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The evolution, function and mechanisms of action for plant defensins

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Abstract

Plant defensins are an extensive family of small cysteine rich proteins characterized by a conserved cysteine stabilized alpha beta protein fold which resembles the structure of insect and vertebrate defensins. However, secondary structure and disulphide topology indicates two independent superfamilies of defensins with similar structures that have arisen via an extreme case of convergent evolution. Defensins from plants and insects belong to the cis-defensin superfamily whereas mammalian defensins belong to the trans-defensin superfamily. Plant defensins are produced by all species of plants and although the structure is highly conserved, the amino acid sequences are highly variable with the exception of the cysteine residues that form the stabilizing disulphide bonds and a few other conserved residues.

The majority of plant defensins are components of the plant innate immune system but others have evolved additional functions ranging from roles in sexual reproduction and development to metal tolerance. This review focuses on the antifungal mechanisms of plant defensins. The activity of plant defensins is not limited to plant pathogens and many of the described mechanisms have been elucidated using yeast models. These mechanisms are more complex than simple membrane permeabilisation induced by many small antimicrobial peptides. Common themes that run through the characterized mechanisms are interactions with specific lipids, production of reactive oxygen species and induction of cell wall stress. Links between sequence motifs and functions are highlighted where appropriate. The complexity of the interactions between plant defensins and fungi helps explain why this protein superfamily is ubiquitous in plant innate immunity.

Introduction

Fungal diseases have an enormous impact on plants, animals and humans. They destroy agricultural crops [1], threaten the extinction of wildlife [2] and can kill immunocompromised humans who are more susceptible to fungal infections [3-5]. All organisms have evolved survival strategies to evade infection from pathogens. Plants have a number of unique defence mechanisms including physical barriers to pathogen invasion as well as a wide range of secondary metabolites and antimicrobial peptides (AMP) [6]. Plant AMPs include thionins, lipid transfer proteins, hevein-like peptides, knottin-like peptides, glycine-rich peptides, homologs of MBP-1 (AMP from maize), snakins, cyclotides, protease inhibitors (PIs) and defensins [7-10]. AMP's in plants are often expressed in seeds and reproductive tissues and are also up-regulated by stress induced by drought, salt, cold, pathogen invasion or wounding [11]. This review will focus on plant defensins which form one of the largest families of plant AMPs.

Defensin evolution and diversity

Plant defensins belong to a large and diverse evolutionary group of proteins called the cis-defensin superfamily. This superfamily was identified using structural similarity and topology to detect remote evolutionary homology [12]. The structures of cis-defensins vary, but are all built around a conserved core scaffold (Fig.1). The structure is referred to as a cystine-stabilised α-helix and β-sheet fold (CSαβ). A pair of cysteines in a CxC motif on the final strand of the antiparallel β-sheet form a pair of disulphide bonds to cysteines on a α-helix and are the origin of the 'cis' nomenclature (Fig.1A). Additional disulphide bonds further constrain the loops. The superfamily encompasses a wide array of related proteins whose functions have diverged to cover a wide array of functions throughout the eukaryotes. This includes homologous antimicrobial defensins from plants, fungi, and invertebrate animals that use elaborations of the same basic fold with varied disulphide bond number and loop lengths (Fig.2A). Additional superfamily members have diverged to perform functions ranging from self/non-self-recognition to venom neurotoxicity.

The cis-defensins share many features with the evolutionarily unrelated α-defensins and β-defensins from vertebrates (Fig.2B). This unrelated trans-defensin superfamily is similarly as diverse as the cis-defensins [13]. Vertebrate β-defensins have a similarly short α-helix and β-sheet structure stabilised by three disulphide bonds, but are constructed with a different topology to the cis-defensins, and lack the Csαβ motif [6, 12, 14-17]. They also share an analogous range of activities, and are involved in host defence (both by direct antimicrobial activity and chemoattractant signalling), toxins in several taxa, and enzyme inhibition [15]. It is worth noting that the term 'defensin' is sometimes used as a generic term to mean any protein with host defence activity [18]. This review will focus on the biological activity of plant defensins particularly their most well characterised function, anti-fungal activity.

Plant defensins

Plant defensins are major components of the innate immune system of plants [11]. In general, they are non-toxic to mammalian or plant cells. However, they are extremely active against fungal pathogens of both plants and humans. Plant defensins are small, cationic proteins that are common in seeds, but are also located in other parts of the plant including leaves, roots, bark, pods, tubers, fruit, and floral organs [6, 11, 19, 20]. Concentrated in epidermal cells and stomatal cells, they are produced in areas that are

likely to be the initial points of contact with pathogens. Plant defensins are comprised of 45-54 amino acid residues and are held together by four disulphide bonds. These disulphide bonds render the molecule highly stable to proteases and extremes of pH and temperature [21]. The spacing and connectivity of the eight highly conserved cysteines defines the plant defensin family [11, 16]. The *Petunia hybrida* defensins (PhD1 and PhD2) from the flowers of petunia are exceptions to this, with an extra pair of cysteines forming a fifth disulphide bond [22].

A sequence alignment of the 1292 known plant defensin sequences revealed that only the eight cysteine residues are completely conserved [12]. Apart from the cysteines, residues that are common are two glycine residues (positions 12 and 32), an aromatic residue (position 10) and a glutamate (position 27) (position numbers are relative to NaD1) [9] all of which contribute to the defensin fold. The sequence of plant defensins can be divided into seven loops, defined as the regions between conserved cysteine residues (Fig.3). The sequence variability of these loops determines the various biological functions and modes of action displayed by different members of the defensin family [16].

Plant defensins are further divided into classes I and II. These classes are defined by the structure of the precursor proteins predicted from cDNA clones [11]. Class I plant defensins comprise an endoplasmic reticulum (ER) signal sequence and a mature defensin domain. In contrast, class II defensins are produced from larger precursors with an ER signal sequence, together with a mature defensin domain and a C-terminal pro-peptide (CTPP) of 27-33 amino acids [6, 11]. The class I defensins are directed to the secretory pathway. They lack signals for post-translational modifications or subcellular targeting and accumulate in cell walls and the extracellular space. Class II defensins are targeted to the vacuole by the CTPP. In the vacuole they are proteolytically processed to release the mature defensin and are stored [11, 23]. Most class II defensins are produced by solanaceous species where they are expressed in floral tissues and fruit. However, a small number have been found in other species such as ZmESR-6 (*Zea mays* Embryo Surrounding Region-6) from maize [23, 24]. The CTPP also protects plant cells from the cytotoxic activity of this class of defensins during transport to the vacuole [23].

Lipid binding

Lipid binding is a common feature of plant and insect defensins [25] and the specificity of binding is often mediated by the loop 5 region. Defensins have been described that bind preferentially to lipid II [25], the fungal sphingolipids, mannosyldiinositol phosphorylceramide ($M(IP)_2C$) [26] and glucosylceramide (GlcCer) [27], phosphatidic acid (PA) [28, 29] and phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) [30, 31]. The loop 5 sequence can be used to predict the lipid binding specificity of a defensin [16], although defensins with different loop 5 sequences can bind to the same lipid. X-ray crystallography has been used to solve the structures of two defensins in complex with $PI(4,5)P_2$. These class II defensins from the Solanaceae form dimers [23] which have a cleft lined with cationic amino acids, predominantly from loop 5. This cleft termed the cationic grip has high affinity for PI(4,5)P2 (Fig 4).

Chimeric defensins and mutagenesis studies have revealed that the amino acid residues in loop 5 have a crucial role in antifungal activity. Substitution of loop 5 from MtDef4 onto the MsDef1 backbone produced a chimeric protein with similar activity to MtDef4 and not MsDef1 [32]. That is, the chimera gained the ability to inhibit glucosylceramide-deficient *F. graminearum*, which the native MsDef1 could

not. Similarly, a loop 5 swap from the weak antifungal NaD2, onto the potent antifungal NaD1 backbone changed the lipid binding preference from PI(4,5)P_2 to PA and resulted in reduced antifungal activity compared to wild type NaD1 [33].

Plant defensins have more than one function

The large variation in sequence that is displayed on the surface loops confers different functions to different defensins [16]. These functions include protein synthesis inhibition through interactions with nucleic acids [34], antibacterial activity, trypsin and α-amylase inhibition which interfere with insect digestion [35], roles in heavy metal tolerance [36] and plant development [37, 38] and sexual reproduction as well as inhibition of ion channels in mammalian and plant cells [39, 40]. These functions are described briefly below. The best characterised and most common function of plant defensins is their antifungal activity which is also described in detail in this review.

Protein synthesis inhibition in cell free systems

Prior to 1995, plant defensins were commonly called □-thionins [41] and were reported to inhibit protein synthesis in cell free systems. Two plant defensins from barley endosperm called □-hordothionin and□□-hordothionin displayed similar inhibitory activity on *in vitro* eukaryotic protein synthesis. Three different cell free systems, two of which were derived from mammalian cells and the third from crustaceans were used to test the inhibitory activity of the two hordothionins [42]. Both inhibited translation in all three systems probably by inhibiting polypeptide chain initiation and elongation [35, 42]. Interestingly, □-hordothionin inhibited activity of *in vitro* plant translation systems, but □-hordothionin did not [35]. *Vigna radiata* plant defensin 1 (VrD1), the defensin from mung bean, kills fungi and bacteria by inhibiting protein synthesis and arresting growth [43].

Trypsin or α-amylase inhibitory activity

Plant defensins with α-amylase inhibitory activity were first discovered in *Sorghum bicolor.* The three isoforms from sorghum, SIα-1, SIα-2 and SIα-3, are strong inhibitors of the digestive α-amylases from cockroaches and locusts. They appear specific for insect amylases, because they are only weakly inhibitory towards α-amylases from human saliva and the fungus *Aspergillus oryzae* and have no inhibitory activity against α-amylases from porcine pancreas, barley or *Bacillus spp* [11, 44, 45]. These defensins are likely to hamper nutrient acquisition by inhibiting starch digestion in the insect gut. More recently the plant defensins VrD1 and TvD1 were reported to inhibit the α-amylase from *Tenebrio molitor* (mealworm beetle) larvae [43, 46].

The first defensin that was reported to have trypsin inhibitory activity is produced in the seeds of *Cassia fistula*. The seeds have two defensins of 5.1 kDa and 5.5 kDa, but only the 5.5 kDa defensin is a trypsin inhibitor [47, 48]. A bifunctional defensin from cowpea (Cp-thionin) targets α-amylases from weevils and also inhibits trypsin [48-50].

Antibacterial activity

Antibacterial activity is a common feature of the vertebrate trans-defensins, but is less common in the cis-defensins from plants. Exceptions are the fabatins from the broad bean, *Vicia faba* that are active against Gram negative *P. aeruginosa* and have moderate activity against Gram negative *E. coli* and Gram positive *Enterococcus hirae.* They appear specific for bacteria and have no activity against *S. cerevisiae* or

C. albicans [37]. In contrast, defensins from other members of the *Fabaceae* such as VaD1 from azuki bean and plant defensin Ct-AMP1 from the butterfly pea (*Clitoria terna*) are toxic to both fungal and bacterial species [51, 52].

Heavy metal tolerance

Defensins from *Arabidopsis halleri* (AhPDF1.1-1.4) confer zinc tolerance to this species which, unlike *A. thaliana,* is a zinc hyperaccumulator. The role for AhPDF in metal tolerance was confirmed when transfer of the *A. halleri* defensin AhPDF1 into *A. thaliana* conferred tolerance to zinc. Further evidence for the role of AhPDF in zinc tolerance came from the observation that *A. halleri* plants increased the levels of AhPDF mRNA and protein in response to exposure to Zn [36].

Ion channel blocking

Some defensins inhibit sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) ion channels. Two defensins from *Zea mays,* ?1- and ?2-zeathionin displayed rapid, reversible and repeatable blocking of the Na^+ ion channel in a rat tumour cell line when tested with a whole cell patch clamp technique [53].

Another example is MsDef1, an antifungal defensin that inhibits the $Ca_v1.2$ (L-type) channel in mammalian cells. It is predicted to interfere with Ca^{2+} homeostasis in fungal cells leading to growth arrest, although activity on fungal calcium channels has not been demonstrated [39].

Certain plant defensins inhibit mammalian voltage-gated potassium channels (K_v) by physically blocking the channels, and preventing the passage of K^+ ions. The structure of plant defensins is similar to other proteins that block sodium or potassium channels such as the βαββ?structured scorpion toxins [54, 55]. Although plant defensins and channel-blocking scorpion toxins have evolved from a common ancestor, they have diverged markedly since then and most characterised defensins are not ion channel blockers [40]. Plant defensin and defensin-like proteins have homologous structures to the scorpion toxins, but generally lack the surface loop sequences required for interaction with specific ion channels [15]. In particular, the homologous scorpion toxins bind K_v channels via a conserved KCXN motif [56, 57]. Most plant defensins do not have this motif, apart from *Arabidopsis thaliana* defensin AtPDF2.3 which has part of the signature sequence at the homologous structural location. When examined further this plant defensin inhibited two different subtypes of mammalian K_v channels. AtPDF2.3 variants bearing a KC, a CXN or a KCXN toxin signature were also prepared and tested for inhibitory activity. They all inhibited K_v channels to some degree, but the variant KCXN was the most active [40]. Almeida and colleagues noted the similarity of the surface topology and charge of Psd1, the defensin from Pea, to the proteins that inhibit K^+ ion channels. Psd1 is known to block Ca^{2+} channels, but has yet to be tested on K_v channels [58].

Antiproliferative activity on cancer cells

The class II solanaceous defensins, NaD1 and TPP3 bind phospholipid $PI(4,5)P_2$ and disrupt the plasma membranes of mammalian cancer cells leading to rapid cell death. Poon and colleagues [30] hypothesised that these defensins pass through the membrane, possibly by endocytosis, and then attack the inner leaflet of the plasma membrane where $PI(4,5)P_2$ is located. Dimer formation is essential for $PI(4,5)P_2$ binding which is rapidly followed by oligomerisation. Sequestration of the $PI(4,5)P_2$ disrupts the connections between the plasma membrane and the cytoskeleton, resulting in membrane blebbing and fracturing of the membrane [30, 31, 59]. The class I defensin limyin from lima beans also has

antiproliferative activity against human liver hepatoma cells)(Bel-7402) and neuroblastoma cells (SHSY5Y) [60].

Root growth inhibition and effects on plant development

Plant defensins can have negative effects on the growth and development of plants. RsAFP2, MsDef2 and MsDef1 inhibit *A. thaliana* root growth in a dose dependent fashion over a similar concentration range as reported for antifungal activity [61]. No inhibition was observed in *Medicago truncatula* indicating that there is some species specificity [7, 61].

The biological rationale for this activity against roots may be for protection against invading parasitic plants. Sunflowers for example, overexpress the Ha-DEF1 defensin when infected by the parasitic plant *Orobanche cumana* [62]. This sunflower defensin inhibits root growth of both *O. cumana* and *O. ramosa,* but does not inhibit the parasitic plant *Striga hermonthica* or the non–parasitic plant *Arabidopsis thaliana* [63].

Plant defensins target many fungal species by varied mechanisms

Many plant defensins have antifungal activity against a wide variety of plant and animal pathogens and model species. Some oomycetes also succumb to defensin activity [64]. **Error! Reference source not found.** lists plant defensins with potent antifungal activity and the pathogens they affect.

The antimicrobial activity of defensins is largely directed at fungi while bactericidal activity is relatively rare. Defensins that inhibit both fungi and bacteria are DmAMP1, Ah-AMP1 and Br-AMP1 from the seeds of the *Asteraceae, Hippocastanaceae* and *Brassicaceae* families respectively [7, 51]. Rarely do defensins exhibit anti-bacterial activity against both Gram-positive and Gram-negative bacteria. However, Ns-D1 and Ns-D2 defensins from blackseed of the *Nigella sativa* plant have antifungal activity as well as anti-bacterial activity against both Gram-positive and Gram-negative *spp* [65].

Dahlia merkii antimicrobial peptide 1 – DmAMP1

DmAMP1 from the seed of *Dahlia merkii,* is active against a wide range of fungal species. [51, 66-68]. The antifungal activity is mediated via irreversible binding to the sphingolipid mannosyldiinositol phosphorylceramide ($M(IP)_2C$) [27, 66, 69, 70]. This was discovered using genetic complementation studies with *S. cerevisiae* which revealed that mutants defective in the *IPTI* and *SKN1* genes were less sensitive to DmAMP1. *IPTI* encodes the enzyme inositolphosphate transferase which is required for the final conversion of mannosyl-inositolphosphorylceramide (MIPC) to $M(IP)_2C$, a major sphingolipid in the membranes of
S. cerevisiae [27, 69, 71]. *SKN1* has an important role in the biosynthesis of $M(IP)_2C$ in
S. cerevisiae and functions to restore defects in cell wall synthesis and anchorage of cell wall proteins [72]. Sphingolipids, together with sterols, form membrane rafts (lipid rafts) and are enriched in specific domains in the outer leaflet of fungal plasma membranes [26, 27]. Therefore, DmAMPI may interact with sphingolipids which are concentrated in the lipid rafts.

The binding of DmAMP1 to $M(IP)_2C$ sparks a number of rapid responses from the fungus, including an increase in K^+ efflux and Ca^{2+} uptake, as well as changes in membrane potential and an increase in the uptake of membrane impermeable fluorescent dyes such as SYTOX Green [69]. It remains unclear

whether DmAMPI moves into the cytoplasm or remains in the membrane, controlling cellular processes that lead to fungal cell death. Other defensins like MsDef1 also exert their toxic effect at the plasma membrane whereas NaD1 and PsD1 both progress into the cytoplasm and intracellular targets [17, 32, 73].

Fungi activate the cell wall integrity (CWI) pathway after exposure to DmAMPI. The observation that DmAMP1 and NaD1 (described in a later section) activate different signalling cascades supports the hypothesis that different plant defensins have different mechanisms of action in killing fungi. The mode of action of DmAMPI is summarised in Fig.5.

Raphanus sativus antifungal peptides 1 & 2 – RsAFP1 and RsAFP2

RsAFP1 and RsAFP2 are defensins from the seeds of radish (*Raphanus sativus)*. They are nearly identical in sequence apart from variations at positions 5 glutamate/glutamine and 27 asparagine/arginine in RsAFP1 and 2 respectively [74]. RsAFP2 is 2-30 fold more potent than RsAFP1 towards filamentous fungi causing hyper-branching and growth reduction in hyphal tips [41]. This higher activity has been attributed to the higher overall net positive charge generated by the amino acid residues glutamine and arginine [74]. Like DmAMP1, RsAFP2 produces rapid membrane responses in fungi involving Ca^{2+} uptake and K^{+} efflux and changes to the membrane potential [75]. Two adjacent sites on the defensins were identified as important for the antifungal activity through mutational analysis of amino acids [76].

Studies on the interaction of *C. albicans*, *Pichia pastoris* and *Neurospora crassa* with RsAFP2 revealed that GlcCer is the specific target required for initiation of antifungal activity [27]. This explains why RsAFP2 is not active on *S. cerevisiae* which lacks GlcCer. Camelid antibodies that bind to fungal GlcCer also have antifungal activity and protective effects against fungal disease when sprayed on plants, indicating that binding to GlcCer on the fungal surface is sufficient to inhibit fungal cell growth [77]. However, the antibodies have less inhibitory activity than RsAFP2, consistent with a more complicated mechanism of action for RsAFP2 than simple binding to sphingolipid.

ROS production is induced after RsAFP2 docks onto the GlcCer in the membrane. Stressed cells produce ROS which in turn damages proteins, lipids and DNA inducing programmed cell death or apoptosis [78]. RsAFP2 also activates mitogen activated protein kinase (MAPK) signalling cascades of the CWI pathway in *Fusarium graminearum* [71, 79]. The CWI pathway is activated by cell wall damage (Fig.6). In *C. albicans* RsAFP2 induces the accumulation of long chain ceramides in the plasma membrane, this in turn leads to septin mislocalisation and consequently inhibition of the yeast to hyphal transition [79]. Finally, RsAFP2 activates fungal caspases to induce programmed cell death, but this activation is independent of metacaspase CaMca1p [80]. The mechanism of action of RsAFP2 is summarised in Fig.7.

Nicotiana alata defensin 1 – NaD1

NaD1, is a class II solanaceous defensin (see section 0), that is produced in the flowers of the ornamental tobacco, *Nicotiana alata*. It is active against several pathogenic fungi ([19, 81] and functions to protect the reproductive tissues against damage from potential fungal pathogens [82].

NaD1, like DmAMP1 and RsAFP2, requires the presence of the cell wall to initiate its specific and lethal effect on fungal cells. Van der Weerden and co-workers (2008) discovered that the filamentous fungus *Fusarium oxysporum* became resistant to NaD1 when the glycoprotein or 1,3-β-glucan layers were

damaged by incubation with proteinase K or 1,3-β-glucanase [17]. As mentioned earlier, NaDI forms dimers that bind tightly to phosphatidylinositol 4, 5-bisphosphate ($PI(4,5)P_2$) which is located on the inner leaflet of the plasma membrane This lipid interaction is essential for the cytotoxic effect of NaD1 on tumour cells which is associated with extensive membrane blebbing and cell lysis [30]. However interaction with $PI(4,5)P_2$ may not be essential for the antifungal mechanism because NaD1 variants that do not bind $PI(4,5)P_2$ still kill fungal cells albeit slightly less efficiently than wt NaD1 [33]. How NaD1 moves through the cell wall and the membrane to gain access to the inner leaflet where $PI(4,5)P_2$ is located, remains to be defined.

NaD1 enters the cytoplasm of the plant pathogen *Fusarium oxysporum* where interaction with intracellular targets leads to the production of reactive oxygen species (ROS), permeabilisation of the plasma membrane, granulation of the cytoplasm and cell death [17, 81]. Induction of oxidative stress by NaD1 has also been recorded for *C. albicans* [83]. Consistent with this, *S. cerevisiae* strains with mitochondrial gene deletions are more resistant to NaD1 than the wild type [84]. The HOG1 pathway is also activated in response to sub-lethal concentrations of NaD1, but unlike RsAFP2 and DmAMPI, NaD1 does not activate the CWI pathway [68]. The HOG1 pathway is activated by osmotic and oxidative stress along with other stresses (Fig.8).

Polyamine uptake has been linked to the antifungal mechanism of NaD1 [84]. Yeast cells lacking Agp2, a plasma membrane protein that regulates uptake of the polyamines spermidine and carnitine [84, 85], are more resistant to NaD1 compared to wild type cells. In addition, the Agp2 deletion mutant failed to import fluorescently tagged NaD1 as efficiently as the wild type control. This effect was not restricted to NaD1. Agp2 deficient cells were also less susceptible than wild type cells to other cationic antimicrobial peptides, which was attributed to an increase in positive charge on the outer surface of the mutant cells when the import of polyamines is impaired. This hypothesis was supported by the observation that cytochrome C, another positively charged protein of similar size, bound to the surface of wt but not the *agp2Δ* mutant cells [84]. The mechanism of the antifungal activity of NaD1 is summarised in Fig.9.

Medicago sativa defensin 1 – MsDef1

MsDef1, a 45 amino acid plant defensin from alfalfa seed (*Medicago sativa*) is also active against several fungal pathogens [86]. MsDef1 is morphogenic, that is, it causes hyphal branching in *F. graminearum.* It also inhibits conidial germination and mycelial growth in *N. crassa* [87].

The residues required for antifungal activity and the importance of signalling cascades have been described for MsDef1. Chimeras produced from MsDef1 and MsDef2 revealed that residues 31-45 in loops 4 and 5 (four of which are positive), and residues 1-15 in loop 1 of MsDef1 are essential for the antifungal activity. MsDef1 disrupts Ca^{2+} signalling and Ca^{2+} gradients in the hyphal tips leading to the characteristic hyper-branching that occurs with this morphogenic defensin [39, 88]. In another study, a knockout of the glucosylceramide gene, *gcs1*, in *F. graminearum*, Fg*gcs1,* blocked the activity of MsDef1 revealing the involvement of GlcCer in the mode of action as occurs with RsAFP2 [86]. MsDef1 activates two MAPK signalling pathways. Insertional mutation or gene replacement of *Gpmk1* and *Mgv1* genes in *F. graminearum* led to hypersensitivity to MsDef1 along with several other defensins, including RsAFP2. These genes are involved in regulating the cell wall integrity pathway, sexual reproduction and

pathogenicity. They are activated in *F. graminearum* with MsDef1 treatment consistent with a role for these MAP kinase signalling cascades in protection of the fungus against the toxic effects of MsDef1 [86, 89]. In contrast, the HOG1 pathway which is turned on in response to oxidative and osmotic stress is not activated by MsDef1 [89]. The mechanism of action of MsDef1 is outlined in Fig.10 (i).

Medicago truncatula defensin 4 – MtDef4

MtDef4 a 47 amino acid protein from the model legume *Medicago truncatula* is active against the filamentous fungal pathogen, *F. graminearum* [86]. Unlike MsDef1, which is from the same genus (*Medicago spp*), MtDef4 is non-morphogenic and is more potent against this pathogen. Like MsDef1, MtDef4 also inhibits the conidial and mycelial growth of *N. crassa* [32].

The contribution of loop 5 towards the antifungal activity of MtDef4 was determined using chimeras of MtDef4 and MsDef1 [32]. Loop 5 from MtDef4 (RGFRRR) was swapped with loop 5 of MsDef1 (RDDFR), known as MsDef1-⍰4 and the morphological responses in the fungus and the antifungal activity compared with that of the parent defensins. The MsDef1-⍰4 chimera was not morphogenic like its parent MsDef1, suggesting loop 5 is essential for the hyperbranching phenotype of MsDef1. [32]. The molecular target for loop 5 from MsDef 1 has not been elucidated, but loop 5 from MtDef4 binds to phosphatidic acid (PA), and is essential for MtDef4 entry into fungal cells [90]. In a later study, the interaction of fluorescently tagged MtDef4 with two different fungal species was examined using high resolution confocal microscopy. This defensin inhibited the growth of both fungi, but the mechanism for transport across the plasma membrane and the intracellular targets differed between the two fungal species, leading to the hypothesis that a single defensin can have different mechanisms of action on different fungal species [91].

Like MsDef1, MtDef4 disrupts Ca^{2+} signalling and/or homeostasis leading to inhibition of hyphal growth and fusion, but they appear to do this via different mechanisms [39, 87]. The mechanism of action of MtDef4 is outlined in Fig.10 (ii).

Pisum sativum defensin 1 – Psd1

Psd1 is constitutively expressed in the epidermal tissues and vascular bundles of pea pods *(Pisum sativum)* indicating a role in pea defence mechanisms [58, 92]. It has antifungal activity against human and plant pathogens [64]. Interestingly, like RsAFP2, it binds to the sphingolipid GlcCer and has no activity against *F. oxysporum* or *S. cerevisiae* [92, 93].

Loop 1 of Psd1 is important for antifungal activity and may function as a potassium channel blocker [58]. This potential function was identified when the solution structure was solved using high resolution NMR, together with *in silico* analysis of the surface topology and comparison with several proteins that inhibit potassium channels. Channel blocking activity has not been verified experimentally [58], but other defensins have been reported with this activity. Loop1 was also recognised as important for membrane binding, when a conformational change occurred in this region upon binding to GlcCer [94].

A study with micelles composed of lipids from different sources revealed that Psd 1 binds to GlcCer produced by the fungus *Fusarium solani* and not to GlcCer from plants or animal sources [93, 94]. It also binds ergosterol, another lipid found only in fungi. Further studies revealed that GlcCer is a target for antifungal activity, but is not essential. A *C. albicans* strain deficient in GlcCer production was about two

fold more resistant to 10 μM Psd1, but the antifungal activity had not been eliminated suggesting other molecules are targeted by this defensin [95]. Ergosterol is likely to be one of these.

Cyclin F has been identified as a binding partner for Psd1 using a yeast two-hybrid screen with a cDNA library from *N. crassa* [73]. Consistent with this, Psd1 impedes progression from the S to G2 phase of the cell cycle in retinal tissue from neonatal rats [73]. The proposed mechanism of action of Psd1 is presented in Fig.11.

Heuchera sanguinea antifungal peptide 1 – HsAFP1

HsAFP1 is a peptide from the seeds of the dicotyledonous plant species, *Heuchera sanguinea* (Coral Bells). Its antifungal activity is characterised by multiple budding and swelling of *F. culmorum* germ tubes and hyphae. It is also active against yeast and other fungal species but has no effect on bacteria [51, 96-98].

Potential fungal targets for HsAFP1 and responses were identified by screening the non-essential gene deletion library of *S. cerevisiae* for mutants with altered sensitivity to HsAFP1. Roles in mitochondrial function, cell wall biosynthesis and maintenance and stress response signalling, amongst others not discussed here, were implicated through the annotation of the 84 mutants that were sensitive or resistant to HsAFP1 [96]. Mitochondria appear to be important targets for this defensin and this was supported by the observation that HsAFP1 induces apoptosis or programmed cell death through the production and accumulation of ROS and other key markers of apoptosis. This was also supported by the observation that HsAFP1 requires functional mitochondria and a functional respiratory chain for antifungal activity [96]. The screen also identified the involvement of MAPK pathways including the CWI pathway (Fig.6) and HOG1 (Fig.8) pathway in yeast tolerance to HsAFP1, but this has not been verified experimentally.

Loop 5 and adjacent regions are important for the antifungal activity of HsAFP1. The activity of synthetic peptides of 24 amino acid residues covering the entire HsAFP1 molecule were tested for antifungal activity and only the peptide that spanned loop 5 and the adjacent amino acids was active [98]. The proposed mechanism of action for HsAFP1 is presented in Fig.12.

Conclusions

Plant defensins are a large and highly sequence-diverse family of innate immunity molecules with a highly conserved and stable structure and a wide range of biological functions. They share many features with the animal defensins such as size, charge and secondary structure, but this is an extreme example of convergent evolution, because they have a different disulphide bond topology. Thus, plant defensins have been classified as part of the as cis-defensins (also containing fungal and invertebrate defensins) as separate from the trans-defensins (containing mostly vertebrate defensins), since the two superfamilies have not evolved from a common ancestor. Potent antifungal activity is the most common and best described function for plant defensins, and it is likely that other functions such as metal binding and roles in development and sexual reproduction have evolved from their basic role in defence. There are more than 1200 plant defensin sequences in the public data-bases, but only a handful of those with antifungal activity have been studied in detail. Surprisingly the antifungal activity is often achieved by different mechanisms. They can be broadly divided into two groups; those that pass through the cell wall and the

plasma membrane and enter the cytoplasm and those that appear to mediate their toxic effects without entering the cell. All ultimately lead to disruption of the plasma membrane, but this may arise from direct interaction with specific lipids in the inner or outer leaflet of the membrane or membrane perturbation caused indirectly by impaired mitochondrial function, production of reactive oxygen species or induction of programmed cell death. Their complex mechanisms of action explain why these molecules have persisted as effective antifungal molecules over the millennia and why they are attractive targets for the development of antifungal agents for use in agriculture and the clinic.

References

1. Pennisi, E., *Armed and dangerous.* Science (New York, NY), 2010. **327**(5967): p. 804.
2. Fisher, M.C., et al., *Emerging fungal threats to animal, plant and ecosystem health.* Nature, 2012. **484**(7393): p. 186-194.
3. Bastert, J., et al., *Current and future approaches to antimycotic treatment in the era of resistant fungi and immunocompromised hosts.* International journal of antimicrobial agents, 2001. **17**(2): p. 81-91.
4. Shoham, S. and S.M. Levitz, *The immune response to fungal infections.* British journal of haematology, 2005. **129**(5): p. 569-582.
5. Gow, N.A. and M.G. Netea, *Medical mycology and fungal immunology: new research perspectives addressing a major world health challenge.* Phil. Trans. R. Soc. B, 2016. **371**(1709): p. 20150462.
6. Tavares, L.S., et al., *Biotechnological potential of antimicrobial peptides from flowers.* Peptides, 2008. **29**(10): p. 1842-1851.
7. Carvalho, A.D. and V.M. Gomes, *Plant Defensins and Defensin-Like Peptides - Biological Activities and Biotechnological Applications.* Current Pharmaceutical Design, 2011. **17**(38): p. 4270-4293.
8. Broekaert, W.F., et al., *Antimicrobial peptides from plants.* Critical Reviews in Plant Sciences, 1997. **16**(3): p. 297-323.
9. van der Weerden, N., M. Bleackley, and M. Anderson, *Properties and mechanisms of action of naturally occurring antifungal peptides.* Cellular and Molecular Life Sciences, 2013: p. 1-26.
10. Cools, T.L., et al., *Antifungal plant defensins: increased insight in their mode of action as a basis for their use to combat fungal infections.* Future Microbiology, 2017. **12**(5): p. 441-454.
11. Lay, F. and M. Anderson, *Defensins-components of the innate immune system in plants.* Current Protein and Peptide Science, 2005. **6**(1): p. 85-101.
12. Shafee, T.M., et al., *The defensins consist of two independent, convergent protein superfamilies.* Molecular biology and evolution, 2016: p. msw106.
13. Hollox, E.J. and R. Abujaber, *Evolution and Diversity of Defensins in Vertebrates*, in *Evolutionary Biology: Self/Nonself Evolution, Species and Complex Traits Evolution, Methods and Concepts*. 2017, Springer. p. 27-50.
14. de Oliveira Dias, R. and O.L. Franco, *Cysteine-stabilized αß defensins: From a common fold to antibacterial activity.* Peptides, 2015. **72**: p. 64-72.
15. Shafee, T.M., et al., *Convergent evolution of defensin sequence, structure and function.* Cellular and Molecular Life Sciences, 2017. **74**(4): p. 663-682.
16. van der Weerden, N.L. and M.A. Anderson, *Plant defensins: Common fold, multiple functions.* Fungal Biology Reviews, 2013. **26**(4): p. 121-131.
17. van der Weerden, N.L., F.T. Lay, and M.A. Anderson, *The plant defensin, NaD1, enters the cytoplasm of Fusarium oxysporum hyphae.* Journal of Biological Chemistry, 2008. **283**(21): p. 14445-14452.
18. Undheim, E.A., M. Mobli, and G.F. King, *Toxin structures as evolutionary tools: Using conserved 3D folds to study the evolution of rapidly evolving peptides.* BioEssays, 2016. **38**(6): p. 539-548.
19. Lay, F.T., F. Brugliera, and M.A. Anderson, *Isolation and properties of floral defensins from ornamental tobacco and petunia.* Plant Physiology, 2003. **131**(3): p. 1283-93.
20. De Coninck, B., B.P.A. Cammue, and K. Thevissen, *Modes of antifungal action and in planta functions of plant defensins and defensin-like peptides.* Fungal Biology Reviews, 2013. **26**(4): p. 109-120.
21. Woytowich, A.E. and G.G. Khachatourians, *Plant antifungal peptides and their use in transgenic food crops.* Applied Mycology and Biotechnology, 2001. **1**: p. 145-164.
22. Janssen, B.J.C., et al., *Structure of Petunia hybrida defensin 1, a novel plant defensin with five disulfide bonds.* Biochemistry, 2003. **42**(27): p. 8214-8222.
23. Lay, F.T., et al., *The C-terminal propeptide of a plant defensin confers cytoprotective and subcellular targeting functions.* BMC plant biology, 2014. **14**(1): p. 1.
24. Balandín, M., et al., *A protective role for the embryo surrounding region of the maize endosperm, as evidenced by the characterisation of ZmESR-6, a defensin gene specifically expressed in this region.* Plant molecular biology, 2005. **58**(2): p. 269-282.
25. Wilmes, M., et al., *Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation.* Natural product reports, 2011. **28**(8): p. 1350-1358.

26. Thevissen, K., et al., *DmAMP1, an antifungal plant defensin from dahlia (Dahlia merckii), interacts with sphingolipids from Saccharomyces cerevisiae.* FEMS Microbiology Letters, 2003. **226**(1): p. 169-173.
27. Thevissen, K., et al., *Interactions of antifungal plant defensins with fungal membrane components.* Peptides, 2003. **24**(11): p. 1705-1712.
28. Payne, J.A., et al., *The plant defensin NaD1 introduces membrane disorder through a specific interaction with the lipid, phosphatidylinositol 4, 5 bisphosphate.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2016. **1858**(6): p. 1099-1109.
29. Kvansakul, M., et al., *Binding of phosphatidic acid by NsD7 mediates the formation of helical defensin–lipid oligomeric assemblies and membrane permeabilization.* Proceedings of the National Academy of Sciences, 2016. **113**(40): p. 11202-11207.
30. Poon, I.K., et al., *Phosphoinositide-mediated oligomerization of a defensin induces cell lysis.* Elife, 2014. **3**: p. e01808.
31. Baxter, A., M. Hulett, and I.K. Poon, *The phospholipid code: a key component of dying cell recognition, tumor progression and host-microbe interactions.* Cell death and differentiation, 2015. **22**(12): p. 1893-1905.
32. Sagaram, U.S., et al., *Structure-Activity Determinants in Antifungal Plant Defensins MsDef1 and MtDef4 with Different Modes of Action against Fusarium graminearum.* Plos One, 2011. **6**(4).
33. Bleackley, M.R., et al., *Nicotiana alata defensin chimeras reveal differences in the mechanism of fungal and tumour cell killing and an enhanced antifungal variant.* Antimicrobial Agents and Chemotherapy, 2016: p. AAC. 01479-16.
34. Colilla, F.J., A. Rocher, and E. Mendez, *gamma-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm.* FEBS Lett, 1990. **270**(1-2): p. 191-4.
35. Méndez, E., et al., *Primary Structure of ω-Hordothionin, a Member of a Novel Family of Thionins from Barley Endosperm, and Its Inhibition of Protein Synthesis in Eukaryotic and Prokaryotic Cell-Free Systems.* European journal of biochemistry, 1996. **239**(1): p. 67-73.
36. Mirouze, M., et al., *A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, Arabidopsis halleri, confers zinc tolerance.* The Plant Journal, 2006. **47**(3): p. 329-342.
37. Zhang, Y. and K. Lewis, *Fabatins: new antimicrobial plant peptides.* FEMS microbiology letters, 1997. **149**(1): p. 59-64.
38. Bircheneder, S. and T. Dresselhaus, *Why cellular communication during plant reproduction is particularly mediated by CRP signalling.* Journal of Experimental Botany, 2016: p. erw271.
39. Spelbrink, R.G., et al., *Differential antifungal and calcium channel-blocking activity among structurally related plant defensins.* Plant Physiology, 2004. **135**(4): p. 2055-2067.
40. Vriens, K., et al., *The antifungal plant defensin AtPDF2. 3 from Arabidopsis thaliana blocks potassium channels.* Scientific Reports, 2016. **6**: p. 32121.
41. Terras, F.R., et al., *Small cysteine-rich antifungal proteins from radish: their role in host defense.* The Plant Cell, 1995. **7**(5): p. 573-588.
42. Mendez, E., et al., *Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, γ-hordothionin, from barley endosperm.* European journal of biochemistry, 1990. **194**(2): p. 533-539.
43. Liu, Y.J., et al., *Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids.* Proteins: Structure, Function, and Bioinformatics, 2006. **63**(4): p. 777-786.
44. Bloch, C. and M. Richardson, *A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds or sorghum (Sorghum bicolor (L) Moench) have sequence homologies with wheat γ-purothionins.* FEBS letters, 1991. **279**(1): p. 101-104.
45. Franco, O.L., et al., *plant α-amylase inhibitors and their interaction with insect α-amylases.* European journal of biochemistry, 2002. **269**(2): p. 397-412.
46. Vijayan, S., et al., *Enhanced antifungal and insect α-amylase inhibitory activities of Alpha-TvD1, a peptide variant of Tephrosia villosa defensin (TvD1) generated through in vitro mutagenesis.* Peptides, 2012. **33**(2): p. 220-229.
47. Wijaya, R., et al., *Defense proteins from seed of Cassia fistula include a lipid transfer protein homologue and a protease inhibitory plant defensin.* Plant Science, 2000. **159**(2): p. 243-255.

48. Pelegrini, P.B. and O.L. Franco, *Plant γ-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins.* The international journal of biochemistry & cell biology, 2005. **37**(11): p. 2239-2253.
49. Melo, F.R., et al., *Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking.* Proteins: Structure, Function, and Bioinformatics, 2002. **48**(2): p. 311-319.
50. Pelegrini, P.B., et al., *Novel insights on the mechanism of action of α-amylase inhibitors from the plant defensin family.* Proteins: Structure, Function, and Bioinformatics, 2008. **73**(3): p. 719-729.
51. Osborn, R.W., et al., *Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae.* FEBS letters, 1995. **368**(2): p. 257-262.
52. Chen, G.-H., et al., *Cloning and characterization of a plant defensin VaD1 from azuki bean.* Journal of agricultural and food chemistry, 2005. **53**(4): p. 982-988.
53. Kushmerick, C., et al., *Functional and structural features of γ-zeathionins, a new class of sodium channel blockers.* FEBS letters, 1998. **440**(3): p. 302-306.
54. Mouhat, S., et al., *Diversity of folds in animal toxins acting on ion channels.* Biochemical Journal, 2004. **378**(3): p. 717-726.
55. Tarr, D.E.K., *Establishing a reference array for the CS-αβ superfamily of defensive peptides.* BMC Research Notes, 2016. **9**(1): p. 490.
56. Zhu, S., et al., *Experimental conversion of a defensin into a neurotoxin: implications for origin of toxic function.* Molecular biology and evolution, 2014. **31**(3): p. 546-559.
57. Banerjee, A., et al., *Structure of a pore-blocking toxin in complex with a eukaryotic voltage-dependent K+ channel.* Elife, 2013. **2**: p. e00594.
58. Almeida, M.S., et al., *Solution structure of Pisum sativum defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action.* Journal of molecular biology, 2002. **315**(4): p. 749-757.
59. Baxter, A.A., et al., *The tomato defensin TPP3 binds phosphatidylinositol (4, 5)-bisphosphate via a conserved dimeric cationic grip conformation to mediate cell lysis.* Molecular and cellular biology, 2015. **35**(11): p. 1964-1978.
60. Wang, S., P. Rao, and X. Ye, *Isolation and biochemical characterization of a novel leguminous defense peptide with antifungal and antiproliferative potency.* Applied microbiology and biotechnology, 2009. **82**(1): p. 79-86.
61. Allen, A., et al., *Plant defensins and virally encoded fungal toxin KP4 inhibit plant root growth.* Planta, 2008. **227**(2): p. 331-339.
62. Letousey, P., et al., *Molecular analysis of resistance mechanisms to Orobanche cumana in sunflower.* Plant Pathology, 2007. **56**(3): p. 536-546.
63. de Zélicourt, A., et al., *Ha-DEF1, a sunflower defensin, induces cell death in Orobanche parasitic plants.* Planta, 2007. **226**(3): p. 591-600.
64. Carvalho, A.d.O. and V.M. Gomes, *Plant defensins-prospects for the biological functions and biotechnological properties.* Peptides, 2009. **30**(5): p. 1007-1020.
65. Rogozhin, E.A., et al., *Novel antifungal defensins from Nigella sativa L. seeds.* Plant Physiology and Biochemistry, 2011. **49**(2): p. 131-137.
66. Thevissen, K., et al., *Specific binding sites for an antifungal plant defensin from Dahlia (Dahlia merckii) on fungal cells are required for antifungal activity.* Molecular plant-microbe interactions, 2000. **13**(1): p. 54-61.
67. Thevissen, K., et al., *Therapeutic potential of antifungal plant and insect defensins.* Drug discovery today, 2007. **12**(21): p. 966-971.
68. Hayes, B.M., et al., *Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against Candida albicans.* Antimicrobial agents and chemotherapy, 2013. **57**(8): p. 3667-3675.
69. Thevissen, K., et al., *A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of Saccharomyces cerevisiae to an antifungal plant defensin from dahlia (Dahlia merckii).* Proceedings of the National Academy of Sciences, 2000. **97**(17): p. 9531-9536.
70. Im, Y.J., et al., *IPT1-independent sphingolipid syringomycin E and biosynthesis and yeast inhibition by plant defensin DmAMP1.* Fems Microbiology Letters, 2003. **223**(2): p. 199-203.
71. Vriens, K., B. Cammue, and K. Thevissen, *Antifungal plant defensins: mechanisms of action and production.* Molecules, 2014. **19**(8): p. 12280-12303.

72. Thevissen, K., et al., *SKN1, a novel plant defensin-sensitivity gene in Saccharomyces cerevisiae, is implicated in sphingolipid biosynthesis.* FEBS letters, 2005. **579**(9): p. 1973-1977.
73. Lobo, D.S., et al., *Antifungal Pisum sativum defensin 1 interacts with Neurospora crassa cyclin F related to the cell cycle.* Biochemistry, 2007. **46**(4): p. 987-996.
74. Terras, F., et al., *Analysis of two novel classes of plant antifungal proteins from radish (Raphanus sativus L.) seeds.* Journal of Biological Chemistry, 1992. **267**(22): p. 15301-15309.
75. Thevissen, K., et al., *Fungal membrane responses induced by plant defensins and thionins.* Journal of Biological Chemistry, 1996. **271**(25): p. 15018-15025.
76. De Samblanx, G.W., et al., *Mutational analysis of a plant defensin from radish (Raphanus sativus L.) reveals two adjacent sites important for antifungal activity.* Journal of Biological Chemistry, 1997. **272**(2): p. 1171-1179.
77. De Coninck, B., et al., *Fungal Glucosylceramide-Specific Camelid Single Domain Antibodies Are Characterized by Broad Spectrum Antifungal Activity.* Frontiers in microbiology, 2017. **8**.
78. Aerts, A.M., et al., *The antifungal activity of RsAFP2, a plant defensin from Raphanus sativus, involves the induction of reactive oxygen species in Candida albicans.* Journal of molecular microbiology and biotechnology, 2007. **13**(4): p. 243-247.
79. Thevissen, K., et al., *The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in Candida albicans.* Molecular Microbiology, 2012. **84**(1): p. 166-180.
80. Aerts, A.M., et al., *The antifungal plant defensin RsAFP2 from radish induces apoptosis in a metacaspase independent way in Candida albicans.* FEBS letters, 2009. **583**(15): p. 2513-2516.
81. van der Weerden, N.L., R.E.W. Hancock, and M.A. Anderson, *Permeabilization of Fungal Hyphae by the Plant Defensin NaD1 Occurs through a Cell Wall-dependent Process.* Journal of Biological Chemistry, 2010. **285**(48): p. 37513-37520.
82. Lay, F.T., et al., *Dimerization of plant defensin NaD1 enhances its antifungal activity.* Journal of Biological Chemistry, 2012. **287**(24): p. 19961-19972.
83. Hayes, B.M., et al., *Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins.* Cellular and molecular life sciences, 2014. **71**(14): p. 2651-2666.
84. Bleackley, M.R., et al., *Agp2p, the plasma membrane transregulator of polyamine uptake, regulates the antifungal activities of the plant defensin NaD1 and other cationic peptides.* Antimicrobial agents and chemotherapy, 2014. **58**(5): p. 2688-2698.
85. Aouida, M., et al., *AGP2 encodes the major permease for high affinity polyamine import in Saccharomyces cerevisiae.* Journal of Biological Chemistry, 2005. **280**(25): p. 24267-24276.
86. Ramamoorthy, V., et al., *Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of Fusarium graminearum.* Molecular Microbiology, 2007. **66**(3): p. 771-786.
87. Muñoz, A., et al., *Specific domains of plant defensins differentially disrupt colony initiation, cell fusion and calcium homeostasis in Neurospora crassa.* Molecular microbiology, 2014. **92**(6): p. 1357-1374.
88. Jackson, S. and I. Heath, *Roles of calcium ions in hyphal tip growth.* Microbiological reviews, 1993. **57**(2): p. 367-382.
89. Ramamoorthy, V., et al., *Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defensins in Fusarium graminearum.* Cellular microbiology, 2007. **9**(6): p. 1491-1506.
90. Sagaram, U.S., et al., *Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: identification of an RGFRRR motif governing fungal cell entry.* PLoS One, 2013. **8**(12): p. e82485.
91. El-Mounadi, K., et al., *Antifungal mechanisms of a plant defensin MtDef4 are not conserved between the ascomycete fungi Neurospora crassa and Fusarium graminearum.* Molecular Microbiology, 2016.
92. Almeida, M.S., et al., *Characterization of two novel defense peptides from pea (Pisum sativum) seeds.* Archives of Biochemistry and Biophysics, 2000. **378**(2): p. 278-286.
93. Gonçalves, S., et al., *Evaluation of the membrane lipid selectivity of the pea defensin Psd1.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2012. **1818**(5): p. 1420-1426.
94. de Medeiros, L.N., et al., *Backbone dynamics of the antifungal Psd1 pea defensin and its correlation with membrane interaction by NMR spectroscopy.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2010. **1798**(2): p. 105-113.

95. Neves de Medeiros, L., et al., *Psd1 binding affinity toward fungal membrane components as assessed by SPR: The role of glucosylceramide in fungal recognition and entry.* Peptide Science, 2014. **102**(6): p. 456-464.
96. Aerts, A.M., et al., *The antifungal plant defensin HsAFP1 from Heuchera sanguinea induces apoptosis in Candida albicans.* Front Microbiol, 2011. **2**: p. 1-9.
97. De Brucker, K., B.P. Cammue, and K. Thevissen, *Apoptosis-inducing antifungal peptides and proteins.* Biochemical Society Transactions, 2011. **39**(5): p. 1527-1532.
98. Vriens, K., et al., *Synergistic activity of the plant defensin HsAFP1 and caspofungin against Candida albicans biofilms and planktonic cultures.* PloS one, 2015. **10**(8): p. e0132701.
99. Shafee, T.M., et al., *Structural homology guided alignment of cysteine rich proteins.* Springerplus, 2016. **5**: p. 27.
100. Dracatos, P.M., et al., *Inhibition of cereal rust fungi by both class I and II defensins derived from the flowers of Nicotiana alata.* Molecular plant pathology, 2014. **15**(1): p. 67-79.
101. Zhu, Y.J., R. Agbayani, and P.H. Moore, *Ectopic expression of Dahlia merckii defensin DmAMP1 improves papaya resistance to Phytophthora palmivora by reducing pathogen vigor.* Planta, 2007. **226**(1): p. 87-97.
102. Mello, E.O., et al., *Antifungal activity of PvD1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ROS in fungi cells.* Current microbiology, 2011. **62**(4): p. 1209-1217.
103. Anderson, J.A., et al., *Emerging agricultural biotechnologies for sustainable agriculture and food security.* Journal of agricultural and food chemistry, 2016. **64**(2): p. 383-393.
104. Moreno, M., A. Segura, and F. GARCÍA-OLMEDO, *Pseudothionin-St1, a potato peptide active against potato pathogens.* European Journal of Biochemistry, 1994. **223**(1): p. 135-139.
105. Vijayan, S., L. Guruprasad, and P. Kirti, *Prokaryotic expression of a constitutively expressed Tephrosia villosa defensin and its potent antifungal activity.* Applied microbiology and biotechnology, 2008. **80**(6): p. 1023-1032.
106. De Beer, A. and M.A. Vivier, *Vv-AMP1, a ripening induced peptide from Vitis vinifera shows strong antifungal activity.* BMC plant biology, 2008. **8**(1): p. 1.
107. Levin, D.E., *Cell wall integrity signaling in Saccharomyces cerevisiae.* Microbiology and molecular biology reviews, 2005. **69**(2): p. 262-291.
108. Rodríguez-Peña, J.M., et al., *The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways interplay: a yeast dialogue between MAPK routes.* Yeast, 2010. **27**(8): p. 495-502.

Parisi, K., Shafee, T. M., Quimbar, P., van der Weerden, N. L., Bleackley, M. R., & Anderson, M. A. (2019, April). The evolution, function and mechanisms of action for plant defensins. In *Seminars in cell & developmental biology* (Vol. 88, pp. 107-118). Academic Press. doi:10.1016/j.semcdb.2018.02.004

Figures and tables

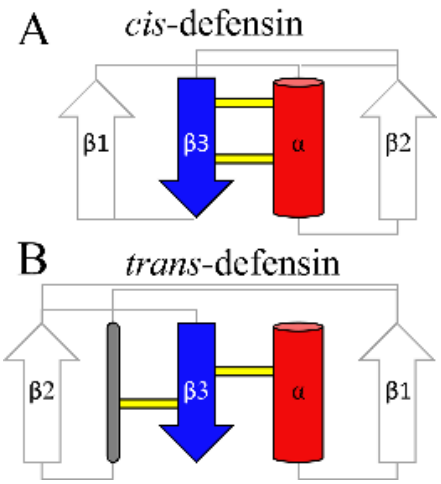

Fig.1 Conserved disulphide bridges of the defensins.

A) The most conserved disulphide bridges of the cis-defensin superfamily (in yellow) point from the central β-strand to the same α-helix in a 'cis' orientation. B) The most conserved disulphides of the trans-defensin superfamily (in yellow) point from the central β-strand to two different secondary structure elements in a 'trans' orientation. Non-conserved disulphide bonds are shown as dashed lines.

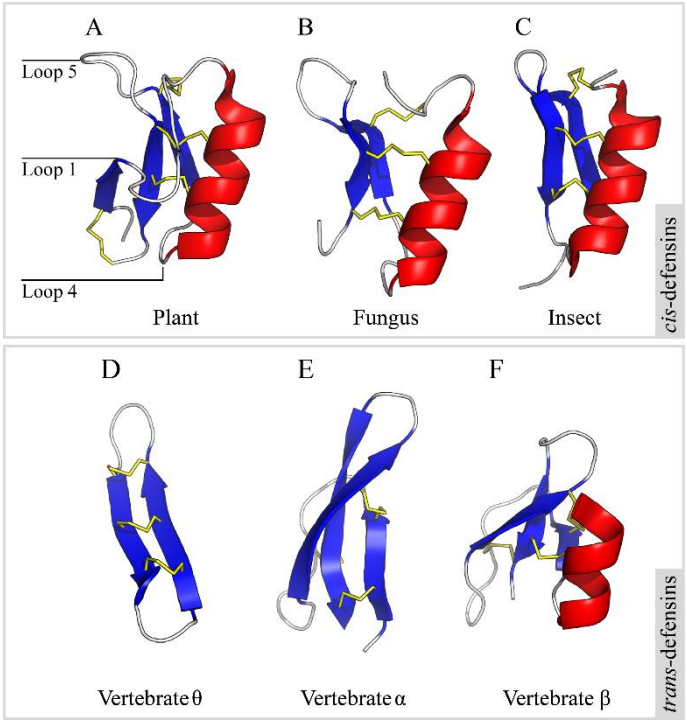

Fig.2 Structural similarity between defensins from different species

Example structures from the cis-defensin superfamily. A) Plant defensin NaD1 from *Nicotiana alata* PDB:1MR4 with key inter-cysteine loops indicated, B) Fungal defensin micasin from *Microsporum canis* PDB:2LR5, C) Insect defensin nasonin from *Nasonia vitripennis* PDB:2KOZ. Example structures from the trans-defensin superfamily: D) Vertebrate θ-defensin BTD-2 from *Papio anubis* PDB:2LYE, E) Vertebrate α-defensin HD5 from *Homo sapiens* PDB:2LXZ, F) Vertebrate β-defensin HBD1 from *Homo sapiens* PDV:1IJV, α-helices in red, β-strands in blue, disulphide bonds in yellow.

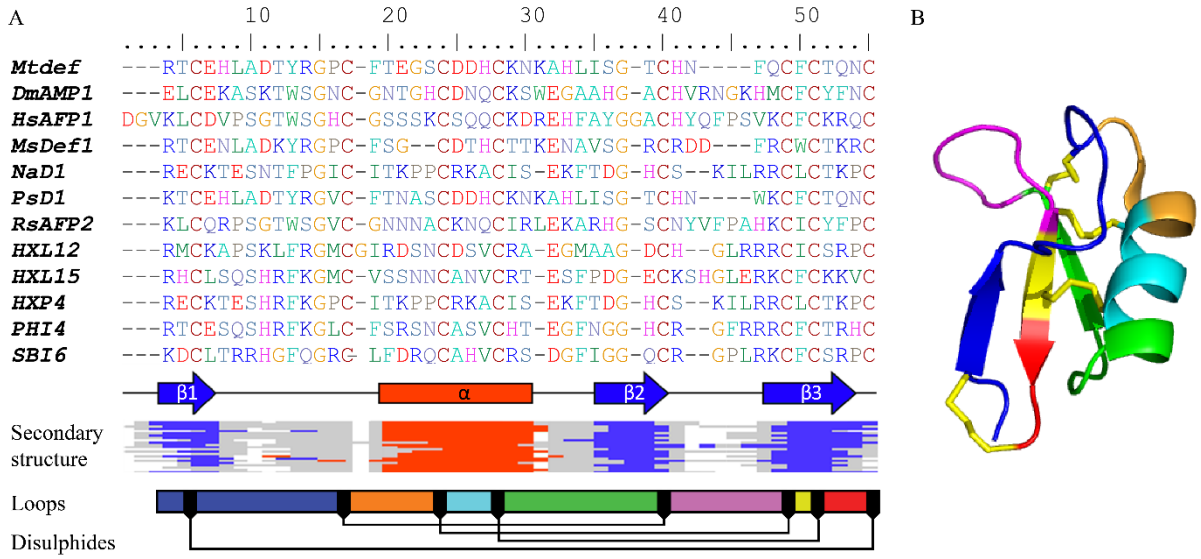

Fig.3 Sequence alignment reveals lack of sequence conservation in the plant defensins

Alignment of 14 plant defensin sequences. The conserved cysteine residues are in black, the conserved glycine residues in yellow, the common glutamic acid in purple and the conserved aromatic residue in orange. The relative location of the α-helix and β-strands is indicated below the alignment by a structure-guided sequence alignment of 24 cis-defensin proteins with α-helices in red, β-strands in blue [99]. The location of each loop, defined as the regions between conserved cysteine residues, is also indicated. B) Structure of NaD1 with loops coloured as in panel A (PDB:1MR4, sequence highlighted in blue in panel A). Loop 1 in dark blue comprises the first β-strand and most of the loop connecting β-strand 1 and the α- helix. Loop 2 in orange comprises the remaining loop (connecting β-strand 1 and α-helix) and the initial part of the α- helix. Loop 3 in light blue is short and encompasses a small portion in the middle of the α- helix. Loop 4 in green involves the remainder of the α- helix and β-strand 2. Loop 5 in purple comprises the flexible region between β-strands 2 and 3

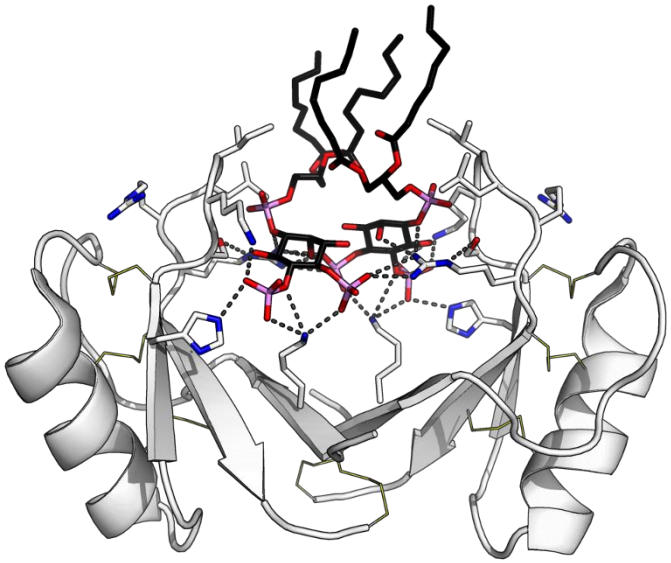

Fig.4 Plant defensin dimerisation

Dimer of the plant defensin NaD1 bound to two molecules of $PI(4,5)P_2$ (PDB:4CQK). Loop 5 residues shown as white sticks. Lipids shown as black sticks. Phosphorus in purple, oxygen in red, nitrogen in blue. Ionic interactions between the defensins and lipids shown as dotted lines.

Table 1 A list of defensins and susceptible pathogens

Defensin Name	Plant species	Tissue	Activity on Fungal pathogen	References
NaD1	*Nicotiana alata*	flowers	*Aspergillus nidulans, Botrytis cinerea, Candida albicans, Colletotrichum graminicola, Cryptococcus gattii, Cryptococcus neoformans, Fusarium graminearum, F. oxysporum, Puccinia coronate, P. sorghi, Saccharomyces cerevisiae, Thielaviopsis basicola, Verticillium dahliae*	[19, 68, 81, 84, 100]
RsAFP2	*Raphanus sativa*	seed	*Alternaria longipes, A. solani, A.brassicola, Ascochyta pisi, Aspergillus flavus, B. cinerea, C. albicans, C.krusei. C. glabrata, Cercospora beticola, Cladosporium sphaerosperm, Colletotrichum lindemuthianum, Fusarium culmorum F. graminearum, F. solani, F. oxysporum, Leptosphaeria maculans, Mycosphaerella fijiensis, N. crassa, Nectria haematococca, Penicillium digitatum, Phoma betae, Phytoophthora infestans, Pyrenophora tritici-repentis, Pyricularia oryzae, Rhizoctonia solani, Sclerotinia sclerotiorum Septoria nodorum, Septoria tritici, Trichoderma hamatum, Trichophyton mentagrophytes, Trichoderma viride, Verticilium alboatrum, V. dahlia, Venturia inaequalis*	[39, 67, 74, 76, 101]
Psd1	*Pisum sativum*	seed	*Aspergillus niger, Avicularia. versicolor, Fusarium solani, F. moniliformae, F. oxysporum, Neurospora crassa, S. cerevisiae, T. mentagrophytes*	[92]
HsAFP1	*Heuchera sanguinea*	seed	*A. flavus, B. cinerea, C. albicans, C. glabrata, Candida krusei, Cladsporium sphaerospermum, F. culmorum, F. solani, Leptosphaeria maculans, N. crassa, Penicllium digitatum, Septoria tritici, Verticillium albo-atrum, T. viride*	[51]
PvD1	*Phaseolus vulgaris*	seed	*C. albicans, Candida tropicalis, F. oxysporum, F. solani, Fusarium laterithium, Kluyveromyces marxiannus, S. cerevisiae*	[102]
MsDef1	*Medicago sativa*	seed	*F. graminearum, N. crassa V. dahlia*	[32, 39, 86]
MtDef4	*Mendicago trucatula*	seed	*F. graminearum, N. crassa, Puccinia tritici*	[32, 91, 103]
DmAMP1	*Dahlia merkii*	seed	*Alternaria brassicicola, A. flavus, B. cinerea, C. albicans, C. glabrata, C. krusei, C. sphaerospermum, F. solani, F. culmorum, L. maculans, N. crassa, P. digitatum, S. cerevisiae, S. tritici, Trichoderma viride, V. albo-atrum*	[51, 66-68]
NsD1, NsD2	*Nigella sativa*	seed	*A. niger, B. cinerea, Bipolaris sorokiniana, F. culmorum, F. graminearum, F. oxysporum*	[65]
PhD1, PhD2	*Petunia hybrida*	flowers	*B. cinerea, F. oxysporum*	[19]
Zm-ESR6	*Zea mays*	seed	*B. cinerea, F. oxysporum sp, Plectosphaerella cucumerina*	[24]
PTH1	*Solanum tuberosum*	tuber	*Clavibacter michiganesis, F. solani, Pseudomonas solanacearum*	[104]
TvD1	*Tephrosia villosa*	leaf	*Alternaria helianthi, B. cinerea, Curvularia sp, Fusarium moniliforme, F. oxysporum, Pheaoisariopsis personata*	[105]
Vv-AMP1	*Vigna unguiculata*	berry	*Alternaria longipes, B. cinerea, F. graminearum, F. oxysporum, F. solani, V. dahliae*	[106]

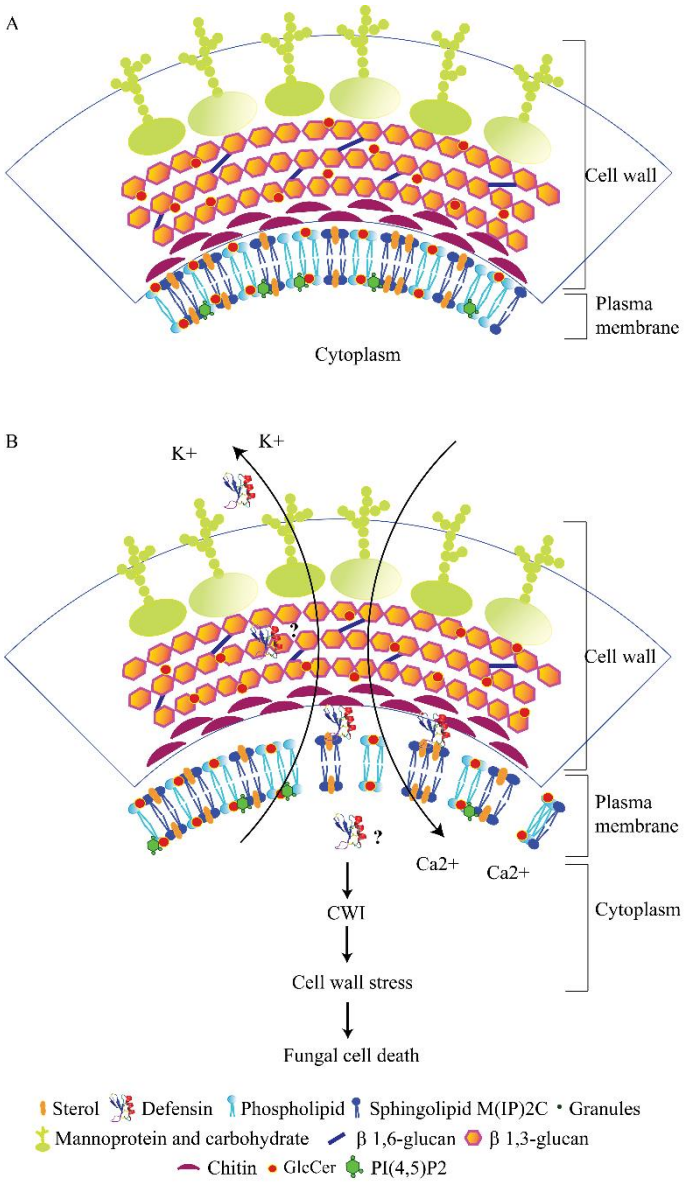

Fig.5. The predicted mode of action of DmAMP1 on fungal cells

A. depicts the normal fungal cell and B. illustrates the consequences of treatment with DmAMP1. DmAMP1 binds to sphingolipids in the cell wall and potentially in the outer plasma membrane. Insertion into and permeabilisation of the membrane results in potassium efflux and calcium uptake. The CWI pathway is activated after exposure to DmAMPI and fungal growth arrest occurs. Modified from Vriens et al [71]. The '**?**' stipulates unknown processes.

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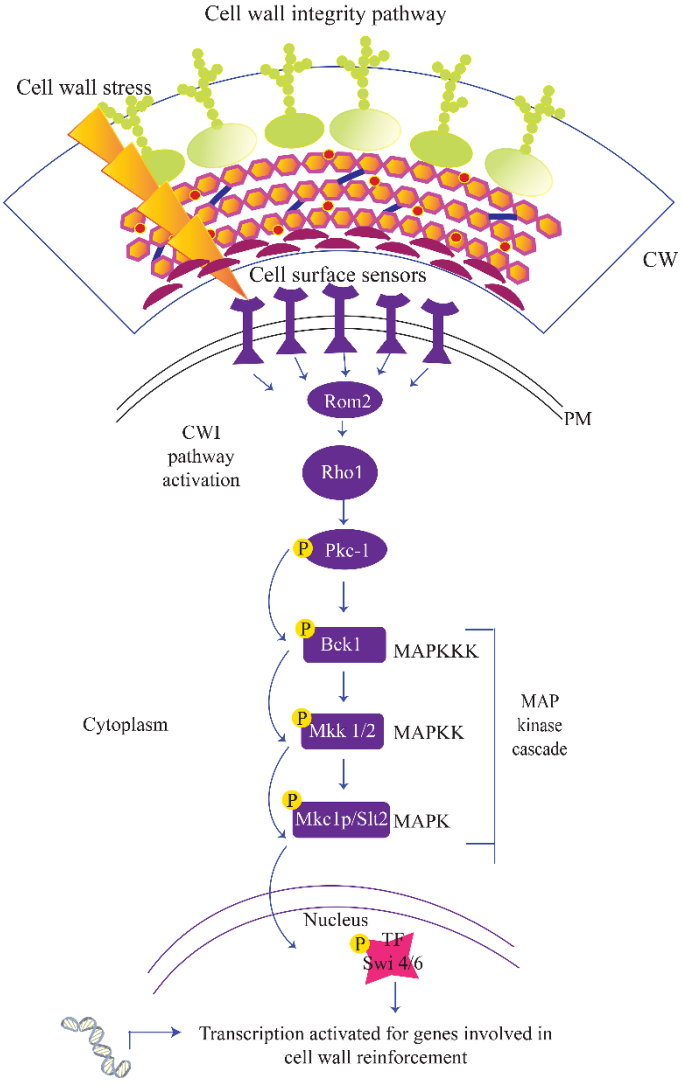

Fig.6 Cell wall integrity pathway, response to cellular stresses in *S. cerevisiae*

The cell wall integrity pathway is activated by cell wall stress. The signalling is initiated via cell surface receptors (Wsc1, -2, -3, Mid2 and Mtl1) in the plasma membrane. These sensors activate the guanosine nucleotide exchange factor Rom2 that stimulates nucleotide exchange on Rho1. Rho1then activates the effector Pck1 which activates the MAP kinase cascade in the CWI (Bck1, Mkk1/2, Mkcp1/Slt2) via phosphorylation to target and activate the transcription factors (Swi4/Swi6) in the nucleus which turn on the genes for reinforcement of the cell wall. Some pathway components have been omitted for clarity [83, 107, 108].

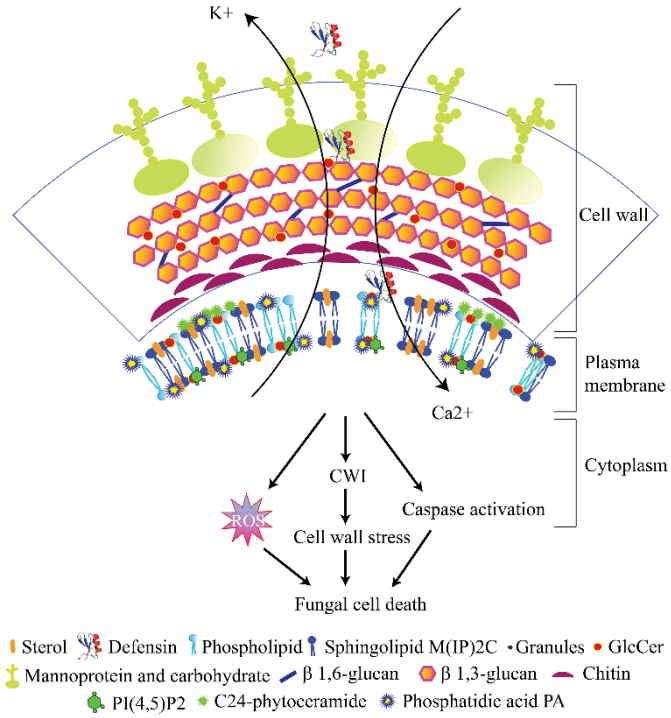

Fig.7 Mechanism of action of RsAFP2

RsAFP2 binds to GlcCer in the cell wall and plasma membrane and does not enter the cell. The CWI MAP kinase cascade is activated via cell wall stress. There is production of ROS, induction of ion fluxes, accumulation of phyto C24-ceramides and activation of caspases all of which contribute to fungal cell death [71].

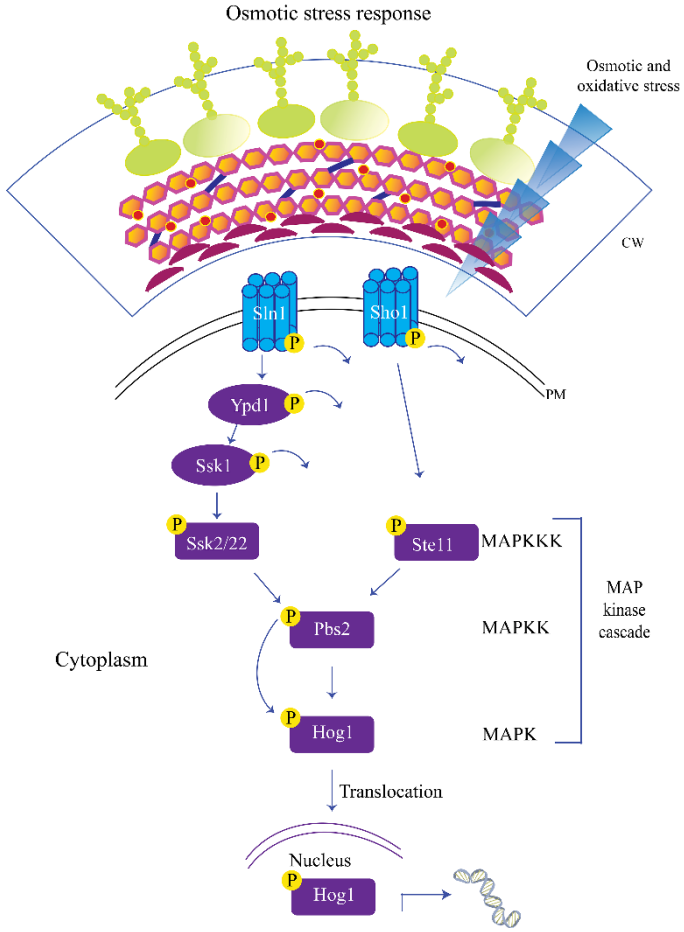

Fig.8 Hog1, an osmotic stress response pathway in *C. albicans*

The HOG pathway has two upstream osmo-sensors (Sln1 and Sho1) each with a downstream MAPK cascade (Ssk2/Ssk22 and Ste11 MAPKKKs, Pbs2 MAPKK, and Hog1 MAPK). The cascade is activated when the osmo-sensors are dephosphorylated and the cascade kinases are phosphorylated (A yellow phosphate on the right indicates de-phosphorylation and the yellow phosphate on the left indicates phosphorylation). Activation of the HOG pathway leads to rapid translocation of Hog1 into the nucleus, which stimulates expression of osmo-responsive genes from osmotic or oxidative stress. Some pathway components have been omitted for clarity (adapted from Hayes and colleagues [83].

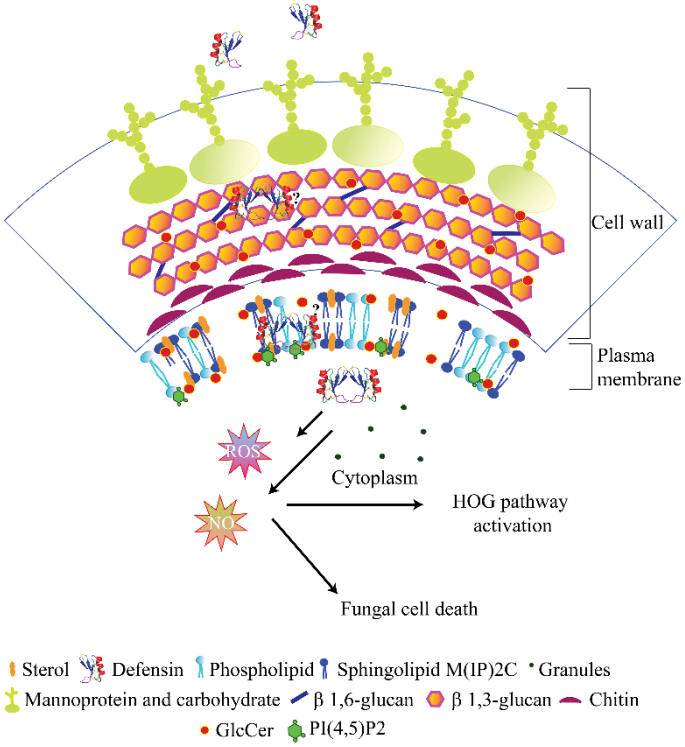

Fig.9 NaD1 proposed mechanisms of action

NaD1 binds to the cell wall where it may dimerise. The dimer moves to the cytoplasm and triggers the production of ROS and NO which damage the membranes. This damage may be compounded by an interaction between NaD1 and $PI(4,5)P_2$ on the inner leaflet of the plasma membrane. This is followed by granulation of the cytoplasm and cell death. A depiction of the normal cell can be viewed in Fig.5.

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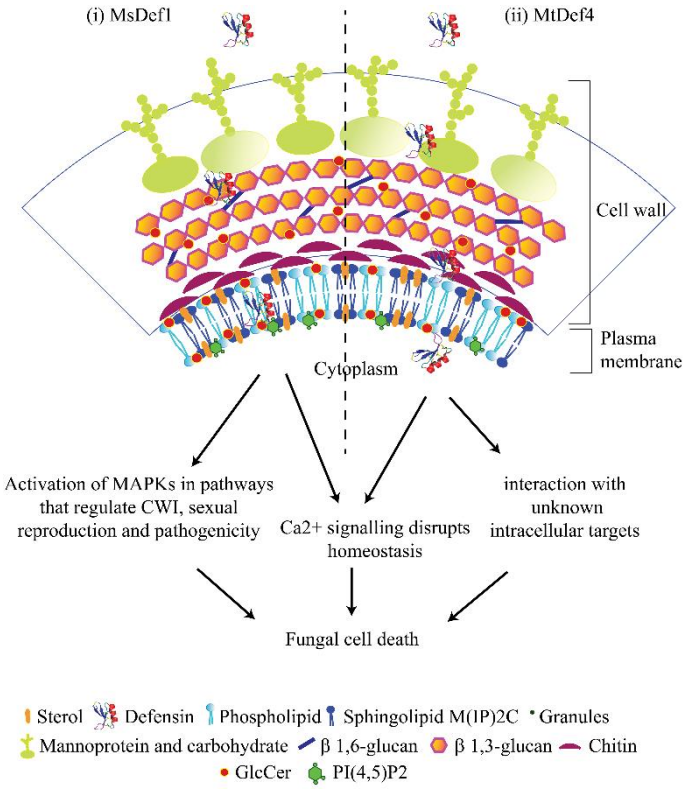

Fig.10 Proposed mechanisms of action for MsDef1 and MtDef4

An illustration depicting the proposed mechanisms of action for the plant defensins (i) MsDef1 and (ii) MtDef4. . MsDef1 interacts with GlcCer in the cell wall and membrane. The MAPK cascade in the CWI pathway is activated and Ca2+ signalling/and homeostasis is disrupted, all contributing to fungal cell death. (ii) MtDef4 is internalised into the fungal cell. MtDef4 binds to PA via loop 5. An interaction with intracellular targets has been proposed but has not been defined. MtDef4 disrupts Ca^{2+} and homeostasis in a different way to MsDef1. A depiction of the normal cell is presented in Fig.5.

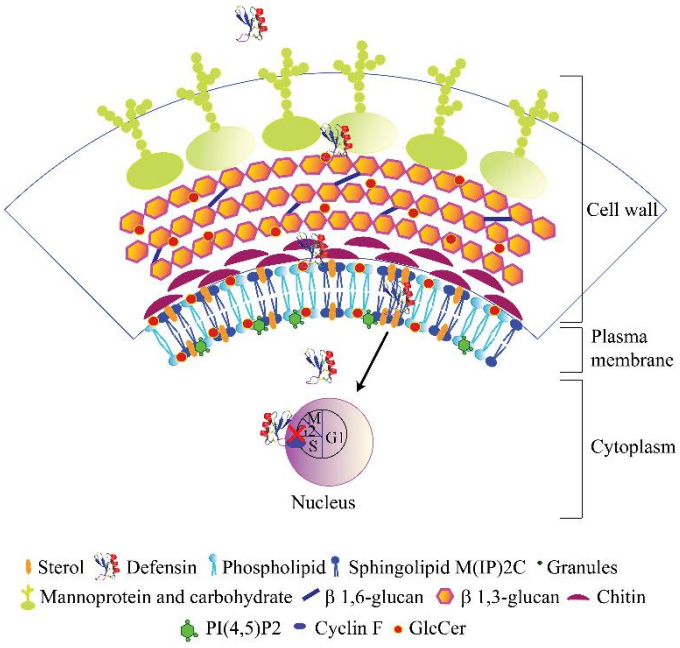

Fig.11 The proposed mechanism of action for Psd1

Psd1 binds to GlcCer and potentially ergosterol in the plasma membrane of fungal cells. Psd1 moves to the cytoplasm and interacts with cyclin F in the nucleus which results in cell cycle arrest and fungal cell death. Refer to Fig.5 for a depiction of the normal cell.

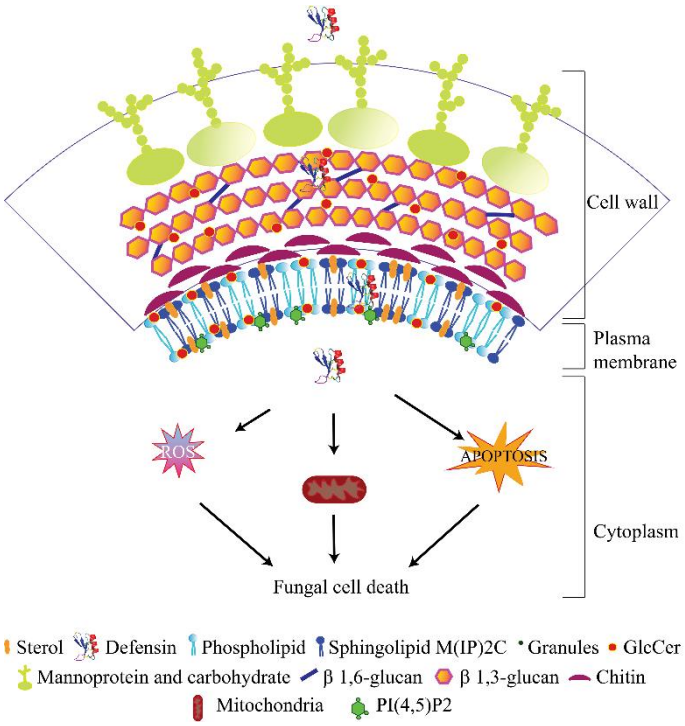

Fig.12 The proposed mechanism of action for HsAFP1

HsAFP1 binds to the fungal cell wall and plasma membrane via loop 5. It penetrates the cytoplasm and targets mitochondria, produces ROS, induces programmed cell death and causes fungal cell death. A depiction of the normal cell is presented in Fig.5