Title: Engineering bacterial blight-resistant plants through CRISPR/Cas9-targeted editing of the *MeSWEET10a* promoter in cassava

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Abstract:

Editing of the promoter in the disease-susceptibility gene *MeSWEET10a* of SC8 cassava confers resistance to cassava bacterial blight (CBB). All mutated cassava lines had normal morphological and yield-related traits as the wild type. The results lay a research foundation for breeding cassava resistant to bacterial blight.

Dear Editor,

Cassava (*Manihot esculenta* Crantz) is a major food crop in tropical and subtropical regions that provides food security for 800 million people. However, cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) results in severe yield losses and is the most destructive bacterial disease in all worldwide cassava planting regions (McCallum et al., 2017). The vast majority of cassava cultivars are not resistant to CBB. *Xam* pathogen infection secretes transcription activator-like effectors (TALEs) into cassava host cells through the type III secretion system (Boch and Bonas, 2010). The repeat variable diresidues (RVDs) in TALEs recognize the effector-binding elements (EBEs) on the host susceptibility (S) gene promoters and activate S gene expression which facilitates bacterial growth and disease symptom formation.

Inhibition of S gene expression by genetic engineering is an attractive strategy to improve plant disease resistance (Zaidi et al., 2018). *Mildew resistance locus O* (*Mlo*) is one of the best-known S genes and encodes a conserved membrane-associated protein. *Mlo* is required for the penetration of powdery mildew (PM) fungi into host epidermal cells. Mutation of *Mlo* through CRISPR-Cas9 has conferred PM resistance in wheat (Wang et al., 2014). In addition, mutations of other S genes such as *eIF4E* and *MPK* enhances disease resistance (Gomez et al., 2019; Xie and Yang, 2013). However, these mutations often affect plant fitness with reduced growth, fertility, yield, and abiotic stress tolerance (Zaidi et al., 2018). Genetic engineering of the core S gene promoter elements recognized by pathogenic TALEs improves disease resistance without affecting plant characteristics in citrus and rice (Peng et al., 2017; Xu et al., 2019). *CsLOB1* is a citrus canker S gene and plays a key role in promoting pathogen growth and blistering pustule formation. Deletion of the *EBE_{PthA4}* sequence from the *CsLOB1* gene promoter by CRISPR/Cas9 has conferred high resistance to citrus canker (Peng et al., 2017). Sugars will eventually be exported transporters (*SWEET*) genes are the other best-known S genes encoding sugar transporters. Some

SWEET genes can be hijacked by pathogenic bacteria to induce sucrose transport from the interior of plant cells to the apoplasts and provide carbon sources for the bacteria. Disruption of EBEs in promoters of three major target rice *SWEET* genes by CRISPR-Cas9 avoids recognition by TALEs from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and improves bacterial blight resistance (Xu et al., 2019).

Generally, *Xam* contains 1-5 TALEs which determine its virulence (Bart et al., 2012). TALE20 contributes toward most of the aggressiveness and virulence of *Xam* in cassava (Cohn et al., 2014) by targeting a *SWEET* gene. *MeSWEET10a* expression rapidly increases after infection with *Xam*_{TALE20} (*Xam* containing TALE20) which induces sucrose translocation to the infection site and facilitates the multiplication and spread of *Xam* in susceptible cassava varieties, but not in resistant varieties (Mora et al., 2019). *Xam668* is a highly virulent *Xam* strain to cassava which contains five TALEs including TALE20 (Bart et al., 2012). TALE20 disruption in *Xam668* (*Xam668*Δ*TAL20*) reduces cassava water-soaking symptom development, while reintroducing TALE20 into the plasmid-cured strain fully complemented the symptom defect phenotype (Cohn et al., 2014). TALE20_{*Xam668*} has 20 RVDs, and directly targets the EBE in the *MeSWEET10a* promoter (Cohn et al., 2014). Thus, editing the EBE in the *MeSWEET10a* promoter may potentially confer resistance to CBB in cassava (Lin et al., 2019).

Xam11 is a highly pathogenic subspecies from Hainan, China that infects the cassava SC8 cultivar with marked pathogenic symptoms (Figure S1). In this study, the effect of genetically modifying the EBE in the *MeSWEET10a* promoter on CBB resistance in cassava after *Xam11* infection was investigated. TALE20 was cloned based on the genome sequencing information of *Xam11*. TALE20_{*Xam11*} (TALE20 in *Xam11*) has 20 RVD with variable amino acids at positions 12 and 13 in each repeat (Figure S2). The promoter sequence of *MeSWEET10a* in SC8 cassava was cloned, which is identical to the reported sequence in Mora et al. (2019). EBE_{TALE20} is targeted by TALE20_{*Xam11*} and is located in the -92 to -112 bp region before the

MeSWEET10a start codon (Figure S3). The dual luciferase reporter assay demonstrated that TALE20_{Xam11} activated the *MeSWEET10a* promoter and initiated downstream expression of firefly luciferase; however, TALE20_{Xam11} could not activate the *MeSWEET10a* promoter lacking the EBE_{TALE20} region (Figure 1A).

CRISPR-P v2.0 was used to design the sgRNA (TGGGCGAGAAGCGTTTATATAGG) targeting EBE_{TALE20} based on the sequence characteristics of the *MeSWEET10a* promoter in SC8 cassava. The pCAMBIA1301-Cas9-EBE-sgRNA plasmid was constructed and transformed into the SC8 cassava genome by *Agrobacterium*-mediated fragile embryonic callus (EFC) transformation. In total, 57 independent transgenic lines were regenerated after hygromycin (20 µg/mL) selection and confirmation by PCR analysis for the *Cas9* gene (Figure S4). The EBE_{TALE20} regions from all the transgenic lines subjected to Sanger sequencing and high throughput tracking of mutation (Hi-TOM) sequencing (Figure S5, Table S1) showed that Cas9 activity efficiency was 98.2%. Three types of mutations were obtained among the transgenic lines: homozygous mono-allelic (19.30%), homozygous bi-allelic (61.40%), and heterozygous (17.54%) (Table S1). The EBE_{TALE20} regions in the four mutant types of transgenic cassava (#1, #2, #3, #4) and wild type SC8 cassava (WT) were shown in Figure 1B. The effects of EBE_{TALE20} modification on *MeSWEET10a* expression in the mutant cassava lines were investigated after inoculation with *Xam11*. The relative expression level of *MeSWEET10a* in mutant lines showed no obvious difference from that of the wild type at 0 and 0.5 day post-inoculation (dpi); however, markedly lower levels were observed in mutant lines compared with that of the wild type at 1–7 dpi (Figure 1C). This showed that EBE_{TALE20} mutations repressed *MeSWEET10a* expression after *Xam11* infection. Wild type had obvious water-soaked symptoms at 3 dpi which were not observed in mutant lines (#1, #2, #3, #4). At 7 dpi, the wild type had obvious pustule symptoms while the mutant lines showed slightly water-soaked symptoms; at 14 dpi, the wild type injection sites were withered and turned yellow, while the

mutant injection sites started to wither and the plaque expanded slowly; at 40 dpi, whole leaves of the wild type were withered and become yellow whereas the leaves of the mutant lines only withered near the plaques (Figure 1D). Diseased lesions in mutant lines were significantly smaller at 3 dpi, 7 dpi, and 14 dpi (Figure 1E) suggesting that EBE_{TALE20} mutations limited the expansion of CBB symptoms in cassava. The bacterial growth assay showed that *Xam11* populations in the four mutant lines were significantly smaller than that in the wild type at 3 dpi and 7 dpi (Figure 1F). Callose is deposited on the cassava cell wall during the early stages of *Xam* pathogen attack which restricts pathogen growth (Wei et al., 2021). The leaves of the mutant cassava lines displayed significantly more callose depositions at 3 dpi than that in the wild type (Figure 1G). These results revealed that EBE_{TALE20} modification of *MeSWEET10a* in the four edited cassava lines had strong and stable resistance to CBB.

There were no significant differences in tuber root traits and starch content between the EBE_{TALE20} mutated cassava lines and the wild type plants when grown under normal conditions (Figure 1H and 1I). This indicated that the EBE_{TALE20} mutation in the *MeSWEET10a* promoter has no effect on yield-related traits in cassava.

In conclusion, editing of the promoter in the disease-susceptibility gene *MeSWEET10a* of SC8 cassava confers resistance to CBB. All mutated cassava lines had normal morphological and yield-related traits as the wild type. The results lay a research foundation for breeding cassava resistant to bacterial blight.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant* Online.

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AUTHOR CONTRIBUTIONS

Y.Y., J.G., and Y.C. designed the experiments. Y.W., M.G., R.P., X.Z., R.L., and J.L. performed the experiments. T.Z. and Y.C. conducted the cassava transformation. Y.Y., Y.W., M.G., and R.P. analyzed the data and wrote the manuscript. J.G. and Y.C. revised the manuscript. All authors read and approved the final manuscript.

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The authors declare no conflict of interest.

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200

201 **Figures**

202
203 **Figure 1. Engineering CBB resistant plants through CRISPR/Cas9-targeted**
204 **editing of the *MeSWEET10a* promoter in cassava.** (A) TALE20_{Xam11} directly
205 activates *MeSWEET10a* expression through the EBE region. *MeSWEET10a*
206 promoter-luciferase fusion and 35S-driven TALE20_{Xam11} constructs were delivered
207 into *Nicotiana benthamiana* cells through infection by *Agrobacterium tumefaciens*.
208 Luciferase activity was quantified after 1 dpi. SK: pGreen II62-SK vector (control
209 vector); TAL20: pGreen II62-SK-TALE20_{Xam11} vector; pSWEET10a: pGreen
210 II0800-pMeSWEET10a-LUC vector; pSWEET10a-EBE: pGreen
211 II0800-pMeSWEET10a-EBE_{TAL20}-LUC vector (deletion of EBE_{TAL20} from the
212 *MeSWEET10a* promoter). Data are displayed as mean technical replicates ± standard
213 deviation. Experiments were repeated three times with similar results (Student's t-test,
214 ***P < 0.005). (B) Hi-TOM sequencing of the EBE_{TAL20} regions of wild type (WT)
215 non-transgenic SC8 cassava compared with the engineered lines #1, #2, #3, and #4.
216 The EBE_{TAL20} region of the TALE20_{Xam11} target is marked in a red box in WT; the
217 sgRNA sequences are underlined; the protospacer adjacent motif (PAM) sites are

marked in blue text; the hyphens are missing bases, and the red letter is the inserted base. **(C)** Time course of *MeSWEET10a* expression in mutant lines and WT after *Xam11* inoculation. *MeSWEET10a* transcript levels in leaves determined by qPCR at 0, 0.5, 1, 3, and 7 dpi. Three biological repeats were performed for each data point (Student's t-test, *** $P < 0.005$). **(D)** Symptom development after *Xam11* inoculation. Scale bar, 5 mm. **(E)** and **(F)** Time course of leaf disease areas and bacterial numbers after *Xam11* inoculation. At least 10 leaves were harvested for the diseased areas with the bacterial number assay performed using three biological replicates (Student's t-test, * $P < 0.05$, *** $P < 0.005$). **(G)** Callose depositions in leaves. Scale bar, 50 μm . **(H)** Tuber root phenotypic characterizations. Scale bar, 1 cm. **(I)** Yield-related traits shown as the mean $\pm$ SD from five plants of each line. P values were calculated using Student's t-test. The values marked with the same letter (a) represented no significant difference.

Supplemental Figures

Figure S1. *Xam11* infects cassava cultivar SC8 with marked pathogenic symptoms. Red arrows indicate the region affected in the infected cassava.

Figure S2. Analysis of TALE20 protein sequence from *Xam11*. TALE20_{*Xam11*} has 20 RVD with variable amino acids at positions 12 and 13 in each repeat (highlighted in red).

AACTTTTAGAATGAGCCCTTGAAATGAAACCTCTAAGCTTGTAGGTTCTAATAGGGT
TACTTAATTAAATGATAGACCTAACCCTTATAATTTGATTATGACTTTTTATAATAG
AGATGAACGAAACCACAAAATTCTCCAGTTACCCAATAAACGTGGGAATGAGAGTGT
GTGGTTAGGAATCCATGTGCATGGCAAGAGAGAAAATCACAGGACTTTCTTTCAAAT
TAATTCTTGGTTAATCATGATTACATATTAATCACCTTGTCTTTTACGTGCAATCTT
CAAGTTTTTCAACCATTATTAATTACGTTGTGGACGTGGACAACCTTGAAGAAATCG
TAAAGGGCCGTCAACAAGCATTCAACAAGAGCTGTTATCAGCGAGGGGGAATGAGACA
ACGGCGGCTGATGTGTTATGGTGGTAAACTGGGAAATACTAGGCACGGTTTCTTGTA
TGGGAAAAACAGCATATCCTCTGCCATGATGCTGAGCTCGTTTTCTCCACCGTTTGAT
TGAATGTTCTGATTTGTTTCATCATCATCAAATGCGATTACACATCAGAAAGCTTGT
TGATGGGATTCTGTAACAAATGAAGTAAGCTTCAAAGCCTTTTGGATCCCTACTATG
GGAATGATCCAAGTGGGTAACTACTATTAGTTAGTTGACCTCATCCACTGCGCTAT
AGGCTCATATAGGTCCAGTTAACTCGATCTCTGAAATTAAATTTCTATTTTTTAAAT
GAAATTAAGTCAAATGCATTATACGTACCTTCCCTCAACACCCTATATTATATTAAC
AAAAATCTTTATATTGCATTGCATCCAGTAAATGATAAAAAAAAAAATCAGGGTATA
ATTTTCTTAATCCATGCATGATAATCTGATTCATTTTCATGTATAAATTTAAATATAA
TTTGTGAGAAAGAGCAGGAGTCCCTCTGGAATAAAATATGAGTTGTAACCTTAAA
ACCACTTGGTGGGTGACACATCAGAAAGAACAAAGTGGGACCCTTTGAATTTTGTGC
ACGTACTAGGAATGGTACCTACAAGTAGGGCTACTTTTGTATAGTGAGAGTTTACCT
GAAGAAAAATTAACCATAGATGGATAAATATAGGGCATTAGGTTTAGAAAGCTGTTC
GGATCATTTTAAACGTAAAAAGCACTTAGTAGGACCAACTTAAAAGTAACCATAAAAT
TAATATTGTTTTCTTTCTTCAAAAAAATAATAAAAGAAACAAGGCCACTGTTAC
ATTGACATATTTTATTTCACTTTAATCATGCATGCAACTTGACTTCATTCCGTTCCCT
GGATTCCCTCCCCT**ATATAAACGCTTCTCGCCCA**TCCATCATTCGACAACATAGC
TAGAGTTTCCTCTTGAGAAAGAGAGTTTCTCTGCACAAGGGAAAGAGAGTCTCTAC
TATAGCCGGAGAA**ATG**GCCTTGCACTTGTCATTGGACTTCGTTTTTGGCGTTTTAGG
TACTA

244

245 **Figure S3. *MeSWEET10a* promoter sequence.** EBE_{TALE20} (bold letters) is located in
246 the -92 to -112 bp region before the start codon (red letters) of *MeSWEET10a*, which
247 is recognized by TALE20_{Xam11}.

248

249 **Figure S4. Transgenic lines confirmed by PCR analysis of the *Cas9* gene.** M:
250 DL2000 marker; 1-70, transgenic lines; +, positive control; -, negative control. 13, 23,
251 19, and 20 represent mutant lines #1, #2, #3, and #4.

Figure S5. Sanger sequencing peak map of target fragments in mutant lines #1, #2, #3, #4 and wild type (WT). Red boxes represent the protospacer adjacent motif (PAM), and underlines represent the target sequences.