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Review

Plant defensins: Common fold, multiple functions

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ABSTRACT

Plant defensins represent a large class of structurally similar peptides found throughout the plant kingdom. Despite a conserved cysteine spacing pattern and three-dimensional structure, their sequences are highly divergent and they display a range of activities including antifungal and antibacterial activities, enzyme inhibitory activities as well as roles in heavy metal tolerance and development. The vast number of sequences along with their diverse range of activities makes it impossible to test the activity and assign function to all plant defensins. However, as the number of characterized defensins increases, in depth sequence analysis may allow us to predict the function of newly identified peptides. In this review, we analyze the sequences of defensins whose activities have been described and group these based on similarity using a maximum-likelihood phylogenetic tree. We also compare the amino acids that have been described as essential for the activity of various plant defensins between these groups. While many more plant defensins will need to be characterized before we can develop rules to predict the activity of novel sequences, this approach may prove useful in identifying structure–function relationships.

1. Introduction

The search for natural peptide inhibitors of pathogens for use in agricultural and medicine has resulted in an ever increasing interest in defense peptides produced by plants. One group of peptides that is of particular interest is the plant defensins. Plant defensins are small (45–54 amino acids), basic, cysteine-rich proteins that are found ubiquitously throughout the plant kingdom. They share structural and functional homology to defensins from insects, mammals and fungi. Many are growth inhibitory toward fungi; however numerous other activities have also been described. These include antibacterial activity (Zhang and Lewis, 1997), trypsin and α-amylase inhibition (Melo *et al.*, 2002; Bloch and Richardson, 1991), protein synthesis inhibition (Colilla *et al.*, 1990) as well as roles in heavy metal tolerance (Mirouze *et al.*, 2006) and development (Wilson *et al.*, 2005; Laitinen *et al.*, 2005). This ever increasing list of activities has led to the hypothesis that the stable plant defensin structure may provide a scaffold for the display of various activities. As more information becomes available, it is becoming clear that even defensins with similar activities, such as the antifungal defensins, are likely to act *via* different modes of action.

Genome sequencing programs have led to a rapid increase in the number of reported plant defensins. Small cysteine-rich peptides such as defensins have also been under-predicted so far because of their small size and tremendous sequence diversity. As better gene prediction programs become available the

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number of identified defensin sequences is likely to increase dramatically. Of the defensins identified so far, only a relatively small number have been isolated from plant material or expressed in quantities sufficient for biological studies. The large number of defensins being identified makes it impractical to test each for their primary function. Furthermore, mutagenesis to identify essential amino acids is an extremely time consuming task that is often hindered by difficulties expressing recombinant protein. To overcome these difficulties, a bioinformatic approach could assist in predicting the activity of new defensins and in detecting conserved amino acids that are essential for biological function.

The original classification of defensins into functional groups as proposed by Broekaert *et al.* (1995) was based on a defensin's ability or inability to inhibit fungal growth, as well as the effect it had on fungal morphology during growth inhibition. This was based on 12 defensin sequences, four of which differed by no more than seven amino acids. Basing the classification purely on antifungal characteristics failed to account for the many other activities identified for defensins. These defensins were simply placed into the 'non-antifungal' group. A further classification based on percent sequence identity was later proposed by Harrison *et al.* (1997) to account for newly identified defensins. However, this was based on seventeen sequences and is therefore not appropriate for the classification of the large number of sequences available to date.

This paper describes the sequence-based analysis of 139 plant defensins described in the NCBI protein database. An alignment and phylogenetic tree were constructed and this was used to group defensins based on sequence identity. Further analysis of these groups identified functional similarities between defensins within these groups.

2. Methodology used

A maximum-likelihood phylogenetic analysis was performed. Sequences of known defensins were downloaded from the NCBI protein database using the search string 'plant defensin'. Other sequences identified in the literature, but not available in the database, were also added manually. A complete list of peptides including their source and accession number is included in Table 1. A TCOFFEE (Tree based Consistency Objective Function For AlignmEnt Evaluation) (Poirot *et al.*, 2003) alignment was performed on the mature defensin domain sequences of 139 peptides and the resulting alignment file was used to generate a maximum-likelihood phylogenetic tree using MEGA-5 (Tamura *et al.*, 2007). The integrity of the tree was estimated by 1000 bootstrap replicates, values greater than 20 % are indicated at nodes.

3. Proposed grouping of plant defensins based on phylogenetic analysis

The recent discovery of large numbers of plant defensins and the increase in the number that are functionally characterized has made it clear that the classification groups proposed for defensins over 10 y ago are no longer appropriate. A phylogenetic tree of 139 plant defensin sequences was constructed and is presented in Fig 1. Eighteen groups can be distinguished. In some instances (eg *Raphanus sativus* and *Triticum kiharae*), defensins from a single plant species clustered together in the tree, while in others, they are separated throughout the tree (*Arabidopsis thaliana*, *Nicotiana alata*, *Vigna radiata*). It is likely that as more defensin sequences are discovered, characterization of them as described here will lead to a different number of groups. For this reason, the number of the group a defensin falls into is clearly not as informative as the defensins within the group.

4. Functional annotation of the plant defensin phylogenetic tree

Group 1

To investigate whether this method of analysis could be used to predict the function of novel defensins, reported functions of peptides were mapped onto the phylogenetic tree (Fig 2). The first proposed group (Group 1) contains many defensins that are inhibitors of either α-amylase (SIα2−3, aainhi21) or trypsin (Cpthio1). Since bifunctional inhibitors of these two enzymes are relatively common (Franco *et al.*, 2002) some of these peptides may be able to act on both enzymes but this has not been tested. Three defensins (γ-purothionin 1-2 and γ-hordothionin) inhibit protein synthesis *in vitro* (Colilla *et al.*, 1990; Mendez *et al.*, 1990) and one is able to block sodium channels (γ-Z1). In this group, the defensins that have been reported to be protein synthesis inhibitors, α-amylase inhibitors and sodium channel blockers share over 90 % identity and form a distinct subgroup (1.1, see Fig 1). In each case, the peptides have only been examined for one activity and, if tested, their activity profiles may overlap. Other members of group 1 exhibit antifungal (MtDef4, EGAD1, SD2, JI-2) and antibacterial (TaDef) activity. Whether protein synthesis inhibition or inhibition of enzymes is responsible for the antifungal and antibacterial activities reported for defensins of this group is not known. The only member of this group that has been characterized for its antifungal activity is MtDef4. This defensin inhibits growth of *Fusarium graminearum* without causing increased hyphal branching. It permeabilises the plasma membrane of fungal hyphae but, unlike some group 9 and group 10 defensins, does not require the sphingolipid glucosylceramide for its activity (Ramamoorthy *et al.*, 2007). Interestingly, another antifungal member of group 1 from oil palm (EGAD1) is significantly overexpressed in inflorescences displaying a 'mantled' phenotype (Tregear *et al.*, 2002), suggesting it may also have a role in flower development.

Groups 2−6

Group 2 contains two defensins from Capsicum. One of these (J1-1) is up-regulated during fruit ripening and wounding suggesting a role in defense although this hypothesis has not been tested (Meyer *et al.*, 1996). Group 3 contains defensins from Spruce and *Gingko biloba* trees, one of which (PgD1) has antifungal activity *in vitro*. Group 4 contains a defensin with α-amylase inhibitory (SIα1) activity and a sodium channel inhibitor (γ-Z2). Interestingly, these separate into a distinct group from their functional homologs in group 1 (SIα2−3 and

Table 1 – Source and accession numbers of plant defensins used to construct phylogenetic tree.

Peptide	Source	Accession number	Peptide	Source	Accession number	Peptide	Source	Accession number
aainhi21	*Sorghum bicolor*	Q09198	**γ-puro1**	*Triticum turgidum*	P20158	**PgD1**	*Picea glauca*	AY494051
AdAFP	*Arachis diogoi*	AAO72633	**γ-puro2**	*Triticum turgidum*	P20159	**PhD1**	*Petunia hybrida*	Q8H6Q1
AdDef	*Arachis diogoi*	AAP92330	**g-thionin**	*Nicotiana paniculata*	O24115	**PhD2**	*Petunia hybrida*	Q8H6Q0
AFP1	*Arabidopsis thaliana*	P30224	**γ-Z1**	*Zea mays*	P81008	**PmDef**	*Plantago major*	CAH58740
AFP2B	*Sinapis alba*	Q10989	**γ-Z2**	*Zea mays*	P81009	**PPT**	*Petunia inflata*	L27173
AhAMP1	*Aesculus hippocastanum*	AAB34970	**HcAFP1**	*Heliophila coronopifolia*	AER45491	**PsD1**	*Pisum sativum*	P81929
AhPDF1.1	*Arabidopsis halleri*	AAY27736	**HcAFP3**	*Heliophila coronopifolia*	AER45489	**PsD2**	*Pisum sativum*	P81930
AhPDF1.2	*Arabidopsis halleri*	AAY27737	**HsAFP1**	*Heuchera sanguinea*	AAB34974	**RsAFP1**	*Raphanus sativus*	P69241
AhPDF1.4	*Arabidopsis halleri*	AAY27739	**HvAMP1**	*Hardenbergia violacea*	n/a	**RsAFP2**	*Raphanus sativus*	P30230
Artv1	*Artemisia vulgaris*	Q84ZX5	**JI-1**	*Capsicum annuum*	X95363	**RsAFP3**	*Raphanus sativus*	CAA65984
At2g26010	*Arabidopsis thaliana*	O80995	**JI-2**	*Capsicum annuum*	X95730	**RsAFP4**	*Raphanus sativus*	O24331
At2g26020	*Arabidopsis thaliana*	O80994	**LCR66**	*Arabidopsis thaliana*	Q9C947	**SaAFP1**	*Sinapis alba*	P30231
AtAFP	*Arabidopsis thaliana*	P30224	**LCR67**	*Arabidopsis thaliana*	NP_565119	**SaAFP2a**	*Sinapis alba*	P30232
AtAMP1	*Arabidopsis thaliana*	AAM45086	**LCR68**	*Arabidopsis thaliana*	Q9ZUL7	**SD2**	*Helianthus annuus*	AF178634
AtAMP1.1	*Arabidopsis thaliana*	AAL36289	**LCR69**	*Arabidopsis thaliana*	Q39182	**SIa1**	*Sorghum bicolor*	P21923
AX1	*Beta vulgaris*	P81493	**LCR70**	*Arabidopsis thaliana*	Q41914	**SIa2**	*Sorghum bicolor*	P21924
AX2	*Beta vulgaris*	P82010	**LCR72**	*Arabidopsis thaliana*	Q9ZUL8	**SIa3**	*Sorghum bicolor*	P21925
BnAFP	*Brassica napus*	Q39313	**LCR73**	*Arabidopsis thaliana*	P82782	**SoD2**	*Spinacia oleracea*	P81571
BnDef1.2	*Brassica napus*	AAX35338	**LCR74**	*Arabidopsis thaliana*	Q9FFP8	**SPI1B**	*Picea abies*	AAN40688
BoDef	*Brassica oleracea*	CAC37558	**LCR77**	*Arabidopsis thaliana*	NP_199255	**TaDef**	*Triticum aestivum*	AB089942
BoPCP	*Brassica oleracea*	CAA06465	**LCR78**	*Arabidopsis thaliana*	P82787	**TfAFP**	*Trigonella foenum-graecum*	AAO72632
Brazzein	*Pentadiplandra brazzeana*	P56552	**LmDef**	*Lepidium meyenii*	AAV85992	**TkAMPD1**	*Triticum kiharae*	P84963
BSD1	*Brassica campestris*	L47901	**MsDef1.1**	*Medicago sativa*	AAV85437	**TkAMPD1.1**	*Triticum kiharae*	P84965
CaDef1	*Cicer arietinum*	ABC59238	**MsDef2.1**	*Medicago sativa*	AAV85438	**TkAMPD1.2**	*Triticum kiharae*	P84964
CaDef2	*Capsicum annuum*	AAL35366	**MsDef3.1**	*Medicago sativa*	AAT66095	**TkAMPD2**	*Triticum kiharae*	P84968
CaDef3	*Cicer arietinum*	ABC02867	**MsDef3.2**	*Medicago sativa*	AAT66096	**TkAMPD3**	*Triticum kiharae*	P84970
CcDef	*Cajanus cajan*	AAP49847	**MtDef2.1**	*Medicago truncatula*	AAQ91290	**TkAMPD4**	*Triticum kiharae*	P84971
Ccgth	*Capsicum chinense*	AAD21200	**MtDef2**	*Medicago truncatula*	AY313169	**TkAMPD5**	*Triticum kiharae*	P84966
CfD1	*Cassia fistula*	n/a	**MtDef3.1**	*Medicago truncatula*	AAT66097	**TpDef**	*Tephrosia platycarpa*	AAX86993
CfD2	*Cassia fistula*	n/a	**MtDef3.1a**	*Medicago truncatula*	AAT69983	**TPP3**	*Solanum lycospersicum*	AAA80496
Cpthio1	*Vigna unguiculata*	P83399	**MtDef4**	*Medicago truncatula*	n/a	**Tvdef**	*Tephrosia villosa*	AAX86993
Cpthio2	*Vigna unguiculata*	P84920	**MtDef1.1**	*Medicago truncatula*	AAQ91287	**VaD1**	*Vigna angularis*	n/a
CtAMP	*Clitoria ternatea*	AAB34971	**NaD1**	*Nicotiana alata*	Q8GTM0	**VrD1**	*Vigna radiata*	AAR08912
DmAMP1	*Dahlia merckii*	AAB34972	**NaD2**	*Nicotiana alata*	n/a	**VrD2**	*Vigna radiata*	2GL1_A
DRR39	*Pisum sativum*	Q01784	**NatD1**	*Nicotiana attenuata*	AAS13436	**Vudef**	*Vigna unguiculata*	ACJ06538
EGAD1	*Elaeis guineensis*	AF322914	**Nethio1**	*Nicotiana excelsior*	BAA21114	**WT1**	*Wasabi japonica*	BAB19054
Fabatin1	*Vicia faba*	A58445	**Nethio2**	*Nicotiana excelsior*	BAA21113	**Zmdef**	*Zea mays*	NP001146963
Fabatin2	*Vicia faba*	B58445	**Npthio1**	*Nicotiana paniculata*	O24115	**ZmES1**	*Zea mays*	AAK08132
FST	*Nicotiana tabacum*	P32026	**p322**	*Solanum tuberosum*	P20346	**ZmES2**	*Zea mays*	AAK08133
GbDef	*Ginkgo biloba*	AAU04859	**PCP-A1**	*Brassica oleracea*	CAA06464	**ZmESR6**	*Zea mays*	CAH61275
γ-hordo	*Hordeum vulgare*	P20230	**PDF1.1**	*Arabidopsis halleri*	AAY27736			
GmPI	*Glycine max*	AAC97524	**Pedef(SP E10)**	*Pachyrhizus erosus*	3PSM			

γ-Z1), despite originating from the same plant species. Two defensins from sugar beet (AX1 and AX2) are the only members of group 5. These proteins both inhibit the growth of filamentous fungi but not bacteria and are expressed in leaves. A defensin from *Arabidopsis* that has not been characterized is the only member of group 6.

Group 7

Group 7 contains only defensins from Solanaceous plants. All of the peptides tested from this group display antifungal activity and are expressed only in floral tissues. These defensins are also all class II defensins as they possess a C-terminal pro-peptide (CTPP) that is cleaved to release the mature defensin domain. NaD1, the class II defensin from the flowers of *N. alata*, is located in the vacuole and the CTPP is proposed to play a role in targeting of the peptide to this location (Lay *et al.*, 2003a). NaD1 is also the only member of this group for which the mechanism of antifungal activity has been investigated. The activity of this peptide involves specific interaction with the fungal cell wall, permeabilization of the plasma membrane and entry of the peptide into the cytoplasm of the hyphae as well as production of reactive oxygen species (ROS) (van der Weerden *et al.*, 2008, 2010). NaD1 has also been examined for inhibition of trypsin, chymotrypsin and α-amylase activity and it does not significantly affect any of these enzymes (Fung Lay, unpublished data). The tomato defensin DEF2 is predicted to have a role in both defense and

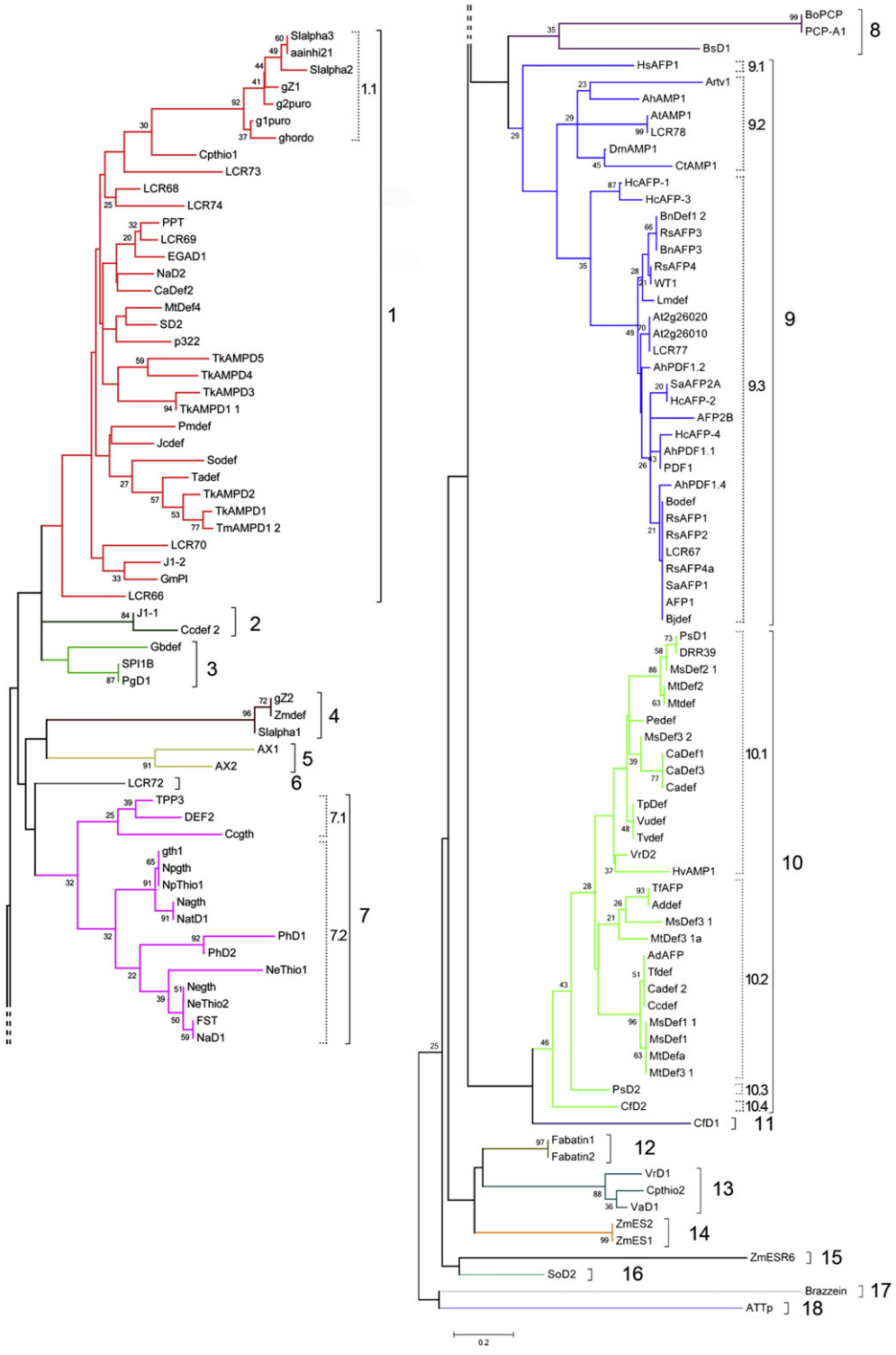


Fig 1 — Proposed classification of plant defensins. Maximum-likelihood phylogenetic tree of plant defensin mature domains constructed using MEGA 5.0. Bootstrap replicates greater than 20 % are indicated. Defensins were separated into groups and subgroups (indicated on right) based on branch length. Branch scale = substitutions per residue.

development since overexpression of the protein enhances resistance to *Botrytis cinerea* while either overexpression or silencing of the endogenous gene has severe effects on seed set and pollen viability (Stotz *et al.*, 2009). A role in development has not been investigated for other members of this group.

Group 8

Group 8 is composed of an antifungal peptide expressed in the stamen of *Brassica campestris* (BsD1) and two defensins from *Brassica* species (BoPCP, PCP-A1) termed pollen coat

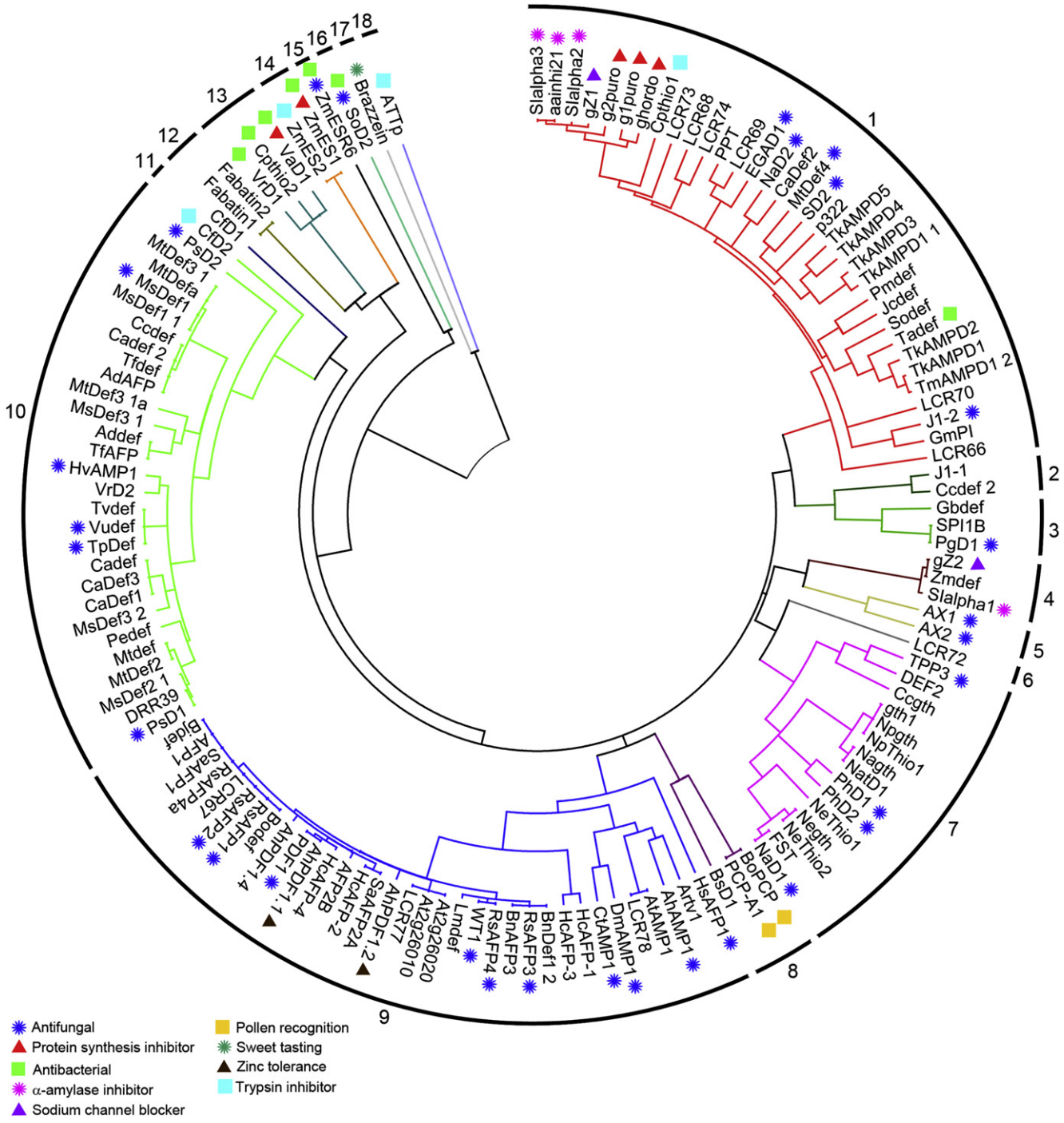


Fig 2 – Plant defensin phylogenetic tree indicating known functions of individual peptides. Circular view of the phylogenetic tree from Fig 1. Branches are colored to represent different groups which are indicated on the outer circle. Known functions of individual peptides are indicated by stars colored according to activity.

proteins (PCP) that have been implicated in pollen recognition. *Brassica* species also express a family of structurally similar cysteine-rich peptides (S-locus cysteine-rich proteins, SCR) that are predicted to be the male component of self-incompatibility. These peptides exhibit an altered cysteine spacing and are therefore not classified as defensins. Vanoosthuyse *et al.* (2001) describe the identification of homologs of both PCP and SCR families in *Arabidopsis*. A total of 86 PCP-like genes were identified in this screen and these included the 13 previously identified *Arabidopsis* defensins. However no evidence exists for any role in pollen development. Two defensin-like molecules were recently identified as the chemoattractants responsible for attracting an incoming pollen tube to the ovule in the flowering plant

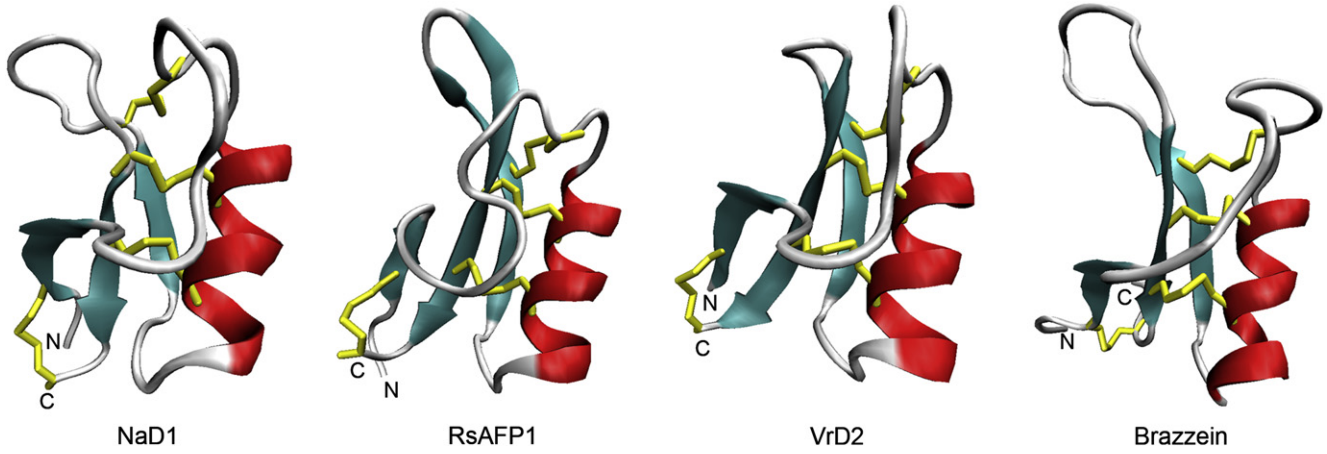


Fig 3 – Conserved structure among functionally discreet plant defensins. Comparison of the three-dimensional structure of plant defensins from groups 6 (NaD1: 1MR4), 1 (RsAFP1: 1AYJ), 9 (VrD2: 2GL1) and 2 (Brazzein: 1BRZ). All peptides share a conserved triple-stranded β-sheet (cyan) tethered to an α-helix (red) by three disulfide bonds (yellow) with a fourth disulfide bond joining the N- and C-termini.

Torenia fournieri (Okuda *et al.*, 2009). These peptides (named LUREs) are expressed in the synergid cell and secreted to the surface of the egg apparatus.

Group 9

The most well characterized defensins to date are the antifungal defensins belonging to group 9. These defensins can be broadly separated into three subgroups. Of these, groups 9.1 and 9.3 represent the morphogenic defensins, causing increased hyphal branching and swelling in treated hyphae, while group 9.2 is non-morphogenic (Osborn *et al.*, 1995; De Samblanx *et al.*, 1997). All the defensins present in group 9.3 are from plants in the Brassicaceae family. The more distantly related groups 9.1 and 9.2 also include members of the Asteraceae and Saxifragaceae families. Defensins in groups 9.2 and 9.3 require the presence of fungal sphingolipids for their activity (Thevissen *et al.*, 2004). The dahlia defensin DmAMP1 from group 9.2 binds specifically to mannosediinositolphosphorylceramide (MI_2PC) and RsAFP2, a radish defensin from group 9.3, binds to glucosylceramide (GlCer). Defensins from within the DmAMP1 subgroup compete for binding to the sphingolipid while those from other subgroups do not (Thevissen *et al.*, 2000). HsAFP2, the only defensin in group 9.1, also interacts with high affinity binding sites on the fungal cell membrane (Thevissen *et al.*, 1997) but this interacting partner has not yet been identified. The interaction between these defensins and lipids was initially proposed to occur at the plasma membrane. However, recent data on the activity of RsAFP2 (group 9.3) suggests that this defensin interacts with GlcCer in the cell wall (Thevissen *et al.*, 2012). Members from group 9 including RsAFP2 (9.3) and HsAFP2 (9.1) induce apoptosis in yeast cells *via* a mitochondrion-dependent mechanism (Aerts *et al.*, 2011). *Arabidopsis* defensins belonging to group 9.3 (AhPDF1.1–1.4) are up-regulated in response to zinc and confer zinc tolerance to both *Arabidopsis* plants and yeast cells.

Groups 10 and 11

Group 10 contains mostly antifungal defensins, all of which are isolated from the Fabaceae family. A defensin from pea belonging to group 10.1 (Psd1) moves into the nucleus of treated fungi and is predicted to halt the cell cycle through

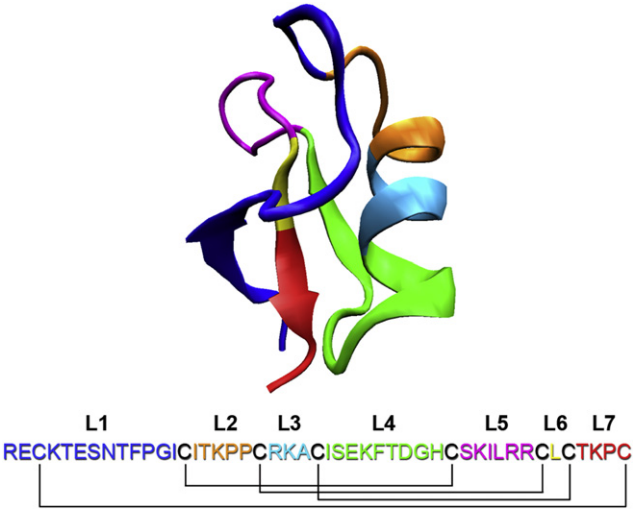


Fig 4 – Representation of 'loops' based on the structure of NaD1. Loops are defined as the region between cysteine residues. Loop 1 encompasses the first β-strand and a majority of the loop connecting the β-strand and the α-helix. Loop 2 encompasses the remainder of the connecting loop and the beginning of the α-helix. Loop 3 is a small region in the α-helix and loop 4 encompasses the remainder of the α-helix along with β-strand 2. Loop 5 is the flexible region between β-strands 2 and 3 and loops 6 and 7 together make up β-strand 3. Sequence and structure = NaD1 (PDB 1MR4).

interaction with a cell cycle control protein (Lobo *et al.*, 2007). Another member of this group (MsDef1) is able to block mammalian Ca^{2+} channels and is predicted to interfere with Ca^{2+} homeostasis in fungal cells leading to growth arrest (Spelbrink *et al.*, 2004). This peptide also requires the sphingolipid GlcCer for its activity against *F. graminearum*. The location of MsDef1 in treated fungi has not yet been investigated. A defensin from *Cassia fistula* (CfD2), the only member of subgroup 10.4, is a trypsin inhibitor although its antifungal activity has not been investigated. A second defensin from *C. fistula* that does not inhibit trypsin is the only member of Group 11.

Groups 12–14

All the defensins in groups 12 and 13 that have been functionally characterized display antibacterial activity. Two defensins from group 13 also possess α-amylase inhibitory activity and antifungal activity (VaD1, VrD1). Group 14 consists of defensins from *Zea mays* that are expressed in the female gametophyte and interact with a potassium channel on pollen tubes causing the pollen tube to burst (Amien *et al.*, 2010).

Groups 15–18

Groups 15–18 contain only one member each. ZmESR6 from corn (group 14) is active against both fungi and bacteria and, unlike all defensins outside of group 7, is expressed with a CTPP. This protein is expressed in the embryo surrounding region of developing corn kernels and accumulates in the placentochalaza cells. The CTPP may play a role in targeting the peptide to these cells. SoD2 (group 16) is an antibacterial and antifungal peptide from spinach. Brazzein (group 17) is an extremely sweet tasting peptide from the fruit of *Pentadiplandra brazzeana* (Ming and Hellekant, 1994) and ATTp (group 18) is a trypsin inhibitor from *Arabidopsis* (Zhao *et al.*, 2002).

Overall, functional analysis of these groups revealed that defensins which clustered displayed similar activities while those that separated did not. This trend also applied to defensins from a single plant species. For example, the radish defensins RsAFP1–4 all fall into group 9.3 and display antifungal activity. In addition the *V. radiata* defensin, VrD1 (group 13) is an α-amylase inhibitor while VrD2 (group 10) is not. This supports the idea that phylogenetic analysis may prove a useful tool for predicting the functions of novel defensins.

5. Alignment and structure–function analysis of plant defensins

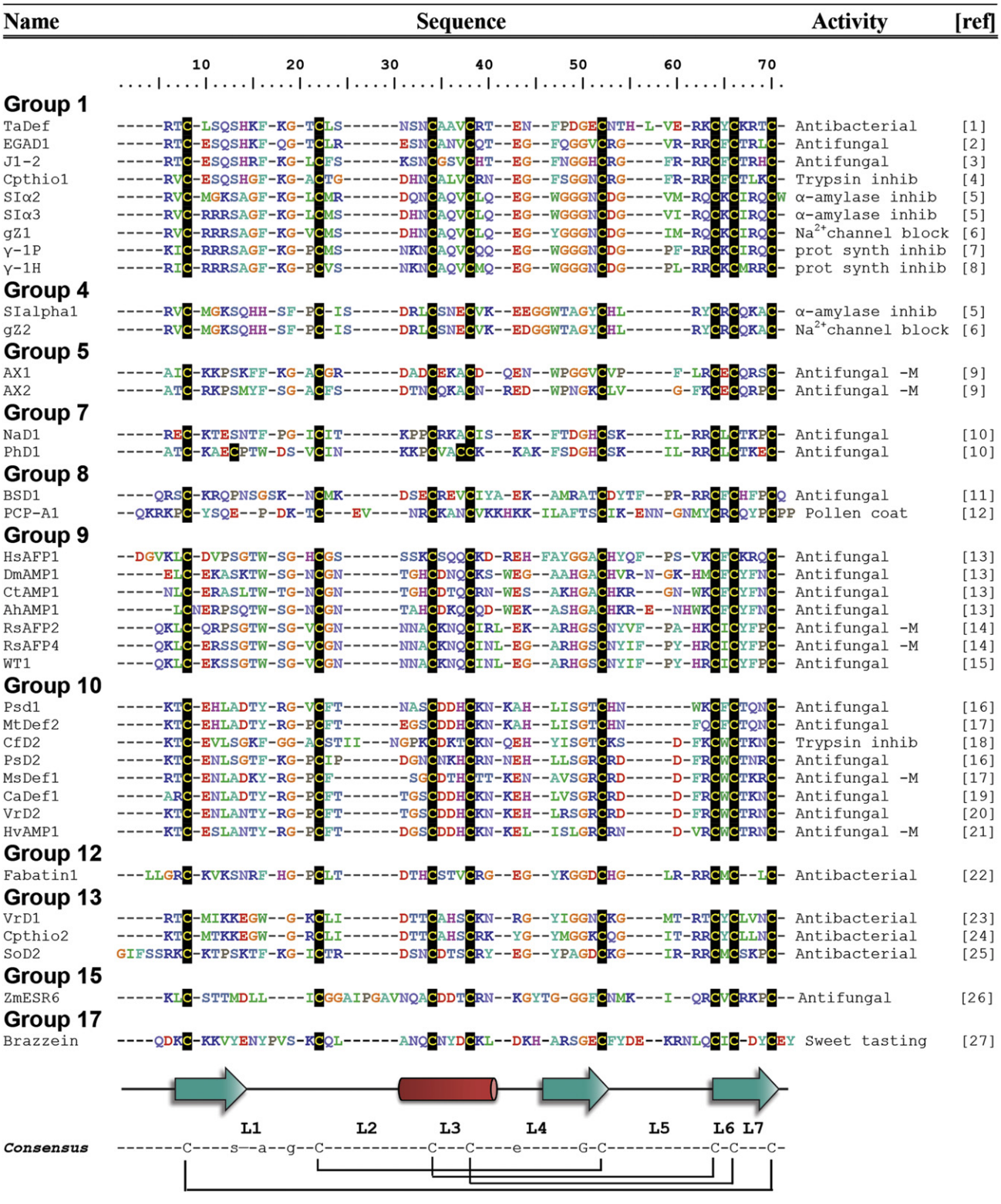
The structures of at least 11 defensins have been solved. They display a common fold consisting of a triple-stranded, antiparallel β-sheet connected to an α-helix by three disulfide bonds forming a cysteine-stabilized αβ motif (CSαβ). A fourth disulfide joins the N- and C-termini creating an extremely stable protein (Lay *et al.*, 2003b). Fig 3 shows the structure of four defensins, each from a different group. Despite the common fold, the overall level of sequence identity between these defensins is very low (less than 35 %) and they also differ in their described activities. Two are antifungal (NaD1, RsAFP1), one inhibits α-amylase (VrD2) and one is a sweet tasting protein (Brazzein).

A sequence alignment of characterized representatives from each group (Fig 5) allowed for the comparison of conserved amino acids both within and between groups. Within groups there is a relatively high degree of amino acid conservation. However, between groups, very little sequence identity exists, even between defensins with similar activities (Fig 6). Overall, only the eight cysteine residues are completely conserved, although the glycine at position 32 is only absent from two sequences (BsD1, PCP-A1). For the sake of this sequence analysis, the defensin sequence is broken in 'loops' defined as the regions between cysteine residues (Fig 4).

Amino acids essential for the antifungal activity of some defensins have been reported. The amino acids Thr-10, Ser-12, Leu-28, Tyr-38, Phe-40, Ala-42, Lys-44, Ile-46 and Phe-49 are essential for the antifungal activity of RsAFP2 (Group 9.3) (De Samblanx *et al.*, 1997). Analysis of antifungal defensins from outside group 9 revealed that these residues are not conserved and therefore are not likely to be responsible for the activity of all antifungal defensins (Fig 6). Overall, comparison of the sequences of all the antifungal defensins revealed that there is no sequence conservation beyond that observed for all defensins. This supports the idea that different mechanisms of action are involved for defensins from different groups.

The region defined by loops 4–7 is known as the γ-core, a conserved motif described by Yount and Yeaman (2004) that is present in all cysteine-rich antimicrobial peptides identified to date. This region, particularly the flexible loop 5, often contains amino acids essential for the activity of plant defensins. Indeed, 7 out of the 10 amino acids that are essential for the activity of RsAFP2 are located in this γ-core region. Except for HsAFP1, the sequences of the defensins from group 9 are similar in all loops apart from loop 5. The loop 5 region of DmAMP1, CtAMP1 and AhAMP1 is similar and, interestingly, these defensins compete for the same binding sites on the *Neurospora. crassa* cell surface (Thevissen *et al.*, 2000). Defensins from group 9 that belong to a distinct sub-group (9.3, RsAFP2 & 4) do not compete for these binding sites which may reflect the substantial differences in loop 5 of these defensins. It may be this loop that interacts with the cell surface.

The antifungal defensins of group 10 can be broken into two groups based on the level of sequence similarity in loop 5. This is reflected in their separation into distinct subgroups in the phylogenetic tree and it is possible that they mediate their antifungal activity by different mechanisms. Transferring the loop 5 region of MtDef4 (group 1) onto the MsDef1 (group 10) backbone resulted in a chimeric defensin with similar activity to MtDef4 (Sagaram *et al.*, 2011). Strikingly, the chimeric defensin no longer caused the increased hyphal branching exhibited by MsDef1 and was able to inhibit the growth of a glucosylceramide deficient *F. graminearum* strain which is resistant to MsDef1. Loop 5 on SOD2 and Fabatin1, antibacterial members of groups 16 and 12, is similar to loop 5 on the trypsin inhibitory/antifungal defensins from group 1. SOD2 is also active against filamentous fungi and Fabatin1 has not been tested for this activity. It would be interesting to know if these defensins do in fact share the same spectrum


Name
Sequence
Activity
[ref]
10
20
30
40
50
60
70
Group 1
TaDef Antibacterial [1]
EGAD1 Antifungal [2]
J1-2 Antifungal [3]
Cpthio1 Trypsin inhib [4]
SIα2 α-amylase inhib [5]
SIα3 α-amylase inhib [5]
gZ1 Na2+channel block [6]
γ-1P prot synth inhib [7]
γ-1H prot synth inhib [8]
Group 4
SIalpha1 α-amylase inhib [5]
gZ2 Na2+channel block [6]
Group 5
AX1 Antifungal -M [9]
AX2 Antifungal -M [9]
Group 7
NaD1 Antifungal [10]
PhD1 Antifungal [10]
Group 8
BSD1 Antifungal [11]
PCP-A1 Pollen coat [12]
Group 9
HsAFP1 Antifungal [13]
DmAMP1 Antifungal [13]
CtAMP1 Antifungal [13]
AhAMP1 Antifungal [13]
RsAFP2 Antifungal -M [14]
RsAFP4 Antifungal -M [14]
WT1 Antifungal [15]
Group 10
Psd1 Antifungal [16]
MtDef2 Antifungal [17]
CfD2 Trypsin inhib [18]
PsD2 Antifungal [16]
MsDef1 Antifungal -M [17]
CaDef1 Antifungal [19]
VrD2 Antifungal [20]
HvAMP1 Antifungal -M [21]
Group 12
Fabatin1 Antibacterial [22]
Group 13
VrD1 Antibacterial [23]
Cpthio2 Antibacterial [24]
SoD2 Antibacterial [25]
Group 15
ZmESR6 Antifungal [26]
Group 17
Brazzein Sweet tasting [27]
L1
L2
L3
L4
L5
L6 L7
Consensus

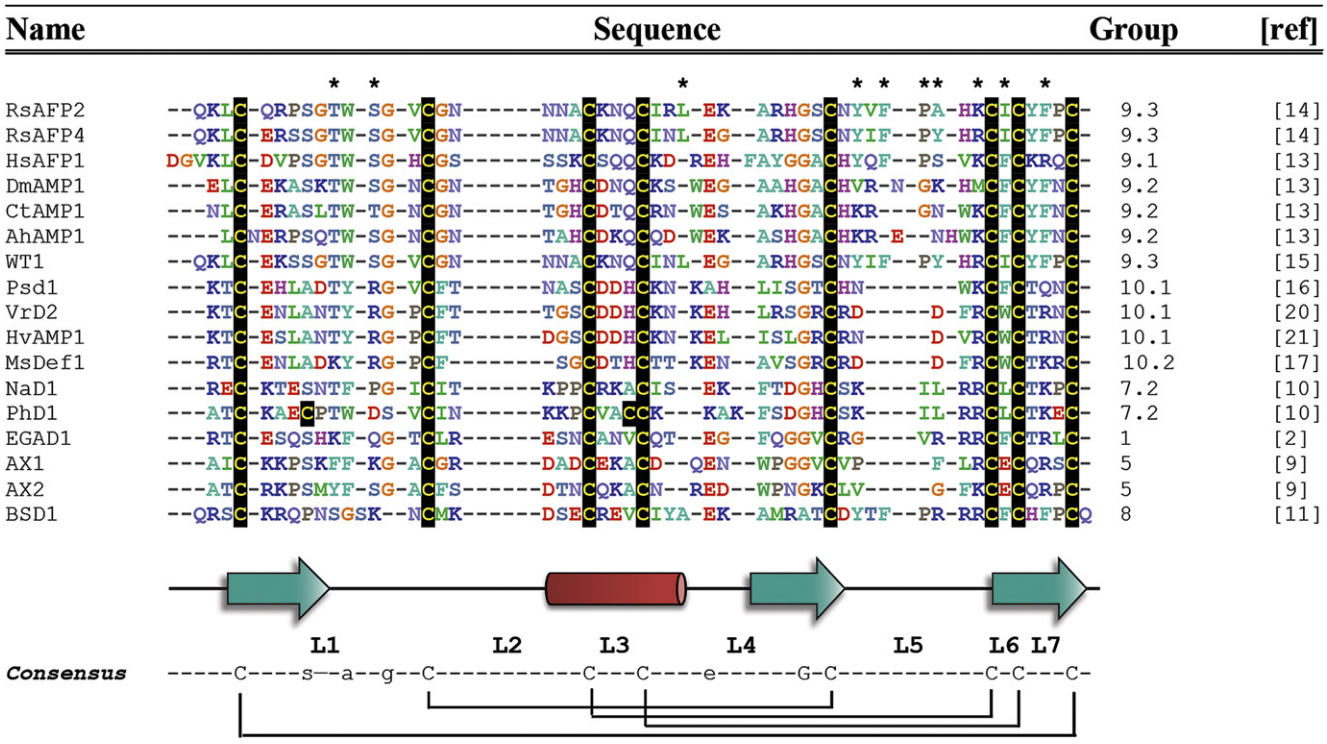


Fig 6 – Alignment of antifungal plant defensin sequences. Sequence alignment of antifungal plant defensins. Amino acids previously proposed as important for antifungal activity of RsAFP2 are indicated (*). Gaps have been inserted to maximize alignment and amino acids are colored according to properties. Position of β-strands and α-helix are indicated below alignment. Loops are defined as regions between cysteine residues (L1–L7).

of activities as defensins in group 1 as expected if loop 5 was responsible for activity.

Among the α-amylase inhibitors, Na^{2+} channel blockers and protein synthesis inhibitors in group 1, loops 4–7 are highly conserved between all the sequences. The amino acids in loop 5 are also similar to those in VrD1, an α-amylase inhibitor from group 13. This region of the peptide is probably responsible for its α-amylase inhibitory activity as a graft of this region onto the non-α-amylase inhibitor VrD2 conferred inhibitory activity onto VrD2 (Lin *et al.*, 2007). Molecular modeling suggested that the positively charged residues in loop 5 of VrD1 interacted electrostatically with the negatively charged active site of the enzyme and that the negatively charged amino acids in the loop of VrD2 prevented this interaction.

The identification of over 300 defensin-like genes was recently reported for both *Medicago* and *Arabidopsis* (Silverstein *et al.*, 2005; Graham *et al.*, 2004). This suggests that plant defensins are members of large gene families with a variety of activities. The phylogenetic analysis undertaken here revealed defensins with similar activities often cluster together. This method of analysis may, therefore, prove useful in determining the activities of as yet uncharacterized defensins; however, real trends will only become apparent when the functions of more defensins have been established. Another limiting factor in the prediction of defensin function is that many of the defensins reported to date have only been tested for one or two activities. In some instances, the reported activity of a peptide may not reflect its primary function. Purification of protein and testing for the handful of activities that have already been defined is unlikely to resolve the function of most of the defensin-like genes identified by genome analysis. For these, a gene knockdown approach may be useful although this is also unlikely to

Fig 5 – Alignment of plant defensin sequences with known functions according to groups. Sequence alignment of plant defensins representing proposed classification groups. Known activities are indicated. Gaps have been inserted to maximize alignment. The position of the β-strands and the α-helix is indicated below the alignment. Amino acids are colored according to properties with hydrophobic in green, polar in light blue, basic in blue, acidic in red, glycine in orange, proline in gray and histidine in magenta. Loops (L1-L7) are defined as regions between cysteine residues and the disulfide connectivities are indicated by bold lines below the consensus sequence. M = morphogenic. References: ([1] Koike *et al.*, 2002; [2] Tregear *et al.*, 2002; [3] Meyer *et al.*, 1996; [4] Melo *et al.*, 2002; [5] Bloch and Richardson, 1991; [6] Kushmerick *et al.*, 1998; [7] Colilla *et al.*, 1990; [8] Mendez *et al.*, 1990; [24] Franco *et al.*, 2006; [23] Liu *et al.*, 2006; [22] Zhang and Lewis, 1997; [25] Segura *et al.*, 1998; [9] Kragh *et al.*, 1995; [18] Wijaya *et al.*, 2000; [16] Almeida *et al.*, 2000; [17] Hanks *et al.*, 2005; [19] Hassairi *et al.*, 2005; [20] Lin *et al.*, 2007; [21] Harrison *et al.*, 1997; [13] Osborn *et al.*, 1995; [14] Terras *et al.*, 1992; [15] Saitoh *et al.*, 2001; [11] Park *et al.*, 2002; [10] Lay *et al.*, 2003a; [26] Balandin *et al.*, 2005; [12] Dickinson *et al.*, 1998; [27] Ming and Hellekant, 1994).

uncover function unless an obvious change in phenotype can be detected in the screening process.

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