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Review

Modes of antifungal action and *in planta* functions of plant defensins and defensin-like peptides

Barbara DE CONINCK, Bruno P. A. CAMMUE, Karin THEVISSEN*

Centre of Microbial and Plant Genetics, KU Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium

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ABSTRACT

Plant defensins are small basic peptides that are inhibitory against a range of plant and human pathogens. Their *in vitro* antimicrobial activity and structural similarity with human and insect defensins indicated an important role for plant defensins in the innate immune system of plants. Regarding their mode of antimicrobial action, most plant defensins interact with a specific microbial surface receptor, resulting in microbial cell death *via* e.g. induction of apoptosis. However, accumulating evidence suggests additional *in vivo* functions of these plant defensins, and by extension of the more recently discovered defensin-like peptides, in general plant development. In this review we will discuss both, the functional roles of defensins in the plant and their modes of antimicrobial action.

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1. Introduction

Since the discovery of the first plant defensins in wheat and barley seed by Mendez and coworkers (Colilla *et al.*, 1990; Mendez *et al.*, 1990), originally termed γ-thionins, these intriguing plant peptides have often been the focus of pioneering research in diverse biological domains. They belong to a superfamily of structurally related peptides with representatives in vertebrates, invertebrates, plants and fungi, suggesting that they predate the evolutionary divergence in eukaryotes. As such, their main characteristics have also been extensively reviewed during the last decade (e.g. Lay and Anderson, 2005; Stotz *et al.*, 2009a; Carvalho and Gomes, 2009, 2011; Wilmes *et al.*, 2011). In the present manuscript, we focus on their increasing number of potential *in planta* functions and specificity of antifungal activity, based on most recent findings in these domains.

2. Plant defensin structure

The primary structure of plant defensins generally consists of an N-terminal acidic signal peptide and a basic mature peptide containing 45–54 amino acids. However, plant defensins with alternative structures have been identified including those from floral organs of different plant species (e.g. *Nicotiana alata* NaD1, *Petunia hybrida* PhD1 and PhD2) and ZmESR6 isolated from developing maize kernels, which contain an extra acidic C-terminal prodomain (Lay *et al.*, 2003; Balandín *et al.*, 2005; Fig 1). The function of this additional domain is

* *Corresponding author*. Tel.: +32 16 32 96 82; fax: +32 16 32 19 66.
E-mail addresses: Barbara.deconinck@biw.kuleuven.be (B. De Coninck), Bruno.cammue@biw.kuleuven.be (B. P. A. Cammue), Karin.thevissen@biw.kuleuven.be (K. Thevissen).

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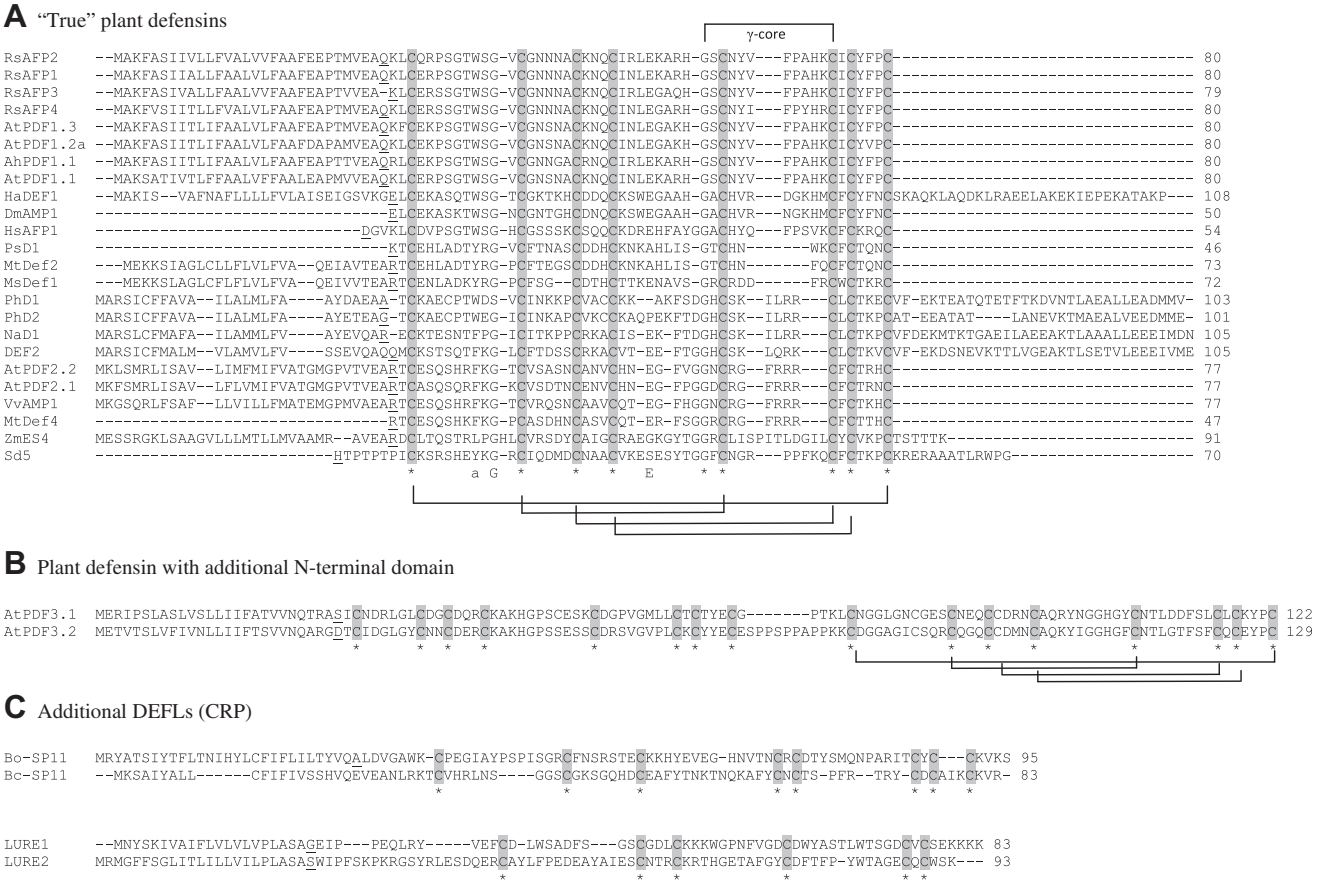

Fig 1 – Alignment of the amino acid sequences of plant defensins and defensin-like peptides mentioned in this review. Sequences have been aligned with ClustalW. Cysteine residues are marked in gray and indicated with an asterisk. Connecting lines between cysteine residues represent disulfide bonds. The start of the mature peptide is underlined. A "True" plant defensins. The conserved amino acids glycine (G), a glutamic acid (E) and an aromatic residue (a) are indicated below the sequences. B Defensin-like peptides with an additional N-terminal domain. C Defensin-like peptides with various structures. Numbers at the end of the sequences indicate the length of the peptide. Sequences were obtained from different databases. UniProtKB/Swiss-Prot: *Arabidopsis thaliana* AtPDF1.1 (P30224), AtPDF1.2a (Q9F123), AtPDF1.3 (O80995), AtPDF2.1 (Q41914), AtPDF2.2 (Q39182), AtPDF3.1 (P82789), AtPDF3.2 (P82773); *Arabidopsis halleri* AhPDF1.1 (Q29SA6); *Brassica campestris* Bc-SP11 (Q9ST12), *Brassica oleracea* Bo-SP11 (Q6F493); *Dahlia merckii* DmAMP1 (P0C8Y4); *Heuchera sanguinea* HsAFP1 (P0C8Y5); *Medicago sativa* MsDef1 (Q9FPM3); *Medicago truncatula* MtDef2 (Q5YLG7); *Nicotiana alata* NaD1 (Q8GTM0); *Petunia hybrida* PhD1 (Q8H6Q1), PhD2 (Q8H6Q0); *Pisum sativum* Psd1 (P81929); Radish RsAFP2 (P30203), RsAFP1 (P69241), RsAFP3 (O24332), RsAFP4 (O24331); *Saccharum officinarum* defensin 5 (Sd5, F2Z241), *Torenia fournieri* TfCRP1 (LURE1, B9ZZY1), TfCRP3 (LURE2, B9ZZY3); *Zea mays* ZmES4 (Q9AY28). GenBank ID: *Helianthus annuus* HaDEF1 (AAM27914). Other: DEF2 from *Solanum lycopersicum* (Stotz *et al.*, 2009b), *Vitis vinifera* VvAMP1 (de Beer and Vivier, 2008); *Medicago truncatula* MtDef4 (Sagaram *et al.*, 2011).

not known but it has been suggested to be involved in vacuolar targeting or in eliminating potential detrimental effects caused by the basic nature of the defensin. There are indications that plant defensins with other additional domains exist. For example, from the 15 plant defensins originally identified in *Arabidopsis thaliana* two (AtPDF3.1 and AtPDF3.2) contained an extra acidic cysteine-rich domain between the signal peptide and the mature defensin domain (Fig 1; Thomma *et al.*, 2002). It still needs to be determined whether this additional domain is a prodomain and as such potentially involved in targeting or in neutralizing the potential toxic activity of the basic effector domain during protein synthesis. Alternatively, the plant defensin domain might function as part of a fusion protein with still unknown activity. Like previously reported floral plant defensins containing an extra acidic C-terminal domain, AtPDF3.1 and AtPDF3.2 have also been detected in flowers and more specifically in the central cell of the female gametophyte (Steffen *et al.*, 2007; Wuest *et al.*, 2010).

The overall tertiary structure of plant defensins is defined by the presence of one α-helix and three antiparallel β-sheets stabilized by four disulfide bridges formed by eight conservative cysteine residues (Fig 1). In addition to this cysteine-stabilized αβ motif (CSαβ) defensins are also characterized by the occurrence of a γ-core motif GXC(X_{3-9})C, conserved among disulfide-containing antimicrobial peptides and characterized by the presence of two antiparallel β-sheets with an interposed short turn region (Yount and Yeaman, 2004).

While both motifs are highly conserved within plant defensins, overall amino acid sequence conservation is very poor and restricted to a glycine, a glutamic acid and an aromatic residue at positions 13, 29 and 11, respectively, with respect to the mature radish seed RsAFP2 identified as the first plant defensin (Terras *et al.*, 1992, 1995) which occur in most of the sequences (Fig 1).

3. Tissue and subcellular localization of plant defensins

Initially, most plant defensins were isolated from seeds but based on both protein localization and gene expression studies it is now clear that plant defensins can occur in all tissues of the plant including fruits, flowers, pollen, shoots, leaves, cotyledons, roots, bark (reviewed by Carvalho and Gomes, 2009, 2011). Some plant defensins are exclusively expressed in very specific parts of a tissue. The maize defensins ZmES1-4, for example, are exclusively expressed in the female gametophyte (Amien *et al.*, 2010), while ZmESR6 is expressed in the endosperm of immature kernels (Balandín *et al.*, 2005). Other plant defensins are constitutively expressed in a wide variety of tissues. For example, the *A. thaliana* AtPDF2.2 is expressed in seedlings, leaves, flowers, roots, siliques, stems and even in specific structures formed during interaction with other organisms such as syncytia in nematode-challenged roots (Siddique *et al.*, 2011).

Based on their predicted N-terminal signal peptide and the absence in their primary sequence of any known internal retention signal, most plant defensins are thought to be secreted. This extracellular localization was confirmed by immunolocalization studies for several plant defensins from seeds including *Raphanus sativus* RsAFP2 and *Medicago sativa* MsDef1 (Terras *et al.*, 1995; Gao *et al.*, 2000). More recently, using GFP-fusion protein experiments, de Beer and Vivier (2008) demonstrated that the predicted signal peptide of the *Vitis vinifera* defensin VvAMP1 results in protein accumulation in the apoplast. However from this experiment, it cannot be excluded that the full-length defensin contains internal retention signals. Accumulating evidence indeed suggests that some plant defensins are not transported to the apoplastic region but are retained intracellularly. A first convincing report described the vacuolar location of the flower-specific NaD1 in tobacco (Lay *et al.*, 2003). As mentioned before, NaD1 contains an additional C-terminal domain, which has been postulated to contain a vacuolar-sorting determinant (VSD). Recently Oomen *et al.* (2011) demonstrated that AhPDF1.1, a defensin from *Arabidopsis halleri* is not secreted in the plant. AhPDF1.1 enters the endomembrane pathway but on its way to the lytic vacuole it is retained in intracellular compartments. Vacuolar localization, however, could not be detected (Oomen *et al.*, 2011). In contrast to NaD1, AhPDF1.1 does not contain a C-terminal prodomain. More generally, it has been postulated that redirection of it to the vacuole requires a VSD, of which two types are currently known. The first is the sequence-specific VSD (ssVSD) prevalently characterized by the "NPIR" consensus sequence. The C-terminal VSD (ctVSD) lacks any consensus sequence but shows overrepresentation of hydrophobic amino acids, and is strictly C-terminally located (Zouhar and Rojo, 2009; Robinson *et al.*, 2005). Neither of the two VSDs was found in AhPDF1.1 (Oomen *et al.*, 2011). A possible explanation could be that to date relatively few VSDs have been characterized and new types remain to be discovered. Furthermore, based on proteomic analyses, Carter *et al.* (2004) demonstrated that many proteins are targeted to the vacuole *via* mechanisms that do not rely on only amino acid sequence or distribution patterns.

A potential vacuolar localization of plant defensins does not exclude a role in defense responses. Like chitinases, vacuolar defensins can be released only when plant cells are damaged by pathogens (Collinge *et al.*, 1993), thereby resulting in the concentration of defensins at the site of cell damage and slowing down the generation of plant defensin-resistance in the pathogen as a result of continuous exposure in the intercellular space. Moreover, an intracellular localization of plant defensins could potentially be related to (additional) *in vivo* roles of defensins not linked to plant defense (see section 6).

4. Large multigene families

Initial reports on plant defensins indicated the presence of such peptides predominantly in the seed of specific plant species, (Colilla *et al.*, 1990; Mendez *et al.*, 1990; Terras *et al.*, 1992, 1993; Osborn *et al.*, 1995; Almeida *et al.*, 2000). During the last decade however, it is proven that defensins are present in probably every plant species and in all types of organs and tissues (reviewed in Carvalho and Gomes 2009, 2011). Through the increasing availability of bioinformatics tools and resulting genomic data from various plants it is becoming clear that the number of peptides in general is significantly underestimated in plants. Recent studies led to the discovery of several additional cysteine-rich peptide (CRP) families in plants including at least 300 defensin-like peptides in *Medicago truncatula* (Fedorova *et al.*, 2002; Mergaert *et al.*, 2003; Graham *et al.*, 2004) and *A. thaliana* (Silverstein *et al.*, 2005, 2007) and 93 defensin-like peptides in *Oryza sativa* (Silverstein *et al.*, 2007). Most of the defensin-like peptides are expressed in nodules, seeds and reproductive organs. Compared to "true" plant defensins, plant defensin-like peptides also contain an N-terminal signal sequence, comparable intron size and position and conserved cysteine residues (Mergaert *et al.*, 2003; Graham *et al.*, 2004; Silverstein *et al.*, 2005, 2007). However the number and arrangement of cysteines can differ from the eight residues defined for "true" plant defensins (Fig 1), as was summarized by Silverstein *et al.* (2007). It should be noted that some authors also use the term CRP to indicate plant defensin-like peptides. We will further use the latter since CRPs refer to a much wider range of peptides including e.g. lipid transfer proteins, RALFs-like peptides and thionins (Silverstein *et al.*, 2007; Marshall *et al.*, 2011), which are not the focus of this review.

5. Antifungal activity

Several *in vitro* biological activities have been attributed to plant defensins (reviewed by Carvalho and Gomes, 2009,

2011). They have been reported to function as protein translation inhibitors, α-amylase and protease inhibitors or ion channel blockers. Several plant defensins reduce *in vitro* the activity of HIV1 reverse transcriptase and exhibit an antiproliferative activity effect toward several types of breast cancer cells. Only a few plant defensins have been shown to inhibit bacterial growth (reviewed by Carvalho and Gomes, 2009, 2011). Their best characterized activity, however, is their ability to inhibit the growth of a broad range of fungi and yeasts (reviewed by Carvalho and Gomes, 2009, 2011).

We will focus here on studies aimed at unraveling the modes of antifungal action of plant defensins as well as determining the protection or tolerance mechanisms of the fungus against the inhibitory or killing activity of plant defensins. Most of these studies make use of fungal or yeast mutants that are either resistant to plant defensins and are affected in plant defensin targets, or alternatively, are hypersensitive to plant defensins, and consequently, are affected in protection or tolerance mechanisms against plant defensins. Nearly two decades of research have yielded considerable insight into the modes of antifungal action of plant defensins. Several aspects have been investigated in this context: (i) their interaction with fungal-specific plasma membrane components, (ii) putative uptake of plant defensins and identification of intracellular targets, (iii) downstream signaling pathways activated by plant defensins with emphasis on the induction of apoptosis, and (iv) tolerance mechanisms of susceptible yeast and fungal species against the plant defensin activity. Many of these topics have been discussed in recent reviews (Aerts *et al.*, 2008; De Brucker *et al.*, 2011; Wilmes *et al.*, 2011). In the following paragraphs, we will present a short update on these different elements related to the antifungal modes of action of plant defensins (Fig 2).

In contrast to what was known for insect and human defensins, the first studies on the modes of action of plant defensins reported more than a decade ago pointed to interaction with fungal-specific membrane components (Thevissen *et al.*, 1997, 2000, 2004), being complex sphingolipids such as inositol phosphoryl-containing sphingolipids (M(IP)2C) and glucosylceramides (GlcCer). Sphingolipids are not only important structural components of eukaryotic membranes, but also fulfill an important role as secondary messengers, regulating the delicate balance between cell death and survival (Thevissen *et al.*, 2006). GlcCer distribution is not limited to fungal membranes, since large amounts of this glycosphingolipid have been also found in the fungal cell wall (Nimrichter and Rodrigues, 2011; Thevissen *et al.*, 2012), underscoring the important role of the cell wall in the plant defensin killing process. GlcCer is produced by most fungal pathogens (Barreto-Bergter *et al.*, 2004), and was recently shown to be required for virulence in *Candida albicans* (Noble *et al.*, 2010).

Up until now, the important role for sphingolipids in the killing process of fungi by plant defensins has been established for five different plant defensins, namely DmAMP1 from *Dahlia merckii* (Thevissen *et al.*, 2000), RsAFP2 from radish (Thevissen *et al.*, 2004), MsDef1 from *M. sativa* (Ramamoorthy *et al.*, 2007a), Sd5 from *Saccharum officinarum* (de Paula *et al.*, 2008), and Psd1 from pea (de Medeiros *et al.*, 2010). Evidence for the crucial role of sphingolipids in the killing/inhibitory process induced by these plant defensins was gathered

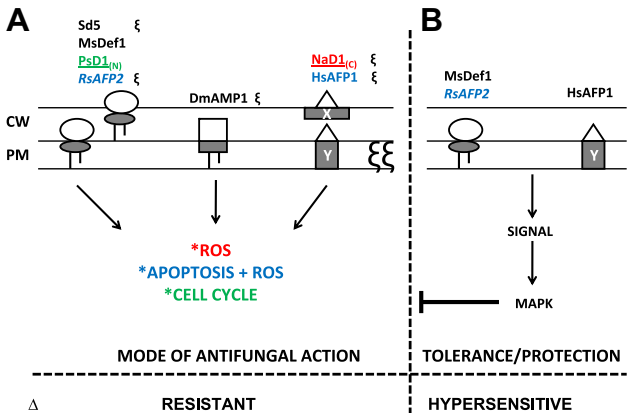

Fig 2 – Schematic representation of the plant defensin modes of antifungal action A and tolerance mechanisms against different plant defensins B. Plant defensins interact with various types of receptors, present in the fungal plasma membrane (PM) and/or in the cell wall (CW). Plant defensins that interact with GlcCer (gray ovals) are represented by white ovals; plant defensins that interact with $M(IP)_2C$ (gray rectangle) are represented by white rectangles; plant defensins that interact with as yet unknown receptors (gray rectangles represented by X or Y) are represented by white triangles. Plant defensins that can permeabilize plasma membranes are indicated with ξ. Upon interaction, some plant defensins stay in the extracellular space and are not taken up by fungal cells (italic), whereas others are taken up intracellularly (underlined), localizing in the nucleus (N) or cytoplasm (C). For the other defensins, uptake by fungal cells has not been proven. Plant defensins that induce reactive oxygen species (ROS) are depicted in red; plant defensins that induce apoptosis and ROS are depicted in blue, and plant defensins that interfere with cell cycle in green. Yeast or fungal deletion mutants in genes encoding for compounds of the above cascades (△) are either plant defensin resistant, thereby affected in plant defensin targets or components in the signaling cascades leading to killing or inhibitory effect of plant defensins, or alternatively, are plant defensin hypersensitive, thereby affected in compounds that are part of cascades leading to protection or tolerance mechanisms of the fungus against the action of plant defensins.

indirectly, *via* the observation that fungal mutants affected in sphingolipid metabolism are hypersensitive to these plant defensins, and/or directly, *via* binding studies of these plant defensins with purified fungal sphingolipids. To gain a better molecular insight into the interaction between plant defensins and their respective sphingolipid interaction partners, the backbone dynamics of Psd1 and Sd5 were probed and their interaction with membrane vesicles containing phosphatidylcholine or dodecylphosphocholine with fungal GlcCer was investigated further (de Medeiros *et al.*, 2010; de Paula *et al.*, 2011). These data indicated that the dynamic properties of Sd5 were completely different from those of Psd1, demonstrating that although defensins share similar structures, their dynamics can be extremely diverse. Hence, these studies

suggested that specific regions of the plant defensins are responsible for their ability to interact with GlcCer, ensuring anchorage to fungal membranes. Sagaram *et al.* (2011) recently demonstrated that the major determinants of the antifungal activity and morphogenicity of MsDef1 and MtDef4 from *M. truncatula* (Ramamoorthy *et al.*, 2007a) reside in their γ-core motif. Interestingly, the membrane interaction of Psd1 was found to be mediated in part by this γ-core motif.

Very recently, we demonstrated that RsAFP2 interacts with GlcCer present in the *C. albicans* cell wall, but is not taken up intracellularly (Thevissen *et al.*, 2012). This is in contrast to the intracellular localization of the plant defensins NaD1 and Psd1. It has been shown that NaD1 is taken up and localized to the cytoplasm of susceptible fungi, resulting in granulation of the cytoplasm and cell death (van der Weerden *et al.*, 2008). Apparently, NaD1 permeabilized fungal cells *via* a novel mechanism, which required the presence of the fungal cell wall (van der Weerden *et al.*, 2010). In the latter study, the authors hypothesized that a yet unidentified NaD1-receptor may be located in the proteinaceous layer of the cell wall. An intracellular accumulation was also demonstrated for Psd1. This plant defensin was demonstrated to localize to the nucleus of the fungus *Neurospora crassa* where it interacts with the cell cycle control protein Cyclin F (Lobo *et al.*, 2007).

Various plant defensins have been shown to induce apoptosis or programmed cell death of susceptible yeast and fungal species. RsAFP2 induces the accumulation of intracellular reactive oxygen species (ROS) and apoptosis in cells of the human pathogenic yeast *C. albicans* (Aerts *et al.*, 2007a, 2009). This RsAFP2-induced killing of *C. albicans* cells requires caspase or caspase-like proteases but is independent of metacaspase 1. Moreover, we recently demonstrated that RsAFP2 induces septin mislocalization and accumulation of apoptosis-inducing molecules, i.e., ceramides, in membranes of *C. albicans* (Thevissen *et al.*, 2012). Likewise, treatment of *C. albicans* cells with another plant defensin from *Heuchera sanguinea*, HsAFP1, resulted in ROS accumulation and the induction of apoptosis (Aerts *et al.*, 2011). In line with this, sodium azide, which blocks the respiratory electron transport chain, antagonized HsAFP1 antifungal activity, suggesting that a functional respiratory chain is indispensable for HsAFP1 antifungal action (Aerts *et al.*, 2011). Similarly, treatment of the plant pathogenic fungus *Fusarium oxysporum* with NaD1 was shown to result in increased accumulation of ROS (van der Weerden *et al.*, 2008). Whether NaD1 concomitantly induces apoptosis was not investigated further.

To defend themselves against the action of plant defensins, susceptible yeast and fungal species make use of various tolerance mechanisms. In order to identify genes that are involved in governing tolerance to HsAFP1 we recently screened a deletion mutant library of the model yeast *Saccharomyces cerevisiae* for mutants that showed hypersensitivity to HsAFP1. Many of the corresponding genes, so-called HsAFP1-tolerance genes, implicated the MAPK pathway playing a key role in maintaining cell wall integrity in distinct environmental conditions (Aerts *et al.*, 2011). This pathway is induced in periods of polarized growth and responds to heat, hypo-osmotic shock, cell wall damage, and oxidative stress (Martin *et al.*, 2005). Apparently, the MAPK cell wall integrity pathway is also involved in protection of the plant pathogenic fungus *Fusarium graminearum* to the radish RsAFP2 and to the alfalfa MsDef1 defensins (Ramamoorthy *et al.*, 2007b). Very recently, we could demonstrate a direct activation of the *C. albicans* MAPK cell wall integrity pathway by RsAFP2 (Thevissen *et al.*, 2012). Furthermore, we identified one HsAFP1-tolerance gene, *PTC1*, that has been implicated in the osmosensing high osmolarity glycerol (HOG) MAPK pathway that responds to osmotic stress, heat shock, oxidative stress and citric acid (Martin *et al.*, 2005).

6. *In vivo* role of plant defensins

Role in defense response

Based on their above-mentioned *in vitro* antifungal activity and predominantly extracellular localization, plant defensins were thought to play a major role in the plant defense response. The following evidence also supports this function.

(i) Firstly, as mentioned previously, defensins were initially and mainly isolated from seeds (reviewed by Carvalho and Gomes, 2009, 2011) and supposed to play a role in protection of the vulnerable germinating seed against pathogens (Terras *et al.*, 1995). For example, RsAFP1 and RsAFP2 from radish seeds are preferentially released during seed germination after disruption of the seed coat and the amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is suppressed (Terras *et al.*, 1995). In developing maize kernels, two antifungal defensins, ZmESR6a and ZmESR6b, were shown to be specifically produced shortly after pollination in the embryo-surrounding region (Balandín *et al.*, 2005).

(ii) Secondly, over the last decade transgenic overexpression of plant defensins in several plants resulted in increased resistance of those plants against several fungal diseases indicating their *in planta* potential to act as resistance traits against phytopathogenic fungi (Table 1). Interestingly, overexpression of an insect defensin in tobacco and a human defensin HBD2 in *A. thaliana* rendered plants more resistant against *Golovinomyces cichoracearum* and *Sclerotinia minor* (Langen *et al.*, 2006) and *Botrytis cinerea* (Aerts *et al.*, 2007b), respectively. Hence, apart from the known structural homology, additional functional homology exists between defensins originating from different eukaryotic kingdoms. As can be also deduced from Table 1 most studies were performed on plant defensins which were heterologously expressed. More indicative for their role *in planta* are studies on the effect of the modulation of plant defensin gene expression in their plant of origin. However, such reports are limited to (i) a study in tomato, demonstrating reduced susceptibility to *B. cinerea* in plants overexpressing the tomato defensin gene *DEF2* (Stotz *et al.*, 2009b), and (ii) the more recent observation that overexpression of *AtPDF1.1* in *A. thaliana* leads to increased resistance toward the non-host pathogen *Cercospora beticola* but not to the host pathogen *B. cinerea* (De Coninck *et al.*, 2010). However a direct *in vivo* role of plant defensins in defense response has not yet been

Table 1 – Overview of transgenic plants overexpressing a plant defensin and their resulting phenotype

Species of origin	Defensin name	Transformed plant	Phenotype (increased resistance/tolerance to the indicated stressor)	Reference
Arabidopsis halleri	AhPDF1.1	*Arabidopsis thaliana*	Zn	Oomen *et al.*, 2011
Arabidopsis thaliana	AtPDF1.1	*Arabidopsis thaliana*	*Cercospora beticola*	De Coninck *et al.*, 2010
Brassica campestris	BsD1	*Nicotiana tabacum*	*Phytophthora parasitica*	Park *et al.*, 2002
Brassica juncea	BjD	*Arachis hypogaea*	*Pheaoisariopsis personata*, *Cercospora arachidicola*	Swathi *et al.*, 2008
		Nicotiana tabacum	*Fusarium moniliforme*, *Phytophthora parasitica* pv. *nicotianae*	
Brassica rapa	BrD1	*Oryza sativa*	*Nilaparvata lugens*	Choi *et al.*, 2009
Dahlia merckii	DmAMP1	*Carica papaya*	*Phytophthora palmivora*	Zhu *et al.*, 2007
		Oryza sativa	*Magnaporthe oryzae*, *Rhizoctonia solani*	Jha *et al.*, 2009
		Solanum melongena	*Botrytis cinerea*, *Verticillium albo-atrum*	Turrini *et al.*, 2004a
Medicago sativa	MsDEF1/alfAFP	*Solanum lycopersicum*	*Fusarium oxysporum* f.sp. *lycopersici*	Abdallah *et al.*, 2010
		Solanum tuberosum	*Verticillium dahliae*	Gao *et al.*, 2000
Nicotiana megalosiphon	NmDef02	*Solanum tuberosum*	*Phytophthora infestans*, *Alternaria solani*	Portieles *et al.*, 2010
		Nicotiana tabacum	*Phytophthora parasitica* var. *nicotianae*, *Peronospora hyoscyami* f.sp. *tabacina*	
Orychophragmus violaceus	Ovd	*Brassica napus*	*Sclerotinia sclerotiorum*	Wu *et al.*, 2009
Pisum sativum	DRR230	*Brassica napus*	*Leptosphaeria maculans*	Wang *et al.*, 1999
Raphanus sativus	RsAFP2	*Nicotiana tabacum*	*Alternaria longipes*	Terras *et al.*, 1995
		Oryza sativa	*Magnaporthe oryzae*, *Rhizoctonia solani*	Jha and Chattoo, 2010
		Solanum lycopersicum	*Fusarium oxysporum* f.sp. *lycopersici*, *Botrytis cinerea*	Kostov *et al.*, 2009
		Triticum aestivum	*Fusarium graminearum*, *Rhizoctonia cerealis*	Li *et al.*, 2011
Solanum lycopersicum	DEF2	*Solanum lycopersicum*	*Botrytis cinerea*, reduced seed setting, pollen viability, growth changes	Stotz *et al.*, 2009b
Wasabia japonica	WT1/WjAMP1	*Colocynthis citrullus*	*Alternaria solani*, *Fusarium oxysporum*	Ntui *et al.*, 2010
		Oryza sativa	*Magnaporthe grisea*	Kanzaki *et al.*, 2002
		Phalaenopsis orchid	*Erwinia carotovora*	Sjahril *et al.*, 2006
		Solanum tuberosum	*Botrytis cinerea*	Khan *et al.*, 2006
Zea mays	ZmDEF1	*Nicotiana tabacum*	*Phytophthora parasitica*	Wang *et al.*, 2011

established, since in the same studies knock-down plants of the corresponding single plant defensin genes did not result in plants with an altered disease phenotype (Stotz *et al.*, 2009b; De Coninck *et al.*, 2010). This can probably be explained by functional redundancy of several (similar) plant defensins. Indeed, in *A. thaliana* two more genes (*AtPDF1.2b* and *AtPDF1.2c*) encode for the same mature peptide as *AtPDF1.2a*, and a fourth gene (*AtPDF1.3*) encodes for a peptide that only differs in one amino acid. These four genes have been detected in leaves and accumulate after *B. cinerea* inoculation (De Coninck; unpublished data). Therefore, at the moment we are evaluating the effect of RNAi-cosilencing of all four above-mentioned *AtPDF1*-genes on the resistance of *A. thaliana* to different pathogens. In the context of plant defensin gene redundancy, the discovery of more than 300 defensin-like peptides in *A. thaliana* adds further complexity to the potential involvement of plant defensins in plant defense (Silverstein *et al.*, 2005, 2007).

In the framework of the potential application of plant defensins in genetically modified crops, it is important to evaluate their effect not only on pathogens but also on beneficial fungi. Turrini *et al.* (2004a, 2004b) investigated the effect of DmAMP1 on arbuscular mycorrhizal (AM) fungi which are crucial for soil fertility and plant nutrition. *Solanum melongena* plants overexpressing DmAMP1 were more resistant to *B. cinerea*. Interestingly, with respect to the AM fungus *Glomus mosseae* neither pre-symbiotic hyphal growth nor mycorrhizal colonization was affected by the presence of DmAMP1 in *S. melongena* (Turrini *et al.*, 2004a, 2004b).

(iii) Third, plant defensins from several plants are induced by a wide range of biotic stresses (reviewed by Lay and Anderson, 2005) and plant hormones involved in stress signaling. This paragraph highlights some examples demonstrating such induction of defensins under several conditions. Originally plant defensins could be detected in different plant species specifically in their seed (Terras

et al., 1992, 1993; Osborn et al., 1995) but not in vegetative tissues. Terras et al. (1995) reported the first detected induction of plant defensins (RsAFP3 and RsAFP4) in radish leaves after inoculation with *Alternaria brassicicola*. The best-known induced plant defensin in plants is the *A. thaliana* defensin *AtPDF1.2a*. The latter is induced by, for instance, necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Penninckx et al., 1996; Manners et al., 1998) and herbivoric insects (Moran and Thompson, 2001; Abe et al., 2008). Further analysis of the signaling pathways leading to pathogen-induced expression of *AtPDF1.2a* also indicated induction by the plant hormones ethylene (Et) and methyl jasmonate (MeJa). Consistently, *AtPDF1.2a* transcripts fail to accumulate after inoculation with *A. brassicicola* of *A. thaliana* mutants involved in methyl jasmonate (*coi1*) and ethylene signaling (*ein2*) (Penninckx et al., 1996, 1998). Therefore, *AtPDF1.2a* is now considered a general marker gene in *A. thaliana* for MeJa/Et-mediated plant responses. On the contrary, *AtPDF1.2a* is not induced by the salicylic acid-mediated signaling pathway (Penninckx et al., 1996; Manners et al., 1998) which is generally linked with the plant response to biotrophic pathogens; though it is reported to be induced by the biotrophic non-host pathogen *Blumeria graminis* f. sp. *hordei* (Zimmerli et al., 2004). Interestingly, in a recent study Ahmad et al. (2011) correlated MeJa responsiveness of *AtPDF1.2a* in different accessions of *A. thaliana* with enhanced basal resistance against the necrotrophic fungus *Plectosphaerella cucumerina* and the herbivoric cotton leafworm *Spodoptera littoralis*. On the other hand, while *AtPDF1.2a*, was not found to be induced by nematodes such as *Heterodera schachtii*, another *Arabidopsis* defensin, *AtPDF2.1*, appears highly induced by this cyst nematode in root-specific structures called syncytia (Szakasits et al., 2009; Siddique et al., 2011). The latter seems specific since transcriptome analysis of the syncytia showed a repression of the general defense response. The two reported cases on the induction of *AtPDF1.2a* and *AtPDF2.1* from *A. thaliana* exemplifies a possible specific regulation of different defensins during various stress conditions. The reported occurrence in *A. thaliana* of 15 plant defensins (Thomma et al., 2002) and of more than 300 plant defensin-like peptides greatly enforces this complexity of defensin-involvement in stress responses.

Plant defensins not only play a role in defense against fungi and nematodes but strikingly are also involved in the defense response against parasitic plants. The sunflower root defensin HaDef1, for example, is highly induced by the dicotyledonous parasite *Orobanche cumana* in a resistant sunflower genotype (Letousey et al., 2007). The induction of HaDef1 coincides with the appearance of necrotic symptoms on *O. cumana* (Letousey et al., 2007). Treatment of *O. cumana* seedlings with HaDef1, at concentrations necessary for antifungal activity, caused browning of the radical apex resulting from localized cell death. However, HaDef1 did not induce browning of another parasitic plant nor the non-parasitic plant *A. thaliana* (de Zélicourt et al., 2007), again indicating the specificity of biocidal activities of plant defensins. This is the first report of a plant defensin that causes cell death of a plant cell. Similar to the studies on antifungal plant defensins interacting with specific sphingolipids in the fungal membrane, it would be very interesting to investigate whether HaDef1 can interact with specific targets in the plasma membrane of *O. cumana*.

Role in symbiotic interactions

Recent data indicate that plant defensins and defensin-like peptides are involved in symbiotic interactions with both mycorrhizal fungi and nitrogen-fixing bacteria.

While a plant defensin was induced in roots of *M. truncatula* upon infection by the AM fungus *Glomus versiforme* (Hanks et al., 2005; Liu et al., 2007), a defensin from birch was downregulated by *Paxillus involutus* (Johansson et al., 2004). As mentioned earlier some plant defensins were reported to have antifungal activity but no "anti-AM fungus" activity.

Interesting data are available on the roles of plant defensin-like peptides in symbiotic interactions with nitrogen-fixing bacteria. As mentioned before, a large family of cysteine-rich peptides which resemble antimicrobial defensins, by other authors referred to as defensin-like peptides, were found in nodules of *M. truncatula* (Mergaert et al., 2003; Graham et al., 2004). These nodule-specific cysteine-rich peptides (NCRs), including defensin-like peptides, play an important role in the symbiosis between legume plants and *Rhizobium* bacteria as some of them are reported to be transported to the bacteroid membrane and cytosol (Van de Velde et al., 2010). The high amount and diversity of NCRs in nodules opened an intriguing new field of research on the role of defensin-like peptides. Identifying molecular targets of specific NCRs and potential defensin-like peptides will be the most important step in unraveling the mode of action of NCRs.

Involvement of plant defensins in other stresses

Plant defensins were reported to be induced by a wide variety of other stresses including wounding (van den Heuvel et al., 2001; Pervieux et al., 2004; Shen et al., 2005; Bahramnejad et al., 2009), cold (Koike et al., 2002; Carvalho et al., 2006), salt and drought stresses (Do et al., 2004). However, reports on a functional relationship between abiotic stress and plant defensins are restricted to the intracellular defensin *AhPDF1.1* from the zinc hyper-accumulating plant *A. halleri*, which was identified by performing a functional screening for Zn tolerance in yeast (Mirouze et al., 2006). *AhPDF1.1* is induced by $ZnCl_2$ treatment and overexpression of *AhPDF1.1* in *A. thaliana* resulted in enhanced tolerance to both Zn and selenite (Mirouze et al., 2006; Tamaoki et al., 2008).

Effect of defensins on root development

Accumulating evidence suggests that plant defensins and defensin-like peptides not only play a role in defense response against phytopathogenic fungi but that they are also involved in plant growth and development. Since supporting data are most pronounced for such a role in roots and reproductive organs, this will be further discussed in this review.

In plant roots, defensins and defensin-like peptides have been reported to be constitutively present (Szakasits *et al.*, 2009; Siddique *et al.*, 2011) or to be induced by various triggers mostly related to interaction with other organisms including parasitic plants (de Zélicourt *et al.*, 2007), nematodes (Szakasits *et al.*, 2009; Siddique *et al.*, 2011), AM fungi (Hanks *et al.*, 2005; Liu *et al.*, 2007) and nitrogen-fixing bacteria (Van de Velde *et al.*, 2010). Interesting evidence on a possible function of plant defensins on root development was gained by Allen *et al.* (2008). Based on earlier observations that the antifungal MsDef1 from alfalfa is able to block mammalian L-type calcium channels (Spelbrink *et al.*, 2004) and that calcium influx is essential for growth of plant root hairs and fungal hyphae (Schiefelbein *et al.*, 1992), they investigated whether plant defensins can also affect root hair growth. MsDef1 indeed inhibited irreversibly root development and extension of root hairs of the model plant *A. thaliana* when applied at the same concentration necessary for antifungal activity. Intriguingly, application of other plant defensins such as MtDef2 or RsAFP2, which do not affect calcium channels, resulted in a similar inhibitory effect on root hairs, suggesting that the Ca-channel blockage is not essential in this activity. Similar observations obtained by application of other plant defensins to roots (Vijayan *et al.*, 2008; Oomen *et al.*, 2011) supported the data of Allen *et al.* (2008). Surprisingly, overexpression of *MsDef1* in *A. thaliana* did not alter root morphology (Allen *et al.*, 2008). Before concluding that plant defensins have a role in root development further research is required. For example, it will be important to define potential targets of plant defensins in roots and to determine whether the reported effects are also observed upon modulation of *in planta* defensin expression.

Role of defensins in reproductive organs

Accumulating evidence demonstrates that plant defensin-like peptides have evolved specific functions during plant reproduction. As a basis for understanding these functions, crucial components necessary for sexual reproduction can be summarized as follows (reviewed by Yadegari and Drews, 2004). The female gametophyte consists of four different cell types including two synergid cells, the egg cell, the central cell and three antipodal cells. The pollen tube, germinated from the male gametophyte and containing two sperm cells, is directed to the ovule and grows into one of the two synergid cells, which undergoes cell death upon pollen tube arrival. One sperm cell fertilizes the egg cell leading to the development of the embryo and the other sperm cell fuses with the central cell, giving rise to the endosperm. The female gametophyte is crucial in this reproductive process since it directs both the pollen tube growth and the sperm cells and controls seed development. A number of plant defensins and defensin-like peptides were reported to be expressed specifically in the cells of the female gametophyte of *A. thaliana* (Punwani *et al.*, 2007; Jones-Rhoades *et al.*, 2007; Steffen *et al.*, 2007; Wuest *et al.*, 2010), *Zea mays* (Cordts *et al.*, 2001; Amien *et al.*, 2010) and *Torenia fournieri* (Okuda *et al.*, 2009). For example, in *Z. mays* the defensin ZmES4 is demonstrated to be expressed in the synergid cells and to be required for pollen tube burst (Amien *et al.*, 2010). Interestingly, ZmES4 application induced membrane depolarization and opening of a specific potassium channel KZM1, present in the pollen tube (Amien *et al.*, 2010). As such, one can observe similar working mechanisms of plant defensins in their growth inhibitory activity against fungal hyphae, plant roots and pollen tubes. In addition to such an inhibitory activity, Okuda *et al.* (2009) recently identified two defensin-like peptides (called LUREs; Fig 1) in the synergid cells of *T. fournieri*, that rather act as pollen tube attractants. Plant defensin-like peptides have also been reported to be expressed in the male gametophyte. In Brassicaceae, for example, they are involved in the self-incompatibility system, developed by plants to prevent self-fertilization (Higashiyama, 2010; Marshall *et al.*, 2011). More specifically, the *Brassica* plant defensin-like SCR/SP11 (sterility-locus Cys-rich; Fig 1) functions as the male determinant in the pollen coat and is perceived by the female determinant, the S-locus receptor kinase (SRK), as such inhibiting self-pollen germination and pollen tube growth (Takayama *et al.*, 2000, 2001; Higashiyama, 2010).

7. Conclusions

The involvement of plant defensins during the plant defense response has been established based on (i) their stress-related induction (ii) *in vitro* antimicrobial activity and (iii) the increased resistance of plants expressing a specific heterologous plant defensin. Research during the past decade revealed that plant defensins bind to specific membrane receptor targets in order to fulfill their antimicrobial mode of action. The antimicrobial activity and high stability of plant defensins make them ideal tools for applications in the agricultural and medicinal sector. For more detailed information on those biotechnological applications, the authors refer the reader to the recent reviews from Carvalho and Gomes (2009, 2011) and Kaur *et al.* (2011).

Taking into account all recent reports on (i) the intracellular/vacuolar localization of some plant defensins, (ii) the inhibitory effect of several plant defensins on root growth, (iii) the induced plant cell death caused by HaDef1, (iv) the abiotic stress tolerance phenotype associated with AhPDF1.1, and (iv) the high amount of defensin-like peptides found in nodules and reproductive organs, it can be postulated that plant defensins acquired additional functions besides their role in plant defense. This phenomenon, called protein promiscuity, has been reported for several peptides in plant defense response (Franco, 2011) and has been postulated as being essential for peptide evolution. Based on above-mentioned examples, it can be stated that most functional roles of plant defensins are due to specific interactions with components of the plasma membrane.

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Prof. Dr. Bruno Cammue (1961, Belgium) obtained his PhD in 1989 at the Faculty of Bioscience Engineering from the KU Leuven. Currently, he is full professor at the Department of Microbial and Molecular Systems of the KU Leuven and heading the Center of Microbial and Plant Genetics. Within the latter he is leading the Plant–Fungi Interactions group (PFI), known for its pioneering research on antifungal plant defensins and stress-response plant peptides in general, including both their function in disease resistance of plants as well as the elucidation of their mode of biological activity, which formed the basis for various drug discovery programs.

Dr. Karin Thevissen (1969, Belgium) received her PhD in 1997 at the Faculty of Bioscience Engineering from the KU Leuven. Her PhD focused on the modes of action of plant defensins. Her postdoctoral work at the Center of Microbial and Plant Genetics, KU Leuven, was directed to the unraveling of the modes of action of various natural and synthetic antifungal compounds. In 2005, she obtained a position as Research Manager from the Industrial Research Fund, KU Leuven. In collaboration with the Centre of Drug Design & Discovery, KU Leuven, she identified various novel classes of antifungal and antibiofilm small molecules.

Dr. Barbara De Coninck (1980, Belgium) received her PhD in 2007 at the Faculty of Science (Biology) from the KU Leuven. Currently, she is a senior postdoctoral researcher at the Plant–Fungi Interactions group within the Center of Microbial and Plant Genetics. She studies the interaction between necrotrophic pathogens and the model plant *Arabidopsis thaliana* with a focus on stress-induced peptides, including plant defensins.