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Review

Antifungal proteins: More than antimicrobials?

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ABSTRACT

Antimicrobial proteins (AMPs) are widely distributed in nature. In higher eukaryotes, AMPs provide the host with an important defence mechanism against invading pathogens. AMPs of lower eukaryotes and prokaryotes may support successful competition for nutrients with other microorganisms of the same ecological niche. AMPs show a vast variety in structure, function, antimicrobial spectrum and mechanism of action. Most interestingly, there is growing evidence that AMPs also fulfil important biological functions other than antimicrobial activity. The present review focuses on the mechanistic function of small, cationic, cysteine-rich AMPs of mammals, insects, plants and fungi with antifungal activity and specifically aims at summarizing current knowledge concerning additional biological properties which opens novel aspects for their future use in medicine, agriculture and biotechnology.

1. Introduction

Small proteins with antimicrobial activity, so called antimicrobial proteins (AMPs), are produced by organisms throughout all kingdoms comprising prokaryotes, lower and higher eukaryotes. AMPs are secreted proteins that efficiently inhibit the growth of viruses, bacteria, fungi and parasites. In unicellular organisms, AMPs might provide their hosts the advantage to successfully compete with organisms that possess similar nutritional and ecological requirements. In multicellular organisms, AMPs constitute a primitive mechanism of innate immunity and form the first line of defence to protect their hosts from microbial attack. The innate immunity represents an evolutionarily ancient and widespread defence mechanism found in plants, insects and vertebrates. In addition, vertebrates developed the adaptive immune system – a sophisticated mechanism that uses antibodies and killer cells to recognize and eliminate invading microorganisms and allows immunological memory and self versus non-self recognition. The innate immune response is fast, and complements the adaptive immunity. Thus, both mechanisms combine to form an optimal and efficient defence system that supports the fitness of the host. The fact that closely related AMPs are widely distributed over different eukaryotic kingdoms, i.e. the class of defensins, suggests that ancestral AMP genes existed in basal eukaryotes even before fungal and insect lineages diverged (Lehrer and Ganz, 1999; Lehrer, 2007; Zhu, 2008).

AMPs are gene-encoded and they are either constitutively expressed or rapidly transcribed upon induction. In higher eukaryotes invading microbes and their products, e.g. lipopolysaccharides (Mendez-Samperio *et al.*, 2007; Amlie-Lefond *et al.*, 2005), or host cellular compounds, such as butyrate (Murakami *et al.*, 2002), cytokines (Wolk *et al.*, 2004; Wilson

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et al., 2007; Lai and Gallo, 2009), and vitamins (Schauber *et al.*, 2006) stimulate AMP production.

Due to the vast variety in function, structure, expression pattern, target organisms and producing hosts, the classification of AMPs is difficult and somewhat arbitrary to date. Mostly, AMPs are classified according to their functional and/or structural properties. Both characteristics are determined by the primary sequence of the protein which very often shows a high number of certain amino acids such as glycine, cysteine, histidine, proline, tyrosine, arginine, lysine and serine.

Although only a defined number of AMPs has been structurally analysed by nuclear magnetic resonance (NMR), calorimetric dichroism (CD) or X-ray crystallography, the secondary and tertiary structure of numerous AMPs has been predicted from primary sequence homologies. The most common classes contain proteins with α-helical, β-sheet or mixed α-helical/β-sheet structures (Zhu, 2008; Dimarcq *et al.*, 1998; Giangaspero *et al.*, 2001; Tossi *et al.*, 2000).

The best functionally characterized AMPs are bactericidal, whereas the properties of antifungal AMPs and their mode-of-action are less well studied. In the online database of AMPs at http://aps.unmc.edu/AP/main.php around 1900 AMPs of different origin are registered. From these, more than 1500 AMPs (79 %) have been assigned antibacterial activity compared to 648 antifungal AMPs (34 %). This classification, however, is redundant and antibacterial AMPs may also show antifungal activity that has not been investigated so far.

Most interestingly, the number of reports that document new additional functions of AMPs beyond their antimicrobial activity is constantly increasing. These features might arise from signalling functions of AMPs that accompany the activity of AMPs to interfere with the cell proliferation of microbes. For example, antimicrobial peptides from bacteria are part of the quorum-sensing mechanism that helps microorganisms to communicate and co-ordinate their behaviour by accumulating signalling molecules in the extracellular environment. This microbial communication system regulates e.g. symbiosis, biofilm formation, conjugation, sporulation, virulence, motility and the production of various secondary metabolites (Raina *et al.*, 2009; Maroti *et al.*, 2011). In fungi, plants and insects, AMPs have been related to development and differentiation (Stotz *et al.*, 2009a,b; Eigentler *et al.*, 2012; Hegedus *et al.*, 2011a), symbiotic interaction (Maroti *et al.*, 2011) and root hair extension (Allen *et al.*, 2008). In vertebrates, especially mammals, most diverse biological effects of AMPs have been reported, such as endotoxin neutralization (Rosenfeld *et al.*, 2010), signalling (Salzet, 2002), regulation of the immune response by chemotactic and immunomodulating activities (Wiesner and Vilcinskas, 2010; Yang *et al.*, 2002), induction of angiogenesis and wound repair (Baroni *et al.*, 2009) and protection against cancer (Yang *et al.*, 2002).

This review focuses on the properties and mode-of-action of small (5–8 kDa), cysteine-rich antifungal AMPs produced in mammals, insects, plants and fungi of related structure and highlights additional biological functions apart from antimicrobial activity. We want to apologize in advance for not being able to refer to all excellent publications available in this field due to space limitation. Readers specifically interested in antibacterial AMPs are directed to other excellent reports and reviews (Lehrer, 2007; Wiesner and Vilcinskas, 2010; Bulet and Stocklin, 2005; Yang *et al.*, 2004; Selsted and Ouellette, 2005; Lay and Anderson, 2005; Wong *et al.*, 2007; Papagianni, 2003; Boman, 2003; Brogden, 2005; Reddy *et al.*, 2004; Guani-Guerra *et al.*, 2010; Taylor *et al.*, 2008; Mygind *et al.*, 2005).

2. A short general overview on the structure and mode-of-action of antifungal cysteine-rich AMPs

The most prominent group within the antifungal AMPs with a close structural relationship constitutes defensins from plants, insects and mammals (Taylor *et al.*, 2008; Aerts *et al.*, 2008). Defensins contain six to eight cysteines which form intramolecular disulfide bonds and stabilize an antiparallel β-sheet conformation flanked by an α-helical segment, also called cysteine stabilized α/β motif (CSα/β) (Bulet and Stocklin, 2005; Selsted and Ouellette, 2005; Taylor *et al.*, 2008). Defensin-like antifungal AMPs of fungal origin are steadily increasing in number and show similar structural features as defensins but lack the α-helix (Campos-Olivas *et al.*, 1995; Batta *et al.*, 2009). The compact structure of defensins and defensin-like AMPs confers resistance towards extreme temperature, pH and protease-mediated degradation (Batta *et al.*, 2009; Bulet *et al.*, 1999; Landon *et al.*, 1997; Hajji *et al.*, 2010).

Based on their cationic character, defensins are thought to interact with negatively charged plasma membrane components of sensitive microorganisms. Two general models try to explain the mechanism of antimicrobial action of defensins: (A) the permeabilization of the cell membrane by (i) the carpet model and (ii) the pore model (Brogden, 2005). Whereas in (i) several protein molecules insert into the membrane forming a pore, in (ii) the protein molecules oligomerize and form a multimeric pore. Both models are primarily based on AMP-bacterial interaction and describe the disintegration of the plasma membrane, cell leakage and cell death by necrosis. (B) Alternatively, the membrane interaction of defensins and defensin-like AMPs may not primarily damage the plasma membrane of target cells. Instead, antifungal protein interaction with specific lipid and/or protein components of the plasma membrane leads to a selective ion permeability of the membrane and to the formation of transient pores and/or results in (active) protein transport into the host cell where these antifungals interact with intracellular targets (Brogden, 2005; Marx *et al.*, 2008; Thevissen *et al.*, 2003a). This antifungal activity increases the intracellular level of reactive oxygen species (ROS) and triggers programmed cell death (PCD) (Leiter *et al.*, 2005; Aerts *et al.*, 2011, 2009). Thus, plasma membrane leakage could occur at a later time point after protein contact with the fungal cell as a secondary effect of extensive intracellular ROS formation, as proposed for plant defensins (Thevissen *et al.*, 2003a).

3. Mammalian AMPs

Among mammalian AMPs, the defensins are best characterized and categorized in three subfamilies, α-, β- and θ-

defensins. Whereas the θ-defensins were primarily found in primates (Schutte *et al.*, 2002), α- and β-defensins are produced by humans and differ in the position of the disulfide bridges (Schneider *et al.*, 2005). α-defensins are products of neutrophils and Paneth cells of the small intestine (Mallow *et al.*, 1996; Ganz *et al.*, 1985) and human β-defensins 1–4 (hBD1, hBD2, hBD3, hBD4) are secreted by epidermal, epithelial and mesenchymal cells (Ali *et al.*, 2001; Varoga *et al.*, 2006; Zhao *et al.*, 1996). We will focus on this latter defensin group hBD1-4 that shows the closest homology to insect and plant defensins. Six conserved cysteine residues form three disulfide bridges and arrange three antiparallel β-sheets flanked by an α-helical segment (Pazgier *et al.*, 2006). Apart from their toxicity against gram-positive and gram-negative bacteria and viruses, these proteins possess antifungal activity against *Candida albicans*, *Candida krusei* and *Candida parapsilosis* (Feng *et al.*, 2005; Hoover *et al.*, 2003; Hazrati *et al.*, 2006; Sun *et al.*, 2005). β-defensins form the first line of defence against microbial infection and link innate with adaptive immunity. They are constitutively expressed or are induced by microbes, cytokines (TNF-α, IL-1) or even by the status of cellular differentiation (Liu *et al.*, 2002). Near the site of microbial invasion β-defensins form chemotactic gradients to recruit immature dendritic cells and memory T cells (Yang *et al.*, 2002, 1999). Immunomodulation is regulated *via* the Toll signalling pathway. For example, the murine β-defensin 2 (mDF2beta) is an endogenous ligand to the Toll-like receptor 4 and induces dendritic cell maturation (Biragyn *et al.*, 2002).

Apart from functions in the immune response, β-defensins regulate other most diverse cellular processes. For example, β-defensins affect sperm function including the initiation of motility and capacitation (Tollner *et al.*, 2004; Yudin *et al.*, 2005; Zhou *et al.*, 2004). hBD2 activates and triggers the degranulation of mast cells, which results in the release of histamine and prostaglandin D_2, two messenger molecules that regulate many physiological processes (Befus *et al.*, 1999; Niyonsaba *et al.*, 2001). hBD2 also stimulates the migration, proliferation and tube formation of endothelial cells in wounds and accelerates wound closure (Baroni *et al.*, 2009). Furthermore, hBD2–4 promote wound healing by supporting the migration and proliferation of keratinocytes (Niyonsaba *et al.*, 2007).

Notably, human β-defensins also have been associated with the pathogenesis of diseases. For example, hBD2 and hBD3 were originally purified from inflammatory skin disease psoriasis and proinflammatory cytokines induce their transcription (Harder *et al.*, 2001), whereas the loss of hBD1 favours cancer development (Sun *et al.*, 2006). hBD2 has been implicated in Crohn's disease (Fellermann *et al.*, 2006).

Thus defensins act not only as potent antimicrobial agents, but also as immunological adjuvants, similar to chemokines (Salzet, 2002), and as signalling molecules which regulate important cellular functions, ultimately contributing to the fitness of the host.

4. Insect AMPs

The antibacterial features of insect defensins are well documented (Bulet *et al.*, 1999; Cociancich *et al.*, 1993; Lehrer and Ganz, 1996; Otvos, 2000), whereas the number of antifungal insect defensins comprises only four members which show a high structural relatedness to mammalian and plant defensins: drosomycin from the fruit fly *Drosophila melanogaster* (Fehlbaum *et al.*, 1994; Zhang and Zhu, 2009), heliomicin from the tobacco budworm *Heliothis virescens* (Lamberty *et al.*, 1999), termicin from the fungus-growing termites *Pseudocanthothermes spiniger* and *Reticulitermes flavipes* (Lamberty *et al.*, 2001) and gallerimycin from the greater wax moth larvae *Galleria mellonella* (Lee *et al.*, 2004; Schuhmann *et al.*, 2003). Most insect AMPs are produced after microbial challenge in the fat body, equivalent to the mammalian liver, and certain haemocytes before they are released into the hemolymph.

Only modest information exists on gallerimycin and heliomicin. Gallerimycin has antifungal activity against *Aspergillus niger* and the entomopathogenic fungus *Metharhizium anisopliae*, but not against yeasts and bacteria (Schuhmann *et al.*, 2003; Mak *et al.*, 2010). Similarly, heliomicin is inactive against bacteria, but toxic against yeasts like *C. albicans*, *Pichia pastoris* and *Cryptococcus neoformans* and filamentous fungi such as *Neurospora crassa*, *Fusarium* sp., *Aspergillus fumigatus*, *Trichoderma viridae* (Lamberty *et al.*, 1999). In analogy to plant defensins (RsAFP2), heliomicin directly binds to fungal plasma membrane ceramides, i.e. glucosylceramides. Consequently, yeast deletion mutants in the glucosylceramide biosynthesis gene GCS1 were 20-fold less sensitive than the wild-type strains (Thevissen *et al.*, 2004).

The mode-of-action of drosomycin and termicin is better described. Although drosomycin is strictly antifungal at micromolar concentrations against filamentous fungi (*N. crassa*, *Fusarium* sp., *Alternaria* sp., *Botrytis cinerea*) and yeasts (*Saccharomyces cerevisiae*) (Zhang and Zhu, 2009; Tian *et al.*, 2008), bacterial challenge can induce drosomycin gene expression. The systemic gene expression is regulated by the Toll pathway (Zhang and Zhu, 2009). In addition, a local response in epithelia situated in the respiratory, digestive and reproductive tracts can also induce drosomycin expression independently of the Toll pathway (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000), whereby the inducible response in the respiratory tract is controlled by the Immune Deficiency (IMD) pathway (Zhang and Zhu, 2009).

Drosomycin lyses fungal hyphae suggesting a mode-of-action based on membrane permeabilization and pore formation in susceptible fungi (Fehlbaum *et al.*, 1994). Membrane components, such as sphingolipids, seem to play a central role for the interaction of drosomycin with its target organisms (Gao and Zhu, 2008). Indeed, a *N. crassa* mutant (Mut16) displaying clear differences in sphingolipid composition compared to the wild-type strain, was less susceptible to drosomycin (Ferket *et al.*, 2003). Most interestingly, drosomycin closely interferes with the *D. melanogaster* voltage-gated sodium channel ($DmNa_v1$). This observation and the detection of drosomycin transcripts in the *Drosophila* brain and thoracic-abdominal ganglia (Jiggins and Kim, 2005) further support the assumption that drosomycin might act as neuropeptide in the fly's central nervous system (Cohen *et al.*, 2009).

Tian *et al.* (2008) discovered for the first time that the antifungal drosomycin also exhibits antiparasitic activity. The observation that drosomycin inhibited the development of the ookinetes of *Plasmodium berghei* undoubtedly opens a new research field for the design of novel anti-malaria agents.

The termite antifungal defensin termicin inhibits the growth of the ubiquitous soil and entomopathogenic fungus *M. anisopliae* (Hamilton *et al.*, 2011). The termite *Reticulitermes* produces termicin together with antifungal glucanases, e.g. the gram-negative bacteria binding protein 2 (GNBP2), which efficiently breaks down β-1,3-glucans of fungal cell walls (Bulmer *et al.*, 2009). Termicin and GNBP2 are constitutively expressed in the salivary gland, stored in reservoirs and constitutively secreted and disseminated by self- and allo-grooming. The secretion of termicin and synergistically acting enzymes prevent *M. anisopliae* from attaching and penetrating the cuticle where it can effectively evade the insect immune defense (Hamilton and Bulmer, 2012). Furthermore, *Reticulitermes* incorporates termicin and GNBP2 in nest materials that may act as an external recognition system. The release of digested fungal cell wall material may trigger the immune defence of termites that are working in infested areas of their nests or support the accession of foraging grounds (Bulmer *et al.*, 2009). Thus the secretion of antifungal proteins, such as termicin, could have been crucial in overcoming strong pathogenic pressures during social evolution in insects (Hamilton and Bulmer, 2012).

5. Plant AMPs

The plant defensin family is quite diverse in its primary structure as only eight structure-stabilizing cysteines are conserved among all plant defensins (Thevissen *et al.*, 2003a). Reflecting the structural diversity, plant defensins display most diverse biological activities. Unlike the insect and mammalian defensins, which are mainly active against prokaryotes, only a few plant defensins have been shown to possess antibacterial activity (Stotz *et al.*, 2009b; Koike *et al.*, 2002; Segura *et al.*, 1998). Most plant defensins are active against numerous filamentous ascomycetes (Carvalho Ade and Gomes, 2009), including phytopathogenic fungi such as *Fusarium graminearum* (Sagaram *et al.*, 2011), *B. cinerea* (Stotz *et al.*, 2009a), and *Alternaria brassicicola* (de Zelicourt *et al.*, 2007). Some plant defensins inhibit the growth of baker's yeast and opportunistic human pathogenic fungi such as *C. albicans* (Thevissen *et al.*, 2004; de Zelicourt *et al.*, 2007). Plant defensins are divided into two groups: (i) antifungal activity is accompanied by changes in morphology such as hyperbranching of hyphae; (ii) antifungal activity occurs without hyphal distortions (Thomma *et al.*, 2002). Consequently, it is assumed that differences exist in the mode-of-action of these closely related antifungal proteins.

Defensins are expressed by various organs and tissues, e.g. in seeds and in peripheral cell layers of fruits and floral organs, including the style, the ovary and the stamen filaments, of different plants like Arabidopsis (Penninckx *et al.*, 1996), pea (Almeida *et al.*, 2000), spinach (Segura *et al.*, 1998), tobacco (Allen *et al.*, 2008; Lay *et al.*, 2003a,b) and radish (Terras *et al.*, 1992). The peripheral location of plant defensins in different generative tissues reflects their defence role against microbial invasion, either as inducible antimicrobial agents within vegetative tissues or as part of the constitutive defence barriers, especially in storage and reproductive organs (Thevissen *et al.*, 2003a). In addition some defensin genes are systemically induced upon fungal infection or wounding of vegetative tissues such as *pI39* and *pI230* in pea (Chiang and Hadwiger, 1991).

Among all antifungal defensins, the mode-of-action of plant defensins has been exceptionally well characterized at the molecular level. The following examples are given: the seed defensins DmAMP1 from dahlia (*Dahlia merckii*) (Osborn *et al.*, 1995), RsAFP2 from radish (*Raphanus sativus*) (Terras *et al.*, 1992), HsAFP1 from coral bells (*Heuchera sanguinea*) (Osborn *et al.*, 1995), MsDef1 from alfalfa (*Medicago sativa*) (Gao *et al.*, 2000), MtDef2 from barrel medic (*Medicago truncatula*) (Spelbrink *et al.*, 2004) and Psd1 from pea (*Pisum sativum*) (Almeida *et al.*, 2000). There is growing evidence that excludes a direct interaction of the cationic defensins with the negatively charged phospholipids of the plasma membrane as well as pore formation (Thevissen *et al.*, 1996; Caaveiro *et al.*, 1997). Instead, recent data underscore the existence of specific binding domains on the fungal plasma membrane, so called lipid rafts, which are specifically enriched in sphingolipids and sterols and which allow a high local concentration of membrane bound defensins (Thevissen *et al.*, 2003a). For example, DmAMP1 and RsAFP2 interact with sphingolipids of the fungal plasma membrane, DmAMP1 with mannosyldiinositolphosphoryl-ceramide $M(IP)_2C$, an acid complex sphingolipid (Thevissen *et al.*, 2000, 2005), and RsAFP2 with a neutral sphingolipid class of glucosylceramides (GlcCer) (Thevissen *et al.*, 2004). Notably, equimolar concentrations of ergosterol and sphingolipids enhance the interaction of DmAMP1, but not of RsAFP2 (Thevissen *et al.*, 2004, 2003b). Similar binding activities as for RsAFP2 have been proposed for MsDef1 (Ramamoorthy *et al.*, 2007a). HsAFP1 also interacts with specific, so far unidentified high affinity binding sites on plasma and microsomal membranes (Thevissen *et al.*, 1997). The interaction of DmAMP1 and RsAFP2 with sphingolipid components of the fungal plasma membrane results in an increased Ca^{2+} influx and K^+ efflux (Thevissen *et al.*, 1996). The question whether fungal growth arrest is a direct consequence of increased membrane permeability or of the interaction of plant defensins with intracellular targets has not been fully answered yet. Nevertheless, existing data suggest that more complex mechanisms than membrane permeabilization are involved in cell death induction. RsAFP2 and HsAFP1 for example, increase the level of intracellular ROS in *C. albicans* (Aerts *et al.*, 2011, 2007) which contributes to the induction of PCD (Aerts *et al.*, 2008). Environmental stress signalling cascades, such as the mitogen-activated protein kinase (MAPK) pathway may also be activated (Aerts *et al.*, 2011). The Gpmk1 and Mgv1 MAPK signalling were involved in the response to RsAFP2, MsDef1 and MtDef2 in *F. graminearum* (Aerts *et al.*, 2008; Ramamoorthy *et al.*, 2007b). Using a yeast two-hybrid screen the cell cycle control related protein cyclin F was found to interact with the *P. sativum* defensin Psd1 and further experiments proved the co-localization of Psd1 with the nucleus and the inhibition of cell division by Psd1 (Lobo *et al.*, 2007).

Apart from their antimicrobial defence function, defensins also fulfil other important roles in plant physiology. An exceptionally well researched overview on the complex functions of plant defensins is presented by Carvalho Ade and Gomes (2009). For example, plant defensins mediate resistance

against abiotic stress. The *Arabidopsis halleri* defensin AhPDF1.1 confers zinc- and a slight selenite-tolerance in *Arabidopsis* sp. (Oomen *et al.*, 2011; Mirouze *et al.*, 2006; Tamaoki *et al.*, 2008).

Other features of plant defensins are related to the regulation of growth, development and fertilization. The supplementation of the culture medium with defensins MsDef1, MtDef2, RsAFP2 or AhPDF1.1 inhibits plant root growth (Allen *et al.*, 2008; Oomen *et al.*, 2011). The *Solanum lycopersicon* DEF2 is produced during early flower development and promotes meiosis. As such it plays a central role in regulating the growth of different plant organs. During later stages of flower differentiation the inactivation of DEF2 is crucial for survival and development of pollen grains. Consequently, antisense suppression or constitutive overexpression of DEF2 negatively interferes with pollen viability and seed production (Stotz *et al.*, 2009a,b).

Fertilization in flowering plants involves distinct pollen–pistil interactions that include determinants of self-incompatibility, factors for pollen germination and tube growth, pollen tube attractants and bursting (Amien *et al.*, 2010; Higashiyama, 2010; Okuda *et al.*, 2009; Schopfer *et al.*, 1999; Takayama *et al.*, 2001). The *Zea mays* defensin ZmES4 plays a central role in inter-gametophyte signalling. It mediates pollen tube bursting by activating the K^+ channel KZM1 (Amien *et al.*, 2010).

Antifungal plant defensins are also involved in the defence against parasitic plants and insects. The sunflower defensin Ha-DEF1 specifically combats invasion of the parasitic plant *Orobanche cumana*. When Ha-DEF1 is exogenously applied to the parasitic plant embryo, it causes browning symptoms at the radicle apex of *Orobanche* seedlings during the germination step, but has necrotic effects neither on other parasitic plants, e.g. *Striga hermonthica*, nor on the host plant *Arabidopsis thaliana* (de Zelicourt *et al.*, 2007). The defensin VrD1 from *Vigna radiate* seeds exhibits insecticidal activity by inhibiting the insect α-amylase that digests plant starch in the insect gut (Carvalho Ade and Gomes, 2009; Liu *et al.*, 2006).

Finally, the inhibitory potential of antifungal plant defensins towards certain human cancer cell types and viruses has also been reported. Sesquin from the ground bean *Vigna sesquipedalis* shows anti-proliferative activity towards leukaemia M1 cells and breast cancer (MCF-7) cells and inhibits human immunodeficiency virus-type 1 reverse transcriptase (Wong and Ng, 2005). The *Phaseolus limensis* defensin Limyin has anti-cancer activity (Wang *et al.*, 2009).

6. AMPs of fungal origin

The number of identified small, cysteine-rich defensin-like AMPs in ascomycetes is constantly increasing, but only few have been characterized in more detail, e.g. AFP from *Aspergillus giganteus* strain IfGB0203 (Wnendt *et al.*, 1994) and AFP_{NN5353} from *A. giganteus* strain A3274 (Binder *et al.*, 2011), PAF from *Penicillium chrysogenum* strain Q176 (Marx *et al.*, 1995), PgAFP from *P. chrysogenum* strain RP42C (Rodríguez-Martín *et al.*, 2010), strain bubble protein (BP) from *Penicillium brevicompactum* Dierckx (Seibold *et al.*, 2011), ANAFP from *A. niger* (Gun Lee *et al.*, 1999), NAF from *Penicillium nalgiovense* (Geisen, 2000), AcAFP and AcAMP from *Aspergillus clavatus* (Skouri-Gargouri and Gargouri, 2008) and NFAP from *Neosartorya fisheri* (Kovacs *et al.*, 2011). All these proteins exclusively exhibit antifungal activity against filamentous ascomycetes at micromolar concentrations, including opportunistic plant- and animal pathogens, such as *Fusarium* sp., *Botrytis* sp., and *A. fumigatus* (Marx, 2004; Meyer, 2008). Moreover, the *P. chrysogenum* PAF inhibits the growth of selected Zygomycete species (Galgoczy *et al.*, 2005). Further exceptions are (i) the *A. clavatus* AcAMP with antibacterial activity (Hajji *et al.*, 2010) and (ii) the *A. niger* AnAFP with anti-yeast activity (Gun Lee *et al.*, 1999). (iii) The recently characterized *P. brevicompactum* BP only weakly affects yeast growth (Seibold *et al.*, 2011). To date, PAF is one of the best characterized antifungal proteins of fungal origin besides its orthologous AFP from *A. giganteus* (Marx, 2004; Meyer, 2008; Marx *et al.*, 2008), and both exhibit functional similarities with plant defensins to a certain extent.

Although direct evidence is lacking, it is believed that the toxicity of many of these fungal AMPs is based on their interaction with molecules/receptors situated in the cell wall and/or the plasma membrane of target fungi from where signalling cascades are activated. In the case of PAF, heterotrimeric G-protein and cAMP/protein kinase A signalling are involved, whereas AFP activates the cell wall integrity pathway (Leiter *et al.*, 2005; Binder *et al.*, 2011, 2010a; Hegedus *et al.*, 2011b; Hagen *et al.*, 2007; Ouedraogo *et al.*, 2011). The interaction of PAF with the sensitive *Aspergillus nidulans* leads to an immediate hyperpolarization of the plasma membrane at hyphal tips accompanied by K^+ efflux and Ca^{2+} influx (Leiter *et al.*, 2005; Binder *et al.*, 2010b; Kaiserer *et al.*, 2003) which parallels the effect observed with plant defensins RsAFP2 and DmAMP1 (Thevissen *et al.*, 1996). Changes in the membrane potential might influence the activity of ion channels/pumps/transporters or be a result of their activation. We could prove that the *P. chrysogenum* PAF and the *A. giganteus* AFP_{NN5353} trigger a rapid Ca^{2+} influx which leads to a sustained elevation of the cytoplasmic Ca^{2+} resting level in the sensitive fungi *N. crassa* and *A. niger*, respectively. This Ca^{2+} influx, however, does not result from unspecific pore formation in the plasma membrane (Binder *et al.*, 2011, 2010b). It is well known that a sustained perturbation of Ca^{2+} homeostasis may trigger PCD. Intracellular ROS and apoptotic markers are indeed increased in PAF-treated sensitive fungi (Leiter *et al.*, 2005). Interestingly, supplementation of the growth medium with Ca^{2+} ions, decreases the toxicity of antifungal proteins and counteracts the perturbation of the intracellular Ca^{2+} resting level. This points towards the induction of adaptive responses that may be induced by ion/Ca^{2+} signalling (Binder *et al.*, 2011, 2010b; Ouedraogo *et al.*, 2011). Even though PAF and AFP exhibit close similarities in their structure, function and antimicrobial spectrum, they also show significant differences in their mechanisms of action. AFP modulates the cell wall composition of sensitive fungi by enhancing α-1,3-glucan synthase A (*agsA*) and the chitin synthase D (*chsD*) gene expression (Hagen *et al.*, 2007) which suggests an attempt by the fungus to counteract AFP attack. As evidenced by the close AFP relative AFP_{NN5353}, *agsA* induction is mediated *via* protein kinase C (Pkc)/MAPK A. Instead, PAF fails to trigger the cell wall integrity pathway (CWIP) and PkcA/MAPK A signalling is not activated (Binder *et al.*, 2010a).

Table 1 – Summary of the current knowledge regarding the mechanisms of antifungal action and additional biological functions of selected antifungal defensins and defensin-like AMPs from fungi, plants, insects and mammals.

	AMP producing organism	Interaction molecules of the fungal envelope	Cellular uptake	Mechanism of action and signalling pathways involved in fungi–AMP interaction	Biological functions beyond antimicrobial activity	References
Fungi	PAF *Penicillium chrysogenum* Q176	n.d.	+	Plasma membrane hyperpolarization, K^+ efflux, Ca^{2+} influx, perturbation of the intracellular Ca^{2+} homeostasis, cAMP/PkA signalling, generation of intracellular ROS, apoptosis	Signalling, supports asexual development	(Hegedus *et al.*, 2011a; Leiter *et al.*, 2005; Binder *et al.*, 2010a; Binder *et al.*, 2010b; Kaiserer *et al.*, 2003; Oberparleiter *et al.*, 2003)
	AFP *Aspergillus giganteus* lfGB0203	n.d.	±	Plasma membrane permeabilization, activation of the cell wall integrity pathway	n.d.	(Hagen *et al.*, 2007; Ouedraogo *et al.*, 2011; Theis *et al.*, 2005, 2003)
	AFP_{NN5353} *Aspergillus giganteus* A3274	n.d.	+	Ca^{2+} influx, perturbation of the intracellular Ca^{2+} homeostasis, MAPK signalling, activation of the cell wall integrity pathway	n.d.	(Binder *et al.*, 2011)
	Anisin1 *Aspergillus nidulans*	n.d.	n.d.	n.d.	Support of asexual development, oxidative stress signalling	(Eigentler *et al.*, 2012)
Plants	DmAMP1 *Dahlia merckii*	Sphingolipid $M(IP)_2C$	n.d.	K^+ efflux, Ca^{2+} uptake, alkalinization of the medium, membrane potential changes, membrane permeabilization	n.d.	(Thevissen *et al.*, 1996, 2000, 2005, 2003b, 1999)
	RsAFP2 *Raphanus sativus*	Sphingolipid GlcCer	n.d.	K^+ efflux, Ca^{2+} uptake, alkalinization of the medium, membrane potential changes, plasma membrane permeabilization, induction of ROS accumulation, MAPK signalling	Plant root growth inhibition	(Allen *et al.*, 2008; Thevissen *et al.*, 1996, 2004, 1999; Aerts *et al.*, 2007; Ramamoorthy *et al.*, 2007b)
	HsAFP1 *Heuchera sanguinea*	Interaction with plasma and microsomal membranes	n.d.	Plasma membrane permeabilization, ROS accumulation, apoptosis, MAPK signalling	n.d.	(Aerts *et al.*, 2011; Thevissen *et al.*, 1997, 1999)
	MsDef1 *Medicago sativa*	Sphingolipid GlcCer	n.d.	MAPK signalling	Mammalian Ca^{2+} channel blocker, plant root growth inhibition	(Allen *et al.*, 2008; Spelbrink *et al.*, 2004; Ramamoorthy *et al.*, 2007a,b)
	MtDef2 *Medicago truncatula*	n.d.	n.d.	MAPK signalling	Plant root growth inhibition	(Allen *et al.*, 2008; Ramamoorthy *et al.*, 2007b)
	Psd1 *Pisum sativum*	n.d.	+	Interaction with cyclin F and interferes with cell division	n.d.	(Lobo *et al.*, 2007)
	AhPDF1.1 *Arabidopsis halleri*	n.d.	n.d.	n.d.	Tolerance against zinc and selenite, inhibition of plant root growth	(Oomen *et al.*, 2011; Mirouze *et al.*, 2006; Tamaoki *et al.*, 2008)
	DEF2 *Solanum lycopersicon*	n.d.	n.d.	n.d.	Influence of pollen viability, seed production, and the growth of various organs of the producing plant	(Stotz *et al.*, 2009a)
	ZmES4 *Zea mais*	n.d.	n.d.	n.d.	Inter-gametophype signalling, induction of pollen tube burst	(Amien *et al.*, 2010)

(*continued on next page*)

Table 1 (*continued*)

	AMP producing organism	Interaction molecules of the fungal envelope	Cellular uptake	Mechanism of action and signalling pathways involved in fungi–AMP interaction	Biological functions beyond antimicrobial activity	References
	Ha-DEF1 *Helianthus inbred line* (LR1)	n.d.	n.d.	Plasma membrane permeabilization	Parasitic plant defence	(de Zelicourt *et al.*, 2007)
	VrD1 *Vigna radiata*	n.d.	n.d.	n.d.	Insect inhibitory activity	(Liu *et al.*, 2006; Chen *et al.*, 2004, 2002)
	Sesquin *Vigna sesquipedalis*	n.d.	n.d.	n.d.	Inhibitory activity against cancer cells, inhibitory effect towards HIV reverse transcriptase	(Wong and Ng, 2005)
	Limyin *Phaseolus limensis*	n.d.	n.d.	n.d.	Inhibitory activity against cancer cells	(Wang *et al.*, 2009)
Insects	Drosomycin *Drosophila melanogaster*	Sphingolipids	n.d.	Plasma membrane permeabilization, pore formation	Interaction with voltage-gated sodium channel ($DmNa_v1$), neuropeptide activity?	(Gao and Zhu, 2008; Ferket *et al.*, 2003; Jiggins and Kim, 2005)
	Heliomicin *Heliothis virescens*	Sphingolipid GlcCer	n.d.	n.d.	n.d.	(Lamberty *et al.*, 1999; Thevissen *et al.*, 2004)
	Termicin *Pseudocanthothermes spiniger, Reticulitermes flavipes*	Cell wall	n.d.	Acts synergistically with glucanases to break down β-glucans of the fungal cell wall	Immune defence, external recognition system, accession of foraging grounds	(Bulmer *et al.*, 2009; Hamilton and Bulmer, 2012)
Mammals	hBD1 *Homo sapiens*	n.d.	n.d.	Plasma membrane permeabilization, pore formation	Chemotactic for immature dendritic cells, suppression of cancer development	(Sun *et al.*, 2005; Yang *et al.*, 1999, 2000)
	hBD2 *Homo sapiens*	n.d.	n.d.	Plasma membrane permeabilization, pore formation	Chemotactic for $CD45R0^+$ memory T cells, chemotactic for immature dendritic cells, degranulation of mast cells, migration and proliferation of keratinocytes, promotion of wound healing	(Baroni *et al.*, 2009; Yang *et al.*, 1999, 2000; Befus *et al.*, 1999; Niyonsaba *et al.*, 2001; Niyonsaba *et al.*, 2006)
	hBD3 *Homo sapiens*	n.d.	n.d.	Plasma membrane permeabilization, pore formation	Chemotactic for immature dendritic cells, mast cell activation, migration and proliferation of keratinocytes, promotion of wound healing	(Harder *et al.*, 2001; Niyonsaba *et al.*, 2006; Chen *et al.*, 2007)
	hBD4 *Homo sapiens*	n.d.	n.d.	Plasma membrane permeabilization, pore formation	Mast cell activation, migration and proliferation of keratinocytes, promotion of wound healing	(Niyonsaba *et al.*, 2006; Chen *et al.*, 2007)
	mDF2beta *Mus musculus*	n.d.	n.d.	Plasma membrane permeabilization, pore formation	Maturation of dendritic cells	(Biragyn *et al.*, 2002)

n.d. – not determined.
cellular uptake: – no uptake; + intracellular localization.

Diverging data also exist about the localization of these two antifungal proteins. Whereas Theis *et al.* (2005, 2003) reported AFP to localize to the cell wall and/or the plasma membrane of sensitive fungi, a time- and concentration-dependent internalization and cytoplasmic localization of AFP was shown by Martin-Urdiroz *et al.* (2009). Another study showed the uptake of Alexa-labelled AFP and its co-localization with the nucleus (Moreno *et al.*, 2006). Instead, we detected the energy-dependent endocytic uptake of the *P. chrysogenum* PAF into the cytoplasm of sensitive fungi, but no co-localization with the nucleus (Batta *et al.*, 2009; Oberparleiter *et al.*, 2003). A similar uptake mechanism and staining pattern in sensitive fungi was shown for the AFP relative AFP_{NN5353} (Binder *et al.*, 2011).

Most interestingly, there is increasing evidence that antifungal proteins, similar to defensins from plants, insects and mammals, exhibit additional functions that go beyond antimicrobial activity. The *P. chrysogenum paf* and the *A. giganteus afp* exhibit a temporal and spatial regulation during asexual differentiation (Hegedus *et al.*, 2011a; Meyer *et al.*, 2002). Both genes are exclusively expressed in the vegetative mycelium at the developmental stage. No gene expression occurs in conidiophores or conidia (Hegedus *et al.*, 2011a; Meyer, 2008; Meyer *et al.*, 2002). Key regulators for asexual development and/or secondary metabolism such as StuA and VelA (Adams *et al.*, 1998; Adams and Yu, 1998) were suggested to play a role in *afp* and *paf* gene expression. As for *afp*, StuA is positively involved in *afp* expression in *A. giganteus* (Meyer, 2008), whereas for *paf*, the *P. chrysogenum* VelA seems to play a central role in *paf* gene induction (Hegedus *et al.*, 2011a; Hoff *et al.*, 2010). Notably, depletion of PAF results in the down-regulation of *brlA* that encodes the central regulator for mitospore development and spore production is severely reduced in the Δ*paf* strain compared to the wild type.

Moreover, expression of the *A. giganteus afp* is inducible by alkaline growth conditions, carbon starvation, excess NaCl and ethanol and heat-shock all of which point towards a stress-related expression pattern (Meyer *et al.*, 2002; Meyer and Stahl, 2002).

Taking advantage of a growing number of sequenced fungal genomes and using a computional approach, Zhu (2008) postulated the existence of 25 fungal CSα/β-defensins. We characterized one of these potential fungal defensins, Anisin1 from *A. nidulans*. Depletion of Anisin1 negatively affects *brlA* expression and asexual spore production. Furthermore, the deregulation of the major scavenger against H_2O_2, CatB, in a Δ*anisin1* mutant suggests a role of Anisin1 in sensing/signalling elevated H_2O_2 levels (Eigentler *et al.*, 2012). Although the antimicrobial activity of Anisin1 has not been investigated to date, we provided evidence that Anisin1 has an important function in improving the fitness of *A. nidulans* by linking stress signalling with developmental regulation (Eigentler *et al.*, 2012).

7. Conclusion

As summarized in Table 1, antifungal defensins and defensin-like AMPs exhibit complex biological functions in addition to antimicrobial toxicity. The multitude of biological functions undoubtedly underscores the great impact of these proteins on our life. They gain great medical and biotechnological significance as attractive candidates for drug development and combinatorial therapies. In addition, they allow crop improvement by the generation of transgenic plants with enhanced resistance against phytopathogenic fungi, insects and plant parasites (Terras *et al.*, 1995). Notably, the recent discovery that antifungal AMPs of fungal origin also cover sensing/signalling functions contributes to our knowledge on the regulation of fungal development and may be of extraordinary importance for the optimization of fermentation processes and for the production of secondary metabolites and other fungal products.

It is, however, most puzzling how one protein may display such functional diversity. One explanation could be its specific protein structure. The disulfide bonds between the cysteine residues not only stabilize a compact structure, but they may be sensitive to specific environmental or cellular redox conditions. These may influence the redox status of the protein itself and finally determine its conformation and binding affinity with target molecules and even promote protein dimerization or oligomerization (Batta *et al.*, 2009; Wu *et al.*, 2003; Hoover *et al.*, 2000, 2001). In this respect it may be assumed that the increase of ROS concentrations in the cytoplasm of sensitive fungi is triggered by the redox activity of antifungal defensins and defensin-like AMPs (Hegedus *et al.*, 2011b).

Especially the occurrence of antifungal AMPs in biotechnologically important fungi suggests a simple protein preparation from easy-to-handle microorganisms that are partially considered as “safe” by the Food and Drug Administration. However, increasing knowledge about the structure–function relation of defensins and defensin-like AMPs opens new opportunities to improve their function and their (selective) antimicrobial activity, or to introduce new activities (Francois *et al.*, 2005), for example, by the substitution of single amino acids or by the *de novo* synthesis of non-natural peptides. The latter uncouples protein production from biological systems and a nice example is given by characterization of the synthetic antifungal hexapeptide PAF26 (RKKWFW) (Munoz *et al.*, 2006, 2007, 2012; Lopez-Garcia *et al.*, 2007).

AMPs belong to a fast growing scientific field and the identification of novel antifungal proteins may be expected in the future. By a detailed characterization of the biological functions of antifungal AMPs, we will undoubtedly gain deeper insight into their modes-of-action. This will not only complete our understanding of specific cellular/biological processes but also provide an excellent basis for new approaches in medicine, agriculture and biotechnology.

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