Journal of Experimental Botany, Vol. 69, No. 21 pp. 4997–5011, 2018
doi:10.1093/jxb/ery294 Advance Access publication 7 August 2018

REVIEW

The role of antimicrobial peptides in plant immunity

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Received 9 May 2018; Editorial decision 31 July 2018; Accepted 31 July 2018

Editor: Katherine Denby, York University, UK

Abstract

Selective pressure imposed by millions of years of relentless biological attack has led to the development of an extraordinary array of defense strategies in plants. Among these, antimicrobial peptides (AMPs) stand out as one of the most prominent components of the plant immune system. These small and usually basic peptides are deployed as a generalist defense strategy that grants direct and durable resistance against biotic stress. Even though their name implies a function against microbes, the range of plant-associated organisms affected by these peptides is much broader. In this review, we highlight the advances in our understanding on the role of AMPs in plant immunity. We demonstrate that the capacity of plant AMPs to act against a large spectrum of enemies relies on their diverse mechanism of action and remarkable structural stability. The efficacy of AMPs as a defense strategy is evidenced by their widespread occurrence in the plant kingdom, an astonishing heterogeneity in host peptide composition, and the extent to which plant enemies have evolved effective counter-measures to evade AMP action. Plant AMPs are becoming an important topic of research due to their significance in allowing plants to thrive and for their enormous potential in agronomical and pharmaceutical fields.

Keywords: AMPs, plant defense, plant immune system, plant defense responses, pest, plant–pathogen interaction.

Introduction

In their natural ecosystems, plants co-exist with a wide variety of micro-organisms and pests. In order to survive, plants have evolved sophisticated mechanisms that allow them to mount an effective defense response against harmful agents such as bacteria, fungi, nematodes, insects, and large herbivores. Among these mechanisms are physical barriers such as waxy cuticular layers and trichomes capable of deterring initial agent infection (Glas *et al.*, 2012; Malinovsky *et al.*, 2014), intricate cell surveillance systems that recognize specific foreign threats (Spoel and Dong, 2012; Gust *et al.*, 2017), a complex network of plant hormones that interact to trigger the most advantageous defense responses (Pieterse *et al.*, 2012; Campos *et al.*, 2014), a myriad of transcriptional pathways that are wired to finely tune plant development in response to attack (Tsuda and Somssich, 2015; Chae *et al.*, 2016; Birkenbihl *et al.*, 2017), and a cocktail of diverse proteins and secondary metabolites capable of providing a toxic barrier to the threat (Howe and Jander, 2008). Together, these mechanisms compose the defensive layers that are crucial for plant survival, the so-called 'plant immune system' (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Spoel and Dong, 2012; Dangl *et al.*, 2013; Campos *et al.*, 2014).

Research on the plant immune system and plant immunity has extensively focused on the molecular mechanisms involved with microbial pathogen recognition and activation of proper defense responses. Insights from decades of plant–pathogen interaction studies have demonstrated that the initial alert for the presence of an intruding organism and rapid activation of basal resistance is mediated by plant transmembrane pattern-recognition receptors (PRRs) that are capable of detecting slowly evolving microbial-associated molecular patterns (MAMPs) such as fragments of the bacteria cell wall or flagellum, components of the fungi cell surface, and secreted growth factors (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Boller and He, 2009; Dangl *et al.*, 2013; Wang *et al.*, 2014). To circumvent this MAMP-triggered immunity, pathogenic microbes produce polymorphic effector proteins that can be secreted at the pathogen–plant cell interface or directly injected inside the plant cell through needle-like protein complexes (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Dangl *et al.*, 2013; Liu *et al.*, 2013; Wang *et al.*, 2014). Those effectors promote virulence by mimicking or inhibiting plant cellular functions. To counteract this effector-triggered susceptibility, plants employ disease resistance (R) proteins that specifically recognize microbial effectors and activate more robust defense responses such as hypersensitive cell death at the site of infection (Jones and Dangl, 2006; Boller and He, 2009; Spoel and Dong, 2012; Dangl *et al.*, 2013). As in an evolutionary arms race, natural selection drives pathogens and plants to constantly develop new effector or R proteins to promote effector-triggered susceptibility or effector-triggered immunity, respectively. This model of plant immunity has been constantly extended to include specific herbivore and damage-derived danger signals that are also recognized by associated PRRs, demonstrating that the mechanism for how plants perceive and mount defenses against complex attackers is remarkably similar to those observed for microbial organisms (Felton and Tumlinson, 2008; Howe and Jander, 2008; Mousavi *et al.*, 2013; Campos *et al.*, 2014). Additionally, it is now becoming evident that, upon attack, conserved signaling components of the plant immune system interact to finely tune its activity and the fitness cost of unnecessary defense responses (Hatsugai *et al.*, 2017; Nobori *et al.*, 2018).

While there is a wealth of knowledge on recognition of attackers and the early steps in the activation of the plant defense responses, a less understood part of the plant immune system involves the action of more generalist host defense strategies that are used to provide direct and durable resistance against a large spectrum of pests and pathogens. These chemical and morphological defense traits are characterized by a high level of heteromorphism among plant species and heterogeneous mechanisms of action (Table 1). One of the most prominent generalist chemical barriers employed to fend off infective agents is that provided by antimicrobial peptides (AMPs). These are distinguished by their overall basic nature and small size (up to 100 amino acid residues). Most plant AMPs present an 'amphipathic design', a conformation where the hydrophobic and cationic amino acids are clustered into distinct segments of the peptide (Jenssen *et al*, 2006; Fjell *et al.*, 2012; Fox, 2013; Wang *et al.*, 2016). Interestingly, some AMPs adopt this amphipathic conformation only when interacting with their targets (Zasloff *et al.*, 2002). Moreover, AMPs can be derived from single gene-encoded precursor molecules (the pre-peptide), from inactive precursor proteins (zymogens), or from the internal sections of mature proteins (encrypted AMPs), all of which are cleaved and frequently post-translationally modified to generate the mature peptide (Brogden *et al.*, 1997; Tailor *et al.*, 1997; Silverstein *et al.*, 2005; Toke, 2005; Utkina *et al.*, 2013; Tam *et al.*, 2015; Ramada *et al.*, 2017).

The ever-increasing number of AMPs isolated from plants, the wide range of plant attackers whose development is influenced by these peptides, the novel findings on their diverse mechanisms of actions, and the recent observation that plant signaling peptides may have evolved from ancient AMPs all provide an impetus to consider immunity from the perspective of these molecules (Lay and Anderson, 2005; Gruber *et al.*, 2008; Pelegrini *et al.*, 2011; Tavormina *et al.*, 2015; Bircheneder and Dresselhaus, 2016; Bolouri Moghaddam *et al.*, 2016; Wang *et al.*, 2016; Ageitos *et al.*, 2017). In this review, we focus on the advances in our understanding of the role of AMPs in plant immunity. We highlight evidence to support the proposal that these chemical shields compose an essential and constantly evolving branch of the plant immune system. We show that AMP efficiency as a defense barrier is achieved by an astonishing heterogeneity in host peptide composition and a vast diversity in mechanisms of action, allowing plants to utilize these molecules as weapons to combat a broad spectrum of pests and pathogens. Readers are also referred to a wealth of excellent review articles focusing on the structural properties of plant AMPs as well as the signaling hubs involved with plant immunity (Tossi and Sandri, 2002; Sels *et al.*, 2008; Howe and Jander, 2008; Desai *et al.*, 2010; Maróti *et al.*, 2011; Pelegrini *et al.*, 2011; Dangl *et al.*, 2013; Viana *et al.*, 2013; Campos *et al.*, 2014; Kim *et al.*, 2014; Conrath *et al.*, 2015; Tam *et al.*, 2015; Bolouri Moghaddam *et al.*, 2016; Ageitos *et al.*, 2017; Ramirez-Prado *et al.*, 2018).

AMPs comprise a fundamental section of the plant immune system

A number of features underscore the role of AMPs in plant defense against pest and pathogen attack, further emphasizing how these peptides comprise a distinct and elementary branch of the plant immune system. First, spatiotemporal analysis of AMP gene expression demonstrates that some are constitutively found in all plant organs, whereas others are detected only in a condition- and/or tissue-specific manner (Broekaert *et al.*, 1997; Berrocal-Lobo *et al.*, 2002*b*; Silverstein *et al.*, 2007; Pelegrini *et al.*, 2011; Tam *et al.*, 2015). This heterogeneous pattern of expression indicates that while some AMPs are immediately available at any site of infection, other are deployed only upon attack to deter organ-specific invaders. The observation that these different expression mechanisms operate alongside one another is probably a strategy to maximize resistance against constantly evolving harmful agents. This idea is further corroborated by the observation that AMP-mediated defense is not achieved by the presence of a unique AMP in the damaged organ but rather by a complex cocktail of peptides with different expression patterns and action mechanisms (Zasloff, 2002; Spelbrink *et al.*, 2004; Barbeta *et al.*, 2008; Poth *et al.*, 2011).

Table 1. Overview of the main classes of plant antimicrobial peptides

Class name	Structural hallmark	Size and mass	Mode of action	References
α/β-thionins	Two antiparallel α-helices and one antiparallel double-stranded β-sheet. Three to four disulfide bonds.	45–48 aa, ~5 kDa	Interaction with membrane lipids followed by increase in cell membrane permeability and lysis.	Thevissen *et al.*, 1996; Stec *et al.*, 2004; Stec, 2006; Tam *et al.*, 2015.
Defensins (γ-thionins)	One α-helix and three antiparallel β-sheets. Four to five disulfide bonds.	45–54 aa, ~5–7 kDa	Interaction with specific membrane components to trigger intracellular signaling cascades that hinder pathogen growth. Can also inhibit the action of insect digestive proteins.	Pelegrini and Franco, 2005; Pelegrini *et al.*, 2008*a*; Lacerda *et al.*, 2014.
Heveins	One antiparallel β-sheet and sporadic short α-helices. Three to five disulfide bonds.	30–45 aa, ~5 kDa	Inhibit bacterial and fungal growth through interaction with the machinery involved with microbial cell wall biosynthesis and pathogenicity. Also promote defense against large mammals by working as allergens.	Koo *et al.*, 1998; Blanco, 2003; Odintsova *et al.*, 2009; Porto *et al.*, 2012; Slavokhotova *et al.*, 2014.
Knottins	Three antiparallel β-sheets connected by hypervariable loops. Three disulfide bonds forming a conserved 'knotted' structure.	28–37 aa, ~4 kDa	Bind to various molecular targets including microbial membrane and intracellular components. Also work as α-amylase or protease inhibitors.	Hwang *et al.*, 2010; Cândido *et al.*, 2014; Nguyen *et al.*, 2014.
Cyclotides	Characterized by the same 'knotted' arrangement found in knottins but with the N- and C-terminals covalently joined by a peptide bond to form a circular structure.	28–37 aa, ~4 kDa	Can disrupt the biological membranes of specific pathogens, interact with specific membrane lipids to internalize into the target cells to modify the activity of internal cellular components and alter the physiological properties of arthropod digestive systems.	Gruber *et al.*, 2008; Burman *et al.*, 2015; Weidmann and Craik, 2016; Craik and Du, 2017.
Lipid transfer proteins	Four α-helices linked by flexible loops held in a compact fold by four disulfide bonds. A large and internal tunnel-like cavity along the axis of the molecule forms a lipid-binding site.	70–90 aa, ~9–10 kDa	Possibly interact with microbial membranes to 'cage' their lipid molecules into the peptide lipid-binding site. Such interactions would lead to loss of membrane integrity and increase membrane permeabilization.	Maldonado *et al.*, 2002; Carvalho and Gomes, 2007; Yeats and Rose, 2008; Conrath *et al.*, 2015; Safi *et al.*, 2015.
Snakins	Helix–turn–helix domain and a short helical region located between two large loops, which are held in place by three disulfide bonds.	60–70 aa, ~7 kDa	Mechanism of action remains to be elucidated. Capacity to disrupt microbial membranes is ruled out due to their inability to interact with artificial lipid membranes.	Porto and Franco, 2013; Yeung *et al.*, 2016; Oliveira-Lima *et al.*, 2017.
α-harpinins	Helical hairpin structure where both α-helices are oriented antiparallel and connected by two disulfide bonds.	31–50 aa, ~4–5 kDa	Mechanism of action remains to be elucidated. Present antimicrobial and trypsin-inhibitory activity.	Nolde *et al.*, 2011; Rogozhin *et al.*, 2012; Tam *et al.*, 2015.
2S albumins	Five α-helices arranged in a right-handed superhelix. Three to four disulfide bonds.	Up to 100 aa, ~3–10 kDa	Mechanism of action remains to be elucidated. Present antimicrobial and allergenic activity.	Pantoja-Uceda *et al.*, 2004, Maria-Neto *et al.*, 2011.
Short non-disulfide rich peptides/ Glycine-rich proteins	Few or no cysteine residues. May present a high percentage of glycines in their primary sequence. Structure varies from simple random coils to complex peptides with more than 10 helices.	7–50 aa, <7 kDa	Interact with multiple targets such as the microbial cell surface, internal cell structures, and the nuclei to modulate the metabolism of pathogens.	Pelegrini *et al.*, 2008*b*; Tavares *et al.*, 2012; Zottich *et al.*, 2013; Cândido *et al.*, 2014; Santana *et al.*, 2015.

A fundamental feature of the vertebrate immune system involves responses that are capable of adjusting to the attacking organisms. This adaptive immune system relies on somatic cells that employ antigen receptors not encoded in the germ line but generated *de novo* in each individual upon contact with the pathogen (Iwasaki and Medzhitov, 2010). Even though plants lack this type of somatic adaptive defense—a major difference between plant and animal immune systems—their immune system does show a form of 'adaptation to attack' as many morphological and chemical defense shields can be raised (i.e. have their production increased) when the plant is challenged. The majority of AMP genes show this type of adaptive response, given that their expression is quickly up-regulated upon microbial or herbivore attack (Lee *et al.*, 2000; Berrocal-Lobo *et al.*, 2002*b*; Lay and Anderson, 2005; Jenssen *et al.*, 2006; Utkina *et al.*, 2013; Chapman *et al.*, 2016; Herbel

et al., 2017). The effectiveness of this strategy is observed in transgenic plants where the overexpression of AMP genes is associated with enhanced tolerance to pathogen attack (Almasia et al., 2008; Maróti et al., 2011; Mohan et al., 2014; Ji et al., 2015).

The plant immune system is usually described as a set of danger-recognition systems in which an input signal—the stressful condition—is recognized and translated by a conserved core signaling module to activate the appropriate defense outputs (Fig. 1) (Campos et al., 2014; Bolouri Moghaddam et al., 2016). According to this model, danger signals generated by biotic stressors are initially perceived by PRRs located at the plant cell surface. The relevance of this initial step of recognition for plant defense is evidenced by the vast diversity of danger signals whose cognate plant receptors have already been identified and are currently under study (Mousavi et al., 2013; Campos et al., 2014; Choi et al., 2014; Kim et al., 2014; Saijo et al., 2018; Wang et al., 2018). PRRs are coupled to a network of signaling cascades whose activation converts the danger signals into the most suitable defense responses. A characteristic of this core signaling module is the convergent utilization of ubiquitously occurring cellular messengers, such as reactive oxygen species (ROS), calcium sensors, nitric oxide (NO), mitogen-activated protein kinases (MAPKs), electric signals, and plant hormones, to orchestrate large-scale transcriptional reprogramming that ultimately leads to the production of a wide array of defense traits (Pedley and Martin, 2005; Kim et al., 2014; Tsuda and Somssich, 2015; Bolouri Moghaddam et al., 2016; Gilroy et al., 2016). Recent research has indicated that AMP genes are integrated within this immunity-signaling network, as their expression appears to be an output governed by the same core-signaling module responsible for the control of several other plant defense responses (Fig. 1). For example, plant hormones that act as central regulators of plant immune responses, such as jasmonic acid, ethylene, and salicylic acid, are frequently described as potent up-regulators of AMP gene expression in numerous plant species (Lee et al., 2000; Kiba et al., 2003; Nahirñak et al., 2012; Tesfaye et al., 2013; Bolouri Moghaddam et al., 2016; Herbel et al., 2017). These hormones are known modulators of transcription factors whose activity is essential for AMP responses upon pathogen attack (Berrocal-Lobo et al., 2002a; Hiruma et al., 2011). Indeed, the correlation between defense hormones and AMP induction is so evident that some AMP genes, such as *Thi2.1* and *PDF1.2*, are established as important marker genes to study the activation of plant hormonal and immune system signaling pathways (Zander et al., 2010).

Plant AMPs also interact with ROS and MAPK signaling cascades to regulate defense responses, although in a fashion that is still poorly understood (Bolouri Moghaddam et al., 2016). For example, microbial pathogen recognition by plant PRRs activates multiple MAPK signaling cascades that culminate in up-regulation of AMP genes (Asai et al., 2002; Meng et al., 2013). At the molecular level, it has been demonstrated that MAPKs are responsible for activation of transcription factors involved with the expression of plant AMPs (Meng et al., 2013). In agreement with this observation, MAPK-knockout mutant plants show significant reductions in the expression patterns of specific AMP genes, even after treatment with potent AMP elicitors such as jasmonic acid, which may lead to increased susceptibility to pathogen infection (Petersen et al., 2000; Meng et al., 2013; Bolouri Moghaddam et al., 2016). Furthermore, components of a ROS-activated MAPK signaling cascade can physically interact with AMPs as a potential strategy to regulate plant defense processes (Damon et al., 2012; Bolouri Moghaddam et al., 2016). Alternatively, plant AMPs can also work as modulators of the cellular redox status,

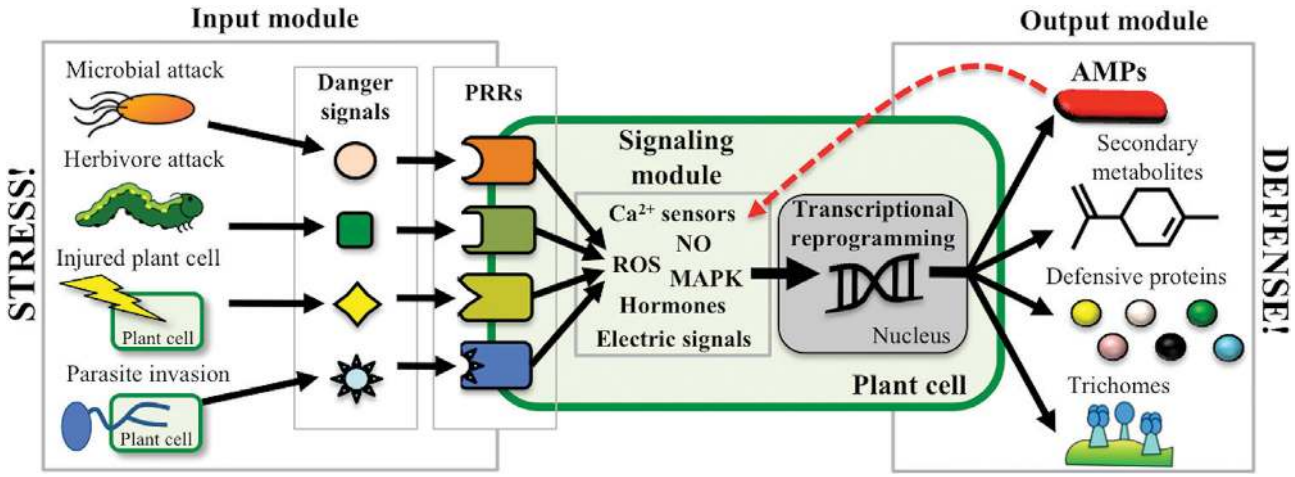

Fig. 1. Antimicrobial peptide (AMP) genes are integrated into the plant immune system signaling cascade. Stressful conditions induced by biotic attack engender danger signals that are perceived by specific pattern-recognition receptors (PRRs) located at the plant cell surface. PRRs are coupled to a conserved signaling module that utilizes ubiquitously occurring cellular messengers (such as ROS, MAPK, Ca^{2+} sensors, nitric oxide, electric signals, and plant hormones) to translate the danger input signals into a large-scale transcriptional reprogramming that ultimately leads to the production of the most appropriate defense responses. AMP genes are integrated within this immunity-signaling network, as their expression appears to be governed by the same signaling cascade responsible for the control of other plant defense responses. Interestingly, AMPs can also function as signaling molecules that modulate the action of some components of the immunity-signaling module (dashed red line and arrow), suggesting that some of these peptides may play a fundamental role as components of feedback loops that regulate the duration and intensity of a plant defense response. Abbreviations: NO, nitric oxide; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase.

whether by converting plant-generated ROS to other less reactive compounds or, in an opposite fashion, by inducing the accumulation of ROS (and NO) in microbial cells as a strategy to trigger apoptosis and eliminate the threat (Aerts *et al.*, 2007; Huang *et al.*, 2008; van der Weerden *et al.*, 2008; Mello *et al.*, 2011). Surprisingly, it has been reported that, besides their role as an antimicrobial shield, some AMPs can also act as antioxidant enzymes, thus being directly involved in the control of redox status (Huang *et al.*, 2008). However, the mechanisms utilized by AMPs to modulate ROS are still poorly understood. Taken together, these findings indicate that the same signaling cascade utilized by plants to orchestrate the immune responses to pest and pathogen attack governs the expression and activity of AMPs. Interestingly, recent findings that plant AMPs can further regulate the activity of the aforementioned cellular messengers involved with the immune system signaling module (reviewed by Bolouri Moghaddam *et al.*, 2016)—for example, by activating MAPK and ROS signaling cascades—suggest that these peptides may play a role as components of feedback loops that regulate the duration and intensity of a plant defense response (Fig. 1).

The efficacy of a specific immune response is reflected in the extent to which pathogens and pests have evolved effective counter-measures that allow them to evade that particular defensive barrier. Given the effectiveness of plant AMPs as chemical shields, it is not surprising that many plant-harming agents have developed inducible mechanisms to evade interaction with plant AMPs. For example, as we discuss later, the antibacterial action of AMPs is highly dependent on electrostatic interactions between the plant AMP (positively charged) and the outermost layer of the bacterial cells (negatively charged). Upon contact with the plant peptides, some Gram-positive and Gram-negative bacteria can activate the expression of specific regulons whose gene products are involved in the process of remodeling cell wall lipopolysaccharides and membrane lipids (Rio-Alvarez *et al.*, 2012; Pandin *et al.*, 2016). Such modifications on their outermost layers alter the bacterial surface charge in order to avoid AMP interactions, thus leading to resistance to the peptide and promotion of pathogenesis (Gunn, 2008; Rio-Alvarez *et al.*, 2012; Pandin *et al.*, 2016). Modification of the electrostatic environment is actually a recurring strategy of AMP resistance in bacteria, as it has been demonstrated that some of these micro-organisms can secrete cationic exopolysaccharides that cause charge repulsion of AMPs or anionic exopolymers that sequester and aggregate the peptides away from the bacteria (Otto, 2006). Eukaryotic organisms have also evolved mechanisms to avoid or detoxify plant AMPs upon interaction. For example, plant AMPs are capable of inhibiting the action of digestive enzymes present in the gastrointestinal tract of herbivores in order to lower nutritional gain from herbivory (see section below). Insects combine multiple strategies to overcome this mechanism of action: they can overproduce the existing digestive enzymes, increase the expression of inhibitor-insensitive protease isoforms, and even activate the production of enzymes that hydrolyse and disarm plant inhibitors (Zhu-Salzman and Zeng, 2015). A more indirect counter-measure utilized by many pathogens and arthropods to avoid AMPs (and also other plant defense responses) involves the hijacking and modulation of plant hormonal pathways involved with the activation of the immune system (Thatcher *et al.*, 2009; Rahman *et al.*, 2012; Campos *et al.*, 2014; Zhang *et al.*, 2017). This mechanism relies on the observation that the regulation of plant AMP genes is largely dependent on plant hormones, as already discussed (Fig. 1).

Finally, from a holistic perspective, the relevance of AMPs for immunity is highlighted by their ubiquitous presence not only in land plants but throughout all kingdoms of life (Tossi and Sandri, 2002; Zasloff, 2002; Jenssen *et al.*, 2006; Wang *et al.*, 2016). This widespread occurrence indicates that these peptides are ancient weapons of defense that appeared early in the history of life and still play a fundamental role in the battle against pest and pathogen attack.

AMPs are ancient, widespread, and dynamically evolving weapons of defense

Millions of years of constant interactions with harmful organisms have led to the evolution of an astonishing collection of defense strategies in plants. Among these, AMPs excel as one of the most efficient and prevalent chemical weapons utilized to provide resistance against pest and pathogen attack (Perron *et al.*, 2006; Peschel and Sahl, 2006). This view is based on the widespread incidence of AMPs in plant genomes. In *Arabidopsis thaliana*, rice, and alfalfa, for example, it is estimated that AMP-coding genes comprise up to 3% of the whole gene repertoire (Mergaert *et al.*, 2003; Silverstein *et al.*, 2005, 2007). Indeed, the diversity of AMPs discovered in plants is so striking that it is difficult to categorize them except on the basis of their tridimensional structure: Table 1 provides a broad overview of the main classes of plant AMPs (Broekaert *et al.*, 1997; Cândido *et al.*, 2014; Nawrot *et al.*, 2014; Tam *et al.*, 2015; Goyal and Mattoo, 2016). This diversity becomes more impressive when it is observed that many AMP genes are taxon-specific, appearing only in particular botanical families or groups (Silverstein *et al.*, 2005, 2007; Gruber *et al.*, 2008). Comprehensive information on hundreds of AMPs identified from several plant families can be found in antimicrobial peptide databases such as APD (http://aps.unmc.edu/AP/) and PhytAMP (http://phytamp.hammamilab.org/main.php), whilst recent use of computational prediction tools points to a tremendous increase in this number in the near future (Silverstein *et al.*, 2005, 2007; Hammami *et al.*, 2009; Niarchou *et al.*, 2013; Tam *et al.*, 2015; Wang *et al.*, 2016; Porto *et al.*, 2017).

The ubiquitous occurrence of these AMPs not only in plant species but also throughout all kingdoms of life is strong evidence that these peptides are ancient weapons of defense (Tossi and Sandri, 2002; Zasloff, 2002; Brogden, 2005; Toke, 2005; Jenssen *et al.*, 2006; Perron *et al.*, 2006; Peschel and Sahl, 2006; Wang *et al.*, 2016). Indeed, it is reasonable to speculate that the origin of these AMPs precedes the transition of plants from water to land. Despite their ancient lineage, AMP genes have evolved in a particular manner in plants, possibly as a consequence of the unique evolutionary pressures experienced by these organisms and distinctive dynamics in their genome evolution (e.g. high tolerance of changes in

chromosome number). For example, it has been demonstrated that sequence-related subgroups of AMP genes are clustered in specific regions of the genome of plants as an outcome of successive rounds of local duplications (Silverstein *et al.*, 2005, 2007). These studies have also demonstrated that AMP mature sequences, secondary structures, and sizes are usually hypervariable whereas there is strong conservation in the sequence of the AMP signal peptide, the intron position, and the cysteine motifs.

Plant AMPs are characterized by an unusually high content of cysteine residues (Zasloff, 2002; Silverstein *et al.*, 2005, 2007; Hammami *et al.*, 2009; Poth *et al.*, 2011; Maróti *et al.*, 2015; Tam *et al.*, 2015). These cysteine motifs are conserved among AMP classes, allowing the formation of an unusual and highly stabilized topology that confers high thermal, chemical, and enzymatic stability to the peptides (Colgrave and Craik, 2004; Wang *et al.*, 2009; Tam *et al.*, 2015). As we discuss below, this structural rigidity provided by the multiple disulfide bonds (see Table 1) is crucial for plant AMPs to act as a defensive shield. Interestingly, the high content of cysteine residues may also explain the enormous diversity of AMP genes in plants. In a genome-wide analysis performed in different species, Silverstein *et al.* (2007) demonstrated that distinct classes of plant AMPs are subjected to frequent internal duplications and rearrangements of their cysteine motifs. Such events would permit a peptide to accept different types of beneficial mutations while still folding to its native structure (Bloom *et al.*, 2006), thus allowing a recurrent emergence and maintenance of new AMPs in plant genomes. This theory can be extended to a more comprehensive perspective, as it is now becoming clear that the frequent appearance of plant peptides with novel and non-defense related functions may be a consequence of gene-duplication and neo-functionalization events that occurred in polymorphic AMP ancestors. In fact, it has been demonstrated that signaling peptides involved with plant development, reproduction, metal tolerance, and even communication with symbiotic bacteria evolved from ancient plant AMPs (Silverstein *et al.*, 2005; Stotz *et al.*, 2009; Van de Velde *et al.*, 2010; Maróti *et al.*, 2011, 2015; Marshall *et al.*, 2011; Bircheneder and Dresselhaus, 2016; Arnold *et al.*, 2017; Parisi *et al.*, 2018). For example, in the Brassicaceae and Poaceae, self-incompatibility responses between the male and female reproductive organs are mediated by peptides of the defensin family, which utilize a signaling cascade to arrest pollen tube growth that is remarkably similar to the one utilized by other AMPs to halt the growth of fungal hyphae (Takayama *et al.*, 2001; Amien *et al.*, 2010; Marshall *et al.*, 2011; Bircheneder and Dresselhaus, 2016). In *Medicago truncatula*, nodule-specific defensin-like peptides are able to control the differentiation of bacterial endosymbionts into nitrogen-fixing bacteroides while still retaining some antimicrobial activity (Stotz *et al.*, 2009; Tiricz *et al.*, 2013; Maróti *et al.*, 2015). The observation that some plant AMPs still retain a direct defense role but are also able to interact with plant transcription factors whose activity is associated with both defense and non-defense-related responses may represent an intermediary step in the evolutionary transition between a defense-related and a development-related peptide (Damon *et al.*, 2012).

Plant AMPs confer resistance to a large spectrum of plant attackers

Classical studies on the plant immune system often rely on attacker challenge assays and phenotypic characterization of loss-of-function mutants. Similar experiments performed in single-AMP knockout or knockdown plants fail to detect any altered phenotype, even after pest or pathogen attack (Stotz *et al.*, 2009; De Coninck *et al.*, 2010). These observations suggest a high degree of functional redundancy among AMP genes and further support the idea that plants utilize a complex cocktail of peptides to optimize defense (Zasloff, 2002; Spelbrink *et al.*, 2004; Barbeta *et al.*, 2008; Poth *et al.*, 2011). For this reason, much of the knowledge about the protective effects of AMPs comes through homologous and heterologous overexpression of single genes and/or purification of the peptide and evaluation of its activity *in vitro* (Spelbrink *et al.*, 2004; de Zélicourt *et al.*, 2007; Ji *et al.*, 2015). These studies have demonstrated the crucial role of AMPs in plant immunity: even though the fundamental principle of AMPs is to present activity against microbial pathogens, the number of plant-associated organisms whose development is affected by these peptides is much broader than that (Table 2). Among them are Gram-positive and Gram-negative bacteria, phytopathogenic fungi/oomycetes with different lifestyles (e.g. the necrotrophic *Rhizoctonia solani* and the hemibiotroph *Phytophthora infestans*), nematodes, mollusks, piercing-sucking insects (aphids), leaf-chewing insects, and even the parasitic plant *Orobranche cumana*. In fact, the anti-infective action of AMPs is so broad that some authors favor the term 'host-defense peptides' when discussing the role of those molecules in immune systems (Mayer *et al.*, 2010).

The chemical barrier: mechanisms of action of plant AMPs

The ability of plant AMPs to function as a chemical barrier that grants resistance against a large spectrum of attackers is based on two fundamental principles: (1) their remarkable structural stability, and (2) the diversity in their mechanisms of action (Table 1). The compact structure and the prevalence of disulfide bonds allow plant AMPs to maintain their conformation and activity even in harsh environments such as inside the plant vacuole or the digestive systems of herbivores (Montesinos, 2007; Pelegrini *et al.*, 2011; Tam *et al.*, 2015). Moreover, one of the most fascinating features of plant AMPs is their ability to assume different functions depending on the different conditions or targets with which they interact. This 'peptide promiscuity' (reviewed by Franco, 2011) allows AMPs to operate through different mechanisms of action in order to exploit different weak spots depending on the attacking organism.

The capacity to interact with bacterial membranes is a classical feature of microbial, animal, and plant AMPs. This mechanism relies on the perturbation of the so-called 'bacterial Achilles heel', their cellular membrane (Zasloff *et al.*, 2002; Toke, 2005). In contrast to the outermost layer of the bacterial membranes, which maintains a negative transmembrane

Table 2. Plant-associated organisms whose development is affected by antimicrobial peptides (AMPs) produced by plants

Organism	Source plant (Family)	AMP	Type of experiment	References
Phytopathogenic bacteria (Gram-negative)				
Burkholderia plantarii	Oat (Poaceae)	Thionin	Heterologous expression	Iwai *et al.*, 2002
Pseudomonas syringae	Wheat (Poaceae)	β-purothionin	Heterologous expression	Oard and Enright, 2006
Pectobacterium carotovorum	Potato (Solanaceae)	Snakin-1	Overexpression	Almasia *et al.*, 2008
Pectobacterium atrosepticum	Potato (Solanaceae)	Snakin-2	Overexpression	Mohan *et al.*, 2014
Phytopathogenic bacteria (Gram-positive)				
Clavibacter michiganensis	Potato (Solanaceae)	Snakin-2	*In vitro* challenge	Berrocal-Lobo *et al.*, 2002*b*
	Buckwheat (Plygonaceae)	*Fa*-AMP1/*Fa*-Amp2	*In vitro* challenge	Fujimura *et al.*, 2003
Curtobacterium flaccumfaciens	Buckwheat (Plygonaceae)	*Fa*-AMP1/*Fa*-Amp2	*In vitro* challenge	Fujimura *et al.*, 2003
Phytopathogenic fungi/Oomycetes				
Alternaria brassicicola	Sunflower (Asteraceae)	HaDEF1	*In vitro* challenge	de Zélicourt *et al.*, 2007
Alternaria solani	*Nicotiana megalosiphon* (Solanaceae)	NmDef02	*In vitro* challenge	Portieles *et al.*, 2010
Fusarium graminearium	Alfalfa (Fabaceae)	MsDef1	*In vitro* challenge	Spelbrink *et al.*, 2004
Fusarium oxysporum	Wheat (Poaceae)	β-purothionin	Heterologous expression	Oard and Enright, 2006
Pythium graminicola	Rice (Poaceae)	OsTHI7	Overexpression	Ji *et al.*, 2015
Phytophthora infestans	*Nicotiana megalosiphon* (Solanaceae)	NmDef02	Heterologous expression	Portieles *et al.*, 2010
Rhizoctonia solani	Potato (Solanaceae)	Snakin-1	Overexpression	Almasia *et al.*, 2008
Verticillium dahliae	*Nicotiana megalosiphon* (Solanaceae)	NmDef02	*In vitro* challenge	Portieles *et al.*, 2010
	Alfalfa (Fabaceae)	alfAFP	Heterologous expression	Gao *et al.*, 2000
Nematodes				
Meloidogyne spp.	*Capsicum annuum* (Solanaceae)	CaSn	*In vitro* challenge	Mao *et al.*, 2011
	Colocasia esculenta (Araceae)	CeCPI	Heterologous expression	Chan *et al.*, 2010
	Rice (Poaceae)	OsTHI7	Overexpression	Ji *et al.*, 2015
Molluscs				
Pomacea canaliculata	*Oldenlandia affinis* (Rubiaceae)	Kalata B1	*In vitro* challenge	Plan *et al.*, 2008
	Oldenlandia affinis (Rubiaceae)	Kalata B2	*In vitro* challenge	Plan *et al.*, 2008
	Viola odorata (Violaceae)	Cycloviolacin O1	*In vitro* challenge	Plan *et al.*, 2008
Insects				
Aphis gossypii	Pea (Fabaceae)	PA1b	Artificial feeding assay	Gressent *et al.*, 2007
Callosobruchus chinensis	Mungbean (Fabaceae)	VrCRP	Artificial feeding assay	Chen *et al.*, 2002
Diatraea saccharalis	*Palicourea rigida* (Rubiaceae)	Parigidin-br1	Artificial feeding assay	Pinto *et al.*, 2012
Helicoverpa armigera	*Clitoria ternatea* (Fabaceae)	Cter M	Artificial feeding assay	Poth *et al.*, 2011
	Oldenlandia affinis (Rubiaceae)	Kalata B1	Artificial feeding assay	Barbeta *et al.*, 2008
Sytophilus oryzae	Pea (Fabaceae)	PA1b	Artificial feeding assay	Louis *et al.*, 2004
Parasitic plants				
Orobranche cumana	Sunflower (Asteraceae)	HaDEF1	*In vitro* challenge	de Zélicourt *et al.*, 2007

potential and is predominately composed of negatively charged phospholipid headgroups, AMPs typically have a positive charge (Shai, 1995; Stec *et al.*, 2004; Toke, 2005; Fjell *et al.*, 2012; Tam *et al.*, 2015). This electrostatic difference promotes the association between AMPs and the bacterial membrane, which is followed by perturbation of surface tension, dislocation of lipids, and modification of the membrane organization (Fig. 2A). Alternatively, after a threshold concentration is achieved, AMPs can also form a cylindrical structure similar to a pore or staves in a barrel (Bocchinfuso *et al.*, 2009; Bobone *et al.*, 2012; Harris *et al.*, 2016). In both cases, AMP–membrane interaction leads to leakage of cellular components and fatal disruption of the microbial membrane. Moreover, AMP–membrane associations can be additionally promoted by the capacity of some peptides to adopt an amphipatic design, which further allows them to interact with and permeate lipid layers (Matsuzaki *et al.*, 1995; Shai, 1995; Tossi *et al.*, 2000; Tam *et al.*, 2015). The significance of those electrostatic interactions for AMP antimicrobial activity is evidenced by the observation that these peptides act without requiring a membrane receptor, and also the relatively lower activity of AMPs on the membranes of plants and animals, which are composed of lipids with no net charge, which maintain weaker membrane potential, and which contain cholesterol, a sterol capable of stabilizing the lipid bilayer and reducing peptide interactions (Matsuzaki *et al.*, 1995; Zasloff *et al.*, 2002; Fox, 2013).

Plant AMPs can also target more specific structural components of the cell surface, such as certain types of lipids of the plasma membranes or building blocks of the cell walls (Wilmes *et al.*, 2011). This binding specificity allows certain classes of AMPs to act in a more directed manner against particular groups of pathogens. For example, the defensins—a widespread group of AMPs found in plants—act as potent antifungal peptides by interacting with specific sphingolipids present in the fungal cell membrane (Thevissen *et al.*, 2000; Wilmes *et al.*, 2011; Hegedüs and Marx, 2013; Lacerda *et al.*, 2014). Differently to

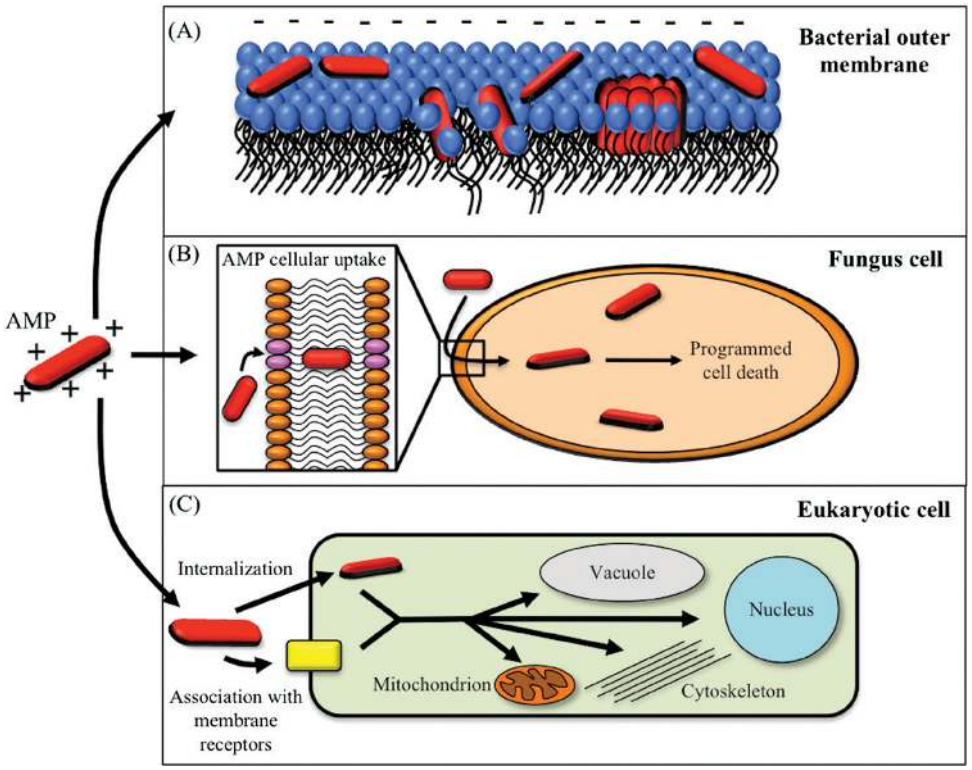

Fig. 2. Action mechanisms of plant antimicrobial peptides (AMPs). (A) Electrostatic differences between AMPs (positively charged) and the bacterial outer membrane (negatively charged) promote interaction. Upon association, AMPs can dislocate membrane lipids to modify the microbial membrane structure or form structures similar to pores in the membrane. Both mechanisms lead to leakage of cellular components and fatal disruption of the microbial membrane. (B) Interaction with specific membrane targets such as types of sphingolipids in fungus cells (indicated in purple in the inset) can lead to cellular uptake of AMPs, which further interact with internal cell components to activate signaling cascades that culminate in programmed cell death. (C) AMPs can also associate with membrane receptors or traverse biological membranes (internalization) to target the internal components of eukaryotic cells such as the vacuole, the mitochondrion, the nucleus, and components of the cytoskeleton.

bacterial membrane interactions, AMP preference for certain membranes or lipid types is usually not dependent on charge differences but rather on the structural features and amino acid sequence of the peptide (Fjell *et al.*, 2012). This type of interaction results in the formation of transient pores that allow AMPs to easily translocate across the membrane and further interact with intracellular components. Besides being a membrane component, sphingolipids also play an important role as secondary messenger molecules involved in the regulation of the cell cycle (Cheng *et al.*, 2001; Lobo *et al.*, 2007; Wilmes *et al.*, 2011). Indeed, a proposed mechanism of action for defensins suggests that, upon cellular uptake, these peptides can interact with sphingolipids to trigger downstream signaling cascades that ultimately lead to programmed cell death of fungi (van der Weerden *et al.*, 2008; Wilmes *et al.*, 2011) (Fig. 2B). Binding of plant AMPs to sphingolipids can also influence the influx and efflux of ions in the pathogen cell. Fungal development is dependent on the maintenance of intracellular Ca^{2+} concentration gradients, which are responsible for driving polarized (tip) growth (Jackson and Heath, 1993). Plant defensins such as the radish Rs-AFP2 and dahlia Dm-AMP1 bind to specific types of fungal membrane sphingolipids to trigger a drastic and rapid increase of Ca^{2+} influx into the fungus cell, thus leading to dissipation of the gradients and inhibition of pathogen cell growth (Thevissen *et al.*, 1996, 2003, 2004; Muñoz *et al.*, 2014; Bolouri Moghaddam *et al.*, 2016). The link between alteration in Ca^{2+} fluxes and AMP antifungal activity is supported by the observation that a variant of Rs-AFP2 that displays enhanced antifungal activity (V39R) also stimulates a stronger Ca^{2+} uptake, whereas a variant that is virtually devoid of antifungal activity (Y38G) does not stimulate Ca^{2+} influx (De Samblanx *et al.*, 1997). Unlike insect and mammal defensins, Rs-AFP2 and Dm-AMP1 do not form ion-permeable pores, and nor do they change the electrical properties of artificial lipid bilayers, indicating that plant AMP-triggered alteration in Ca^{2+} fluxes results from a distinctive but still not clearly understood mechanism (Thevissen *et al.*, 2000, 2003, 2004).

Many of the studies described so far that have dealt with AMP action mechanisms have focused on the capacity of these peptides to associate with structural lipids of biological membranes (Fig. 2A, B). However, as a strategy to diversify their weapons of defense, plants have also evolved AMPs that act by specifically disturbing the function of internal components of the cells of their attackers (Fig. 2C). This mechanism of action can be initiated by different processes, including peptide interactions with specific membrane receptors that transduce the signal to internal cell mediators, AMP internalization pathways that also utilize membrane receptors, or commonly occurring endocytic uptakes that demand energy expenditure (Lichtenstein *et al.*, 1988; Lobo *et al.*, 2007; Nguyen *et al.*, 2011; Marcos *et al.*, 2012; Hayes *et al.*, 2013, 2018; El-Mounadi *et al.*, 2016). For example, Koo *et al.* (2004) showed that Pn-AMP1,

an antifungal AMP produced in the seeds of morning glory (*Ipomoea nil*), causes a rapid depolarization of the actin cytoskeleton that is correlated with arrest of fungal growth. The authors demonstrated that Pn-AMP1 associates with membrane receptors such as cell wall integrity sensors present in the fungal plasma membrane, which in turn transduce the external peptide signal into an internal signaling cascade that modifies the status of the actin filaments. PsD1, a defensin constitutively produced in seeds and leaves of pea (*Pisum sativum*) is capable of crossing fungal membranes (via an unknown mechanism) to interact with nuclear proteins involved with the regulation of fungal cell division and control of the cell cycle, thus inhibiting pathogen cell growth (Almeida *et al.*, 2000; Lobo *et al.*, 2007). The precise mechanism utilized by plant AMPs to disturb the function of internal cell components is still not clearly understood; however, the list of internal targets is constantly expanding. Examples of internal cell components whose activity appears to be modulated by plant AMPs are the machinery involved with the initiation and elongation steps of protein synthesis (Méndez *et al.*, 1996), the nucleus itself (Zottich *et al.*, 2013) the mitochondria (Esmaeili *et al.*, 2016), the vacuole, and other as yet unidentified targets located in the cytoplasm (Hayes *et al.*, 2013; El-Mounadi *et al.*, 2016).

Finally, in addition to directly targeting pest and pathogen cells, plants can also employ AMPs as passive weapons of defense. For example, one of the most fascinating and frequently studied properties of plant AMPs concerns their capacity to indirectly modulate physiological properties of the gastrointestinal tract of insects and other herbivores. The rationale behind this strategy is to reduce the nutritional gain of herbivory in order to impair herbivore growth. Plant AMPs can inhibit the action of enzymes involved with the digestive process, such as trypsin, chemotrypsin and α-amylase (Melo *et al.*, 2002; Pelegrini *et al.*, 2008*a*), disrupt the cells of the insect midgut epithelium (Barbeta *et al.*, 2008), and even alter the electrophysiology of intestinal cells to reduce nutrient absorption (Chouabe *et al.*, 2011). Interestingly, AMP action in the gastrointestinal tract appears to be target specific: it has been demonstrated that VuD1, a defensin from cowpea (*Vigna unguiculata*), shows strong inhibition of the activity of α-amylases from insect pests but not from fungi and mammals (Pelegrini *et al.*, 2008*a*). Other examples of passive action of plant AMPs are their capacity to elicit allergenic responses in mammals (Pastorello *et al.*, 1999; Blanco, 2003; Petersen *et al.*, 2015) and their ability to inhibit the action of secreted proteins involved with fungal pathogenicity in plants (Slavokhotova *et al.*, 2014).

Conclusions and perspectives

Despite the fact that plants are continuously exposed to a myriad of pests and pathogens, we still live in a world that is dominated by these green organisms. This observation implies that plants have evolved highly effective mechanisms of defense that are deployed to hamper the development of attackers that threaten their tissues. In recent years, considerable progress in deciphering the genetic and molecular basis of the plant immune system has allowed researchers to visualize a conceptual framework of how a stressful condition generated by a biological threat is perceived by the plant and ultimately translated into an optimal defense strategy. In this context, AMPs comprise one of the most prevalent barriers utilized by plants to fend off attack. Their ubiquitous occurrence among plant species is explained by the observation that these small molecules provide rapid, direct, and durable resistance against a large spectrum of pests and pathogens. Indeed, plant AMPs are now becoming a hot topic of research due to their importance in ensuring that plants thrive in natural environments, and also for their enormous potentials in the agronomical and pharmaceutical fields (Porto *et al.*, 2018).

Their small size and the high number of disulfide bonds found in plant AMPs allow these peptides to fold into a compact size, with remarkable physical stability. This rigid topological configuration is maintained among plant AMP families by strong conservation of cysteine residues while still allowing high tolerance to variations in other regions of the molecule. As such, AMPs are apt to evolve dynamically, which often results in the presence of multiple AMP gene families with different modes of action in a single plant species (Bloom *et al.*, 2006; Silverstein *et al.*, 2007). This dynamically evolving phenomenon has a profound impact on the plant immune system since it allows the emergence and maintenance of new AMPs that are constantly changing to adapt to biotic stressors. It also permits neo-functionalization of AMP genes, expanding the repertoire of plant signaling molecules involved with responses to different environmental conditions (Bircheneder and Dresselhaus, 2016; Arnold *et al.*, 2017). In this context, computer-assisted design strategies are now being widely used to perform *in silico* evolution on AMP genes, aimed at the development of new molecules with a specific desired activity (Fjell *et al.*, 2012; Porto *et al.*, 2012, 2017). For example, the guava peptide Pg-AMP1 was recently used as a template for the *de novo* design of Guavanin-2, a potent AMP that presents a more specific spectrum of activity and lower toxicity towards human cells when compared to its native counterpart (Porto *et al.*, 2018). It is reasonable to speculate that artificial optimization of plant AMPs will soon represent a rapid and cost-efficient strategy to develop new natural pesticides designed to combat pests and pathogens of agronomic relevance. Moreover, the fitness costs associated with the induction of a plant immune response often result in a negative impact on plant growth and in a reduction in yield (Huot *et al.*, 2014). This 'growth versus defense' paradigm is physiologically explained by trade-offs in the allocation of limited resources to growth or defense processes and the existence of a complex cascade of signaling networks that ultimately regulate plant development in response to environmental conditions (Campos *et al.*, 2016; Züst and Agrawal, 2017; Guo *et al.*, 2018). As small, single gene-encoded protein elements, we hypothesize that AMPs are manufactured quickly and at relatively low metabolic cost when compared to other defensive traits that demand the activation of large and very specific metabolic/biosynthetic pathways, such as secondary metabolites and glandular trichomes (Gershenzon, 1994; Zasloff, 2002; Tam *et al.*, 2015; Huchelmann *et al.*, 2017; Guo *et al.*, 2018). In agreement with this 'low cost of production' theory, there are few reports that associate the overexpression of a plant AMP with obvious negative impacts on plant growth

processes (Epple *et al.*, 1997; Montesinos, 2007; Mohan *et al.*, 2014; Ji *et al.*, 2015), which is an entirely different scenario compared to the overproduction of 'costly' defense barriers, where growth is severely impacted (Strauss *et al.*, 2002; Campos *et al.*, 2016; Züst and Agrawal, 2017). Thus, in our opinion, the heterologous expression of AMP genes may represent an attractive strategy to increase defense responses with relatively little impact on plant development. Furthermore, AMPs can be seen as one of the most elementary chemical barriers produced by plants.

The evident role of AMPs as chemical shields that defend plants against a wide range of pests and pathogens leads to obvious speculation regarding the possibility of using those molecules to treat human diseases. In fact, plant AMPs are beginning to be evaluated for their potential to act against a large number of viruses, micro-organisms, and parasites of medical relevance (Chiche *et al.*, 2004; Hayes *et al.*, 2013, 2018; Nascimento *et al.*, 2015; da Cunha *et al.*, 2017). Interestingly, such studies are also indicating that these 'natural antibiotics' may display other important pharmaceutical properties such as anti-inflammatory, anti-cancer, and immunomodulatory activities (Harris *et al.*, 2016; Guzmán-Rodríguez *et al.*, 2015; Molesini *et al.*, 2017; Leite *et al.*, 2018). In this context, AMPs can be considered as promising alternatives for use as complementary molecules in traditional therapies (da Silva and Machado, 2012; Leite *et al.*, 2018). In addition, their ultra-stability and high tolerance to sequence substitution have motivated the development of AMPs as bioengineering scaffolds in the pharmaceutical industry (Wang *et al.*, 2009; Craik and Du, 2017). Unfortunately, despite their promising healthcare potential and ongoing clinical trials, no plant-derived AMP has yet reached the status of becoming a clinically approved drug (da Cunha *et al.*, 2017; Porto *et al.*, 2018).

Their vital role in the plant immune system means that AMPs are being subject to ever-increasing research. Their wide spectrum of activities, dynamic ability to evolve, and broad mechanisms of action are characteristics that make AMPs excellent weapons for plant defense and also very important candidates for agricultural and pharmaceutical purposes, clearly indicating a promising future for research into these molecules.

Acknowledgements

This work was supported by fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (MLC and CMS) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (KBSO). This manuscript was also granted by FUNDECT, FAPDF, CNPq and CAPES. The authors declare that they have no conflicts of interest.

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