

## Note

# DNA marker development by the allele-specific detection of powdery mildew resistance loci derived from Japanese domestic tobacco cultivar ‘Kokubu’

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Japanese domestic tobacco (*Nicotiana tabacum* L.) cultivar ‘Kokubu’ shows high powdery mildew resistance controlled by recessive alleles at two loci, and these alleles have been widely used as a resource for powdery mildew resistance in tobacco breeding. However, the introduction of this trait by conventional breeding takes much work because of the requirement for test crosses with the parental strains and inoculation tests using active fungi to confirm the introduction of two recessive alleles during back-crossing. Recently, we found that powdery mildew resistance in ‘Kokubu’ is caused by splice site mutations of two *MILDEW LOCUS O* genes, *NtMLO1* and *NtMLO2*. Here, we report DNA markers that detect mutations of the *NtMLO1/2* genes based on the cleaved amplified polymorphic sequence (CAPS) or allele-specific polymerase chain reaction (AS-PCR) methods. These markers can be used as co-dominant markers that detect heterozygotes of the *NtMLO* genes at the seedling stage in back-crossed progenies, and will contribute to the simplification of breeding.

**Key Words:** tobacco (*Nicotiana tabacum*), powdery mildew, cultivar ‘Kokubu’, DNA marker, CAPS, allele-specific PCR.

## Introduction

Powdery mildew is a fungal disease affecting various grains and crops, causing serious yield losses in environments with high humidity and moderate temperatures. Infected plants display white powdery spots on the leaves and stems. Powdery mildew diseases are caused by many different species of fungi in the order Erysiphales, and each fungal species infects its specific plant host. *Golovinomyces cichoracearum* var. *cichoracearum* (syn. *Erysiphe cichoracearum*) is the powdery mildew pathogen of tobacco (*Nicotiana tabacum* L.), and causes losses of yield and quality in southern Africa, southeastern Europe, the Middle East and Asia. Several genes for powdery mildew resistance have been found in various *Nicotiana* species and have been used in tobacco breeding. The wild tobacco species *N. debneyi*, *N. glutinosa*, and *N. tomentosiformis* harbor a single dominant locus for powdery mildew resistance (Davis and Nielsen 1999). The Japanese domestic tobacco cultivar ‘Kokubu’ shows powdery mildew resistance controlled by recessive alleles at two loci (Wan 1962). These loci have been used as a genetic resource for breeding powdery mildew resistance and introduced into

several tobacco cultivars in Japan (Hamamura *et al.* 1981). However, the introduction of this trait in breeding takes much work. Since the powdery mildew-resistant alleles of ‘Kokubu’ are recessive, the heterozygotes of these alleles, such as the progenies of crosses between resistant and susceptible plants, are susceptible to powdery mildew. Therefore, to determine the zygosity and isolate heterozygotes, test crosses (crossing between backcross progenies and the donor parent) and subsequent inoculation tests on the progeny population are required in addition to the ordinary backcrosses (crossing between backcross progenies and the recurrent parent). Moreover, maintenance of tobacco plants infected with powdery mildew is required to keep fresh conidiospores for the inoculation tests, because the powdery mildew fungi are obligate parasites, requiring live hosts to grow and reproduce.

Recently, the causative genes for powdery mildew resistance in ‘Kokubu’ were identified (Fujimura *et al.* 2016). This resistance is due to splice site mutations in two *MLO* genes, *NtMLO1* and *NtMLO2*. The *MLO* genes encode a plant-specific seven-transmembrane domain protein localized in the plasma membrane (Devoto *et al.* 2003). *MLO* genes have been found in various plant species and consist of gene families. Loss-of-function mutants of *MLO* alleles show powdery mildew resistance in various dicot and monocot species such as pea (Pavan *et al.* 2011), *Arabidopsis* (Consonni *et al.* 2006), tomato (Bai *et al.* 2008), cucumber (Nie *et al.* 2015), and barley (Büsches

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*et al.* 1997). Phylogenetically, MLO proteins are divided into seven clades, and all MLO proteins from dicot species that are known to be associated with powdery mildew susceptibility belong to clade V (Acevedo-Garcia *et al.* 2014). Tobacco contains 15 *MLO* genes, which are classified into six clades, and the proteins NtMLO1 and NtMLO2 are the sole MLO proteins belonging to clade V (Appiano *et al.* 2015). It is expected that discovery of the DNA sequences responsible for powdery mildew resistance in ‘Kokubu’ should pave the way to developing DNA markers useful for the breeding of resistance. In this study, we developed co-dominant DNA markers using the cleaved amplified polymorphic sequence (CAPS) and allele-specific PCR (AS-PCR) methods for the detection of powdery mildew resistance genes derived from ‘Kokubu’. These easy-to-use and reliable methods should contribute to saving both labor and materials in the breeding of powdery mildew resistance in tobacco.

## Materials and Methods

### Materials

Japanese domestic tobacco cultivar ‘Kokubu’ and flue-cured tobacco cultivar ‘Tsukuba 1’, whose *NtMLO* genes contain splice site mutations, were used as the powdery mildew-resistant cultivars. As powdery mildew-susceptible cultivars, domestic tobacco cultivar ‘Ibusuki’ and flue-cured tobacco cultivar ‘K326’ were used. ‘Kokubu’ and ‘Tsukuba 1’ were crossed with ‘Ibusuki’ and ‘K326’, respectively and the F<sub>1</sub> hybrids obtained were used as the heterozygotes of *NtMLO* genes. These F<sub>1</sub> hybrids showed susceptibility to powdery mildew because the powdery mildew resistance of ‘Kokubu’ and ‘Tsukuba 1’ is controlled by two recessive loci. Genomic DNA was extracted from green leaves using a Gentra Puregene Cell kit (QIAGEN) according to the manufacturer’s instructions. The genomic DNA obtained was used directly as a template for DNA fragment amplification using the CAPS and AS-PCR methods.

### Cleaved amplified polymorphic sequence (CAPS)

The primer pairs were designed for the amplification of DNA fragments containing polymorphic sequences of each *NtMLO* gene (Table 1). Amplification of DNA fragments with the QIAGEN Multiplex PCR Kit was performed according to the manufacturer’s instructions. PCR was carried out in 10 µl reactions that consisted of 5 ng genomic DNA, 5 µl of QIAGEN Multiplex PCR Master Mix, and 0.1 µM of each primer. After preheating at 95°C for 15 min, 40 PCR cycles (94°C for 30 sec, 60°C for 90 sec and 72°C for 60 sec) were performed. The last cycle was followed by a final 72°C incubation for 10 min. Restriction enzymes that recognize the polymorphic sequences of each *NtMLO* gene were identified using Genetyx v. 10 software (Genetyx Corporation, Tokyo, Japan). The following enzymes were selected for the digestion of the DNA: *Bfa*I (New England Biolabs) or *Bci*T130I (Takara) for *NtMLO1* fragment digestion; *Bse*RI (New England Biolabs) for *NtMLO2* fragment digestion. *Bfa*I and *Bci*T130I digest the mutant and wild-type *NtMLO1*, respectively; *Bse*RI digests the mutated *NtMLO2*. All digestion reactions were carried out according to the manufacturer’s instructions. Ten µl of reaction solution was consisted of 3 µl of PCR product and 2.5–5.0 units of appropriate restriction enzyme. After one-hour digestion at 37°C, the restriction enzyme was denatured by heating. The DNA fragments were electrophoresed by 3% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer, and the patterns of bands were confirmed by GelRed (Biotium Inc.) staining.

### Allele-specific PCR (AS-PCR)

In the AS-PCR method, PCR primer pairs were designed for the separate amplification of wild-type or mutant DNA fragments (Table 1) referring to the previous report (Liu *et al.* 2012). For the amplification of *NtMLO1* DNA fragments, the substituted dinucleotide that was observed at the 3'-end of intron 7 was placed on the 3'-end of the reverse primers, and the DNA fragments were amplified separately using the same forward primer and either wild-type-specific or mutant-specific reverse primer. Amplification of *NtMLO2* fragments were carried out using primer pairs that

**Table 1.** Sequences of primers for cleaved amplified polymorphic sequence (CAPS) and allele-specific PCR of *NtMLO* genes

Primer pairs for CAPS		
<i>NtMLO1</i>	F-primer	5'-TTCAGAATTATATTCTTCCCTCCC-3'
	R-primer	5'-TGACAGTATTGGTAAAAAGTTTCTG-3'
<i>NtMLO2</i>	F-primer	5'-CCAGAGAGGTTTCAGGTTTGAAGAG-3'
	R-primer	5'-TTTTGAACCCCTTCGTGAAGATCC-3'
Primer pairs for allele-specific PCR		
<i>NtMLO1</i>	F-primer	5'-TCAAATTTTCAGAATTATATTCTTCCCTCCC-3'
	Wild type R-primer	5'-CCTGATTCTGTGGAGTTAAATGTGCCTGG-3'
	Mutant R-primer	5'-TCTGTGGAGTTAAATGTGCTAGG-3'
<i>NtMLO2</i>	Wild type F-primer	5'-TGCTGCTCTGGATAGTCAGTC-3'
	Mutant F-primer	5'-GTGCTGCTCTGGATAGTCTC-3'
	R-primer	5'-ACCCCTTCGTGAAGATCC-3'

contained an allele-specific region at the 3'-end of the forward primer. PCR were carried out as a duplex reaction by the two primer pairs that amplify wild-type or mutant DNA fragments of both *NtMLO* genes. The reaction volume was 10  $\mu$ l including 5 ng genomic DNA, 5  $\mu$ l of QIAGEN Multiplex PCR Master Mix, and 0.1  $\mu$ M of each primer. After preheating at 95°C for 10 min, 36 PCR cycles (94°C for 30 sec, 60°C for 20 sec and 72°C for 30 sec) were performed, and followed a final 72°C incubation for 10 min. Amplified DNA fragments were separated by 0.8% agarose gel electrophoresis in 0.5  $\times$  TBE buffer and detected by GelRed staining.

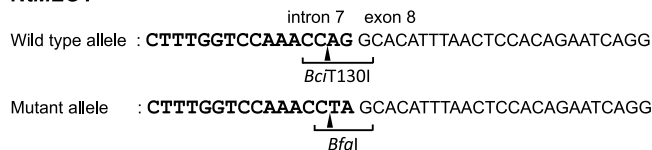
## Results

### CAPS

The CAPS method detects polymorphism caused by nucleotide changes in the restriction endonuclease recognition sites in a gene. In this method, DNA fragments containing the polymorphic sequence of a target gene are amplified and then digested with the specific restriction enzymes. After the enzyme reaction, DNA fragments are separated by electrophoresis and the genotypes are indicated by the pattern of bands. For the detection of *NtMLO1* gene polymorphism, DNA fragments of 283 bp that include the junction region of intron 7 and exon 8 of *NtMLO1* were amplified by PCR, and the fragments obtained were then digested by *Bci*T130I or *Bfa*I. *Bci*T130I recognizes the sequence 5'-CCWGG-3' and *Bfa*I recognizes the sequence 5'-CTAG-3'; these recognition sequences coincide with the sequence of the junction region. Therefore, *Bci*T130I and *Bfa*I digest the DNA fragments amplified from the wild-type (susceptible) and mutant (resistant) *NtMLO1* gene, respectively (Fig. 1). Electrophoresis results (Fig. 2) showed that the DNA fragments derived from resistant cultivars (lane 1: 'Kokubu' and lane 4: 'Tsukuba 1') were digested by *Bfa*I and produced two fragments (183 bp and 100 bp). In contrast, the DNA fragments derived from susceptible cultivars (lane 2: 'Ibusuki' and lane 5: 'K326') were not digested by *Bfa*I. *Bci*T130I recognized and digested the DNA fragments of the wild-type *NtMLO1* gene and generated two fragments (183 bp and 100 bp). The DNA fragments derived from the heterozygote, an F<sub>1</sub> hybrid of powdery mildew-resistant and susceptible cultivars (lane 3: 'Kokubu'  $\times$  'Ibusuki' and lane 6: 'Tsukuba 1'  $\times$  'K326'), showed three fragments of different lengths in electrophoresis after digestion by each restriction enzyme. These DNA fragments comprised one longer undigested fragment and two shorter digested fragments derived from wild-type and mutant genes. Thus, using the CAPS markers we developed, we were able to distinguish the wild-type, mutant, or heterozygote of *NtMLO1* gene by the electrophoretic pattern of digested DNA fragments.

For the detection of *NtMLO2* gene polymorphism, DNA fragments that included the junction region of exon 6 and intron 6 of *NtMLO2* were amplified by PCR, and the frag-

### *NtMLO1*



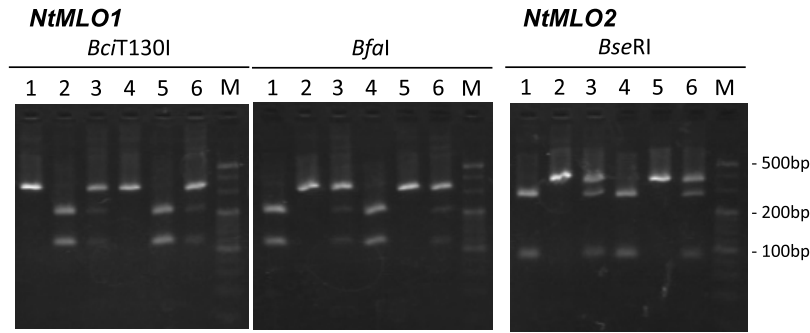
### *NtMLO2*



**Fig. 1.** Alignment of *NtMLO* genomic DNA sequences and restriction enzyme recognition sites used in the cleaved amplified polymorphic sequence (CAPS) method. The *NtMLO1* gene of powdery mildew-resistant cultivars harbors two-base transitions (AG to TA) at the 3' end of intron 7. In the *NtMLO2* gene, the fourth and fifth nucleotide (AG) of intron 6 are deleted in powdery mildew-resistant cultivars. The CAPS method detects these polymorphisms by restriction enzyme digestion patterns. *Bci*T130I and *Bfa*I recognize and digest the wild-type (powdery mildew-susceptible) and mutant (powdery mildew-resistant) *NtMLO1* genomic DNA sequence, respectively. *Bse*RI recognizes and digests the mutant (powdery mildew-resistant) *NtMLO2* genomic DNA sequence. Intron sequences are shown in bold letters. Arrow heads indicate the digestion site of each enzyme.

ments obtained were digested by *Bse*RI. *Bse*RI recognizes the sequence 5'-CTCCTC-3' and digests asymmetrically at the eight-base position upstream of the recognition site. *Bse*RI recognizes the two-base deleted sixth intron 5'-splice site sequence 5'-GTCTCCTC-3' of the mutant (resistant) *NtMLO2* gene but the same region of the wild-type (susceptible) *NtMLO2* gene (5'-GTCAGTCC-3') is not recognized by this enzyme (Fig. 1). Unfortunately, enzymes that specifically recognize the wild-type *NtMLO2* DNA sequence were not among the commercially available restriction enzymes. Electrophoresis results (Fig. 2) showed that the DNA fragments of resistant cultivars (lanes 1 and 4) were digested by *Bse*RI to give two fragments of 257 bp and 81 bp. The DNA fragments derived from susceptible cultivars (lanes 2 and 5) were not digested by *Bse*RI, and the DNA fragments derived from heterozygotes (lanes 3 and 6) showed three fragments of different sizes (340 bp, 257 bp and 81 bp) in the electrophoresis profile after digestion. Thus, a co-dominant CAPS marker detecting *NtMLO2* polymorphism was also successfully developed.

These results showed that resistance to powdery mildew is detectable by the electrophoresis profiles. The amplified DNA fragments derived from powdery mildew-resistant tobacco plants were digested by *Bfa*I (*NtMLO1*) and *Bse*RI (*NtMLO2*) and not digested by *Bci*T130I (*NtMLO1*). Detection of heterozygotes is essential for the utilization of DNA markers in backcrossing. Heterozygotes of the *NtMLO* genes are detectable by the electrophoresis profiles that show three DNA fragments in all three enzyme digestions. As all three restriction enzyme digestions were able to distinguish the DNA fragments derived from wild-type,



Genotype	Phenotype	Number of DNA fragments		
		<i>NtMLO1</i>		<i>NtMLO2</i>
		<i>BciT130I</i>	<i>BfaI</i>	<i>BseRI</i>
Mutant	Resistant	1	2	2
Heterozygote	Susceptible	3	3	3
Wild type	Susceptible	2	1	1

**Fig. 2.** Agarose gel electrophoresis profiles of *NtMLO* alleles detected using the cleaved amplified polymorphic sequence (CAPS) method. Amplified DNA fragments were digested by *BciT130I* or *BfaI* for *NtMLO1*, and *BseRI* for *NtMLO2*. *BciT130I* and *BfaI* digests the wild-type (susceptible) and mutant (resistant) *NtMLO1* fragments, respectively, and *BseRI* digests mutant (resistant) *NtMLO2* fragments. In the heterozygotes, three (digested and undigested) DNA fragments are generated by the restriction enzyme treatment. 1: ‘Tsukuba 1’ (resistant); 2: ‘K326’ (susceptible); 3: F<sub>1</sub> hybrid of ‘Tsukuba 1’ × ‘K326’ (susceptible); 4: ‘Kokubu’ (resistant); 5: ‘Ibusuki’ (susceptible); 6: F<sub>1</sub> hybrid of ‘Kokubu’ × ‘Ibusuki’ (susceptible).

mutant and heterozygote of *NtMLO1* or *NtMLO2*, these CAPS markers effectively function as co-dominant markers.

### Allele-specific PCR

Allele-specific PCR (AS-PCR) detects polymorphism caused by a single nucleotide change in genomic DNA by analyzing PCR products. In this approach, the allele-specific DNA fragments are amplified using specific primers differing in the 3'-end nucleotides. In our assay, we designed allele-specific primers that harbored different dinucleotides in each *NtMLO* gene at the three- and four-base position upstream of the 3' end (Fig. 3). In addition, the opposite primer for each *NtMLO* gene was arranged at positions that amplified DNA fragments of different sizes for *NtMLO1* (134 bp) and *NtMLO2* (260 bp). This primer pair arrangement enabled duplex PCR and detected both fragments in a single electrophoresis gel. Therefore, the genotypes of both *NtMLO* genes were distinguishable by two PCR events that detected the wild-type or mutant alleles of both genes. In powdery mildew-resistant cultivars (lane 1: ‘Kokubu’ and lane 4: ‘Tsukuba 1’ of Fig. 4), two DNA fragments of different sizes, derived from *NtMLO1* and *NtMLO2*, were amplified using mutant *NtMLO* gene-specific primer pairs, and no DNA fragment was amplified by wild-type *NtMLO* gene-specific primer pairs. In contrast, powdery mildew-susceptible cultivars (lane 2: ‘Ibusuki’ and lane 5: ‘K326’) showed the opposite results from the resistant cultivars after duplex PCR using mutant and wild-type *NtMLO* gene-specific primer pairs. In the

### *NtMLO1* reverse primer

intron 7 exon 8  
 Wild type allele : CTTTGGTCCAAACCAG GCACATTTAACTCCACAGAATCAGG  
 GGTC CGTGTA AATTGAGGTGTCTTAGTCC  
 Mutant allele : CTTTGGTCCAAACCCTA GCACATTTAACTCCACAGAATCAGG  
 GGAT CGTGTA AATTGAGGTGTCT

### *NtMLO2* forward primer

exon 6 intron 6  
 Wild type allele : GCCGGTGCTGCTCTGGATA GTCAGTCCTCTCCCCCTCTC  
 GTGCTGCTCTGGATA GTCAGTC  
 Mutant allele : GCCGGTGCTGCTCTGGATA GTC--TCCTCTCCCCCTCTC  
 GTGCTGCTCTGGATA GTC--TC

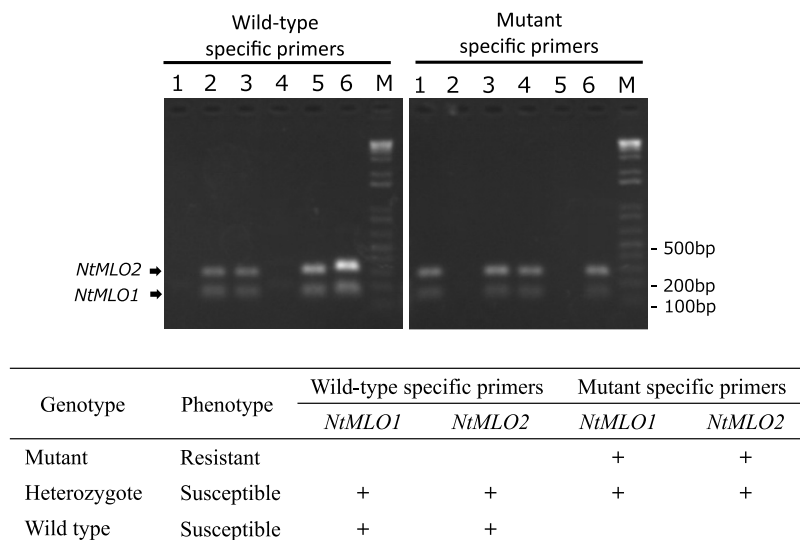
**Fig. 3.** Alignment of the genomic DNA sequences and specific primers for the detection of resistant or susceptible alleles of *NtMLO* genes by allele-specific PCR. Specific primers (shaded sequences) differing in the 3'-end nucleotides were used for the amplification of allele-specific DNA fragments. For each *NtMLO* gene, the opposite direction primers were the same sequence for the amplification of both alleles (Table 1).

heterozygotes between powdery mildew-resistant and susceptible cultivars (lane 3: ‘Kokubu’ × ‘Ibusuki’ and lane 5: ‘Tsukuba 1’ × ‘K326’), two DNA fragments of different sizes were amplified by both primer pairs. These results suggest that the duplex PCR successfully determined the genotype for both *NtMLO* genes.

## Discussion

Recessive powdery mildew-resistant loci derived from





**Fig. 4.** Allele-specific PCR electrophoresis profiles of powdery mildew-resistant and susceptible cultivars. As the size of the amplified DNA fragment differs between *NtMLO1* (134 bp) and *NtMLO2* (260 bp), the determination of powdery mildew-resistant/susceptible genotype is performed by duplex PCR with wild-type-specific and mutant-specific primers, then subsequent electrophoresis. The relation between the electrophoresis profile and genotype or phenotype is summarized in the table. Lane 1: ‘Tsukuba 1’ (resistant); 2: ‘K326’ (susceptible); 3: F<sub>1</sub> hybrid of ‘Tsukuba 1’ × ‘K326’ (susceptible); 4: ‘Kokubu’ (resistant); 5: ‘Ibusuki’ (susceptible); 6: F<sub>1</sub> hybrid of ‘Kokubu’ × ‘Ibusuki’ (susceptible).

Japanese cultivar ‘Kokubu’ have been widely used and introduced into various tobacco cultivars. However, the introduction of these loci has required considerable effort. In the traditional breeding procedure for the introduction of recessive traits, two types of crossing experiments have been performed in each backcross program, namely backcrossing with the recurrent parental cultivar for the transfer of the desired trait and test crosses with the resistant parental cultivar for the confirmation of the genotype. Then, the test-cross progenies are cultivated and the phenotype of each progeny is investigated to determine the genotype of each backcrossed plant. Theoretically, a backcrossed plant harboring two heterozygote recessive loci generates test-cross progenies showing the desired trait at a rate of one fourth. Moreover, specific examinations, such as pathogen inoculation tests, are needed for genotype determination, depending on the desired trait. Therefore, for the introduction of powdery mildew resistance by traditional breeding, special facilities such as a containment greenhouse are required to cultivate the test-crossed progenies infected artificially with powdery mildew. Thus, the spread of the disease to other tobacco plants can be prevented.

In this study, we provide two methods of detecting recessive powdery mildew-resistant loci of tobacco derived from mutations of the *NtMLO1* and *NtMLO2* genes, which originated in the Japanese domestic cultivar ‘Kokubu’. These two methods have several advantages compared with the standard procedure of recessive trait breeding: 1) test crosses for the pathogen inoculation tests are not needed; 2) genotypes can be determined in young seedlings; 3) assays can be carried out using common genetic experiment devices such as a PCR machine and electrophoresis equip-

ment. In the method described in this manuscript, we used a commercially-available genomic DNA extraction kit, but the genomic DNA extracted by easier and less expensive ways that does not affect amplification reaction might be available as the template of PCR. In addition, the small amount of genomic DNA extracted from young seedlings grown on cell culture trays is sufficient for DNA amplification in our methods. Therefore, using our DNA markers, the genotype determination and the heterozygote selection are practicable at the early growth stage of young seedlings, and the space needed for the cultivation of backcrossed plants is minimized. Both DNA markers described in this manuscript are co-dominant markers that can distinguish three genotypes, namely homozygous mutant, heterozygote and wild type; this capability is useful for breeding to introduce recessive traits.

The CAPS method is superior as the co-dominant marker detecting three genotypes by one electrophoresis operation, but this method entails digestion by restriction enzymes after DNA fragment amplification. In contrast, our AS-PCR method is able to determine the genotypes for two *NtMLO* genes by the electrophoresis profiles of duplex PCR products. Therefore, the AS-PCR method might be simpler and less expensive than the CAPS method for application in breeding programs. However, since the primers for AS-PCR method are designed based on the two-nucleotide difference between wild-type and mutant *NtMLO* genes, PCR in this method is subject to the quality of genomic DNA solution. On the other hand, amplification of *NtMLO* DNA fragments by the CAPS method is relatively stable regardless of the genomic DNA quality. Therefore, the CAPS method seems to be more reliable than the

AS-PCR method. In order to check the PCR process in the AS-PCR method, the co-amplification of known house-keeping genes designed to amplify the fragments of different size from amplified *NtMLO* DNA fragments will be effective. In either method, we recommend the application of the powdery mildew resistance and susceptible cultivar samples as the control to verify that each step is going well.

The two methods described in this paper will make it possible to breed tobacco powdery mildew resistance more speedily, easily, reliably and inexpensively.

### Author Contribution Statement

TK and HU contributed the development of AS-PCR method. SS contributed the development of CAPS method. TT and MA designed this study. MA drafted the manuscript. All authors read and approved the final manuscript.

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