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DEVELOPMENT OF A dCAPS MARKER BASED ON A MINOR RESISTANCE GENE RELATED TO HEAD SMUT IN BIN8.03 IN MAIZE

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Abstract

Head smut can cause maize yield losses of up to 80% in the maize-growing region of Northern China. Molecular markers based on candidate resistance genes have proved to be useful for highly sensitive selection of resistance in maize breeding programs. In the present study, a SNP (single-nucleotide polymorphism) in resistant and susceptible maize lines was identified within the nucleotide-binding site (*NBS*)-leucine-rich repeat (*LRR*) gene *GRMZM2G047152* in bin 8.03. Using this SNP, we developed the derived cleaved amplified polymorphic sequence marker *DNdCAPS8.03-1*. The molecular coincidence rate of the combination of markers *DNdCAPS8.03-1* and *LSdCAP2* (related to the QTL *qHS2.09*) was higher than that of a single marker in a set of 56 inbred maize lines belonging to five heterotic groups. These results provide information and tools that will prove to be useful in marker-assisted selection (MAS) in breeding maize for head smut resistance.

Key words: Maize; Head smut resistance; NBS gene family; Marker-assisted selection.

Introduction

Head smut is a destructive disease in both commercial maize (*Zea mays*) production and seed production in the maize-growing regions in Northern China (Wang *et al.*, 2012). The causal agent is the fungus *Sporisorium reilianum* (Kühn) Clint and the disease leads to significant yield losses (Bernardo *et al.*, 1992; Lu *et al.*, 1999; Yu *et al.*, 2014). Breeding for resistance to head smut is now seen as the most resource-efficient and ecologically sustainable way to manage this disease.

Resistance to head smut is quantitatively inherited and is controlled by several genes (Bernardo *et al.*, 1992). Quantitative Trait Loci (QTL) have been identified on chromosomes 1, 2, 3, 5, 6, 8, 9, and 10 (Lu *et al.*, 1999; Lübberstedt *et al.*, 1999; Chen *et al.*, 2008; Li *et al.*, 2015). Our previous study found five QTL on chromosomes 1, 2, 3, and 8 using an $F_{2:3}$ population developed from a susceptible inbred line (Huangzao4) and a resistant inbred line (Mo17). 43.7% of the phenotypic variance in this population was explained by the key QTL identified in bin 2.09, *qHS2.09* (Li *et al*., 2008). Six dCAPS markers linked closely to *qHS2.09* were reported and the efficiency of them had been evaluated in maize inbred lines (Di *et al.*, 2015). We propose that minor QTL should also be considered useful for highly sensitive selection in maize breeding programs.

Wang *et al*., (2012) revealed 18 candidate genes associated with resistance to maize head smut in a genome-wide association study (GWAS) based on 45,868 SNPs in a set of 144 inbred lines. This set of candidate genes included genes related to disease resistance, disease response, and other plant disease-related processes. The candidate gene *GRMZM2G047152* in bin 8.03 was identified as the subject of this study by synthesizing results from QTL gene functional analyses.

Our objectives of the current study include the following: (i) to establish dCAPS markers based on SNPs in the candidate gene *GRMZM2G047152*, and (ii) to assess the efficacy of new markers in maize inbred lines and a recombinant inbred line (RIL) population derived from Mo17 × Huangzaosi. The findings from this study will improve the efficiency of MAS for head smut resistance breeding in maize.

Materials and Methods

Plant materials: Huangzao4 is an elite line of Chinese inbred maize but extremely susceptible to head smut; approximately 75% of field-grown plants are susceptible to this disease (Chen *et al*., 2008). The resistant lines Mo17 and Qi319 have almost complete resistance to the fungus. The genome of inbred maize line B73 has been sequenced and serves as the reference genome for maize (https://www.maizegdb.org/). The B73 reference genome and the sequenced genomes of Mo17 and Qi319 were used to develop SNP markers for head smut resistance. To assess the efficacy of dCAPS markers for selection of head smut resistance, we analyzed 56 lines of inbred maize from five subgroups: LAN (Lancaster), SPT (derived from Si-ping-tou, a Chinese landrace), PA (derived from modern US hybrids), PB (derived from US hybrid 78599), and BSSS (derived from the Iowa Stiff Stalk Synthetic population) (Supplementary Table 1) (Di *et al*., 2015; Yong *et al*., 2017).

The inbred lines Mo17 and Huangzaosi were crossed to generate the F_1 hybrid and a random set of 191 F_2 individuals (Li *et al*., 2008). F_2 individuals were selfed and lines developed by single-seed descent were used to generate an F_9 RIL population consisting of 151 families. In 2011 and 2012, the 56 inbred maize lines and the Mo17 × Huangzaosi RIL population were grown outdoors at the experimental farm of the Northeast Agricultural University to investigate their resistance to head smut (Supplementary Table 2).

Table 1. Primers and enzymes used for dCAPS marker development.

Markers	Forward primer (5'to 3')[a]	Reverse primer (5'to 3')[a]	Types[b]	Enzyme	Recognition site	Expected product size[c]	SNPs[d]	
							Location	SNP
DNdCAPS8.03-1	AGCTCAACGCAATTCATT***g***TGC	CGCTCACTGCCAAGTTGTGC	S	*BsiHKA*I	GWGCWC	137/23,114	23	C/T
LSdSCAP2	TACATTGATATTTGCCACACGAAT	CAAATCAACGAGTGATTTAC***t***GCA	S	*Pst*I	CTGCAG	93/7320	69	T/C

a. Italicized lower case letters indicate mismatched base
b. S denotes that the PCR product from susceptible maize plants could be digested
c. Expected size of PCR product and size after digestion
d. Number corresponds to position of SNP site relative to the 5' end of the PCR product

Table 2 Analysis of consistency between molecular detection and field phenotypic identification.

Groups	Resistance level[a]	Field phenotypic identification	LSdCAP2		LSdCAP2+DNdCAPS8.03-1	
			Marker detection[b]	Consistency	Marker detection[b]	Consistency
LAN	R	13	13	100.0	13	100.0
	S	2	0	0.0	2	100.0
	Total	15	13	86.7	15	100.0
PB	R	9	9	100.0	9	100.0
	S	3	2	66.7	2	66.7
	Total	12	11	91.7	11	91.7
SPT	R	0	0	100.0	0	100.0
	S	11	10	90.9	11	100.0
	Total	11	10	90.9	11	100.0
PA	R	4	4	100.0	4	100.0
	S	8	3	37.5	4	50.0
	Total	12	7	58.3	8	66.7
BSSS	R	4	3	75.0	4	100.0
	S	2	1	50.0	2	100.0
	Total	6	4	66.7	6	100.0
All inbred lines	R	30	29	96.7	30	100.0
	S	26	16	61.5	21	80.8
	Total	56	45	80.4	51	91.1
Mo17 × Huangzaosi RIL	R	5	4	80.0	5	100.0
	S	146	128	87.7	136	93.2
	Total	151	132	87.4	141	93.4

a. R, disease incidence of inbred lines ranging from 0 to 10%; S, disease incidence of inbred lines ranging from 10.1 to 100%
b. Numbers of inbred lines whose dCAPS assay results agreed with field phenotypic identification of disease incidence

Table 3. Information for PCR amplification of the two overlapping fragments of the *GRMZM2G047152* gene.

Fragments	Primer name	Primer sequences	PCR product sizes	Annealing temperature
1	8.03-1-F: 8.03-1-R:	5' TCGGTCCTCACTGTCCTGTA 3' 5' CACAATCAAACCGAGAAACCT 3'	1625 bp	62℃
2	8.03-2-F: 8.03-2-R:	5' GCGTCGGAGTTACTATTACA 3' 5' AGTACACCACGCACCTTT 3'	1902 bp	62℃

Sequence analysis of candidate gene *GRMZM2G047152*: Sequence analysis was conducted in 2014. The candidate gene sequence *GRMZM2G047152* was obtained from the maize reference genome B73 RefGen_v2 at MaizeGDB (https://www.maizegdb.org/). Fgenesh software (www.softberry.com/berry.phtml) was used for sequence analysis, and ORF Finder (http://www. ncbi.nlm.nih.gov/ projects/gorf/) and Conserved Domain Search Service (http://www.ncbi.nlm.nih.gov/Structure/ cdd/wrpsb.cgi/) was used for prediction of conserved protein motifs. BLAST searches were run using the National Center for Biotechnology Information (NCBI) (http://www.ncbi. nlm.nih.gov/) and MaizeGDB (http:// www.maizegdb.org/) databases. Predicted protein sequences were aligned and a phylogenetic tree was built with the neighbor-joining (NJ) method and a bootstrap value of 1000 in MEGA 6.0 (http://www.megasoftware. net/).

Amplification of *GRMZM2G047152*: The uppermost two leaves were harvested from three-leaf stage maize seedlings and genomic DNA was extracted following the CTAB (cetyl trimethylammonium bromide) procedure (Murray *et al*., 1980). Primer sequences to amplify the two overlapping fragments of gene *GRMZM2G047152* were designed using Primer 5.0 software (http://simgene. com/Primer5). Primer sequences, approximate PCR product sizes, and annealing temperatures used for amplification of gene sequences appear in Table 3. PCR was conducted in 20-μL reactions comprised of 1 U Taq polymerase with 1× PCR buffer with 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 2.5 μM of each primer, and 50 ng of template DNA. PCR conditions consisted of initial denaturation for 5 min at 94°C, then 35 cycles of 45 s at 94°C, 45 s at 62°C, and 1.5 min at 72°C, and final extension was performed for 10 min at 72°C. Amplification

products were resolved on 1% agarose gels, then bands were excised, and an Agarose Gel DNA Purification Kit (Tiangen Biotechnology CO, Beijing, China) was used to extract DNA fragments from the gel. Fragments were subsequently inserted into the pGEM-T vector (pGEM-T kit, Promega CO., Beijing, China) and sequenced (Sangon Biotech Co., Shanghai, China). DNAMAN 4.0 (Lynnon Biosoft, San Ramon, CA, USA) was used to identify SNP markers by comparing DNA sequences amplified from the smut-resistant and smut-susceptible lines.

SNPs conversion to dCAPS markers and classification: dCAPs Finder (http://helix.wustl.edu/dcaps/dcaps.html) was used to determine the forward primer, the enzyme, and the recognition site in the sequences of SNPs linked to the candidate resistance gene *GRMZM2G047152*. The reverse primer in the region 140–160 bp downstream of the forward primer was then designed using Primer 5.0 software. PCR reactions were performed following the protocol described by Di *et al*., (2015). PCR cycling was as follows: 94°C for 3 min, then 34 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final 72°C for 10 min. Amplified PCR products were then subjected to an enzyme digestion in a 20 μl volume with standard methods. Restricted DNA products were then separated by using a native 8% polyacrylamide gel. Information about the dCAPS marker *LSdCAP2* associated with the QTL *qHS2.09* is shown in Table 2.

Evaluation of maize resistance to head smut in the field: Maize was artificially inoculated with head smut by covering seeds with 0.1% (w:w) *S. reiliana* teliospores mixed with soil. *S. reiliana* teliospores were collected from a maize crop of the previous season which was infected with head smut. Diseased plant materials were stored in in a ventilated dry environment in cloth bags. The experimental field was located at Harbin in Heilongjiang Province, China (45.8°N, 126.5°E) and experiments were conducted from April to September in 2011 and 2012. The field incidence of head smut was determined at crop maturity in either ears or tassels in three replicates per plot, by a method previously described by Di *et al*., (2015).

Results

Characterization of the candidate gene *GRMZM2G047152*: The 3572-bp sequence of the *GRMZM2G047152* gene was identified in bin 8.03 of B73 RefGen_v2 of the MaizeGDB database. A transcription start site (*TSS*) and a poly-A tail were identified in the 5000-bp sequences upstream and downstream of the gene using Fgenesh (Fig. 1). A complete open reading frame (ORF) of 3336 bp in *GRMZM2G047152* was predicted using ORF-Finder. It encoded a protein of approximately 124.5 kD which was composed of 1051 amino acids (Supplementary Fig. 1). The putative secondary structure of the predicted protein includes 532 amino acids in an α-helix conformation (47.88%), 329 amino acids in random coil conformation (29.61%), 167 amino acids in extended strands (15.03%), and 83 amino acids in β-turns (7.47%). CD-Search showed that the predicted GRMZM2G047152 protein has conserved NB-ARC and LRR domains (Fig. 2).

GRMZM2G047152 shows high nucleotide and amino acid homology to plant *NBS*-containing *R* genes. *GRMZM2G047152* shows 76% similarity to the *Setaria italica* resistance RPP13-like protein and its amino acid sequence was also highly similar to other R proteins (Fig. 3). Phylogenic trees were constructed to compare the GRMZM2G047152 predicted protein sequence to those from *Aegilops tauschii* (XP_020193514.1, XP_020188482.1), *Aegilops ventricosa* (AAF19148.1), *Brachypodium distachyon* (XP_003568448.1, XP_003580978.1), *Dichanthelium oligosanthes* (OEL12658.1, OEL20572.1), *Elaeis guineensis* (XP_019708115.1, XP_019704043.1), *Hordeum vulgare* (BAJ94946.1), rice (*Oryza sativa*) (XP_015692749.1, ALO70115.1, XP_015640238.1, XP_015636780.1), *Panicum hallii* (PAN19519.1, PAN26784.1), *Setaria italica* (XP_004962082.1, XP_004971089.1, KQL15471.1), *Sorghum bicolor* (XP_002439805.2), and *Triticum urartu* (EMS50526.1).

Development of the dCAPS marker *DNdCAPS8.03-1*: DNA sequences corresponding to the candidate gene *GRMZM2G047152* were amplified from the susceptible maize line Huangzao4 and resistant lines Mo17 and Qi319 by PCR using primers designed to amplify the 1625-bp and 1902-bp overlapping fragments containing the ORF.

When the candidate gene *GRMZM2G047152* sequences were aligned, a (G→A) mutation at nucleotide position 3169 that results in a frameshift mutation was revealed between susceptible Huangzao4 and resistant Mo17 and Qi319. This SNP was converted into a dCAPS marker by designing appropriate primers and screening corresponding restriction enzymes (Table 1). The DNA fragment containing the expected SNP and the sequences of the forward and reverse primers were successfully amplified and sequenced. The enzyme *BsiHKA*I could digest the PCR products from the susceptible line Huangzao4, but could not digest PCR products from the resistant lines B73, Mo17, and Qi319.

Identification of dCAPS markers in five maize subgroups: In our previous study, we showed that *LSdCAP2*, linked closely to QTL *qHS2.09*, was an efficient marker for selection of head smut resistance (Di *et al*., 2015). Fifty-six maize inbred lines from five maize subgroups could be efficiently distinguished using the dCAPS markers *DNdCAPS8.03-1* and *LSdCAP2*. Figure 4 shows the PAGE profiles of PCR products from 24 inbred lines, including the susceptible line Huangzao4 and resistant lines B73, Mo17, and Qi319. The SPT heterotic group showed the highest incidence of smut (88.4%), while the LAN, PB, and BSSS heterotic groups showed the lowest incidence of smut (0%) (Supplementary Table 1). In the Mo17 × Huangzaosi RIL group, the incidence of smut of most lines was over 10% with the exception of five lines (Supplementary Table 2). Using *LSdCAP2* and *DNdCAPS8.03-1* in combination increased the correlation between molecular detection of resistance in the lab and phenotypic identification of resistance in the field from 80.4% and 91.1% in the 56 maize inbred lines and the Mo17 × Huangzaosi RILs, respectively, to 87.4% and 93.4%, compared to selection using a single marker (Table 2). MAS using these markers was the most effective in lines with smut incidence under 10% or above 40%.

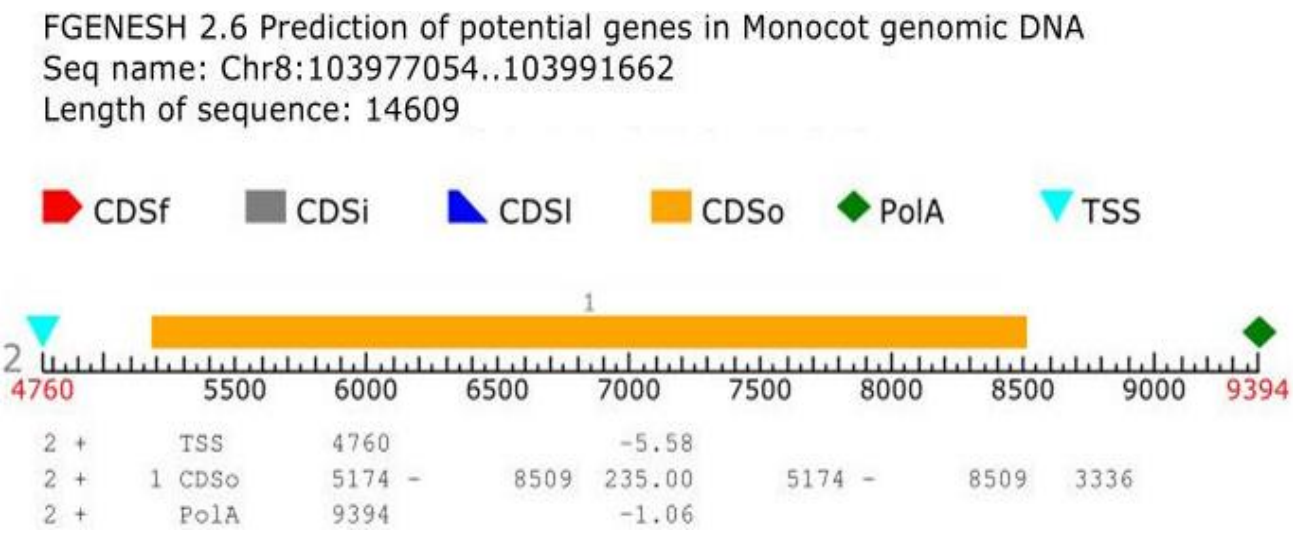

Fig. 1. Gene prediction using Fgenesh. TSS: transcription start site; CDSo: single exon; PolA: poly-A tail.

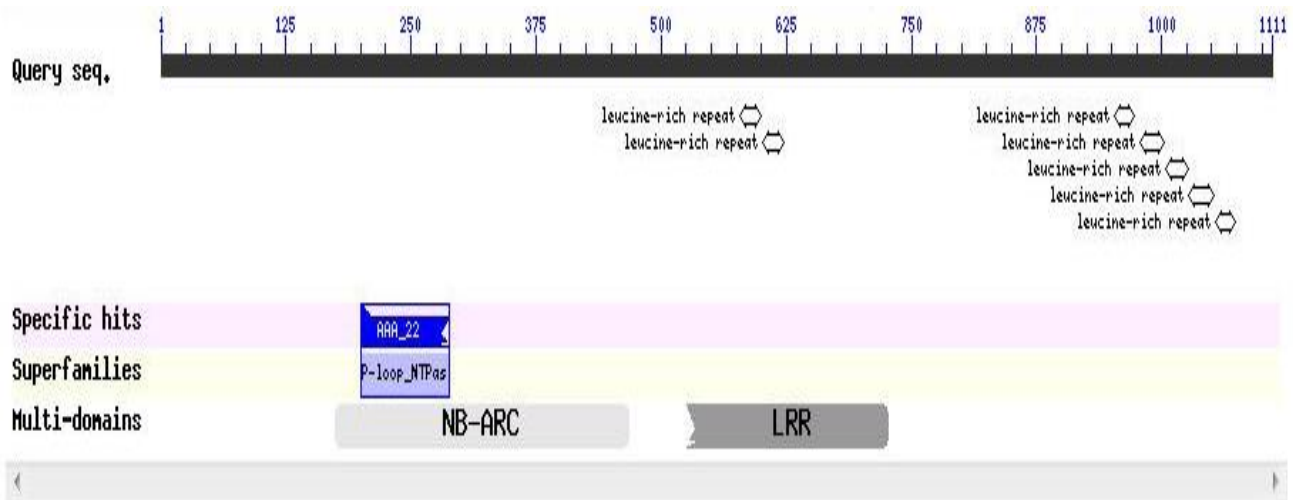

Fig. 2. Conserved domains in the predicted protein encoded by *GRMZM2G047152*.

Discussion

Breeding for resistance in maize often depends on introgression of genes or QTL from resistant donor germplasm into susceptible genotypes. When successively backcrossing to the recipient genotype, using MAS hastens the retrieval of the recipient genetic background (Michelmore, 1995; Hospital *et al*., 1997; Munir *et al*., 2020). Thus, high-efficient MAS-mediated breeding programs must be able to detect QTL or genes that control the genetic variability of complex traits (Hospital *et al*., 1997).

Several previous studies indicated that maize head smut resistance was controlled by a particular QTL in bin 2.09 from different mapping populations (Chen *et al.*, 2008; Li *et al.*, 2008). Several other types of molecular markers for head smut resistance have also previously been identified, including SSR, CAPS, STS, and SNP markers (Chen *et al*., 2008), as well as SCAR markers (Shi, 2009). Xu *et al*., (2010) established STS markers associated with the major QTL *qHSR1* in Ji1037, and Di *et al*., (2015) reported six dCAPS markers in the resistant line Mo17. The wall-associated kinase gene *ZmWAK* (Zuo *et al*., 2015, Konlasuk *et al*., 2015) and the NBS–LRR gene *ZmNL* (Di *et al*., 2018) were reported from a similar region.

Disease resistance based on several genes tends to be more stable than resistance controlled by single genes, so plant breeders try to combine the most effective resistance genes and combinations of genes to ensure durable disease resistance in crops (Palloix *et al.*, 2009). Beyond the major QTL *qHS2.09*, the QTL in bin 1.04, bin 3.04-3.05, and bin 8.02/8.03 also contribute to maize head smut resistance in different crosses and experiments (Lübberstedt *et al.*, 1999; Li *et al.*, 2008). The QTL in bin 8.02-8.03 was found to contribute to up to 6% and 7.2% of the phenotypic variance in the F3 (IF2) generation of D32×D145 crosses (Lübberstedt *et al.*, 1999) and in F2:3 populations from Mo17 and Huangzao4 (Li *et al.*, 2007), respectively. Other reports have shown that the QTL in bin 8.02-8.03 is also related to maize northern leaf blight (Chung *et al.*, 2011), maize southern leaf blight (Balint-Kurti *et al.*, 2007), and maize rough dwarf disease (Shi *et al.*, 2012). These reports indicated that one or more genes in bin8.02-8.03 were capable of the same broad-spectrum disease resistance as observed for the *R* gene.

Wang *et al*., (2012) used the Illumina MaizeSNP50 BeadChip to conduct a GWAS for the genetic control of head smut resistance and identified 18 candidate genes taking part in the complex mechanism of maize resistance to *S. reiliana*. Because so many genes and markers in bin 8.02-8.03 are associated with a variety of maize diseases, the development of markers closely linked to the candidate *R* gene *GRMZM2G047152* in bin 8.03 was the objective of the present study.

R genes, which contain a NBS (nucleotide-binding site) domain and C-terminal LRR (leucine-rich repeats) (Dangl *et al.*, 2001), confer resistance against a variety of pathogens in plants (Hammond-Kosack *et al.,* 1997; Ellis *et al*., 1998; Williamson, 1999; Meyers *et al.,* 1999). Few *R* genes have been reported in maize compared to other grains, such as rice and common wheat (Di *et al.,* 2017; Johal *et al.,* 1992). *R* genes reported in maize include *Hm1* (*Cochliobolus carbonum* race 1 resistance; Johal *et al.,* 1992), *Rp1-D* (common rust resistance; Collins *et al.,* 1999), and *Rxo1* (*Burkholderia andropogonis* resistance; Zhao *et al.,* 2005).

In the present study, the GRMZM2G047152 protein was predicted to be an *R* gene containing conserved NB-ARC and LRR domains. A dCAPS marker *DNdCAPS8.03-1* was developed from a SNP in this gene that caused a frameshift mutation between the resistant and susceptible lines. Using *DNdCAPS8.03-1* combined with *LSdCAP2* as markers related to the major QTL *qHS2.09* increases selection efficiency compared to using either marker in isolation. Further study is necessary to more precisely determine the function of *GRMZM2G047152* and the protein it encodes.

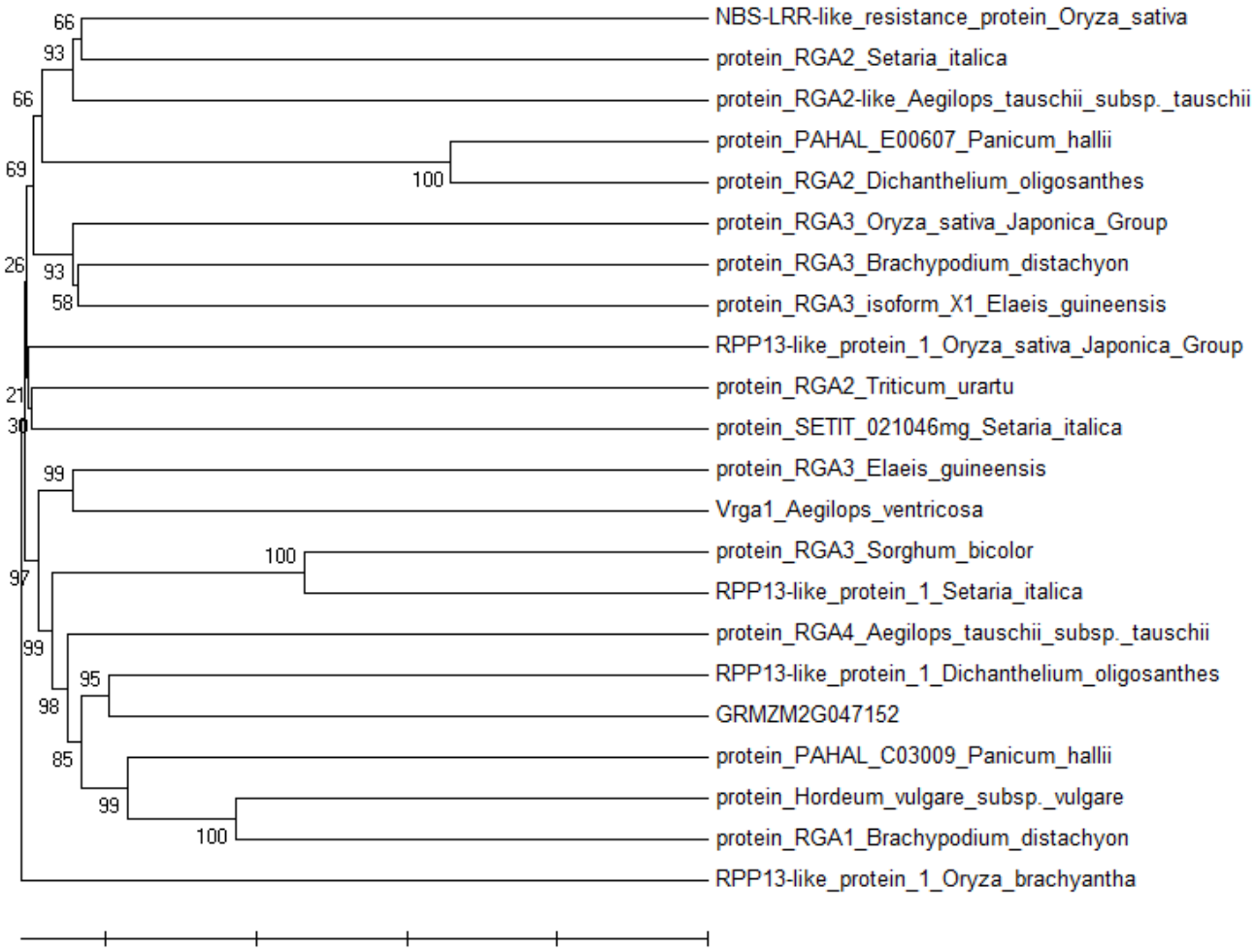

Fig. 3. Comparison of the amino acid sequence of the predicted GRMZM2G047152 protein with homologous proteins from other plants. Phylogenetic tree based on alignment of the deduced amino-acid sequences of GRMZM2G047152 and other known or putative R proteins from different plant species. The scale bar represents conversion of branch length to genetic distance between clades (0.1 = 10% genetic distance). The protein sequences used for phylogenetic analysis include: RGA4 (XP_020193514.1) from *Aegilops tauschii*, RGA2-like protein (XP_020188482.1) from *Aegilops tauschii*, Vrga1 (AAF19148.1) *Aegilops ventricosa*, RGA1 (XP_003568448.1) from *Brachypodium distachyon*, RGA3 (XP_003580978.1) from *Brachypodium distachyon*, RPP13-like protein 1 (OEL12658.1) from *Dichanthelium oligosanthes*, RGA2 (OEL20572.1) from *Dichanthelium oligosanthes*, RGA3 (XP_019708115.1) from *Elaeis guineensis*, RGA3 isoform X1 (XP_019704043.1) from *Elaeis guineensis*, predicted protein (BAJ94946.1) from *Hordeum vulgare*, RPP13-like protein 1 (XP_015692749.1) from rice (*Oryza sativa*), NBS-LRR-like resistance protein (ALO70115.1) from rice (*Oryza sativa*), RGA3 (XP_015640238.1) from rice (*Oryza sativa*), RPP13-like protein 1 (XP_015636780.1) from rice (*Oryza sativa*), PAHAL (PAN19519.1) from *Panicum hallii*, PAHAL (PAN26784.1) from *Panicum hallii*, RPP13-like protein 1 (XP_004962082.1) from *Setaria italic*, RGA2 (XP_004971089.1) from *Setaria italic*, SETIT (KQL15471.1) from *Setaria italic*, RGA3 (XP_002439805.2) from *Sorghum bicolor* (XP_002439805.2), and RGA2 (EMS50526.1) from *Triticum urartu.*

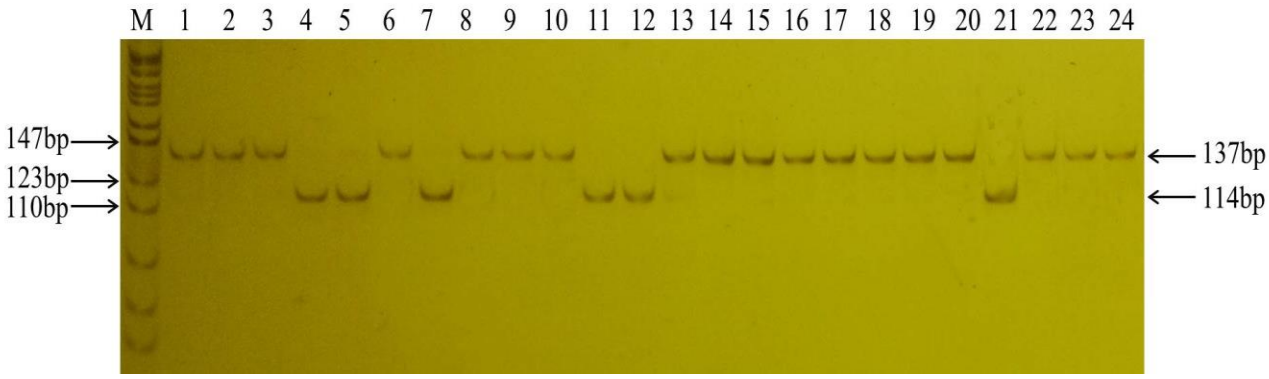

Fig. 4. Products of digestion of *DNdCAPS8.03-1* with *BsiHKA*I separated by electrophoresis on PAGE gel.
Part of 56 maize inbred lines detected by *DNdCAPS8.03-1* was shown in Figure 1. Digested and undigested products 137 bp and 114 bp in size, shown in lanes 1-24 respectively. The figure was Lane M pBR322/*Msp*I, Lane 1 B73, Lane 2. Mo17, 3. Qi319, 4. Huangzao4, 5. K10, 6. Yu12, 7. Qi318, 8. Zheng58, 9. Longkang11, 10. He344, 11. Lu2548, 12. Huangye4, 13. Ji419, 14. 501, 15. Huotanghuang, 16. K22, 17. 803, 18. Ji412, 19. Han23, 20. 4f1, 21.Shen5003, 22.Moqun17, 23. B84, 24. Hai014.

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