controlling plant diseases and to be an alternative to economically costly and environmentally undesirable chemical control. One promising approach to achieve enhanced disease-resistance has been through the expression of genes encoding antimicrobial peptides (AMPs) in transgenic plants. Hence, this study aimed to investigate the feasibility of using the novel insect AMP *Et*Def, a defensin from drone fly *Eristalis tenax* and the well-known AMP thanatin from spined soldier bug *Podisus maculiventris* to engineer disease resistance in the model plant Arabidopsis.

A prerequisite for the utilization of these peptides is a precise knowledge about their biological activity. Thus, *in vitro* antifungal activity of the chemically synthesized *Et*Def and thanatin was evaluated against the devastating phytopathogens *F. culmorum*, *B. cinerea* and *P. parasitica* using spore germination inhibition assays. Results of these assays revealed that synthetic *Et*Def led to total inhibition of spore germination and mycelial growth of all tested fungi, with minimum inhibitory concentrations (MICs) varying between 1 – 2 for *B. cinerea* and 5 – 10 µM for *F. culmorum* and *P. parasitica*. Synthetic thanatin showed higher efficacy as compared to *Et*Def regarding inhibitory effects. There the MICs ranged between 0.5 – 1 µM for *B. cinerea*, 5 – 10 µM for *F. culmorum*, and 2 – 5 µM for *P. parasitica*.

Concomitantly, a protocol for the production of recombinant *Et*Def in *E. coli* expression system was established by inserting the sequence for mature *Et*Def peptide in frame downstream of the multiple tag TrxA - His -S of pET32a(+) vector. The resulting recombinant THS-*Et*Def protein was then refolded and its *in vitro* biological activity was evaluated against *B. cinerea* using spore germination inhibition assay. It was observed that THS-*Et*Def showed also a similar antifungal activity to the chemically synthesized counterpart, with IC_{50} occurred at 0.5 µM. This indicates that the presence of the tag, which is bigger than the AMP peptide, didn't much alter the activity of *Et*Def *in vitro*.

Because of their promising antimicrobial properties, *Et*Def (with its putative signal peptide) and the chimeric thanatin (containing *Hv*Chi26 signal peptide) were introduced into Arabidopsis via Agrobacterium-mediated transformation and expressed under the

control of the constitutive CaMV35S promoter. Molecular characterization analysis revealed that both *Et*Def and thanatin genes were efficiently transcribed into mRNA, although the levels of expression varied among transformants.

Due to the signal peptides both AMPs are thought to enter the secretory pathway. Therefor intercellular washing fluids (IWFs) from individual transgenic plants expressing either *Et*Def or thanatin were isolated. Spore germination of *B. cinerea* was inhibited to various degrees, indicating that the expression of these peptides was functional and localized to extracellular space in all transgenic lines tested.

The degree of resistance achieved by expressing either *Et*Def or thanatin were then evaluated *in planta* against the fungal pathogens *G. orontii* and *B. cinerea* and the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000. *Et*Def and thanatin transgenic Arabidopsis plants displayed remarkably reduced conidial sporulation, hyphal spread and proliferation of *G. orontii* on the rosette leaves, mediating enhanced disease resistance in these plants. This suppressive effect against *G. orontii* was correlated with RNA expression level. Three independent *Et*Def transgenic lines namely 395, 396, and 405 and thanatin transgenic lines 407, 410, and 411 showed high RNA expression levels, and correspondingly high resistance degree to *G. orontii*. In contrast, transgenic expression of *Et*Def and thanatin in Arabidopsis was clearly less active against *B. cinerea*. Nevertheless, two *Et*Def transgenic lines 396 and 405 and two thanatin transgenic lines 410 and 411 were consistently more resistant against *B. cinerea*. When challenged with *Pst*, all transgenic *Et*Def lines were as sensitive as the control plants, except for transgenic line 405. Transgenic expression of thanatin in Arabidopsis could provide, however, a higher degree of resistance against *Pst*. Thanatin transgenic lines 407, 410, and 411, showed the highest resistance to *Pst*. In summary, plants of the *Et*Def transgenic lines 395, 396, 398 and 405 and those of Thanatin 407, 410 and 411 seem to be promising candidates to evaluate their potential *in planta* against other phytopathogens. Finally, data presented here indicate that transgenic expression of *Et*Def and Thanatin could be utilized to improve disease resistance of other economically important crops.

Zusammenfassung

Gentechnische Methoden haben sich als wichtiges Werkzeug zur Kontrolle von Pflanzenkrankheiten erwiesen und bilden eine Alternative zum kostenintensiven und ökologisch unerwünschten Einsatz von Chemikalien. Als viel versprechender Ansatz zur Steigerung der Resistenz gegen Krankheiten hat sich die Expression von Genen in transgenen Pflanzen erwiesen, die für antimikrobielle Peptide (AMPs) kodieren. Ziel der hier vorgestellten Studie war es daher zu untersuchen, inwieweit sich *Et*Def, ein neues Peptid aus der Schwebfliege *Eristalis tenax*, und das gut untersuchte AMP Thanatin aus der Raubwanze *Podisus maculiventris* zur Erhöhung der Krankheitsresistenz der Modellpflanze *Arabidopsis thaliana* nutzen lassen.

Voraussetzung für den Einsatz dieser Peptide ist ein präzises Wissen um deren biologische Wirkung. Aus diesem Grund wurde zu Beginn in Sporenkeimungstests die antimykotische Wirkung von synthetisch hergestelltem *Et*Def und Thanatin auf die phytopathogenen Pilze *F. culmorum*, *B. cinerea* und *P. parasitica* untersucht. Synthetisches *Et*Def führte hierbei zu einer vollständigen Inhibierung der Sporenkeimung und des Myzelwachstums bei allen getesteten Pilzen mit miminimalen Hemm-Konzentrationen (MHK) von 1 – 2 µM für *B. cinerea* und 5 – 10 µM für *F. culmorum* und *P. parasitica*. Für synthetisches Thanatin wurde eine größere inhibitorische Wirksamkeit als für *Et*Def beobachtet. Die minimalen Konzentrationen zur vollständigen Hemmung lagen hier bei 0,5 – 1 µM für *B. cinerea*, 5 – 10 µM für *F. culmorum* und 2 – 5 µM für *P. parasitica*.

Parallel zu diesen Experimenten wurde ein Protokoll zur Produktion von rekombinantem *Et*Def in *E. coli* etabliert. Hierzu wurde die Sequenz des *Et*Def Peptids *in frame* abwärts des TrxA - His – S *Tags* des pET32a(+) Vektors inseriert. Die biologische Aktivität des hergestellten THS-*Et*Def Proteins wurde *in vitro* überprüft, wofür wiederum die Inhibierung der Sporenkeimung bei *B. cinerea* untersucht wurde. Es konnte eine ähnliche antimykotische Wirkung für THS-*Et*Def wie bei synthetischem *Et*Def gezeigt werden. Das deutet darauf hin, dass die Aktivität von THS-EtDef *in vitro* nur gering durch den *Tag*, der größer als das AMP selbst ist, beeinflusst wird.

Aufgrund der viel versprechenden antimikrobiellen Eigenschaften wurden mittels Agrobacterium-vermittelter Transformation Arabidopsis-Pflanzen erstellt, die *Et*Def (mit

seinem putativen Signalpeptid) oder chimäres Thanatin (mit dem pflanzlichen Signalpeptid *Hv*Chi26) unter Kontrolle des konstitutiven CaMV35S Promotors exprimieren.
Molekularbiologische Tests zeigten, dass sowohl das *Et*Def-, als auch das Thanatingen effizient in mRNA transkribiert wurden, wobei zwischen einzelnen Transformanten variierende Expressionslevel nachgewiesen wurden.
Die vorgeschalteten Signalpeptide sollten zur Sekretion der AMPs in den Apoplasten führen. Deshalb wurden von individuellen transgenen Pflanzen, die entweder *Et*Def oder Thanatin exprimierten *intercellular washing fluids (IWFs)* isoliert. Die Sporenkeimung von *B. cinerea* wurde im Vergleich zur Kontrolle bei den verschiedenen Linien in unterschiedlichem Ausmaß inhibiert. Das deutet darauf hin, dass die Peptide in allen untersuchten Linien funktionell und im extrazellulären Raum lokalisiert waren.
Im Folgenden wurde der Grad der Resistenz, der durch die Expression von entweder *Et*Def oder Thanatin hervorgerufen wurde, *in planta* untersucht. Hierfür wurden die pilzlichen Pathogene *G. orontii* und *B. cinerea* und das bakterielle Pathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) verwendet. Transgene Arabidopsis Pflanzen mit entweder *Et*Def oder Thanatin zeigten eine deutlich verringerte Sporulation der Konidien, verringertes Myzelwachstum und Vermehrung von *G. orontii* auf Rosettenblättern, was zu einer erhöhten Resistenz der Pflanzen gegen diesen Pilz führte. Der Effekt auf *G. orontii* korrelierte mit der Transcriptmenge in den Pflanzen. Drei unabhängige transgene *Et*Def- (395, 396 und 405) und drei Thanatin-Linien (407, 410 und 411) wiesen hohe RNA-Expressionslevel auf, was mit einem hohen Grad an Resistenz gegen *G. orontii* einherging. Im Gegensatz dazu zeigte die Expression von *Et*Def und Thanatin in Arabidopsis eine deutlich geringere Wirkung auf *B. cinerea*. Gleichwohl zeigten zwei transgene *Et*Def- (396 und405) und zwei transgene Thanatin-Linien (410 und 411) eine gesteigerte Resistenz gegen diesen Pilz. In den *Et*Def-Linien konnte eine erhöhte Resistenz gegenüber *Pst* nur in Linie 405 beobachtet werden. Die Sensitivität der übrigen Linien gegenüber dem Bakterium war nicht signifikant unterschiedlich zu den Kontrollpflanzen. Jedoch vermittelte die transgene Expression von Thanatin eine deutlich erhöhte Resistenz der Pflanzen gegen *Pst*. Hier zeigten wiederum die transgenen Linien 407, 410 und 411 die stärkste Wirkung. Zusammengefasst erscheinen die Linien 395, 396, 398 und 405 und 407, 410 und 411 am besten geeignet, um in weiteren Untersuchungen das Potential von *Et*Def bzw. Thanatin gegen andere Phytopathogene *in planta* zu testen. Die in dieser Studie

präsentierten Ergebnisse deuten darauf hin, dass die transgene Expression von *Et*Def und Thanatin genutzt werden könnte, um eine gesteigerte Resistenz gegenüber Krankheiten auch in anderen, ökonomisch wichtigen, Pflanzen zu erzielen.

7 Refferences

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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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