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*Commentary*

# *Musa* Methylated DNA Sequences Associated with Tolerance to *Mycosphaerella fijiensis* Toxins

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**Abstract.** DNA methylation is an epigenetic phenomenon associated with gene silencing in transgenic plants, retrotransposons and virus infection. Expression analysis of specific genes in Arabidopsis methylation mutants showed an association between DNA methylation and gene expression. To determine whether DNA methylation is associated with resistance to black Sigatoka (BS) and *Mycosphaerella fijiensis* (MF), we used an *in vitro* assay of mesophyll cell suspensions of reference cultivars with known resistance to BS. Methylation of $CC^{m}GG$ sequences was evaluated by methylation-sensitive amplification polymorphism (MSAP) markers of reference cultivars and somaclonal variants to identify molecular markers associated with resistance to MF toxins and BS. Four MSAP markers were associated with resistance (MAR) to MF toxins. The MSAP markers show a high degree of sequence similarity with resistance gene analog and with retrotransposon sequences. The MSAP markers are useful as molecular indicators of tolerance to MF toxins and resistance to BS.

**Key words:** black Sigatoka, MSAP, methylation, *Musa*, *Mycosphaerella fijiensis* toxins, somaclonal variants

**Abbreviations:** AFLP, amplified fragment length polymorphism; BA, N-6-benzyladenine; BS, black Sigatoka; MAR, methylation associated with resistance; MAS, methylation associated with susceptibility; MF, *Mycosphaerella fijiensis*; MSAP, methylation-sensitive amplification polymorphisms; TGS, transcriptional gene silencing; SAMPL, selective amplification of microsatellite polymorphic loci; TI, tolerance index.

## Introduction

Feinberg (2001) proposed the term "methylome" to define the complete set of DNA methylation modifications of a cell. This concept underscores the importance of methylation in genetic control at different levels and serves to formally unite the study of DNA methylation with the power of modern genomics.

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DNA methylation is a covalent modification of cytosine, heritable by somatic cells after cell division, and is generally associated with transcriptional silencing. It occurs at CpG dinucleotides, as DNA methyltransferases that maintain DNA methylation recognize hemimethylated DNA at this site after DNA replication. Approximately 70% of CpG sites are methylated, with the notable exception of CpG-rich sequences termed "CpG islands," which are generally unmethylated. Some CpG islands also show increased methylation with age, and both methylation processes (of the CpG sites and the CpG islands) may lead to deregulated gene expression and senescence (Feinberg, 2001).

In Arabidopsis two floral homeotic transformations are due to hypermethylation of the *SUPERMAN* and *AGAMOUS* genes, which regulate flower development. No transcripts were detected from either hypermethylated gene, suggesting that these genes are transcriptionally silenced (Jacobsen et al., 2000).

Since Vos et al. (1995) develop the amplified fragment length polymorphism (AFLP), many applications and variations have been devised. Methylation-sensitive amplification polymorphism (MSAP) markers represent a modification of AFLP markers for methylation studies (Reyna-López et al., 1997). MSAP is based on selective PCR amplification and comparison between fragments generated by a pair of isoschizomer enzymes with different sensitivity to methylation. This technique combines the advantages of AFLP, allowing visualization of a large number of markers per sample, and the examination of cytosine methylation status of the restriction site. This method has been used for surveying CpG methylation at the CCGG site in the genome of rice cultivars (Xiong et al., 1999); characterizing methylation changes associated with micropropagation of banana (Peraza-Echeverria et al., 2001) and apple (Xu et al., 2000); and studying banana dwarfism (Engelborghs and Swennen, 1999) and oil palm somaclonal variation (Matthes et al., 2001).

Although the MSAP technique had been applied to *Musa* studies (Engelborghs and Swennen, 1999; Peraza-Echeverria et al., 2001), this is the first report describing DNA methylation associated with resistance to *Mycosphaerella fijiensis* (MF) toxins.

## Materials and methods

### *Induction and selection of somaclonal variants*

Somaclonal variation was induced in *Musa* (AAA) cv. Williams by adventitious shoot induction under high levels of N-6-benzyladenine (BA) (20 mg/L). Fifty plants were randomly selected from a population of 2,000 plants obtained *in vitro* by adventitious shoot induction. SAMPL markers and flow cytometry analyses were performed to select the five somaclonal variants used in this research (VS1, VS2, VS3, VS6, and VS13) (Giménez et al., 2005).

### *Toxicity assay*

A MF toxin extract was obtained using a monosporic MF mycelia cultured in MID medium for 40 days. The mycelium was separated from the medium by

*Table 1.* Primer combinations used for MSAP amplifications.

| Primer combinations | Primer combinations |
|---|---|
| *Msp*-1AT + *Eco*-1T | *Msp*-1AA + *Eco*-1C |
| *Msp*-1CG + *Eco*-1T | *Msp*-1GG + *Eco*-1C |
| *Msp*-1GA + *Eco*-1G | *Msp*-1CC + *Eco*-1C |
| *Msp*-1TA + *Eco*-1G | *Msp*-1CG + *Eco*-1C |
| *Msp*-1CT + *Eco*-1AA | *Msp*-1AC + *Eco*-1C |
| *Msp*-1TC + *Eco*-1AA | *Msp*-1GC + *Eco*-1C |
| *Msp*-1A + *Eco*-1TC | *Msp*-1CC + *Eco*-1G |
| *Msp*-1A + *Eco*-1AG | *Msp*-1GG + *Eco*-1G |
| *Msp*-1A + *Eco*-1GT | *Msp*-1CG + *Eco*-1G |
| *Msp*-1TT + *Eco*-1C | *Msp*-1GC + *Eco*-1G |

filtration, and toxins were extracted with chloroform : isoamyl alcohol (2:1) as described in Giménez and Colmenares (2004).

Toxicity assays were performed on the MF toxin extract by cell viability estimation with Evans Blue (1%), employing suspensions of mesophyll cells from different *Musa* cultivars and somaclonal variants: Pisang Mas (AA) (susceptible), Yangambi km5 (AAA) (highly resistant), Williams (AAA) (susceptible) and five somaclonal variants of Williams (AAA) selected by SAMPL markers (Giménez and Colmenares, 2004; Giménez et al., 2005).

A tolerance index (TI), was calculated using the following equation:

$$\mathrm{T1} = \frac{\%\,\text{live cells control} - \%\,\text{live cells toxins}}{\%\,\text{live cells control} + \%\,\text{live cells toxins}} \times 100$$

This index represents the percentage of total cells affected by the MF toxins. With this index it is possible to standardize all measurements and to compare different cell suspensions (Giménez and Colmenares, 2004).

### *MSAP markers*

MSAP were carried out following Reyna-López et al. (1997) with minor modifications reported by others (Xiong et al., 1999; Xu et al., 2000) using twenty combinations of selective oligonucleotides (Table 1).

### *Genomic DNA purification*

Genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1990), as modified by Weising and Kahl (1997). The DNA samples were dissolved in 200 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Quantification was by SYBR green staining of bands in agarose gels. λ-DNA was used as a mass reference, and the quantification was made with the Volume Tool of the Quantity One software (Bio-Rad).

### *DNA restriction digests and adaptor ligations*

DNA manipulations were performed with standard methods. Two enzyme reactions were set up at the same time for each DNA. For the first reaction, 200 ng of

genomic DNA was digested with *Eco*RI plus *Hpa*II. A second reaction substituted *Msp*I for *Hpa*II. Adapters were then ligated to digested fragments. The *Eco*RI adapter sequence is (5′-CTCGTAGACTGCGTACC-3′/3′-CATCTGACGCATTGG TTAA-5′); the *Msp*I-*Hpa*II adapter sequence is (5′-CGACTCAGGACTCAT-3′/3′-TGAGTCCTGAGTAGCAG-5′).

*Preamplification*

Preamplifications were performed using 5 μL of the ligated samples with 0.5 μL *Eco*RI (10 μM) primer with no selective base (Eco-10 primer: 5′-AGACTGCGT-ACCATTCA-3′), 0.5 μL *Msp*I/*Hpa*II (10 μM) primer without selective bases (Msp-10 primer: 5′-GATGAGTCCTGAGTCGG-3′), 1 μL 10× PCR Buffer (Invitrogen), 1.5 μL dNTPs (2.5 mM) (Invitrogen), 1.5 μL $MgCl_2$ (25 mM), and 0.25 U Taq DNA polymerase (Invitrogen) in a final volume of 10 μL. Thermal cycling was performed in a Bio-Rad thermal cycler (iCycler) as follows: an initial denaturing step at 94°C for 5 min and 25 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min.

*Selective amplifications*

Preamplification reactions were diluted 1:10 with TE. Selective amplifications were carried out with the same preamplification conditions, but using 2 μL of the diluted preamplification reaction as template and using 0.3 μL of selective oligonucleotide primer *Eco*R1-XY (10 μM) and 1 μL of *Msp*1-XY (10 μM). Twenty different oligonucleotide combinations were tested for MSAP amplifications (Table 1). The PCR thermal cycling reactions were performed as described by Palacios et al. (2002).

*Gel electrophoresis and silver staining*

Denaturing 4% polyacrylamide sequencing gels (45% urea) were prepared in a Bio-Rad sequencing chamber, and preheated at approximately 45°C by pre-running with 1× TBE buffer for 1 h. The PCR products were dried and dissolved in 6 μL of denaturing loading buffer. Samples were denatured at 96°C for 3 min and chilled on ice just before loading. The gels were run at a constant temperature of 45°C (1800-2200 V) for 2.5-3 h and were silver stained following the manufacturer's instructions for the Silver Sequence™ DNA Sequencing System (Promega Q4130).

*MSAP data analysis*

Paired bands from *Eco*RI-*Msp*I/*Hpa*II digests were registered as absence/presence (0/1) data, and this information was transformed in a secondary matrix to describe methylation information of each band pair as a single digit. The final codes are as follows: 0 (0,0): sequence absence or both cytosines methylated ($C^mC^mGG$); 1 (0,1): sequence non-methylated (CCGG); 2 (1,0): internal cytosine methylated ($CC^mGG$); and 3 (1,1): mix of methylated and non-methylated alleles (Figure 1).

These methylation conditions were used to calculate an index to estimate whether methylation is associated with resistance to MF toxins or BS in *Musa* spp. Two hypotheses were tested:

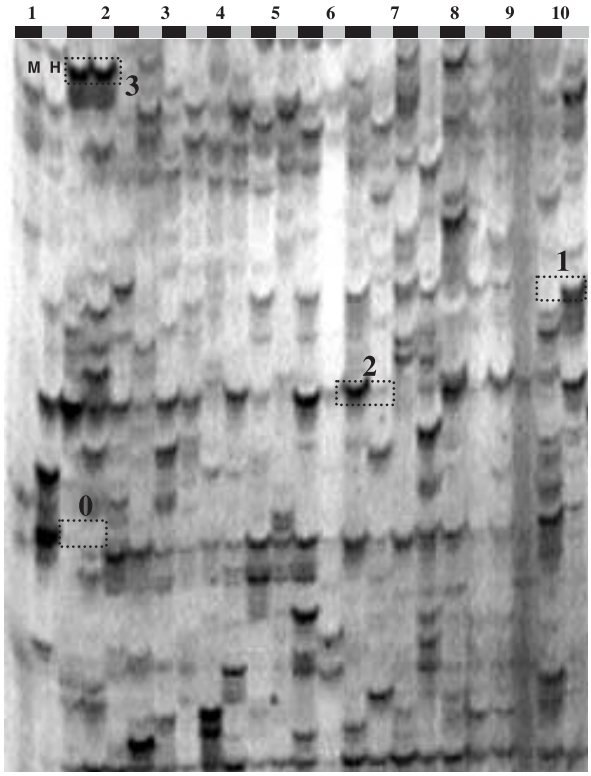


*Figure 1.* MSAP markers profiles and its methylation codes: 0) sequence absence or both cytosine methylation ($C^mC^mGG$), 1) non-methylated sequence (CCGG), 2) internal methylated cytosine ($CC^mGG$), and 3) mix of methylated and non-methylated alleles. M: digestion with *Msp*I, H: digestion with *Hpa*II.

1. Methylation is associated with resistance (MAR). If the tested plant has the resistant phenotype and if the methylation condition codes 2 or 0, then 1 will be added to the MAR equation; otherwise, if the plant is susceptible with the same methylation conditions, then 0 will be added to the equation. For the methylation condition coded 1, if the tested plant is susceptible, then 1 will be added to the equation; otherwise, if the plant is resistant with the same methylation condition, then 0 will be added to the equation.
2. Methylation is associated with susceptibility (MAS). This means that the opposite conditions to the MAR equation describe the MAS equation. If the tested plant has the susceptible phenotype and if the methylation condition codes 2 or 0, then 1 will be added to the MAS equation; otherwise, if the plant is resistant with the same methylation conditions, then 0 will be added to the equation. For the methylation condition coded 1, if the tested plant is resistant, then 1 will be added to the equation; otherwise, if the plant is susceptible with the same methylation condition, then 0 will be added to the equation.

The summations of values associated with susceptibility and resistance (0 and 1, respectively) were divided by the total number of tested plants to calculate the respective percentages associated with the two proposed hypotheses:

$$\text{MAR or MAS} = \frac{\sum \text{Resistant} + \sum \text{Susceptible}}{12} \times 100$$

$$\text{MAS} \begin{cases} \text{If code 2 or 0 and susceptible, add 1 each} \\ \text{Otherwise, if code 2 or 0 and resistant, add 0 each} \\ \text{If code 1 and resistant, add 1 each} \\ \text{Otherwise, if code 1 and susceptible, add 0 each} \end{cases}$$

$$\text{MAR} \begin{cases} \text{If code 2 or 0 and resistant, add 1 each} \\ \text{Otherwise, if code 2 or 0 and susceptible, add 0 each} \\ \text{If code 1 and susceptible, add 1 each} \\ \text{Otherwise, if code 1 and resistant, add 0 each} \end{cases}$$

*Cloning and Sequencing of MSAP markers*

The MSAP markers were recovered from polyacrylamide gels following the protocol of Chalhoub et al. (1997). Recovered bands were reamplified using the same PCR conditions described for selective amplifications, and with the same selective primers.

PCR products were resolve on agarose gels and purified using the purification kit QG from QIAGEN. Then, the purified PCR product was ligated using pCR®II-TOPO® (Invitrogen) and transformed into *E. coli* DH-5α. To identify possible sequence variations in the PCR fragments, forty independent clones of each cloned PCR fragment were amplified and analyzed by restriction digest.

Sequencing was done with an automated sequencer. Sequence analyses were done using the European Bioinformatics Services EBI, and NCBI-Blast2, for query databases, using defaults parameters.

## Results and Discussion

*In vitro evaluation of tolerance to MF toxins*

Tolerance to MF toxins were evaluated for all the somaclonal variants selected by SAMPL markers. As a result of these in vitro assays, three somaclonal variants (VS3, VS6 and VS13) that show similar tolerance indices to MF toxins as Yangambi km5 and two highly susceptible somaclones (VS1 and VS2) were selected (Table 2).

Giménez and Colmenares (2004) reported that MF toxin extracts (0.05 µg/mL) could lead to cell permeability in just 24 h. Using Evans Blue dye, they evaluated cell membrane integrity with different toxin concentrations in reference cultivars (Williams, Pisang Mas), and reported that the tolerant cultivars (FHIA-21 and Yangambi km5) resisted up to 50 µg/mL of toxin extract, while susceptible cultivars (Williams and Pisang Mas) were affected by as little as 0.05

*Table 2. Musa* spp. relative nuclear DNA content (Rc) and tolerance to toxins and fungus.

| | Cultivar or somaclone | R | TI | Tolerance to toxins or fungus |
|---|---|---|---|---|
| 1 | Pisan Mas (AA) | 3 | 30±5 | Toxin susceptible |
| 2 | Poyo (AAA) | 3 | ND | Fungus susceptible |
| 3 | Yangambi km5 (AAA) | 3 | 0 | Toxin tolerant |
| 4 | FHIA–02 (AAAA) | 4 | ND | Fungus tolerant |
| 5 | Williams (AAA) | 3 | 17 | Toxin susceptible |
| 6 | Calcuta IV (AA) | 2 | ND | Fungus tolerant |
| 7 | *M. balbisiana* (BB) | 2 | ND | Fungus tolerant |
| 8 | VS1 | 3.5 | 19±3 | Toxin susceptible |
| 9 | VS2 | 3.9 | 14±4 | Toxin susceptible |
| 10 | VS3 | 4.3 | 2.0±1 | Toxin tolerant |
| 11 | VS6 | ND | 5.0±2 | Toxin tolerant |
| 12 | VS13 | ND | 4.5±1 | Toxin tolerant |

ND: Non determinate. Fungus tolerance for cultivars was determined by field test (Fouré, 1982; García et al., 2000). *In vitro* tolerance index (TI) 48 h after MF toxins extracts (25 µ g/mL) inoculation. This data is an average of three different tests at different times.

µg/mL. Thus, the TI for the resistant cultivars was 6 times higher than that of the susceptible cultivars. Somaclonal variant evaluations were performed using 25 µg/mL MF toxin extracts, which causes 50% cell lethality under in vitro conditions. Three somaclonal variants (VS1 to VS3) were selected by their DNA content and SAMPL markers analysis (Table 2) (Giménez and Colmenares, 2004). The tetraploid-like somaclonal variant (VS3, Table 2, Rc: 4.3) was the most tolerant to MF toxins; its TI was 8.9 times higher than its parental cultivar, Williams. Other Rcvariants (VS1 and VS2) show similar susceptibility to MF toxins as their parent cultivar, Williams. In this report, we studied the MSAP association at two levels, the resistance to fungus toxins and to black Sigatoka.

### *MSAP markers associated with tolerance to MF toxins*

We performed MSAP amplifications with 20 primer combinations on twelve DNA samples from different cultivars and somaclonal variants of Williams. With these MSAP amplifications we surveyed 11,424 CCGG sequences, of which 16.2% had methylated internal cytosines ($CC^{m}GG$) and 14% were non-methylated sites (CCGG) (Table 3).

To correlate the significance of the CCGG methylation sequences in the context of tolerance to MF toxins and field resistance to black Sigatoka, we tested all of the logical possibilities for methylation and tolerance phenotype relations as follows: We assumed that DNA methylation or absence of a promoter sequence inhibits gene expression (Bender, 2004), which implies that methylation of both cytosines or absent sequence (condition 0, Figure 1) have the same genetic implications, inhibition of gene expression or functional protein changes. The first possibility, then, is that methylation is associated with resistance (MAR), which means, in terms of the MSAP codes, that the methylation of the internal cytosine

*Table 3.* Percentage of different conditions for DNA methylation.

| Sequence | Code | N° Amplified bands | % of condition |
|---|---|---|---|
| $C^mC^mGG$ | 0 | 5,893 | 51.6 |
| CCGG | 1 | 1,679 | 14.7 |
| $CC^mGG$ | 2 | 1,846 | 16.2 |
| Mixes 1 and 2 | 3 | 2,006 | 17.6 |
| Total bands: | | 11,424 | 100 |

*Table 4.* Index for MSAP markers association with tolerance to MF toxins in *Musa* spp.

| | Primer combinations | MAR | MARcv | MARsom |
|---|---|---|---|---|
| *MSAP1* | *Msp*-1GG + *Eco*-1C | 87.5 | 80 | 100 |
| *MSAP2* | *Msp*-1GG + *Eco*-1C | 87.5 | 80 | 100 |
| *MSAP3* | *Msp*-1CC + *Eco*-1C | 73 | 86 | 71 |
| *MSAP4* | *Msp*-1CC + *Eco*-1C | 73 | 86 | 71 |
| | Primer combinations | MAS | MAScv | MASsom |
| *MSAP5* | *Msp*-1AT + *Eco*-1T | 73 | 83 | 75 |

(code 2, Figure 1), both methylated cytosines and the complete sequence absent (code 0, Figure 1) were related with resistance (Table 4).

The second and opposite hypothesis is that methylation of CCGG sequence is associated with susceptibility (MAS) (Table 4), which means that the no methylation condition (code 1, Figure 1) is correlated with resistance. An association index that quantifies the correlation to these two hypotheses (MAR or MAS) was calculated for all 11,424 bands. The association indices, MAR or MAS, correlate the methylation condition with resistance to BS or MF toxins (Table 2).

The relationship between tolerance to MF toxins or BS and the methylation condition was studied for each polymorphic MSAP band. If the phenotype matched the hypotheses (MAR or MAS), the resulting output for the specific plant adds one (+1) to the MAR or MAS index (Table 4), but if the phenotype did not match, it adds zero (0). For these analyses, the mix of methylated and non-methylated alleles (code 3, Figure 1) was not included because it is impossible to infer the exact methylation status.

We determined that four MSAP markers correlated highly with the MAR hypothesis: MSAP1 and 2 (87.5%) and MSAP3 and 4 (73%). One MSAP marker, MSAP5, (73%) was associated with the MAS hypothesis (Table 4). This MAR index indicates that 73% or 87.5% of cultivars or somaclonal variants that were tolerant to BS or MF toxins have condition 2 or 0, and the susceptible varieties present condition 1 (Figure 1). For the other condition, MAS, 73% of cultivars or somaclonal variants that were tolerant to BS or MF toxins have the condition 1, and the susceptible plants present condition 2 or 0. If this association index is calculated employing data from only cultivar (MARcv) or somaclonal variants (MARsom) exclusively, then two of the MAR markers fit 100% to this hypothesis

*Table 5.* BLAST evaluation of MSAP-4 (DQ300173) protein amino acid sequence.

| Access Nº | Description | Weight | % Identity | % Positives | E () |
|---|---|---|---|---|---|
| Q53MN2_ORYSA | Retrotransposon protein, putative, unclassified | 1058 | 40 | 64 | $5e^{-5}$ |
| Q53MU3_ORYSA | Retrotransposon protein, putative, Ty3-gypsy sub-class | 1401 | 40 | 65 | $6e^{-5}$ |
| Q8S7X4_ORYSA | Putative retroelement | 756 | 39 | 59 | $2e^{-5}$ |

*Table 6.* BLAST evaluation of MSAP-5 (DQ264397) protein amino acid sequence.

| Access Nº | Description | Weight | % Identity | % Positives | E () |
|---|---|---|---|---|---|
| Q8LJV5_MUSAC | NBS/LRR resistance-like (fragment) | 109 | 45 | 62 | $3e^{-6}$ |
| Q5ZAY2_ORYSA | Putative resistance protein RPS2 | 909 | 41 | 58 | $1e^{-5}$ |
| Q9ZTI4_MAIZE | Resistance gene analog PIC21 (fragment) | 167 | 39 | 59 | $2e^{-5}$ |
| Q7X8S5_ORYSA | OSJNBb0070J16.6 protein (OSJNBb0072M01.2 protein) | 302 | 36 | 58 | $2e^{-4}$ |
| O48972_HORVU | NBS-LRR type resistance protein (fragment) | 484 | 33 | 55 | $4e^{-4}$ |

(Table 4), indicating that the marker is methylated in all of the plants tested that are tolerant to the MF toxins, and unmethylated in susceptibles.

This is the first report demonstrating an association between $C^mG$ methylation and tolerance to MF toxins and black Sigatoka. Five MSAP markers out of 11,424 polymorphic bands show association with the expected phenotype. However, these markers demonstrate that only one $C^mG$ methylation of the complete gene sequence shows this association, and do not offer any additional information about the methylation state of the complete sequence, specially at regulatory regions. In *Musa*, how does methylation regulate the expression of this phenotype, and what genes are responsible? The answers to these questions are completely unknown, but sequence analysis of these markers could lead to new hypotheses.

### *Sequence analysis of MSAP markers*

All of the MSAP markers sequenced (Table 4) show open reading frames, although only two have significant homology to sequences in protein databases. MSAP4 (DQ300173) (Table 5) shows significant homology with the Ty3-gypsy sub-class of retrotransposons. One hypothesis could be the inactivation of the gene expression by the methylation in this retrotransposon sequence. In plants a large part of the genome is occupied by retrotransposons. However, only a small fraction of spontaneous mutations in plants has been shown to be caused by retrotransposons (Wessler et al., 1995), because most plant retrotransposons are highly methylated, and are probably not transcribed (Hirohiko et al., 2000). In plants the presence of many retrotransposons in the genome could markedly influence the transcription of flanking genes. Thus, suppression of the promoter–

enhancer activity by methylation should also be important for ensuring proper transcription of nearby genes. Suppression of transcription may be more important than suppression of transposition (Hirohiko et al., 2000).

The second mechanism of transcriptional gene silencing (TGS) reduces gene expression by suppressing transcriptional initiation, and is often correlated with cytosine methylation of promoter regions (Trevor et al., 2000). In the context of MSAP markers, this suggests that the main gene candidate could be a resistance gene. The MSAP5 (DQ264397) marker show significant homology with resistance genes of *Oriza sativa*, *Zea mays*, *Hordeum vulgare*, etc. (Table 6). Bender (2004) and Yuko (2005) report that in plants hypermethylation could inhibit gene expression. Thus, the methylation of this resistance gene analogue (MSAP5) and the inhibition of its expression could be responsible for the tolerance to MF toxins or resistance to black Sigatoka. This seems to be contradictory because the methylation of MSAP5 is associated with susceptibility. However, it is important to remember that this methylation does not indicate anything about the methylation status of the complete sequence of the candidate gene. To contrast these hypotheses it is necessary to perform expression analysis of these MSAP markers to demonstrate the association between gene expression and the resistance to MF toxins or tolerance to BS. The methylation status of the complete gene should then be determined to correlate this condition with the control of gene expression. In conclusion, the MSAP markers are useful as molecular markers associated with tolerance to MF toxins and resistance to BS.

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