Appears in -
Mycotoxin Prevention and Control in Agriculture
Chapter 9, pp 119–142 ACS Symposium Series, Vol. 1031, 2009.

Chapter 9

Transgenic Expression of Lytic Peptides in Food and Feed Crops to Control Phytopathogens and Preharvest Mycotoxin Contamination

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Transgenic crops are widely cultivated in several countries to control crop losses due to insects and weeds. However, disease resistant transgenic crops that can withstand infections due to fungal and bacterial pathogens are not yet available due to several reasons. The primary reasons are 1) host plant-pathogen interaction is a very complex phenomenon and it is often crop/variety or pathogen/strain–specific.; 2) natural antimicrobial proteins and peptides are subject to digestion by proteases, lack specificity and may be toxic to non-target plant and animal species; 3) large scale production of antimicrobial proteins and peptides are very expensive and 4) microbial pathogens can develop resistance to natural proteins and peptides. Recent advances in combinatorial chemistry and automated peptide synthesis have paved the way for rational design of stable, potent, and novel synthetic peptides with target-specific biological activity. Some of these lytic synthetic peptides have been already expressed in transgenic plants with varying degrees of success towards control of phytopathogens including some fungal pathogens that produce mycotoxins. This review gives a brief account of recent developments regarding the use of lytic peptides in transgenic crops to control yield losses due to pathogens and mycotoxin contamination.

The welfare of humanity is inextricably linked with the efficient cultivation of food and feed crops. Microbial plant diseases account for more than 16% loss in agricultural production (*1*). History of agricultural civilization includes stark examples of devastating crop losses due solely to unexpected microbial phytopathogens (*1, 2*). In addition to reduction in crop yield and quality, some fungal pathogens such as *Aspergillus* spp. and *Fusarium* spp. cause food and feed safety concerns because of their ability to produce potent mycotoxins - aflatoxins and fumonisins, respectively (*3-6*). Susceptibility of cultivated crops to plant diseases is exacerbated by several factors such as monoculture of genetically uniform high yielding varieties under large acreage; lack of disease resistant genotypes in the germplasm, and development of resistance in microbial populations to commonly used chemical pesticides. In this regard, development of transgenic crops is very attractive as they maximize crop productivity and quality and is less dependent on use of toxic chemicals that can cause irreparable damage to human health and the environment in the long run. Overexpression of native or heterologous antifungal peptides to enhance host plant protection has been the subject of several reviews (*1, 5, 6, 7*). Several of these natural peptides possess nonspecific toxicity to non-target organisms and are subject to proteolytic degradation. The advent of automated peptide synthesizers and combinatorial peptide chemistry over the past decade has made it possible for rational synthesis of stable and target-specific peptides to overcome some of the problems associated with lytic peptides. More than twenty years ago, it was recognized that certain single genes, encoding for potent natural antimicrobial peptides (lytic peptides), might offer a means to improve the disease resistance of plants utilizing current molecular techniques (*8, 9*). It was first achieved in the early 90's (*10, 11*) and since that time numerous papers have been published demonstrating the efficacy of this approach in enhancing plant disease resistance (*2, 7, 12*). In spite of these efforts so far, success has not resulted in release of commercially viable disease-resistant crops although several field tests have been conducted, as listed by the USDA-APHIS Biotechnology Regulatory Services (http://www.isb.vt.edu/cfdocs/fieldtests1.cfm). The advantages of lytic peptides to control broad-spectrum microbial pathogens in agriculture is very appealing and we provide here a brief discussion on the structure of lytic peptides as related to their antimicrobial activity, advantages and disadvantages of lytic peptides, construction of vectors for both nuclear and plastid transformation and antimicrobial effects of transgenic plants expressing lytic peptides.

Lytic Peptides

Lytic peptides are small proteins that are major components of the antimicrobial defense systems of numerous species. They are a ubiquitous feature of nearly all multi-cellular and some single-cellular life forms. They generally consist of between 10-40 amino acid sequences, which have potential for forming discrete secondary structures. Often, they exhibit the property of amphipathy. An amphipathic α-helix may be depicted as a cylinder with one curved face composed primarily of nonpolar amino acids while the other face is

composed of polar amino acids. Most of the lytic peptides that have been described in the literature seem to fall into one of three different classes based on the arrangement of amphipathy and high positive charge density within the molecule:

1) Cecropins (35 amino acids in length and derived from the Giant Silk Moth), N-terminal half amphipathic while the C-terminal half mostly hydrophobic (*13*);

2) Magainins (23 amino acids in length and derived from the African Clawed Frog), amphipathic the full-length of the molecule (*14*); and

3) Melittin (26 amino acids in length and derived from the Honeybee), C-terminal half amphipathic with the N-terminal half primarily hydrophobic (*15*).

The conservation of these physical properties is requisite for activity, but the requirements seem to be somewhat nonspecific in terms of amino acid sequence. For example, we have synthesized highly sequence divergent analogs for each of the peptide classes and have found some of them to be more active and less toxic than their natural counterparts (Jaynes, unpublished data).

Decades ago, the original intent in our laboratory was to utilize the gene encoding a close homolog of Cecropin B (SB-37) to augment bacterial disease resistance in plants. However, during the course of our studies, a new highly sequence divergent peptide was synthesized (Shiva-1) and was shown to possess a more potent lytic activity than SB-37 (*16*). The enhanced bioactivity of Shiva-1 was the first indication that modifications made in the primary sequence of lytic peptides would not destroy the peptide's activity provided certain physical characteristics of the peptide were conserved. Indeed, this was a paradigm-shifting moment in understanding of the structure/function relationship of these incredibly interesting natural molecules and allowed us to pursue the design of novel molecules with enhanced activities. Another example of sequence modification resulting in increased potency of natural peptides is provided in MSI-99, an analog of magainin-II that displayed more positive charge and antibacterial and antifungal activity than its predecessor (*17, 18*).

Amphipathy, Hydrophobicity, & Charge Density: Some of the Physical Properties that Unify Protein Structure and Function

To best illustrate the physical connections between proteins and peptides, it is necessary to display their sequences in ways that make it easier to visualize structural differences and similarities. There are a number of physical features that appear to be important in modulating the activity of peptides:

1. Degree of amphipathy
2. Length of amphipathy
3. Heterogeneity of amphipathic section
4. Placement of amphipathic section (N or C terminal)
5. “+” Charge density (less or more)

6. Hydrophobicity of amphipathic section
7. Presence of hydrophobic tail
8. Length of hydrophobic tail
9. Hydrophobicity of tail
10. Placement of hydrophobic tail (N or C terminal)
11. Absence, presence, & position of "+" charged center
12. Absence or presence & position of flanking sequence
13. Predominating secondary structure
14. Termini modification (N-acetylation, C-amidation)
15. Surface area of hydrophilic and hydrophobic faces
16. Steric or volume considerations.

One can distinguish these characteristics by viewing the amino acids in ways that visually accentuate the differences in their physical attributes. In this respect, it is instructive to ponder the evolution of protein structure and the fact that, generally speaking, only 20 different amino acids are found in proteins. These are: alanine (A), arginine (R), aspargine (N), aspartic acid (D), cysteine (C), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tyrosine (Y), valine (V), and tryptophan (W). There are a few exceptions, but these 20 are the only ones that are represented in the genetic code (and are called the protein amino acids). So, they are the only ones that matter, at least for the sake of this discussion.

Why did life select these 20 when there are at least 500 other amino acids that have been found to occur in the natural world? Why are these so special? Living processes are never profligate; energy is never expended for little purpose. The simple answer is that these 20 amino acids provide enough unique physico-chemical information that no more are needed for protein structure *(19)*. Chemically speaking, these amino acids differ in very subtle, profound, and important ways. The remarkable diversity of life found on the Earth, in large measure, is merely a reflection of the differences in these molecular building blocks when they are assembled into proteins. With the addition of water, vitamins, minerals, lipids, carbohydrates, and nucleic acids, an individual organism is built, from bacterium to human being. The sheer majesty of the physical world that derives from this relatively simple chemical alphabet is overwhelming. Each living entity produces sets of molecules unique to its type or specie and arranges them in novel ways. During a normal life span, a human may produce as many as 100,000 unique protein transcripts that are constructed from the 20 protein amino acids *(20)*. These separate proteins come together, some, only at specific stages of life, to form a singular, functional human being.

Taking these special 20 amino acids and viewing just two of their seemingly simple properties: hydrophobicity and volume differences can give one an appreciation of the significant chemical refinements that they must represent (Table 1).

Table 1. Hydrophobicity/Hydrophilicity and volume of amino acids

AA	Volume*	D (Å)	Hydrophobicity	Hydrophobicity /Hydophilicity %	Luminosity
F	189.9	7.13	3.7	100	128
M	162.9	6.78	3.4	92	138
I	166.7	6.83	3.1	84	149
L	166.7	6.83	2.8	76	159
V	140.0	6.44	2.6	70	166
C	108.5	5.92	2.0	54	187
W	227.8	7.58	1.9	51	190
A	88.6	5.53	1.6	43	201
T	116.1	6.05	1.2	32	214
G	60.1	4.86	1.0	27	221
S	89.0	5.54	0.6	16	235
P	122.7	6.16	-0.2	2	254
Y	193.6	7.18	-0.7	6	249
H	153.2	6.64	-3.0	25	225
Q	143.9	6.50	-4.1	34	213
N	117.7	6.08	-4.8	39	206
E	138.4	6.42	-8.2	67	170
K	168.6	6.85	-8.8	72	164
D	111.1	5.96	-9.2	75	159
R	173.4	6.92	-12.2	100	128

The single letter code for the amino acids (AA) is shown on the previous page *Note: the total volume, in cubic angstroms, is derived from the van der Waals' radii occupied by the amino acid when it is in a protein. Hydrophobicity is in kcal/mol and is the amount of energy necessary to place the amino acid, when in an α-helical protein, from the membrane interior to its exterior. Luminosity helps assign the density of cyan (hydrophobic amino acids) or magenta (hydrophilic amino acids) to each glyph of the "molecular" font (Molly) that was developed and is described in the text Data derived from *(21)*.

The structural clues they provide in determining protein functionality are available, if we just look at them in the right way. For at least the last 2 billion years, life has found 20 amino acids, combined in different ways, to be adequate to meet all the challenges that it has faced on this planet. All the protein questions that will ever be asked can be answered by natural selection; and life, since the dawn of "biological" time, has been "compelled" to solve just a miniscule number of structural problems. By this it is meant that there might be, let's say, a trillion different proteins that have ever existed on the earth (there probably have been far fewer). By applying combinatorial mathematics to the 20 amino acids, we derive, in practical terms, an almost infinite number of possible combinations that becomes an even bigger number as the length of the protein is increased. For example, if we assume the maximum length for a protein is 200 amino acids, then, the total number of different proteins possible can be derived from the formula *(22)* found below (sum of a finite geometric series):

$$\sum_{i=2}^{200} 20^{i} = a^{1}\frac{1-r^{n}}{1-r}$$

"a^1" is the first term, "n" is the number of terms, and "r" is the common ratio of the series increase, i.e., it goes up by a factor of 20 each time (the number of different protein amino acids). When one goes through the arithmetic, the number of possible combinations of proteins, from two amino acids in length to 200, is 8.458×10^{257}. A huge number to say the least, particularly, when one considers that the total number of atoms of matter in the universe is estimated to be less than 10^{100}! *(23)*. Also, it should be noted that there are many proteins far larger than 200 amino acids in length. The point of this exercise is that life, in 2 billion years of existence, has not significantly diminished the total number of possible assembled amino acid combinations (proteins) that can do all of the different jobs required by all living organisms. That is the power of evolution; biology will derive suitable answers to any question, given enough time. By studying the predominating 20 protein amino acids in certain ways, we can gain insight into the structural principles that govern all of protein biochemistry and then, as our awareness increases, subtle connections are discovered and seeming disparities can be replaced by recognizable physical commonalties. The unity of the protein structure/function paradigm will continue to emerge as our understanding deepens. After all, every protein that ever existed has been tempered in the "forge" of natural selection. The recognizable similarities of protein structure, even taken from widely divergent species, should not come as a surprise---all of life's processes are interconnected throughout their numerous levels of complexity.

In order to visualize differences and similarities in protein structure more easily, a molecular font was designed (Molly) that is more representative of the chemical nature of the amino acids (see below). To do this, spheres were substituted with circles with diameters equal to:

$$2\sqrt[3]{\frac{Volume}{4/3\pi}}$$

for each particular amino acid. The equation is a rearrangement of the formula for the volume of a sphere: $4/3\ \pi\ r^3$. Then, setting the largest volume to 1, the smaller ones were proportionally reduced. Thus, the size of the circle is directly related to amino acid volume and, the differences shown between the amino acids in Molly, then, are visually accurate. To increase the information of the representation, the hydrophobicity or hydrophilicity of each amino acid was converted to a color scale. The most hydrophobic amino acids are the most intense cyan color while those that are less hydrophobic are proportionally less concentrated cyan. Conversely, those amino acids that are most hydrophilic possess the deepest magenta color. Likewise, a graduated scale of less intense magenta color is used for those amino acids of lower hydrophilic character. From this scale, it can be seen that, as amino acids become less hydrophobic or less hydrophilic, they become less pigmented and, therefore, more likely to be "exchangeable" within the protein structure. Also, implicit in this scheme is that, within a particular hue, i.e., amongst hydrophobic amino acids or hydrophilic amino acids, of very similar properties, exchanges would be more likely to occur (generating the variability one observes in proteins of similar function from evolutionarily distant organisms). Of course, changes would be within the specific structural constraints imposed on each particular protein for it to retain its functionality---natural selection, at it again. Most of the amino acid glyphs possess a mnemonic symbol that further characterizes its chemical properties. For example, charged amino acids have a "+" or a "-" sign incorporated within their glyph, the thickness of which, is related to the dissociation constant of their ionizable protons, other symbols aid in identifying the rest of the amino acids (Figure 1).

With the above in mind, we will discuss in the next section lytic peptides and the basic design parameters that have guided in the construction of novel peptides.

Figure 1. Amino acids in lytic peptides. The symbols represent a molecular alphabet
(see page 1 of color insert)

Lytic Peptide Design Parameters

Lytic peptides are small basic proteins that appear to be major components of the antimicrobial defense systems of a number of animal species including insects, amphibians, and mammals. They consist of around 12 to less than 50 amino acids, which have potential for forming amphipathic α-helices or partial β-pleated sheets (locked by disulfide linkages); and thus, can interact with all cell types at the membrane surface. This interaction can result in no observable cellular effect, temporary cell impairment, death, cell proliferation, or other activities (Jaynes, unpublished). That is why these molecules are more than lytic peptides. Four distinct types of lytic peptides have been discovered over the last several decades; examples of each type are melittin, cecropins, magainins, and defensins. The properties of naturally occurring lytic peptides suggest at least three distinct α-helical classes consisting of different arrangements of amphipathic and hydrophobic regions (Figure 2).

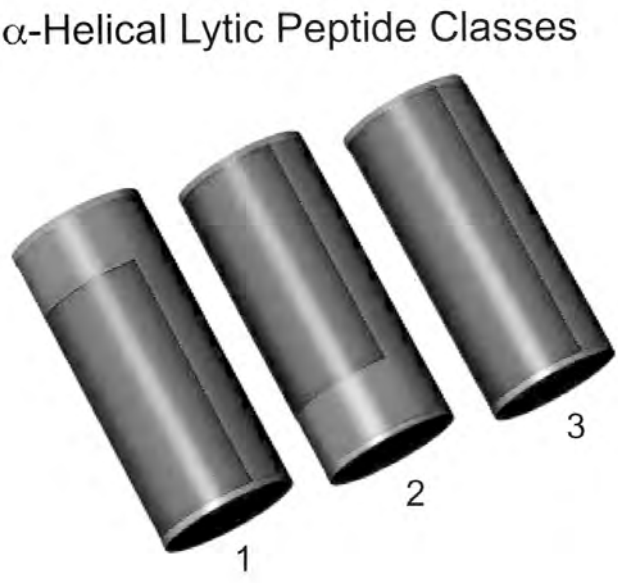

Figure 2. α-helical lytic peptide classes
(see page 2 of color insert)

The green band on the cylinders indicates the amino-terminus of the peptide while the gray band represents the carboxy-terminus. The cyan color represents regions that are predominately hydrophobic and the magenta color signifies regions that are hydrophilic. Representative examples of natural peptides, which fit this classification system are: melittin-class 1, cecropins-class 2, and magainins-class 3 (note, more than 90% of all the known natural peptides fall within this classification system, data not shown). Therefore, separate synthetic peptides can be subdivided into distinct classes based on what has been observed in Nature.

Some examples of natural lytic peptides and their sequence as cast in the Molly motif are listed below, along with representative optimized analogs. These are shown in a typical linear array and are read from left to right (Figure 3).

There are several natural lytic peptides that assume a β-conformation, examples of which are the defensins and protegrins. These peptides can assume this shape because of intra-disulfide linkages that lock them into this form, an absolute requisite for activity. We have designed a novel class of peptides that form β-sheets without the necessity of disulfide linkages. An example, D4E1 is shown in Figure 4.

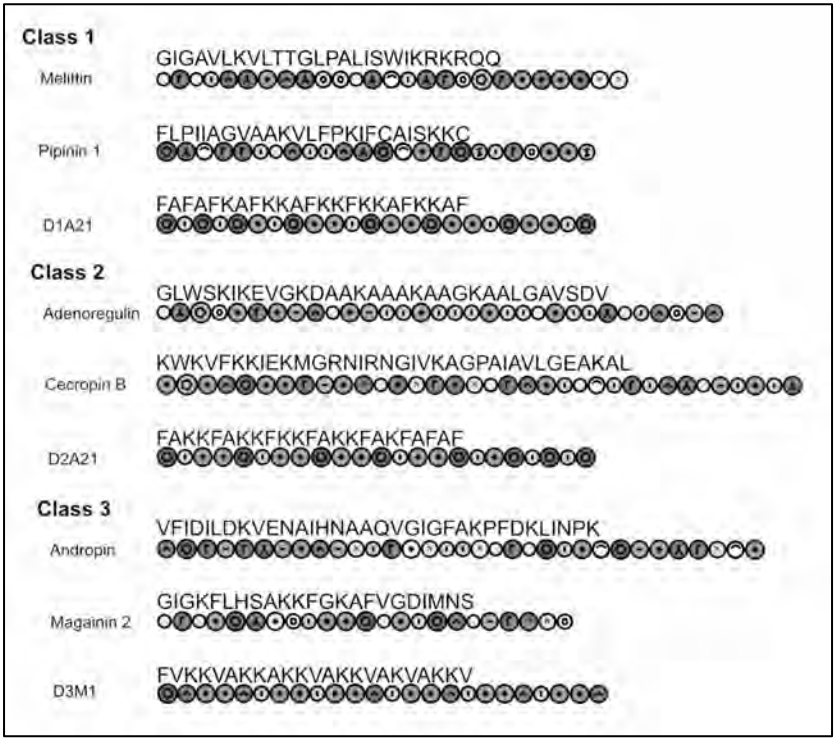

Figure 3. Natural lytic peptides
(see page3 of color insert)

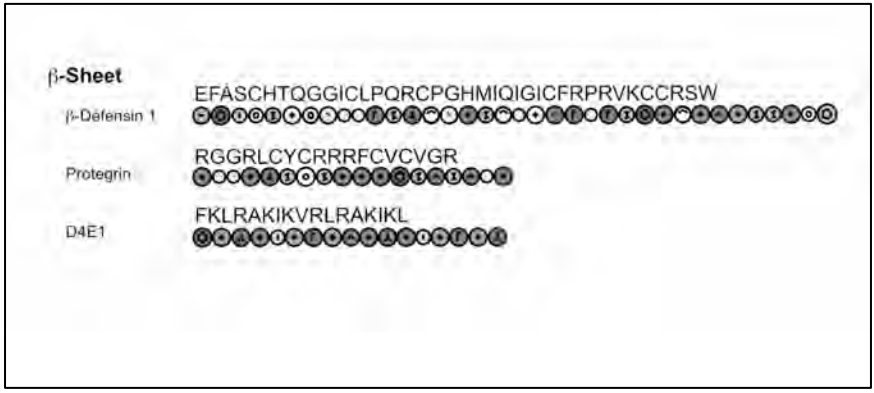

Figure 4. β-sheet peptides
(see page 3 of color insert)

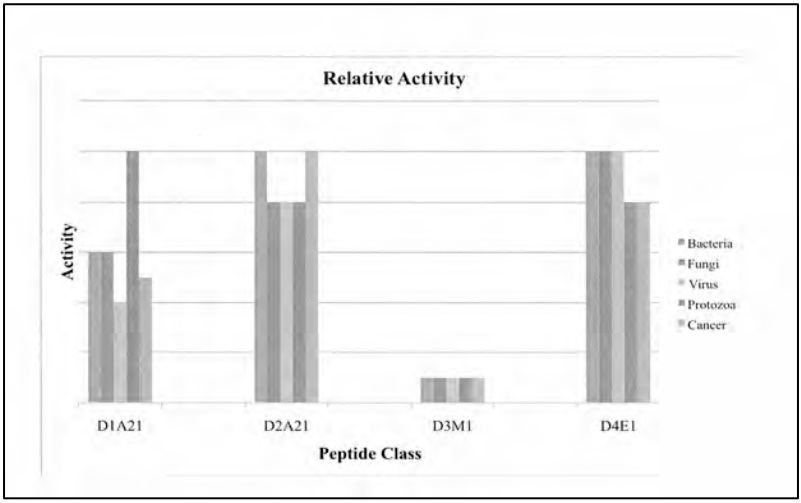

Figure 5. Relative Activity of selected synthetic lytic peptides (Jaynes, unpublished)
Note: Activity is the concentration of the peptide necessary to kill 50 % of the microorganisms. Therefore, the longer the histogram the higher the activity, the longest yielding 100% kill. The concentrations of the peptides are around 1 μM to 25 μM.
(see page 4 of color insert)

It was surmised that simply alternating hydrophobic and hydrophilic amino acids would render an amphipathic beta sheet, which indeed, is the case (*24*). The columnar array of hydrophobic and positive charged amino acids is apparent when the peptide adopts an amphipathic β-form. However, the width of the columns is narrower but overall length is greater than a peptide that adopts an amphipathic α-helix conformation of the same number of amino acids.

All classes of lytic peptides differ somewhat in activity (note that Class 3, magainin class, is far less active in all cases than are the other lytic peptide classes). Figure 5 illustrates, in general terms, their activity spectrum. For the most part, the idealized designed peptides exhibit higher levels of activity with reduced toxicity (*25-28*).

Genetic Engineering of Food crops

Gene Constructs for Plant Transformation

Well-characterized antimicrobial peptides from different sources as indicated above or synthetic peptides can be transferred to other food and feed crops to enhance host plant resistance to phytopathogens. Whenever possible, it is useful to evaluate the antimicrobial traits of unknown peptides by assaying the chemically synthesized pure peptides against as many microbial pathogens as

possible (e.g., *29*) prior to actual transgenic work that is usually very time-consuming. Antifungal genes coding for promising peptides can be transferred to other crops by any one of the available transformation methods (*30, 31*). The most widely used nuclear transformation method employs a soil-borne *Agrobacterium* (*32*) which itself has been transformed to include a binary vector containing the gene of interest (antifungal genes) and selectable markers (Figure 6A). Alternatively, plasmids containing the gene of interest can be delivered into plant cell nucleus or organelles (e.g., chloroplast) by a biolistic device or "gene gun" (*33*). The latter method has become the method of choice in transforming chloroplasts (Figure 6B) and other organelles. Other methods of transformation (e.g., electroporation, microinjection, somatic hybridization and others) are rarely employed outside laboratory conditions and, as such, not discussed in detail here.

When engineering a plant transformation vector the investigator has a number of different classes of promoters to choose from depending on which tissues and at what developmental stage expression of the transgene is desired (reviewed in *34*). Often the gene is placed under the control of a constitutive promoter such as the CaMV 35S (*35*) or potato ubiquitin 3 promoter (*36*). Placing the gene under control of a constitutive promoter usually ensures that all tissues will harbor the antifungal protein/peptide though there is some difference in levels of transgene expression in different tissues and at different developmental stages. In many cases it is desirable to place expression of the antifungal gene under a tightly-controlled promoter that will allow inducible or tissue-specific expression of the transgene thus reducing the metabolic load on the host plant imparted by constitutive promoters hence decreasing the chances for reduced plant growth and yield. Examples of tissue-specific promoters include the cottonseed α-globulin B gene promoter (*37*) and the barley lemma (*lem1*) gene promoter (*38*) that demonstrate seed-specific expression. Seed is often the target of infection of mycotoxigenic fungi and therefore seed-specific expression of the antifungal gene should provide the greatest levels of protection. There are a number of classes of inducible promoters including the pathogen/wound-inducible promoters such as the maize proteinase inhibitor (*mpi*) gene promoter (*39*) and the poplar *win3.12T* gene promoter (*40*). These promoters respond to mechanical and insect damage to plant tissues and also to fungal infection. In the case of aflatoxin contamination in crops such as peanut, maize, tree nut and cottonseed, entry of the aflatoxigenic fungus *Aspergillus flavus* to the seed is usually facilitated by boring insects so these types of inducible promoters would provide activation of antifungal gene expression at a very early stage of fungal invasion and only at the site of wounding/infection thus reducing the chances of any deleterious effects on plant growth and development.

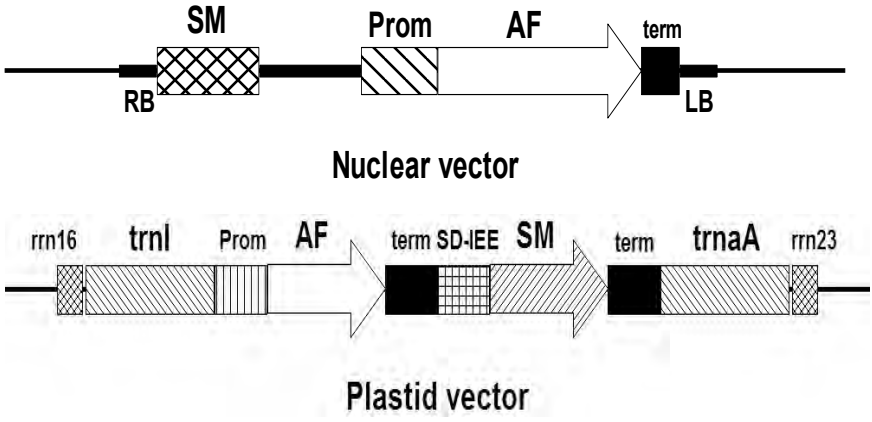

Figure 6. Generic plasmid vectors for nuclear and plastid transformation
Schematic diagram of generic plasmid vectors used for nuclear and plastid transformation of plant tissues.
[A] Binary vector for nuclear transformation: the main elements for these vectors 1) Left and right T-DNA border sequences (LB and RB) required for integration of the transgenes into the plant genome; 2) SM, selectable marker gene; Examples of SMs include antibiotic resistance genes such as nptII (kanamycin resistance), hptII (hygromycin resistance), or resistance genes to various herbicides such as glyphosate, sulfonylurea, imidazolinones, and phosphonothricin; 3) Promoter: These may include constitutive promoters such as enhanced CaMV 35S or ubiquitin control regions and wound or pathogen inducible promoters such as PINII or mpi; 4) transcriptional terminator (term) such as nopaline synthase (nos) terminator sequence; AF- antifungal peptide gene of interest as listed in the text.
[B] Plastid vector: the main elements in a plastid transformation vector usually include 1) Regions of homologous plastid DNA flanking the transgenes; The plastid rrn16-trnI and trnA-rrn23 gene regions are often used as flanking DNA for integration of the transgenes into the trnI-trnA intergenic region of the plastid genome via homologous recombination; 2) plastid promoter: The choice of plastid promoter can vary but two commonly used sequences are the Prrn ribosomal RNA operon promoter which drives gene expression in both green and non-green tissues or the psbA photosystem II D1 gene promoter that drives high level gene expression under light conditions; 3) 3' UTR and transcriptional terminators (term); 4) SD-IEE region: sequence that provides a Shine-Dalgarno region and intercistronic expression element that allow for the generation of stable, translatable monocistronic mRNAs thus facilitating transgene stacking in operons (for a description of vectors for plastid transformation, see Lutz et al. (41); 5) SM, selectable marker gene: This is usually the aadA gene encoding spectinomycin resistance or the nptII gene encoding kanamycin resistance; AF- antifungal peptide gene of interest as listed in the text.

Often, despite the plethora of available promoters and transformation vectors, there is still the problem of lack of significant levels of expression of the transgene and hence lack of enough production of the antifungal protein/peptide to be efficacious in inhibiting the growth of the mycotoxigenic fungus. This can often be traced to events such as gene silencing due to multiple integrations of the transgene or position effects due to integration of the transgene within regions of the genome that are transcriptionally inactive. These events are common to nuclear transformation. An alternative to nuclear transformation is the transformation of the plastid genome in plants (*42, 43*). Plastids are present in both green and non-green plant tissues. Plastid transformation eliminates many of the drawbacks associated with nuclear transformation (Table 2). Gene silencing does not occur in plastids nor is there any position effect as integration is site-specific due to homologous recombination of flanking plastid DNA sequences that are present in the vector (*43*). Another advantage of plastid transformation is the ability to generate significant levels of the transgene product due to high numbers of plastids and hence plastid genomes present in each cell, resulting in a very high number of functional transgene copies. Additionally, multiple genes can be expressed in an operon-like fashion from one promoter thus allowing for "stacking" of genes in a single transformant. From an environmental standpoint, plastid transformation is preferable to nuclear transformation as in most angiosperm plant species plastid genes are maternally inherited and therefore transgenes present in these plastid genomes are not susceptible to dispersal to other plant species via pollen dissemination (*42, 44*). One of the major hurdles left with respect to plastid transformation technology is the development of efficient transformation systems for specific plant species. Though plastid transformation has been achieved in many plant species there are still a great number that have not been transformed up to this time (*43*). This is especially true for many crop plants that are susceptible to mycotoxin contamination such as maize, peanut, wheat, barley, grape, and tree nuts. In many cases difficulty in transforming these species arises because non-green tissues must be used as targets for biolistic transformation. Little is know about the regulation of the transcriptional and translational machinery in pro-plastids present in these tissues and pro-plastids are much smaller than that of chloroplasts found in green tissues thus making successful integration of the transgene more difficult (*43*). Given time, plastid transformation protocols will most probably be optimized for these plant species.

Table 2. Comparison of Chloroplast and Nuclear Genetic Engineering

Transgenic Traits	Chloroplast Genome	Nuclear Genome
Transgene copy number	up to 10,000 copies/cell	usually less than 10 copies/cell
Level of gene expression	high abundance w/ high accumulation of foreign protein	gene expression often too low to be efficacious
Gene transcription	genes can be arranged in operon-form allowing multiple genes to be expressed from one promoter	difficult to stack multiple genes into one construct. Requires multiple promoters
Position effect	site-specific recombination eliminates positional effects on transgene expression	random insertion into genome results in variable transgene expression levels
Gene containment	maternal inheritance results in high level of containment-transgene not carried by pollen	possible outcrossing via pollen drift
Toxicity of foreign proteins	potential for minimization of adverse effects of transgenic proteins due to compartmentation	accumulation of toxic proteins in the cytosol may be deleterious to host

Genetic Engineering for disease resistance

Genetic engineering of host plants for resistance to microbial diseases has been an ongoing area of investigation that has seen many small-scale laboratory/greenhouse successes but success with respect to wide-scale commercialization is yet to be seen (*2, 7, 12, 45-47*). Several reports are available on the efficient nuclear expression of peptides for controlling microbial plant pathogens. It is noteworthy that expression of lytic peptides did not alter the morphology or flowering of transgenic plants (*18, 48, 49*). We provide below some selected reports on antimicrobial effects of transgenic plants expressing peptides of plant or non-plant origin including synthetic peptides.

Peptides of Plant–origin

Huffaker et al. (*50*) identified peptide elicitors derived from the plant itself that activate defensive genes against pathogens. For example, they isolated a 23-aa peptide from *Arabidopsis*, called AtPep1, which activates transcription of the defensive gene defensin (PDF1.2) and activates the synthesis of H_2O_2, both being components of the innate immune response. The peptide is derived from a 92-aa precursor encoded within a small gene that is inducible by wounding, methyl jasmonate, and ethylene. Constitutive expression of the AtPep1 precursor gene PROPEP1 in transgenic *Arabidopsis* plants causes a constitutive transcription of PDF1.2. The transgenic plants exhibited increased root development compared to control plants and an enhanced resistance toward the

root pathogen *Pythium irregulare*. Kanzaki et al. (*51*) overexpressed the wasabi (Japanese horseradish) defensin gene, a plant defensin effective against the rice blast fungus, in transgenic rice (*Oryza sativa* cv. Sasanishiki). Transformants exhibited resistance to rice blast caused by *Magnaporthe grisea* in T2 and T3 generations from which they were able to detect the 5-kDa peptide, corresponding to the processed form of the wasabi defensin in the total protein fraction extracted from the T3 progeny. Enhanced quantitative resistance to *Leptosphaeria maculans* (causal agent of blackleg disease) was observed by Kazan et al. (*52*) in canola (*Brassica napus* L.) by transgenic expression of a novel antimicrobial peptide MiAMP1, originally isolated from the seeds of *Macadamia integrifolia*. Ko et al. (*53*) transformed apple scion cultivar `Galaxy' and the apple rootstock M.26 with a cDNA clone of the gene encoding attacin gene (from *Cecropia* moth) to enhance resistance to *Erwinia amylovora*, the bacterium that causes fire blight. Although *in vitro* assays indicated that attacin was partially degraded in the intercellular fluid of apple leaves they observed enhanced resistance to fire blight. Constitutive over-expression of a seed specific hevein-like antimicrobial peptide from *Pharbitis nil* in transgenic tobacco plants enhanced resistance to the oomycete *Phytophthora parasitica*, the causal agent of black shank disease (*54*). Francois et al. (*55*) developed a method for expression in *Arabidopsis* of a transgene encoding a cleavable chimeric polyprotein. The polyprotein precursor consists of a leader peptide and two different antimicrobial proteins (AMPs), DmAMP1 originating from *Dahlia merckii* seeds and RsAFP2 originating from *Raphanus sativus* seeds, which are linked by an intervening sequence ("linker peptide") originating from a natural polyprotein occurring in seed of *Impatiens balsamina*. The chimeric polyprotein was found to be cleaved in transgenic *Arabidopsis* plants and the individual AMPS were secreted into the extracellular space. Both AMPS were found to exert antifungal activity *in vitro*. They also observed that the amount of AMPs produced in plants transformed with some of the poly-protein transgene constructs was significantly higher compared with the amount in plants transformed with a transgene encoding a single AMP, indicating that the polyprotein expression strategy may be a way to boost expression levels of small proteins. Recently, Swathi Anuradha et al. (*56*) expressed defensin gene from mustard in transgenic plants to obtain fungal resistance – *Fusarium moniliforme* and *Phytophthora parasitica* pv. *nicotianae* in transgenic tobacco and *Pheaoisariopsis personata* and *Cercospora arachidicoloa* which cause late leaf spot disease in peanut.

Peptides of non-plant origin

Several small MW peptides isolated from organisms other than plants have been shown to be effective antifungal agents (*57-60*). Some examples include the cecropins (*61*) and magainins (*14*) of insect and amphibian origins respectively, and their synthetic analogs (*18, 29, 48 ,62, 63*). The antimicrobial peptide, cecropin, from Cecropia moth has been introduced into several crops – tobacco (*11, 64*), potato (*65*), rice (*66*), apple (*67*) for the control of bacterial pathogens. Attacin gene from the same moth species has been incorporated into

apple (*16, 68*) and potato (*65*) for bacterial pathogen control. Li et al. (*62*) introduced a magainin analog into tobacco and demonstrated both fungal and bacterial control. As a possible approach to enhance plant resistance, a DNA coding for a modified esculentin-1, a 46-residue antimicrobial peptide present in skin secretions of *Rana esculent*a, was introduced into *Nicotiana tabacum* (*69*). The antimicrobial peptide was isolated from the intercellular fluids of healthy leaves of transgenic plants, suggesting that it was properly processed, secreted outside cells and accumulated in the intercellular spaces. The morphology of transgenic plants was unaffected. Challenging these plants with bacterial or fungal phytopathogens demonstrated enhanced resistance up to the second generation. Moreover, transgenic plants also displayed insecticidal properties.

Synthetic peptides

Certain small lytic peptides, redesigned from that of natural peptides, have demonstrated convincing inhibitory activity against fungal species including *Aspergillus flavus* (Figure 7) and show promise for transformation of plants to reduce infection of seed. In our laboratory, we (*29, 48*) reported that a synthetic lytic peptide (D4E1) gene, when transformed into tobacco, greatly enhances resistance *in planta* to *Colletotrichum destructivum*. The broad spectrum antifungal activity of the synthetic peptide D4E1 is given in Table 3 (*29*). Treatment of germinating *A. flavus* spores with tobacco leaf extracts from plants, transformed with the D4E1 gene, significantly reduced spore viability (colony forming units) relative to results obtained using extracts from non-transformed (control) plants. Similarly, in recent tests with cottonseed expressing the D4E1 gene, we demonstrated resistance to penetration of seed coats by a GFP reporter gene-containing *A. flavus* strain (*70, 71*). In addition to inhibiting the germination of *A. flavus* spores, D4E1 caused severe abnormal lytic effects on mycelial wall, cytoplasm, and nuclei. The expression of D4E1 gene in the progeny of transgenic cotton was sufficient enough to inhibit the growth *in vitro* of *Fusarium verticillioides* and *Verticillium dahliae* or *in planta* of *Thielaviopsis basicola* (*70*) and provide a good germination stand in a field infected with *Fusarium oxysporum* fsp. *vasinfectum* (*72*). Transformation of peanut with another antifungal peptide, D5C, has been reported (*73*). Although the pure D5C showed strong activity *in vitro* against *A. flavus*, it was shown that the transgenic peanut callus showed poor recovery of plants because of possible phytotoxicity of the peptide. Puterka et al. (*74*) observed that the biology and behavior of pear psylla, *Cacopsylla pyricola* Foerster, on a transgenic clone of 'Bartlett' pear, *Pyrus communis* L,, containing a synthetic antimicrobial gene, D5C1. The purpose of the original transformation was to enhance pear resistance to the bacterial disease fireblight caused by *Erwinia amylovora* (Burr.). During the conduct of the study, they observed that that the insect pest's biology and behavior were initially enhanced on transgenic pear clone. However, chronic exposure of psylla populations to transformed pear plants that express the *npt*II marker and lytic peptide genes had detrimental effects on pear psylla.

Mentag et al. (*75*) demonstrated bacterial disease resistance of transgenic hybrid poplar (*Populus tremula* L. x *Populus alba* L.) expressing the synthetic antimicrobial peptide D4E1. The transgenic poplar lines were tested for resistance to *Agrobacterium tumefaciens*, *Xanthomonas populi* pv. *populi* and *Hypoxylon mammatum* (Wahl.) Miller. One transgenic poplar line, Tr23, bearing the highest transcript accumulation for the D4E1 gene, showed a significant reduction in symptoms caused by *A. tumefaciens* and *X. populi*. However, none of the transgenic poplar lines showed a significant difference in disease response to the fungal pathogen *H. mammatum*.

Transgenic expression of a synthetic substitution analog of magainin, MSI-99 imparted disease resistance in both tobacco (*Nicotiana tabacum* L.) and banana [*Musa* spp. cv. Rasthali (AAB)]. Transgenic tobacco showed enhanced resistance to *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Botrytis cinerea* where as transgenic banana plants showed resistance to *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola* (*63)*. Alan et al (*76*) transformed tomato with MSI-99 and they observed that transgenic tomato plants were more inhibitory against a bacterial pathogen *Pseudomonas syringae* pv. *tomato* (bacterial speck pathogen) than against the fungal pathogens - *Alternaria solani* (early blight) and the oomycete pathogen *Phytophthora infestans* (late blight) possibly due to proteolytic degradation and lower expression of the peptide.

Figure 7. D4E1 effects on Aspergillus flavus spores. Transmission electron micrographs of pre-germinated A. flavus spores exposed to the antifungal peptide D4E1. A) Control in potato dextrose broth; B) Spores exposed to 10 μM D4E1 for 1h. C) Note cytoplasmic degradation due to exposure to D4E1 leading to eventual lysis at 25 μM D4E1 for 1h (K. Rajasekaran, unpublished).

Table 3. Broad spectrum antimicrobial activity of D4E1 in vitro

Phytopathogen	IC_{50} (μM)	MIC (μM)
Alternaria alternata	12.39	>25.0
Aspergillus flavus	7.75	25.0
Aspergillus flavus 70-GFP	11.01	25.0
Cercospora kikuchii	8.67	>25.0
Colletotrichum destructivum	13.02	>25.0
Claviceps purpurea	1.60	20.0
Fusarium graminearum	2.10	25.0
Fusarium moniliforme	0.88	12.5
Fusarium oxysporum	2.05	12.5
Penicillium italicum	5.92	>25.0
Phytophthora cinnamomi	nd	4.67
Phytophthora parasitica	nd	4.67
Pseudomonas syringae pv. *tabaci*	0.52	2.25
Pythium ultimum	nd	13.33
Rhizoctonia solani	nd	26.7
Thielaviopsis basicola	0.52	6.0
Verticillium dahliae	0.60	5.25
Xanthomonas campestris pv. *malvacearum*	0.19	1.25

nd = not determined ; Source: reproduced from (*29*)

In addition to nuclear transformation, we are in the process of exploring the possibility of expressing antifungal genes in plastids with the objective of higher expression and preventing transgene escape through pollen, as indicated in the previous section. For example, the antimicrobial peptide MSI-99, an analog of magainin 2, was expressed via the chloroplast genome (*18*) of tobacco. Leaf extracts from T2 generation plants showed 96% inhibition of growth against the bacterial pathogen *P. syringae* pv. *tabaci*. In addition, leaf extracts from T1 generation plants inhibited the growth of pregerminated spores of three fungal species, *A. flavus*, *Fusarium verticillioides*, and *Verticillium dahliae*, by more than 95%, compared with non-transformed control plant extracts. *In planta* assays with the bacterial pathogen *P. syringae* pv *tabaci* resulted in areas of necrosis around the point of inoculation in control leaves, whereas transformed leaves showed no signs of necrosis, demonstrating high-dose release of the peptide at the site of infection by chloroplast lysis. *In planta* assays with the fungal pathogen, *Colletotrichum destructivum*, showed necrotic anthracnose lesions in non-transformed control leaves, whereas transformed leaves showed no lesions. In addition to lytic peptide genes, a variety of other candidate antifungal genes from bacterial, plant, and mammalian sources have a good probability of being active against *A. flavus* upon transformation into plants.

Potential Problems Associated with Antimicrobial Peptides

In general, the majority of antimicrobial peptides function primarily by compromising the membrane of the target organism (lytic activity) though some

also appear to function at the DNA or protein level [reviewed in Marcos et al. (*7*). With respect to their use in agriculture, many natural antimicrobial peptides have undesirable properties such a nontarget toxicity, poor activity, and susceptibility to protease degradation. Generation of hybrid peptides, rational design of peptide analogs, and synthetic peptide combinatorial chemistry have been used in an effort to increase antimicrobial peptide activity and stability while diminishing nontarget toxicity (*7, 77, 78*). Hybrid peptides and their analogs such as cecropin::mellitin have demonstrated resistance to bacterial and fungal pathogens in transgenic potato (*77, 79*) while cecropin::magainin analogs displayed increased bactericidal/tumoricidal activity without inducing hemolysis (*80*). Rational design of substitution analogs of the naturally occurring cecropin-B peptide identified, Shiva-1, an analog that retained only 46% homology to cecropin-B that conferred enhanced resistance to bacterial wilt in tobacco (*11*). Our studies on the synthetic peptide D4E1 have shown that it was more resistant to plant and fungal protease degradation than the natural peptide, cecropin-A (*24*). Additionally, introduction of a gene encoding an antimicrobial peptide into the plastid genome and its subsequent production and compartmentalization within the plastid improved antimicrobial activity while reducing proteolytic degradation (*18*). López-Garcia et al. (*81*) screened a synthetic peptide combinatorial library in a positional scanning format to identify improved versions of the hexapeptide PAF19. They identified a number of bioactive peptides with improved activity against a group of fungal phytopathogens responsible for postharvest decay in fruits yet these did not demonstrate increased, nontarget toxicity to *Escherichia coli* and *Saccharomyces cerevisiae*. In some cases substitution of just one amino acid residue could result in loss of activity against a particular fungal pathogen. Combinatorial chemistry was also used to increase antibacterial activity while maintaining low cytotoxicity of cyclic decapeptides (*82*).

Conclusion

The importance of peptides, either natural or synthetic, in control of microbial pathogens is well established from the steady stream of publications in the last decade or so. Modern technology in automated peptide synthesis and combinatorial chemistry have made the task of designing novel, environmentally benign, yet target pathogen(s)-specific potent peptides more attainable than before. In addition, availability of transgenic technology in several crops via both nuclear and/or organelle transformation provides much needed expertise in facilitating a rapid development of disease-resistant, commercially-useful germplasm or varieties of food and feed crops. In addition, identification of potent antifungal peptides from different sources will be valuable in transferring them to susceptible crops to combat fungal infection resulting in preharvest mycotoxin contamination compromising food and feed safety. More importantly, the combination of technologies presented in this review provides a means of enhancing the host-plant resistance of susceptible crop species in a relatively short time frame compared to conventional breeding.

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Figure 9.1. Amino acids in lytic peptides. The symbols represent a molecular alphabet

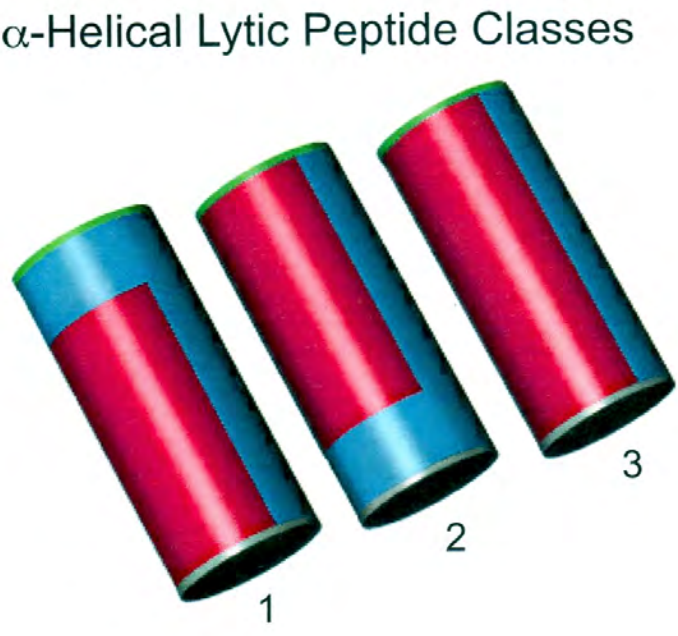

Figure 9.2. α-helical lytic peptide classes

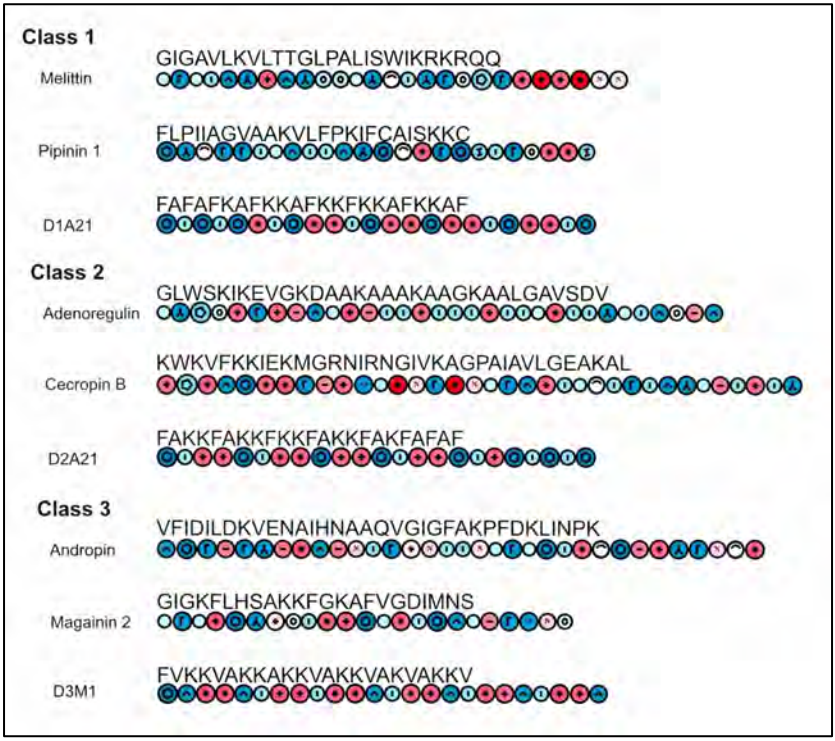

Figure 9.3. Natural lytic peptides.

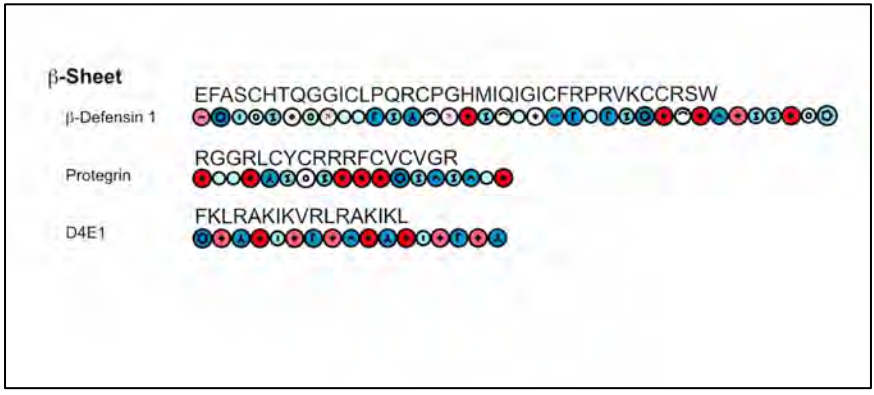

Figure 9.4. β-sheet peptides

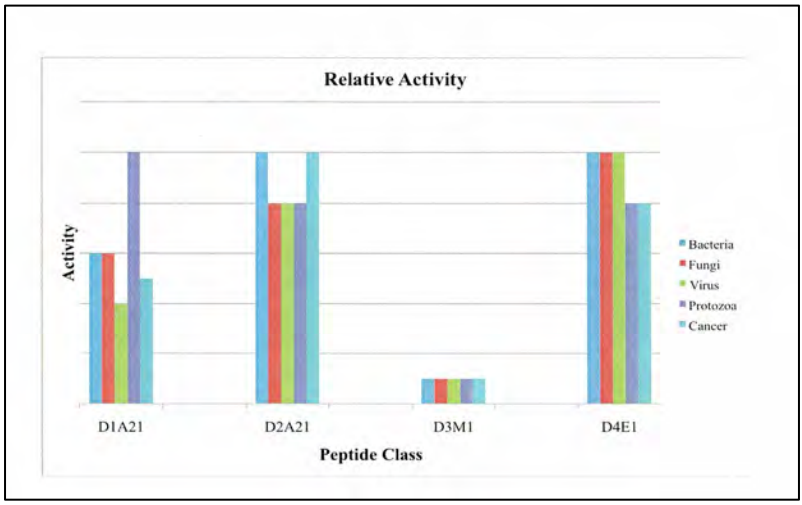

Figure 9.5. Relative Activity of selected synthetic lytic peptides (Jaynes, unpublished)