Identification of a putative protease inhibitor involved in three different *Puccinia - Triticum aestivum* interactions

by

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“Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life”.

Marcus Aurelius

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Abbreviations

A

AP	Aspartic peptidase
ARF	ADP-ribosylation factor

B

BBI	Bowman-Birk protease inhibitor
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BIK1	Botrytis-induced kinase 1
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin

C

CDC	Cell division control protein gene
cDNA	Complementary deoxyribonucleic acid
Co-IP	Co-immunoprecipitation
CR	Control resistant
CS	Control susceptible

D

DAMP	Danger-/damage-associated molecular pattern
DEPC	Diethyl pyrocarbonate
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

E

EDTA	Ethylenedinitrilotetraacetic acid
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility

F

FLS2	Flagellin-sensitive 2 receptor-like kinase
FHA	Forkhead-associated

G

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLP	Germin-like protein
GLPI	Germin-like protease inhibitor
GPCR	G-protein-coupled receptor
G-proteins	Guanine nucleotide binding proteins
GTP	Guanosine-5'-triphosphate

H

HAMP	Herbivore-associated molecular pattern
hpi	Hours post inoculation
HR	Hypersensitive response

I

Ig	Immunoglobulin
IR	Induced response/Infected resistant
IS	Infected susceptible
ISR	Induced systemic resistance

J

JA	Jasmonic acid

K

KLH	Keyhole limpet hemocyanin

L

LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LRR	Leucine-rich repeat
LRR-RLK	Leucine-rich repeat receptor-like protein kinase

M

M-value	Average expression stability value
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeJA	Methyl jasmonate
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments

N

NBS	Nucleotide-binding site
NBT	Nitro blue tetrazolium chloride
NO	Nitric oxide
NP-40	Nonyl phenoxypolyethoxylethanol

P

PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
Pgt	*Puccinia graminis* f. sp. *tritici*
PI	Protease inhibitor
PR	Pathogenesis-related
PRR	Pattern recognition receptor
Ps	*Puccinia striiformis*
PSI	Plant specific insert
Pt	*Puccinia triticina*
PTI	Pattern-triggered immunity

Q

qPCR	Quantitative (Real-time) PCR

R

R genes / R proteins	Resistance genes / resistance proteins
RIN	RNA integrity value
RLK	Receptor-like protein kinase
RLCK	Receptor-like cytoplasmic kinase
RLI	RNase L inhibitor-like protein gene
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction

S

SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSH	Suppression subtractive hybridisation

T

TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween™ 20
Tris	Tris (hydroxymethyl)-aminomethane
TUBB	Beta-tubulin
Tween™ 20	Polyoxyethylene sorbitan monolaurate

U

UTR	Untranslated region

Y

Yop	Yersinia outer protein

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Chapter 1

Introduction

Approximately 8000 years ago, following the hybridisation of *Triticum turgidum* L. (AABB) and *Aegilops tauschii* Coss. (DD), hexaploid wheat (*T. aestivum* L.) was formed with a genome constitution of AABBDD (2n = 6x = 42; Feldman, 2001). Archaeological evidence suggests that wheat was first domesticated between 7500 and 6500 B.C. in an area known as the 'fertile crescent' in Western Asia (Zohary and Hopf, 2000).

Wheat is currently one of the most important food crops in the world. It is consumed in 175 countries with an estimated world consumption of 679 million tonnes for 2012/2013 (http://www.igc.int/en/grainsupdate/sd.aspx?crop=Wheat). According to the Food and Agriculture Organization of the United Nations, wheat is the leading crop regarding land area usage (http://faostat.fao.org/site/567/default.aspx#ancor). With an estimated 217 million hectares in 2010, it is followed by maize, rice and potatoes. It is adapted to a wide range of environments; while the optimum cultivation temperature is 25°C, it is grown in temperatures ranging from 3 to 32°C with annual precipitation of between 250 to 1750 mm.

Many diseases affect the growth and survival of wheat. Wiese (1987) identified over 150 diseases in wheat caused by fungal, viral and bacterial infections. Forty of these were due to fungi which cause some of the most important diseases of wheat. The occurrence of rust diseases in wheat caused by *Puccinia* species has played a major role in the development of human civilisation and is currently responsible for some of the largest yield losses (Hovmøller *et al*., 2010).

Puccinia is the largest genus in the family *Pucciniaceae.* It has an estimated 3000-4000 species, all of which are obligate biotrophs that cause rust diseases in many plants (Littlefield, 1981). Rust spores are aerially transported, causing a rapid spread of disease epidemics between different continents (Aylor, 2003). The majority of *Puccinia* species have a complex life cycle with five distinct spore stages, namely teliospores (diploid), basidiospores (haploid), pycniospores (haploid), aeciospores (dikaryotic) and urediniospores (dikaryotic). Certain species require two unrelated hosts for a complete life cycle, while others require only one (Kolmer *et al.*, 2009).

The rust diseases of wheat are caused by *Puccinia triticina* Eriks. (*Pt*)*, Puccinia graminis* Pers. f. sp. *tritici* Eriks. and Henn. (*Pgt*) and *Puccinia striiformis* Westend. (*Ps*). These three pathogens are the causal agents of leaf, stem and yellow rust respectively. All three diseases have the ability to severely affect wheat production. Although the annual yield losses from *Pt*

infection are normally greater than from the other two rusts, it is usually less damaging (Huerta-Espino *et al.,* 2011). The yield losses are due to reduced kernel weight and a reduced number of kernels per head.

Leaf rust produces red-brown, oval shaped urediniospores that penetrate the leaf stomata upon germination. It has a heteroecious life cycle that includes the five different spore stages and two hosts. *Thalictrum speciosissimum* L. (Meadow rue) is the alternate host on which sexual reproduction takes place. The sexual stage is not required and some epidemics are caused by re-infection with urediniospores (Bolton *et al.,* 2008).

Asexual reproduction of stem rust is characterised by the five distinct spore stages on its cereal hosts. *Berberis* species act as the alternate host for sexual reproduction. The disease symptoms of stem rust in cereals mainly occur on the stems and leaf sheaths and occasionally on the leaf blades. The brick-red uredinial pustules are usually diamond shaped and may become up to 10 mm in length (Leonard and Szabo, 2005). It is considered a serious threat to wheat production in the world. The severity of epidemics largely depends on climatic conditions. Researchers at the 2011 ICARDA international wheat stripe rust symposium reported that aggressive new strains of stem and stripe rust have destroyed approximately 40% of farmers' wheat fields in recent harvests (http://www.scidev.net/en/opinions/fight-against-wheat-rust-needs-sustained-investment-1.html). Additionally, in 2005 Nobel Laureate Dr Norman E. Borlaug warned the world about the severity of the Ug99 race of stem rust. It is rapidly spreading into parts of Africa, Asia and the Middle East, threatening worldwide wheat production and posing a serious threat to food security (Singh *et al.,* 2011).

Yellow rust, caused by *P. striiformis* is heteroecious with wheat as the primary host while *Berberis* has recently been identified as an alternate host for both yellow and stem rust (Jin *et al.,* 2010). It appears as yellow powdery pustules arranged in stripes along the leaf veins, reducing the grain quality by affecting the photosynthetic area of leaves and subsequent amount of carbohydrates in the developing seed.

Wheat and other plants employ a diverse range of defence strategies to combat disease. These include structural barriers and chemical defences which may be passive or induced. Passive defences include amongst others the cuticle, cell wall and constitutively expressed antimicrobial compounds. Induced defences rely on a vast array of receptors and signalling molecules to perceive and respond appropriately to invading pathogens. This induced

response is regulated by various defence hormones and is generally more intense (Pastor *et al.,* 2012). The perception of pathogenic effector molecules may activate certain signal transduction pathways that lead to the expression of defence-associated genes. The encoded proteins of these genes are directly involved in counteracting a pathogen infection (Singh *et al.*, 2008) and include hydrolytic enzymes, defensins, oxidases, proteases and protease inhibitors.

Protease inhibitors are responsible for the inhibition of specific proteases and are essential for the normal function of organisms. In plants, protease inhibitors play important roles in many diverse processes including the regulation of endogenous proteases and responses to abiotic and biotic stresses (Mosolov *et al.,* 2001).

During the PhD study of Huang (2008), suppression subtractive hybridisation (SSH) was used to identify genes that were differentially expressed in wheat infected with *Puccinia triticina.* One of the clones, *LRW222,* showed homology to *wali5* (accession number L11882, e-value: 8e-45), a predicted Bowman-Birk-type protease inhibitor. *Wali5* was previously shown to be induced by aluminium stress and wounding (Snowden *et al.,* 1995).

The aim of this study was to examine the expression levels and role of *LRW222* in *Pt-*, *Ps-* and *Pgt-*infected wheat. This was done by first validating a suitable set of reference genes for qPCR (quantitative polymerase chain reaction) gene expression analysis whereafter the expression of *LRW222* was quantified during all three wheat-*Puccinia* interactions. Reference gene validation and expression analysis of *LRW222* was done according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin *et al.,* 2009). Co- immunoprecipitation was used in an attempt to reveal the role of *LRW222* in wheat by identifying proteins that interact with the LRW222 protein.

Chapter 2

Literature review

2.1 Introduction

Plants provide an essential source of nutrients for most organisms on earth. Humans in particular, either directly or indirectly, depend on plants for nutrition. Although these vital food sources are constantly under pathogen attack, plants have evolved an arsenal of structural and chemical defences to detect pathogens and resist infection. These include multiple layers of preformed and inducible defences (Hammond-Kosack and Kanyuka, 2007). The plant's innate immune system confers broad spectrum immunity against the vast majority of pathogens (Figure 2.1).

Interactions in which plants are innately immune are called non-host resistance and can be divided into type I and II non-host resistance. Type I does not produce any visible symptoms, while type II is always associated with localised cell death during the hypersensitive response (HR; Mysore and Ryu, 2004). Host interactions result when pathogens bypass the innate immune system. This interaction is compatible if disease occurs and incompatible if the plant's active immune system effectively prevents disease. The active immune system is capable of directed and specific responses against invading pathogens (Hammond-Kosack and Jones, 1997). The current view of plant immunity is summarised in a zigzag model (Figure 2.2) proposed by Jones and Dangl (2006), which illustrates innate immunity, active immunity and the co-evolution of pathogenic effector and plant resistance (R) proteins.

2.1.1 Pattern-triggered immunity

The plant innate immune system provides protection against the vast majority of pathogens using preformed and inducible structural or chemical barriers. These include the cell wall, cytoskeleton and antimicrobial compounds that have to be overcome by pathogens in order to invade the plant and multiply. The first inducible layer of defence uses pattern recognition receptor (PRR) proteins that respond to entire classes of microorganisms. This is done through the recognition of conserved microbe-associated molecular patterns (MAMPs) such as chitin, flagellin and glycoproteins (Jafary *et al.*, 2006). Perception leads to a chain of signalling events that result in a non-specific defence response via an increase in extracellular pH, deposition of callose and the release of reactive oxygen species (ROS; Jones and Dangl, 2006; Chinchilla and Boller, 2012).

Pattern-triggered immunity (PTI) occurs when a plant successfully defends itself following

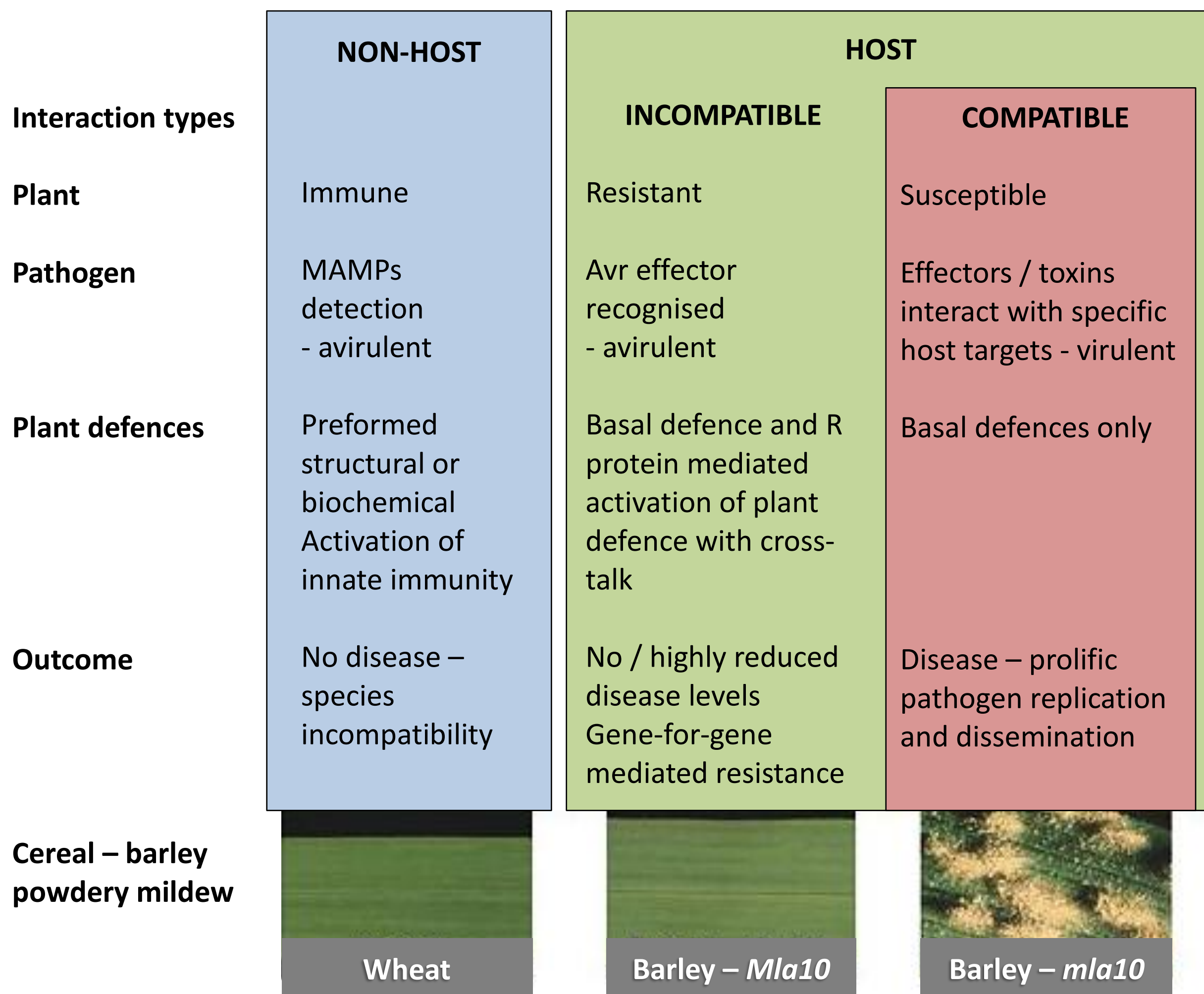

Figure 2.1 Plant resistance mechanisms as illustrated by interactions between barley powdery mildew and wheat and resistant and susceptible barley respectively (adapted from Hammond- Kosack and Kanyuka, 2007). Avr - Avirulence; R - Resistance; MAMPs - Microbe-associated molecular pattern.

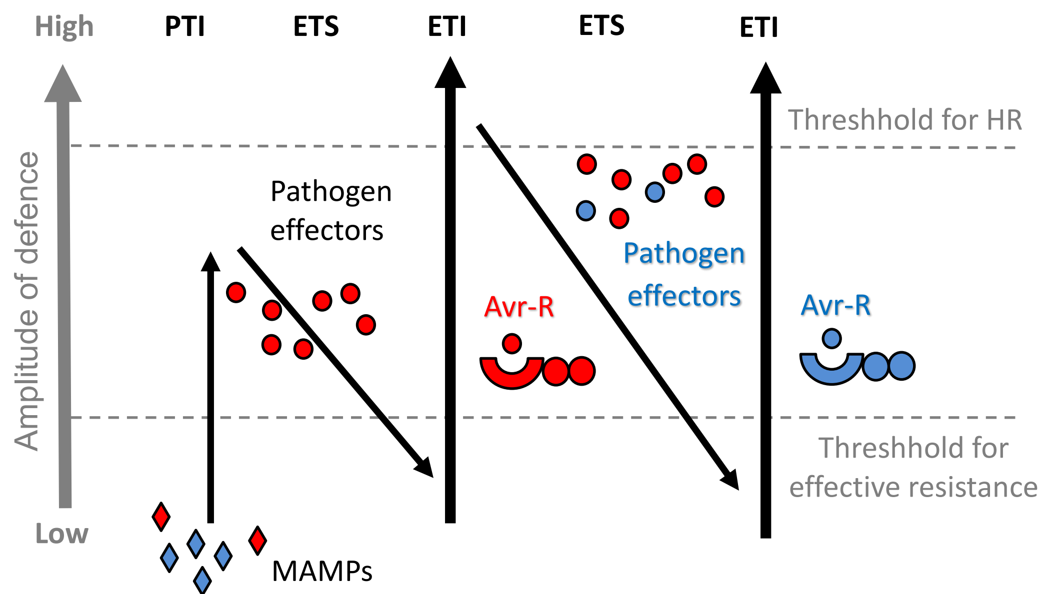

Figure 2.2 Zigzag model of plant pathogen interactions (adapted from Jones and Dangl, 2006). MAMPs - Microbe-associated molecular patterns, PTI - Pattern-triggered immunity, ETS - Effector- triggered susceptibility, ETI - Effector- triggered immunity, HR - Hypersensitive response, Avr-R - Avirulence-resistance protein-protein interaction.

the recognition of the MAMPs (Belkhadir *et al*., 2012). PTI was originally defined as pathogen-associated molecular pattern (PAMP) triggered immunity while MAMPs were formerly known as PAMPs. The change was due to the fact that PTI is not necessarily triggered only by pathogens, but by any microbe (pathogenic or not) that possess these molecular patterns (Chinchilla and Boller, 2012). In case of herbivore damage, the term HAMP (herbivore-associated molecular pattern) is often used, while the term DAMP (danger- or damage-associated molecular pattern; previously known as endogenous elicitors) is now often used in association with both herbivores and pathogens. DAMPs are endogenously released by plants and mediate defence responses following infection or tissue damage (Boller and Felix, 2009).

The most well-known plant PRRs include Flagellin Sensing 2 (FLS2), EFR and CERK1 that respond to MAMPs in bacterial flagellin, elongation factor Tu and fungal chitin respectively (Gómez-Gómez and Boller, 2000; Kunze *et al.,* 2004; Miya *et al*., 2007). FLS2 is a leucine-rich repeat receptor-like protein kinase (LRR-RLK) that is activated by bacterial flagellin (Gómez-Gómez and Boller, 2000). It recognises the flg22 conserved region in the N-terminus of flagellin. While a number of studies have found orthologs of FLS2 in other plants (Chinchilla *et al.,* 2007; Boller and Felix, 2009), they most likely exist in all higher plants. The kinase domain of FLS2 associates with Botrytis-induced Kinase 1 (BIK1), a receptor- like cytoplasmic kinase (RLCK; Lu *et al*., 2010). BIK1 is an important component for signalling initiated by several PRRs including FLS2, ERF and CERK1 (Zhang *et al*., 2010). Upon perception, FLS2 forms a complex with another LRR-RLK, namely BAK1 which results in the rapid phosphorylation of BIK1 and the activation of downstream events such as the production of ROS and nitric oxide (NO) and the activation of mitogen-activated protein kinase (MAPK) cascades (Chinchilla *et al.,* 2007).

2.1.2 Effector-triggered susceptibility

Pathogens have evolved a strategy to overcome innate immunity. By secreting effector molecules into the apoplast or directly into the plant cell (Schwessinger and Ronald, 2012), pathogens are able to successfully suppress PTI, resulting in effector-triggered susceptibility (ETS). These effector molecules thus play a key role in the virulence of pathogens. An immense diversity of effector proteins exists, capable of affecting most aspects of eukaryotic physiology. These include proteases that may cause the alteration of key proteins involved in the host defence response (Dean, 2011).

Hundreds of putative effector molecules have been identified in fungal plant pathogens such as rust and powdery mildew (Caillaud *et al.*, 2011; Schmidt and Panstruga, 2011). Certain bacterial pathogens are capable of delivering multiple effectors into a single host cell that collaborate to suppress host defences, while other effectors are multifunctional to independently affect multiple processes (Galán, 2009).

Effectors are able to evolve rapidly in the co-evolutionary race with plants. Many effector genes are situated in genomic areas such as the chromosome ends that are frequently rearranged. This along with mutation and selection pressure causes a high genetic flexibility in overcoming corresponding resistance (*R*) genes (Stergiopoulos and de Wit, 2009). Research has also shown that pathogens complement their effector arsenal by masking MAMPs with post-translational modifications or mutations, thereby increasing virulence by avoiding detection by PRR proteins (Schwessinger and Ronald, 2012).

2.1.3 Effector-triggered immunity

To resist further multiplication of pathogens, plants have evolved *R* genes that encode R proteins involved in the recognition of specific effector molecules. Plants that possess these R proteins, initiate defence responses that lead to the onset of effector-triggered immunity (ETI) while those that do not, remain susceptible (Figure 2.3). The induced defence responses include the activation of signalling pathways, production of ROS and the onset of the HR (Jones and Dangl, 2006). The HR and systemic acquired resistance (SAR) were once exclusively associated with ETI, but research has shown that in *Arabidopsis*, the recognition of MAMPs may also have this effect (Mishina and Zeier, 2007; Thomma *et al.,* 2011).

Pathogens and plants are thus under continuous pressure to evolve new effector proteins to overcome ETI and new R proteins to re-establish it. Selection pressure could encourage pathogens to lose, change or entirely replace their effector proteins (Thomma *et al.*, 2011). Plant *R* genes encode a number of different protein classes, but the majority belong to the nucleotide-binding site-leucine-rich repeats (NBS-LRR) class. These generally contain both LRR and NBS domains together with variable amino acid terminal domains (Belkhadir *et al.,* 2004). The LRR domain appears to play an important role in recognising pathogen effectors during direct interactions, as demonstrated in various yeast two-hybrid experiments (DeYoung and Innes, 2006).

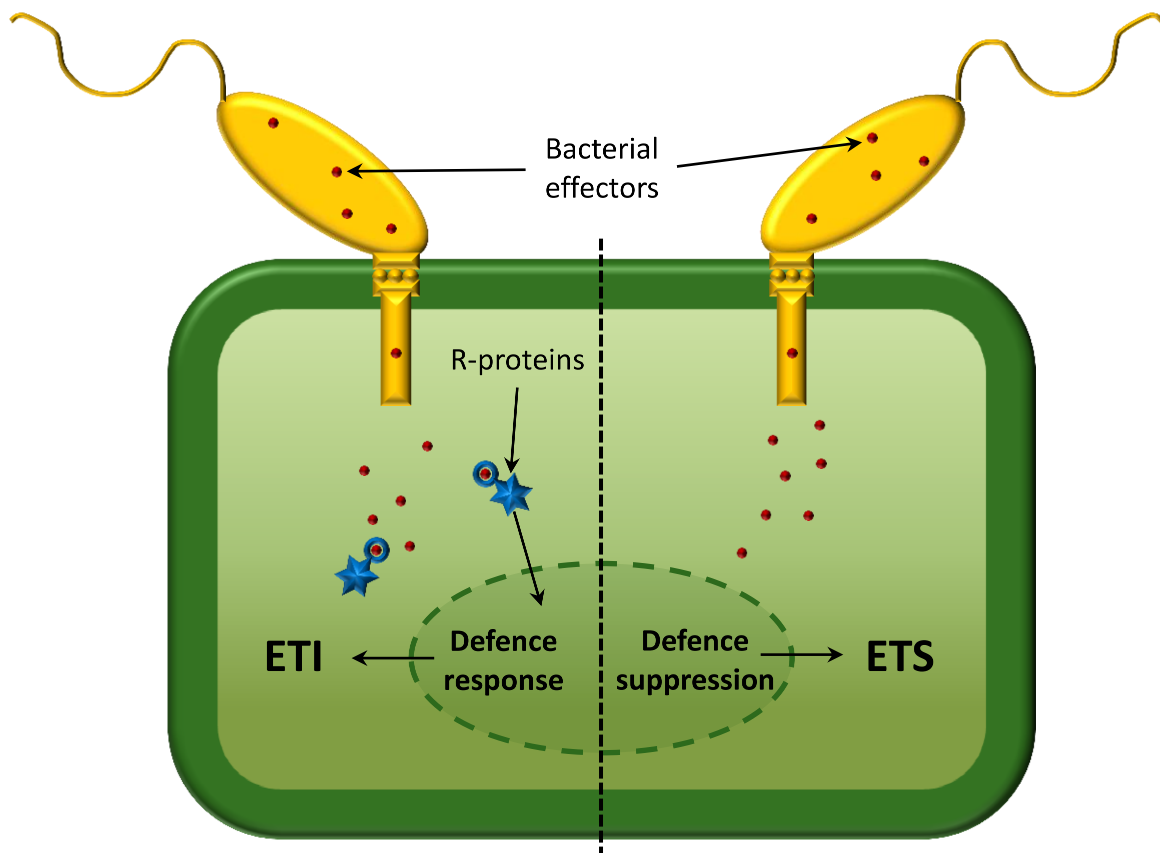

Figure 2.3 The detection of effectors by resistance (R) proteins. Binding results in the activation of a defence response and effector-triggered immunity (ETI). When the appropriate R proteins are not present, effectors suppress host defences, resulting in effector-triggered susceptibility (ETS).

During indirect interactions, as proposed in the guard hypothesis (Van der Biezen and Jones, 1998), certain NBS-LRR proteins monitor the functional activity of effector molecules. It was shown that *Arabidopsis* R proteins RPM1 and RPS2 monitor the phosphorylation of RIN4 by *Pseudomonas syringae* van Hall effectors AvrRpm1, AvrB and AvrRpt2. The detection of phosphorylated RIN4 results in the activation of RPM1-mediated defence responses (Chung *et al.,* 2011; Liu *et al.,* 2011).

The distinction between R proteins and PRRs are occasionally somewhat blurred. Products of classic *R* genes such as *Xa21* and *Cf4* from rice and tomato respectively are able to recognise highly conserved MAMPs and therefore more closely resemble PRRs than R proteins (Lee *et al.,* 2009; Ronald and Beutler, 2010). It has been shown that Xa21 recognises a sulphated peptide, AxY^{S}22, derived from the highly conserved *Xanthomonas* Ax21 protein (Han *et al.,* 2011). Similarly, the distinction between MAMPs and effectors and by implication PTI and ETI is not clear-cut. Certain effectors qualify as MAMPs due to their widespread occurrence (de Jonge *et al.,* 2010; Thomma *et al.,* 2011), while MAMPs may be narrowly conserved and contribute to virulence (Brunner *et al.*, 2002; Lee *et al.*, 2009).

2.2 Signal transduction in plants

Due to the immobility of plants, the efficiency of their defence mechanisms is extremely important. Similar to other organisms, plants have evolved the ability to sense various environmental and internal stimuli and respond appropriately (Krauss, 2003). Biotic and abiotic stresses are sensed by specific receptor proteins, leading to signal transduction and responses that counteract these stress conditions (Fordham-Skelton and Lindsey, 2001; Trewavas, 2002; Pfannschmidt, 2008).

In plants, most signals are perceived at membrane level by transmembrane receptor proteins. These include receptor-like kinases (RLKs) and G-protein-coupled receptors (GPCRs; Tuteja and Sopory, 2008). Transmembrane receptors contain intracellular, transmembrane and extracellular domains, while cytoplasmic receptors lack extracellular and transmembrane domains (Yang *et al.,* 2010). Signal transduction is initiated with the binding of a specific ligand to the extracellular domain (Krauss, 2003). The signal is transmitted across the plasma membrane when the shape or conformation of the intracellular part of the receptor changes (Tiffin and Moeller, 2006). This is usually followed by the generation of second messengers that relay and greatly amplify the signal. Compared to the vast array of possible

stimuli, second messengers are relatively few and mostly include calcium ions and cyclic mononucleotides, NO and phospholipids. Despite their small size and structural simplicity, they are capable of mediating very specific responses within the cell (Rudolf *et al*., 2012). Second messengers may lead to the direct activation of signalling cascades that phosphorylate transcription factors, leading to gene expression.

2.2.1 Receptor-like protein kinases

RLKs play important roles in a variety of processes that regulate growth, development and defence in plants (Figure 2.4; Shiu and Bleecker, 2001; Haffani *et al.*, 2004). They may account for up to 4% of all proteins encoded by the plant genome (Shiu *et al.,* 2004). They were originally defined as transmembrane proteins, but it is now clear that RLKs include both transmembrane and cytoplasmic RLCKs. RLCKs such as the tomato Pto protein kinase do not contain transmembrane or extracellular domains (Yang *et al.,* 2010).

RLKs are grouped into approximately 20 subfamilies based on sequence motifs in their extracellular domains. These may be involved in protein-protein interactions or binding of carbohydrate substrates such as microbial cell wall components or glycoproteins. The largest group of RLKs in plants have extracellular domains that contain between one and 32 LRRs that are often involved in direct protein-protein interactions (Kobe and Deisenhofer, 1994; Lehti- Shiu *et al.,* 2009). These LRR-RLKs can be separated according to function into two major groups. The one group includes BRI1 that is involved in plant growth/development while the other, such as FLS2, is involved in plant defence (Gómez-Gómez and Boller, 2000).

2.2.2 G-protein-coupled receptors

Guanine nucleotide binding proteins (G-proteins) represent a class of proteins that assist in the transmission of external signals to the inside of a cell (Ricart and Millner, 1997). They are subdivided into monomeric GTPases (Guanosine-5'-triphosphate hydrolase enzymes) and heterotrimeric G-proteins. Heterotrimeric G-proteins consist of α, β and γ subunits, while small GTPases are homologous to the α subunit and are capable of independently hydrolysing GTP (Guanosine-5'-triphosphate; Assmann, 2002). Heterotrimeric G-proteins are activated by GPCRs which are located in the cell membrane and responsible for the recognition of external stimuli.

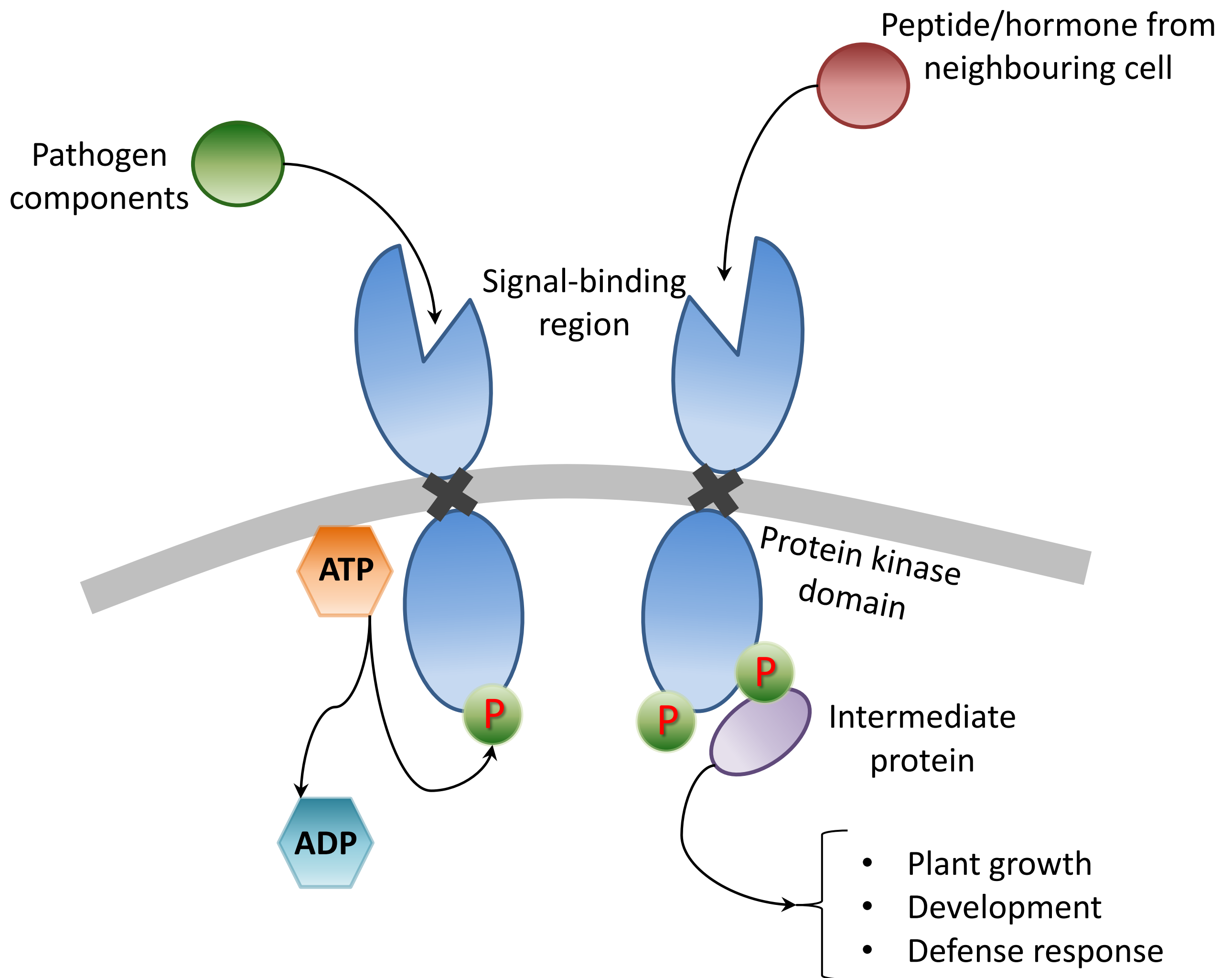

Figure 2.4 Generalised illustration of how receptor-like protein kinases work in plants (adapted from Shiu and Bleecker, 2001).

Stimulus recognition results in a structural change of the GPCR. This activates the G-protein, causing the α-subunit to hydrolyse a molecule of GTP. This subunit dissociates from the other two, exposing certain sites for interaction with other molecules. The subunits detach from the receptor to initiate signalling by means of phosphodiesterases, adenylyl cyclases, phospholipases and ion channels that permit the release of second messenger molecules such as cAMP and cGMP (Ricart and Millner, 1997).

There is evidence that G-proteins participate in several diverse physiological processes such as the regulation of pollen germination and tube growth (Clark *et al.*, 2001). It has been shown that mutant rice lacking the α-subunit has delayed defence responses and decreased resistance to the rice blast fungus *Magnaporthe grisea* (Herbert) Barr. (Suharsono *et al.,* 2002).

2.2.3 Mitogen-activated protein kinases (MAPK)

Pathways regulated by MAPKs are found in all eukaryotic organisms where they transduce developmental and environmental signals through relayed phosphorylation into intracellular responses (Nakagami *et al.*, 2005). These signalling pathways commonly consist of three closely associated protein kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. MAPKKK initially phosphorylates MAPKK, which in turn phosphorylates MAPK. Activated MAPKs are then able to phosphorylate downstream targets such as transcription factors.

Plant MAPKs play key roles in regulating processes during plant development, innate immunity and stress responses (Pitzschke *et al.,* 2009). Sequencing of the *Arabidopsis* genome resulted in the identification of 20 MAPKs, 10 MAPKKs and 60 MAPKKKs and has shown that some MAPK components are shared between many of these processes (Ichimura *et al.,* 2002).

2.3 The plant defence response

During the course of evolution, plants have developed a multitude of mechanisms to counteract abiotic and biotic stresses. Passive defences are often not sufficient to prevent a pathogen invasion, so when pathogens successfully evade the initial defences, the second response layer is activated (Burdon and Thrall, 2009; Dodds and Rathjen, 2010). This induced response (IR) is generally more directed and intense than the initial PTI response. It

is regulated by newly produced defence hormones such as salicylic acid (SA) and jasmonic acid (JA) and is associated with long distance signals that systemically induce long lasting resistance in a plant (Pastor *et al.,* 2012). The IR is currently grouped into two categories, namely SAR and induced systemic resistance (ISR; Grant and Lamb, 2006). SAR is triggered by a local infection and gives long lasting resistance against a broad spectrum of pathogens in distal tissues. It relies on a functional SA synthesis pathway and is associated with the systemic accumulation of SA (Durrant and Dong, 2004). It also involves the systemic expression of certain defence genes such as those encoding pathogenesis-related (PR) proteins 1 to 5 (Li *et al.,* 2009; Puthoff *et al.*, 2010).

Induced systemic resistance develops in response to colonisation of plant roots by non-pathogenic organisms such as growth promoting rhizobacteria and mycorrhizal fungi. MAMPs of these beneficial organisms are perceived, which result in the activation of immune responses in systemic tissues. ISR depends on JA and ethylene (ET) signal transduction pathways (Pieterse *et al*., 1998; Ton *et al.*, 2002).

As discussed earlier, active (or induced) defence involves the perception of biotic or abiotic stimuli via receptors and the activation of signal transduction pathways that eventually lead to the expression of genes that contribute to the overall defence reaction. When these defence genes are expressed in response to a pathogen infection, it is often influenced by abiotic factors such as temperature and humidity, indicating a complex signalling network that plants use for protection against biotic stress (Fraire-Velázquez *et al.,* 2011).

2.3.1 Cell wall reinforcement

Cell wall-associated defence mechanisms play a major role in non-host and incompatible interactions. Cell wall appositions and papillae form impenetrable barriers, preventing penetration by pathogens (Schmelzer, 2002; Collins *et al.,* 2003). Papillae reinforce the cell wall to isolate the pathogen. They serve as sites for the accumulation of antimicrobial compounds and may contain callose and phenolics, lignin, cellulose, pectin, suberin, lipids, ROS and peroxidases (Zeyen *et al*., 2002). During non-host interactions, papilla formation is mostly confined to the epidermal cells, leading to inhibition of pathogen development. However during the incompatible interactions the response also occurs in the adjacent mesophyll cells when the haustorial developmental stage has been reached (Narusaka *et al.,* 2005; Ellis, 2006; Elliott *et al.,* 2008). Callose (β-1,3-glucan) is one of the most

important components of papillae. Its rapid deposition and strong reinforcement resists the penetration by most pathogens, but certain pathogenic fungi release β-1,3-glucanases to overcome this barrier (Romero *et al.,* 2008).

Lignin is a collective term for a large group of polymers that are involved in the strengthening of cell walls. The structure of lignin that is developmentally accumulated differs in composition to lignin deposited in response to a pathogen attack (Nicholson and Hammerschmidt, 1992). The latter is therefore more appropriately termed "defence lignin" (Lange *et al.,* 1995). Defence lignin can be induced upon various biotic and abiotic stress conditions. Some of these include wounding, pathogen infection and metabolic stress (Tronchet *et al*., 2010).

2.3.2 Hypersensitive response

The hypersensitive response, first described by Stakman (1915), is a strategy used by plants to prevent the spread of invading pathogens. It forms part of the innate immune system and is characterised by programmed cell death (PCD) around the area of infection, but also involves an increased synthesis of PR proteins and the synthesis of antimicrobial secondary metabolites (Mur *et al.,* 2008).

The HR is induced upon non-host or incompatible interactions and is regulated by ROS, SA and NO (Amirsadeghi *et al.,* 2007). In many cases the HR is preceded by an oxidative burst that leads to the generation of ROS. It was once assumed that ROS only act as damaging agents within a cell, but research has shown that they are important signalling molecules in the orchestration of the HR (Jaspers and Kangasjärvi, 2010).

There are large dissimilarities between the HR responses of different plant-pathogen interactions. These include variations in the phenotype and timing of the response, possibly resulting from different infection strategies of pathogens (Krzymowska *et al*., 2007). The HR is often used as a visual marker to detect plant-pathogen interactions, but it has been shown that certain abiotic stress conditions (e.g. ozone exposure) may also lead to localised necrotic lesions and production of PR proteins (Joo *et al.,* 2005). There is also clear evidence of overlap between necrotrophic pathogen-induced cell death and HR (Gorvin and Levine, 2000).

2.3.3 Phytoalexins

Antimicrobial compounds found in plants are broadly classified into two groups; phytoalexins and phytoanticipins (Iriti and Faoro, 2009). Phytoalexins are low-molecular-weight secondary metabolites with antimicrobial properties that are synthesised in plants following biotic and abiotic stresses (Paxton, 1981). They are not produced in healthy plants, but require elicitors that result in gene activation. Phytoanticipins on the other hand are constitutively produced over the entire life cycle of most plants.

Members of a certain plant species normally produce similar antimicrobial compounds (Grayer and Harborne, 1994). Interestingly some of these may be phytoalexins in a certain plant species and phytoanticipins in another. It has also been shown that the accumulated concentrations of phytoalexins are similar in both resistant and susceptible hosts, indicating the precise timing required for synthesis at the infection site (Mert-Türk, 2002).

Some of the most common classes of phytoalexins include terpenoids, sesquiterpenes and diterpenes (Smith, 1996). The most important phytoanticipins include saponins, cyanogenic glycosides and glucosinolates (Osbourn, 1996). Phytoalexins and phytoanticipins are components of the coordinated plant defence strategy. Together with structural barriers, the oxidative burst, HR and PR proteins, they contribute to restrict the spread of a pathogen (Iriti and Faoro, 2007).

2.3.4 Pathogenesis-related proteins

The term PR proteins refer to proteins that are exclusively expressed during and directly involved in counteracting a pathogen infection (Singh *et al.*, 2008). These stress conditions may include interactions with viruses, bacteria, fungi, insects or herbivores as well as situations that mimic an attack such as the application of ET, JA, SA or wounding (Van Loon and Van Strien, 1999). The term "inducible defence-related protein" is also used because in the past the term "PR proteins" have erroneously been used in referring to any plant protein that is upregulated following an infection (Van Loon *et al.,* 2006). Five main groups of PR proteins (PR1 to PR5) were originally characterised in tobacco, but 17 groups are currently recognised along with a putative new PR18 group, each containing proteins with similar properties that contribute to plant defence (Christensen *et al.,* 2002; Hoffmann-Sommergruber, 2002; Van Loon *et al.,* 2006). The genes are numbered according to their

discovery, *PR1* being the first and *PR17* the last.

PR proteins with known functions include hydrolytic enzymes, defensins, oxidases, proteases and protease inhibitors, each having an inhibitory effect on the invading pathogen (Table 2.1; Van Loon *et al.,* 2006; Spoel and Dong, 2012). Distinct groups of PR proteins are expressed depending on the infecting pathogen. It has been shown that the *Arabidopsis* PR1, PR2 and PR5 proteins are induced by biotrophic pathogens that cause SA production, while PR3, PR4 and PR12 are induced by necrotrophic pathogens that induce JA production (Thomma *et al.,* 1998).

The PR2 family consists of β-1,3-glucanases. They play a role in plant defence, either by directly decomposing fungal cell walls or indirectly by releasing elicitors through limited hydrolysis of the fungal cell wall (Lawrence *et al*., 2000). Endochitinases, included in groups PR3, PR4, PR8 and PR11 are enzymes that cleave poly-β-1,4-N-acetylglucosamine (chitin) and are distinguished from each other based on their specific activities on a range of substrates (Van Loon and Van Strien, 1999). Thaumatin-like proteins (PR5) play a role in permeabilising fungal membranes, while PR6 protease inhibitors (PI) are involved in various aspects of plant defence against herbivores and microorganisms. These PIs are a subclass of serine proteinase inhibitors and are related to the tomato/potato inhibitor I (Glazebrook, 2005). The PR7 endoprotease has a possible role in the disruption of microbial cell walls and has thus far only been identified in tomato. The peroxidases found in the PR9 class are possibly involved in the catalysis of lignification, thereby reinforcing the cell wall and enhancing resistance (Passardi *et al.,* 2004). The PR10 proteins have weak ribonuclease activity and it is hypothesised that they play a role in cleaving viral mRNA. The defensins, thionins and lipid transfer proteins of groups PR12, PR13 and PR14 respectively all exhibit antifungal and antibacterial activity, while PR15 and PR16 both generate hydrogen peroxide (superoxide dismutase activity) that is harmful to certain invaders (Van Loon *et al.,* 2006). PR17 proteins have not been characterised thus far, but resemble zinc-metalloproteases. They have been identified in tobacco, wheat and barley (Christensen *et al.,* 2002).

Table 2.1 Recognised families of pathogenesis-related proteins (adapted from Van Loon *et al.*, 2006 and Sels *et al.*, 2008).

Family	Properties	Average size (kDa)	Original reference
PR1	Unknown	15	Antoniw *et al.*, 1980
PR2	Β-1,3-glucanase	30	Antoniw *et al.*, 1980
PR3	Chitinase type I, II, IV, V, VI, VII	25-30	Van Loon, 1982
PR4	Chitinase type I, II	15-20	Van Loon, 1982
PR5	Thaumatin-like	25	Van Loon, 1982
PR6	Protease-inhibitor	8	Green and Ryan, 1972
PR7	Endoprotease	75	Vera and Conejero, 1988
PR8	Chitinase type III	28	Métraux *et al.*, 1988
PR9	Peroxidase	35	Lagrimini *et al.*, 1987
PR10	Ribonuclease-like	17	Somssich *et al.*, 1986
PR11	Chitinase type I	40	Melchers *et al.*, 1994
PR12	Defensin	5	Terras *et al.*, 1995
PR13	Thionin	5	Epple *et al.*, 1995
PR14	Lipid-transfer protein	9	García-Olmedo *et al.*, 1995
PR15	Oxalate oxidase	20	Zhang *et al.*, 1995
PR16	Oxalate-oxidase-like	20	Wei *et al.*, 1998
PR17	Unknown	27	Okushima *et al.*, 2000

2.4 Proteases

Proteases (or peptidases) are enzymes that catalyse the hydrolysis of peptide bonds in proteins (Rao *et al.,* 1998). It was originally thought that they are dedicated to protein recycling or digestion of a food source, but it has become clear that they have a wide range of biological functions (Hoge *et al.*, 2010). These include the removal of unwanted proteins, the supply of amino acids and the control of metabolism by reducing certain enzymes and regulatory proteins (Pesquet, 2012). There are more than 800 proteases from 60 families in *Arabidopsis* which point to their importance (van der Hoorn, 2008).

The precise cleaving mechanisms and active sites of proteases vary considerably among different types, providing a basis for their classification (Barrett *et al*., 1998). According to the MEROPS database (merops.sanger.ac.uk), proteases can be broadly grouped into aspartic-, cysteine-, glutamic-, metallo-, aspargine-, serine- and threonine proteases.

Aspartic peptidases (APs) are classified into 16 families based on their amino acid homology and are widely distributed between plants, fungi, vertebrates, protozoa and viruses. They are active at a low pH and contain two aspartic acid residues that are responsible for their catalytic activity (Rawlings and Barrett, 1995). Plant APs are unique due to the presence of an extra domain that shows no homology with either mammalian or microbial APs. This is known as the plant specific insert (PSI). It has been proposed that the PSI plays a role in the processing or degradation of proteins (Simoes and Faro, 2004) and the sorting of proteins to vacuoles since the deletion of the PSI from the phytepsin AP results in secretion, while the wild-type phytepsin accumulates inside the vacuoles (Tormakangas *et al.,* 2001).

Cysteine proteases are involved in many diverse processes. In plants these include protein processing for seed development, mobilisation of storage proteins required for germination, cellular degradation during senescence and degradation of proteins following PCD and HR (Andersson *et al.,* 2004; Grudkowska and Zagdańska, 2004). Certain cysteine proteases play important roles in promoting the virulence of pathogens. They affect processes such as entry to the host, feeding and suppressing immune responses (McKerrow *et al.,* 2006).

One of the largest groups of proteases found across all kingdoms of life is serine proteases representing over one third of all known proteolytic enzymes (Hedstrom, 2002). They also appear to be the largest group in plants, with approximately 250 enzymes listed for

Arabidopsis on the MEROPS database. All members contain a serine amino acid residue that is situated in the active site of the enzyme. They are involved in processes that regulate many diverse aspects of plant development and defence (Schaller, 2004).

2.4.1 Proteases in plant defence

Proteases were once thought to be involved in "housekeeping", but the accumulation of a subtilisin-like serine protease (P69) in viroid-infected tomato plants (Vera and Conejero, 1988) and an increase in leucine-aminopeptidase during insect feeding, implied their involvement in plant defence (Tornero *et al.,* 1996).

It is now common knowledge that proteases of the P69 family play an important role in plant defence (van der Hoorn and Jones, 2004). They are approximately 69 kDa in size and are represented by different isoforms in plants. The P69A and P69D isoforms have a constitutive expression pattern, while the P69B and P69C isoforms are co-ordinately and systemically induced by *Pseudomonas syringae* van Hall infection or SA treatment (Jorda *et al.*, 1999). P69B and P69C have been included into the PR7 class of PR proteins (Van Loon and Van Strien, 1999).

Apoplastic proteases (e.g. in tomato) are also important constituents of the plant defence response. The apoplastic cysteine protease Rcr3 found in tomatoes is required for resistance against certain strains of *Cladosporium fulvum* Cooke (Kruger *et al.*, 2002).

2.4.2 Proteases contributing to virulence of pathogens

Proteases may play key roles in the life cycle and virulence of pathogens (Bird *et al.*, 2009). Some bacterial proteases have the ability of affecting various stages of defence in both plants and animals (Rawlings *et al.*, 2008). One group of notorious virulence proteases of mammalian pathogens is the metallo-proteases produced by *Clostridium botulinum* van Ermengem. Better known as the botulinum toxins, these neurotoxic proteases are some of the most lethal proteins known to man (Lebeda *et al.,* 2010). Other animal pathogens, such as *Streptococcus pyogenes* Rosenbach, produce immunoglobulin (Ig) proteases that directly affect the defence of host organisms by destroying antibodies, a crucial component of its defence machinery (Collin and Olsén, 2003).

Yersina pestis (Lehmann and Neumann) van Loghem, responsible for Justinian plague,

Black Death and modern plague pandemics, injects six Yop (*Yersinia* outer protein) effectors into host cells (Juris *et al*., 2002). One, YopT, is a cysteine protease that has orthologs in a number of plant pathogenic bacteria. One ortholog is AvrPphB, a cysteine protease that is delivered into plant host cells by *P. syringae* pv. *phaseolicola* Burkholder. It promotes disease in pea, soybean and *Arabidopsis* (Tampakaki *et al.,* 2002). HopN1 is another cysteine protease effector produced by *P. syringae.* It suppresses HR cell death in non-host tobacco plants and host tomato plants by inhibiting the production of ROS (López-Solanilla *et al*., 2004; Rodríguez- Herva *et al*., 2012).

It has been proposed that fungal plant pathogens may use proteases in a variety of ways, such as enabling penetration of the cell wall, the destruction of defence-related proteins or the utilisation of cell wall proteins during colonisation (Dobinson *et al.,* 1997). It has been shown that the economically important fungal phytopathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal agent of stem rot in a variety of crops, secretes a range of proteases that seemingly aid in the infection process (Poussereau *et al.,* 2001).

2.5 Protease inhibitors

Proteases are essential for the normal function of organisms, but pose a threat when present at higher concentrations or when introduced by attackers. PIs are molecules that inhibit the function of specific proteases. In plants, PIs play important roles in many diverse processes including the regulation of endogenous proteases and responses to abiotic stress, herbivores and pathogens (Mosolov *et al.,* 2001). It has been shown that a barley cysteine PI (cystatin) and a rice chymotrypsin PI (OCPI1) are both induced by various abiotic stresses and abscisic acid (Huang *et al.,* 2007). Certain PIs in plants may interfere with digestive processes of insects by acting as anti-metabolic proteins, while others may target pathogenic effector proteases (Reeck *et al.,* 1997).

Their role in plant protection was first noticed when trypsin inhibitors present in soybean products disrupted the normal development of *Tribolium confusum* Duval larvae (Lipke *et al*., 1954; Lawrence and Koundal, 2002). Hilder *et al.* (1987) showed that an Alfalfa Bowman-Birk protease inhibitor (BBI) may function as an endogenous insecticide when transferred to tobacco plants. A recently identified *Arabidopsis* PR6 unusual protease inhibitor plays an important role in defence against necrotrophic fungi and insects (Laluk and Mengiste, 2011).

Protease inhibitors are grouped into 48 families according to sequence similarities and inhibitory domains. They can be found as single domain proteins, but are also embedded within proteins that contain single (simple) or multiple (compound) inhibitory domains. Complex inhibitors may contain up to 15 inhibitory domains and are thus difficult to classify according to their catalytic action (Rawlings *et al.,* 2004; Habib and Fazili, 2007). Some of the most important PI families in cereals include the Bowman-Birk, Kunitz, Cereal and Potato type 1 PI families.

2.5.1 Bowman-Birk protease inhibitors

The Bowman-Birk family of PIs are plant serine protease inhibitors that inhibit trypsin or chymotrypsin. The first member of this family was isolated from soybean in 1944 (Bowman, 1944) and characterised 19 years later (Birk *et al.,* 1963). Members are largely restricted to the Fabaceae and Poaceae and are most commonly found in the cytoplasm and apoplast of developing seeds and wounded tissue (Qi *et al.,* 2005). Some of their *in vivo* roles include regulation of proteins during germination and many aspects of protection against abiotic stress, pathogens and herbivores (Otlewski *et al*., 2005). They were found to be wound- inducible in leaves of certain plants (Chen *et al.,* 2004). Additionally, it was shown that BBIs in yeast are involved in cadmium tolerance (Shitan *et al.* 2007), while in wheat certain BBIs (wali3, wali5 and wali6) were induced both by wounding and aluminium stress (Richards *et al.,* 1994; Snowden *et al.,* 1995).

The classification of BBIs is done according to their structure and inhibitory properties. They are referred to as single or double-headed, meaning that they contain either one or two active sites within a single inhibitor molecule. Double-headed BBIs are thought to have evolved via gene duplication (Odani *et al.,* 1986; Chen *et al.,* 1992) and are capable of simultaneously and independently inhibiting trypsin and chymotrypsin (Birk *et al.,* 1967). Their approximate sizes in plants are usually between 7 and 16 kDa (Birk, 1987). BBIs in dicotyledonous plants are double-headed and about 8 kDa in size, while monocot BBIs have lost one active site during the course of evolution. Single-headed BBIs found in monocot plants are therefore 8 kDa in size, while 16 kDa double-headed BBIs most likely evolved via gene duplication (Qi *et al*., 2005). Unique features of BBIs include an unusually high thermal stability, tolerance towards low pH and resistance towards the action of proteases (Yavelow *et al*., 1983), which may result from their high percentage of disulphide bridges (Clemente *et al.,* 2008).

BBIs have a large amount of potential applications and are therefore attracting much attention. They have already been used in transgenic plants to improve resistance against insects. Wheat transformed with a BBI barley trypsin inhibitor type has resistance against *Sitotroga cerealella* Oliv. (Altpeter *et al.,* 1999). Certain studies have indicated its possible role as an anticancer or radio-protective agent (Kennedy and Wan, 2002; Magee *et al.,* 2012), while others have indicated the possibility of treating allergic or anti-inflammatory disorders (Dia *et al.,* 2008; Marín-Manzano *et al.,* 2009).

2.6 Protease inhibitors involved in wheat defence

In recent years, many genes involved in plant-pathogen interactions have been identified and characterised (Thilmony *et al.,* 2006; Ma *et al.,* 2009; Wang *et al.,* 2010; Djami-Tchatchou *et al.,* 2012; Oloriz *et al.,* 2012). However, before the development of high throughput technologies like microarrays and RNA sequencing, studies could not analyse the processes globally. They were confined to the analysis of a small number of genes involved in the host response to pathogen attack. Much progress has been made in the understanding of different wheat-pathogen interactions. The roles of protease inhibitors in these defences have also been demonstrated.

Research has shown that a thermostable germin-like protease inhibitor (GLPI) found in the wheat apoplast inhibits serine proteases in *Septoria tritici* Desm.-infected wheat (Segarra *et al.,* 2003). This fungal infection stimulated the protease activity in resistant plants, while its inhibition was observed in susceptible plants. Although others studies have implicated germin-like proteins (GLPs) as integral parts of basal resistance to biotic stress in cereals, this study was the first to demonstrate the protease inhibitor activity of a germin. A recent study showed that GLPI has at least three different enzymatic activities. These include trypsin inhibition, superoxide dismutase and adenosine diphosphate glucose pyrophosphatase activities (Mansilla *et al.,* 2011).

The role of wheat BBIs in the defence against pathogens has been demonstrated in a number of studies. One study showed the inhibition of a *Botrytis cinerea* (de Bary) Whetzel-produced protease with a Bowman-Birk trypsin inhibitor found in wheat kernels (Chilosi *et al.,* 2000). This BBI appeared to play an important role in defence of seeds during rest and early germination.

In another study cDNA library construction and sequencing was used for the identification of differential gene expression in *Puccinia triticina*-resistant and susceptible wheat containing the *Lr10* resistance gene (Manickavelu *et al.,* 2010). A total of 25 highly differentially expressed unigenes were identified and their expression confirmed with traditional reverse transcription polymerase chain reaction (RT-PCR). Genes involved in the incompatible interactions included the abiotic stress-induced putative BBIs wali5 and WRSI5-1.

Recent research by Gottwald *et al*. (2012) used a GeneChip® wheat genome array to characterise transcriptional changes in resistant and susceptible wheat following infection with *Fusarium graminearum* Schwabe. Amongst others, their results showed an up-regulation of five serine-protease inhibitors. Two of these were functionally annotated as BBIs due to high sequence homology with WRSI5, a salt-responsive gene with a suggested role in plant growth (Shan *et al.,* 2008). Following qPCR analysis, they proposed that one BBI may be a possible resistance candidate due to its high expression levels during early and late stages of fungal colonisation.

Next generation technologies such as RNA sequencing are currently underway and will reveal many more aspects of the interaction between wheat and different pathogens (Bakkeren *et al.,* 2012). These next generation technologies greatly increase the speed and efficiency of genome characterisation, even in a non-model species without much prior molecular information (Cantu *et al.,* 2011). Proteomic approaches are also used to generate genome-wide protein profiles and could be considered a more definitive analysis than the revealing mRNA transcripts. A partially generated proteasome of haustoria from *Puccinia triticina*-infected wheat has already verified many predicted pathogenicity and virulence factors (Song *et al.,* 2011). Together, these new technologies will enhance our knowledge of the roles of known and currently unknown BBIs in the defence response of wheat against fungal pathogens.

Chapter 3

Reference gene selection for qPCR gene expression analysis of rust-infected wheat

Reference gene selection for qPCR gene expression analysis of rust-infected wheat

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3.1 Abstract

Real-time PCR is an effective method to quantify mRNA levels, but requires validated reference genes for data normalisation. The GeNorm-Plus algorithm was used to examine the expression stability of six candidate reference genes in resistant Avocet *Yr1* wheat infected with *Puccinia triticina, P. striiformis* and *P. graminis* f. sp. *tritici* respectively. Results indicated that within the first 48 h after inoculation, the expression stability of the candidate reference genes differed between the three incompatible interactions. The geometric mean of *ARF* and *RLI* showed the best stability in *P. triticina*-infected wheat, *CDC* and *RLI* in *P. striiformis*-infected wheat and *CDC*, *18S* and *TUBB* in *P. graminis* f. sp. *tritici*-infected wheat respectively. This clearly emphasised the need for reference gene validation for each different plant-pathogen interaction.

Keywords:

Reference genes, GeNorm, *Triticum aestivum*, *Puccinia graminis* f. sp. *tritici, Puccinia triticina, Puccinia striiformis*

Abbreviations:

Cq, quantification cycle; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; *Pgt*, *Puccinia graminis* f. sp. *tritici*; *Ps*, *Puccinia striiformis*; *Pt*, *Puccinia triticina*; RIN, RNA integrity value; qPCR, quantitative polymerase chain reaction

3.2 Introduction

Wheat production is frequently threatened by pathogen infection. Rust diseases of wheat account for large yield losses with aggressive new strains of stem and stripe rust destroying approximately 40% of farmers' wheat fields (Marasas *et al*., 2004). In particular, the widespread susceptibility of wheat to the Ug99 stem rust race group is a major concern due to its rapid movement from Africa into parts of Asia and the Middle East (Singh *et al*., 2011). The genetic improvement of wheat to survive rust-induced stress conditions therefore remains a priority (Roche *et al*., 2009). Gene expression studies form part of this process.

Quantitative PCR (qPCR) has become the industry standard for gene expression quantification due to its high sensitivity and reproducibility. However, the absence of strict qPCR requirements has in many cases led to the publication of unreliable and irreproducible results (Gue´nin *et al*., 2009). The MIQE guidelines have been proposed in an attempt to improve the quality of expression results by standardising each step of the qPCR work-flow (Bustin *et al*., 2009). These steps include experimental design, RNA (extraction, storage and quality control), reverse transcription, oligonucleotides (design and optimisation), data normalisation and analysis.

Accurate qPCR data analysis requires an appropriate normalisation strategy to minimise non-biological variation between samples (Huggett *et al.,* 2005). The preferred approach is validated endogenous reference genes whose expression remains stable under all tested conditions (Bustin *et al*., 2009). Additionally the geometric mean of multiple validated reference genes is becoming the minimum requirement for data normalisation (Vandesompele *et al.,* 2009; Lilly *et al*., 2011).

It was shown that the expression stability of traditional reference genes such as Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-Tubulin (*TUBB*) or 18S rRNA (*18S*) may vary significantly between different tissue types and disease states of an organism (Czechowski *et al.,* 2005; Kwon *et al.,* 2009; Tong *et al.,* 2009; Chari *et al.,* 2010; Long *et al.,* 2010). Others have shown that even a minor alteration in experimental conditions may cause a previously suitable reference gene to become unstable (Ferguson *et al.,* 2010). The use of unvalidated reference genes may obscure the true biological variation between samples. It is thus extremely important to carefully select appropriate reference genes for each tissue state using available statistical algorithms. Some of these include GeNorm

(Vandesompele *et al*., 2002), Bestkeeper (Pfaffl *et al*., 2004) and Normfinder (Andersen *et al*., 2004).

Jarosova and Kundu (2010) identified *GAPDH*, *18S* and *TUBB* as the most stable reference genes in wheat infected with Barley yellow dwarf virus. In another study, the cell division control protein (*CDC*), ADP-ribosylation factor (*ARF*) and RNase L inhibitor-like protein (*RLI*) encoding genes were used for data normalisation during various developmental stages of wheat (Paolacci *et al.,* 2009).

The goal of this study was to examine the stability of six candidate reference genes (mentioned above) in Avocet *Yr1* wheat shortly after inoculation with three avirulent *Puccinia* spp. The three rust species were *P. triticina* (*Pt*), *P. striiformis* (*Ps*) and *P. graminis* f. sp. *tritici* (*Pgt*) which are the causal agents for leaf, stripe and stem rust respectively.

3.3 Materials and methods

3.3.1 Wheat cultivation and infection

Using standard inoculation procedures, three different incompatible *Puccinia*-wheat interactions were established when 10 day old resistant Avocet *Yr1* wheat seedlings were inoculated with *Pt* race UVPt19, *Pgt* race UVPgt55 (TTKSF) and *Ps* race 6E22A+ respectively (Prins *et al*., 2011). Leaf tissue was harvested immediately after inoculation and then at either 6 h (*Pgt* and *Pt*) or 12 h (*Ps*) intervals. Each inoculation was done in triplicate.

3.3.2 cDNA synthesis

Total RNA was extracted from harvested tissue using Trizol (Invitrogen) according to manufacturer’s instructions. Residual DNA was removed with a DNaseI treatment (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) while its integrity was confirmed on a 1% (w/v) denaturing agarose gel (Sambrook and Russell, 2001). cDNA was synthesised from 1 µg total RNA using random and 15-mer oligo-dT primers with the Improm II cDNA synthesis kit (Promega) according to the manufacturer’s specifications. The cDNA was diluted 1/50 in Diethyl pyrocarbonate (DEPC) treated water before qPCR analysis.

3.3.3 qPCR analysis

The optimal annealing temperature for each primer set was determined using a temperature gradient. A preliminary standard curve was used to determine the amplification efficiency of each primer set. Each qPCR experiment consisted of three biological replicates, three technical replicates and three time intervals following inoculation with each respective *Puccinia* species. All qPCR reactions were run on a CFX96^{TM} Real-Time PCR detection system (BioRad). Each 10 µl qPCR reaction contained 1 µl 1/50 diluted cDNA, 1 pmol primer mix (Table 3.1) and 1x SybrFast® Readymix (KAPA Biosystems). The cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and the optimal annealing temperature (Table 3.1) for 40 sec. A final melt curve analysis ranging from 65 to 95°C with 0.5^{o}C increments for 5 sec was included.

All quantification cycle (Cq) values were imported into the GeNorm-Plus2 software (Biogazelle) and the average expression stability (M) values calculated. Replicate variability was set to a maximum limit of 0.5 and significant outliers were excluded for each set of three technical replicates.

3.4 Results and discussion

Numerous studies have shown the unreliability of unvalidated reference genes for qPCR data normalisation (Czechowski *et al.,* 2005; Kwon *et al.,* 2009; Tong *et al.*, 2009; Chari *et al.,* 2010; Long *et al.,* 2010). This indicated that universal reference genes do not exist and should be validated for each individual experimental setup. The MIQE guidelines (Bustin *et al.*, 2009) recommend criteria for real-time experiments and publication in order to make them more reproducible. All results in this study were generated according to the MIQE guidelines.

Table 3.1 Primer sequences and qPCR amplification efficiencies of six candidate reference genes.

Gene symbol	Accession number (NCBI)*	Primer sequence (5’-3’)	Amplicon size (bp)	Annealing temp (^{o}C)	qPCR efficiency (%)	References
18S	M82356	F GTGACGGGTGACGGAGAATT R GACACTAATGCGCCCGGTAT	151	60	98.0	Jarosova and Kundu, 2012
GAPDH	AK251456	F TGTCCATGCCATGACTGCAA R CCAAGTGCTGCTTGGAATGATG	105	60	98.3	
TUBB	U76897	F CAAGGAGGTGGACGAGCAGATG R GACTTGACGTTGTTGGGGATCCA	84	60	93.0	
ARF	AB050957	F GCTCTCCAACAACATTGCCAAC R GCTTCTGCCTGTCACATGCGC	165	60	90.3	Paolacci *et al.*, 2009
CDC	EU267938	F CAAATGCGCCATCAGGGAGAACATC R CGCTGCCGAAACCACGAGAC	227	56	100.5	
RLI	AK331207.1	F CGATTCAGAGCAGCGTATTGTTG R AGTTGGTCGGGTCTCTTCTAAATC	242	60	974	

*http://www.ncbi.nlm.nih.gov/

18S - 18S rRNA; *GAPDH* - Glyceraldehyde-3-phosphate dehydrogenase; *TUBB* - Beta-tubulin; *ARF* - ADP-ribosylation factor; *CDC* - Cell division control protein gene; *RLI* - RNase L inhibitor-like protein gene.

Here, the stability of six candidate reference genes was investigated in three different incompatible *Puccinia*-wheat interactions. To eliminate expression variation due to the host genetics, the resistant Avocet *Yr1* wheat cultivar was used. This cultivar carries the *Lr10, Sr26* and *Yr1* disease resistance genes that are effective against *Pt* race UVPt19, *Pgt* race UVPgt55 and *Ps* race 6E22A+ respectively, thus resulting in three incompatible interactions.

The initial defence response of wheat after infection with *Puccinia* spp. is activated approximately 12-48 hours post inoculation (hpi; Yu *et al*., 2010). For that reason, reference gene stability for *Pt* and *Pgt* infected wheat was tested at 0, 12 and 24 hpi and for *Ps* infected wheat at 0, 24 and 48 hpi. RNA extracted from infected leaf tissue displayed no degradation or genomic DNA contamination. RNA integrity (RIN) values of at least seven or higher were obtained using a 2100 Bioanalyzer (Agilent Technologies).

A gradient PCR was used to determine the optimal annealing temperature for all primer pairs which ranged from 56°C for *CDC* to 60°C for the other five primer sets (Table 3.1). A single melting curve peak for each primer set indicated denaturation of 50% of every amplicon at a specific temperature with no abnormalities, confirming the absence of non-specific amplification and primer dimers. R^2 values typically ranged from 0.985 to 0.99. Target amplification efficiencies using a four-fold cDNA dilution series ranged from 90.3 to 100.5% (Table 3.1) with a standard deviation of <10% which can be considered comparable (Livak and Schmittgen, 2001). All primer sequences were successfully verified with DNA sequencing of the amplified DNA fragments.

The most suitable reference gene combination for each *Puccinia*-wheat interaction was established using the qBase-plus2 analysis software (Biogazelle). Results indicated that the most suitable reference gene combinations differed amongst the three incompatible interactions (Figure 3.1). GeNorm analysis selected *ARF* and *RLI* as the best reference gene combination for normalisation in *Pt*-infected wheat with an average M-value of 0.313 (Table 3.2). *CDC* and *RLI* were found to be appropriate for *Ps*-infected wheat ($M_{avg} = 0.279$) while a combination of three reference genes, namely *CDC, 18S* and *TUBB*, was found to be the best suited for normalisation in *Pgt*-infected wheat ($M_{avg} = 0.492$). While some reference genes showed stability in at least two interactions (*RLI* and *ARF*), the traditionally used *GAPDH* reference gene was not stably expressed in any of the interactions. It should be noted that these reference gene combinations are only appropriate for the tested time intervals following

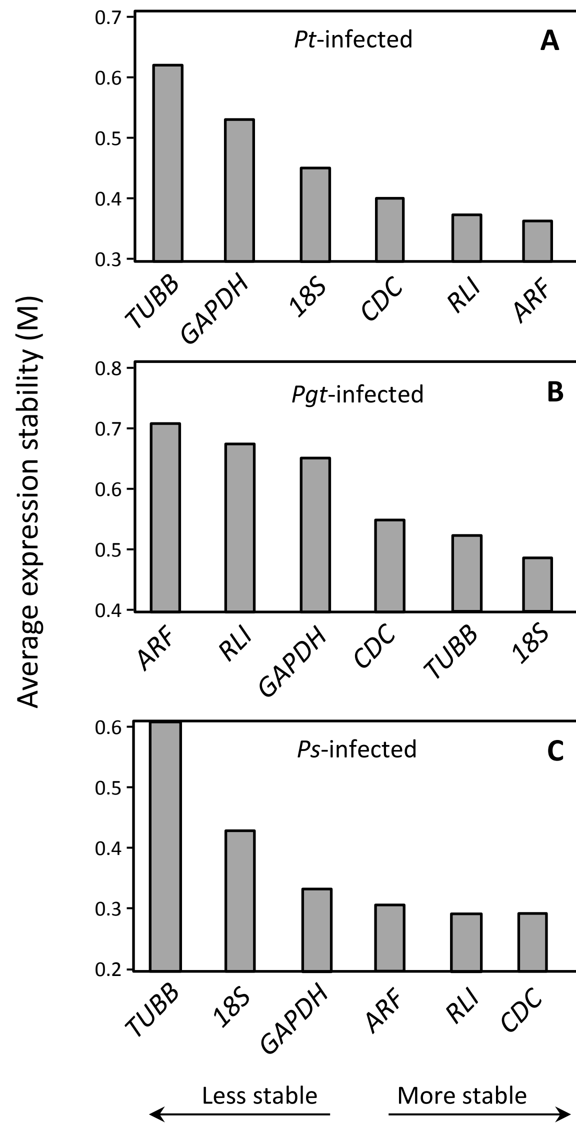

Figure 3.1 Average expression stability (M) of six candidate reference genes in A) *Pt*-, B) *Pgt*- and C) *Ps*-infected Avocet *Yr1* wheat as analysed with GeNorm. *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis*; *GAPDH* - Glyceraldehyde-3-phosphate dehydrogenase; *TUBB* - Beta-tubulin; *18S* - 18S rRNA; *ARF* - ADP-ribosylation factor; *CDC* - Cell division control protein gene; *RLI* - RNase L inhibitor-like protein gene.

Table 3.2 GeNorm stability values (M) of candidate reference genes in *Pt-, Pgt-* and *Ps*-infected wheat.

Gene Symbol	Average expression stability values (M)		
	***Pt*-infected**	***Pgt*-infected**	***Ps*-infected**
18S	0.46	**0.49***	0.43
GAPDH	0.53	0.66	0.34
TUBB	0.62	**0.51***	0.60
ARF	**0.36***	0.70	0.31
CDC	0.41	**0.55***	**0.30***
RLI	**0.39***	0.68	**0.30***
AVG#	**0.31**	**0.49**	**0.28**

[*] Reference genes selected are shown in bold

[#] Average values of selected reference genes

Pt - *Puccinia triticina; Pgt* - *Puccinia graminis* f. sp. *tritici; Ps* - *Puccinia striiformis; 18S* - 18S rRNA; *GAPDH* - Glyceraldehyde-3-phosphate dehydrogenase; *TUBB* - Beta-tubulin; *ARF* - ADP-ribosylation factor; *CDC* - Cell division control protein gene; *RLI* - RNase L inhibitor-like protein gene.

inoculation and will not necessarily be well suited for later times post-inoculation.

The fact that the suitable reference gene combinations differed between the three *Puccinia*-wheat interactions is yet another demonstration that reference gene validation prior to qPCR gene expression analysis is extremely important. Results suggest that inoculation with different *Puccinia* spp. may have vastly different effects on the initial defence response of wheat. For instance, the high instability of *ARF* in *Pgt* infected wheat shortly after infection compared to the other two interactions may point to an altered effect on vesicular traffic, lipid metabolism and microtubule dynamics (Yao *et al*., 2009). Studies have shown that overexpression of *ARF1* in tobacco leaves causes cell death and have demonstrated its involvement in the non-host and *R*-mediated resistance (Coemans *et al.,* 2008).

3.5 Conclusions

In conclusion, we have identified and validated stable reference genes for gene expression studies in three different incompatible *Puccinia*-Avocet *Yr1* interactions. This study confirms the importance of reference gene validation for every experimental setup, enabling more accurate gene expression studies. Our results provide a useful starting point for the analysis of gene expression during different *Puccinia*-wheat interactions.

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Chapter 4

Expression analysis and co-immunoprecipitation of a putative protease inhibitor in rust-infected wheat

Expression analysis and co-immunoprecipitation of a putative protease inhibitor in rust-infected wheat

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4.1 Abstract

Bowman-Birk type protease inhibitors are involved in the response to various stress conditions. *LRW222* was recently identified by means of SSH in *Puccinia triticina*-infected wheat and was found to be homologous to Wali5, a wound- and aluminium stress-induced Bowman-Birk type protease inhibitor. Validated reference genes were used to determine the expression of *LRW222* in wheat infected with three different rust species. Results showed induced expression in both resistant and susceptible wheat, indicating its possible involvement in the early general defence response of wheat. Co-immunoprecipitation and mass spectrometry were used in an attempt to identify LRW222-interacting proteins to shed light on its specific role in plant defence. A number of unrelated proteins were identified, but the results were inconclusive.

Keywords:

Bowman-Birk protease inhibitor, *Triticum aestivum*, *Puccinia graminis* f. sp. *tritici, Puccinia triticina, Puccinia striiformis*

Abbreviations:

BBI, Bowman-Birk protease inhibitor; Cq, quantification cycle; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; *Pgt*, *Puccinia graminis* f. sp. *tritici*; *Ps*, *Puccinia striiformis*; *Pt*, *Puccinia triticina*; RIN, RNA integrity value; qPCR, quantitative polymerase chain reaction

4.2 Introduction

PIs are responsible for the inhibition of specific proteases and are found in a wide variety of organisms. They are grouped into 48 families according to sequence similarity and the presence of inhibitory domains (Rawlings *et al.,* 2004). Protease inhibitors found in plants are involved in many diverse processes such as the regulation of endogenous proteases and responses to abiotic stress, herbivores and pathogens (Mosolov *et al.,* 2001).

The BBI family of plant PIs are serine protease inhibitors that inhibit trypsin and/or chymotrypsin (Ryan, 1981). They are referred to as single or double headed because they contain either one or two active sites within a single molecule (Chen *et al.,* 1992). Certain double headed BBIs are capable of simultaneously and independently inhibiting trypsin and chymotrypsin (Birk *et al.,* 1967; Prakash *et al.,* 1997). Their approximate sizes are usually between 7 and 16 kDa (Birk, 1987). BBIs in dicotyledonous plants have two active sites and are about 8 kDa in size. In monocotyledonous plants, one active site became non-functional during the course of evolution, resulting in 8 kDa BBIs with a single active site (Qi *et al*., 2005). Double headed monocot BBIs evolved after the loss of one active site. Following gene duplication, this resulted in a 16 kDa protein with two active sites (Prakash *et al.,* 1997; Odani *et al.,* 1986).

The role of BBIs in plant protection was first noticed when trypsin inhibitors present in soybean products disrupted the normal development of *Tribolium confusum* larvae (Lipke *et al*., 1954; Lawrence and Koundal, 2002). Since then, BBIs were found to be wound-inducible in the leaves of several plants (Chen *et al.,* 2004). Hilder *et al.* (1987) showed that an alfalfa BBI may function as an endogenous insecticide when transferred to tobacco plants. Certain genes encoding BBIs (wali3, wali5 and wali6) were induced both by wounding and aluminium stress in wheat (Richards *et al.,* 1994; Snowden *et al.,* 1995).

Unique features of BBIs include unusually high thermal stability, tolerance towards low pH and resistance towards the action of proteases (Yavelow *et al*., 1983) which may be due to a high number of disulfide bridges (Clemente *et al.,* 2008). BBIs have several potential applications and are therefore attracting much attention. It has been shown that wheat transformed with barley trypsin inhibitor CMe has improved resistance against *Sitotroga cerealella* (Altpeter *et al.,* 1999). Studies have also indicated BBIs possible role as an anticancer or radio-protective agent in humans (Kennedy and Wan, 2002; Magee *et al.,*

2012), while others have indicated the possibility of using BBIs for treating allergic or anti-inflammatory disorders (Dia *et al.,* 2008; Marín-Manzano *et al.,* 2009).

A recent study by Huang (2008) identified genes induced during the defence response of wheat after leaf rust infection using SSH. Amongst these, clone *LRW222* showed sequence similarity to an expressed sequence tag (EST) isolated from wheat infected with powdery mildew (accession number: CJ956114, e-value: 1e-136). *LRW222* contig reconstruction using overlapping ESTs (accession numbers: CJ955962.1 and AM285535) identified the complete nucleotide sequence of the gene (Figure 4.1). The sequence was translated to obtain the LRW222 polypeptide sequence, which showed near identical homology (e-value: 2e-56; max indent: 97%) to the wound- inducible *wali5* (accession number AAA50850)*,* a predicted BBI (Snowden *et al.,* 1995). The polypeptide also contained several putative defence-associated functional sites, but its precise function remains unknown.

In this study, qPCR was used according to the MIQE guidelines (Bustin *et al.,* 2009) to determine the expression of *LRW222* during three different *Puccinia*-wheat interactions. Co-immunoprecipitation (Co-IP) was further used in an attempt to elucidate the role of LRW222 in *Puccinia triticina* (*Pt*), *P. striiformis* (*Ps*) and *P. graminis* f. sp. *tritici* (*Pgt*) infected wheat by identifying proteins interacting with LRW222.

a)

```
CJ955962.1        ------------------------ACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCA
AM285535          GAATTCGGCACGAGGTACTCCTCCACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCA
LRW222_clone      ------------------------------------------------------------

CJ955962.1        TCAGCAACTATGAAGGGCACCAAGCTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTC
AM285535          TCAGCAACTATGAAGGGCACCAAGCTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTC
LRW222_clone      ------------------------------------------------------------

CJ955962.1        ATGGGAGTCCTCTCGCACGTCAACGCCGACTTCTTCCCCAAGTGCTGCAACAACTGCGGG
AM285535          ATGGGAGTCCTCTCGCACGTCAACGCCGACTTCTTCCCCAAGTGCTGCAACAACTGCAGG
LRW222_clone      ------------------------------------------------------------

CJ955962.1        TCCTTCTCGGGGGTCGACGTCTGCGACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTCG
AM285535          TCCTTCTCGGGGGTCGACGTCTGCGACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTCG
LRW222_clone      ------------------------------------------------------------

CJ955962.1        GCGTGCCGCGTGGTGTCGACGAGCCCCGAAATGTGGCGCTGCGCGGATATGAAATCCACC
AM285535          GCGTGCCGCGTGGTGTCGACGAGCCCCGAAATGTGGCGCTGCGCGGATATGAAATCCACC
LRW222_clone      ------------------------------------------------------------

CJ955962.1        GTCGACGGCACCTGCGGTGGACCCTGCAAGAAGTACTGATCGGTTCATGCCGGCATATGA
AM285535          GTCGACGGCACCTGCGGTGGACCCTGCAAGAAGTACTGATCGGTTCATGCCGGCATATGA
LRW222_clone      ----------------------------------------CGGTTCATGCCGGCATATGA
                                                          ********************

CJ955962.1        TATGTTCCGCCTAAAATTAAATAAAAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTAT
AM285535          TATGTTCCGCCTAAAATTAAATAAAAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTAT
LRW222_clone      TATGTTCCGCCTAAAATAAAATAAAAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTAT
                  ***************** ******************************************

CJ955962.1        GCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTT
AM285535          GCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTT
LRW222_clone      GCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTT
                  ************************************************************

CJ955962.1        TCTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGA
AM285535          TCTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGA
LRW222_clone      TCTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGA
                  ************************************************************

CJ955962.1        ATAAACACCTGTAGTGATTGTGCGTGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAG
LRW222_clone      ATAAACACCTGTAGTGATTGTGCGTGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAG
AM285535          ATAAACACCTGTAGTGATTGTGCGTGGCTGCCAATAATGTGAGTTGNGTNGTCCACGCAG
                  *********************************************   * **********

CJ955962.1        TTACATGTTTCCCGTTGTATTTATATACTCTCCGATATGATATTTGTTGGCGTGTTAAGC
AM285535          TTACATGTTTCCCGTTGTATTTATATACTCTCCGATATGATATTTGTTGGCGTGTTAAGC
LRW222_clone      TTACATGTTTCCCGTTGTATCC--------------------------------------
                  ********************

CJ955962.1        AGTAAAAAAAAAA
AM285535          AGTATCTT-----
LRW222_clone      -------------
```

b)

```
Wali5           MKGTKLAAILILQAVLVMGLLSHVNADFFPKCCNNCRSFSGVDVCDDAHPKCPQGCSACR
LRW222          MKGTKLAAILILQAVLVMGVLSHVNADFFPKCCNNCGSFSGVDVCDDAHPKCPKGCSACR
                *******************:**************** ****************:******

Wali5           VVSTSPEMWRCADMKSTVDGTCGGPCKKY
LRW222          VVSTSPEMWRCADMKSTVDGTCGGPCKKY
                *****************************
```

Figure 4.1 Contig reconstruction for *LRW222*. a) Alignment of the *LRW222* clone with overlapping ESTs (CJ955962.1 and AM285535) with the protein coding sequence highlighted in grey. b) Alignment of the LRW222 polypeptide sequence with Wali5.

4.3 Materials and methods

4.3.1 Biological material and infection

Three different wheat-*Puccinia* interactions were analysed. The rust pathogens were *P. triticina* race UVPt19, *P. graminis* f. sp. *tritici* race UVPgt55 (TTKSF) and *P. striiformis* race 6E22A+. All three *Puccinia* species were avirulent on the resistant Avocet *Yr1* and virulent on the susceptible Avocet S wheat lines respectively. All experiments were done in triplicate. Ten day old resistant and susceptible wheat seedlings were inoculated with either *Pgt* or *Ps* (6 mg/ml urediospores in kerosene oil) or *Pt* (3 mg/ml urediospores in kerosene oil; Prins *et al*., 2011). Mock infected seedlings were sprayed with kerosene oil alone. Leaf tissue was harvested immediately after inoculation and then at either 6 h (*Pgt* and *Pt*) or 12 h (*Ps*) intervals. Harvested tissue was frozen in liquid nitrogen and stored at -80°C.

4.3.2 RNA extraction and cDNA synthesis

Frozen wheat leaves were ground in liquid nitrogen and RNA extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Residual DNA was removed with a DNaseI (Fermentas) treatment. RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific), while its integrity was confirmed on a 1% (w/v) denaturing agarose gel (Sambrook and Russel, 2001). cDNA was synthesised using 1 µg total RNA with the Improm II cDNA synthesis kit (Promega) according to the prescribed instructions. All cDNA samples were diluted 1 in 50 with DEPC water. To evaluate the success of cDNA synthesis, a *GAPDH* amplicon was PCR amplified using 1 µl diluted cDNA, 1 pmol gene specific primers (Table 4.1) and a 1x dilution of KAPA Taq ReadyMix (KAPA Biosystems). PCR conditions were 94°C for 30 sec, 30 cycles of 94°C, 60°C and 72°C (each for 30 sec) and a final extension step at 72°C for 5 min.

4.3.3 qPCR gene expression analysis of *LRW222*

Primers used for qPCR analyses are indicated in Table 4.1. Optimal annealing temperatures and amplification efficiencies for each primer pair were determined using a temperature gradient and a four-fold cDNA dilution series. Each 10 µl qPCR reaction contained 1 µl diluted cDNA, 1 pmol primer mix and 1x SybrFast® Readymix (KAPA Biosystems). All reactions were run on a Biorad C1000 thermal cycler with a CFX96 Real-time attachment.

Table 4.1 Primers used for expression analysis and normalisation of *LRW222*.

Gene symbol	Accession number (NCBI)*	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp (^{o}C)	qPCR efficiency (%)
GAPDH	AK251456	F TGTCCATGCCATGACTGCAA R CCAAGTGCTGCTTGGAATGATG	105	60	98.3
LRW222	CJ956114.1	F ATGGGAGTCCTCTCGCACGTCAA R AGTACTTCTTGCAGGGTCCACCG	262	62	98.6
18S	M82356	F GTGACGGGTGACGGAGAATT R GACACTAATGCGCCCGGTAT	151	60	98.0
TUBB	U76897	F CAAGGAGGTGGACGAGCAGATG R GACTTGACGTTGTTGGGGATCCA	84	60	93.0
ARF	AB050957	F GCTCTCCAACAACATTGCCAAC R GCTTCTGCCTGTCACATGCGC	165	60	90.3
CDC	EU267938	F CAAATGCGCCATCAGGGAGAACATC R CGCTGCCGAAACCACGAGAC	227	56	100.5
RLI	AK331207.1	F CGATTCAGAGCAGCGTATTGTTG R AGTTGGTCGGGTCTCTTCTAAATC	242	60	97.4

* http://www.ncbi.nlm.nih.gov/

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase; *18S* - 18S rRNA; *TUBB* - Beta-tubulin; *ARF* - ADP-ribosylation factor; *CDC* - Cell division control protein gene; *RLI* - RNase L inhibitor-like protein gene.

The cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and the optimal annealing temperature for 40 sec. A final melt curve analysis ranging from 65 to 95°C with 0.5°C increments for 5 sec was included.

Expression of the *LRW222* gene was analysed in three biological replicates shortly after infection with each rust species. Multiple reference genes were used for the quantification of gene expression. These genes were previously validated using GeNorm analysis (Biogazelle) and included *ARF* and *RLI* for *Pt*-infected, *CDC* and *RLI* for *Ps*-infected and *CDC*, *18S* and *TUBB* for *Pgt*-infected wheat (Scholtz and Visser, 2013). The obtained Cq values of the experimental gene were exported into qBase-plus (Biogazelle) software. All qPCR experiments were done according to the MIQE guidelines (Bustin *et al.*, 2009).

4.3.4 Protein extraction

Total protein was extracted for Co-IP using a mild, non-denaturing protocol with some modifications (Roberts *et al.,* 2011). One time interval was selected for each biological replicate (*Pt* – 12 h, *Pgt* – 12 h, *Ps* – 24 h). These time intervals were selected according to increased protein levels found during qPCR analysis. Approximately 0.5 g frozen leaf tissue was homogenised in 500 µl extraction buffer (20 mM Tris (hydroxymethyl)-aminomethane (Tris) pH 8.0, 1 mM Ethylenedinitrilotetraacetic acid (EDTA) and 50 mM NaCl). The mixture was centrifuged at 12000 x *g* (4°C for 5 min) and the supernatant collected. Protein concentration was determined using the Bradford protein assay (Bradford, 1976) and stored at -80°C. A total of 100 µg of each sample was separated in duplicate on tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Schägger, 2006). One gel was Coomassie stained, while the other was transferred to Hybond-C nitrocellulose membranes (GE Healthcare) for Western blot analysis using the anti-LRW222 antibody.

4.3.5 Co-immunoprecipitation

Polyclonal antibodies against LRW222 were manufactured by Genscript (Piscataway, New York, USA). An 11-mer peptide was synthesised, conjugated to the Keyhole limpet hemocyanin (KLH) carrier protein (ADMKSTVDGTC-KLH) and used for the immunisation of two New Zealand white rabbits. Antibodies were finally affinity-purified from the rabbit antiserum.

Co-IP experiments were performed using the Catch and Release® v2.0 kit (Millipore) along with its prescribed protocol. Briefly, 1 mg total protein, 4 µg anti-LRW222 antibody and 10 µl affinity ligand were added to the spin column. Wash buffer (1% (v/v) NP-40 (nonyl phenoxypolyethoxylethanol), 0.25% (w/v) dicholic acid, 15 mM imidazole pH 7.4) was added to a final volume of 500 µl. The mixture was incubated on a shaking platform for 30 min at room temperature. The spin columns were centrifuged at 2000 x *g* (4^oC for 5 min) for every wash and elution step. After multiple washing steps, the protein-antibody complexes were eluted using 1x, 2x and 4x concentrations of the Catch and Release® non-denaturing elution buffer respectively. Ten µl of the eluted protein fractions were separated in duplicate on 10% (w/v) Tricine SDS-PAGE gels (Schägger, 2006) for Coomassie staining and Western blot analysis respectively.

For Coomassie staining, gels were incubated for 20 min in staining solution (0.2% (w/v) Coomassie Blue R250, 0.1% (w/v) Coomassie Blue G250, 10% (v/v) glacial acetic acid and 45% (v/v) methanol) and destained overnight in destaining solution (10% (v/v) glacial acetic acid and 45% (v/v) methanol).

4.3.6 Western blot analysis

Western blot analysis was done to confirm the presence of LRW222 in the Co-IP eluate. Proteins were transferred to Hybond-C nitrocellulose membranes (GE Healthcare) using the Mini Trans-blot Electrophoretic transfer cell (Bio-Rad). Non-specific binding was minimised by blocking the nitrocellulose membranes in 5% (w/v) Bovine serum albumin (BSA) in Tris-buffered saline (TBS) with Tween™ 20 (Polyoxyethylene sorbitan monolaurate; TBST; 0.1% (w/v) Tween™ 20, 137 mM NaCl and 20 mM Tris-HCl pH 7.6) at room temperature for 1 h. The membranes were incubated with a 1 µg/ml dilution of the anti-LRW222 antibody in 5% (w/v) BSA in TBST. This was followed by three 10 min washes with TBST. After washing, the membranes were incubated at room temperature for 1 h with a 1/9500 dilution of alkaline phosphatase-linked goat anti-rabbit IgG (Sigma-Aldrich) secondary antibody in 5% (w/v) BSA in TBST. This was followed by one 5 min wash in TBST, one 10 min wash in TBST with 0.05% (w/v) SDS and two 5 min washes in TBST respectively. The membranes were then incubated in staining buffer (0.1 M NaCl, 5 mM $MgCl_2$ and 0.1 M Tris-HCl pH 9.5) for 10 min followed by incubation with 0.3 mg/ml Nitro blue tetrazolium chloride (NBT) and 0.2 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate (BCIP; Calbiochem) in staining buffer for approximately 1 h.

4.3.7 Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) analysis

LC-MS/MS was used to identify eluted proteins obtained with Co-IP using LRW222. Eluate fractions were pooled, freeze-dried overnight and resuspended in 40 µl 50 mM NH_4HCO_3. Dithiothreitol (DTT) was added to a final concentration of 2 mM, the reaction incubated for 1 h at 37°C followed by the addition of iodoacetamide to a concentration of 8 mM. After incubation for 15 min at room temperature, $CaCl_2$ was added to a final concentration of 1 mM. Trypsin (Promega) was added to each sample in a 1:25 protease-to-protein (w/w) ratio, vortexed and incubated overnight at 37°C. Ten µl of a 10% (w/v) formic acid solution was added to quench the digest. Samples were re-purified with SDS-PAGE to reduce the amount of background noise obtained with in-solution digestion.

Five µl of each digestion was individually injected and concentrated on a C18 reverse phase trapping column and then eluted onto and separated by a custom packed C18 reverse phase column. Peptides were separated and eluted off the column over 2 h with a 10 to 25% (v/v) elution buffer (acetonitrile in 5% (v/v) formic acid) gradient. The eluted peptides were analysed on an AB SCIEX API4000QTRAP hybrid triple quadrupole ion trap mass spectrometer with a nanospray source at 350 nL/min. A survey scan between 400 and 1200 Da were performed to identify eluting peptides. An enhanced resolution scan was performed on peptides to determine the charge state of each peptide before fragmenting the peptides in the collision cell.

The obtained peptide sequence information was analysed by an in-house Mascot server using the Swissprot database (Swissprot 2010_09; Viridiplantae, 29441 sequences). The experiment was repeated three times and the protein identification data from Mascot was further processed using ProteoIQ. ProteoIQ combines the identification data for each slice to create a per lane dataset and calculates relative abundance of each protein based on its spectral abundance.

4.4 Results

Total extracted RNA was separated on a denaturing agarose gel to determine its quality. RNA integrity analysis showed intact 28S and 18S rRNA bands for all RNA samples with no degradation (Figure 4.2). The efficiency of cDNA synthesis was confirmed with the

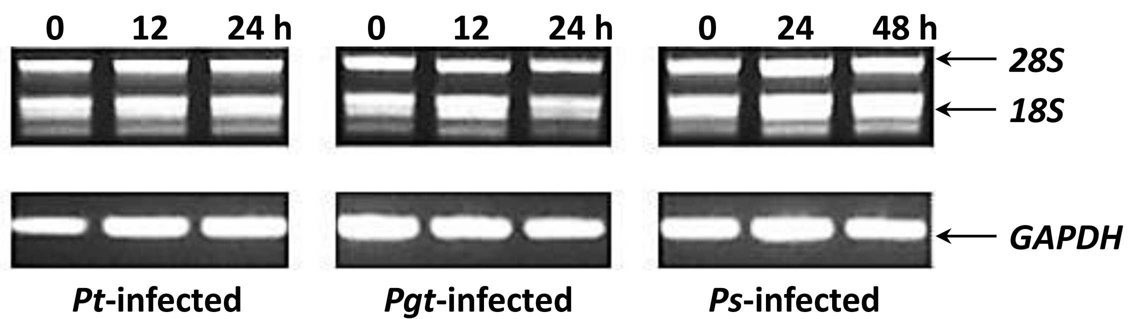

Figure 4.2 Confirmation of the quality of extracted total RNA and synthesised cDNA. Indicated are total RNA (above) and the PCR-amplified *GAPDH* amplicon from *Pt*-infected, *Pgt*-infected and *Ps*-infected wheat (below). One biological replicate of each infection study is presented. *28S* – 28S rRNA; *18S* – 18S rRNA; GAPDH - Glyceraldehyde-3-phosphate dehydrogenase; *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis.*

successful PCR amplification of a 354 bp *GAPDH* amplicon using the synthesised and diluted cDNA as template. Comparable results were found for all time intervals of all three different interactions.

Melt curves for the qPCR reactions indicated the absence of primer dimers and non-specific amplification products for the *LRW222* primer set in all experiments (Figure 4.3). The amplification efficiency for the *LRW222* primer pair was 98.6% (Table 4.1) which falls within the accepted published criteria (Livak and Schmittgen, 2001).

Analysis of *LRW222* expression using qBase-plus (Biogazelle) showed significant induced expression in both resistant and susceptible wheat following infection with the three different fungal pathogens, reaching maximum expression at either 24 or 48 hpi (Figure 4.4). Considerably lower levels of expression were observed in the mock- infected control plants where the maximum expression values were found at 24 hpi. In general, no significant differences were observed between infected resistant and susceptible wheat cultivars, except for the *Pgt*-infected wheat where significantly higher expression was observed in the IR wheat compared to the IS wheat. Induced expression levels of *LRW222* also reached much higher levels at 12 hpi compared to the other two interactions.

Crude protein extracts of the three different incompatible wheat-*Puccinia* interactions were separated on SDS-PAGE gels to determine the efficiency of the prepared antibodies to detect the LRW222 polypeptide. Translation of the *LRW222* gene indicated a protein size of approximately 10 kDa, but the Western blots using the anti-LRW222 antibodies did not indicate the presence of a 10 kDa protein in any of the time intervals post-inoculation (Figure 4.5). A number of larger proteins were however detected on the Western blots, ranging from approximately 20 to 250 kDa.

Following Co-IP, LRW22 was still not observed in Coomassie stained gels, but Western blot analysis confirmed the presence of a 10 kDa protein in the eluate of all samples, as well as the mock-infected control (Figure 4.6). No such polypeptide was evident in the flow- through fraction that represents the total protein extract. This indicated that the antibody was indeed highly specific, but that during the initial Western blot, the LRW222 polypeptide levels were too low to be detected. Co-IP concentrated the LRW222 polypeptides to such an extent that it was visible on the Western blot, but not on the Coomassie stained gel. A 55 kDa polypeptide also cross-reacted with the anti-LRW222 antibody in both infected and mock-

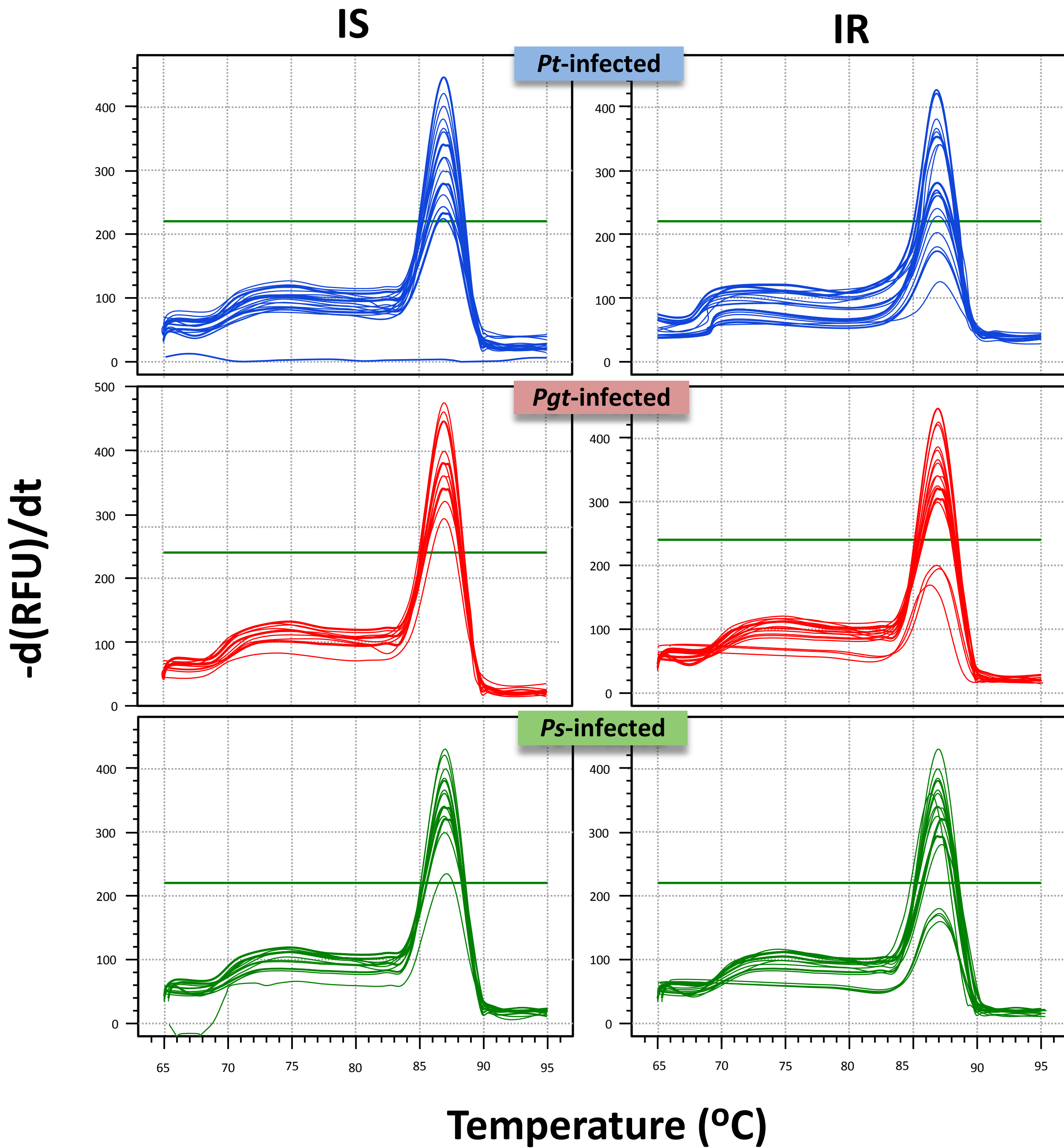

Figure 4.3 qPCR melt curves during *LRW222* expression analysis. One biological replicate of *Pt*-, *Pgt*- and *Ps*-infected susceptible (IS) and resistant (IR) wheat is represented. *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis.*

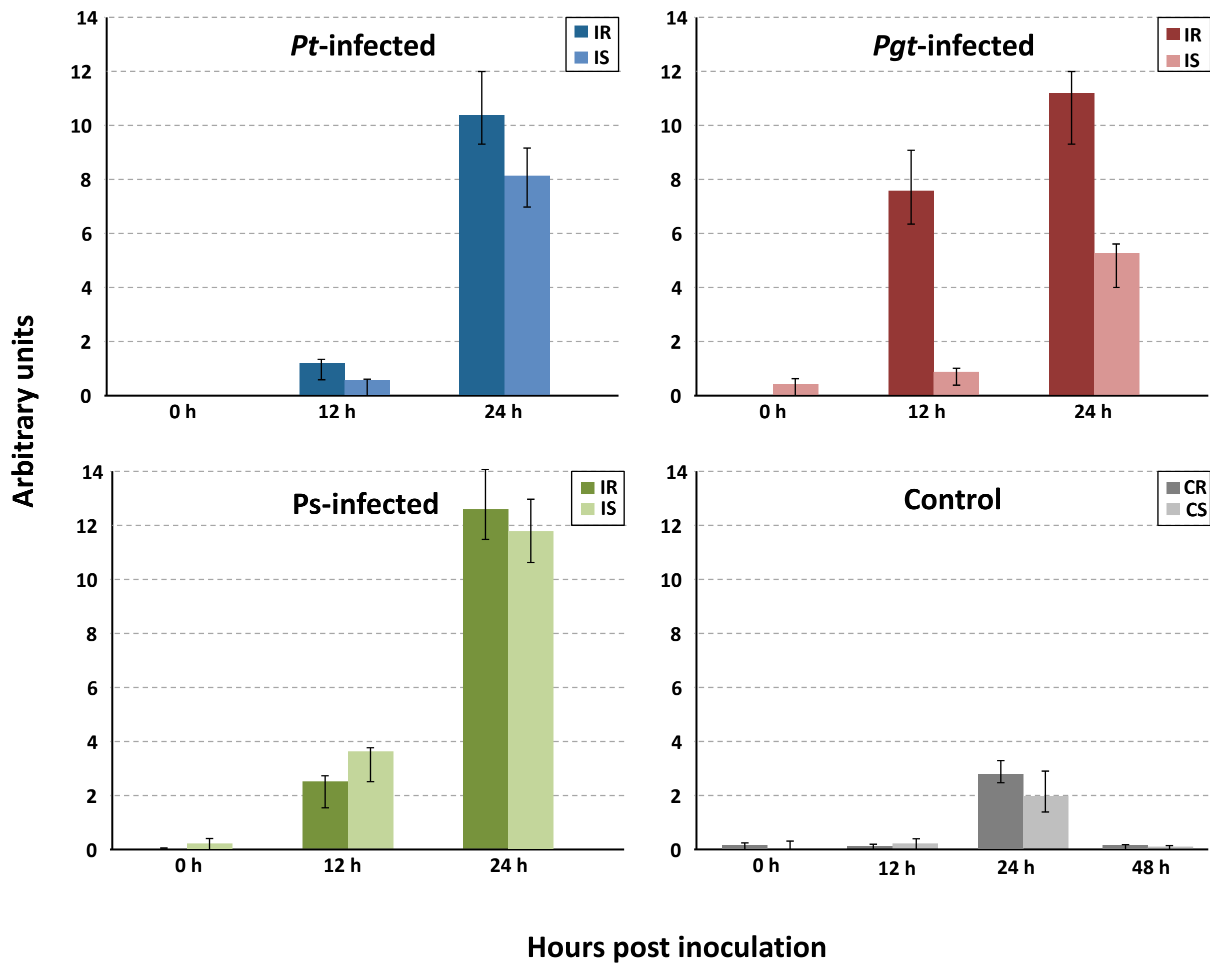

Figure 4.4 Relative gene expression values for *LRW222* in *Pt*-, *Pgt*-, *Ps*- and mock-infected control resistant (IR, CR) and susceptible (IS, CS) wheat. The figure represents averaged expression levels between biological and technical replicates. *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis.*

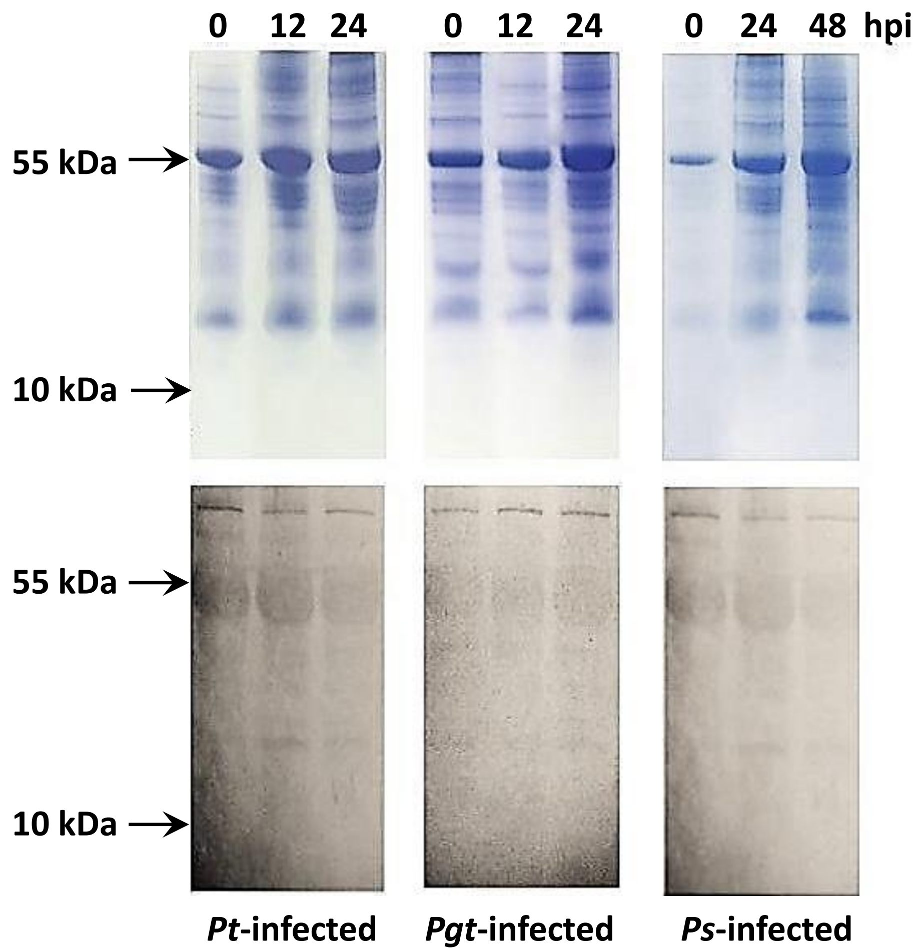

Figure 4.5 Crude protein extracts from *Pt-, Pgt-*, and *Ps*-infected wheat visualised via Coomassie staining and Western blots probed with the anti-LRW222 antibody. Coomassie stained gels (above) and Western blots (below) contained protein extracted for time-intervals 0, 12 and 24 hpi for *Pt-* and *Pgt*-infected wheat and 0, 24 and 48 hpi for *Ps*-infected wheat. A total of 100 µg of protein was loaded for each sample. *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis.*

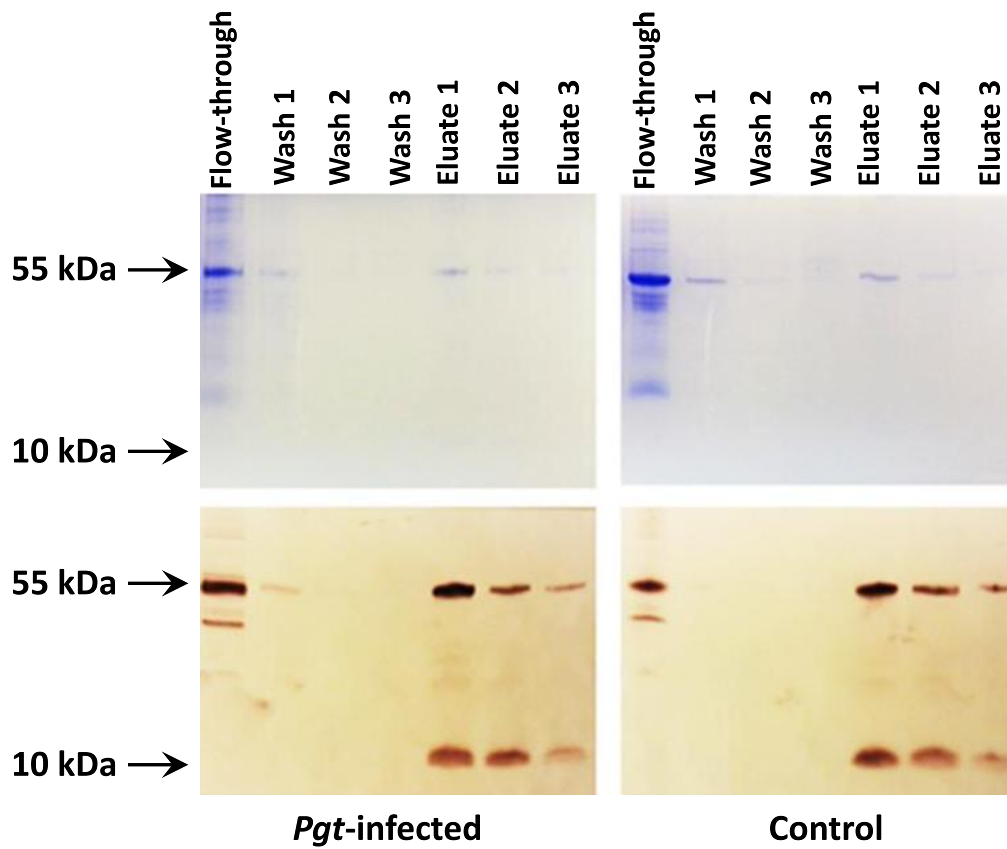

Figure 4.6 Co-immunoprecipitation fractions visualised via Coomassie staining and Western blot analysis using the anti-LRW222 antibody. Coomassie stained gels (above) and Western blot membranes (below) each contained protein obtained from column flow-through, three wash steps and three elution steps. Ten µl of each fraction was loaded. *Pt* - *Puccinia triticina*; *Pgt* - *Puccinia graminis* f. sp. *tritici*; *Ps* - *Puccinia striiformis.*

infected samples. The SDS-PAGE gels and Western blots were nearly identical for each Co-IP experiment and thus only one set of images is displayed.

LC-MS/MS analysis resulted in a wide variety of possible matches. *Pt*-infected samples resulted in possible polypeptide matches that included Casein kinase, COBRA like protein, poliamine oxidase, protein translocase subunit SecA and a pentatricopeptide repeat-containing protein. Identities for *Pgt*-infected samples included the Floral homeotic protein FBP1, DNA-directed RNA polymerase β subunit, Glutamyl-tRNA(Gln) amidotransferase subunit B-2, maturase K and BTB/POZ and MATH domain-containing protein 3. Possible matches for *Ps*-infected wheat included the U6 snRNA-associated Sm-like protein LSm4, ULTRAPETALA 1, 4-coumarate-CoA ligase 2, acetyl-CoA carboxylase and DNA-directed RNA polymerase β subunit. None of the identified proteins were simultaneously present in all three samples as was expected. The probability values of all identified proteins were extremely low.

4.5 Discussion

Gene expression studies during different stress conditions play a major role in the analysis of gene function. qPCR is currently one of the most accurate and sensitive technologies used for mRNA transcript abundance analysis (Bustin *et al.*, 2009). However, the many sources of experimental error increases the possibility of inaccurate results (Taylor *et al.,* 2010). Publication according to MIQE standards ensures standardised and repeatable results (Bustin *et al.*, 2009). It was demonstrated that poor RNA quality may seriously influence qPCR results and is thus a critical first step (Fleige and Pfaffl, 2006).

LRW222 was previously identified in *Pt*-infected wheat by means of SSH. The translated protein showed significant homology to the Wali5 protein. However, the untranslated regions (UTRs) of *LRW222* and *Wali5* were non-homologous. It was therefore concluded that the LRW222 protein is a homologue or allele of Wali5. Previous studies demonstrated the involvement of Wali5 in abiotic stress conditions such as aluminium-stress, wounding and Methyl jasmonate (MeJA) treatment (Richards *et al.,* 1994; Snowden *et al.,* 1995; Garg *et al.,* 2012). Aluminium-stressed wheat showed an induction of *Wali* genes in both sensitive and resistant plants (Snowden and Gardner, 1993; Richards *et al.,* 1994). Snowden *et al.* (1995) also reported an increase in *Wali1, Wali3, Wali4* and *Wali5* expression in wounded leaves and plants treated with high concentrations of an array of heavy metals.

In the current study, qPCR was used to confirm the induced expression of *LRW222* during the infection of the resistant Avocet *Yr1* and susceptible Avocet S wheat lines with three different *Puccinia* species. Results indicated that expression levels of *LRW222* were similar in the resistant and susceptible plants during *Pt* and *Ps* infection. However, the significant differences observed in the *Pgt*-infected plants may indicate a more specific role of LRW222 in the resistant plants in response to a *Pgt* infection. This contradicted the findings by Huang (2008) which showed opposite expression profiles for *Pt* and *Ps*-infected wheat. Similar to our results, *Pt*-infected plants showed a strong induction in both the infected resistant and susceptible plants. However the *Ps*-infected plants showed strong, constitutive expression of *LRW222*. The fact that Huang used RT-PCR for gene expression analysis is the most probable reason for these conflicting results.

A study conducted by Manickavelu *et al.* (2010) compared the gene expression level of *Wali5* in near-isogenic Thatcher lines carrying different resistance genes by means of RT-PCR. Their results indicated that *Wali5* is exclusively expressed in resistant Thatcher *Lr10* cultivars after infection with the Swiss *Pt* race BRW 97512-19. This *Pt* race is avirulent on Thatcher *Lr10* lines. However, traditional RT-PCR cannot be regarded as quantitative and is semi-quantitative at best. Even though this study used RT-PCR to confirm their results, the possibility exists that a qPCR approach with the use of validated reference genes could have yielded a different result.

The low, but elevated levels of gene expression observed in the control plants may have been caused by the use of kerosene during infection or other possible abiotic stress conditions during the infection process such as wounding or a sub-optimal soil pH. Studies have shown that certain plants are sensitive to kerosene and other mineral oils (Nicetic *et al.,* 2010; Kadiri and Eboigbodin, 2012). This may have activated a general defence response in the control plants. Gene expression studies of wheat treated with kerosene oil have not yet been conducted.

Research by Huang (2008) also showed induced expression of *LRW222* following treatment with H_2O_2 and JA, which indicated the regulation of *LRW222* by oxidative stress and its possible role in JA-mediated resistance respectively. It has shown that MeJA treatment and wounding may produce similar expression of PIs (Zhao *et al.*, 1996), which point to the possibility of LRW222 also being involved in abiotic stress conditions like its homologue, Wali5. Numerous other Bowman-Birk PIs have been implicated in biotic and abiotic stress

conditions (Yavelow *et al.,* 1983; Qi *et al.,* 2005; Dramé *et al.,* 2013).

It has been shown that under optimal conditions, *Ps* urediniospores germinate on leaf surfaces at approximately 12 hpi (Mares and Cousen 1977), the germ tube penetrates the stomata after 8-12 hpi, while infection hyphae and haustoria form at 16 hpi (Moldenhauer *et al.*, 2006). The qPCR results of this study indicated that the LRW222 protein plays a role during these very early stages of *Puccinia* infection in both susceptible and resistant cultivars as part of the general defence response of wheat.

A number of functional domains were previously identified on the LRW222 polypeptide sequence which may point to an additional role in the defence signal transduction of wheat. A MAPK phosphorylation site was identified, where LRW222 might be the phosphorylation substrate of MAPK. A MAPK docking motif was found which may facilitate interactions between LRW222 and a MAPK cascade. A 14-3-3 ligand binding site was present and is responsible for mediating signal transduction by binding to phosphoserine or phosphothreonine containing motifs from other proteins. 14-3-3 proteins have been implicated in numerous abiotic and biotic stress responses in plants (Roberts *et al.,* 2002). Manickavelu *et al.* (2010) have found that, in addition to the Wali5 protein, the 14-3-3 protein was induced after infection with *P. triticina.* The 14-3-3 transcripts accumulated in the epidermis of powdery mildew-infected barley, activating plasma membrane ATPase to create a binding site for the fusicoccin toxin (Brandt *et al.,* 1992). A study by Rampitsch *et al.* (2006) analysed the proteomes of wheat and *P. triticina* during a susceptible interaction. Among others, they identified a 14-3-3 protein which aligns well with both plant and fungal 14-3-3 proteins. A forkhead-associated (FHA) phosphopeptide ligand was also present on the LRW222 protein. It has been shown that FHA, together with 14-3-3 proteins are responsible for mediating phosphoprotein interactions in plants (Chevalier *et al.,* 2009).

Western blot analysis, using anti-LRW222 antibodies and crude protein extracts did not indicate the presence of LRW222. Initial expectations were that these should contain increasing amounts of LRW222, as seen during gene expression analysis. This could be explained by insufficiently low levels of LRW222 present in the samples compared to the other proteins or the non-specificity of the anti-LRW222 antibody. The former hypothesis seemed more likely after Co-IP results were observed. Western blots using the eluate from the Co-IP experiments appeared to be sufficiently enriched for LRW222 due to the presence

of a polypeptide with the predicted size of LRW222 (9.52 kDa). LRW222 was most likely present at increasing concentrations in the crude protein extracts, but concentrations were still not high enough for detection via Western blot analysis. Even though anti-LRW222 antibodies should only bind to the LRW222 epitopes, multiple bands were visualised during the Western blot analysis of crude proteins. This indicated the presence of additional LRW222-like epitopes in the denatured state of the proteins, which were absent during non-denaturing Co-IP experiments in which only two proteins were precipitated.

An additional protein of approximately 55 kDa was present in the Co-IP eluates, which corresponds to the size of the IgG heavy chain. Antibodies do not bind to themselves, so this could have resulted from the incomplete dissociation of anti-LRW222 antibodies and the target protein. However, this is unlikely because the antibody-antigen complex would have been approximately 65 kDa in size. As stated earlier, a unique property of BBIs include its high thermal stability (Yavelow *et al*., 1983). Its resistance to denaturation could have caused the reactivity of anti-LRW222 antibodies with the 55 kDa protein and larger proteins detected in the Co-IP eluates and crude protein extracts respectively. LRW222 might be present as a multimeric protein in the samples, being bound to varying combinations of proteases and/or signalling molecules. Komarnytsky *et al.* (2006) have observed that under natural conditions, BBIs exist as numerous multimers of higher molecular masses.

During the initial attempt to uncover interacting proteins by means of LC-MS/MS in the Co-IP eluates, a high amount of background noise made accurate identification of polypeptides by MS impossible. Although various possible sources of experimental error exist, this background noise was most likely due the insufficient removal of storage buffer from the Co- IP column. The trypsin-digested samples were thus re-purified with SDS-PAGE and subjected to a final MS analysis. The LC-MS/MS results contained large differences between the different samples, with the majority of identified proteins having low-scoring probability values. Additionally, no trace of LRW222 was found in the analysis. With the exception of polyamine oxidase, none of the identified proteins were found to be involved in the defence response of plants. The quality of the mass spectrometry results was not sufficient to draw any noteworthy conclusions regarding LRW222 protein interactions. A number of possible causes can be hypothesised. The absence of LRW222 in the LC-MS/MS data could point to the diffusion and loss of trypsin-digested peptides from the SDS- PAGE gel during purification for the second MS attempt.

In conclusion, this study has found that a previously identified homologue of *Wali5*, *LRW222*, is differentially expressed following the infection of wheat with *Pt*, *Pgt* and *Ps*. Wali5 and LRW222 has thus far been implicated in both biotic and abiotic stress conditions, but its specific role in the process of alleviating stress is not yet clear. Differential expression in both resistant and susceptible cultivars indicated a possible role for *LRW222* in the general defence response of wheat. Co-IP experiments and subsequent LC-MS/MS analysis was unsuccessful in identifying interacting proteins to further elucidate the role of LRW222 in wheat.

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Chapter 5

General discussion

During the PhD study of Ju-Chi Huang (2008), SSH was used for the identification and traditional RT-PCR for the gene expression analysis of *LRW222* in *Pt*- infected wheat. The reconstructed LRW222 protein showed significant homology to Wali5, a putative protease inhibitor belonging to the Bowman-Birk family of protease inhibitors. Previous studies have demonstrated the involvement of Wali5 in biotic and abiotic stress conditions (Richards *et al.,* 1994; Snowden *et al.,* 1995; Garg *et al.,* 2012). Due to the unreliability of traditional RT-PCR for gene expression analysis, the first aim of this study was to confirm the differential expression of *LRW222* by means of qPCR. The MIQE guidelines, which ensures standardised and repeatable expression results (Bustin *et al.*, 2009), were used for qPCR reference gene validation and gene expression analysis.

During this study we have successfully identified and validated a set of suitable reference genes for qPCR gene expression analysis in *Puccinia*-infected wheat (Scholtz and Visser, 2013). The expression stability of six candidate reference genes was established in three different incompatible *Puccinia*-wheat interactions. The qBase-plus2 analysis software (Biogazelle; Vandesompele *et al.,* 2002) was used to determine the appropriate reference gene combinations for each *Puccinia*-wheat interaction. GeNorm analysis nominated *ARF* and *RLI* as the best reference gene combination for normalisation in *Pt*-infected wheat. *CDC* and *RLI* were found to be appropriate for *Ps*-infected wheat, while three reference genes, *CDC, 18S* and *TUBB* were found to be the best combination for data normalisation in *Pgt*- infected wheat. Results demonstrated that inoculation with different *Puccinia* spp. may have diverse effects on the initial defence response of wheat, which emphasises the need for reference gene validation for each experimental step. The traditionally used *GAPDH* reference gene was not stably expressed in any of the interactions. A number of studies have revealed similar results, revealing the unreliability of unvalidated reference genes for qPCR data normalisation (Czechowski *et al.,* 2005; Kwon *et al.,* 2009; Tong *et al.,* 2009; Chari *et al.,* 2010; Long *et al.,* 2010).

The obtained reference genes were used in qPCR gene expression analysis to confirm the induced expression of *LRW222* during the infection of the resistant and susceptible wheat lines with three different *Puccinia* species. The expression of *LRW222* was induced in all infected plants. The expression levels were similar in the resistant and susceptible plants during *Pt* and *Ps* infection. However, the differences observed between the IR and IS lines in the *Pgt*-infected plants may indicate a more specific role of LRW222 in the resistant plants in

response to a *Pgt* infection. These results pointed to the involvement of the LRW222 protein in the early general defence response of wheat.

Western blot analysis and Co-IP was used in an attempt to reveal the role of LRW222 in *Pt*-, *Ps*- and *Pgt*-infected wheat by identifying proteins that interact with LRW222. Western blot analysis using anti-LRW222 antibodies, did not show the presence of the LRW222 protein in crude protein extracts. However, Western blots using the eluate from the Co-IP experiments appeared to be sufficiently enriched for LRW222 due to the presence of a polypeptide with the predicted size of LRW222 (9.52 kDa). Additional larger proteins and a 55 kDa band were present in Western bolts of both crude protein extracts and Co-IP eluates respectively. A unique property of BBIs is a high resistance to denaturation. This could have caused the reactivity of anti-LRW222 antibodies with the 55 kDa protein and larger proteins. As seen in other studies where BBIs exist as multimers under natural conditions (Komarnytsky *et al.* 2006), LRW222 might be present as a multimeric protein in the samples, being bound to varying combinations of proteases and/or signalling molecules.

LC-MS/MS was used to identify proteins co-immunoprecipitated with the LRW222 antibodies. The trypsin-digested samples were re-purified with SDS-PAGE following a high amount of background noise during MS analysis. The LC-MS/MS results mostly identified proteins with low-scoring probability values. The LRW222 protein was not found in the analysis, while an abundance of Rubisco was present. The quality of the mass spectrometry results was not sufficient to identify LRW222 protein interactions. The absence of LRW222 in the LC-MS/MS data could point to the diffusion and loss of trypsin-digested peptides from the SDS-PAGE gel during the second purification attempt.

In conclusion, this study identified and validated reference genes for use in future gene expression studies in *Puccinia*-infected wheat. We have also confirmed the elevated mRNA transcript levels of *LRW222* following the infection of wheat with three different *Puccinia* species. High expression levels in both resistant and susceptible wheat pointed to the involvement of the LRW222 protein in the general defence response of wheat. Although Co-IP experiments were inadequate for the identification proteins that interact with LRW222, results have shown that LRW222 may exist as different sized multimers in wheat. Future studies will have to employ a different strategy to identify proteins that interact with LRW222. One of these could include a yeast two hybrid system capable of screening an EST library for novel interactions.

Chapter 6

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Summary

The Bowman-Birk family of plant protease inhibitors are serine protease inhibitors that can simultaneously inhibit trypsin and/or chymotrypsin. Unique features include an unusually high thermal stability, tolerance towards low pH and resistance towards the action of proteases. Bowman-Birk protease inhibitors have been implicated in various biotic and abiotic stress conditions in plants. *LRW222* was recently identified by means of SSH in *Puccinia triticina*-infected wheat and was found to be homologous to Wali5, a wound- and aluminium stress-induced Bowman-Birk type protease inhibitor. The aim of this study was to determine the expression levels and role of the LRW222 protein in *Puccinia*-infected wheat.

This study identified and validated stable reference genes for qPCR gene expression analysis in rust-infected wheat. qPCR is the preferred method to quantify mRNA levels, but requires validated reference genes for data normalisation. As prescribed by the MIQE guidelines, the GeNorm-Plus algorithm was used to examine the expression stability of six candidate reference genes in resistant Avocet *Yr1* wheat infected with *P. triticina, P. striiformis* and *P. graminis* f. sp. *tritici* respectively. These reference genes were used in the gene expression analysis of *LRW222*. Results indicated induced expression in both resistant and susceptible wheat, indicating its possible involvement in the early general defence response of wheat. Co-immunoprecipitation and mass spectrometry was used in an attempt to identify LRW222-interacting proteins to shed light on its specific role in plant defence. Protein identification was inconclusive, but results indicated that under natural conditions, LRW222 may exist as multimers in wheat.

Opsomming

Die Bowman-Birk familie van plant protease inhibeerders is serien protease inhibeerders wat gelyktydig tripsien en/of chimotripsien kan inhibeer. Hul unieke kenmerke sluit 'n buitengewoon hoë termiese stabiliteit, toleransie teenoor lae pH en weerstand teenoor die aksie van proteases in. Bowman-Birk protease-inhibeerders is by verskeie biotiese en abiotiese strestoestande in plante betrokke. LRW222 is onlangs deur middel van SSH geïdentifiseer in *Puccinia triticina*-geïnfekteerde koring en homologie was met 'n wond- en aluminium stres-geïnduseerde Bowman-Birk tipe protease inhibitor, naamlik Wali5, gevind. Die doel van hierdie studie was om die uitdrukking en rol van die LRW222 proteïen in *Puccinia*-geïnfekteerde koring vas te stel.

Hierdie studie het stabiele verwysingsgene vir qPCR geenuitdrukking-analise in roes-geïnfekteerde koring geïdentifiseer en bevestig. qPCR is die voorkeur metode om mRNA transkripsie-vlakke te kwantifiseer, maar vereis die gebruik van stabiele verwysingsgene vir data normalisering. Soos voorgeskryf deur die MIQE riglyne, was die GeNorm-Plus algoritme gebruik om die uitdrukkings-stabiliteit van ses moontlike verwysingsgene in Avocet *Yr1* koring geïnfekteer met *P. triticina, P. striiformis* en *P. graminis* f. sp. *tritici* onderskeidelik, te ondersoek. Hierdie verwysingsgene was tydens die geenuitdrukkings-analise van LRW222 gebruik. Die resultate het geïnduseerde uitdrukking in beide weerstandbiedende en vatbare koringlyne aangedui, wat op die moontlike betrokkenheid van LRW222 in die vroeë algemene verdedigingsrespons van koring dui. Ko-immunopresipitering en massa-spektrometrie was gebruik in 'n poging om proteïene wat interaksie met LRW222 toon te identifiseer, om sodoende lig te werp op sy spesifieke rol in plantverdediging. Proteïen identifikasie was onoortuigend, maar die resultate het aangedui dat LRW222 moontlik as multi-grootte multimere onder natuurlike toestande in koring teenwoordig is.

Appendix 1

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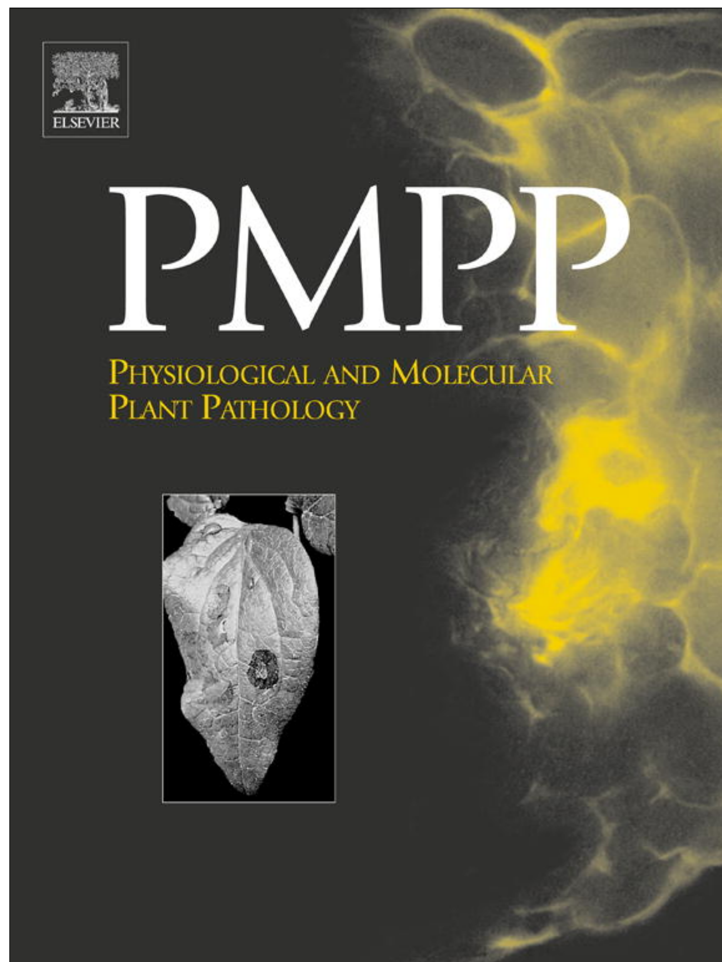

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Reference gene selection for qPCR gene expression analysis of rust-infected wheat

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Puccinia striiformis

ABSTRACT

Real-time PCR (qPCR) is an effective method to quantify mRNA levels, but requires validated reference genes for data normalisation. The GeNorm-Plus algorithm was used to examine the expression stability of six candidate reference genes in resistant Avocet *Yr1* wheat infected with *Puccinia triticina*, *Puccinia striiformis* and *Puccinia graminis* f.sp. *tritici* respectively. Results indicated that within the first 48 h after inoculation, the expression stability of the candidate reference genes differed between the three incompatible interactions. The geometric mean of *ARF* and *RLI* showed the best stability in *P. triticina*-infected wheat, *CDC* and *RLI* in *P. striiformis*-infected wheat and *CDC*, *18S* and *TUBB* in *P. graminis* f.sp. *tritici*-infected wheat respectively. This clearly emphasised the need for reference gene validation for each different plant–pathogen interaction.

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1. Introduction

Wheat production is frequently threatened by pathogen infection. Rust diseases of wheat account for large yield losses with aggressive new strains of stem and stripe rust destroying approximately 40% of farmers' wheat fields [1]. In particular, the widespread susceptibility of wheat to the Ug99 stem rust race group is a major concern due to its rapid movement from Africa into parts of Asia and the Middle East [2]. The genetic improvement of wheat to survive rust-induced stress conditions therefore remains a priority [3]. Gene expression studies form part of this process.

Real-time PCR (qPCR) has become the industry standard for gene expression quantification due to its high sensitivity and reproducibility. However, the absence of strict qPCR requirements has in many cases led to the publication of unreliable and irreproducible results [4]. The MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) have been proposed in an attempt to improve the quality of expression results by standardising each step of the qPCR work-flow [5]. These steps include experimental design, RNA (extraction, storage and quality control), reverse transcription, oligonucleotides (design and optimisation), data normalisation and analysis.

Accurate qPCR data analysis requires an appropriate normalisation strategy to minimise non-biological variation between samples [6]. The preferred approach is validated endogenous reference genes whose expression remains stable under all tested conditions [5]. Additionally the geometric mean of multiple validated reference genes is becoming the minimum requirement for data normalisation [7,8].

It was shown that the expression stability of traditional reference genes such as Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-Tubulin (*TUBB*) or 18S rRNA (*18S*) may vary significantly between different tissue types and disease states of an organism [9–13]. Others have shown that even a minor alteration in experimental conditions may cause a previously suitable reference gene to become unstable [14]. The use of unvalidated reference genes may obscure the true biological variation between samples. It is thus extremely important to carefully select appropriate reference genes for each tissue state using available statistical algorithms. Some of these include GeNorm [15], Bestkeeper [16] and Normfinder [17].

Jarosova and Kundu [18] identified *GAPDH*, *18S rRNA* and *TUBB* as the most stable reference genes in wheat infected with Barley yellow dwarf virus. In another study, the cell division control protein (*CDC*), ADP-ribosylation factor (*ARF*) and RNase L inhibitor-like protein (*RLI*) encoding genes were used for data normalisation during various developmental stages of wheat [19].

The goal of this study was to examine the stability of six candidate reference genes in Avocet *Yr1* wheat shortly after inoculation with three avirulent *Puccinia* spp. The three rust species were *Puccinia triticina* (*Pt*), *Puccinia striiformis* (*Ps*) and *Puccinia graminis* f.sp. *tritici* (*Pgt*) which are the causal agents for leaf, stripe and stem rust respectively.

Abbreviations: Cq, quantification cycle; MIQE, minimum information for publication of quantitative real-time PCR experiments; *Pgt*, *Puccinia graminis* f.sp. *tritici*; *Ps*, *Puccinia striiformis*; *Pt*, *Puccinia triticina*; RIN, RNA integrity value; qPCR, quantitative polymerase chain reaction.

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2. Materials and methods

2.1. Wheat cultivation and infection

Using standard inoculation procedures, three different incompatible *Puccinia*–wheat interactions were established when 10 day old resistant Avocet *Yr1* wheat seedlings were inoculated with *Pt* race UVPt19, *Pgt* race UVPgt55 (TTKSF) and *Ps* race 6E22A+ respectively [24]. Leaf tissue was harvested immediately after inoculation and then at either 6 h (*Pgt* and *Pt*) or 12 h (*Ps*) intervals. Each inoculation was done in triplicate.

2.2. cDNA synthesis

Total RNA was extracted from harvested tissue using Trizol (Invitrogen) according to manufacturer's instructions. Residual DNA was removed with a DNaseI treatment (Fermentas). RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific) while its integrity was confirmed on a 1% (w/v) denaturing agarose gel [25]. cDNA was synthesised from 1 μg total RNA using random and 15-mer oligo-dT primers with the Improm II cDNA synthesis kit (Promega) according to the manufacturer's specifications. The cDNA was diluted 1/50 in DEPC treated water before qPCR analysis.

2.3. qPCR analysis

The optimal annealing temperature for each primer set was determined using a temperature gradient. A preliminary standard curve was used to determine the amplification efficiency of each primer set. Each qPCR experiment consisted of three biological replicates, three technical replicates and three time intervals following inoculation with each respective *Puccinia* species. All qPCR reactions were run on a CFX96™ real-time PCR detection system (BioRad). Each 10 μl qPCR reaction contained 1 μl 1/50 diluted cDNA, 1 pmol primer mix (Table 1) and 1× SybrFast® Readymix (KAPA Biosystems). The cycling conditions were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and the optimal annealing temperature (Table 1) for 40 s. A final melt curve analysis ranging from 65 to 95 °C with 0.5 °C increments for 5 s was included.

All Cq values were imported into the GeNorm-Plus2 software (Biogazelle) and the average expression stability (*M*) values calculated. Replicate variability was set to a maximum limit of 0.5 and significant outliers were excluded for each set of three technical replicates.

3. Results and discussion

Numerous studies have shown the unreliability of unvalidated reference genes for qPCR data normalisation [9–13]. This indicated that universal reference genes do not exist and should be validated for each individual experimental setup. The MIQE guidelines [5] recommend criteria for real-time experiments and publication in order to make them more reproducible. All results in this study were generated according to the MIQE guidelines.

Here, the stability of six candidate reference genes was investigated in three different incompatible *Puccinia*-wheat interactions. To eliminate expression variation due to the host genetics, the resistant Avocet *Yr1* wheat cultivar was used. This cultivar carries the *Lr10*, *Sr26* and *Yr1* disease resistance genes that are effective against *Pt* race UVPt19, *Pgt* race UVPgt55 and *Ps* race 6E22A+ respectively, thus resulting in three incompatible interactions.

The initial defence response of wheat after infection with *Puccinia* spp is activated approximately 12–48 h after inoculation [20]. For that reason, reference gene stability for *Pt* and *Pgt* infected wheat was tested at 0, 12 and 24 hpi and for *Ps* infected wheat at 0, 24 and 48 hpi. RNA extracted from infected leaf tissue displayed no degradation or genomic DNA contamination. RNA integrity (RIN) values of at least 7 or higher were obtained using a 2100 Bioanalyzer (Agilent Technologies).

A gradient PCR was used to determine the optimal annealing temperature for all primer pairs which ranged from 56 °C for *CDC* to 60 °C for the other 5 primer sets (Table 1). A single melting curve peak for each primer set indicated denaturation of 50% of every amplicon at a specific temperature with no abnormalities, confirming the absence of non-specific amplification and primer dimers. R^2 values typically ranged from 0.985 to 0.99. Target amplification efficiencies using a 4 fold cDNA dilution series ranged from 90.3 to 100.5% (Table 1) with a standard deviation of <10% which can be considered comparable [21]. All primer sequences were successfully verified with DNA sequencing of the amplified DNA fragments.

The most suitable reference gene combination for each *Puccinia*-wheat interaction was established using the qBase-plus2 analysis software (Biogazelle). Results indicated that the most suitable reference gene combinations differed amongst the three incompatible interactions (Fig. 1).

GeNorm analysis selected *ARF* and *RLI* as the best reference gene combination for normalisation in *Pt*-infected wheat with an average *M*-value of 0.313 (Table 2). *CDC* and *RLI* were found to be appropriate for *Ps*-infected wheat ($M_{avg} = 0.279$) while a combination of three reference genes, namely *CDC*, *18S* and *TUBB*, was found to be the best suited for normalisation in *Pgt*-infected wheat ($M_{avg} = 0.492$). While some reference genes showed stability in at least two interactions (*RLI* and *ARF*), the traditionally used *GAPDH* reference gene was not stably expressed in any of the interactions. It should be noted that these reference gene combinations are only appropriate for the tested time intervals following inoculation and will not necessarily be well suited for later times post-inoculation.

Table 1
Primer sequences and qPCR amplification efficiencies of six candidate reference genes.

Gene symbol	Accession number (NCBI)[a]	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp (°C)	qPCR efficiency (%)	Ref
18S	M82356	F GTGACGGGTGACGGAGAATT R GACACTAATGCGCCCGGTAT	151	60	98.0	[18]
GAPDH	AK251456	F TGTCCATGCCATGACTGCAA R CCAAGTGCTGCTTGGAATGATG	105	60	98.3	
TUBB	U76897	F CAAGGAGGTGGACGAGCAGATG R GACTTGACGTTGTTGGGGATCCA	84	60	93.0	
ARF	AB050957	F GCTCTCCAACAACATTGCCAAC R GCTTCTGCCTGTCACATACGC	165	60	90.3	[19]
CDC	EU267938	F CAAATACGCCATCAGGGAGAACATC R CGCTGCCGAAACCACGAGAC	227	56	100.5	
RLI	AK331207.1	F CGATTCAGAGCAGCGTATTGTTG R AGTTGGTCGGGTCTCTTCTAAATG	242	60	97.4	

[a] http://www.ncbi.nlm.nih.gov/.

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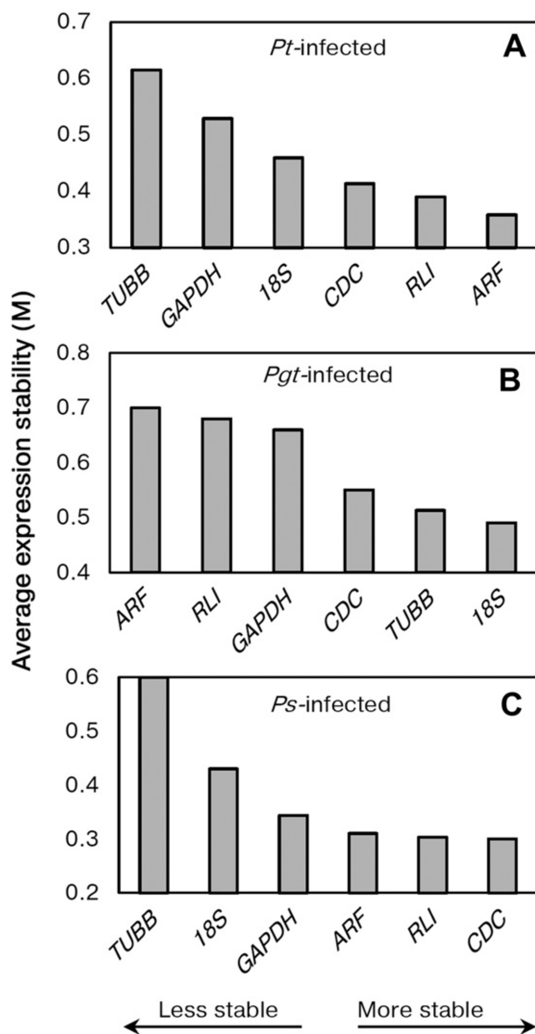

Fig. 1. Average expression stability (*M*) of six candidate reference genes in Avocet *Yr1* wheat infected wheat. A) *P. triticina*, B) *P. graminis* f.sp. *tritici* and C) *P. striiformis* as analysed with GeNorm. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Beta-tubulin (*TUBB*), 18S rRNA (*18S*), ADP-ribosylation factor (*ARF*), cell division control protein (*CDC*), RNase L inhibitor-like protein (*RLI*).

The fact that the suitable reference gene combinations differed between the three *Puccinia*-wheat interactions is yet another demonstration that reference gene validation prior to qPCR gene expression analysis is extremely important. The results suggest that inoculation with different *Puccinia* spp. may have vastly different effects on the initial defence response of wheat. For instance, the high instability of *ARF* in *Pgt* infected wheat shortly after infection compared to the other two interactions may point to an altered effect on vesicular traffic, lipid metabolism and microtubule dynamics [22]. Studies have shown that over-expression of *ARF1* in tobacco leaves causes cell death and have demonstrated its involvement in the non-host and *R*-mediated resistance [23].

Table 2
GeNorm stability values (*M*) of candidate reference genes in *Pt*-, *Pgt*- and *Ps*-infected wheat.

Gene symbol	Average expression stability values (*M*)		
	Pt-infected	*Pgt*-infected	*Ps*-infected
18S	0.46	**0.49**[a]	0.43
GAPDH	0.53	0.66	0.34
TUBB	0.62	**0.51**[a]	0.60
ARF	**0.36**[a]	0.70	0.31
CDC	0.41	**0.55**[a]	**0.30**[a]
RLI	**0.39**[a]	0.68	**0.30**[a]
AVG[b]	**0.31**	**0.49**	**0.28**

[a] Reference genes selected with GeNorm are shown in bold.
[b] Average values of selected reference genes.

4. Conclusions

In conclusion, we have identified and validated stable reference genes for gene expression studies in three different incompatible *Puccinia*-Avocet *Yr1* interactions. This study confirms the importance of reference gene validation for every experimental setup, enabling more accurate gene expression studies. Our results provide a useful starting point for the analysis of gene expression during different *Puccinia*-wheat interactions.

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