# IDENTIFICATION OF MOLECULAR MARKERS FOR SCREENING TOBACCO GERMPLASM RESISTANT TO WHITE MOULD

By

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A thesis submitted in partial fulfilment of the requirements for the Master of Science degree in Crop Science (Plant Breeding)

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December 2014

## DECLARATION

**UNIVERSITY OF ZIMBABWE**

**FACULTY OF AGRICULTURE**

The undersigned certify that they have read and recommended to the Department of Crop Science, the thesis entitled:

**IDENTIFICATION OF MOLECULAR MARKERS FOR SCREENING TOBACCO GERMPLASM RESISTANT TO WHITE MOULD**

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## ABSTRACT

White mould is a fungal disease of tobacco and other crops and is caused by *Golovinomyces cichoracearum*. White mould resistance is introduced into commercial varieties by backcrossing followed by phenotypic selection which lengthens the breeding cycle. In order to reduce the breeding cycle molecular markers have to be identified to help in the screening and selection of germplasm resistant to white mould. The purpose of this study was therefore to identify the DNA markers that can assist breeders when they are selecting for resistant plants. Two resistant parental lines XZ, STNCB and two susceptible parental lines XSR, K51 were used in this study. Backcrosses of XZ and XSR were grown in the greenhouse and DNA extracted for molecular analysis. A total of eight (8) simple sequence repeat markers and ten (10) inter-simple sequence repeat markers were used. These markers were screened on the parental lines and the backcross generations for the differences between resistant and susceptible material. Locus PT30021 was the only marker that showed linkage to white mould resistance gene, and thus managed to distinguish between the resistant and the susceptible materials. Locus PT30021 has the potential of being used in the identification of white mould resistant germplasm in marker assisted backcrossing.

## DEDICATION

To all those who believed in me especially my family Mr and Mrs D.J. Bhiza, Noreen, Nelliosa and Kaylah my niece.

## ACKNOWLEDGEMENTS

My sincere and heartfelt gratitude goes to the Tobacco Research Board and the General Manager Dr D. Garwe for funding my research and studies. The work could not have been completed without the continuous input and supervision from my supervisors Dr E. Gasura (UZ), Mrs F. Mufunda (TRB) and Mr N. Muzhinji (TRB). I would also like to appreciate Mr G. Makombe, Mr T. Sagonda and Ms M. Magombo from the Tobacco Research Board who helped me with information and technical knowledge that made a difference in the research. I cannot thank enough the Molecular Biology Division staff and students for being there during the time I needed help and assistance. I thank my friends Tatenda Goche, Grace Madewe, Gamuchirai Kavhumbura and Malvern Shumbanhete for being friends indeed. My gratitude also goes to my parents Mr and Mrs D.J. Bhiza as well as my sisters Noreen and Nelliosa for their unwavering support, love and also for making me believe that I could make it. Above all I would like to give all the credit to the Almighty God who is the creator and author of my life.

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## LIST OF ABBREVIATIONS

AFLP – Amplified Fragment Length Polymorphism

AGE - Agarose Gel Electrophoresis

BSA – Bulked Segregant Analysis

CHL - Chloroplast primer

DNA – Deoxyribonucleic acid

IAEA – International Atomic Energy Agency

PAGE - Polyacrylamide Gel Electrophoresis

QTL – Quantitative Trait Loci

RAPD - Randomly Amplified Polymorphic DNA

RFLP – Restriction Fragment Length Polymorphism

SSR – Simple Sequence Repeats

TMV - Tobacco Mosaic Virus

TRB – Tobacco Research Board

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# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Tobacco (*Nicotiana tabaccum L.*) is one of the top grown crops worldwide that is cultivated for its leaves (green gold). In Zimbabwe tobacco is grown in all the agricultural regions. It is of importance to the economy at both household and national level because of the foreign currency earnings from the export market (Chivuraise, 2011). Tobacco production contributes 10.7% of the gross domestic products (GDP). Tobacco production has taken over the agricultural industry in Zimbabwe with the largest portion of arable land (97 000 hectares) which is approximately 3% of total arable land falling under its production nationwide (Leaver, 2003). The industry has been shown to be the largest employer in agriculture and is the economic backbone of Zimbabwe because production is labour intensive (Leaver, 2003).

The disease susceptibility of crops generally is a cause for concern to most farmers in the developing countries such as Zimbabwe. The smallholder farmers face challenges in trying to successfully protect their crops from diseases using the recommended chemicals due to financial constraints. Tobacco like any other commercial crop is susceptible to diseases which result in the reduction of yields and leaf quality and therefore grade of the leaves hence making them less valuable on the market (Nyoka, 2005). Examples of some of the pathogens and the diseases they cause in tobacco are tobacco mosaic virus (TMV) (Kozjak and Megli, 2012), bacterial wilt (Agrios, 2004) and fungi causing white mould (Fabro *et al*., 2008). The International Atomic Energy Agency (IAEA) report of 2012 was quoted supporting the fact that fungal diseases are a major cause of yield and quality loss in crops (Kozjak and Megli, 2012).

The solution to the problem of disease susceptibility that has been brought forward by breeders has been the development of disease resistant varieties using resistant genes from wild relatives. The process of developing these resistant varieties is long when done using conventional phenotypic selection. Modern technology has however brought forward a quicker method which uses molecular markers.

This study focused on white mould (Powdery mildew) disease caused by *Golovinomyces cichoracearum* var *cichoracearum* (V.P. Heluta) which was previously known as *Erysiphe cichoracearum* and belongs to the *Ascomycetes* family (Aguiar *et al*., 2012). White mould has been seen as a minor disease in tobacco throughout the world but has been reported to cause extensive yield losses in most tobacco regions including southern Africa (Lahoz *et al*., 2005; Seifi *et al*., 2013).

Plant breeders when developing new varieties with desirable traits such as disease resistance need to know early in the breeding program whether the variety they are producing has the trait of interest. This can be easily achieved through the use of molecular markers in marker assisted breeding (Kulwal *et al*., 2011). Molecular markers are defined in agriculture as an allele that shows the presence of a specific trait in a genome allowing traceability of that DNA region in plants. These genetic markers may be a phenotypic expression of a trait that is closely related to the trait under study and is passed down together with the marker in the progeny due to close linkage (Semagn *et al*., 2006; Indrapratap1, 2013). Molecular markers enable the detection of the presence or absence of a gene early before field trials commence unlike phenotypic based or biochemical based selection which requires observable expression of symptoms to determine

whether the variety is susceptible or resistant therefore taking longer time and utilizing more resources (Negishi, 2003).

In Zimbabwe at the Tobacco Research Board (TRB) there are flue-cured tobacco varieties available that have been bred for resistance to white mould. Resistance has been introgressed from the wild *Nicotiana* based on phenotypic selection which may not be reliable as the establishment of the pathogen in plants is sometimes affected by environmental conditions (Nyoka, 2005). These problems can be solved by the use of molecular markers which enable selection based on the genotype of the plants therefore, there is need to develop molecular markers which will be used in the early stages of any breeding program to screen for varieties that are resistant to white mould. Some local varieties have been reported to lose resistance over time and this necessitates the need to develop molecular markers which can be used to detect genotypic composition before phenotypic expressions in the field.

Molecular marker assisted breeding is advantageous in shortening the period required to complete a phenotype based breeding cycle which may take up to ten generations of backcrossing to introduce a new gene from one variety to the other (Gujaria *et al*., 2011; Goswami *et al*., 2013). Molecular markers can be used in the study of tobacco resistance to white mould as literature shows that there has been successes in resistance studies using markers in other crops such as cereals (Ayliffe, 2004; Jones, 2001) and in tobacco as well (Davalieva *et al*., 2010; Bindler *et al*., 2011). In molecular marker assisted breeding the presence or absence of a marker can help in selection of varieties with desired trait (Collard *et al*., 2005; Kulwal *et al*., 2011).

## 1.2 Statement of the problem

White mould disease causes extensive yield losses and reduces the life quality making the leaves undesirable in the market thereby reducing farmer profits. Introgression of resistance conferring genes is the best control measure where conventional control methods are not completely effective. Selection of white mould resistant varieties have been done by applying physical selection pressure through raising varieties in pathogen inoculated fields and selecting thriving plants with less or no susceptibility to white mould (phenotypic selection). Phenotypic method of selection is time consuming, not reliable, labour intensive and expensive to use when screening for the varieties that are resistant.

## 1.3 Justification

Marker assisted selection (MAS) can be used for resistance screening as it does not have the limitations such as those found with phenotypic selection. Molecular markers have not yet been identified for white mould resistance in tobacco and therefore work has to be done to identify the markers that can be effectively used in breeding. MAS enable screening at seedling stage of plant development therefore makes MAS selection more efficient in terms of resource utilisation when compared to phenotypic selection and this increases precision.

## 1.4 Main objective

To identify DNA markers for screening tobacco germplasm that is resistant to white mould.

### 1.4.1 Specific objective

To identify molecular markers for use in screening tobacco germplasm that is resistant to white mould.

### 1.4.2 Hypothesis

There are molecular markers for white mould resistance genes in tobacco.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 General review of white mould in plants

White mould is not a new disease in the plant kingdom but has caused problems in crop production since the 19th century. There are many species of fungi that are known to cause white mould in a range of crops which shows the extent of the threat it poses to farmers and agriculture in general. This study is focused on a pathogen that affects tobacco and other plants from the *Curcubitae* family which is called *Golovinomyces cichoracearum* (Aguiar *et al*., 2012). Its effects in tobacco were noticed in the 19th century in France where there were yield losses above 30%. It is then that it became an important disease in tobacco production in Zimbabwe then Southern Rhodesia. White mould was among the first diseases that tobacco was protected against and therefore there is not much literature found on the research done on this disease in tobacco as most of the varieties on the market have been shown to possess the resistance gene. In other tobacco growing regions across the globe it has been shown that there are many races of *G. cichoracearum* that are known to cause white mould in tobacco (Torgiano, 2013).

There are not many studies that have been carried out in relation to white mould in tobacco. This can be attributed to a number of factors some of which are the fact that white mould is a seasonal disease which can be rampant in one season when conditions are conducive and absent in another. This makes it a less pertinent problem in tobacco as compared to other diseases such as the *Tobacco Mosaic Virus* and *Potato Virus Y* which are a problem every season and region. These problematic diseases have a lot of attention in research and therefore there is a lot of literature on these diseases.

## 2.2 White mould biology, symptoms and epidemiology

*Golovinomyces cichoracearum* is an obligate biotroph and polyphagous pathogen which affects a wide range of plants including roses, cucurbits and soyabeans causing damage to all organs at all stages of development (Takamatsu *et al*., 2006). It can survive on plant debris and in alternative hosts as cleistothecia (Fabro *et al*., 2008). The fungi can survive for many years in an overwintering stage. White mould reduces the photosynthetic surface of the plant resulting in plant growth retardation and loss in foliage. All the affected plant organs show characteristic symptoms which include white powdery patches which appear first on the leaf's upper surface and these spread to cover the whole leaf lamina (Takamatsu *et al*., 2006). The affected tissues gradually show brown lesions which are a result of cell death. In tobacco production, leaves are the organ of economic importance and therefore attack by *G. cichoracearum* reduces yields.

White mould has been shown to be a seasonal disease which requires specific conditions to proliferate. There are requirements for maximum and minimum temperature above and below which spread can be inhibited. The fungus is very sensitive to temperature which therefore makes it present in one region as compared to others. Humidity is another factor which determines the spread and growth of the disease. The spread is promoted by high humidity but growth is favoured by drier conditions (Administration, 1979).

## 2.3 Control of White mould

The spread of *G. cichoracearum* in tobacco can be controlled by many approaches which are fungicide application, practicing sanitation, proper site selection and use of resistant cultivars. The most commonly used approach is fungicide application using fungicides such as

pencanazole, benomyl and bupirimate (Lahoz *et al*., 2005). The disadvantage of using fungicides for protection against white mould is that once the fungi establishes under favourable conditions it will be difficult to stop the progression of the disease before major plant losses are incurred (Seifi *et al*., 2013).

Practicing sanitation involves the destruction of all possible pathogen hosts including weeds. Early harvesting of tobacco lower leaves can prevent the spread of the disease to upper leaves. Risk of white mould can effectively be lowered by selecting field locations in areas that have sufficient aeration and sunlight as well as avoiding stagnation of water in the plot (Seifi *et al*., 2013).

## 2.4 Resistance to White mould

The most effective method to control white mould has been shown to be the use of resistant cultivars. Breeders have produced hybrids from the *Nicotiana* species that have shown resistance to *G. cichoracearum*. Resistance has been found in many species which are *Nicotiana debneyi, Nicotiana glutinosa* and *Nicotiana formis*. The two types of resistance that have been identified are dominant monogenic and partial resistance (Aguiar *et al*., 2012). The dominant form of resistance is expressed at all stages of plant development and plants with this form of resistance inhibit fungal hyphae growth and haustoria degenerates rapidly (Aguiar *et al*., 2012; Bojórquez-Ramos Cosme, 2012). Another form of resistance has been reported in a Japanese cultivar (Kokobu) which is controlled by two recessive genes (Palakarcheva and Tunkara, 1992). It is this form of resistance that is being used in the TRB breeding programs for white mould resistance in their varieties.

At the TRB there is a parallel program that is run in the Plant Breeding Division that utilizes the dominant form of resistance. The advantage of using the dominant form of resistance is its potential of being identified easily in phenotypic selection. Breeding programs that have dominant genes as compared to recessive genes are usually shorter as there is expression in both homozygote and heterozygote conditions (Nyoka, 2005).

At TRB most of the varieties produced have been bred for white mould resistance being one of the first diseases for which resistance was incorporated in the breeding programs (Torgiano, 2013) . It has however been realised that some of the varieties have lost the resistance to white mould hence the need to reintroduce it through backcrossing to the resistant parent. The loss of resistance in future can be quickly and easily identified using molecular markers (Swati *et al*., 1999).

### 2.5 Molecular markers

In agriculture the conventional phenotypic evaluation of quantitative and qualitative traits is not possible without the use of markers (Poczai *et al*., 2013). The markers act as signs for the presence of a target gene. There are many types of markers that can be used in agriculture for selection of favourable traits (Gholizadeh *et al*., 2012). These markers include the biochemical markers, morphological markers and the molecular markers which are superior to the former markers (Pradesh *et al*., 2013). Molecular markers are independent of the environment effects, abundant in genomes and more importantly can be detected at any developmental stage of the plant. They can be used for many purposes in breeding including germplasm characterisation (Gujaria *et al*., 2011; Goswami *et al*., 2013).

There are various types of DNA based markers that can be used in agriculture (Jiang, 2010). DNA markers arise from different classes of DNA mutations such as substitution mutations, rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA. Ideal DNA markers should have qualities such as high reproducibility, frequency of occurrence in genome and a highly polymorphic nature (Jiang, 2010; Pradesh *et al*., 2013)**.** It is of great importance for a molecular marker to be able to show the difference in alleles within a population (polymorphism) for it to be considered in marker assisted selection (Kumar *et al*., 2009). The various examples of molecular markers are Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPDs) and Simple Sequence Repeats (SSR) also known as microsatellites (Pradesh *et al*., 2013). All these markers have been proved to have the ability to show some level of polymorphism within populations.

There are advantages and disadvantages associated with each of these various molecular markers in relation to trait analysis. The RFLPs have the advantages of reliability, being robust and are transferable across populations. RFLPs have a major disadvantage of not being amenable in marker assisted selection, they are labour intensive, have a low throughput potential for screening and require large amounts of DNA. The disadvantages of using AFLPs are that the methodology is complicated and require large quantities of DNA (Jiang, 2010).

Microsatellites are the marker of choice when it comes to studies that involve related plants with small differences (Kulwal *et al*., 2011). Variability is high in microsatellites which make them appropriate for their use in population studies and marker assisted selection (Kumar *et al*., 2009).

Microsatellites analyze co-dominance in segregating populations which differs from other markers such as RAPDs and AFLPs. SSRs are derived from conserved genes and are not specific to any genome. Scoring of markers is shown as the presence and absence of the marker (Bindler *et al*., 2007).

Inter- simple sequence repeats (ISSR) is a technique in which primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. Microsatellites at the 3' end are used in the amplification of genomic DNA with increased specificity. ISSRs are dominant markers and an unlimited amount of primers can be synthesized using varying combinations of nucleotides (Swati *et al*., 1999). The main advantages of using these markers are transferable between populations, technically simple to use, are robust and reliable. The only disadvantages of using these markers are requirements for polyacrylamide electrophoresis and vast amounts of time required to produce primers.

In tobacco disease resistance studies RAPDs and AFLP markers have been used but they reveal low levels of polymorphisms among tobacco materials (Liu and Zhang, 2008). The use of these molecular markers (RAPDs and AFLPs) was restricted to detecting chromatin from wild relatives. Microsatellite markers have been developed and they have increased rates of polymorphism which are useful in genetic mapping in tobacco (Bindler *et al*., 2007).

Molecular marker development can only be possible in plants when linkage maps are available for the desired trait which is White mould resistance in this study (Yang and Buirchell, 2008).

Linkage maps are important in determining the distances between genes found on a chromosome known as chromosomal locations. The principle behind linkage maps is based on crossing over that occurs during meiosis. Linkage maps can be derived from progeny resulting from two different parents through sexual reproduction (Kulwal *et al*., 2011; Poczai *et al*., 2013). When the frequency of recombination between markers is low, it can be concluded that the markers are closely linked or close together. Conversely, when the frequency of recombination is high it means the markers are further apart on the same chromosome or the markers are on different chromosomes. These are called unlinked genes. Linkage maps can be constructed by producing a mapping population, identifying polymorphism and carrying out the linkage analysis on the markers (Collard *et al*., 2005).

## 2.6 Marker Assisted Breeding

Breeding in present day requires the adoption of the new techniques that are being developed to reduce the time that is required for the release of new varieties that carry all the required traits in crops in agriculture today (Kulwal *et al*., 2011; Tabor *et al*., 2000). Breeders who rely on the conventional method of breeding which is based on phenotypic selection take longer time to complete a breeding program successfully (Brumlop and Finckh, 2011). In tobacco studies, research has been carried out in the use, development and application of molecular markers. Different types of molecular markers have been improved for utilisation in tobacco for cultivar genotyping and disease studies (Davalieva *et al*., 2010; Bindler *et al*., 2011; Xu *et al*., 2010).

In the case of traits that are controlled by recessive genes, when doing conventional breeding where phenotypic selection is done, selfing the plants is a requirement which adds another

season to the breeding program to ensure that the trait is expressed in the progeny. Selfing homogenizes gene expression and increases the expression of recessive traits (Pradesh *et al*., 2013). Marker assisted breeding can circumvent this lengthening of a breeding program and therefore can be of great help to the breeder who requires to release new varieties to the growers market.

In order to identify a linkage group for a particular trait (white mould resistance in this study), a large number of SSR primers are required. These have to be selected at random from all the linkage groups in cases where there is no prior knowledge of the chromosome where the trait is located. Polymorphic bands have to be checked for production from the varieties that have the trait and those that lack the trait. In disease resistance studies this could be the resistant and susceptible varieties. The polymorphism shown by these bands should be between the varieties that contrast in the trait under study. Validation can only be done when the selected polymorphic SSR markers produce bands between two types of DNA bulks (Michelmore *et al*., 1991).

In most studies that have been carried out in recent years for the identification of molecular markers for use in screening germplasm, bulked segregant analysis (BSA) has gained popularity as compared to mapping quantitave trait loci (QTL). Qualitative trait loci are expensive to carry out; labour intensive and time consuming which are disadvantages that can be addressed by adopting the use of the BSA (Barakat, 2012). In plant disease resistance studies, locating the quantitative trait loci (QTL) is of importance for the study to be successful. However, in cases where time, resources and labour supply are in short supply an approach that can be used instead of the QTL location is the bulked segregant analysis (BSA). This analysis minimizes the

disadvantages posed by locating the QTL and is therefore less tedious. Bulked segregant analysis enables the identification of molecular markers linked to the regions of genes that are being studied (Boopathi *et al*., 2013).

Bulked segregant analysis involves the bulking of DNA into two bulks from a segregating population and screening markers among the parents and the bulks (Magwene *et al*., 2011). Linkage to the gene of interest (white mould resistance in this case) is shown by the presence of polymorphic markers (Boopathi *et al*., 2013). These markers can help in distinguishing varieties that are resistant and susceptible.

There are similar studies that have been carried out in tobacco for Granville wilt. Negishi *et al* (2003) highlighted that there were varieties available with resistance to wilt but the linkage group and specific gene had not been identified (Negishi, 2003). Such studies therefore require the screening of many markers because the sequence of the gene will not be available.

## 2.7 Genetics of white mould resistance in tobacco at present day

The tobacco genome has been sequenced using different molecular markers including the recent work that has been completed by Bindler using SSRs (Bindler *et al*., 2011). With this knowledge in mind there has however not been any work that has been done on the molecular level focusing on white mould. At the present moment the gene that codes for white mould resistance is not known as well as the chromosome where it is located. This therefore makes it difficult to accurately select SSR markers that are available to use for this type of study because the search

has to be wide in order to target and cover all the twenty four chromosomes of tobacco. The consequence of not knowing the target chromosome and gene is that the search is blind and a larger number of primers is required if the search is to be successful.

At TRB, white mould resistance is introduced to all the commercial varieties by backcrossing to a donor parent with known resistance (Nyoka, 2005). Selection in this type of breeding program is done by inoculating with the pathogen and selecting the plants that do not develop the symptoms (phenotypic selection). This method is not reliable, is time consuming and is not resource efficient (Mazarire *et al*., 2013). The use of marker assisted selection for a disease resistance program has been shown to have more benefits to breeders. This brings about the use of molecular tools such as the Polymerase Chain Reaction.

### 2.8 Polymerase chain reaction

Polymerase chain reaction (PCR) is a process used in the amplification of DNA from small quantities (Basirnia *et al*., 2014). The process involves three stages which are denaturation, annealing and amplification which make up a cycle. The denaturing stage requires temperatures as high as 95$^{o}$C while the annealing stage have a temperature range between 35-65$^{o}$C. The process is completed in the presence of an enzyme called *Taq* polymerase obtained from bacteria called *Thermus aquaticus* found at the thermal vents in the oceans and is used because of its temperature resistance. Most enzymes are denatured by high temperature and this makes the *Taq* polymerase outstanding and suitable to complete for the polymerase chain reaction. The activity of the *Taq* polymerase is terminated by increasing the temperature after all the cycles are completed.

## 2.9 Gel electrophoresis

The products from PCR can be separated using gel electrophoresis. Electrophoresis is a technique that is used to separate macromolecules such as proteins and nucleic acids that have different sizes and / or charge (Liu and Zhang, 2008). It is a widely used technique in molecular biology and modern agriculture. The charged molecules are placed in an electric field and they migrate toward either the positive or negative pole depending on their charge. Nucleic acids have a consistent negative charge imparted by their phosphate backbone and therefore migrate towards the anode. There are two types of gels used and these are polyacrylamide and agarose.

The two types of gels that are used in electrophoresis differ in clarity after staining and (Jiang, 2010) viewing with ultraviolet radiation. When polyacrylamide gel is used band separation can be clearly seen when compared to agarose gel separation. This can be illustrated when using agarose gel bands may appear as a single bands but when polyacrylamide gel is used these bands may appear as two separate bands. Polyacrylamide Gel Electrophoresis (PAGE) has been proved as a method of choice where separation can be difficult due to similarity of band sizes (Semagn *et al*., 2006).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Germplasm

All the varieties that were used in the study were obtained from the plant breeding division at Kutsaga research station. The parental materials were coded XS and XSR which are susceptible to *Golovinomyces cichoracearum* and XZ which is resistant to the pathogen. The $F_1$ progeny of XSR and XZ backcrossed up to $BC_5$. Other lines that were also used were STNCB and K51 which are resistant and susceptible varieties respectively.

### 3.2 Trial site

The plants were grown in pots in the greenhouse located at Kutsaga Research Station. The greenhouse conditions were maintained at the temperature range of $35^oC$ to $40^oC$.

The laboratory work was done in the Molecular Biology Services Division laboratory at Kutsaga research station.

### 3.3 DNA extraction

DNA extraction was done on six weeks old plants. DNA extraction was done using the modified CTAB protocol used at the Tobacco Research Board (Appendix A). The extracted DNA was suspended in ultra pure water. The chloroplast (CHL) test was done to check if the extraction was successful and to check for the presence of PCR inhibitors.

Table 3.1 List of SSR primers used, their sequences and linkage group

| Primer name | Forward sequence | Reverse sequence | Linkage group |
|---|---|---|---|
| PT20172 | ACACCTCCTTCTTCCTGC | CCAAAATGGTTCACTGGA | 3a |
| PT30021 | CATTTGAACATGGTTGGCTG | CTCAACTCTCGTCGCTCTTG | 4 |
| PT30096 | GAAGTTTCAAAGTAGCACCAACAA | GCACCCTATTTGGTCTCCC | 12 |
| PT30144 | TGATTTGTATTGACAGCGTGAAG | TTGTTTAGTTACCCTATTTGACTTGC | 16 |
| PT30274 | GACAGCAAGCTAATAACAGTAAATG | GGACTTTGGAGTGTCAAATGC | 17 |
| PT30378 | TCAAATGAGGGTTGTAGCCA | TGCAATGGCTACACAAGAAGA | 21 |
| PT30392 | CGAGGACGATGTAAATGCTT | GCTAAGCTTGAAATCACATTCA | 20 |
| PT30480 | AAAGGGAAACATGGACATTG | TAGGCGAGATTGTGGGATTC | 13 |
| PT51635 | TGTTATCACAACTCGACATTTATGAG | GGCCTTGTCATCATATTGGG | 10 |
| PT51984 | TTCGTTAGTTTGCTCAATCACAA | GGACGAAGTAGCTTTCATTGG | 18 |

Table 3.2 List of ISSR primers used and their sequences

| Primer name | Sequences |
|---|---|
| UBC826 | ACACACACACACACACC |
| UBC833 | ATATATATATATATATYG |
| UBC834 | AGAGAGAGAGAGAGAG |
| UBC835 | AGAGAGAGAGAGAGAGYC |
| UBC840 | GAGAGAGAGAGAGAGAYT |
| UBC845 | CTCTCTCTCTCTCTCT |
| UBC855 | ACACACACACACACAC |
| UBC856 | ACACACACACACACACCTA |
| UBC833 | AGAGAGAGAGAGAGAG |

### 3.4 Polymerase chain reaction conditions

#### 3.4.1 PCR mix components and volumes

The PCR mix contained 30.8 µl ultra pure water, 5 µl of 1× PCR buffer, 6µl of 0.25mM (Inqaba Biotech South Africa) and dNTPs (Sigma Aldrich). The primer concentrations were 0.15mM at a volume of 1.5µl and 0.2µl of 0.3U Taq polymerase (Super-Therm 250U). The primers used were adopted from Bindler (2011) and Davalieva *et al* (2010) and are listed in Table 3.1 and Table 3.2. The reaction mix dispensed into the PCR tubes was 7µl per tube and 1µl of the DNA template. The PCR conditions were adopted from Gholizadeh (Gholizadeh, Darvishzadeh, and Mandoulakani 2012).

#### 3.4.2 PCR cycling conditions for SSR markers

The cycling conditions used in this experiment were 4 minutes of denaturation at 94ºC which was followed by 1 minute at 94ºC. The annealing temperatures were 55 ºC for 1.5 minutes which was followed by an extension for 2 minutes at 72 ºC. Final extension took place at 72 ºC for 10 minutes. PCR was completed after 40 cycles. The cycling machine used was the Gene Amp 9700.

#### 3.4.3 PCR cycling conditions for ISSR markers

The cycling conditions used in this experiment were 4 minutes of denaturation at 94ºC which was followed by 1 minute at 94ºC. The annealing temperatures ranged from 52 ºC - 59 ºC for 1.5 minutes which was followed by 2 minutes of extension at 72 ºC. Final extension took place at 72ºC for 7 minutes. PCR was completed after 36 cycles.

### 3.5 Post PCR analysis

Post PCR analysis was done using 2% agarose gel. Gel electrophoresis run at 130V for 45 minutes. The buffer that was used for gel electrophoresis was 1X tris-acetate-EDTA (TAE). Gels were viewed for the presence of the bands using UVI tech trans-illuminator fluorescence. Ethidium bromide was used to stain all the gels.

## CHAPTER FOUR

### 4.0 RESULTS

The chloroplast (CHL) primer showed the presence of amplifiable extracted DNA.

There were markers that showed polymorphism (Fig 3). The other markers could not distinguish between the resistant and susceptible material and these are shown (Fig 5 and Fig 6).

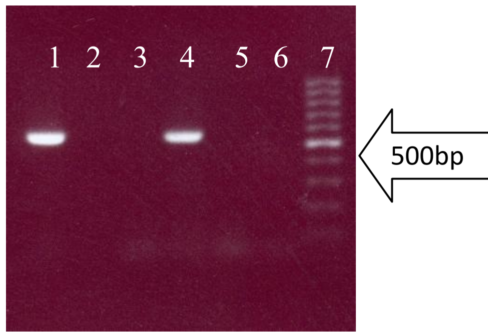


**Figure 1- CHL electrographs**

Lane 1 – XZ; Lane 2 – blank; Lane 3 – blank;
Lane 4 – $F_{1;}$ Lane 5 – 100bp ladder

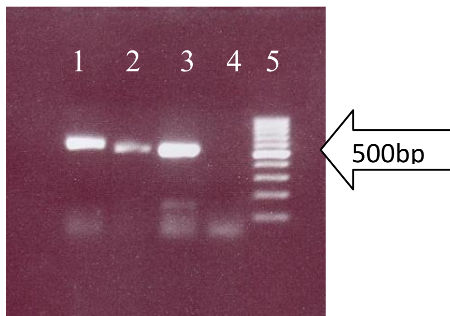


**Figure 2 – CHL electrographs**

Lane 1 – XSR; Lane 2 – $BC_{5;}$ Lane 3 – Corn;
Lane 4 – Water; Lane 5 – 100bp ladder

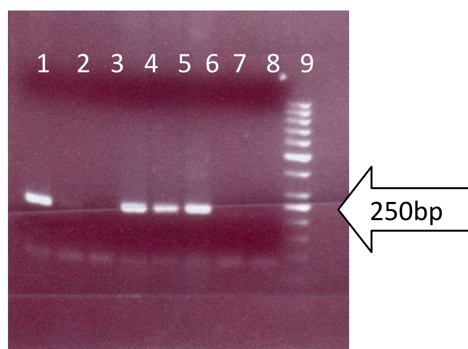


**Figure 3 – PT30021 electrographs**

Lane 1 – K51; Lane 2 – SNTCB; Lane 3 - X; Lane 4 – X;
Lane 5 – X; Lane 6 -T72 (Negative control); Lane 7 – Brondal;
Lane 8 – Water; Lane 9 – 50bp Ladder;
X – Represents germplasm not relevant to this study

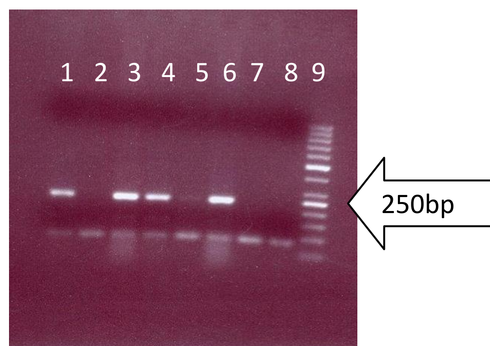


**Figure 4 – PT51635 electrographs**

Lane 1 – K51; Lane 2 – SNTCB; Lane 3 - X; Lane 4 - X
Lane 5 – X; Lane 6 -T72 (Negative control); Lane 7 – Brondal;
Lane 8 – Water; Lane 9 – 50bp Ladder;
X – Represents germplasm not relevant to this study

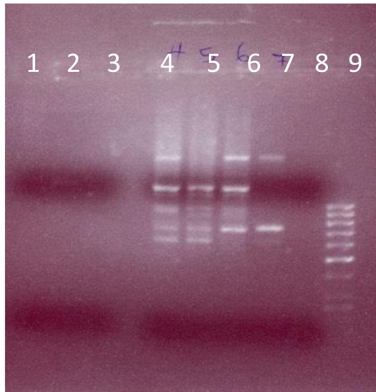


**Figure 5 – UBC833 electrographs**

Lane 1 – K51; Lane 2 – SNTCB; Lane 3 – X; Lane 4 – X;
Lane 5 – X; Lane 6 -T72 (Negative control); Lane 7 – Brondal;
Lane 8 – Water; Lane 9 – 50bp Ladder;
X – Represents germplasm not relevant to this study

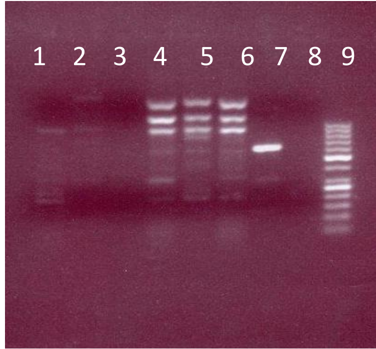


**Figure 6 – UBC835 electrographs**

Lane 1 – K51; Lane 2 – SNTCB; Lane 3 - X; Lane 4 – X;
Lane 5 – X; Lane 6 -T72 (Negative control); Lane 7 – Brondal;
Lane 8 – Water; Lane 9 – 50bp Ladder;
X – Represents germplasm not relevant to this study

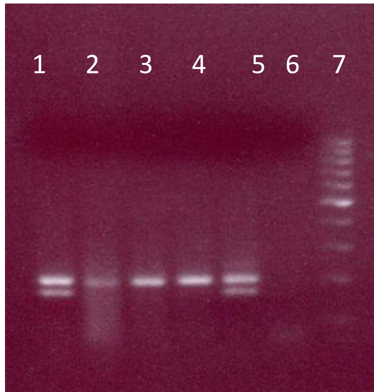


**Figure 7 – PT30392 electrographs**

Lane 1 – XZ; Lane 2 – XSR; Lane 3 – BC5
Lane 4 – F1; Lane 5 – Corn; Lane6 – Water;
Lane 7 – 100bp ladder

The detailed results from the primers are presented in the binary matrix form in Appendix B.

# CHAPTER FIVE

## 5.0 DISCUSSION

Amplifiable DNA was successfully extracted. This was evident by the presence of clearly defined 500bp band. The CHL primers that were used helped in showing the quality of the DNA such that when there are further tests with other primers used in the study there is no question about the amplifying quality of the available DNA. From the electrographs obtained in this study, the DNA extracted was of commendable quality. The CHL test also showed that there were no PCR inhibitors in the extracted DNA.

The marker that was identified for use in the screening of tobacco germplasm from the primers that were used was PT30021. Identification of molecular markers for use in screening germplasm for a trait of interest is possible where the marker is linked to the trait under study. Linkage is the basis for marker assisted selection (MAS) and success is dependent on how closely linked the trait and the marker are (Collard *et al*., 2005). The tightness of the link is determined by the distance (centiMorgans) between the trait and marker (Guo and Elston, 1999). Linkage allows the segregation of the marker and the trait together during the process of meiosis. The identification of PT30021 as a marker linked to white mould resistance shows that there is a linkage and the closeness of the link can be calculated by determining the frequency of recombination (Yang and Buirchell, 2008).

The results that were obtained in these experiments on some electrographs were not clear. This could be attributed to a number of factors one of which is the fact that the use AGE reduced the

accuracy in the separation of bands. AGE can be used when the differences in band size are 100bp or more. The study would have been more precise if PAGE was used as it can show differences for molecules that are less than 100bp (Poczai *et al*., 2013). AGE has been shown in studies by Poczai *et al* (2013) to be unreliable in detection of polymorphism when compared to PAGE. Agarose gel has poor resolution which makes it difficult to identify small differences in bands shown on the gel.

Polyacrylamide gel electrophoresis has been shown to be the most effective for post PCR when used together with radioactivity. Agarose gel electrophoresis has been shown to be the least sensitive method when used with ethidium bromide system (Benemann *et al*., 2012). These post PCR systems affect the end results that are obtained on the electrographs.

The post PCR analysis that was used is not the best to utilize in such studies. Modern technology has brought about other alternatives which can yield better results than the one used. Agarose gel electrophoresis has disadvantages of a high risk of contamination which can either be from the genomic DNA or from PCR amplicons. These contaminants will prevent the DNA from separating into distinct bands that can be scored and may give the impression of a smear. This reduces the possibility of giving available bands distinct band sizes using the ladder.

Another reason for the failure of many markers is that the primers that were available did not cover all the linkage groups (Collard *et al*., 2005). It is possible that the loci for the genes responsible for white mould resistance are on a chromosome that had no marker targeting it. The

primers used in this study targeted the following linkage groups as shown by Bindler *et al* 1, 3, 4, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20 and 21 which leaves the possibility of the trait being linked to the remaining ten linkage groups (Bindler *et al*., 2007; Bindler *et al*., 2011). In studies carried out in other crops for the resistance to powdery mildew, a large pool of primers was used to select for the polymorphic ones to use in the study which makes the research a success (Ek *et al*., 2005).

Many markers need to be screened when studying crops like tobacco which have narrow genetic diversity (Liu and Zhang, 2008). There is great diversity in wild varieties as compared to commercial and bred varieties. In order to find significant differences in varieties with narrow genetic diversity and self pollinating plants, many primers have to be screened because it can be difficult to find differences as they may be too small to notice or may not be as many as those found in genomes that are diverse (Collard *et al*., 2005). In this study only a few markers were used because of the cost for screening. Research has to be continued by screening other primers that were not used in this study such that markers can be made available for the breeders to use in their breeding programs. Generally in tobacco it is difficult to identify genetic polymorphism particularly in cultivated varieties due to similarities. Genetic polymorphism in tobacco is greatly identified in wild types (Liu and Zhang, 2008).

The results that were obtained from the use of the UBC primers were not favourable as compared to those from the PT primers. The electrographs from the post PCR of UBC markers had multiple bands. This made it difficult to compare between the resistant and susceptible material as there was not much clarity from the agarose gel. Some of the bands were not separated

distinctly which made it even hard to determine their band size and therefore made them unsuitable for use in the study to get conclusive results.

UBC markers were more suitable for the study because there is no previously available information on the sequence of the gene that is being researched on (Semagn *et al*., 2006). Use of ISSR markers does not require prior information of the sequences and has been shown to have the advantage of requiring relatively less DNA (Ansari *et al*., 2012). It has the advantage of using random primers.

## CHAPTER SIX

## 6.0 CONCLUSION AND RECOMMENDATIONS

### 6.1 Conclusion

The primer that has been shown to be useful for screening tobacco germplasm resistant to white mould is PT 30021.

### 6.2 Recommendations

There is need to screen more primers which were not used in this study such that breeders can have reliable markers to use for the white mould resistance breeding program.

Identification of markers that can be used to screen for resistance can be further tested on the $F_2$ or backcross generations of the resistant and susceptible parents which can be further tested by applying the bulked segregant analysis (BSA) to get conclusive results.

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# APPENDICES

## Appendix A DNA extraction method

**Reagents**

CTAB (3%, 1%)

Chloroform

Cesium chloride

Isopropanol

Ethanol

Ultra pure water

**Procedure**

The sample was weighed and 100mg were ground in a 2ml epperndorf tube.

The epperndorf tube was filled with 3% CTAB solution (100mM Tris-Cl, pH 8.0, 20mM EDTA, pH 8.0; 1,4M NACl, 3% CTAB) that has been warmed in the microwave for 2 minutes

The contents were vortexed for 15 seconds

Incubation on a heating block was done for 30 minutes at 65°C and tubes were inverted every 10

Samples were allowed to cool at room temperature for 5 minutes

Centrifugation was done at 10481Xg for 10 minutes (12500rpm) in a microfuge and 1000µl of supernatant was moved to a clean 2ml epperndorf tube

The contents were mixed with an equal volume of chloroform and the tubes were shaken vigorously for 30s

Centrifugation at 10481xg for 10 minutes was done in a microfuge and 700µl aqueous phase was transferred to a new 1,5ml epperndorf tube

An equal volume of chloroform was mixed with the sample and shaken vigorously for 30 seconds

Centrifugation was done at 10481xg for 10 minutes in a microfuge and 400µl aqueous phase was transferred to a new tube

There was addition of 1.5-2.0 volume of 1% CTAB solution (50mM Tris-Cl, pH 8.0, 1% CTAB, (800µl for every 400µl of supernatant) which was mixed by inversion until a homogeneous solution was obtained

Incubation at room temperature for 1 hour took place and centrifugation was done for 10 minutes at 10481xg in a microfuge

Supernatant was discarded and the precipitate was dissolved in 400µl of 1M CsCl

A volume of 400µl of Isopropanol was added at room temperature and mixed by inversion until the DNA precipitated

Centrifugation was done at 10481xg for 10 minutes and the supernatant was discarded

There was an addition of 400µl of 70% ethanol at room temperature to wash pellet

Centrifugation was done at 10481xg for 5 minutes and the supernatant was discarded

The pellet was left to dry at room temperature for 30 minutes and dissolved in 50µl of pure water

**Appendix B List of selected primers, genotypes / generations, reaction and marker score**

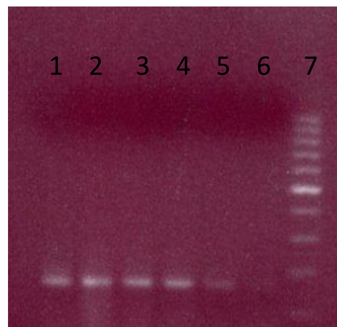
| Primer | Genotype | Reaction | Marker score |
|---|---|---|---|
| PT30021 | XZ | R | 0 |
| | XSR | S | 1 |
| | SNTCB | R | 0 |
| | K51 | S | 1 |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 0 |
| PT51635 | XZ | R | 1 |
| | XSR | S | 1 |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 0 |
| PT51984 | XZ | R | 0 |
| | XSR | S | 1 |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | 1 |
| | $BC_5$ | R | 1 |
| PT20445 | XZ | R | 1 |
| | XSR | S | 1 |
| | SNTCB | R | 1 |
| | K51 | S | 1 |
| | $F_1$ | R | 1 |
| | $BC_5$ | R | 1 |
| PT30144 | XZ | R | 0 |
| | XSR | S | 0 |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 1 |
| UBC958 | XZ | R | 0 |
| | XSR | S | 0 |

| | | | |
|---|---|---|---|
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 0 |
| UBC833 | XZ | R | 1 |
| | XSR | S | 0 |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 0 |
| UBC835 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 1 |
| | K51 | S | 1 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |

| | | | |
|---|---|---|---|
| UBC 826 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 1 |
| | K51 | S | 0 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 834 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 840 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 845 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 0 |
| | K51 | S | 0 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 855 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 1 |
| | K51 | S | 1 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 856 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 1 |
| | K51 | S | 1 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| UBC 886 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 0 |
| | K51 | S | 0 |

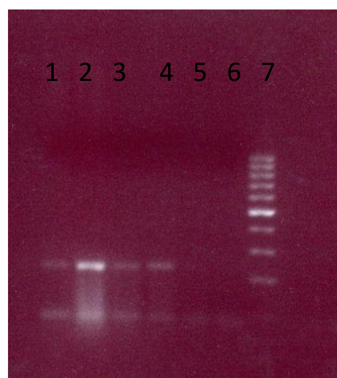
| | | | |
|---|---|---|---|
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| PT54277 | XZ | R | - |
| | XSR | S | - |
| | SNTCB | R | 1 |
| | K51 | S | 1 |
| | $F_1$ | R | - |
| | $BC_5$ | R | - |
| PT54081 | XZ | R | 1 |
| | XSR | S | 1 |
| | SNTCB | R | - |
| | K51 | S | - |
| | $F_1$ | R | 1 |
| | $BC_5$ | R | 1 |
| PT20172 | XZ | R | 1 |
| | XSR | S | 1 |
| | SNTCB | R | - |
| | K51 | S | - |
| | $F_1$ | R | 1 |
| | $BC_5$ | R | 1 |
| PT30096 | XZ | R | 1 |
| | XSR | S | 0 |
| | SNTCB | R | - |
| | K51 | S | - |
| | $F_1$ | R | 0 |
| | $BC_5$ | R | 1 |

## Appendix C Electrographs



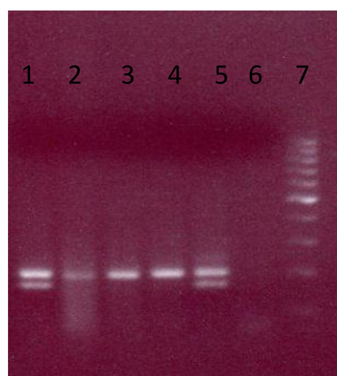

Lane 1 - XZ
Lane 2 - XSR
Lane 3 – BC5
Lane 4 – F1
Lane 5 – Corn
Lane6 – Water
Lane 7 – 100bp ladder

**Figure 8 – PT51635**



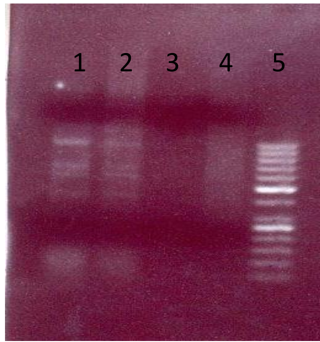

Lane 1 - XZ
Lane 2 - XSR
Lane 3 – BC5
Lane 4 – F1
Lane 5 – Corn
Lane6 – Water
Lane 7 – 100bp ladder

**Figure 9 – PT30096**



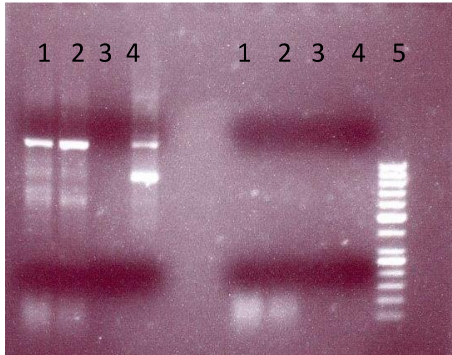

Lane 1 - XZ
Lane 2 - XSR
Lane 3 – BC5
Lane 4 – F1
Lane 5 – Corn
Lane6 – Water
Lane 7 – 100bp ladder

**Figure 10 – PT51984**




Lane 1 – STNCB
Lane 2 – K51
Lane 3 – T3KE1
Lane 4 – Water
Lane 5 – 50bp ladder

**Figure 11 – UBC855**




Lane 1 – STNCB
Lane 2 – K51
Lane 3 – Water
Lane 4 – T3KE1
Lane 5 – blank
Lane 6 – blank
Lane 7 – STNCB
Lane 8 – K51
Lane 9 – Water
Lane 10 – T3KE1
Lane 11 – 50bp ladder

**Figure 12 UBC856 and UBC886**