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Novel Findings of Defensins and their Utilization in Construction of Transgenic Plants

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ABSTRACT

Defensins are small peptides with a common structure, the cysteine-stabilized α/β motif. These molecules are found in diverse organisms, such as plants, mammals and insects and can be active against a wide range of pathogens, including fungi and bacteria. Moreover, defensins are also able to inhibit digestive enzymes, protein synthesis and the activity of ion channels. In this field, defensins have also become an alternative strategy for pest biocontrol, being active against several insects such as bean bruchids. Hence, the development of biotechnology has led to the application of defensins in plant improvement, enhanced pest resistance and increased crop production. Furthermore, they are also being used in the pharmaceutical field for development of novel antibiotics and fungicides active against pathogenic microorganisms. This review describes the latest information in defensin structure and function, and also brings insights for this peptide's biotechnological use, through transgenic strategies, in agriculture and pharmacy.

Keywords: crop resistance, defensins, transgenic plants
Abbreviations: AMPs, antimicrobial proteins; **CSαβ**, cysteine-stabilized-α–β motif; **HTH**, helix turn helix motif

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INTRODUCTION

In the last few years, a variety of biotechnological tools has being developed to decrease the resistance of nocive pests and pathogens that predate plants and mammals towards chemical products administrated against them. One of these amazing techniques consists in the possibility of inserting, silencing or over-expressing different genes in plants, creating an organism with enhanced resistance to pathogens, and clearly improving crop production (Bhargava *et al*. 2007; Sesmero *et al*. 2007). Transgenic technology has brought high technological science to crop science, not only for plant resistance towards pests and pathogens, but also in order to produce biofactors to create novel compounds with importance for industry. One example is the bioplastic polyhydroxyalkanoate, a molecule used to substitute the non-biodegradable petroleum-derived plastic, being now produced in large scale by genetically modified plants (Suriyamongkol *et al*. 2007).

But, which molecules should we choose to study and apply in biotechnology? Plants have a powerful defence mechanism, conferring systemic resistance against pathogens. This resistance is acquired during plant evolution and co-evolution processes (Gatehouse *et al*. 1992), in which proteins and peptides are the most involved molecules. Among these peptides are included plant defensins, small cationic peptides of 45-54 amino acid residues, very stable to temperature and pH variations (Terras *et al*. 1995; Selitrennikoff 2001). Although their primary sequences are not well conserved between different species, they present a very similar three-dimensional structure, composed by an α-helix, followed by three β-sheets and stabilized by four disulfide bonds (Bloch *et al*. 1998; Romagnoli *et al*. 2003; Villa-Perelló *et al*. 2003). This conserved structure is also known as the cysteine-stabilized α/β motif, which was also found in insect defensins and scorpion neurotoxins (Cornet *et al*. 1995; Krezel *et al*. 1995). They have been isolated from seeds, flowers, leaves, roots and stems from a diverse range of plant species, always showing a role in plant defence against different pathogens (Selitrennikoff 2001).

Hence, plant defensins can be divided into two main groups, according to their functions: antimicrobial and enzyme inhibitor peptides. The first group is composed by antifungal and antibacterial defensins and includes most of the peptides isolated so far (Janssen *et al*. 2003; Lay *et al*. 2003; Franco *et al*. 2006). They are able to inhibit one specific microorganism, from Gram-negative and Gram-positive bacteria to yeast and filamentous fungi (Terras *et al*.

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1993; Thevissen *et al*. 1996), and can also inhibit the growth of about 2-5 different pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas syringae* (Franco *et al*. 2006). The second group corresponds to defensins able to inhibit proteinases and/or α-amylases from different sources. Mainly, these defensin inhibitors have demonstrated specificity to enzymes from insect midguts, such as from *Callosobruchus maculatus* and *Tenebrio molitor* (Melo *et al*. 2002; Farias *et al*. 2007).

Although there are many plant defensins described in the literature, their mechanism of action against antimicrobial and/or enzyme inhibitors is not well understood. Some hypotheses have been reported and are gradually being confirmed (Thevissen *et al*. 2005; Liu *et al*. 2006). The application of plant defensins in biotechnology for development of resistant plants and pharmaceutical purposes has helped researchers to explore the way in which these peptides act and also substitute the use of synthetic molecules for products from protein sources in agriculture, medicine and industry. In this review, we describe the main features of plant defensins, focusing on the application of their functions and structural features in biotechnology and genetic engineering.

STRUCTURAL FEATURES OF DEFENSINS

The three dimensional structure of several plant defensins have been elucidated in the last few years by nuclear magnetic resonance (NMR), clearly improving the structure-functional knowledge. Among them, *Pisum sativum* defensin 1 (*Ps*d1) (Almeida *et al*. 2002), *Raphanus sativus* antifungal protein 1 (*Rs*-AFP1) (Fant *et al*. 1998), *Vigna radiata* defensin 1 (*Vr*d1) (Liu *et al*. 2006), γ1-hordothionin (g1-H) from barley and γ1-purothionin (g1-P) from wheat endosperm (Bruix *et al*. 1993), *Petunia hybrida* defensin 1 (*Ph*d1) (Janssen *et al*. 2003) and *Nicotiana alata* defensin 1 (*Na*d1) (Lay *et al*. 2003) structures were solved. Moreover, molecular modelling was also used for defensins structure characterization such as CP-thionin I and II from *V. unguiculata* seeds (Melo *et al*. 2002; Franco *et al*. 2006). It is well known that plant defensins share a common structure, the cysteine-stabilized α/β (CSαβ) motif, which is composed of three antiparallel β-sheets and one α-helix. This motif was also found in insect defensins (Cornet *et al*. 1995) and in scorpion neurotoxins as the potassium channel blocker named agitoxin 2 (Krezel *et al*. 1995). Despite all of these peptides being related and sharing a similar structural core, an enormous functional variation has been observed. *Ps*d1, *Rs*-AFP1, *Ph*d1 and *Na*d1 showed antifungal activities against *Neurospora crassa*, *Alternaria longipes*, *Botrytis cinerea* and *Fusarium oxysporum* respectively (Fant *et al*. 1998; Almeida *et al*. 2002; Janssen *et al*. 2003; Lay *et al*. 2003), insect defensin (idA) and Cp-thionin II showed antibacterial activity against *S. aureus*, *E. coli* and *P. syringae* (Cornet *et al*. 1995; Franco *et al*. 2006) and γ1-H and *Vr*d1 are protein synthesis inhibitors for cell-free organisms, that act against bacteria, protozoa and fungi (Bruix *et al*. 1993; Liu *et al*. 2006). Moreover, *Vr*d1 also acts as an α-amylase inhibitor (Bruix *et al*. 1993) and CP-thionin I is an unusual proteinase inhibitor (Melo *et al*. 2002). So, an important question arises. If all of them have the same structure but show different functions, then how do they in fact act in plant defence?

To answer that question, several efforts have been made in the last few years in order to elucidate it (Almeida *et al*. 2002; Pelegrini *et al*. 2005; Jenssen *et al*. 2006) in spite of the true mechanism of action still being uncharacterised. To illustrate the problem, **Fig. 1** shows a defensin primary structure alignment generated by ClustalW software (Thompson *et al*. 1994) and edited using the Jalview program (Clamp *et al*. 2004). Except for *Ph*d1, plant defensins have four extremely conserved disulphide bonds (Almeida *et al*. 2002; Pelegrini *et al*. 2005). Otherwise, defensins apparently maintain low primary sequence identities, even those with the same function properties (**Fig. 1**). As an exception, *Ph*d1 presents an uncommon feature: a fifth Cys-Cys linkage that confers a more thermodynamic stability (Janssen *et al*. 2003). Therefore, **Table 1** shows the Root Mean Square (RMS) deviation of all defensins structures in relationship to CP-thionin II calculated by PyMOL (de Lano 2002). Lower values indicate a higher similarity. These values demonstrate that all structures are basically identical, indicating a low tertiary structure variation between these peptides. Finally, **Fig. 2A** shows a superimpose image of several defensins, demonstrating once more the tertiary structure similarities between them. These characteristics were also observed by Almeida *et al*. (2002), who tried to correlate function versus structure of *Ps*d1, when compared to different defensins. A profound examination of the tertiary structure (**Fig. 2A**) demonstrates two variable regions observed on loops 1 and 2 (**Fig, 2A-1**, **2A-2**). These sites seem to be extremely important in some specific defensin functions such as α-amylase and proteinase inhibitory activities (Melo *et al*. 2002; Liu *et al*. 2006), potassium channel blockage (Krezel *et al*. 1995) and probably antifungal and bactericidal activities (Fant *et al*. 1998; Almeida *et al*. 2002; Franco *et al*. 2006). Almeida *et al*. (2002) suggest that basic conserved residues in loop 1 and hydrophobic residues on loop 2 may distinguish plant defensins with and without antifungal activity. This property was previously explored by Fant *et al*. (1998), which created an *Rs*-AFP1 mutant, shifting the basic residue to a neutral one ($Lys_{44}Gln$). This

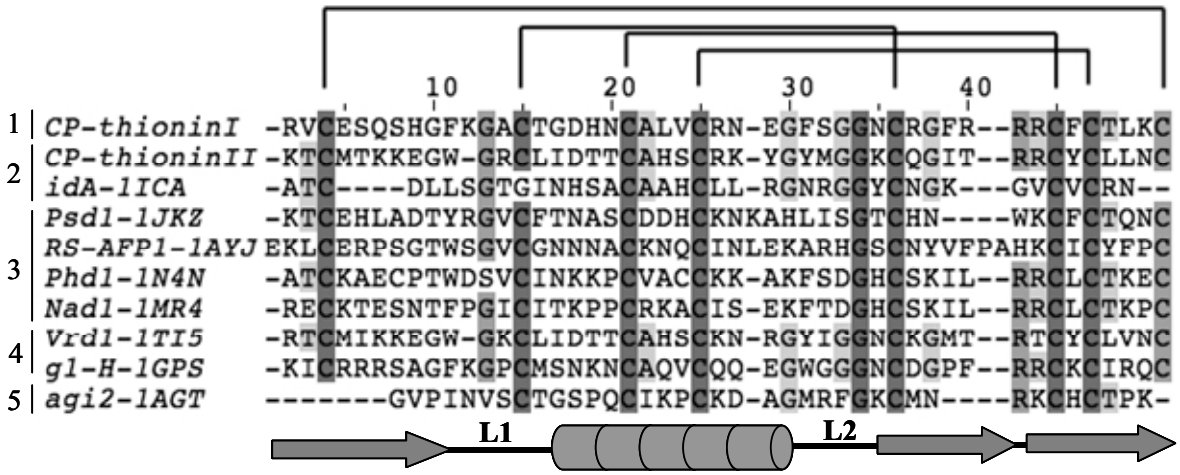

Fig. 1 Primary structure alignment of defensins with activity of 1) proteinase inhibitor, 2) antibacterial, 3) antifungal, 4) protein synthesis inhibitor and 5) neurotoxin potassium channel inhibitor. Black lines at the top indicate disulfide bonds. PDB access codes were provided after protein names. Cylinders indicate α-helices. Arrows indicates β-sheets. Loops are represented by a black line.

Table 1 Root mean square (RMS) deviation values and electrostatic potentials (e-pot) of defensin's tertiary structure. RMS values were calculated in relation to CP-thionin II matched residues only. A lower value corresponds to greater identity. Electrostatic potential were calculated in a vacuum. Both were calculated using PyMOL software.

Defensin	**CP-thioninII**	**idA**	**Psd1**	**Rs-AFP1**	**Phd1**	**Nad1**	**Vrd1**	**G1-H**	**agi2**
RMS	-	1.667	1.974	1.782	1.401	1.974	0.238	1.241	1.031
e-pot	±67.3	±47.5	±54.1	±49.2	±67.1	±54.1	±75.2	±81.8	±73.9

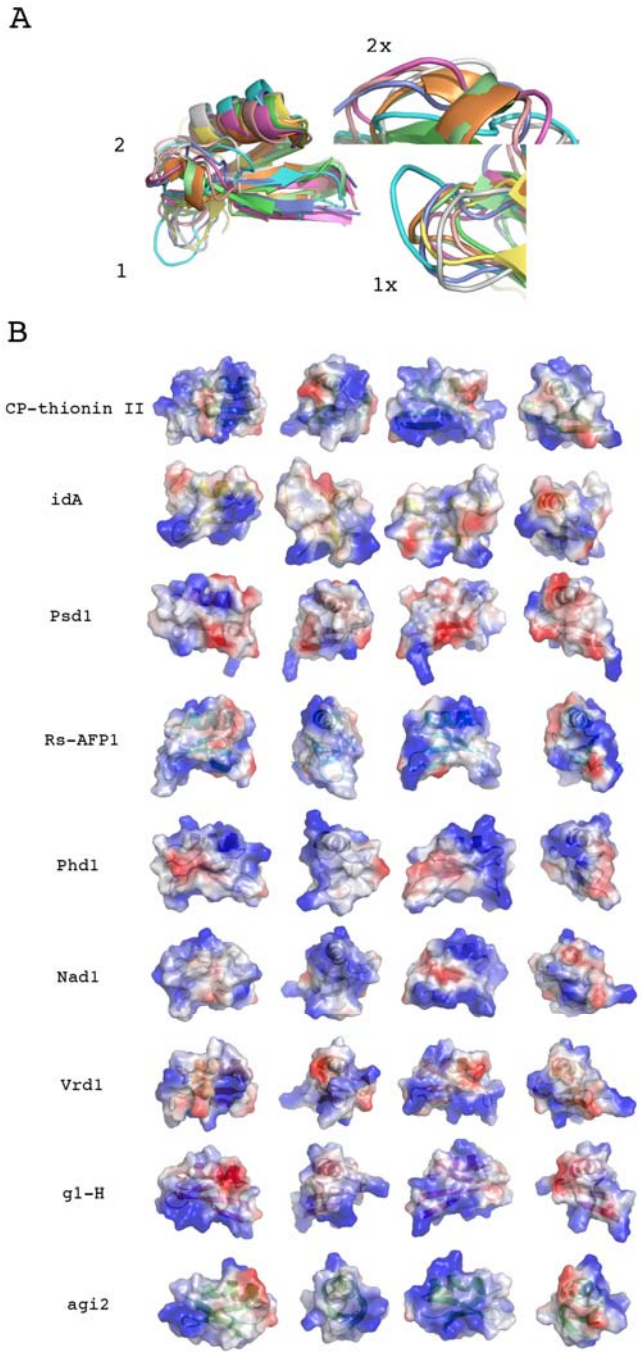

Fig. 1 Defensins tertiary structures. (**A**) Superimposition of all structures. 1 and 1x represents loop 1 and loop closer view, same for 2 and 2x. (**B**) Eletrostatic potential map of defensins. Each one was rotated 90° clockwise. Blue corresponds to positive charged regions. Red corresponds to negative charged regions. PyMOL software was used for image acquiring.

specific alteration leads to a complete loss of *Rs*-AFP1 activity. But, once those plant defensins need to expose basic residues for a crucial activity, what feature may distinguish the function of these peptides? The charge surface of each one could help to elucidate this question.

In **Fig. 2B** several electrostatic potential surface images of each defensin rotated 90° clockwise are shown, calculated using PyMOL. In **Table 1** there are the maximum and minimum values of the electrostatic potential (e-pot) for charged regions, blue, for positive charged, and red, for negative charged, that can be observed in **Fig. 2B**. Inside of each surface there are cartoon defensin images. Analysing each group separately, we could observe that antibacterial types, CP-thionin II and idA, have an e-pot of ±67.3 and ±47.5, respectively. CP-thionin II has an elevated positive charged region at the side and bottom of the defensin. On the contrary, idA shows more non-polar regions. Antifungal defensins *Ps*d1, *Rs*-AFP1, *Ph*d1 and *Na*d1 possess e-pots of ±54.1, ±49.2, ±67.1 and ±59.7, respectively. *Rs*-AFP1 has positive charged regions on the upper right and on the left side of the peptide and *Ph*d1 on the upper right side. *Nad*1 has positive charges on both lower and upper sides. *Ps*d1 is the only defensin that has a more negatively charged region on the lower surface. Protein synthesis inhibitors *Vr*d1 and γ1-H have ±75.2 and ±81.8 e-pot values. Both are similar with respect to their surface structures, but γ1-H has a more concentrated positively charged surface on the lower side. Finally, agi2 neurotoxin has an e-pot value of ±73.9. agi2 is the most amphipatic peptide showing a positively charged side and a non-polar one. This data clearly indicates that for now, it is impossible to drawn a possible function/structure pattern for each group, since almost all properties are observed in all groups. For example, in different groups we have the same charge surface, as observed in the potassium channel inhibitor and antifungal group (**Fig. 2B**), *Vr*d1 has more charged residues then CP-thionin II, but a very similar structure with an RMS of 0.238 (**Table 2**). This implies that almost identical proteins do not have the same functions. Of course those small differences could occur in e-pot values for some peptides, but they are unable to explain major cases. Therefore, the determination of complex activities, such as bactericidal activities, has been considered a Herculean task, especially due to infinity of possible mechanisms of action. Among them we could include peptide aggregation, binding to lipids in micelle-like complexes, as well several models predicted as toroidal pores, carpets and barrel staves (Pelegrini and Franco 2005; Jenssen *et al*. 2006). In those specific cases, an enormous variation in the surface structure can be found. These data could clearly be observed in agi2, which showed almost the same CP-thionin II e-pot and also possesses the same important basic residues on the loops (Krezel *et al*. 1995; Franco *et al*. 2006) but does not have an antibacterial function. Does CP-thionin II act as a potassium channel inhibitor or show another unpublished function? Almeida *et al*. (2002) believe that *Ps*d1 may act also as potassium channel blocker based on similar surfaces with agi2 (**Fig. 2B**), but until now, this is an unsolved question. By using these data, we raised an important hypothesis: defensins may act with multiple functions. For example, *Vr*d1 may act both with α-amylase inhibitor and antifungal and CP-thionin II acts through its antibacterial activity but may possess another function as an inhibitor of the potassium channel in spite of a highly positively charged region equally found on scorpion neurotoxin agi2 (**Fig. 2B**). Thus, how to find a pattern to group them? This is still the main question remaining to be answered, and it is possible that a combination of surface-exposed residues and different protein sizes and shapes could be the solution for this unsolved mystery.

INSECTICIDAL PEPTIDES: DEFENCE PLANT MECHANISMS TOWARDS INSECT-PESTS

Plants are constantly susceptible to pests and pathogens, being both deleterious agents responsible for severe crop losses. These predators have an interest in carbohydrates, proteins and lipids, stored in seeds and other plant tissues, providing them the main sources of metabolic energy (Moreira *et al*. 2005). Nevertheless, plants have developed defense mechanisms to avoid insect intrusion usually being limited by the number of predators with an ability to feed on a specific plant. Actually, this resistance consists of several defense mechanisms, acquired during plant evolution and co-evolution processes (Gatehouse *et al*. 1992). Hence, during the last years, proteins and peptides have been studied as defense molecules. In this field, plant defensins belonging to a selected group of plant proteins with enzyme inhibition activity. They are able to inhibit α-amylase or proteinases from insects and, in some cases, show an ability to inhibit both trypsin and chymotrypsin (as reviewed by Pelegrini and Franco 2005, **Table 2**).

α-Amylase inhibitors

Bruchids are extremely dependent on starch to survive. For this reason, they use their α-amylolytic enzymes to cleave starch into small carbohydrate molecules as observed in the larval midgut of *Tenebrio molitor*, *Callosobruchus maculatus*, *Acanthoscelides obtectus* and *Zabrotes subfasciatus* (Grossi de Sá and Chrispeels 1997; Strobl *et al*. 1998; Franco *et al*. 2005; Pelegrini *et al*. 2006). However, some efforts have been made to inhibit these insect enzymes and enhance plant resistance towards crop losses. Six different α-amylase inhibitor classes, lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like could be used in pest control. These classes of inhibitors show remarkable structural variety, leading to different modes of inhibition and different specificity profiles against diverse α-amylases (Franco *et al*. 2002). Of particular interest is the γ-purothionin-like family, with high similarities to defensins, showing favorable properties such as high insect specificity and low molecular masses. The first plant defensin described with α-amylase inhibitory activity was a peptide isolated from sorghum (*Sorghum bicolor*). It was able to inhibit insect α-amylases, such as those from *Periplaneta americana* and *Schistocerca americana*, but was not able to inhibit mammalian α-amylases (Bloch and Richardson 1991). Moreover, a peptide from papaya seeds (*Carica papaya*), also similar to plant defensins, was able to inhibit α-amylases from cowpea weevil (*C. maculatus*) (Farias *et al*. 2007). Assays using artificial seeds with 0.5-1.0% of papaya inhibitor showed that the peptide was capable of inhibiting 50% of the growth of cowpea weevil larvae (Farias *et al*. 2007).

Furthermore, it has been demonstrated that a defensin from *Vigna radiata*, named *Vr*D1, was able to inhibit *T. molitor* α-amylases (TMA) (Liu *et al*. 2006). It seems that the mechanism of action between plant defensins differs depending on the inhibitor structure, although most of the interactions with the inhibitors occur at the active site of the enzyme. Therefore, *Vr*D1 was able to form hydrogen bonds with three amino acid residues from the *T. molitor* active site (Glu_{222}, Asp_{287}, and Asp_{332}) and also showed that the second loop was important for interaction and enzyme inhibition (Liu *et al*. 2006). The same mechanism was observed by a γ-hordothionin (γ-H) from barley (Mendes *et al*. 1990; Liu *et al*. 2006). It could be observed that Loop 2 from γ-H inserts into the active site of TMA, impeding the entrance of the substrate. Moreover, it was found that positively charged residues in Loops 1 and 2 (Lys_{12} and Arg_{38} in *Vr*D1, Arg_{39} and Arg_{40} in γ-H) are important for interaction with α-amylases (Mendes *et al*. 1990; Liu *et al*. 2006), which did not occur with plant defensins that have a short Loop 2. The mechanism of action for insecticidal plant defensins is not completely understood, but another hypothesis for the mechanism of action has been proposed, being related to the essentiality of the Ca^{2+} ion for some insect α-amylase activity (Pelegrini *et al*. 2006). In this sense some authors have suggested that defensins are able to chelate calcium, destabilizing the enzyme and further causing its inhibition (Castro and Vernon 2003; Pelegrini and Franco 2005).

Proteinase inhibitors

Plant defensins also have activity towards insect proteinases. The first proteinase inhibitor from the defensin family was described in *Cassia fistula* and was able to decrease the activity of insect serine proteases trypsin or chymotrypsin (Wijaya *et al*. 2000). Furthermore, there are other reports describing the inhibitory activity of defensins. These data were verified in a defensin from *N. alata*, *Na*D1, which showed activity towards trypsin and chymotrypsins from *Helicoperva armigera* and *H. punctigera*, in addition to its antifungal activity (Lay *et al*. 2003). The conservative CSαβ motif present in plant thionins may be the key to explain the diversity of *Na*D1's function, as there is no hypothesis to explain clearly how this peptide can work both as an antifungal agent and as a proteinase inhibitor (Lay *et al*. 2003). Moreover, the most studied proteinase inhibitor pertaining to the defensin family was Cp-thionin I, isolated from *V. unguiculata* seeds. It showed activity towards bovine pancreatic trypsin (BPT), but was unable to inhibit chymotrypsin (Melo *et al*. 2002). The mechanism of action of Cp-thionin I is not well understood, but the main hypothesis lies in the fact that this defensin interacts specifically with the enzyme in a water-mediated environment, where the Lys_{11} residue of the inhibitor was identified as being extremely important for its inhibitory activity. This amino acid acts in a canonical-style fashion, by occupying a specific enzyme cavity, blocking its catalytic site and impeding the entrance of the substrate (Melo *et al*. 2002).

In summary, although there are only a few reports describing proteinase and α-amylase inhibitors related to the plant defensin family, they are extremely important for understanding the novel mechanisms of action proposed for this multi-family. Cloning and expression of these peptides in transgenic plants could lead to an enhanced resistance to pest and pathogen predation, by reducing the protein and starch adsorption during digestion processes.

Table 2 Plant defensins with enzyme inhibitory activity.

Activity	Name	Source	Reference
α-Amylase inhibitors	*SI*α	*Sorghum bicolor*	Bloch and Richardson 1991
	γ-hordothionin	*Hordeum vulgareae*	Mendes *et al.* 1990
	αAI	*Vigna unguiculata*	Melo *et al.* 1999
	*Vr*D1	*Vigna radiata*	Chen *et al.* 2004
	CpAI	*Carica papaya*	Farias *et al.* 2006
Proteinase inhibition	*C. fistula* PI	*Cassia fistula*	Wijaya *et al.* 2000
	Cp-thionin I	*Vigna unguiculata*	Melo *et al.* 2002
	*Na*D1	*Nicotiana alata*	Liu *et al.* 2006

PLANT DEFENSINS WITH BACTERICIDAL ACTIVITY

Plants are constantly attacked by predators and exposed to environmental stresses. But different to the majority of organisms, plants are unable to escape from predation by self-movement to a more favorable environment. Deprived of this simple alternative, plants needed to develop sophisticated and complex defense mechanisms throughout their evolution. Among several organic compounds synthesized by plants to reduce pathogen predation, defensins are known to contribute to the plant innate host defense (Fujimura *et al*. 2004; Pelegrini and Franco 2005; Franco *et al*. 2006). Several defensins show potent activity against several microorganisms such as *Candida albicans* and *Pichia pastoris* (Thevissen *et al*. 2004), *Fusarium oxysporum* and *Geotrichum candidum* (Fujimura *et al*. 2005), *Rhizoctonia solani* (Olli and Kirti 2006), insects as *T. molitor* larvae (Liu *et al*. 2006) and bacteria such as *S. aureus*, *E. coli* and *P. syringae* (Franco *et al*. 2006). In fact, phytopathogenic bacteria are not common predators as fungi and insect-pests (Thomma *et al*. 2002). Probably, for this reason, only few plant defensins demonstrate anti-bacterial activity, in comparison to defensins from others organisms (Dhople *et al*. 2006; Elahi *et al*. 2006; Ouhara *et al*. 2006). Among these exceptions, some anti-bacterial defensins have been studied such as pseudo-thionin (Pth-St1) from *Solanum tuberosum* (Moreno *et al*. 1994), So-D1-7 from *Spinacia oleracea* (Segura *et al*. 1998), Pa-AMP-1 from *Phytolacca americana* (Liu *et al*. 2000), Fa-AMP1 and Fa-AMP2 from *Fagopyrum esculentum* (Fujimura *et al*. 2003), *Va*D1 from *Vigna angularis* (Chen *et al*. 2005) and Cp-thionin II from *Vigna unguiculata* (Franco *et al*. 2006). Fa-AMPs, which belong to the defensin family also, were included in the glycine-rich family due their primary structural features, which exhibit both 8 cysteine resides and continuous sequences of cysteines (–CC–), characteristic of defensins, and 10 glycine residues and continuous sequences of glycines (–GGG– and –GG–), characteristic of glycine-rich proteins. Thus, Fa-AMPs were the first to be classified into two families.

Moreover, plant defensins have demonstrated antimicrobial inhibitory activity towards different microorganisms, as mentioned above. Normally, antimicrobial activity was observed against only fungi or bacteria, and occasionally against both pathogens (Garcia-Olmedo *et al*. 1998; Pelegrini *et al*. 2005). These include Pa-AMP-1, which was active against the harmless bacterium *Bacillus megaterium*, with an IC_{50} of 8 $\mu g.mL^{-1}$ concentration, and phytopathogenic fungi *Alternaria panax*, *Fusarium* sp., and *Rhizoctonia solani*, with an IC_{50} of 20 $\mu g.mL^{-1}$ (Liu *et al*. 2000). In the same way, some plant defensins appear to be active towards both Gram-negative and Gram-positive bacteria as well fungi. Furthermore, was also observed that plant defensin So-D1-7, was able to reduce 50%, with 20 $\mu g.mL^{-1}$ concentration, of the development of the bacteria *Clavibacter michiganensis* and *Ralstonia solanacearum*, which are able to cause black rot, as well against some phytopathogenic fungi (*Fusarium culmorum*, *F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium*) at 25 $\mu g.mL^{-1}$ concentration (Segura *et al*. 1998). Moreover, similar data was obtained by *Va*D1, which inhibits the growth of *Staphylococcus epidermidis* – a cause of common infections in immune deficient patients – and *Salmonella typhimurium* – the root cause of intragastric infections – as well the fungus *F. oxysporum*, at IC_{50} of 36.6, 143.4 and 30 $\mu g.mL^{-1}$ concentration, respectively (Chen *et al*. 2005). Fa-AMPs were equally able to inhibit both Gram-negative (*Erwinia carotovora*, *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*), with Fa-AMP1 IC_{50} of 11, 24 and 20 $\mu g.mL^{-1}$ concentration, respectively, and Fa-AMP2 IC_{50} of 15, 17 and 24 $\mu g.mL^{-1}$, respectively, and Gram-positive bacteria (*C. michiganensis* and *Curtobacterium flaccumfaciens*), with Fa-AMP1 IC_{50} at 14 and 13 $\mu g.mL^{-1}$, and Fa-AMP2 IC_{50} of 17 and 15 $\mu g.mL^{-1}$, respectively. Finally, plant fungal pathogens such as *F. oxysporum* and *Geotrichum candidum*, were also affected by Fa-AMP1 with an IC_{50} of 19 and 36 $\mu g.mL^{-1}$, and by Fa-AMP2 with an IC_{50} of at 29 and 25 $\mu g.mL^{-1}$, respectively, (Fujimura *et al*. 2003). On the other hand, some defensins have shown specificity only against bacteria, being unable to control fungal development. In this case, Cp-thionin II showed antibacterial activity toward Gram-negative bacteria *P. syringae* and *E. coli*, as well the Gram-positive *S. aureus*, with a minimum inhibitory concentration of 42, 64 and 128 $\mu g.mL^{-1}$, respectively (Franco *et al*. 2006).

Recently, diverse plant defensins with bactericidal activity have been isolated from bean seeds and characterized. Ground beans (*Vigna sesquipedalis*) are able to synthesize sesquin, which exerts antibacterial activity toward *E. coli*, *Proteus vulgaris*, *Mycobacterium phlei* and *Bacillus megaterium*; and also antifungal activity against *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Wong and Ng 2005). Furthermore, sesquin appears to be able to inhibit about 80% of breast cancer (MCF-7) cells and leukemia M1 cells at approximately 100 $\mu g.mL^{-1}$ concentration. Otherwise, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, individually stops the activity of sesquin, but not its anticancer activity, indicating a different mode of action in the same compound (Wong and Ng 2005). From white cloud bean (*Phaseolus vulgaris*), another antibacterial defensin was isolated, showing activity against *Mycobacterium phlei*, *B. megaterium* and *B. subtilis* at fairly high concentrations with IC_{50} of approximately 86, 121, 101 and 68 μM, respectively, as well as inhibition of fungal growth and induction of a mitogenic response in mouse splenocytes (Wong *et al*. 2006). Moreover, phaseococcin, from runner beans (*Phaseolus coccineus*) was able to inhibit *Bacillus* proliferation, fungal development, and leukemia cell growth and reduce HIV-1 reverse transcriptase activity (Ngai and Ng 2005).

It is easy to observe that some plant defensins present an enhanced biological specificity. Results as Cp-thionin II are uncommon for plant defensins, which frequently appear to be active toward both Gram-positive and Gram-negative bacteria at same time. Usually, plant defensins act uniquely and specifically against one bacterial class (Segura *et al*. 1998; Fujimura *et al*. 2003; Pelegrini *et al*. 2005). Possibly, this selectivity occurs due differences between the cell wall structure of Gram-negative and Gram-positive bacteria, mainly in the thickness of the peptidoglycan layer (Pelegrini *et al*. 2005) and in the presence or absence of lipopolysaccharide (LPS), a constitutive compound of the outer membrane of Gram-negative bacteria. Plant defensins could interact with negative charges of lipid A, a compound of LPS. This interaction results in the death of Gram-negative bacteria and in the neutralization of the LPS effect (Frecer *et al*. 2004; Pelegrini *et al*. 2005). On the other hand, the bacterial membrane is mainly composed by phospholipids such as phosphatidyl glycerol, which have exposed negative charges (Hancock *et al*. 2000). Probably, defensins with antibacterial activity could show this lipid-protein interaction due their cationic properties, leading to an initial depolarization step of the bacterial membrane and further death (Thomma *et al*. 2002; Pelegrini *et al*. 2005). Several mechanisms of action have been suggested in order to explain this process, a hypothesis being postulated in which defensin may interact both with specific and non-specific membrane receptors. However, until now, the complete mechanism has not yet been elucidated (Liu *et al*. 2000; Frecer *et al*. 2004; Pelegrini *et al*. 2005; Stec *et al*. 2006).

All the information above reinforces the fact that plant defensins may be used as an efficient tool to control bacterial pathology by serving as a source of new antibacterial products, as good as the construction of bacterial resistant transgenic plants (discussed in another section), Moreover, these plants could be utilized as bio-factories, producing large quantities of antibiotics with activity toward bacteria with enhanced resistance to classic antibiotic.

PLANT DEFENSINS WITH FUNGICIDAL ACTIVITY

Antimicrobial defensins are also able to inhibit fungal growth. Most plant defensins described inhibit yeasts or filamentous fungi (Thomma *et al*. 2002). Antifungal peptides usually are capable of inhibiting more than one fungal species, such as the defensin isolated from *P. vulgaris*, which showed activity against *F. oxysporum* and *M. arachidicola* (Wang and Ng 2007). Furthermore, a peptide from *Trigonella foenum-graenum* also showed activity towards *R. solani* and *Phaeoisariopsis personata* (Olli and Kirti 2006). However, plant defensins generally present a specific activity towards a unique pathogen, as observed for a peptide from *P. sativum*, which was able to inhibit the activity of *Neurospora crassa* (Lobo *et al*. 2007). Another example is NaD1, a defensin from *N. alata* that was able to inhibit in 56% the growth of *F. oxysporum* at a 2 $\mu g.mL^{-1}$ concentration (Lay *et al*. 2003).

The mechanism of action of antifungal defensins has not yet beenn fully confirmed, but there is strong evidence that they act by interaction with glycosylceramides (GlcCer) at the fungal cell surface (Thevissen *et al*. 2004). Therefore, a defensin from *Raphanus sativus*, called *Rs*-AFP2, showed activity against *Pichia pastoris* and *Candida albicans*. When both fungi were mutated, reducing GlcCer in their membrane, it was observed that the mutants were resistant to *Rs*-AFP2, while the parental lines were susceptible to defensin attack (Thevissen *et al*. 2004). Moreover, assays with *Rs*-AFP2 against *S. cerevisae* and *Shizosaccharomyces pombe* showed that those fungi were resistant to this defensin by the fact that they are unable to express a gene encoding GlcCer in their membranes (Thevissen *et al*. 2004). Another interesting discovery was that *Rs*-AFP2 was specific to just one form of GlcCer, as it was not able to interact with glycosylceramides from other sources, such as soybean and human (Thevissen *et al*. 2004).

In order to evaluate if interaction between *Rs*-AFP2 to GlcCer was sufficient to cause membrane permeabilization and cell death, a study with *Rs*-AFP2 variants was performed (Thevissen *et al*. 2004). A mutant of this peptide, with a substitution of a Tyrosine to a Glycine at position 38, resulted in lost of antifungal activity by *Rs*-AFP2. However, the mutant defensin was able to interact with GlcCer of *P. pastoris*, although it could not lead to membrane permeabilization. In fact these results lead to the conclusion that interaction with GlcCer is important for fungal growth inhibition, but not sufficient to cause membrane damage and consequently cell death (Thevissen *et al*. 2004; Lay and Anderson 2005). Furthermore, it seems that, after binding to GlcCer, plant defensins induce Ca^{2+} influx and K^{+} efflux, leading to cell death, as it was reported to occur with *Rs*-AFP2 and *Dm*-AMP1 from *Dahlia merckii* when assayed against *Neurospora crassa* (Thevissen *et al*. 1996, 1999).

HOW CAN DEFENSINS IMPROVE THE RESISTANCE OF TRANSGENIC PLANTS?

Conscribing all the above information it seems obvious that the potential of defensins as a biotechnological tool for the discovery of new, natural drugs or in the construction of transgenic plants with enhanced resistance toward pests and pathogens exists (Pelegrini and Franco 2005). Due to a wide variation of defensin functions, several efforts in different fields have been made in order to obtain products derived from high technology that could bring a better life as well high production and lower cost to crop farmers.

In fact, not only have plant defensins been cloned and expressed in transgenic plants. Other peptides have also been utilized as a particular resistance source as lipid transfer proteins (Carvalho *et al*. 2006), digestive enzyme inhibitors (Franco *et al*. 2002) and also animal defensins (Langen *et al*. 2006). In the latter, a human β-defensin was expressed in genetically engineered *A. thaliana* Columbia 0 (Aerts *et al*. 2005) and gallerimycin, an antifungal peptide from the greater wax moth *Galleria mellonella*, was used to produce transgenic tobacco cv. 'Xanthi' nc. with resistance to fungal pathogens *Erysiphe cichoracearum* and *Sclerotinia minor* (Langen *et al*. 2006). Nevertheless, plant defensins have occupied the first position in a world race to produce high productive agricultural crops with resistance against biotic and non-biotic stresses. This excellent position could be strictly related to a multi-functionality of defensins that, as described before, could act against bacteria, fungi, insects and non-biotic stresses (Pelegrini and Franco 2005), being a masterpiece in the transgenic puzzle.

In the 1990s, pioneer studies started to visualize the enormous potential of defensins and their production by using transgenic plants. As previously observed, defensins are able to control fungi and bacteria and also are upregulated in the presence of several pathogens (Epple *et al*. 1995) being directly involved in plant defence. Hence, using this natural strategy, a gene coding a thionin, named Thi2.7, showed to be constitutively overexpressed in *Arabidopsis* plants, enhancing the resistance of the susceptible ecotype Columbia (Col-2) against the attack of *Fusarium oxysporum* f. sp. *matthiolae* (Epple *et al*. 1997). This resistance was measured in transgenic lines, which had a reduced loss of chlorophyll after inoculation. Furthermore, fungi on transgenic cotyledons had more hyphae with growth anomalies, including hyperbranching, in comparison to cotyledons of the parental line (Epple *et al*. 1997). In this same report, no transcripts for other pathogenesis-related or defence peptides could be detected in non-inoculated transgenic seedlings, indicating that all of the observed effects of the overexpressing lines are most likely the result of the toxicity of the TH12.1 thionin. These data clearly indicated that an overexpression of a single defensin could, in a first moment, unbalance the plant-pathogen relation, giving more defence artifices to plants.

In this trail, several other researches started to translocate defensins from one species to another. The same TH12.1 thionin, overexpressed in *A. thaliana*, was utilized to produce other transgenic crops such as tomato (*L. esculentum* L. Miller) cv. 'CL5915-93D4-1-0-3' (5915) (Chan *et al*. 2005). This gene was introduced into tomato, taking care to impede transgene expression in fruits by the use of a fruit-inactive promoter (RB7) isolated from tobacco. This procedure aimed to protect roots and leaves, thereby rendering genetically modified tomatoes more palatable. As expected, transgenic lines demonstrated the functionality of TH12.1 thionin was against the fungus *Fusarium oxysporum* f. sp. *lycopersici*. Moreover, a surprisingly result was also obtained. Despite TH12.1 thionin never been tested against bacteria, these same lines were also resistant to *Ralstonia solanacearum* (Chang *et al*. 2005). These data indicated that some peptides could have a wide protective effect against several phytopathogens and some collateral resistances not yet tested could be obtained. But not only *Arabidopsis* defensins have been utilized to construct transgenic crop plants. The alfalfa antifungal peptide (alfAFP) defensin isolated from *Medicago sativa* seeds was also used to produce genetically-modified potatoes cv. 'FR13-08', with resistance against the agronomically important fungal pathogen *Verticillium dahliae*. Expression of the alfAFP peptide in transgenic potato cv. 'FR13-08' plants provided robust resistance in greenhouses, this resistance being maintained under field conditions (Gao *et al*. 2000). To determine the performance of alfAFP under field conditions, trials were conducted in Illinois as well as Wisconsin and Oregon, two major potato producing states in the United States where *Verticillium* is a significant problem for potato production. In 1996, a total of 21 transgenic alfAFP and 25 transgenic control lines were evaluated by planting six cuttings of each line in both infested and uninfested soil. A similar experiment was repeated in 1997 and also in 1998. As result, transgenic potato which had shown high levels of disease resistance in the greenhouse, were confirmed to perform extremely well in the field in terms of fungal resis-

tance (Gao *et al*. 2000).

Moreover, the oat cell-wall-bound thionin was overexpressed in transgenic rice, conferring resistance to both phytopathogenic bacteria *Burkholderia plantarii* and *Burkholderia glumae* (Iwai *et al*. 2002). Moreover, the constitutive CaMV-ω promoter was utilized to drive the expression of the viscotoxin A3 cDNA from *Viscum album* in transgenic lines of *A*. *thaliana* ecotype C24 (Holtorf *et al*. 1998).

Another improvement that defensins could give to genetically modified plants is the resistance to metals (Mirouze *et al*. 2006). A recent work investigated and confirmed the potential of defensins to confer zinc (Zn) resistance. This result was obtained in *A*. *thaliana* Columbia 0 ecotype plants overexpressing the *A*. *halleri* AhPDF1.1 cDNA, which clearly displayed Zn tolerance when compared to control *A*. *thaliana* plants. In addition, defensins not only induced Zn tolerance but the mRNA and protein accumulation of some of these defensins were increased by Zn treatments. Surprisingly, AhPDF1.1 seems to be extremely specific since it confers tolerance to Zn but not to cadmium (Cd), iron (Fe), cobalt (Co) or copper (Cu) and nor to salt treatments (Mirouze *et al*. 2006). The authors involved in this work proposed a hypothesis that defensins could interfere with divalent metal cation trafficking and thus conferring Zn tolerance, probably binding to transport membrane proteins. Further studies, with certainty, will shed some light over the mechanism of action. However, this unforeseen role of defensins opens new horizons for the use of these molecules in the production of transgenic plants with high resistance to non-biotic stresses.

In spite of the use of defensin genes to show extreme effectiveness against phytopathogens and zinc stress, other strategies have also been developed. One example consists in the use of elicitors (Huffaker *et al*. 2006), which are compounds that are able to initiate innate immunity in plants through the recognition of a variety of pathogen-associated molecules. Huffaker *et al*. (2006) reported the isolation and characterization of a peptide with 23 amino acid residues length from *Arabidopsis*, named AtPep1, which activates transcription of the defensive gene defensin (PDF1.2). For this purpose, the gene locus encoding the *At*Pep1 peptide precursor was identified by using *in silico* analyses to search genomic sequences from *A*. *thaliana*. Orthologs were also identified by using the NCBI and The Institute for Genomic Research (TIGR) algorithms. Moreover, in order to obtain gene expression, RNA was isolated and reverse-transcribed with a RETROscript kit. The *Atpro Pep1* forward and reverse primers with the respective sequences generated a 310-bp intron-spanning product. The genomic sequence encoding PROPEP1 was amplified by using a forward primer and a reverse primer to generate a 1,078-bp product. The construct was transformed into chemically competent *E*. *coli* TOP10F′ cells that were plated out on LB-ampicillin (50 $\mu g.ml^{-1}$). A plasmid clone containing the full *PROPEP1* genomic DNA insert with no nucleotide errors was used to generate an *PROPEP1*/pBART construct. A pBART clone containing the *CaMV 35S*/*PROPEP1* construct, and a second clone containing the empty vector, were transformed into *Agrobacterium tumefaciens* strain AGLO cells by electroporation. The transformed cells were grown on 2× yeast tryptone (YT) medium containing 100 $\mu g.ml^{-1}$ spectinomycin, and viable colonies were screened by using RT-PCR with pART F and pART R primers. The constitutive expression of the AtPep1 precursor gene PROPEP1 in transgenic *Arabidopsis* plants induced several events including a related constitutive transcription of PDF1.2. Some morphological modifications are induced by defensin expression such as an increase in root development. Therefore, a remarkable enhancing in the resistance toward the root pathogen *Pythium irregulare* was also obtained (Huffaker *et al*. 2006). In conclusion, this innovative strategy induces an indirect expression of defensins, conferring resistance to transgenic plants. Additionally, some intriguing results have been obtained in transgenic plants expressing totally different protein classes, but that induced the production of unexpected defensins. An important example was observed in transgenic *Arabidopsis* ecotype Columbia Col-0 plants overexpressing an ionotropic glutamate receptor (RsGluR). This alteration in plants resulted in noticeable growth inhibition of *Botrytis cinerea*, a necrotic fungal pathogen, possibly due to up-regulation of the defensins, observed by microarray analyses (Kang *et al*. 2006). These analyses showed that jasmonic acid (JA)-responsive genes including defensins and JA-biosynthetic genes were up-regulated. RsGluR overexpression also inhibited growth of a necrotic fungal pathogen *Botrytis cinerea* possibly due to up-regulation of the defensins. Therefore, an ectopic expression of the cotton non-symbiotic hemoglobin triggered several defence responses and increased tolerance towards *Arabidopsis* diseases (Qu *et al*. 2006). The production of haemoglobin, also induced by the presence of exogenous defensin gene, led to constitutive expression of the defensin PDF1.2 and also the synthesis of other pathogenesis-related protein (PR-1), conferring enhanced resistance to *Pseudomonas syringae* and also tolerance to *Verticillium dahliae* (Qu *et al*. 2006).

Another strategy utilized to produce transgenic plants with enhanced resistance consists in the utilization of polyproteins. By using *Arabidopsis* ecotype Columbia-0 once more, a method for the expression of a transgene encoding a cleavable chimeric polyprotein was developed. The polyprotein precursor consists of a leader peptide and two different antimicrobial proteins (AMPs), DmAMP1 from *Dahlia merckii* seeds and Rs-AFP2 from *Raphanus sativus* seeds, which were linked by a linker peptide originated from a natural polyprotein occurring in seed of *Impatiens balsamina* (François *et al*. 2002). The chimeric polyprotein was cleaved in transgenic *Arabidopsis* ecotype Columbia-0 plants and the individual AMPs were secreted into the apoplast (François *et al*. 2004). Both AMPs were found to exert antifungal activity *in vitro*. It is surprising that the amount of AMPs produced in modified plants with some of the polyprotein transgene constructs was significantly higher, compared with the amount in plants transformed with a transgene encoding a single AMP. This indicated that the polyprotein expression strategy may be a way to boost expression levels of small proteins (François *et al*. 2002). By this way, the polyprotein encoding sequences of plasmid pFAJ3105 was constructed following the two-step recombinant PCR protocol (François *et al*. 2002). Cleavage of the pFAJ3340 polyprotein precursor resulted in the release of a Rs-AFP2 derivative with an additional Ser at its carboxy terminus (DmAMP1 S) and a DmAMP1 derivative with an additional pentapeptide at its amino terminus (DVEPG DmAMP1). The expression cassette in the resulting plasmid, called pFAJ3099, was digested and cloned in the corresponding sites of the plant transformation vector pGPTVbar to yield a new plasmid named pFAJ3105. The expression cassette in the resulting plasmid, was digested and cloned in the corresponding site of the plant transformation vector pFAJ3337, which is a pPZP-RCS2 derivative with a selectable marker gene cassette based on the *Streptomyces hygroscopicus pat* gene (François *et al*. 2002). With this methodology, Arabidopsis plants ecotype Columbia 0 were transformed using recombinant *A*. *tumefaciens* and ELISA assays were set up as competitive type assays. Furthermore, extraction of RNA from Arabidopsis leaves and northern-blot analysis via chemiluminiscent detection were also performed (François *et al*. 2002).

Finally, the production of defensins in plants could also take on a different objective, and not be utilized as a source of resistance. Plant defensins have also been indicated as possible new drugs to control human infections (Pelegrini and Franco 2005; Franco *et al*. 2006), especially when resistant bacteria are involved. In this field, transgenic plants could act as biofactories, producing defensins at a large scale for pharmaceutical use. For this purpose, several techniques have been developed, since production of such drugs often experiences major problems. An important study des-

cribed the transgenic expression of a seed-specific plant defensin gene in their host *A. thaliana* ecotype Columbia-0 using a formerly developed plant expression system. Therefore, both genes were cloned in a matrix attachment region, or MAR, based on a plant transformation vector and expressed in post-transcriptional gene silencing (PTGS)-impaired *A. thaliana* plants (Sels *et al*. 2006). After expression, peptides were purified to homogeneity and the correct folding was confirmed. Finally, expressed defensins were assessed for their inhibitory activity against several yeasts such as *S. cerevisae* BY4741 strain and *P. pastoris* strain GS115, being considered as potent candidates for pharmaceutical or agricultural antimycotics (Sels *et al*. 2006). In this manner, *PDF1.1* and *PDF1.3* gene sequences from *A. thaliana* were amplified from genomic DNA by PCR using specific primers and cloned into a modular vector system. Then, they were inserted into a plant transformation vector pMAR-p35S-*uidA*, where the resulting T-DNA overex-pression vectors contained p35S promoter-driven *PDF*-expression cassette and a selectable marker gene (*pat*), at each border flanked by a MAR sequence. After expression, both PDF1.1 and PDF1.3 were purified from crude leaf ex-tracts and tested against various pathogenic fungi (Sels *et al*. 2006). Probably novel studies will use PTGS-MAR and other expression systems in order to obtain large amounts of bioactive, correctly processed plant defensins from transgenic biofactories.

In summary, the present review clearly demonstrates that defensins from several sources can be successfully used for the genetic engineering of enhanced resistance agricultural plants to important fungal and bacterial pathogens. On a separate path, some studies also showed that plant defensins also could be used to genetically improve animals. With this in mind, a defensin from *Capsicum chinense* was expressed in bovine endothelial cells, conferring impenetrability of *Candida albicans* in transfected cells BVE-E6E7 (Anaya-Lopez *et al*. 2006). Results from this work point to the enormous potential of the use of defensins from plants in the treatment of animal mycoses, in the *in vitro* study of host-pathogen interaction, and in the development of novel drugs. This is only the beginning of the use of all benefits that defensins could bring to farmers, industries and consumers.

CONCLUDING REMARKS

Plant defensins have shown to be an important tool in the construction of transgenic plants as reported in this review. They are able to inhibit enzymes such as proteinases and α-amylases by combating insect pests and reducing the stored crop losses caused by these pathogens. They also have bactericidal and antifungal activity against a variable range of microorganisms, acting as a viable source for the development and production of novel drugs as well to improve the resistance of agricultural plants against these pathogens.

Hence, plant defensins have been used as novel tools for the development of transgenic plants resistant to phytopathogens, as well for the production of new medicines that help treat infections caused by bacteria and fungi (Selitrennikoff 2001; Pelegrini and Franco 2005). In this way, several peptides have been isolated and characterized for use in biotechnology for genetic improvement (Franco *et al*. 2002; Pelegrini and Franco 2005). Furthermore, many experiments have been applied in order to better characterize this group of defence peptides and also understand their mechanism of action towards pathogens and pests. Hence, the use of mutagenesis on DmAMP1, a plant defensin from *Dahlia merckii*, and the identification of a DmAMP1-sensitivity gene in *Saccharomyces cerevisiae*, SKN1, lead to novel insights on the mechanism of action for antifungal defensins (Thevissen *et al*. 2005). Furthermore, NMR for identification of defensins' 3-D structures, such as with VrD1 from *Vigna radiata* could lead to a better understanding of insecticidal peptides towards insect digestive enzymes (Liu *et al*. 2006). *In vivo* assays are also crucial for characterization of defensins function and way of action. Recently, research on DmAMP1 has demonstrated the ability of increasing *Carica papaya* resistance against *Phytophthora* sp., one of the main causes of diseases in this plant (Zhu *et al*. 2007). The construction of a transgenic *C. papaya* with enhanced quantities of DmAMP1 permitted its resistance towards the attack of this fungus (Zhu *et al*. 2007).

Finally, plant defensins can be considered an easy peptide to work with in biotechnology, as it is a small molecule with resistance to temperature (27°C-49°C) and pH variations (4-8), as well because of its diverse application in agriculture and medicine. Future experiments might lead to the use of these plant defence peptides in crop fields and also in medicare, in the form of bio-insecticides and antibiotics.

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