Identification of molecular markers linked to multiple rust resistance traits in Kazakh bread wheat

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A thesis submitted for the Master degree of Biotechnology studies

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Declaration

I certify that this thesis does not content material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not content of any material previously published or written by another person except where due reference is made in the text of this thesis

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Abbreviation

AFLP	Amplified fragment length polymorphism
Bp	Base pairs
CAPS	Cleaved Amplified Polymorphic Sequences
CIMMYT	Centro Internacionale de Mejoramiento de Maiz y Trigo (International Maize and Wheat Improvement Centre)
DNA	Deoxy ribose nucleic acid
dATP	deoxy ribose adenosine tri-phosphate
ECA	Eastern Europe and Central Asia
EDTA	Ethylenediamine tetrachloroacetic acid,-sodium salt
FAM	6-Fluorescein Phosphoramidite
FAO	Food and Agriculture Organisation of the United Nations
GOI	Gene of interest
Ha	Hectare
HEX	6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein succinimidyl ester
IPTG	Isopropylthio-*b*-D-galactoside
LB	Luria-Bertani medium
Lr	Leaf rust
MAS	Marker-Assisted Selection
NCBI	National Central for Biotechnology Information
NEB	Neutral electrophoretic buffer
NRFU	Normalized Relative Fluorescent Unit

PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RUK	Russia, Ukraine and Kazakhstan
SNP	Single Nucleotide Polymorphism
Sr	Stem rust
TBE	Tris-borate EDTA
X-gal	5-Bromo-4-chloro-3-indolyl-*b*-D-galactoside
RFU	Relative fluorescent unit

Abstract:

Bread wheat (*Triticum aestivum*), is considered as the second most important staple crop in the world after rice, providing the food for approximately one third of the world's population. It represents approximately 20% of the global major cereal crop production with 759.5 million tonnes (FAO, 2018), and thus plays a key role in global food security. Due to the increasing food demand of an increasing human population, bread wheat production is only likely to increase in its importance in the future (Tadesse et al. 2016). Of the approximately 46 fungal diseases of wheat that have been documented, rust diseases are the most economically important. Controlling these destructive fungal diseases through rust resistance breeding is the most effective and environmentally friendly approach to minimize the yield losses and bring more financial benefits to plant breeders, farmers and consumers (Singh, 1998; Vida, 2009; Herrera, 2011). To ensure a diverse pool of genetic-based resistance is available for breeding, identification of new and effective major rust resistance genes from different germplasms of global wheat cultivars is essential.

Recent advances in molecular marker technology have created effective tools for selective breeding which has several advantages over traditional phenotype-based trait selection. Marker-assisted selection (MAS) has also been widely used to target wheat rust resistance genes. Molecular markers offer powerful tools to tag rust resistance genes and as a part of MAS can be used in the improvement of plant breeding efficiency. This research project aims to identify PCR-based markers associated with important leaf and stem rust resistance genes, and then develop these markers into highly accurate and easy-applicable PCR-based markers for using in breeding programs. In this study, PCR-based molecular markers used for MAS included; length SSR (Simple sequence repeat) polymorphic markers, Cleaved amplified polymorphic sequences (CAPS), and SNP (Single nucleotide polymorphism). The aims of this research are;

1) To identify the PCR-based markers (length polymorphic marker, SNP and CAPS markers) linked to rust resistance genes in Kazakh wheat varieties the identified disease resistance genes.
2) To develop a MAS strategy using the identified molecular markers to detect the candidate resistance genes in selected Kazakh bread wheat varieties.

The results of recent work provided further information about polymorphic fragments of *Lr22a* and *Sr2* amplified from different homoeologous genomes. The complete sequences of cloned fragments will be useful for genome-specific primer design to avoid the cross-amplification in the PCR of target in paralogs from the same genome and from homologs and paralogs in the homoeologuos genomes. In addition, one SNP identified at recognition site of *Bco*DI restriction enzyme can be used for developing CAPS marker, allowing the differentiation PCR products from target genome than other genomes. Furthermore, the successful development of CAPS marker for *Lr51* revealed an additional easy, cheap and reliably score-able marker which can be routinely used to track *Lr51* in not only Kazakh wheat varieties but also worldwide wheat germplasms. More importantly, the designed Amplifluor-like SNP system showed the level of effectiveness and accuracy in allelic discrimination of *Lr*51, indicating the potential use of this cost-effective genotyping technique in future genotyping studies involving this leaf rust resistance trait.

Chapter 1: Literature review

1.1 . Introduction:

Leaf and stem rusts, caused by the fungal pathogens *Puccinia triticina* Eriks. (Formerly *P. recondita* f. sp. *tritici Rob. ex Desm.)*, and *Puccinia graminis f*. sp. *tritici* West, respectively, are two of the most devastating pathogens of cultivated wheat. They have recently caused up to 50% and 30% yield losses in Europe, respectively (Draz et al, 2015; Leonard and Srabo, 2005). Due to the airborne transmission of rust pathogens, disease outbreaks can spread rapidly, threatening global wheat production. Many studies have shown that selection for disease resistance is not only crucial for breeding programs, but also offers the financial benefits to the major wheat producers in the world.

The main goal of this project is to develop PCR-based molecular markers linked to important leaf and stem rust resistance genes in bread wheat. Major criteria set for the markers are their accuracy, affordability and ease of use. The utilization of well-functioning molecular markers will facilitate the marker-assisted selection (MAS) of rust resistance in modern wheat breeding programmes.

This chapter will provide a justification of work presented here by explaining (1) wheat taxonomy and its importance in global food security, and (2) an overview of fungal rust pathogens and their evolution to fungicide resistance and virulence to resistance genes that negatively affect wheat production and quality. Following this will be a summary of the relevant literature providing (3) the basis of the four types of DNA molecular markers used in this project and (4) their potential applications in MAS as a novel tool for disease resistance breeding. The chapter will conclude with (5) a discussion of the challenges and perspectives for future molecular marker-based breeding.

1.2. Bread wheat (*T. aestivum*) taxonomy and its importance in global food security

1.2.1. Bread wheat (Triticum aestivum) taxonomy

Wheat (*Triticum* spp.), a part of the Poaceae family, is believed to be one of the first cereal grains that humans domesticated approximately 10,000 year B.C (Feldman, 2001). It is divided into groups according to the number of chromosomes in their genomes: diploid (2n=2x=14) (seven pairs e.g. einkorn wheat); tetraploid (2n=4x=28) (14 pairs e.g. durum wheat); and

hexaploid (2n=6x=42) (21 pairs e.g. bread wheat). Bread wheat or common wheat (*T. aestivum*) is a hexaploid species consisting three homeologous genomes (AABBDD, 2n = 42) (Gupta et al., 2002). The genome of bread wheat is much bigger than other cereal crops, being 8x and 40x larger than that of maize and rice, respectively (Arumuganathan and Earle, 1991). Bread wheat originated from hybridisation between cultivated emmer (*T. dicoccum* AABB, 2n = 28) and the wild goat grass *T. tauschii* (DD, 2n = 14) approximately 8000-10000 years ago. The evolution of bread wheat is shown in Figure 1.

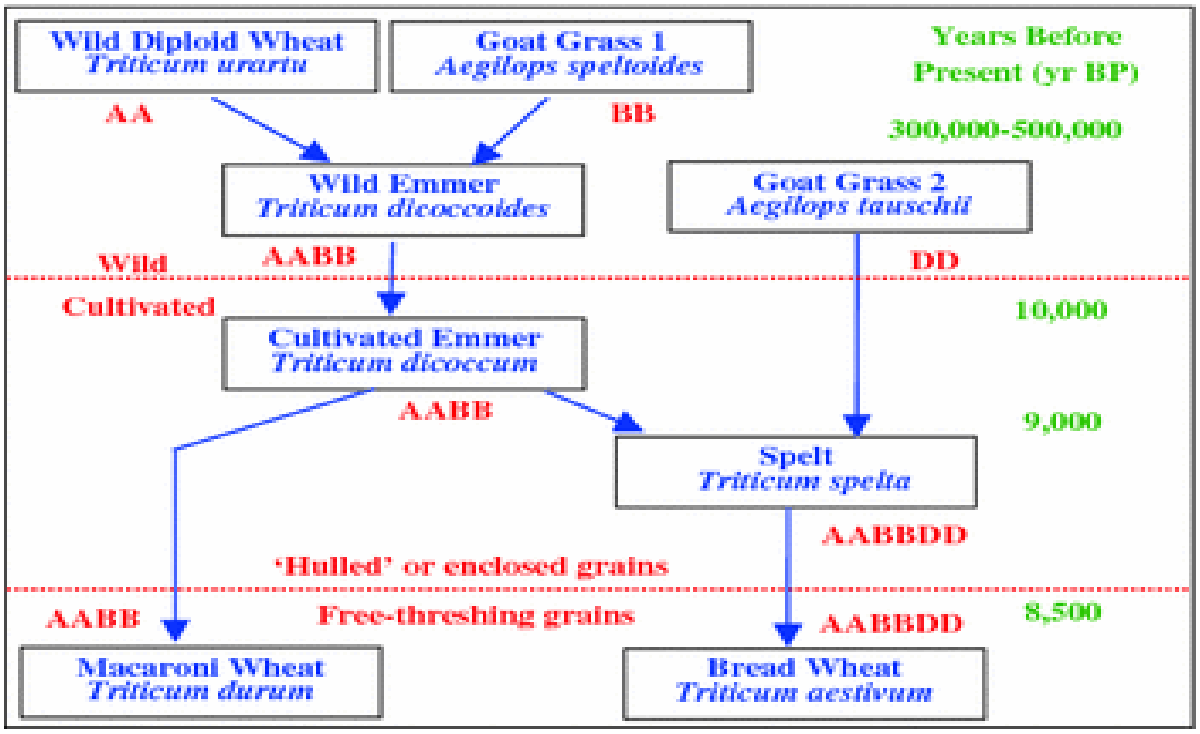

Figure 1. The evolution of cultivated wheat, from the prehistoric grasses to durum and bread wheats (adapted from http://www.newhallmill.org.uk/wht-evol.htm and Peng et al. (2011).

Bread wheat, accounting for 80% of the wheat consumed in the world, is a cultivated wheat species widely grown in a range of weather conditions, from warm, humid to dry and cold environments (Nuttonson, 1957). The optimal temperature for most wheat cultivars is however, approximately 25 °C, with minimum and maximum temperatures of 3-4 °C and 30-32 °C, respectively. The wide adaptation of bread wheat to a range of climatic conditions, geographical regions is related to its large and complex genome (Gupta et al., 2008).

1.2.2. The importance of bread wheat

Wheat is considered as the second most important crop after rice (Tadesse et al., 2016). Rice, wheat, corn and potatoes are the leading food staples and rank in this order of importance with wheat being the national food staple in 43 countries. Wheat is often passed off as merely a starchy food crop. It contains however other valuable nutritive materials, notably proteins, minerals, and vitamins. The minerals and vitamins are of significant nutritional value, especially in foods derived from whole grain products and enriched flour.

According to FAO (FAOSTAT, 2018), in 2017/2018, the global production volume of wheat amounted to approximately 759.75 million metric tons. Eastern Europe and Central Asia (ECA) region is recently considered as bread baskets of the world providing almost a quarter of world wheat exports due to the large wheat production and exports with their immense land and yield reserves (Glauben et al., 2014). With total wheat production of 161.5 million tons in 2017, this region has significant contribution to global food security, accounting for 18% of global wheat production and 22% of world's wheat exports.

Among leading wheat producers in ECA, Kazakhstan is one of major producers and exporters with approximately 17.2 million tons in 2017 and expected to increase to 19.5 million tons in 2018. The acreage was estimated at 217.0 million ha of agriculture land used. (Swinnen et al., 2017). These figures reveal a significant contribution of Kazakhstan for global wheat production in comparison with Australia, an important wheat producer in the world with estimated production of 23.3 tons in 2017/2018 period (according to Department of Agriculture and Water resources, Australia). Collectively, these data emphasises the importance of bread wheat to global food security.

In terms of global food demand, annual wheat demand is proposed to rise at a rate of 1.6%/pa until 2050 (Grafton et al., 2015). Consequently, the average global yield of wheat will need to rise from approximately 3 tons per ha currently, to 5 tons per ha to meet the increasing demand. This means that wheat is an important food crop for the planet, so that we not only need to secure its production but also increase it to meet the demands of a growing population. This is becoming increasingly difficult with a changing climate and more importantly, the constant threat of pests and diseases. Historically, wheat rusts, the most devastating pathogens, have caused significant reduction of yield worldwide (Singh et al., 2005; Park, 2008; Vurro et al., 2010; Fetch et al., 2011). Therefore, how to control these fungal agents

are the central of disease management to date. In practice, the detailed information of pathogen's life cycle, alternative hosts, their genetic evolution against fungicides and a thorough consideration of methods used to control rusts is necessary for better strategy of disease management.

1.3. Fungal diseases in bread wheat and their evolution to fungicide resistance and virulence to resistance genes

1.3.1. The leaf and stem rusts

The stem, leaf and stripe rusts are the most important diseases that cause production losses in wheat. However, this study has focus on leaf rust and stem rust (and their corresponding resistance genes) as they are considered as the most devastating pathogens in Kazakh wheat varieties in the time of study. One or both these rusts can be found wherever wheat is grown and all are an ever-present threat to high productivity of the crop. Wheat rust pathogens belong to the genus *Puccinia*, family Pucciniaceae, order Uredinales and class Basidiomycetes (Bolley, 1889). Both stem and leaf rusts are bio-trophic fungi that attack only living parts or tissues of plant. They are both highly specialized and have narrow host ranges. The prevalence of each varies greatly from year to year and region to region.

While the three types of rusts have many characteristics in common, their differences are sufficient to consider each separately. The following sections provide an overview of their life cycle, alternate hosts and symptoms on leaf and stem.

Leaf rust **Stem rust**

Figure 2. Uredinial stages of leaf rust and stem rust (Source: Cereal rust, USDA). Source: USDA (https://www.ars.usda.gov/midwest-area/stpaul/cereal-disease-lab/docs/cereal-rusts/cereal-rusts/)

Considering the economic importance of rust fungi, a solid understanding of their life cycles is essential. The life cycle of rust fungi can be very complicated with five spore stages: basidospores, pycniospores, aeciospores, urediospores and teliospores. One stage visible to the naked eyes is uredinial (Figure 2) in which the presence of teliospores on leaf or stem surface can be observed.

Characteristics of stem rust

Wheat stem rust is caused by *Puccinia graminis* f. sp. *Tritici* West. Stem rust is called black rust due to the formation of black teliospores at the end of the growing season. Given proper environmental conditions, this fungus can attack all above ground parts of the plant such as leaves, leaf sheaths, stem and spikes. The infection of stem rust leads to the disruption of nutritional transport, root growth reduction and stem breakage (Knott, 1989). On wheat stems or leaf sheaths, stem rust produces dark brownish-red, elongate pustules. The pustules burst through the epidermis and remnants of it give the pustules a ragged appearance. On leaves, the pustules can be of various sizes and shapes but on young leaves of fully susceptible plants they are often diamond shaped. On older leaves the pustules tend to be restricted by the veins. Pustules sporulate on both leaf surfaces but tend to be heavier on the lower surface.

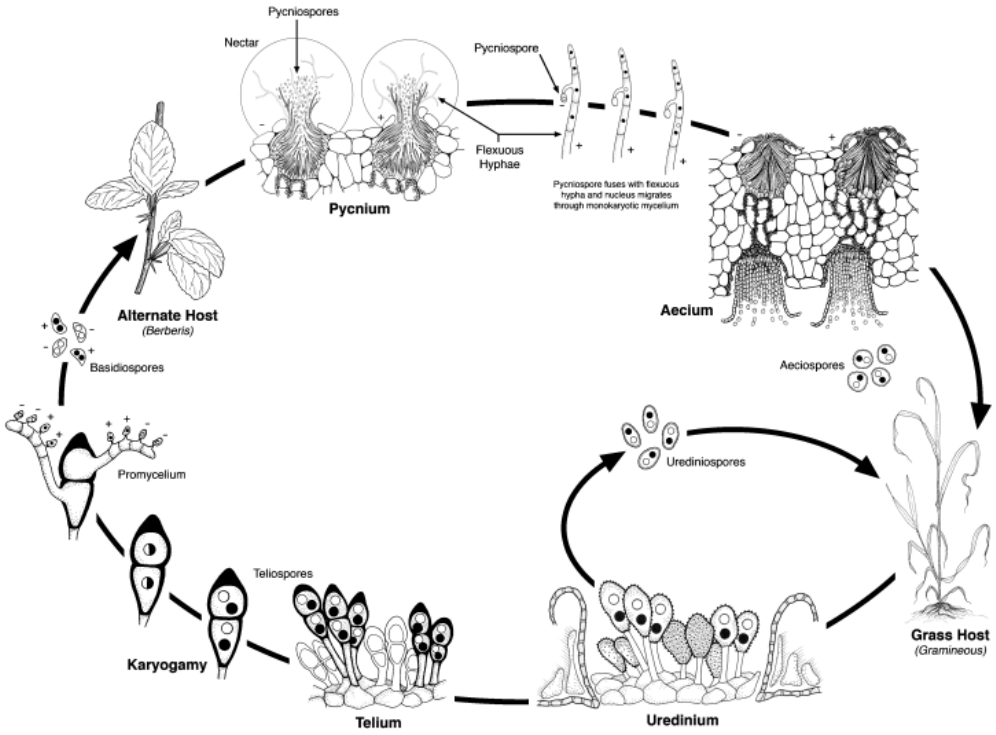

Figure 3. Life cycle of *Puccinia graminis* showing both primary and alternate hosts. Adapted from Leonard and Szabo (2005)

With five spore forms (basidospores, pycniospores, aeciospores, urediospores and teliospores), cereal rusts are heteroecious that attack not only primary host (wheat, barley or grass) but also alternate host (*Berberis*) to complete the sexual cycle. Basically, the host alternation takes place after urediospores and basidospores stages.

Characteristics of Leaf rust

Wheat leaf rust, which is also called brown rust, is caused by *Puccinia triticina* Eriks. This disease is characterized by the development of small, round, brownish-red uredia that occur principally on the leaves. This is the most common and widely distributed disease in wheat that the infection can take place on leaf blades, leaf sheaths and glumes, leading to the premature defoliation of wheat plants (Knott, 1989). The typical symptoms of leaf rust are small round, orange-red pustules, often about 0.2 cm in diameter. The pustules are largely on the upper leaf surface. They are readily distinguishable from stem rust pustules on leaves by their smaller size, round shape, and orange-red colour. The surface layer of spores may darken but it can be wiped off with a finger to reveal the true colour. In a severe epidemic, almost the entire surface of the leaf blades can be covered with pustules. The leaves senesce rapidly and dry out, depriving the plant of much of its photosynthetic area.

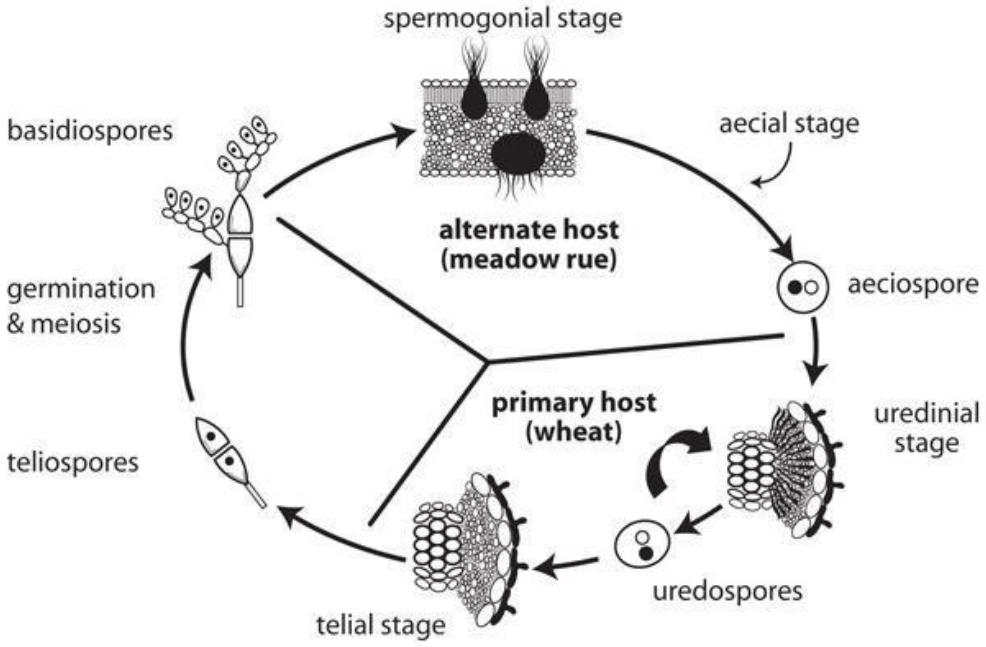

Figure 4. Life cycle of *Puccinia triticinia*, showing both primary and alternate hosts. Adapted from Alexopoulos et al. (1996)

Leaf rust have the five common spore stages with stem rust, however, this pathogen differs from stem rust in alternate hosts. After the basidospores stage, the incomplete spores require alternate (meadow rue) to complete their life cycle.

The following table (Table 1) summarize the similarities and differences between these pathogens which are mentioned above.

Table 1. The causative agent, alternate hosts and symptoms of leaf and stem rust in wheat (Loegering, 1967)

Disease	Pathogen	Primary hosts	Alternate hosts	Symptoms
Leaf rust	*Puccinia triticina*	Bread and durum wheats, triticale	*Thalictrum, Anchusa, Isopyrum, Clematis*	Isolated uredinia on upper leaf surface and rarely on leaf sheaths
Stem rust	*Puccinia graminis* f. sp. *tritici*	Bread and durum wheats, barley, triticale	*Berberis vulgaris*	Isolated uredinia on upper and lower leaf surfaces, stem and spikes

1.3.2. Host-Pathogen interaction

Naturally, plants have their adapted mechanisms to protect themselves from invasion of pathogens such as sticky or waxy surface and the release of anti-microbial compounds. As a result, plants are predominantly resistant to most plant pathogens (Dangle and Jones, 2001). However, pathogens also have evolutionary strategies to infect the host plants, resulting in epidemics worldwide. In molecular level, the occurrence of pathogen infection or plant resistance is governed by the gene-for-gene interaction between disease resistance genes in the host and avirulence/virulence genes in the pathogens (Kolmer 1996). Their relationship is described in Figure 5.

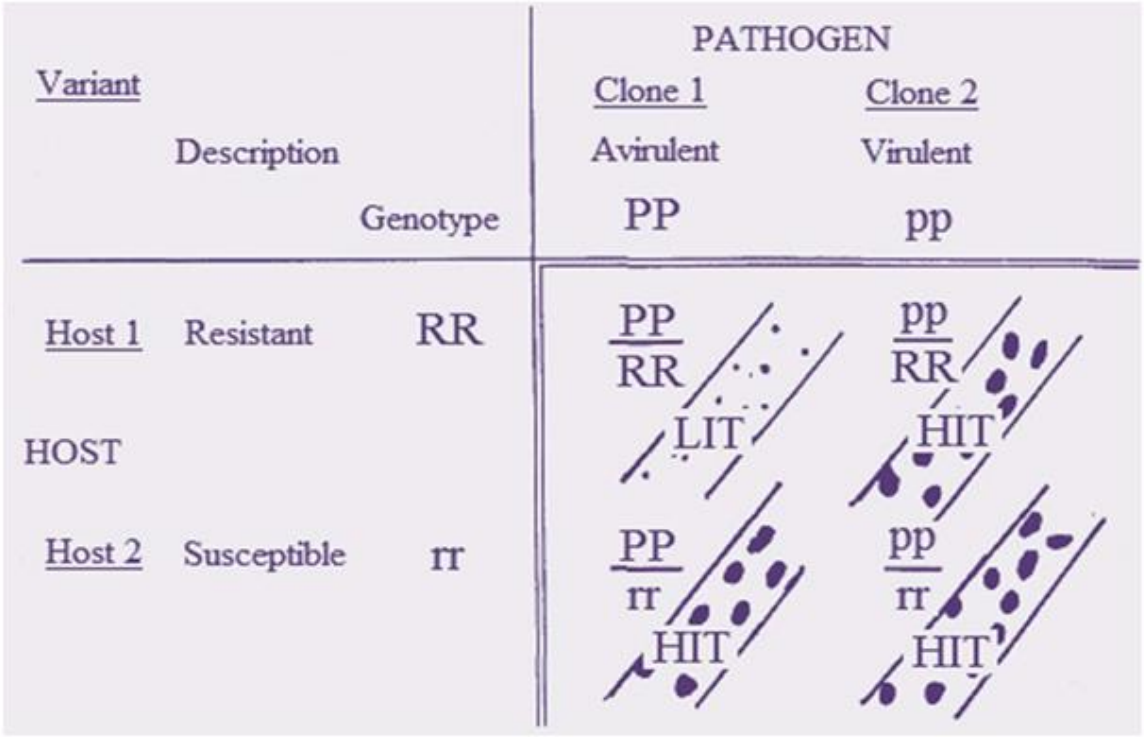

Figure 5. The genetic interactions between host resistance gene and pathogen effector gene and its results. The interaction of host resistance (RR) and complementary avirulence alleles (PP) of the pathogen produce low infection type (LIT) or incompatibility, while high infection type (HIT) or compatibility is due to all other combinations. Source: (McIntosh and Wellings, 1986).

According to the basis of plant-pathogen interaction, there are 2 types of relationship: incompatibility and compatibility. When both avirulence genes and resistance genes are present in the pathogen and host, respectively, the interaction is incompatible or disease resistant. On the other hand, the absence of either avirulence genes or resistance genes and the presence of virulence genes will result in pathogen infection (compatibility). Therefore, the innate defense system of plant can be broken down if pathogens have changes to become virulent.

1.3.3. Variation in rust pathogen

In order to overcome the host-defence system, the genetic variation within pathogen population is required. Beside retaining the common characteristics of the same species, the new individuals produced from a complete sexual spore are expected to different from their parents and each other. To know how rust pathogens break down host-resistance genes, the understanding of mechanisms of their genetic variability is necessary. Historically, the process

of pathogen evolution has been studied, including a) mutation to virulence and b) somatic hybridization (Kiyosawa, 1982)

a) Mutation from avirulence to virulence

Mutation is the most common process of pathogen variation which is diversified from new alleles (CIMMYT, 1988). According to gene-for-gene hypothesis (Flor, 1971), once the pathogen attacks the host plant, the detection of effector proteins released from pathogen by host cell receptor will trigger the plant defence mechanism, resulting in hypersensitive cell death and then restricting pathogen development. McDonald and Linde (2002) have indicated that the mutations occurred in pathogen have changed effector's structure or produced no effector to prevent the recognition of receptor. This converts pathogens from avirulent to virulent. To infect a plant consisting two or more resistance genes, the pathogen therefore must have virulence to overcome the defence coded by these genes.

b) Somatic hybridization

This process involves in the exchange of nuclei between two pathogen pathotypes when the anastomosis (fusion) of germ tubes and hyphae occurs (Park and Welling, 2012). This is a major source of evolution of pathogens in nature. The evidence of somatic hybridization was previously reported (Watson and Luig, 1958; Little and Manners, 1969; Watson, 1981; Park et al., 1999; Cheng, 2012).

As the result of genetic variability, the emergence of new rust races has been recorded. Various annual surveys of wheat leaf rust carried out in both Canada (Pozniak and Clarke, 2016) and the US (Kolmer et al., 2006) have showed the abundance of leaf rust genotypes. Up to 30-50 *P*. *triticina* races have been identified in France (Goyeau et al., 2006) and 10-15 races are detected in Australia (Park, 2008). Recently, 8 new wheat stem rust races were reported in North America (McVey et al., 2002).

1.3.4. Economic importance of wheat rusts

Due to the genetic variation of rust pathogens to overcome resistance in the host and their widespread distribution of rust pathogens, leaf rust and stem rust have caused very distinct losses in wheat yield worldwide and pose a continual threat to all wheat production.

Studies on the effect of rust on yield have shown that even in resistant varieties, heavy infections of rust can cause considerable loss of grain. Reduction of quality also occurs. Heavy

infections usually cause reductions in the protein content of the grain and an increase in carbohydrate content. Heavy leaf rust infections cause a large increase in the rate of transpiration and therefore increase the water requirement of wheat plants. This is due partly to the added use of water by fungus and partly to evaporation from ruptured plant tissues. The use of water at night is much higher for rusted plants than rust-free plants. It has been shown that heavy infections of leaf rust can cause reduction in plant height and straw production. There is a serious loss of fibrous roots when leaf rust develops early and persists to maturity. Heavy leaf rust tends to hasten maturity of the crop, especially when there is a shortage of soil moisture **(**Alexopoulos et al., 1996)

Historically, the major yield losses have recorded in many regions in the world. In the mid-twentieth century, the notable yield losses of 20%–30% caused by stem rust were recorded in Europe (Zadoks, 1967), and similar losses have also been reported in many other countries, including Australia, China and India (Leonard and Szabo, 2005). Moreover, the significant yield reductions of approximately 50% caused by leaf rust were recorded in susceptible cultivars (Draz et al., 2015). In Australia, a stem rust epidemic in 1974 has seriously damaged wheat crops in the southern states (Watson, 1981).

Due to the airborne transmission of rust pathogens, disease outbreaks are expected to spread rapidly through many regions over the world, threatening global wheat production. Therefore, the efforts on how to control these pathogens effectively is on the top priority to ensure the stable wheat production.

1.3.5. Approaches used to control rust diseases in wheat

1.3.5.1. Utilization of fungicides

In some regions growing wheat as main crop such as European Union, China and India, the utilization of fungicides is considered as the main strategy to control rusts (Hodson, 2011). Chemical control can be an important approach when rust epidemics are severe. Nevertheless, in the long-term strategy, this option remains the minor effectiveness in rust management. This is due to the over-changing of rust pathogens with appearance of new races, the rapid rate of rust infection under favourable conditions, and significantly increased production costs for farmers (Hodson 2011). Control strategies based on genetic resistance are considered the most cost-effective, environmentally safe and sustainable long-term option (Ellis et al., 2014).

1.3.5.2. Stem and leaf rust resistance genes

Numerous genetic studies of slow rusting resistance genes have been conducted over the past century by researchers worldwide. More than 120 leaf rust and stem rust resistance genes have been found in wheat, providing wheat breeders over the world available genetic resources for highly effective breeding of rust resistance.

To date, 68 slow leaf rusting genes from *Lr1* to *Lr 68* have been documented in common hexaploid wheat including bread wheat (Volkova et al., 2009; Bansal et al., 2017; Herrera-Foessel et al., 2012). These genes are widely distributed across the wheat genome. Furthermore, molecular markers linked to race-specific and slow leaf rust resistance genes: *Lr1, Lr3, Lr9, Lr10, Lr13, Lr19, Lr21 Lr23, Lr24, Lr25, Lr27, Lr28, Lr29, Lr31, Lr34, Lr35, Lr37, Lr39, Lr46, Lr47, Lr50* and *Lr51* (http://maswheat.ucdavis.edu), have been identified (Goutam et al., 2015; Wei et al., 2015).

For stem rust, several resistance genes were mapped on different chromosomes (McIntosh et al., 2003), in which *Sr2* is considered as the most effective gene, showing remarkable protection to Ug99 race. Currently, there are approximately 15 stem rust resistance genes that have been found (Aktar-Uz-Zaman et al., 2017). More importantly, molecular markers are also available for some stem rust resistance genes such as: *Sr2, Sr22, Sr24, Sr25, Sr36, Sr50* (Rouse et al., 2012; Tsilo et al., 2008; Bansal et al., 2014; Khan et al., 2005). The availability of genetic markers provides a significant assistance to wheat breeders to find the resistant cultivars as well as breeding strategies for rust resistance.

1.3.6. Evolution of wheat rust pathogens to fungicide resistance and virulence to resistance genes

Chemical control, or the use of fungicides, is a commonly used approach to control rust diseases. However, the increasing use of foliar fungicides in wheat has posed a considerable challenge. After the intensive use of fungicides, fungal isolates resistant to fungicides have been reported (Deising et al., 2002; Bayles et al., 2000) tested the exposure of rust isolates of the stripe rust (*P. striiformis*) for sensitivity towards declining fungicide and the results showed that 54 % of the isolates tested being less sensitive than the resistant.

In addition, the emergence and spread of new virulent fungal races of both leaf rust and stem rust have had considerable impacts on wheat production and global effects to control rust epidemics during the past two decades. For stem rust, the new race named Ug99 TTKSK

possessing virulent combinations not recognised by numerous resistance genes, was first detected in Uganda in 1998 (Pretorius et al., 2000). After a few years, the rapid spread of this race throughout 13 countries of Africa and Middle East has caused major production losses and become a global threat to susceptible wheat cultivars (Singh et al., 2015). In recent years, five new Ug99 races were reported in Ethiopia in 2013 (Olivera et al., 2015) and other countries such as Uganda, Rwanda, Eritrea and Egypt in 2014 (Singh et al., 2015), causing serious problems for farmers in these countries and threatening the wheat production of their neighbouring countries. For leaf rust, a new race group rendering ineffective some deployed leaf rust resistance genes was also detected in Mexico (Herrera-Foessel et al., 2013). As a consequence of past epidemics and the constant threat of new ones, many research and breeding programs have focussed on wheat rust resistance due to its economic importance. Although various studies focusing on rust control were conducted from 19th and early 20th centuries, the scientific society in the 21st century is still required to address this global issue (Johnson, 2010). Therefore, global wheat production requires highly effective strategies for rust controlling that reduce the use of fungicides and also develop more race-specific genes for rust resistance breeding. The deployment of new rust resistance genes into commercial wheat cultivars could be greatly assisted by the use of closely linked genetic markers and more specifically molecular or DNA-based markers. For the more effective application of rust resistance genes in wheat, the solid understanding of rust resistance mechanism is necessary.

1.3.7. Rust resistance mechanism

Generally, race-specific resistance genes are defined as operating against some but not all rust races. This adheres to the basis of gene-for-gene model, in which disease resistance relies on a specific genetic interaction between plant-resistance (*R*) genes and pathogen avirulence (*Avr*) genes. In contrast, non-race-specific resistance is effective against multiple pathogen's races. This resistance is normally expressed at late stages of plant development and is therefore referred to as adult plant resistance (APR).

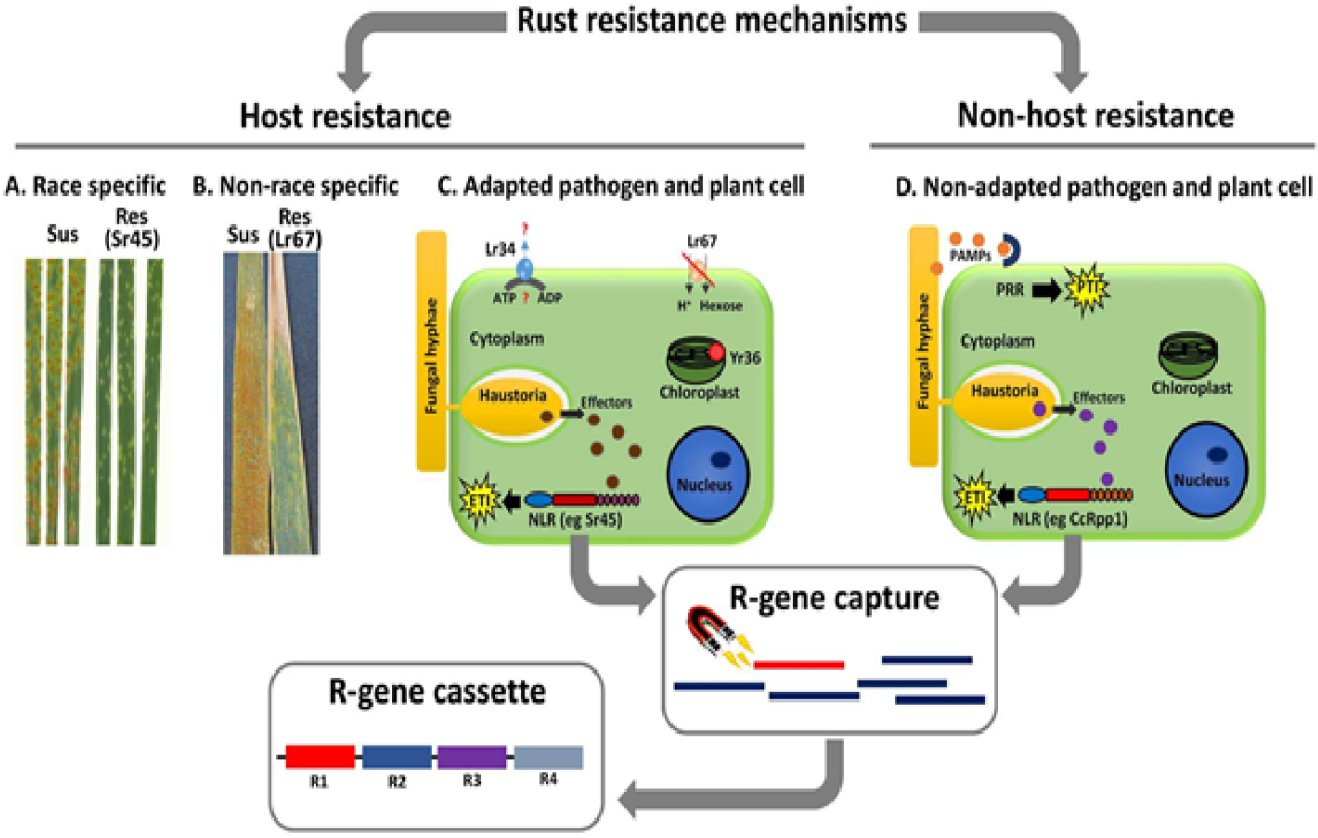

Fig 6. Host and non-host mechanisms of rust resistance. Race-specific and non–race-specific resistances can be phenotypically quite different (A versus B). (A) Strong resistance is conferred by NLR proteins such as stem rust resistance 45 (Sr45) and is associated with a hypersensitive response. (B) Non–race-specific resistance may be characterised by partial resistance or slowed fungal growth coupled to leaf-tip necrosis in the presence of genes such as leaf rust resistance 67 (Lr67). (C) Adapted pathogens deliver effectors that can subvert pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI), but which may be detected in the plant cell by nucleotide-binding and leucine-rich repeat (NLR) proteins, leading to effector-triggered immunity (ETI). (D) PTI can operate in non-host resistance in response to non-adapted pathogens, and ETI can also occur for pathogens that are more compatible to the host plant. R-gene capture methods may be used to detect and identify NLR genes, with the goal of incorporation of these NLRs into R-gene cassettes or stacks to provide durable, long-lasting resistance. Adapted from Periyannan et al. (2017)

With rapid emergence and spread of rust pathogens worldwide, resistance durability of wheat cultivars is therefore urgent necessity to protect wheat production. The tendency of using race-specific resistance genes to break down the conversion of Avirulence (*Avr)* to virulence genes in pathogen and the pleiotropic and broad-spectrum resistance conferred by non-race specific

genes reveals that the most promising approach is dual resistance involving stacking of such genes to generate additive interactions between resistance genes and minimise the possibility of pathogen virulence evolution and safeguard plant resistance durability. Such resistance gene pyramids could be developed using conventional breeding approaches using marker-assisted selection based on cloned gene sequences or through the deployment of resistance gene cassettes in which multiple cloned genes may be combined in a single locus (Fig 6). Understanding the potential for additive interactions between resistance genes is important to identify the most effective combinations to pursue, while identifying rust *Avr* genes is also a priority to monitor pathogen evolution and prioritise resistance genes for deployment.

1.4. Genetic markers applied in plant breeding: why DNA markers?

Genetic markers are the differences of allelic forms of homologous DNA sequences and can be used as the biological tags or probes for plant breeding as they are transmitted from one generation to the next. Historically, the classical markers including morphological markers (differences in visible traits such as the shape or colour of leaf, flower or seed), cytological markers (differences in chromosomal structure) and protein markers (the presence or absence of specific protein affecting expression of particular traits) have been used as an assisting tool to select desirable traits in plant breeding for a long time and laid a foundation for modern molecular marker development. However, classical markers have some disadvantages that make their use limited, such as:

- The limited availability of these markers
- Many of them are not associated with important economic traits (e.g. disease resistance, high yield or quality)
- Some of them are linked to unwanted effects on plant growth or development
- The detection of classical markers is generally limited to some stages of plant growth.

The limitations of classical markers and the advent of molecular techniques has seen the development of more effective genetic markers that are highly polymorphic, abundantly available, suitable for duplication, cost efficient and co-dominant in expression to distinguish homozygotes and heterozygotes. To date, DNA markers are considered as valuable and highly effective alternatives that have been extensively used for not only in modern plant breeding but also in animal breeding and human genetics.

1.5. Molecular markers for rust resistance genes

DNA markers or molecular markers are defined as small fragments of DNA showing polymorphism (deletion, insertion or changes of nucleotides resulting in different genotypes) between different individuals in a population. They can be identified by detection systems including Southern blotting, PCR technique followed by agarose gel or polyacrylamide gel electrophoresis, DNA hybridization and sequencing. In the research presented here, four types of molecular markers will be utilised that include 1) length of SSR fragmernts polymorphism; 2) SNP (Single nucleotide polymorphism); 3) CAPS (Cleaved amplified polymorphic sequences) and 4) Amplifluor-like markers based on SNP. All have been extensively used and show great promise in their application to molecular breeding (Shavrukov, 2014).

1.5.1. Length of amplified polymorphic markers

Length of amplified polymorphic markers are a type of DNA-based genetic marker where the variation in the length of DNA fragments usually generated by PCR can be a target to discriminate the genetic variation of different individuals. This length variation may involve with the deletion or insertion of DNA sequence in introns (non-coding regions) or exons (coding regions) of the gene.

1.5.2. Single nucleotide polymorphism (SNP) markers

A single nucleotide polymorphism, or SNP, is a single change between two DNA sequences at only one nucleotide (Mammadov et al., 2012). The difference can be the transition of a nucleotide (C/T or G/A) or the transversion (T/G, A/T, C/A or C/G) occurring in coding or non-coding regions of a gene. The average frequency of SNPs in the plant genome ranges from one SNP every 100-300 bp (Gupta et al., 2001). In wheat, the estimated frequency is reported to one SNP per 540-569 bp in the individual genomes (Somers et al., 2003, Lai et al., 2012, Paux et al., 2012) and one in every 20 bp between homoeologous genomes (A, B and D genomes). In comparison with barley, the frequencies are one SNP out of 189 bp (Kanazin et al., 2002) and one SNP out of 240 bp (Duran et al., 2009). This means the frequency of SNP in wheat is much lower compared to other plants and can be constrain for development of SNP-related markers.

However, the advance of modern sequencing techniques and availability of sequenced DNA segments published on major databases make SNP detection the simplest form of DNA marker for allelic discrimination. Based on sequencing databases, SNPs are detected by analysing the sequence data. This is a useful tool to detect the desirable genes from the vast data of wheat

genome sequencing and can accelerate the improvement in wheat production by providing insights into their genetic constitution (Mammadov et al., 2012). SNP-based molecular markers offer various advantages over conventional approaches including rapid screening of individuals for target genes from large populations, abundance of genetic variations in wheat genome, ease of use and no electrophoresis required (Michael, 2014).

Table 2. The comparison of different techniques used to identify molecular markers (Nadeem et al., 2018)

Characteristics	RFLP	RAPD	AFLP	ISSR	SSR	SNP
Co-dominant/Dominant	Co-dominant	Dominant	Dominant	Dominant	Co-dominant	Co-dominant
Reproducibility	High	High	Intermediate	Medium–High	High	High
Polymorphism level	Medium	very high	High	High	High	High
Required DNA quality	High	High	High	Low	Low	High
Required DNA quantity	High	Medium	Low	Low	Low	Low
Marker index	Low	High	Medium	Medium	Medium	High
Genome abundance	High	Very high	Very high	Medium	Medium	Very high
Cost	High	Less	High	High	High	Variable
Sequencing	Yes	No	No	No	Yes	Yes
Status	Past	Past	Past	Present	Present	Present
PCR requirement	No	Yes	Yes	Yes	Yes	Yes
Visualization	Radioactive	Agarose gel	Agarose gel	Agarose gel	Agarose gel	SNP-VISTA
Required DNA (ng)	10000	20	500–1000	50	50	50

It should be stressed that advances in modern sequencing techniques have facilitated the discovery of SNPs (Singh et al., 2012) (Duitama et al., 2015). Recently, a huge database of 4 million SNPs in wheat was generated for further studies and investigating the genetic nature of trait variation in wheat (Lai et al., 2015).

1.5.3. Cleaved Amplified Polymorphic Sequences (CAPS) markers

CAPS markers exploit the difference in length of digested fragments created by SNPs or insertion or deletion of nucleotides, causing a change in the recognition site for restriction enzymes (Börner and Khlestkina, 2015). In practice, if one allele includes the recognition site for specific restriction enzyme while another allele does not, the digestion of a PCR products that

spans the restriction site will result in different sized fragments that can be easily detected electrophoretically. According to Shavrukov (2016), CAPS markers are powerful and useful tools to employ in small-scale experiments with highly polymorphic genetic regions containing multiple SNPs. In bread wheat, the frequency of CAPS markers is rare due to the lower frequency of SNP detected (Jiang et al., 2015). However, once CAPS marker is developed, that can be a simple but effective and cost-effective way for genotyping, therefore, it is worth investigating the time and effort to identify them. Historically, some CAPS markers were discovered in wild accessions of wheat, such as *Triticum dicoccoides* and *Aegilops tauschii* Coss (Azhaguvel et al., 2012).

1.5.4. Amplifluor-like SNP marker used in genotyping

Due to the massive and publicly available DNA sequence data for plant species, including important crops like wheat, SNP can be easily detected by comparison of sequenced fragments in the gene family. The application of low and high-throughput technologies based on identified SNP has revolutionized the process of plant genotyping.

Two of the most commonly used methods based on AS-PCR in genotyping are KASP (KBioscience Competitive Allele Specific PCR) and Amplifluor SNP marker which share the similar basic principles. KASP markers are proprietary technique owned and operated by LGC company (www.lgcgroup.com) using robotic system to genotype plants from the first step of DNA extraction from the leaf tissues to final step of genotype analysis. The genotyping service is conducted using confidential master mix and reagent assays providing highly accurate results. However, the prices are relatively expensive, making it less attractive to smaller laboratories.

For the greater flexibility and more reasonable prices for researchers, Millipore company which co-operates with Merck company (http://www.merckmillipore.com) has recently developed the new technique called Amplifluor SNP genotyping with acceptable accuracy. More importantly, the information about components and used for Millipore's genotyping assay were disclosed and available for all researchers to use. Researchers can order the entire service from Millipore or only reagents (Universal probes) for their self-made Amplifuor-like SNP marker assay with much lower cost. Once the stock of universal probes has been purchased, researchers have more flexibility to design their own gene-specific primers and test as many SNPs as they need at the prices 10-20 times less than the KASP technology. These issues are very important for researchers conducting small experiments.

The basic structure of universal probes has been previously described (Nazarenko, 1997), (Myakishev, 2001) consisting of five elements (1) fluorophore or fluorescent dye at 5'-end; (2) a stem; (3) a loop; (4) the Black Hole Quencher attached to modified Thymine nucleotide and (5) specific tail sequence at 3'-end corresponding to identical tail in allele-specific primers (Fig 7)

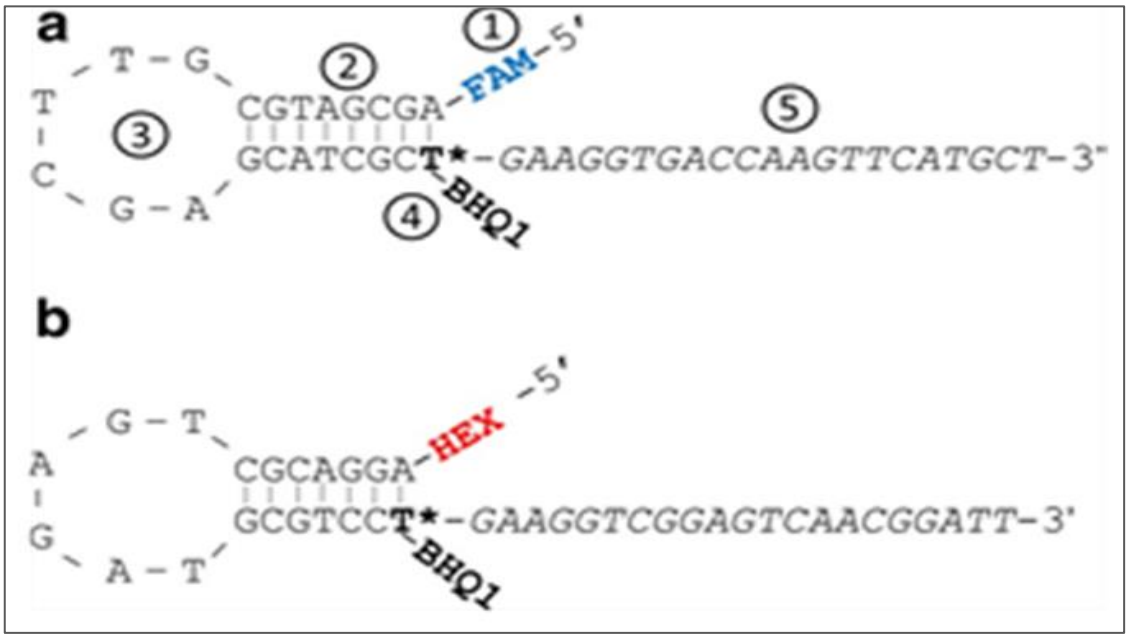

Figure 7. The basic structure of universal probes using two differently labelled fluorophores FAM (a) and HEX (b). Five components of fluorophore, stem, loop, quencher and tail are indicated in order from 1 - 5, respectively (Adapted from Nazarenko, 1997 and Myakishev, 2001). Circled numbers represented five main elements described in the text

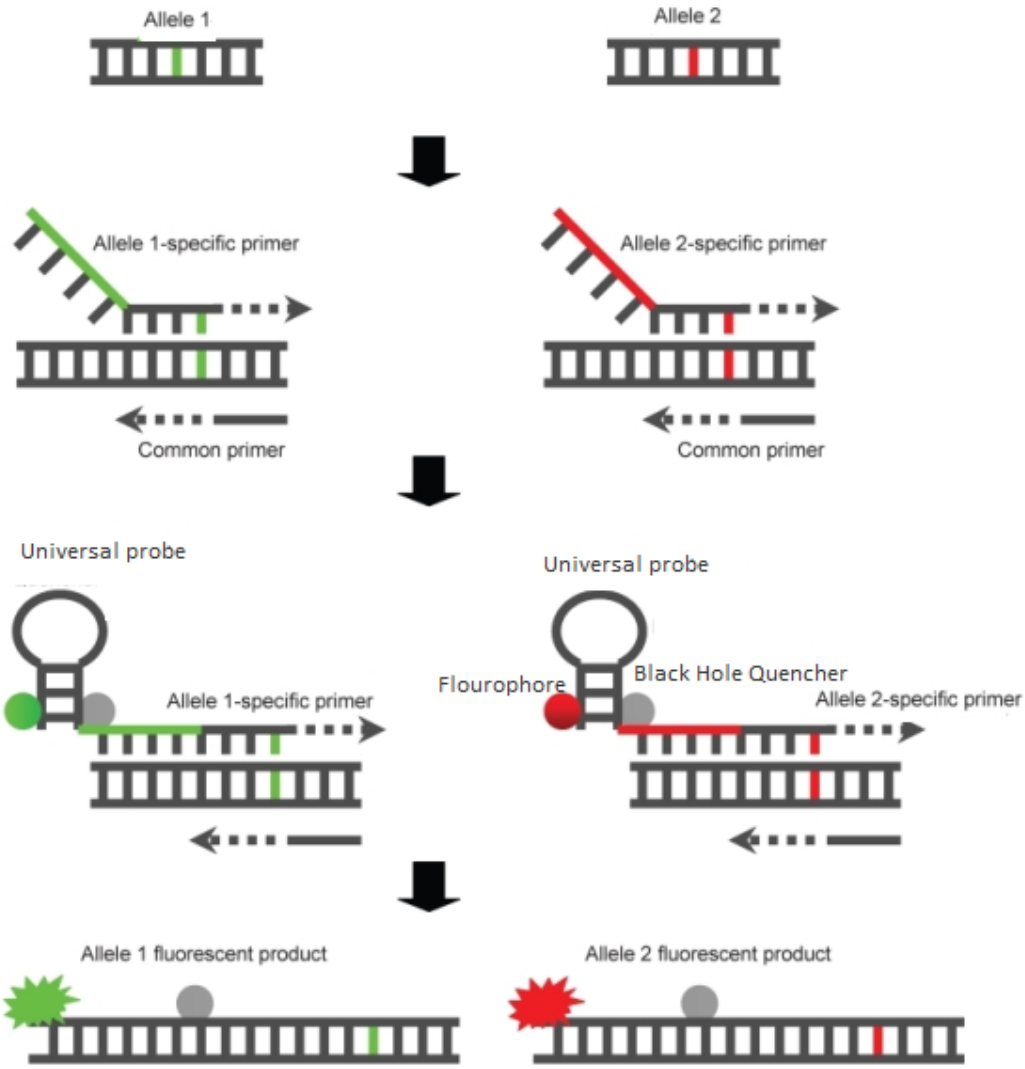

Figure 8. Schematic diagram showing the components and reactions in Amplifluor technique

Adapted from Fuhrman et al., 2008

The allelic difference between individuals can be discriminated by the newly released method named Allele-specific PCR (AS-PCR) platform. In this technique, the two main components (1) the Universal probes (Ups) which are labelled with different fluorescent tags linked to either green or red fluorophore) and (2) Gene-specific primers (GSPs) which are non-labelled and designed to match the SNP flanking region and end up at SNP position at 3'-end. The matching of the gene-specific primer with either one of fluorescent tags and generating PCR products as a result of PCR amplification will separate the fluorophore from the quencher. This process will generate a fluorescent signal which can be detected by a qPCR machine. Homozygotes produce either a green or red signal while heterozygotes produce a yellow signal combined for both fluorophores.

1.6. Marker-Assisted Selection (MAS), a novel tool of breeding for rust resistance in wheat

Control of wheat pests and diseases is an important phase of wheat production. Many diseases can be controlled by the use of fungicides. Even though these means are available, they are not always properly applied. Cultural methods are also useful, but a number of these depend upon community cooperation and are not often carried out successfully. The air-spread diseases such as rust cannot be adequately controlled by the use of fungicides or cultural practices, so genetic control is the most effective, economically viable and environmentally friendly approach (Todorovska et al., 2009). Although resistance is not always effective or lasting because of the constantly changing genotypes of attacking parasites, however, the use of resistant varieties has showed advantages in reducing the use of fungicides, resulting in less production cost and possibility of the rust development resistance to fungicides. That is the reason why farmers over the world much prefer using resistant cultivars. Using resistant varieties to prevent or avoid diseases at early stages, or to minimize disease impact, is considered as the most effective control strategy. To ensure genetic-based resistance, identification of effective resistance genes in different germplasms of global wheat cultivars is essential. Traditional phenotype or trait selection to identify resistance genes by multi-pathotype testing, or gene postulation, is labour-intensive and time consuming. In addition, this approach is limited because screening for one resistance gene interferes with the ability to screen for another, which is a frequent problem in breeding for disease resistance. Furthermore, due to evolution and selection pressure, new virulent races frequently appear, requiring wheat breeders to find new resistance genes to incorporate into elite wheat varieties by selective breeding. This is a very time consuming process, in some cases taking many years to achieve (Acquaah, 2015). Despite this, genetic control remains the best option for the majority of wheat farmers in the foreseeable future and therefore any improvement to the efficiency of rust resistance breeding programs is highly valued.

Recent advances in molecular marker technology have created effective tools for solving such complex problems. Since the discovery and development of various types of molecular markers during the 1980s and 1990s, the integration of molecular markers and traditional selection offers a powerful and reliable tool for modern plant breeding with following advantages:

- MAS allows the selection of desirable gene at early stage of plant growth (e.g. seedling stage) so that it is not necessary to wait to fully developed plant even the desirable trait is expected to express at late stage (mature plant trait). This character of MAS is thus important for breeding because it significantly reduces the long time required for phenotyping and eliminates unwanted genotypes during backcrossing progress.
- The markers linked to target genes are not affected by environmental conditions, MAS thus can be used in any conditions (e.g. glass-house, field or even off-season trials). This feature is useful for selection the disease resistance traits that require favourable condition to express.
- For recessive traits, MAS using co-dominance markers can detect the recessive allele in heterozygotes without requiring selfing scheme to confirm.
- MAS can be used to detect individual gene in the combination of multiple genes controlling a single trait (for the trait controlled by multiple genes) which is a big difficulty for genotypic selection since one gene can overwhelm other in phenotypic expression.

In conclusion, traditional phenotypic selection still plays a key role in plant breeding, however, the use of MAS facilitates the traditional phenotypic selection in terms of time, resources and efforts, and offers higher effectiveness and efficiency for modern plant breeding.

Historically, the use of polymerase chain reaction (PCR)-based DNA molecular markers has several advantages over traditional phenotype trait selection (Todorovska et al., 2009). Marker-assisted selection (MAS) breeding has also been widely used to target rust resistance genes in wheat. These techniques offer the improved selection efficiency in plant breeding, especially by providing pathways to overcome some of the problems associated with classical phenotypic screening approaches (Mohan et al., 1997. Molecular markers offer powerful tools to tag rust resistance genes and as a part of MAS to be used in improvement to the efficiency of selection in plant breeding. The most common markers used for MAS in general are Single nucleotide polymorphism (SNP), and Cleaved amplified polymorphic sequences (CAPS).

1.7. Perspectives and challenges for molecular marker-based breeding in wheat

William et al. (2007) has reviewed the applications of molecular markers used at CIMMYT (International Maize and Wheat Improvement Centre) in wheat breeding and reports that approximately 45,000 MAS data points are generated annually. The adoption of MAS is

increasing in not only private but also public sectors. According to CIMMYT, the close collaboration of breeders and molecular biotechnologists is one of major contributors to the success of utilization of markers in wheat breeding.

Another aspect of marker application is that plant genetic resources are valorised throughout the MAS process. The huge genetic resources stored in gene database could possibly be exploited through the development of new markers based on the available data or using finely mapped Quantitative trait locus (QTL) for efficient use in the improvement of crop varieties.

Although MAS has accelerated the breeding process and considered as preferable approach over traditional phenotypic breeding, there are some challenges for molecular breeding. Naturally, wheat is an inbreeding crop and, therefore, the pedigree system is usually used in breeding programs. Because of the high complexity of wheat homoeologous genomes, the hexaploid wheat is also a relatively difficult species for marker application (Eagles et al. 2001). In addition, there is limited conversion of published data into practical applications. Numerous studies on the development of markers have been published, however, the publications with successful utilization of developed markers in MAS remains limited. In practice, MAS is still mainly utilized to select for traits encoded by single gene (monogenic traits) such as leaf rust and stem rust resistance since their high heritability. For quantitative traits encoded by multiple genes (oligogenic traits) with lower heritability and strong influence of environment such as yield or grain colour, the complexity of breeding process requires more appropriate molecular platforms and closer collaboration between the breeders and biotechnologists.

1.8. Summary of Aims, Hypotheses and Biotechnological Significance

In summary, the aims of the current work are as follows:

3) To identify the PCR-based markers (length polymorphic marker, SNP and CAPS markers) linked to rust resistance genes in Kazakh wheat varieties the identified disease resistance genes.
4) To develop a MAS strategy using the identified molecular markers to detect the candidate resistance genes in selected Kazakh bread wheat varieties.

The specific hypotheses to be tested in current work is that there are PCR-based markers linked to specific rust resistance genes in bread wheat varieties selected in Kazakhstan that show traits of rust resistance.

The successful development a set of PCR-based markers in this study including Cleaved Amplified Polymorphic Sequences (CAPS) marker and self-designed Amplifluor-like technique for genotyping of leaf rust resistance gene *Lr51*, and length polymorphic markers for *Lr22a* and *Sr2,* offers an additional cost-effective, easy to use and reliably score-able marker which can be routinely used to track these genes in not only Kazakh wheat varieties but also worldwide wheat germplasms to combat an ever-changing rust pathogen population and protect wheat from rust diseases.

This research also contributes to an ongoing need to constantly improve a recycle resistance specificities in marker assisted selection and accelerates the incorporation of several resistance genes for more durable and effective rust resistance in new commercial bread wheat varieties.

Chapter 2: Materials and Methods

2.1. Plant materials

Plant materials used for the study were two cultivars provided from Kazakhstan, Altaiskaya Zhnitza (Barnaul Breeding Station, Russia) and Karabalykskaya 90 (Karabalyk Breeding Station, Kazakhstan), which were phenotypically scored as resistant and susceptible to some leaf and stem rusts, respectively, and segregating population F_2 and F_3 of their reciprocal crosses. Approximately 200 progenies each cross was produced by Dr. Grigory Sereda (Karaganda, Kazakhstan). The parental cultivars Altaiskaya and Karabalykskaya will subsequently be referred as Alt and Kar.

2.2. DNA extraction

DNA samples of parents and progenies were extracted from tissue of seedlings (at the tillering stage) using phenol-chloroform method and kindly provided by Kazakh breeders (Karaganda, Kazakhstan). The DNA extraction method was previously described by Shavrukov et al., (2016). The leaves collected from five random plants for each sample were placed in a 10mL tube and rapidly frozen by liquid nitrogen then stored at -80°C prior to DNA extraction. The frozen leaf samples were ground to a fine powder using 9mm stainless ball bearings, followed by phenol-chloroform method as described by Sharp et al (1988). Genomic DNA quantity was then estimated using spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific) and checked on 1.0% agarose gel. The DNA samples were then diluted to make the working concentration of 20 ng/ul for further use.

2.3. Primer design for PCR amplification

Published primers for leaf and stem rust resistant genes in wheat were tested and reported in previous studies (Arruda et al., 2016; Aktar-Uz-Zaman et al., 2017; Yu et al., 2017). In the work presented here, primers for PCR amplification of 10 and 14 well-studied stem rust and leaf rust resistance genes, respectively, were selected based on previous publication and the website of Marker Assisted Selection in Wheat (http://maswheat.ucdavis.edu/protocols/index.htm)

Amplification of markers linked to resistance genes by Polymerase chain reaction (PCR): Based on a total of 24 selected genes responsible for SR and LR resistance, 24 gene-specific primer pairs (Reverse and Forward primers) were designed to use for PCR. The sequences and details

of these primers are available via website http://wheat.pw.usda.gov/SNP/new/index.shtml. The primers were commercially purchased from Sigma Company.

Table 3. PCR-based markers associated with stem rust resistance genes (*Sr*) effective to *Puccinia graminis* f. sp. tritici race Ug99 (Source: http://maswheat.ucdavis.edu)

Target gene	Oligo name	Sequence (5'-3')	Size (bp)	Reference
Sr2	gwm533-F	5' GTTGCTTTAGGGGAAAAGCC 3'	120/149	Hayden et al. (2004)
	gwm533-R	5' AAGGCGAATCAAACGGAATA 3'		
Sr22	WMC633 F	5'- ACACCAGCGGGGATATTTGTTAC -3'	117	Olson et al. (2010)
	WMC633 R	5'- GTGCACAAGACATGAGGTGGATT -3'		
Sr24-Lr24	Sr24#12	5'- CACCCGTGACATGCTCGTA -3'	500	Mago et al. (2005)
	Sr24#12	5'- AACAGGAAATGAGCAACGATG T -3'		
Sr25-Lr19	PSY-D1-F	5' TTGCAGTGCAATGGTTTTCCA -3'	175	Zhang and Dubcovsky (2008)
	PSY-D1_R	5' GACTCCTTTGACGATGTCTTC -3'		
Sr26	Sr26#43-F	5'- AATCGTCCACATTGGCTTCT -3'	207	Mago et al. (2005)
	Sr26#43-R	5'- CGCAACAAAATCATGCACTA -3'		
Sr28	wPt-7004-PCR-F	5'- CTC CCA CCA AAA CAG CCT AC -3'	194	Rouse et al. (2012)
Sr28	wPt-7004-PCR-R	5'- AGA TGC GAA TGG GCA GTT AG -3'		
Sr39	Sr39#22r-F	5'AGAGAAGATAAGCAGTAAACATG -3'	487	Mago et al. (2009)
	Sr39#22r-R	5'TGCTGTCATGAGAGGAACTCTG -3'		
Sr47	Xgpw4043-F	5'- ACATATGCACGCACGCAC -3'	95+155	Klindworth et al. (2012)
	Xgpw4043-R	5'- CATTGACACCCCTGACACTC -3'		
SrCad	FSD	5'- GTTTTATCTTTTTATTTC -3'	275	Laroche et al. (2000)
	RSA	5'- CTCCTCCCCCCA -3'		

Table 4. PCR-based markers associated with leaf rust resistance genes (*Lr*) effective to *Puccinia triticina* (Source: http://maswheat.ucdavis.edu)

Target gene	Marker	Sequence (5'-3')	Size (bp)	Reference
Lr9	J13/2	5' - TCCTTTTATTCCGCACGCCGG -3'	1110	Schachermayr et al. (1994)
		5'-CCACACTACCCCAAAGAGACG-3'		
Lr10	Lrk10D1	5'-GAAGCCCTTCGTCTCATCTG-3'	282	Schachermayr et al. (1997)
	Lrk10D2	5'-TTGATTCATTGCAGATGAGATCACG -3'		
Lr19	GbF	5'-CATCCTTGGGGACCTC-3'	130	Prins et al. (2001)
	GbR	5'-CCAGCTCGCATACATCCA-3'		
Lr20	STS638	5'-GCGGTGACTACACAGCGATGAAGCAATGAAA -3'	542	Gul'tyaeva et al. (2009)
	STS638	5'-GCGGTGACTAGTCCAGTTGGTTGATGGAAT-3'		
Lr21	D14	5' - CCAAAGAGCATCCATGGTGT -3'	885	Huang and Gill (2001)
	D14	5'- CGCTTTTACCGAGATTGGTC -3'		
Lr22a	Xgwm296	5'- AATTCAACCTACCAATCTCTG -3'	131 + 121	Hiebert et al. (2007)
	Xgwm296	5'- GCCTAATAAACTGAAAACGAG -3'		
Lr24	J09-1	5'-TCTAGTCTGTACATGGGGGC-3'	350	Schachermayr et al. (1995)
	J09-2	5'-TGGCATGAACTCCATACG -3'		
Lr25	Lr25-R20	5' CCACCCAGAGTATACCAGAG -3'	1800	Procunier et al., 1995
	Lr25-R20	5' CCACCCAGAGCTCATAGAA -3'		
Lr26	IB-267L	5'- GCAAGTAAGCAGCTTGATTTAGC -3'	267	Mago et al. (2002)
	IB-267R	5'- AATGGATGTCCCGGTGAGTGG-3'		
Lr29	Lr29F24	5'- GTGACCTCAGGCAATGCACACAGT-3'	900	Procunier et al., 1995
	Lr29R24	5'- GTGACCTCAGAACCGATGTCCATC -3'		
Lr34	csLV34F	5'- GTTGGTTAAGACTGGTGATGG-3'	150	Lagudah et al. (2009)
	csLV34R	5'- TGCTTGCTATTGCTGAATAGT -3'		
Lr35	Lr35F	5'- AGAGAGAGTAGAAGAGCTGC -3'	900	Gold et al. (1999)
	Lr35R	5'- AGAGAGAGAGCATCCACC -3'		
Lr37	URIC	5'- GGTCGCCCTGGCTTGCACCT -3'	285	Helguera et al. (2003)
	LN2	5'- TGCAGCTACAGCAGTATGTACACAAAA -3'		
Lr51	S30-13L	5'- GCATCAACAAGATATTCGTTATGACC -3'	422+397	Helguera et al. (2005)
	AGA7-759R	5'- TGGCTGCTCAGAAAACTGGACC -3'		

The PCR reactions were performed in 96-well plates in total volume of 15 µL including 1.5 µL of DNA template (final concentration of 20ng/µl), 3 µL of 5X GoTaq flexi green buffer, 1.5 µL of 2 mM dNTPs (Roche), 1.2 µL of 25 mM $MgCl_2$ (Promega), 0.75 µL GoTaq polymerase 5U/µL, 2 ng/µL forward and 2 ng/µL reverse primer (5µM) was used for the DNA amplification process. Distilled MilliQ H_2O was then used to bring the volume up to 15 µL.

PCR was conducted with the following cycling conditions: conditions were an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C (15 sec), 55 °C annealing (15 sec) and 72 °C extension (45 sec), with a final extension at 72 °C for 2 min. The amplification products were resolved by electrophoresis system (Biorad). The direct detection of bands containing as little as 1-10 ng of DNA using ultraviolet light was conducted by Gel Doc™ EZ Gel Documentation System (BioRad).

2.4. Detection of length polymorphic markers

PCR products were initially visualized on a 1.0% agarose gel.

In case of length polymorphisms where the size difference ranged from 100 - 200bp, the fragments were separated on 2.5% agarose gels, run with 1X TAE buffer at 120 volts for 2 hours using Bio-Rad electrophoresis system. A 100 bp DNA ladder (Bioline, Australia) was included on agarose gels to estimate the size of the amplified fragments.

If better discrimination of polymorphic fragments, PCR products were also resolved on 12% polyacrylamide gels in separate experiments.

2.5. Cloning of length polymorphic DNA fragments linked to *Lr22a* and *Sr2* using the pGEM T-easy system (Promega).

Genomic DNA extracted from wheat leaf was used for PCR amplification of *Lr22a* gene. The PCR protocol was followed: 1 cycle if initial denaturation at 95^{o}C for 2 min, followed by 39 cycles of 95^{o}C for 15 sec, 55^{o}C for 15 sec, 72^{o}C for 1 min and final extension at 72^{o}C for 2 min.

PCR products were visualized on a 1.5% agarose gel and then purified using FavorPrep PCR purification mini kit (Favorgen Biotech Corp, Taiwan). The concentration of purified PCR products was measured by a Nanodrop spectrophotometer and used for ligation ratio calculations. In order to ligate PCR products with the pGEM T-easy vector, a 10 ul ligation reaction consisting of 5µls of 2x rapid ligation buffer, 50 ng of pGEM T-easy vector, 10 ng of PCR product and 3 Weiss units of T4 DNA ligase was incubated overnight at 4^{o}C. For transformation,

2ul of a ligation reaction and 50ul of chemically treated competent cells (*E.coli*, strain JM109) were gently mixed and incubated on ice for 20 min, followed by a heat shock at 42^{o}C for 50 sec and then an ice treatment for 2 min.

Approximately 475ul of room temperature SOC medium was added to a centrifuge tube containing the bacterial cells transformed with a ligation reaction. The transformation culture was then incubated at 37^{o}C for 1.5 hr with shaking (150 rpm) and transferred to LB/ampicillin/X-Gal/IPTG plates followed by an overnight incubation at 37^{o}C.

After blue/white colony selection, the white colonies which were expected to contain recombinant plasmids were analysed by colony PCR screening. The PCR conditions for this were an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C (30 sec), 55°C annealing (30 sec) and 72 °C extension (1 min), with a final extension at 72 °C for 5 min.

By using the Wizard Plus SV Minipreps DNA purification System (Promega, USA), the plasmid DNA from bacterial cells was purified and then sent for sequencing using M13 reverse and forward primers. Sequencing was carried out at Australian Genome Research Facility Ltd., Adelaide by capillary separation in the Applied Biosystems 3730xl DNA analyser (Applied Biosystems, CA, USA).

2.6. Detection of SNP markers

For the primers generating good PCR amplification but no length polymorphisms, amplified products were purified using FavorPrep PCR purification mini kit (Favorgen Biotech Corp, Taiwan). Prior to sequencing, 0.15 uL of forward primers (100uM) was added to purified PCR products and then the mixture was sent for sequencing by the AGRF. The sequences of submitted samples were visualised using software Chromas lite 2.1 (Technelysium Pty Ltd). By aligning and comparing these DNA sequences using EMBOSS needle website (https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html), the position of SNPs was identified. To double check SNPs, sequencing was done using the M13 reverse primer and analysed with both EMBOSS needle and Chromas software. That was solidly confirmed the real SNP and eliminate interfering SNPs causing by low quality DNA or errors at beginning of sequences.

2.7. Develop the CAPS markers from identified SNP marker

Identification of specific restriction enzyme (RE) sites: A recognition site containing an SNP or could be created by a small mutation (insertion or deletion) or a base substitution. Such a SNP could then be used for CAPS marker development.

The suitable restriction enzymes affected by a SNP can be identified by using bioinformatics tools, for example http://tools.neb.com/NEBcutter2. The restriction enzymes that cuts a SNP-containing PCR product only once are the first of choice. By using the NEB website, the restriction enzyme cutting DNA fragment at SNP position was identified as *Hind*III (New England Biolabs, Ipswich, GB).

For the CAPS marker based on an SNP found in this study, the genomic DNA was amplified using the S30-13L forward primer and AGA7-759R reverse primers. The PCR conditions were 95°C for 2 min followed by 39 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 1 min. The final extension step was at 72°C for 2 min. Digestion of the total 15 uL PCR product was performed by adding 5 units of restriction enzyme *Hind*III and 2 uL of CutSmart buffer (New England Biolabs, Ipswich, GB) and incubating at 37^{o}C for 2 hours. The digested products were separated on a 1.0% (w/v) agarose gel and visualized by Gelred stain using Gel Doc^{TM} EZ Gel documentation System (Bio Rad).

2.8. Development of self-designated Amplifluor-like SNP marker for genotyping based on identified SNP

For developing a co-dominant Amplifluor-like assay for genotype determination of parents and their cross progenies, the sequence information of *Lr51* in both parents Alt and Kar was aligned using web-based software EMBOSS needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). By aligning two sequences, a 'G/T' polymorphism at 519 bp position in the coding region of this gene was found in which 'G' was specific for the homozygous resistant genotype, while 'T' revealed the heterozygous resistant ones. This SNP was utilized for developing the Amplifllluor-like assay to detect the genotypes of all progenies. According to the protocol described by Shavrukov et al. (2016) with modifications, the primer mixture with two allele-specific forward primers (TaLr51-SNP-F1-T and TaLr51-SNP-F2-G) and one common reverse primer was prepared.

The primers used for SNP Amplifluor genotyping were designed base on the SNP position located in polymorphic fragment linked to *Lr51*. The 3' end of the forward primers F1 and F2 end up at the SNP position with a length of 19-20 bps, while the common reverse primer was 22bp. The primers were designed with similar annealing temperatures to ensure no hair-pin formation. The GC content, melting temperature and self-complementarity were checked by using the web-based software Oligo Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Three allele-specific primers (forward primers F1 and F2 and common reverse primer) were synthesized by Sigma-Aldrich Company (NSW, Australia). Two Universal probes fluorescently-labelled with either FAM or HEX (distinctive fluorophores, blue or green, respectively) were purchased from DNA Synthesis Company (Moscow, Russia).

The details for the designed primers are described in Table 5 and Appendix 7.

Table 5: Sequence of allele-specific primers used for Amplifluor-like SNP genotyping

Name	**Sequence (5' – 3')**
TaLr51-SNP-F1	**GAAGGT**GACCAAGTTCATGCTGAACTGGCTGACCAAGCT***T***
TaLr51-SNP-F2	**GAAGGT**CGGAGTCAACGGATTGAACTGGCTGACCAAGCT***G***
TaLr51-SNP-R	CAACAGGGGCACATGATAAAGT

The tails are underlined with the identical sequence indicated in Bold. The nucleotide in Bold and Italics indicates the SNP position.

Two universal probes contain either FAM or HEX fluorophore at the 5'- end and the Black Hole Quencher, BHQ-1, was added in the middle part with one of two specific tails at 3'-end. The two forward primers shared the identical sequence in the middle with six nucleotides in the 5' tail that were different. They also differed from each other by one single nucleotide at the 3'-end in which the PCR amplification with either the first or second gene-specific forward primer is dependent on allele of the SNP.

Amplifluor SNP analysis was based on the principals of the published information (Myakishev et al., 2001; Rickert et al., 2004; Giancola et al., 2006; Khripin, 2006; Hamilton et al., 2010; Löfström et al., 2015). Reactions were performed in 96-well plates, in a final reaction

volume of 10 µL. Reaction mixtures contained a final concentration of 40 ng of template DNA, 1xPCR Buffer, 1.8 mM $MgCl_2$, 0.25 µM each fluorescent labelled probe, 0.2 mM each of dNTPs, 0.15 µM of each forward primer, 0.78 µM of reverse primer and 0.5 units of Go-Taq flexi-DNA polymerase (Promega, USA). Thermocycling was performed using CFX96 Real-time PCR Detection System (BioRad, Gladesville, Australia) designed for FAM and HEX discrimination. The PCR program consisted of initial denaturation for 94°C for 2 min; 40 cycles of 94°C for 15s, 55°for 20 s, and 72°C for 45 s; and final extension for 72°C for 1 min. Digital single-channel and multi-channel pipettes (Eppendorf, Germany) were used for accurate loading of Master-Mix and DNA samples, respectively. The Amplifluor-like assay was carried out to genotypes of 7 progenies. Parents with valid sequencing information and SNP detection were used as reference genotypes and reaction reagents mixed with distilled water (no DNA template) was used as negative control for the assay.

Genotyping calls were assigned automatically by software accompanying the qPCR machine. However, the confidence of genotype determination was also checked through amplification curves and allelic discrimination.

Chapter 3: Results

Electrophoresis through agarose gels is the standard method used to separate and identify DNA fragments generated by PCR amplification. Due to its simplicity, time-saving performance and capacity of resolving DNA fragments that cannot be separated adequately by other procedures such as density gradient centrifugation, agarose electrophoresis was the first but crucial step in this study to find potential PCR-based markers. Furthermore, the location of DNA within the gel can be detected using stains with low concentration of the fluorescent dye ethidium bromide or Gelred.

Screening of all 24 markers linked to leaf and stem rust resistance genes through standardized PCR amplification and 1.0% agarose electrophoresis was the first step in this study. The results showed some primer sets generating good PCR amplifications with sharp bands on the gel at the expected size, whilst some were non-specific or generated no product at all. PCR optimization with modifications was performed especially for primers generating faint bands on the gel. Optimized PCR amplification was seen in *Sr47* and *Srcad* (Appendix 1), however, in each of these cases no length polymorphism was detected.

Based on the PCR amplification and electrophoresis results, good candidate markers to look for length polymorphisms and SNPs were thoroughly examined and selected. For length polymorphisms, two markers linked to *Lr22a* and *Sr2* which generated clear polymorphic bands distinguished between parents and progenies were chosen. For SNP markers, the first of choice were PCR products with high quality single bands on a gel suitable of DNA sequencing. A total of six products including those linked to four leaf rust resistance genes *Lr29, Lr34, Lr37, Lr51* and two stem rust resistance genes *Sr22* and *Sr28* were selected as candidates meeting the requirements listed above and thus suitable for further analysis.

3.1. Length polymorphic markers

3.1.1. Length polymorphic marker for Leaf rust gene Lr22a

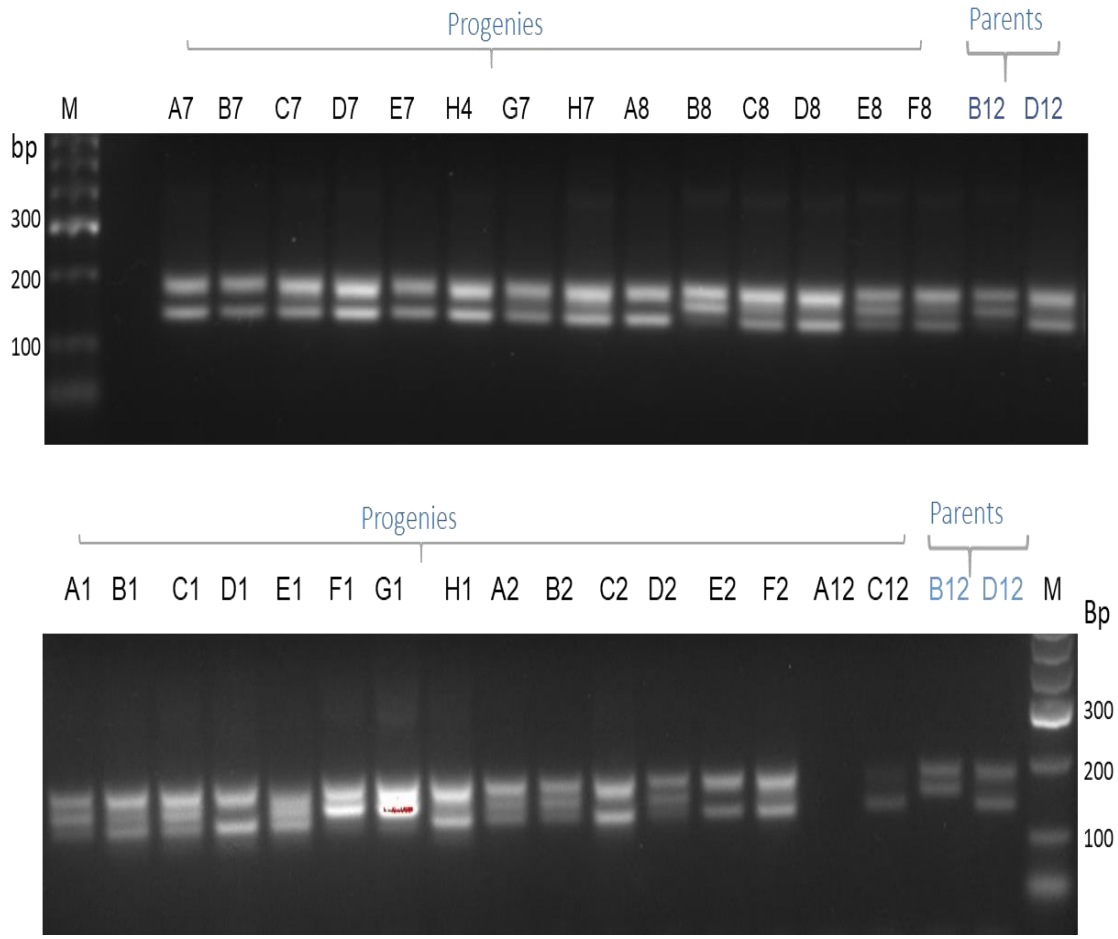

Figure 9. Electrophoresis of amplified *Lr22a* gene tested for 28 F_2 progenies (indicated from A1 to C12) and 2 parents (B12 and D12) on 2.5% agarose gel. M indicates Hyper Ladder 100bp (Bioline).

It can be clearly seen that the amplification of the *Lr22a*-linked marker generated polymorphic bands where the size ranged from approximately 120 – 180 bp. All parents and progenies shared the same size band of 180 bp, however, they differed from each other in the bands of smaller sizes. Beside the shared band of 180 bp, some individuals showed another band either approximately 160 bp or 140 bp in length while others showed both fragments.

For the better scoring, the polymorphic fragments were then separated on 12% polyacrylamide gel (Fig. 10).

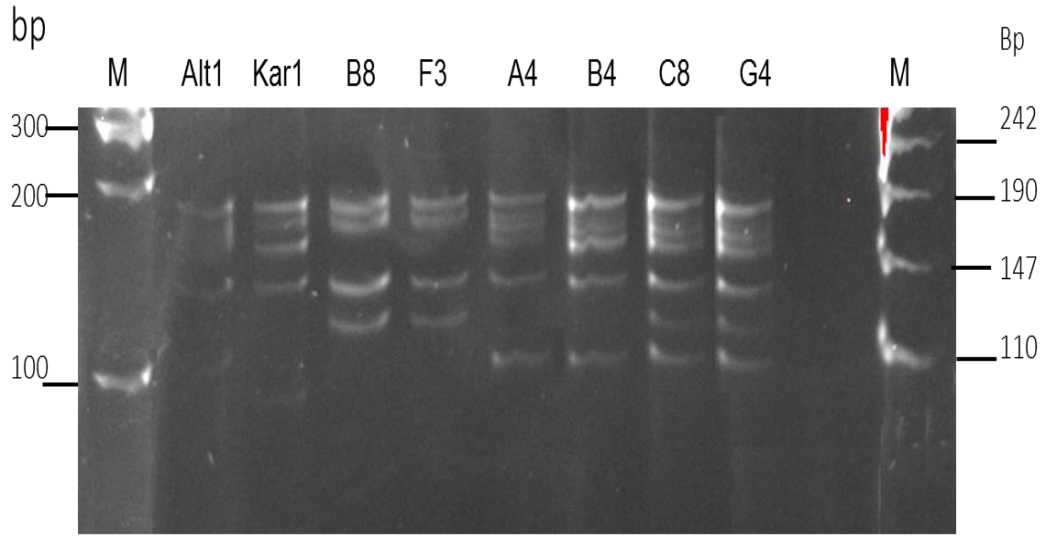

Figure 10. Electrophoregram of PCR products with *Lr22a*-specific primers separated on a 12% polyacrylamide gel. Alt1 and Kar1 are parental forms. Plants from the F_2 progeny derived from a cross of these parents are identifies in the top of the Figure with positions in 96-well plate. Lambda-DNA ladder is indicated (M) and size of fragments are shown.

Figure 10 highlights the variation in fragment lengths, even more so than that separated on anagarose gel. One attempt was made to genotype the plants based on the polymorphic patterns. Plants B6 and F3 have bands 120 bp with homozygote genotype '*aa*'. Plants A4 and B4 have bands 110 bp with homozygote genotype '*bb*'. Last two plants, C8 and G4 have both bands 120 and 110 bp with heterozygote genotype '*ab*'. Bands for 130 bp are non-polymorphic. Presence of bands for 180 bp and absence of bands of 160 bp are associated with '*aa*' genotypes in plants B8 and F3.

3.1.2. Length polymorphic marker lined to stem rust resistance gene Sr2

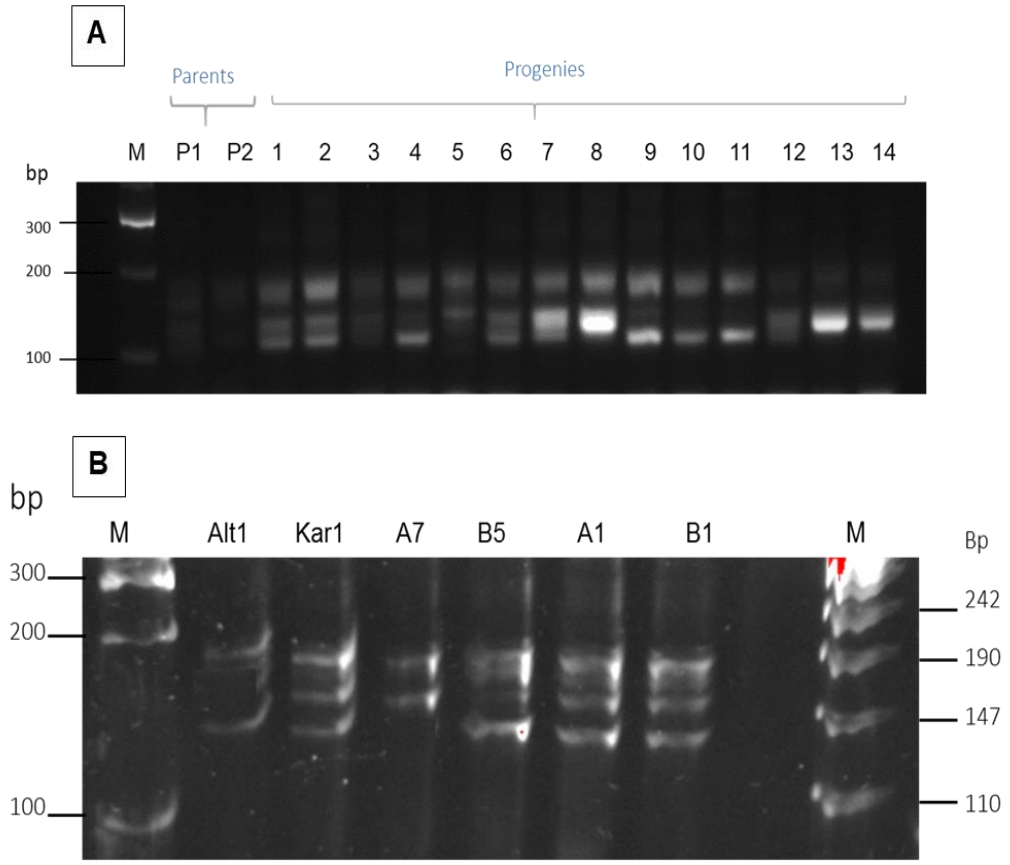

Figure 11. Electrophoretic separation of PCR products with *Sr2*-specific primers on a 2.5% agarose gel (A) and a 12% acrylamide gel (B). Alt1 and Kar1 are parental forms. Plants from F_2 progeny are identifies in the top of the Figure with positions in 96-well plate. The 100bp Lambda marker is indicated as M.

Plant A7 has band at 160 bp with the homozygote genotype '*aa*'. Plant B5 has a very faint band at 160bp and a band at 140 bp with homozygote genotype '*bb*'. The last two plants, A1 and B1 have both bands at 160bp and 140 bp with the heterozygote genotype '*ab*'. Bands for 200bp are non-polymorphic.

Figures 9 - 11 outline the length variation of amplified fragments linked to leaf and stem rust resistance genes. In hexaploid bread wheat, PCR products amplified from genomic DNA may produce multiple fragments of similar size, which are derived from more than one of the component genomes (Figures 1 and 2). In these cases, the bands can be used as length SSR polymorphic markers, however, for future studies developing molecular markers based on allelic polymorphism in one of the homoeologous genomes of wheat, a strategic way to focus

of the polymorphic fragment is needed. To do this, the sequence of the different homeoalleles is needed to design a set of gene-specific primers that amplifies only the target allele (Helguera et al., 2000). Given the technical difficulty of either, cutting out the narrow bands with similar sizes on the agarose gel for PCR purification, or using a mixture of PCR products for Sanger sequencing that will yield unmeaningful results, the best way to distinguish amplicons corresponding to those homoeologous genomes is to ligate and clone all the PCR products into a plasmid vector, and identify the different products by DNA sequencing.

3.1.3. Cloning of length polymorphic fragments

A considerable amount of effort was invested in cloning of polymorphic fragments. After checking the presence of the plasmid in bacterial cells by plasmid purification and agarose gel visualization, five colonies including three containing the plasmids with inserts, one positive control and one negative control were digested with the restriction enzyme *Eco*RI, which is likely to cut the plasmid twice either side of the insert. The results for the plasmid purification and digestion are shown in Figure 12.

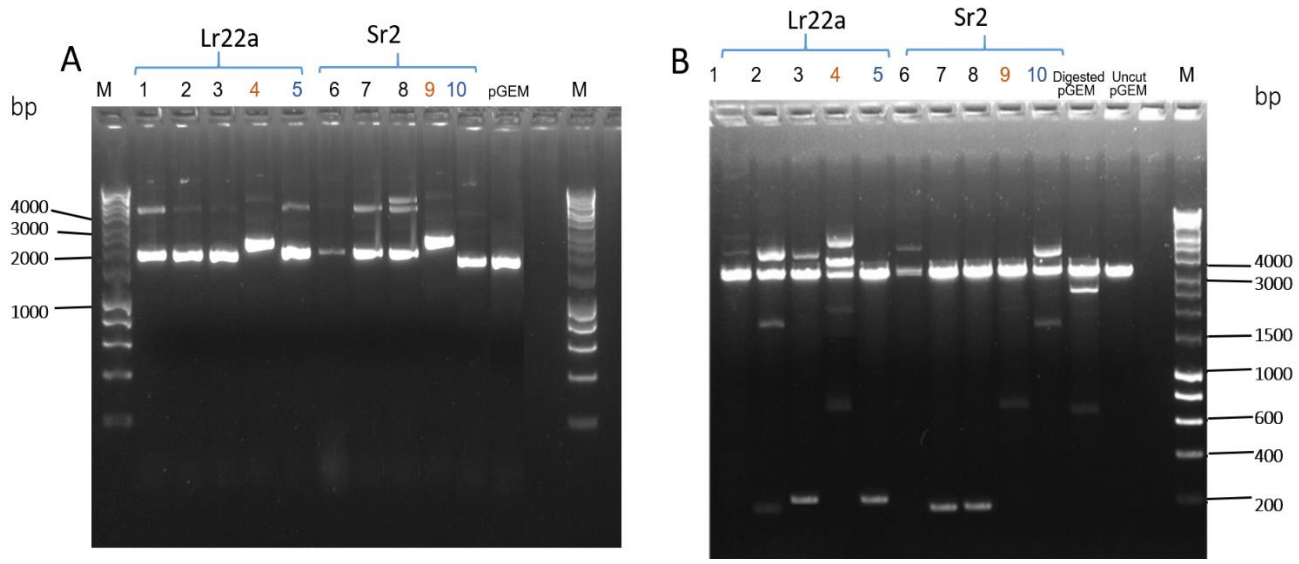

Figure 12. Electrophoresis of plasmid purified from JM109 baterial cells (A) and plasmid digestion with *Eco*RI restriction enzyme to release the inserts (B) on 1% agarose gel. Numbers coloured in red are positive control with control insert of 542 bp. Numbers colorred in blue are negative control (blue colonies). M indicates the 1kb Lamda DNA ladder (Bioline, Australia).

Before the plasmid purification step, colony PCR was conducted to screen the presence of inserts in 40 white colonies for each marker of interest (*Lr22a* or *Sr2*). This step was aimed to save time

and resources for plasmid purification and the data is shown in appendix 8. The plasmids in screened colonies that showed the presence of inserts were then purified using Wizard Plus SV Minipreps DNA purification System (Promega) and digested with the *Eco*RI restriction enzyme. The results shown in Figure 10 highlighted the successful ligation of inserts into the pGEM-T vector and the appropriate digestion of plasmids to release the inserts of interest. Three inserts with sizes ranging from approximately 140 – 190 bp and two inserts with similar size of 150 bp were recorded for markers linked to *Lr22a* and *Sr2*, respectively. The positive controls (sample 4 and 9) released the control inserts with the expected size of 542 bp. However, unexpectedly a band from the negative control (sample 5), which theoretically contains no insert, was also detected.

Four samples including three fragments for *Lr22a* and one fragment for *Sr2* were chosen for sequencing using M13 primers provided by AGRF. The sequencing result is shown in Figure 13.

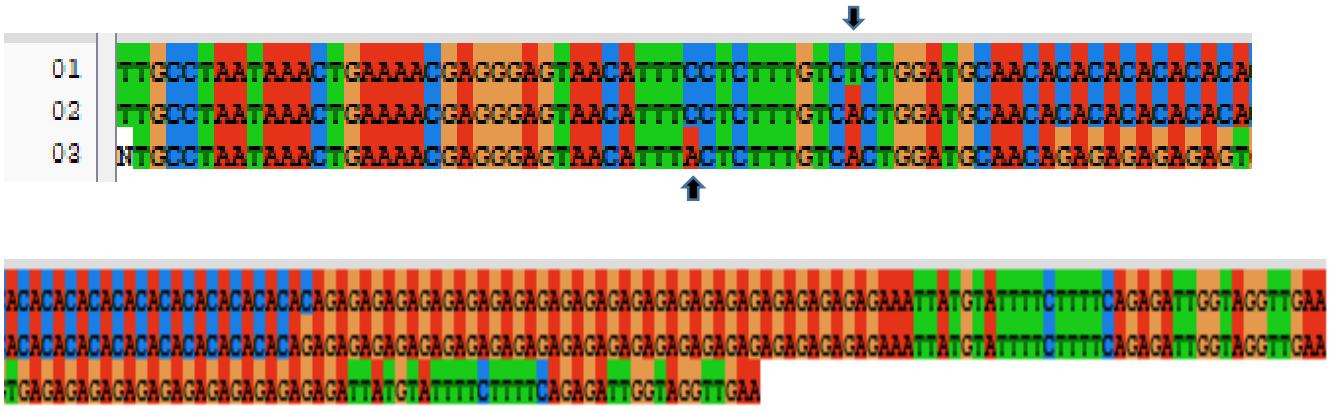

Figure 13. Sequence alignment of three polymorphic fragments lined to *Lr22a*. Fragments 1 and 2 share the same size of 181 bp while fragment 3 is 132 bp in length. Nucleotide difference between sequences is indicated by black arrowhead. The sequences are aligned using ClustalX 2.1 programme with multiple alignment mode.

The sequencing result of cloned fragments showed the exact sizes of 181 bp for both fragments 1 and 2, and 132 bp for fragment 3. The difference in length between these fragments is due to the expansion of 18 units of AC dinucleotides and 24 units of AG dinucleotides in 181 bp fragments compared to 131 bp fragment. A BLAST search was performed against all available sequences in Gene bank database. A high degree of collinearity between the sequence of interest and the wheat cultivar, Chinese Spring sequence, was recorded. The result of the BLAST search also showed the likely location of fragments linked to *Lr22a* was on the short arm of chromosome 2D, corresponding to previous findings (Dyck, 1979; Hiebert et al., 2007; Ingala,

2012; Thind et al., 2017). This is in the encoding region of the intracellular immune receptor homologous to the *Arabidopsis thaliana* RPM1 protein. This gene is an adult plant resistance gene which is usually expressed at the adult stage of plant development (Mishra, 2015). For *Sr2*, The BLAST search for one of the sequences indicated the location of this fragment on the short arm of chromosome 3B.

Moreover, the result of sequence alignment also highlighted the sequence diversity of fragments. Two single nucleotide polymorphisms were detected. One was T/A transition between fragments 1 and 2 at position 46 and another one was A/C transition at position 36 different from fragment 3 to others.

Interestingly, by checking the recognition site for any restriction enzyme cutting the fragment at SNP position using NEBcutter website (http://nc2.neb.com/NEBcutter2/), one recognition site for *Bco*DI restriction enzyme was detected. This enzyme cuts fragment 1 at GTCTC sequence at T/A SNP position, revealing a potential use of this enzyme to distinguish the same size fragments that might be amplified from different genomes.

3.2. Identification of SNP marker for leaf rust resistance genes *Lr51*

After screening 24 primer sets with standard PCR conditions and three of them were determined as length polymorphic markers, six candidate PCR fragments with good PCR amplifications but no length polymorphism were chosen for sequencing.

The sequencing results of the six selected fragments showed clear and good quality results. Using the EMBOSS needle website to compare the sequences in pairs, the potential SNP were indicated in the two parental cultivars sued in this study. However, when the sequences were double-checked with Chromas Lite which visualizes the nucleotide sequence and also showing the reliability of putative SNP, five of six sequenced genes showed no SNPs. Interestingly, most of sequences contained the continuously repetitive nucleotides which are the potential for development of Simple Sequence Repeat (SSR) marker (Appendix 4).

Among 6 sequenced fragments, Lr51-linked segment was the promising one. The visualization of the PCR products on 1.0% gel showed a good PCR amplification with clear and sharp bands on the gel, but there was no difference in size of these bands, indicating no length polymorphism (Figure 14).

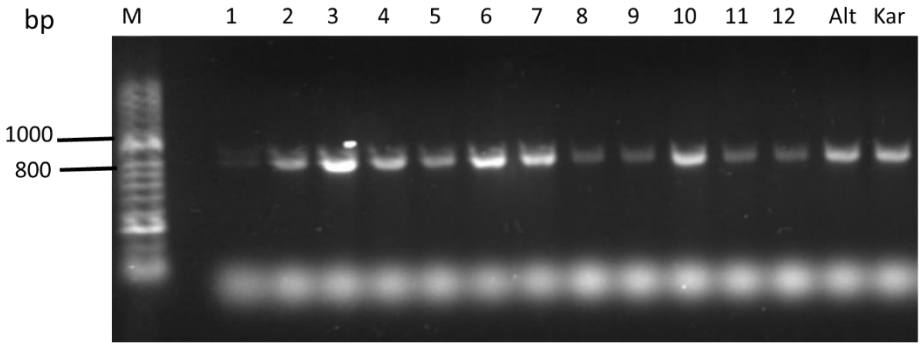

Figure 14. PCR amplification of Lr51-linked fragment visualized on 1.0% agarose gel. Alt and Kar are parental forms while their progenies are indicated from 1-12. M represents the 100 bp Lamda DNA ladder (Bioline, Australia).

Interestingly, a rare but clear SNP was detected at the 519 bp position of the *Lr51*-linked fragment. The G nucleotide present in the sequence of the Altaiskaya (Alt) parent fragment was replaced by a T nucleotide at the same position in the Karabalykskaya (Kar) parent. The SNP was checked with both forward and reverse directions confirming the greater reliability (Fig 15).

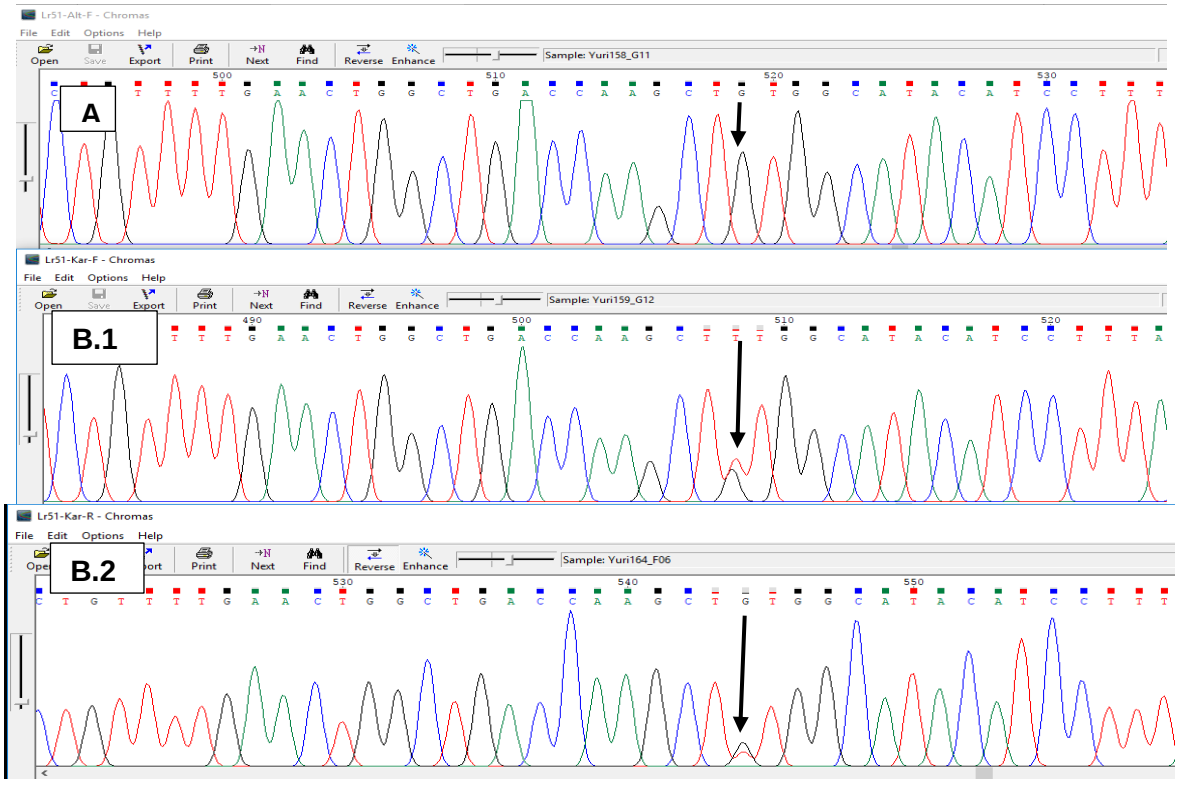

Figure 15. The identification of SNP marker of *Lr*51 in both parental forms using Chromas Lite. program. The transition of nucleotide G in one parent Altaiskaya (A) is replaced by nucleotide T in another parent Karabalykskaya (B). The black arrow indicates the position of SNP which is checked in both directions in the Karabalykskaya cultivar with both forward (B.1) and reverse (B.2) primers.

The identified SNP is a substitution of nucleotide G for T in position of 519 bp. The comparison of two putative amino acid sequences translated from the nucleotide sequences using ExPASy translate tool (https://web.expasy.org/translate/), the result showed that the SNP was in the triplet nucleotides coding for the same amino acid (both triplets CTG and CTT code for Leucine amino acid). This reveals that the identified SNP does not alter the amino acid sequence of protein.

3.3. Development of a CAPS Marker for *Lr51*

Due to the very low frequency of SNPs found from sequencing results of the Lr51-linked PCR product, there was no guarantee that the identified SNP would be suitable for use as a molecular marker unless its location was in any restriction enzyme recognition site. Following the identification of SNP, the NEBCutter website was employed as an online bioinformatics tools to detect the restriction enzymes cutting the DNA fragment at SNP position. Surprisingly and fortuitously, the identified SNP affected the recognition site of one restriction enzyme within the PCR fragment: *Hind*III. This enzyme cuts the sequence at AAGCTT recognition site. The affected recognition site at SNP was only present in fragment amplified from the Altaiskaya parent which is phenotypically recorded as resistant to some leaf and stem rusts, but not present in the fragment from the other parent, Karabalykskaya, which is sensitive to most rust infections.

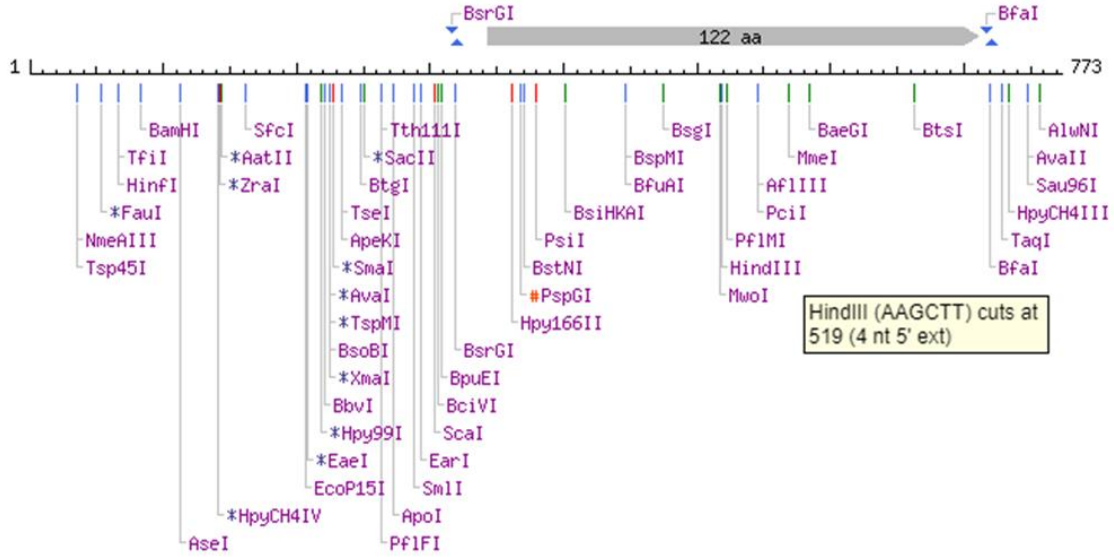

Figure 16. Mapping of restriction enzymes cutting the sequence of the *Lr51*-linked fragment using NEBcutter online bioinformatics tool. *Hind*III is detected as restriction enzyme that cuts the sequence at AAGCTT recognition site at 519 bp SNP position.

Based on the results of restriction enzyme determination, the development of CAPS marker corresponding to the SNP marker was already investigated and shown in Figure 17. The *Hind*III restriction enzyme digestion of the PCR products amplified by specific *Lr51* primers was conducted to develop a novel diagnostic CAPS marker.

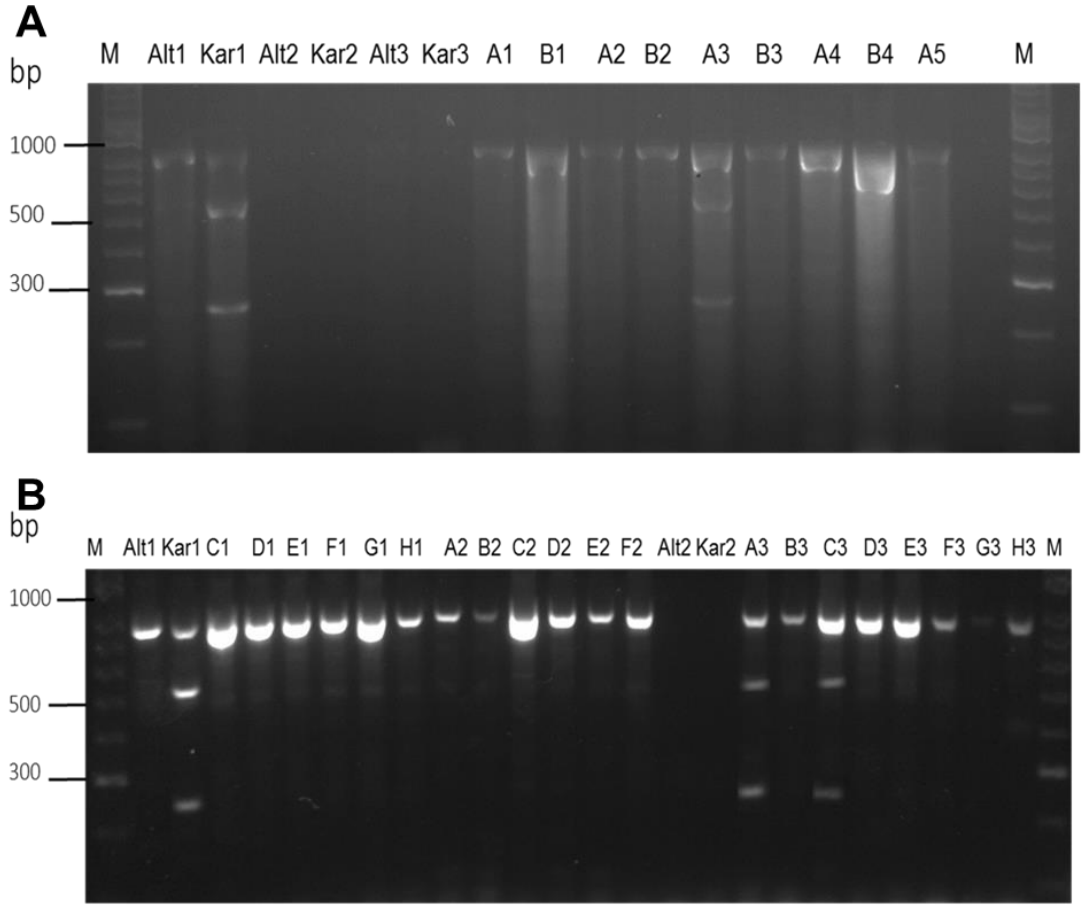

Figure 17. Electrophoregram of *Hind*III digested PCR products separated on a 1% (w/v) agarose gel. The digestion of PCR product results in two smaller fragments of approximately 519 bp and 281 bp while the undigested product remains the size of 800 bp. Altaiskaya1 (Alt1) and Karabalykskaya1 (Kar1) are parental forms. Plants from F_2 progeny are identifies in the top of the Figure with positions in 96-well plate. The 100 bp ladder (Bioline) was included as a size standard (Lane M) with the sizes shown listed in base pairs.

Figure 17 outlines the results of electrophoretic separation of the Lr51-linked PCR products digested with *Hind*III. The forward primer S30-13L and reverse primer AGA7-759R amplified a PCR product linked to *Lr51* with size of approximately 800 bp. Genotypic interpretation of this result is that the plant, Alt1, has a proposed homozygote genotype '*aa*' with a single band of 800 bp (there is no recognition site of *Hind*III) while plant Kar1 is a heterozygote genotype '*ab*' with three bands 800+519+281 (the recognition site of *Hind*III is occurred due to the SNP in

allele '*b*'). Plants A3 (A) and A3 and C3 (B) showed three bands identical to those in paternal plant Kar1 with the heterozygote genotypes '*ab*'. Genotypes with allele '*a*' and allele '*b*' are indicated as sensitive and resistant to *Lr51* strain pathogen, respectively. The distribution of the two alleles amongst the progeny is not equal relating to possibility of linkage disequilibrium. No bands were produced with other DNA samples of parental forms due to low template or poor DNA quality.

3.4. Application of Amplifluor-like SNP assay for genotyping of *Lr51*

In order to develop a more rapid and high-throughput tool based on the SNP identified in the *Lr51*-linked PCR product, an Amplifluor-like assay was developed. This assay showed the successful detection of the G/T transition in the identified SNP. The assay depends on the interaction between a quencher molecule in the middle of a Universal probe, in close proximity to the fluorophore at 3'-end whereby the fluorescence resonance energy transfer quenches the fluorescence. The allele-specific primers were added to the PCR cocktail during the annealing phase of the PCR. The primers hybridized to the strands of the PCR products and during the extension phase of PCR, the 5'-3' exonuclease activity of DNA polymerase extended the newly synthesized strand from the perfectly matched and annealed primers. Followed by the denaturation in the next cycles of PCR, then the annealing or matching of the allele-specific primer's tail complementary to universal probe's tail, subsequently the extension phase opened the stem-loop structure and separated the fluorophore from the quencher. Once the distance between the quencher and fluorophore was created, the fluorescence signal increases with subsequent cycles of the PCR.

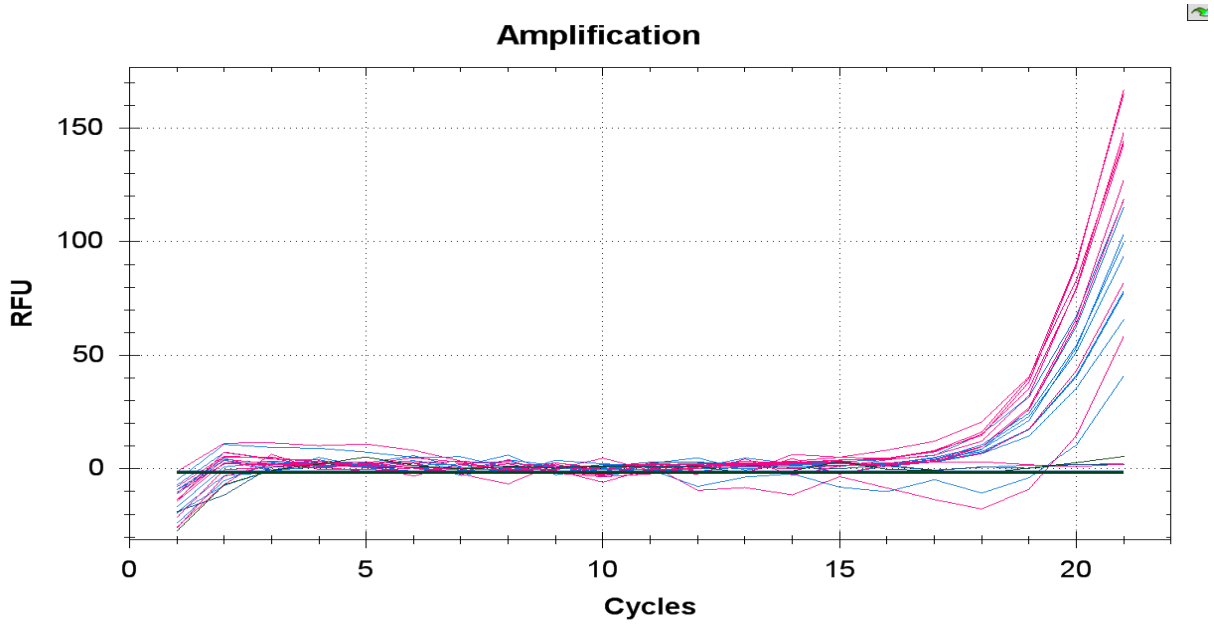

Figure 18. PCR amplification curves for Amplifluor-like universal primers labelled with FAM and HEX corresponding to the number of PCR cycles. Fluorescent signals are detected as different colours, blue and purple, for FAM and HEX, respectively. Relative fluorescent unit (RFU) is shown in Y-axis.

The Figure 18 shows amplification plots produced for the chosen samples and reference genotypes. Amplification was recorded over a range of PCR cycles from the starting point to 21 cycles. Figure 18 illustrates that at a threshold cycle, the fluorescence rises to a detectable level of relative fluorescence unist (RFU) and increase as more PCR products accumulate. The optimal number of PCR cycles for detection of fluorescence in the current assay was 18 cycles. At lower cycles, there is insufficient FAM or HEX signal to distinguish between samples. However, too many cycles can lead to the false-detection as the mismatched primer can be over-amplified.

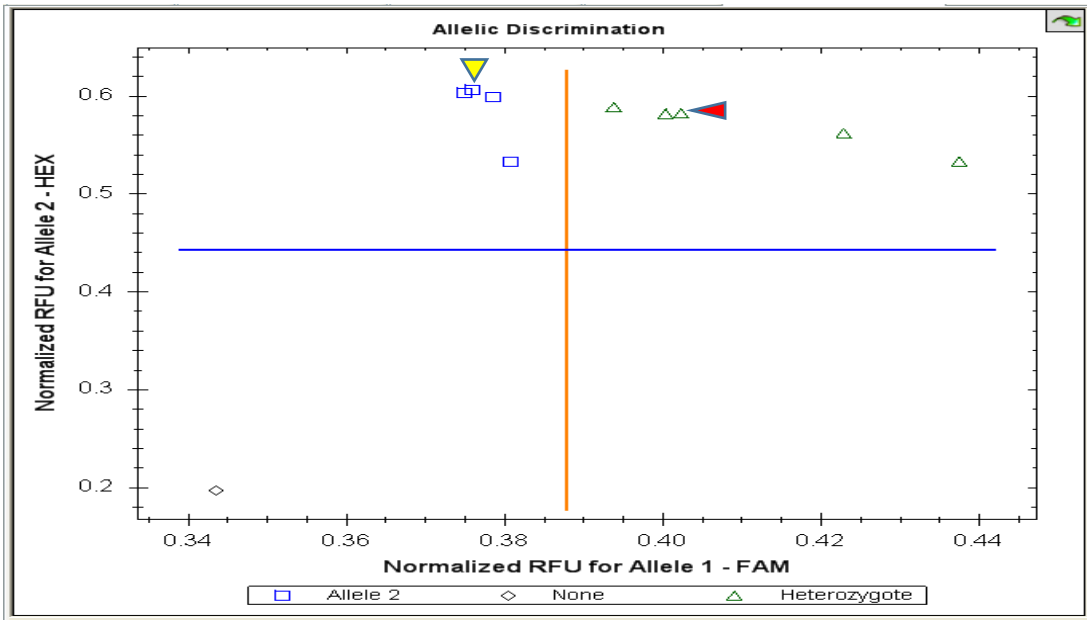

Figure 19. Genotyping results of *Lr51*-linked – Amplifluor SNP marker scored in the parents and group of seven F_2 segregating progenies from their reciprocal crosses. The parents were used as reference genotypes with homozygous parent indicated with yellow arrowhead and heterozygous indicated with red arrowhead. X- and Y- axes represent Relative Fluorescent Unit (RFU) for FAM and HEX fluorescent signals, respectively. The blue and green dots call for homozygotes and heterozygotes, respectively, while black rhombus in the bottom left quadrant is negative control (No Template Control)

Figure 19 shows the clear allelic discrimination generated by the self-designed Amplifluor-like method. The co-dominant Lr51_T/G Amplifluor-linked assay successfully categorized the zigousity of the homozygous resistant (blue rectangles), heterozygous resistant (green triangles) and undetermined (black rhombus) genotypes. Since the forward primers matching to T and G nucleotide corresponding to HEX and FAM fluorophore, respectively, the comparison of allelic fluorescence signals between two fluorophores will illustrate the zygosity.

In CAPS marker screening with the parents and 27 F2 progeny plants, there were two samples (A3 and C3) showing the heterozygous genotype which was also observed in one reference parent Kar1. For this reason, these samples were included in Amplifluor-like assay. When normalised to the heterozygous calibrator, five of the nine plants including the tested genotypes in CAPS assay and one reference parent were classified as heterozygotes. Four samples were identified as homozygotes with allele2 and only one plant was scored as null (negative control) and most likely the result of degraded and poor template DNA.

Chapter 4: Discussion

The identification of some commonly used molecular markers (SNP, CAPS and Amplifluor-like markers) linked to rust resistance genes and the applications of the identified markers for plant genotyping are the overarching aims of this work. The CAPS markers have been successfully identified and applied for genotype analysis in previous studies (Azhaguvel et al., 2012; Raats et al., 2014). However, in this project, it is the first time CAPS marker have been applied in Kazakh bread wheat varieties.

One of the main aims of this study was to clarify the effectiveness of the self-designed Amplifluor-like technique for genotyping that was developed by Yuri Shavrukov and his colleagues (provide a reference here). Given the high efficiency of this technique tested for drought tolerance genes in barley and wheat (Jatayev et al., 2017), an attempt to prove and establish a reliable protocol for SNP genotyping based on fluorescence amplification was made. This is a crucial step to achieve an accurate determination of genotypes and facilitate further development of more reliable and cost-effective genotyping technology.

4.1. Length SSR polymorphisms for *Lr22a* and *Sr2* and their characterizations

Because of the great abundance (>80%) of repetitive sequences in the wheat genome (Blake et al., 2004), the length polymorphic bands generated from *Lr22a* and *Sr2* showed a large portion of repeated sequences. For development of PCR primers that are more specific and efficient, their design should target the conserved genic regions (Blake et al., 2004). In practice, exonic sequence in protein coding genes are usually more conserved with fewer polymorphisms than introns and untranslated regions (Haga et al., 2002). To design the conserved primers, tools in comparative genomics are employed. The alignment of sequenced fragments against ESTs can indicate the exonic and intronic regions. Therefore, the complete sequence of *Lr22a* and *Sr2*-linked fragments obtained from sequencing of clones are beneficial for conserved primer design. In addition, they are also useful for the mapping of these loci on a genetic map for future attempts to isolate and clone these genes.

The reason for length polymorphic fragments shown in products linked to *Lr22a* and *Sr2* is the polyploid structure of the wheat genome. The PCR products with multiple fragments of similar lengths are amplified from more than one of component wheat genomes.

For the *Lr22a*-linked marker, the length polymorphism between fragments 1, 2 and 3 was visualized on agarose and polyacrylamide gels. The insertion of AC and AG dinucleotide repeat in 181bp fragment made it different in length from 131 bp fragment. Di-nucleotide repeats are subject to slippage during DNA replication which leads to expansion and contraction, hence their frequent length polymorphism. Furthermore, the result of sequencing emphasised the sequence diversity of fragments. In addition to the length polymorphism detected on gels, two SNPs were detected. Among them, one SNP in fragment 1 is useful for developing CAPS marker using *Bco*DI restriction enzyme. The digestion of the product amplified from one genome will generate two fragments with expected sizes of 46bp and 135 bp, allowing the differentiation of the PCR products from target genome than that of the other genomes. Another application of the sequence information from the inter-homoeologous fragments is to assist in the design the genome-specific primers. The perfect match of designed primers for one nucleotide difference between homoeologous sequences allows the amplification of products from the target genome. This greatly simplifies the assay and contributes to solving one of the main problems of working with such complex genomes as hexaploid wheat: avoiding the cross-amplification in the PCR of target in paralogs from the same genome and from homologs and paralogs in the homoeologuos genomes.

4.2. Characterization of *Lr51*

The result of the BLAST search for the sequence of *Lr51* on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_32812835) showed the 100% identity of the ADP-glucose pyrophosphorylase (AGP2) gene family from *Triticum aestivum* that has been published in Chinese Spring and Neepawa cutivars (GenBank # X14350.1). The gene codes for a large subunit of ADP-glucose pyrophosphorylase (AGPase) which comprises two small and two large subunits. The large subunits play a regulatory role while the small subunits have catalytic roles. This protein functions as a key enzyme in the endosperm starch synthesis pathway in wheat which catalyses the conversion of glucose-1-Pi to ADP-glucose. AGPase is usually expressed in leaves and endosperm (Olive, 1989). It is highly unlikely that ADP-glucose pyrophosphorylase is involved in leaf rust resistance and this most likely represents a closely

linked sequence to *Lr51*. The gene coding for AGPase protein is highly conserved that can be found in any wheat species, however, *Lr51* was not originally present in bread wheat genomes. This is the result of introgression process from wild species to domesticated wheat.

Leaf rust resistance gene *Lr51* has been introgressed from chromosome 1S of diploid *Aegilops speltoides* (2n = 2x = 14, genome SS) to chromosome 1B of hexaploid bread wheat *Triticum aestivum* L. (2n = 6x = 42). This gene is present on an interstitial segment (approximately 14-32 cM long) translocated to long arm of chromosome 1B (Helguera et al., 2005). Wheat breeders are introgressing the useful rust resistance genes word wide from wild accessions including *Aegilops speltoides*.

Historically, *Lr51* has shown high level of resistance to new races of leaf rust. All the plants homozygous for the *Ae. speltoides* chromosome segment carrying the *Lr51* gene, have very low leaf rust infection (Drovak and Knott, 1980). In a study conducted by Dvorak (1977), plants homozygous for *Lr51* were tested for resistance to *P.triticina* (strain 5), and the results showed a high level of resistance with hyper-sensitive flecks, whilst heterozygotes showed a lower resistance with obvious symptoms of infections including small pustules surrounded by necrosis and chlorosis. Recently, *Lr51* was tested on a wide range of wheat cultivars, showing a low level of rust infection in the presence of *Lr51* (Helguera, 2005). All mentioned studies prove the importance of *Lr51* as such a robust and broad-spectrum wheat rust resistance gene.

4.3. SNP marker for *Lr51*

In such a hexaploid genome as bread wheat, SNP discovery is classified into two types. The first are called inter-homoeologous polymorphisms, whereby in the same individual variation is between the homoeologous genomes that are not totally identical. The different genomes (A, B and D genomes) share approximately 96-98% sequence identity (Dvorak et al., 2006). The second is an allelic polymorphism between different individuals or referred as a varietal SNP. This type of polymorphism is evenly much less frequent than that of inter-homoeologous polymorphisms (Trick et al., 2012).

Considering the low frequency of inter-variety SNPs in wheat much efforts has been made to identify SNP markers linked to genes of interest. In this study, the screening of a total of 24 genes with sorting in those with good PCR amplifications but no length polymorphism was a time-consuming process that required the PCR optimization and further high-resolution electrophoresis to ascertain the quality of PCR products. Moreover, after choosing 6 genes for

sequencing, only one single SNP was identified in an 850 bp PCR product. This revealed and confirmed the very low frequency of SNPs in hexaploid wheat. The final frequency of SNP found in this study was estimated at 1 SNP per 2,5 kb. The calculation was based on the presence of one SNP out of total length of six sequenced PCR products. However, all studied wheat germplasms in this study originated from a single geographic location (Kazakhstan) with limited genetic variability. The frequency of SNP detection in wheat reported in other studies is estimated at a frequency of one SNP every 540-569 bps (Somers et al., 2003; Lai et al., 2012; Paux et al., 2012).

SNPs can lead to the change of amino acid sequence in the corresponding polypeptide and subsequently an enormous alteration in the subsequent protein's structure or function. However, the results showed that in the case of the *Lr51*-linked marker that shared similarity to the large subunit of ADP-glucose pyrophosphorylase, the identified SNP did not affect the sequence of amino acids, Thus, the SNP is likely to link with other regulatory mechanisms such as the recognition site for alternative slicing factor or changes in the promoter regions, so that the level of gene expression can be affected. An additional explanation can be related to the nature of any genetic marker, which can be just closely linked and associated with the gene of interest (in this case *Lr51*) but changes occur in another gene located nearby.

The SNP marker linked to *Lr51* can be used as the effective diagnostic tool for genotyping through CAPS or SNP-based markers which are thoroughly described later. The putative SNPs can be identified by comparing the published information without primarily sequencing required. However, sequences of global wheat germplasms are not all available online, including Kazakh wheat cultivars, therefore, one's own experiment for finding a SNP is required in this study. Moreover, although the genotyping methods used in this study could be successfully conducted based on the putative SNPs, it should be confirmed that the sequenced SNP obtained verifying its presence, facilitating the development of CAPS marker and self-designed Amplifluor-like technique. More importantly, the sequenced SNP can be used as reference for both mentioned methods and for further genotyping studies.

4.4. Development of CAPS marker for *Lr51*

Historically, the first utilization of CAPS markers in plant biology was described by Konieczny and Ausubel (1993). Subsequently, this type of marker has been widely used as an effective tool in plant breeding for selection of economically important traits, or in genomic studies to

achieve high-resolution mapping of valuable loci. Previously, CAPS markers have been reported in wild diploid wheat *Ae. tauschii* Coss (Azhaguvel et al., 2012) and tetraploid wild emmer wheat *T. dicoccoides* (Raats et al., 2014). Here, the discussion of functional CAPS marker used for rust resistance selection in hexaploid bread wheat is the focus.

The availability of identified restriction enzyme and the size difference of the digested products are the main criteria for development of a well-functioning CAPS marker. This study was initially aimed to develop a CAPS marker that can be easily applied to distinguish the genetic difference between wheat cultivars in Kazakhstan, and in a larger range of wheat cultivars in other wheat growing regions over the world. Here, the CAPS marker developed for *Lr51* leaf rust resistance gene has met the major criteria for a functional molecular marker with two main features:

1) The proper detectable lengths of digested products: The digestion of PCR products resulted in two differently sized fragments: 519 bp and 281 bp. These fragments are neither too small to be lost during electrophoresis or confused with primer's dimers, nor too large so as to migrate close to the size of the undigested band. In addition, the *Hind*III restriction enzyme cuts the sequence once at the SNP position, not at multiple sites, resulting in clear detection of digested segments. This is important since another study (Clarinde van Hierden, 2014, Master thesis,) has attempted but failed to develop CAPS marker using *Hind*III restriction enzyme in tomato due to the multiple recognition sites for enzyme at not only the SNP position but also in other conserved genetic regions which are shared between the compared individuals. Another study to develop a CAPS marker for *Lr51* in five different wheat varieties has been conducted by Helguera et al. (2005) to distinguish the amplicons from different genomes. The successful digestion of PCR products with the *Pst*I restriction enzyme revealed a separation of two digested fragments. However, their sizes were similar (422 bp and 397bp) and thus difficult to resolve on an agarose gel. This properly can lead to the confusion with non-specific PCR products generated from inappropriate PCR amplification. Therefore, the co-dominant CAPS marker system developed in this study is sufficient for differentiating the homozygous from heterozygous genotypes.

2) The *Hind*III restriction enzyme used for the CAPS marker in this study is a robust and relatively cheap enzyme which can be found in most biotechnology laboratories used for various purposes. In addition, *Hind*III can be easily ordered from many companies providing another advantage of the chosen restriction enzyme. Due to the availability and inexpensive cost of *Hind*III, the development of CAPS marker based on the identified SNP and its utilization

for testing various samples in this study and other wheat populations are straightforward and cost-effective.

In addition to the mentioned benefits, the CAPS marker also has another advantage: the ease of use. CAPS markers do not require specific instruments or high-throughput platforms. In this study, the ordinary equipment including agarose electrophoresis and Gel-Image Systems were sufficient for developing a novel CAPS marker. Therefore, the CAPS marker can be applied in small less well-equipped laboratories. More importantly, even in case of the absence of genetic sequences and the SNPs are putative and obtained by comparing the available sequences of interest from published sequence data, the assignment of developed of CAPS markers can properly be done by using a set of available restriction enzymes to find a suitable one. This approach was successfully used in barley, for example, by Řepková et al. (2009).

Nevertheless, the CAPS marker developed in this research project still has some limitations that need to be minimized for its proper utilization.

Due to the genetic variation among wheat populations, there is no guarantee that the CAPS marker developed in this study can be applied to various cultivars other than those from Kazakhstan. As mentioned above, cultivars consist of different nucleotide polymorphisms, therefore, the determination of restriction enzymes cutting the sequence at a SNP position can vary. The use of *Hind*III and *Pst*I restriction enzymes for CAPS markers in this research project and in Helguera's study, respectively, for the same gene PCR product linked to *Lr51* is a good example. However, the complete sequence of the *Lr51*-linked fragment in the two Kazakh bread wheat varieties can contribute to the greater wheat genomic sequence database, and subsequently contribute to the development of CAPS marker based on comparative genomic analysis.

Another inevitable disadvantage of the development of CAPS markers is the low-throughput technology involving in multiple steps including PCR optimisation, PCR product purification, DNA sequencing, using bioinformatics tools for restriction enzyme determination, enzymatic digestion and electrophoresis throughout the long procedure. In summary, the development of CAPS marker for *Lr51* in this study has fulfilled the main aim of project to establish a cheap but effective co-dominant marker for genotyping Kazakh wheat varieties. Although large amount of time and effort was applied, it is worth investing to find such an efficient molecular marker.

4.5. Advantages of Amplifluor-like assay used for genotyping of *Lr51*

The Amplifluor-like genotyping used in this study shared the common principles with KASP technology. More details are provided in Appendix 6 indicate the identical tails used for both mentioned approaches. Due to the business, the structure of Universal probes in KASP technique evenly was described with no information about the quencher. However, by searching from LGC Genomics website of KASP (www.lgcgroup.com), the available information reveals the use of FRET (Fluorescence resonance energy transfer) with two different fluorophores HEX and FAM in KASP technique. It is presumable that if the Universal probes in both methods are not the same, their general structures are relatively similar to each other.

The first advantage of the self-designed Amplifluor-like method is that it is much cheaper compared to the commercial KASP genotyping service. It is an undeniable that KASP technology has recently been widely used in plant genotyping worldwide as the increasing number of publications using this technique. The reagents, optimized structure of Universal probes for better accuracy and efficiency, the convenience as well as strategic marketing and business makes the KASP genotyping service a good choice for breeders and researchers despite its high costs. However, for small experiments with limit budget, the effort to reduce the expenses but retain reliable results is a high priority. In this case, the Amplifluor-like technique with a lower cost would be a considerable alternative method.

Table 6. Comparison of KASP and Amplifluor marker characteristics, cost and publications with references to these methods

	KASP	Amplifluor
Automatic/robotic system	Low- and high- throughput	Possible but not included
Sequence of probes and primer design	Unavailable	Available (disclosed)
Cost of low- and high-throughput equipment line (US$)	450 K–550 K	Not available
Cost of reagents in bulk per reaction, 5 or 10 µl (US$)	3.0–6.0	0.06–0.12
Number of publications using the technology. Scopus database (including those with plants)	151 (61)	27 (5)

Source: Jatayev et al., 2017

From the experience obtained in this study, another advantage of the Amplifluor-like technique is its flexibility and convenience of using once ordered Universal probes for long term studies, as these probes are common for all experiments using the Amplifluor method. Fortunately, the probes are easily ordered and purchased from many companies (Sigma Aldrich, Roche, etc.). In this work, the Universal probes were ordered in bulk from DNA Synthesis Company in Moscow, Russia and shared to use for various experiments related to SNP genotyping using Amplifluor-like technique. This means once the Universal probes are obtained, they can be used for further Allele-Specific PCR experiments without re-ordering. It also should be noted that the probes are stable indefinitely if covered in foil and storage at -20^{o}C.

Due to the competitively lower cost and convenience for long term use, this method is well-suited for experiments where the throughput and cost-efficiency are crucial, e.g, experiments require testing various putative SNPs or samples for successful study.

Another advantage of this promising technique is that the genotyping analysis can be conducted by many types of instruments: from the proper Real-time qPCR systems manufactured by BioRad or ThermoFisher, to regular PCR machine that are used in most biotechnology laboratories if the instruments consist two channels for absorbance detection corresponding to different wavelengths of fluorescent dyes (FAM and HEX). Therefore, in the situation of lacking proper equipment, genotyping could still be undertaken using ordinary PCR machines. From the absorbance results, the SNP allele discrimination can be analysed by manual conversion of absorbed wavelengths to fluorophore's spectra.

This *Lr51*_T/G genotyping assay is diagnostic, highly accurate and less time consuming in comparison with gel-based marker such as CAPS marker which requires more time for digestion and electrophoresis. Genotyping by this assay thus can be a good choice for large-scale genotyping of hundreds or even thousand individuals as it can be performed using a wide range of platforms including qPCR machine, PCR blocks and plate readers with 96- or 384-well plates.

For better allelic discrimination and avoiding false-positive scores for heterozygotes, the PCR protocol plays a key role as a minor change of any PCR component can significantly affect the amplification. The best PCR condition is to avoid the risk of masking true results by false amplification as the gene-specific primers used are different at only one nucleotide.

Choosing of PCR cycles used for determination of alleles is also important. The more cycles, the more risk of over-amplification leading to a false conclusion. However, an insufficient number

of cycles can also result in the inadequate discrimination. In this case, reference genotypes with known sequences are crucial. In this study, SNP position which was detected in the parents' sequences was then validated through the CAPS assay leading to genotyping with a high level of reliability.

The double validation of a SNP marker in this study illustrates the reliable use of SNP-related markers for *Lr51* genotyping in Kazakh wheat germplasms for future studies.

4.6. Development of leaf and stem rust resistant wheat germplasm using identified molecular markers

Historically, *Sr2* gene has been reported as the most extensively studied adult-stage stem rust resistance gene over the last 50 years (McIntosh 1988; Rajaram et al. 1988; Roelfs 1998; Heyden 2004; Mago, 2010). It provides an effective broad-spectrum resistance to stem rust. This is verified by the presence of *Sr2* in many current wheat varieties grown all over the world.

Lr22a is also an adult-plant stage leaf rust resistance gene that confers the resistance to leaf rust from 25 days of age (Thind et al., 2017). In spite it is less importance to rust resistance compared to that of *Sr2*, this gene has showed a high level of resistance to a wide range of leaf rust pathogens (Hiebert et al., 2007).

In contrast to *Lr22a* and *Sr2* which are expressed in adult stage of plant, *Lr51* is developed in a seedling stage. Previous studies have shown the high level of resistance conferred by *Lr51* to predominant leaf rust strains (Kolmer, 2005; Ahmed, 2010), confirming its importance in rust resistance selection.

Due to *Lr51* having a short-lived resistance to leaf rust which is effective for only the seedling stage, the combination of both leaf rust and stem rust resistance genes for longer and greater durable resistance against increased number of pathogens is a strategic approach.

In this study, a set of PCR-based markers for effective selection of important rust resistance genes *Lr51, Lr22a* and *Sr2* has been developed. They could be used reliably in breeding programmes to accelerate the deployment of *Lr22a, Sr2* and *Lr51* in new commercial bread wheat varieties and keep them in track during the introgression progress.

Recommendations for future works

A phenotyping scoring of three genes (*Lr22a, Sr2* and *Lr51*) linked to the molecular markers that have been developed in this study in the Kazakh varieties is necessary to examine the importance of those to rust resistance.

In validating the CAPS molecular marker developed in the current work, a diverse set of wheat cultivars grown in Kazakhstan and Australia should be tested in future work. The CAPS markers developed here were found and tested in only two Kazakh cultivars, Altaiskaya and Karabalykskaya, which are phenotypically scored as resistant and susceptible to some leaf and stem rusts, respectively, and their breeding lines. The validation of these markers in a wide range of wheat germplasm will verify their efficiency and effectiveness.

The application of the self-designed Amplifluor-like technique for genotyping in this project has shown many advantages over commercial genotyping technologies including the greater flexibility for researchers, time-saving feature compared to CAPS marker-based genotyping that require multiple steps of PCR performance, restriction enzyme digestion and electrophoresis separation. Although the genotyping results were in a level of accuracy, it still remains a disadvantage: the low specificity that need to be improved. The genotypes of samples tested are determined based on fluorescent signal (Relative Fluorescence Unit) of either HEX or FAM fluorophore. The better discrimination of fluorescent signal in different genotype groups, the greater reliability and accuracy the assay has. From a researcher's point of view, the spreading of scattered signals grouped in same cluster reveal the room for improvements to make the *Lr51*_T/G genotyping assay more specific and accurate. Jatayev et al. (2017) mentioned that the minor change in PCR components can lead to the huge effect in final result of genotyping, therefore, more experiments to optimize the PCR conditions are required. In addition, the strategic primer design can generate a higher specificity rate to minimize the amplification of mismatches (Lie et al. 2012). This emphasises that more consideration is needed for allele-specific primer testing. Further work of designing and testing the specificities of range of self-designed primers is required for the improved Amplifluor-like genotyping technique.

Overall this has been a successful project in identifying useful molecular markers to assist in rust resistance breeding in Kazakhstan wheat varieties. This research contributes to an ongoing need to constantly improve a recycle resistance specificities in our crop plants to combat an ever changing pathogen population. This need is only going to increase as world populations grow and climatic conditions become more extreme.

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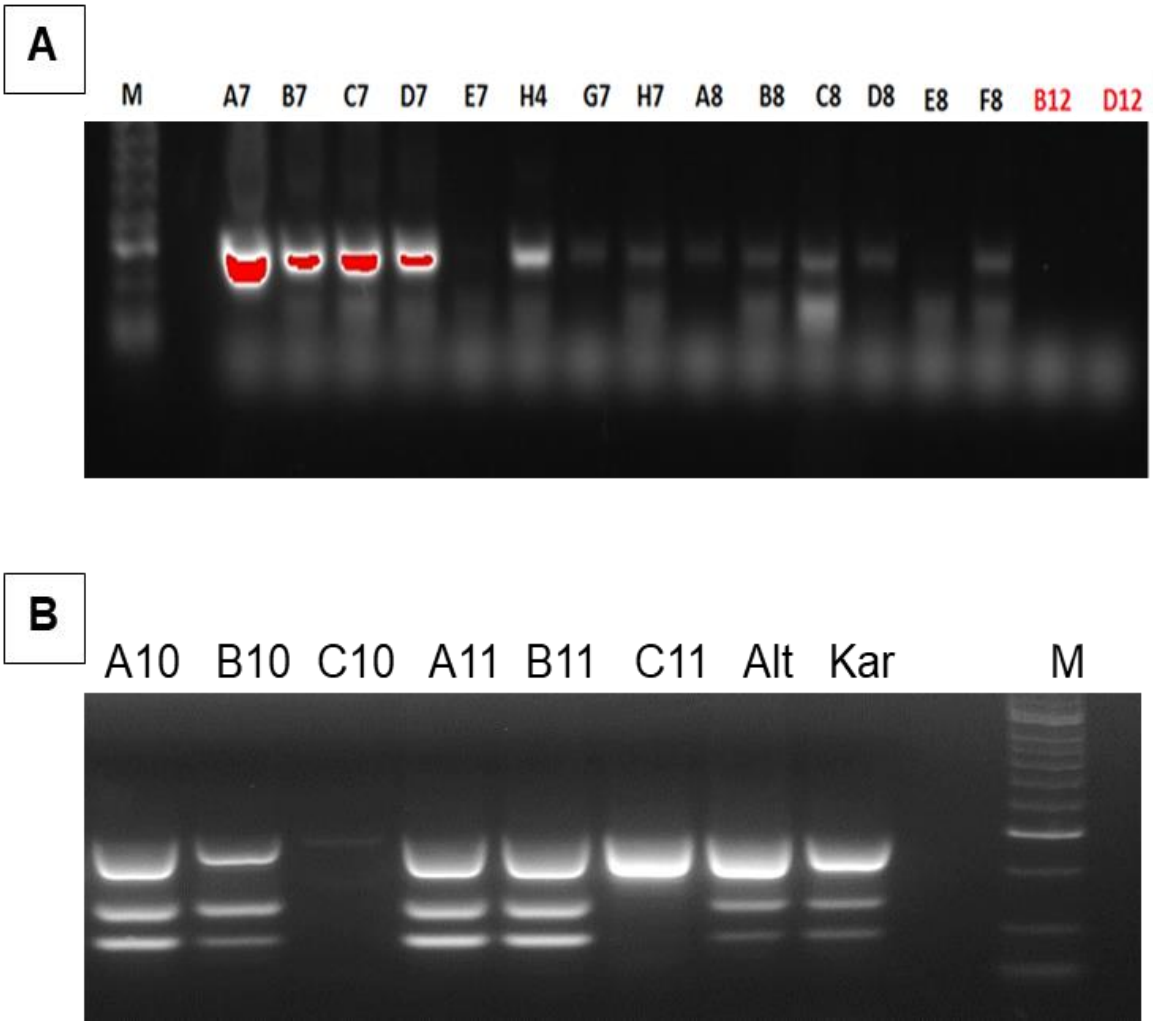
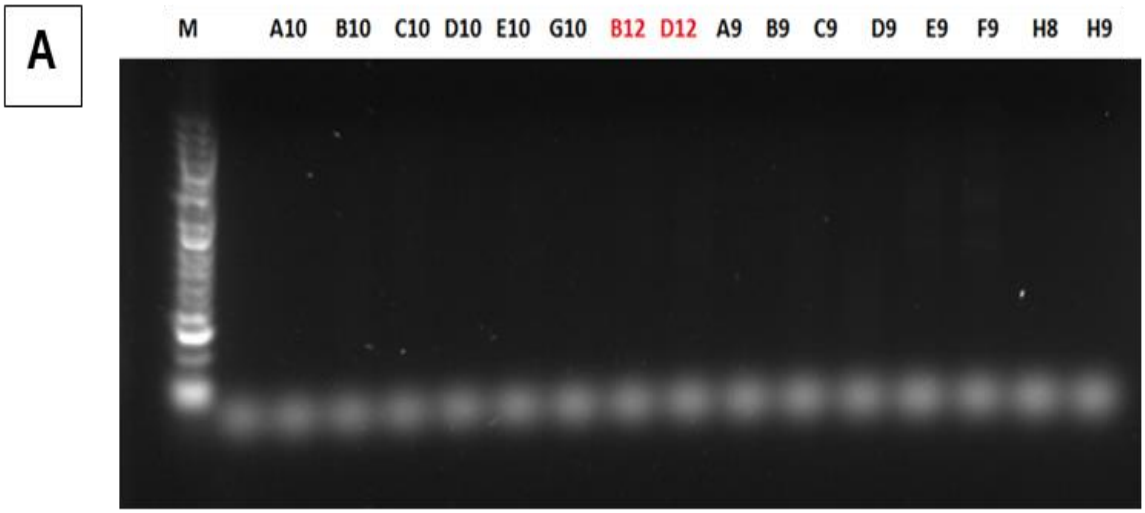
Appendices

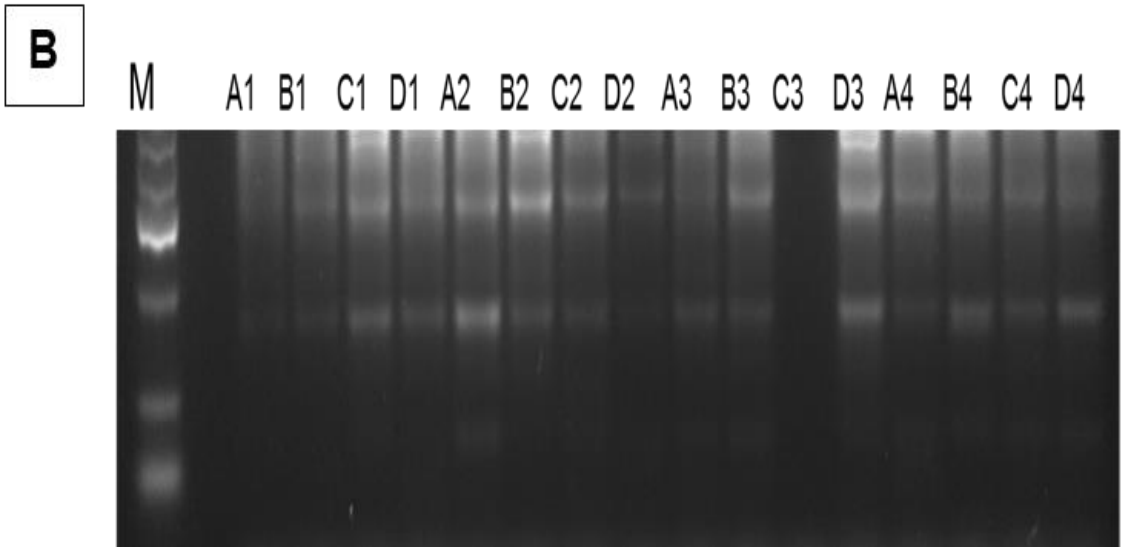
Appendix 1: PCR optimization for *Sr47* and *Srcad* genes

Electrophoresis of PCR products of *Sr47* before (A) and after (B) of PCR optimization



Electrophoresis of PCR products of *Srcad* before (A) and after (B) of PCR optimization

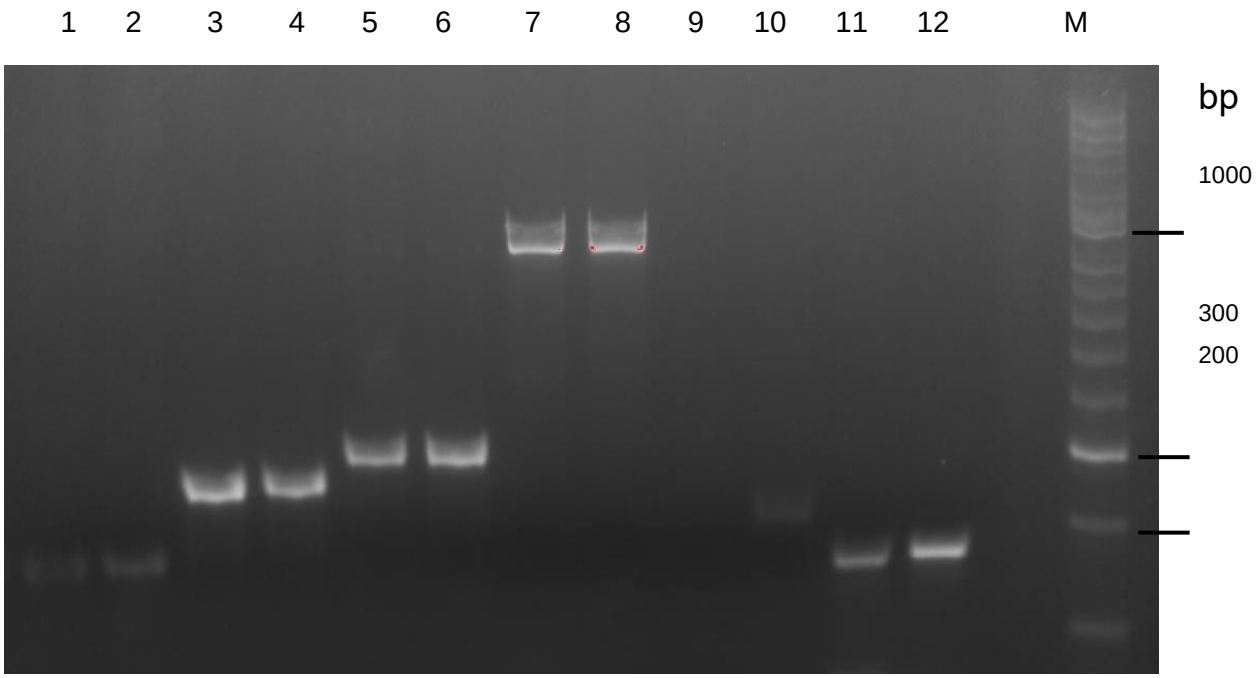




Appendix 2: Preparation of purified PCR products for sequencing of six rust resistance genes generating good PCR amplification but no length polymorphism

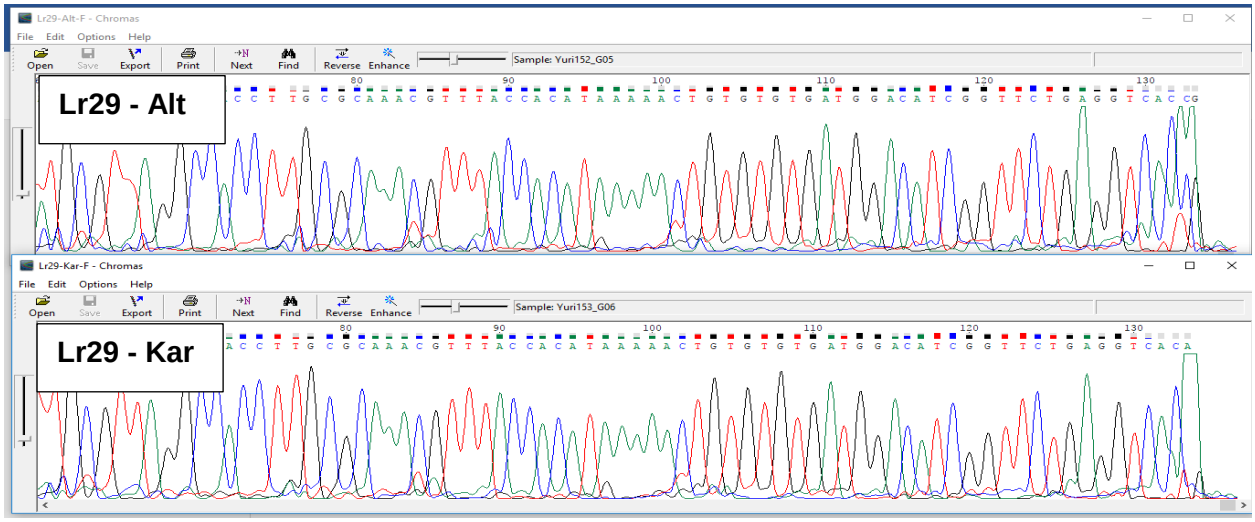
Order	Sample description	DNA concentration (ng/ul)
1	Lr29-F-Alt	7.9
2	Lr29-F-Kar	12.6
3	Lr34-F-Alt	17.6
4	Lr34-F-Kar	18.2
5	Lr37-F-Alt	11.1
6	Lr37-F-Kar	14.6
7	Lr51-F-Alt	11.0
8	Lr51-F-Kar	11.8
9	Sr22-F-Alt	3.2
10	Sr22-F-Kar	4.3
11	Sr28-F-Alt	11.9
12	Sr28-F-Kar	14.6

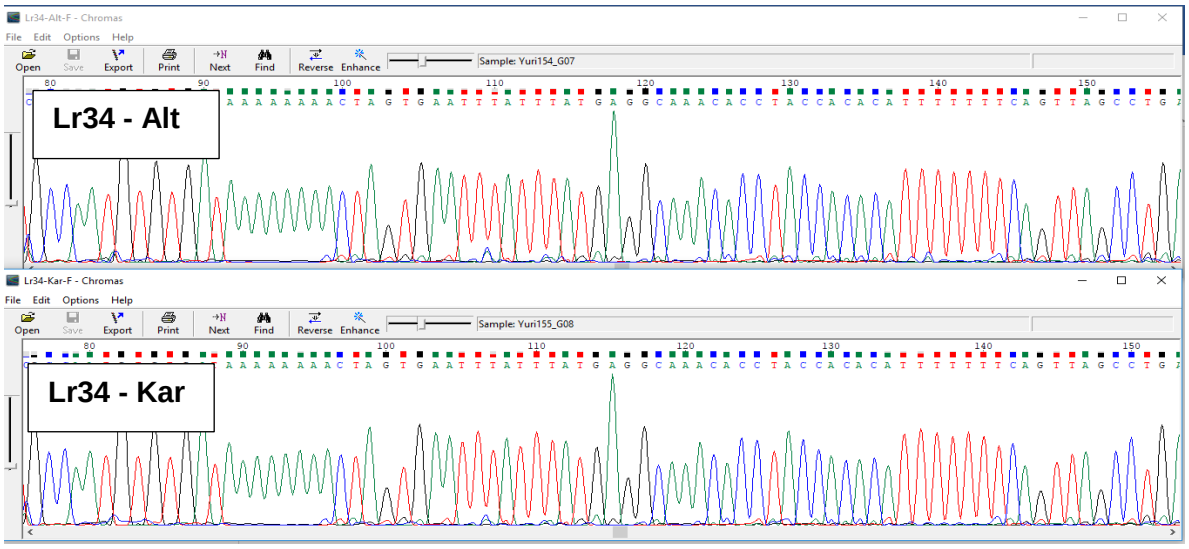
Appendix 3: Electrophoresis of purified PCR products run on a 1.0% agarose gel. The PCR product purification is conducted using FavorPrep PCR purification mini kit. Lane 1 - 12 show the amplification of six genes with both parental forms. 5uL of purified PCR products is loaded in each well. M indicates the Hyper 100bp DNA ladder (Bioline).

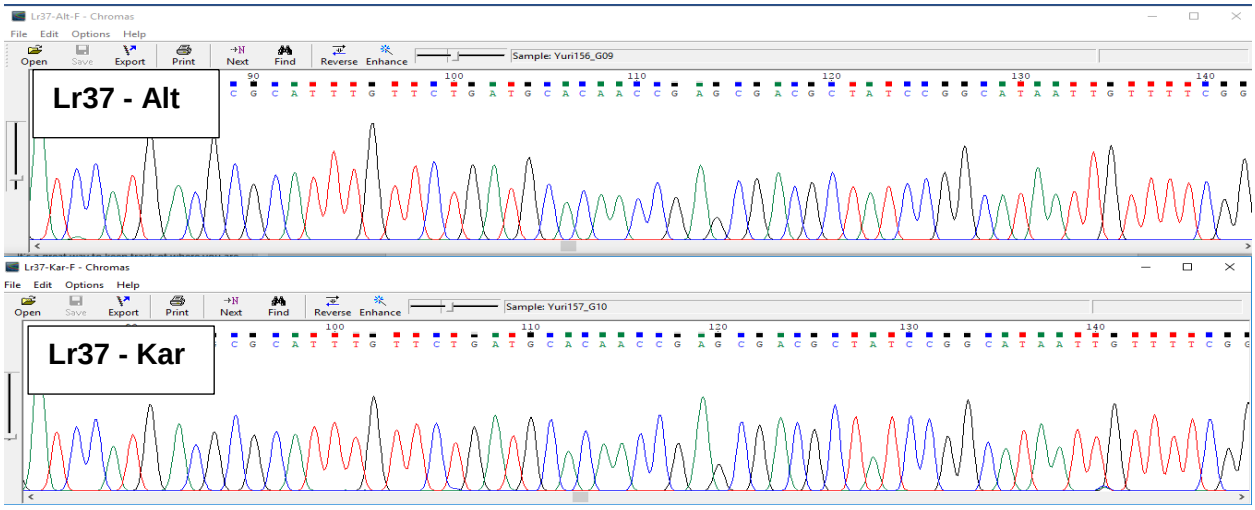


1, Lr29-Alt;; 3, Lr34-Alt; 5,Lr37-Alt 7, Lr51-Alt 9, Sr22-Alt 11,Sr28-Alt
2, Lr29-F-Kar 4,Lr34-Kar 6,Lr37-Kar 8, Lr51-Kar 10, Sr22-Kar 12,Sr28-Kar

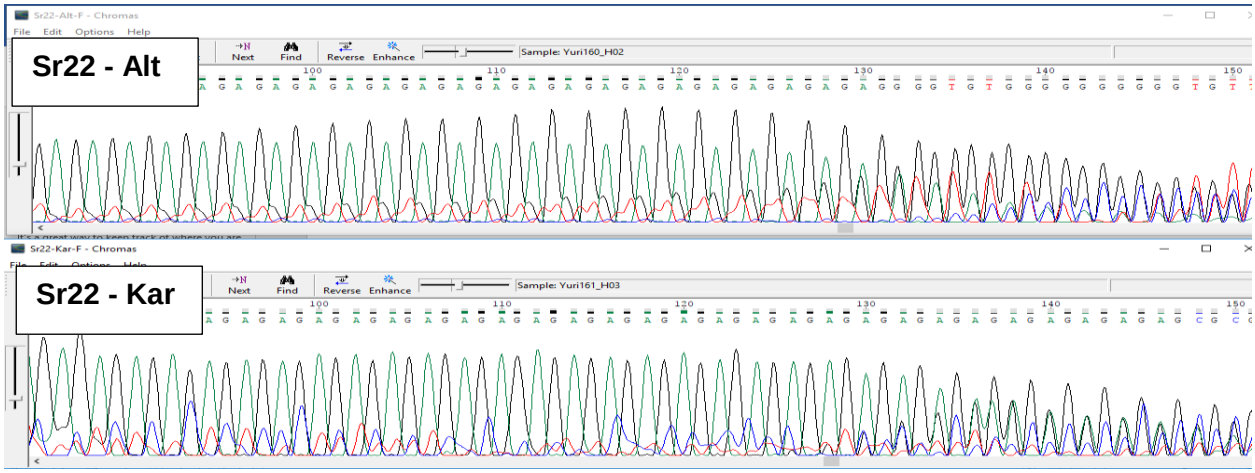
Appendix 4: Sequencing result of Leaf rust resistance genes

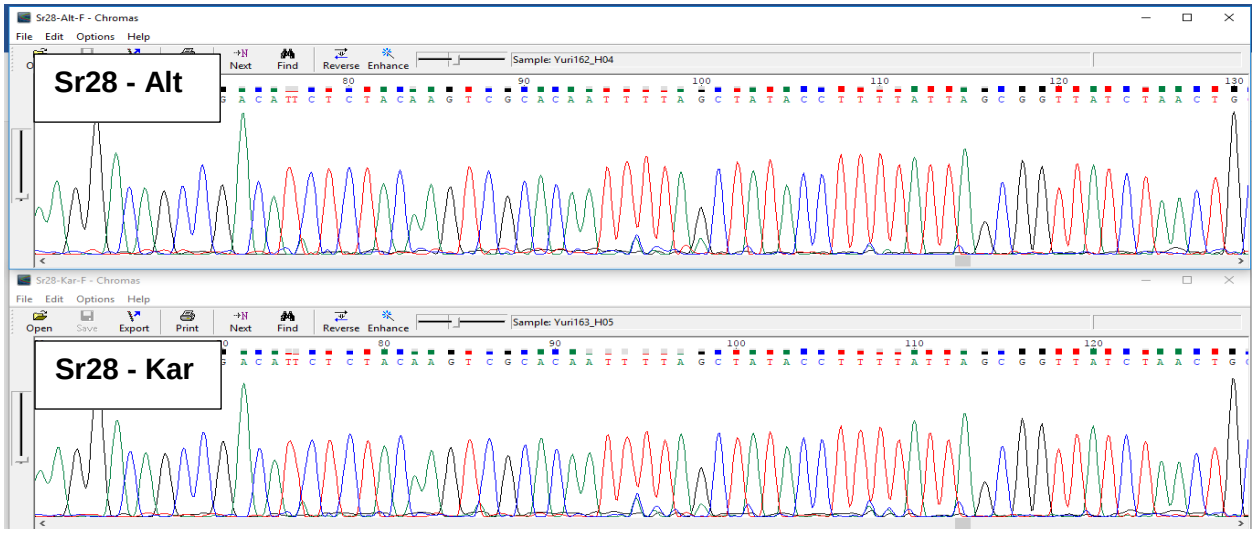






Sequencing result of stem rust resistance genes



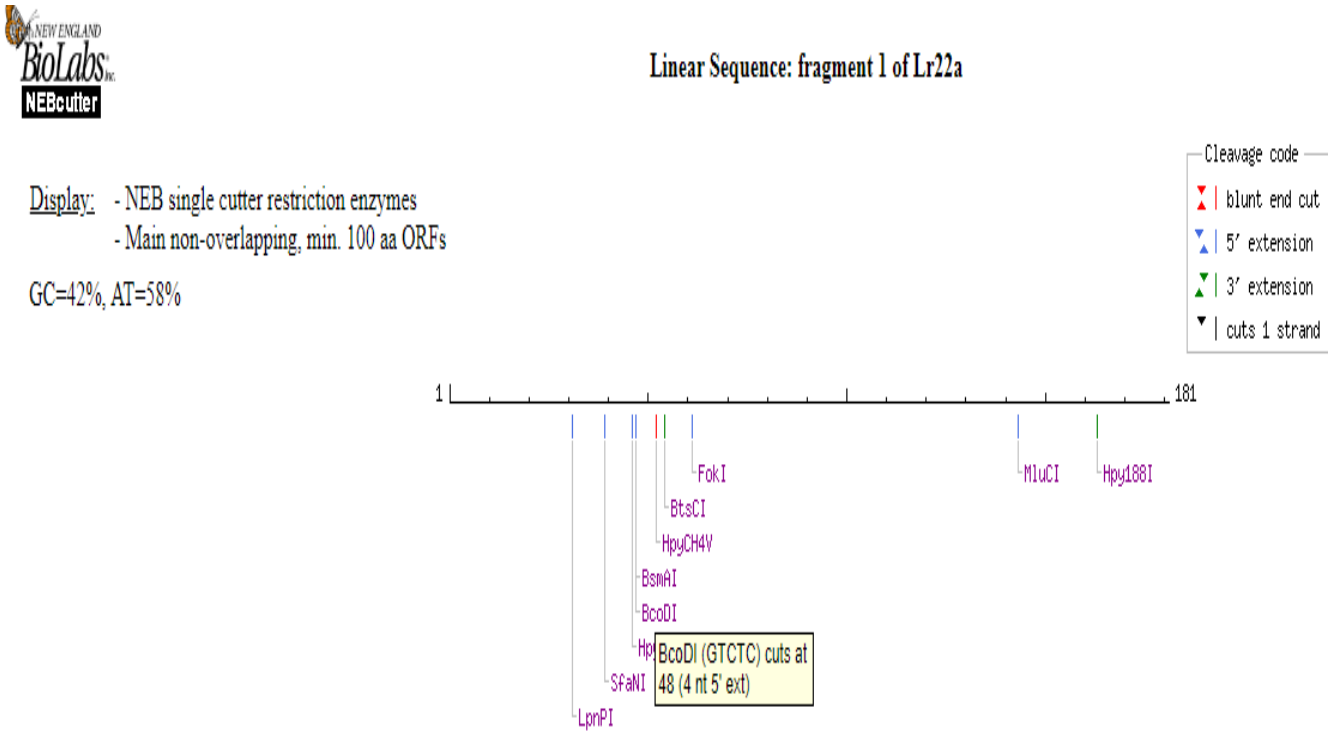


Appendix 5: **Nucleotide sequence and derived amino acid sequence (5'3' Frame 1) of Lr51 using ExPASy website (https://web.expasy.org/cgi-bin/translate/dna_aa). The triplet and amino acid affected by SNP are highlighted in yellow and green, respectively. Due to the Leucine amino acid is encoded by either CTT or CTG triplet, the SNP (T/G) does not lead to the change in amino acid sequence according to 5'3' Frame 1 reading.**

```
attcaccgcacctacctcggcgggggaatcaatttcactgatggatccgttgaggtaatt
 I  H  R  T  Y  L  G  G  G  I  N  F  T  D  G  S  V  E  V  I
gatctcagcttattaatttatacttgatttttcatcttgacgtcttgggcttttagctg
 D  L  S  L  L  I  Y  T  -  F  F  H  L  D  V  L  G  F  -  L
actacagcacacattgactgatacacctttttgcaacaaataggtattggccgcgacgca
 T  T  A  H  I  D  -  Y  T  F  L  Q  Q  I  G  I  G  R  D  A
aatgcccggggaggctgctggatggttccgcggaacagcggacgccgtcagaaaatttat
 N  A  R  G  G  C  W  M  V  P  R  N  S  G  R  R  Q  K  I  Y
ctgggtgcttgaggtgagaagagtactatctggatacttgtacatctgcatgtgttaaag
 L  G  A  -  G  E  K  S  T  I  W  I  L  V  H  L  H  V  L  K
taatgctacttgtgttatgtgtacctctccaggactattataagaataaatccatagagc
 -  C  Y  L  C  Y  V  Y  L  S  R  T  I  I  R  I  N  P  -  S
acattttgatcttgtcgggcgatcagctttatcgcatggattacatggagcttgtgcagg
 T  F  -  S  C  R  A  I  S  F  I  A  W  I  T  W  S  L  C  R
tttgtgatgttctctttaaccagctccagtgtatgttctgttttgaactggctgaccaag
 F  V  M  F  S  L  T  S  S  S  V  C  S  V  L  N  W  L  T  K
ctgtggcatacatcctttacagaaacatgtggatgacaatgctgacattactttatcatg
 L  W  H  T  S  F  T  E  T  C  G  -  Q  C  -  H  Y  F  I  M
tgcccctgttggagagaggtattaactattgttctacatcccagatccttatttatgagt
 C  P  C  W  R  E  V  L  T  I  V  L  H  P  R  S  L  F  M  S
gtatcttccttttgttgaagttgcactgcatcctgatttgcttttctttgttttggaagc
 V  S  S  F  C  -  S  C  T  A  S  -  F  A  F  L  C  F  G  S
cgggcatctgagtacgggctagtgaagttcgacagttcaggccgtgtggtccagttttct
 R  A  S  E  Y  G  L  V  K  F  D  S  S  G  R  V  V  Q  F  S
gaacnacccaaa
 E  X  P  K
```

Amino Acid	SLC	DNA codons
Leucine	L	CTT, CTG , CTC, CTA, TTA, TTG

Appendix 6: Determination of restriction enzyme based on the SNP position located in cloned fragment 1 of *Lr22a*



Appendix 7: Primer design for Amplifluor-like genotyping of *Lr51*

Example of design for non-labelled Gene-specific primers (GSP), TaLr51 for *Lr51*. SNP position in the sequence was coded 'S', designating mixed nucleotides 'T' and 'G', and highlighted in red and green, respectively. Two forward primers and one common reverse primer are shown in Bold and highlighted in blue and purple, respectively. Amplicon size is indicated. Two sets of forward primers with 'standard' and short tails (Table 5), and common reverse primer were developed. The tails are shown in normal case.

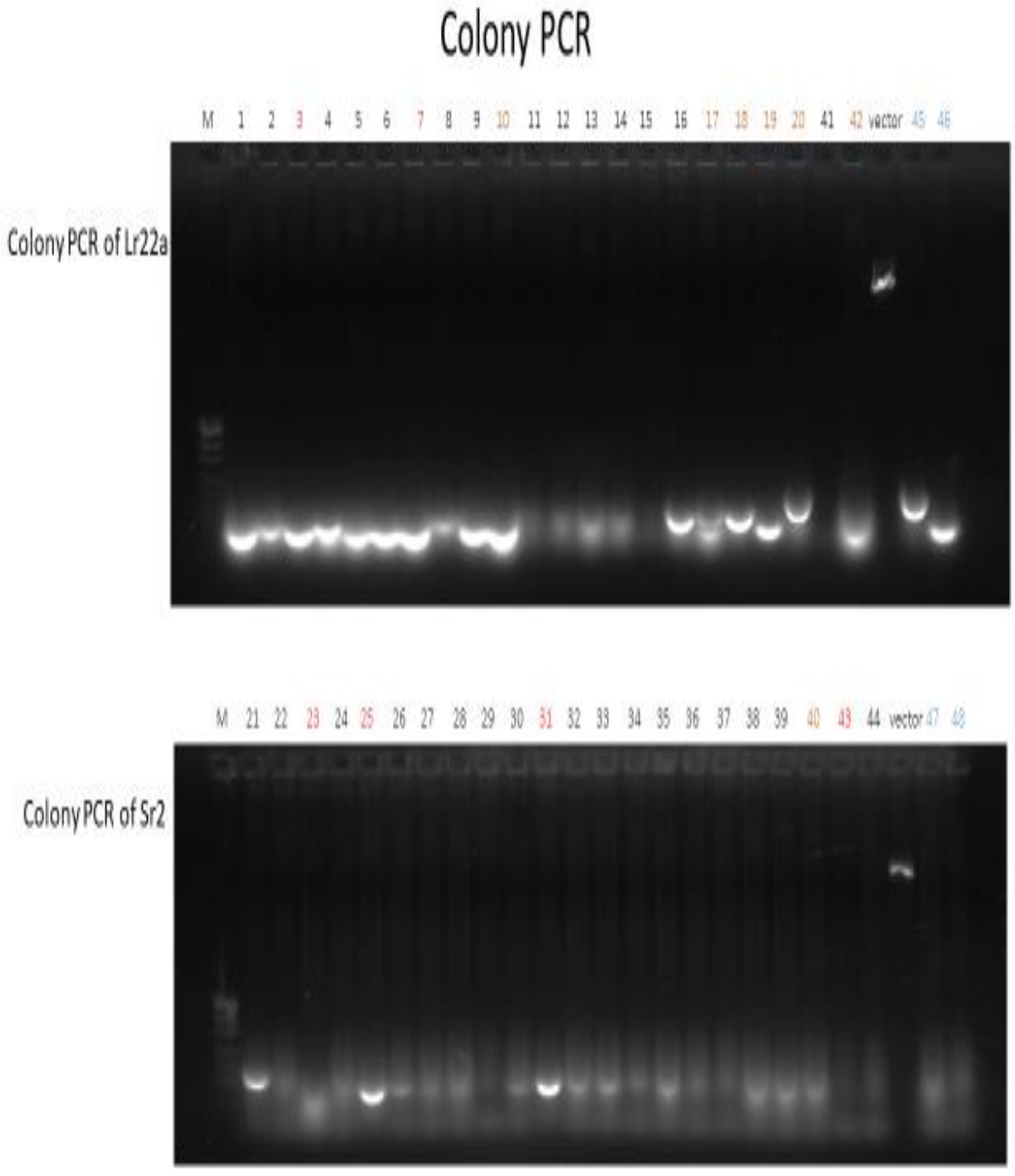
Sequence:
5' – TAACCAGCTCCAGTGTATGTTCTGTTTTGAACTGGCTGACCAAGCTSTGGCATACATCCTTTACAGA
AACATGTGGATGACAATGCTGACATT ACTTTATCATGTGCCCCTGTTGGAGAGAGGT- 3'

Expected PCR product size: 88bp
T – Karab90 – **F1-HEX**
G – AltZhn – **F2-FAM**

Primers:	**Length**	**GC content**	**Tm**
TaLr51-SNP-F1: TGAACTGGCTGACCAAGCTT	20 bp,	50%,	51.8 °C.
TaLr51-SNP-F2: GAACTGGCTGACCAAGCTG	19 bp,	58%,	53.2 °C.
TaLr51-SNP-R: CAACAGGGGCACATGATAAAGT	22 bp,	45%,	53.0 °C.
TaLr51-SNP-R (Rev-Com): ACTTTATCATGTGCCCCTGTTG			

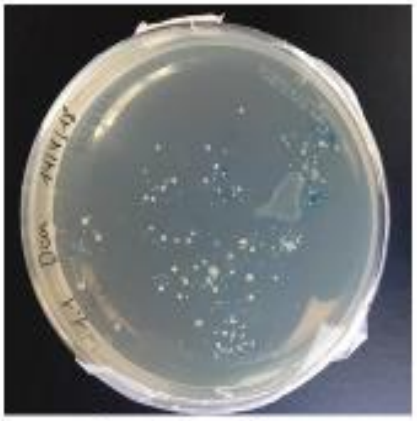
Ordered Primers:
TaLr51-SNP-F1: 5' – GAAGGTGACCAAGTTCATGCT***TGAACTGGCTGACCAAGCTT** – 3'
TaLr51-SNP-F2: 5' – GAAGGTCGGAGTCAACGGATT***GAACTGGCTGACCAAGCTG** – 3'
TaLr51-SNP-R: CAACAGGGGCACATGATAAAGT

Appendix 8: Cloning of polymorphic fragments of *Lr22a* and *Sr2* using pGEM-T plasmid

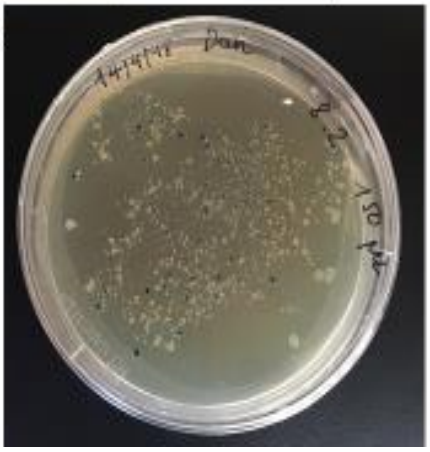


Blue/white screening

Positive control using insert control



50 uL of transformed cells was plated on LB/Ampicilin/IPTGX-Gal plate



150 uL of transformed cells was plated on LB/Ampicilin/IPTGX-Gal plate