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CONSTRUCTION OF NOVEL RECOMBINANT RECEPTOR PROTEINS TO IMPROVE DISEASE RESISTANCE IN WHEAT

By

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"Research is to see what everybody else has seen and to think what nobody else has thought "

Albert Szent-Györgi

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ABBREVIATIONS

A

ATP	Adenosine-5' triphosphate
avr	avirulence

B

bp	Basepair
BRI1	Brassinosteroid insensitive1
BRs	Brassinosteroids
BSA	Bovine serum albumin

C

CaM	Calmodulin
CC	Coiled-coil
CD	Common docking
cDNA	Complementary DNA
CDPK	Ca^{2+}-dependent protein kinase
CERK1	Chitin elicitor receptor kinase1
CHRK1	Chitinase-related receptor-like kinase1

D

DAMPs	Damage-associated molecular patterns
DMPC	Dimethylpyrocarbonate
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DSP	Dual specificity protein phosphatase
DTT	Dithiothreitol

E

EDTA	Ethylenedinitrilotetraacetic acid
EF-Tu	Elongation factor-Tu
EGF	Epidermal growth factor
EILP	Elicitor-induced LRR receptor-like protein
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility

F

flg22	Flagellin
FLS2	Flagellin sensing2

G

G	Guanine triphosphate protein
GPCR	G protein coupled receptors
GTP	Guanine triphosphate

H

H_2O_2	Hydrogen peroxide
HR	Hypersensitive response

I

IDT	Integrated DNA Technologies
INA	2,6-Dichloroisonicotinic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside

ISR	Induced systemic resistance

J

JA	Jasmonic acid

K

kb	Kilobase pair
kDa	Kilodalton

L

LB	Luria-Bertani
LecRK1	Lectin receptor kinase1
LMW	Low molecular weight
LPS	Lipopolysaccharides
LRK10	Leaf rust resistance protein kinase10
LRR	Leucine-rich repeat
LysM RLK1	Lysine motif receptor like protein kinase1
LZ	Leucine zipper

M

MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeJA	Methyl jasmonate
MKP	MAPK phosphatase
MS	Murashige and Skoog

MW	Molecular weight

N

NB-LRR	Nucleotide binding-leucine-rich repeat
NO	Nitric oxide
NPR1	Non-expressor of PR-1
NTF2	Nuclear transport factor2

O

O_2^-	Superoxide
OD	Optical density
OGs	Oligogalacturonides
OH^-	Hydroxyl free radical

P

PAMPs	Pathogen-associated molecular patterns
PERK1	Proline-rich extensin-like protein kinase1
PEG	Polyethylene glycol
PG	Polygalacturonase
PGIPs	Polygalacturonase inhibiting proteins
PGN	Peptidoglycan
PIPES	Piperazine-1, 2-bis (2-ethanesulfonic acid)
PP2C	Protein phosphatase 2C
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
PTP	Protein tyrosine phosphatase

R

R gene	Resistance gene
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like protein kinase
RLP	Receptor-like protein
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species

S

SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SLG	Self-incompatibility locus glycoprotein
STPP	Serine/threonine protein phosphatase

T

TIR	Toll interleukin repeat
TM	Trans-membrane
TMV	Tobacco mosaic virus
TNFR	Tumor necrosis factor receptor-like repeat class
Tris-HCl	Tris-hydroxymethylaminomethane-HCl
Triton X-100	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
Tween 20	Polyoxyethylesorbitanmonolaurat

V

Vir	Virulence

W

WAK1	Wall-associated protein kinase1

X

X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

TABLES AND FIGURES

CHAPTER 1

INTRODUCTION:

1. Introduction:

People and animals worldwide rely on wheat (*Triticum aestivum L.*) as a source of food. Wheat, which belongs to the poaceae family, is the most produced cereal after maize and rice and has higher protein content compared to the other two crops. It is thought that wheat originated in the Fertile Crescent region in the Near East over 10000 years ago. Wheat is used for making bread, biscuits, pasta (Cauvain and Cauvain, 2003), fermentation (Palmer, 2001) and recently as a biofuel (Neill, 2002). In South Africa, wheat production occurs in both summer and winter rainfall regions and is affected by variable climatic conditions and low soil fertility that could result in yield losses.

Wheat yield can also be lowered by fungal, bacterial and viral diseases. The most common fungal diseases include the rust diseases caused by *Puccinia* spp. which are spread via airborne spores. Symptoms associated with this fungus are pustules which develop randomly on the leaf blades or the stem of the plant that may form patches in serious cases (Singh *et al.*, 2008). These rust diseases can result in a yield loss of 5-20% and up to 80% in the case of stem rust. Generally, plants are resistant to most pathogens but in time this resistance can be overcome by successful pathogens making these plants susceptible (Heath, 2000; Thordal-Christensen, 2003). An example is the stem rust race Ug99 that overcame two widely used and effective resistance genes, Sr7 and Sr34 (Park *et al.*, 2010). The Ug99 lineage comprises of seven different pathotypes and wheat plants have decreased resistance against them (Jin *et al.*, 2008; 2009; Pretorius *et al.*, 2010).

Plants have developed different ways in which they can activate their defence responses. This can be done on two levels depending on the nature of the genetic interaction between the plant and pathogen (Zipfel and Felix, 2005). Firstly, pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), thus inhibiting colonization of the plant by the pathogen (Zipfel and Felix, 2005). This immunity is known as PAMP-triggered immunity (PTI). Pathogens can however employ effector molecules encoded by virulence (*Vir*) genes in order to suppress PTI and this results in effector-triggered susceptibility (ETS) (Abrahamovitch *et al.*, 2006; Grant *et*

al., 2006; Zhou and Chai, 2008; Boller and He, 2009; Cui *et al.*, 2009). However, during the evolution of plants and pathogens, plants have acquired resistance (*R*) genes whose encoded products act as receptor proteins that recognize the effector proteins from the pathogens resulting in effector-triggered immunity (ETI), an efficient form of resistance that includes the hypersensitive response (HR) (Jones and Dangl, 2006). Should the pathogen overcome this resistance, the plant again has to adapt to ensure its survival. This continual arms-race between plant and pathogen thus form the motivation for the current study.

The overall hypothesis of the study is that by combining the general PAMP binding capability of extracellular PRRs with the specific kinase signalling capability of a receptor-like protein kinase (RLK) *R* gene, infection of a plant by any fungal pathogen would activate an effective defence response in the form of the HR, thereby preventing disease. The specific aim of this part of the study was therefore to construct a new class of "resistance" proteins in the form of chimeric RLKs. This was done by combining the kinase domain encoding portion of the *LRK10 R* gene with the extracellular domain encoding gene portions of several different PRR genes. Expression of these genes in wheat would hopefully lead to enhanced disease resistance that is not dependent on a specific avirulence (*avr*) gene product, but rather on general PAMPs that are produced during infection.

CHAPTER 2

LITERATURE REVIEW:

2. Introduction

Plants are sessile organisms which cannot escape any stressful conditions they are exposed to. Therefore, plants use different strategies to overcome these stresses. The most effective mechanism is genetic resistance through the recognition of stress factors (Agrios, 1988; Cornelissen and Melchers, 1993). Stress factors have one of two origins; they can either be endogenous or exogenous. Endogenous factors are generally produced by stressed or damaged plant cells in the form of damage-associated molecular patterns (DAMPs) (Lotze *et al.*, 2007). Exogenous factors include PAMPs or microbe-associated molecular patterns (MAMPs) (Ausubel, 2005; Bittel and Robatzek, 2007; Boller and Felix, 2009), enzymes (Beliën *et al.*, 2006), effector molecules (Kamoun, 2006; Tör, 2008) and non-microbial or abiotic stress factors such as toxic compounds, UV-B light, ozone and pollutants (Kamoun, 2006).

Defence mechanisms against these stresses can either be inducible or pre-existing within the plant (Heath, 2000). Inducible defence mechanisms can occur either locally, meaning that they are restricted to a certain area of the plant or systemically, thereby occurring throughout the plant. The focus of this literature study will be on the interaction between plants and pathogens where two types of plant resistance responses are employed, namely non-host and host specific resistance.

2.1. Non-host resistance

During an attack by a pathogen, the survival of multi-cellular organisms depends on their ability to activate an effective immune response (Jones and Dangl, 2006). There are two types of immune responses, namely adaptive and innate immunity. Plants lack the adaptive immunity of vertebrates and thus rely on innate immunity for defence (Ausubel, 2005).

Most pathogens enter the plant by either penetrating the leaf or root surface directly or through wounds or plant openings such as the stomata (Nürnberger *et al.*, 2004). Once

inside the plant, the plant cell wall, a rigid cellulose-based support surrounding every cell, is the next obstacle that the pathogen must overcome. As soon as these obstacles have been bridged, the pathogen will be subjected to multiple molecular surveillance systems employed by the plant cell (Shiu and Bleecker, 2003).

Plant defence activation or immunity can be achieved on two levels, depending on the nature of the genetic interaction between the plant and the pathogen (Zipfel and Felix, 2005). The first level of immunity is known as PTI (Bray *et al.*, 2007; Xiang *et al.*, 2008), while the second is called ETI (Jones and Dangl, 2006).

2.1.1. Pathogen-associated molecular patterns

PAMPs are highly conserved motifs within molecules or the molecules themselves that are characteristic of specific pathogenic classes. These molecules are also referred to as MAMPs as they are not restricted to pathogens only but also to microbes (Abramovitch *et al.*, 2006; Chisholm *et al.*, 2006; Bittel and Robatzek, 2007; Bray *et al.*, 2007). Different types of PAMPs include lipopolysaccharides (LPS) (Newman *et al.*, 2007), peptidoglycans (PGN) (Gust *et al.*, 2007), flagellin (flg22) (Zipfel and Felix, 2005), elongation factor-Tu (EF-Tu) (Nürnberger *et al.*, 2004) and chitin (Iriti and Faoro, 2007). Of all these, the involvement of only flg22, LPS and EF-Tu from Gram negative bacteria, chitin and glucans from fungi and oomycetes (Nürnberger *et al.*, 2004; Zipfel and Felix, 2005) and sulphated peptides for example *Ax21* from *Xanthomonas oryzae oryzae* (Lee *et al.*, 2009) have been proven in the activation of plant responses. These PAMPs are recognized by PRRs which distinguish them from other conserved microbial structures shared by different pathogens (Figure 2.1) (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Imler and Hoffman, 2001; Underhill and Ozinsky, 2002).

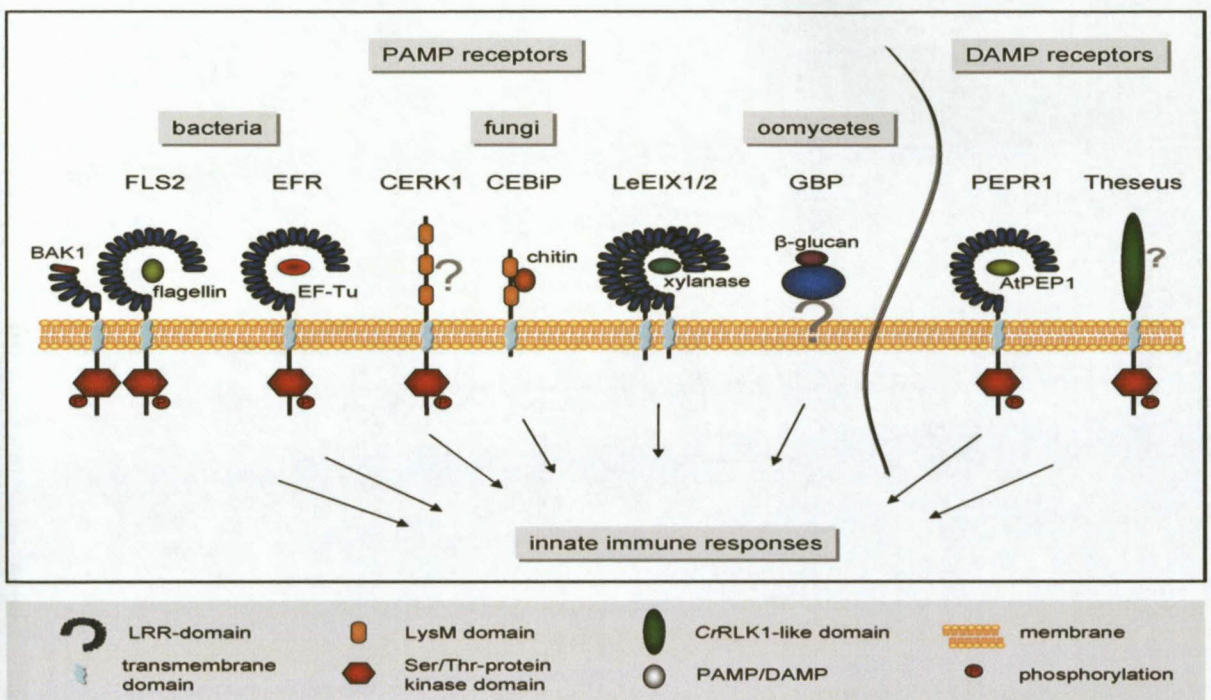

Figure 2.1. Different types of PAMPs/MAMPs and DAMPs and the plant PRRs which recognizes them (Postel and Kemmerling, 2009). PAMP: pathogen-associated molecular pattern, MAMP: Microbe-associated molecular pattern, DAMP: damage-associated molecular pattern and PRRs: Pattern recognition receptors.

2.1.2. Damage-associated molecular patterns

In addition to PAMPs/MAMPs, mechanical injury and insect or herbivore damage releases endogenous signalling molecules known as DAMPs which help to elicit plant immune responses (Figure 2.1) (Lotze *et al.*, 2007; Denoux *et al.*, 2008; Boller and Felix, 2009). Examples of DAMPs include plant cell wall-derived oligogalacturonide fragments, cellulose fragments or cutin monomers (Darvill *et al.*, 1994; Fauth *et al.*, 1998; Aziz *et al.*, 2007). DAMPs are recognized in a similar way to PAMPs/MAMPs by PRRs at the outer surface of healthy cells (Lotze *et al.*, 2007).

2.1.3. Pattern recognition receptors

Most PRRs are plasma membrane bound (extracellular PRRs) but they can also be localized in the endosomal compartment or can even be cytoplasmic (intracellular PRRs) (Nürnberger *et al.*, 2004). Most identified PRRs are RLKs (Shiu and Bleecker, 2003), receptor-like proteins (RLPs) (Wang *et al.*, 2008) and polygalacturonase inhibiting proteins (PGIPs) (Gomez-Gomez and Boller, 2000; Di Matteo *et al.*, 2003; Shiu *et al.*, 2004; Kaku *et al.*, 2006; Zipfel *et al.*, 2006).

2.1.3.1. Extracellular PRRs

2.1.3.1.1. Receptor-like protein kinases

RLKs are proteins which contain both receptor and signalling capabilities within a single molecule. It recognizes and interacts with extracellular signals in the form of ligands (Shiu and Bleeker, 2001a). These RLKs have four main regions; a signal peptide, the extracellular domain, a trans-membrane (TM) domain and an intracellular catalytic protein kinase domain.

The signal peptide consists of 20 hydrophobic amino acids followed by three positively charged amino acids. Its presence targets the polypeptide to the plasma membrane. The

extracellular domain contains different conserved motifs that are involved in the binding of various ligands. The TM domain, which consists of a short region of acidic amino acids followed by several basic amino acids, links the extracellular domain to the catalytic kinase domain. Finally, the catalytic kinase domain which forms the intracellular portion of the protein has 11 blocks of conserved amino acid sequences or sub-domains characteristic of protein kinases (Shiu and Bleecker, 2003) (Figure 2.2).

Ligand binding promotes both homo- and heterodimerization of RLKs (Wang *et al.*, 2005a; Wang *et al.*, 2008). An example is the FLS2/BAK1 interaction during flagellin (flg22) perception (Russinova *et al.*, 2004). Another possible interaction is between RLKs and RLPs, such as CERK1/CEBiP interaction during chitin binding (Miya *et al.*, 2007; Wan *et al.*, 2008). Ligand binding promotes conformational changes that generate docking sites for adaptor molecules such as BAK1 for brassinosteroid insensitive1 (BRI1) (Shpak *et al.*, 2005) and promotes phosphorylation of residues at the juxta-membrane domain, a region between the kinase and TM domains of RLKs. Depending on the structure of their extracellular domains and the presence of conserved motifs, RLKs are classified into several groups (Figure 2.2) (Walker, 2004).

2.1.3.1.1.1. The S-domain class

The S-domain class is related to the *Brassica* self-incompatibility locus glycoproteins (SLGs) (Nasrallah *et al.*, 1994). This class is characterized by the presence of 12 cysteines of which ten are conserved in the following sequence: $CX_5CX_5CX_7CXCX_NCX_7CX_NCX_3CX_3CXCX_NC$ (Torii and Clark, 2000). In addition, the S-domain possesses the PTDT box which has a conserved WQSFDXPTDTFL sequence with X being a non-conserved amino acid and F an aliphatic amino acid. An example of an RLK which belongs to this class is ZmPK1, the first RLK that was discovered in maize (Walker and Zhang, 1990).

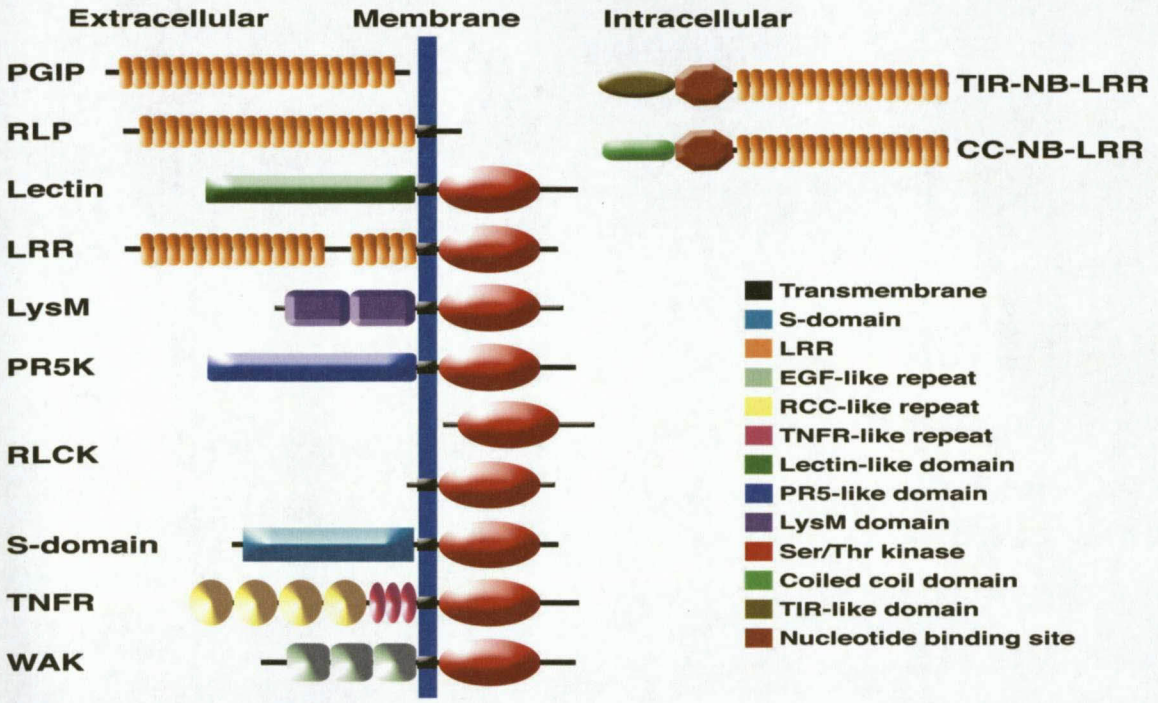

Figure 2.2. Different types of PRRs involved in the perception of PAMPs/MAMPs and DAMPs (Tör *et al.*, 2009). PGIP: Polygalacturonase inhibiting proteins, RLP: Receptor-like protein, LRR: leucine-rich repeats, LysM: Lysine motif, PR5K: PR5-like receptor kinase, RLCK: Receptor-like cytoplasmic receptor kinase, S-domain: Self-incompatibility domain, TNFR: Tumour necrosis factor receptor-like repeat class, WAK: Wall-associated protein kinase, TIR-NB-LRR: Toll interleukin repeat-nucleotide binding-leucine rich repeat, CC-NB-LRR: Coiled-coil-nucleotide binding-leucine rich repeat.

2.1.3.1.1.2. The leucine-rich repeat class

The leucine-rich repeat (LRR) class contains the largest number of RLKs (Torii and Clark, 2000). The LRR motifs are commonly involved in signalling and they also mediate protein-protein interactions (Kobe and Diesenhofer, 1994; Shiu and Bleecker 2001a; 2001b). There are approximately eight different LRR domain organizations in the *Arabidopsis* LRR-RLK family with variations in both the number and arrangement of the LRR motifs (Shiu and Bleecker 2001a; 2001b). LRR-RLKs contain between three (Stracke *et al.*, 2002) and 28 (Takemoto *et al.*, 2000) tandem LRR repeats with a consensus sequence of $LX_2LX_2LX_2LXLX_2NLXGXIPX_2$. Paired cysteines that are six to eight amino acids apart, surround the LRR motif (Torii and Clark, 2000).

RLKs which belong to this group and function as PRRs include the *A. thaliana* FLS2 which binds flg22 (Chinchilla *et al.*, 2006). The exact binding site of FLS2 for flg22 is unknown. What is known is that upon binding, FLS2 forms a heterodimer with BAK1, a related LRR-RLK that positively regulates FLS2 functioning (Chinchilla *et al.*, 2007). EFR is an abundant protein in *Arabidopsis* and the *Brassicaceae* (Kunze *et al.*, 2004) which recognizes the 18 amino acid elf18 motif from the bacterial EF-Tu. FLS2 and EFR are highly homologous as they belong to the same subfamily (Zipfel *et al.*, 2006). CERK1 was shown to be required for chitin perception (Miya *et al.*, 2007; Wan *et al.*, 2008) and binds directly to chitin *in vitro* (Iizasa *et al.*, 2010). CERK1 plays a role in disease resistance to *Pseudomonas syringae* and therefore mediates the perception of an unknown bacterial PAMP (Gimenez-Ibanez *et al.*, 2009).

RLK-PRRs which recognize DAMPs include PEPR1 and Theseus (Huckelhoven, 2007). PEPR1 recognizes AtPEP1, a 23 amino acid peptide released during wounding that triggers weak antifungal activity in plants (Yamaguchi *et al.*, 2006). This peptide is derived from the C-terminus of a 92 amino acid precursor protein AtPROPEP1 (Huffakar *et al.*, 2006). Theseus is a *Cr*RLK1L protein which is involved in cell elongation, damage associated processes and monitoring of the cell wall integrity via the perception of an unknown signal (Hèmaty *et al.*, 2007).

2.1.3.1.1.3. The epidermal growth factor-like repeat class

RLKs belonging to this group include the wall-associated receptor kinases (WAKs) from *Arabidopsis*. These proteins differ from other RLKs by the presence of epidermal growth factor (EGF) motifs in their extracellular domain (Kohorn *et al.*, 1992; He *et al.*, 1999; Shiu and Bleecker, 2001a). Initially, five members of the WAK gene family were identified namely *WAK1-WAK5* (He *et al.*, 1999). These genes are expressed in various tissues and developmental stages with *WAK1* and *WAK2* being the most abundant (He *et al.*, 1999; Larry *et al.*, 2001; Wagner and Kohorn, 2001). The extracellular domain of WAKs physically links the plasma membrane to the cell wall matrix (He *et al.*, 1996; Anderson *et al.*, 2001; Verica and He, 2002) and it is through this link that WAKs have the potential to signal cellular events directly through their cytoplasmic kinase domains.

Initially, WAKs were identified in *Arabidopsis* where they play important roles in cell expansion, heavy-metal stress tolerance and resistance against pathogenic bacteria (He *et al.*, 1998; Larry *et al.*, 2001; Hou *et al.*, 2005). However, recently *WAK* genes were also found in the rice genome and the proteins are known as *Oryza sativa* WAKs (*Os*WAKs). The function of these genes still has to be determined (Zhang *et al.*, 2005).

2.1.3.1.1.4. The tumour necrosis factor receptor-like repeat class

The tumour necrosis factor receptor-like repeat (TNFR) class has a conserved arrangement of six cysteines and seven repeats of approximately 39 amino acids each. The maize CR4 possesses a TNFR motif that is required for normal cell differentiation of the epidermis (Becraft *et al.*, 1996).

2.1.3.1.1.5. The lectin class

The lectin class includes the lectin receptor kinase1 (*LecRK1*) gene from *Arabidopsis* which possesses an extracellular domain homologous to carbohydrate-binding proteins of the legume family (Hervè *et al.*, 1996). The biological function of LecRK1 is not known

but its structural features suggest its involvement in the oligosaccharide-mediated signal transduction. The *Arabidopsis* genome contains more than 30 genes belonging to this class (McCarty and Chory, 2000).

2.1.3.1.2. Receptor-like proteins

A second group of receptors is the RLPs which consists of an extracellular domain and a membrane spanning domain but lacks the intracellular activation domain (Figure 2.2) (Fritz-Laylin *et al.*, 2005). Examples of RLPs which act as PRRs include two fungal proteins namely LeEIX1 and LeEIX2 that are required for ethylene-inducing xylanase perception in tomato (Ron and Avni, 2004). For chitin binding, there is a protein identified in rice known as the CEBiP (Kaku *et al.*, 2006). CEBiP, a plasma membrane glycoprotein, contains two extracellular LysM motifs and a TM domain but lacks any obvious intracellular domains for signal transduction, indicating it may require additional factors for signalling through the plasma membrane into the cytoplasm. CEBiP plays an important role in chitin signalling in rice and specifically binds chitin oligosaccharides at the cell surface (Kaku *et al.*, 2006).

2.1.3.1.3. Polygalacturonase inhibiting proteins

PGIPs are proteins which have an extracellular domain but lack the TM and kinase domains (Figure 2.2) (Di Matteo *et al.*, 2003). As mentioned before, the majority of pathogens have to breach the cell wall in order to have access to the plant cell. These pathogens produce enzymes that degrade the cell wall polymers (De Lorenzo and Ferrari, 2002). Pathogenic fungi produce polygalacturonases (PGs) that cleave the bonds between D-galacturonic acid residues in non methylated homogalacturonan, a major component of pectin (Cervone *et al.*, 1989; Leckie *et al.*, 1999; De Lorenzo and Ferrari, 2002). PGs are produced by bacteria, fungi, nematodes and insects (Girard and Jouanin, 1999; De Lorenzo and Ferrari, 2002; Jaubert *et al.*, 2002) and are amongst the first enzymes secreted by pathogenic fungi. Their activation outside the cell wall is a prerequisite for further wall degradation by other degrading enzymes.

Plants have therefore developed many PGIPs with different and specific recognition capabilities against many of these PGs (De Lorenzo *et al.*, 2001; D'Ovidio *et al.*, 2004). The most common known ligands for PGIPs are polygalacturonases and pectin (Di Matteo *et al.*, 2003). The interaction between PGs and PGIPs limits the destructive potential of PGs and leads to accumulation of oligogalacturonide elicitors. These oligogalacturonides activate plant defence responses such as phytoalexin synthesis, lignin and ethylene production, proteinase inhibitor gene expression, β-1, 3-glucanase and reactive oxygen species (ROS) production (Ridley *et al.*, 2001).

2.1.3.2. Intracellular PRRs

Most intracellular plant PRRs are nucleotide binding-leucine rich repeat (NB-LRR) proteins (Figure 2.2) (Meyer *et al.*, 2003) and most of them are encoded by *R* genes. There numerous plant NB-LRR proteins with approximately 150 in *Arabidopsis* and over 400 in *O.sativa* (Meyer *et al.*, 2003; Monosi *et al.*, 2004). The NB-LRR proteins reside in the cytoplasm but they can translocate into the nucleus, mitochondrion or chloroplast and can associate with WRKY transcriptional factors in the nucleus (Shen *et al.*, 2007).

2.1.4. PAMP and effector-triggered immunity

During the activation of PTI, PAMPs/MAMPs are recognized by PRRs and this inhibits colonization of the plant by the pathogen (Figure 2.3) (Zipfel and Felix, 2005). This inhibition is due to the activation of mitogen-activated protein kinase (MAPK) signalling, induction of pathogenesis-related (*PR*) and defence-related gene expression, production of ROS, callose deposition, increases in ion fluxes across the plasma membrane and cell wall reinforcement (Van Loon *et al.*, 2006).

In order to suppress PTI, pathogens employ effectors which results in ETS (Abramovitch *et al.*, 2006; Grant *et al.*, 2006; Zhou and Chai, 2008; Boller and He, 2009; Cui *et al.*, 2009).

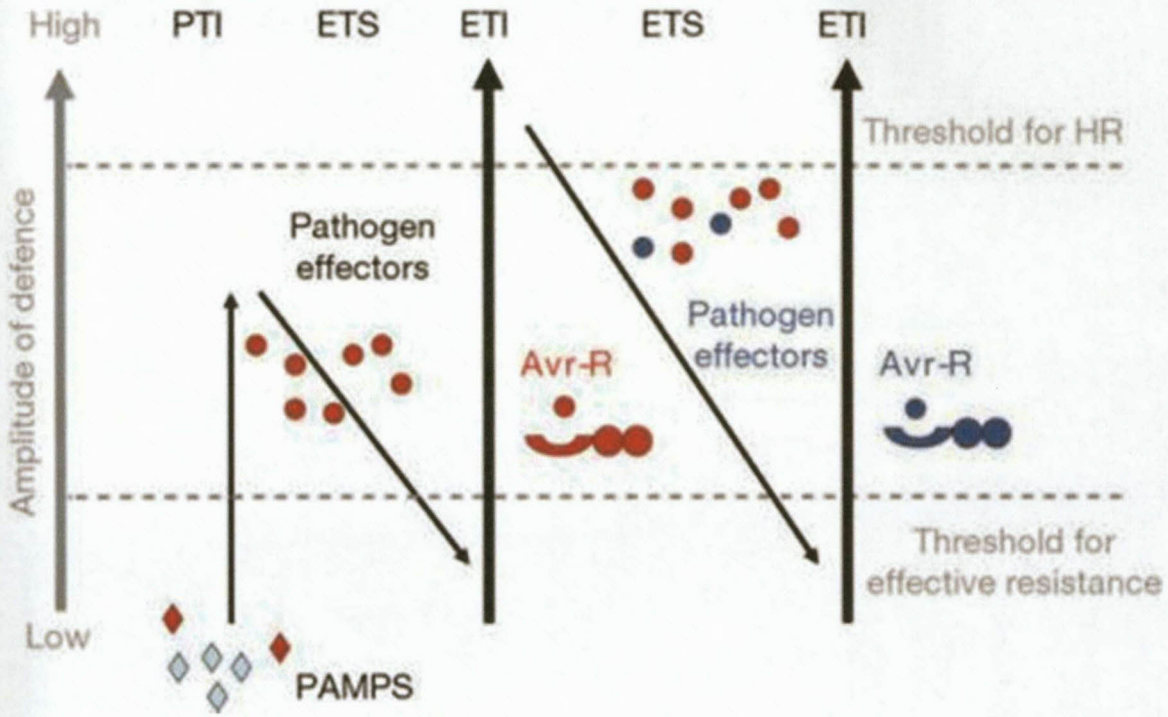

Figure 2.3. The PAMP-triggered and effector-triggered immunity model (Jones and Dangl, 2006). PAMPs: pathogen-associated molecular patterns, PTI: PAMP-triggered immunity, ETS: effector-triggered susceptibility, ETI: effector-triggered immunity, *avr*: avirulence, *R*: resistance and HR: hypersensitive response.

During the evolution of plant-pathogen interactions, plants developed R proteins which recognize pathogenic avr proteins, resulting in ETI (Jones and Dangl, 2006). PTI and ETI have similar signalling networks (Tsuda *et al.*, 2009) and activate an overlapping set of genes (Navarro *et al.*, 2004).

2.2. Host specific resistance

The breakdown of non-host resistance by successful phytopathogens results in the establishment of a host-pathogen interaction (Xiao *et al.*, 2006). This interaction was explained by Flor in his study in the 1940's, where he proposed the gene-for-gene hypothesis to explain his detailed genetic study of both host and pathogen in the interaction between flax (*Linum ultissimum*) and flax rust fungi (*Melamposora lini*) (Flor, 1947). Flor proposed that for resistant plants, individual host *R* genes function to prevent infections by fungal strains that carry the corresponding *avr* gene.

ETI is not expressed in all genotypes within a plant species and this leads to the phenomenon that there are two most likely outcomes from different host-pathogen interactions. Firstly, during the compatible interaction the pathogen is able to suppress host defences and colonize the plants. The second outcome is the incompatible interaction where the pathogen is detected by plants carrying the relevant *R* genes and the plant is resistant (Xiao, 2006). Therefore *R* genes which are genetically defined are polymorphic determinants of host resistance.

The inducible defence response is triggered by the specific recognition of the pathogenic invasion by highly specific receptors. These receptors are encoded by constitutively expressed *R* genes whose encoded proteins are located either on the plasma membrane or in the cytosol (Edreva, 1991; Martin, 1999; McDowell and Dangl, 2000). Several *R* genes with recognition-specificity for known *avr* genes have been isolated from different plant species including monocots and dicots (Chisholm *et al.*, 2006). These R proteins possess domains which share homology with animal proteins involved in innate immunity and /

or apoptosis (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001; Ausubel, 2005).

The first *R* gene, *Hm1* was isolated from maize in 1992 (Johal and Briggs, 1992). This gene confers resistance to race 1 strains of the fungal pathogen *Cochliobolus carbonum* and encodes an NADPH-dependent reductase that inactivates the potent phytotoxin produced by these fungal strains (Johal and Briggs, 1992; Meeley *et al.*, 1992). Most isolated *R* genes seem to activate common defence responses within areas infected by pathogens. Those responses include the transcriptional induction of *PR* and other defence related genes, production of ROS, fortification of the cell wall, synthesis of antimicrobial compounds and HR (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001; Nürnberger *et al.*, 2004). Primary local resistance triggered by these *R* genes can also lead to the activation of the secondary defence response known as systemic acquired resistance (SAR) in uninfected tissues. This is a longer lasting defence response that is effective throughout the plant against a broad range of other pathogens (Durrant and Dong, 2004).

The majority of *R* genes that comply with the gene-for-gene interaction encode proteins that mediate avr protein recognition and are divided into several distinct classes. Only a few *R* genes cannot be placed into one of these classes (Martin *et al.*, 2003; Nimchuk *et al.*, 2003).

Class 1

Proteins which belong to this class are characterized by the lack of an LRR region (Ellis and Jones, 1998; Martin, 1999). The first *avr* gene-specific *R* gene to be isolated, *Pto*, confers resistance against *P. syringae* pv. *tomato* (Martin *et al.*, 1993) and belongs to this class. This protein contains a myristoylation motif which is crucial for *AvrPto* functioning (Loh *et al.*, 1998; Shan *et al.*, 2000) and provides a membrane anchor for this primary hydrophilic protein (Martin *et al.*, 1993). Another example of this class is the

Arabidopsis PBS1 gene which encodes a member of a conserved protein kinase family (Swiderski and Innes, 2001).

Class 2

This is the largest class of *R* genes and codes for NB-LRR proteins containing a nucleotide-binding (NB) and a leucine-rich repeat (LRR) motif (NB-LRR proteins) (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001). These proteins confer resistance to various pathogens and are divided into two sub-groups based on their N-terminal features (Meyer *et al.*, 2003). The first subgroup contains an N-terminal domain which resembles the cytoplasmic signalling domain of the *Drosophila* Toll and human interleukin-1 receptors (TIR) and is known as the TIR-NB-LRR proteins (Whitham *et al.*, 1994; Lawrence *et al.*, 1995). It has been found that approximately 1% of all *A. thaliana* genes are TIR-NB-LRR genes (Meyer *et al.*, 2003).

This sub-group includes the *N* gene, which is a tobacco mosaic virus resistance gene (Hammond-Kosack and Jones, 1997), *L6 which is* a flax rust resistance gene (Hammond-Kosack and Jones, 1997), *M* which is a flax rust resistance gene (Anderson *et al.*, 1997) and *RPP5* which is a downy mildew resistance gene (Parker *et al.*, 1997). One exception is the RRS1 protein from *Arabidopsis* because it has a WRKY domain (Deslandes *et al.*, 2002). This domain is found in a group of transcriptional factors involved in signal transduction of *R* genes (Deslandes *et al.*, 2002). The *RRS1* structural feature implies a direct link between *avr*-recognition and transcriptional activation of defence genes (Deslandes *et al.*, 2003).

The second sub-group of NB-LRR proteins contains a CC domain in most cases and is therefore referred to as the CC-NB-LRRs (Bent *et al.*, 1994; Grant *et al.*, 1995). This group includes RPM1 that confers the ability to recognize *P. syringae* pathogens carrying either the *AvrRpm1* or *AvrB* genes (Stahl *et al.*, 1999) and *RPS2* which confers resistance to *P. syringae2* (Caicedo *et al.*, 1999).

Class 3

This class includes the tomato *Cf* protein which confers resistance to the tomato fungal pathogen *Cladosporium fulvum* (Jones *et al.*, 1994; Hammond-Kosack and Jones, 1997; Sun *et al.*, 2004). The most common example of this class is the *Cf-9* gene, which confers resistance to *C. fulvum9*. It was demonstrated in 2004 that RLPs are also used in some plant species for pathogen recognition. The *Arabidopsis* RPP27 protein confers resistance to *Hyaloperonospora parasitica* (previously referred to as *Perenospora parasitica*) (Tör *et al.*, 2004) that causes downy mildew disease (Slusarenko and Schlarch, 2003). Another example is the apple *HcrVf2* gene which confers resistance against scab caused by *Ventura inequalis* (Belfanti *et al.*, 2004).

Class 4

This class is represented by RLKs including *Xa21*, a gene from rice which encodes an LRR that confers resistance to *X. oryzae oryzae* (Song *et al.*, 1995), a fungus which causes bacterial blight. Another example is *Xa26* which confers resistance to multiple strains of *X. oryzae oryzae* (Sun *et al.*, 2004). The *LRK10* gene which is involved in leaf rust resistance in wheat (Feuillet *et al.*, 1997) also belongs to this group.

Class 5

Proteins belonging to this group are small proteins containing an N-terminal TM and CC domain and include RPW8 which is effective against broad-spectrum powdery mildew (Xiao *et al.*, 2001). RPW8 functionality requires EDS1, the *R* gene-signalling component employed by several NB-LRR R proteins (Aarts *et al.*, 1998; Xiao *et al.*, 2001).

Class 6

This class includes all resistance proteins lacking an extracellular domain thus forming receptor–like cytoplasmic kinases (RLCKs) (Goring and Walker, 2004). This class is

represented by Rpg1, a cytoplasmic protein from barley which is probably anchored to the plasma membrane and contains two tandem protein kinase domains. This protein confers resistance against *Puccinia graminis* f.sp. *tritici* of *Hordeum vulgare* (Brueggeman *et al.*, 2002).

Class 7

Finally, there are two recently cloned rice *R* genes, *Xa27* and *Xa13*, which confers resistance to certain strains of *X. oryzae oryzae*. These genes encode novel proteins which do not show any homology to any other R proteins (Gu *et al.*, 2005; Chu *et al.*, 2006).

2.3. Signal transduction during plant defence

Recognition of an *avr* gene product of the pathogen by a corresponding plant *R* gene product results in the activation of signal transduction events that lead to both basal and specific defence response activation (Dangl and Jones, 2001). This includes the activation of protein kinases, protein phosphatases, phospholipases, guanine triphosphate proteins commonly known as G-proteins, NADPH oxidases, ion channels and production of metabolites such as reactive oxygen intermediates (ROI), nitric oxide (NO), salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Nürnberger and Scheel, 2001).

2.3.1. Calcium ion (Ca^{2+}) changes

Changes in plasma membrane permeability leading to calcium ion (Ca^{2+}) and proton (H^+) influx and K^+ and Cl^- efflux are the earliest defence reactions of plant cells (McDowell and Dangl, 2000). Changes in Ca^{2+} have been reported in response to various signals such as hormones, light, abiotic stress and microbial elicitors (Sanders *et al.*, 1999; Reddy, 2001; Rudd and Frankling-Tong, 2001; White and Broadley, 2003). These changes result in altered protein phosphorylation/dephosphorylation and gene expression patterns (Rudd and Frankling-Tong, 2001; Sanders *et al.*, 2002). Therefore, Ca^{2+} plays an important role

in activating the plant's defence system against attempted microbial invasion (Nürnberger and Scheel, 2001).

There are two main classes of Ca^{2+}-binding proteins in plants namely sensor relays (Luan *et al.*, 2002; Sanders *et al.*, 2002; Reddy and Reddy, 2004) and the Ca^{2+}-dependent protein kinases (CDPKs) (Harmon *et al.*, 2001; Zhang and Lu, 2003; Harper *et al.*, 2004). The sensor relays include calmodulin (CaM) and CaM-related proteins. CaM is a highly conserved and broadly distributed Ca^{2+}-binding protein which connects Ca^{2+} signals to the activation of other cellular components (Zielinski, 1998; Chin and Means, 2000). The CDPKs is the second group of Ca^{2+}- binding proteins (Harmon *et al.*, 2001; Zhang and Lu, 2003; Harper *et al.*, 2004). CDPKs are encoded by 34 different genes in *Arabidopsis* and they constitute one of the largest families of Ca^{2+} sensors in plants (Harmon *et al.*, 2000; Cheng *et al.*, 2002; Hrabak *et al.*, 2003). These proteins are activated in response to various stimuli known to trigger Ca^{2+} concentration changes including osmotic stress, drought, low temperature and pathogen-derived elicitors (Cheng *et al.*, 2002; Lee and Rudd, 2002).

2.3.2. Reactive oxygen species

Ion fluxes induce the production of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl free radical (OH^-) and NO. NO and ROS together act as messengers for HR induction and defence gene expression (Piffanelli *et al.*, 1999) and alone ROS acts as direct toxicants to pathogens, thereby resulting in pathogen death (Baker and Orlandi, 1995).

2.3.3. Plant MAPK cascade in signal transduction

MAPK activation is also one of the earliest responses following ion fluxes. MAPK cascades are key signalling apparatus in eukaryotic cells in response to abiotic and biotic stimuli (Lewis *et al.*, 1998; Madhani and Fink, 1998; Zhang and Klessig, 1998 a; 1998b; Romeis *et al.*, 1999; Schaeffer and Weber, 1999; Widmann *et al.*, 1999; Cardinale *et al.*,

2000; Nühse *et al.*, 2000). MAPK pathways serve as central regulators of growth, death, differentiation, proliferation and stress responses and their activation is dependent on Ca^{2+} influx (Ligterink *et al.*, 1997; Romeis *et al.*, 1999; Yang *et al.*, 2001).

A MAPK cascade typically consists of three protein kinases, a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK, which phosphorylates corresponding downstream substrates. An activated MAPK phosphorylates various proteins such as transcription factors, other protein kinases and metabolic enzymes (Treisman, 1996). Phosphorylation of MAPK substrates occur firstly on a serine or threonine amino acid, and then on a proline residue (Gonzalez *et al.*, 1991).

2.3.3.1. MAPK kinase kinases

MAPKKKs are serine/threonine kinases which phosphorylate MAPKKs. This family consists of an extensive number of genes that often possess a variety of other functional domains in addition to the protein kinase domain (Ichimura *et al.*, 2002). Unlike MAPKs and MAPKKs, this family is classified into three groups, namely A, B and C, in *Arabidopsis*. Group B MAPKKKs have an extended N-terminal domain whereas group A members have a shorter one. The most well known MAPKKKs, namely CTR1, which is a negative regulator of ethylene signal transduction and EDR1, belong to group B. Functions of MAPKKKs which belong to group C are not well understood except for HT1 which was shown to regulate stomatal opening under low CO_2 conditions (Hashimoto *et al.*, 2006). The activation mechanisms of MAPKKKs are diverse, ranging from phosphorylation by protein kinase C to activation via interaction with G proteins (Fanger *et al.*, 1997; Wurgler-Murphy and Saito, 1997).

2.3.3.2. MAPK kinases

MAPKKs activate MAPKs through double phosphorylation of the T-X-Y motif in the activation loop (Zhang *et al.*, 1995). The MAPKK group consists of a relatively small

number of genes as compared to MAPKs and MAPKKKs. There are ten known MAPKK genes in *Arabidopsis*, eight in *O. sativa* and 11 in poplar (MAPK group, 2002).

Plant MAPKKs are likely to be activated by several MAPKKKs; therefore MAPKKs may be used as a phosphorylation relay module between MAPKKK and MAPK regardless of the stimuli. MAPKKs like MAPKs are divided into four groups: A, B, C and D (Ichimura *et al.*, 2002). MAPKKs in group A, C and D have short N and C terminal extensions, whereas group B MAPKKs possess a nuclear transport factor2 (NTF2) domain at the C-terminal region (Steggerda and Paschal, 2002).

2.3.3.3. MAP kinases

MAPKs, also referred to as MPKs, are the last components of MAPK cascades; therefore they are likely to specify downstream components. Due to phosphorylation by MAPKKs, MAPKs change their intracellular localization resulting in their translocation into the nucleus (Lenormand *et al.*, 1993; Ligterink *et al.*, 1997). There are about 20 MAPK genes in *Arabidopsis* (Ichimura *et al.*, 2002), 15 in *O.sativa* (Hamel *et al.*, 2006) and 21 in the poplar genome (Liu and Xue, 2007). They are divided into four groups; A, B, C and D.

MAPKs in groups A, B and C contains a conserved phosphorylation motif TEY which is generally phosphorylated by MAPKKs, while group D contains a TDY motif in the same position. Group D MAPKs differ from the others due to the presence of an extended C-terminal region. Groups A and B contain an evolutionary conserved common docking (CD) domain in their C-terminal extension. This domain serves as a binding site for MAPKKs (Tanoue *et al.*, 2000). Group C has a modified CD domain and group D lacks a CD domain (Ichimura *et al.*, 2002).

Arabidopsis MPK3 and MPK6 together with their tobacco orthologs WIPK and SIPK are the best studied plant MAPKs (Ichimura *et al.*, 2002; Nakagami *et al.*, 2005; Seo *et al.*, 2007). They are involved in multiple plant responses to signals caused by stress,

pathogen determinants, JA, SA, wounding, ROS, ET and environmental cues. Stress responses activate at least three MAPKs, namely MPK3, MPK4 and MPK6 in *Arabidopsis* which results in the altered expression of various stress responsive genes (Nakagami *et al.*, 2005). These MAPKs are also activated by bacterial and fungal PAMPs (Desikan *et al.*, 2004).

2.3.4. Protein phosphatases

Protein phosphatases are negative regulators of MAPK cascades (Schweighofer *et al.*, 2004; Farkas *et al.*, 2007). There are about 122 genes encoding protein phosphatases in the *Arabidopsis* genome and these are classified into five distinct classes: protein phosphatase 2C (PP2C), dual specificity protein phosphatase (DSP, also referred to as MAPK phosphatase (MKP)), serine/threonine protein phosphatase (STPP), protein tyrosine phosphatase (PTP) and low molecular weight (LMW) PTP (Kerk *et al.*, 2002; Schweighofer *et al.*, 2004). PTPs and DSPs are negative regulators of MAPKs and MAPKKs in yeast and animals (Martin *et al.*, 2005; Tamura *et al.*, 2006; Owens and Keyse, 2007).

During the wounding and pathogen responses of *Arabidopsis*, AP2C1, a PPC2-type phosphatase, is a negative regulator of MPK4 and MPK6 by binding to both MAPKs and reducing their kinase activity (Schweighofer *et al.*, 2007). Plants over-expressing AP2C1 produce less ET and JA and are more sensitive to *Botrytis cineria*, a necrotrophic fungus that affects many plant species (Schweighofer *et al.*, 2004; Kerk, 2006). Therefore the phosphorylation and dephosphorylation status of MPK4 and MPK6 that is controlled by AP2C1 are key regulatory mechanisms in JA, wounding and pathogen signalling pathways (Schweighofer *et al.*, 2007). Another phosphatase that appears to be a negative regulator of MPK3, MPK4 and MPK6 is the *Arabidopsis* MKP1 (Ulm *et al.*, 2001; 2002).

2.3.5. Guanine triphosphate binding proteins

Ligands are most likely perceived at membrane level and TM events are the likely routes for signal generation and transduction. The guanine triphosphate (GTP) binding proteins, commonly known as G proteins, form an important part of the signalling cascade. These proteins are so called because they bind GTP (Temple and Jones, 2007). G-proteins couple the activation of a specific class of cell-surface receptors known as G protein coupled receptors (GPCRs) to the intracellular signalling cascades leading to corresponding cellular responses (Assmann, 2002; Temple and Jones, 2007). G proteins are divided into two major subfamilies, namely heterotrimeric G-proteins and small G-proteins.

2.3.5.1. Heterotrimeric G-proteins

Heterotrimeric G-proteins and protein phosphorylation and dephosphorylation are involved in transferring elicitor signals from the receptor to calcium channels that activates downstream reactions such as the oxidative burst and phytoalexin accumulation (Ma, 1994; Bischoff *et al.*, 1999). Heterotrimeric G-proteins consist of alpha, beta and gamma subunits. The alpha subunit has GTPase activity, converts GTP to guanine diphosphate (Hildebrandt, 1997; Knall *et al.*, 1998) and has a receptor-binding region (Gilman, 1987). Activation of heterotrimeric G-proteins involves the exchange of GTP for bound GDP thereby resulting in the dissociation of α subunit and formation of a $\beta\gamma$ dimer.

2.3.5.2. Small G-proteins

Small G-proteins are monomeric proteins with a molecular mass of 20-30 kilodaltons (kDa). Unlike heterotrimeric G proteins, these proteins consist of the alpha subunit only (Knall et al., 1998; Hildebrandt, 1997).

2.4. Hypersensitive response

The activation of cell death which results in the formation of necrotic spots at and around the infection site is a common feature of disease resistance and it is known as the HR (Agrios, 1988; Goodman and Novacky, 1994). HR is an active process that is genetically controlled and requires activated gene transcription and translation (He *et al.*, 1994). HR can be triggered by a variety of pathogens and is a fast response, occurring within a few hours after infection. Following the avr/R protein interaction, the HR is induced and disease resistance follows (Bent, 1996). There is evidence of ROS involvement (Jones, 1994) and the reinforcement of cell walls around the infected sites during the HR. The requirements for cell death may depend on the nature of the plant-pathogen interaction, because pathogens have developed various strategies for growth and reproduction. The impact of the HR may also vary depending on the lifestyle of the pathogen. Two parameters that must be taken into consideration is whether the parasite is intra- or extracellular and whether it is biotrophic or necrotrophic.

2.4.1. Biotrophic and obligate pathogens

Intracellular obligate parasites like viruses require the host cell machinery to replicate and their multiplication can be inhibited by the HR in invaded cells. Biotrophic fungal pathogens require a living host and have developed structures called haustoria which develop inside the host cells to absorb nutrients. Activation of the HR in these invaded cells and its subsequent death would deprive the pathogen of nutrients and thus result in the death of the pathogen (Bushnell, 1981; Chen and Heath, 1991; Bennet *et al.*, 1996; Naton *et al.*, 1996).

2.4.2. Necrotrophic pathogens

Most necrotrophic pathogens utilize the matrix of plant cells as a nutrient source and are thus extracellular pathogens. These pathogens are able to live on dead tissues and they

often trigger nutrient leakage from the host cells. The HR induction is therefore not really a problem for necrotrophic pathogens (Johal *et al.,* 1995).

2.5. Systemic resistance

There are two different types of systemic resistance involved in plant defence, namely SAR and induced systemic resistance (ISR) (Ross, 1966).

2.5.1. Systemic acquired resistance

SAR is activated towards a broad spectrum of micro-organisms in a manner comparable to mammalian immunization. Systemic resistance is induced by pathogens that usually infect leaves or stems of plants (Grant and Lamb, 2006). This resistance is expressed locally at the site of infection, systemically around the infection site and finally throughout the plant.

SAR is dependent on SA signalling (Gaffney *et al.*, 1993) and defence pathways dependent on this hormone is not only activated by microbial pathogens but also by insects (Van Loon *et al.,* 2006). SAR development within a plant takes several hours depending on the interaction between the plant and the pathogen and is accompanied by the production of PR proteins (Van Loon *et al.*, 2006). SAR requires the nonexpressor of PR-1 (NPR1) protein, which functions downstream of SA as a major regulatory factor in SAR expression, PR-protein accumulation and priming (Pieterse and Van Loon, 2004; 2007).

2.5.2. Induced systemic resistance

ISR is a phenomenon where resistance is systemically induced by localized infection or treatment with microbial components. ISR induction is also associated with certain plant growth-promoting rhizobacteria (De Vleesschauwer and Höfte, 2009), but these bacteria do not elicit necrotic responses or cause any visible disease symptoms. ISR is dependent

on JA, ET and NO signalling pathways but their accumulation does not result in systemic expression of *PR* genes (Van Loon *et al.*, 1998). Plants expressing ISR as in the case of SAR, are also primed to express additional defence responses upon any subsequent infection (Conrath *et al.*, 2006) and they require NPR1 as well (Pieterse and Van Loon, 2004; 2007). Combined, these specified defence mechanisms enable the plant to survive multiple stress factors they are exposed to.

CHAPTER 3

MATERIALS AND METHODS:

3.1. Materials

3.1.1. Cultivation and harvesting of plants

3.1.1.1. *Arabidopsis thaliana* and *Nicotiana tabacum*

Arabidopsis thaliana and *Nicotiana tabacum* seeds were first surface-sterilized with 70% (v/v) ethanol for 30 s and then with 10 ml bleach solution [0.047% (w/v) sodium hypochlorite containing one drop of polyoxyethylenesorbitanmonolaurat (Tween 20)] for 20 min with vigorous shaking. Seeds were rinsed six times with sterile distilled water, resuspended in 1 ml sterile distilled water and spread onto the growth medium [0.5x Murashige and Skoog (MS) salt mix, 2% (w/v) sucrose, 0.7% (w/v) phytoagar, 1 mg/ml thiamine and 100 mg/ml inositol, pH 5.8]. Seeds were incubated in the dark at 4°C for three days and then in a growth cabinet at full light intensity (16 h day cycle, 8 h night cycle at 24°C).

3.1.1.2. *Triticum aestivum*

In the case of wheat, seeds of cultivars contain the Lr10 gene, which including Federation, JIC24, Thatcher + *Lr10* and Waldren were soaked in sterile water to remove contaminants and pre-germinated overnight at 25°C in a growth cabinet. Pre-germinated seeds were planted in a pot containing potting soil and peat (1:1). Seeds were grown in the glasshouse (16 h day cycle, 8 h night cycle at 24°C) with regular watering until the leaves of the plants were harvested. When harvested, plant leaves were immediately frozen in liquid nitrogen and stored at -80°C. The tissue was ground to a fine powder in liquid nitrogen, transferred to micro-centrifuge tubes and stored at -80°C until used.

All solutions used for the extraction and analyses of total RNA were treated overnight with 0.1% (v/v) dimethylpyrocarbonate (DMPC) and autoclaved at 121°C for 20 min the next morning. Mortars, pestles and spatulas were first washed in soap, then in 10% (w/v) sodium dodecyl sulphate (SDS), rinsed with DMPC treated water and autoclaved. The

mortars and pestles were finally sprayed with 100% (v/v) ethanol and set alight before use.

3.2. Methods

3.2.1. Bio-informatic analyses

3.2.1.1. Bio-informatic analyses of RLK extracellular domains

The nucleotide sequences of genes encoding different RLKs were downloaded from the Genbank website (www.ncbi.nlm.gov/PubMed). The first selected gene was the *Arabidopsis* Brassinosteroid insensitive (*BRI1*) (AF017056) gene. BRI1 binds brassinosteroids (BRs), a class of growth-promoting regulators that play important roles in plant development (Li and Chory, 1997; Kinoshita *et al.*, 2005; Wang *et al.*, 2005a). BRI1 is involved in plant growth and development and is expressed throughout the plant in response to different light conditions.

Secondly, the chitin elicitor receptor kinase1 (*CERK1*) (NM_113058), a gene from *Arabidopsis*, was selected (Miya *et al.*, 2007). *CERK1* has an extracellular domain with three LysM domains and is also referred to as Lysine motif receptor like protein kinase1 (*LysM RLK1*) (Wan *et al.*, 2008). This gene is induced by fungal pathogens, while the encoded protein binds to chitin and plays an important role in fungal PAMP perception and chitin elicitor signalling (Miya *et al.*, 2007).

The third gene was the chitinase-related receptor-like kinase1 (*CHRK1*) (AF088885) from *N. tabacum* whose encoded protein product binds chitin oligosaccharides (Kim *et al.*, 2000). CHRK1 activation induces defence responses like the oxidative burst, phosphorylation of specific proteins, biosynthesis of phytoalexin and transcriptional activation of defence genes (Benhamou, 1996). *CHRK1* gene expression is stimulated by fungal pathogens and tobacco mosaic virus (TMV) infection (Kim *et al.*, 2000).

The elicitor-induced LRR receptor-like gene (*EILP*) (AB029327) from *N. tabacum* is an exception amongst the other genes because it encodes an RLP instead of an RLK (Takemoto *et al.*, 2000). EILP is induced by *P. syringae* pv. *glycinea* and binds fungal elicitors. This gene is involved in both pre-existing and inducible disease resistance.

The *Arabidopsis* Flagellin sensing2 (*FLS2*) gene (AF088885) (Gomez-Gomez and Boller, 2000), which after binding flg22 activates the defence response (Chinchilla *et al.*, 2006), was also selected. The main building block of the bacterial flagellum is flg22 and it acts as a PAMP that triggers innate immunity in plants. Perception of flg22 by FLS2 induces the oxidative burst, callose deposition and the expression of *PR-1* and *PR-5*.

The next gene, lectin receptor kinase1 (*LecRK1*) (AF001168) from *Arabidopsis*, binds CaM in a Ca^{2+} dependent manner. *LecRK1* is induced by ET, SA, mechanical wounding and cold treatment (Hanks and Quinn, 1991).

The proline-rich extensin-like receptor kinase1 (*PERK1*) (AY028699) from *Brassica napus* binds extensin (Silva and Goring, 2002). The gene is induced by wounding and pathogen attack and plays an important role in mediating the early events of the plant defence response following mechanical injury.

Finally, the wall-associated protein kinase1 (*WAK1*) (NM_101978) from *Arabidopsis* which is tightly bound to the cell wall (He *et al.*, 1996) and covalently bound to pectin, was chosen (Maleck *et al.*, 2000). *WAK1* is up-regulated during SAR (Maleck *et al.*, 2000) and is induced by *P. syringae* infection, the exogenous application of SA and its analogue 2,6-Dichloroisonicotinic acid (INA) (He *et al.*, 1998), methyl jasmonate (MeJA), ET (Schenk *et al.*, 2000) and aluminium treatment (Sivaguru *et al.*, 2003). The *WAK1* gene is required for cell elongation and vacuolar invertase activity (Kohorn *et al.*, 2006 a; 2006b), communication between the cell wall and cytoplasmic events and is also involved in the response to pathogens (He *et al.*, 1998).

The portion of each gene coding for the extracellular domain was searched for a BamHI palindrome using the Webcutter tool (www.users.tools/cut2.html). When absent, an artificial BamHI palindrome was created as part of the reverse primer using PCR mutagenesis. Where an internal BamHI palindrome was present, a search for an EcoRV palindrome was similarly done. Two primers were finally designed for each gene fragment using Webprimer (www.yeastgenomic.org/cgi-bin/web-primer) where the two palindromes were incorporated in the respective reverse primers (Table 3.1). The forward primers were designed to include the start codon of each gene. Gene sequences, including the changed nucleotide sequences of the reverse primers, were translated (www. expasy.org/tools/dna/html) to determine its effect on the amino acid sequence.

3.2.1.2. Bio-informatic analysis of the intracellular protein kinase domain of *LRK10*

The nucleotide sequence of the *T. aestivum* leaf rust protein kinase10 (*LRK10*) gene (U51330) (Feuillet *et al.*, 1997) was downloaded from Genbank (www.ncbi.nlm.gov/PubMed). The portion of the gene encoding the TM and intracellular protein kinase domains was identified and screened for either BamHI or EcoRV palindromes using Webcutter (www.users.tools/cut2.html). Two gene specific primers were designed to amplify this gene fragment including a 15 bp segment upstream of the TM coding region (Table 3.1). The forward primer contained either a BamHI or EcoRV palindrome.

3.2.1.3. Bio-informatic analyses of the recombinant genes

Putative recombinant genes were constructed *in silico* by linking the respective extracellular domain encoding DNA fragments with the LRK10 encoding gene sequence using either of the two palindromes. The recombinant genes were then translated using a Web-based translation tool (www.expasy.org/tool/dna.html) to ensure that the ligated recombinant DNA fragments will produce a full length active polypeptide. Once confirmed, primers were ordered from Integrated DNA Technologies (IDT).

3.2.2. Construction of recombinant genes

3.2.2.1. Genomic DNA extraction

Genomic DNA was extracted from 100 mg frozen plant leaves using the GenElute plant genomic DNA miniprep kit (Sigma) according to manufacturer's specifications. The DNA was finally dissolved in 200 µl DMPC treated water. DNA concentration was determined using spectrophotometry and the concentration expressed as ng/µl (Sambrook *et al.*, 1989). To evaluate the quality and quantity, 100 ng DNA was separated on a 1% (w/v) agarose gel containing 0.05 µg/ml Goldview (Beijing SBS Genetech). The gel was prepared in 0.5x TAE [20 mM Tris-hydroxymethylamino-methane-HCl (Tris-HCl) pH 8.0, 0.5 mM ethylenedinitrilotetraacetic acid (EDTA), 0.28% (v/v) acetic acid]. The DNA was mixed with DNA loading buffer (0.25% (w/v) Orange G, 0.375 M ficoll), loaded on the gel and separated for 30 min at 10 V/cm. The gel was photographed under UV illumination using the Quantity-one 4.6 software on the BioRad gel documentation system.

3.2.2.2. Total RNA extraction

Total RNA was extracted from 100 mg frozen and ground plant leaves using Trizol reagent (Invitrogen) according to manufacturer's specifications. The plant material was resuspended in 500 µl Trizol reagent, tubes vortexed for 30 s and incubated at room temperature for 10 min. To precipitate the DNA and proteins, 100 µl chloroform was added and the tubes were inverted several times to mix. The tubes were incubated at room temperature for 5 min and then centrifuged at 12000 *g* for 15 min at 4°C.

Table 3.1. Nucleotide sequences of primers used in the study. The incorporated palindromes are indicated in blue and the start codon in red. N- Any nucleotide and V- pyrimidine.

Name of gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Fragment size (bp)	Annealing temperature (°C)
BRI1	Bovis 291 CACAAACTCTTGAGAAATGAAGACTTT	Bovis 292 AAGGGATCCTGGTCTCCTT	2392	55.1
BRI1 internal	Bovis 380 GCTGAGATCAATCGAAGTAACTTTG	Bovis 381 GCTCGATGGGAGGATTCCT		
CERK1	Bovis 387 ATGAAGCTAAAGATTTCTCTAATCG	Bovis 388 CCATTTGGATCCCTTCCA	690	51.0
CHRK1	Bovis 339 ATGTCTTCCAAAAACCTCTTCTCC	Bovis 340 CCTTCGGTTGGATCCGCTT	2166	51.0
EILP	Bovis 341 ATGATGATGGTTGGCAAATTA	Bovis 342 CTCGGATCCCCTATCATTGC	2388	51.6
FLS2	Bovis 350 ATGAAGTTACTCTCAAAGACCTTTTTG	Bovis 346 AATAATCAGGATGACTCTGGATCCC	2433	51.9
LecRK1	Bovis 347 ATGTCTCGTGAACTTATTATTCTCTGCC	Bovis 348 AGTTTTGGATCCATTAGATGTTTTC	840	51.0
*LRK*10 (BamH1)	Bovis 378 ATTTGCAGGATCCCATATCAAAG	Bovis 294 TAGTCCGCGCGACTCTACAGAAT	1249	51.7

LRK10 (EcoRV)	Bovis 401 CCTCATGGATCCGATATCAAAG	Bovis 402 CAGAATTCAGGACATCAGCTCGTT	1249	58.5
PERK1	Bovis 349 AAGAAGCAGACATGTCCTCGG	Bovis 345 TCCTGTGGATCCACCGTCGGA	422	54.3
WAK1	Bovis 343 ATGAAGGTGCAGGAGGGTTT	Bovis 344 TACAAGGATATCTGTAGTCCATGC	1020	51.9
Oligo-dT	Bovis 27 TTTTTTTTTTTTTTTTNV			37
pUC/M13 forward	GTAAAACGACGGCCAGT			

The supernatant was transferred to a sterile micro-centrifuge tube and 250 µl isopropanol added to precipitate the RNA. The tube was vortexed, incubated at room temperature for 10 min and then centrifuged for 10 min at 12000 *g* at 4°C to pellet the RNA. After washing the pellet with 500 µl 70% (v/v) ethanol, the air-dried pellet was resuspended in 200 µl DMPC treated water. The concentration and quality of the RNA was determined as described (3.2.2.1).

3.2.2.3. Complementary DNA (cDNA) synthesis

cDNA was synthesized from 100 ng total RNA using Im-PromII reagents (Promega). The RNA was denatured in the presence of 10 pmol oligo-dT primer (Table 3.1) at 70°C for 5 min. After a 2 min incubation on ice, cDNA was synthesized using 20 U Im-Prom II reverse transcriptase in the presence of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates (dNTP's). Synthesis was carried out at 25°C for 5 min, 37°C for 60 min with a final denaturation step for 15 min at 70°C.

3.2.2.4. PCR amplification of selected gene fragments

Synthesized cDNA and extracted genomic DNA were used as templates for amplification of the different gene fragments. To determine the optimal annealing temperature for each primer pair, a gradient PCR was performed. Six reactions containing 20 ng template DNA or cDNA, 1x KapaReady mix (Kapabiosystems) and 10 pmol of each primer were run at different annealing temperatures in a gradient thermal PCR block. Amplification started with one cycle of denaturation at 94°C for 30 s, followed by 30 cycles of a 94°C denaturation step for 30 s, the gradient step ranging from 45°C to 60°C for 30 s and an elongation step at 72°C for 30 s. For longer gene fragments (>2000 bp), the elongation time was increased to 2 min 30 s. The final elongation step was at 72°C for 5 min. The PCR products were separated on a 1% (w/v) agarose gel and photographed (3.2.2.1). The optimum annealing temperature was chosen based on which temperature gave the best amplification without any secondary amplification products being formed.

The respective DNA fragments were then amplified using the determined optimum annealing temperature (Table 3.1). The different gene fragments were amplified using 20 ng cDNA or

genomic DNA, 10 pmol of each primer, 1.75 mM $MgCl_2$ except for *LRK10* where 2.5 mM was used, 0.3 mM dNTP's, 1x Kapa LongRange Buffer (without Mg^{2+}) and 1.25 units of Kapa LongRange Taq polymerase. The amplification regime was as follows: a one cycle denaturation at 94°C for 2 min, 30 cycles for all the gene fragments except for *LRK10* which used 40 cycles of denaturation at 94°C for 30 s, the specific annealing temperature for 30 s (Table 3.1), elongation at 72°C for 30 s and 2 min 30 s for longer gene fragments, with a final elongation step at 72°C for 5 min.

3.2.2.5. Cloning of amplified DNA fragments

The PCR products were first purified using the FavorPrep Gel/PCR purification kit (FAVORGEN) according to manufacturer's recommendations. A 10 µl ligation reaction was prepared for each amplified fragment by mixing 50 ng pGemT-Easy vector (Promega), 1 unit T_4 DNA ligase, 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM adenosine triphosphate (ATP), 1% (v/v) polyethylene glycol (PEG) and 1-2 µl of the PCR products to a final volume of 10 µl. After mixing, the reactions were incubated overnight at 4°C.

3.2.2.6. Transformation of competent *Escherichia coli* cells

3.2.2.6.1. Preparation of competent cells

Luria-Bertani (LB) medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl pH 7.0] 5 ml was inoculated with *Escherichia coli* DH5α cells from the freezer and the cells grown overnight at 37°C. This culture was used to inoculate 25 ml SOB medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.05% (w/v) NaCl pH 7.0] (Inoue *et al.*, 1990) which was incubated at 37°C for 8 h. The 25 ml culture was used to inoculate three 250 ml SOB cultures with 10, 4 and 2 ml respectively which were then incubated at 18-22°C overnight. When an optimal density of 0.55 at 600 nm (OD_{600}) was reached, cells were placed on ice for 10 min and then harvested at 2500 *g* for 10 min at 4°C. The pellet was gently resuspended in 80 ml ice-cold transformation buffer [55 mM $MnCl_2$,15 mM $CaCl_2$, 250 mM KCl, 0.5 M piperazine-1,2-bis(2-ethanesulfonic acid) (PIPES) pH 6.7] (Inoue *et al.*, 1990). After centrifugation, the pellet was resuspended in 20 ml transformation buffer, 1.5 ml dimethyl sulfoxide (DMSO) added and cells placed on ice for 10 min. The cells were finally aliquot into tubes, flash frozen in liquid nitrogen and stored at -80°C.

3.2.2.6.2. Transformation of chemical competent *E. coli* cells

Chemically competent *E. coli* DH5α cells (100 µl) were mixed with 2.5 µl of the ligation reaction and the tubes incubated for 30 min on ice. The cells were heat shocked at 42°C for 90 s, cooled on ice for 5 min where after 900 µl SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl pH 7.0, 10 mM $MgCl_2$, 20 mM glucose] was added. The tubes were incubated at 37°C for 45 min with vigorous shaking. The cells were finally plated on LB plates [LB with 7% (w/v) bacterial agar] containing 50 µg/ml ampicillin, 2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 2% (w/v) isopropyl-β-D-thiogalactopyranoside (IPTG) to allow for blue/white screening. The plates were incubated upside down overnight at 37°C (Sambrook *et al.*, 1989).

White colonies were inoculated in 5 ml LB medium containing 50 µg/ml ampicillin and incubated on a shaker overnight at 37°C. Bacterial cells were harvested from 1.5 ml of the culture by centrifugation at 12000 *g* for 2 min at 4°C. The pellet was resuspended in 100 µl DEPC treated water, boiled for 5 min in a water bath and then centrifuged at 12000 *g* for 10 min. To confirm the presence of the cloned fragments, 1 µl of the supernatant was used as template in a PCR reaction as described (3.2.2.4). The amplified products were separated on a 1% (w/v) agarose gel (3.2.2.1). Once the presence of the insert was confirmed, recombinant plasmid DNA was extracted from liquid LB cultures using the Gene JET plasmid miniprep kit according to manufacturer's specifications (Fermentas).

3.2.2.7. DNA sequencing

The cloned DNA fragments for each recombinant plasmid were amplified in a 25 µl PCR reaction (3.2.2.4). The ABI PRISM ® BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used for sequencing. Each sequencing reaction contained either 10-50 ng PCR product or 200-500 ng plasmid DNA in a 10 µl reaction containing 1 µl Reaction ready mix, 2 µl Big-dye sequencing buffer and 3.2 pmol sequencing primer. The sequencing regime was as follows: one denaturation cycle at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and elongation at 60°C for 4 min. Sequencing was done using both the original forward and reverse primers and where necessary, specially designed internal sequencing primers (Table 3.1).

To purify the sequenced DNA fragments, the 10 µl sequencing reaction was mixed with 10 µl water, 5 µl 125 mM EDTA and 60 µl 100% (v/v) ethanol. The tubes were vortexed and incubated at room temperature for 15 min. After centrifugation at 12000 *g* for 15 min at 4°C, the pellet was washed with 60 µl 70% (v/v) ethanol, re-centrifuged and the pellet dried overnight in the dark. The sequenced DNA fragments were finally separated on an Applied Biosystems 3130xl Genetic Analyzer at the Department of Microbiological, Biochemical and Food Biotechnology at the University of the Free State.

For the visual confirmation of the DNA sequences, Chromas lite (version 2.01) (www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml) was used. When the reverse primer was used, the nucleotide sequence was first reverse complemented (www.bioinformatics.org/sms/rev_comp.html) before alignment. Both the forward and reverse complimented reverse sequences were then aligned with the original gene sequence to confirm the sequence of the cloned fragments using pair wise alignment (www.ebi.ac.uk/Tools/emboss/align/index.html). Any nucleotide differences between the original DNA fragment and the sequenced fragments were resolved using Chromas lite where possible.

3.2.2.8. Construction of recombinant genes

A total of 200 ng recombinant plasmid DNA for each gene fragment (*BRI1*, *CERK1*, *LecRK1*, *PERK1* and *LRK10*) was digested with 1 U BamHI in the presence of 10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.02% (v/v) polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100), 0.1 mg/ml Bovine serum albumin (BSA) at 37°C for 2 h. Similarly, 200 ng recombinant plasmid DNA containing the *WAK1* and *LRK10* gene fragments was digested with 1 U EcoRV at 37°C for 10 min according to manufacturer's instructions (Fermentas). To confirm digestion, 100 ng digested plasmid DNA was separated on an agarose gel (3.2.2.1).

Ligation reactions containing 20 ng of the respective digested plasmids (Figure 4.3) and 330 mM Tris-acetate pH 7.8, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT, 10 mM ATP and 1 U Fast-link DNA ligase were prepared and incubated at room temperature for 15 min. Before the recombinant genes were amplified, a gradient PCR using the ligation mix as template was performed (3.2.2.4) using the respective extracellular

domain specific forward primer and the *LRK10* kinase domain specific reverse primer (Table 3.1). The respective recombinant genes were then amplified using the KAPA LongRange Taq polymerase at the optimal annealing temperature (3.2.2.4). The amplicons were finally cloned as described (3.2.2.5) and plasmid DNA was extracted (3.2.2.6.2).

3.2.2.9. *In vitro* transcription and translation of selected recombinant genes

In vitro transcription and translation was done using the TNT® Quick Coupled Transcription/Translation System (Promega). The *LecRK1-LRK10* recombinant gene was amplified from recombinant plasmid DNA using the *LRK10* gene specific reverse primer in combination with the pUC / M13 Universal forward primer (Table 3.1) that anneals to pGemT-Easy (3.2.2.4). Approximately 100 ng of the PCR product was mixed with the T7 Quick master mix, T7 PCR enhancer and 10 μCi [^{35}S] methionine according to the manufacturer's instructions and incubated at 30°C for 90 min. Equal volumes of Laemmli buffer [0.5 M Tris-HCl pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue] were added and the samples denatured at 95°C for 5 min. The resulting polypeptides were separated on a 12% (w/v) SDS-PAGE gel at 230 V for 1 h using 0.1 M Tris-HCl pH 8.3, 0.15 M glycine, 0.1% (w/v) SDS as running buffer (Laemmli, 1970). The gel was fixed in 10% (v/v) acetic acid for 20 min, rinsed with tap water and shaken in Amplify (BioRad) for 15 min. After drying the gel at 70°C for 2 h, it was exposed overnight to an X-ray film which was then developed.

CHAPTER 4

RESULTS:

4.1. Bio-informatic analyses of genes

In order to simplify the results and prevent repetition, the bio-informatics analyses of only the *PERK1-LRK10* gene combination will be described as an example in this section. Since the same applies to the other four gene combinations, these results are given in Appendices 1 to 4.

4.1.1. Bio-informatic analysis of the extracellular domain of *PERK1*

The nucleotide and amino acid sequences of the *Brassica napus PERK1* gene were obtained from Genbank (Figure 4.1). The start and stop codons, as well as the different protein domains (signal peptide, extracellular, TM and kinase domains) of the gene and its encoded polypeptide were identified and indicated.

The gene was searched for a possible palindrome that could be incorporated through PCR mutagenesis into the nucleotide sequence close to the end of the extracellular domain encoding portion of the gene. An artificial BamHI site (5'-GGATCC-3') was created by changing the nucleotide sequence from 5'-acggttatcaacagga-3' to 5'-acggtggatccacagga-3'. The resulting gene sequence was translated to determine whether a change in amino acid sequence would occur. A change from a hydrophobic leucine (L) to a polar hydrophilic glycine (G) was found. Two gene specific primers were finally designed to anneal on the ends of the gene fragment (Table 3.1) with the reverse primer having the incorporated BamHI palindrome. The two primers would amplify a 422 base pairs (bp) gene fragment.

The other four gene sequences were similarly analysed. The results are shown in Appendix 1 to 4. In the case of *WAK1* where an internal BamHI was present within the gene fragment, an EcoRV palindrome was created in the same way (Appendix 4).

Figure 4.1. Nucleotide (a) and encoded amino acid (b) sequences of the *PERK1* gene from *Brassica napus*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon.

4.1.2. Bio-informatic analysis of the kinase domain of *LRK10*

The nucleotide and amino acid sequences of the *T. aestivum LRK10* gene were obtained from Genbank. The start and stop codons, as well as the different domains were identified and indicated (Figure 4.2). The portion of the gene encoding the TM and intracellular protein kinase domain was screened for either BamHI or EcoRV palindromes. When absent, an artificial palindrome was created just upstream of the portion of the gene encoding the TM domain. The changed nucleotide sequences were translated to determine whether amino acid changes were caused. The original sequence changed from 5'-gcaggttcacatatcaaagt-3' to 5'-gcaggatcccatatcaaagt-3'. The palindrome incorporation did not change the amino acid sequence of LRK10. Two gene specific primers were thus designed to amplify this gene fragment with the forward primer incorporating the BamHI palindrome and the reverse primer included several nucleotides after the stop codon. The gene fragment amplified by these primers is 1249 bp in length. The same was done for the *LRK10* gene fragment containing the EcoRV palindrome (Appendix 5).

4.1.3. Bio-informatic analysis of the recombinant gene

The portion of the gene encoding the *PERK1* extracellular domain was linked *in silico* to the *LRK10* gene fragment encoding the TM and kinase domains through the BamHI palindrome (Figure 4.3). This figure illustrates how the gene fragments will be amplified (a), digested (b) and ligated to each other (c). The recombinant gene will therefore code for the start codon, signal peptide and extracellular domain of *PERK1* and the TM and kinase domains and stop codon of *LRK10*. The complete recombinant gene was translated (Figure 4.4) to ensure that the ligated recombinant DNA fragments will produce a full length active polypeptide. The recombinant protein is predicted to have a molecular weight (MW) of 53.8 kDa. The other recombinant genes are presented in Appendixes 6 to 9.

Figure 4.2. Nucleotide (a) and amino acid (b) sequences of the *LRK10* gene from *Triticum aestivum*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon.

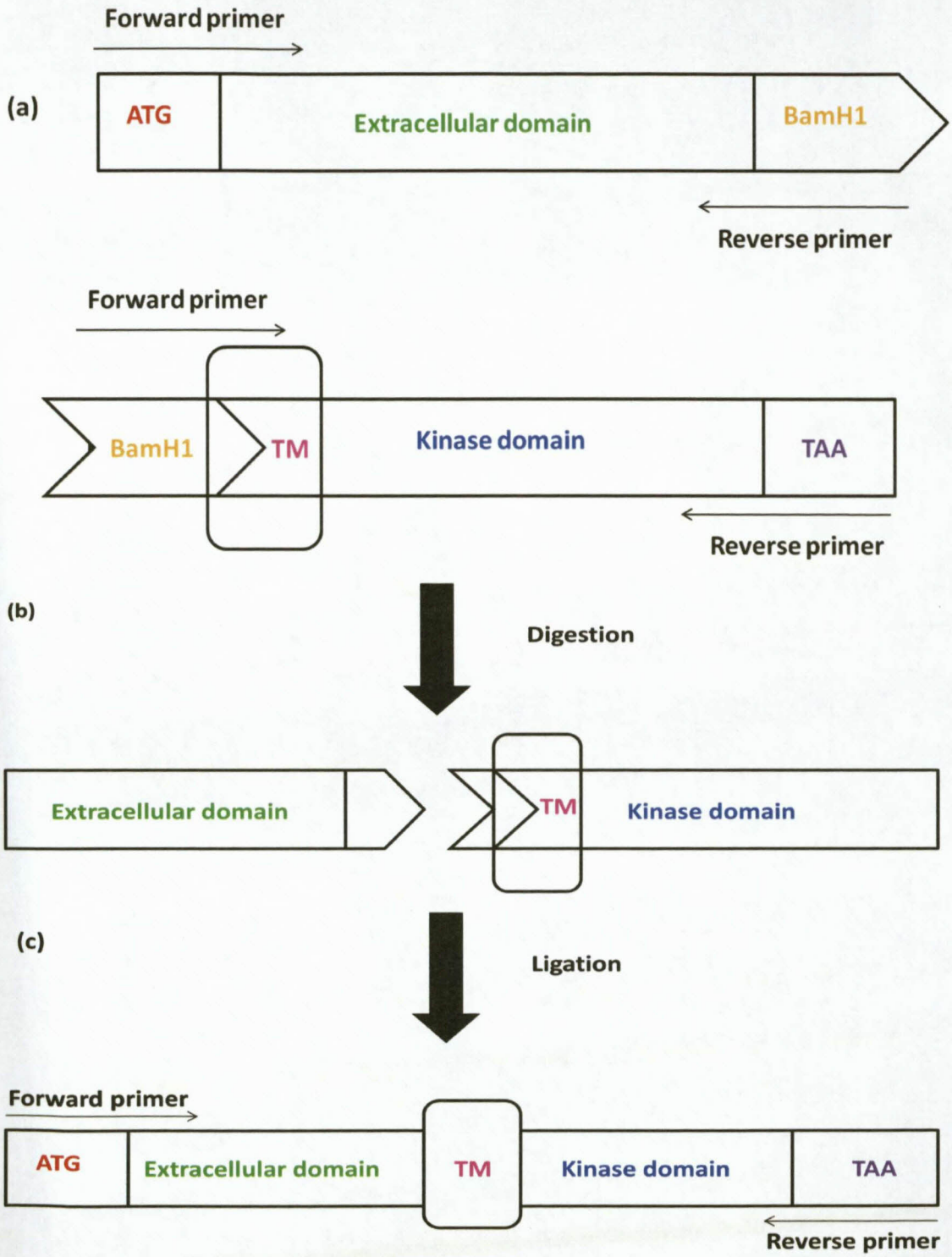

Figure 4.3. Construction of the different recombinant genes. The gene fragments were PCR amplified (a), digested (b) and ligated (c) together to form the recombinant gene. ATG: start codon, TM- TM domain, TAA: stop codon.

(a)

PERK1 forward primer

5′-AAGAAGCAGA CATGTCCTCG G-3′

5′-aagaagcaga catgtcctcg gcgccgtctc cgggg

actggttcgc ctccatctcc accatcaaac tccacaacca ccactcctcc tccagcttcc gctcctcctc ccaccacacc

ttcttctcct ccgccgccat ccactattcc gacatctcct cctccttctt ctcgctctac accttctgct cctcctccat

ctccaccaac tccatctacg ccgggatctc cacctcctct tcctcagccg tctccacccg ctccaactac gcccggatct

ccacccgcac ctgttactcc tcctactcga aaccctccac cttcagtccc aggaccaccg tccaatcctt cacgcgaagg

aggatctcct cgacctccat cttctccctc gccgccgtct ccttcttccg acggtgg atccatatc aaagtcattg

cagctacatc atcagtggcc gcgtttgttg ctcttttgtt gacggtggcc actgtgcttt atctttcact caagacaaga

tataatgcgg agatacatat gaaggttgaa atgtttctca agacatatgg aacatcaaaa ccgacaaggt acactttctc

tgaagttaag aagatggcaa gacggtttaa ggaaaaagta gggcagggag gatttggaag tgtgtacaaa ggtgagctac

caaatggagt gcctgtggca gtcaagatgc tagagaactc tacaggagag ggagaatcgt tcatcaatga agttgcaacc

atcggactaa tccaccatgc caatattgtc cgcctcctgg gattttgttc cgaaggaatg aggcgggctc ttatttatga

attcatgcct aatgagtcac tggagaaata catattctct gacgactcta atatttttca gaatcttcta gtaccagaga

agctgctaga tattgcttta ggcatcgcgc gaggaatgga gtacttgcat caagggtgca accagcgcat cctccacttt

gacatcaagc ctcacaatat cctgcttgac tacaacttca atccaaagat ctcagacttt gggcttgcaa agctgtgcgc

gagggaccaa agcatcgtca ccttaactgc agcaagaggc acgatgggct acattgcacc ggagctatat tcccggaact

ttgggggagt atcgtacaag gcagacgtgt acagtttcgg catgctggtg ctagaaatgg tgagcgggag gaggaattca

gacccaagaa tcgggagcca ggacgatgtt tacctcccag agtggatcta cgagaaagtg atcaatgggg aggagttggc

gcttactttg gaaacgactc aggaagagaa agacaaggtg aggcagctgg caatggttgc actgtggtgt atccagtgga

acccgagaaa ccgtccgtcg atgacgaagg ttgttaacat gctaacaggg aggttgcaga gtctgcagat gcccccgaag

cccttcgtct catctgaaaa tgaacttatg tcataaattc tgtagagtcg cgcggacta-3′

5′-TAAG ACATCTCAGC GCGCCTGAT-3′

LRK10 reverse primer

(b)

M S S A P S P G T G S P P S P P S N S T T T T P P P A S A P P P T T P S S P P P P S T I

P T S P P P S S R S T P S A P P P S P P T P S T P G S P P P L P Q P S P P A P T T P G S

P P A P V T P P T R N P P P S V P G P P S N P S R E G G S P R P P S S P S P P S P S S D

G G S H I K V I A A T S S V A A F V A L L L T V A T V L Y L S L K T R Y N A E I H M K

V E M F L K T Y G T S K P T R Y T F S E V K K M A R R F K E K V G Q G G F G S V Y K G E

L P N G V P V A V K M L E N S T G E G E S F I N E V A T I G L I H H A N I V R L L G F C

S E G M R R A L I Y E F M P N E S L E K Y I F S D D S N I F Q N L L V P E K L L D I A L

G I A R G M E Y L H Q G C N Q R I L H F D I K P H N I L L D Y N F N P K I S D F G L A K

L C A R D Q S I V T L T A A R G T M G Y I A P E L Y S R N F G G V S Y K A D V Y S F G M

L V L E M V S G R R N S D P R I G S Q D D V Y L P E W I Y E K V I N G E E L A L T L E T

T Q E E K D K V R Q L A M V A L W C I Q W N P R N R P S M T K V V N M L T G R L Q S L Q

M P P K P F V S S E N E L M S Stop I L Stop

Figure 4.4. Nucleotide (a) and amino acid (b) sequences of the *PERK1-LRK10* recombinant gene with the different regions indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon.

4.2. Construction of recombinant receptor genes

4.2.1. Cloning of the extracellular domain encoding gene fragments

4.2.1.1. Genomic DNA and total RNA isolation

Genomic DNA was extracted from *Arabidopsis thaliana* and *Brassica oleracea* plants due to the unavailability of *Brassica napus* (Figure 4.5). The extracted DNA was separated on a 1% (w/v) agarose gel to determine its quality and quantity. It was clear that the DNA was intact with no degradation in the form of a smear visible. In addition, no contaminating RNA was evident, indicating that the DNA was of good quality that could be used further.

Similarly, total RNA was separated on an agarose gel (Figure 4.6). Clearly visible on the gel were the 28S and 18S rRNA fragments and the background smear that represented the mRNA. Since the rRNA bands were intact, it indicated that the RNA was of good quality with no contaminating genomic DNA evident.

4.2.1.2. PCR amplification of the different gene fragments

Before the individual fragments were amplified, the optimal annealing temperature for each primer set was determined (Figure 4.7). The gradient PCR was done using either cDNA synthesized from total RNA or genomic DNA as template (3.2.2.4). The optimal annealing temperature was where an intense amplicon was evident with no obvious secondary products. The optimal annealing temperatures indicated in red (Figure 4.7) were then used for all subsequent PCR reactions. The initial gradient PCR reactions for *CHRK1*, *EILP* and *FLS2* were unsuccessful. Despite increasing the $MgCl_2$ concentration, number of PCR cycles or amount of template DNA, the gene fragments could not be amplified and they were left out of the study.

Using these optimal annealing temperatures, the five remaining gene fragments were successfully amplified (Figure 4.8). All produced a single amplicon with the correct size which was then further used.

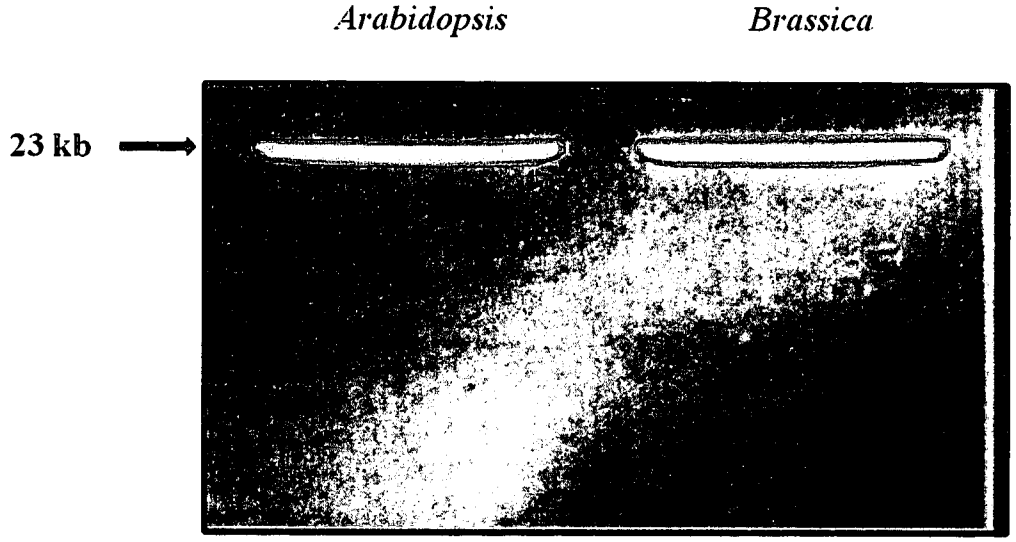

Figure 4.5. Genomic DNA extracted from *Arabidopsis thaliana* and *Brassica oleracea*. The size of the DNA fragments is indicated in kilobase pairs (kb).

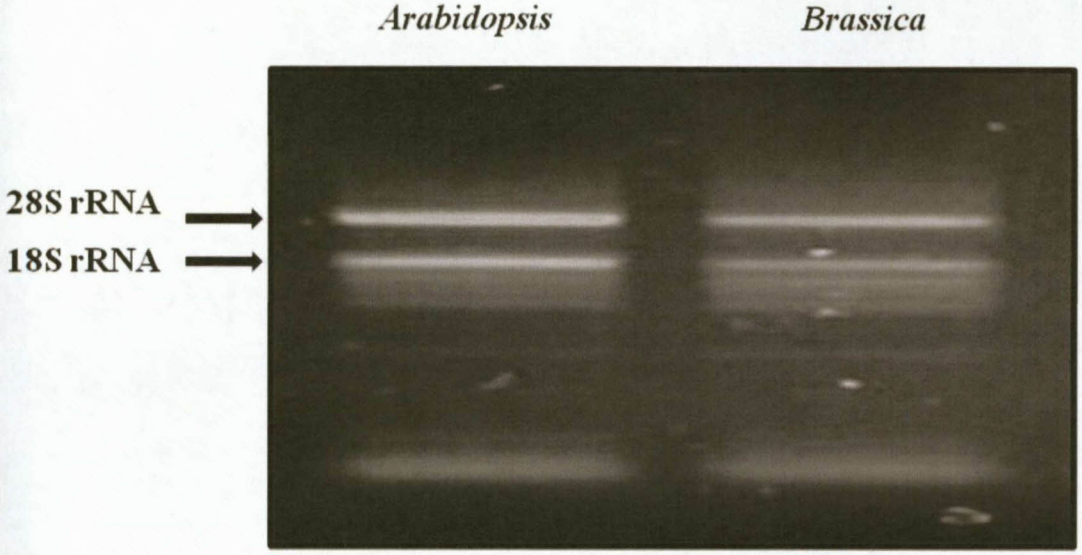

Figure 4.6. Total RNA extracted from *Arabidopsis thaliana* and *Brassica oleracea*.

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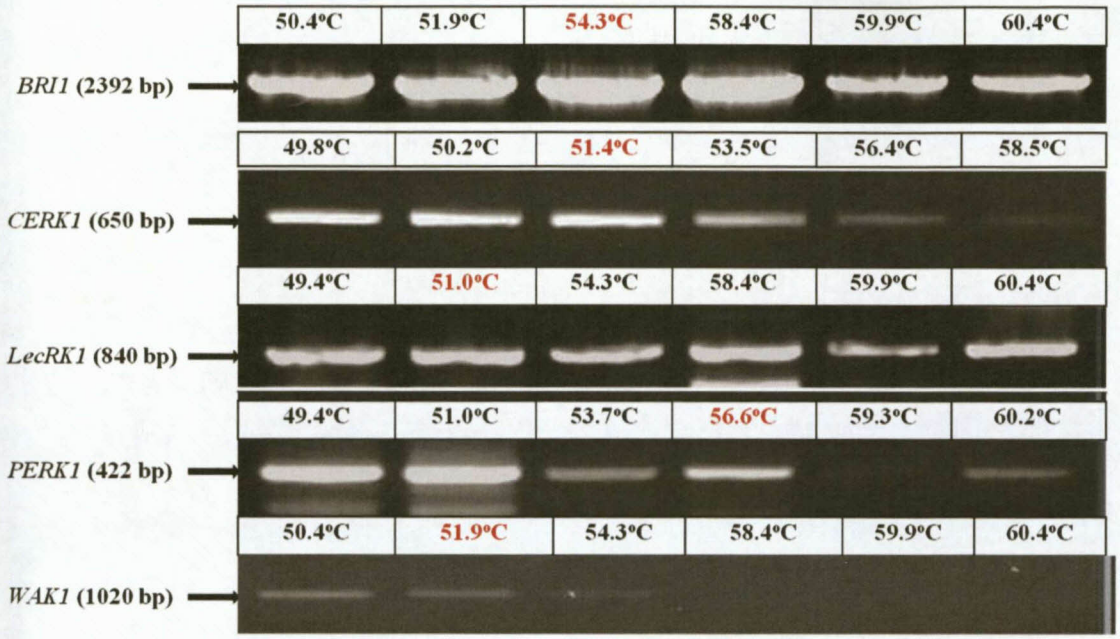


Figure 4.7. Determination of the optimal annealing temperatures for primer sets amplifying the different extracellular domain gene fragments. The optimal annealing temperature is indicated in red. Fragment sizes are as indicated.

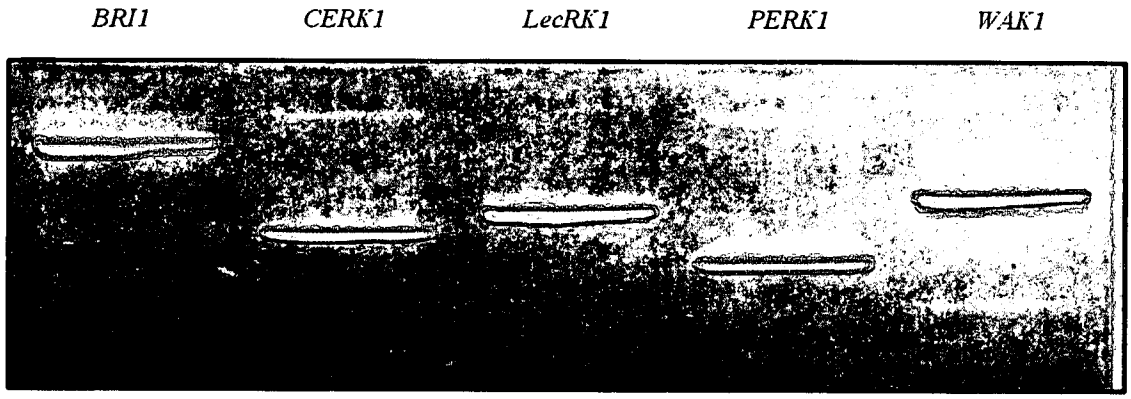


Figure 4.8. Amplification of the five different extracellular domain encoding gene fragments. The sizes of the gene fragments were as follows: *BRI1* (2392 bp), *CERK1* (650 bp), *LecRK1* (840 bp), *PERK1* (422 bp) and *WAK1* (1020 bp).

4.2.1.3. Cloning of PCR amplified gene fragments

The five PCR amplified gene fragments were cloned into the pGEM-T-Easy vector (3.2.2.5). *E. coli* colonies containing recombinant plasmid DNA were selected based on their inability to convert X-gal to a blue product. Plasmid DNA was extracted from selected colonies and PCR was used to confirm the presence of the correct inserts in the vector. As stated earlier, only the results for *PERK1* are given (Figure 4.9). The correct sized *PERK1* gene fragment (422 bp) were amplified from clones 4-9, while clones 1 and 3 showed no amplification products and clone 2 showed an amplicon with the wrong sized. Colonies containing the correct sized gene fragments were selected for DNA sequencing.

4.2.1.4. Sequencing of the clones

To confirm that there were no PCR induced mutations in the amplified gene fragments, at least six cloned inserts for each gene fragment was sequenced using both the gene specific forward and reverse primers (3.2.2.7). Internal sequencing primers were developed for the longer *BRI1* gene fragments to ensure that the complete gene fragment was sequenced (Table 3.1).

The results shown are for the *PERK1* gene fragment (Figure 4.10). Despite sequencing several different cloned inserts, the two indicated nucleotide changes were observed everytime. This might be due to the fact that cauliflower (*Brassica oleracea*) leaf tissue was used for RNA and DNA extraction instead of rape (*Brassica napus)* leaf tissue. The fragment was translated to see if the changes will have an impact on the amino acid sequence, but since no changes were observed the cloned gene fragment was therefore used as it is for further analyses. For each of the other four gene fragments, at least one recombinant clone was obtained where the cloned sequence was identical to the original Genbank gene sequence (results not shown).

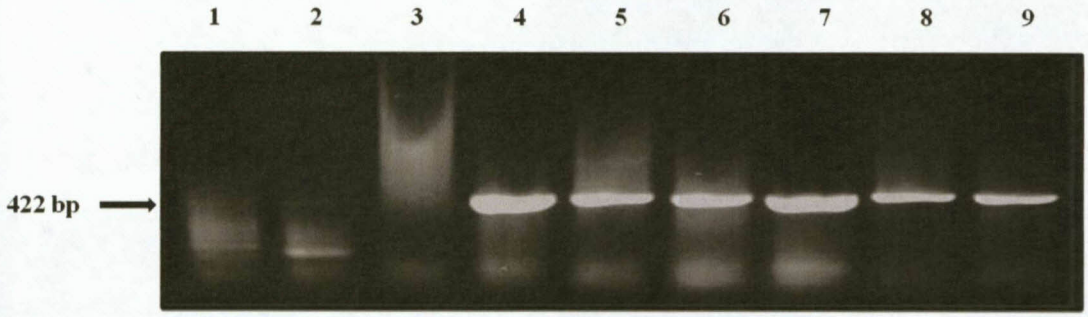


Figure 4.9. Confirmation of the presence of *PERK1* inserts in recombinant pGemT-Easy plasmid vectors in different *E. coli* colonies (1-9).

Figure 4.10. The sequence alignment of *PERK1* clone 7 with the original *PERK1* gene sequence. The start codon and nucleotide changes are indicated in red.

4.2.1.5. Restriction enzyme digestion of cloned gene fragments

In preparation for recombinant gene construction, the extracted plasmid DNA harbouring the different gene fragments was digested with BamH1 in the case of *BRI1*, *CERK1*, *LecRK1* and *PERK1* and EcoRV in the case of *WAK1* (Figure 4.11). A single digested DNA fragment with the combined size of the insert and vector was seen for each cloned gene fragment, indicating successful digestion.

4.2.2. Cloning of the *LRK10* kinase domain encoding region

4.2.2.1. Genomic DNA isolation from wheat

Genomic DNA extracted from the different *Triticum aestivum* cultivars (Federation, JIC24, Thatcher + *Lr*10 and Waldren) was of good quality with no degradation or contaminating RNA being visible (Figure 4.12).

4.2.2.2. PCR amplification of the *LRK10* kinase domain encoding gene fragment

The optimal annealing temperature of the *LRK10* kinase domain specific primers was determined (Figure 4.13) by doing a gradient PCR using genomic DNA as a template (3.2.2.4). The optimal annealing temperature was where an intense amplicon was evident with no obvious secondary products. The optimal annealing temperature indicated in red was used for all subsequent PCR reactions. However, there was no amplification when the normal PCR was repeated. This was improved by using 2.5 mM $MgCl_2$ instead of 1.75 mM $MgCl_2$ and by increasing the cycle number from 30 to 40. PCR amplification was then successful from JIC24, Thatcher + *Lr10* and Waldren cultivars but not for Federation (Figure 4.14).

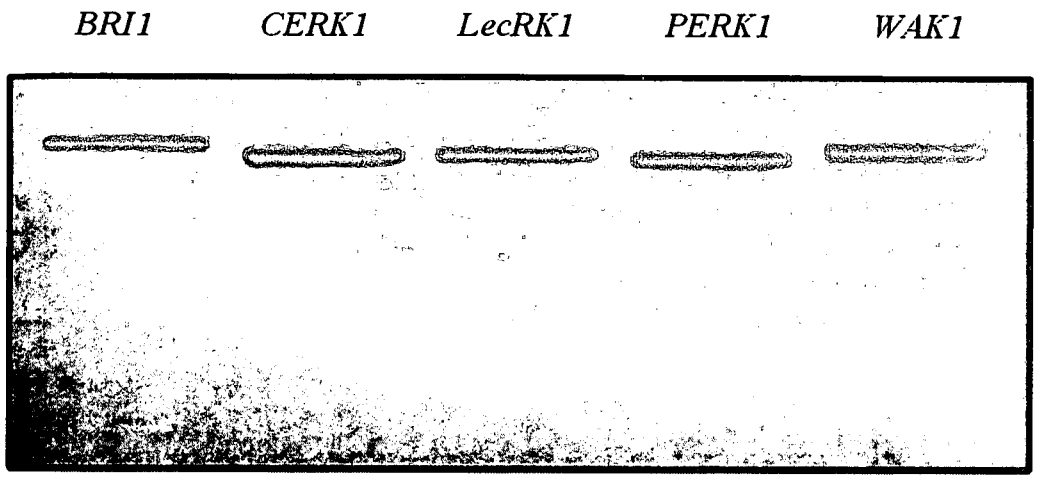


Figure 4.11. Digestion of recombinant plasmid DNA harbouring the gene fragments encoding the extracellular domains of *BRI1* (5407 bp), *CERK1* (3665 bp), *LecRK1* (3855 bp), *PERK1* (3437 bp) and *WAK1* (4035 bp).

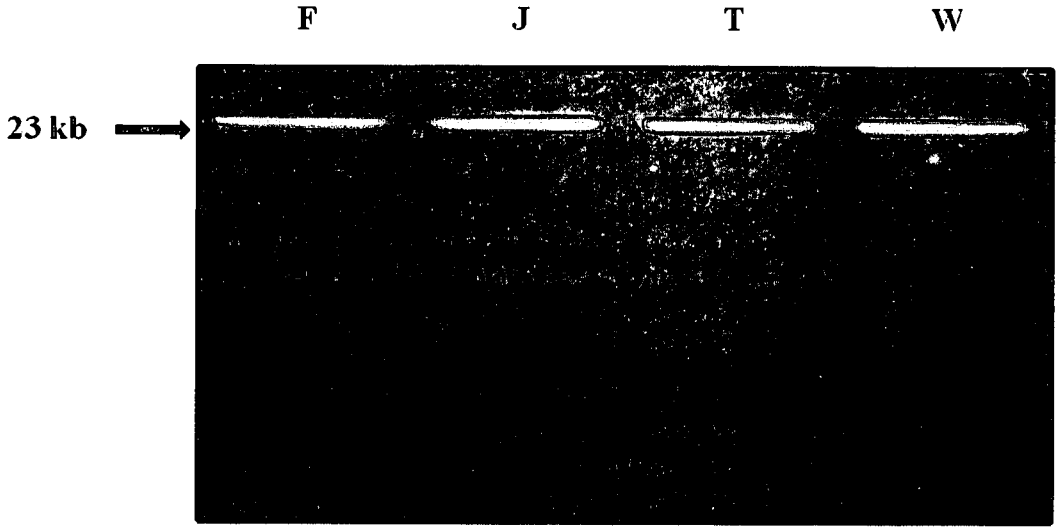


Figure 4.12. Genomic DNA extracted from different wheat cultivars. F: Federation, J: JIC24, T:Thatcher + *Lr*10 and W: Waldren. The sizes of the DNA fragments are as indicated.

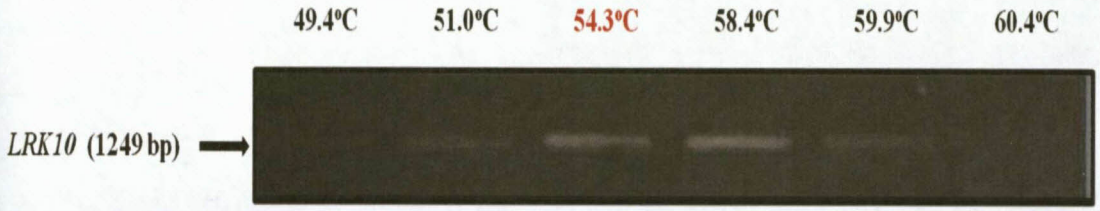


Figure 4.13. Determination of the optimal annealing temperature for the primer set amplifying the *LRK10* kinase domain encoding gene fragment. The optimal annealing temperature is indicated in red. The fragment size is as indicated.

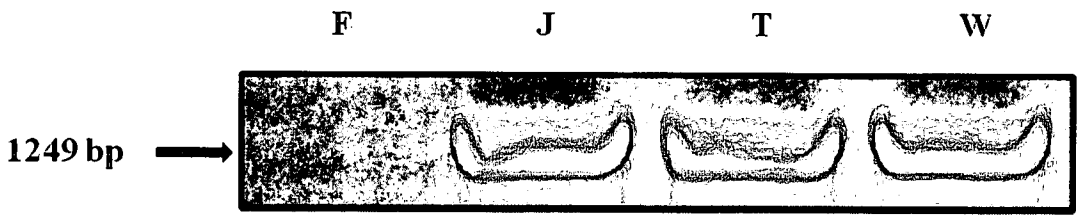


Figure 4.14. PCR amplification of the *LRK10* kinase domain encoding gene fragment. F: Federation, J: JIC24, T: Thatcher + *Lr*10 and W: Waldren.

4.2.2.3. Cloning of the amplified *LRK10* kinase domain encoding gene fragment

The PCR amplified *LRK10* gene fragments from the different cultivars were cloned into pGEM-T-Easy vector and blue/white screening was used to select for recombinants. For insert confirmation, PCR was used to amplify the inserts from extracted plasmid DNA (Figure 4.15). In this case, all clones except 2 and 7 contained the correct sized insert.

4.2.2.4. Sequencing of the cloned *LRK10* gene fragments

Recombinant pGemT-Easy plasmids containing the LRK10 kinase domain encoding gene fragments were sequenced using the *LRK10* specific forward and reverse primers (Table 3.1). Despite sequencing several cloned inserts from the different cultivars, a particular region within the gene fragment gave poor results (Figure 4.16a). This region indicated the presence of multiple guanines which differed from the original Genbank sequence. Following this region, the quality of the sequence reaction deteriorated significantly.

It was initially thought that the problem arose during amplification of the gene fragment from the genomic DNA. Efforts to solve this problem included using different Taq DNA polymerases like Kapa LongRange HotStart Polymerase (Kapabiosystems), High Fidelity PCR mix (Fermentas) and *Pfu* Turbo DNA polymerase (Stratagene), the inclusion of 5% (v/v) DMSO to each PCR reaction and increasing the initial denaturation step from 30 s to 2 min 30 s.

When these steps were unsuccessful, it was thought that the problem arose from the sequencing reaction itself. Attempts to test the sequence reaction included using a full sequencing reaction instead of one eighth of a reaction, the primer annealing temperature was increased from 50 to 55°C and the denaturation time was increased, but still without success. Finally, two recombinant plasmids were sequenced by the DNA sequencing unit at the University of Stellenbosch (Figure 4.16b). From these results, it was clear that G/C rich region was part of the amplified and cloned fragment, implying that the problem lay with its original amplification and not the sequence reaction itself.

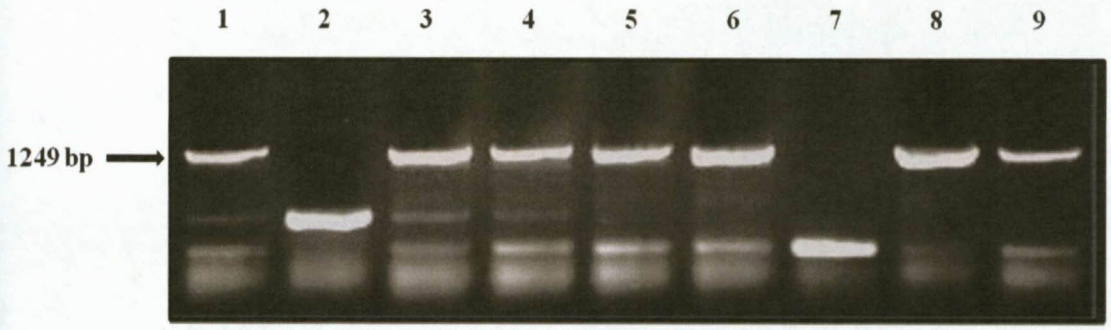
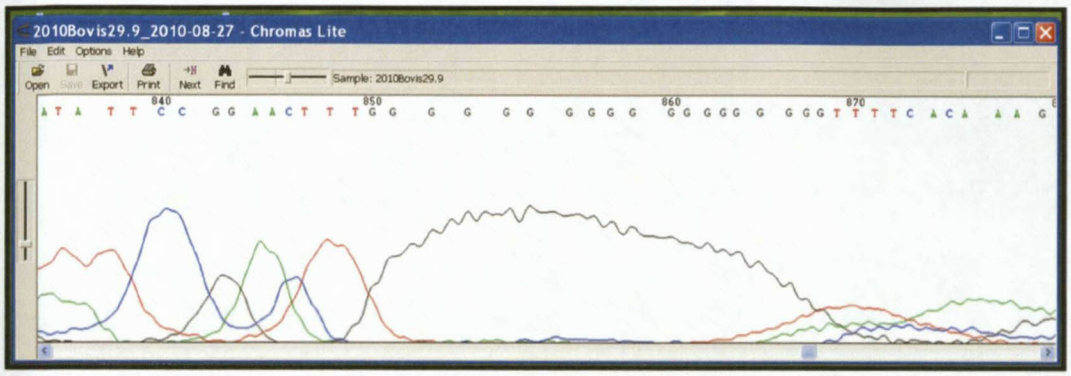


Figure 4.15. Confirmation of the presence of *LRK10* inserts in recombinant pGemT-Easy plasmid vectors in different *E. coli* colonies (1-9).

(a)



(b)

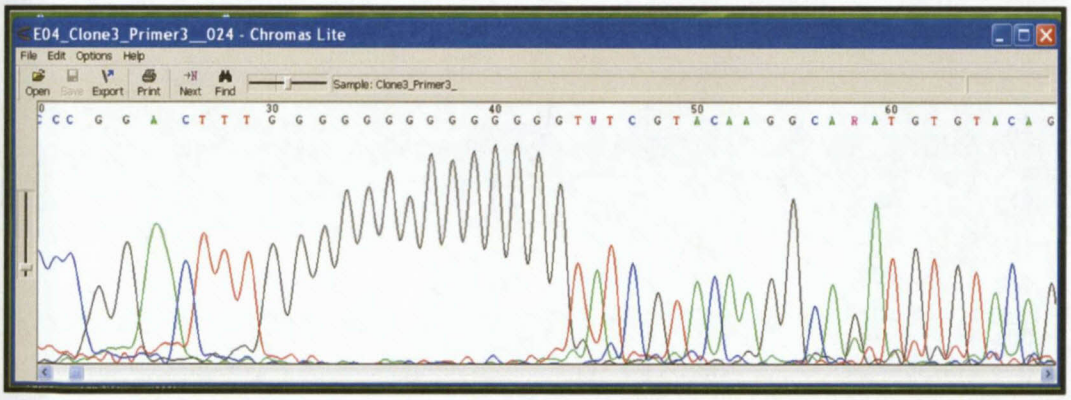


Figure 4.16. Sequencing of a G/C rich region within the *LRK10* kinase domain encoded gene fragment. In (a) the sequence results done at the UFS using the forward *LRK10* primer is indicated and in (b) results from the University of Stellenbosch using the same primer.

Finally, due to all the problems associated with cloning of this fragment, it was decided to rather synthesize the *LRK10* kinase domain encoding gene fragment by GeneArt (www.geneart.com/gene-synthesis). The sequence was designed to include the two primer annealing sites with the BamH1 palindrome included in the forward primer annealing site. The codon usage was optimized for expression in wheat, while taking care not to introduce any BamHI or EcoRV palindromes within the sequence.

The cloned *LRK10* gene fragment was received within the pMAT plasmid vector (Figure 4.17). A second set of *LRK10* specific primers that incorporated the EcoRV palindrome within the forward primer, were designed (Table 3.1). Using the two primers, a gradient PCR followed by PCR amplification of the gene fragment was done (results not shown). This fragment was cloned into pGEM-T-Easy and recombinant plasmids selected as described (results not given). Recombinant pMAT and pGemT-Easy plasmid vectors containing the *LRK10* kinase domain encoding gene fragments were digested with BamHI and EcoRV respectively (Figure 4.18).

4.2.3. Construction of the recombinant genes.

4.2.3.1. PCR amplification and cloning of recombinant genes

To construct the recombinant genes, the different BamHI digested recombinant plasmids containing the extracellular domain encoding gene fragments (Figure 4.11) were individually ligated to the BamH1 digested recombinant plasmid containing the *LRK10* kinase domain encoding gene fragment (Figure 4.18). Similarly, the two EcoRV digested recombinant plasmids were ligated. These ligated products were used as templates for a gradient PCR using the respective extracellular domain specific forward primer and the *LRK10* kinase domain specific reverse primer (Figure 4.3; Figure 4.19). Using this optimal annealing temperature, the different recombinant genes were amplified using the proofreading Kapa LongRange Taq polymerase (Figure 4.20) and cloned into pGemT-Easy. Despite various attempts, the *WAK1-LRK10* recombinant gene could not be amplified from the ligation reaction, most probably due to the difficulties of ligating blunt ended DNA fragments. This recombinant gene was thus eliminated from the study.

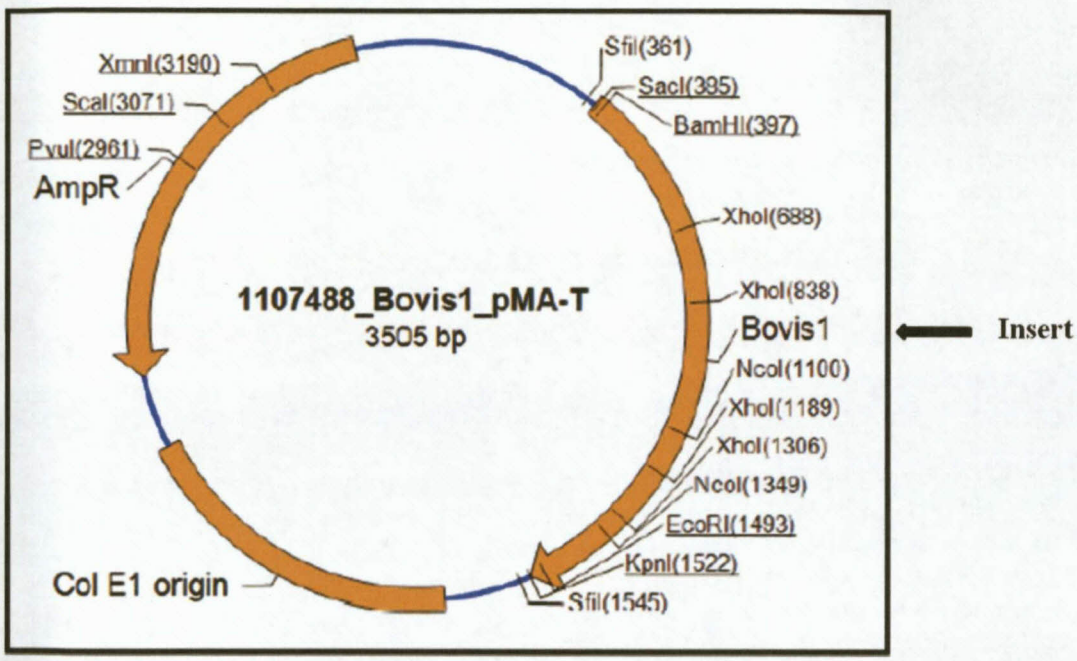


Figure 4.17. The pMAT vector containing the synthesised *LRK10* kinase domain encoding gene fragment indicated as Bovis 1.

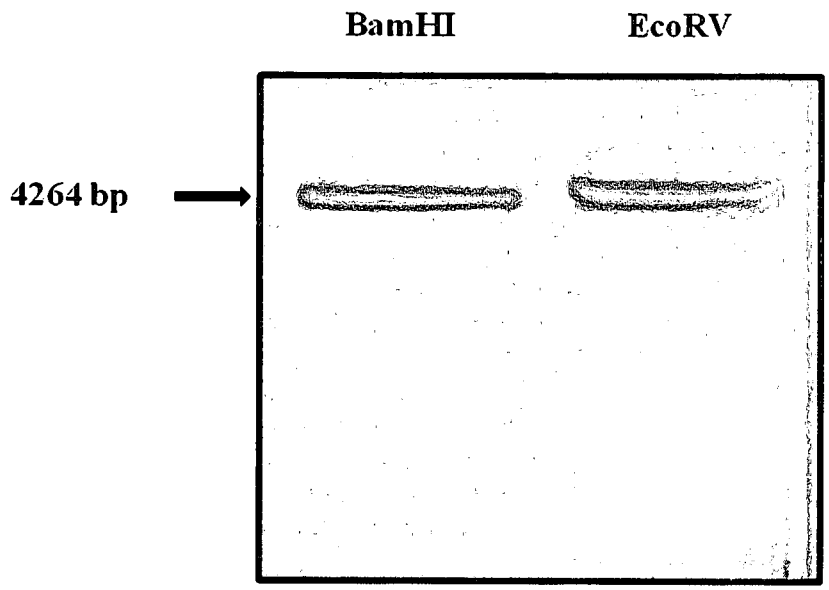


Figure 4.18. Digestion of recombinant plasmid DNA containing the *LRK10* kinase domain encoding gene fragments with BamHI and EcoRV respectively. The approximate size of the fragments is indicated.

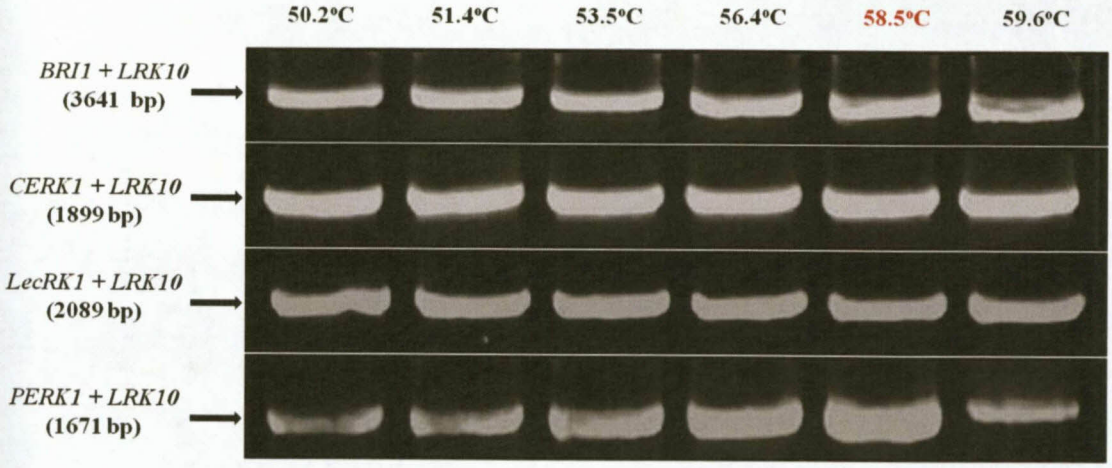


Figure 4.19. Determination of the optimal annealing temperatures of primer sets amplifying the different recombinant genes. The different fragment sizes are indicated in brackets.

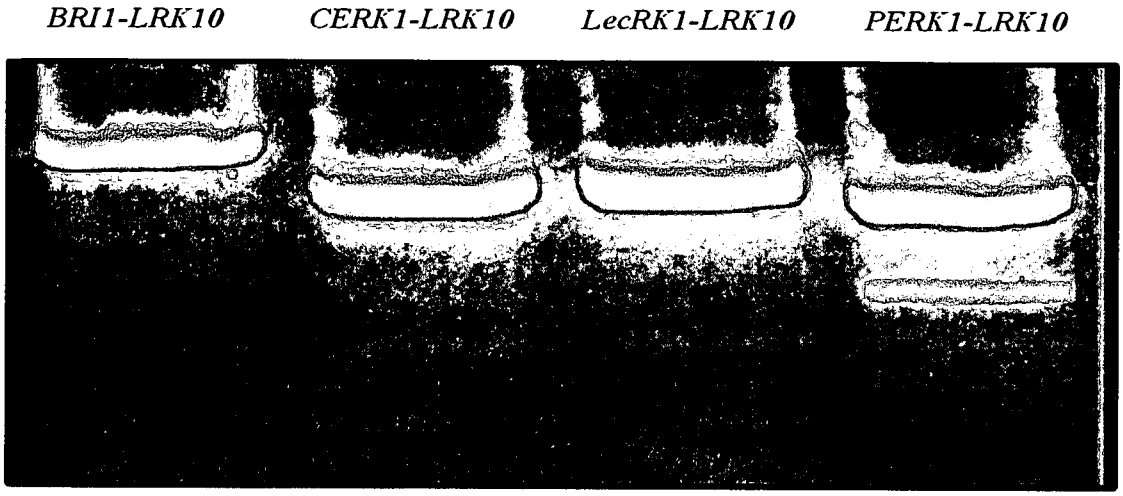


Figure 4.20. PCR amplification of the different recombinant genes; *BRI1-LRK10* (3641 bp), *CERK1-LRK10* (1899 bp), *LecRK1-LRK10* (2089 bp) and *PERK1-LRK10* (1671 bp).

4.2.3.2. *In vitro* expression analyses of a selected recombinant gene

To verify the success of recombinant gene construction, the *LecRK1-LRK10 (LL)* recombinant genes was transcribed and translated *in vitro* (Figure 4.21b). The genes were amplified from recombinant pGemT-Easy plasmids using the pUC/M13 universal forward and *LRK10* kinase domain specific reverse primers (Table 3.1) so as to include the plasmid T7 promoter region on the amplicons (Figure 4.21a). Multiple amplicons were found for *LL*, most probably because the PCR reaction was not completely optimized. When this amplicon was transcribed and translated using the TNT® Quick Coupled Transcription/Translation System, four polypeptides were evident for LL with the most prominent being approximately 71 kDa in size. These correspond to the predicted size for this recombinant receptor (Appendixes 6 to 9).

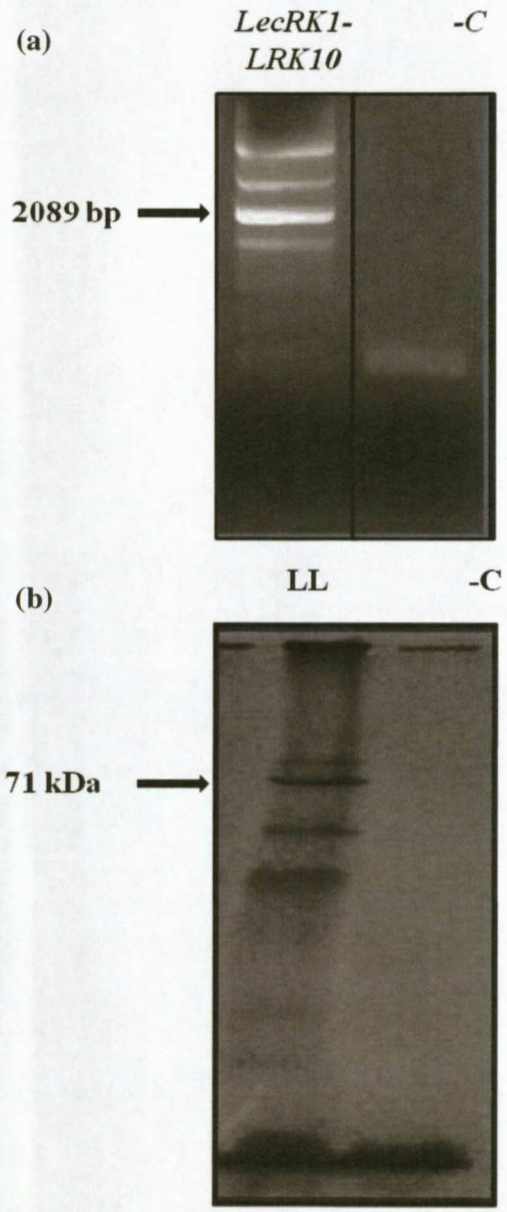


Figure 4.21. *In vitro* expression of recombinant gene *LecRK1-LRK10* (LL). In (a) the PCR amplification for LL (2089 bp) and the negative control (-C) is indicated and in (b) the synthesized polypeptides. Size is as indicated.

CHAPTER 5

DISCUSSION:

The continual growth of the human population will place increased pressure on wheat production in the future.To meet this increased demand, agricultural practices must ensure the optimal production of wheat. Severe challenges could however prevent this, leading to world-wide food shortages. These challenges include both abiotic and biotic factors.

Abiotic factors include climate change, salinity and the availability of water and nutrients for plants. Plants have an optimal temperature for growth and reproduction and unlike animals, plants cannot maintain an optimal temperature for growth (McKersie, 2001). Plants can withstand temperatures 5-10°C above their optimal temperature without being stressed. Temperatures out of this range may negatively affect physiological and biochemical processes needed for plant growth. Climate change is thus expected to impact heavily on crop production.

The challenges posed by biotic factors include plant pathogens and pests (fungi, bacteria, nematodes, insects and viruses) (Sun *et al.*, 2009). While control measures in the form of fungicides and pesticides are frequently used, they pose serious environmental concerns. Pollutants emitted from agricultural operations pose a threat to people living and working in rural areas because only roughly 85% of sprayed pesticides reach their target destination (Pimental and Levitan, 1986). The remaining 15% is deposited elsewhere. A World Health Organization estimate suggests that over 3 million farmers in developing countries experience severe poisoning from pesticides and another 25 million suffer mild pesticide poisoning.

The safest and cheapest option to manage biotic stress in the long run is to exploit the natural defence response of the plants themselves. Plants have an effective innate defence response to overcome pathogen infection. This response includes both physical and chemical barriers as well as the activation of an inducible defence response (Hammond-Kosack and Jones, 1996).

In general, plants can overcome attacks by most pests and pathogens by means of PTI (Zipfel and Felix, 2005). This defence relies on PAMPs/MAMPs being recognized by PRRs that activate the basal defence response which is usually very effective. However, pathogens can overcome this defence response through ETS where an effector protein is produced and transferred to the plant cell where it suppresses the plant defence response.

To gain the upper-hand, plants employ race-specific *R* genes to activate the effective HR based defence response (Jones and Dangl, 2006). In order to improve resistance of crops against pathogens, plant breeders have developed wheat cultivars carrying specific *R* genes (Lagudah *et al.*, 2006; Leonard *et al.*, 2008). These *R* genes are often found in wild relatives of wheat and are transferred into commercial cultivars through breeding.

One major problem with the continual use of specific *R* genes in successful cultivars is the development of new pathogenic races that acquired virulence against the particular *R* gene (Bolton *et al.*, 2008). One such an example is the Ug99 race of *Puccinia graminis* f.sp *tritici* that causes stem rust in wheat (Pretorius *et al.*, 2000). Races within the Ug99 lineage overcame three different resistance genes within 10 years since the first detection of Ug99 (Jin *et al.*, 2008).

The major problem with this is that only a limited number of effective *R* genes are still available for transfer into commercial cultivars. Since race-specific *R* genes are only effective against particular races of the pathogen, increasing numbers of virulent races would definitely harm wheat cultivation in the future. One solution for the problem is the development of new classes of *R* genes.

The specificity of the *R-avr* interaction is also its weak point. Since this interaction relies on only two proteins for recognition of the pathogen by the plant, even a slight change to either of them or the production of a totally new effector protein would lead to a virulent pathogen. Several cloned *R* genes code for RLKs that bind pathogen specific avirulence gene products. These include *Xa21* (Song *et al.*, 1995), *Xa26* (Sun *et al.*, 2004) and *LRK10* (Feuillet *et al.*, 1997). Several PRRs are also RLKs including FLS2 (Chinchilla *et al.*, 2006), EFR (Kunze *et al.*, 2004) and CERK1 (Miya *et al.*, 2007; Wan *et al.*, 2008). Their extracellular domains bind PAMPs or DAMPs that are general components of either the pathogen or the plant which are unlikely to change.

The general structure of these RLK proteins (Shiu and Bleecker, 2003) makes it easy to manipulate. While the actual signalling is initiated by the binding of the ligand to the extracellular domain of the RLK, it is the interaction of the kinase domain with specific intracellular proteins that activates the defence response. Thus, by combining a DAMP binding extracellular domain to the kinase domain of a resistance protein should theoretically

generate an "R" protein specific resistance response in the form of the HR upon binding of a general signalling molecule in the form of a DAMP or PAMP. Infection by any species of pathogen would in theory thus activate the highly effective HR based defence response within the plant.

While the overall aim of the current study is to produce crops with durable resistance, this part only focussed on the actual production of these chimeric receptor genes. A total of eight different DAMP and MAMP/PAMP binding RLKs and one RLP were initially identified (3.2.1.1). These receptors perceive various types of signals such as BRs (Li and Chory, 1997), chitin (Miya *et al.*, 2007; Kim *et al.*, 2000), fungal elicitors (Takemoto *et al.*, 2000), flg22 (Gomez-Gomez and Boller, 2000), lectin (Hanks and Quinn, 1991), extensin (Silva and Goring, 2002), pectin and wounding (Maleck *et al.*, 2000). The choice for the kinase domain fell on *LRK10*, an *R* gene that confers resistance to wheat against leaf rust (*Puccinia triticina*) in a species specific manner (Feuillet *et al.*, 1997). LRK10 interacts with specific downstream intracellular proteins to activate the HR specific defence response. Only four chimeric receptor genes were eventually successfully produced, mainly due to problems amplifying and ligating the gene fragments, as well as time constraints. They included *BRI1-LRK10*, *CERK1-LRK10*, *LecRK1-RLK10* and *PERK1-LRK10*.

When the *LecRK1-LRK10* recombinant gene was expressed *in vitro*, a polypeptide with the correct size was produced (Figure 4.21b). Even though only one recombinant gene was tested, it indicated that the general approach and methodology was correct so as to produce chimeric proteins. Future work will include testing all the recombinant genes, as well as to confirm their kinase activity.

Chimeric receptors have been used mostly in animals to understand the mechanisms of signal perception (Tauszig *et al.*, 2000; Tsujita *et al.*, 2004) with little progress being made in plants. One of the first chimeric receptors in plants was derived from the extracellular and TM domains of *Arabidopsis* BRI1 and the kinase domain of *O. sativa* Xa21 (He *et al.*, 2000). While both proteins are RLKs, BRI1 recognizes and binds BRs and is involved in plant development (Li and Chory, 1997; Kinoshita *et al.*, 2005; Wang *et al.*, 2005a). Xa21 confers resistance to rice against the bacterial pathogen *X. oryzae oryzae* in rice (Song *et al.* 1995). In this study, the BRI1 extracellular domain perceived the presence of BRs thereby initiating plant defence through the Xa21 kinase domain of the chimeric receptor (He *et al.*, 2000).

A recent domain swapping study was done by Brutus *et al.* (2010) where they investigated whether WAKs perceive oligogalacturonides (OGs) as signal molecules. The WAK family comprises five members, WAK1 to WAK5 (He *et al.*, 1996). *Arabidopsis thaliana* WAK1 is a putative receptor for OGs and is induced by wounding, SA, INA, bacterial infection and aluminium treatment. Upon binding OGs, WAK1 activates a defence response in the plant.

A test-of-concept study was first done to assess the possibility of obtaining functional chimeric receptors when two unrelated RLKs were fused (Brutus *et al.*, 2010). Two chimeras comprising the extracellular domain of *FLS2* and the TM and kinase domains of *EFR* were made. The design of these two proteins allowed for correct ligand-induced conformational change (Moriki *et al.*, 2001). The only difference between these two chimeras was the junction point which was either immediately upstream of or within the TM region.

EFR normally induces ET production (Zipfel *et al.*, 2006) and this was used to assess the ability of the chimeric receptors to activate the downstream response upon treatment with flg22. The ET accumulation was higher in plants treated with flg22 as compared to elf18 (Robatzek *et al.*, 2007). Following flg22 treatment, the highest ET levels were found in the chimeric receptor where the junction point was upstream of the EFR TM region, emphasizing the importance of an intact kinase domain within the chimeric receptor.

The ligand-dependent dimerisation and activation of receptors are dependent of the TM region (Bennasroune *et al.* 2005; Bocharov *et al.*, 2008). Therefore, it is important to have the TM domain correctly incorporated into the chimeric receptors. During the current study (section 4.1), care was taken to ensure that the intact LRK10 TM and kinase domains formed part of the chimeric receptor by incorporating the palindromes upstream of the TM domain. This should ensure accurate anchoring of the receptor protein on the plasma-membrane with the subsequent successful activation of the kinase domain specific defence response.

Two further chimeric WAK1 receptors were made (Brutus *et al.*, 2010). The first consisted of the EFR extracellular domain and the WAK1 TM and kinase domains, while the second had the WAK1 extracellular domain and the EFR TM and kinase domains. The obtained results confirmed that binding of the elf18 PAMP to the EFR extracellular domain, led to the activation of the WAK1 kinase domain with the subsequent activation of the defence response. A similar response was observed with the second chimeric receptor.

This study by Brutus et al (2010) emphasized two important points. The first was that the improvement of durable resistance against pathogens and pests is dependent on the correct engineering of the chimeric receptors. It also showed that the enhanced resistance is not only obtained when two closely related receptors are combined but the combination of unrelated receptors also works. Therefore, the construction of the new PRR based chimeric receptors controlled by various signals including PAPMs/MAMPs and DAMPs is feasible.

RLPs lack the intracellular kinase domain (Fritz-Laylin *et al.*, 2005), but most probably interact with other intracellular proteins. The ability of a chimeric receptor consisting of the RLP CEBiP and the Xa21 kinase domain to transfer an effective signal was tested by Kishimoto *et al* (2010). CEBiP is a receptor for chitin (Kaku *et al.*, 2006) and *Xa21* is an *R* gene which confers resistance against bacterial leaf blight caused by *X. oryzae* pv. *oryzae* (Song *et al.*, 1995). For the construction of the chimeric receptor, the *CEBiP* gene was fused with the *Xa21* intracellular domain encoding region. Transgenic plants showed a stronger response to chitin. When transgenic plants were inoculated with *Magnaporthe oryzae*, improved resistance was observed (Kishimoto *et al.*, 2010). The use of chimeric receptors in several studies demonstrated that the specificity of the ligand perception is determined by the extracellular domain and the output of the signal by the intracellular domain (He *et al.*, 2000; Brutus *et al.*, 2010; Kishimoto *et al.*, 2010).

Since the current chimeric receptor protein design is similar to that done by Brutus *et al.*, (2010), it is believed that the general strategy using the LRK10 kinase domain will work. The CERK1-LRK10, LecRK1-LRK10 and PERK1-LRK10 receptors should function when infection by a pathogen produces the relevant ligand (chitin, lectin and pectin). These chimeric receptors will then bind the ligand and activate an effective defence response in the form of the HR. In the case of BRI1-LRK10, treatment with a plant activator that contains BR as the active compound, should prime the plant sufficiently to survive infection by any pathogen.

Future work will include the production of transgenic wheat expressing one or various combinations of the chimeric receptors. Their effectiveness will then be tested in wheat infected with different fungal pathogens. If successful, this project will have a huge impact in agriculture in terms of the successful production of wheat.

CHAPTER 6

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SUMMARY

The aim of the current study was to construct novel recombinant *RLK* genes encoding chimeric receptor proteins to broaden disease resistance in wheat. Eight different RLKs were initially chosen as extracellular domain donors based on their ability to bind PAMPs/MAMPs or DAMPs. The kinase domain donor was LRK10, an R protein that renders wheat resistant against *Puccinia triticina*.

These recombinant genes were first designed *in silico* using a bio-informatic approach where the gene portions encoding the different extracellular domains were linked to the *LRK10* kinase domain encoding gene fragment. Translation of the different recombinant genes confirmed that full length polypeptides will be formed. General PAMP binding capacity was therefore combined with the highly specific defence signalling ability of LRK10 within a single protein.

Due to several problems encountered, only four recombinant genes were successfully produced, namely *BRI1-LRK10, CERK-LRK10, LecRK1-LRK10* and *PERK1-LRK10*. To confirm the success of the approach, *LecRK1-RLK10* was successfully transcribed and translated *in vitro*, thus producing the correct sized polypeptide. Future work will include the production of transgenic wheat to test the ability of these chimeric proteins to increase wheat resistance against pathogen infection.

Keywords: Chimeric receptors, domain-swopping, recombinant genes.

OPSOMMING

Die doel van die huidige studie was om rekombinante *RLK* gene wat vir ´n nuwe klas chimeriese reseptorproteïene kodeer te produseer om sodoende die siekteweerstand van koring te verbreed. Agt verskillende RLKs was aanvanklik gekies omdat hulle ekstrasellulêre domeine verskillende PAMPs/MAMPs en DAMPs bind. Die geenstreke wat vir die ekstrasellulêre domeine kodeer sal aan die geenstreek van *LRK10* wat vir die kinasedomein kodeer, geheg word. *LRK10* is ´n weerstandsgeen wat aan koring weerstand teen *Puccinia triticina* verleen.

Die rekombinante gene is *in silico* met behulp van bio-informatika ontwerp deur die ekstrasellulêre domein geenstreke aan die *LRK10* kinasedomein spesifieke geenstreek te heg. Translasie van die verskillende rekombinante gene het bevestig dat elk van die gene vir ´n volledige polipeptied kodeer. Die algemene vermoë om PAMPs/MAMPs en DAMPs te bind is dus binne ´n enkele proteïen met die spesifieke verdedigingsseinvermoë van LRK10 gekoppel.

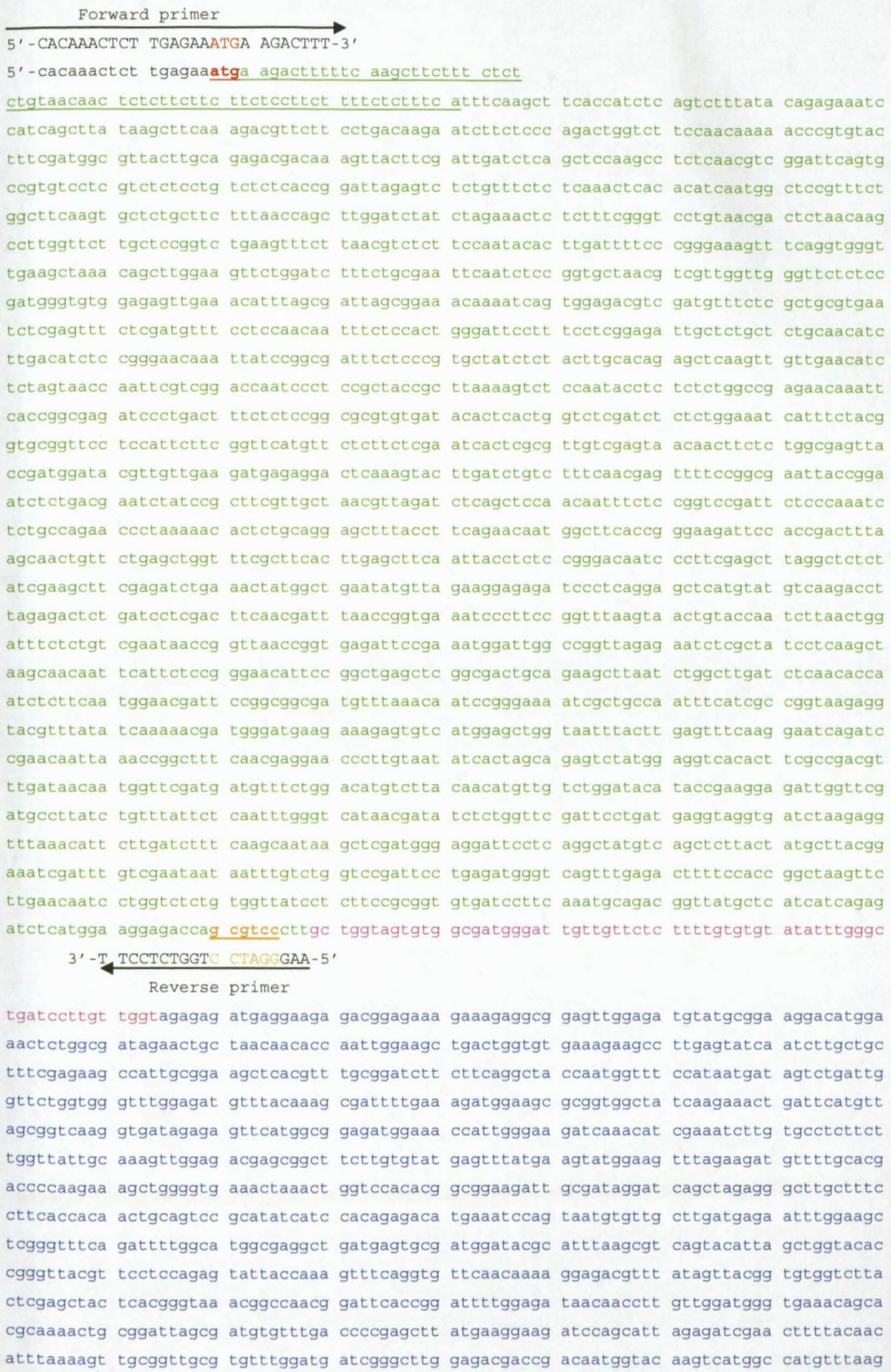
As gevolg van verskeie probleme, is daar slegs vier verskillende rekombinante gene geproduseer. Hulle was *BRI1-LRK10, CERK-LRK10, LecRK1-LRK10* en *PERK1-LRK10.* Om die sukses van die benadering te toets, is die *LecRK1-RLK10* rekombinante geen suksesvol getranskribeer en getransleer om ´n polipeptied met die korrekte grootte te vorm. Toekomstige werk sal die maak van transgeniese koring insluit om sodoende die vermoë van die chimeriese proteïene om aan koring weerstand teen patogeeninfeksie te verleen, te toets.

Sleutelwoorde: Chimeriese reseptore, domein-omruiling, rekombinante gene.

APPENDICES

APPENDIX 1

(a)


Forward primer
5′-CACAAACTCT TGAGAAATGA AGACTTT-3′
5′-cacaaactct tgagaaatga agacttttttc aagcttcttt ctct
ctgtaacaac tctcttcttc ttctccttct tttctctttc atttcaagct tcaccatctc agtctttata cagagaaatc
catcagctta taagcttcaa agacgttctt cctgacaaga atcttctccc agactggtct tccaacaaaa acccgtgtac
tttcgatggc gttacttgca gagacgacaa agttacttcg attgatctca gctccaagcc tctcaacgtc ggattcagtg
ccgtgtcctc gtctctcctg tctctcaccg gattagagtc tctgtttctc tcaaactcac acatcaatgg ctccgtttct
ggcttcaagt gctctgcttc tttaaccagc ttggatctat ctagaaactc tctttcgggt cctgtaacga ctctaacaag
ccttggttct tgctccggtc tgaagtttct taacgtctct tccaatacac ttgattttcc cgggaaagtt tcaggtgggt
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atctctgacg aatctatccg cttcgttgct aacgttagat ctcagctcca acaatttctc cggtccgatt ctcccaaatc
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ttgaacaatc ctggtctctg tggttatcct cttccgcggt gtgatccttc aaatgcagac ggttatgctc atcatcagag
atctcatgga aggagaccag cgtcccttgc tggtagtgtg gcgatgggat tgttgttctc ttttgtgtgt atatttgggc
3′-T TCCTCTGGTC CTAGGGAA-5′
Reverse primer
tgatccttgt tggtagagag atgaggaaga gacggagaaa gaaagaggcg gagttggaga tgtatgcgga aggacatgga
aactctggcg atagaactgc taacaacacc aattggaagc tgactggtgt gaaagaagcc ttgagtatca atcttgctgc
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gagatacaag ccgggtcagg gatagattca cagtcaacga tcagatcaat agaggatgga gggttcagta caatagagat
ggttgatatg agtataaaag aagttcctga aggaaaatta **tga**-3'

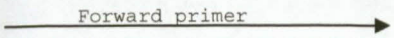
(b)

M K T F S S F F L S V T T L F F F S F F S L S F Q A S P S Q S L Y R E I H Q L I S F K D
V L P D K N L L P D W S S N K N P C T F D G V T C R D D K V T S I D L S S K P L N V G F
S A V S S S L L S L T G L E S L F L S N S H I N G S V S G F K C S A S L T S L D L S R N
S L S G P V T T L T S L G S C S G L K F L N V S S N T L D F P G K V S G G L K L N S L E
V L D L S A N S I S G A N V V G W V L S D G C G E L K H L A I S G N K I S G D V D V S R
C V N L E F L D V S S N N F S T G I P F L G D C S A L Q H L D I S G N K L S G D F S R A
I S T C T E L K L L N I S S N Q F V G P I P P L P L K S L Q Y L S L A E N K F T G E I P
D F L S G A C D T L T G L D L S G N H F Y G A V P P F F G S C S L L E S L A L S S N N F
S G E L P M D T L L K M R G L K V L D L S F N E F S G E L P E S L T N L S A S L L T L D
L S S N N F S G P I L P N L C Q N P K N T L Q E L Y L Q N N G F T G K I P P T L S N C S
E L V S L H L S F N Y L S G T I P S S L G S L S K L R D L K L W L N M L E G E I P Q E L
M Y V K T L E T L I L D F N D L T G E I P S G L S N C T N L N W I S L S N N R L T G E I
P K W I G R L E N L A I L K L S N N S F S G N I P A E L G D C R S L I W L D L N T N L F
N G T I P A A M F K Q S G K I A A N F I A G K R Y V Y I K N D G M K K E C H G A G N L L
E F Q G I R S E Q L N R L S T R N P C N I T S R V Y G G H T S P T F D N N G S M M F L D
M S Y N M L S G Y I P K E I G S M P Y L F I L N L G H N D I S G S I P D E V G D L R G L
N I L D L S S N K L D G R I P Q A M S A L T M L T E I D L S N N N L S G P I P E M G Q F
E T F P P A K F L N N P G L C G Y P L P R C D P S N A D G Y A H H Q R S H G R R P A S L
A G S V A M G L L F S F V C I F G L I L V G R E M R K R R R K K E A E L E M Y A E G H G
N S G D R T A N N T N W K L T G V K E A L S I N L A A F E K P L R K L T F A D L L Q A T
N G F H N D S L I G S G G F G D V Y K A I L K D G S A V A I K K L I H V S G Q G D R E F
M A E M E T I G K I K H R N L V P L L G Y C K V G D E R L L V Y E F M K Y G S L E D V L
H D P K K A G V K L N W S T R R K I A I G S A R G L A F L H H N C S P H I I H R D M K S
S N V L L D E N L E A R V S D F G M A R L M S A M D T H L S V S T L A G T P G Y V P P E
Y Y Q S F R C S T K G D V Y S Y G V V L L E L L T G K R P T D S P D F G D N N L V G W V
K Q H A K L R I S D V F D P E L M K E D P A L E I E L L Q H L K V A V A C L D D R A W R
R P T M V Q V M A M F K E I Q A G S G I D S Q S T I R S I E D G G F S T I E M V D M S I
K E V P E G K L Stop

Nucleotide (a) and amino acid (b) sequences of the *BRI1* gene from *Arabidopsis thaliana*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The palindrome incorporation resulted in a hydrophobic alanine (A) changing to a hydrophilic glycine (G) and the designed primers will amplify a 2392 bp fragment.

APPENDIX 2

(a)



5′-ATGAAGCTAA AGATTTCTCT AATCG-3′

5′-atgaagctaa agatttctct aatcgctccg attcttcttc tcttctcatt cttcttcgcc gtggaatcta agtgcaggac

tagctgtcct ttagctctag cttcgtacta tctcgagaac ggaacaacac tctccgtcat caaccaaaac ctcaattctt

caatcgcgcc ttacgatcaa atcaatttcg atccaatcct caggtacaac agtaacatta aagacaaaga tagaatccag

atgggctcta gggttcttgt acctttccct tgcgaatgtc aacctggtga tttcctaggg cacaatttca gctacagtgt

tcgacaggaa gatacttacg aaagagtcgc gattagtaat tacgcgaatc tcacgacgat ggagtcgtta caggcgagga

atccttttcc ggcgactaac atacctctct ctgcgacgct taatgtattg gtgaattgtt cttgtggtga tgagagtgtt

tcgaaagatt ttggtttgtt tgttacgtat ccgcttcgtc ctgaagacag tctcagttct attgcgagat cttccggtgt

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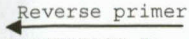

3′-ACCTTCCC

TAGGTTTACC-5′

atccaaatgg tgcatttcca ccattcaaat caagtaaaca agatggtgtt ggtgctggag ttattgctgg tatagttata

ggagtgattg tggctttgtt gttgatcttg tttatcgtat attatgctta ccggaagaat aagtcgaagg gtgattcgtt

ttcttcttct attccgttgt ctactaaggc tgatcatgct tcttctacta gtctccaaag tggaggtttg ggtggtgccg

gagtgtctcc tggcattgct gccataagcg tggacaaatc tgttgagttt tcgttggagg aactagcaaa ggctactgat

aatttcaatt tgtcttttaa gattgggcaa ggtggttttg gggctgttta ctatgcagag ctgagaggag aaaaagctgc

gattaagaag atggacatgg aggcatcgaa acagttcttg gcggaactaa aagtcttaac gcgtgtacat catgtcaacc

tggttcgcct gattggatat tgtgttgagg gatcactttt cttggtgtat gaatatgttg agaatggtaa ccttggacaa

catttacatg ggtcaggacg agaaccatta ccgtggacta agagagtgca gattgcacta gactcagcta gaggtttaga

atatatccac gagcacacgg ttccagttta tgtccatagg gacattaaat ctgccaatat tttgatagac cagaaattcc

gagcaaaggt agcagatttc gggttaacaa aactgacaga agttggaggt tcagcaactc ggggtgcaat gggtacattt

ggttacatgg caccagagac tgtttatgga gaagtgtctg caaaagtaga tgtatatgca tttggagttg tcctttacga

attgatttct gcgaaaggtg cggttgtcaa aatgacagaa gccgttggtg aatttagagg ccttgttggt gtgttcgaag

aatcattcaa ggaaaccgac aaagaagaag cactacgcaa gattatagac ccgaggctcg gtgatagtta cccgtttgat

tcggtataca agatggcgga attagggaaa gcatgtacac aagagaatgc gcagctacgt ccgagtatga gatacattgt

ggttgcttta tccactctct tttcgtctac cggaaattgg gatgttggaa acttccaaaa cgaagattta gtcagtctta

tgtccggccg gtag-3′

(b)

M K L K I S L I A P I L L L F S F F F A V E S K C R T S C P L A L A S Y Y L E N G T T L

S V I N Q N L N S S I A P Y D Q I N F D P I L R Y N S N I K D K D R I Q M G S R V L V P

F P C E C Q P G D F L G H N F S Y S V R Q E D T Y E R V A I S N Y A N L T T M E S L Q A

R N P F P A T N I P L S A T L N V L V N C S C G D E S V S K D F G L F V T Y P L R P E D

S L S S I A R S S G V S A D I L Q R Y N P G V N F N S G N G I V Y V P G R D P N G A F P

P F K S S K Q D G V G A G V I A G I V I G V I V A L L L I L F I V Y Y A Y R K N K S K G

D S F S S S I P L S T K A D H A S S T S L Q S G G L G G A G V S P G I A A I S V D K S V

E F S L E E L A K A T D N F N L S F K I G Q G G F G A V Y Y A E L R G E K A A I K K M D

M E A S K Q F L A E L K V L T R V H H V N L V R L I G Y C V E G S L F L V Y E Y V E N G

N L G Q H L H G S G R E P L P W T K R V Q I A L D S A R G L E Y I H E H T V P V Y V H R

D I K S A N I L I D Q K F R A K V A D F G L T K L T E V G G S A T R G A M G T F G Y M A

P E T V Y G E V S A K V D V Y A F G V V L Y E L I S A K G A V V K M T E A V G E F R G L

V G V F E E S F K E T D K E E A L R K I I D P R L G D S Y P F D S V Y K M A E L G K A C

T Q E N A Q L R P S M R Y I V V A L S T L F S S T G N W D V G N F Q N E D L V S L M S G

R Stop

Nucleotide (a) and amino acid (b) sequences of the *CERK1* gene from *Arabidopsis thaliana*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The palindrome incorporation did not result in any change in amino acid sequence and the primers will amplify a 650 bp fragment.

APPENDIX 3

(a)

Forward primer

5′-ATGTCTCGTG AACTTATTAT TCTCTGCC-3′

5′-atgtctcgtg aacttattat tctctgccaa ccaattcttg ttctgttcct tactctgttt tacaattctc atggctactt

cgtctcacaa ggatctgtag gaatcggctt caacggttac ttcactttaa ccaacaccac aaaacacaca ttcggtcaag

ctttcgaaaa cgagcacgtt gaaatcaaga actcatcgac aggtgtcatc tcatctttct cagtcaactt cttctttgcg

atcgtccctg aacataacca gcaaggctca cacggtatga ctttcgtcat ctctcccaca agaggccttc ccggagcttc

ctccgatcaa tacctcggaa tctttaacaa aacaaacaac ggtaaagcct cgaataacgt tatagctatc gagttagata

ttcataaaga cgaagagttt ggagatattg atgataatca tgttgggatt aatattaacg gtttgagatc tgttgcctct

gcttctgctg gttactatga tgataaagat ggaagcttta aaaaactttc tttgatcagc agagaggtaa tgaggctttc

catcgtttat agtcaacctg atcaacagct caatgttact ttattccctg ctgagatccc tgttccgcca ctaaaaccgc

tcttgtcttt gaaccgagat ctctcgccgt atttgcttga gaaaatgtat cttggattca ctgcatcgac ggggtcagtt

ggagcaattc attacttgat gggttggtta gttaatggtg taatcgagta tccgagattg gagcttagta taccagtcct

tcctccatat ccaaagaaaa catctaatag aacgaaaact gttttagccg tctgcttaac ggtatctgtg tttgctgcgt

3′-CTTTT GTAGATTAC CTAGGTTTTGA-5′

Reverse primer

ttgtcgcttc gtggatcggt ttcgtcttct atttgaggca taagaaggtt aaagaggttc ttgaagaatg ggagattcaa

tatggacctc ataggtttgc ttataaggag cttttcaatg ccacaaaggg tttcaaggag aaacaacttc ttggtaaagg

aggctttggt caagtctata aaggaacact tccgggttct gatgcagaga tcgctgtgaa gcggacttct catgattcaa

gacaagggat gagcgagttt ctagccgaga tatcgaccat tggtcgtctc agacatccaa atttagtcag gcttttagga

tactgtaggc ataaagagaa tctctacttg gtgtatgact atatgcctaa tggaagcctt gacaagtatc taaaccgtag

cgagaatcaa gaacggctta cttgggaaca acgtttcagg atcatcaaag atgttgcaac tgctctacta cacctgcatc

aagaatgggt acaagtcatc attcatcgag atatcaaacc agctaatgtt ttaatcgaca atgaaatgaa tgcgaggctc

ggggatttcg gactggcgaa actgtatgat cagggattcg atcctgaaac atctaaagta gcgggaacat tcggatatat

cgcaccagag tttctaagaa caggaagagc aaccacaagc actgatgttt acgcctttgg gttggtaatg cttgaagtag

tgtgtggtag aaggataatt gagagacgtg cagcagaaaa tgaagaatat cttgtggatt ggatcttaga gctttgggaa

aatgggaaaa ttttcgatgc agcagaggaa agtatacgtc aagaacaaaa caggggacaa gttgagcttg ttttgaagct

aggtgtgttg tgttcgcatc aagctgcatc gataagaccg gctatgagtg tggtaatgag gattttgaat ggagtttcac

agctaccaga taatcttctt gatgttgtaa gggctgagaa atttagagaa tggcctgaga catcaatgga attactacta

cttgatgtga atacatcgag ttcattggag ttaaccgact cttcttttgt ctcccacggc cgctga-3′

(b)

M S R E L I I L C Q P I L V L F L T L F Y N S H G Y F V S Q G S V G I G F N G Y F T L T

N T T K H T F G Q A F E N E H V E I K N S S T G V I S S F S V N F F F A I V P E H N Q Q

G S H G M T F V I S P T R G L P G A S S D Q Y L G I F N K T N N G K A S N N V I A I E L

D I H K D E E F G D I D D N H V G I N I N G L R S V A S A S A G Y Y D D K D G S F K K L

S L I S R E V M R L S I V Y S Q P D Q Q L N V T L F P A E I P V P P L K P L L S L N R D

L S P Y L L E K M Y L G F T A S T G S V G A I H Y L M G W L V N G V I E Y P R L E L S I

P V L P P Y P K K T S N R T K T V L A V C L T V S V F A A F V A S W I G F V F Y L R H K

K V K E V L E E W E I Q Y G P H R F A Y K E L F N A T K G F K E K Q L L G K G G F G Q V

Y K G T L P G S D A E I A V K R T S H D S R Q G M S E F L A E I S T I G R L R H P N L V

R L L G Y C R H K E N L Y L V Y D Y M P N G S L D K Y L N R S E N Q E R L T W E Q R F R

I I K D V A T A L L H L H Q E W V Q V I I H R D I K P A N V L I D N E M N A R L G D F G

L A K L Y D Q G F D P E T S K V A G T F G Y I A P E F L R T G R A T T S T D V Y A F G L

V M L E V V C G R R I I E R R A A E N E E Y L V D W I L E L W E N G K I F D A A E E S I

R Q E Q N R G Q V E L V L K L G V L C S H Q A A S I R P A M S V V M R I L N G V S Q L P

D N L L D V V R A E K F R E W P E T S M E L L L L D V N T S S S L E L T D S S F V S H G

R Stop

Nucleotide (a) and amino acid (b) sequences of the *LecRK1* gene from *Arabidopsis thaliana*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The palindrome incorporation resulted in a change from a polar positively charged arginine (R) to a hydrophilic glycine (G) and a hydrophilic tyrosine (T) to a hydrophilic serine (S). The primers will amplify a 840 bp fragment.

APPENDIX 4

(a)

Forward primer

5′-ATGAAGGTGC AGGAGGGTTT-3′

5′-atgaaggtgc aggagggttt gttcttggtg gctattttct tctcccttgc gtgt

acgcagctgg tgaaggggca acatcaacct ggtgagaatt gccaaaataa atgtggcaac atcacaatag agtacccttt

tggcatttct tcaggttgtt actatcccgg aaatgaaagt ttcagtatca cctgtaagga agataggcca catgtcttaa

gcgacattga agtggcaaac tttaatcaca gcggccagct acaagttctg cttaatcgat cctctacttg ctacgacgag

caaggaaaaa aaactgagga ggacagttct tttacactgg aaaatttatc tctttccgcc aacaacaagt taactgcagt

aggctgtaac gctttatcac ttctggacac ttttggaatg caaaactact caactgcatg cttgtcatta tgcgattctc

ccccagaggc tgatggagaa tgtaatggta gaggttgctg cagagtcgac gtttctgccc cgttggatag ctatacattc

gaaactacat caggtcgcat caagcacatg acttcttttc acgactttag tccttgcacc tacgcttttc tcgttgaaga

tgataagttc aacttcagtt ctacagaaga tcttctgaat ctgcgaaatg tcatgaggtt ccctgtgtta ctagattggt

ctgttggaaa tcagacatgc gagcaagttg gaagcacaag catatgcggt gggaacagca cttgtctcga ttctactcct

agaaacgggt atatctgcag atgcaatgaa ggctttgatg ggaatccata cctttcagct ggttgccaag acgtcaatga

gtgtactact agtagtacta tccatagaca taactgttcg gatcccaaaa cctgtagaaa caaggttgga ggcttctatt

gtaagtgtca atctggttac cgcttagata ccaccactat gagctgcaag cgtaaagagt ttgcatggac tacaattctt

Reverse primer 3′-CGTACCTG ATGTCTATAG

GAACAT-5′

cttgtaacca ccatcggctt cttggtcatt ctgcttggcg ttgcctgtat acaacagaga atgaagcacc tgaaggacac

caagctccga gaacaattct tcgagcaaaa tggtggcggc atgttgacac aacgactctc aggagcaggg ccgtcaaatg

ttgatgtcaa aatctttact gaggatggca tgaagaaagc aacaaatggt tatgctgaga gcaggatcct gggtcagggt

ggccaaggaa cagtgtacaa agggatattg ccggacaact ccatagttgc tataaagaaa gcccgacttg gagacagtag

ccaagtagag cagttcatca atgaagtgct cgtgctttca caaatcaacc ataggaacgt agtcaagctc ttgggctgct

gtctagagac tgaagttccc ttgttggtct atgagttcat caccaatggc acccttttcg atcacttgca tggttccatg

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tgcttctatt ccaatcatcc atcgggatat caaaactgca aatattcttc tggatgtaaa cttaactgca aaagtagctg

actttggtgc ttcaaggctg ataccaatgg ataaagaaga gctcgaaact atggtgcaag gcactctagg ttacctagac

ccagaatatt acaacacagg gttgttaaac gaaaagagcg atgtttatag ttttggggtc gtcctaatgg aactgctctc

aggtcaaaag gcattgtgct ttaaacggcc acagtcctca aaacatctgg tgagttactt tgcgactgcc acaaaagaga

ataggttgga tgagattatt ggcggcgaag tgatgaacga ggataatctg aaggagatcc aggaagctgc aagaattgct

gcagagtgta caaggctaat gggagaggaa aggccaagga tgaaagaagt agctgcaaag ctagaagcct tgagggtcga

aaaaaccaaa cataagtggt cggatcagta ccctgaggag aatgaacact tgattggtgg tcacatcttg tcagcacaag

gcgaaaccag tagcagcatt ggctatgaca gcatcaagaa tgtagcaata ttggacattg aaactggccg ctga-3′

(b)

M K V Q E G L F L V A I F F S L A C T Q L V K G Q H Q P G E N C Q N K C G N I T I E Y P

F G I S S G C Y Y P G N E S F S I T C K E D R P H V L S D I E V A N F N H S G Q L Q V L

L N R S S T C Y D E Q G K K T E E D S S F T L E N L S L S A N N K L T A V G C N A L S L

L D T F G M Q N Y S T A C L S L C D S P P E A D G E C N G R G C C R V D V S A P L D S Y

T F E T T S G R I K H M T S F H D F S P C T Y A F L V E D D K F N F S S T E D L L N L R

N V M R F P V L L D W S V G N Q T C E Q V G S T S I C G G N S T C L D S T P R N G Y I C

R C N E G F D G N P Y L S A G C Q D V N E C T T S S T I H R H N C S D P K T C R N K V G

G F Y C K C Q S G Y R L D T T T M S C K R K E F A W T T I L L V T T I G F L V I L L G V

A C I Q Q R M K H L K D T K L R E Q F F E Q N G G G M L T Q R L S G A G P S N V D V K I

F T E D G M K K A T N G Y A E S R I L G Q G G Q G T V Y K G I L P D N S I V A I K K A R

L G D S S Q V E Q F I N E V L V L S Q I N H R N V V K L L G C C L E T E V P L L V Y E F

I T N G T L F D H L H G S M I D S S L T W E H R L K I A I E V A G T L A Y L H S S A S I

P I I H R D I K T A N I L L D V N L T A K V A D F G A S R L I P M D K E E L E T M V Q G

T L G Y L D P E Y Y N T G L L N E K S D V Y S F G V V L M E L L S G Q K A L C F K R P Q

S S K H L V S Y F A T A T K E N R L D E I I G G E V M N E D N L K E I Q E A A R I A A E
C T R L M G E E R P R M K E V A A K L E A L R V E K T K H K W S D Q Y P E E N E H L I G
G H I L S A Q G E T S S S I G Y D S I K N V A I L D I E T G R **Stop**

Nucleotide (a) and amino acid (b) sequences of the *WAK1* gene from *Arabidopsis thaliana*. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domin, EcoRV, kinase domain and stop codon. The palindrome incorporation resulted in two changes; a hydrophilic isoleucine (I) to a polar negative aspartic acid (D) and a hydrophobic leucine (L) to a hydrophobic isoleucine (I). The primers will amplify a 1020 bp fragment.

APPENDIX 5

(a)

5′-atgagtaaat tacttgtcat

agccctcctg ctgctgcctc tgatcaacca cggaatctac ttggccacgg catgggatga tcaagatttc ttcaaatact
gcccaccttc caagtgcagc caacatggcc caatgatcag gtatcctctc tgccttgaat ccagcaatac atcatcatca
tcgtcatgtg ggtgtgccgg cagatcaatc tggaagttag catgctctgg tcaagacacc atcctagtcc acccagttct
tggcccatac agtgtcagcg ccatagatta cagacgttct tccatgaaga tcaccccgct tgtagacccc tgtttggtgc
tccagcagaa gctcatcatc tccagaagct cgtcatctcc acaggttgat gttatcaacg atgaaaagcc aagttttgac
gaaaatttct ttgagagttc atctgcaacc atagtacact gttcaagaga gttcacgcct gctgctgccc atgccgatag
cattgcaggc ccagtctcct gccttagcaa cacaacccac ttcttttatt tggtgaatag tgatgaagac atgtctattc
ttccgttgga ctgcaaggtc gtcccagtct cagatcgagg tggcatctcg ttaccgcata tgcttaaaga ccaaatgttc
tataacttca cggaaaccgc aaaaaaaatc cccagttttg ctgagacggc agtgtcttgg gatgagggag actgcagaga
atgtgaactc agtgggcgac gctgcgcgtt cagctcacaa agggatagag aattctgcat

Forward primer

5′-ATTT GCAGGTTCAG ATATCAAAG-3′

gcctgaattt gcaggttcac atatcaaagt cattgcagct acatcatcag tggccgcgtt tgttgctctt ttgttgacgg
tggccactgt gctttatctt tcactcaaga caagatataa tgcggagata catatgaagg ttgaaatgtt tctcaagaca
tatggaacat caaaaccgac aaggtacact ttctctgaag ttaagaagat ggcaagacgg tttaaggaaa aagtagggca
gggaggattt ggaagtgtgt acaaaggtga gctaccaaat ggagtgcctg tggcagtcaa gatgctagag aactctacag
gagagggaga atcgttcatc aatgaagttg caaccatcgg actaatccac catgccaata ttgtccgcct cctgggattt
tgttccgaag gaatgaggcg ggctcttatt tatgaattca tgcctaatga gtcactggag aaatacatat tctctgacga
ctctaatatt tttcagaatc ttctagtacc agagaagctg ctagatattg ctttaggcat cgcgcgagga atggagtact
tgcatcaagg gtgcaaccag cgcatcctcc actttgacat caagcctcac aatatcctgc ttgactacaa cttcaatcca
aagatctcag actttgggct tgcaaagctg tgcgcgaggg accaaagcat cgtcacctta actgcagcaa gaggcacgat
gggctacatt gcaccggagc tatattcccg gaactttggg ggagtatcgt acaaggcaga cgtgtacagt ttcggcatgc
tggtgctaga aatggtgagc gggaggagga attcagaccc aagaatcggg agccaggacg atgtttacct cccagagtgg
atctacgaga aagtgatcaa tggggaggag ttggcgctta ctttggaaac gactcaggaa gagaaagaca aggtgaggca
gctggcaatg gttgcactgt ggtgtatcca gtggaacccg agaaaccgtc cgtcgatgac gaaggttgtt aacatgctaa
cagggaggtt gcagagtctg cagatgcccc cgaagccctt cgtctcatct gaaaatgaac
ttatgtcata aattctgtag agtcgcgcgg acta-3′

3′-TAAGACATC TCAGCGCGCC TGAT-5′

Reverse primer

(b)

M S K L L V I A L L L L P L I N H G I Y L A T A W D D Q D F F K Y C P P S K C S Q H G P
M I R Y P L C L E S S N T S S S S S C G C A G R S I W K L A C S G Q D T I L V H P V L G
P Y S V S A I D Y R R S S M K I T P L V D P C L V L Q Q K L I I S R S S S S P Q V D V I
N D E K P S F D E N F F E S S S A T I V H C S R E F T P A A A H A D S I A G P V S C L S
N T T H F F Y L V N S D E D M S I L P L D C K V V P V S D R G G I S L P H M L K D Q M F
Y N F T E T A K K I P S F A E T A V S W D E G D C R E C E L S G R R C A F S S Q R D R E
F C M P D P H G S D I K V I A A T S S V A A F V A L L L T V A T V L Y L S L K T R Y N A
E I H M K V E M F L K T Y G T S K P T R Y T F S E V K K M A R R F K E K V G Q G G F G S
V Y K G E L P N G V P V A V K M L E N S T G E G E S F I N E V A T I G L I H H A N I V R
L L G F C S E G M R R A L I Y E F M P N E S L E K Y I F S D D S N I F Q N L L V P E K L
L D I A L G I A R G M E Y L H Q G C N Q R I L H F D I K P H N I L L D Y N F N P K I S D
F G L A K L C A R D Q S I V T L T A A R G T M G Y I A P E L Y S R N F G G V S Y K A D V
Y S F G M L V L E M V S G R R N S D P R I G S Q D D V Y L P E W I Y E K V I N G E E L A
L T L E T T Q E E K D K V R Q L A M V A L W C I Q W N P R N R P S M T K V V N M L T G R
L Q S L Q M P P K P F V S S E N E L M S Stop

Nucleotide (a) and amino acid (b) sequences of the *LRK10* gene from *Triticum aestivum* with the EcoRV palindrome. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, EcoRV, kinase domain and stop codon. Palindrome incorporation resulted in a single amino acid change; from hydrophilic cysteine (C) to a hydrophilic glycine (G). The primers will amplify a 1249 bp fragment.

APPENDIX 6

(a)

Forward primer

5′-CACAAACTCT TGAGAAATGA AGACTTT-3′

5′-cacaaactct tgagaaatga agacttttttc aagcttcttt ctct

ctgtaacaac tctcttcttc ttctccttct tttctctttc atttcaagct tcaccatctc agtctttata cagagaaatc

catcagctta taagcttcaa agacgttctt cctgacaaga atcttctccc agactggtct tccaacaaaa acccgtgtac

tttcgatggc gttacttgca gagacgacaa agttacttcg attgatctca gctccaagcc tctcaacgtc ggattcagtg

ccgtgtcctc gtctctcctg tctctcaccg gattagagtc tctgtttctc tcaaactcac acatcaatgg ctccgtttct

ggcttcaagt gctctgcttc tttaaccagc ttggatctat ctagaaactc tctttcgggt cctgtaacga ctctaacaag

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atttctctgt cgaataaccg gttaaccggt gagattccga aatggattgg ccggttagag aatctcgcta tcctcaagct

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taaattctgtag agtcgcgcgg acta-3′

3′-TAAGACATC TCAGCGCGCC TGAT-5′

Reverse primer

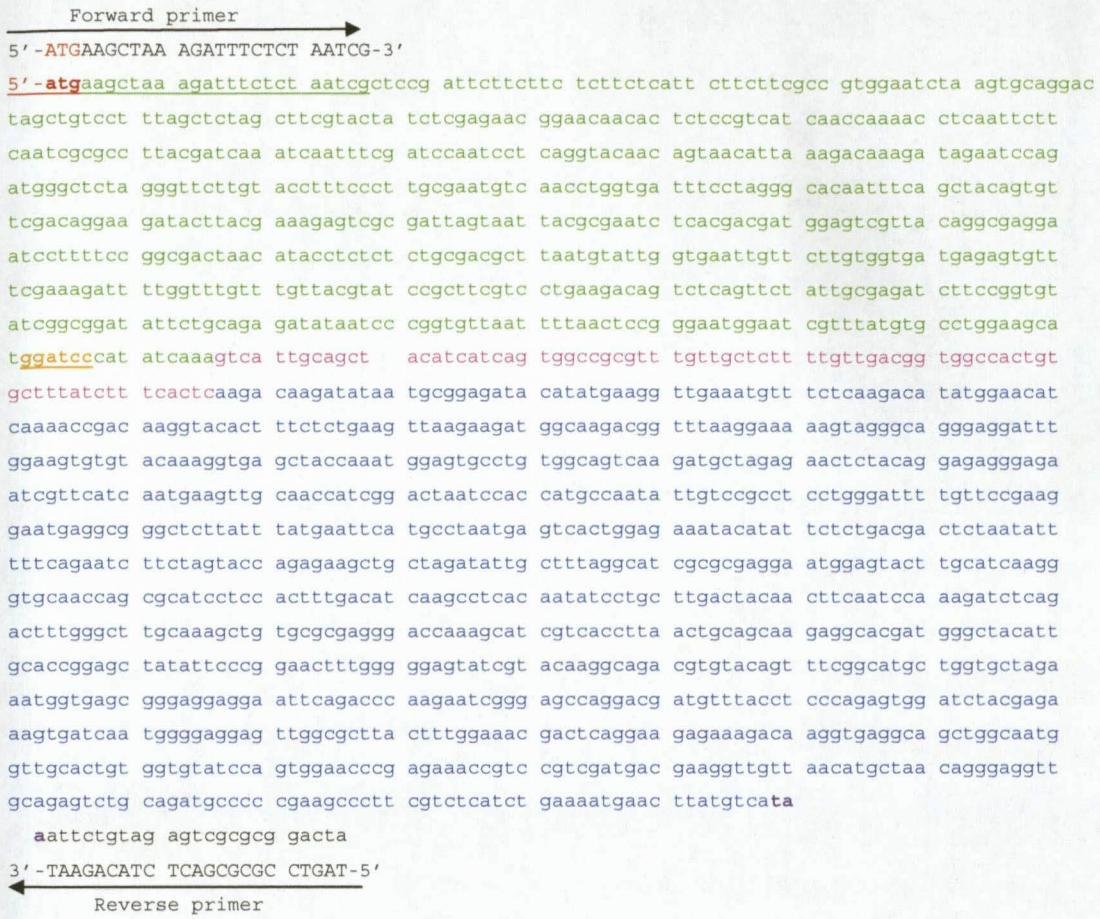
(b)

MKTFSSFFLSVTTLFFFSFFSLSFQASPSQSLYREIHQLISFKDVLP
DKNLLPDWSSNKNPCTFDGVTCRDDKVTSIDLSSKPLNVGFSAVSSS
LLSLTGLESLFLSNSHINGSVSGFKCSASLTSLDLSRNSLSGPVTTL
TSLGSCSGLKFLNVSSNTLDFPGKVSGGLKLNSLEVLDLSANSISGA
NVVGWVLSDGCGELKHLAISGNKISGDVDVSRCVNLEFLDVSSNNFS
TGIPFLGDCSALQHLDISGNKLSGDFSRAISTCTELKLLNISSNQFV
GPIPPLPLKSLQYLSLAENKFTGEIPDFLSGACDTLTGLDLSGNHFY
GAVPPFFGSCSLLESLALSSNNFSGELPMDTLLKMRGLKVLDLSFNE
FSGELPESLTNLSASLLTLDLSSNNFSGPILPNLCQNPKNTLQELYL
QNNGFTGKIPPTLSNCSELVSLHLSFNYLSGTIPSSLGSLSKLRDLK
LWLNMLEGEIPQELMYVKTLETLILDFNDLTGEIPSGLSNCTNLNWI
SLSNNRLTGEIPKWIGRLENLAILKLSNNSFSGNIPAELGDCRSLIW
LDLNTNLFNGTIPAAMFKQSGKIAANFIAGKRYVYIKNDGMKKECHG
AGNLLEFQGIRSEQLNRLSTRNPCNITSRVYGGHTSPTFDNNGSMMF
LDMSYNMLSGYIPKEIGSMPYLFILNLGHNDISGSIPDEVGDLRGLN
ILDLSSNKLDGRIPQAMSALTMLTEIDLSNNNLSGPIPEMGQFETFP
PAKFLNNPGLCGYPLPRCDPSNADGYAHHQRSHGRRPHGSHIKVIAA
TSSVAAFVALLLTVATVLYLSLKTRYNAEIHMKVEMFLKTYGTSKPT
RYTFSEVKKMARRFKEKVGQGGFGSVYKGELPNGVPVAVKMLENSTG
EGESFINEVATIGLIHHANIVRLLGFCSEGMRRALIYEFMPNESLEK
YIFSDDSNIFQNLLVPEKLLDIALGIARGMEYLHQGCNQRILHFDIK
PHNILLDYNFNPKISDFGLAKLCARDQSIVTLTAARGTMGYIAPELY
SRNFGGVSYKADVYSFGMLVLEMVSGRRNSDPRIGSQDDVYLPEWIY
EKVINGEELALTLETTQEEKDKVRQLAMVALWCIQWNPRNRPSMTKV
VNMLTGRLQSLQMPPKPFVSSENELMS Stop

Nucleotide (a) and amino acid (b) sequences of the *BRI1-LRK10* recombinant gene. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The primers will amplify a 3641 bp fragment which will code for a polypeptide of 126 kDa.

APPENDIX 7

(a)


Forward primer
5′-ATGAAGCTAA AGATTTCTCT AATCG-3′
5′-atgaagctaa agatttctct aatcgctccg attcttcttc tcttctcatt cttcttcgcc gtggaatcta agtgcaggac
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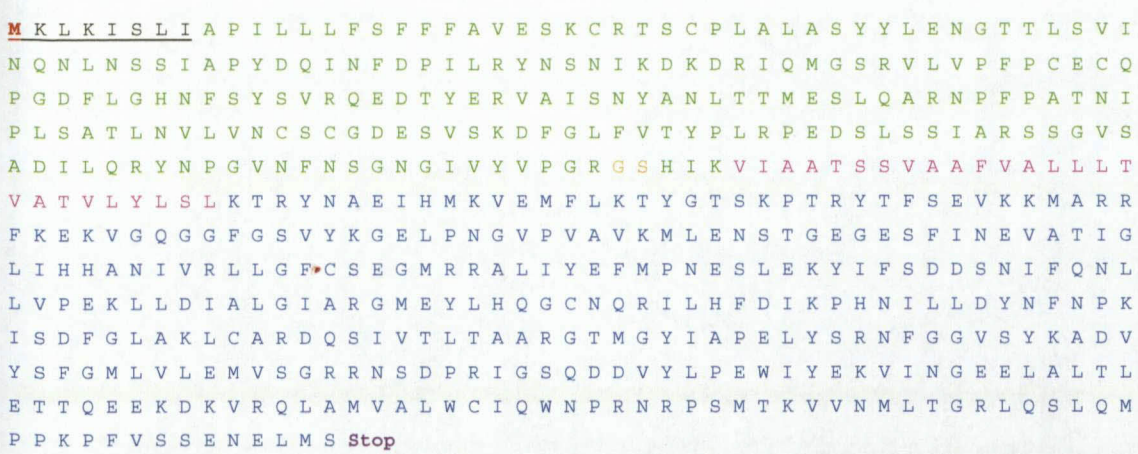
gcagagtctg cagatgcccc cgaagccctt cgtctcatct gaaaatgaac ttatgtcata
aattctgtag agtcgcgcg gacta
3′-TAAGACATC TCAGCGCGC CTGAT-5′
Reverse primer

(b)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N Q N L N S S I A P Y D Q I N F D P I L R Y N S N I K D K D R I Q M G S R V L V P F P C E C Q
P G D F L G H N F S Y S V R Q E D T Y E R V A I S N Y A N L T T M E S L Q A R N P F P A T N I
P L S A T L N V L V N C S C G D E S V S K D F G L F V T Y P L R P E D S L S S I A R S S G V S
A D I L Q R Y N P G V N F N S G N G I V Y V P G R G S H I K V I A A T S S V A A F V A L L L T
V A T V L Y L S L K T R Y N A E I H M K V E M F L K T Y G T S K P T R Y T F S E V K K M A R R
F K E K V G Q G G F G S V Y K G E L P N G V P V A V K M L E N S T G E G E S F I N E V A T I G
L I H H A N I V R L L G F C S E G M R R A L I Y E F M P N E S L E K Y I F S D D S N I F Q N L
L V P E K L L D I A L G I A R G M E Y L H Q G C N Q R I L H F D I K P H N I L L D Y N F N P K
I S D F G L A K L C A R D Q S I V T L T A A R G T M G Y I A P E L Y S R N F G G V S Y K A D V
Y S F G M L V L E M V S G R R N S D P R I G S Q D D V Y L P E W I Y E K V I N G E E L A L T L
E T T Q E E K D K V R Q L A M V A L W C I Q W N P R N R P S M T K V V N M L T G R L Q S L Q M
P P K P F V S S E N E L M S Stop

Nucleotide (a) and amino acid (b) sequences of the *CERK1-LRK10* recombinant gene. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The primers will amplify a 1899 bp fragment which will code for a polypeptide of 64.6 kDa.

APPENDIX 8

(a)

Forward primer

5′-ATGTCTCGTGAACTTATTATTCTCTGCC-3′

5′-atgtctcgtg aacttattat tctctgccaa ccaattcttg ttctgttcct tactctgttt tacaattctc atggctactt

cgtctcacaa ggatctgtag gaatcggctt caacggttac ttcactttaa ccaacaccac aaaacacaca ttcggtcaag

ctttcgaaaa cgagcacgtt gaaatcaaga actcatcgac aggtgtcatc tcatctttct cagtcaactt cttctttgcg

atcgtccctg aacataacca gcaaggctca cacggtatga ctttcgtcat ctctcccaca agaggccttc ccggagcttc

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tcttgtcttt gaaccgagat ctctcgccgt atttgcttga gaaaatgtat cttggattca ctgcatcgac ggggtcagtt

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gaaggttgtt aacatgctaa cagggaggtt gcagagtctg cagatgcccc cgaagccctt cgtctcatct gaaaatgaac

ttatgtcata attctgtag agtcgcgcgg acta-3′

3′-TAAGACATC TCAGCGCGCC TGAT-5′

Reverse primer

(b)

M S R E L I I L C Q P I L V L F L T L F Y N S H G Y F V S Q G S V G I G F N G Y F T L T N T T

K H T F G Q A F E N E H V E I K N S S T G V I S S F S V N F F F A I V P E H N Q Q G S H G M T

F V I S P T R G L P G A S S D Q Y L G I F N K T N N G K A S N N V I A I E L D I H K D E E F G

D I D D N H V G I N I N G L R S V A S A S A G Y Y D D K D G S F K K L S L I S R E V M R L S I

V Y S Q P D Q Q L N V T L F P A E I P V P P L K P L L S L N R D L S P Y L L E K M Y L G F T A

S T G S V G A I H Y L M G W L V N G V I E Y P R L E L S I P V L P P Y P K K T S N H G S H I K

V I A A T S S V A A F V A L L L T V A T V L Y L S L K T R Y N A E I H M K V E M F L K T Y G T

S K P T R Y T F S E V K K M A R R F K E K V G Q Q G G F G S V Y K G E L P N G V P V A V K M L E

N S T G E G E S F I N E V A T I G L I H H A N I V R L L G F C S E G M R R A L I Y E F M P N E

S L E K Y I F S D D S N I F Q N L L V P E K L L D I A L G I A R G M E Y L H Q G C N Q R I L H

F D I K P H N I L L D Y N F N P K I S D F G L A K L C A R D Q S I V T L T A A R G T M G Y I A

P E L Y S R N F G G V S Y K A D V Y S F G M L V L E M V S G R R N S D P R I G S Q D D V Y L P

E W I Y E K V I N G E E L A L T L E T T Q E E K D K V R Q L A M V A L W C I Q W N P R N R P S

M T K V V N M L T G R L Q S L Q M P P K P F V S S E N E L M S Stop

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BLOEMFONTEIN
BIBLIOTEEK - LIBRARY

Nucleotide (a) and amino acid (b) sequences of the *LecRK1-LRK10* recombinant gene. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, BamHI, kinase domain and stop codon. The primers will amplify a 2089 bp fragment which will code for a polypeptide of 71 kDa.

APPENDIX 9

(a)


Forward primer
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5′-atgaaggtgc aggagggttt gttcttggtg gctattttct tctcccttgc gtgt
acgcagctgg tgaaggggca acatcaacct ggtgagaatt gccaaaataa atgtggcaac atcacaatag agtacccttt
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cagatgcccc cgaagccctt cgtctcatct gaaaatgaac ttatgtcata attctgtag agtcgcgcgg acta
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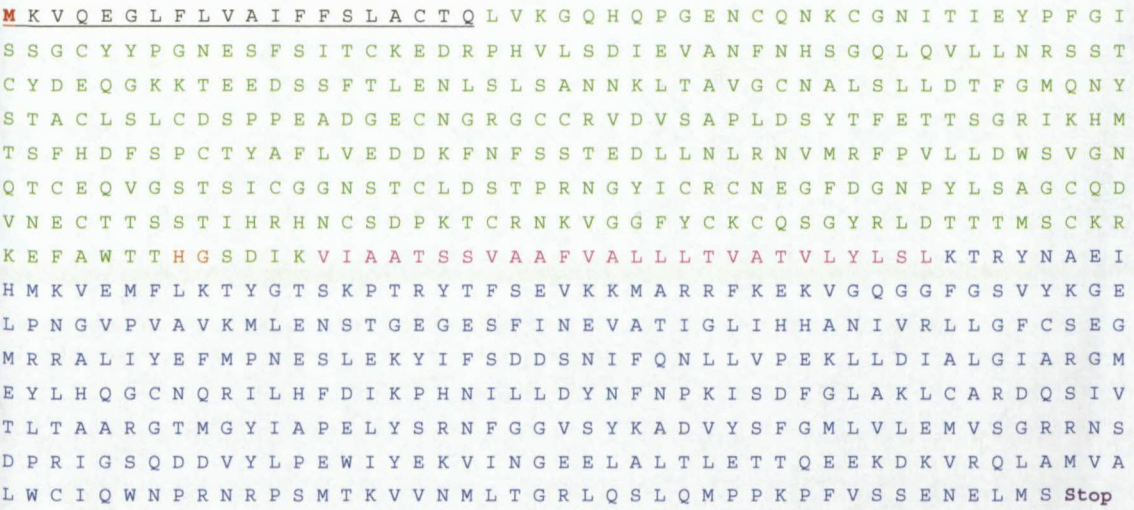
Reverse primer

(b)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S S G C Y Y P G N E S F S I T C K E D R P H V L S D I E V A N F N H S G Q L Q V L L N R S S T
C Y D E Q G K K T E E D S S F T L E N L S L S A N N K L T A V G C N A L S L L D T F G M Q N Y
S T A C L S L C D S P P E A D G E C N G R G C C R V D V S A P L D S Y T F E T T S G R I K H M
T S F H D F S P C T Y A F L V E D D K F N F S S T E D L L N L R N V M R F P V L L D W S V G N
Q T C E Q V G S T S I C G G N S T C L D S T P R N G Y I C R C N E G F D G N P Y L S A G C Q D
V N E C T T S S T I H R H N C S D P K T C R N K V G G F Y C K C Q S G Y R L D T T T M S C K R
K E F A W T T H G S D I K V I A A T S S V A A F V A L L L T V A T V L Y L S L K T R Y N A E I
H M K V E M F L K T Y G T S K P T R Y T F S E V K K M A R R F K E K V G Q G G F G S V Y K G E
L P N G V P V A V K M L E N S T G E G E S F I N E V A T I G L I H H A N I V R L L G F C S E G
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E Y L H Q G C N Q R I L H F D I K P H N I L L D Y N F N P K I S D F G L A K L C A R D Q S I V
T L T A A R G T M G Y I A P E L Y S R N F G G V S Y K A D V Y S F G M L V L E M V S G R R N S
D P R I G S Q D D V Y L P E W I Y E K V I N G E E L A L T L E T T Q E E K D K V R Q L A M V A
L W C I Q W N P R N R P S M T K V V N M L T G R L Q S L Q M P P K P F V S S E N E L M S Stop

Nucleotide (a) and amino acid (b) sequences of the *WAK1-LRK10* recombinant gene. The different regions are as indicated: signal peptide, start codon, extracellular domain, TM domain, EcoRV, kinase domain and stop codon. The primers will amplify a 1671 bp fragment which will code for a polypeptide of 78 kDa.