RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
56 (1) 18-28, 2024
http://doi.org/10.54910/sabrao2024.56.1.2
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

SEQUENCE VARIABILITY AND *IN VITRO* CLEAVAGE OF CHITINASE HOMOLOGS TOWARD CRISPR/CAS9-BASED GENOME-EDITING IN PHILIPPINE BANANAS

R.R. GARDOCE[1*], D.A. RAMIREZ[1], H.F. GALVEZ[1,2], F.M. DELA CUEVA[1], A.C. LAURENA[1,2], and C.E. REAÑO[2]

[1]Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines, Los Baños, Philippines
[2]Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines, Los Baños, Philippines
*Corresponding author's email: rrripalda@up.edu.ph
Email addresses of co-authors: dar1998@gmail.com, hfgalvez@up.edu.ph, fmdelacueva@up.edu.ph, aclaurena@up.edu.ph, cereano@up.edu.ph

SUMMARY

The technology based on CRISPR/Cas9, one of the potential solutions recognized for addressing the worsening situation of fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* 'Tropical Race 4' (TR4) plaguing the Philippine banana industry, still lacks information on its use to target host resistance in Philippine bananas. This study reports the elucidation of the gene sequence of chitinase homologs *chit6* and *chac* in the leading Philippine dessert banana varieties, 'Lakatan' and 'Latundan,' and the design, selection, and pre-validation of sgRNAs using *in vitro* cleavage assay. Multiple sequence alignment revealed the conserved sites, SNPs, and indels. Evolutionary analysis disclosed that the pattern of nucleotide substitution resulted in an overall bias in favor of adenine and thymine changing to cytosine and guanine. It said transitions outnumber transversions typical of clonally propagated crops. The *in silico* prediction initially identified a total of 58 sgRNAs for *chit6* and 68 sgRNAs for *chac*, with 58.62% and 58.97% found across 'Latundan' and 'Lakatan'/'Mapilak' backgrounds, respectively. A design criteria imposition for plants and consideration of the predicted efficiency and localization of sgRNAs along the targets narrowed down the sgRNAs. Four selected efficient sgRNAs were demonstrated to cleave all targets under *in vitro* assay with Cas9, showing its potential for gene editing by the SDN-1 or SDN-2 mechanism. With unavailable protocols for embryogenic cell suspension and corresponding delivery systems for these varieties, this *in vitro* approach provides a strategy to identify potential sgRNAs to streamline resources for the gene-editing pipeline and a guide to employing CRISPR/Cas9 for elucidation of the functional role of *chit6* and *chac* in host resistance response to TR4 in bananas.

Keywords: Banana, chitinase, CRISPR/Cas9, *in vitro* cleavage assay, *Foc* TR4, sequence variation

Communicating Editor: Dr. Gwen Iris Descalsota-Empleo

Manuscript received: August 1, 2023; Accepted: November 15, 2023.

Citation: Gardoce RR, Ramirez DA, Galvez HF, Dela Cueva FM, Laurena AC, Reaño CE (2024). Sequence variability and *in vitro* cleavage of chitinase homologs toward CRISPR/Cas9-based genome-editing in Philippine bananas. *SABRAO J. Breed. Genet.* 18-28. http://doi.org/10.54910/sabrao2024.56.1.2.

Key findings: This reports the elucidation of the full-length sequence of the chitinase gene homologs *chit6* and *chac* in the leading Philippine dessert banana varieties 'Lakatan,' its mutant derivative 'Mapilak,' and 'Latundan,' revealing conserved sites, point mutations, and their classification. The selection criteria employing *in silico* prediction resulted in designing and selecting efficient sgRNAs that exhibited cleavage of designated targets in all genomic backgrounds evaluated under *in vitro* cleavage assay, offering valuable information for prospective research harnessing CRISPR/Cas9 to manipulate *chit6* and *chac* for breeding TR4 resistance or functional studies in 'Lakatan'/'Mapilak' and 'Latundan.'

INTRODUCTION

Bananas are essential contributors to the Philippine economy, livelihood, and people's diet. For several years, the country has consistently placed second among the leading global exporters of bananas (FAO, 2022). In 2022, however, the national volume of production suffered due to the rising costs of agricultural inputs and, most importantly, with the devastation from diseases mainly by fusarium wilt, from the soil-borne fungus *Fusarium oxysporum* f. sp*. cubense* 'Tropical Race 4' (commonly referred to as *Foc* TR4 or simply TR4). Fusarium wilt is the most destructive disease of bananas that has spread in the Philippines' major banana-growing regions in Mindanao despite enforcement of quarantine regulations (Ploetz, 2015; Petersen and Luis, 2023). 'Lakatan' and 'Latundan' are the leading domestic varieties of dessert bananas under the threat of TR4 (Solpot *et al.*, 2019). Unlike the export variety 'Cavendish,' efforts to improve TR4 resistance of Philippine dessert bananas have been essentially non-existent but equally crucial to prevent the downfall of the local banana industry and prevent the spread of TR4 in unaffected plantations and its incursion to other major island groups in the Philippines.

Chitinases are among the plant's first line of defense against fungal pathogens. It hydrolyzes the β-1,4-glycosidic bonds that link the N-acetyl-D-glucosamine units of chitin, which are integral components of the fungal cell wall (Hamid *et al.*, 2013). Chitinases also perform essential physiological processes in plants (Vaghela *et al.*, 2022). Transcriptome studies have identified two chitinase genes as differentially expressed in resistant and susceptible bananas during infection of TR4 (Zhang *et al.*, 2019). The chitinase 6 (*chit6*) and the acidic endochitinase (*chac*) genes became upregulated after TR4 infection.

Precise mutagenesis or induction of desired traits with minimal disturbance in the genomic background is achievable through genome editing. The widely used method based on CRISPR/Cas9 technology in crops was able to work in bananas, particularly in deactivating the endogenous banana streak virus (BSV) in plantains (Tripathi *et al.*, 2019a) and in modifying the phytoene desaturase gene for enhancing the beta-carotene content of triploid bananas (Naim *et al.*, 2018). Establishing a protocol for CRISPR/Cas technology-based genome editing in bananas opens the possibility of developing disease-resistant and climate-smart bananas, circumventing the need for hybridization (Tripathi *et al.*, 2019b; Tripathi *et al.*, 2021). However, studies reported that editing efficiency varies across gene sequences with CRISPR technology. The nucleotide sequence of the target site affects CRISPR editing efficiency (Doench *et al.,* 2014; Liang *et al.*, 2016; Moon *et al.*, 2018). The single guide RNA (sgRNA)'s properties also play a role in successful editing (Liang *et al.*, 2016). Direct evaluation of editing success is possible after the delivery of constructs. However, the practice of pre-validation using *in vitro* methods aims at selecting highly efficient targets to streamline resources in a genome-editing pipeline. Gel-based assays have been the standard for pre-validation due to their relatively simple requirements, versatility, and robustness (O'Connell *et al.*, 2014; Bente *et al.*, 2020). Previous studies also reported assays employing electrochemiluminescence or fluorescence-based isothermal amplification to ascertain sgRNA functionality in a CRISPR/Cas9 system (Liu *et al*., 2016; Zhang *et al.*, 2016).

Identifying *chit6* and *chac* homologs as likely targets for manipulating host response to TR4 offers a starting point to work on fusarium wilt resistance in Philippine bananas. However, information on these genes in the well-liked dessert varieties 'Lakatan' and 'Latundan' are lacking, and essential protocols on using CRISPR/Cas9 in these varieties and infrastructure need establishment. This research proceeded to explore the sequence variation of chitinase gene homologs in 'Lakatan'/'Mapilak' and 'Latundan' to employ *in silico* prediction to identify optimum target sites and design efficient guide RNA for genome-editing through the SDN-1 or SDN-2 mechanism of CRISPR-Cas9 and pre-validate editing success using *in vitro* cleavage assay. The information generated by this study hopes to guide future efforts in genome-editing chitinase genes for functional studies and breeding for TR4 resistance in Philippine dessert bananas.

MATERIALS AND METHODS

Sequence verification of chitinase homologs in 'Lakatan' and 'Latundan'

The two chitinase genes identified by Zhang and colleagues (2019) based on an earlier version of the *M. acuminata* Doubled Haploid (DH) Pahang reference genome sequence pinpointed in the *M. acuminata* DH Pahang v4.3 assembly used the JBrowse and BLAST tools in the Banana Genome Hub (https://banana-genome-hub.southgreen.fr/content/m-acuminata-dh-pahang-version-2). These are the Chitinase 6 (*chit6*) and the Acidic Endochitinase (*chac*) genes in chromosomes 5 and 8, respectively. PCR amplification and capillary sequencing determined the DNA sequences in different genome backgrounds, namely, 'Lakatan' and its mutant BBTV-tolerant derivative 'Mapilak' and 'Latundan.' The 'Latundan,' 'Lakatan,' and 'Mapilak' plants maintained at the Institute of Plant Breeding - University of the Philippines Los Baños bore sampling for flag leaf. High-molecular-weight genomic DNA was isolated from fresh leaf tissue with the Doyle and Doyle (1990) protocol with minor modifications. Designing primers employed Primer 3 (https://primer3.ut.ee/) (Table 1), with the PCR conducted to generate overlapping amplification fragments for outsourced bidirectional Sanger sequencing. Raw sequences' trimming and processing had a minimum of 75% high quality (HQ) call considered for the assembly of consensus

Table 1. Primer sequence information.

Gene name and Banana Genome Hub ID	Primer name	Sequence (5' to 3')	Expected product length (bp)[a]
Chitinase 6 (Macma4_05_g18860.1)	CHIT6 1-F	GCGTTTTCCACTTTCTTCCCT	765
	CHIT6 1-R	GCGGGTGTAGAAGCTCTTG	
	CHIT6 3-F	CAGAGCTTCTTCGACGGGAT	750
	CHIT6 3-R	ATAGCACGCCACAAACACTT	
	CHIT6 4-F	GGTCCACAACACGATGCTTC	530
	CHIT6 4-R	TCCGTGTTGTTCTCATCGCA	
	CHIT6 5-F	TTCTCGCATGCAAAGACTGG	593
	CHIT6 5-R	TGCGACGAGTACTTGAAGGA	
Acidic endochitinase (Macma4_08_g29480.1)	CHAC 1-F	CCCATTTATTCCCAGCAGCC	1039
	CHAC 1-R	GAGGTAGTTGGCCCACAGAT	
	CHAC 2-F	GTACGTGATGCTGGCTTTCC	899
	CHAC 2-R	CTCTGACCACCTCAATGCCT	
	CHAC 3-F	CATCCCACCCTCCTTCCCTA	558
	CHAC 3-R	CTTCGCCAGCTCGTTGTAGT	
	CHAC 4-F	TGTCTTCATCCCAAAGCGCA	616
	CHAC 3-R	CTTCGCCAGCTCGTTGTAGT	

A corresponds to the number of base pairs of the expected amplification product based on the gene sequence in the reference genome *Musa acuminata* DH Pahang v.4.3.

sequences in the Geneious Prime version 2022.2.2 program. Multiple sequence alignment, performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), helped identify structural variations in the homologs between the *M. acuminata* DH Pahang reference, 'Latundan,' and 'Lakatan'/'Mapilak' varieties. Nucleotide substitution analysis employed the Maximum Composite Likelihood and the Tamura-Nei model in MEGA X software (Tamura *et al.*, 2004; Kumar *et al.*, 2018; Stecher *et al.*, 2020). The MEGA X also obtained transition-transversion rate ratios for purines (k_1) and pyrimidines (k_2) and the transition to transversion rate bias (R).

Design and synthesis of guide RNA

In silico prediction employment to identify suitable targets for the SDN1 mechanism prioritized the first exon or the 5' UTR of chitinase genes. Identification of target sites used selection criteria that combined the parameters of Doench and colleagues (2014) and Liang and team (2016). For assessment using the Liang *et al.* (2016) parameters, predicting the RNA structure of the candidate site with the scaffold sequence used mfold (Zuker, 2003). The obtained efficiency score for the top-ranking sgRNAs utilized the CRISPR Efficiency Predictor (https://www.flyrnai.org/evaluateCrispr/), which calculates the cumulative *p*-value for each sgRNA based on the nucleotide sequence. The Alt-R® CRISPR-Cas9 system of IDT (Integrated DNA Technologies, Inc., USA) synthesized the gRNA in the format of crRNA:tracrRNA for the assembly of CRISPR-RNP complexes (https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). The crRNA is the 20-nucleotide (nt) sequence identified by the selection criteria, and the tracrRNA is a 67-nt oligonucleotide containing the RNA scaffold sequence necessary for binding the duplex to the Cas9 endonuclease.

Cleavage template generation, *in vitro* RNP complex assembly, and DNA cleavage

Generation of cleavage templates used PCR. Purifying products comprised the GF-1 AmbiClean kit (Vivantis) and checking absorbance values (A260/A280) using a microplate spectrophotometer (Agilent, California, USA) to ascertain quality. Concentration adjustment to 30 nM consisted of the nuclease-free water. Assembling RNP complexes continued in a 30 uL reaction consisting of 1X NEBuffer r3.1, 30 nM gRNA, 30 nM Cas9 nuclease, *S. pyogenes* (NEB #M0386S), and nuclease-free water at room temperature, then, incubated at 25 °C for 10 min. A molar ratio set at 10:1 Cas9-RNP complex to template DNA aided in obtaining the best cleavage efficiency. The cleavage template's addition to the reaction (3 nM final concentration) and incubation at 37 °C for 15 min followed the manufacturer's recommendation. Resolution of cleavage proceeded by electrophoresis in 1.5% agarose gel with nucleic acid stain in 1% TBE buffer under 90 V for 40 min and viewed with the UV light.

RESULTS AND DISCUSSION

Chitinase gene sequence variation in 'Lakatan'/'Mapilak' and 'Latundan' varieties

The full-length gene sequences of the chitinase homologs *chit6* and *chac* were elucidated in the 'Latundan' and 'Lakatan'/'Mapilak' varieties. The *chit6* homolog in 'Lakatan'/'Mapilak' is of the same length as the *M. acuminata* DH Pahang reference at 902 bp, while 'Latundan' *chit6* is shorter by several bases, at 899 bp. The *chac* homologs in 'Lakatan'/'Mapilak' and 'Latundan' are 1058 bp, identical in length to the DH Pahang reference sequence. Multiple sequence alignment revealed 42 SNPs with one 3-bp deletion in *chit6* and 27 SNPs in *chac*.

These point mutations and their classification according to type are available in Tables 2 and 3.

In constructing the neighbor-joining tree for the nucleotide substitution analysis, the set partial deletion option was at 95%, eliminating sites below 95% coverage, resulting in 894 base positions considered for analysis in *chit6* and 1047 in *chac*. The pattern of nucleotide substitution recognition used the Maximum Composite Likelihood method with the Tamura-Nei model (Tamura *et al.*, 2004) for *chit6* and *chac*. For *chit6*, the nucleotide frequencies are 19.02% (A), 17.49% (T/U),

Table 2. SNPs in *chitinase 6* gene. The SNP position and type of mutation are with reference to the *M. acuminata* DH Pahang *chitinase 6* gene, here indicated as 'Reference.'

SNP position	'Latundan'	'Lakatan'/ 'Mapilak'	Reference	Type of mutation
9 to 11	---	TGG	TGG	Deletion
12	T	C	C	Transition
15	C	T	T	Transition
16	G	A	A	Transition
17	G	C	C	Transversion
18	T	A	A	Transition
20	C	A	A	Transversion
24	A	C	C	Transversion
25	C	G	G	Transversion
35	C	T	T	Transition
37	G	C	C	Transversion
162	A	G	G	Transition
211	A	G	G	Transition
217	T	C	C	Transition
251	G	A	G	Transition
271	T	C	C	Transition
277	A	Y(T/C)	C	Transition/Transversion
428	G	G	A	Transition
432	A	G	A	Transition
436	A	C	A	Transversion
451	C	C	G	Transversion
453	A	A	C	Transversion
454	G	T	G	Transversion
459	C	T	C	Transition
470	T	T	C	Transition
515	C	T	C	Transition
521	G	G	T	Transversion
528	G	G	A	Transition
539	T	C	C	Transition
545	C	C	A	Transversion
546	A	A	C	Transversion
547	A	A	G	Transition
554	G	G	A	Transition
584	T	C	C	Transition
593	G	G	A	Transition
602	G	A	G	Transition
608	C	C	G	Transversion
626	R(A/G)	A	G	Transition
702	A	G	A	Transition
792	R(A/G)	G	G	Transition
812	T	G	G	Transversion
822	W(A/T)	A	A	Transversion
823	Y(T/C)	C	C	Transition

Table 3. SNPs in *acidic endochitinase* gene. The SNP position and type of mutation are with reference to the *M. acuminata* DH Pahang *acidic endochitinase* gene, here indicated as 'Reference.'

SNP position	'Latundan'	'Lakatan'/ 'Mapilak'	Reference	Type of mutation
2	T	W(A/T)	T	Transversion
6	C	S(G/C)	C	Transversion
12	C	S(G/C)	C	Transversion
17	C	Y(C/T)	C	Transition
77	C	Y(C/T)	T	Transition
94	T	T	G	Transversion
95	T	T	G	Transversion
105	C	T	T	Transition
419	A	A	C	Transversion
429	G	S(G/C)	C	Transversion
444	C	C	T	Transition
459	C	C	T	Transition
493	G	C	C	Transversion
645	C	M(A/C)	C	Transversion
674	G	G	C	Transversion
676	G	G	A	Transition
780	A	A	G	Transition
858	A	A	C	Transversion
910	G	G	A	Transversion
984	T	T	A	Transversion
1022	T	W(A/T)	T	Transversion
1032	G	C	G	Transversion
1034	T	W(A/T)	T	Transversion
1035	G	K(G/T)	G	Transversion
1037	G	C	G	Transversion
1053	G	R(A/G)	G	Transition

34.34% (C), and 29.16% (G), and the estimated Transition/Transversion bias is R = 1.484. For *chac*, the nucleotide frequencies are 18.05% (A), 18.27% (T/U), 34.86% (C), and 28.81% (G), and the estimated Transition/Transversion bias is R = 0.668. The transition/transversion rate ratios k_1 = 3.879 (purines) and k_2 = 2.587 (pyrimidines) for *chit6,* and k_1 = 1.6 (purines) and k_2 = 1.305 (pyrimidines) for *chac*.

In both chitinase homologs, the pattern of nucleotide substitutions results in an overall bias in favor of adenine and thymine changing to cytosine and guanine (A:T → C:G). It leads to the overall higher nucleotide frequencies observed for C and G. Contrasted the wild *M. acuminata* DH Pahang, both chitinase homologs of the cultivars 'Lakatan' and 'Latundan' possess point mutations in the form of transitions and transversions. Transitions are mutations that involve purine-to-purine or pyrimidine-to-pyrimidine replacement, which maintain the ring structure in the affected base position. Transversions involve the substitution of purine with pyrimidine and vice versa. Transversions have more significant biological impact than transitions, as these directly influence DNA structure and help gene regulation. Transversion mutations in the minor groove of the DNA alter its width and roll, as well as the overall shape, which influences the binding of transcription factors (Guo *et al.*, 2017). The ratio of transition to transversion (Ti/Tv ratio) is also an indicator of the molecular divergence of sequences and the prediction of their evolution over time (Kimura, 1980; Strandberg and Salter, 2004). An excess of transition over transversion commonly occurs in spontaneous mutations (Wakely, 1997; Lusiyanto *et al*., 2021; Martanti *et al*., 2022). Asexual propagation leads to developing and accumulating somatic mutations in bananas (Mertens *et al.*, 2021; Sardos *et al.*, 2022; Ji *et al.*, 2023).

With the 'Lakatan' and 'Latundan' varieties as clonally propagated, existing point mutations in the genes examined, especially transitions, are predictable. Transitions are also less likely to result in changes in amino acid and may persist as silent mutations with no drastic effect on protein function. However, several transversions in the chitinase homologs prompt further studies to assess its potential impact on gene regulation, which can provide valuable insights into the host response of 'Lakatan' and 'Latundan' to *Foc* TR4. The elucidation of the gene sequence of *chit6* and *chac* homologs in 'Lakatan' and 'Latundan' provides prospects for further functional studies and a crucial step to identifying target gene regions for editing with CRISPR/Cas9 and other modern tools.

Designed efficient gRNAs based on selection criteria

Using the parameters by Doench *et al.* (2014), there are 58 sgRNAs for *chit6* identified in 'Latundan' (29 sgRNAs) and 'Lakatan'/'Mapilak' (29 sgRNAs), with 58.62% found in both backgrounds. For *chac*, 68 sgRNAs were detected in 'Latundan' (33 sgRNAs) and 'Lakatan'/'Mapilak' (35 sgRNAs) and had 58.97% in common. With the superimposition of the Liang *et al.* (2016) parameters, the top five sgRNAs in order of rank and priority based on the Doench *et al.* (2016) parameters were evident, with the efficiency score predicted (Table 4). The final two sgRNAs selected for synthesis depended on their presence across the genomic backgrounds sampled, an efficiency score of at least 5.00, and their proximity to the 5' terminus of the gene. For *chit6*, these were 'Latundan' *chit6 sgRNA 3* and 'Lakatan'/'Mapilak' *chit6 sgRNA 2* (5'-GGGGTGGAGGAGGAGTAGCA-3') and 'Latundan' *chit6 sgRNA 5* and 'Lakatan'/'Mapilak' *chit6 sgRNA 5* (5'-TCCATCATCCCCAGCAATGG-3'), referred to, hereon, as *chi 1s* and *chi 2s*, respectively. For *chac*, the final sgRNAs were 'Latundan' *chac sgRNA 1* and 'Lakatan'/'Mapilak' *chac sgRNA 1* (5'-CAGATACGTCGCCACGCTCG-3') and 'Latundan' *chac sgRNA 8* and 'Lakatan'/'Mapilak' *chac sgRNA 8* (5'-CCAGTACACGGCGATGCTGC-3') from here on named as *cha 1s* and *cha 2s*, respectively.

***In vitro* cleavage assay**

The *in vitro* cleavage assay with *S.p.* Cas9 validated the four sgRNAs. Ribonucleoprotein (RNP) complexes harboring single sgRNAs exhibited cleavage of the corresponding targets successfully in 'Latundan' and 'Lakatan'/'Mapilak' (Figures 1 and 2A). Cleavage of targets also manifested when employing the sgRNAs in combination (Figure 2B). The results showed that the RNPs access the target sites regardless of the sgRNAs for single incorporation or combination, and the Cas9 can execute a double-strand break accordingly. The size of the resulting cleavage products was also congruent to the expected size when the Cas9 achieved the double-strand break three bases upstream of the PAM site (Table 5). Previous studies demonstrated the utility of *in vitro* cleavage assays to pre-select efficient sgRNAs for forwarding to transformation (Mehravar *et al.*, 2019; Bente *et al.*, 2020). One should note, however, that the current selection guidelines cannot guarantee complete gene-editing success *in vivo* (Sagarbarria and Caraan, 2023). It can be apparent that a higher number of sgRNAs tested in this study may have offered a comprehensive observation of editing efficiency in the different genomic backgrounds. Ultimately, the evaluation of editing success can conclude post-transformation or after the delivery of constructs. The currently established CRISPR/Cas9-mediated genome editing protocol in bananas uses embryogenic cell suspension coupled with *Agrobacterium*-mediated transformation (Poerba *et al*., 2019; Tripathi *et al.*, 2019a, 2019b). The protocols for producing embryogenic cell suspension in 'Lakatan' and 'Latundan' and the corresponding transformation or construct delivery system still need complete establishment in the Philippines.

Table 4. Details on the top five sgRNAs that satisfied the design criteria.

Genomic Background	Gene	Target code	Target (20 bp) + *NGG* PAM + 3 bp (5'-3')	%GC	Mammalian search string[a]	Mfold prediction[b]	RNA folding parameters[c]			Score[d]			
						Initial ΔG	RAR intact	SL 2 intact	SL 3 intact	IBP (<=6)	CBP (<=7)	TBP (<=12)	
Latundan	*chit 6*	*sgRNA 1*	CTACTGCGATGAGAACAACA *CGG*AGT	50	N N W N N N N N N N N N N N C N N N A *C G G* A N N	-22.60	Yes	Yes	Yes	0	5	5	3.98071
		sgRNA 3	GGGGTGGAGGAGGAGTAGCA *CGG*TCC	69	N N D N N N N N N N N N N N H N N N A *C G G* T H H	-30.90	Yes	Yes	Yes	0	4	9	6.22464
		sgRNA 5	TCCATCATCCCCAGCAATGG *CGG*TCC	62	N N N N N N N N N N N N N N N N N N G *C G G* T H H	-25.80	Yes	Yes	Yes	5	4	6	6.30584
		sgRNA 6	CTCCCGCTTGGAGTCGTCGG *CGG*TTC	69	N N N N N N N N N N N N N N N N N N G *C G G* T H H	-27.90	Yes	Yes	Yes	4	4	7	7.08871
		sgRNA 7	AACGGCRACCTCGAGTGCGA *CGG*TAA	60	N N N N N N N N N N N N N N N N N N A *C G G* T H H	-26.50	Yes	Yes	Yes	4	5	9	6.81837
	Chac	*sgRNA 1*	CAGATACGTCGCCACGCTCG *CGG*CGT	69	N N D N N N N N N N N N N N N N N N G *C G G* C G T	-27.70	Yes	Yes	Yes	0	3	10	7.07705
		sgRNA 2	GAAGTCGATGCCGTCGAGGA *CGG*CGT	65	N N D N N N N N N N N N N N N N N N A *C G G* C G T	-25.00	Yes	Yes	Yes	0	4	9	6.46735
		sgRNA 5	CGCCACGCTCGCGGCGTCGT *CGG*AGG	81	N N N N N N N N N N N N N N N N N N T *C G G* A N N	-28.40	Yes	Yes	Yes	3	3	7	7.63038
		sgRNA 6	CCCAGCCTGGCCGCCCAAGT *CGG*AGA	73	N N N N N N N N N N N N N N N N N N T *C G G* A N N	-26.60	Yes	Yes	Yes	0	6	6	6.01142
		sgRNA 8	CCAGTACACGGCGATGCTGC *CGG*CGT	69	N N N N N N N N N N N N N N N N N N N *C G G* C G T	-29.50	Yes	Yes	Yes	0	4	8	5.30642
Lakatan/ Mapilak	*chit 6*	*sgRNA 1*	CTACTGCGACGAGAACAACA *CGG*AGT	54	N N W N N N N N N N N N N N C N N N A *C G G* A N N	-23.20	Yes	Yes	Yes	0	4	7	5.89504
		sgRNA 2	GGGGTGGAGGAGGAGTAGCA *CGG*TCC	69	N N D N N N N N N N N N N N H N N N A *C G G* T H H	-30.90	Yes	Yes	Yes	0	4	9	6.22464
		sgRNA 5	TCCATCATCCCCAGCAATGG *CGG*TAC	58	N N N N N N N N N N N N N N N N N N G *C G G* T H H	-25.80	Yes	Yes	Yes	3	4	6	6.30584
		sgRNA 6	CTCGCGCTTGGAGTCGTCGG *CGG*TTC	69	N N N N N N N N N N N N N N N N N N G *C G G* T H H	-27.50	Yes	Yes	Yes	0	4	7	7.32254
		sgRNA 7	AACGGCGACCTCGAGTGCGA *CGG*TAA	62	N N N N N N N N N N N N N N N N N N A *C G G* T H H	-27.90	Yes	Yes	Yes	3	5	10	7.43509
	Chac	*sgRNA 1*	CAGATACGTCGCCACGCTCG *CGG*CGT	69	N N D N N N N N N N N N N N N N N N G *C G G* C G T	-27.70	Yes	Yes	Yes	0	3	11	7.07705
		sgRNA 2	GAAGTCGATGCCGTCGAGGA *CGG*CGT	65	N N D N N N N N N N N N N N N N N N A *C G G* C G T	-25.00	Yes	Yes	Yes	0	4	9	6.46735
		sgRNA 5	CGCCACGCTCGCGGCGTCGT *CGG*AGG	81	N N N N N N N N N N N N N N N N N N T *C G G* A N N	-28.20	Yes	Yes	Yes	3	3	6	7.63038
		sgRNA 6	CCCAGCCTGGCCGCCCAAGT *CGG*AGA	73	N N N N N N N N N N N N N N N N N N T *C G G* A N N	-26.60	Yes	Yes	Yes	0	6	7	6.01142
		sgRNA 8	CCAGTACACGGCGATGCTGC *CGG*CGT	69	N N N N N N N N N N N N N N N N N N N *C G G* C G T	-29.50	Yes	Yes	Yes	0	4	8	5.30642

a Sequence of 20-bp target, PAM and immediate 3-bp based on the parameters of Doench *et al.* (2014)

b Gibbs free energy of RNA structures predicted using mfold program by Zuker (2003)

c Liang *et al.*, 2016; IBP = internal base pairs; CBP = consecutive base pairs; TBP = total base pairs

d Predicted efficiency score reflecting cumulative p-value, higher values are better (DRSC/TRiP Functional Genomics Resources, https://www.flyrnai.org/evaluateCrispr/)

Table 5. Expected sizes of cleavage templates generated by PCR and cleavage products from *in vitro* assay.

Genomic Background	sgRNA Code	PCR Primer	Cleavage Template Length (bp)		Cleavage Product Length (bp)
'Latundan'	*chi 1s*	CHIT6 1F/4R	1,011	644	367
	chi 2s	CHIT6 1F/4R	1,011	480	531
	cha 1s	CHAC 4F/2R	1,172	481	691
	cha 2s	CHAC 4F/2R	1,172	202	970
'Lakatan'/ 'Mapilak'	*chi 1s*	CHIT6 1F/4R	1,012	645	367
	chi 2s	CHIT6 1F/4R	1,012	478	534
	cha 1s	CHAC 3F/2R	1,171	481	690
	cha 2s	CHAC 3F/2R	1,171	202	969

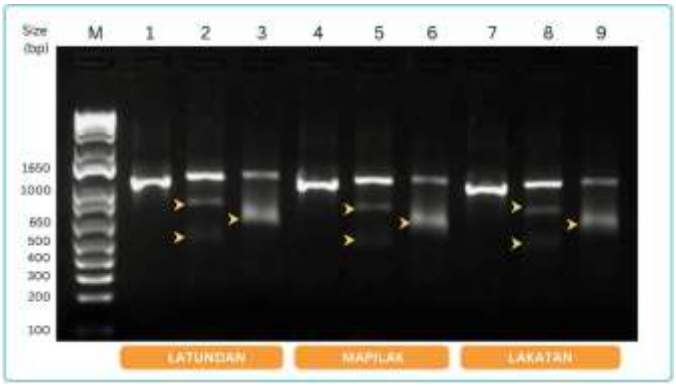

Figure 1. *In vitro* cleavage assay with RNPs harboring single sgRNA. Resolving cleavage products ran in 1.5% agarose gel in 1X TBE buffer. The reflected genomic background of the cleavage template comprised orange-colored labeled boxes corresponding to the target gene *chit6*. The molecular weight marker used is 1Kb plus DNA ladder. Yellow arrows indicate the cleavage products. The uncut/control cleavage template (Lanes 1, 4, and 7) precede cleavage products (Lanes 2, 3, 5, 6, 8, and 9).

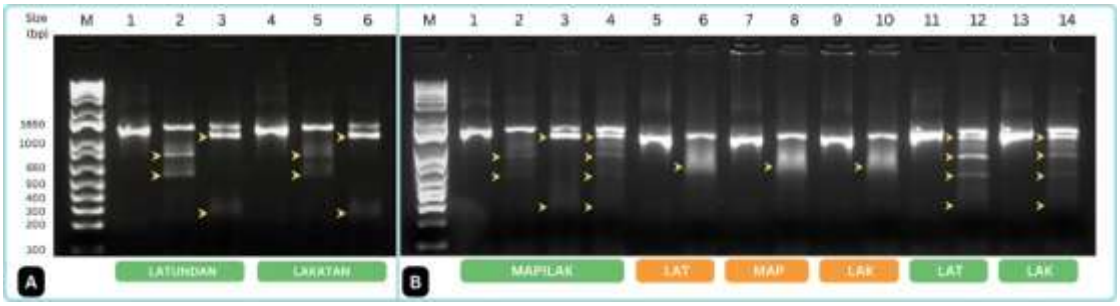

Figure 2. *In vitro* cleavage assay with RNPs harboring sgRNAs incorporated singly and in combination. Cleavage products from *in vitro* assay with single sgRNA *cha 1s* and *cha s* (A - Lanes 2, 3, 5, and 6, and B - Lanes 2 and 3) preceded by uncut/control cleavage template (A - Lanes 1 and 4, and B - Lane 1). Cleavage with sgRNAs employed in combination: *chi 1s/chi 2s* (B - Lanes 6, 8, and 10) preceded by uncut/control cleavage template (B - Lanes 5, 7, and 9); and *cha 1s/cha 2s* (B - Lanes 4, 12, and 14) preceded by uncut/control cleavage template (B - Lanes 1, 11, and 13). Cleavage products' resolving used 1.5% agarose gel in 1X TBE buffer. The genomic background of the cleavage template, reflected in labeled boxes, had colors corresponding to the target gene specifically orange for *chit6* and green for *chac*. The molecular weight marker used is 1Kb plus DNA ladder. Yellow arrows indicate the cleavage products.

CONCLUSIONS

The identification of the *chit6* and *chac* gene sequences in the chief Philippine dessert banana varieties 'Latundan' and 'Lakatan'/'Mapilak' provides valuable information for the design and validation of efficient sgRNAs that will guide future works that employ CRISPR/Cas9 technology to elucidate the functional role of these chitinase homologs in the host resistance response to *Foc* TR4. The sgRNAs validated by the *in vitro* cleavage assay can benefit gene-editing *chit6* and *chac* by the SDN-1 or SDN-2 mechanism of CRISPR/Cas9. The successful cleavage of the targets across all genomic backgrounds sampled demonstrates the viability of the method described to design, pre-select, and validate sgRNAs before gene editing with CRISPR/Cas9.

ACKNOWLEDGMENTS

This research was part of the Doctoral Dissertation of the chief author conducted with funding support from the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) University Consortium (UC) Grant for Research Activities and a dissertation grant from the Philippine Department of Science and Technology - Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD). The authors acknowledge the Institute of Plant Breeding for facilities and equipment use, the UPLB Graduate School, and Darlon V. Lantican for technical advice.

REFERENCES

Bente H, Mittelsten Scheid O, Donà M (2020). Versatile *in vitro* assay to recognize Cas9-induced mutations. *Plant Direct* 4(9): e00269.

Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE (2014). Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation. *Nat. Biotechnol* 32(12): 1262-1267.

FAO (2022). Banana Market Review – Preliminary results, 2022. Rome.

Guo C, McDowell IC, Nodzenski M, Scholtens DM, Allen AS, Lowe WL, Reddy TE (2017). Transversions have larger regulatory effects than transitions. *BMC Genom.* 18: 1-9.

Hamid R, Khan MA, Ahmad M, Ahmad MM, Abdin MZ, Musarrat J, Javed S (2013). Chitinases: An update. *J Pharm Bioallied Sci* 5(1): 21.

Ji Y, Chen X, Lin S, Traw MB, Tian D, Yang S, Wang L, Huang J (2023). High level of somatic mutations detected in a diploid banana wild relative *Musa basjoo*. *Mol. Genet. Genom.* 298(1): 67-77.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution* 16: 111-120.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* 35:1547-1549.

Liang G, Zhang H, Lou D, Yu D (2016). Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. *Sci. Rep.* 6(1): 1-8.

Liu W, Yu H, Zhou X, Xing D (2016). *In vitro* evaluation of CRISPR/Cas9 function by an electrochemiluminescent assay. *Anal. Chem.*, 88(17): 8369-8374.

Lusiyanto, Nurhasanah, Sunaryo W (2021). *In vitro* regeneration of banana genotypes possessing distinct genomes by using male flower explants. *SABRAO J. Breed. Genet.* 53(2): 322-333.

Martanti D, Poerba YS, Witjaksono, Herlina, Ahmad F (2022). Male flower characteristics of induced tetraploid, mixoploid, and diploid banana *Musa acuminata* (AA) cv. Pisang Rejang. *SABRAO J. Breed. Genet*. 54(3): 617-626. http://doi.org/10.54910/sabrao2022.54.3.14

Mehravar M, Shirazi A, Mehrazar MM, Nazari M (2019). *In vitro* pre-validation of gene editing by CRISPR/Cas9 ribonucleoprotein. *AJMB*, 11(3): 259-263.

Moon SB, Lee JM, Kang JG, Lee NE, Ha DI, Kim SH, Yoo K, Kim D, Ko JH, Kim YS (2018). Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3'-overhang. *Nat. Commun.* 9(1): 1-11.

Naim F, Dugdale B, Kleidon J, Brinin A, Shand K, Waterhouse P, Dale J (2018). Gene editing the phytoene desaturase alleles of Cavendish banana using CRISPR/Cas9. *Transgenic Res.* 27(5): 451-460.

O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516(7530): 263-266.

Petersen E, Luis J (2023). Impact assessment of integrated management of Fusarium wilt of bananas in the Philippines and Australia. ACIAR Technical Report No. 105, Australian Centre for International Agricultural Research, Canberra.

Ploetz RC (2015). Fusarium wilt of banana. *Phytopathology* 105, 1512-1521.

Poerba YS, Martanti D, Handayani T, Witjaksono (2019). Morphology and reproductive function of induced autotetraploid banana by chromosome doubling. *SABRAO J. Breed. Genet.* 51(2): 175-190.

Sagarbarria MGS, Caraan JAM (2023). Usefulness of current sgRNA design guidelines and *in vitro* cleavage assays for plant CRISPR/Cas genome editing: A case in eggplant (*Solanum melongena* L.). *bioRxiv* 2023.03.19.532877.

Sardos J, Breton C, Perrier X, Van den Houwe I, Carpentier S, Paofa J, Rouard M, Roux N (2022). Hybridization, missing wild ancestors and the domestication of cultivated diploid bananas. *Front. Plant Sci.* 13: 969220.

Solpot T, Cumagun CJ (2019). Control of *Fusarium oxysporum* f. sp. *cubense* (EF Sm.) Snyder and Hansen tropical race 4 causing Fusarium wilt in banana cv. "Lakatan". *JARDET 1*(1): 21-30.

Stecher G, Tamura K, Kumar S (2020). Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Mol. Biol. Evol.* 37(4): 1237-1239.

Strandberg AK, Salter LA (2004). A comparison of methods for estimating the transition: Transversion ratio from DNA sequences. *Mol. Phylogenet. Evol.* 32(2): 495-503.

Tamura K, Nei M, Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS* 101:11030-11035.

Tripathi JN, Ntui VO, Ron M, Muiruri SK, Britt A, Tripathi L (2019a). CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of *Musa* spp. overcomes a major challenge in banana breeding. *Commun. Biol.* 2(1): 1-11.

Tripathi L, Ntui VO, Tripathi JN (2019b). Application of genetic modification and genome editing for developing climate-smart banana. *Food Energy Secur.* 8(4): e00168.

Tripathi L, Ntui VO, Tripathi JN, Kumar PL (2021). Application of CRISPR/Cas for diagnosis and management of viral diseases of banana. *Front Microbiol*, *11*, p.609784.

Vaghela B, Vashi R, Rajput K, Joshi R (2022). Plant chitinases and their role in plant defense: A comprehensive review. *Enzyme Microb. Technol* 159:110055.

Wakeley J (1996). The excess of transitions among nucleotide substitutions: New methods of estimating transition bias underscore its significance. *Trends Ecol. Evol.* 11: 158-162.

Zhang K, Deng R, Li Y, Zhang L, Li J (2016). Cas9 cleavage assay for pre-screening of sgRNAs using nicking triggered isothermal amplification.

Zhang L, Cenci A, Rouard M, Zhang D, Wang Y, Tang W, Zheng SJ (2019). Transcriptomic analysis of resistant and susceptible banana corms in response to infection by *Fusarium oxysporum* f. sp. *cubense* tropical race 4. *Sci. Rep*. 9(1): 8199.

Zuker M (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31(13): 3406-3415.