APPLICATION OF ANTIMICROBIAL PEPTIDES TO CONTROL LEAF RUST (*PUCCINIA TRITICINA*) INFECTION IN BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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ABSTRACT

Screening and developing novel antifungal agents with minimal environmental impact are needed to maintain and increase crop production constantly threatened by various pathogens. Small peptides with antimicrobial and antifungal activities have been known to play an important role in plant defense both at the pathogen level by suppressing its growth and proliferation as well as at the host's level through activation or priming of the plant's immune system for faster, more robust response against fungi. Rust fungi (*Pucciniales*) are plant pathogens that can infect key crops, threaten global food security and are capable of overcoming the resistance genes introduced in elite wheat cultivars. We performed an in vitro screening of 19 peptides predominantly of plant origin with antifungal or antimicrobial activity for their ability to inhibit leaf rust (*Puccinia triticina*, CCDS isolate) urediniospores germination. Nine peptides demonstrated significant fungicidal properties compared to the control. Foliar application of the top three candidates, β-purothionin, Purothionin-α2 and Defensin-2, decreased the severity of the leaf rust infection in wheat (*Triticum aestivum* L.) seedlings. Additionally, increased pathogen resistance was paralleled by elevated expression of the defense-related genes.

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AB - Alberta

ABA - abscisic acid

alfAFP - alfalfa antifungal peptide

AMPs - Antimicrobial Peptides

Avr - Avirulence

BAK1 - Brassinosteroid-insensitive 1-associated Receptor Kinase 1

BTH - benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester

BTB/POZ - Broad-Complex, Tramtrack and Bric-a-brac/Pox Virus and Zinc Finger

bZIP10 - Basic Leucine Zipper 10

CAPE1 - CAP-derived peptide 1

CDPK - Calcium-Dependent Protein Kinase

CEBiP - Chitin Elicitor-Binding Protein

CERK1 - Chitin Elicitor Receptor Kinase 1

cDNA - complementary Deoxyribonucleic Acid

Cmm - *Clavibacter michiganensis* subsp. *michiganensis*

Cr-ACP1 - Cycas Revoluta Anticancer Peptide 1

CSP - Cold Shock Protein

ddPCR - Droplet Digital Polymerase Chain Reaction

DND1 - Defense Not Death 1

DNA - Deoxyribonucleic Acid

EDS1- *ENHANCED DISEASE SUSCEPTIBILITY 1*

EF-Tu - Elongation Factor Tu

ETI - Effector-Triggered Immunity

FAM™ - Fluorescein Amidite

Fa-AMP1 - *Fagopyrum esculentum* AMP1

Fa-AMP2 - *Fagopyrum esculentum* AMP2

FHB - Fusarium Head Blight

FLS2 - Flagellin Sensing 2

gDNA - Genomic Deoxyribonucleic Acid

GRP7 - Glycine-rich Protein 7

GSH/GSSG - Reduced Glutathione/Oxidized Glutathione

ha - hectare

hCAP18/LL-37 - human cathelicidin antimicrobial peptide

HEX™ - Hexachloro-Fluorescein

HR - Hypersensitive Response

HPLC - High-Performance Liquid Chromatography

ICS1 - *Isochorismate Synthase* 1

INA - 2,6-dichloroisonicotinic acid

IκB - Inhibitor of Kappa-light-chain-enhancer of Activated B Cells

ISR - Induced systemic resistance

LPS - Lipopolysaccharide

LRR-RLK - Leucine-Rich Repeat Receptor-Like Kinase

LTP - Lipid transfer proteins

LysM - Lysine motif

MAPK - Mitogen-Activated Protein Kinase

MAS - Marker-Assisted Selection

MB - Manitoba

mRNA - Messenger Ribonucleic Acid

NADPH - Nicotinamide adenine dinucleotide phosphate hydrogen

NB-LRR - Nucleotide-binding leucine-rich repeat

NPR1 - *Non-expressor of Pathogenesis-Related Genes 1*

PAD3 - Phytoalexin Deficient 3

PAMP - Pathogen-associated molecular patterns

PAGE - Polyacrylamide Gel Electrophoresis

PCD - Programmed Cell Death

PGN - Peptidoglycan

Pgt - *Puccina. graminis f.* sp. *Tritici*

Pol II - Polymerase II

PR - Pathogenesis-related protein

PR-1 - Pathogenesis-related protein-1

PRR - Pattern-recognition receptors

PTI - Pattern-Triggered Immunity

PAMP - Pathogen-associated molecular pattern-triggered immunity

Pst DC3000 - *Pseudomonas syringae* pv. tomato DC3000

Pt - *Puccinia. triticina*

qPCR - Quantitative Polymerase Chain Reaction

Rboh - Respiratory Burst Oxidase Homolog

RLK - Receptor-Like Kinase

RLP - Receptor-Like Protein

RNA - Ribonucleic Acid

RNA Pol II - RNA Polymerase II

RIN4 - RPM1 Interacting Protein 4

ROS - Reactive Oxygen Species

RT-qPCR - Reverse Transcription Quantitative Polymerase Chain Reaction

SA - Salicylic Acid

SAR - Systemic Acquired Resistance

SCF^CUL3 - Skp, Cullin, F-box containing complex with Cullin 3

Ser – serine

sid2 - SA induction-deficient 2

SK - Saskatchewan

SOD - Superoxide Dismutase

SN2 - Snakin-2

StSN1 - Snakin-1

PR1- *PATHOGENESIS-RELATED GENE* 1

TGA - TGACG-Binding

TIR - Toll/Interleukin-1 Receptor

TIR - Toll/interleukin-1

TLP - Thaumatin-Like Proteins

TMV - Tobacco Mosaic Virus

TRX – thioredoxins

1. INTRODUCTION

1.1 Wheat production

Wheat (*Triticum aestivum* L.) as a staple crop plays a crucial role in human nutrition and food security. It includes the yearly intake of 20% of the overall calories and protein for a population of 7.9 billion people (FAO, 2020). The market equilibrium of wheat production can be challenging when the world's population is projected to reach 10 billion by 2050. The inevitable change in population growth will continue to create escalating demand for increased production.

Canada is a prominent global exporter of wheat , with an average yearly export value of $7 billion and a harvested area of over 10 million ha (Statistics Canada, 2019). The primary fungal diseases that have a substantial influence on wheat production are stem rust , leaf rust , stripe rust, fusarium head blight (FHB). The powdery mildew and leaf spot complex mostly caused by tan spot, *Septoria spp*. and spot blotch are commonly seen on winter wheat (Aboukhaddour, Fetch et al. 2020). In Canada, wheat is susceptible to twenty different fungal pathogens (Bailey and Society 2003), but only five diseases are regarded as high breeding priorities in Western Canada. These are classified as "Priority 1" comprises stem rust ,leaf rust , stripe rust FHB and common bunt. These pathogens have the capacity to induce extensive epidemics and significant economic losses. Wheat production in western Canada was mostly effected by frost, smut (covered and loose), and grasshoppers (Estey 1994). Covered smut, in particular, was especially detrimental, with the potential to devastate up to fifty percent of the crop in certain North American regions (Güssow and Conners 1927, Cherewick 1953, Johnson 1961). Furthermore the occurrence of stem and leaf rust became prominent issue at the turn of the 20th century, resulting in catastrophic outbreaks in the early 1900s (Johnson 1961).

Since 1927, the Canadian Phytopathological Society has published annual reports on disease incidence and severity on its website (https://phytopath.ca/publication/cpds/). These data suggest the prevalence of stem and leaf rust in commercial wheat fields in southern Manitoba and southeast Saskatchewan (Craigie 1944, Johnson 1961). Over the last 120 years, the wheat industry in Saskatchewan, Manitoba and Alberta has evolved markedly. The shift has been influenced by contributions from farmers, agricultural service providers, grain handlers, and various research and extension organizations (Slinkard and Knott 1995).

Most Canadian wheat cultivars have been introduced by research centers of Agriculture and Agri-Food Canada and several universities (Slinkard and Knott 1995).The new rule in western Canada recommends the certified wheat lines should possess minimum level of resistance to priority 1 diseases. (ECONOMIC and AFFAIRS. 2023). The variability of crop yields is caused by unpredictable and extreme weather conditions. Additionally losses from biological threats directly affect the productivity and diminish quality, taste, nutrition, and food safety.

1.2 Leaf Rust in wheat

Today, leaf and stripe rust are the most destructive and widespread cereal rust diseases worldwide. However, in the early 1900s, the widespread influence stem and leaf rust epidemics was identified in North America and Australia (Ellis, Lagudah et al. 2014). Provided, with the ideal growing conditions(high temperature and humidity), the leaf rust pathogen thrives in all wheat-growing regions. In contrast, the stem rust pathogen prefers warm and humid conditions(Figueroa, Hammond-Kosack et al. 2018). While stem rust inflicted more severe losses in the early Northern American era, it probably overshadowed the effect of leaf rust (Samborski 1985).Prior to the introduction of the stem rust-resistant cultivar Thatcher in 1935, the incidence

of leaf rust in Canada was comparable to that of stem rust. Leaf rust had a longer duration of moderate to severe damage (40 years) compared to stem rust (22 years) over the past century. Nevertheless, stem rust outbreaks had a greater extend of continuous distribution taking place prior to 1935 and between 1953 and 1955 (Peturson 1958). The stem rust had a catastrophic impact on farmers in Prairie region, unlike any other cereal disease. The 1954 outbreak was particularly severe, resulting in an estimated loss of 4 million tons of wheat (Peturson 1958).Since 1956, the infection rate of leaf rust was controlled due to the introduction genetic resistance in commercial wheat in North America. However, the worldwide incidence of leaf rust persists with recent leaf rust epidemics in North America occurred between 2000 and 2005, resulting in losses estimated at over $350 million (Huerta-Espino, Singh et al. 2011).

In Canada, wheat leaf rust is predominantly prevalent in Saskatchewan and Manitoba. The inoculum enters Canada via the Great Plains, where leaf rust is the most common of the three rusts (Aboukhaddour, Fetch et al. 2020). Temperatures ranging from 15° to 20°C and high humidity are ideal environmental conditions for spore germination (Wegulo and Byamukama 2012). Leaf rust (*Pt*) infections in wheat plants were moderate to severe in the 1920s and 1930s, generating millions of dollars in annual losses in Western Canada (McCallum, Hiebert et al. 2016). *Pt* can cause yield losses ranging from 7 to 50%, depending on the plant stage at the time of infection, host susceptibility, and environmental conditions (Huerta-Espino, Singh et al. 2011). Losses usually result in yield, protein content, and seed weight reductions (Huerta-Espino, Singh et al. 2011, McCallum, Hiebert et al. 2016). The most successful strategies for reducing *Pt* in Canada are foliar fungicide spraying and resistant wheat cultivars with resistance genes such as *Lr16, Lr21,* and *Lr34* (Randhawa, Asif et al. 2013).

Leaf rust can infect plants at any stage of development. Typically, the upper surface of the leaves is affected, although it can also be observed on the leaf sheath (Agrios 2005). The disease symptoms include brown to reddish pustules that are round to oval and usually found on the plant's leaf surface. Pustules contain thousands of urediniospores that the wind distributes (Agrios 2005). After being infected, the fungus starts to multiply, and by the 14^{th} day, fully mature urediniospores can be found on the plant leaves. Urediniospores are crucial for transmitting the pathogen, especially in high temperatures and late in the growing season, leading to widespread infection. As the season continues, the life cycle progresses with the development and sprouting of teliospores. Teliospores are crucial for the fungus's persistence in unfavorable conditions, such as winter, as they function as the stage for overwintering. After germination, teliospores generate basidia and basidiospores. Basidiospores play a crucial role in the sexual reproduction of the fungus, promoting genetic variation and facilitating the infection of new host plants. The fungus can survive the winter in both the host plant and other hosts, enabling it to endure during difficult seasons. Although *Thalictrum* spp. from the *Ranuculaceae* family is *Pt*'s alternate host, *Anchusa italica* (*Boraginaceae* family) has been found as *Pt*'s alternate host in the Mediterranean regions (Bouftass, Ezzahiri et al. 2010). During the spring season, the progression of diseases is comparatively slow. However, when conditions become increasingly conducive, the fungus recommences its life cycle, resulting in fresh infections. The enduring nature and flexibility of the leaf rust pathogen highlight its capacity to consistently invade plants and propagate effectively across different periods of growth, presenting a substantial obstacle to the plant health and productivity of crops.

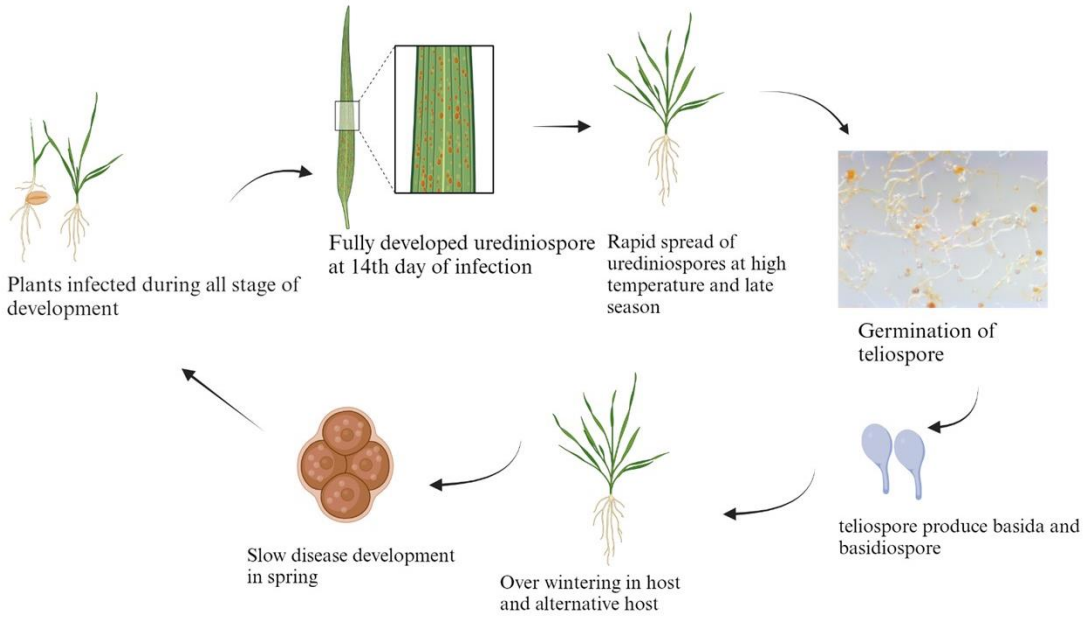


Figure 1. The life cycle of the fungal pathogen *Puccinia triticina* in wheat and its alternative host. The wheat plants are susceptible to infection at any point throughout their growth, and fully developed urediniospores may be seen 14 days following the infection. The dissemination of these spores is increased under elevated temperatures and throughout the latter part of the season. Following germination, teliospores give rise to basidia and basidiospores. The fungus survives the winter in both the host plant and other hosts, resulting in a gradual disease progression in the spring.

1.3 Leaf rust control

Plant disease control is considered one of the major issues these days especially given the rising crop losses caused by pathogens. These methods include the application of chemical pesticides, the implementation of integrated pest management (IPM) strategies, alterations in cropping patterns to disrupt disease cycles, the use of bio-pesticides derived from natural organisms, and the development of genetically resistant plant varieties. Each of these approaches

contributes to a more sustainable and effective strategy for managing plant diseases (Agrios 2005), as it is cost-effective and a reliable source for short-term solutions. Regardless of its benefits, the negative aspects cannot be ignored. Deterioration of the environment, causing adverse effects on human health and ultimately affecting the ecosystem, including climate change, are the major highlights that need to be focused on as the result of optimum use of pesticides.

Managing plant diseases involves implementing various strategies to cure (curative strategies) or prevent (preventative strategies) the damage caused or that can be caused to plants. These strategies take into consideration three crucial factors (the disease triangle) necessary for the development of disease: (1) the presence of the pathogen, (2) the host plant species, and (3) the environmental conditions. Preventive measures include avoidance, exclusion, protection, and resistance, whereas curative measures rely primarily on therapy (eradication).

Using chemical compounds toxic to pathogens has played a vital role in plant protection and therapy for centuries and in controlling plant diseases. Several hundreds of chemicals have been developed to combat plant diseases by killing or inhibiting the growth of invading pathogens; these chemicals can be applied to soil, seed, foliage, flowers, or crops (Agrios 2005). Depending on the target pathogen, these compounds are classified as bactericides, fungicides, virocides, protocides, or nematicides (Agrios 2005). Only a few exhibit therapeutic properties, and the overwhelming majority can only protect plants before infection but cannot cure a disease that has already taken hold. Copper and sulphur compounds, quinones and other benzene derivatives, heterocyclic compounds, and the antibiotics streptomycin, tetracyclines, cycloheximide, and griseofulvin are among the most frequently used plant protection chemicals. Some are toxic to various pathogens, whereas others are more specific.

In recent decades, large quantities of chemicals have been used to maintain high crop production. However, pesticide accumulation in soils and water has deleterious effects on the environment and consumer health (Margni, Rossier et al. 2002, Karabelas, Plakas et al. 2009). In addition, it has been reported that these compounds induce resistance in some plant pathogens (Loper and Buyer 1991). As a result, several nations have enacted regulatory measures and prohibited some pesticides, making plant disease management more difficult due to a lack of effective, sustainable compounds. Consequently, plant protection has been gradually refocused on the rational use of pesticides and the search for new compounds that do not induce phytopathogen resistance and have low adverse effects on the host organism, the environment, and public health.

2. LITERATURE REVIEW

2.1. Plant defenses against microbial pathogens

Various microorganisms, such as bacteria, viruses, fungi, and nematodes, may invade plants. Depending on their parasitic lives, these pathogens are categorized as either biotrophs or necrotrophs. Biotrophs are organisms that rely on keeping their host alive and healthy as a means to obtain nutrients. They include both facultative saprophytes, which may also live off dead organic matter, and obligate parasites, which rely only on their host for survival. Facultative saprophytes, such as *Aspergillus niger*, can alternate between parasitic and saprophytic modes. They can break down organic matter without relying on a host but may also cause illnesses like black mold rot in plants that are already compromised. Obligate parasites, such as *Puccinia triticina* (wheat leaf rust), depend wholly on live hosts for their survival and reproduction. They use specialized structures called haustoria to absorb nutrients from the cells of their hosts. Conversely, necrotrophs such as, like *Botrytis cinerea*, cause cell death by releasing toxins or enzymes that degrade tissues, leading to the fast release of nutrients. Both biotrophic and necrotrophic pathogens can severely impact plant growth and overall survival. Pathogenic bacteria pose a substantial risk causing 10% to 20% reduction in worldwide agricultural production (Oerke 2006). Plants have developed advanced natural defensive systems to protect themselves from microbial diseases, as a result of the selection pressure imposed by these pathogens. Understanding the molecular pathways involved in plant disease resistance is essential for enhancing future crop advancement.

The defense mechanisms of plants can be categorized into passive and active mechanisms. Each mechanism serves distinct purposes and exhibits specific responses when faced with pathogen attacks. Passive defense mechanisms refer to the inherent structural and chemical barriers that plants possess to hinder the invasion of pathogens. Physical barriers, such as the secondary cell wall, offer enhanced fortification and safeguarding by obstructing the entry of pathogens. Chemical barriers, such as the development of saponins, include the synthesis of antibiotic substances that disturb the cell membranes of pathogens, thus impeding infection (Vaahtera, Schulz et al. 2019, Bacete and Hamann 2020, Wan, He et al. 2021).

Plants activate defense systems in response to the detection of disease. Swift and vigorous defense responses involve alterations in membrane permeability, which hinder the infiltration or propagation of pathogens, and hypersensitive cell death, wherein localized cell demise at the site of infection curtails pathogen proliferation and dissemination. In addition, plants can quickly restore damaged tissues caused by pathogen attacks to prevent further infection. Delayed active defense responses encompass protracted tactics, such as reinforcing the cell walls of plants near the infection site by depositing supplementary structural proteins and chemicals. This fortification impedes the penetration of pathogens. Pathogenesis-related (PR) proteins are crucial in the defense against pathogens. These proteins either break down the cell walls of pathogens, hinder their growth, or activate other defense processes (Dos Santos and Franco 2023).

Systemic Acquired Resistance (SAR) is a plant defensive mechanism that is activated across the whole plant after it has been exposed to a pathogen in a specific area.

It involves the creation of signaling molecules that prepare other areas of the plant to defend against future attacks (Fu and Dong 2013).

In summary, Figure 2 depicts the complex and diverse nature of plant defensive mechanisms, which involve intrinsic structural protections and response biochemical methods to safeguard against pathogen invasions.

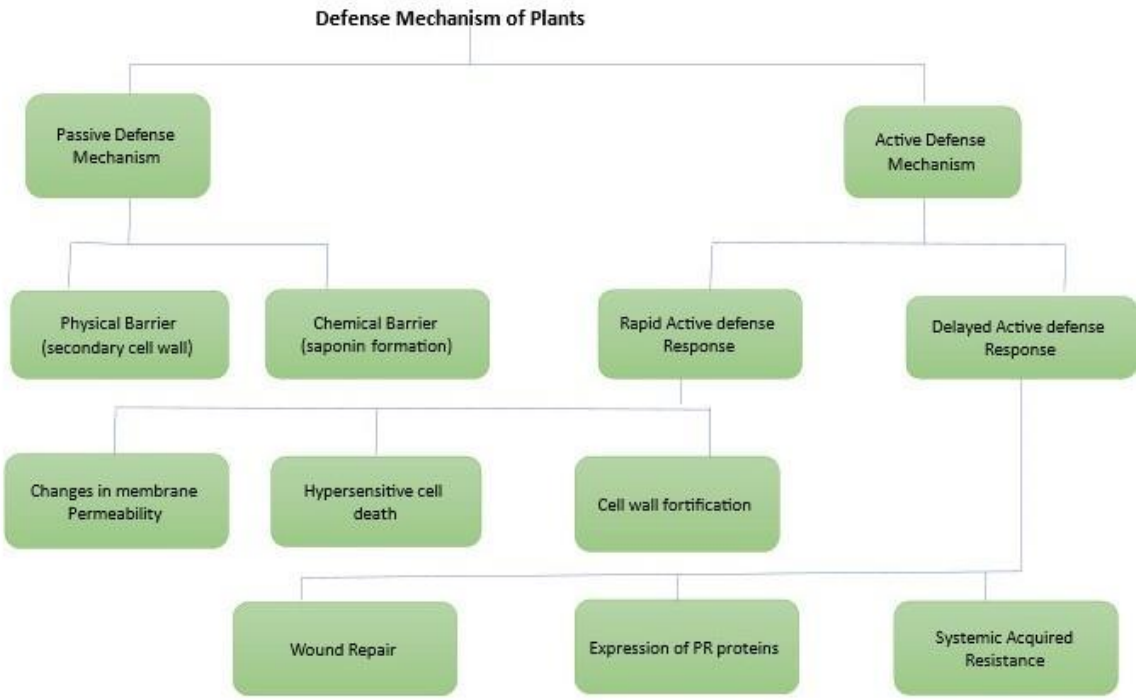


Figure 2. Active and passive plant defense mechanisms. Passive defenses include physical barriers such as the secondary cell wall and chemical barriers such as saponin production. These barriers hinder the entrance of pathogens and interfere with cell membranes. Active defenses enclose swift reactions, such as alterations in membrane permeability, hypersensitive cell death, and wound healing, as well as delayed reactions like cell wall reinforcement, production of pathogenesis-related proteins, and SAR. Together, these strategies safeguard plants against infections using structural, chemical, and systemic responses.

2.1.1 Physical barriers

Direct physical contact is necessary to create a parasitic connection between a pathogen and a host. Fungal infections such as *Botrytis cinerea* invade plant cells and obtain nutrition through hyphae, while bacterial pathogens like *Pseudomonas syringae* dwell outside the cells and alter the host's physiology to obtain nutrients (Freeman and Beattie 2008).

2.1.1.1 Plant cuticle

Plants have developed a strong physical barrier, the cuticle on their outer surface, to prevent pathogen invasion. It is composed of hydrophobic insoluble cutin, which is saturated and enveloped by cuticular waxes which effectively prevents the infiltration of germs into wounds (Riederer and Müller 2007). *Penicillium digitatum*, a type of blue mold that affects citrus, cannot penetrate the plant's surface. However, it can enter the fruit through any wounds present (Vilanova, Viñas et al. 2014).

Plant cells are protected from pathogens by physical separation and the cuticle, but spread in the field through the dissemination of spores. The adherence of spores to the plant surface is crucial for disease onset, which happens in the presence of high humidity. Moisture on the surface helps disease-causing spores to develop and spread the disease, however cuticle layer decreases the amount of surface moisture, hence decreasing the likelihood of spore adhesion and germination (Brown, Ogle et al. 1997). While this structural barrier limits pathogen infection, it does not fully prevent pathogen from invading the plants, and overtime, several microbial pathogens have adapted the by pass through evolution.

Despite being protected by a cuticle, plant cells are not entirely insulated from their external surroundings. Some infections specifically use these natural holes to invade the host (Melotto, Underwood et al. 2006). *Puccinia graminis*, a wheat pathogen, can develop a specialized structure called an appressorium, which allows the penetration of fine hyphae into wheat leaves through

stomata, subsequently developing sub-stomatal vesicles. Infection hyphae originate from sub-stomatal vesicles and propagate within wheat leaves. Pathogens such as *Botrytis cinerea* penetrate the cuticle directly by inserting the sharp penetration peg into the plant's surface (Brown and Swinburne 1982).

2.1.1.2. Plant cell wall

Pathogens that enter host plants by natural holes, wounds, or direct penetration must first overcome the cell wall, which serves as an additional structural barrier, before they can access the cellular contents. The plant cell wall is a resilient layer that envelops the plant cell membrane, offering structural reinforcement and the ability to resist stretching and tearing in plant cells. The primary constituents of the plant cell wall consist of polysaccharides, specifically cellulose (including hemicellulose and pectin), phenolic compounds (such as ferulic and coumaric acids), and glycoproteins. The pectin matrix, containing crosslinked cellulose microfibrils, structural proteins, and phenolic polymers, acts as a barrier between plant cells and invading pathogens (Underwood 2012). The composition and density of the plant cell wall impact the plant's vulnerability to diseases. While there has been speculation about this for many years, it has only been observed through experiments in recent times. Tomato plants with inhibited expression of two plant cell wall hydrolase genes (endo-b-1,4-glucanases Cel1 and Cel2) exhibit reduced susceptibility to *Botrytis cinerea* compared to the wild type. Tomato fruits with lower levels of polygalacturonases (enzymes that break down pectin) show increased resistance to *Geotrichum candidum* and *Rhizopus stolonifer*. Arabidopsis plants that have an increased amount of Extensin-1, a gene responsible for producing a glycoprotein that forms the cell wall structure, show a decrease in the ability of *Pseudomonas syringae* to invade them (Cantu, Vicente et al. 2008). To

overcome this obstacle, several infections, particularly necrotrophs, have evolved a variety of enzymes that can degrade cell walls (Kubicek, Starr et al. 2014). While the structural barrier successfully restricts pathogenesis, it is not flawless. Enhanced and targeted immune responses are necessary to effectively combat diseases caused by phytopathogens.

2.1.2 Chemical barrier

2.1.2.1 Antimicrobial substances

Plant-derived compounds, such as lactones, saponins, cyanogenic glucosides, and terpenoids, exhibit antibacterial properties (Lucas 1998). These defensive chemicals are either present before plants are exposed to infections or are created after they are infected by pathogens. The chemicals that are already present in plants before they are exposed to bacteria or are created after infection using existing components are called phytoanticipins. These molecules are low-molecular-weight compounds that have antimicrobial properties. Phytoalexins are plant antibacterial chemicals produced *de novo* following pathogen infection (VanEtten, Mansfield et al. 1994).

2.1.2.1.1 Phytoanticipin - chemical barrier

Phytoanticipins, such as avenacin, are pre-formed and stored in anticipation of possible pathogen invasions. For instance, young oat roots contain four structurally linked avenacins which are triterpenoids that exhibit antifungal activity. These compounds form complexes with sterols on the fungal cell membrane, leading to pore development and compromising the cell membrane's

integrity. *Gaeumannomyces graminis var. tritici*, a fungus, poses a significant threat to wheat and barley crops. Nevertheless, it cannot infect oat roots since it is susceptible to avenacins. Interestingly, another fungus, *G. graminis var. avenae*, which infects oats, possesses a detoxifying enzyme that specifically acts on the primary avenacin, avenacin A-1 (Abdelrahman and Jogaiah 2020).

2.1.2.1.2. Phytoalexins - iinducible plant antibiotics

Phytoalexins are inducible plant antibiotics that accumulate in response to pathogen infection. Camalexin, scientifically referred to as 3-thiazol-2'-yl-indole, is among the most extensively researched phytoalexins was initially, it was obtained from the leaves of the *Camelina sativa* that were infected with *Alternaria brassicae* (Glawischnig 2007). *Arabidopsis* leaves produce camalexin in response to bacterial, viral, fungal, and oomycete infections, accumulating excessively near the site of infection and inhibits the growth of certain pathogens, particularly fungi. The inhibitory impact is more pronounced in fungi compared to Gram-negative bacteria. The *Arabidopsis phytoalexin deficient 3* (*pad3*) mutant, which lacks an enzyme necessary for camalexin production, is more fvulnerable to *Alternaria brassicicola* and *Leptosphaeria maculans* compared to the wild-type control. However, the *pad3* mutant and the wild-type control show no discernible variation in their susceptibility to *Pseudomonas syringae pv maculicula,* (Schuhegger, Rauhut et al. 2007, González-Lamothe, Mitchell et al. 2009).

2.1.2.2 Protein-based protectors

Plant biochemical defense known as pathogenesis-related (PR) proteins are also activated upon the pathogen invasion. Due to the energy and resource requirements, the production of

defense proteins is only increased or initiated once the plant's immune system is active. These proteins, first discovered in tobacco leaves infected by tobacco mosaic virus, discovery of the inducible PR proteins occurred in tobacco leaves exposed to the tobacco mosaic virus are now classified into 17 families based on their diverse function (Van Loon, Pierpoint et al. 1994) (van Loon, Rep et al. 2006).

Certain PR proteins exhibit hydrolytic enzymatic activity, including chitinases, glucanases, and lysozymes. Chitinases and glucanases break down chitin and glucans, which are the primary constituents of the fungal cell wall. Tomato plants that express both tobacco PR-2e (which encodes glucanase) and PR-3d (which encodes chitinase) are more resistant to *Fusarium oxysporum f.sp. lycopersici* than wild-type plants (Melchers and Stuiver 2000). In contrast to the fungal cell wall, the bacterial cell wall comprises peptidoglycan (PGN), a polymer consisting of cross-linked polysaccharide chains with attached amino acids. Certain members of the PR-8 gene family have been proposed to encode proteins that exhibit lysozyme activity. They produce an antibacterial action by accelerating the hydrolysis of PGN (van Loon, Rep et al. 2006).

Defensin belongs to the PR-12 family, a kind of PR protein. These proteins are small, with a molecular weight of approximately 5 kilodaltons and consisting of 45 to 54 amino acids. They are rich in cysteine, containing 8 cysteine residues and have potent antifungal action (Thomma, Cammue et al. 2002). RsAFP2, a type of radish defensin has shown to disrupt cell membranes in certain fungi and yeast through the stimulation of reactive oxygen species (ROS) production in *Candida albicans*.. The alfalfa defensin (MsDef1) hinders the elongation of hyphae by obstructing the signaling of the L-type calcium channel, which is necessary for the growth of the tip (Stotz et al., 2009).

2.1.3 Immunity activated by pathogen-associated molecular patterns

Beyond physical and chemical barriers ,plants also have an innate immune system that produce defense related compounds upon pathogen invasion. This immune response involves the localized and/or systemic production of defense-related proteins and compounds through *de novo* biosynthesis. The innate immune responses are exclusively triggered by pathogen infection and have a limited duration, with its duration varying based on the specific pathogen invasion. (Jones and Dangl 2006).

Pathogen-associated molecular pattern-triggered immunity (PTI) is a rapid and temporary immune response that enables plants to respond quickly. Once the host plant cells detect the presence of invading pathogens, a sequence of immunological responses is initiated to halt the progression of pathogenesis (Bittel and Robatzek 2007). Interestingly, even non-pathogenic organism can trigger PTI. Both host and non-host microorganisms exhibit transcriptional activation of a similar group of host genes and elicit comparable physiological responses (Thilmony, Underwood et al. 2006). This implies that the initiation of PTI relies on recognition of molecular patterns seen in foreign microorganisms rather than the specific identity of individual pathogens.

2.1.3.1 Microbial/Pathogen associated molecular patterns for PTI initiation

The plant immune system can be triggered by pathogen-associated molecular patterns (PAMPs), which are frequent microbial signatures. Recognized PAMPs include flagellin, harpins, elongation factor Tu (EF-Tu), PGN, lipopolysaccharide (LPS), cold shock protein, chitin, oomycete necrosis-inducing phytophthora proteins, cryptogein, and fungal elicitins (Nicaise, Roux et al. 2009).

Flagellin is the fundamental component of a bacterial motility structure called a flagellum, contains conserved regions that serve as PAMP epitopes which are recognized by a wide range of higher plants, including tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum L.*), rice (*Oryza sativa L.*), and *Arabidopsis thaliana* (Boller and Felix 2009). The epitope flg22 is well-defined and consists of a sequence of 22 amino acids located at the N-terminus of flagellin. The synthetic flg22 peptide can induce PTI reactions at low concentration of one nanomole (Felix, Duran et al. 1999). Besides flg22, the bacterial flagellin also possesses other epitopes, including flgII-28, another conserved area in the N-terminus of flagellin. PTI induction with flgII-28 has been seen in tomato and other *Solanaceae* species (Cai, Lewis et al. 2011).

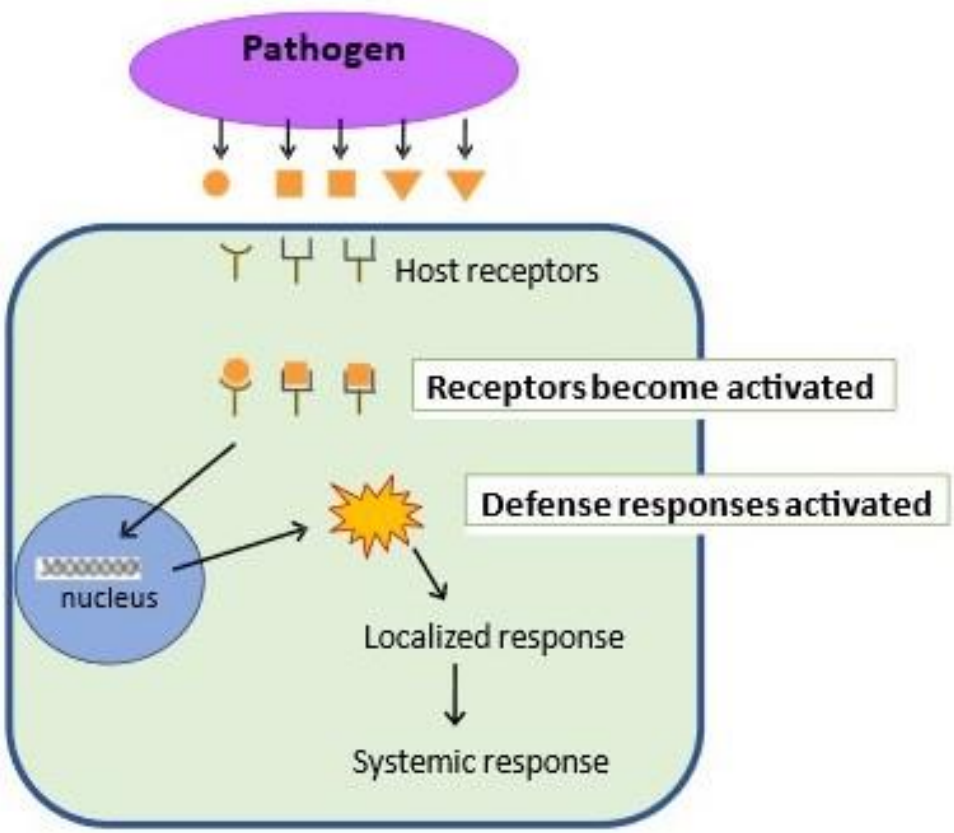


Figure 3. Defense response in plants upon pathogen detection. Upon binding to particular receptors on the surface of plant cells, pathogen-associated molecular patterns (PAMPs) activate

these receptors, initiating a signal transduction cascade inside the cell. The signal is sent to the nucleus, causing the activation of defense-related genes. Initially, the plant exhibits a confined reaction at the site of infection, where it generates antimicrobial chemicals, strengthens cell walls, and triggers programmed cell death to confine the pathogen. In addition, the plant can trigger a systemic reaction, referred to as SAR, which strengthens the plant's defensive mechanisms throughout all its tissues, preparing it for any future infection. This procedure highlights the plant's capacity to perceive and promptly react to pathogenic dangers accurately.

The plant immune system recognizes and targets the microbial cell wall PGNs, also known as PAMPs. *Arabidopsis* immune system can detect the PGN fragments produced by Gram-positive and Gram-negative bacteria (Gust, Biswas et al. 2007). Lipopolysaccharide (LPS), a primary component of the outer membrane in gram-negative bacteria, is also recognized as a pathogen-associated molecular pattern (PAMP) (Newman, Sundelin et al. 2013). Chitin is the most extensively researched pathogen-associated molecular pattern (PAMP) found in the fungal cell wall. It can induce PTI response in rice and *Arabidopsis* (Shibuya and Minami 2001).

2.1.3.2 Recognition of pathogen-associated molecular patterns (PAMPs) at the plasma membrane

PAMPs are detected with great sensitivity by a wide range of receptors located on the surface of cells, specifically known as pattern-recognition receptors (PRRs). PRRs, which are now recognized, can be classified as either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel 2014).

A receptor-like kinase (RLK) comprises an extracellular domain responsible for binding ligands, a transmembrane region that spans the cell membrane, and an intracellular domain that

functions as a kinase. The conformation of RLK undergoes modifications when a ligand binds to it. This change enables the kinase domain to undergo autophosphorylation (Zhang, Shao et al. 2007), and/or the phosphorylation of downstream signaling components that interact with the kinase domain. Thus, an extracellular signal is converted into a sequence of intracellular molecular processes. In *Arabidopsis* the flg22 receptor, known as Flagellin Sensing 2 (FLS2), is an RLK involved in the perception of PAMPs. The *FLS2* gene locus was discovered via genetic screening to identify *Arabidopsis* mutants that exhibit insensitivity to flg22. The *FLS2* gene encodes a widely distributed receptor-like kinase (RLK) that belongs to subfamily XII of leucine-rich repeats receptor-like kinase (LRR-RLK) (Gómez-Gómez and Boller 2000). The subsequent publication provides evidence of a particular interaction between flg22 and FLS2, confirming that the FLS2 protein is the genuine receptor for flg22 (Chinchilla, Zipfel et al. 2007). When flg22 binds to FLS2, it causes FLS2 to quickly form hetero-complexes with its signaling partner, Brassinosteroid-insensitive 1-associated Receptor Kinase 1 (BAK1). This is followed by the phosphorylation of their kinase domains (Schulze, Mentzel et al. 2010).

RLPs exhibit a general structure similar to RLKs but lack the intracellular domain. Thus, it is believed that RLPs operate by interacting with RLKs to facilitate intracellular signaling (Zipfel 2014). The recognition of fungal chitin in rice involves two specific proteins: Chitin Elicitor-binding Protein (CEBiP), which contains a lysine motif (LysM), and Chitin Elicitor Receptor Kinase 1 (CERK1), which is a receptor-like kinase (RLK) (Kaku, Nishizawa et al. 2006). CEBiPs undergo dimerization when they bind to chitin and subsequently form hetero-oligomeric complexes with CERK1 to activate intracellular signaling (Hayafune, Berisio et al. 2014).

RLKs and RLPs are a diverse group of plant proteins, with 610 RLKs and 56 RLPs found in the *Arabidopsis* genome. Only a few proteins have been thoroughly studied, and their functions

have been understood. Several unpaired PAMPs are waiting to be matched with their PRR counterparts (Bittel and Robatzek 2007, Zipfel 2014).

2.1.3.3 Downstream signaling and physiological responses

While our understanding of the specific molecular pathways involved in intracellular signaling during PTI is still limited, many significant signaling processes have been identified and studied. Ion channels are commonly affected by different pathogen-associated molecular patterns (PAMPs). The perception of PAMPs triggers a fast (within 30-40 seconds) elevation in the concentration of calcium ions in the cytosol. The cytosolic calcium spike is detected by calmodulin, calcineurin B-like proteins, and calcium-dependent protein kinases (CDPKs) to initiate the appropriate downstream signaling pathways (Bittel and Robatzek 2007). After calcium enters the cell, there is an elevated production of reactive oxygen species (ROS) by the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases located in the plasma membrane. These oxidases are similar to the respiratory burst oxidase homologs (Rbohs). The rapid and short-lived occurrence, referred to as oxidative burst, is used to measure the initiation of PTI in bioassays (Torres, Dangl et al. 2002). CDPKs can phosphorylate the N-termini of Rbohs. This has led to the suggestion that CDPKs can influence the formation of ROS by controlling the phosphorylation status of Rbohs (Gao, Brodhagen et al. 2009). The reactive oxygen species (ROS) created by Rboh as a result of PAMP stimulation strengthen the cell wall by facilitating the bonding of polymers and proteins. Additionally, they function as signals to initiate the reprogramming of gene transcription (Torres, Jones et al. 2006).

Mitogen-activated protein kinases (MAPKs) play a crucial role in the signaling network of PTI. In *Arabidopsis*, FLS2 triggers the activation of two opposing MAPK cascades that have

contrasting impacts on PTI signaling. One cascade has a beneficial impact, while the other has a negative impact (Bittel and Robatzek 2007). This enables the precise adjustment of defense reactions triggered during PTI. CDPKs and MAPKs work together to control transcriptional reprogramming by adding phosphate groups to enzymes and transcription factors involved in PTI signaling processes. This phosphorylation helps regulate the events during PTI signaling (Meng and Zhang 2013).

Additional physiological reactions observed during PTI encompass the formation of callose, the generation of phosphatidic acid, protein degradation facilitated by ubiquitination, and the closure of stomata (Macho and Zipfel 2014). A comprehensive investigation is required to further our comprehension of the molecular mechanism that underlies the PTI responses.

2.1.4 Effector-triggered immunity (ETI)

Effector-triggered immunity (ETI) is the secondary component of the plant's innate immune system. PTI, or pattern-triggered immunity, is a general defense mechanism that responds to bacteria in a non-specific manner. On the other hand, ETI can only be activated by pathogens that possess specific chemicals called effectors, which plants recognize (Jones and Dangl 2006). The host plant's detection of this chemical identity triggers both local and systemic immune responses. The hypersensitive response (HR) is a quick and localized form of programmed cell death (PCD) that typically occurs at the site of infection. This reaction is commonly related to ETI (Morel and Dangl 1997). The localized self-sacrifice of plant cells restricts the growth and propagation of biotrophic and hemi-botrophic diseases, as these pathogens rely on living hosts for sustenance. Simultaneously, the healthy tissue can initiate systemic acquired resistance (SAR) as a precaution against possible pathogen invasions (Fu and Dong 2013). Avirulent pathogens elicit

ETI, which triggers the activation of the plant's immune defenses, making them less harmful. The proteins that activate ETI are referred to as avirulence (Avr) proteins.

2.1.4.1 Effectors

Due to the co-evolution of phytopathogens with plants, certain pathogens have developed the capability to manipulate the physiology of their hosts to promote pathogenesis. They achieve this by releasing effectors that have various functions. Despite the significant growth in the knowledge of known effectors due to rigorous bioinformatics studies in recent decades, our understanding of their targets, functions, and dynamics remains restricted (Abramovitch, Kim et al. 2003, Chisholm, Coaker et al. 2006).

Given that PTI reactions inhibit infections, it is clear that pathogens would naturally evolve to suppress PTI. Altering one's identity is a potential strategy to evade detection by the plant immune system. Nevertheless, the process is arduous and time-consuming because PTI focuses on the extremely preserved characteristics of infections, which are typically unable to tolerate mutations because of their critical role in the survival and/or proliferation of the pathogen. While certain subspecies of *Xanthomonas campestris pv. campestris* have been found to have a single amino acid polymorphism in flg22, which explains the differences in how these pathogens are recognized by their hosts, most pathogens do not tolerate mutations in their PAMPs (Ngou, Ding et al. 2022).

Another method of inhibiting basal defense is to disrupt the crucial signaling pathways associated with PTI. Bacterial and fungal infections release several effectors that target the components involved in these processes (Buscaill and van der Hoorn 2021).

Effectors can impact both the creation and breakdown of PRRs, decreasing the amount of these proteins in plants. PTI is triggered by the binding of pathogen-associated molecular patterns (PAMPs) to cell surface PRRs, which leads to the internalization of PRRs and the activation of transcription for many PRRs. The purpose of this is to stimulate the regrowth of PRRs on the cells' surface without any ligands. Additionally, it aims to enhance the responsiveness to various pathogen-associated molecular patterns (PAMPs) (Boller and Felix 2009). The effector HopU1 from *Pseudomonas syringae pv. tomato* DC3000 (Pst DC3000) specifically modifies an RNA-binding protein, Glycine-rich Protein 7 (GRP7), by ADP-ribosylation in *Arabidopsis*. This alteration disrupts the interaction between GRP7 and the mRNAs that code for FLS2, a crucial step in translating FLS2 mRNAs. The decreased production of FLS2 leads to a decrease in the host plant's ability to resist *Pseudomonas syringae* (Jeong, Lin et al. 2011). Moreover, effectors such AvrPtoB derived from *Pseudomonas syringae* facilitate the breakdown of PRRs. The interaction between AvrPtoB and tomato ubiquitin was significant in the yeast-two-hybrid screening approach (Abramovitch, Kim et al. 2003). The C-terminus of AvrPtoB possesses E3 ligase activity. This activity involves the tagging of PRRs, such as FLS2 and CERK1, for degradation through the ubiquitin-mediated, 26S proteasome-dependent pathway, as shown by (Göhre, Spallek et al. 2008, Gimenez-Ibanez, Hann et al. 2009).

Effectors can also directly suppress the activity of PRRs. The N-terminus of AvrPtoB described above functions as an inhibitor of the cytoplasmic kinase domain of Bti9, which is the tomato equivalent of CERK1. The direct interaction between AvrPtoB and Bti9 eliminates the plant immunological responses triggered by Bti9. The primary region of AvrPtoB possesses the ability to attach to other molecules. The interaction between AvrPtoB and the PRRs, FLS2 and BRI1-Associated Receptor Kinase (BAK1) hinders the formation of the FLS2-BAK1 complex,

which is crucial for plant immunity and immune responses (Shan, He et al. 2008). vrPtoB's interaction with these two PRRs hinders the formation of the FLS2-BAK1 complex. Similarly, the AvrPto effector of *Pseudomonas syringae* directly attaches to the kinase domain of FLS2, inhibiting FLS2 from adding phosphate groups to its substrates (Xiang, Zong et al. 2008). The effectors also target the signaling components downstream of PRRs. The HopAI1 protein derived from Pst DC3000 suppresses the plant immune response by regulating the MAPK cascade initiated by PAMPs. HopAI1 directly targets MPK3 and MPK6 in *Arabidopsis*. HopAI1 exhibits a distinctive phosphothreonine lyase function, enabling it to eliminate the phosphate group from a phosphorylated threonine residue. HopAI1 catalyzed the dephosphorylation of MPK3/6, inhibiting the MPK3/6-mediated signaling pathway (Zhang, Shao et al. 2007). In addition, the eukaryotic vesicle trafficking system is necessary to release defense proteins into the apoplastic region. The bacterium *Xanthomonas campestris pv. vesicatoria* secretes the effector XopB, which reduces PTI (pathogen-associated molecular pattern-triggered immunity) via disrupting vesicle trafficking, as demonstrated by (Schulze, Kay et al. 2012).

2.1.4.2 Recognition of effectors by plant resistance genes

Pathogens that carry effectors are more harmful to sensitive host plants compared to pathogens that do not have effectors. Plants have developed a surveillance system to detect the presence of harmful effectors in response to the advent of pathogenic effectors (Jones and Dangl 2006).

The "gene-for-gene" idea elucidates the interaction between pathogens and hosts in the context of disease resistance (Flor 1971). According to this idea, host resistance occurs when a product of the resistance (R) gene in the host enables the particular recognition of a product of an

Avr gene in the avirulent pathogen. The interaction between the *Avr* gene and the *R* gene is deemed adequate to initiate immunological responses in the resistant plant. This concept has directed research in contemporary plant immunity in the subsequent decades. The idea is supported by successfully cloning the first *Avr* gene (Staskawicz, Dahlbeck et al. 1984) and the first *R* gene (Martin, Brommonschenkel et al. 1993) in the 1980s.

R genes often code for nucleotide-binding leucine-rich repeat (NB-LRR) proteins, which consist of an N-terminal coiled-coil (CC) or Toll/interleukin-1 (TIR) domain, C-terminal LRRs, and an NB domain in between. The basic logic is that NB-LRRs identify Avr proteins through direct protein-protein interaction, as demonstrated in the instance of the flax R protein L6 (Dodds, Lawrence et al. 2006). Nevertheless, the occurrence of direct contact between R and Avr proteins has been infrequently proven. However, the literature predominantly consists of reports on the indirect recognition of Avr effectors by R proteins. The *Arabidopsis* R protein RPM1 and the Avr protein AvrRpm1 derived from *Pseudomonas syringae pv. maculicola* serves as an excellent illustration. AvrRpm1 does not have any propensity to bind with RPM1. However, it does physically interact with RIN4, which is a positive regulator of plant immunity and is known as RPM1 interacting protein 4. The connection between RIN4 and AvrRpm1 leads to the excessive phosphorylation of RIN4, which may be detected by RPM1 (Mackey, Holt et al. 2002, Liu, Elmore et al. 2011). To understand this indirect occurrence, two prominent scientists in plant immunology put forward a "guard hypothesis" (Dangl and Jones 2001, Jones and Dangl 2006).

According to the notion, R proteins in the host monitor the integrity of the molecular targets of effectors rather than the effectors themselves. A matching R protein can detect the disturbance of a host target by effector molecules, resulting in the onset of ETI (Jones and Dangl 2006). Numerous R proteins have been studied and described in recent decades. However, the specific

molecular mechanisms by which these NB-LRRs are activated, how they facilitate signaling transmission, and the recipient of the signal are yet unknown (Jones and Dangl 2006, Bonardi, Tang et al. 2011).

2.1.5 The hypersensitive response

ETI induction is frequently linked to HR, a localized cell death occurring at and near the site of infection. This phenomenon has been documented in certain plant species, such as wheat, since the 1900s. This decisive yet measured activity is strongly associated with the ability to resist fungal infections which was classified as HR (Stakman 1915). Subsequent morphological studies demonstrated that HR resembles the three extensively researched forms of cell death in the animal system. HR exhibits the characteristic traits of both non-programmed cell death (mitochondrial swelling known as oncosis) and programmed cell death (cytoplasmic shrinkage, chromatin condensation as seen in apoptosis, and vacuolization as observed in autophagy) (Mur, Kenton et al. 2008). HR undeniably plays a crucial function in restricting pathogens' proliferation by secreting chemicals and defense proteins into the extracellular environment. The beginning of HR occurs due to the activation of the host resistance (R) proteins by the pathogenic avirulence (Avr) proteins. This is corroborated by the study, which states that AvrPtoB cannot induce HR in tomato plants when the R protein Prf is absent (Rathjen, Chang et al. 1999). AvrRpm1 triggers HR that relies on the presence of RIN4 and RPM1.

Our understanding of the signaling events that occur after the activation of R proteins is very limited. Nevertheless, certain crucial chemical constituents implicated in HR have been identified (Brodersen, Petersen et al. 2002). In contrast to the analysis of form and structure, the molecular evidence indicates that HR is predetermined to a certain extent. *Arabidopsis* has

undergone genetic screening to identify mutants that exhibit either impaired or spontaneous HR following the introduction of an effector. A mutant lesion called *lsd1* showed a phenotype characterized by uncontrolled cell death when exposed to HR-eliciting bacteria (Dietrich, Richberg et al. 1997). These findings indicate that the LSD1 protein is involved in HR. Additional research has shown that LSD1 suppressed cell apoptosis by stimulating the transcription of *Cu/Zn superoxide dismutase* (*SOD*) genes, thereby diminishing the buildup of superoxide radicals (Kliebenstein, Dietrich et al. 1999). Research has demonstrated that LSD1 inhibits HR by interfering with the nuclear localization of the basic leucine zipper 10 (bZIP10) transcription factor (Kaminaka, Näke et al. 2006). Such research suggests that LSD1 plays a crucial role as a cellular "switch" in the process of HR.

In addition to proteins, the chemical signals generated during HR have also been well investigated. Calcium influx is a crucial signal for the commencement of HR. During HR in cowpea plants infected with a rust fungus, there was a noticeable and continuous rise in the concentration of calcium ions in the cytoplasm (Xu and Heath 1998). In addition, inhibiting or eliminating a calcium cyclic nucleotide-gated channel expressed by the *Defense Not Death* (*DND1*) gene eliminates HR in *Arabidopsis* (Clough, Fengler et al. 2000, Ali, Ma et al. 2007). ROS has also been proposed as a beneficial indicator in HR. The delay of tobacco mosaic virus (TMV)-elicited HR can be efficiently achieved by infiltrating ROS scavengers, such as SOD or catalase (Doke and Ohashi 1988).

The defense hormone salicylic acid (SA) is a crucial signal for activating the HR. SA accumulates excessively at the site of infection and functions as a stimulator of HR. The content of SA is much higher in the HR zone of tobacco leaves that are challenged with TMV compared to the HR-free area. The lesion exhibits a gradient, with the highest concentration of SA in the

middle and the lowest at the edge (Enyedi et al., 1992). The strong association between SA and HR suggests that SA plays a significant role in HR. While exogenous SA did not cause cell death in intact plants, it significantly speeds up HR-like programmed cell death in soybean suspension cells (Shirasu, Nakajima et al. 1997). The beneficial impact of SA on HR has been validated by utilizing mutants lacking SA. Two non-pathogenic strains of *Peronospora parasitica* do not trigger HR in various *Arabidopsis* mutants that lack SA, but tobacco plants deficient in SA exhibit a delayed HR in response to TMV (Mur, Bi et al. 1997, Nawrath and Métraux 1999). In addition, there are spontaneous cell death mutants known as *lsd6* and *accelerated cell death 11*. The individuals with the genetic variant *acd11* exhibit abnormally high levels of SA. The excessive expression of the bacterial SA hydrolase gene *nahG* in *lsd6* or *acd11* knockout mutants results in the complete removal of free SA and effectively prevents programmed cell death (PCD). The inhibition of programmed cell death (PCD) can be reversed by using SA agonists such as 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Weymann, Hunt et al. 1995, Brodersen, Malinovsky et al. 2005). The interaction between SA and NPR1 also contributes to the occurrence of HR. Unlike SA, NPR1 functions as a suppressor of HR. Although SA and NPR1 play a crucial role in HR, there is limited understanding of how they regulate HR.

Due to insufficient information, it is challenging to establish the precise sequence and causal relationship between the molecular events occurring during HR. Thus, it remains uncertain if HR is a genuine disease resistance mechanism plants employ to combat pathogens, or only an exaggerated form of physiological hypersensitivity triggered by pathogen infection.

2.1.6 Systemic acquired resistance

Systemic acquired resistance (SAR) refers to a plant's ability to develop resistance against pathogens across its entire system rather than only in the area directly affected by the pathogen. By the early 1900s, it was already extensively reported that plants could develop immunity against initial parasite infections. Plants develop acquired immunity, which allows them to resist reinfection effectively. In 1961, Ross used the phrase "systemic acquired resistance" to describe the phenomena where entire tobacco plants become immune to localized TMV infection and gain resistance to future reinfection (Ross 1961). Once activated, SAR can persist for a period ranging from weeks to months. SAR provides the host plant with resistance against a wide range of diseases. Simultaneously, the plant is prepared for a hypersensitive condition, enabling quicker and more powerful responses to subsequent pathogen infections (Conrath 2011).

2.1.6.1 Biological indicators

The process of characterizing SAR commenced with the exploration of biomarkers. The expression of a certain group of genes in tobacco is strongly associated with the induction of SAR. The SAR genes in question do not encompass all defense-related genes but represent a specific subset. The gene expression profile mentioned here is a distinctive marker of SAR induction and sets it apart from other plant physiological responses (Ward, Uknes et al. 1991). Other plant species, including tomato, wheat, and the model plant *Arabidopsis*, have gene expression patterns that are quite similar to those generated by SAR (Ryals, Neuenschwander et al. 1996). Most of these SAR genes encode PR proteins crucial for preserving disease resistance. During SAR, the activation of at least nine families of PR proteins significantly increases in tobacco. The *in vitro* antibacterial activity of these PR proteins has been proven, and the overexpression of some SAR genes protects against many infections in tobacco (Ryals, Neuenschwander et al. 1996). The

enhanced expression of SAR genes is strongly linked to the deployment of SAR. Collectively, these genes are regarded as indicators of SAR. The *PR-1* gene in *Arabidopsis* is commonly employed to assess the occurrence of SAR.

Distinct alterations in plant metabolism could potentially function as an indicator of SAR. For instance, SA is strongly linked to SAR. Following pathogen injection, there is an excessive accumulation of SA in both the infected and systemic tissue. Thus, it is proposed that SA is a SAR signal, as indicated by (Rasmussen, Hammerschmidt et al. 1991, Shulaev, Silverman et al. 1997). The genetic data unequivocally corroborate this. SA does not build up in either the *Arabidopsis* plants transformed with the *nahG* gene (referred to as NahG henceforth) or in the *SA induction-deficient 2* (*sid2*) mutants that lack a functional enzyme called *isochorismate synthase 1* (*ics1*), which is crucial for SA production. The lack of SA in *NahG* and *sid2* mutants leads to weak SAR formation and the inability to activate SAR genes (Friedrich, Lawton et al. 1996, Wildermuth, Dewdney et al. 2001). In addition, the administration of exogenous SA or SA agonists is enough to trigger the SAR response and activate the expression of SAR genes (Ward, Uknes et al. 1991, Uknes, Mauch-Mani et al. 1992, Vernooij, Friedrich et al. 1994). Collectively, these pieces of data suggest that the accumulation of SA is necessary for activating SAR. Therefore, SA is a legitimate chemical indicator, given its crucial function in SAR signaling (Fu and Dong 2013). The several crucial regulatory elements involved in the signaling pathways of SAR include NPR1, TGA, and WRKY transcription factors.

2.1.6.2 NPR1-mediated transcriptional reprogramming

Nonexpresser of Pathogenesis-Related Genes 1 (NPR1) is present in the cytoplasm as a group of molecules linked by disulphide bonds. These bonds are broken by thioredoxins (TRXs)

when triggered by signals such as SA, separating the molecules into individual units. NPR1, in its monomeric form, moves to the nucleus and binds with TGA transcription factors to initiate the transcription of PR genes. The phosphorylation of NPR1 is essential for its transcriptional activity. WRKY transcription factors can attach to the *NPR1* promoter to control its transcription, suggesting a feedback loop. The NPR1-TGA complex, in conjunction with the phosphorylation status of NPR1, stimulates the activity of RNA Polymerase II (Pol II) to initiate the transcription of certain genes, such as *PR1*, which are crucial for the plant's defensive mechanism. The NPR1 protein undergoes ubiquitination by the Skp, Cullin, F-box containing complex with Cullin 3 (SCF-CUL3) complex and subsequent degradation by the 26S proteasome (Mou, Fan et al. 2003, Spoel, Mou et al. 2009). This process prevents excessive accumulation of NPR1 and ensures the timely control of defensive responses. The system plays a crucial role in regulating the equilibrium between activating and suppressing plant immune responses.

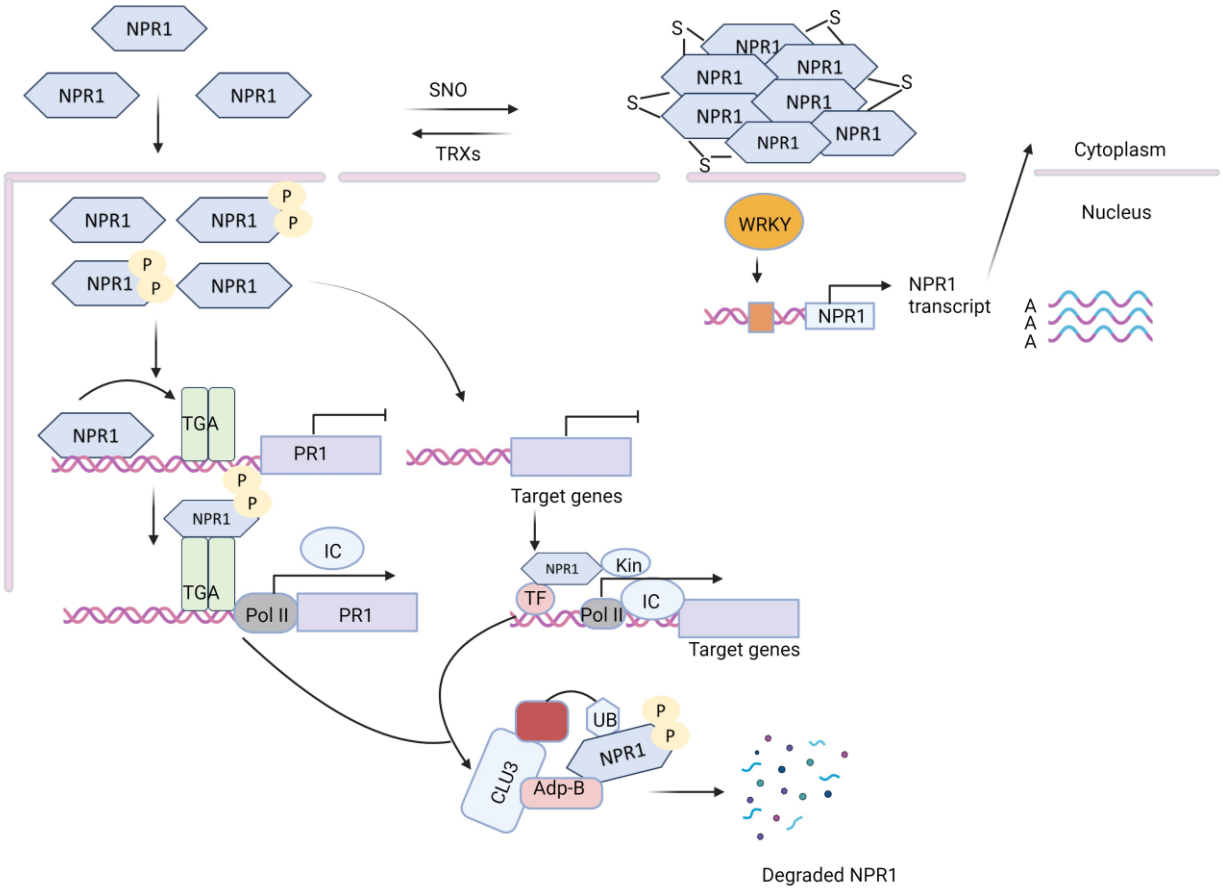


Figure 4. Schematic representation of the involvement of the NPR1 (Nonexpressor of Pathogenesis-Related Genes 1) protein in the plant's immune response. Phosphorylation is necessary for the function of NPR1, and WRKY transcription factors may regulate the transcription of NPR1. The NPR1 protein undergoes ubiquitination by the SCF-CUL3 complex and subsequent degradation by the 26S proteasome, which regulates defensive response. The immune system effectively defends against pathogens by maintaining a balance between activating and suppressing immunological responses (Mou et al., 2003; Spoel et al., 2009; Wang & Qian, 2022).

Given SA's essential role in SAR, there is a special interest in studying the SA signaling pathway. Genetic analysis was conducted to ascertain the crucial constituents of the SA-dependent signaling pathway. During the early 1990s, screenings were conducted to identify *Arabidopsis* mutants that exhibit insensitivity to SA (Cao, Bowling et al. 1994, Delaney, Uknes et al. 1994, Shah, Tsui et al. 1997). Remarkably, all of these SA-insensitive mutants, which were acquired by separate screenings, possess recessive mutations in a singular gene locus known as *NPR1/NIM1* (Non-inducible Immunity)/*SAI1* (Salicylic Acid-insensitive). The expression of SAR genes in the *npr1* mutants was not induced upon pathogen attack, demonstrating the crucial involvement of NPR1 in regulating SAR. SA can nevertheless build up in *npr1* mutants after inoculation, albeit somewhat greater than in plants with normal genetic makeup (Rasmussen, Hammerschmidt et al. 1991). This indicates that NPR1 acts as a pivotal controller of SAR following the signaling of SA.

The *Arabidopsis* NPR1 protein is composed of a plant-specific NPR1-like C-terminus, an N-terminal BTB/POZ (Broad complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) protein-protein interaction domain, and intervening ankyrin repeats (Rochon, Boyle et al. 2006). Due to the remarkable similarity between the ankyrin repeats and the mammalian transcription factor inhibitor IκB (Inhibitor of Kappa-light-chain-enhancer of Activated B Cells), it was initially hypothesized that NPR1 operates by interacting with transcription factors (Ryals, Weymann et al. 1997). This is corroborated by the necessity of nuclear localization of NPR1 for the expression of SAR genes triggered by SA. When NPR1 is confined to the cytoplasm, the expression of the marker gene *PR-1* is not induced by either SA or INA (SA agonist) (Kinkema, Fan et al. 2000).

Contrary to IκB, which keeps the transcription factor NF-κB in the cytoplasm to prevent gene transcription, NPR1 is responsible for activating or releasing genes in the nucleus. According to reports, NPR1-GFP fusion proteins are primarily found in the cytoplasm when SA is not present,

but they relocate to the nucleus when SA is present. The cytosolic NPR1-GFP proteins form oligomers by intermolecular disulfide bonds at the Cys82 and Cys216 sites. Following INA treatment, a portion of the NPR1-GFP oligomer is converted into active monomers, which then go into the nucleus and facilitate the transcriptional activation of *PR-1* (Mou, Fan et al. 2003). The redox state of NPR1 is regulated by many reducing and oxidizing agents, including GSH/GSSG, S-nitrosoglutathione, and thioredoxin (Mou, Fan et al. 2003, Tada, Spoel et al. 2008). Thus, the NPR1 oligomer undergoes continuous fluctuations with the monomers, which are affected by the particular redox circumstances in which NPR1 is present, as in uninfected *Arabidopsis* leaf cells (Després, DeLong et al. 2000).

A subset of transcription factors belonging to the bZIP protein family (TGA2, TGA3, TGA5, TGA6) has been discovered using yeast-two-hybrid screens. These transcription factors are known to interact with NPR1. They demonstrate a distinct and strong attraction to NPR1, as observed in the yeast system and *in vitro* pull-down tests (Zhang, Fan et al. 1999). NPR1 selectively interacts with these TGA transcription factors. Within yeast, TGA2 and TGA3 exhibit a significant interaction with NPR1, while TGA5 and TGA6 have a weak affinity for interacting with NPR1 (Zhang, Tessaro et al. 2003). Genetic studies have examined the involvement of TGAs in activating NPR1-dependent SAR genes at the transcriptional level. Disrupting a single *TGA* gene through knockout mutation in *Arabidopsis* does not result in any observable changes in the organism's physical characteristics due to the presence of redundant genes. Nevertheless, a mutant lacking the *tga2*, *tga5*, and *tga6* genes exhibits impaired SAR response after infection and inability to activate the *PR-1* gene following treatment with SA (Zhang, Tessaro et al. 2003). This provides evidence that NPR1 has a function in controlling SAR via recruiting TGA transcription factors.

TGA2/5/6 operate as inhibitors of the *PR-1* gene, as the production of PR-1 is approximately 50 times higher in the tga2/5/6 mutant compared to wild-type *Arabidopsis*. Additionally, the overexpression of TGA2/5/6 does not lead to the transcriptional activation of *PR-1* (Zhang, Tessaro et al. 2003). In addition, TGA2 inhibits the activation of a reporter gene in plant transcription tests, as demonstrated by (Rochon, Boyle et al. 2006). On the other hand, NPR1 contains a hidden transactivation domain at its C-terminus, indicating that NPR1 may be involved in activating genes. Contrary to expectations, NPR1 does not stimulate the activity of a reporter gene controlled by the *PR-1* promoter (Rochon, Boyle et al. 2006). Additional analysis uncovered the combined impact of TGA2 and NPR1 on the stimulation of *PR-1* gene expression. Individually, neither TGA2 nor NPR1 can cause the activation of the reporter gene controlled by the *PR-1* promoter. Nevertheless, the combined action of TGA2 and NPR1 induced the expression of the reporter gene in a manner that depended on SA. This suggests that TGA2 and NPR1 collaborate to create an enhanceosome on the *PR-1* promoter, activated by the presence of SA (Rochon, Boyle et al. 2006).

Based on gel filtration research, it is probable that the stoichiometry of this complex consists of two NPR1 molecules, two TGA2 molecules, and one DNA molecule. The interaction between the BTB/POZ domain of NPR1 and the N-terminus of TGA2 was confirmed by gel shift experiments. This interaction specifically enables the binding of the TGA2 dimer to the *PR-1* promoter by preventing other forms of TGA2 from binding to its corresponding DNA sequence (Rochon, Boyle et al. 2006, Boyle, Le Su et al. 2009). The enhanceosome stimulates the expression of the *PR-1* gene, maybe via utilizing the transactivation domain of NPR1. This domain's transactivation activity depends on the presence of the Cys521 and Cys529 residues. Replacing

these two cysteine residues with serine (Ser) eliminates the activity of the transactivation domain (Rochon, Boyle et al. 2006).

Furthermore, it has been shown that TGA1, TGA4 and the TGAs listed above exhibit a binding affinity for NPR1 in *Arabidopsis* treated with SA. However, this interaction is not observed in yeast (Lindermayr, Sell et al. 2010). The likely cause of this mismatch is the variation in the redox state of TGA1 at the Cys260 and Cys266 residues. NPR1 interacts specifically with the reduced form of TGA1 rather than the oxidized form of TGA1, where Cys260 and Cys266 are involved in an intramolecular disulfide bond (Lindermayr, Sell et al. 2010). The substitution of Cys260 and Cys266 with asparagine (Asn) and serine (Ser) facilitates the interaction between NPR1 and TGA1, both in yeast and *in planta*. The presence of NPR1 greatly amplifies the ability of TGA1 to bind to its specific DNA binding sites (Lindermayr, Sell et al. 2010). Altogether, NPR1 acts as a transcriptional coactivator alongside TGA transcription factors to facilitate the transcriptional activation of SAR genes (Rochon, Boyle et al. 2006).

2.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are short chains of amino acids that can kill or inhibit the growth of microorganisms. AMPs are usually positively charged molecules with both hydrophobic and hydrophilic regions. They vary in size from 10 to 100 amino acids and possess a wide range of amino acid sequences. This diversity results from the host adapting to the various microbial pathogens in their environment (Zasloff 2002, Hancock and Sahl 2006, Nawrot, Barylski et al. 2014).

AMPs in mammals can be categorized into four distinct classes based on their three-dimensional structures, amino acid makeup, and number of disulphide linkages. The classes

mentioned are α-helical peptides, β-sheet peptides, extended peptides, and loop peptides (Boman 1995, Hancock and Lehrer 1998, Hancock and Sahl 2006). The initial two categories are the predominant AMPs discovered in animals. Animals typically generate numerous peptides from various groups to carry out overlapping or complementary actions to protect against various potential pathogens in different tissues (Hancock and Diamond 2000).

The α-helical peptide group is characterized by its α-helical structure, which exhibits a little bend in the center of the molecule as a distinctive structural trait (Powers and Hancock 2003). Some of the animal-derived peptides are cecropins from insects, porcine cecropin P1 from the swine gut, magainins from frog skin, and cathelin-associated peptides from the blood cells of humans, cattle, pigs, mice, rabbits, and sheep (Hancock and Lehrer 1998). The β-sheet peptide group is characterized by a β-sheet conformation and occasionally a secondary helical shape, which is stabilized by two to four disulphide bonds (Powers and Hancock 2003)This group includes defensins from different vertebrates and invertebrates, protegrins from pigs, and tachyplesins from horseshoe crabs (Hancock and Lehrer 1998, Hancock and Sahl 2006).

Plant AMPs all have the characteristics of an overall positive charge and the existence of disulphide bonds (Barbosa Pelegrini, Del Sarto et al. 2011). They can be categorized into seven families depending on their basic structure: defensins, lipid transfer proteins, hevein-like proteins, knottins, snakins, and cyclotides. Plants, unlike animals, do not have linear α-helix. AMPs with a helix structure are distinguished by the presence of either eight or six cysteines. They are present in the majority of plant groups. Plant defensins are characterized by eight or more cysteines with unique spacing and disulphide linkages that differ from thionins but are more similar to insect defensins (Terras, Schoofs et al. 1992).

Plant defensins and thionins differ in their structural composition. Thionins consist of two α-helices and two strands, whereas plant defensins consist of one α-helix and three β-sheets. Lipid transfer proteins (LTPs) consist of bigger molecules composed of 90 to 93 amino acids, which contain eight cysteine residues (Barbosa Pelegrini, Del Sarto et al. 2011). The thionins, defensins, and lipid transfer proteins categories of plant AMPs have received more extensive research attention than the hevein-like proteins, knottins, snakins, and cyclotides. Hevein-like AMPs and knottins are distantly related peptides that include either six or eight cysteine residues. These peptides can attach to chitin, a substance found in the exoskeleton of insects and crustaceans (Barbosa Pelegrini, Del Sarto et al. 2011). Snakins possess 12 conserved cysteines resembling a protein produced by a gibberellic-acid-stimulated transcript found in *A. thaliana* (Su, Han et al. 2020). Cyclotides are a class of short cyclic peptides consisting of 27 to 37 amino acids. They are distinguished by their unique structure, which includes a cyclic peptide connected by three disulphide linkages to form a cysteine-knot (Su, Han et al. 2020). Furthermore, some of the plant AMPs do not fall into any of these categories. The four cysteine-type AMPs, known for their tiny size, consist of MBP-1 from maize and Ib-AMP from *Impatiens balsamina* (Barbosa Pelegrini, Del Sarto et al. 2011). Like animal AMPs, the diverse spectrum of AMPs in plants enables them to function synergistically within the same tissues and complementarily in separate tissues or at various times. This allows plant defenses to effectively target a wider array of microorganisms, hence expanding their protective capabilities.

2.2.1 Mode of action of antimicrobial peptides

Regardless of their variety, all AMPs seem to operate by attacking microbial membranes using the AMP's cationic amphipathic structure. Eukaryotic AMPs with a positive charge are

typically drawn to prokaryotic membranes with a negative charge. These prokaryotic membranes do not have cholesterol and a high concentration of anionic phospholipids, unlike eukaryotic membranes with neutral phospholipids (He and Deber 2024). Due to their amphipathic nature, they can be incorporated into microbial membranes, which can immediately lead to membrane disruption through hole creation, membrane thinning, and/or alterations in membrane permeability. AMPs carry positive charges, often due to amino acid residues like arginine or lysine, while pathogens, including fungi, typically feature negatively charged components on their cell surfaces. Upon contact, electrostatic interactions between the positively charged AMPs and the pathogen's negatively charged surface components facilitate binding. Once bound, AMPs disrupt the integrity of the pathogen's cell membrane, leading to cellular content leakage, disturbance of essential processes, and, ultimately, cell death. In fungi, this interaction triggers an influx of calcium ions (Ca^{2+}), disrupting cellular signaling pathways crucial for growth and development, thereby inhibiting fungal proliferation, which enhances the antimicrobial effect of AMPs (Jackson and Heath 1993).

Modifications in the membrane's permeability can indirectly affect the entry of AMPs into the microbial cell and enable their interaction with targets inside the cell (Zasloff 2002, Hancock and Sahl 2006). Their interaction with membranes has been strongly linked to their biological activity. It is widely believed that the disruption of the cytoplasmatic membrane is their primary antimicrobial mechanism. AMPs can inhibit several cellular processes, including nucleic acid and protein synthesis, enzymatic activity, and cell wall synthesis (Yeaman and Yount 2003, Brogden 2005, Hancock and Sahl 2006, Jenssen, Hamill et al. 2006, Nicolas 2009). AMPs that interfere with intracellular processes must traverse the cytoplasmatic cell membrane to reach their site of action, highlighting the importance of peptide-membrane interaction in AMP activity. The vast

majority of studies on the mechanism of action of antimicrobials with microorganisms have centred on bacteria.

The structural properties are the major criteria essential for the way AMPs work. Their capacity to adapt amphipathic structures allows them to be integrated into the hydrophobic core of microbial membranes (Tossi, Sandri et al. 2000, Hancock 2001, Shai 2002, Jenssen, Hamill et al. 2006). Before accessing the cytoplasmic bacterial membranes, AMPs must pass through the bacterial envelope. This envelope is rich in negatively charged components, such as lipopolysaccharide (LPS) in Gram-negative bacteria and wall-associated teichoic acids in Gram-positive bacteria. Lipopolysaccharide (LPS) is the main constituent of the outer layer of the outer membrane in Gram-negative bacteria. It is commonly held together by divalent cations like Ca^{2+} and Mg^{2+}. Cationic peptides exhibit a higher attraction towards LPS compared to native divalent cations. As a result, they displace the divalent cations and induce a localized disturbance in the outer membrane of bacteria. This self-promoted uptake process enables the peptides to pass through the outer membrane more easily (Hancock 2001). Therefore, AMPs penetrate the cytoplasmic membranes of microorganisms and disturb their normal functioning.

Various hypotheses have been presented to explain the interaction and disruption of membranes by AMPs, which rely on interfering with the membrane to exert their antimicrobial activity. However, the exact mechanism of action remains unknown (Pouny, Rapaport et al. 1992, Huang 2006, Leontiadou, Mark et al. 2006), and the most prominent modes of action are the barrel-stave, carpet, toroidal pore, and disordered toroidal pore processes. These models consider the structural changes in the membrane due to AMP-mediated disruption, including the creation of pores, the destruction of cells, and the movement of peptides into the cytoplasm. Generally, these theories propose that AMPs initially bind with the negatively charged lipid-head groups of the

microbial cytoplasmic membrane. They accumulate alongside the lipid bilayer until they reach a critical concentration level. At this point, they spontaneously organize themselves into a route allowing permeation.

I.Barrel-stave design

This mechanism is believed to account for the construction of stable pores by a predetermined number of peptide molecules. Peptides bind to the membrane's surface, either as monomers or aggregates. Then, they insert perpendicularly into the lipid core of the membrane, enlisting additional monomers and spanning it to form a uniformly sized channel. Pore-forming amphiphylic AMPs have a well-ordered structure, with hydrophobic patches confronting the lipid tails and hydrophilic patches lining the pore. At low peptide:lipid (P:L) ratios, this model predicts membrane permeation to occur. This model has been postulated for the cyclic peptide gramicidin S (Yang, Harroun et al. 2001).

II.Carpet construction

This model predicts the membrane's detergent-like disintegration. Peptides accumulate in a carpet-like formation on the surface of the membrane, altering the fluidity and decreasing the membrane's barrier properties. At a certain threshold peptide concentration, the integrity of a membrane is compromised, leading to micellization. This mechanism predicts a high membrane coverage of peptides. It has been postulated that Dermaseptin S, cecropin, melittin, and ovisporin follow this mechanism (Brogden 2005, Jenssen, Hamill et al. 2006).

III.Toroidal pore design

This model predicts that AMPs will form transient, short-lived pore structures. Initially, peptides lie parallel to the interface of the outer leaflet of the membrane, with their polar regions interacting with the lipid-head groups and their hydrophobic regions being inserted into the membrane (snorkel effect). This interaction induces mechanical stress on the lipid bilayer, which thins and develops a local disorder of the lipid tails. Upon reaching a threshold peptide concentration, AMPs change their orientation from parallel to perpendicular, forming a cylindrical hydrophilic pore. In toroidal pores, lipids are curved inwards towards the pore in a spiral fashion, a continuous manner from the membrane's surface. The lifetime of these transient apertures is believed to vary. They open and close, and peptides end up in both bilayer leaflets. The barrel-stave and carpet mechanisms do not account for the phospholipid flip-flop and the translocation of the peptide into the cytoplasm, but this model does. Melittin, magainin, and LL-37 are AMPs believed to exert this mechanism (Brogden 2005, Jenssen, Hamill et al. 2006).

This mechanism accounts for transient apertures in which peptides adopt a less rigid conformation and orientation. This model, predicted by molecular dynamic simulations, has been proposed for magainin and melittin (Leontiadou, Mark et al. 2006, Sengupta, Leontiadou et al. 2008). It differs from the conventional toroidal pore in terms of the pore's geometry and the conformation of the peptides that compose it. While the toroidal model proposes a cylindrical pore with a defined number of helical peptides aligned perpendicular to the plane of the membrane, the disordered toroidal pore has a chaotic lumen shape formed primarily by the lipid head-groups and a diffuse distribution of peptides with less-defined conformations. The "Droste mechanism" has been postulated for melittin as an extrapolation of this model. This occurs when many disordered toroidal pores are generated nearby, resulting in the dissolution or micellation of the membrane (Sengupta, Leontiadou et al. 2008).

2.2.2 Antimicrobial peptides as biocontrol agents

The production of AMPs has been attributed to the inhibitory activity of several microorganisms that act against fungal and bacterial plant pathogens. They operate by disrupting the cell membranes of pathogens, thus preventing infections and supporting plant health (Tang, Tan et al. 2023).

In the study, pre-treatment maize leaves and stalks with ZmPep1 peptide upregulated the expression of several defense genes and significantly increased the resistance to leaf and stalk fungal pathogens (Huffaker, Dafoe et al. 2011). Similarly, pre-treatment of tomato plants with either CAP-derived peptide 1 (CAPE1), thanatin or synthetic peptides resulted in increased resistance to bacterial and fungal pathogens (Ijaz, Ejaz et al. 2017, Chen, Lin et al. 2023). The peptide maSAMP, developed by Invaio Sciences (https://www.invaio.com/), is utilized to manage citrus Huanglongbing, a highly damaging disease (Huang, Araujo et al. 2021). The peptide eradicates *Liberobacter asianticum Jagoueix*, the bacteria responsible for Huanglongbing illness, while also stimulating the plant's immune system to ward off future infections. The maSAMP's spi rapidly infiltrates the bacterial membrane, resulting in its lysis within a 30-minute timeframe. Due to the absence of efficient disease control solutions, maSAMP is anticipated to serve as a potent weapon. The PREtec technology, developed by the American Plant Health Care (PHC) company, was granted a patent in the United States in 2019 (https://www.planthealthcare.com). The immune-inducing peptides of this product, along with its combinations with other substances, have gained recognition for enhancing plant resistance against diseases and stress while also stimulating plant growth. All PREtec peptides are derived from natural proteins and undergo quick environmental degradation, resulting in the absence of any detrimental residues on the crop or the surrounding

environment. In 2021, PHC introduced PHC279 in Brazil, incorporating PREtec technology, and marketed it as Saori™. This chemical serves as a seed treatment to mitigate the occurrence of Asian soybean rust.

Recently, several immune induction peptides have been developed. The study conducted by (Hou, Wang et al. 2014) found that PIP1 and PIP2 can boost immune responses and increase resistance to pathogens in *Arabidopsis*. The study conducted by (Wen, Sun et al. 2021) found that *Nicotiana tabacum* NbPPI1 can activate the immune response and improve the plant's resistance against *Phytophthora*. The immunological signal peptide Zip1 derived from maize lowers the virulence of the maize smut fungus (Ziemann, van der Linde et al. 2018). The compound known as Inceptin plays a role in the defensive mechanisms of cowpeas and kidney beans against herbivores by stimulating the production of SA and jasmonic acid, which are plant hormones associated with defense responses (War, Taggar et al. 2018). Applying Inceptin to plants results in the production of volatile organic compounds, including indole and methyl salicylate. These compounds attract the natural predators of *Spodoptera frugiperda*, facilitating indirect defense. Thirty newly discovered CAPE1-like peptides, found in *Vitinaceae*, *Solanaceae*, *Fabaceae*, *Brassicaceae*, and *Nicotiana tabacum,* play a role in the defensive mechanisms of different plants (Huffaker, Pearce et al. 2006). The protein Pep-13, derived from the pathogen *Phytophthora sojae*, induces an immune response in parsley (Brunner, Rosahl et al. 2002).

Using transgenic technology to express AMPs in plants is a powerful and efficient approach to boosting the plant's resistance against phytopathogens. In wheat plants, overexpression of anti-fungal defensins from radish and wheat (RsAFP2 and TAD1, respectively) led to increased resistance to *Fusarium graminearum* and other pathogens (Li, Zhou et al. 2011). The coding DNA (cDNA) sequence of the target AMPs was fused with the carrier plasmids,

specifically pMON22659 and pSAI4, and then introduced into the target plant using *Agrobacterium*-mediated transformation. The researchers effectively introduced the alfalfa antifungal peptide (alfAFP) defensin into potato plants, leading to robust resistance against fungal infections like *Phytophthora cactorum* and *Fusarium solani,* as well as the bacteria *Erwinia carotovora* (Gao, Hakimi et al. 2000, Osusky, Zhou et al. 2000). This resistance was observed in both controlled greenhouse conditions and field experiments. The transgenic plants demonstrated a significant decrease of six times in the presence of fungi compared to the non-transgenic ones. Expression of diverse peptides derived from plants and animals in plants confers resistance against bacteria and fungi in multiple plant families. This resistance has been documented in tomato (Chen, Lee et al. 2014). Similarly, Snakin-1 from potato combats *Clavibacter michiganensis* and *Botrytis cinerea* through transgenic expression tested at the lab scale (Segura, Moreno et al. 1999). Transgenic lines of *Musa spp.* (banana) were created by expressing floral defensins from *Petunia hybrida*. These lines exhibited notable resistance against the filamentous fungus *Fusarium oxysporum f. sp. cubense,* as reported by (Ghag, Shekhawat et al. 2012). Snakin-2 (SN2) was produced in *Solanum lycopersicum* (tomato) plants, resulting in transgenic lines that showed improved resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) bacteria (Balaji and Smart 2012). Lactoferricin B, sourced from bovine origins, was produced in *Nicotiana tabacum* (tobacco). The transgenic plants produced showed enhanced resistance against bacterial and fungal infections (Buziashvili and Yemets 2023). The expression of PmAMP1, derived from *Pinus monticola* (western white pine), in *Brassica napus* (canola) resulted in enhanced resistance against *Alternaria brassicae, Leptosphaeria maculans,* and *Sclerotinia sclerotiorum* pathogens (Verma, Yajima et al. 2012). Researchers successfully incorporated the human cathelicidin antimicrobial peptide (hCAP18/LL-37) into *Brassica rapa* (Chinese cabbage), resulting in

genetically modified plants that exhibited different levels of resistance against bacterial and fungal diseases (Jung, Lee et al. 2012). The antimicrobial peptide SN1 derived from the *Solanum tuberosum* (potato) was produced in the wheat species *Triticum aestivum*. The transgenic wheat plants exhibited enhanced resistance to *Gaeumannomyces graminis* var. *tritici* (Almasia, Bazzini et al. 2008). Thanatin, AMP derived from the insect species *Podisus maculiventris*, was genetically engineered in *Arabidopsis thaliana* plants. The transgenic plants developed resistance to different types of plant-damaging fungi and bacteria. Additionally, Thanatin(S) demonstrated antifungal and antibacterial activity in laboratory tests (Rivero, Furman et al. 2012). According to (Bundo, Montesinos et al. 2014), rice seeds that have been genetically modified to contain a synthetic peptide Cecropin A, have shown resistance to both fungal and bacterial diseases. AMP obtained from the *pro-SmAMP2* gene of chickweed was genetically produced in *Solanum tuberosum* (potato), which provided increased resistance against phytopathogens. However, its effects were shown only in potato cultivars that were already resistant and not in susceptible potato cultivars (Vetchinkina, Komakhina et al. 2016). The synthetic peptide D2A21 was produced in the *Carrizo citrange* plants, which exhibited notable resistance to canker compared to the control plants (Hao, Zhang et al. 2017). LF chimera, obtained from bovine sources, was produced in tobacco. The protein extracts obtained from these genetically modified plants showed inhibitory properties against the growth of both clinical and phytopathogenic bacteria (Fukuta, Kawamoto et al. 2012). LL-37, a human antimicrobial peptide, was successfully produced in *Hordeum vulgare* (barley) and demonstrated notable antibacterial properties (Mirzaee, Holásková et al. 2021).

Plant-derived peptides, like tannins from *Sapium baccatum,* demonstrate efficacy against *Ralstonia solanacearum* in lab and greenhouse trials (Vu, Kim et al. 2017). Cecropin A, derived from the silkmoth *Hyalophora cecropia*, targets pathogens like *Fusarium oxysporum* and *Dickeya*

dadantii. This peptide has been tested using *in vitro* killing assays and transgenic expression, extending from lab to greenhouse scale trials (Cavallarin, Andreu et al. 1998, Bundo, Montesinos et al. 2014). AlfAFP from alfalfa is notable for its field-scale testing against *Verticillium dahlia* (Gao, Hakimi et al. 2000). Human-derived peptides, such as hCAP18/LL-37 target *Pectobacterium carotovorum*, were tested in lab-scale *in vitro* assays and transgenic experiments (Jung, Lee et al. 2012, Holásková, Galuszka et al. 2018). Snakin-1, derived from the potato species *Solanum tuberosum*, comprises 88 amino acid residues and has 6 disulphide linkages. It demonstrates antibacterial properties by quickly causing the clumping together of both Gram-positive and Gram-negative bacteria (Segura, Moreno et al. 1999). Snakin-2, derived from the same source, consists of 104 amino acid residues and has 6 disulphide bridges. These bonds enhance its biochemical stability, allowing it to withstand biotic and abiotic stresses. Additionally, Snakin-2 plays a crucial role in hormone crosstalk with abscisic acid (ABA) and SA, contributing to several physiological processes (Almasia, Bazzini et al. 2008). Antimicrobial Peptide 1 (AMP1_COCNU), Antimicrobial Peptide 2 (AMP 2), and Antimicrobial Peptide 3 (AMP 3) derived from *Cocos nucifer* (coconut) are composed of 9, 11, and 8 amino acid residues, respectively. These peptides do not include disulphide linkages and have been shown to possess antibacterial properties (Mandal, Dey et al. 2009). The Cycas Revoluta Anticancer Peptide 1 (Cr-ACP1) generated from the sago palm, *Cycas revoluta*, consists of 9 amino acid residues and lacks disulfide connections. It suppresses cell growth and triggers apoptosis in cancer-derived cell lines (Mandal, Migliolo et al. 2012). Pharbitis Nil AMP1 (Pn-AMP1) and Pharbitis Nil AMP2 (Pn-AMP2) are proteins found in *Ipomoea nil* (Japanese morning glory) consisting of 41 amino acid residues and 5 disulphide bonds which can bind to chitin and provide defense against fungal diseases and Gram-positive bacteria that contain chitin (Lee, Lee et al. 2003). *Fagopyrum esculentum* AMP1 (Fa-AMP1) and

Fagopyrum esculentum AMP2 (Fa-AMP2) are peptides derived from the buckwheat plant, *Fagopyrum esculentum;* each peptide consists of 40 amino acid residues and has 5 disulphide bonds. These peptides have demonstrated activity against plant pathogenic fungi and Gram-negative and Gram-positive bacteria (Fujimura, Minami et al. 2003). Ac-AMP1 and Ar-AMP are two proteins derived from the plants *Amaranthus caudatus* (love-lies-bleeding) and *Amaranthus retroflexus* (redroot pigweed), respectively. Ac-AMP1 consists of 29 amino acid residues and has 3 disulfide bonds, while Ar-AMP consists of 89 amino acid residues and also has 3 disulfide bonds; both proteins exhibit chitin-binding and antifungal activities (Broekaert, Mariën et al. 1992). AMP-1.1a, AMP-1.2a, and SmAMP3 peptides derived from the *Stellaria media* (chickweed) plants consist of 167, 167, and 35 amino acid residues, respectively. They also contain 7, 7, and 3 disulphide links; accordingly, it has been observed that the peptides possess antifungal properties (Gao, Hakimi et al. 2000). Cowpea Thionin (Cp-thionin II) derived from *Vigna unguiculata* (cowpea) consists of 46 amino acid residues and is characterized by the presence of 4 disulphide linkages. The peptide has antifungal and antibacterial properties (Franco, Murad et al. 2006). Thionin 2.4, derived from *Arabidopsis,* is composed of 134 amino acid residues and has 3 disulfide linkages. It has been found to possess antifungal properties (Asano, Miwa et al. 2013). The compound Cycloviolacin O2, derived from the plant species *Viola odorata*, specifically sweet violet, comprises 30 amino acid residues and has 3 disulphide linkages. It has been observed to possess antifungal and antibacterial properties (Ireland, Colgrave et al. 2006). Cycloviolacin O8, derived from the same source, consists of 118 amino acid residues and has 3 disulphide linkages. It demonstrates comparable antibacterial properties (Narayani, Chadha et al. 2017).

Synthetic peptides like MsrA1, targeting a wide range of pathogens, including *Phytophthora cactorum* and *Fusarium solani*, were evaluated both in lab and greenhouse settings

(Osusky, Zhou et al. 2000). Cyclic lipopeptides have been identified as the key factors enabling *Bacillus amyloliquefaciens* to control *Fusarium oxysporum* (Wang, Qiu et al. 2022).

Pep3, a cecropin-melittin hybrid, is active against two *Fusarium* species, *Phytophthora infestans* and *Thielaviopsis basicola* (Cavallarin, Andreu et al. 1998). The analogue of cecropin B, D4E1, inhibits *Thielaviopsis basicola*, *Verticillium dahliae*, *Fusarium moniliforme*, two species of *Phytophthora*, and the bacterial pathogens *Pseudomonas syringae* pv. *tabaci* and *Xanthomonas campestris* pv. *malvacearum* (Rajasekaran, Stromberg et al. 2001).

2.2.3 Defensin

The initial documentation of plant defensins occurred in wheat (*Triticum turgidum*) and barley (*Hordeum vulgare*) seeds (Höng, Austerlitz et al. 2021). They were classified as a novel constituent of the thionine family because of their comparable molecular weight, amino acid sequence, and cysteine count. Nevertheless, subsequent research (Bruix, Jiménez et al. 1993) unveiled variations in the disulphide bridge patterns, thereby establishing the dissimilarity between these two peptide families. They were reclassified as "plant defensins" after a comparative analysis of their structural and functional similarity to AMPs previously identified in mammals and invertebrates (Broekaert, Terras et al. 1995). Defensins are small cationic peptides consisting of 45–54 amino acid residues, distinguished by their signature of cysteines, capable of forming three to four disulphide bridges. Defensins found in plants possess a tertiary structure that is conserved. This structure comprises one α-helix and a triple-stranded antiparallel β-sheet, joined together by disulphide bridges to maintain compact form. A cysteine-stabilized α-helix β-sheet motif (CSα/β) is formed by these bridges (Mak and Jones 1976, Cornet, Bonmatin et al. 1995).

The function of defensins in plant defense mechanisms has been extensively documented. Multiple studies indicate that defensins constitute an essential component of the innate immune system in plants. Most characterized plant defensins exhibit a constitutive pattern of expression that is upregulated in response to pathogen attack, injury, and certain abiotic stresses (Sher Khan, Iqbal et al. 2019). The involvement of defensin peptides in plant defense is evident from various characteristics. Their distribution aligns with their hypothesized function as a defense mechanism. Significant roles in safeguarding germinating seeds and developing progeny have been attributed to their identification in various plant parts, including leaves, tubers, flowers, pods, and seeds (García-Olmedo, Molina et al. 1995). Furthermore, it has been observed that plant defensins exhibit localization in parenchyma cells, xylem, stomata, and stomata cells, among other peripheral regions (Kragh, Nielsen et al. 1995, Segura, Moreno et al. 1998). Identifying these peptides in various tissues is consistent with their potential defensive function, given that these locations represent the initial point of contact with a potential pathogen (Carvalho Ade and Gomes 2011).

Moreover, plant defensins also exhibit a wide range of antimicrobial activity when tested *in vitro*. Multiple reports present the generation of transgenic plants that constitutively express foreign defensins. These defensins are small cysteine-rich peptides that disrupt microbial cell membranes, providing an effective defense mechanism against fungal and bacterial infections. Therefore, they exhibit many biological activities, including antimicrobial, insecticidal, protein synthesis inhibitory, abiotic stress mediator, zinc tolerance, and digestive enzyme inhibitory properties (Carvalho Ade and Gomes 2011). As a result of their diverse biological activities, these defense peptides are categorized as promiscuous proteins (Franco 2011). To illustrate, consider the defensin family derived from *Vigna unguiculata*; various homologous forms of these

compounds have demonstrated activity as enzyme inhibitors, antifungals, and antibacterials (Franco 2011). Despite exhibiting various functionalities, the predominant antimicrobial effect of plant defensins is observed against fungi.

2.2.3.1. Action mechanism of defensins

The mechanism of action of antimicrobial defensins is attempted to be explained by two main hypotheses: the carpet model and the pore model. Both models depict defensins interacting with negatively charged molecules located at the cell membrane of pathogens, resulting in an augmentation of permeability. This, in turn, induces cell leakage and necrosis-induced cell death. The pore model demonstrates that peptides form oligomers, creating numerous pores in the cell membrane. In contrast, the carpet model emphasizes the formation of pores by multiple peptides in the membrane. On the contrary, an alternative theory posits that defensins function by interacting with phospholipids, thereby increasing ion permeability or potentially transporting these peptides to the intracellular environment rather than causing damage to the cell membrane (Wilmes, Cammue et al. 2011, Hegedüs and Marx 2013). Therefore, they can induce programmed cell death (PCD) and increase the accumulation of ROS.

It has also been shown that positively charged amino acid residues in loops and β-sheet regions are very important for killing fungi. A positive charge was found on the concave face of the VI β-turn that came from Rs-AFP1. This supports the idea of the electrostatic interactions between peptide and pathogenic fungi (De Samblanx, Goderis et al. 1997, Fant, Vranken et al. 1998). Upon structural study of plant defensins NaD1, the positively charged residues in the loop between β2 and β3 were very important for identifying pathogens and killing fungi (Lay, Brugliera et al. 2003). *In vitro* tests showed that MtDef4 antifungal activity came from its positively charged

residues, while alfAFP inability to kill filamentous fungi was due to its negatively charged residues. The antifungal activity significantly dropped when the positively charged residues were switched out for alanine in mutagenesis studies of the MtDef4 region RGFRRR. Also, MtDef4 had stronger antifungal effects and selectively absorbed SYTOX Green (SG), indicating damaged fungus membranes (Sagaram, El-Mounadi et al. 2013).

The mechanism of action of defensins is also associated with electrostatic interaction. Antifungal plant defensins like Phd1, Rs-AFP1, and VrD2 have an electrostatic surface area connected to the γ-core region, which is crucial for antifungal action. A sugarcane defensin called Sd5 has a hydrophobic core that is important for contact and membrane permeabilization. Studies of the structure of Sd5 showed that changes in the hydrogen bond distances in the β-sheet and α-helix regions make it easier for the membrane to connect and for vesicles to leak (Hein, Kvansakul et al. 2022).

Recently, research has shown that the shape of dimeric defensins has a big effect on how well they kill fungi (Song, Zhang et al. 2011). The pattern Arg36-Trp42-Arg40 in SPE10 from *Pachyrrihizus erosusis* is very important for making dimers and killing fungi. However, the NaD1 dimer has a β-sheet/β-sheet shape and still has antifungal action. This shows how plant defensins with positively charged surfaces can interact with fungal cell walls.

Defensins from *Nicotiana alata* (NaD1), *Petunia hybrida* (PhD1 and PhD2), and maize kernels (ZmESR6) are all different in how they are structured. One of them has an extra acidic C-terminal prodomain that might help target vacuoles or lessen harmful effects (De Coninck, Cammue et al. 2013). Using NMR and crystallography to study the structures of peptides from *Petunia hybrida* (PhD1), *Nicotiana alata* (NaD1), *Pachyrrhizus erosus* (SPE10), *Pisum sativum* (Psd1), *Raphanus sativus* (Rs-AFP1), and *Saccharum officinarum* (Sd5) have shown that they are

antifungal (Fant, Vranken et al. 1998, Almeida, Cabral et al. 2002, Janssen, Schirra et al. 2003, Lay, Brugliera et al. 2003). Antifungal defensins do not have any conserved amino acid sequences other than cysteine and glycine residues. However, they have a conserved γ-core signature linked to antifungal action because of the positively charged residues at the second β-turn (Pelegrini and Franco 2005, van der Weerden and Anderson 2013). The γ-core motif is important for killing microbes because it has two antiparallel β-sheets and a patched turn region (Yount and Yeaman 2004). It was proven through mutation studies that the antifungal action depends on positively charged amino acids in the γ-core (De Samblanx, Goderis et al. 1997, Fant, Vranken et al. 1998). The antifungal qualities of MtDef1 were due to positively charged amino acids in the γ-core region. These amino acids were not present in MtDef2, which is not antifungal (Spelbrink, Dilmac et al. 2004).

2.2.3.1. Antifungal properties of defensins

Rs-AFP2 exhibited the lowest protein concentration necessary to inhibit fungal growth by 50% (IC_{50}) when tested against *Pyricularia oryzae*. The IC_{50} varied between 0.08 µm and 5 M.

Four AMP isolated from *Clitoria ternatea, Dahlia merckii, Aesculus hippocastanum,* and *Heuchera sanguinea* (Ah-AMP1, Dm-AMP1, and Hs-AFP1, respectively) were evaluated against eight distinct fungi in the presence or absence of inorganic ions (Osborn, De Samblanx et al. 1995). When Ah-AMP1 was evaluated against *Cladosporium sphaerospermum, Leptosphaeria maculans,* and *Septoria tritici*, an IC50 value of approximately 0.1 M was determined.

The membrane permeabilization result is consistent with the findings of a substantial number of earlier investigations concerning the mechanism of action of plant defensins (Thevissen, Terras et al. 1999). Recently, it was reported that two peptides resembling plant defensins

possessed this disruptive ability. The initial one, derived from *Phaseolus vulgaris*, among other fungi, permeabilizes the membrane of *Mycosphaerella arachidicola* (Wu, Sun et al. 2011). It was reported that the second compound, derived from *Picea glauca*, inhibited the permeability of *Verticillium dahlia* membranes (Osborn, De Samblanx et al. 1995, Picart, Pirttilä et al. 2012).

The transgenic potato plants expressing AlfAFP, an antifungal peptide derived from *Medica sativa* that exhibited activity against *Verticillium dahliae*, provided enhanced resistance to this filamentous fungus (Gao, Hakimi et al. 2000). The IC_{50} value of 1 M for AlfAFP against *Verticillium dahlia* is approximately ten times greater than that of the previous AMP described in this study. Nevertheless, the efficacy of chemical methods in greenhouse and field conditions was surpassed by the resistance of transgenic potatoes expressing AlfAFP against *Verticillium dahliae*.

In contrast to certain findings that indicate the significance of the N-terminus in defensin activity, the carboxy-terminal region of a defensin derived from *Medicago sativa* (MsDef1) is the primary determinant of its antifungal activity (Almeida, Cabral et al. 2002, Spelbrink, Dilmac et al. 2004). Six distinct defensin chimeras derived from molecular combinations of MsDef1, which are active against *Fusarium graminearum*, were assessed. Additionally, they examined MtDef2, a defensin derived from *Medicago truncatula*, which exhibited no inhibitory effect on F*usarium graminearum.* Out of the six chimeras examined only those that contained the MsDef1 segment at their C-terminus exhibited any discernible activity against *Fusarium graminearum* (Spelbrink, Dilmac et al. 2004). The discrepancy between results indicating that the C-terminus and N-terminus are crucial for plant defensin activity demonstrates the lack of clarity regarding the relationship between the structure and function of such molecules and, furthermore, regarding the mechanism by which they modulate activity.

2.2.4 Antifungal activity of thionins

Anti-fungal γ-thionins are more numerous when compared to bactericidal γ-thionins (Vigers, Roberts et al. 1991, Selitrennikoff 2001, Li, Hu et al. 2021). However, some anti-fungal γ-thionins also present toxicity to other organisms. One example is observed in peptides isolated from bulbs of tulip and buckwheat, already described before (Fujimura, Ideguchi et al. 2004). These peptides showed activity against bacteria and fungi (*Fusarium oxysporum* and *Geotrichum candidum*), although their anti-fungal activity is higher than anti-bacterial. Anti-fungal mechanism of action seems to be occasioned by an interaction with a specific receptor as an ion channel or a sphingolipid (Vriens, Cammue et al. 2014). Electrostatic interaction of γ - thionins-cell membrane could be reduced by divalent cations, such as Ca^{2+} (Lay, Brugliera et al. 2003). Hordothionins from barley can interact with the fungal lipid bilayer, linking to the membrane surface (but not inserting into it), leading to permeabilization and disrupting the membrane organization. Consequently, the fungicide mechanism may not occur through a direct protein-protein interaction but via lipid membrane receptor (Thevissen, Terras et al. 1999). One possibility was described recently where γ-thionins might bind to glycolipids at the fungal membrane surface (Thevissen, Warnecke et al. 2004). In this case, glycolipids will work as membrane receptors despite two or more proteins being involved, and consequently, a pore will be formed, leading to ion influx/efflux. This mechanism, which blocked ion Ca^{2+} influx through fungi cells, was observed (Spelbrink, Dilmac et al. 2004). Glucosylceramides and sphingolipids are frequently described as cell membrane receptors for γ-thionins. It was demonstrated that glycolipids knockout increases fungi resistance against γ-thionins deleterious effects (Kuźniak and Gajewska 2024). Otherwise, it was observed that fungi susceptibility might be related to specific sphingolipid mannosyl diinositolphosphoryl ceramide biosynthesis. Yeast mutants revealed that the expression of two

genes involved in sphingolipids biosynthesis, *IPT1* and *SKN1*, increased susceptibility to γ-thionin DmAMP1 (Manzanares, Giner-Llorca et al. 2024). Another poorly answered question is how the pores are maintained open into the fungal cell membranes. The repulsion of γ-thionins positive charges or their hydrophobic interaction with the phospholipids bilayer could facilitate this process. Consequently, if the positive charges of γ- thionins maintain an open pore, ions potassium and/or calcium will be repulsed to cells outside, as observed in the proposed mechanism of action scheme. The attraction of γ-thionins to fungi membrane receptors could also be made by ionic interaction between γ-thionin side chain residues and charged glycolipids from the cell surface (Almeida, Cabral et al. 2002). Other purothionins also demonstrate a wide range of anti-fungal activity. Purothionin purified from wheat was shown to be capable of killing both fungi and mammal cells by forming monovalent cation-selective ion channels in cell membranes, affecting the osmotic permeability of fungal cells (Hughes, Dennis et al. 2000). Purothionins have also presented lytic activity against *Rhizoctonia solani*, alone or in association with other anti-fungal proteins (Oard, Rush et al. 2004). Cell disruption increases the possibility of lyses and, consequently, enhances cell death. Ion channels can also be formed by 1-, 2-Purothionins and 1-hordothionin in artificial bilayers and biological membranes, promoting similar cell damage in fungi (Hughes, Dennis et al. 2000). Likewise, a peptide isolated from barley grain (BCP-2) appears to have great similarity to thionin from barley, having anti-microbial activity, especially against fungi. BCP-2 bounds to constitutive polysaccharides, such as glucosylceramides and sphingolipids, from fungal cell walls (Oita, Ohnishi-Kameyama et al. 2000), but these interactions are not very well understood.

3. HYPOTHESIS

We hypothesize that antibacterial peptides lessen the severity of leaf rust (*Puccinia triticina*) disease in wheat (*Triticum aestivum* L.) by directly inhibiting the urediniosporesgermination and activating wheat endogenous pathogen-responsive genes.

4. OBJECTIVES

1. Screen antimicrobial and antifungal peptides *in vitro* for their ability to suppress the leaf rust urediniospores germination.

2. Test response of wheat plants pre-treated with the most promising peptides to leaf rust infection.

3. Test the expression level of pathogen response following the foliar application of selected peptides.

5. MATERIALS AND METHODS

5.1 Selection of candidate peptides for antirust activity screening

Literature sources were examined for suitable peptide candidates with proven antifungal/antibacterial properties in cereals and other plant species (Hammami, Ben Hamida et al. 2009). Nineteen promising candidates were synthesized (CanPeptide, QC, Canada), and most peptides had purity above 90%, as confirmed with HPLC, mass spectrometry, and PAGE analysis (Table 1).

Table 1. List of Antimicrobial peptides used for the study.

Antifungal/ antibacterial peptides selected for this study	Species origin	Peptide family	Peptide function	Sequence length, AA	Purity of the synthetic peptide, %	References
Alliumin	*Allium sativum*	Alliumin	Antibacterial, Anticancer, Antifungal	10	96.64	(Xia and Ng 2005)
β-purothionin	*Triticum aestivum*	Thionin	Antibacterial, Antifungal, Antiyeast, Toxin	45	96.83	(Fernandez de Caleya, Gonzalez-Pascual et al. 1972)
CaThi	*Capsicum annuum*	Thionin	Antifungal	59	95.96	(Taveira, Mello É et al. 2017)
Chain A	*Triticum kiharae*	NA	Antibacterial, Antifungal	43	91.19	(Odintsova, Vassilevski et al. 2009)
Coccinin	*Pisum sativum*	NA	Antifungal, Antiproliferative HIV-1-reverse-transcriptase-inhibition	10	98.8	(Ngai and Ng 2004)
Cp-thionin II	*Vigna unguiculata*	Defensin	Antibacterial	46	95.21	(Schmidt, Arendt et al. 2019)
Defensin TM-AMP D1.2	*Triticum monococcum*	Defensin	Antimicrobial, Antifungal	49	90.47	(Odintsova, Egorov et al. 2007)

Defensin-2	*Pisum sativum*	Defensin	Antifungal	47	95.3	(Almeida, Cabral et al. 2000)
Ep-AMP1	*Echinopsis pachanoi*	Cyclotide	Antibacterial, Anticancer, Antifungal	35	97.4	(Aboye, Strömstedt et al. 2015)
Kalata B2	*Oldenlandia affinis*	Cyclic Peptide	Antibacterial, Anticancer, Antifungal, Nematocide, Molluscicidal, Membrane-Binding, Insecticidal, Hemolytic, Antiviral, Antiparasitic	29	95.45	(Craik, Daly et al. 1999)
MBP-1	*Zea mays*	Hairpin-like peptide	Antimicrobial, Antibacterial, Antifungal	33	92.14	(Duvick, Rood et al. 1992)
NCR044	*Medicago truncatula*	Cysteine-rich	Antifungal	36	96.67	(Velivelli, Czymmek et al. 2020)
Psacotheasin	*Psacothea hilaris*	Knottin-type	Antimicrobial	34	94.37	(Hwang, Hwang et al. 2010)
Purothionin-α2	*Triticum aestivum cv. Maniton*	Thionin	Toxin, Antifungal	45	91.88	(Castagnaro, Maraña et al. 1994)
Rs-AFP2	*Raphanus sativus*	Defensin	Antifungal	51	96.09	(Aerts, François et al. 2007)
Systemin	*Lycopersicon esculentum*	NA	Defense gene activator	18	97.22	(Pearce, Strydom et al. 1991)
Tk-AMP-X1	*Triticum kiharae, Triticum aestivum, Aegilops tauschii, Triticum timopheevii*	Hairpin-like peptide	Antifungal	31	98.57	(Utkina, Andreev et al. 2013)
WAMP-1a	*Triticum kiharae, Triticum aestivum, Aegilops tauschii*	Hevein	Antibacterial, Antifungal	44	86.54	(Odintsova, Vassilevski et al. 2009)
ZmD32	*Zea mays*	Defensin	Antibacterial, Antifungal	47	93.08	(Kerenga, McKenna et al. 2019)

5.2 Evaluation of the antirust activity of selected peptides in *in vitro* study

The fungicidal activity of peptides was evaluated in an *in vitro* experiment through the germination of leaf rust (*Puccinia triticina*, CCDS isolate) urediniospores on 2% solid water-based agar medium supplemented with peptides at a concentration of 100 µg/ml. Special cavity slides were layered with a 2% water-based agar solution supplemented with peptides. Urediniospores were heat shock treated at 40°C for at least 6 minutes before use to promote germination. Following agar solidification, the spores were applied to the medium. Slides were kept covered in Petri dishes to maintain humidity at room temperature. A nonanol solution was also applied to the filter paper placed in the lid to increase the germination rate. After 4 hours, pictures were taken using a Leica MZF LIII stereomicroscope equipped with a Leica MC170 HD camera and compared to the spore's germination rate without peptides. Germinated and non-germinated spores were manually counted via LAS v 4.5 software. Three of the most promising peptides (β-purothionin, Purothionin-α2, Defensin-2)were further evaluated in the dose-dependent response experiment with three concentrations – 25, 50 and 100 µg/ml. Each experiment was repeated at least twice, and the average percent of germination was calculated for five field views.

5.3 Assessment of leaf rust infection severity following foliar application of the peptides

To promote the adhesion of peptides to the wheat leaves' surface, they were diluted in 0.5% water-based industrial grade methylated seed oil (MSO™ concentrate with Leci-Tech, Loveland Products Canada Inc.) surfactant solution at 100 µg/ml (Velivelli, Czymmek et al. 2020). Leaves of the 2-weeks-old wheat seedlings, cultivar Fielder (cv. Fielder), were sprayed with peptide solution (around 400 µl) 24 hours before inoculation with leaf rust spores (*Puccinia triticina*,

isolate CCDS) and 24 hours post-infection. Infection with rust spores was done using a spray applicator by randomly combining the peptide-treated and non-treated plants in groups (technical replicates). The plants were maintained in the dew chamber for 24 hours to maintain humidity. Fourteen days after infection (DAI), the infection types and severity were scored using the infection scale from (Park and Karakousis 2002), and leaves were collected for gDNA isolation and fungal genomic DNA (gDNA) quantification using quantitative PCR (qPCR). Untreated and mock-treated plants (0.5% MSO, cv. Fielder) were used as controls. The experiment was repeated three times (biological replicates) with nine plants per treatment group (technical replicates).

5.4 qPCR quantification of gene expression and rust gDNA in infected wheat plants

To quantify gene expression, total RNA was isolated from leaves of seedlings treated either with 0.5% MSO (mock treatment), selected peptides in 0.5% MSO or no-treatment control using Nucleospin RNA Plant Kit (cat. # 740949, Macherey-Nagel, Germany) according to the manufacturer's instructions. The total RNA was treated with DNase I (RNase-free, cat. # M0303L, NEB), and the absence of gDNA contamination was verified with PCR and *TaGA3PD* primers (Table 2). For every duplex reaction, 50 ng of RNA was combined with 1xLuna Universal Probe One-Step Reaction Mix (cat. # E3006; NEB), 400 nM of each primer pair (for the target and reference gene), 200 nM of each probe (target and reference gene, table 2) and 1xLuna WarmStart® RT Enzyme Mix in 20 µl final reaction volume. The probes were either 5′ FAM (6-fluorescein)- or 5′ HEX (hexachloro-fluorescein) labelled and contained ZEN and Iowa Black Hole Quencher 1 (Integrated DNA Technologies, Coralville, IA, USA). qPCR was run on CFX96 Real-Time PCR Detection System (Bio-Rad). The reverse transcription was done at 55°C for 10 min followed by initial denaturation at 95°C for 1 min and 40 cycles with 95°C for 1 min and 60°C

for 30 sec. *TaGA3PD* (TraesCS7A02G313100, TraesCS7B02G213300, and TraesCS7D02G309500) was used as an endogenous reference (Garrido, Aguilar et al. 2020). At least four plants were used for analysis for every group, and reactions were run in duplicates. Average $2^{-\Delta\Delta CT}$ was calculated using CFX manager, v. 3.1 (Bio-Rad).

Pt gDNA was quantified in infected leaves using qPCR. Total gDNA was isolated from non-infected and infected leaves using the Warner et al. method (Warner, Karakousis et al. 2002). The qPCR sample reaction contained 50 ng of gDNA, 1x Luna® Universal Probe qPCR Master Mix (cat. #M3004L; NEB), 400 nM of each primer pair (specific for the *Pt* and wheat genome, respectively), 200 nM of each probe (Suppl. table 1). The *Pt*- and wheat-specific primers/probes were designed to amplify single copy genes *PtRTP1* (Pt15 chromosome 18B, GenBank: CP110454.1 (Panwar, Jordan et al. 2018)) and *TaPUROINDOLINE-b* (*TaPINb-D1b* gene (Collier, Dasgupta et al. 2017)), respectively. Similarly, the probes were 5′ FAM- or 5′ HEX-labeled, and the same cycling conditions were used, except for the initial reverse transcription step. Normalization was done against the *TaPINb-D1b* gene with four independent plants/treatment groups and two technical replicates. Average $2^{-\Delta\Delta CT}$ was calculated using CFX manager, v. 3.1 (Bio-Rad).

5.5 Estimation of MIC, IC_{50} and statistical analysis of the data

Calculation of the minimal inhibitory concentration (MIC) and inhibitory concentration 50 (IC_{50}) was done using the *drc* package in R (Ritz, Baty et al. 2015). A significant difference between treatment groups was validated using an unpaired Student's t-test with $p < 0.05$. Below we provide the schematic outline of this study (Figure 5).

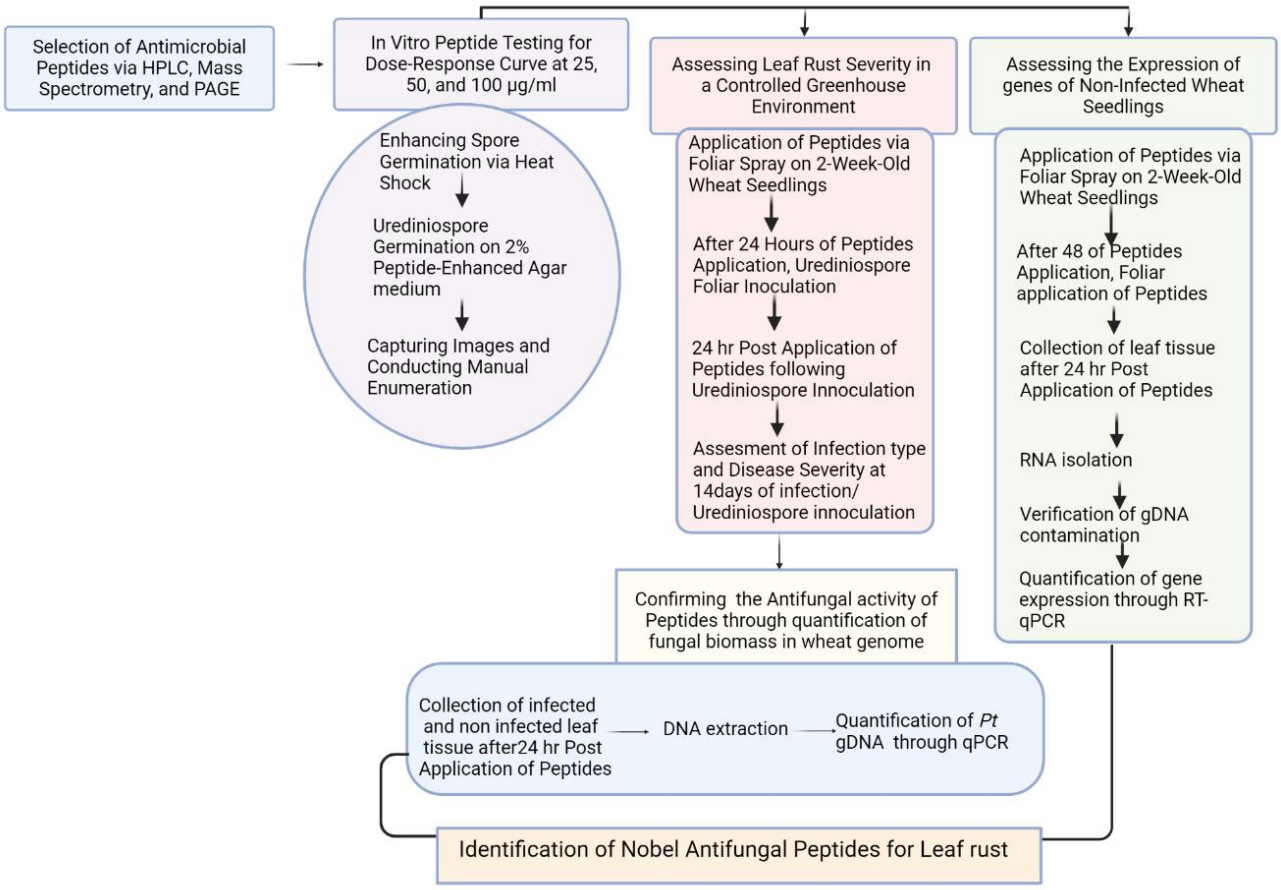


Figure 5. The outline of the procedure used to identify antifungal peptides against leaf rust infection (*Puccinia triticina*) in bread wheat (*Triticum aestivum* L.). Initially, antimicrobial peptides selected from the literature search were synthesized (CanPeptide, QC, Canada) and the quality was verified by High-Performance Liquid Chromatography (HPLC), Mass Spectrometry, and Polyacrylamide Gel Electrophoresis (PAGE). These peptides were then tested in vitro at concentrations of 25, 50, and 100 µg/ml. The testing involved enhancing spore germination through heat shock, followed by germination of urediniospores on a 2% peptide-containing agar medium. The germination process was captured through images and manually enumerated to assess peptide effectiveness. Peptides were applied as a foliar spray on two-week-old wheat seedlings. After 24 hours, the plants were inoculated with urediniospores, and the severity of leaf

rust infection was assessed 24 hours post-inoculation and again at 14 days post-infection. The antifungal activity is confirmed by qPCR through the amplification of single-copy genes PtRTP1 (Pt15 chromosome 18B, GenBank: CP110454.1 (Panwar, Jordan et al. 2018)) and TaPUROINDOLINE-b (TaPINb-D1b gene (Collier, Dasgupta et al. 2017)) for leaf rust and wheat, respectively. Additionally, gene expression in non-infected wheat seedlings was assessed by applying peptides, followed by RNA isolation, verification of gDNA contamination, and quantification of gene expression through Reverse Transcription quantitative PCR (RT-qPCR).

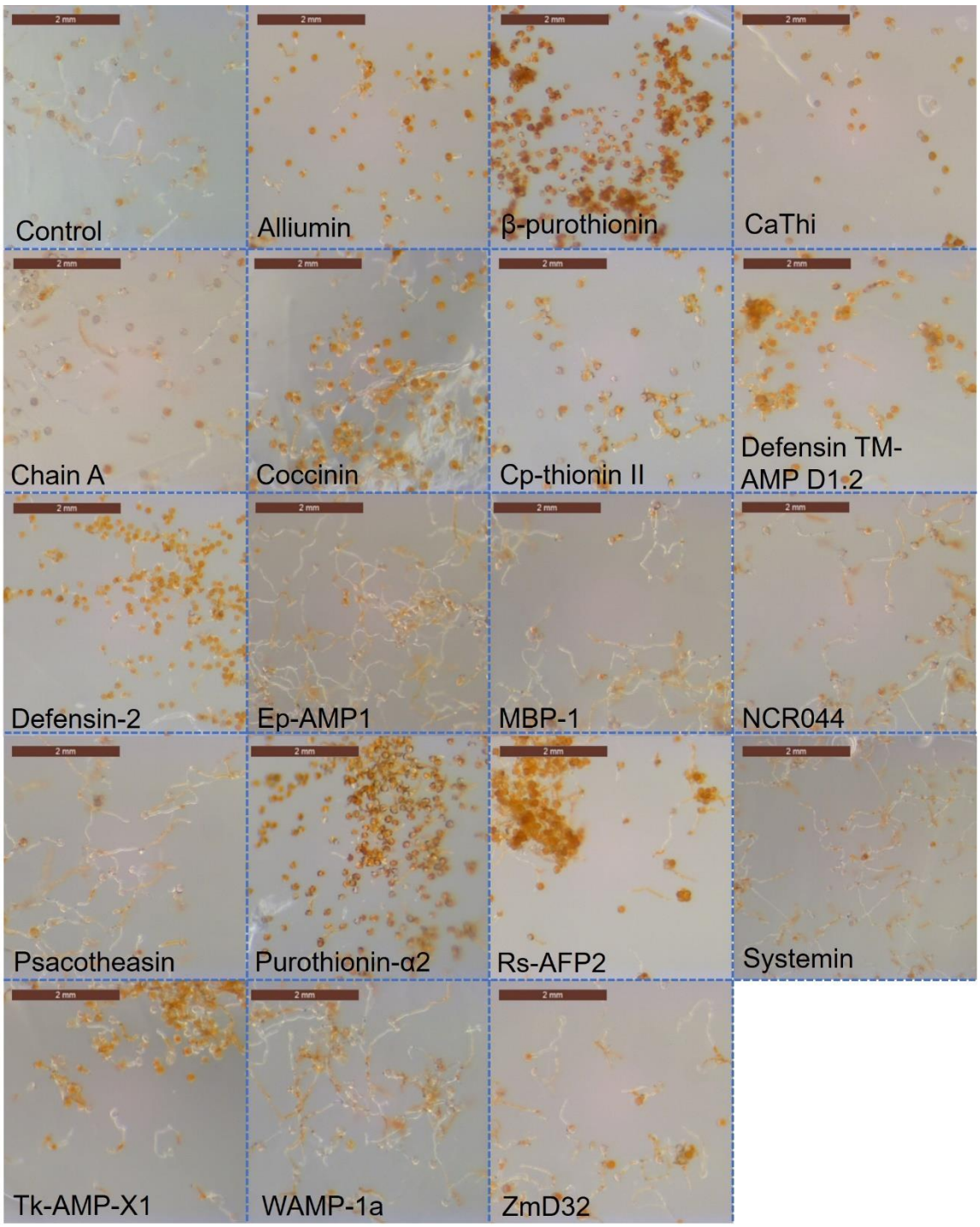
6. RESULTS

Eighteen synthetic peptides (Figure 6 and Table 1) of plant origin and one from the yellow-spotted longicorn beetle *Psacothea hilaris* were selected to evaluate antirust properties in an *in vitro* experiment. The peptides represented different families, including alliumin, thionins, defensins, cyclic peptides, hairpin-like peptides, cysteine-rich, knottin-type, and hevein. The peptides were chemically synthesized (CanPeptide, QC, Canada) with a purity above 90%.

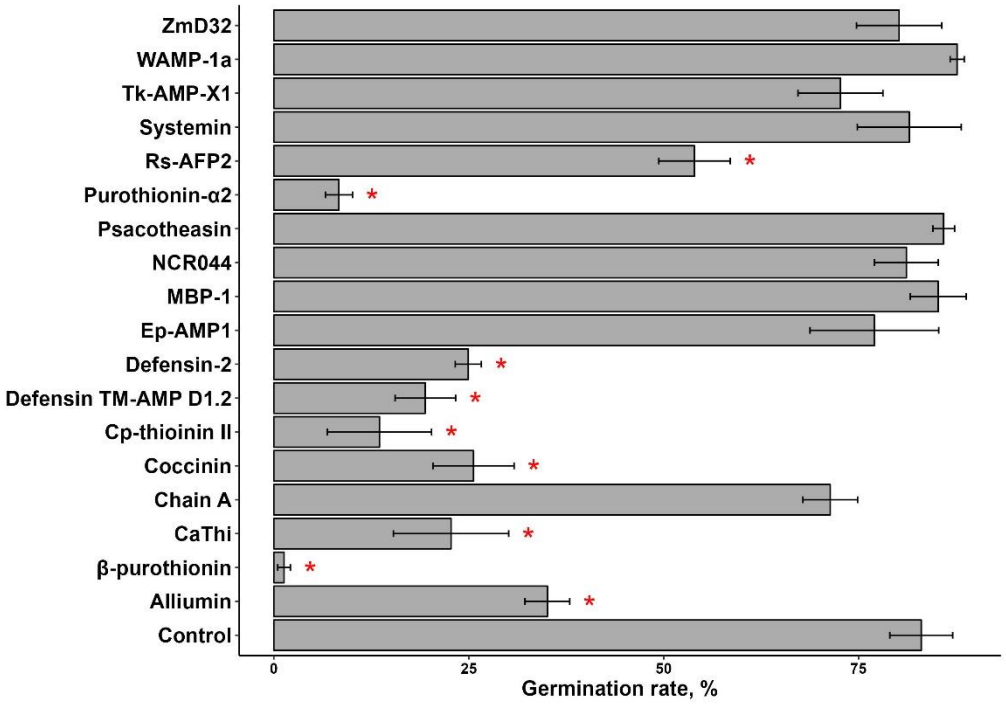
6.1 Evaluation of antirust properties of selected peptides in *in vitro* assay

Leaf rust (CCDS isolate) urediniospores were germinated on a 2% water-based agar medium supplemented with selected peptides at a final 100 µg/ml concentration. Germination of urediniospores was evaluated four hours post-treatment and compared to the control treatment without peptides (Figure 6A). Nine peptides demonstrated significant fungicidal properties compared to the control ($p < 0.05$, unpaired Student's t-test, Figure 6B).

A


Control
Alliumin
β-purothionin
CaThi
Chain A
Coccinin
Cp-thionin II
Defensin TM-AMP D1.2
Defensin-2
Ep-AMP1
MBP-1
NCR044
Psacotheasin
Purothionin-α2
Rs-AFP2
Systemin
Tk-AMP-X1
WAMP-1a
ZmD32

B


ZmD32
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Systemin
Rs-AFP2
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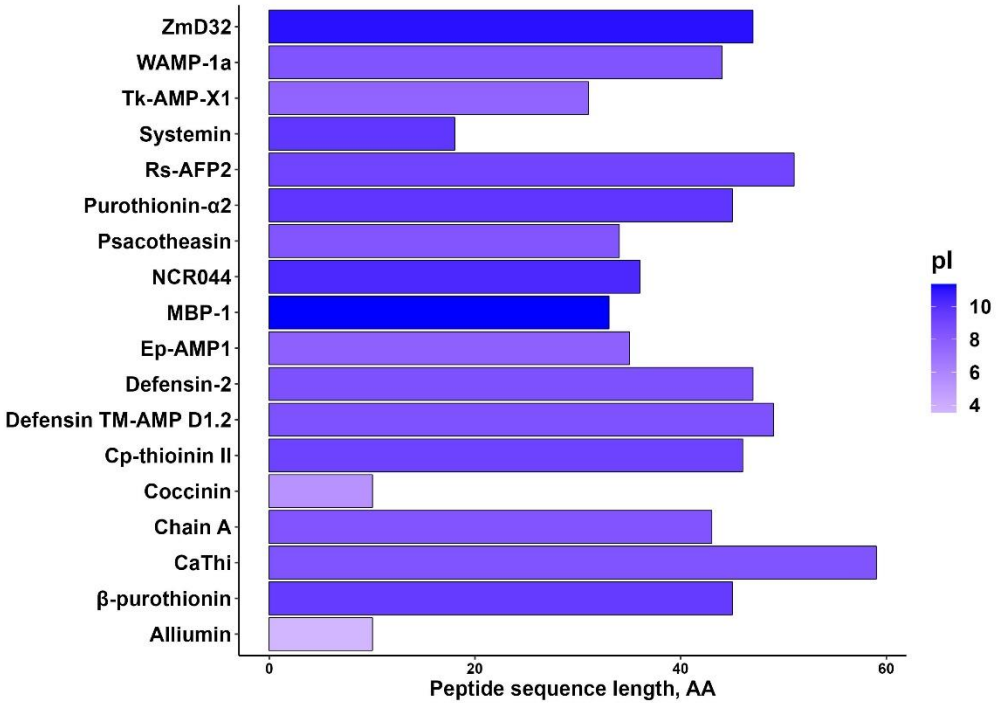
Psacotheasin
NCR044
MBP-1
Ep-AMP1
Defensin-2
Defensin TM-AMP D1.2
Cp-thioinin II
Coccinin
Chain A
CaThi
β-purothionin
Alliumin
Control
0
25
50
75
Germination rate, %

C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WAMP-1a
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Systemin
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Purothionin-α2
Psacotheasin
NCR044
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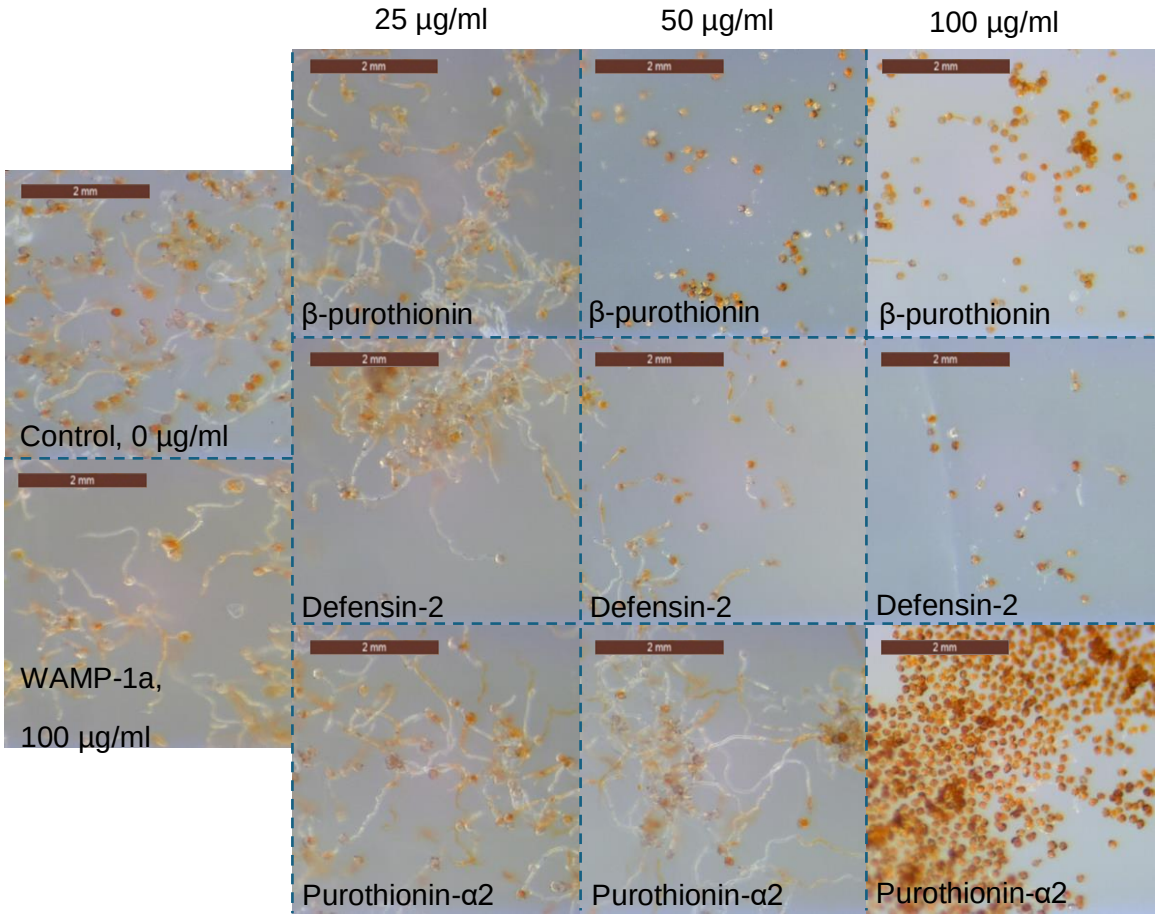
Ep-AMP1
Defensin-2
Defensin TM-AMP D1.2
Cp-thioinin II
Coccinin
Chain A
CaThi
β-purothionin
Alliumin
0
20
40
60
Peptide sequence length, AA
pI
10
8
6
4

Figure 6. Effect of selected peptides on germination of leaf rust (*Puccinia triticina*, CCDS isolate) urediniospores on 2% solid agar medium. (A) shows the representative images of the leaf rust spores germinated on agar medium either without (Control) or with peptides (100 µg/ml). Photos were taken 4 hours after spores germination at room temperature. The scale bar is 2 mm. (B) shows the average spore viability on the solid agar media supplemented with different peptides at 100 µg/ml. At least five measurements were used to calculate the mean values, and error bars represent the standard error. * - $p < 0.05$ as compared to Control (unpaired Student's t-test). (C) shows the length distribution of tested peptides and predicted isoelectric points (pI - colour gradient for isoelectric point).

We selected β-purothionin and Purothionin-α2 peptides, which demonstrated the highest antirust activity (germination rate, 1.31% ± 0.81 and 8.35% ± 1.74, respectively), a peptide with the average activity, Defensin-2 (19.43% ± 3.88) and one peptide with minimal inhibitory activity, WAMP-1a (87.63% ± 0.90) for further evaluation. We tested the dose-dependent response of the rust germ tube elongation for the first three peptides and observed the concentration-dependent inhibition of spores germination *in vitro* (Figure 7).

A


25 µg/ml
50 µg/ml
100 µg/ml
β-purothionin
β-purothionin
β-purothionin
Control, 0 µg/ml
Defensin-2
Defensin-2
Defensin-2
WAMP-1a,
100 µg/ml
Purothionin-α2
Purothionin-α2
Purothionin-α2

B

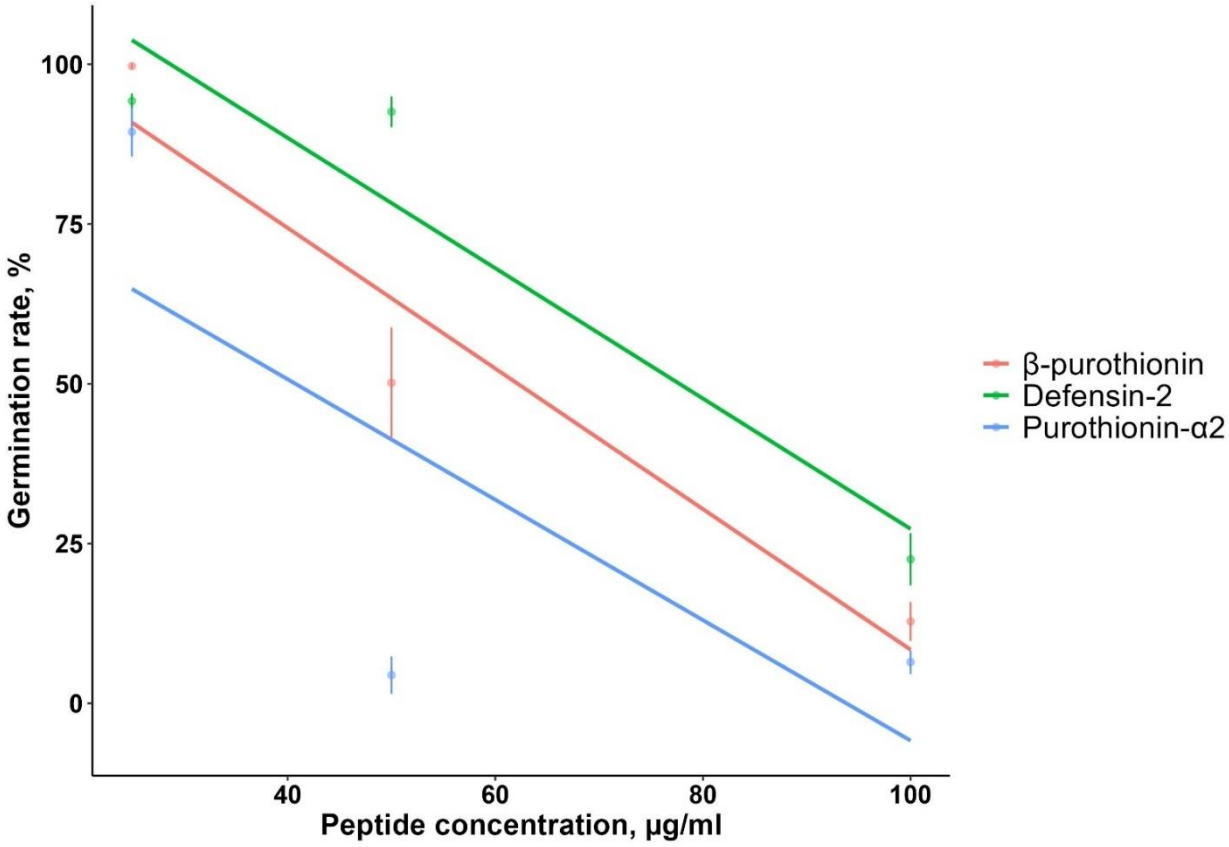

100
75
50
25
0
Germination rate, %
40
60
80
100
Peptide concentration, µg/ml
β-purothionin
Defensin-2
Purothionin-α2

Figure 7. Dose-response effect of selected peptides on germination of leaf rust (*Puccinia triticina*, CCDS isolate) urediniospores on 2% solid agar medium. (A) shows the representative images of the leaf rust spores germinated on agar medium either without (Control) or with selected peptides at three different concentrations. The WAMP-1a peptide at 100 µg/ml was used as a peptide control. Photos were taken 4 hours after spores were applied to the media at room temperature. The scale bar is 2 mm. (B) shows the dose-response graph of germinated spores on the solid agar media supplemented with selected peptides at three different concentrations – 25, 50, and 100 µg/ml. At least five measurements were used to calculate the mean values, and error bars represent the standard error.

β-purothionin demonstrated the highest activity (IC_{50} = 28.4 µg/ml, MIC = 43.5 µg/ml) followed by Defensin (IC_{50} = 46.5 µg/ml, MIC = 227.6 µg/ml) and Purothionin-α2 (IC_{50} = 82.2 µg/ml, MIC = 197.7 µg/ml). Similar to the previous reports (reviewed in (Lintz, Dubrulle et al. 2022)), the inhibitory effect of selected peptides on the rust spores' germination was observed at a concentration above 25 µg/ml.

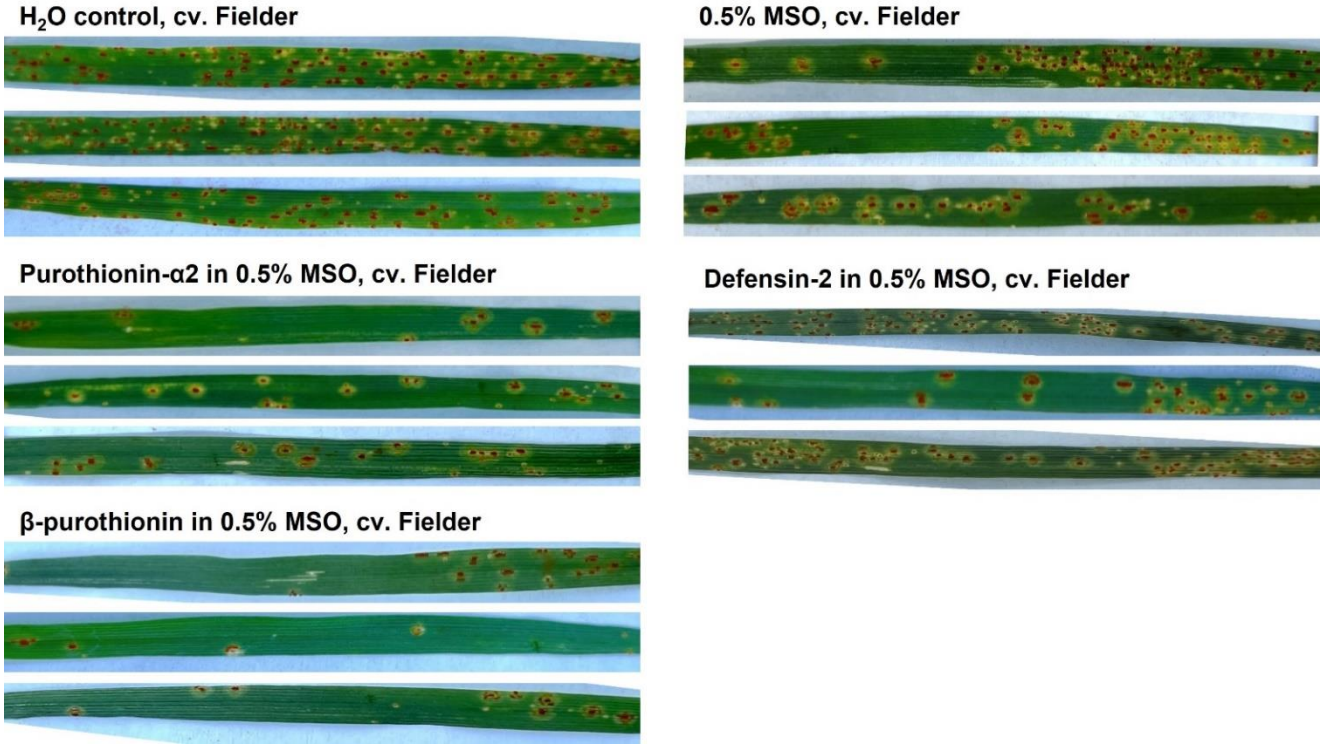
6.2 Foliar application of selected anti-rust peptides (ARPs) decreases the severity of leaf rust infection in wheat, cv. Fielder

We further evaluated the potential of these three peptides to increase wheat seedlings' resistance to leaf rust following foliar application. Peptides were diluted in 0.5% MSO surfactant and applied to 2-week-old plants (cv. Fielder) 24 hours prior- and 24 hours post-inoculation with leaf rust. The severity of infection was scored 14 days after inoculation with water, and 0.5%

MSO-treated plants were used as the mock-treatment controls (Figure 8 and Supplementary figure 1). WAMP-1a was a control peptide with minimal antifungal activity.

The severity of the infection was examined visually, and the am ount of *Pt* gDNA to wheat gDNA was quantified using qPCR. Three studied peptides (β-purothionin, Defensin-2, and Purothionin-α2) significantly decreased the severity of infection as compared to untreated and MSO-treated infected control ($p < 0.05$, unpaired Student's t-test, Figure 8B). At the same time, we also observed a prominent, but to a lower extent, decrease in infection for plants treated with just 0.5% MSO ($p < 0.05$, unpaired Student's t-test). Stock MSO solution composed of at least 70% methylated soybean seed oil provides enhanced penetration and droplet adhesion without cuticle disruption. The MSO surfactant (0.5%) was used previously in solutions with stable antimicrobial peptides (SAMPs) to control Citrus Huanglongbing (HLB), caused by a vector-transmitted phloem-limited bacterium *Candidatus Liberibacter asiaticus* (CLas) in citrus (Huang, Araujo et al. 2021). It is possible that foliar-applied MSO could interfere with the urediniospores germination on the leaf surface, decreasing the severity of infection. Nevertheless, the treatment groups with peptide in 0.5% MSO solution demonstrated significantly lower pathogen accumulation than the MSO-alone plants ($p < 0.05$, unpaired Student's t-test).

A


H_2O control, cv. Fielder
0.5% MSO, cv. Fielder
Purothionin-α2 in 0.5% MSO, cv. Fielder
Defensin-2 in 0.5% MSO, cv. Fielder
β-purothionin in 0.5% MSO, cv. Fielder

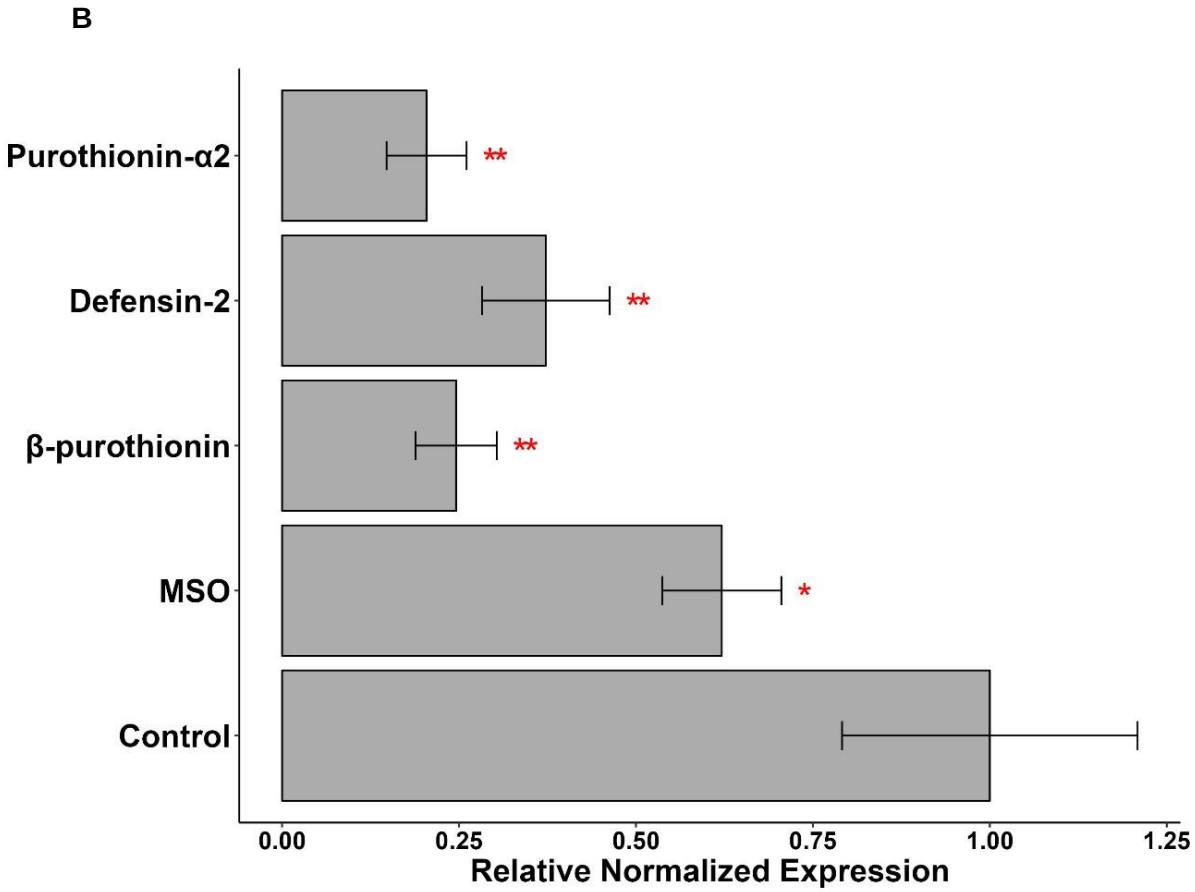


Figure 8. Evaluation of wheat (cv. Fielder) susceptibility to leaf rust (*Puccinia triticina*, isolate CCDS) following foliar application of selected peptides. (A) representative images of leaf rust infection either in the presence or absence of peptides application at 14 days after infection (DAI). Peptide solutions (100 µg/ml in 0.5% methylated seed oil) were applied to 2-weeks-old wheat seedlings 24 hr before inoculation with rust spores and 24 hr post-infection. (B) qPCR quantification of rust gDNA in infected leaves treated either with aqueous 0.5% MSO solution or peptides in 0.5% MSO. The bar plots represent the mean values of the relative normalized expression calculated from at least four plants per experimental group with two technical

replicates. The error bars represent the standard error of the mean. The Pt- and wheat-specific primers/probes were designed to amplify single copy genes PtRTP1 and TaPINb-D1b, respectively. ** - $p < 0.05$ compared to the MSO group and * - $p < 0.05$ compared to the Control (unpaired Student's t-test).

6.3 Selective upregulation of the expression of the defense-related genes in the peptide-treated plants

Endogenous elicitors, such as active peptides produced following a pathogen attack, can induce an immune response in plants (Boller and Flury 2012). Therefore, we decided to test the expression of the *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*TaNPR1*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*TaEDS1*), and *PATHOGENESIS-RELATED GENE 1* (*TaPR1*) following foliar application of selected peptides.

Leaves of the two-week-old seedlings, cv. Fielder was sprayed with the selected set of peptides with non-treated and mock-treated plants used as the controls. Twenty-four hours post-treatment, the leaf tissue was collected, RNA isolated, and gene expression was quantified for three sub-genomes of *TaNPR1*, subgenome A of *TaEDS1*, and subgenomes B and D of the *TaPR1* homeologs using qPCR (Figure 9).

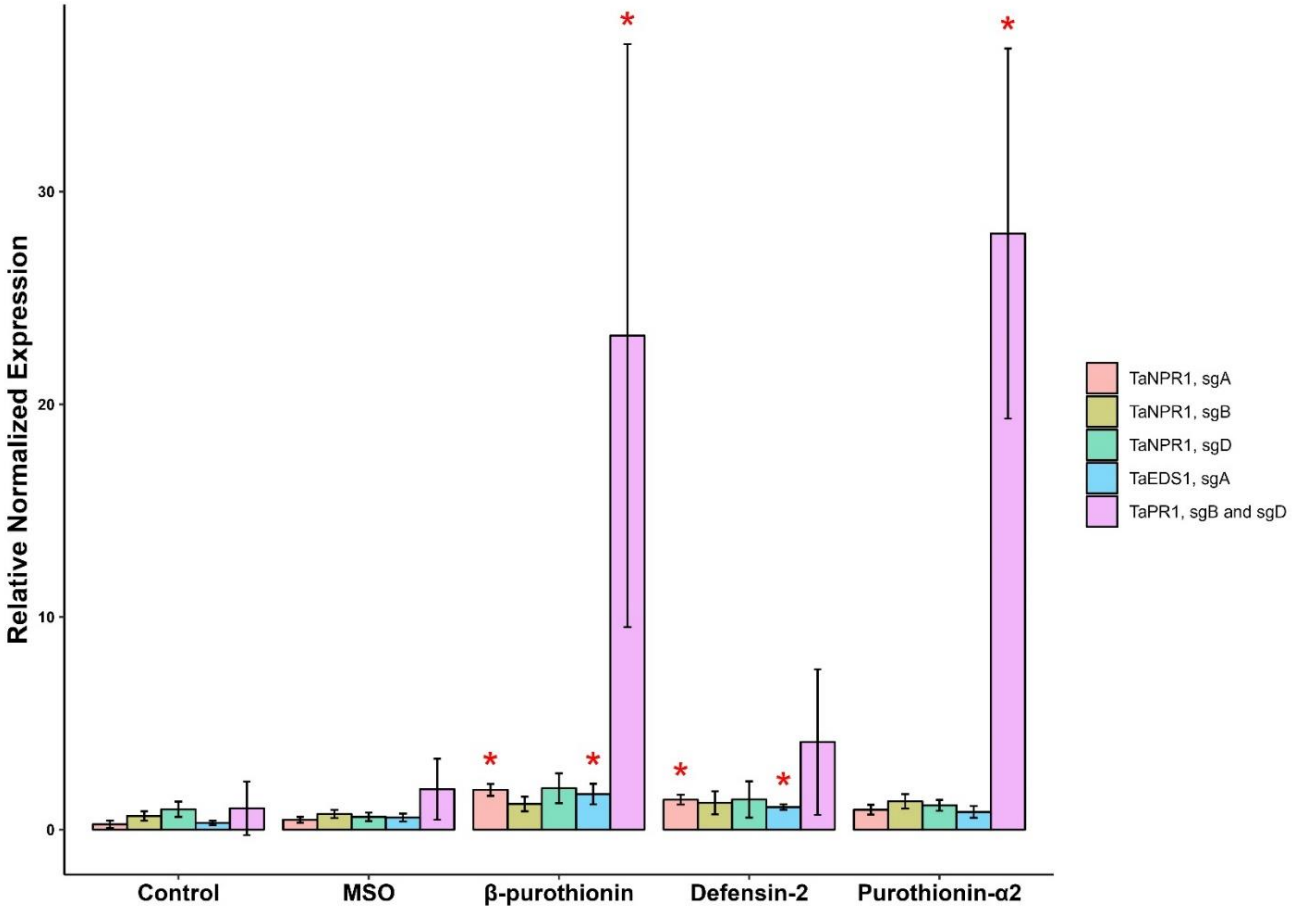


Figure 9. Differential gene expression of the defense-related genes following foliar application of selected peptides. The following treatment groups were examined: Control – no treatment control, MSO – mock treatment with 0.5% MSO solution, β-purothionin, Defensin-2 and Purothionin-α2 – plants treated with respective peptide solution (100 µg/ml) in 0.5% MSO. Leaf samples from (cv. Fielder) were collected from plants at the Z13 stage (3-4 leaves), followed by RNA isolation and gene expression analysis using qPCR. Normalization was done against the TaGA3PD gene. The bar graphs represent the mean values calculated for four plants per treatment group in two technical repeats. The error bars represent the standard error of the mean with the asterisk denoting a significant difference compared to the control ($p < 0.05$, unpaired Student's t-

test). TaNPR1 - NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1, TaEDS1 - ENHANCED DISEASE SUSCEPTIBILITY 1, TaPR1 - PATHOGENESIS-RELATED GENE 1.

The expression of the *TaNPR1*, *sgA* and *TaEDS1*, *sgA* genes were significantly upregulated in plants treated with the β-purothionin and Defensin-2 as compared to untreated and mock-treated controls ($p < 0.05$, unpaired Student's t-test). Similarly, the combined *TaPR1*, *sgB,* and *sgD* gene expression increased in plants treated with the β-purothionin and Purothionin-α2 ($p < 0.05$, unpaired Student's t-test).

7. DISCUSSION

Overall, the mechanism of anti-rust peptides (ARP) action remains largely unknown (Lintz, Dubrulle et al. 2022), with only two of them (EC 3.2.1.14 (Mathivanan, Kabilan et al. 1998) and NaD1 (van der Weerden, Lay et al. 2008, van der Weerden, Hancock et al. 2010)) shown to act either through chitinase activity or binding to the cell wall and permeabilizing the plasma membrane. Purothionin-α2 and β-purothionin belong to the thionin peptide family (Table 1). Thionins are found exclusively in plants and are first expressed as preproproteins that are processed to produce basic peptides of around 5 kDa in size, with three or four disulphide bridges and having two consecutive cysteine residues at positions 3 and 4 (Höng, Austerlitz et al. 2021).

The purothionins and related peptides were isolated and sequenced from the endosperm of maize, barley and oat and are present in other species of the *Poaceae* family (Bohlmann, Clausen et al. 1988, Bohlmann and Apel 1991). Purothionins-α2 and β were originally crystallized from the endosperm of bread wheat (*Triticum aestivum* L.) and were shown to have antimicrobial activity against plant phytopathogens during germination of seed (Fernandez de Caleya, Gonzalez-Pascual et al. 1972). The purothionin-α2 peptide is part of the protein coded by the *THI1.2* (Castagnaro, Marana et al. 1994) gene on the D subgenome (TraesCS1D02G405700, Suppl. figure 2), whereas the β-purothionin, originates from the A subgenome homeologue (TraesCS1A02G398200). The homeologs are expressed explicitly in grain, with the highest expression observed in the endosperm (Suppl. figure 3). In addition to grain, the thionins could also be expressed in leaves, with cultivated (*Hordeum vulgare* L.) and wild barley species containing a large multigene family for closely related leaf thionins (Bohlmann, Apel et al. 1987, Gausing 1987, Bohlmann, Clausen et al. 1988). The leaf thionins in barley are light-regulated, and the expression of thionin genes in etiolated barley seedlings drops drastically following illumination (REIMANN-PHILIPP, BEHNKE et al. 1989). Curiously, the genes are also stress-responsive, and watering of plants with a 1 mM $ZnCl_2$ solution or inoculation of the barley seedlings with powdery mildew (*Erisyphe graminis* f.sp. *hordei*) results in a rapid transient increase in the transcript levels of the genes (Bohlmann, Clausen et al. 1988). Overall, along with other AMPs and larger proteins (e.g., chitinases, glucanases, Thaumatin-Like Proteins (TLPs), proteinase inhibitors, peroxidases, etc.), they belong to the PR proteins activated during the pathogen attack (Buziashvili and Yemets 2022). Thionins are highly toxic to bacteria and fungi, including plant pathogenic and small animals (Nakanishi, Yoshizumi et al. 1979, Bohlmann, Clausen et al. 1988, Florack, Visser et al. 1993, Molina, Goy et al. 1993). Therefore, it is not surprising that three thionins tested in our study (β-

purothionin, CaThi, and purothionin-α2) were highly effective against leaf rust spores in *in vitro* germination and *in vivo* assays (Figures 6, 7, and 8). The peptides contain 8 (β-purothionin and purothionin-α2) or 9 (CaThi) cysteine residues and belong to the class 1 thionins (Bohlmann, Apel et al. 1994, Höng, Austerlitz et al. 2021). Tyrosine at position 13, a phospholipid binding site, was found to be indispensable for toxicity (Stec, Markman et al. 2004) and is present in all known structures of thionins with AMP activity. Two possible mechanisms were proposed for their action – withdrawal of phospholipids that disturb the membrane's fluidity with subsequent lysis [55], insertion of the peptides in the membrane, and acting as the water channels causing local membrane disruption (Oard 2011). Although both β-purothionin and purothionin-α2 contain tyrosine at position 13 (Table 1 and Suppl. figure 2, position 40 in the aminoacid multiple alignment figure), CaThi, which originates from hot pepper *Capsicum annuum*, has no tyrosine at this position (Table 1), although was also found in our study to have a strong inhibitory effect on rust spores germination *in vitro* (Figure 1, germination rate 22.73% ± 7.39, $p < 0.05$, unpaired Student's t-test).

Similarly to thionins, we have also observed strong antirust properties of Defensin-2 originating from peas (*Pisum sativum*, Table 1). Defensins are a group of peptides (45-54 amino acids long) that belong to the family of small cysteine-rich proteins (CRPs) and are highly active against an extensive range of microorganisms (Van der Weerden, Bleackley et al. 2013). Although divergent in sequence, the defensins are structurally similar, having β-hairpin structures stabilized by three/four disulphide bonds. Fungi secret several defensins with AFP properties (Hegedüs and Marx 2013). Identical to the defensins tested in our study, some demonstrate activity against plant fungal pathogens and no off-target cytotoxicity to bacterial, plant or animal cells (Lee, Shin et al. 1999, Moreno, Martínez del Pozo et al. 2006, Chen, Ao et al. 2013, Silva, Gonçalves et al. 2014).

Priming the plant immune system for earlier and more robust activation of pathogen-responsive genes can significantly increase crops' resistance (Prime, Conrath et al. 2006). *NPR1* is a master regulator of pathogen response and is involved in the initiation of systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse, van Wees et al. 1998). SAR is a long-lasting, broad-spectrum plant resistance mechanism (Prime, Conrath et al. 2006, Fu and Dong 2013). Similarly, the *EDS1* gene acts in the SA signal transduction pathway, and the mutant is impaired in the *PR* gene expression (Falk, Feys et al. 1999). Overexpression of the *NPR1* gene enhances resistance to various biotrophic and necrotrophic pathogens in many crops, including cotton, apple, pearl millet, Brassica, carrot, tomato, tobacco, rice and wheat (Cao, Li et al. 1998, Lin, Lu et al. 2004, Malnoy, Jin et al. 2007, Potlakayala, DeLong et al. 2007, Yuan, Zhong et al. 2007, Meur, Budatha et al. 2008, Wally, Jayaraj et al. 2009, Parkhi, Kumar et al. 2010, Ramineni, Sadumpati et al. 2014). Resistance to *Fusarium* head blight (FHB) was achieved in transgenic wheat lines overexpressing rye *ScNPR1* (*Secale cereale-NPR1*) (Yu, Zhang et al. 2017). Curiously, we observed only significant upregulation of the *TaNPR1* coded on sgA for β-purothionin and Defensin-2 (over sevenfold, Figure 9) and no response for sgB and sgD, suggesting the difference in the cis-regulatory elements in the promoter regions (not examined in this study). The *TaEDS1* gene at sgA was also significantly upregulated compared to control for plants treated with β-purothionin and Defensin-2 (over 5 and 3-fold, respectively). The highest increase in expression, though, was observed for the *TaPR1* genes at sgB and sgD with over 23 folds as compared to control, indicating that reduced severity of the leaf rust infection in the peptide-treated plants could be due to a combination of the direct inhibition of the spores germination on the leaf surface and upregulation of the host defense genes. Indeed, a recent report provides direct evidence for the involvement of the *TaPR1* gene in protecting wheat plants against leaf rust (Wang, Yuan et al.

2020). Purified TaPR1 protein significantly reduced the germination of urediniospores and the growth of germ tubes in an *in vitro* assay. Furthermore, virus-induced gene silencing (VIGS)-mediated downregulation of the *TaPR1* gene significantly increased plants' susceptibility to *Pt*.

The large-scale production of AMPs for field application remains too expensive (Bahar and Ren 2013, Marcos López, Gandía Gómez et al. 2020). The peptides could be expressed in heterologous expression systems, such as bacteria, yeast, filamentous fungi or plants (Ingham, Moore et al. 2007, Parachin, Mulder et al. 2012, Marcos López, Gandía Gómez et al. 2020). At the same time, the preliminary screening of the antifungal properties of selected peptides could be done *in vitro,* followed by strategic engineering of the peptide sequence in the crop's genome using gene editing tools, like the base or prime editors. Increasing knowledge of the structure-function relationship of AFP could aid in improving their function in antifungal activity with reduced off-target toxicity on other organisms.

8. CONCLUSION

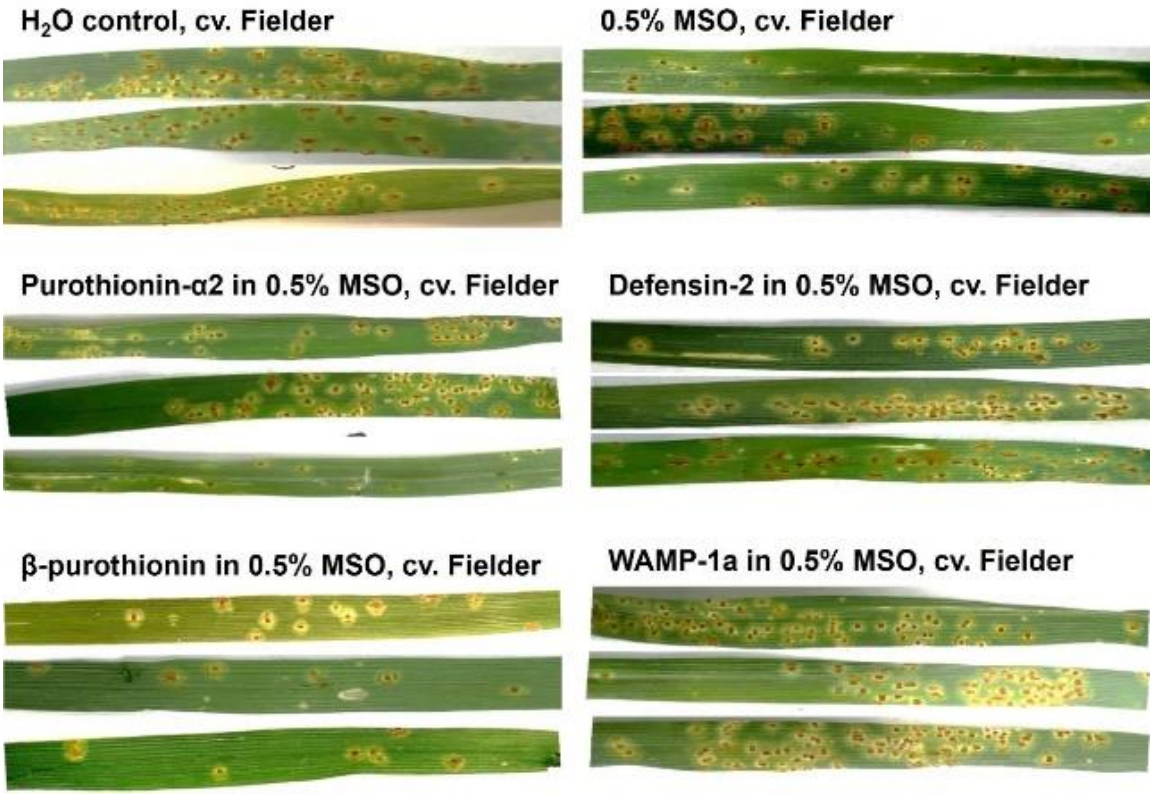
The findings of this study provide evidence that specific AMPs are efficient in preventing leaf rust in wheat. More specifically, nine out of eighteen AMPs that were examined were able to considerably suppress the germination of urediniospores of *Puccinia triticina*. Of them, β-purothionin, Defensin-2, and Purothionin-α2 had the most potential to lower infection rates when administered as foliar sprays both before and after exposure to pathogens in biotic stress and non-biotic stress conditions. This suggests that these peptides essentially prime the immune system through the regulation of the *NPR1* gene. Defensin and purothionin are primarily expressed in wheat seed, which can be prolonged into the two-leaf stage of the wheat seedling through cisgenic

or gene-editing techniques.

The manufacture of AMPs on a wide scale and the cost-effectiveness of using them in real agricultural applications should be the primary focus of future research. It may be possible to increase the feasibility of AMP synthesis by doing research on heterologous expression systems, such as those found in bacteria, yeast, filamentous fungi, or plants. In addition, more research into the structure-function connection of antifungal peptides has the potential to result in enhanced antifungal efficacy with fewer off-target consequences. In order to successfully incorporate these AMPs into crop genomes, it will be essential to make ongoing research and development efforts in the field of gene editing tools, such as base or prime editors. For the purpose of widening the applicability of these results and improving the overall sustainability of agriculture, it will be vital to expand the scope of this study to encompass additional types of crops and diseases. Furthermore, it is vital to conduct large field experiments in order to assess the effectiveness and environmental impact of AMPs in real-world settings. Additionally, it is essential to ensure that the usage of AMPs complies with regulatory standards and safety criteria. It will be possible to fully fulfill the promise of AMPs in agricultural disease control if these future research areas are addressed. This will result in crop production systems that are more sustainable and robust.

SUPPLEMENTARY MATERIALS

A



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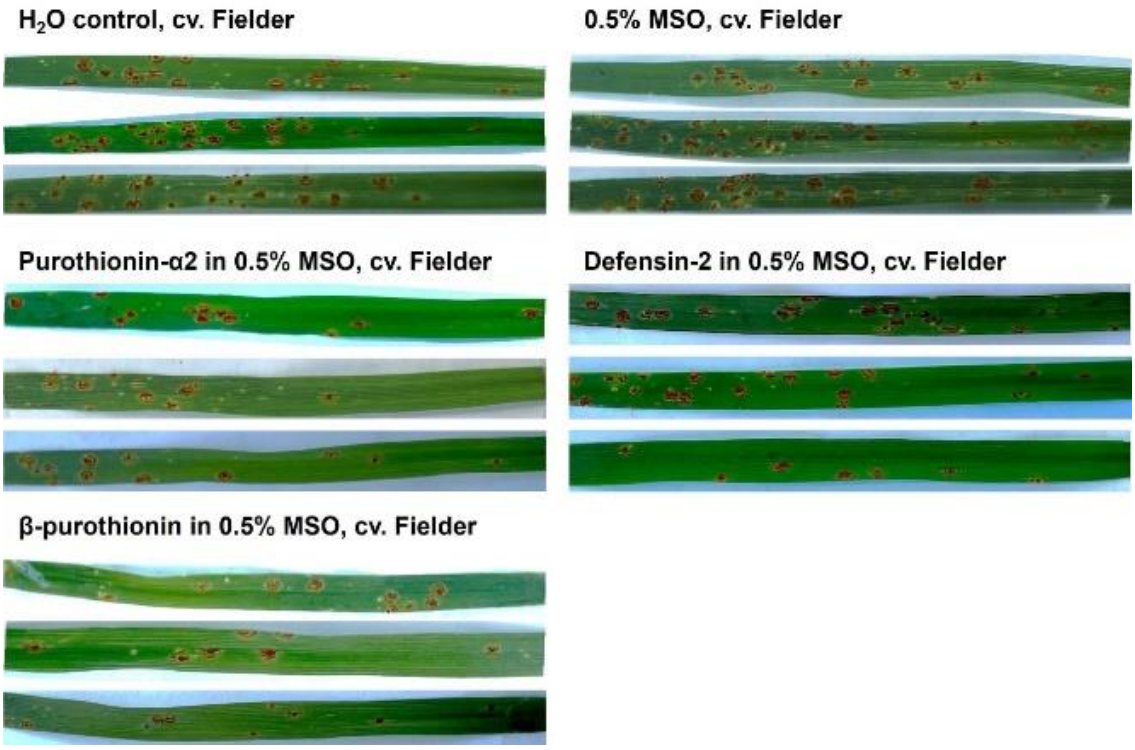
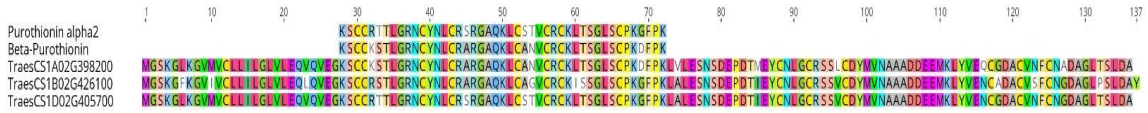
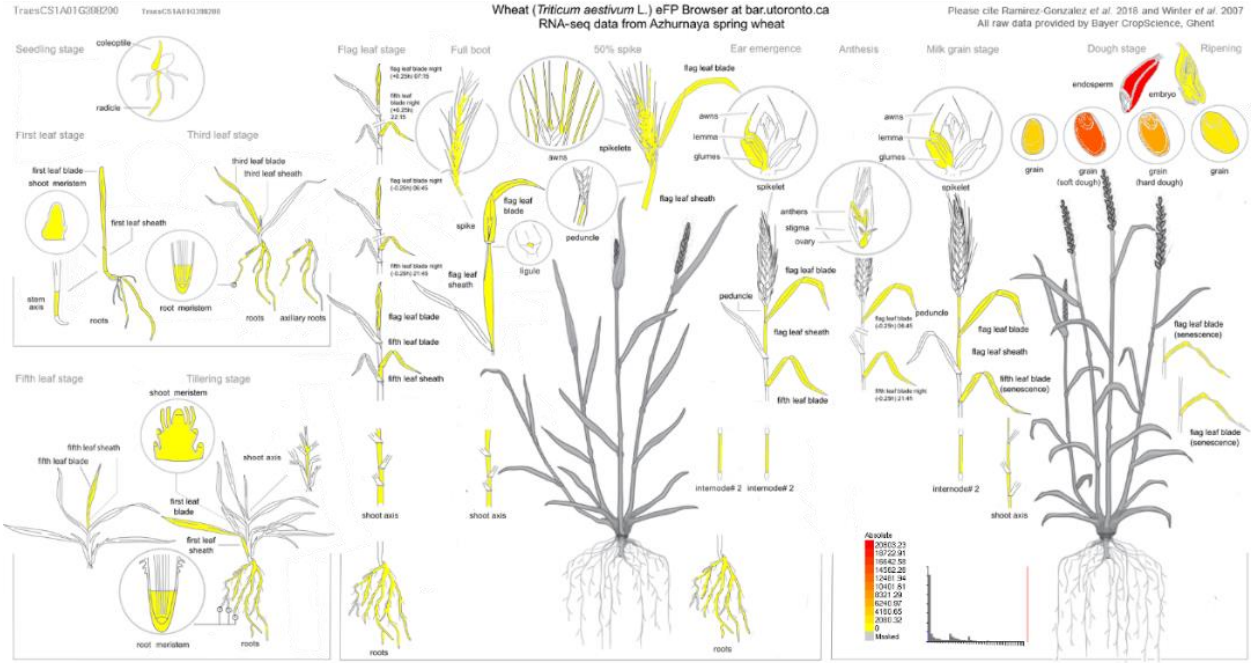


Supplementary figure 1. Evaluation of susceptibility to leaf rust (*Puccinia triticina*, isolate CCDS) of cv. Fielder following foliar application of selected peptides. Representative images of leaf rust infection (second and third biological replicates) either in the presence or absence of peptides application at 14 days after infection (DAI). Peptide solutions (100 µg/ml in 0.5% MSO) were applied to 2-weeks-old wheat seedlings 24 hr before inoculation with rust spores and 24 hr post-infection. A and B are the second and the third biological replicates, respectively. Water and MSO-treated plants are mock controls. WAMP1 was used as the peptide-treatment control. MSO - methylated seed oil.



Supplementary figure 2. Amino acid alignment of the β-purothionin and purothionin-α2 peptide sequences used in this study with the wheat (*Triticum aestivum* L., cv. Chinese Spring) purothionin proteins coded by three homeolog genes. Alignment was done using Clustal Omega 1.2.2 in Geneious Prime 2023.0.2

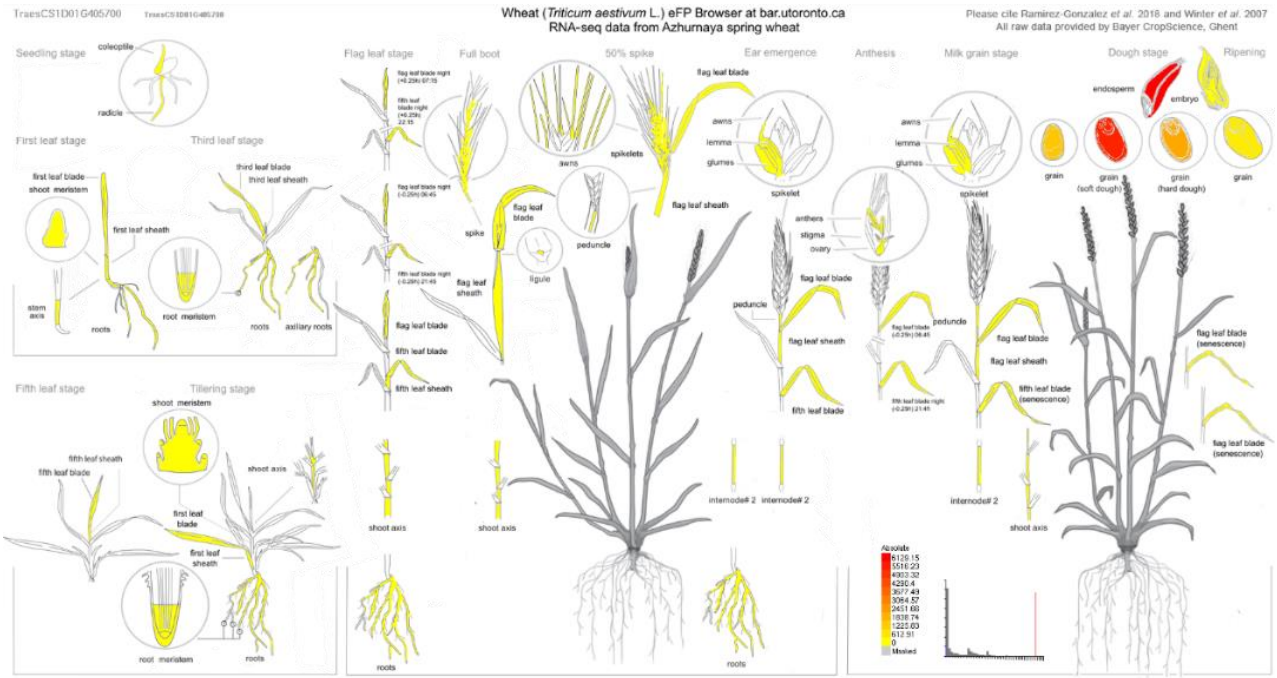
A



Wheat eFP Browser (*Triticum aestivum*) cv. Azhurnaya Developmental Time Course by Y. Khedikar, A. Pasha and N. Provart. Wheat plants from Bayer Crop Science Ukranian spring wheat cultivar Azhurnaya were grown in growth cabinets with 16.8 hours day night length at 25:15 °C. Three biological replicates, consisting of five individual plants each, were sampled at the developmental times and tissues shown above. All tissues were harvested between 7.5 and 8.5 h into the day and immediately frozen in liquid nitrogen upon collection. Seedling root samples were collected from wheat plants cultivated in agar. RNA was extracted from 100 mg fresh weight material using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and DNA contamination removed using the RNase-Free DNase Set (Qiagen). Quality checks were performed with a BioAnalyser. Sequencing libraries were prepared with 250-350 bp insert size and sequenced on an Illumina HiSeq 2500 using 2 x 125 bp paired-end strand-specific chemistry v4. Kallisto v0.42.3 (Bray et al., 2016) to map the RNA-seq samples to the Chinese Spring RefSeqv1.0+UTR transcriptome reference. Details of this transcriptome reference are provided elsewhere (IWGSC, 2017). Default parameters previously shown to result in accurate homoeolog-specific read mapping in polyploid wheat were used (Borrill et al., 2016). Expression levels were summarized as TPM from the transcript level to the gene level using tximport v1.2.0.

Seedling stage: Zadoks 7, 9, and 11 ● Third leaf stage: Zadoks 13 ● Fifth leaf stage: Zadoks 15 ● Tillering stage: Zadoks 22 ● Flag leaf stage: Zadoks 37 ● Full boot: Zadoks 45 ● 50% spike: Zadoks 54 ● Ear emergence: Zadoks 60 ● Anthesis: Zadoks 64 ● Milk grain stage: Zadoks 73 ● Dough stage: Zadoks 85 and 87 ● Ripening: Zadoks 90

B



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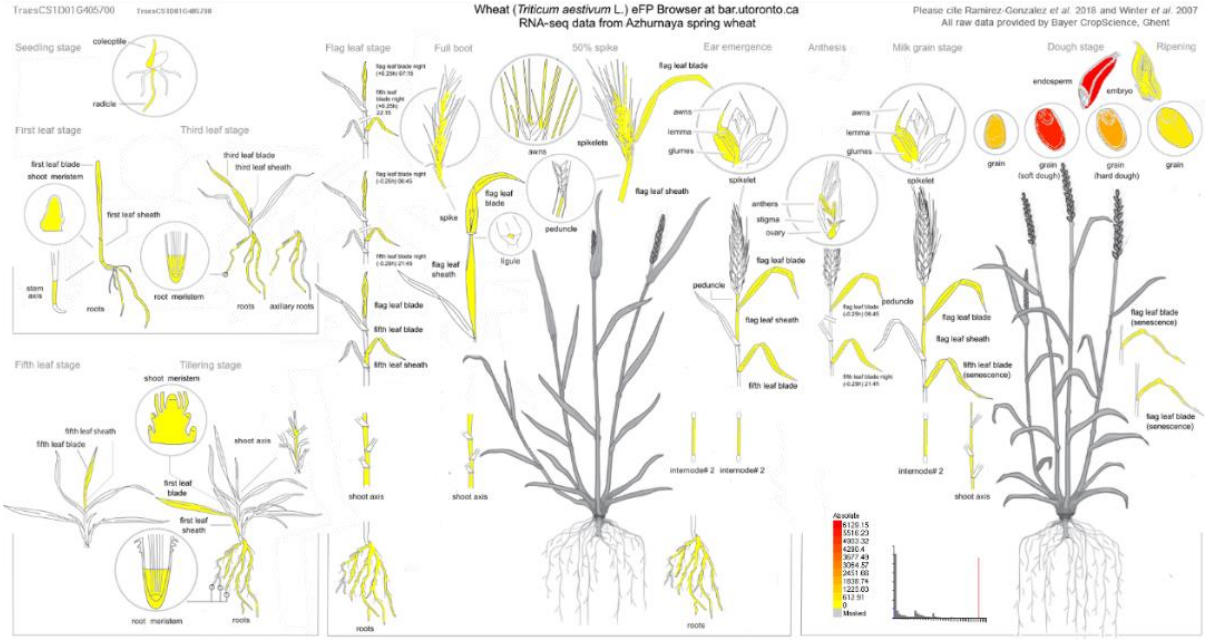

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Supplementary figure 3. Gene expression of the purothionin peptide-coding homeologs in wheat tissues. **A, B,** and **C** represent expressions of the TraesCS1A02G398200, TraesCS1B02G426100, and TraesCS1D02G405700 homeologs, respectively. Taken from http://bar.utoronto.ca

Supplementary table 1. Primers used in this study

qPCR primers/probes name	Primer sequence	Primer description
TaGA3PD	TATGTTTGTGGTTGGTGTCA	qPCR primers for probe-based detection of TaGA3PD housekeeping gene. Universal for three sub-genomes. Forward primer.
	GTGGTCATCAAACCCTCAAT	Reverse primer.
	TAGCTGCACCACTAACTGCC	5′ HEX™ (hexachloro-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaNPR1, sgA	GCACTATGCTGTAGAACACT	qPCR primers for probe-based detection of *TaNPR1*, sgA (TraesSTA3A01G115400). Forward primer.
	CGAGCACCTTTGGTTAAAAG	Reverse primer.
	TCTTCACATCGCTGCTAGGC	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaNPR1, sgB	TCGTTGTCTCCCTTTTAACC	qPCR primers for probe-based detection of *TaNPR1, sgB* (TraesSTA3B01G149400). Forward primer.
	CGGCTTTCCTTCTTCAGTAT	Reverse primer.
	TGCCCGGCCTTCAGATTTTA	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaNPR1, sgD	TCGTTGTCTCCCTTTTAACC	qPCR primers for probe-based detection of *TaNPR1, sgD* (TraesSTA3D01G137300). Forward primer.
	AATCCCCATGTTTTGTGAGT	Reverse primer.
	CGGCCTTCGGATTTAACATTTGA	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaEDS1, sgA	AAGTGCTCACTGGGAATATG	qPCR primers for probe-based detection of *TaEDS1, sgA* (TraesSTA5A03G02656710). Forward primer.
	AATTGCTGTCCCATTCATCT	Reverse primer.
	TCATCATCCCTTCTTGGCACA	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaPR1, sgB and D	GGTTAACCTTTCCCAAGCC	qPCR primers for probe-based detection of *TaPR1, sgB and sgD* (TraesSTA5B03G02873170, and TraesSTA5D03G03101330). Forward primer.
	CTTGCAGTCGTTGATCCTC	Reverse primer.
	CTACCTCTCGCCTCACAACG	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
PtRTP1	GAAAGCTGTTTAGACGAGGA	qPCR primers for quantification of *Puccinia triticina* (Pt, leaf rust) gDNA (Pt15 chromosome 18B, GenBank: CP110454.1)

	AAAAGTACCGGGATAGCAAG	Reverse primer.
	TCGGTACTCCCAGATCCGAG	5′ FAM™ (6-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).
TaPINb-D1b	AGTTGGCGGCTGGTACAATG	qPCR primers and probe for single copy wheat gene PINb-D1b to check transgene copy number using ddPCR. Reference: Collier, R., et al., Accurate measurement of transgene copy number in crop plants using droplet digital PCR. Plant J, 2017. 90(5): p. 1014-1025.
	ACATCGCTCCATCACGTAATCC	Reverse primer.
	TCAACAATGTCCGCAGGAGCG	5′ HEX™ (hexachloro-fluorescein)-labelled probe, double-quenched with ZEN™ and Iowa Black Hole Quencher® 1 (Integrated DNA Technologies, Coralville, IA, USA).

Supplementary table 2. Concentration of the peptides in µM used for the study.

SN	Peptide name	Concentration of peptides, µg/ml	Molecular weight, Da	Concentration of peptides, µM
1.	Alliumin	100	1013.15	98.70
2.	Beta-purothionin	100	4925.9	20.30
3.	CaThi	100	6701.09	14.92
4.	Chain A	100	4269.83	23.42
5.	Coccinin	100	1180.29	84.72
6.	Cp-thionin II	100	5241.27	19.08
7.	Defensin TM-AMP D1.2	100	5699.35	17.55
8.	Defensin-2	100	5403.12	18.51
9.	Ep-AMP1	100	3608.22	27.71
10.	MBP-1	100	4130.69	24.21
11.	NCR044	100	4317.16	23.16
12.	Psacotheasin	100	3479.96	28.74
13.	Purothionin -alpha	100	4928.9	20.29
14.	Rs-AFP2	100	5734.65	17.44
15.	Systemin	100	2010.28	49.74
16.	Tk-AMP-X1	100	3865.3	25.87
17.	WAMP-1a	100	4444.06	22.50
18.	ZmD32	100	5465.29	18.30

REFERENCES

Abdelrahman, M. and S. Jogaiah (2020). Saponins Versus Plant Fungal Pathogens. Bioactive Molecules in Plant Defense: Saponins. Cham, Springer International Publishing**:** 37-45.

Aboukhaddour, R., T. Fetch, B. D. McCallum, M. W. Harding, B. L. Beres and R. J. Graf (2020). "Wheat diseases on the prairies: A Canadian story." **69**(3): 418-432.

Aboye, T. L., A. A. Strömstedt, S. Gunasekera, J. G. Bruhn, H. El-Seedi, K. J. Rosengren and U. Göransson (2015). "A cactus-derived toxin-like cystine knot Peptide with selective antimicrobial activity." Chembiochem **16**(7): 1068-1077.

Abramovitch, R. B., Y. J. Kim, S. Chen, M. B. Dickman and G. B. Martin (2003). "Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death." Embo j **22**(1): 60-69.

Aerts, A. M., I. E. François, E. M. Meert, Q. T. Li, B. P. Cammue and K. Thevissen (2007). "The antifungal activity of RsAFP2, a plant defensin from raphanus sativus, involves the induction of reactive oxygen species in Candida albicans." J Mol Microbiol Biotechnol **13**(4): 243-247.

Agrios, G. N. (2005). Plant Pathology, Elsevier Science.

Ali, R., W. Ma, F. Lemtiri-Chlieh, D. Tsaltas, Q. Leng, S. von Bodman and G. A. Berkowitz (2007). "Death don't have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity." Plant Cell **19**(3): 1081-1095.

Almasia, N. I., A. A. Bazzini, H. E. Hopp and C. Vazquez-Rovere (2008). "Overexpression of snakin-1 gene enhances resistance to Rhizoctonia solani and Erwinia carotovora in transgenic potato plants." Mol Plant Pathol **9**(3): 329-338.

Almeida, M. S., K. M. Cabral, E. Kurtenbach, F. C. Almeida and A. P. Valente (2002). "Solution structure of Pisum sativum defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action." J Mol Biol **315**(4): 749-757.

Almeida, M. S., K. M. Cabral, R. B. Zingali and E. Kurtenbach (2000). "Characterization of two novel defense peptides from pea (Pisum sativum) seeds." Arch Biochem Biophys **378**(2): 278-286.

Asano, T., A. Miwa, K. Maeda, M. Kimura and T. Nishiuchi (2013). "The Secreted Antifungal Protein Thionin 2.4 in Arabidopsis thaliana Suppresses the Toxicity of a Fungal Fruit Body Lectin from Fusarium graminearum." PLOS Pathogens **9**(8): e1003581.

Bacete, L. and T. Hamann (2020). "The Role of Mechanoperception in Plant Cell Wall Integrity Maintenance." Plants (Basel) **9**(5).

Bahar, A. A. and D. J. P. Ren (2013). "Antimicrobial peptides." **6**(12): 1543-1575.

Bailey, K. L. and C. P. Society (2003). Diseases of Field Crops in Canada, Canadian Phytopathological Society.

Balaji, V. and C. D. Smart (2012). "Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to Clavibacter michiganensis subsp. michiganensis in transgenic tomato (Solanum lycopersicum)." Transgenic Res **21**(1): 23-37.

Barbosa Pelegrini, P., R. P. Del Sarto, O. N. Silva, O. L. Franco and M. F. Grossi-de-Sa (2011). "Antibacterial peptides from plants: what they are and how they probably work." Biochem Res Int **2011**: 250349.

Bittel, P. and S. Robatzek (2007). "Microbe-associated molecular patterns (MAMPs) probe plant immunity." Curr Opin Plant Biol **10**(4): 335-341.

Bohlmann, H., K. Apel and F. J. P. m. b. r. Garcia-Olmedo (1994). "Thionins."

Bohlmann, H. and K. J. A. r. o. p. b. Apel (1991). "Thionins." **42**(1): 227-240.

Bohlmann, H., K. J. M. Apel and G. G. MGG (1987). "Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (Hordeum vulgare L.)." **207**(2): 446-454.

Bohlmann, H., S. Clausen, S. Behnke, H. Giese, C. Hiller, U. Reimann-Philipp, G. Schrader, V. Barkholt and K. J. T. E. j. Apel (1988). "Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defense mechanism of plants." **7**(6): 1559-1565.

Boller, T. and G. Felix (2009). "A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors." Annual Review of Plant Biology **60**(Volume 60, 2009): 379-406.

Boller, T. and P. Flury (2012). Peptides as Danger Signals: MAMPs and DAMPs. Plant Signaling Peptides. H. R. Irving and C. Gehring. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 163-181.

Boman, H. G. (1995). "Peptide antibiotics and their role in innate immunity." Annu Rev Immunol **13**: 61-92.

Bonardi, V., S. Tang, A. Stallmann, M. Roberts, K. Cherkis and J. L. Dangl (2011). "Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors." Proc Natl Acad Sci U S A **108**(39): 16463-16468.

Bouftass, F., B. Ezzahiri and A. Ziouti (2010). "Patterns of virulence diversity in Puccinia recondita on wheat in Morocco in 2005 and 2006." Phytopathologia Mediterranea **48**(3): 430-438.

Boyle, P., E. Le Su, A. Rochon, H. L. Shearer, J. Murmu, J. Y. Chu, P. R. Fobert and C. Després (2009). "The BTB/POZ domain of the Arabidopsis disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function." Plant Cell **21**(11): 3700-3713.

Brodersen, P., F. G. Malinovsky, K. Hématy, M. A. Newman and J. Mundy (2005). "The role of salicylic acid in the induction of cell death in Arabidopsis acd11." Plant Physiol **138**(2): 1037-1045.

Brodersen, P., M. Petersen, H. M. Pike, B. Olszak, S. Skov, N. Odum, L. B. Jørgensen, R. E. Brown and J. Mundy (2002). "Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense." Genes Dev **16**(4): 490-502.

Broekaert, W. F., W. Mariën, F. R. Terras, M. F. De Bolle, P. Proost, J. Van Damme, L. Dillen, M. Claeys, S. B. Rees, J. Vanderleyden and et al. (1992). "Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins." Biochemistry **31**(17): 4308-4314.

Broekaert, W. F., F. R. Terras, B. P. Cammue and R. W. Osborn (1995). "Plant defensins: novel antimicrobial peptides as components of the host defense system." Plant Physiol **108**(4): 1353-1358.

Brogden, K. A. (2005). "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" Nature Reviews Microbiology **3**(3): 238-250.

Brown, A. E. and T. R. Swinburne (1982). "Iron-chelating agents and lesion development by Botrytis cinerea on leaves of Vicia faba." Physiological Plant Pathology **21**(1): 13-21.

Brown, J., H. Ogle and M. Dale (1997). Disease management: general concepts**:** 333-340.

Bruix, M., M. A. Jiménez, J. Santoro, C. González, F. J. Colilla, E. Méndez and M. Rico (1993). "Solution structure of gamma 1-H and gamma 1-P thionins from barley and wheat endosperm determined by 1H-NMR: a structural motif common to toxic arthropod proteins." Biochemistry **32**(2): 715-724.

Brunner, F., S. Rosahl, J. Lee, J. J. Rudd, C. Geiler, S. Kauppinen, G. Rasmussen, D. Scheel and T. Nürnberger (2002). "Pep-13, a plant defense-inducing pathogen-associated pattern from Phytophthora transglutaminases." Embo j **21**(24): 6681-6688.

Bundo, M., L. Montesinos, E. Izquierdo, S. Campo, D. Mieulet, E. Guiderdoni, M. Rossignol, E. Badosa, E. Montesinos, B. San Segundo and M. Coca (2014). "Production of cecropin A antimicrobial peptide in rice seed endosperm." BMC Plant Biol **14**: 102.

Buscaill, P. and R. A. L. van der Hoorn (2021). "Defeated by the nines: nine extracellular strategies to avoid microbe-associated molecular patterns recognition in plants." Plant Cell **33**(7): 2116-2130.

Buziashvili, A. and A. Yemets (2022). "Lactoferrin and its role in biotechnological strategies for plant defense against pathogens." Transgenic Research.

Buziashvili, A. and A. Yemets (2023). "Lactoferrin and its role in biotechnological strategies for plant defense against pathogens." Transgenic Res **32**(1-2): 1-16.

Cai, R., J. Lewis, S. Yan, H. Liu, C. R. Clarke, F. Campanile, N. F. Almeida, D. J. Studholme, M. Lindeberg, D. Schneider, M. Zaccardelli, J. C. Setubal, N. P. Morales-Lizcano, A. Bernal, G. Coaker, C. Baker, C. L. Bender, S. Leman and B. A. Vinatzer (2011). "The Plant Pathogen Pseudomonas syringae pv. tomato Is Genetically Monomorphic and under Strong Selection to Evade Tomato Immunity." PLOS Pathogens **7**(8): e1002130.

Cantu, D., A. R. Vicente, J. M. Labavitch, A. B. Bennett and A. L. Powell (2008). "Strangers in the matrix: plant cell walls and pathogen susceptibility." Trends Plant Sci **13**(11): 610-617.

Cao, H., S. A. Bowling, A. S. Gordon and X. Dong (1994). "Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance." Plant Cell **6**(11): 1583-1592.

Cao, H., X. Li and X. Dong (1998). "Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance." Proc Natl Acad Sci U S A **95**(11): 6531-6536.

Carvalho Ade, O. and V. M. Gomes (2011). "Plant defensins and defensin-like peptides - biological activities and biotechnological applications." Curr Pharm Des **17**(38): 4270-4293.

Castagnaro, A., C. Marana, P. Carbonero and F. Garcia-Olmedo (1994). "cDNA Cloning and Nucleotide Sequences of [alpha]1 and [alpha]2 Thionins from Hexaploid Wheat Endosperm." Plant Physiology **106**(3): 1221-1222.

Castagnaro, A., C. Maraña, P. Carbonero and F. García-Olmedo (1994). "cDNA cloning and nucleotide sequences of alpha 1 and alpha 2 thionins from hexaploid wheat endosperm." Plant Physiol **106**(3): 1221-1222.

Cavallarin, L., D. Andreu and B. San Segundo (1998). "Cecropin A-derived peptides are potent inhibitors of fungal plant pathogens." Mol Plant Microbe Interact **11**(3): 218-227.

Chen, Y.-L., C.-Y. Lee, K.-T. Cheng, W.-H. Chang, R.-N. Huang, H. G. Nam and Y.-R. Chen (2014). "Quantitative Peptidomics Study Reveals That a Wound-Induced Peptide from PR-1 Regulates Immune Signaling in Tomato " The Plant Cell **26**(10): 4135-4148.

Chen, Y.-L., F.-W. Lin, K.-T. Cheng, C.-H. Chang, S.-C. Hung, T. Efferth and Y.-R. Chen (2023). "XCP1 cleaves Pathogenesis-related protein 1 into CAPE9 for systemic immunity in Arabidopsis." Nature Communications **14**(1): 4697.

Chen, Z., J. Ao, W. Yang, L. Jiao, T. Zheng, X. J. A. m. Chen and biotechnology (2013). "Purification and characterization of a novel antifungal protein secreted by Penicillium chrysogenum from an Arctic sediment." **97**(24): 10381-10390.

Cherewick, W. J. (1953). "Smut diseases of cultivated plants in Canada." Smut diseases of cultivated plants in Canada.

Chinchilla, D., C. Zipfel, S. Robatzek, B. Kemmerling, T. Nürnberger, J. D. Jones, G. Felix and T. Boller (2007). "A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defense." Nature **448**(7152): 497-500.

Chisholm, S. T., G. Coaker, B. Day and B. J. Staskawicz (2006). "Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response." Cell **124**(4): 803-814.

Clough, S. J., K. A. Fengler, I.-c. Yu, B. Lippok, R. K. Smith and A. F. Bent (2000). "The Arabidopsis dnd1 “defense, no death” gene encodes a mutated cyclic nucleotide-gated ion channel." Proceedings of the National Academy of Sciences **97**(16): 9323-9328.

Collier, R., K. Dasgupta, Y. P. Xing, B. T. Hernandez, M. Shao, D. Rohozinski, E. Kovak, J. Lin, M. L. P. de Oliveira, E. Stover, K. F. McCue, F. G. Harmon, A. Blechl, J. G. Thomson and R. Thilmony (2017). "Accurate measurement of transgene copy number in crop plants using droplet digital PCR." Plant J **90**(5): 1014-1025.

Conrath, U. (2011). "Molecular aspects of defense priming." Trends Plant Sci **16**(10): 524-531.

Cornet, B., J.-M. Bonmatin, C. Hetru, J. A. Hoffmann, M. Ptak and F. Vovelle (1995). "Refined three-dimensional solution structure of insect defensin A." Structure **3**(5): 435-448.

Craigie, J. (1944). "Increase in production and value of the wheat crop in Manitoba and eastern Saskatchewan as a result of the introduction of rust resistant wheat varieties." Scientific Agriculture **25**(2): 51-64.

Craik, D. J., N. L. Daly, T. Bond and C. Waine (1999). "Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif." J Mol Biol **294**(5): 1327-1336.

Dangl, J. L. and J. D. G. Jones (2001). "Plant pathogens and integrated defense responses to infection." Nature **411**(6839): 826-833.

De Coninck, B., B. P. A. Cammue and K. Thevissen (2013). "Modes of antifungal action and in planta functions of plant defensins and defensin-like peptides." Fungal Biology Reviews **26**(4): 109-120.

De Samblanx, G. W., I. J. Goderis, K. Thevissen, R. Raemaekers, F. Fant, F. Borremans, D. P. Acland, R. W. Osborn, S. Patel and W. F. Broekaert (1997). "Mutational analysis of a plant defensin from radish (Raphanus sativus L.) reveals two adjacent sites important for antifungal activity." J Biol Chem **272**(2): 1171-1179.

Delaney, T. P., S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessmann, E. Ward and J. Ryals (1994). "A central role of salicylic Acid in plant disease resistance." Science **266**(5188): 1247-1250.

Després, C., C. DeLong, S. Glaze, E. Liu and P. R. Fobert (2000). "The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors." Plant Cell **12**(2): 279-290.

Dietrich, R. A., M. H. Richberg, R. Schmidt, C. Dean and J. L. Dangl (1997). "A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death." Cell **88**(5): 685-694.

Dodds, P. N., G. J. Lawrence, A. M. Catanzariti, T. Teh, C. I. Wang, M. A. Ayliffe, B. Kobe and J. G. Ellis (2006). "Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes." Proc Natl Acad Sci U S A **103**(23): 8888-8893.

Doke, N. and Y. Ohashi (1988). "Involvement of an O2− generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus." Physiological and Molecular Plant Pathology **32**: 163-175.

Dos Santos, C. and O. L. Franco (2023). "Pathogenesis-Related Proteins (PRs) with Enzyme Activity Activating Plant Defense Responses." Plants (Basel) **12**(11).

Duvick, J. P., T. Rood, A. G. Rao and D. R. Marshak (1992). "Purification and characterization of a novel antimicrobial peptide from maize (Zea mays L.) kernels." J Biol Chem **267**(26): 18814-18820.

ECONOMIC, U. N. D. F. and S. AFFAIRS. (2023). World population prospects 2022: Summary of results, UN.

Ellis, J. G., E. S. Lagudah, W. Spielmeyer and P. N. Dodds (2014). "The past, present and future of breeding rust resistant wheat." Frontiers in plant science **5**: 116802.

Estey, R. H. (1994). Essays on the Early History of Plant Pathology and Mycology in Canada, McGill-Queen's University Press.

Falk, A., B. J. Feys, L. N. Frost, J. D. Jones, M. J. Daniels and J. E. J. P. o. t. N. A. o. S. Parker (1999). "EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases." **96**(6): 3292-3297.

Fant, F., W. Vranken, W. Broekaert and F. Borremans (1998). "Determination of the three-dimensional solution structure of Raphanus sativus antifungal protein 1 by 1H NMR." J Mol Biol **279**(1): 257-270.

Felix, G., J. D. Duran, S. Volko and T. Boller (1999). "Plants have a sensitive perception system for the most conserved domain of bacterial flagellin." Plant J **18**(3): 265-276.

Fernandez de Caleya, R., B. Gonzalez-Pascual, F. García-Olmedo and P. Carbonero (1972). "Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro." Appl Microbiol **23**(5): 998-1000.

Figueroa, M., K. E. Hammond-Kosack and P. S. Solomon (2018). "A review of wheat diseases-a field perspective." Mol Plant Pathol **19**(6): 1523-1536.

Flor, H. H. (1971). "Current Status of the Gene-For-Gene Concept." Annual Review of Phytopathology **9**(Volume 9, 1971): 275-296.

Florack, D. E., B. Visser, P. M. De Vries, J. Van Vuurde and W. J. N. J. o. P. P. Stiekema (1993). "Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria." **99**(5): 259-268.

Franco, O. L. (2011). "Peptide promiscuity: an evolutionary concept for plant defense." FEBS Lett **585**(7): 995-1000.

Franco, O. L., A. M. Murad, J. R. Leite, P. A. Mendes, M. V. Prates and C. Bloch, Jr. (2006). "Identification of a cowpea gamma-thionin with bactericidal activity." Febs j **273**(15): 3489-3497.

Freeman, B. and G. Beattie (2008). "An Overview of Plant Defenses against Pathogens and Herbivores." The Plant Health Instructor **149**.

Friedrich, L., K. Lawton, W. Ruess, P. Masner, N. Specker, M. G. Rella, B. Meier, S. Dincher, T. Staub, S. Uknes, J.-P. Métraux, H. Kessmann and J. Ryals (1996). "A benzothiadiazole derivative induces systemic acquired resistance in tobacco." The Plant Journal **10**(1): 61-70.

Fu, Z. Q. and X. Dong (2013). "Systemic acquired resistance: turning local infection into global defense." Annu Rev Plant Biol **64**: 839-863.

Fujimura, M., M. Ideguchi, Y. Minami, K. Watanabe and K. Tadera (2004). "Purification, characterization, and sequencing of novel antimicrobial peptides, Tu-AMP 1 and Tu-AMP 2, from bulbs of tulip (Tulipa gesneriana L.)." Biosci Biotechnol Biochem **68**(3): 571-577.

Fujimura, M., Y. Minami, K. Watanabe and K. Tadera (2003). "Purification, characterization, and sequencing of a novel type of antimicrobial peptides, Fa-AMP1 and Fa-AMP2, from seeds of buckwheat (Fagopyrum esculentum Moench.)." Biosci Biotechnol Biochem **67**(8): 1636-1642.

Fukuta, S., K.-i. Kawamoto, Y. Mizukami, Y. Yoshimura, J.-i. Ueda and M. Kanbe (2012). "Transgenic tobacco plants expressing antimicrobial peptide bovine lactoferricin show enhanced resistance to phytopathogens." Plant Biotechnology **29**: 383-389.

Gao, A. G., S. M. Hakimi, C. A. Mittanck, Y. Wu, B. M. Woerner, D. M. Stark, D. M. Shah, J. Liang and C. M. Rommens (2000). "Fungal pathogen protection in potato by expression of a plant defensin peptide." Nat Biotechnol **18**(12): 1307-1310.

Gao, X., M. Brodhagen, T. Isakeit, S. H. Brown, C. Göbel, J. Betran, I. Feussner, N. P. Keller and M. V. Kolomiets (2009). "Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to Aspergillus spp." Mol Plant Microbe Interact **22**(2): 222-231.

García-Olmedo, F., A. Molina, A. Segura and M. Moreno (1995). "The defensive role of nonspecific lipid-transfer proteins in plants." Trends Microbiol **3**(2): 72-74.

Garrido, J., M. Aguilar and P. Prieto (2020). "Identification and validation of reference genes for RT-qPCR normalization in wheat meiosis." Sci Rep **10**(1): 2726.

Gausing, K. J. P. (1987). "Thionin genes specifically expressed in barley leaves." **171**(2): 241-246.

Ghag, S. B., U. K. Shekhawat and T. R. Ganapathi (2012). "Petunia floral defensins with unique prodomains as novel candidates for development of fusarium wilt resistance in transgenic banana plants." PLoS One **7**(6): e39557.

Gimenez-Ibanez, S., D. R. Hann, V. Ntoukakis, E. Petutschnig, V. Lipka and J. P. Rathjen (2009). "AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants." Curr Biol **19**(5): 423-429.

Glawischnig, E. (2007). "Camalexin." Phytochemistry **68**(4): 401-406.

Göhre, V., T. Spallek, H. Häweker, S. Mersmann, T. Mentzel, T. Boller, M. de Torres, J. W. Mansfield and S. Robatzek (2008). "Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB." Curr Biol **18**(23): 1824-1832.

Gómez-Gómez, L. and T. Boller (2000). "FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis." Mol Cell **5**(6): 1003-1011.

González-Lamothe, R., G. Mitchell, M. Gattuso, M. S. Diarra, F. Malouin and K. Bouarab (2009). "Plant antimicrobial agents and their effects on plant and human pathogens." Int J Mol Sci **10**(8): 3400-3419.

Güssow, H. T. and I. L. Conners (1927). Smut Diseases of Cultivated Plants: Their Cause and Control, Department of Agriculture.

Gust, A. A., R. Biswas, H. D. Lenz, T. Rauhut, S. Ranf, B. Kemmerling, F. Götz, E. Glawischnig, J. Lee, G. Felix and T. Nürnberger (2007). "Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in Arabidopsis." J Biol Chem **282**(44): 32338-32348.

Hammami, R., J. Ben Hamida, G. Vergoten and I. Fliss (2009). "PhytAMP: a database dedicated to antimicrobial plant peptides." Nucleic Acids Res **37**(Database issue): D963-968.

Hancock, R. E. (2001). "Cationic peptides: effectors in innate immunity and novel antimicrobials." Lancet Infect Dis **1**(3): 156-164.

Hancock, R. E. and G. Diamond (2000). "The role of cationic antimicrobial peptides in innate host defenses." Trends Microbiol **8**(9): 402-410.

Hancock, R. E. and R. Lehrer (1998). "Cationic peptides: a new source of antibiotics." Trends Biotechnol **16**(2): 82-88.

Hancock, R. E. W. and H.-G. Sahl (2006). "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies." Nature Biotechnology **24**(12): 1551-1557.

Hao, G., S. Zhang and E. Stover (2017). "Transgenic expression of antimicrobial peptide D2A21 confers resistance to diseases incited by Pseudomonas syringae pv. tabaci and Xanthomonas citri, but not Candidatus Liberibacter asiaticus." PLOS ONE **12**(10): e0186810.

Hayafune, M., R. Berisio, R. Marchetti, A. Silipo, M. Kayama, Y. Desaki, S. Arima, F. Squeglia, A. Ruggiero, K. Tokuyasu, A. Molinaro, H. Kaku and N. Shibuya (2014). "Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization." Proceedings of the National Academy of Sciences **111**(3): E404-E413.

He, S. and C. M. Deber (2024). "Interaction of designed cationic antimicrobial peptides with the outer membrane of gram-negative bacteria." Sci Rep **14**(1): 1894.

Hegedüs, N. and F. Marx (2013). "Antifungal proteins: More than antimicrobials?" Fungal Biol Rev **26**(4): 132-145.

Hegedüs, N. and F. J. F. b. r. Marx (2013). "Antifungal proteins: more than antimicrobials?" **26**(4): 132-145.

Hein, M. J. A., M. Kvansakul, F. T. Lay, T. K. Phan and M. D. Hulett (2022). "Defensin-lipid interactions in membrane targeting: mechanisms of action and opportunities for the development of antimicrobial and anticancer therapeutics." Biochem Soc Trans **50**(1): 423-437.

Holásková, E., P. Galuszka, A. Mičúchová, M. Šebela, M. T. Öz and I. Frébort (2018). "Molecular Farming in Barley: Development of a Novel Production Platform to Produce Human Antimicrobial Peptide LL-37." Biotechnology Journal **13**(6): 1700628.

Höng, K., T. Austerlitz, T. Bohlmann and H. Bohlmann (2021). "The thionin family of antimicrobial peptides." PLoS One **16**(7): e0254549.

Hou, S., X. Wang, D. Chen, X. Yang, M. Wang, D. Turrà, A. Di Pietro and W. Zhang (2014). "The Secreted Peptide PIP1 Amplifies Immunity through Receptor-Like Kinase 7." PLOS Pathogens **10**(9): e1004331.

Huang, C.-Y., K. Araujo, J. N. Sánchez, G. Kund, J. Trumble, C. Roper, K. E. Godfrey and H. Jin (2021). "A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing." Proceedings of the National Academy of Sciences **118**(6): e2019628118.

Huang, H. W. (2006). "Molecular mechanism of antimicrobial peptides: The origin of cooperativity." Biochimica et Biophysica Acta (BBA) - Biomembranes **1758**(9): 1292-1302.

Huerta-Espino, J., R. Singh, S. German, B. McCallum, R. Park, W. Q. Chen, S. Bhardwaj and H. Goyeau (2011). "Global status of wheat leaf rust caused by Puccinia triticina." Euphytica **179**: 143-160.

Huffaker, A., N. J. Dafoe and E. A. Schmelz (2011). "ZmPep1, an ortholog of Arabidopsis elicitor peptide 1, regulates maize innate immunity and enhances disease resistance." Plant Physiol **155**(3): 1325-1338.

Huffaker, A., G. Pearce and C. A. Ryan (2006). "An endogenous peptide signal in Arabidopsis activates components of the innate immune response." Proc Natl Acad Sci U S A **103**(26): 10098-10103.

Hughes, P., E. Dennis, M. Whitecross, D. Llewellyn and P. Gage (2000). "The Cytotoxic Plant Protein, β-Purothionin, Forms Ion Channels in Lipid Membranes*." Journal of Biological Chemistry **275**(2): 823-827.

Hwang, B., J. S. Hwang, J. Lee and D. G. Lee (2010). "Antifungal properties and mode of action of psacotheasin, a novel knottin-type peptide derived from Psacothea hilaris." Biochem Biophys Res Commun **400**(3): 352-357.

Ijaz, R., J. Ejaz, S. Gao, T. Liu, M. Imtiaz, Z. Ye and T. Wang (2017). "Overexpression of annexin gene AnnSp2, enhances drought and salt tolerance through modulation of ABA synthesis and scavenging ROS in tomato." Scientific Reports **7**(1): 12087.

Ingham, A. B., R. J. J. B. Moore and a. biochemistry (2007). "Recombinant production of antimicrobial peptides in heterologous microbial systems." **47**(1): 1-9.

Ireland, D. C., M. L. Colgrave and D. J. Craik (2006). "A novel suite of cyclotides from Viola odorata: sequence variation and the implications for structure, function and stability." Biochem J **400**(1): 1-12.

Jackson, S. L. and I. B. Heath (1993). "Roles of calcium ions in hyphal tip growth." Microbiol Rev **57**(2): 367-382.

Janssen, B. J., H. J. Schirra, F. T. Lay, M. A. Anderson and D. J. Craik (2003). "Structure of Petunia hybrida defensin 1, a novel plant defensin with five disulfide bonds." Biochemistry **42**(27): 8214-8222.

Jenssen, H., P. Hamill and R. E. Hancock (2006). "Peptide antimicrobial agents." Clin Microbiol Rev **19**(3): 491-511.

Jeong, B. R., Y. Lin, A. Joe, M. Guo, C. Korneli, H. Yang, P. Wang, M. Yu, R. L. Cerny, D. Staiger, J. R. Alfano and Y. Xu (2011). "Structure function analysis of an ADP-ribosyltransferase type III effector and its RNA-binding target in plant immunity." J Biol Chem **286**(50): 43272-43281.

Johnson, T. (1961). "Rust research in Canada and related plant disease Investigations."

Jones, J. D. and J. L. Dangl (2006). "The plant immune system." Nature **444**(7117): 323-329.

Jones, J. D. G. and J. L. Dangl (2006). "The plant immune system." Nature **444**(7117): 323-329.

Jung, Y. J., S. Y. Lee, Y. S. Moon and K. K. Kang (2012). "Enhanced resistance to bacterial and fungal pathogens by overexpression of a human cathelicidin antimicrobial peptide (hCAP18/LL-37) in Chinese cabbage." Plant Biotechnol Rep **6**(1): 39-46.

Kaku, H., Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami and N. Shibuya (2006). "Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor." Proc Natl Acad Sci U S A **103**(29): 11086-11091.

Kaminaka, H., C. Näke, P. Epple, J. Dittgen, K. Schütze, C. Chaban, B. F. Holt, 3rd, T. Merkle, E. Schäfer, K. Harter and J. L. Dangl (2006). "bZIP10-LSD1 antagonism modulates basal defense and cell death in Arabidopsis following infection." Embo j **25**(18): 4400-4411.

Karabelas, A. J., K. V. Plakas, E. S. Solomou, V. Drossou and D. A. Sarigiannis (2009). "Impact of European legislation on marketed pesticides — A view from the standpoint of health impact assessment studies." Environment International **35**(7): 1096-1107.

Kerenga, B. K., J. A. McKenna, P. J. Harvey, P. Quimbar, D. Garcia-Ceron, F. T. Lay, T. K. Phan, P. K. Veneer, S. Vasa, K. Parisi, T. M. A. Shafee, N. L. van der Weerden, M. D. Hulett, D. J. Craik, M. A. Anderson and M. R. Bleackley (2019). "Salt-Tolerant Antifungal and Antibacterial Activities of the Corn Defensin ZmD32." Frontiers in Microbiology **10**.

Kinkema, M., W. Fan and X. Dong (2000). "Nuclear localization of NPR1 is required for activation of PR gene expression." Plant Cell **12**(12): 2339-2350.

Kliebenstein, D. J., R. A. Dietrich, A. C. Martin, R. L. Last and J. L. Dangl (1999). "LSD1 regulates salicylic acid induction of copper zinc superoxide dismutase in Arabidopsis thaliana." Mol Plant Microbe Interact **12**(11): 1022-1026.

Kragh, K. M., J. E. Nielsen, K. K. Nielsen, S. Dreboldt and J. D. Mikkelsen (1995). "Characterization and localization of new antifungal cysteine-rich proteins from Beta vulgaris." Mol Plant Microbe Interact **8**(3): 424-434.

Kubicek, C. P., T. L. Starr and N. L. Glass (2014). "Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi." Annu Rev Phytopathol **52**: 427-451.

Kuźniak, E. and E. Gajewska (2024). "Lipids and Lipid-Mediated Signaling in Plant–Pathogen Interactions." International Journal of Molecular Sciences **25**(13): 7255.

Lay, F. T., F. Brugliera and M. A. Anderson (2003). "Isolation and properties of floral defensins from ornamental tobacco and petunia." Plant Physiol **131**(3): 1283-1293.

Lee, D. G., S. Y. Shin, C.-Y. Maeng, Z. Z. Jin, K. L. Kim, K.-S. J. B. Hahm and b. r. communications (1999). "Isolation and characterization of a novel antifungal peptide from Aspergillus niger." **263**(3): 646-651.

Lee, O. S., B. Lee, N. Park, J. C. Koo, Y. H. Kim, D. T. Prasad, C. Karigar, H. J. Chun, B. R. Jeong, D. H. Kim, J. Nam, J. G. Yun, S. S. Kwak, M. J. Cho and D. J. Yun (2003). "Pn-AMPs, the hevein-like proteins from Pharbitis nil confers disease resistance against phytopathogenic fungi in tomato, Lycopersicum esculentum." Phytochemistry **62**(7): 1073-1079.

Leontiadou, H., A. E. Mark and S. J. Marrink (2006). "Antimicrobial Peptides in Action." Journal of the American Chemical Society **128**(37): 12156-12161.

Li, J., S. Hu, W. Jian, C. Xie and X. Yang (2021). "Plant antimicrobial peptides: structures, functions, and applications." Botanical Studies **62**(1): 5.

Li, Z., M. Zhou, Z. Zhang, L. Ren, L. Du, B. Zhang, H. Xu and Z. Xin (2011). "Expression of a radish defensin in transgenic wheat confers increased resistance to Fusarium graminearum and Rhizoctonia cerealis." Functional & integrative genomics **11**: 63-70.

Lin, W. C., C. F. Lu, J. W. Wu, M. L. Cheng, Y. M. Lin, N. S. Yang, L. Black, S. K. Green, J. F. Wang and C. P. Cheng (2004). "Transgenic tomato plants expressing the Arabidopsis NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases." Transgenic Res **13**(6): 567-581.

Lindermayr, C., S. Sell, B. Müller, D. Leister and J. Durner (2010). "Redox Regulation of the NPR1-TGA1 System of Arabidopsis thaliana by Nitric Oxide " The Plant Cell **22**(8): 2894-2907.

Lintz, J., G. Dubrulle, E. Cawston, S. Duplessis and B. Petre (2022). "A Short Review of Anti-Rust Fungi Peptides: Diversity and Bioassays." **4**.

Liu, J., J. M. Elmore, Z. J. Lin and G. Coaker (2011). "A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor." Cell Host Microbe **9**(2): 137-146.

Loper, J. E. and J. S. Buyer (1991). "Siderophores in microbial interactions on plant surfaces." Mol. Plant-Microbe Interact **4**(1): 5-13.

Lucas, J. A. (1998). "Plant Pathogens and Plant Diseases." Plant Pathology **47**(4): 542-542.

Macho, A. P. and C. Zipfel (2014). "Plant PRRs and the activation of innate immune signaling." Mol Cell **54**(2): 263-272.

Mackey, D., B. F. Holt, 3rd, A. Wiig and J. L. Dangl (2002). "RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis." Cell **108**(6): 743-754.

Mak, A. S. and B. L. Jones (1976). "The amino acid sequence of wheat β-purothionin." Canadian Journal of Biochemistry **54**(10): 835-842.

Malnoy, M., Q. Jin, E. E. Borejsza-Wysocka, S. Y. He and H. S. Aldwinckle (2007). "Overexpression of the apple MpNPR1 gene confers increased disease resistance in Malus x domestica." Mol Plant Microbe Interact **20**(12): 1568-1580.

Mandal, S. M., S. Dey, M. Mandal, S. Sarkar, S. Maria-Neto and O. L. Franco (2009). "Identification and structural insights of three novel antimicrobial peptides isolated from green coconut water." Peptides **30**(4): 633-637.

Mandal, S. M., L. Migliolo, S. Das, M. Mandal, O. Franco and T. Hazra (2012). "Identification and characterization of a bactericidal and proapoptotic peptide from Cycas revoluta seeds with DNA binding properties." Journal of cellular biochemistry **113**: 184-193.

Manzanares, P., M. Giner-Llorca, J. F. Marcos and S. Garrigues (2024). "Fighting pathogenic yeasts with plant defensins and anti-fungal proteins from fungi." Applied Microbiology and Biotechnology **108**(1): 277.

Marcos López, J. F., M. Gandía Gómez, S. Garrigues, P. Manzanares and M. Coca (2020). "Antifungal peptides and proteins with activity against fungi causing postharvest decay."

Margni, M., D. Rossier, P. Crettaz and O. Jolliet (2002). "Life cycle impact assessment of pesticides on human health and ecosystems." Agriculture, Ecosystems & Environment **93**(1): 379-392.

Martin, G. B., S. H. Brommonschenkel, J. Chunwongse, A. Frary, M. W. Ganal, R. Spivey, T. Wu, E. D. Earle and S. D. Tanksley (1993). "Map-based cloning of a protein kinase gene conferring disease resistance in tomato." Science **262**(5138): 1432-1436.

Mathivanan, N., V. Kabilan and K. Murugesan (1998). "Purification, characterization, and antifungal activity of chitinase from Fusarium chlamydosporum, a mycoparasite to groundnut rust, Puccinia arachidis." Can J Microbiol **44**(7): 646-651.

McCallum, B. D., C. W. Hiebert, S. Cloutier, G. Bakkeren, S. B. Rosa, D. G. Humphreys, G. F. Marais, C. A. McCartney, V. Panwar and C. Rampitsch (2016). "A review of wheat leaf rust research and the development of resistant cultivars in Canada." Canadian Journal of Plant Pathology **38**(1): 1-18.

Melchers, L. S. and M. H. Stuiver (2000). "Novel genes for disease-resistance breeding." Curr Opin Plant Biol **3**(2): 147-152.

Melotto, M., W. Underwood, J. Koczan, K. Nomura and S. Y. He (2006). "Plant stomata function in innate immunity against bacterial invasion." Cell **126**(5): 969-980.

Meng, X. and S. Zhang (2013). "MAPK cascades in plant disease resistance signaling." Annu Rev Phytopathol **51**: 245-266.

Meur, G., M. Budatha, T. Srinivasan, K. R. Rajesh Kumar, A. Dutta Gupta and P. B. Kirti (2008). "Constitutive expression of Arabidopsis NPR1 confers enhanced resistance to the early instars of Spodoptera litura in transgenic tobacco." Physiol Plant **133**(4): 765-775.

Mirzaee, M., E. Holásková, A. Mičúchová, D. J. Kopečný, Z. Osmani and I. Frébort (2021). "Long-Lasting Stable Expression of Human LL-37 Antimicrobial Peptide in Transgenic Barley Plants." Antibiotics (Basel) **10**(8).

Molina, A., P. A. Goy, A. Fraile, R. Sánchez-Monge and F. García-Olmedo (1993). "Inhibition of bacterial and fungal plant pathogens by thionins of types I and II." Plant Science **92**(2): 169-177.

Morel, J.-B. and J. L. Dangl (1997). "The hypersensitive response and the induction of cell death in plants." Cell Death & Differentiation **4**(8): 671-683.

Moreno, A. B., Á. Martínez del Pozo, B. J. A. m. San Segundo and biotechnology (2006). "Biotechnologically relevant enzymes and proteins." **72**(5): 883-895.

Mou, Z., W. Fan and X. Dong (2003). "Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes." Cell **113**(7): 935-944.

Mur, L. A., Y. M. Bi, R. M. Darby, S. Firek and J. Draper (1997). "Compromising early salicylic acid accumulation delays the hypersensitive response and increases viral dispersal during lesion establishment in TMV-infected tobacco." Plant J **12**(5): 1113-1126.

Mur, L. A., P. Kenton, A. J. Lloyd, H. Ougham and E. Prats (2008). "The hypersensitive response; the centenary is upon us but how much do we know?" J Exp Bot **59**(3): 501-520.

Nakanishi, T., H. Yoshizumi, S. Tahara, A. Hakura and K. Toyoshima (1979). "Cytotoxicity of purothionin-A on various animal cells." Gann = Gan **70**: 323-326.

Narayani, M., A. Chadha and S. Srivastava (2017). "Cyclotides from the Indian Medicinal Plant Viola odorata (Banafsha): Identification and Characterization." J Nat Prod **80**(7): 1972-1980.

Nawrath, C. and J. P. Métraux (1999). "Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation." Plant Cell **11**(8): 1393-1404.

Nawrot, R., J. Barylski, G. Nowicki, J. Broniarczyk, W. Buchwald and A. Goździcka-Józefiak (2014). "Plant antimicrobial peptides." Folia Microbiol (Praha) **59**(3): 181-196.

Newman, M.-A., T. Sundelin, J. T. Nielsen and G. Erbs (2013). "MAMP (microbe-associated molecular pattern) triggered immunity in plants." Frontiers in Plant Science **4**.

Ngai, P. H. and T. B. Ng (2004). "Coccinin, an antifungal peptide with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from large scarlet runner beans." Peptides **25**(12): 2063-2068.

Ngou, B. P. M., P. Ding and J. D. G. Jones (2022). "Thirty years of resistance: Zig-zag through the plant immune system." The Plant Cell **34**(5): 1447-1478.

Nicaise, V., M. Roux and C. Zipfel (2009). "Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm." Plant Physiol **150**(4): 1638-1647.

Nicolas, P. (2009). "Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides." The FEBS Journal **276**(22): 6483-6496.

Oard, S., M. C. Rush and J. H. Oard (2004). "Characterization of antimicrobial peptides against a US strain of the rice pathogen Rhizoctonia solani." Journal of Applied Microbiology **97**(1): 169-180.

Oard, S. V. J. B. e. B. A.-B. (2011). "Deciphering a mechanism of membrane permeabilization by α-hordothionin peptide." **1808**(6): 1737-1745.

Odintsova, T., T. Egorov, A. Musolyamov, M. Odintsova, V. Pukhalsky and E. Grishin (2007). "Seed defensins from T. kiharae and related species: Genome localization of defensin-encoding genes." Biochimie **89**: 605-612.

Odintsova, T. I., A. A. Vassilevski, A. A. Slavokhotova, A. K. Musolyamov, E. I. Finkina, N. V. Khadeeva, E. A. Rogozhin, T. V. Korostyleva, V. A. Pukhalsky, E. V. Grishin and T. A. Egorov (2009). "A novel antifungal hevein-type peptide from Triticum kiharae seeds with a unique 10-cysteine motif." The FEBS Journal **276**(15): 4266-4275.

Oerke, E. C. (2006). "Crop losses to pests." The Journal of Agricultural Science **144**(1): 31-43.

Oita, S., M. Ohnishi-Kameyama and T. Nagata (2000). "Binding of barley and wheat alpha-thionins to polysaccharides." Biosci Biotechnol Biochem **64**(5): 958-964.

Osborn, R. W., G. W. De Samblanx, K. Thevissen, I. Goderis, S. Torrekens, F. Van Leuven, S. Attenborough, S. B. Rees and W. F. Broekaert (1995). "Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae." FEBS Lett **368**(2): 257-262.

Osusky, M., G. Zhou, L. Osuska, R. E. Hancock, W. W. Kay and S. Misra (2000). "Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens." Nat Biotechnol **18**(11): 1162-1166.

Panwar, V., M. Jordan, B. McCallum and G. Bakkeren (2018). "Host-induced silencing of essential genes in Puccinia triticina through transgenic expression of RNAi sequences reduces severity of leaf rust infection in wheat." Plant Biotechnol J **16**(5): 1013-1023.

Panwar, V., M. Jordan, B. McCallum and G. Bakkeren (2018). "Host-induced silencing of essential genes in Puccinia triticina through transgenic expression of RNAi sequences reduces severity of leaf rust infection in wheat." Plant Biotechnology Journal **16**(5): 1013-1023.

Parachin, N. S., K. C. Mulder, A. A. B. Viana, S. C. Dias and O. L. J. P. Franco (2012). "Expression systems for heterologous production of antimicrobial peptides." **38**(2): 446-456.

Park, R. F. and A. Karakousis (2002). "Characterization and mapping of gene Rph19 conferring resistance to Puccinia hordei in the cultivar 'Reka 1' and several Australian barleys." **121**(3): 232-236.

Parkhi, V., V. Kumar, L. M. Campbell, A. A. Bell, J. Shah and K. S. Rathore (2010). "Resistance against various fungal pathogens and reniform nematode in transgenic cotton plants expressing Arabidopsis NPR1." Transgenic Res **19**(6): 959-975.

Pearce, G., D. Strydom, S. Johnson and C. A. Ryan (1991). "A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins." Science **253**(5022): 895-897.

Pelegrini, P. B. and O. L. Franco (2005). "Plant gamma-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins." Int J Biochem Cell Biol **37**(11): 2239-2253.

Peturson, B. (1958). "Wheat rust epidemics in western Canada in 1953, 1954 and 1955." Canadian Journal of Plant Science **38**(1): 16-28.

Picart, P., A. M. Pirttilä, D. Raventos, H.-H. Kristensen and H.-G. Sahl (2012). "Identification of defensin-encoding genes of Picea glauca: characterization of PgD5, a conserved spruce defensin with strong antifungal activity." BMC Plant Biology **12**(1): 180.

Pieterse, C. M., S. C. van Wees, J. A. van Pelt, M. Knoester, R. Laan, H. Gerrits, P. J. Weisbeek and L. C. van Loon (1998). "A novel signaling pathway controlling induced systemic resistance in Arabidopsis." Plant Cell **10**(9): 1571-1580.

Potlakayala, S. D., C. DeLong, A. Sharpe and P. R. Fobert (2007). "Conservation of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 function between Arabidopsis thaliana and Brassica napus." Physiological and Molecular Plant Pathology **71**(4): 174-183.

Pouny, Y., D. Rapaport, A. Mor, P. Nicolas and Y. Shai (1992). "Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes." Biochemistry **31**(49): 12416-12423.

Powers, J. P. and R. E. Hancock (2003). "The relationship between peptide structure and antibacterial activity." Peptides **24**(11): 1681-1691.

Prime, A. P. G., U. Conrath, G. J. Beckers, V. Flors, P. Garcia-Agustin, G. Jakab, F. Mauch, M. A. Newman, C. M. Pieterse, B. Poinssot, M. J. Pozo, A. Pugin, U. Schaffrath, J. Ton, D. Wendehenne, L. Zimmerli and B. Mauch-Mani (2006). "Priming: getting ready for battle." Mol Plant Microbe Interact **19**(10): 1062-1071.

Rajasekaran, K., K. D. Stromberg, J. W. Cary and T. E. Cleveland (2001). "Broad-spectrum antimicrobial activity in vitro of the synthetic peptide D4E1." J Agric Food Chem **49**(6): 2799-2803.

Ramineni, R., V. Sadumpati, V. R. Khareedu and D. R. Vudem (2014). "Transgenic pearl millet male fertility restorer line (ICMP451) and hybrid (ICMH451) expressing Brassica juncea Nonexpressor of pathogenesis related genes 1 (BjNPR1) exhibit resistance to downy mildew disease." PLoS One **9**(6): e90839.

Randhawa, H. S., M. Asif, C. Pozniak, J. M. Clarke, R. J. Graf, S. L. Fox, D. G. Humphreys, R. E. Knox, R. M. DePauw, A. K. Singh, R. D. Cuthbert, P. Hucl and D. Spaner (2013). "Application of molecular markers to wheat breeding in Canada." Plant Breeding **132**(5): 458-471.

Rasmussen, J. B., R. Hammerschmidt and M. N. Zook (1991). "Systemic Induction of Salicylic Acid Accumulation in Cucumber after Inoculation with Pseudomonas syringae pv syringae." Plant Physiol **97**(4): 1342-1347.

Rathjen, J. P., J. H. Chang, B. J. Staskawicz and R. W. Michelmore (1999). "Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of avrPto." The EMBO journal.

REIMANN-PHILIPP, U., S. BEHNKE, A. BATSCHAUER, E. SCHÄFER and K. J. E. j. o. b. APEL (1989). "The effect of light on the biosynthesis of leaf-specific thionins in barley, Hordeum vulgare." **182**(2): 283-289.

Riederer, M. and C. Müller (2007). Biology of the Plant Cuticle.

Ritz, C., F. Baty, J. C. Streibig and D. Gerhard (2015). "Dose-Response Analysis Using R." PLoS One **10**(12): e0146021.

Rivero, M., N. Furman, N. Mencacci, P. Picca, L. Toum, E. Lentz, F. Bravo-Almonacid and A. Mentaberry (2012). "Stacking of antimicrobial genes in potato transgenic plants confers increased resistance to bacterial and fungal pathogens." J Biotechnol **157**(2): 334-343.

Rochon, A., P. Boyle, T. Wignes, P. R. Fobert and C. Després (2006). "The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines." Plant Cell **18**(12): 3670-3685.

Ross, A. F. (1961). "Systemic acquired resistance induced by localized virus infections in plants." Virology **14**(3): 340-358.

Ryals, J., K. Weymann, K. Lawton, L. Friedrich, D. Ellis, H. Y. Steiner, J. Johnson, T. P. Delaney, T. Jesse, P. Vos and S. Uknes (1997). "The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B." Plant Cell **9**(3): 425-439.

Ryals, J. A., U. H. Neuenschwander, M. G. Willits, A. Molina, H. Y. Steiner and M. D. Hunt (1996). "Systemic Acquired Resistance." Plant Cell **8**(10): 1809-1819.

Sagaram, U. S., K. El-Mounadi, G. W. Buchko, H. R. Berg, J. Kaur, R. S. Pandurangi, T. J. Smith and D. M. Shah (2013). "Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: identification of an RGFRRR motif governing fungal cell entry." PLoS One **8**(12): e82485.

Samborski, D. (1985). Wheat leaf rust. diseases, distribution, epidemiology, and control, Elsevier: Academic Press.

Schmidt, M., E. K. Arendt and T. L. C. Thery (2019). "Isolation and characterisation of the antifungal activity of the cowpea defensin Cp-thionin II." Food Microbiology **82**: 504-514.

Schuhegger, R., T. Rauhut and E. Glawischnig (2007). "Regulatory variability of camalexin biosynthesis." J Plant Physiol **164**(5): 636-644.

Schulze, B., T. Mentzel, A. K. Jehle, K. Mueller, S. Beeler, T. Boller, G. Felix and D. Chinchilla (2010). "Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1." J Biol Chem **285**(13): 9444-9451.

Schulze, S., S. Kay, D. Büttner, M. Egler, L. Eschen-Lippold, G. Hause, A. Krüger, J. Lee, O. Müller, D. Scheel, R. Szczesny, F. Thieme and U. Bonas (2012). "Analysis of new type III effectors from Xanthomonas uncovers XopB and XopS as suppressors of plant immunity." New Phytol **195**(4): 894-911.

Segura, A., M. Moreno, F. Madueño, A. Molina and F. García-Olmedo (1999). "Snakin-1, a peptide from potato that is active against plant pathogens." Mol Plant Microbe Interact **12**(1): 16-23.

Segura, A., M. Moreno, A. Molina and F. García-Olmedo (1998). "Novel defensin subfamily from spinach (Spinacia oleracea)." FEBS Lett **435**(2-3): 159-162.

Selitrennikoff, C. P. (2001). "Antifungal proteins." Appl Environ Microbiol **67**(7): 2883-2894.

Sengupta, D., H. Leontiadou, A. E. Mark and S.-J. Marrink (2008). "Toroidal pores formed by antimicrobial peptides show significant disorder." Biochimica et Biophysica Acta (BBA) - Biomembranes **1778**(10): 2308-2317.

Shah, J., F. Tsui and D. F. Klessig (1997). "Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene." Mol Plant Microbe Interact **10**(1): 69-78.

Shai, Y. (2002). "Mode of action of membrane active antimicrobial peptides." Biopolymers **66**(4): 236-248.

Shan, L., P. He, J. Li, A. Heese, S. C. Peck, T. Nürnberger, G. B. Martin and J. Sheen (2008). "Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity." Cell Host Microbe **4**(1): 17-27.

Sher Khan, R., A. Iqbal, R. Malak, K. Shehryar, S. Attia, T. Ahmed, M. Ali Khan, M. Arif and M. Mii (2019). "Plant defensins: types, mechanism of action and prospects of genetic engineering for enhanced disease resistance in plants." 3 Biotech **9**(5): 192.

Shibuya, N. and E. Minami (2001). "Oligosaccharide signaling for defense responses in plant." Physiological and Molecular Plant Pathology **59**(5): 223-233.

Shirasu, K., H. Nakajima, V. K. Rajasekhar, R. A. Dixon and C. Lamb (1997). "Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms." Plant Cell **9**(2): 261-270.

Shulaev, V., P. Silverman and I. Raskin (1997). "Airborne signaling by methyl salicylate in plant pathogen resistance." Nature **385**(6618): 718-721.

Silva, P. M., S. Gonçalves and N. C. J. F. i. m. Santos (2014). "Defensins: antifungal lessons from eukaryotes." **5**: 97.

Slinkard, A. and D. R. Knott (1995). Harvest of gold: the history of field crop breeding in Canada, Saskatoon: University Extension Press, University of Saskatchewan.

Song, X., M. Zhang, Z. Zhou and W. Gong (2011). "Ultra-high resolution crystal structure of a dimeric defensin SPE10." FEBS Letters **585**(2): 300-306.

Spelbrink, R. G., N. Dilmac, A. Allen, T. J. Smith, D. M. Shah and G. H. Hockerman (2004). "Differential antifungal and calcium channel-blocking activity among structurally related plant defensins." Plant Physiol **135**(4): 2055-2067.

Spoel, S. H., Z. Mou, Y. Tada, N. W. Spivey, P. Genschik and X. Dong (2009). "Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity." Cell **137**(5): 860-872.

Stakman, E. (1915). "VOL. IV." Journal of Agricultural Research **4**: 193.

Staskawicz, B. J., D. Dahlbeck and N. T. Keen (1984). "Cloned avirulence gene of Pseudomonas syringae pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr." Proc Natl Acad Sci U S A **81**(19): 6024-6028.

Stec, B., O. Markman, U. Rao, G. Heffron, S. Henderson, L. Vernon, V. Brumfeld and M. J. T. J. o. p. r. Teeter (2004). "Proposal for molecular mechanism of thionins deduced from physico-chemical studies of plant toxins." **64**(6): 210-224.

Su, T., M. Han, D. Cao and M. Xu (2020). "Molecular and Biological Properties of Snakins: The Foremost Cysteine-Rich Plant Host Defense Peptides." J Fungi (Basel) **6**(4).

Tada, Y., S. H. Spoel, K. Pajerowska-Mukhtar, Z. Mou, J. Song, C. Wang, J. Zuo and X. Dong (2008). "Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins." Science **321**(5891): 952-956.

Tang, R., H. Tan, Y. Dai, L. a. Li, Y. Huang, H. Yao, Y. Cai and G. Yu (2023). "Application of antimicrobial peptides in plant protection: making use of the overlooked merits." Frontiers in Plant Science **14**.

Taveira, G. B., O. Mello É, A. O. Carvalho, M. Regente, M. Pinedo, L. de La Canal, R. Rodrigues and V. M. Gomes (2017). "Antimicrobial activity and mechanism of action of a thionin-like peptide from Capsicum annuum fruits and combinatorial treatment with fluconazole against Fusarium solani." Biopolymers **108**(3).

Terras, F. R., H. M. Schoofs, M. F. De Bolle, F. Van Leuven, S. B. Rees, J. Vanderleyden, B. P. Cammue and W. F. Broekaert (1992). "Analysis of two novel classes of plant antifungal proteins from radish (Raphanus sativus L.) seeds." J Biol Chem **267**(22): 15301-15309.

Thevissen, K., F. R. Terras and W. F. Broekaert (1999). "Permeabilization of fungal membranes by plant defensins inhibits fungal growth." Appl Environ Microbiol **65**(12): 5451-5458.

Thevissen, K., D. C. Warnecke, I. E. François, M. Leipelt, E. Heinz, C. Ott, U. Zähringer, B. P. Thomma, K. K. Ferket and B. P. Cammue (2004). "Defensins from insects and plants interact with fungal glucosylceramides." J Biol Chem **279**(6): 3900-3905.

Thilmony, R., W. Underwood and S. Y. He (2006). "Genome-wide transcriptional analysis of the Arabidopsis thaliana interaction with the plant pathogen Pseudomonas syringae pv. tomato DC3000 and the human pathogen Escherichia coli O157:H7." Plant J **46**(1): 34-53.

Thomma, B. P., B. P. Cammue and K. Thevissen (2002). "Plant defensins." Planta **216**(2): 193-202.

Torres, M. A., J. L. Dangl and J. D. G. Jones (2002). "Arabidopsis gp91^{phox} homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response." Proceedings of the National Academy of Sciences **99**(1): 517-522.

Torres, M. A., J. D. G. Jones and J. L. Dangl (2006). "Reactive Oxygen Species Signaling in Response to Pathogens." Plant Physiology **141**(2): 373-378.

Tossi, A., L. Sandri and A. Giangaspero (2000). "Amphipathic, α-helical antimicrobial peptides." Peptide Science **55**(1): 4-30.

Uknes, S., B. Mauch-Mani, M. Moyer, S. Potter, S. Williams, S. Dincher, D. Chandler, A. Slusarenko, E. Ward and J. Ryals (1992). "Acquired resistance in Arabidopsis." Plant Cell **4**(6): 645-656.

Underwood, W. (2012). "The plant cell wall: a dynamic barrier against pathogen invasion." Front Plant Sci **3**: 85.

Utkina, L. L., Y. A. Andreev, E. A. Rogozhin, T. V. Korostyleva, A. A. Slavokhotova, P. B. Oparin, A. A. Vassilevski, E. V. Grishin, T. A. Egorov and T. I. Odintsova (2013). "Genes encoding 4-Cys antimicrobial peptides in wheat Triticum kiharae Dorof. et Migush.: multimodular structural organization, instraspecific variability, distribution and role in defense." Febs j **280**(15): 3594-3608.

Vaahtera, L., J. Schulz and T. Hamann (2019). "Cell wall integrity maintenance during plant development and interaction with the environment." Nature Plants **5**(9): 924-932.

van der Weerden, N. L. and M. A. Anderson (2013). "Plant defensins: Common fold, multiple functions." Fungal Biology Reviews **26**(4): 121-131.

Van der Weerden, N. L., M. R. Bleackley, M. A. J. C. Anderson and m. l. sciences (2013). "Properties and mechanisms of action of naturally occurring antifungal peptides." **70**(19): 3545-3570.

van der Weerden, N. L., R. E. Hancock and M. A. Anderson (2010). "Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process." J Biol Chem **285**(48): 37513-37520.

van der Weerden, N. L., F. T. Lay and M. A. Anderson (2008). "The plant defensin, NaD1, enters the cytoplasm of Fusarium oxysporum hyphae." J Biol Chem **283**(21): 14445-14452.

Van Loon, L., W. Pierpoint, T. Boller and V. Conejero (1994). "Recommendations for naming plant pathogenesis-related proteins." Plant Molecular Biology Reporter **12**: 245-264.

van Loon, L. C., M. Rep and C. M. Pieterse (2006). "Significance of inducible defense-related proteins in infected plants." Annu Rev Phytopathol **44**: 135-162.

VanEtten, H. D., J. W. Mansfield, J. A. Bailey and E. E. Farmer (1994). "Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins"." Plant Cell **6**(9): 1191-1192.

Velivelli, S. L. S., K. J. Czymmek, H. Li, J. B. Shaw, G. W. Buchko and D. M. Shah (2020). "Antifungal symbiotic peptide NCR044 exhibits unique structure and multifaceted mechanisms of action that confer plant protection." Proceedings of the National Academy of Sciences **117**(27): 16043-16054.

Velivelli, S. L. S., K. J. Czymmek, H. Li, J. B. Shaw, G. W. Buchko and D. M. Shah (2020). "Antifungal symbiotic peptide NCR044 exhibits unique structure and multifaceted mechanisms of action that confer plant protection." Proc Natl Acad Sci U S A **117**(27): 16043-16054.

Verma, S. S., W. R. Yajima, M. H. Rahman, S. Shah, J. J. Liu, A. K. Ekramoddoullah and N. N. Kav (2012). "A cysteine-rich antimicrobial peptide from Pinus monticola (PmAMP1) confers resistance to multiple fungal pathogens in canola (Brassica napus)." Plant Mol Biol **79**(1-2): 61-74.

Vernooij, B., L. Friedrich, A. Morse, R. Reist, R. Kolditz-Jawhar, E. Ward, S. Uknes, H. Kessmann and J. Ryals (1994). "Salicylic Acid Is Not the Translocated Signal Responsible for Inducing Systemic Acquired Resistance but Is Required in Signal Transduction." Plant Cell **6**(7): 959-965.

Vetchinkina, E. M., V. V. Komakhina, D. A. Vysotskii, D. V. Zaitsev, A. N. Smirnov, A. V. Babakov and R. A. Komakhin (2016). "[Expression of plant antimicrobial peptide pro-SmAMP2 gene increases resistance of transgenic potato plants to Alternaria and Fusarium pathogens]." Genetika **52**(9): 1055-1068.

Vigers, A. J., W. K. Roberts and C. P. Selitrennikoff (1991). "A new family of plant antifungal proteins." Mol Plant Microbe Interact **4**(4): 315-323.

Vilanova, L., I. Viñas, R. Torres, J. Usall, G. Buron-Moles and N. Teixidó (2014). "Acidification of apple and orange hosts by Penicillium digitatum and Penicillium expansum." International Journal of Food Microbiology **178**: 39-49.

Vriens, K., B. P. A. Cammue and K. Thevissen (2014). "Antifungal Plant Defensins: Mechanisms of Action and Production." Molecules **19**(8): 12280-12303.

Vu, T. T., H. Kim, V. K. Tran, H. D. Vu, T. X. Hoang, J. W. Han, Y. H. Choi, K. S. Jang, G. J. Choi and J. C. Kim (2017). "Antibacterial activity of tannins isolated from Sapium baccatum extract and use for control of tomato bacterial wilt." PLoS One **12**(7): e0181499.

Wally, O., J. Jayaraj and Z. K. Punja (2009). "Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (Daucus carota L.) expressing an Arabidopsis NPR1 gene." Planta **231**(1): 131-141.

Wan, J., M. He, Q. Hou, L. Zou, Y. Yang, Y. Wei and X. Chen (2021). "Cell wall associated immunity in plants." Stress Biology **1**(1): 3.

Wang, F., S. Yuan, W. Wu, Y. Yang, Z. Cui, H. Wang and D. Liu (2020). "TaTLP1 interacts with TaPR1 to contribute to wheat defense responses to leaf rust fungus." PLOS Genetics **16**(7): e1008713.

Wang, J., J. Qiu, X. Yang, J. Yang, S. Zhao, Q. Zhou and L. Chen (2022). "Identification of Lipopeptide Iturin A Produced by Bacillus amyloliquefaciens NCPSJ7 and Its Antifungal Activities against Fusarium oxysporum f. sp. niveum." Foods **11**(19): 2996.

War, A. R., G. K. Taggar, B. Hussain, M. S. Taggar, R. M. Nair and H. C. Sharma (2018). "Plant defense against herbivory and insect adaptations." AoB PLANTS **10**(4).

Ward, E. R., S. J. Uknes, S. C. Williams, S. S. Dincher, D. L. Wiederhold, D. C. Alexander, P. Ahl-Goy, J. P. Metraux and J. A. Ryals (1991). "Coordinate Gene Activity in Response to Agents That Induce Systemic Acquired Resistance." Plant Cell **3**(10): 1085-1094.

Warner, P., A. Karakousis, A. Schiemann, J. Eglinton, P. Langridge and A. Barr (2002). "An investigation of a rapid DNA extraction method for routine MAS in the S.A. Barley Improvement Program."

Wegulo, S. N. and E. Byamukama (2012). "Rust diseases of wheat." University of Nebraska–Lincoln: Lincoln, NE, USA.

Wen, Q., M. Sun, X. Kong, Y. Yang, Q. Zhang, G. Huang, W. Lu, W. Li, Y. Meng and W. Shan (2021). "The novel peptide NbPPI1 identified from Nicotiana benthamiana triggers immune responses and enhances resistance against Phytophthora pathogens." J Integr Plant Biol **63**(5): 961-976.

Weymann, K., M. Hunt, S. Uknes, U. Neuenschwander, K. Lawton, H. Y. Steiner and J. Ryals (1995). "Suppression and Restoration of Lesion Formation in Arabidopsis lsd Mutants." Plant Cell **7**(12): 2013-2022.

Wildermuth, M. C., J. Dewdney, G. Wu and F. M. Ausubel (2001). "Isochorismate synthase is required to synthesize salicylic acid for plant defense." Nature **414**(6863): 562-565.

Wilmes, M., B. P. Cammue, H. G. Sahl and K. Thevissen (2011). "Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation." Nat Prod Rep **28**(8): 1350-1358.

Wu, X., J. Sun, G. Zhang, H. Wang and T. B. Ng (2011). "An antifungal defensin from Phaseolus vulgaris cv. 'Cloud Bean'." Phytomedicine **18**(2-3): 104-109.

Xia, L. and T. B. Ng (2005). "Isolation of alliumin, a novel protein with antimicrobial and antiproliferative activities from multiple-cloved garlic bulbs." Peptides **26**(2): 177-183.

Xiang, T., N. Zong, Y. Zou, Y. Wu, J. Zhang, W. Xing, Y. Li, X. Tang, L. Zhu, J. Chai and J. M. Zhou (2008). "Pseudomonas syringae effector AvrPto blocks innate immunity by targeting receptor kinases." Curr Biol **18**(1): 74-80.

Xu, H. and M. C. Heath (1998). "Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus." Plant Cell **10**(4): 585-598.

Yang, L., T. A. Harroun, T. M. Weiss, L. Ding and H. W. Huang (2001). "Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores." Biophysical Journal **81**(3): 1475-1485.

Yeaman, M. R. and N. Y. Yount (2003). "Mechanisms of antimicrobial peptide action and resistance." Pharmacol Rev **55**(1): 27-55.

Yount, N. Y. and M. R. Yeaman (2004). "Multidimensional signatures in antimicrobial peptides." Proceedings of the National Academy of Sciences **101**(19): 7363-7368.

Yu, G., X. Zhang, J. Yao, M. Zhou and H. Ma (2017). "Resistance against Fusarium Head Blight in Transgenic Wheat Plants Expressing the ScNPR1 gene." Journal of Phytopathology **165**(4): 223-231.

Yuan, Y., S. Zhong, Q. Li, Z. Zhu, Y. Lou, L. Wang, J. Wang, M. Wang, Q. Li, D. Yang and Z. He (2007). "Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility." Plant Biotechnol J **5**(2): 313-324.

Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature **415**(6870): 389-395.

Zhang, J., F. Shao, Y. Li, H. Cui, L. Chen, H. Li, Y. Zou, C. Long, L. Lan, J. Chai, S. Chen, X. Tang and J. M. Zhou (2007). "A Pseudomonas syringae effector inactivates MAPKs to suppress PAMP-induced immunity in plants." Cell Host Microbe **1**(3): 175-185.

Zhang, Y., W. Fan, M. Kinkema, X. Li and X. Dong (1999). "Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene." Proceedings of the National Academy of Sciences **96**(11): 6523-6528.

Zhang, Y., M. J. Tessaro, M. Lassner and X. Li (2003). "Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance." Plant Cell **15**(11): 2647-2653.

Ziemann, S., K. van der Linde, U. Lahrmann, B. Acar, F. Kaschani, T. Colby, M. Kaiser, Y. Ding, E. Schmelz, A. Huffaker, N. Holton, C. Zipfel and G. Doehlemann (2018). "An apoplastic peptide activates salicylic acid signaling in maize." Nature Plants **4**.

Zipfel, C. (2014). "Plant pattern-recognition receptors." Trends Immunol **35**(7): 345-351.