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

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20 Cassava is a major food crop in tropical and subtropical regions that provides food
21 security for 800 million people. However, cassava bacterial blight (CBB) caused by
22 *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) results in severe yield losses and is the
23 most destructive bacterial disease in all worldwide cassava planting regions
24 (McCallum *et al.*, 2017). The vast majority of cassava cultivars are not resistant to
25 CBB. *Xam668* is a highly virulent *Xam* strain to cassava which contains five
26 transcription activator-like effectors (TALEs) including TALE20$_{Xam668}$ (Bart *et al.*,
27 2012). The repeat variable diresidues (RVDs) in TALE20$_{Xam668}$ recognize the
28 effector-binding element (EBE) on the *MeSWEET10a* promoter from cassava 60444
29 cultivar and activate *MeSWEET10a* gene expression which facilitates sucrose
30 transport from the interior of plant cells to the apoplasts and provide carbon sources
31 for the bacteria (Cohn *et al.*, 2014). Thus, editing the EBE in the *MeSWEET10a*
32 promoter may potentially confer resistance to CBB in cassava.

33 *Xam11* is a highly pathogenic subspecies from Hainan, China that infects the
34 Chinese local planting cassava SC8 cultivar with marked pathogenic symptoms
35 (Figure 1A). *TALE20*$_{Xam11}$ was cloned based on the genome sequencing information of
36 *Xam11*. TALE20$_{Xam11}$ has 20 RVD, and EBE$_{TALE20}$ is targeted by TALE20$_{Xam11}$ and is
37 located in the -92 to -112 bp region before the *MeSWEET10a* start codon (Figure 1B).
38 The dual luciferase reporter assay demonstrated that TALE20$_{Xam11}$ activated the
39 *MeSWEET10a* promoter from SC8 cultivar and initiated downstream expression of
40 firefly luciferase; however, TALE20$_{Xam11}$ could not activate the *MeSWEET10a*
41 promoter lacking the EBE$_{TALE20}$ region (Figure 1C).

42 CRISPR-P v2.0 was used to design the sgRNA targeting EBE$_{TALE20}$ of the
43 *MeSWEET10a* promoter in SC8 cassava. The pCAMBIA1301-Cas9-EBE-sgRNA
44 plasmid was constructed and transformed into the SC8 cassava genome by
45 *Agrobacterium*-mediated fragile embryonic callus (EFC) transformation. In total, 57
46 independent transgenic lines were regenerated. The EBE$_{TALE20}$ regions from all the
47 transgenic lines subjected to high throughput tracking of mutation (Hi-TOM)

48 sequencing showed that Cas9 activity efficiency was 98.2%. Three types of mutations
49 were obtained among the transgenic lines: homozygous mono-allelic (19.30%),
50 homozygous bi-allelic (61.40%), and heterozygous (17.54%) (Figure 1D). The
51 EBE_{TALE20} regions in the four mutant types of transgenic cassava (#1, #2, #3, #4) and
52 wild type SC8 cassava (WT) were shown in Figure 1E. The effects of EBE_{TALE20}
53 modification on *MeSWEET10a* expression in the mutant cassava lines were
54 investigated after inoculation with *Xam11*. The relative expression level of
55 *MeSWEET10a* in mutant lines showed no obvious difference from that of the wild
56 type at 0 and 0.5 day post-inoculation (dpi); however, markedly lower levels were
57 observed in mutant lines compared with that of the wild type at 1–7 dpi (Figure 1F).
58 This showed that EBE_{TALE20} mutations repressed *MeSWEET10a* expression after
59 *Xam11* infection. Wild type had obvious water-soaked symptoms at 3 dpi which were
60 not observed in mutant lines (#1, #2, #3, #4). At 7 dpi, the wild type had obvious
61 pustule symptoms while the mutant lines showed slightly water-soaked symptoms; at
62 14 dpi, the wild type injection sites were withered and turned yellow, while the
63 mutant injection sites started to wither and the plaque expanded slowly; at 40 dpi,
64 whole leaves of the wild type were withered and become yellow whereas the leaves of
65 the mutant lines only withered near the plaques (Figure 1G). Diseased lesions in
66 mutant lines were significantly smaller at 3 dpi, 7 dpi, and 14 dpi (Figure 1H)
67 suggesting that EBE_{TALE20} mutations limited the expansion of CBB symptoms in
68 cassava. The bacterial growth assay showed that *Xam11* populations in the four
69 mutant lines were significantly smaller than that in the wild type at 3 dpi and 7 dpi
70 (Figure 1I). Callose is deposited on the cassava cell wall during the early stages of
71 *Xam* pathogen attack which restricts pathogen growth (Wei *et al.*, 2021). The leaves
72 of the mutant cassava lines displayed significantly more callose depositions at 3 dpi
73 than that in the wild type (Figure 1J). These results revealed that EBE_{TALE20}
74 modification of *MeSWEET10a* in the four edited cassava lines had strong and stable
75 resistance to CBB.

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

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

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92 **Conflict of interest**

93 The authors declare no conflicts of interest.

94 **Author contributions**

95 Y.Y., J.G. and Y.C. designed the experiments. Y.W., M.G., R.P., T.Z., X.Z., Y.C., R.L.
96 and J.L. performed the experiments. Y.W., M.G. and R.P. wrote the manuscript. Y.Y.,
97 J.G. and Y.C. revised the manuscript.

98 **References**

99 Bart, R., Cohn, M., Kassen, A., McCallum, E.J., Shybut, M., Petriello, A., Krasileva,
100 K. *et al.* (2012) High-throughput genomic sequencing of cassava bacterial blight
101 strains identifies conserved effectors to target for durable resistance. *Proc. Natl*
102 *Acad. Sci. USA,* **109**, E1972-E1979.

103 Cohn, M., Bart, R.S., Shybut, M., Dahlbeck, D., Gomez, M., Morbitzer, R., Hou,
104 B.-H. *et al.* (2014) *Xanthomonas axonopodis* virulence is promoted by a
105 transcription activator-like effector–mediated induction of a SWEET sugar
106 transporter in cassava. *Mol. Plant Microbe Interact.* **27**, 1186-1198.
107 McCallum, E.J., Anjanappa, R.B., and Gruissem, W. (2017) Tackling agriculturally
108 relevant diseases in the staple crop cassava (*Manihot esculenta*). *Curr. Opin.*
109 *Plant Biol.* **38**, 50-58.
110 Wei, Y., Zeng, H., Liu, W., Cheng, X., Zhu, B., Guo, J. and Shi, H. (2021)
111 Autophagy-related genes serve as heat shock protein 90 co-chaperones in disease
112 resistance against cassava bacterial blight. *Plant J.* **107**, 925-937.
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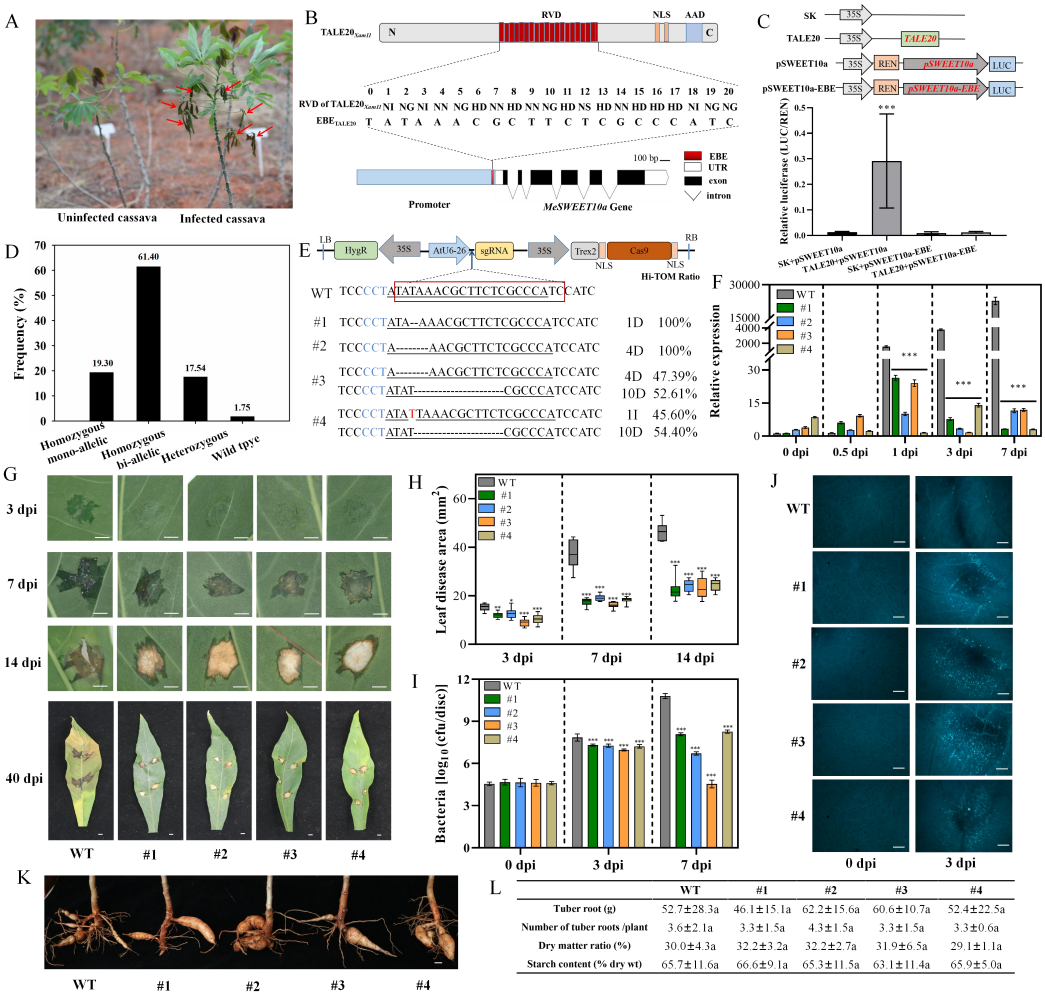
Figures



	WT	#1	#2	#3	#4
Tuber root (g)	52.7±28.3a	46.1±15.1a	62.2±15.6a	60.6±10.7a	52.4±22.5a
Number of tuber roots /plant	3.6±2.1a	3.3±1.5a	4.3±1.5a	3.3±1.5a	3.3±0.6a
Dry matter ratio (%)	30.0±4.3a	32.2±3.2a	32.2±2.7a	31.9±6.5a	29.1±1.1a
Starch content (% dry wt)	65.7±11.6a	66.6±9.1a	65.3±11.5a	63.1±11.4a	65.9±5.0a

Figure 1. Engineering CBB resistant plants through CRISPR/Cas9-targeted editing of the *MeSWEET10a* promoter in cassava. (A) *Xam11* infects the cultivar cassava SC8 with marked pathogenic symptoms. **(B)** Schematic representation of RVD in TALE20$_{Xam11}$ recognize the EBE$_{Xam11}$ on the *MeSWEET10a* promoter from cultivar cassava SC8. The C-terminus of TALE20$_{Xam11}$ has two nuclear localization signals (NLS) and an acidic activation domain (ADD). **(C)** TALE20$_{Xam11}$ directly activates *MeSWEET10a* expression through the EBE region. *MeSWEET10a* promoter-luciferase fusion and 35S-driven TALE20$_{Xam11}$ constructs were delivered into *Nicotiana benthamiana* cells through infection by *Agrobacterium tumefaciens*. Luciferase activity was quantified after 1 dpi. SK: pGreen II62-SK vector (control vector); TAL20: pGreen II62-SK-TAL20$_{Xam11}$ vector; pSWEET10a: pGreen II0800-pMeSWEET10a-LUC vector; pSWEET10a-EBE: pGreen

128 II0800-pMeSWEET10a-EBE_{TAL20}-LUC vector (deletion of EBE_{TAL20} from the
129 *MeSWEET10a* promoter). Data are displayed as mean technical replicates ± standard
130 deviation. Experiments were repeated three times with similar results (Student's t-test,
131 ***P < 0.005). **(D)** CRISPR/Cas9-induced mutation types and frequency. **(E)**
132 Hi-TOM sequencing of the EBE_{TALE20} regions of wild type (WT) non-transgenic SC8
133 cassava compared with the engineered lines #1, #2, #3, and #4. The EBE_{TALE20} region
134 of the $TALE20_{Xam11}$ target is marked in a red box in WT; the sgRNA sequences are
135 underlined; the protospacer adjacent motif (PAM) sites are marked in blue text; the
136 hyphens are missing bases, and the red letter is the inserted base. **(F)** Time course of
137 *MeSWEET10a* expression in mutant lines and WT after *Xam11* inoculation.
138 *MeSWEET10a* transcript levels in leaves determined by qPCR at 0, 0.5, 1, 3, and 7 dpi.
139 Three biological repeats were performed for each data point (Student's t-test, ***P <
140 0.005). **(G)** Symptom development after *Xam11* inoculation. Scale bar, 5 mm. **(H)**
141 and **(I)** Time course of leaf disease areas and bacterial numbers after *Xam11*
142 inoculation. At least 10 leaves were harvested for the diseased areas with the bacterial
143 number assay performed using three biological replicates (Student's t-test, *P < 0.05,
144 ***P < 0.005). (**J**) Callose depositions in leaves. Scale bar, 50 μm. **(K)** Tuber root
145 phenotypic characterizations. Scale bar, 1 cm. **(L)** Yield-related traits shown as the
146 mean ± SD from five plants of each line. *P* values were calculated using Student's
147 t-test. The values marked with the same letter (a) represented no significant
148 difference.
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