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Potential of marker-assisted selection for *Tobacco mosaic tobamovirus* resistance in tobacco breeding

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Tobacco mosaic tobamovirus **(TMV) is one of the most destructive virus threatening worldwide tobacco production. Use of host resistance is the best method of control. The *N*-gene was introgressed into tobacco from *Nicotiana glutinosa* to confer hypersensitive resistance to TMV. Phenotypic selection of TMV resistant germplasm is expensive, slow and unreliable. Use of *N*-gene specific primers is efficient in selecting TMV resistant germplasm in marker-assisted breeding. This study aimed at assessing the utility of *N*-gene specific primers in flue cured and dark-fire cured tobacco breeding materials in Zimbabwe. Four specific primers namely N1/N2, AS1/AS2, E1/E2 and SD1/SD2 were used to detect the *N*-gene in flue cured and dark-fire cured tobacco. DNA was extracted from young leaves of tobacco plants and quantified by a spectrophotometer. Polymerase chain reaction (PCR) mix and amplifying conditions for the four specific primer pairs were optimized. Results show that out of the four sets of primers used, AS1/AS2 and SD1/SD2 did not produce expected band products, while N1/N2 and E1/E2 detected the *N*-gene in flue cured and dark-fire cured tobacco. Therefore, the use of N1/N2 and E1/E2 primers will be relatively cheap, effective and quick in the foreground selection of the *N*-gene.**

Key words: *N*-gene, specific primers, resistance, molecular markers, *Nicotiana tabacum*.

INTRODUCTION

In Zimbabwe, flue cured tobacco (*Nicotiana tabacum* L.) accounts for 45% of the total agricultural earnings (Jeffee, 2003). Furthermore, there is an increase in the demand of dark-fire cured tobacco by the major tobacco merchants. However, tobacco yield and quality are greatly affected by diseases especially those caused by viruses (Yadal et al., 2005). Out of the 16 viruses that infect tobacco (Nielsen and Davis, 1999), eight of them including the *Tobacco mosaic tobamovirus* (TMV) are of economic importance. TMV is a single-stranded, positive-sense RNA virus distributed world-wide (Goelet et al., 1982) which causes leaf mottling, vein chlorosis, deformed and wrinkled leaves in tobacco (Dawson, 1999). TMV infection greatly reduces tobacco yield and quality and further reduces seed viability.

Use of resistant cultivars provides an effective means of TMV management (Ishikawa et al., 1986). Resistance to viruses is defined as the ability of the plant to reduce and overcome viral movement and replication (Dawson, 1999). The *N*-gene that confers hypersensitive resistance to TMV in the heterozygous state (Whitham et al., 1996; Gooding, 1969; Dinesh-Kumar et al., 2000) was introgressed into tobacco cultivars from *N. glutinosa* (Holmes, 1960). A hypersensitive reaction is characterized by localized cell death at the point of pathogen ingress that prevents further systemic spread of the pathogen (Wright et

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al., 2000). The *N*-gene is a member of the Toll-interleukin-1 receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance (R) genes (Wright, 2000). The *R* genes in plants are hypothesized to encode receptors that interact directly or indirectly with ligands produced by the corresponding invading pathogen avirulence (Avr) genes. Erickson (1999) pointed out that initial recognition event triggers defense responses that halt pathogen spread. This R-Avr-mediated defense response in plants is termed 'gene-for-gene' type of resistance. The TIR-NBS-LRR domain is highly conserved and permits the development of gene specific primers that mainly target this conserved region.

At Kutsaga Research Station, the tobacco breeding program is aimed at developing new disease-resistant cultivars with superior agronomic and chemical properties and better quality and aroma than the existing commercial cultivars. Conventionally, the presence of the *N*-gene in tobacco cultivars is phenotypically determined as the absence of TMV symptoms on tobacco leaves. The time from inoculation to assessment can take up to five weeks and is expensive since a large number of progenies which take up massive greenhouse space must be screened, a process requiring substantial labour. Phenotypic screening of resistance to TMV is also subjective and unreliable. For example, TMV symptoms on tobacco overlap with symptoms caused by *Cucumber mosaic cucumovirus.* Furthermore, some nutrient deficiencies may exhibit symptoms which are similar to TMV and some infected plants may fail to express the disease symptoms (Yi et al., 1998). Therefore, the *N*-gene can be tagged using primers specific to this gene in order to circumvent the afore-mentioned challenges.

The use of molecular techniques has advantages which include: easy recognition of phenotypes from all alleles, the gene is detectable at all growth stages and it is not affected by environmental conditions. The *N*-gene specific primers used in this study were developed elsewhere but their efficacy with Kutsaga Research Station germplasm had not been determined. The objective of the study was to assess the utility of *N*-gene specific primer for marker-assisted breeding of tobacco in Zimbabwe.

MATERIALS AND METHODS

A total of 12 flue cured tobacco varieties namely KRK1, KRK26, KRK26R, KRK28R, KRK28, T29, T29R, T64, T64R, T66, T71 and T72 were used together with eight dark-fire cured backcross one materials namely: (Western 86 x BAZR 1-3-22) x Western 86, (Western 86 x BAZR 1-3-14) x Western 86, (Western 86 x BAZR 1-3-3) x Western 86, (Piet Retief x BAZR 1-3-3) x Piet Retief, (Piet Retief x BAZR 1-3-22) x Piet Retief, (Piet Retief x BAZR 1-3-14) x Piet Retief, (Galpao x BAZR 1-3-3) x Galpao and (Galpao x BAZR 1-3-14) x Galpao. Two flue cured tobacco materials KM 10 (resistant to TMV) and K51 (susceptible to TMV) were used as negative and positive controls, respectively. The materials were kindly provided by the Plant Breeding Division of the Kutsaga Research Station in Zimbabwe.

Phenotypic assays for TMV resistance

The TMV inoculations were done at four weeks after transplanting. The TMV infested sap was extracted from the susceptible variety, K51. The sap was then rubbed on plant leaves. Reaction to the TMV was rated either as presence or absence of local lesions due to a hypersensitive response.

Molecular assays

DNA extraction and quality test

DNA was extracted from five-week old tobacco material following the modified CTAB method (Doyle and Doyle, 1987). DNA quantity was obtained by measuring absorbance at 260 and 280 nm using the UV spectrophotometer. DNA purity (based on the ratio of absorbance at 260 nm: absorbance at 280 nm) was determined and this ratio was expected to be between 1.5 and 2.0 for pure DNA. The extracted DNA samples were checked for the presence of PCR inhibitors using the chloroplast (CHL) test. The CHL primers target a conserved region in the chloroplast DNA and yielded an approximately 550 bp product suggesting that the DNA quality was good.

PCR amplification

Four pairs of specific primers (Table 1) were used for TMV resistance gene screening on both flue cured tobacco and dark-fire cured varieties. The PCR reaction mix is shown in Table 2.

The PCR conditions for N1/N2 primer were: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min and a final extension time of 10 min at 72°C while the PCR conditions for E1/E2 primer were initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 7 min. The PCR conditions for the other two primers are not reported because no product was produced. The PCR products obtained were mixed with 2 µl volume of orange loading dye. A volume of 12 µl was loaded in each well for electrophoresis in 1.5% agarose gel in 0.5X TBE buffer (89 mM Tris-base, 89 mM boric acid, and 2 mM ETDA) stained with 0.5 mg/ml of ethidium bromide at 130 V for 45 min. The size of the amplification products was estimated from a 50 bp ladder. DNA bands were photographed using a UV documentation system (UVTech, Cambridge, United Kingdom). Bands of DNA fragments were scored as present (+) or absent (-).

RESULTS AND DISCUSSION

The resistant tobacco materials showed local lesions typical of hypersensitive reaction. Two primer pairs N1/N2 F/R (Forward / Reverse) and E1/E2 F/R resulted in amplification of expected band sizes of 359 and 545 bp, respectively (Lewis et al., 2005) for both flue cured (Figures 1 and 2) and dark-fire cured tobacco (Figures 3 and 4). No product amplification occurred with the primer pair AS1/AS2 whilst the SD1/SD2 primer combination produced multiple bands without the expected band size for both flue cured and dark-fire cured tobacco (Tables 3 and 4).

The *N*-gene, a member of the nucleotide binding site/leucine-rich repeat class of disease resistance genes

Table 1. Details of *N*-gene primers used.

Primer	Sequence (5'-3')	Expected band size (bp)	Source
SD1/SD2	F(GCATCTTCTTCTTCTTCTTC) R(GAGCCTTTGAGATTGGCCGC)	450	Whitham et al., 1996
E1/E2	F(ACCAGAATGATATGTTCCAC) R(GGACTCAACGTTAATTCTCTG)	545	Lewis et al., 2005
N1/N2	F(CGTCGACACATTATGCCATC) R(GAGGGGTCTTACCCCATTGT)	359	Lewis et al., 2005
AS1/AS2	F(GGTGGGGTTGGGAAGACAACG) R(CCACGCTAGTGGCAATCC)	550	Leister et al., 1996

Table 2. PCR reaction mixture (ul) for N1/N2 primer pair and E1/E2 primer pair.

*Reagent	N1/N2 primer	E1/E2 primer
Ultrapure water	29.75	31.75
10x Buffer	5	5
dNTPs (2.5 mM)	4	4
$MgCl_2$ (25 mM)	3	3
Primer 1 (10 µl)	3	2
Primer 2 (10 µl)	3	2
Taq polymerase (5 U/ul)	0.25	0.25
DNA template	2	2
Total volume(µl)	50	50

*Primers were supplied by Inqaba Biotech, South Africa and Taq Polymerase by Seperation Scientific, South Africa.

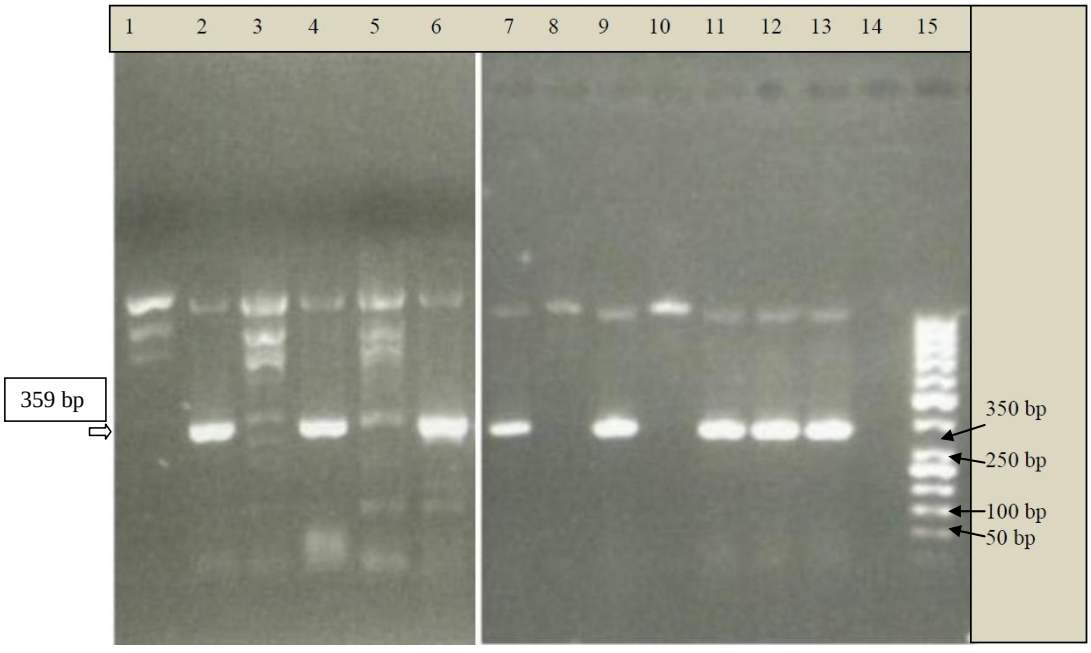

Figure 1. Amplified products of flue cured tobacco with N1/N2 primer after electrophoresis. Lane 1, KRK1; Lane 2, T29R; Lane 3, T29; Lane 4, T64R; Lane 5, T64; Lane 6, T72; Lane 7, KRK26R; Lane 8, KRK26; Lane 9, KRK28R; Lane 10, KRK28; Lane 11, T66; Lane 12, T71; Lane 13, KM10; Lane 14, Water; Lane 15, 50 bp ladder; expected band size, 359 bp.

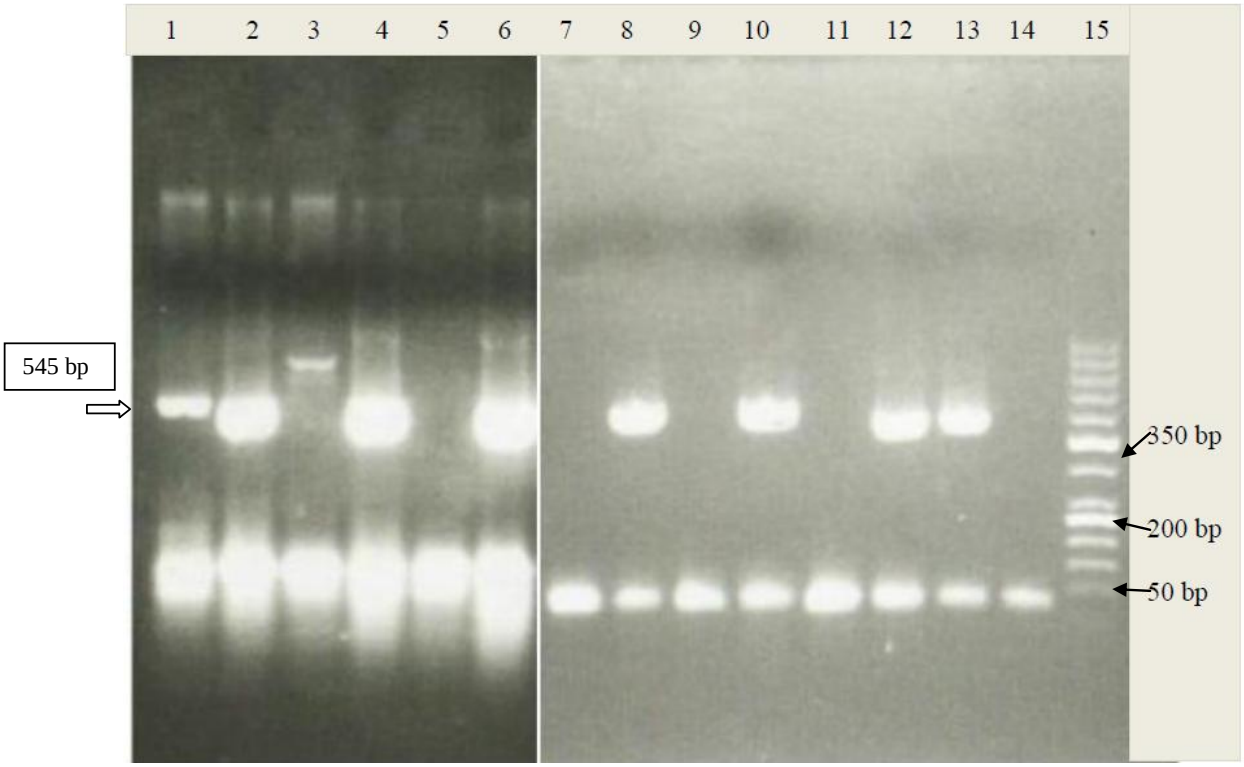

Figure 2. Gel picture result of flue cured tobacco with the primer E1/E2. Lane 1, T71; Lane 2, RK26R; Lane 3, KRK26; Lane 4, KRK 28R; Lane 5, KRK28; Lane 6, T66; Lane 7, T29; Lane 8, T29R; Lane 9, T64; Lane 10, T64R; Lane 11, KRK1; Lane 12, T72; Lane 13, KM10; Lane 14, Water; Lane 15, 50 bp ladder; expected band size, 545 bp.

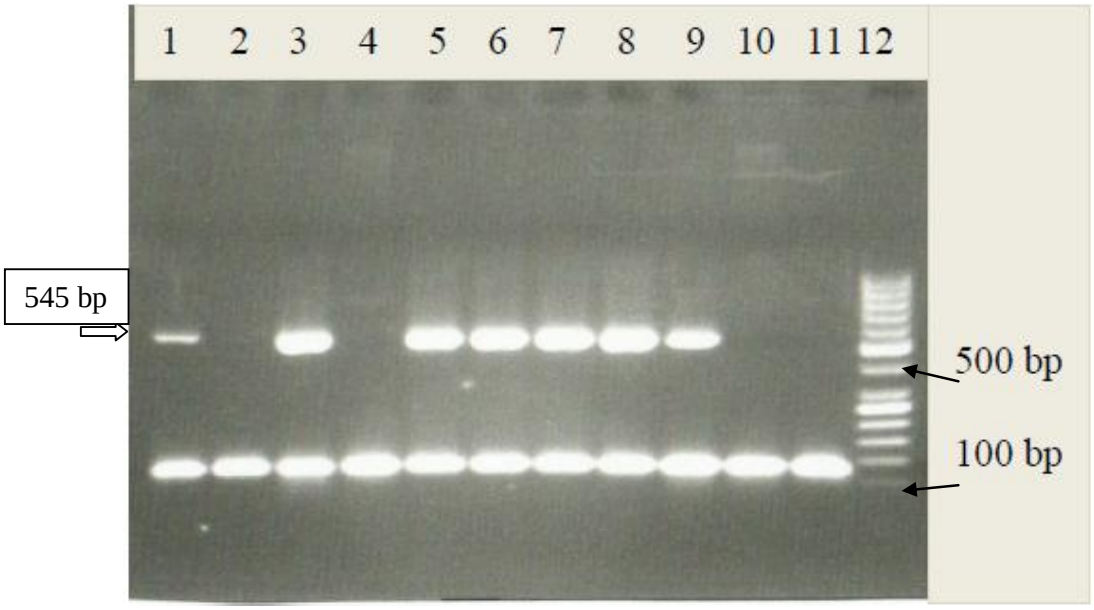

Figure 3. Amplified products of dark-fire cured tobacco with E1/E2 primer after electrophoresis**.** Lane 1, (Western 86 x BAZR 1-3-14) x Western 86; Lane 2, (Western 86 x BAZR 1-3-3) x Western 86; Lane 3, (Western 86 x BAZR 1-3-22) x Western 86; Lane 4, (Piet Retief x BAZR 1-3-3) x Piet Retief; Lane 5, (Piet Retief x BAZR 1-3-22) x Piet Retief; Lane 6, (Piet Retief x BAZR 1-3-14) x Piet Retief; Lane 7, (Galpao x BAZR 1-3-3) x Galpao; Lane 8, (Galpao x BAZR 1-3-14) x Galpao; Lane 9, KM10 (Positive control); Lane 10, K51 (Negative control), Lane 11, Water; Lane 12, 50bp ladder; expected band size, 545 bp.

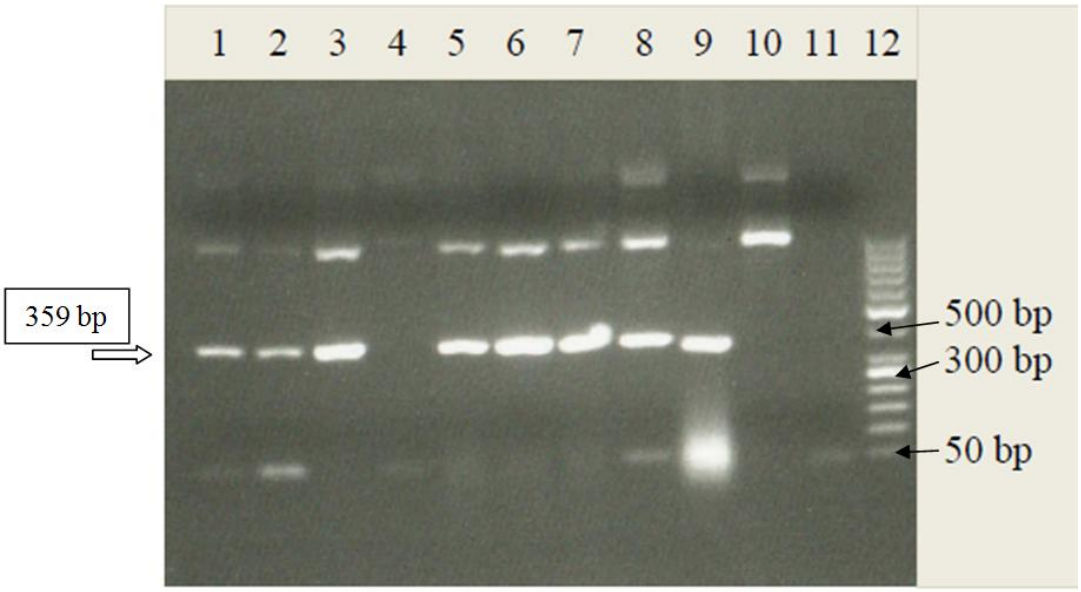

Figure 4. Amplified products of dark-fire cured tobacco with N1/N2 primer after electrophoresis. Lane 1, (Western 86 x BAZR 1-3-14) x Western 86; Lane 2, (Western 86 x BAZR 1-3-3) x Western 86; Lane 3, (Western 86 x BAZR 1-3-22) x Western 86; Lane 4, (Piet Retief x BAZR 1-3-3) x Piet Retief; Lane 5, (Piet Retief x BAZR 1-3-22) x Piet Retief; Lane 6, (Piet Retief x BAZR 1-3-14) x Piet Retief; Lane 7, (Galpao x BAZR 1-3-3) x Galpao; Lane 8, (Galpao x BAZR 1-3-14) x Galpao; Lane 9, KM10 (Positive control); Lane 10, K51 (Negative control), Lane 11, Water; Lane 12, 50bp ladder; expected band size, 359 bp.

Table 3. Phenotypic and molecular results of flue cured tobacco.

Cultivar	Reaction to TMV	N1/N2	E1/E2	AS1/AS2	SD1/SD2
KRK1	S	-	-	*	**
KRK26	S	-	-	*	**
KRK28	S	-	-	*	**
T29	S	-	-	*	**
T64	S	-	-	*	**
KRK26R	R	+	+	*	**
KRK28R	R	+	+	*	**
T29R	R	+	+	*	**
T64R	R	+	+	*	**
T66	R	+	+	*	**
T71	R	+	+	*	**
T72	R	+	+	*	**
KM10	R	+	+	*	**

+, Band present; -, band absent; *no amplification; **unexpected band size; S, susceptible; R, resistant.

can be used to confer hypersensitive resistance to TMV. Although Witham et al. (1996) used the specific primer SD1/SD2 in the amplification of the *N*- gene in transgenic tomato successfully, the primer failed to produce the expected product of 450 bp in both flue cured and dark-fire cured tobacco. Instead, the primer produced multiple bands without the product of interest in both flue cured and dark fire cured tobacco. This result showed that the primer did not work well with the study material. The reason could be that some *N*-gene domains such as NBS-LRR could undergo mutation resulting in the primer failing to anneal. Lewis et al. (2005) pointed out that the *N*-gene in flue cured tobacco is on chromosome H and this might be a mutation hot spot region. Selvaraj et al. (2011) used the AS1/AS2 primer pair for the tobacco *N* gene. However, in this study, primer pair AS1/AS2 did not

Table 4. Phenotypic and molecular results of dark-fire cured tobacco.

Genotype	Reaction to TMV	N1/N2	E1/E2	AS1/AS2	SD1/SD2
(Western 86 x BAZR 1-3-22) x Western 86	R	+	+	*	**
(Western 86 x BAZR 1-3-14) x Western 86	R	+	-	*	**
(Western 86 x BAZR 1-3-3) x Western 86	R	+	+	*	**
(Piet-Retief x BAZR 1-3-3) x Piet Retief	R	-	-	*	**
(Piet-Retief x BAZR 1-3-22) x Piet Retief	R	+	+	*	**
(Piet-Retief x BAZR 1-3-14) x Piet Retief	R	+	+	*	**
(GALPAO x BAZR 1-3-3) x GALPAO	R	+	+	*	**
(GALPAO x BAZR 1-3-14) x GALPAO	R	+	+	*	**
K51 (Control -Susceptible)	S	-	-	*	**
KM 10 (Control - Resistant)	R	+	+	*	**

+, Band present; -, band absent; *no amplification; **unexpected band size; S, susceptible; R, resistant.

amplify at all. This primer pair may require further optimization. The primer pairs that produced unexpected results or failed to amplify the *N*-gene cannot be used for TMV resistance gene screening in Kutsaga tobacco materials.

In this study, two pairs of primers E1/E2 and N1/N2 used on flue cured and dark-fire cured tobacco worked well. The expected band products of 359 (with primer N1/N2) and 545 bp (with primer E1/E2) were observed in the resistant genotypes and as anticipated, there was no amplification with the susceptible tobacco control. Lewis et al. (2005) also used these two primer pairs successfully to determine the cloned *N*-gene sequence derived from *N. glutinosa*. The results obtained with these two primer sets indicate that they can be successfully used for the selection of TMV-resistant germplasm in breeding programmes. However, in dark-fire cured tobacco, the backcross (Piet Retief x BAZR 1-3-3) x Piet Retief failed to produce the expected amplification products with both primers. This genotype might have lost the gene due to meiotic recombination but phenotypically considered resistant probably due to disease escapes in the inoculations. Gooding (1969) described a reaction to TMV infection as symptomless in the plants which display no visible signs of infection. In a study conducted by Gooding (1969), 25 lines exhibited no visual symptoms to the virus after inoculations but after re-infection assays on the symptomless plants, the results revealed that the virus was present in each of the inoculated parent. To this regard, the two primers, E1/E2 and N1/N2 can be used for TMV resistance gene screening at Kutsaga Research Station.

Marker assisted selection has been widely used in other crops that include rice (*Oryza sativa* L.), maize (*Zea mays* L.) and potato (*Solanum tuberosum* L.) (Kumar et al., 2009). DNA marker technology used in commercial plant breeding programs has proved helpful for the rapid and efficient transfer of resistant genes like the *N*-gene into agronomically desirable varieties which enables the movement of products to the market faster (Tanksley et al., 1989). Marker-assisted selection also allows pyramiding of several resistance genes in the same genotype. The use of molecular techniques will reduce the workload, environmental influence on phenotype, can identify the resistance of plants in early stage of breeding, increase selection veracity and efficiency and can shorten the breeding cycle. Furthermore, *N*-gene specific markers would assist breeders to rapidly select for material that is resistant to TMV without necessarily waiting for phenotypic expression of the diseases.

Conclusions

The two specific primers namely, N1/N2 and E1/E2 clearly detected flue cured tobacco and dark-fire cured tobacco with the *N*-gene. These primers can be considered for use at Kutsaga Research Station to accelerate the breeding process. Use of the two markers is adequate to circumvent the hardships involved in phenoltypic screening currently experienced at Kutsaga Research Station. Primer pair AS1/AS2 and SD1/SD2 that failed to produce the expected band sizes needs fur-ther optimization if they are to be exploited in the marker-assisted breeding program at Kutsaga Research Station.

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