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Clade V *MLO* Genes Are Negative Modulators of Cucumber Defence Response to *Meloidogyne incognita*

Xiaoxiao Xie[1,2,3] | Jian Ling[1] | Shaoyun Dong[1] | Mingjuan Zhai[1] | Junru Lu[1] | Jianlong Zhao[1] | Xueyong Yang[1] | Xin Dong[1] | Yan Li[1] | Richard G. F. Visser[2] | Yuling Bai[2] | Zhenchuan Mao[1] | Shengping Zhang[1] | Bingyan Xie[1]

[1]State Key Laboratory of Vegetable Biobreeding, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China | [2]Plant Breeding, Wageningen University & Research, Wageningen, the Netherlands | [3]Graduate School Experimental Plant Sciences, Wageningen University and Research, Wageningen, the Netherlands

Correspondence: Bingyan Xie (xiebingyan@caas.cn)

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ABSTRACT

Cucumber production is seriously constrained by *Meloidogyne incognita*. Because no resistance resources to the pathogen have been reported, disabling susceptibility genes may represent a novel breeding strategy to introduce resistance against this nematode in cucumber. Here, we studied the clade V *MLO* genes for their involvement in the interaction between cucumber and *M. incognita*. Our results showed that *Arabidopsis* clade V *MLO* mutants were resistant to *M. incognita*. Cucumber has three clade V *MLO* genes, *CsaMLO1*, *CsaMLO8* and *CsaMLO11*, with upregulated expression upon inoculation with *M. incognita*. Heterologous overexpression of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in *Arabidopsis* mutants restored susceptibility to varying degrees. Silencing and knockout of individual clade V *MLO* genes in cucumber reduced susceptibility to *M. incognita*. The cucumber CRISPR mutants produced similar fruits as the wild type (WT) did. Although the yields of two single mutants ($M11^1$ and $M11^2$) and two double mutants ($M8^1$ $M11^1$ and $M8^1$ $M11^2$) were reduced compared to WT, the yields of $M8^1$ and $M8^2$ were not decreased. In summary, clade V *MLO* genes function as susceptibility genes for *M. incognita* in cucumber. Among them, *CsaMLO8* may be the most promising candidate for *M. incognita* resistance breeding in cucumber.

1 | Introduction

Cucumber (*Cucumis sativus*) is an economically important vegetable crop with a global production of more than 90 million tonnes in 2021 (https://www.fao.org/). Cucumber growth and production are seriously constrained by the root-knot nematode *Meloidogyne incognita*, which severely affects cucumber yield and quality and causes large economic losses (Fassuliotis 1970; Mukhtar et al. 2013). Chemical nematicides, the most popular control measure against nematodes in agriculture, have negative effects on the environment and may compromise food safety (Hassan et al. 2013). To protect against nematode infestation, the use of resistant cucumber varieties is thus preferable. However, the breeding of varieties with resistance to *M. incognita* is currently difficult to achieve, as resistant cucumber materials and cucumber resistance genes (*R* genes) are lacking (Mukhtar et al. 2013; Wang et al. 2018).

To achieve durable, broad-spectrum disease resistance, a novel breeding strategy has been proposed: disabling of plant disease susceptibility genes (*S* genes) (Pavan et al. 2010). For example, the barley *mlo* mutant, developed through recessive mutations

Xiaoxiao Xie, Jian Ling and Shaoyun Dong contributed equally to this work.

of the best-known *S* gene *MLO* (mildew resistance locus o), exhibits broad-spectrum durable resistance to powdery mildew and has been extensively grown for more than 45 years (Jørgensen 1992; Lyngkjær et al. 2000). *S* genes are plant genes that are exploited by pathogens and pests to infect hosts successfully (van Schie and Takken 2014).

To date, an increasing number of *MLO* homologues have been detected with 7 to 39 *MLO* genes per plant species that can be divided into seven phylogenetic clades (Acevedo-Garcia et al. 2014; Kusch et al. 2016). In dicotyledonous species, specific members in clade V have known functions as *S* genes towards powdery mildew (Appiano et al. 2015; Zheng et al. 2013). For example, T-DNA insertion mutations in three *MLO* homologues (*AtMLO2*, *AtMLO6* and *AtMLO12*) have been found to reduce susceptibility to powdery mildew in *Arabidopsis*, with the mutation of *AtMLO2* having the largest effect (Consonni et al. 2006; Devoto et al. 2003). Membrane-anchored MLO proteins facilitate powdery mildew penetration of epidermal cells (Büschges et al. 1997; van Schie and Takken 2014). When functional MLO proteins are lacking, powdery mildew fungi fail to enter their hosts (Zheng et al. 2013). The role of MLO proteins in powdery mildew susceptibility has been confirmed in various vegetables including tomato, pepper and cucumber (Appiano et al. 2015; Bai et al. 2008; Berg et al. 2015; Nie et al. 2015; Zheng et al. 2013).

The functions of *MLO* genes are not restricted to plant–powdery mildew interactions (Acevedo-Garcia et al. 2014). For instance, cucumber *CsMLO1* (*CsaMLO1*; *Csa1G085890.1*) and *CsMLO2* (*CsaMLO8*; *Csa5G623470.1*) have been reported to negatively regulate the defence response to *Corynespora cassiicola* (Yu et al. 2019). Likewise, pepper *CaMLO2* is involved in bacterial and oomycete proliferation (Kim and Hwang 2012). Interestingly, *CaMLO2* has additional functions in response to the phytohormone abscisic acid and drought stress. *MLO* genes may have multiple roles in plant development and response to stresses (Lim and Lee 2014).

Several *S* genes associated with root-knot nematodes or cyst nematodes have been reported in *Arabidopsis*. The knockout of *CCS52* interferes with the development of galls and syncytia in *Arabidopsis* roots, resulting in smaller feeding sites (de Almeida Engler et al. 2012; Vinardell et al. 2003). In addition, nematode infection tests of *CCS52* RNAi knockdown lines revealed that fewer juveniles of root-knot nematodes and cyst nematodes were able to mature compared with infected wild-type (WT) plants (de Almeida Engler et al. 2012). Nematode infection tests of *WRKY23* knockdown lines resulted in lower infection of the cyst nematodes (Grunewald et al. 2008). Transgenic plants overexpressing *PME3* and *SPDS2* exhibited increased susceptibility to cyst nematodes in *Arabidopsis* (Hewezi et al. 2008, 2010). Despite those findings, no reports have appeared regarding the involvement of *MLO* genes in nematode infection.

Among the 13 *MLO* genes identified in cucumber, *CsaMLO1* (*Csa1M085890.1*), *CsaMLO8* (*Csa5M623470.1*) and *CsaMLO11* (*Csa6M292430.1*), which cluster in clade V, are known MLO-like susceptibility genes promoting powdery mildew infection (Schouten et al. 2014). In addition, *CsaMLO1* and *CsaMLO8* are both negative modulators of the defence response to Corynespora leaf spot (Yu et al. 2019). Therefore, *MLO* genes in cucumber may have additional functions. In this study, we examined whether *MLO* genes also act as negative regulators of resistance to *M. incognita* by analysing these genes in *Arabidopsis* mutants and CRISPR cucumber mutants.

2 | Results

2.1 | Phylogenetic Analysis of *MLO* Genes From Cucumber and *Arabidopsis*

A phylogenetic analysis was carried out using full-length sequences of 28 MLO proteins from cucumber and *Arabidopsis* (Figure 1). In the resulting phylogenetic tree, all proteins were distributed into five clades based on the classification described in a previous study (Devoto et al. 2003). The results were consistent with the results of previous studies (Schouten et al. 2014; Yu et al. 2019). There are seven, five and eight MLO proteins in Clade I, Clade II and Clade III, respectively. Three CsaMLOs (CsaMLO1, CsaMLO8 and CsaMLO11) along with AtMLO2, AtMLO6 and AtMLO12 from *Arabidopsis* belong to Clade V. However, only two MLO proteins (AtMLO3 and CsaMLO2) cluster together and define Clade VI.

2.2 | Expression Analysis of *CsaMLO* Genes Using Reverse Transcription-Quantitative PCR

To analyse the expression of *CsaMLO* genes in cucumber roots at the early stage of *M. incognita* infection, root samples were collected from a susceptible cucumber cultivar Xitaimici before nematode infection (0) and 1, 2 and 3 days post-inoculation (dpi) (Figure 2). We amplified 18S rRNA sequences of *M. incognita* in cucumber roots by PCR and examined nematodes in roots at 1, 2 and 3 dpi using a microscope. As revealed by the PCR results and light microscopic images, *M. incognita* had successfully invaded cucumber roots (Figure S1). The successfully inoculated root tissues were used for gene expression analyses, thereby confirming the correlation between the expression patterns of candidate genes and nematode infection. *CsaMLO1*, *CsaMLO11* and *CsaMLO13* were strongly expressed in roots 1–3 dpi, while expression levels of *CsaMLO4*, *CsaMLO5*, *CsaMLO6*, *CsaMLO7*, *CsaMLO8* and *CsaMLO10* were increased to varying degrees. Interestingly, the expression of two genes (*CsaMLO2* and *CsaMLO9*) decreased significantly after inoculation. Taken together, the results suggested that most *CsaMLO* genes play a role in the cucumber response to the early stage of *M. incognita* parasitism. The expression of all of the clade V *MLO* genes (*CsaMLO1*, *CsaMLO8* and *CsaMLO11*) in cucumber roots was induced by *M. incognita*. We therefore hypothesised that clade V *MLO* genes may be involved in the cucumber response to *M. incognita* infection.

2.3 | Phenotypic Characterisation of *Arabidopsis* Mutants Following *M. incognita* Infection

To further understand the functional contribution of clade V *MLO* genes during *M. incognita* infection, nine *Arabidopsis* mutants (in the genetic background of Col-0; Figure S2) were

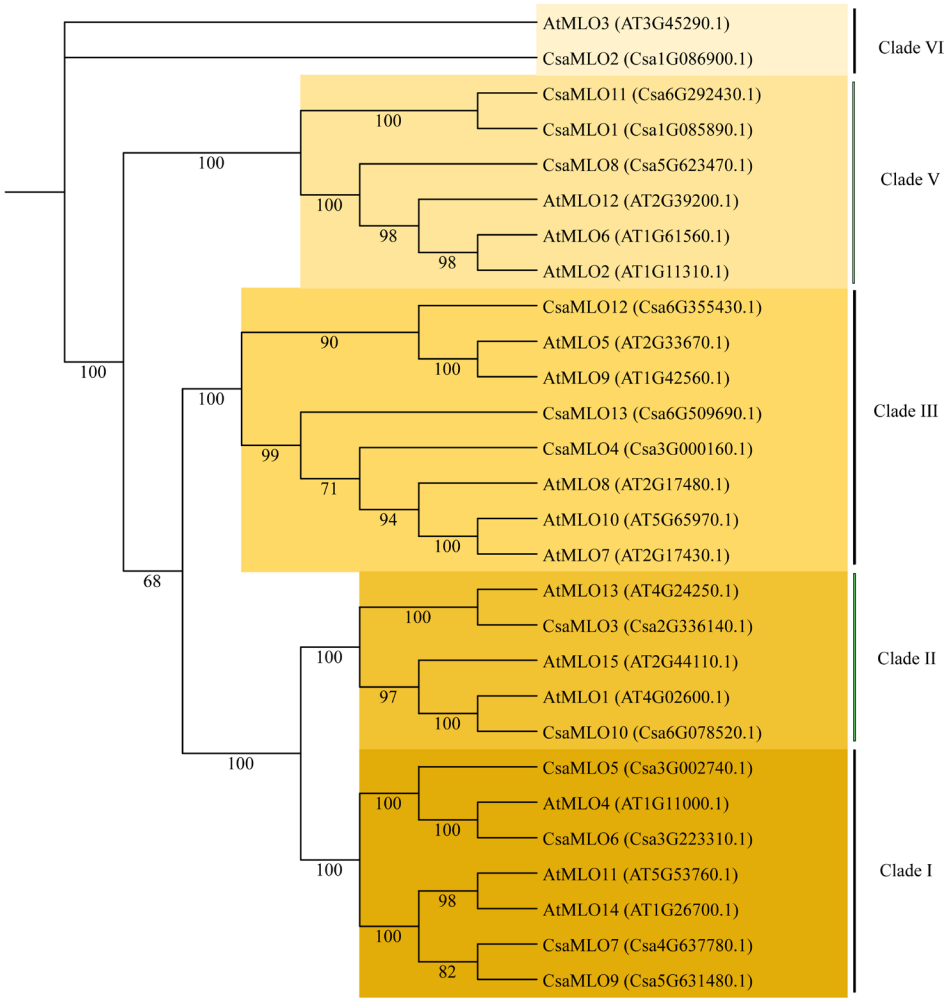

FIGURE 1 | Phylogenetic relationships of MLOs in cucumber and *Arabidopsis*. At, *Arabidopsis thaliana*; Csa, *Cucumis sativus*.

collected. The mutants and the control (Col-0) were inoculated with *M. incognita*, and phenotypic observations were carried out 35 days later. Compared with Col-0, seven mutants were identified as resistant to *M. incognita* ($p < 0.0001$; Table 1). The average number of galls formed on the roots of the three mutants (*atmlo2-5*, *atmlo9* and *atmlo12*) was approximately 6, 7 and 8, respectively. The number of galls on the remaining resistant lines (*atmlo1-5*, *atmlo6-2*, *atmlo7* and *atmlo8*) ranged between 10 and 20. In contrast, the average number of galls on *atmlo3* (~38) was not significantly different ($p = 0.28$) from that of Col-0 (~33). The mean number of galls on *atmlo13* (~33) was the same as that on Col-0 roots.

2.4 | Impaired Resistance to *M. incognita* in *CsaMLO1*, *CsaMLO8* and *CsaMLO11*-Overexpressing *Arabidopsis* Mutants

To further confirm whether defence responses to *M. incognita* are affected by *CsaMLO1*, *CsaMLO8* or *CsaMLO11* in cucumber, the correspondence between 15 *Arabidopsis MLO* genes and 13 cucumber *MLO* genes was determined through BLAST analysis of amino acid sequences from version 2 of the Cucurbit Genomics Database (http://cucurbitgenomics.org/; Cucumber [Chinese Long] v2 Genome) (Table S2) (Devoto et al. 2003; Huang et al. 2009; Schouten et al. 2014). According to the analysis, the homologous genes of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in cucumber were *AtMLO12*, *AtMLO2* and *AtMLO6* in *Arabidopsis*, respectively. Overexpression vectors containing *CsaMLO1*, *CsaMLO8* or *CsaMLO11* fused with GFP were constructed. PCR analysis and GFP observation verified that p35S:GFP-*CsaMLO1*, p35S:GFP-*CsaMLO8* and p35S:GFP-*CsaMLO11* were integrated into the genomes of *atmlo12* (SALK_041042C), *atmlo2-5* (SAIL_878_H12) and *atmlo6-2* (SAIL_523_D09), respectively (Figures S3 and S4A–C). Finally, two independent T_3 transgenic *Arabidopsis* lines expressing *CsaMLO1*, *CsaMLO8* or *CsaMLO11* were generated (p35S:GFP-*CsaMLO1*-1/2, p35S:GFP-*CsaMLO8*-1/2 and p35S:GFP-*CsaMLO11*-1/2).

To determine whether these T_3 transgenic lines exhibit any developmental irregularities, root length was measured 10 days after sowing. The root lengths of *Arabidopsis* mutants and transgenic plants were compared with Col-0 under normal conditions, and the results are summarised in Figure S4. The root length of *atmlo12* and transgenic plants expressing *CsaMLO1* was not significantly different from that of Col-0. However, when the function of the *AtMLO2* gene was lost, the *Arabidopsis* mutants showed a marked decrease in root length, whereas the complemented transgenic plants exhibited partial restoration. Similarly, the root length of *atmlo6-2* was significantly shorter

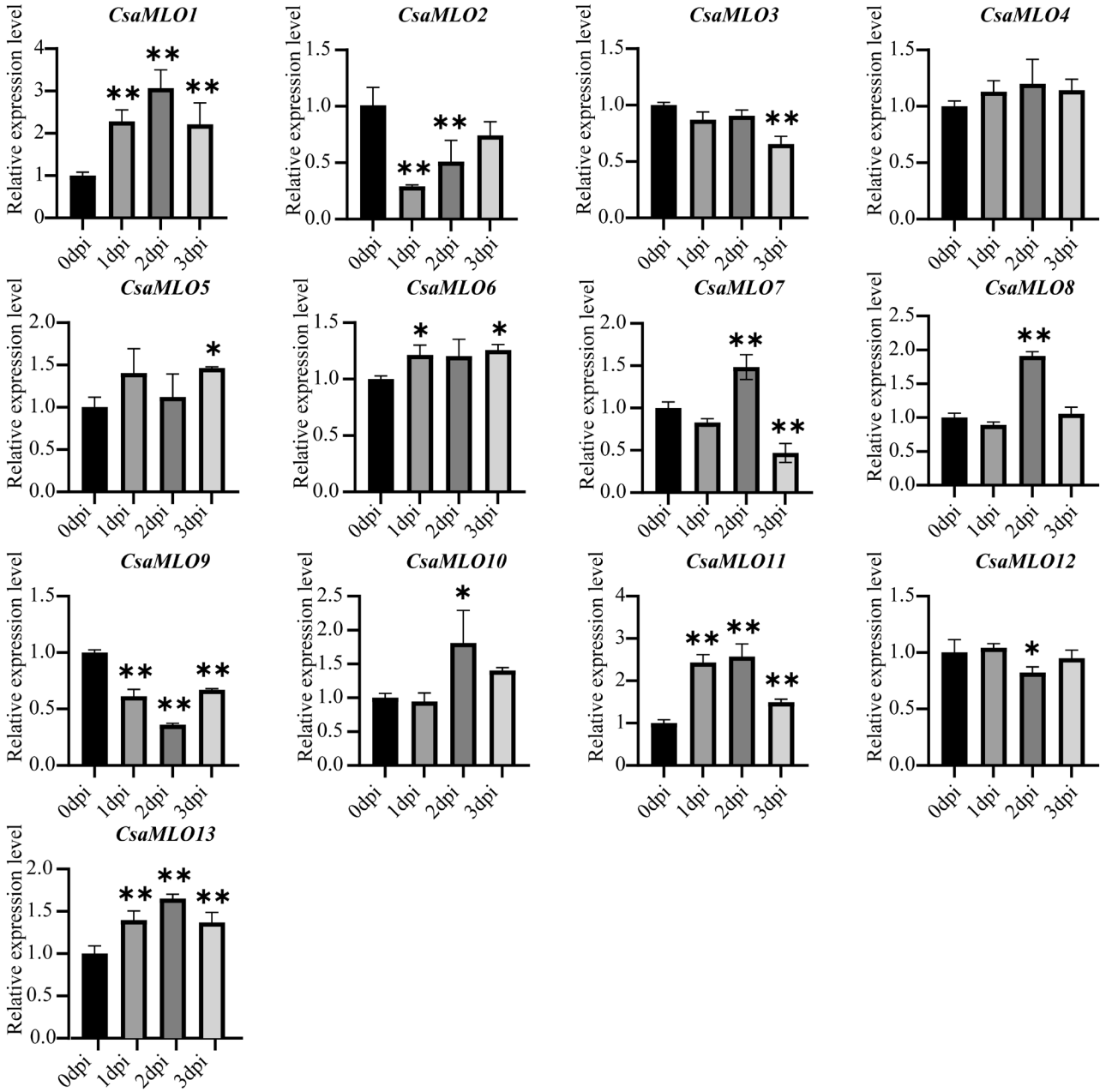

FIGURE 2 | Expression patterns of *CsaMLO* genes in roots of cucumber cultivar Xitaimici inoculated with *Meloidogyne incognita*. Relative expression levels of 13 cucumber *MLO* genes at 0, 1, 2 and 3 days post-inoculation (dpi) based on the $2^{-\Delta\Delta Ct}$ method is shown. The relative gene expression levels were initially normalised to the levels of the reference genes cucumber *UBI*, followed by further normalisation to the cucumber samples at 0 dpi. Data are means ± *SD* of three biological replicates ($n = 3$). Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$; one-way ANOVA followed by Dunnett's multiple comparisons test).

than that of the WT, but overexpression of *CsaMLO11* completely restored root length.

The transgenic lines and *Arabidopsis* mutants were then subjected to *M. incognita* infection with three biological replicates of 7–10 plants each. Following infection, the number of galls and egg masses on the three *Arabidopsis* mutants was significantly lower than that on the susceptible control (Col-0) (Figure 3). These results confirmed that the loss-of-function mutations in *AtMLO2*, *AtMLO6* and *AtMLO12* enhanced the resistance of *Arabidopsis* to *M. incognita*. We also observed a significant increase in the susceptibility of cucumber ectopic expression lines with clade V *MLO* genes compared with the *Arabidopsis* mutants (Figure 3). As judged by the number of galls per plant, overexpression of *CsaMLO8*, followed by *CsaMLO1* and then *CsaMLO11*, indicated the best-restored susceptibility to *M. incognita* (Figure 3A–C). Among the three *CsaMLO* genes, only the overexpression of *CsaMLO8* in *atmlo2-5* significantly increased the number of egg masses ($p < 0.01$). The number of egg masses on *CsaMLO8* transgenic plants (~10.1/6.7) was approximately half that on Col-0 (~16.8) (Figure 3E). In contrast, the number of egg masses on transgenic plants with *CsaMLO1* or *CsaMLO11* was not significantly different from those on *atmlo12* and *atmlo6-2* ($p = 0.66$–0.99) and was significantly less

TABLE 1 | Phenotypic observations of different *Arabidopsis* mutants exposed to *Meloidogyne incognita*.

Genotype	Mutant ID	Number of plants	Number of galls	*p*
Col-0	—	30	33.3 ± 11.8	Control
atmlo1-5	SALK_117153	25	11.6 ± 6.4	< 0.0001****
atmlo2-5	SAIL_878_H12	24	5.9 ± 3.2	< 0.0001****
atmlo3	SALK_027770C	26	38.0 ± 14.1	0.28
atmlo6-2	SAIL_523_D09	13	12.0 ± 5.5	< 0.0001****
atmlo7	SALK_027128C	32	14.3 ± 6.1	< 0.0001****
atmlo8	SALK_024893C	30	10.6 ± 7.9	< 0.0001****
atmlo9	SALK_073198	23	8.3 ± 4.5	< 0.0001****
atmlo12	SALK_041042C	24	6.8 ± 3.5	< 0.0001****
atmlo13	SALK_076325C	29	33.0 ± 11.3	0.99

Note: Data are presented as means ± *SD*.
****$p < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparisons test).

than that on Col-0 ($p < 0.01$) (Figure 3D,F). Overall, our analysis indicated that the overexpression of *CsaMLO8* completely restored cucumber susceptibility to gall formation and partially restored nematode egg-mass generation ability (Figures 3 and S5). Although the overexpression of *CsaMLO1*, *CsaMLO8* or *CsaMLO11* did not completely restore susceptibility to both galls and egg masses in mutants, these results demonstrated the essential role of cucumber clade V *MLO* genes in modulating *M. incognita* parasitism.

2.5 | Improved Resistance to *M. incognita* Through *CsaMLO1*, *CsaMLO8* or *CsaMLO11* Silencing in Cucumber

To determine whether silencing of *CsaMLO1*, *CsaMLO8* or *CsaMLO11* enhances cucumber resistance to *M. incognita*, the cucumber green mottle mosaic virus vector (pV190) was used to construct three silencing vectors (pV190::*CsaMLO1*, pV190::*CsaMLO8* and pV190::*CsaMLO11*) (Figure S6A). We then inoculated *CsaMLO1*-, *CsaMLO8*- or *CsaMLO11*-silenced plants with *M. incognita* and examined their numbers of galls and egg masses per gram. Three independent replicates were performed, with 8–12 plants per replicate. The silencing efficiency of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* was first confirmed by reverse transcription-quantitative PCR (RT-qPCR). Compared with pV190 control plants, *CsaMLO1*, *CsaMLO8* and *CsaMLO11* expression levels in the corresponding silenced plants were reduced by approximately 20%–40%, 40%–50% and 50%–90% in replicates 1, 2 and 3, respectively (Figure S6C–E).

To confirm the role of the three genes in cucumber defence response to *M. incognita*, the number of *M. incognita*-induced galls and egg masses on roots and recorded root fresh weights were counted. The number of galls (Figure 4A–C) and egg masses (Figure 4D–F) per gram of roots was calculated. Although results from the three replicates were slightly biased due to different conditions, the overall trends were similar. Overall, the number of galls per gram on *CsaMLO1*-, *CsaMLO8*- and *CsaMLO11*-silenced plants in the three replicates was significantly reduced compared with the control ($p < 0.01$). In addition, the number of egg masses per gram on *CsaMLO1*-, *CsaMLO8*- and *CsaMLO11*-silenced plants after *M. incognita* inoculation was significantly lower than that on pV190 plants ($p < 0.05$). Taken together, these results indicated that *CsaMLO1*, *CsaMLO8* and *CsaMLO11* silencing enhanced cucumber resistance to *M. incognita* by varying degrees.

2.6 | Enhanced Resistance to *M. incognita* in the *CsaMLO* Cucumber Mutants

To further confirm the functions of cucumber clade V *MLO* genes, four knockout mutant lines were obtained using the CRISPR/Cas9 system. Three homozygous *CsaMLO* mutants ($M8^1$ with a 1-bp deletion, $M11^1$ with a 1-bp insertion and $M11^2$ with a 16-bp deletion) resulting in premature stop codons were identified (Figures 5B and S7). In another homozygous *CsaMLO8* mutant line, $M8^2$, a 6-bp deletion caused a two-amino acid deletion. The two double mutants ($M8^1$ $M11^1$, $M8^1$ $M11^2$) were generated by crossing $M8^1$ with $M11^1$ and $M11^2$, respectively. The number of galls per gram was about 89 on WT (CU2) roots. In marked contrast, all single and double mutants only attained 44–67 galls per gram (Figure 5C). Similarly, the number of egg masses per gram on the six mutants (1.2–3.4) was significantly less than that in the WT (5.5) (Figure 5D). As a general trend, all single and double mutants showed enhanced resistance to the nematodes compared to the WT. In addition, the loss of function of *Csamlo8* and *Csamlo11* increased the resistance to *M. incognita*, similar to that observed in *CsaMLO8*- and *CsaMLO11*-silenced plants. However, there was no significant difference in *M. incognita* resistance between the double mutants and the single mutants. Unfortunately, we did not obtain *CsaMLO1* mutants in this study. However, combining the results of gene editing and gene silencing, we concluded that the loss of function of cucumber clade V *MLO* genes causes an increase in *M. incognita* resistance.

To evaluate the possibility of using these *MLO* genes in *M. incognita* resistance breeding without severe pleiotropic effects,

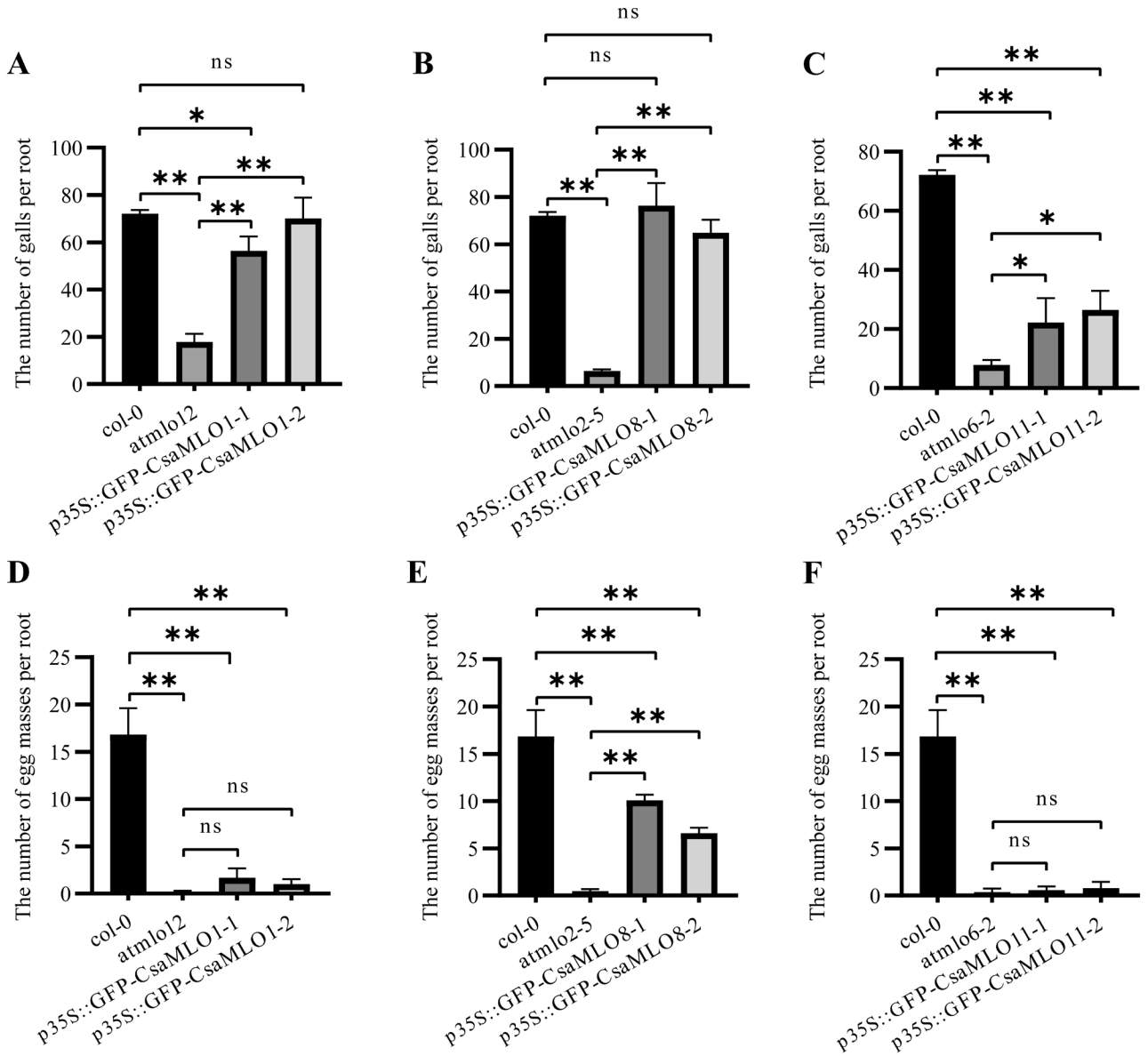

FIGURE 3 | Results of complementation of *Arabidopsis* mutants with *CsaMLO1*, *CsaMLO8* or *CsaMLO11* 40 days after inoculation with *Meloidogyne incognita*. (A–C) Number of galls per plant on susceptible control Col-0, three *Arabidopsis* mutants and all transgenic plants. (D–F) Number of egg masses per plant on Col-0, mutants and transgenic plants. Data are presented as means ± *SD*. $^{*}p < 0.05$, $^{**}p < 0.01$; ns, not significant (Student's *t* test).

we analysed some important plant traits of cucumber mutants and WT, such as plant growth, yield and fruit size. Statistical analysis revealed that the plant height (Figure 5E) and internode length (Figure 5F) of all cucumber mutant plants were not significantly different from those of WT at Beijing, China, in autumn 2022 (A-2022-B). A similar trend was observed at Hainan, China, in spring 2023 (S-2023-H). The leaf length of $M8^1$ $M11^2$ in A-2022-B, and $M8^2$ in S-2023-H was shorter than that of WT (Figure 5G). The leaf length of the other mutants did not differ from that of WT. Only the leaf width of $M8^2$ in S-2023-H was shorter (Figure 5H). The stem diameter of $M8^2$ decreased in both A-2022-B and S-2023-H compared to the WT (Figure 5I). In addition, the stem diameter of $M11^2$ in A-2022-B showed the same trend. The traits affecting fruit size, including fruit length, diameter and seed cavity diameter, were not significantly different from those of WT (Figure 5J–L). Moreover, there was no significant difference in single fruit weight between WT and the mutants (Figure 5M). Finally, we measured the cucumber yield from 43 DAS (days after sowing) to 61 DAS and calculated the number and weight of harvested fruits per plant. The results showed that the number and weight of picked fruits per plant were not affected in the two *CsaMLO8* mutants (Figure 5N,O). The yield of the remaining four mutants showed varying degrees of reduction. These results indicated that some traits related to plant growth were altered in only a few knockout mutants, and all mutants produced fruits similar to those of the WT. Most importantly, neither the number nor weight of picked fruits per plant was reduced in the two *CsaMLO8* mutants. In conclusion, *mlo*-based resistance, especially *CsaMLO8* mutants, may have potential application in cucumber resistance breeding to *M. incognita*.

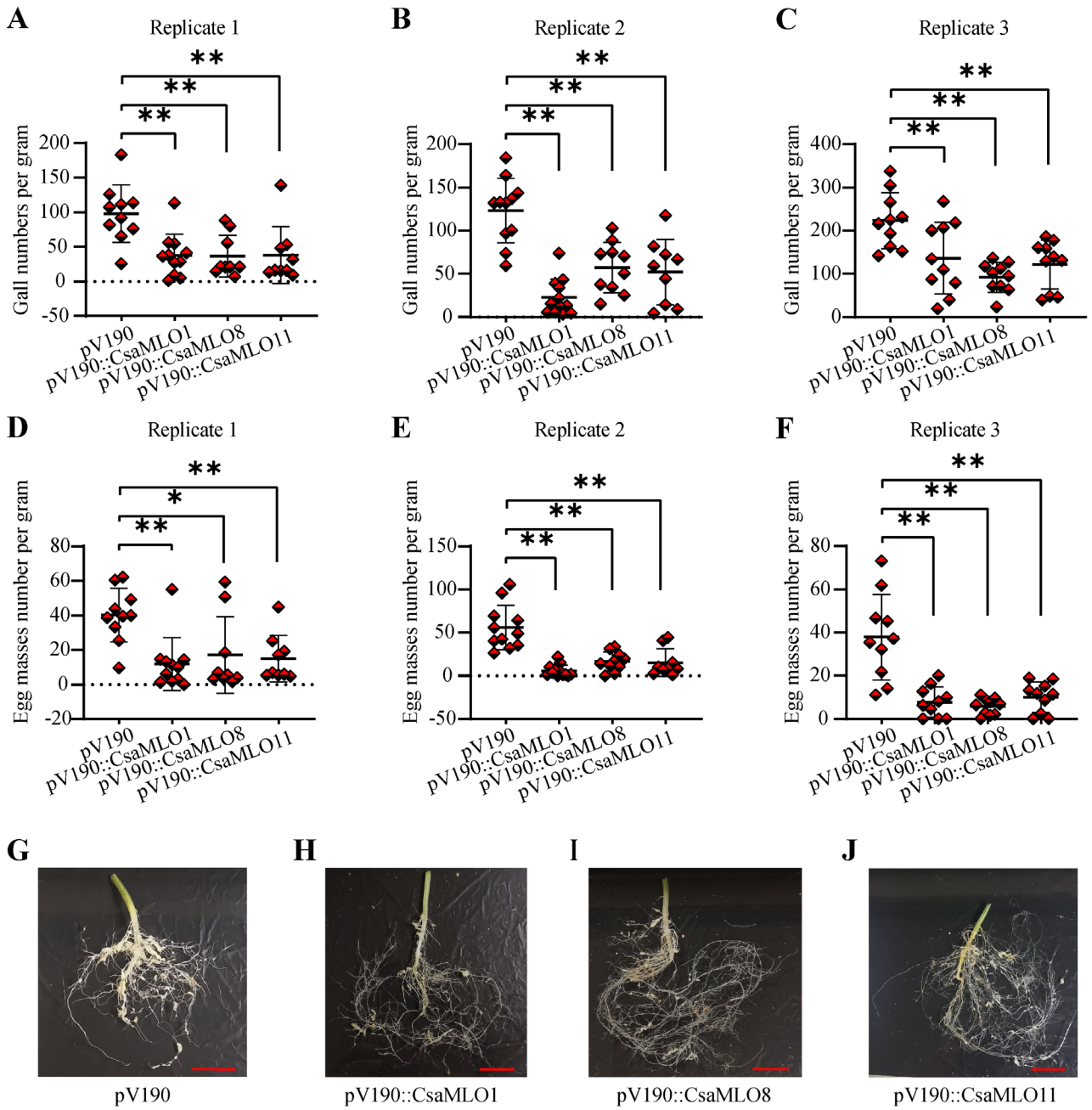

FIGURE 4 | Enhanced cucumber resistance to *Meloidogyne incognita* due to silencing of *CsaMLO1*, *CsaMLO8* or *CsaMLO11*. (A–F) Number of galls per gram (A–C) and number of egg masses per gram (D–F) of virus-induced gene-silenced plants 35 days post-inoculation. Data are presented as means ± *SD*. $*p < 0.05$, $**p < 0.01$; ns, not significant (one-way ANOVA followed by Dunnett's multiple comparisons test). (G–J) Roots of gene-silenced plants. Scale bars = 2 cm.

3 | Discussion

Cucumber, an economically important cultivated vegetable globally, is subject to serious nematode infection, especially from *M. incognita* (Ji et al. 2022). The most environmentally friendly, cost-effective strategy to control *M. incognita* is the development of resistant cultivars. At present, however, no cucumber cultivars have been found that are immune or highly resistant to *M. incognita* (Mukhtar et al. 2013; Walters et al. 1992; Wang et al. 2018; Wehner et al. 1991). Exploiting the resistance that arises from the loss of function of *S* genes may provide a useful strategy to reduce damage caused by *M. incognita* in cucumber.

Many examples demonstrating the potential usefulness of *S* genes in resistance breeding have indeed been reported (van Schie and Takken 2014). In particular, resistance arising from functional defects in *S* genes is potentially robust. For instance, the barley *mlo* mutant providing durable powdery mildew resistance has been cultivated for many decades (van Schie and Takken 2014). According to their action during different stages of infection, *S* genes are divided into three groups: early pathogen establishment, host defence modulation and pathogen sustenance (van Schie and Takken 2014). The first group includes genes involved in host entry; for example, inactivation of *MLO* prevents fungal penetration into host cells (Appiano et al. 2015; van Schie and Takken 2014).

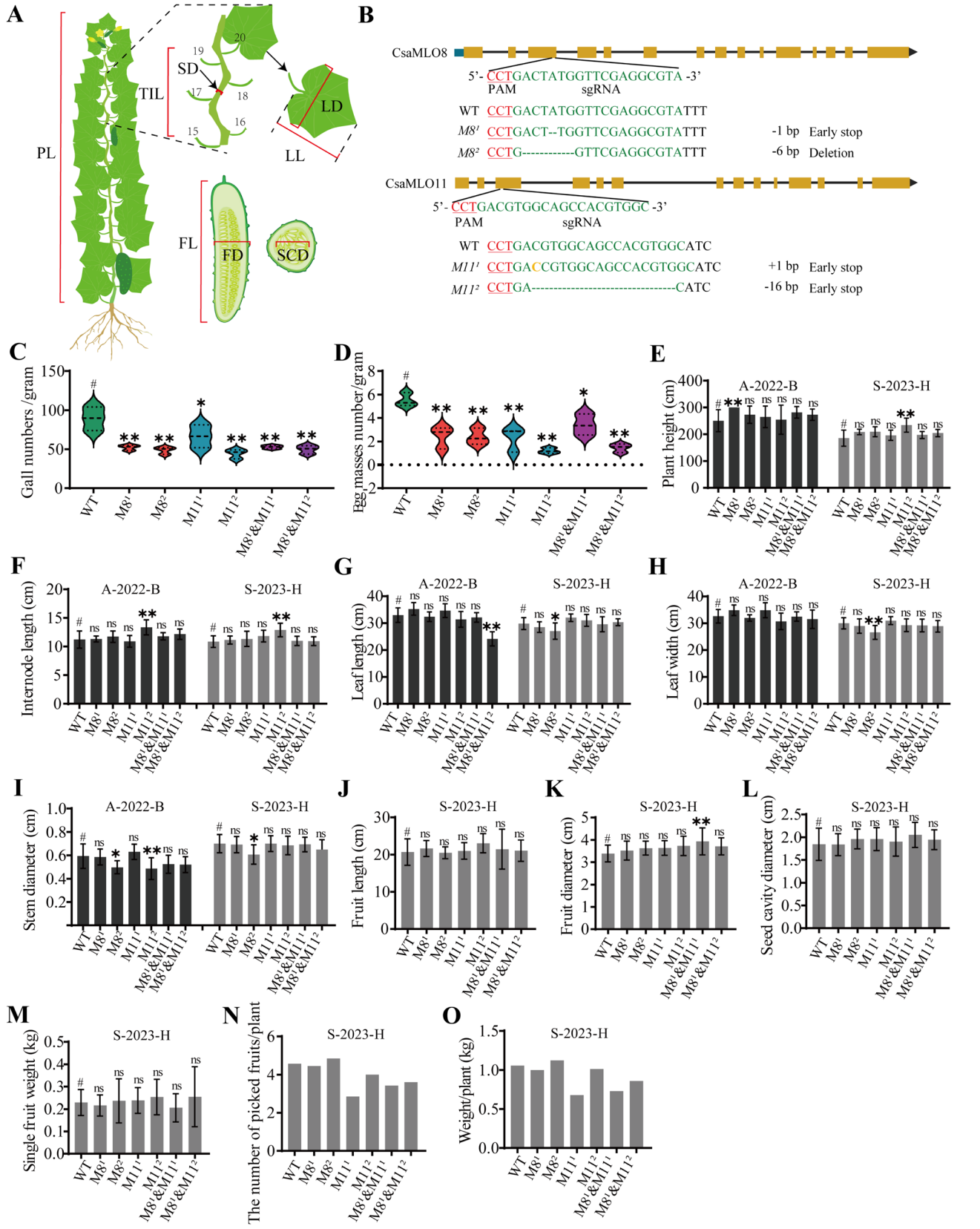

FIGURE 5 | Legend on next page.

Plant-parasitic nematodes must overcome the plant's basal immunity to complete infection. *MLO* genes may facilitate this process, making the host susceptible. Studies have shown that *MLO* genes are regulated by and regulate reactive oxygen species (ROS) and calcium signalling (Jacott et al. 2021; Kim et al. 2002; Lorek et al. 2010). ROS play a key role in host defence against nematodes (Rutter et al. 2022). Nematode infection can trigger a host pathogen-associated molecular

FIGURE 5 | Editing type and phenotype of wild type (WT) (CU2) and *CsaMLO* mutants. (A) Schematic diagram of phenotype including plant length (PL), total length from 15th internode to 20th internode (TIL), leaf length (LL), leaf width (LD), stem diameter (SD), fruit length (FL), fruit diameter (FD) and seed cavity diameter (SCD). (B) The single guide RNA (sgRNA) target site for the CRISPR/Cas9 system in *CsaMLO8* and *CsaMLO11*. The sgRNAs are highlighted by green. Green dash indicates deletion. The protospacer adjacent motif (PAM) sites are underlined and in red, and the yellow letter indicates insertion. The text on the right indicates the editing type. (C, D) Number of galls per gram (C) and number of egg masses per gram (D) of WT and *CsaMLO* mutants at 35 days post-inoculation. (E–I) Comparison of plant height (E), internode length (=TIL/5) (F), leaf length (G), leaf width (H) and stem diameter (I) at adult stage of WT and *CsaMLO* mutants at Beijing, China, in autumn 2022 (A-2022-B) and at Hainan, China, in spring 2023 (S-2023-H). As all measured plant heights of $M8^1$ are consistently 300 cm, the error bar is 0 (E). (J–M) The fruit length (J), diameter (K), seed cavity diameter (L) and single fruit weight (M) of WT and *CsaMLO* mutants (S-2023-H). (N, O) The number of picked fruits (N) and fruit weight (O) per plant of WT and *CsaMLO* mutants (S-2023-H). Data are presented as means ± *SD*. $^*p < 0.05$, $^{**}p < 0.01$; ns, not significant; #, WT (the control) (one-way ANOVA followed by Dunnett's multiple comparisons test).

pattern (PAMP)-triggered immunity (PTI)-dependent ROS burst (Mendy et al. 2017). ROS are potentially toxic to nematodes, and they also play a role in strengthening cell walls and modulating the expression of defence-related genes (Kadota et al. 2015; Rutter et al. 2022). Many root-knot nematode effectors inhibit ROS production, promote host scavenging of ROS or suppress defence downstream of ROS (Rutter et al. 2022). Resistant tomato with the resistance gene (*Mi-1.2*) demonstrates a strong and prolonged production of ROS upon infection by *M. incognita*, whereas susceptible tomato exhibits only a weak and transient ROS response (Melillo et al. 2006, 2011; Zhou et al. 2018). These studies indicate that ROS play a key role in plant defence against nematodes. *MLO* genes may be involved in regulating the production of ROS to mediate plant susceptibility to root-knot nematodes. Additionally, nematodes have virulence effectors that regulate proteins in the calcium signalling pathway to reduce host defence responses (Galaud et al. 2024). Transcriptional results suggest that calcium/calmodulin-mediated defence signalling plays an important role in helping hosts resist nematode infestation (Kumar et al. 2019; Li et al. 2021; Ling et al. 2017; Zhang et al. 2017). A report also found calcium is involved in the resistance gene-mediated resistance against *Meloidogyne chitwoodi* in potato (Davies et al. 2014). Therefore, it is also possible that MLOs regulate host susceptibility against root-knot nematodes through calcium signalling. However, these hypotheses cannot be confirmed by our results, and future studies are needed to decipher the putative function of *MLO* genes in mediating plant susceptibility to root-knot nematodes.

Several *S* genes including *AtWRKY23*, *CCS52*, *PME3* and *SPDS2* associated with root-knot nematodes or cyst nematodes have been reported in *Arabidopsis* (de Almeida Engler et al. 2012; Grunewald et al. 2008; Hewezi et al. 2008, 2010; Vinardell et al. 2003). However, no report was found for *MLO*. In this study, we uncovered the first-known relationship between *MLO* genes and *M. incognita* infection on cucumber roots. Firstly, the inoculation with *M. incognita* led to upregulation of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in cucumber roots. Secondly, the loss-of-function mutations in clade V *MLO* genes (*AtMLO2*, *AtMLO6* and *AtMLO12*) showed resistance to *M. incognita* in *Arabidopsis*. Thirdly, heterologous overexpression of *CsaMLO1* or *CsaMLO8* in *Arabidopsis mlo* mutants restored susceptibility to *M. incognita* to varying degrees. Finally, both knockdown by virus-induced gene silencing (VIGS) and knockout by CRISPR of *CsaMLO1*, *CsaMLO8* or *CsaMLO11* genes increased the resistance to *M. incognita*. Therefore, it is concluded that clade V *MLO* genes contribute to the susceptibility of cucumber to *M. incognita*.

The *S* gene approach has been considered as an alternative to the *R* gene approach in breeding programmes (van Schie and Takken 2014). However, some mutants are prone to pleiotropic effects (Li et al. 2022; van Schie and Takken 2014). In terms of plant growth, most *CsaMLO* mutants were unchanged compared with the WT. The fruits of all mutants were similar to those from the WT. As measured by the number of picked fruits and weight, the yields of the two types of *CsaMLO8* mutants were similar to that of WT plants. These findings suggested that the loss-of-function mutations with clade V *MLO* genes, especially *CsaMLO8*, may be feasible for cucumber *M. incognita* resistance breeding.

Despite these findings, our conclusions are limited. In particular, the different degrees of phenotypic response to *M. incognita* in *CsaMLO*-complemented *Arabidopsis* remain unexplained. In addition, our study has served as a preliminary exploration of the negative regulatory mechanism of Clade V *MLO* genes in cucumber response to *M. incognita*. However, the specific role of these genes needs to be further explored. Furthermore, other *MLO* genes, such as *CsaMLO*13, may also play a role in nematode response, as the expression of this gene was induced in cucumber, and the *Arabidopsis* mutant harbouring its closest homologue (*atmlo8*) exhibits resistance as well.

Our study is the first to demonstrate the negative regulatory role of Clade V *MLO* genes in cucumber's response to *M. incognita*. To integrate resistance arising from functional defects of *MLO* genes into cucumber cultivars, further research is needed to gain a comprehensive understanding of *MLO* gene functions. Gene-editing technology can then be used to improve the resistance of cucumber cultivars to *M. incognita*. Overall, our findings can serve as a foundation for future cucumber breeding for nematode resistance.

4 | Experimental Procedures

4.1 | Plant Materials and Treatments

Seeds of cucumber inbred cultivar Xintaimici, which exhibits susceptibility to *M. incognita*, were obtained from the Institute of Vegetables and Flowers of CAAS (Beijing, China). After germination, seedlings were grown in a glass room under day/night

temperatures of 28°C/18°C, relative humidity of approximately 80%, and a day length of approximately 14 h.

Arabidopsis mutants *atmlo1-5* (SALK_117153), *atmlo3* (SALK_027770C), *atmlo7* (SALK_027128C), *atmlo8* (SALK_024893C), *atmlo9* (SALK_073198), *atmlo13* (SALK_076325C) and *atmlo12* (SALK_041042C) were acquired from Capital Normal University (Beijing, China). Mutant seeds of *atmlo2-5* (SAIL_878_H12) and *atmlo6-2* (SAIL_523_D09) were obtained from the Arabidopsis Biological Resource Center (https://www.arabidopsis.org/). *Arabidopsis* wild-type (Col-0) seedlings and mutants (all in the Col-0 background) were grown in round pots (diameter 4 cm) in a controlled environment room at 22°C and 80%–90% relative humidity under a 16 h light/8 h dark photoperiod.

4.2 | Nematodes and Inoculation

Individual seedlings of water spinach (*Ipomoea aquatica*) were inoculated with 1000 second-stage juveniles (J2s) of *M. incognita*. Two months later, the plants were uprooted and the egg masses of *M. incognita* were extracted from infected roots and placed in distilled water to hatch. J2s that emerged from the eggs were stored at 4°C and used for inoculation after recovery.

The plants were grown in plastic pots filled with a 1:1 volume ratio of vermiculite and peat. A hole approximately 3 cm deep was drilled around each plant, and nematodes were inoculated through this hole. Individual *Arabidopsis* plants were inoculated with 500 J2s. Approximately 35–40 days later, the inoculated plants were uprooted. The roots were gently rinsed to remove residual soil and then placed on plates with water for counting galls and egg masses. Cucumber seedlings were challenged with 600 J2s per plant. After 35 days, the plants were cut off at the stem. The roots were carefully collected and washed, and the number of galls and egg masses was recorded.

4.3 | Sequences and Phylogenetic Analyses

Fifteen *Arabidopsis* MLO amino acid sequences were obtained from the Arabidopsis Information Resource (arabidopsis.org). The protein sequences of 13 cucumber MLOs were extracted from the Cucumber (Chinese Long) v2 Genome (cucurbitgenomics.org). All protein sequences of cucumber and *Arabidopsis* MLOs were aligned in MAFFT v. 7.429 (Rozewicki et al. 2019) and subjected to a maximum-likelihood phylogenetic analysis using IQ-TREE v. 1.6.12 (Hoang et al. 2018). Node support in the resulting phylogenetic tree was assessed using 1000 fast bootstrap replicates. The tree was visualised in iTOL (https://itol.embl.de/).

4.4 | RNA Extraction and Quantification of Gene Expression

Total RNA was isolated from cucumber roots using a MiniBEST Plant RNA Extraction kit (TaKaRa). The extracted RNA was subjected to cDNA synthesis and RT-qPCR analysis using a PrimeScript RT reagent kit with gDNA Eraser and TB Green Premix Ex Taq II (TaKaRa). RT-qPCR amplifications were performed on a CFX96 instrument (Bio-Rad) as previously reported (Zhao et al. 2021). The RT-qPCR results were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *Arabidopsis UBP22* (*At5g10790*) and cucumber *UBI* (*Csa2G036600*) were used as internal controls for the normalisation of RT-qPCR data as previously described (Zhao et al. 2019). Three biological experiments with three or four technical replicates were performed.

4.5 | Complementation Testing of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in *Arabidopsis*

Total RNA was extracted using an RNAiso Plus extraction kit (TaKaRa) and used for cDNA synthesis with a FastQuant RT kit (Tiangen). Coding sequences (CDSs) of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* were amplified by PCR with KOD FX (Toyobo) and introduced into the pCAMBIA1300::GFP vector to form p35S:GFP-*CsaMLO1*, p35S:GFP-*CsaMLO8* and p35S:GFP-*CsaMLO11*, respectively. The three constructs were introduced into *Agrobacterium tumefaciens* GV3101. The *A. tumefaciens* strains with p35S:GFP-*CsaMLO1*, p35S:GFP-*CsaMLO8* and p35S:GFP-*CsaMLO11* were respectively transformed into *atmlo12* (SALK_041042C), *atmlo2-5* (SAIL_878_H12) and *atmlo6-2* (SAIL_523_D09) plants by the floral dip method (Zhang et al. 2006). The positively transformed plants were selected by screening successive generations on Murashige and Skoog (MS) plates containing 50 mg/L hygromycin. After hygromycin resistance screening, seeds from T_2 transgenic plants were harvested. T_3 transgenic lines were considered homozygous and confirmed by PCR. In addition, the roots of 1-week-old seedlings of homozygous T_3 transgenic *Arabidopsis* lines were used for GFP fluorescence under a laser confocal fluorescence microscope (LSM 700; Zeiss). Then, control (Col-0), mutant and transgenic plants were inoculated with *M. incognita*. Inoculation and phenotyping were performed as described in Experimental Procedures (4.2).

4.6 | Functional Validation of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in Cucumber

We carried out VIGS using the cucumber green mottle mosaic virus vector pV190, which has been successfully used for functional verification of target genes in several cucurbitaceous crop species (Bi et al. 2022; Liu et al. 2020; Xu et al. 2021). The vectors, pV190 and pV190::*CsaPDS*, were provided by Professor Qinsheng Gu of the Zhengzhou Fruit Research Institute (Zhengzhou, China). Briefly, a 276-bp fragment (CDS positions 1038–1313) of *CsaMLO1*, a 144-bp fragment (positions 426–569) of *CsaMLO8* and a 124-bp fragment (positions 317–440) of *CsaMLO11* were separately cloned into the pV190 vector to yield pV190::*CsaMLO1*, pV190::*CsaMLO8* and pV190::*CsaMLO11*, respectively. The three constructs were introduced into *A. tumefaciens* GV3101. The transformed strains were individually cultured and prepared as described previously and then diluted to an OD_{600} value of 0.8–1.0 (Liu et al. 2020; Xu et al. 2021). Using a 1-mL syringe, cotyledons of two-leaf-stage cucumber seedlings were infiltrated with one of the *A. tumefaciens* suspensions. Seedlings infiltrated with an *Agrobacterium* culture

carrying the empty pV190 vector served as a control. After about 14 days, expression levels of the three genes were measured by RT-qPCR. Next, cucumber seedlings were inoculated around the roots with 600 J2s per plant. The number of galls and egg masses on each cucumber root was counted 35 days later. In total, three independent replicates were performed, with 11 to 18 plants per replicate. Here, the phytoene desaturase (*PDS*) was used as a control.

To generate the CRISPR/Cas9 constructs, the sgRNA targets were designed using CRISPR-GE (http://skl.scau.edu.cn/) (Xie et al. 2017). The sgRNA targets were chosen from the third exon of *CsaMLO8* (*Csa5G623470*) and *CsaMLO11* (*Csa6G292430*) sequences from Cucumber (Chinese Long) v2 Genome (Figure 5B). In this study, the wild-type CU2 was used as the cucumber recipient material. Vector construction, transformation and regeneration procedures were performed as previously described (Xin et al. 2022). The 3-week-old cucumber seedlings were inoculated with 500 J2s per plant. The number of galls and egg masses and fresh weight per root were measured 35 days later. Three replicate experiments were performed; each replicate had more than 14 plants.

To evaluate the potential of these *MLO* genes in the development of *M. incognita* resistance in cucumber, we analysed some important traits like plant growth, yield and fruit size of cucumber mutants and WT. Firstly, cucumber plant height, internode length, leaf length, leaf width and stem diameter were quantified at 40 days after sowing (DAS). In this study, the plant height was measured with a ruler with a measuring range from 1 to 300 cm. The plant height of plants higher than the maximum range was recorded as 300 cm. Two independent replicates in the north (Beijing) and south (Hainan) of China were conducted, with 10 to 15 plants per replicate. The cucumber fruits at 9 days after flowering (DAF) were considered commercial fruit. Commercial fruits were harvested from 43 DAS to 61 DAS, with intervals of 1–4 days, depending on the time needed to reach the harvesting point. Fruits per plant were counted and weighed to estimate yield values. Yield was demonstrated in numbers of fruits picked per plant and weight per plant. Meanwhile, the independent factors that can influence fruit size, including cucumber fruit length, diameter and seed cavity diameter, were measured. Due to the low fruit production of cucumber CU2 in northern China, including Beijing, yield (10 to 14 plants) and fruit size (14 to 15 fruits) were only surveyed in Hainan, China.

4.7 | Primers

Primers were designed using the online software tool Primer3Plus (https://www.primer3plus.com). All primers used in this study are summarised in Table S1.

4.8 | Statistical Analysis

Data were recorded and analysed using Microsoft Excel 2021 and GraphPad Prism (v. 8). Analysis of significance was performed with Dunnett's multiple comparisons test or Student's *t* test.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data is available in the paper and its Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.