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Targeted knockout of early nodulin-like 3 (*MusaENODL3*) gene in banana reveals its function in resistance to Xanthomonas wilt disease

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Summary

Nodulins and nodulin-like proteins play an essential role in the symbiotic associations between legumes and *Rhizobium* bacteria. Their role extends beyond the leguminous species, as numerous nodulin-like proteins, including early nodulin-like proteins (ENODL), have been identified in various non-leguminous plants, implying their involvement in functions beyond nodulation, such as nutrient transport and growth modulation. Some ENODL proteins have been associated with plant defense against pathogens, as evident in banana infected with *Xanthomonas campestris* pv. *musacearum* (Xcm) causing banana Xanthomonas wilt (BXW) disease. Nonetheless, the specific role of ENODL in plant defense remains to be fully elucidated. The *MusaENODL3* gene was found to be repressed in BXW-resistant banana progenitor '*Musa balbisiana*' and 20-fold upregulated in BXW-susceptible cultivar 'Gonja Manjaya' upon early infection with Xcm. To further unravel the role of the *ENODL* gene in disease resistance, the CRISPR/Cas9 system was employed to disrupt the *MusaENODL3* gene in 'Gonja Manjaya' precisely. Analysis of the *enodl3* edited events confirmed the accurate manipulation of the *MusaENODL3* gene. Disease resistance and gene expression analysis demonstrated that editing the *MusaENODL3* gene resulted in resistance to BXW disease, with 50% of the edited plants remaining asymptomatic. The identification and manipulation of the *MusaENODL3* gene highlight its potential as a critical player in plant-pathogen interactions, offering new opportunities for enhancing disease resistance in crops like banana, an important staple food crop and source of income for resource-poor farmers in the tropics. This study provides the first evidence of the direct role of the *ENODL3* gene in developing disease-resistant plants.

Introduction

Nodulins and nodulin-like genes are induced in legumes upon nodulation by *Rhizobium* bacteria and play a pivotal role in symbiotic interactions. The initiation of symbiosis between leguminous plants and *Rhizobium bacteria* involves specific nodulation (Nod) factors (NFs) or lipochitooligosaccharide (LCO) signalling molecules that are secreted by the bacteria into the soil. These molecules cause root hair deformation and curling, activate early nodulin (*ENOD*) gene expression, and trigger the division of pericycle/cortical cells in the root cortex to form the nodule primordium (Chan *et al.*, 2020). *ENOD* genes are shown to be activated in the root cells upon *Rhizobium leguminosarum* infection, and activation may be as early as the onset of infection thread penetration through the root cortex. *ENOD* genes have been reported to be expressed in developing nodules at early stages before nitrogen fixation.

Several nodulin and nodulin-like genes in non-leguminous plant species, including monocot plants, have been identified, which suggests that these genes play additional roles besides nodulation (Denancé *et al.*, 2014). There are seven nodulin-like gene families present, including *MtN3/saliva/SWEET, MtN21/EamA-like/UMAMIT, Early Nodulin-Like, Major Facilitator Superfamily, Sec14p-nodulin domain proteins, NOD26-like intrinsic proteins*, and *Vacuolar Iron Transporter/nodulin-like* family. Nodulin-like proteins in non-nodulating plants showcased their involvement in transporting nutrients, amino acids, hormones, and solutes required for growth development in the plant (Denancé *et al.*, 2014).

Early nodulin-like proteins (ENODL) were first identified in Arabidopsis. They are chimeric arabinogalactan proteins (AGPs) related to the phytocyanin family, i.e., blue copper proteins that bind a single copper atom and function as electron transporters (Mashiguchi *et al.*, 2004). However, ENODL lacks vital amino acid residues responsible for copper binding and is involved in processes without binding with copper. Phosphoproteomic examination of Arabidopsis plasma membranes treated with elicitors of plant immunity identified ENODL1, 13–15, and 17, suggesting their involvement in plant disease resistance (Benschop *et al.*, 2007). Transcript profiling of the banana Xanthomonas wilt (BXW)-susceptible banana cultivar 'Pisang Awak' compared with the BXW-resistant wild-type progenitor '*Musa balbisiana*' during early infection with *Xanthomonas campestris* pv. *musacearum* (Xcm) showed repression of *MusaENODL1* and *MusaENODL3* in the BXW-resistant banana genotype (Tripathi *et al.*, 2019). This report strongly suggests the

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involvement of ENODL in the plant-pathogen interaction, specifically, in this context, resistance against Xcm. However, no study has documented the specific role of *ENODL* in disease resistance in plants.

Banana Xanthomonas wilt, a bacterial disease caused by Xcm, poses a significant threat to banana cultivation in East and Central Africa (Tripathi *et al*., 2009). This devastating disease affects all banana types and can drive up to 100% yield losses (Tripathi *et al*., 2022). The impact of BXW has been so severe that entire plantations have been wiped out in affected regions, creating economic havoc. BXW disease first emerged in Ethiopia in the 1930s, initially infecting Ensete (*Ensete ventricosum*) and then banana, and later spread to Uganda, the Democratic Republic of Congo, Rwanda, Burundi, Tanzania, and Kenya (Blomme *et al*., 2017). The economic impact of BXW is potentially disastrous because it reduces the yield of banana, and the farmers do not have the option of relocating to new planting sites that are infection-free. It is estimated that over a decade, the disease has resulted in economic losses ranging from US$ 2–8 billion (Abele and Pillay, 2007). Xcm is transmitted by insects that perch on the male inflorescence, contaminated tools, and infected planting material. Although sources of complete resistance to the pathogen have been identified in the wild-type banana progenitor '*Musa balbisiana*' (Nakato *et al*., 2018), all the cultivated varieties remain highly susceptible to the disease.

Plants possess a natural defense mechanism against invading pathogens by activating an immune response upon detection of invasion patterns of the pathogen (Schellenberger *et al*., 2019). However, most pathogens have developed strategies to suppress the plant immune system by interacting with the host mechanism, activating specific host genes that enable them to grow and cause disease symptoms (Boch *et al*., 2014). Numerous approaches, including following phytosanitary practices, such as cutting and burying infected plants, restricting the movement of banana materials from BXW-affected areas, decapitating male buds, and using "clean" tools, have been attempted to manage the disease. Developing resistant varieties using conventional breeding is challenging because of the lack of known resistance in the *Musa* germplasm, polyploidy, lengthy production cycle, and sterility of many cultivars (Tripathi *et al*., 2019). Nonetheless, hope lies in the complete resistance exhibited by '*Musa balbisiana*'. Regrettably, this valuable genetic resource has not been fully leveraged within breeding programs to combat BXW. The transgenic approach has made some progress using defense genes such as *Pflp, Hrap*, and *Xa21* (Tripathi *et al*., 2014a,b). However, the evolution of new pathogenic strains and the ability of the bacterium to overcome the control measures pose a challenge in eradicating the disease. By embracing cutting-edge technologies, harnessing the untapped potential of wild-type banana varieties, and remaining vigilant in the face of evolving pathogens, we can overcome this destructive disease.

Targeted genome editing has emerged as a game-changing tool in plant genetic engineering. Among the arsenal of genome editing technologies, the clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) is at the forefront due to its simplicity and design flexibility. It is based on the type II CRISPR immune system in bacteria that protects against invading DNA viruses and/or plasmids. The CRISPR/Cas9 system holds immense potential to revolutionize crop improvement by enabling precise genetic alterations in plant species, including banana. The availability of reference genome sequences and CRISPR/Cas9-editing tool for banana has made it possible to precisely edit endogenous genes for disease resistance (Ntui *et al*., 2020; Tripathi *et al*., 2019, 2022).

To confirm the role of ENODL in disease resistance, the *MusaENODL3* was precisely knocked out from the BXW-susceptible banana cultivar 'Gonja Manjaya' using the CRISPR/Cas9 system. Sequence analysis of the generated *enodl3* edited events showed precise manipulation of the *MusaENODL3* gene. Disease resistance assay and quantitative real-time PCR (qRT-PCR) analysis showed that editing the *MusaENODL3* gene resulted in resistance to BXW, with 50% of the events remaining asymptomatic. To our knowledge, this study represents the first documented evidence of the direct role of the *ENODL* gene in developing disease-resistant plants. The identification and manipulation of the *ENODL3* gene demonstrate its remarkable potential as a key player in plant-pathogen interactions, unravelling new avenues for enhancing disease resistance in crucial crops like banana, which is an important staple food crop feeding over 400 million people and a source of income for resource-poor farmers in more than 136 tropical and sub-tropical countries. The findings hold promise for tackling BXW disease and can be applied to enhance plant immunity against bacterial pathogens in other crops.

Results

Phylogenetic analysis of early nodulin gene

MusaENODL3 was chosen for this study based on the comparative transcriptomic analysis between the BXW-susceptible cultivar 'Pisang Awak' and the resistant wild-type progenitor *Musa balbisiana* upon early infection with Xcm (Tripathi *et al*., 2019). That analysis revealed significant repression of *MusaENODL3* in the BXW-resistant banana genotype *Musa balbisiana* compared to the susceptible cultivar. In regard to that finding, this study aimed to knockout *MusaENODL3* in the BXW-susceptible cultivar 'Gonja Manjaya' (AAB genome), commonly grown in East and Central Africa. To provide in-depth knowledge, this study commenced with the phylogenetic relationship between *MusaENODL3* and its homologues in other plant species.

The analysis involved aligning nodulin-like protein sequences from *Arabidopsis thaliana* (132), *Oryza sativa* (87), *Medicago truncatula* (118), *Musa acuminata* (69), and *Musa balbisiana* (65) (Figure 1). Phylogenetic analysis using Clustal Omega revealed *MusaENODL3* from *Musa accuminata*, gene ID Ma11_g13300.1, and *Musa balbisiana*, gene ID Mb11_g12710.1, clustered in same clade with Arabidopsis homologue AtENODL9; four homologues in *Oryza sativa* OsENODL5, OsENODL6, OsENODL7, and OsENODL18; four homologues in *Medicago truncatula* G7JFO9, B7FJT6, G7IJN7, and G7JTU3; three in *M. balbisiana* Mba11_g12710.1, Mba01_g08460.1, and Mba09_g21280.1; and two in *M. accuminata* homologues Ma03_g24350.1 and Ma09_g25350.1, indicating *MusaENODL3* is a member of nodulin-like gene family of non-leguminous plants (Figure 1). Clustal Omega uses seeded guide trees and Hidden Markov Model (HMM) profile-profile techniques to generate alignments between multiple sequences. The phylogenetic tree was constructed from the multiple sequence alignment using the neighbour joining method and visualized using the ggtree R package.

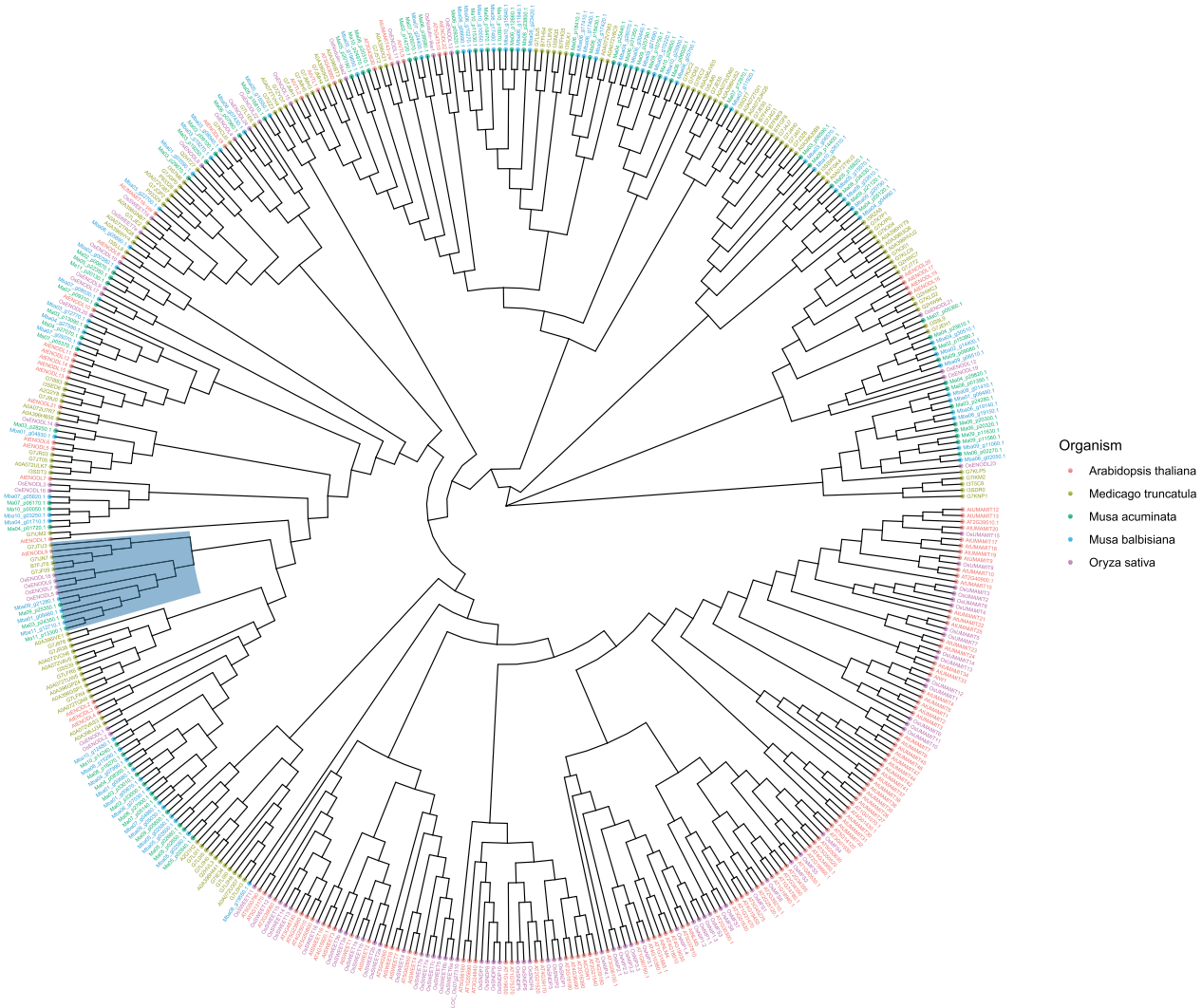

Figure 1 Phylogenetic tree of nodulin-like protein family. Nodulin-like protein sequences for *Arabidopsis thaliana* and *Oryza sativa* were downloaded from The Arabidopsis Information Resource (TAIR – https://www.arabidopsis.org/) and the rice genome annotation project (http://rice.uga.edu/), respectively. ENODL protein sequences for *Medicago truncatula*, *Musa acuminata*, and *Musa balbisiana* were downloaded from Interpro (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR003245/) and the Banana Genome Hub (https://banana-genome-hub.southgreen.fr/), respectively. The protein sequences were aligned using the Clustal Omega multiple sequence alignment program. The alignments were used for inferring phylogenetic trees using the ggtree package in R.

Relative expression of *MusaENODL3* in susceptible and resistant banana

The differential expression of *MusaENODL3* in the susceptible banana cultivar 'Gonja Manjaya' and the resistant wild-type progenitor *Musa balbisiana* upon early infection with Xcm shed light on its role in the plant-pathogen interaction. At 12 h post-inoculation (hpi), the infected plants of the susceptible cultivar 'Gonja Manjaya' exhibited a significant upregulation in the relative expression level of *MusaENODL3*, reaching an 18-24-fold increase compared to the non-infected plants (Figure 2). This result confirms the activation of *MusaENODL3* in response to Xcm, underscoring its significant role in plant-pathogen interaction. However, in the BXW-resistant *Musa balbisiana* plants, the expression of *MusaENODL3* was significantly suppressed upon early infection with Xcm (Figure 2), suggesting *MusaENODL3* may play a potential role in Xcm infection and subsequent disease development. This result aligns with the transcriptomic analysis reported by Tripathi *et al*. (2019) and supports the rationale for selecting *MusaENODL3* for further studies.

Plasmid construction

'Gonja Manjaya' is a triploid (AAB) with two non-identical copies of most of its genes in A and B genomes. The *MusaENODL3* gene (Ma11_g13300) in A genome is 951 bp, whereas Mb11_g12710.1 in B genome is 716 bp, each containing two exons and three introns (Figure 3a,b). The sequences of the *MusaENODL3* gene from *Musa accuminata* (AA genome, gene ID Ma11_g13300) and *Musa balbisiana* (BB genome, gene ID Mb11_g12710.1) were aligned to identify the conserved region (Figure S1). To edit all the alleles of *MusaENODL3*, two gRNAs with a trinucleotide 5′-NGG-3′, the protospacer-adjacent motif (PAM), at the 3′-end were strategically designed in the conserved region of the gene sequences within exon 2 of both A and B

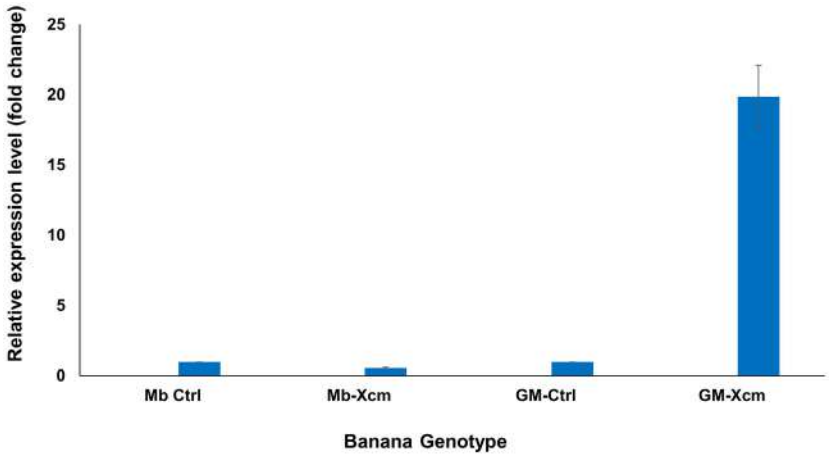

Figure 2 Expression of *MusaENODL3* (Ma11_t13300.1) in BXW-resistant wild-type banana progenitor *Musa balbisiana* and BXW-susceptible banana cultivar 'Gonja Manjaya' at 12 h post inoculation with *Xanthomonas campestris* pv. *musacearum* (Xcm). Mb-Ctrl, *Musa balbisiana* uninoculated control; Mb-Xcm, *Musa balbisiana* inoculated with Xcm; GM-Ctrl, Gonja Manjaya uninoculated control; GM-Xcm, Gonja Manjaya inoculated with Xcm.

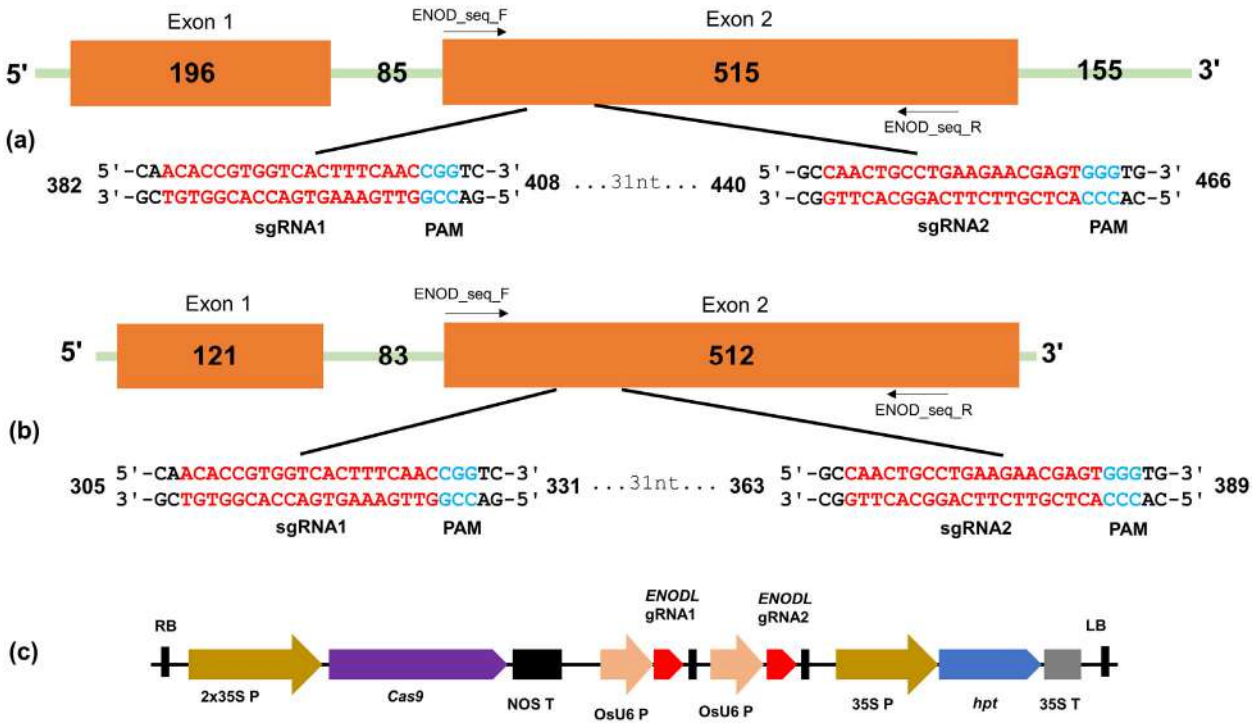

Figure 3 Schematic representation of the *MusaENODL3* gene and CRISPR/Cas9 construct used to edit banana. (a, b) Schematic structure of *MusaENODL3* gene in A and B genomes in *Musa accuminata* and *Musa balbisiana*, respectively, showing positions of gRNAs. Orange boxes represent exons; digits indicate exon numbers while lines indicate introns. gRNAs and PAM (Protospacer Adjacent Motif) sequences are indicated in red and blue, respectively. Primer names used for sequencing are displayed. (c) Schematic map of T-DNA region of binary vector pMDC32-Cas9-MusaENODL3. 2 × 35S P, double CaMV35S promoter; 35S P, CaMV35S promoter; 35S T, CaMV35S terminator; hpt, hygromycin phosphotransferase gene; LB, left border; NOST, Nopaline synthase terminator; OsU6 p, *Oryza sativa* U6 promoter; RB, Right border.

genome (Figure 3a,b; Figure S1). The allelic variations in the target sites of *MusaENODL3* in cultivar 'Gonja Manjaya' were ruled out by sequencing the cloned fragment of the gene.

We targeted to introduce mutations spanning a fragment (>35 bp), necessitating the selection of gRNAs positioned at a suitable distance from each other (Figure 3a,b). The gRNAs were selected predicting the highest editing efficiency with minimal potential for off-target effects. The gRNAs with an on-target score of greater than 70% (the higher the score, the higher the efficiency) from the conserved regions and off-target score of more than 70% from a score of 0–100 (the higher the score, the lower the off-target risk) were selected for cloning. The selected gRNAs, together with high-GC plant codon-optimized *Cas9* gene driven by the double CaMV35S, were integrated into the T-DNA region of the binary vector pMDC32, resulting in the CRISPR/Cas9 construct pMDC32-Cas9-MaENODL3, in which each gRNA is regulated by OsU6 promoter (Figure 3c).

Regeneration of mutant plants

The CRISPR/Cas9 plasmid pMDC32-Cas9-MusaENODL3 was delivered into the embryogenic cell suspension of 'Gonja Manjaya' through *Agrobacterium*-mediated transformation. A

Figure 4 Regeneration of genome-edited events of 'Gonja Manjaya'. (a) Embryogenic cell suspension used for delivery of CRISPR/Cas9 plasmid; (b) Embryos on embryo maturation medium; (c) Germination of embryos in selective medium containing hygromycin; (d) Well-rooted plantlets in proliferation medium; (e) Acclimatization of genome-edited plants in small plastic cups; (f) Potted plants of genome-edited events in the greenhouse for disease assay.

total of 42 transgenic events were regenerated in the selective media (Figure 4). The events were micropropagated in a medium containing 5 mg/L 6-benzyl aminopurine and then rooted and maintained in MS medium without phytohormone. Well-rooted plantlets were transferred to soil in the pots and acclimatized in the greenhouse for disease assay.

PCR analysis of mutants to detect band shift

The hygromycin-resistant putative transgenic events were validated for the presence of the *Cas9* gene. PCR analysis using the Cas9-specific primers confirmed the presence of the *Cas9* gene in all the events tested, as evidenced by the amplification of a 450-bp amplicon (Figure S2a). To further investigate the efficiency of Cas9 in inducing double-stranded breaks (DSBs) at both target sites, the possibility of band shift in PCR gel was examined. Since two gRNAs positioned at a distance from each other were used, simultaneous DSBs induced by Cas9 should result in a deletion >35 bp, which could be detected as a band shift on the PCR gel image. To confirm this, we analysed PCR band shift using the gene-specific primers (Table S1), flanking both gRNAs. Among the events analysed, five events (E1, E11, E17, E38, and E39) showed the anticipated band shift (Figure S2b), indicating that Cas9 probably created DSB at both target sites simultaneously.

Sequence analysis of edited events to detect targeted mutations

To validate the targeted mutations induced by CRISPR/Cas9, all the events used for PCR band shift analysis were subjected to Sanger sequencing. The sequencing results showed a mutation rate of 100%, with all edited events showing indels. The sequencing data revealed a diverse array of mutations, including deletions, insertions, substitutions, and deletions accompanied by insertions (Figure 5). Most of the detected deletions were large, ranging from Δ23 bp to Δ180 bp. Only a few events (E6, E13, E32) showed small deletions of Δ1 bp to Δ4 bp. Two events (E11 and E20) showed small insertions (+1 to +3 bp). A 1-bp substitution was also detected in events E6 and E32 positioned 2–4 base pairs upstream of PAM.

The sequencing results further validated the simultaneous cleavage of both target sites, as events E1, E17, and E38 exhibited >35 bp deletions, consistent with the band shift results. Event E13 showed the same indels for all the replicates sequenced, confirming the homozygous mutations, whereas all the other events showed variations in *Musa*ENODL3 manipulations. For example, event E38 showed four different types of indels (Δ6/Δ32, Δ61, Δ6/Δ101, and Δ6/Δ46), confirming chimeric mutations. Most events (7/11) showed mutations at both target sites of the *MusaENODL3* gene, confirming that both gRNAs were effective. However, gRNA2 was more effective as all the events showed editing in target site 2 (gRNA2) (Figure 5).

Growth analysis of the edited events

To evaluate the impact of *MusaENODL3* gene disruption on plant morphology, ten edited events along with control wild-type plants with three replicates were assessed for their growth in the greenhouse at 90 days. Most of the edited events exhibited

	gRNA1 PAM	gRNA2 PAM		
C	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAACGAGTCGG	WT	Target site
E1	ACACCGTGGTCACTTTCAA------....31....	---------- TCGG	−58	g1–g2
	ACACCGTGGTCACTTTCA--------....31....	---------- GTCGG	−58	g1–g2
	ACACCGTGGTCACTTTCAA-------....31....	----------GG	−60	g1–g2
E6	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAACG-GTCGG	−1	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAA--AA TCGG	−2, S1	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAA----TCGG	−4	g2
E11	ACACCGTGGTCACTTTCAAACG--GGTC.31....	.GCCAACTGCCTGAAGAACG-GTCGG	+3,−2, −1	g1/g2
	ACACCGTGGTCACTTTCAAACG--GGTC.31....	.GCCA----------	+3,−2, −164	g1/g2
	ACACCGTGGTCACTTTCAAACG--GGTC.31....	.GCCA----------	+3,−2, −187	g1/g2
	ACACCGTGGTCACTTTCAAACG--GGTC.31....	.GCCAACTGCCT----------	+3,−2, −180	g1/g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	.GCCAACTG----------	−186	g2
E13	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAAC - AGTCGG	−1	g2
E17	ACACCGTGGTCACTTT---------....31....	----------TCGG	−61	g1–g2
	ACACCGTGGTCACTTTC---------....31....	----------CGG	−61	g1–g2
	ACACCGTGGTCACTTTCAA------....31....	----------G	−61	g1–g2
	ACACCGTGGTCACTTTC---------....31....	---------- G	−63	g1–g2
E20	ACACCGTGGTCACTTTCAAACCGGTC....31....	GCCAACTGCCTGAAGAAC - AGTCGG	+1, −1	g1, g2
	ACACCGTGGTCACTTTCAAACCGGTC....31....	GCCAACTGCCTGAAGAAC--------	+1, −36	g1, g2
E26	ACACCGTGGTCACTTTCAACCGGTC....31....	----------AGTCGG	−41	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------AGTCGG	−43	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAA ----------AGTCGG	−14	g2
	ACACCGTGGTCACTTTCAA--GGTC....31....	----------AGTCGG	−2/−44	g1/g2
E32	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAAC - AGTCGG	−1	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	GCCAACTGCCTGAAGAAC A AGTCGG	S1	g2
E38	ACACCGTGGTCACTTT------GTC....31....	----------TCGG	−6/−32	g1/g2
	ACACCGTGGTCACTTT--------- ....31....	----------TCGG	−61	g1–g2
	ACACCGTGGTCACTTT------GTC....31....	---------- ---------	−6/−101	g1–g2
	ACACCGTGGTCACTTT------GTC....31....	----------TCGG	−6/−46	g1/g2
E39	ACACCGTGGTCACTTT---------....31....	----------	−116	g1–g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−101	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−146	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−83	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−129	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−50	g2
	ACACCGTGGTCACTTTCAACCGGTC....31....	----------	−104	g2

Figure 5 Sequence analysis of *enodl3* edited events of 'Gonja Manjaya'. Red nucleotides indicate the gRNAs and purple nucleotides indicate Protospacer Adjacent Motif (PAM). Black dashes (--) represent deletions. Green nucleotides indicate insertion. Blue nucleotides indicate substitution. C, Non-edited control sequences; 1–39, independent edited events. The number of nucleotides deleted, inserted, or substituted is indicated on the right panel. The target site where modification occurred is also indicated in the right panel by g1, g2, or g1/g2.

normal development with no morphological or phenotypical differences compared to control plants. This was evident in growth parameters such as plant height, pseudostem thickness, number of leaves, and total leaf area, where the edited events were similar to the control plants (Figure 6a–d). Only one edited event, E2, exhibited relatively lower values for plant height, pseudostem thickness, and total leaf area than the control plants. These results demonstrated that disruption of the *MusaENODL3* gene did not negatively affect plant morphology.

Evaluation of *MusaENODL3* edited events for disease resistance

To assess the response of edited events against the bacterial pathogen Xcm, ten edited events along with control wild-type plants were evaluated in the greenhouse for disease resistance. Upon inoculation, the non-edited control plants exhibited pronounced disease symptoms like drooping, wilting, necrosis, and complete wilting of plants. Notably, within 20.6 ± 2.0 days post inoculation (dpi), the non-edited control plants displayed symptoms such as dropping and wilting of the inoculated leaves with a 100% disease incidence rate (Table 1). These symptoms progressively spread to the entire plant and all three replicates succumbed to complete wilting within 40.3 ± 3.2 dpi with a disease severity index (DSI) of 100% (Figure 7a,b, Table 1), confirming their susceptibility to Xcm infection. In contrast, four edited events (E1, E11, E17, and E38) exhibited partial resistance, showcasing DSI ranging from 33.3% to 46.7% (Figure 7a,b, Table 1). These partially resistant events showed delayed

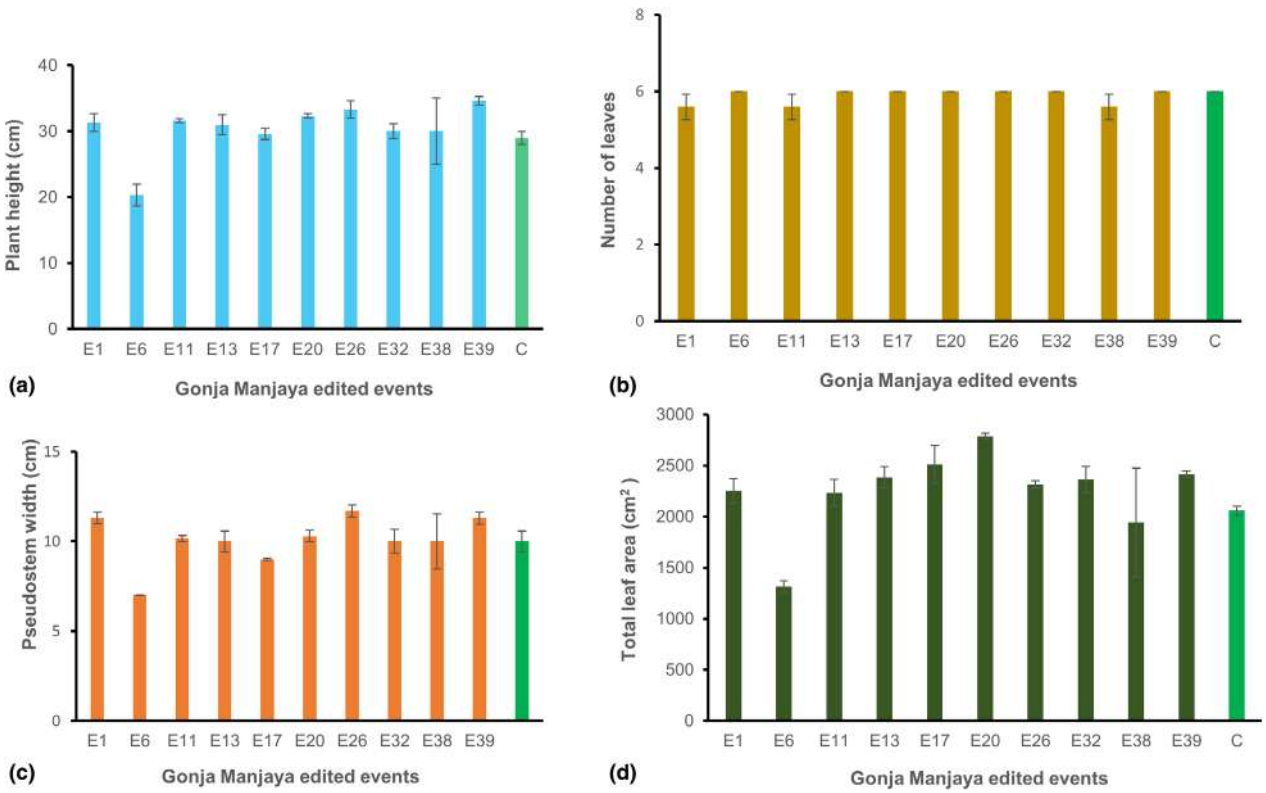

Figure 6 Growth analysis of *enodl3* edited events of 'Gonja Manjaya'. (a) Plant height; (b) Pseudostem girth; (c) Number of leaves; (d) Total leaf area. The data were analysed using Analysis of variance (ANOVA) and the Tukey family test at $P = 0.001$. Data are presented as Mean ± Standard error.

Table 1 Evaluation of *enodl3* events for banana Xanthomonas wilt (BXW) disease resistance under greenhouse conditions

Plant ID	Appearance of first disease symptom on leaves	No. of days for appearance of first symptom	Disease progression on whole plant	No. of days for complete wilting	Disease severity 0–5 scale	Disease incidence %	Resistance %
E1	Drooping of inoculated leaf	32.6 ± 2^{b}	Wilting of 2 ± 0^{b} leaves	NCW	2 ± 0^{b}	100^{a}	62.3 ± 4.0^{b}
E6	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0^{a}
E11	Wilting of inoculated leaf	40.6 ± 5.7^{b}	Wilting of 2.7 ± 2.9^{a} leaves	44.0 ± 0^{b}	2.3 ± 2.3^{b}	100^{a}	55.3 ± 47.9^{b}
E13	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0
E17	Wilting of inoculated leaf	20 ± 0^{C}	Wilting of 2.0 ± 3.4^{b} leaves	38 ± 0^{d}	1.7 ± 2.9^{b}	33^{b}	66.7 ± 57.5^{b}
E20	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0^{a}
E28	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0^{a}
E32	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0^{a}
E38	Wilting of inoculated leaf	41.3 ± 2.8^{b}	Wilting of 2.7 ± 2.9^{a} leaves	50 ± 0^{a}	2.3 ± 2.3^{b}	100^{a}	55.3 ± 47.9^{b}
E39	No disease symptom	–	No disease symptom	NCW	0^{c}	0^{c}	100 ± 0^{a}
C	Wilting of inoculated leaf	20.6 ± 2.0^{C}	Wilting of all leaves and plant died	40.3 ± 3.2^{c}	5 ± 0^{a}	100^{a}	0^{c}

Data are presented as Mean ± Standard error. Means with the same letter are not significantly different from each other ($P \leq 0.05$). Disease severity was recorded on a scale of 0–5 with 0—no symptoms, 1—only the leaf inoculated wilted, 2—two to three leaves wilted, 3—four to five leaves wilted, 4—all the leaves wilted but the plant still alive, 5—whole plant died.

E1-39, *enodl3* edited events of banana cultivar 'Gonja Manjaya'; NCW, No complete wilting.

symptom onset and lower disease severity. The remaining six edited events (E6, E13, E20, E28, E32, and E39) showed complete resistance, with no disease symptoms across any replicates (Figure 7a,b, Table 1). The edited events showing complete resistance to Xcm at 60 dpi are potential promising events for further evaluation in field conditions to assess the durability of their disease-resistant trait.

Expression of *MusaENODL3* in edited events

To assess the effect of mutations in the *MusaENODL3* gene on its expression, the transcript accumulation of *MusaENODL3* was checked in eight *enodl3* events (E1, E6, E11, E13, E17, E32, E38, and E39) compared with the wild-type plants by qRT-PCR. The relative expression of *MusaENODL3* in all the tested *enodl3* events

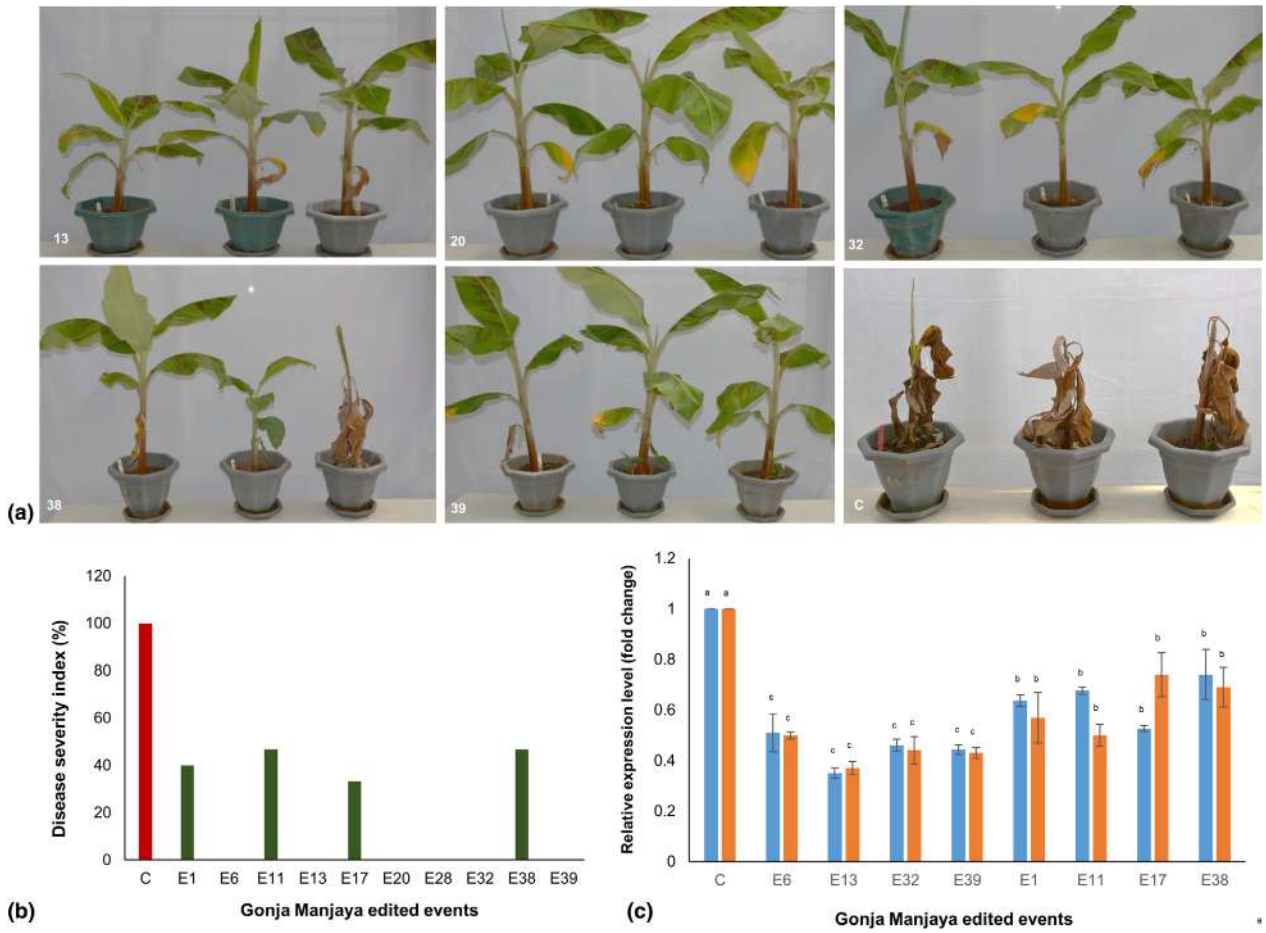

Figure 7 Evaluation of *enodl3* edited events of 'Gonja Manjaya' for banana Xanthomonas wilt (BXW) disease resistance under greenhouse conditions. (a) Response of genome-edited events (E13, E20, E32, E38, E39) and non-edited control plants (C) to *Xanthomonas campestris* pv. *musacearum*. Photographs were taken 60 days post-inoculation. (b) Disease severity index calculated at 60 days post-inoculation. (c) Relative expression of *ENODL3* gene in edited events compared to control plants without (blue bars) and with infection (orange bars) with *Xanthomonas campestris* pv. *musacearum* (Xcm). Data are presented as Mean ± Standard error. Means with the same letter are not significantly different from each other ($P \leq 0.05$).

was significantly lower than in the wild-type control (Figure 7c). Even upon infection with Xcm, the relative expression of the *MusaENODL3* gene in the edited events remained lower than that of the wild-type control plants (Figure 7c). The complete disease-resistant events (E6, E13, E32, and E39) showed much lower relative expression of *MusaENODL3* than partial resistant events (E1, E11, E17, and E38). These results indicate that the mutations introduced in the edited events successfully led to a repression of the *MusaENODL3* gene.

Discussion

The battle against BXW disease is of immense importance, as it not only threatens the livelihoods of millions of smallholder farmers but also jeopardizes the availability of this vital food crop in the affected regions. Efforts to combat this devastating disease include cultural practices and ongoing research for developing resistant banana varieties that can withstand the onslaught of Xcm. By understanding the intricate mechanisms of host-pathogen interactions and harnessing the power of science, we can hope to overcome BXW and safeguard the future of banana cultivation in East and Central Africa. While traditional approaches using resistance (R) genes have shown promise, they come with certain limitations. Recent studies have highlighted the potential of gene editing techniques, such as CRISPR/Cas9, to confer disease resistance. Recently, loss-of-function mutations in susceptibility 'S' genes or negative regulators of plant defense have been shown to confer disease resistance (Tripathi *et al.*, 2022). Previously, editing of *MusaDMR6* in banana resulted in resistance to BXW disease (Tripathi *et al.*, 2021). However, it is crucial to recognize that a single-gene knockout in a plant may not suffice to provide durable resistance in the field, given that pathogens evolve to overcome the resistance mechanism. To generate robust and durable resistance to BXW, it is important to identify potential genes that could be multiplexed. This approach not only enhances durability but also broadens the spectrum of resistance. The probability of a pathogen strain evolving to simultaneously defeat resistance conferred by multiple genes is considerably lower. This study focused on understanding whether the inactivation of the endogenous *MusaENODL3* gene in banana genome could provide resistance to BXW disease with the goal of developing durable resistance by multiplexing key candidate genes. The regenerated edited events exhibited significant resistance to BXW.

Nodulin and nodulin-like proteins play important roles in legumes during nodule formation. These proteins are categorized as early (*ENOD*) and late nodulin genes based on their expression

timing during root nodule development. *ENOD*s are induced shortly after infection, while late nodulins require more time. Interestingly, nodulin-like proteins have been identified in non-nodulating plants, indicating a possible ancestral role for nodulin-like genes in plant physiology (Denancé *et al*., 2014). It has been observed that nodulin-like proteins are also involved in the interaction of plants with pathogenic microbes, suggesting its role in solute transport in plant innate immunity (Denancé *et al*., 2014). The repression of *ENODL3* in BXW-resistant *Musa balbisiana* at early infection with Xcm further supports its role in plant defense (Tripathi *et al*., 2019). By suppressing *MusaENODL3* in banana, the colonization of Xcm may be restricted, thereby enhancing resistance to BXW. We further confirmed the hypothesis in this study by knocking out *MusaENODL3* in the BXW-susceptible cultivar 'Gonja Manjaya'.

MusaENODL3 (Ma11_g13300.1 and Mba11_g12710.1) is a phytocyanin domain-containing protein belonging to a plant-specific subfamily of copper-binding Cupredoxin (IPR008972), which is a major characteristic of ENODL. Phylogenetic analysis of genomic sequence alignment with ENODL homologues in *Arabidopsis thaliana*, *Medicago trancatula*, and *Oryza sativa* classified *MusaENODL3* within the ENODL cluster alongside some Arabidopsis and rice homologues (Figure 1). This result suggests that *MusaENODL3* is a part of the early nodulin-like gene family of non-leguminous plants. *MusaENODL3* clustered with *AtENODL9* of Arabidopsis. *AtENODL9* is involved in carbohydrate transport, which might help pathogens to colonize. The upregulation of *MusaENODL3* to approximately 20-fold in the BXW-susceptible cultivar 'Gonja Manjaya' and its downregulation in BXW-resistant '*Musa balbisiana*' (Figure 2) during early infection with Xcm further supports its role in plant-pathogen interaction.

Nodulin-like genes are expressed in all organs, including flowers, leaves, roots, and stems. Their expressions are modulated by external factors such as biotic or abiotic stress, or treatment with hormones or plant immunity elicitors, and during various developmental stages such as pollen formation, secondary cell wall deposition, germination, and senescence (Denancé *et al*., 2014). We checked the expression of *Musa*ENODL3 in the leaves of *enodl3* events before and after Xcm inoculation. Artificial infection was done by inoculating Xcm culture in the midrib of the leaf, simulating the way farmers infect the plants using contaminated cutting tools. The expression study showed that *MusaENODL3* expression was suppressed in the *enodl3* events before and after Xcm infection. The edited events were resistant to BXW, implicating the role of ENODL in plant immunity beyond nodulation. The disruption of *MusaENODL3* probably activated some defense-related genes in order to provide resistance to edited events against the pathogen. However, further studies are required to check the interaction between *MusaENODL3* and defense-related genes.

In order to gain further insights into the role of *MusaENODL3* in plant immunity, CRISPR/Cas9 tool was employed to engineer targeted mutations in the *MusaENODL3* gene of banana cultivar 'Gonja Manjaya', which is a polyploid with two distinct genomes (A and B). To achieve desirable traits effectively, it is essential to simultaneously disrupt all the alleles of the MusaENODL3 gene. In this study, we multiplexed two gRNAs targeting the conserved region of *MusaENODL3* in exon 2. This strategy yielded a remarkably high mutation frequency of 100%, resulting in the knockout of all the alleles of the gene. The use of multiple gRNAs proved highly efficient, increasing the likelihood of generating large deletions leading to knockout with high mutation efficiency as previously demonstrated (Cui *et al*., 2019). While most edited events showed mutations at both target sites, some events exhibited mutations only at target site 2.

The *enodl3* events yielded a spectrum of potential mutations, including nucleotide insertions and substitutions and short- or long-fragment deletions, particularly around the PAM region, as reported in previous studies (Li *et al*., 2018). The sequencing results of edited events showed various types of mutations, including homozygous, heterozygous, and chimeric types of mutations. Interestingly, all the sequenced events showed mutations in all the alleles, with only one mutant (E13) exhibiting homozygous mutations, while the rest showed either heterozygous or chimeric mutations. This variability in the mutation patterns may be attributed to allelic differences or the continuous activity of CRISPR/Cas9 in somatic cells, leading to various indels, a phenomenon previously observed in CRISPR/Cas9 edited T0 generation rice (Zhang *et al*., 2014).

The functional loss of *MusaENOD3* resulted in a significant reduction in the transcript level of the gene, which could potentially lead to lower MusaENOD3 protein levels. The *enodl3* events showed enhanced resistance against Xcm in the greenhouse compared to control plants, confirming the role of *MusaENODL3* in disease susceptibility. During early infection with Xcm, the expression of the *MusaENODL3* gene was repressed in the edited events compared to control plants, similar to that of the BXW-resistant banana '*Musa balbisiana*', leading to disease resistance. These results strongly suggest *ENODL* genes play an important role in plant immunity, and their accumulation might be required for pathogen colonization, infection, and symptom development.

The precise mechanism by which *ENODL* genes are involved in plant defense remains unclear. It has been reported that nod factors (NFs) induce a modest defense response upon pathogen recognition, including the production of reactive oxygen species (ROS) and the upregulation of defense genes (Peleg-Grossman *et al*., 2009; Grundy *et al*., 2023). However, in non-leguminous plants, some nod factors spike Ca2+, activating the expression of *ENOD* genes, thus facilitating pathogen infection and disease progression (Chaulagain and Frugoli, 2021). In Arabidopsis, NFs attenuated defenses via LYSIN MOTIF-CONTAINING RECEPTOR-LIKE KINASE 3 (AtLYK3). Chitinases were implicated in this host-specific recognition via NF degradation activity (Grundy *et al*., 2023). The disruption of *MusaENODL3* in banana probably activated chitinases, which degraded the NFs, resulting in low *MusaENODL3* accumulation and hence BXW resistance.

Banana Xanthomonas wilt poses a significant threat to banana production, emphasizing the need to develop resistant cultivars for effective mitigation. The *enodl3* mutants offer a valuable source of genetic diversity, holding promise for enhancing the resistance of existing banana varieties against BXW. Leveraging the allelic diversity of these mutants can provide novel genetic traits for elite germplasm, particularly in cases where preferred varieties are susceptible to BXW. By introgressing the advantageous traits found in *enodl3* mutants into breeding parents, we can streamline the breeding process and create BXW-resistant banana varieties that align with farmers' preferences. This approach offers a pragmatic and accessible solution to combat BXW while improving banana crops.

In summary, our results provide strong evidence that *ENODL* plays an essential role in plant-pathogen interaction in non-leguminous crops. The disruption of the *MusaENODL3* gene

did not exert any noticeable adverse effects on plant morphology. The *enodl3* events exhibited normal growth and development, reaffirming the robustness and resilience of the banana plants even in the absence of a functional *MusaENODL3* gene. Nonetheless, comprehensive field studies are needed to thoroughly assess the potential implications of *MusaENODL3* knockout on plant growth and development. Despite this, these results hold promising implications for the possible use of *MusaENODL3* editing in disease-resistant banana breeding programs aimed at developing resistance to BXW, as they suggest that modifications in this gene do not compromise overall plant morphology and development. This study demonstrates that CRISPR/Cas9 -mutagenesis of *MusaENODL3* reduced its expression and activated the defense pathway, resulting in increased resistance to BXW. While the edited events showed disease resistance under controlled greenhouse conditions, they need to be validated in field trials. Manipulating the *MusaENODL3* gene opens new avenues for enhancing disease resistance in banana and offers insights into boosting plant immunity against bacterial diseases.

Materials and methods

Phylogenetic analysis of early nodulin gene

Nodulin-like protein sequences for *Arabidopsis thaliana* (132 proteins) and *Oryza sativa* (87 proteins) already identified in the earlier study by Denancé *et al*., (2014) were downloaded from the Arabidopsis Information Resource (TAIR—https://www.arabidopsis.org/) and the rice genome annotation project (http://rice.uga.edu/), respectively. For *Medicago truncatula* (118 proteins), *Musa acuminata* (69 proteins), and *Musa balbisiana* (65 proteins), only the proteins identified containing the Interpro domain (IPR003245), which is the phytocyanin-containing domain, were downloaded from Interpro (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR003245/) and the Banana Genome Hub (https://banana-genome-hub.southgreen.fr/), respectively. The protein sequences were aligned using the Clustal Omega (Madeira *et al*., 2022) multiple sequence alignment program, and a phylogenetic tree was displayed using the ggtree package in R (Guangchuang, 2022).

Relative expression of *MusaENODL3* transcript in susceptible and resistant banana

The second fully open leaf of 1-month-old *in vitro* plantlets of the BXW-susceptible banana cultivar 'Gonja Manjaya' and the BXW-resistant wild-type progenitor '*Musa balbisiana*' were inoculated with Xcm. The inoculated leaf sample was collected 12 h post-inoculation (hpi). Total RNA was extracted from the leaves using a Qiagen Plant RNeasy Kit according to the manufacturer's instructions to determine transcript accumulation of *MusaENODL3*. cDNA was synthesized from 1 μg of total RNA using LunaScript™ RT SuperMix (NEB, Cat. E3010L) according to the instruction in the user manual. qRT-PCR was performed with the primers ENODL_qPCR_F and ENODL_qPCR_R (Table S1), designed from the conserved region of Ma11_g13300.1 and Mb11_g12710.1, using SYBR Green Master Mix (Applied Biosystems; www.lifetechnologies.com) in a QuantStudio5 real time PCR system (Applied biosystems, Thermo Fisher Scientific). *Musa* primers, Musa 25S_F and Musa 25S_R were used as an internal control (Table S1). The $2^{-\Delta Ct}$ method was used to calculate the relative gene expression levels (Livak and Schmittgen, 2001).

Preparation of early nodulin-like plasmid construct

Banana homologue of early nodulin-like protein 3 (*MusaENODL3*), Ma11_g13300.1, and Mb11_g12710.1 gene sequences were downloaded from banana genome A (*Musa accuminata*) and genome B (*Musa balbisiana*) from the banana genome hub (http://banana-genome-hub.southgreen.fr) (Droc *et al*. 2022), respectively. The *MusaENODL3* gene sequences from A and B genomes were aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/) to identify conserved regions. Two gRNAs, gRNA1: ACACCGTGGTCACTTTCAAC and gRNA2: CAACTGCCTGAAGAACGAGT were designed using Alt-R Custom Cas9 crRNA Design Tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). The gRNAs, together with their corresponding reverse sequences, were synthesized as oligos after adding the appropriate adaptors to the 5′ end (Table S1) to enable cloning to the expression plasmids. The CRISPR/Cas9 construct containing the gRNAs was prepared as described by Ntui *et al*. (2020). The gRNA expression vectors pYPQ131 (for gRNA1) and pYPQ132 (for gRNA2) were linearized with the enzymes BsmBI to produce 4 bp overhang. Forward and reverse oligos of each gRNA were phosphorylated and annealed using T4 polynucleotide kinase according to Lowder *et al*. (2015) and modified by Ntui *et al*. (2020). The phosphorylated and annealed oligos were ligated to the linearized expression vectors and sequenced. The two gRNAs with confirmed sequences were assembled into pYPQ142 by Golden Gate Assembly. The pYPQ142 together with the Cas9 entry vector pYPQ167 was cloned into the Gateway binary vector pMDC32 by LR clonaseTM (Invitrogen, New Zealand) recombination reaction. After confirmation, the plasmid construct pMDC32_Cas9_MaENODL3 was transformed into *Agrobacterium* strain EHA105 by electroporation and confirmed by PCR. One positive colony was used for banana transformation.

Generation of mutant events

The embryogenic cell suspensions (ECS) of 'Gonja Manjaya' were developed using multiple buds as described by Tripathi *et al*. (2012). The CRISPR/Cas9 plasmid pMDC32_Cas9_MusaENODL3 (Figure 1c) was delivered to ECS of 'Gonja Manjaya' through *Agrobacterium*-mediated transformation and transgenic events were generated based on the protocol described by Tripathi *et al*. (2012). The transgenic events were regenerated from the *Agrobacterium*-infected ECS on media containing hygromycin 25 mg/L, as described by Tripathi *et al*. (2015). The regenerated events were maintained and multiplied by sub-culturing every 6–8 weeks on proliferation medium (PM) at 26–28 °C and a photoperiod of 16 h/8 h for other analysis.

Mutant detection by molecular characterization

PCR analysis to confirm integration of Cas9 gene

Genomic DNA was extracted from 100 mg of fresh leave samples collected from regenerated and wild-type plants using the cetyltrimethylammonium bromide (CTAB) method (Stewart and Via, 1993). All hygromycin-resistant transgenic events were characterized by PCR using the primers 35S_F and Cas9_R (Table S1). The PCR was performed in a 20 μL reaction volume comprising of 1 μL genomic DNA (100 ng/μl), 2 μL of 10× PCR buffer, 0.4 μL of dNTP mix, 0.5 μL of 10 μM of each primer, 0.1 μL HotStar Taq polymerase, and 15.5 μL nuclease-free water.

PCR amplification conditions were the initial denaturation step at 95 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. After amplification, 10 μL of PCR product was resolved on 1% agarose gel stained with gel red.

PCR analysis to detect band shift

The mutations in the *MusaENODL3* gene were confirmed by PCR band shift analysis. Genomic DNA from wild-type and Cas9-positive plants was used for PCR amplification of the target regions with primers ENOD_seq_F and ENOD_seq_R flanking the target sites (Table S1). The PCR reaction was set up as described above. PCR amplification conditions were as follows: initial denaturation step at 95 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. PCR amplicons were assessed for band shift by resolving 10 μL of PCR products on 1% agarose gel, stained with gel red.

Sequence analysis to detect targeted mutations in the MusaENDOL3 gene

PCR products derived from band shift analysis were prepared for Sanger sequencing using the BigDye Terminator v3.1 sequencing system (Thermo Fisher). The PCR products were purified with QIAquick PCR purification Kit (Qiagen) and subjected to sequencing as follows: A 10 μL reaction mixture containing 2.5 μL PCR product, 1 μL of 5× sequencing buffer, 0.5 μL of Big Dye Terminator, 1 μL of 100 ng/μl of either ENOD_seq_F or ENOD_seq_R primer (Table S1), and 5 μL of nuclease-free water was prepared. The reaction was amplified in a thermal cycler using the following conditions: initial denaturation step at 96 °C for 2 min, followed by 40 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s, extension at 60 °C for 4 min, and final extension at 72 °C for 4 min. After PCR amplification, the sequencing product was purified in a solution of ethanol, EDTA, and sodium acetate, resuspended in 10 μL HiDi formamide and sequenced using ABI 3130 DNA sequencer (Applied Biosystems, California). The sequence chromatograms were analysed using SnapGene software (www.snapgene.com). Ten replicates per transgenic event were sequenced.

Evaluation of the edited plants for plant growth

Well-rooted plantlets of ten edited events and wilt-type control plants with three replicates each were transferred to disposable plastic cups (10 cm diameter) containing autoclaved soil for weaning and hardening. The plants were maintained in a greenhouse in a humidity chamber for 4 weeks. After 4 weeks, the plants were transplanted to bigger pots (30 cm diameter) containing autoclaved soil. Plant growth parameters (plant height, girth at middle of pseudostem, total number of developed green leaves, length and width of third leaf) were recorded from the edited events and control wild-type plants 90 days after potting. The total leave area (TLA) in cm^2 of the plant was estimated using the formula (Kumar *et al*., 2002; Tripathi *et al*., 2014a,b).

$$\text{Total leaves area} = 0.8 \times L \times W \times N$$

where: L = length of third fully developed middle leaf (cm); W = width of the same leaf (cm); N = total number of green leaves in a plant.

Evaluation of edited events for BXW resistance in greenhouse conditions

Three-month-old plants of ten edited events and control wild-type plants were evaluated for resistance to BXW disease under greenhouse conditions. Midribs of the second fully open leaves of 90-day-old plants were injected with 100 μL of the bacterial culture (10^8 cfu/mL) (Tripathi *et al*., 2010). Three replicates were used for each edited event. Appearance of first disease symptoms such as leaf drooping or wilting, necrosis, and complete wilting of the plantlets was recorded up to 60 dpi. Disease severity was recorded on a scale of 0–5 with 0—no symptoms, 1—only the leaf inoculated wilted, 2—two to three leaves wilted, 3—four to five leaves wilted, 4—all the leaves wilted but the plant still alive, 5—whole plant died. A disease severity index (DSI) was calculated for all the edited events along with control plants.

$$\text{DSI}\ (\%) = [\sum(\text{Disease severity scale} \times \text{no. of plants in each scale})]/ [(\text{total number of plants}) \times (\text{maximal disease severity scale})] \times 100$$

Relative resistance was calculated according to Tripathi *et al*. (2014a).

$$\text{Resistance}(\%) = (\text{Reduction in wilting in event}/ \text{Proportion of leaves wilted in control}) \times 100$$

Expression of *MusaENODL3* transcript in edited events

qRT-PCR was performed with nine edited events (E1, E6, E11, E13, E17, E32, E37, E38, and E39) and wild-type plants. RNA was extracted from leaf samples of 1-month-old edited and wild-type *in vitro* plantlets challenged with Xcm. Leaves were collected at 12 hpi based on the previous comparative transcriptomic study (Tripathi *et al*., 2019), where upon pathogen attack, *MusaENODL3* was found to be differentially expressed at 12 hpi in BXW resistant and susceptible banana genotypes. cDNA was synthesized, and qRT-PCR was performed using the primers ENOD_qPCR_F and ENOD_qPCR_R (Table S1). The relative expression levels were calculated as described under the section relative expression of *ENODL3* transcript in susceptible and resistant banana.

Statistical analysis

All the data for disease assay and plant growth analysis were analysed by Minitab statistical program. Analysis of variance (ANOVA) was performed and significant differences between the means of three independent groups were taken using Tukey family analysis at $P = 0.001$.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

LT conceived the idea; LT, VON, and JNT planned the experiments; JNT generated the mutants, evaluated for plant

growth, and performed phenotyping; VON designed the guides, prepared the construct, and performed molecular analysis of the mutants; TS worked on phylogenetic analysis; all authors wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Nucleotide sequence alignment of *MusaENODL3* from reference genome of *Musa accuminata* (AA genome), gene ID Ma11_g13300, and *Musa balbisiana* (BB genome), gene ID Mb11_g12710. Conserved regions are shown in black, and mismatches are shown in blue. Green nucleotides indicate primers flanking the gRNAs, which were used for sequencing, red nucleotides indicate gRNAs, and purple nucleotides indicate PAM (protospacer adjacent motif) segment.

Figure S2 PCR analysis of *enodl3* edited events of 'Gonja Manjaya'. (a) PCR analysis to confirm the presence of the Cas9 gene in edited events. PCR was done with the primers 35S_F and Cas9_R (Table S1). (b) PCR analysis to detect band shifts in edited events. If Cas9 cleaved the two gRNAs simultaneously, edited events were supposed to have a band shift of about >35 bp compared to the wild-type control plant. M, molecular marker; EW, empty well; C, Non-edited control plants; NT, no template control; 1–39, independent edited events.

Table S1 Primer sequences used in the study.