

A STABLE SIMPLE SEQUENCE REPEAT MARKER FOR RESISTANCE TO WHITE MOULD IN TOBACCO

N.R. BHIZA^{1,2}, E. GASURA¹, G. KUJEKE¹, D. GARWE², F. MUFUNDA², N. MUZHINJI²,
and C. KASHANGURA²

¹Department of Crop Science, University of Zimbabwe, P. O. Box 167, Mt Pleasant, Harare, Zimbabwe

²Kutsaga Research Station, Airport Ring Road, P. O. Box 1909, Harare, Zimbabwe

Corresponding author: egasura@agric.uz.ac.zw; gasurae@yahoo.com

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ABSTRACT

White mould, caused by *Golovinomyces cichoracearum*, is a major fungal disease of tobacco. Breeding for resistance to white mould is slow due to the intensive labour needed in artificial screening and the huge effect of the environment. In order to improve selection efficiency, molecular markers need to be identified to help in the rapid selection of germplasm resistant to white mould. The purpose of this study was, therefore, to identify the neutral, simple sequence repeat markers that can assist breeders in selecting for resistant plants. Two resistant parental lines XZ and STNCB, and three susceptible parental lines XSR, K51 and T71, were used in this study. Backcrosses of the hybrid from XZ and XSR were grown in a greenhouse, and DNA was extracted for molecular analysis. Eight simple sequence repeat markers and ten inter-simple sequence repeat markers were used. These markers were screened on the parental lines and the backcross generations for the differences between resistant (score 1) and susceptible (score 5) material. Single marker analysis was done using a t-test and significant ($P < 0.001$) differences were found between the means of resistant and susceptible plants based on the marker PT30021. Marker PT30021 showed linkage to the white mould resistance gene, and thus managed to distinguish between resistant and susceptible materials in parental and advanced generations. Locus PT30021 has the potential of being used to identify white mould resistant germplasm in marker assisted backcrossing.

Key Words: *Golovinomyces cichoracearum*, marker-assisted backcrossing, molecular markers, resistance

RÉSUMÉ

La moisissure blanche, causée par *Golovinomyces cichoracearum*, est une maladie fongique très importante dans la culture du tabac. La sélection de variétés de tabac résistantes à la moisissure est encore à un stade préliminaire, ceci à cause du travail intense nécessaire à la sélection artificielle mais aussi la forte influence de l'environnement. Dans le but d'une sélection variétale plus efficaces, il est nécessaire que des marqueurs moléculaires soient identifiés afin d'aider à la sélection rapide de variétés de tabac résistantes à la moisissure blanche. L'objectif de la présente étude était donc d'identifier un marqueur SSR neutre capable d'aider les plante-généticiens dans leurs efforts de sélection de variétés de tabac résistantes à la moisissure blanche. A cet effet, deux lignées parentales résistantes XZ et STNCB, avec trois lignées parentales susceptibles XSR, K51 et T71, ont été utilisées. Des croisements en retour d'hybrides obtenus à partir du croisement de XZ et XSR ont été cultivés en serre, et leur ADN extraits pour une analyse moléculaire. Huit marqueurs SSR et dix ISSRs ont été utilisés. Ces marqueurs ont été essayés sur des lignées parentales et produits de backcross afin de déterminer la différence entre résistants (score 1) et susceptibles (score 5). Simple analyse de marqueurs a été réalisée au travers du t-test. En se basant sur le marqueur PT30021, il a été révélé des différences significatives ($P < 0.001$) entre plantes résistantes et susceptibles. Le marqueur PT30021 s'est montré lié au gène de résistance, et donc permet de distinguer les individus résistants des individus susceptibles au sein des lignées parentales ainsi que dans les générations avancées. Le locus

PT30021 a le potentiel d'aider à l'identification des accessions de tabac résistantes à la moisissure blanche, dans un processus de sélection assistée par les marqueurs.

Mots Clés: *Golovinomyces cichoracearum*, croisement en retour assisté au marqueurs, marqueurs moléculaires, résistance

INTRODUCTION

Tobacco (*Nicotiana tabaccum* L.) is grown worldwide as a cash crop (Chivuraise, 2011). In Zimbabwe tobacco has taken over the agricultural industry and as a result, the largest portion of arable land (Leaver, 2003). However, tobacco is susceptible to some diseases which result in yield reduction and poor leaf quality (Nyoka, 2005). Poor leaf quality results in low produce grade on the market leading to low prices.

Fungal diseases are the major causes of yield and quality loss in tobacco. In southern Africa, the white mould disease, caused by *Golovinomyces cichoracearum* is a major threat to tobacco production, causing up to 30% yield loss (Nyoka, 2005). *Golovinomyces cichoracearum* is an obligate biotroph that affects a wide range of plants including roses, cucurbits and soyabean. It causes damage to all organs at all stages of development. White mould reduces the photosynthetic surface of the plant, resulting in plant growth retardation and loss of foliage. All affected plant organs show characteristic symptoms, which include white powdery patches which appear first on the upper surface of leaves and finally covers the whole leaf blade (Marte *et al.*, 1993). The affected tissues gradually show brown lesions, which are a result of cell mortality. In the tobacco industry, leaves are the organ of economic importance and, therefore, attack by *Golovinomyces cichoracearum* reduces yields drastically.

Chemical control is ineffective in most situations because it is often expensive or may be applied when damage has been inflicted already. The most effective method to control white mould has been through the use of resistant cultivars. However, resistance to white mould is not found in the primary gene pool of tobacco (Marte *et al.*, 1993). Fortunately, resistance to white mould was found in the wild relatives that include *Nicotiana debneyi*, *Nicotiana glutinosa*

and *Nicotiana formis* (Valleau, 1952). This resistance was shown to be dominant monogenic and was introgressed into cultivated tobacco through backcrossing (Smith, 1968). The dominant form of resistance was expressed at all stages of plant development, and plants with this form of resistance inhibited hyphae growth and haustoria degenerated rapidly. It is this type of resistance that is used at the Kutsaga Research Station in Zimbabwe. Partial resistance was reported in a Japanese cultivar "Kokobu", and genetic studies have shown that this partial resistance is controlled by two separate recessive genes.

Resistance controlled by recessive genes is difficult to introgress and is not being used at Kutsaga Research Station. At the Station, the dominant monogenic type of resistance is being used in breeding for resistance, because it has the advantage of being identified easily in phenotypic selection. Breeding programmes that have dominant genes, compared to recessive genes, are usually shorter since dominant genes express themselves in both homozygote and heterozygote condition (Nyoka, 2005).

At Kutsaga Research Station, 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 programme. 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. Phenotypic backcrossing is, however, made difficult by the fact that the expression of the disease is influenced by the environment. Furthermore, artificial inoculations of the white mould are relatively difficult and make the whole breeding programme expensive. To this regard, the use of molecular markers for the foreground selection of the resistant gene is encouraged. This process, known as marker assisted backcrossing, has been shown to be effective in many breeding situations

(Poczai *et al.*, 2013) because it can be applied during early stages of plant growth and is independent of the environment.

The simple sequence repeat markers are preferred owing to their co-dominance nature and high reproducibility across laboratories (Diwan and Cregan, 1997). However, molecular markers have not yet been found for resistance to white mould, yet this is essential in marker-assisted backcrossing. The objective of this study was to identify a stable simple sequence repeat marker for use in foreground selection of resistance to white mould.

MATERIALS AND METHODS

Plant materials. All the tobacco varieties used in the study were obtained from the Plant Breeding Division at Kutsaga Research Station in Zimbabwe. The parental materials were coded XS and XSR, which are susceptible to *Golovinomyces cichoracearum* and XZ, which is resistant to the pathogen. The F₁ progeny of XSR and XZ were backcrossed up to BC₅. Other lines that were used were STNCB a resistant variety while T72 and K51 are susceptible varieties. At Kutsaga Research Station, the reaction to white mould is recorded on a scale of 1-5; where 1 is resistant and 5 is susceptible. The effect of the environment actually causes this range of phenotypes from a single dominant gene. Thus, for the purpose of this study, extreme situations were chosen to reflect reaction of plants to white mould. A score of 1 was used to reflect resistant plants, while a score of 5 was used to reflect susceptibility.

DNA extraction and polymerase chain reaction.

DNA extraction was done on six weeks old tobacco plants. DNA extraction was done using the modified CTAB protocol (Mazarire *et al.*, 2013). 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 (Mazarire *et al.*, 2013). The PCR mix contained 30.8 µl ultra pure water, 5 µl of 1× PCR buffer, 6 µl of 0.25 mM (Inqaba Biotech South Africa) and dNTPs (Sigma Aldrich). The primer concentrations were 0.15 mM at a volume of 1.5 and 0.2 µl 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 Tables 1 and 2. The reaction mix dispensed into the PCR tubes was 7 µl per tube and 1 µl of the DNA template. The PCR conditions were adopted from Gholizadeh *et al.* (2012).

The cycling conditions for SSR primers (Table 1) were 4 minutes of denaturation at 94 °C, which was followed by 1 minute at 94 °C. The annealing temperatures were 55 °C for 1.5 minutes, which was followed by an extension for 2 minutes at 72 °C. Final extension took place at 72 °C for 10 minutes. PCR was completed after 40 cycles. The cycling machine used was the Gene Amp 9700. The cycling conditions for the ISSR (Table 2) were 4 minutes of denaturation at 94 °C, which was followed by 1 minute at 94 °C. The annealing temperatures ranged from 52 - 59 °C for 1.5 minutes, which was followed by 2 minutes of extension at 72 °C. Final extension took place at 72 °C for 7 minutes. PCR was completed after 36 cycles.

TABLE 1. Name, sequences and linkage group of SSR primers used in this study

Primer name	Forward sequence	Reverse sequence	Linkage group
PT20172	ACACCTCCTTCTCCTGC	CCAAAATGGTTCACCTGGA	3a
PT30021	CATTTGAACATGGTTGGCTG	CTCAACTCTCGTCGCTCTTG	4
PT30096	GAAGTTTCAAAGTAGCACCAACAA	GCACCCTATTTGGTCTCCC	12
PT30144	TGATTTGATTGACAGCGTGAAG	TTGTTTAGTTACCCTATTTGACTTGC	16
PT30274	GACAGCAAGCTAATAACAGTAAATG	GGACTTTGGAGTGTCAAATGC	17
PT30378	TCAAATGAGGGTTGTAGCCA	TGCAATGGCTACACAAGAAGA	21
PT30392	CGAGGACGATGTAATGCTT	GCTAAGCTTGAAATCACATTCA	20
PT30480	AAAGGGAAACATGGACATTG	TAGGCGAGATTGTGGGATTG	13
PT51635	TGTTATCACAACCTCGACATTTATGAG	GGCCTTGTATCATATTGGG	10
PT51984	TTCGTTAGTTTGCTCAATCACAA	GGACGAAGTAGCTTTCATTGG	18

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.

Data analysis. Single marker analysis was done using a non-paired t-test with GenStat Software Version 13 (GenStat, 2010) to compare the mean of genotypes with and without the marker product (presence or absence of the bands) for all the markers used.

RESULTS

Amplifiable DNA of high quality was successfully extracted as evidenced by the presence of clearly defined 500 bp band (Fig. 1), amplified by CHL

TABLE 2. Name and sequence of ISSR primers used

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

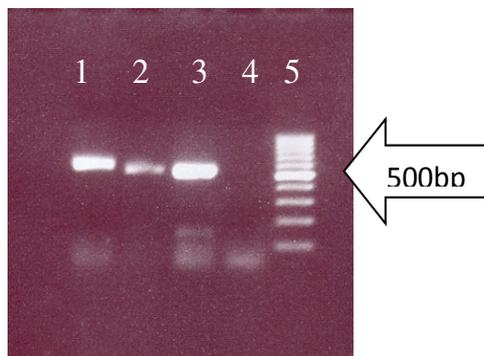


Figure 1. CHL electrographs Lane 1 – XSR; Lane 2 – BC₅; Lane 3 – XZ; Lane 4 – Water; Lane 5 – 100bp ladder.

primers. Furthermore, the CHL test showed that there were no PCR inhibitors in the extracted DNA. A simple sequence repeat marker, PT30021, grouped the resistant and susceptible genotypes, based on the presence and absence of the bands (Fig. 2). A non-paired t-test showed that there was a significant difference ($P < 0.001$) between these two groups in terms of their reaction to the white mould disease. Indeed, all the resistant genotypes (score 1) had no band; while a 250 pb

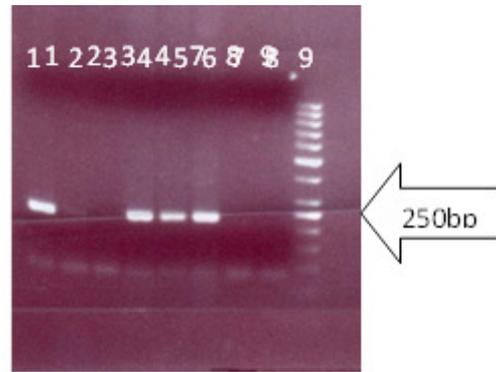


Figure 2. PT30021 electrographs. Lane 1 – K51 variety (susceptible); Lane 2 – SNTCB variety (resistant); Lane 3 – F₁ (resistant); Lane 4 – T72 variety (susceptible), Lane 5 – XSR variety (susceptible); Lane 6 – XS variety (susceptible); Lane 7 – BC₅ (resistant); Lane 8 – XZ variety (resistant) and Lane 9 – 50bp ladder.

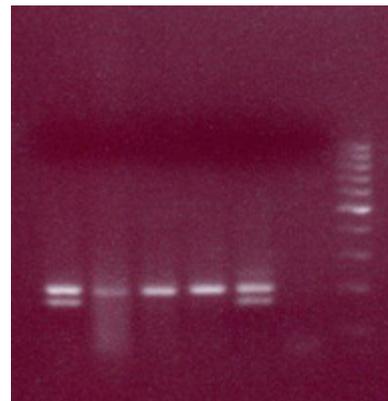


Figure 3. Lane 1 – K51 variety (susceptible); Lane 2 – SNTCB variety (resistant); Lane 3 – F₁ (resistant); Lane 4 – T72 variety (susceptible), Lane 5 – XSR variety (susceptible); Lane 6 – XS variety (susceptible); Lane 7 – BC₅ (resistant); Lane 8 – XZ variety (resistant) and Lane 9 – 50bp ladder.

band was observed in all susceptible genotypes (score 5). Given that the resistance was dominant, all the F_1 hybrids were resistant and did not show the presence of the band. The chloroplast (CHL) primer showed the presence of amplifiable extracted DNA (Fig. 1). There were markers that showed polymorphism (Fig. 3), but could not distinguish between the resistant and susceptible material. An example of such markers is shown in Figure 3.

DISCUSSION

The ability of marker PT30021 to discriminate resistant and susceptible parents shows that this is an ideal marker for white mould resistance. An ideal marker should be linked to the gene of interest (Collard *et al.*, 2005) as observed for PT30021 that is linked to the gene controlling white mould resistance. The marker was co-inherited with resistance to white mould even at advanced backcross generations (BC_3). The tightness of the link is determined by the genetic distance between the trait and marker (Poczai *et al.*, 2013). In future, there is need to saturate this region where the marker has been found in order to find markers that are even more linked to the gene of white mould resistance. The ideal situation would be to find the flanking markers that can improve the selection efficiency especially when used simultaneously.

In plant disease resistance studies, locating the quantitative trait loci (QTL) is of importance. 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 single marker analysis using a t-test or regression analysis as highlighted by Mazarire *et al.* (2013).

The reason why some markers did not work could be due to the fact that they targeted linkage groups that lacked the gene for white mould resistance (Collard *et al.*, 2005). The tobacco linkage map was completed based on different molecular markers (Bindler *et al.*, 2011). However, no work was done on mapping the chromosomal location of white mould resistance. 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 is that the search is blind and a larger number of primers is required if the search is to be successful. The primers used in this study targeted linkage groups 1, 3, 4, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20 and 21; while the other 10 linkage groups were not sampled (Bindler *et al.*, 2007; Bindler *et al.*, 2011). The resistance targeted in this study is controlled by a single dominant gene. This gene could be located on linkage group 4 where primer PT30021 was linked to it is found. This means that all other markers used on different chromosome were mis-targeted. However, given the fact that resistance to white mould was not mapped to linkage groups, therefore many markers are needed to cover the entire genome.

The high variability in microsatellites makes them appropriate for use in population studies and for marker assisted selection (Semagan *et al.*, 2006). Microsatellites analyse co-dominance in segregating populations, which differs from other markers such as ISSR (Bindler *et al.*, 2007). The main advantages of using these markers are that they are transferable between populations and are technically simple to use, are robust and reliable. Results obtained from the use of the UBC primers were not favourable compared to those from the PT primers, and are not presented in this study. The electrographs from the post PCR of UBC markers had multiple bands. This made it difficult to compare between the resistant and susceptible materials. Some of the bands were not separated distinctly, which made it even harder to determine their band size, thus making them unsuitable for use in the study to get conclusive results. However, the use of ISSR markers does not require prior information of the sequences and has the advantage of requiring relatively less DNA (Ansari *et al.*, 2012). Also, it has the advantage of using random primers.

Breeding presently requires the adoption of new techniques that reduce time required for the release of new varieties. Relying on the conventional methods of breeding, based on phenotypic selection, takes longer to complete a breeding programme successfully. Molecular markers such as the PT30021, are independent of the environmental effects; and more importantly can be detected at any developmental stage of

the plant (Davalieva *et al.*, 2010; Bindler *et al.*, 2011). The use of PT30021 in marker-assisted backcrossing will greatly improve the efficiency of breeding programmes aimed at improving resistance to white mould in tobacco.

CONCLUSION

The primer PT30021 is linked to resistance to white mould in tobacco and, thus can be used in foreground selection for the gene of resistance using the marker-assisted backcrossing scheme.

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