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# Alpha-momorcharin enhances *Nicotiana benthamiana* resistance to tobacco mosaic virus infection through modulation of reactive oxygen species

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## Abstract

Alpha-momorcharin (α-MMC), a member of the plant ribosomal inactivating proteins (RIPs) family, has been proven to exhibit important biological properties in animals, including antiviral, antimicrobial, and antitumour activities. However, the mechanism by which α-MMC increases plant resistance to viral infections remains unclear. To study the effect of α-MMC on plant viral defence and how α-MMC increases plant resistance to viruses, recombinant DNA and transgenic technologies were employed to investigate the role of α-MMC in *Nicotiana benthamiana* resistance to tobacco mosaic virus (TMV) infection. Treatment with α-MMC produced through DNA recombinant technology or overexpression of α-MMC mediated by transgenic technology alleviated TMV-induced oxidative damage and reduced the accumulation of reactive oxygen species (ROS) during TMV-green fluorescent protein infection of *N. benthamiana*. There was a significant decrease in TMV replication in the upper leaves following local α-MMC treatment and in *α-MMC*-overexpressing plants relative to control plants. These results suggest that application or overexpression of α-MMC in *N. benthamiana* increases resistance to TMV infection. Finally, our results showed that overexpression of α-MMC up-regulated the expression of ROS scavenging-related genes. α-MMC confers resistance to TMV infection by means of modulating ROS homeostasis through controlling the expression of antioxidant enzyme-encoding genes. Overall, our study revealed a new crosstalk mechanism between α-MMC and ROS during resistance to viral infection and provides a framework to understand the molecular mechanisms of α-MMC in plant defence against viral pathogens.

**KEYWORDS**

alpha-momorcharin (α-MMC), *Nicotiana benthamiana*, overexpression, reactive oxygen species (ROS), resistance, tobacco mosaic virus (TMV)

## 1 | INTRODUCTION

Plants are constantly exposed to multiple biotic stresses, such as bacteria, oomycetes, fungi, viruses, nematodes, and herbivores. To defend themselves, plants have evolved complex and efficient defence mechanisms and strategies (Katagiri, 2018; Saijo and Loo, 2019; Wang *et al*., 2020). For example, microbial (or pathogen)-associated molecular patterns (MAMP/PAMP)-triggered immunity (MTI/PTI), effector-triggered immunity (ETI), systemic acquired resistance (SAR), and gene silencing are the major plant defence mechanisms (Spoel and Dong, 2012; Muthamilarasan and Prasad, 2013; Miller *et al*., 2017; Han, 2019; Nobori and Tsuda, 2019). In addition, some plants produce defensive chemicals (organic molecules and proteins) that are thought to play a key role in their defence mechanisms against pathogenic invaders (Calixto, 2000; Song *et al*., 2000; Wang *et al*., 2016). For instance, numerous studies have suggested that ribosomal inactivating proteins (RIPs) encoded by plant genes confer disease resistance and tolerance of environmental stresses in plants (Huang *et al*., 2008; Dowd *et al*., 2012).

RIPs were named due to their ability to inactivate ribosomes, thereby inhibiting protein synthesis (de Virgilio *et al*., 2010). They are *N*-glycosidases that can damage ribosomes by irreversibly inhibiting protein synthesis by removing one or more adenine residues from ribosomal (r)RNA (Puri *et al*., 2012; Fabbrini *et al*., 2017). Many RIPs have been proven to exhibit unique bioactive properties, for instance antitumour activity, antibacterial activity, antifungal activity, broad-spectrum antiviral activity, and insecticidal activity (Puri *et al*., 2009; Kaur *et al*., 2011; Zhu *et al*., 2018). Furthermore, RIPs enhance plant resistance against different biotic stresses (Zhu *et al*., 2013; Hamshou *et al*., 2016). The results of many studies have suggested that RIPs play a protective role in transgenic plants by inducing the overexpression of various foreign RIP genes (Fabbrini *et al*., 2017). Coexpressing foreign RIP genes in transgenic tobacco, tomato, potato, and rice plants enhanced their tolerance and resistance to various biotic stresses, including fungi, viruses, and insects. For example, overexpression of PhRIP I in transgenic potato plants significantly increased resistance against *Botrytis cinerea* and *Rhizoctonia solani* infection (Gonzales-Salazar *et al*., 2017). Overexpression of alpha-momorcharin (α-MMC), an RIP isolated from *Momordica charantia* seeds, enhanced resistance to rice blast caused by *Magnaporthe grisea* in transgenic rice plants (Qian *et al*., 2014). The expression of type I or type II RIPs from apple in transgenic tobacco plants increased resistance against an insect pest, *Spodoptera exigua* (Hamshou *et al*., 2017). Overexpressing curcin 2 (an RIP) increased resistance to tobacco mosaic virus (TMV) infection in transgenic tobacco plants (Huang *et al*., 2008).

α-MMC, a member of the RIP family, has been proven to exhibit important biological properties in animals including antiviral, antimicrobial, and antitumour activities (Fang *et al*., 2012; Pan *et al*., 2014). Zhu *et al*. (2013) demonstrated that foliar spraying of α-MMC on tobacco plants increased their resistance to various phytopathogenic viruses, including chilli veinal mottle virus (ChiVMV), cucumber mosaic virus (CMV), TMV, and turnip mosaic virus (TuMV). Furthermore, application of α-MMC in *M. charantia* led to a significant increase of jasmonic acid (JA), indicating that the antiviral activities of α-MMC in *M. charantia* may be mediated through a JA-related signalling pathway (Yang *et al*., 2016). Another study's results suggested that α-MMC can enhance tobacco$^{NN}$ plants' genetic resistance against TMV by manipulating JA- and salicylic acid (SA)-related signalling pathways (Yang *et al*., 2018). However, the mechanism by which α-MMC increases plant resistance against viral infection remains unclear.

Reactive oxygen species (ROS) are continuously produced in plants as by-products of various physiological and metabolic pathways, and were initially recognized as toxic molecules that cause oxidative damage to proteins, DNA, and lipids in plants (Apel and Hirt, 2004). The main types of ROS include free radicals such as superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), as well as non-radicals such as hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$). Increasing evidence indicates that ROS also function as important signalling molecules in plants and that they are involved in regulating a broad range of processes, such as growth, development, defence, and responses to various abiotic and biotic stresses (Radwan *et al*., 2010; Sharma *et al*., 2012; Bechtold *et al*., 2013; Baxter *et al*., 2014; Shi *et al*., 2014; Vuleta *et al*., 2016; Mignolet-Spruyt *et al*., 2016; Xu *et al*., 2019). To use ROS as signalling molecules, ROS must be maintained at nontoxic levels by a delicate equilibrium between production and scavenging pathways (Baxter *et al*., 2014). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, encoded by respiratory burst oxidase homologs (RBOHs), play an important role in the network of ROS generation in plants (Suzuki *et al*., 2011). Plants have evolved complicated scavenging and regulation systems to monitor ROS redox homeostasis in order to avoid the excessive accumulation of ROS in plant cells (Foyer and Noctor, 2009; Das and Roychoudhury, 2014). Redox homeostasis in plants is maintained by an efficient antioxidative system composed of enzymatic and nonenzymatic antioxidants. The enzymatic components include catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), guaiacol peroxidase (GPX), and enzymes related to the ascorbate–glutathione cycle, such as monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) (Gill and Tuteja, 2010; Miller *et al*., 2010; Das and Roychoudhury, 2014). Ascorbic acid (AA), α-tocopherol, reduced glutathione (GSH), flavonoids, carotenoids, phenolics, and proline serve as potent nonenzymatic antioxidants (De Gara *et al*., 2003; Das and Roychoudhury, 2014).

In this study, we further investigated the antiviral mechanisms of α-MMC in *Nicotiana benthamiana*. The α-MMC-overexpressing (OE) transgenic *N. benthamiana* plants exhibited enhanced systemic resistance to TMV infection. Our data indicated that α-MMC regulates the systemic resistance responses to TMV infection by adjusting the redox homeostasis state through controlling the expression of antioxidant enzyme-encoding genes. These findings indicate that α-MMC is a crucial regulator in systemic resistance responses and has great potential for engineering crops with enhanced resistance against pathogen infections.

## 2 | RESULTS

### 2.1 | Prokaryotic expression and purification of α-MMC and preparation of polyclonal antibodies

In our previous study, we focused on developing a prokaryotic expression system for producing the α-MMC protein in *Escherichia coli* and the preparation of polyclonal antibodies that recognized it. As shown in Figure S1, a soluble recombinant His-tagged α-MMC protein with a molecular weight of approximately 29.7 kDa was successfully induced at 37°C and a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Furthermore, the α-MMC recombinant protein was successfully purified by Ni–nitrilotriacetic acid (Ni-NTA) resin affinity chromatography (Figure S2). Finally, the His-tagged α-MMC proteins were used to immunize rabbits and obtain serum antibodies. Western blotting results showed that the anti-α-MMC polyclonal antibodies had good specificity and sensitivity, and could be used to detect the expression of the α-MMC protein in bitter melon (Figure S3).

### 2.2 | Treatment with α-MMC alleviated oxidative damage during TMV-GFP infection of *N. benthamiana* plants

The soluble recombinant α-MMC protein obtained from the *E. coli* prokaryotic expression system was used to spray *N. benthamiana* plants before TMV-green fluorescent protein (GFP) infection. The cell membranes could be adversely affected by oxidative damage induced by pathogen infection. The lipid peroxidation, cell death, and penetrability of cell membranes can be analysed by malondialdehyde (MDA) accumulation and electrolyte leakage, used as oxidative stress parameters (Diaz-Vivancos *et al*., 2008; Zhang *et al*., 2012). Therefore, MDA content and electrolyte leakage were investigated in α-MMC-treated *N. benthamiana* plants infected with TMV-GFP at 3 days postinoculation (dpi) (the TMV-GFP-inoculated leaves were sampled) (Figure 1). No obvious differences of MDA content or electrolyte leakage were measured in α-MMC- treated *N. benthamiana* plants and water-treated plants (CK) without TMV-GFP (Figure 1). However, the MDA content was significantly increased by TMV-GFP infection in α-MMC-treated *N. benthamiana* plants and water-treated plants (Figure 1a). Interestingly, α-MMC-treated *N. benthamiana* plants had less MDA formation than control plants after TMV-GFP infection, indicating that α-MMC-treated *N. benthamiana* plants alleviated the lipid peroxidation of cell membranes under TMV-GFP infection (Figure 1a). Furthermore, the level of leakage was also significantly reduced in α-MMC-treated plants compared with control plants after TMV-GFP inoculation, implying that α-MMC induced protection of the cell membranes during TMV-GFP infection (Figure 1b). Overall, the results indicate that α-MMC treatment alleviates TMV-induced oxidative damage and that α-MMC plays a positive role in *N. benthamiana* resistance to TMV infection.

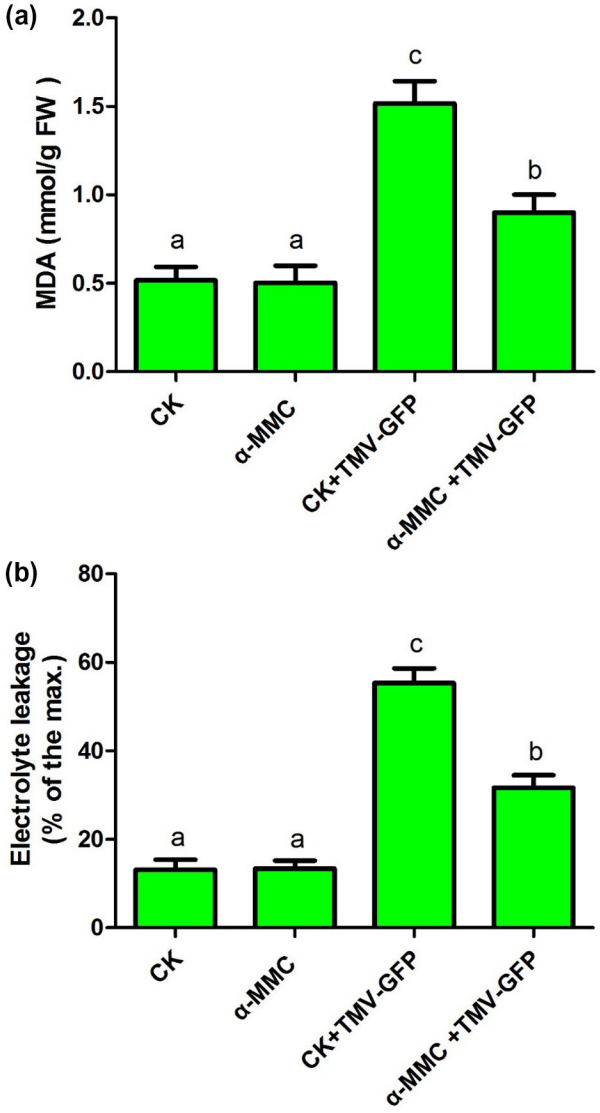


**FIGURE 1** Malondialdehyde (MDA) content (a) and electrolyte leakage (b) were measured in α-MMC-treated *Nicotiana benthamiana* plants infected with TMV-green fluorescent protein (GFP) (at 3 days postinoculation, dpi). The TMV-GFP-inoculated leaves were collected. CK, *N. benthamiana* plants pretreated with only water; α-MMC, *N. benthamiana* plants pretreated with only 0.5 mg/ml α-MMC obtained from the *Escherichia coli* prokaryotic expression system; CK + TMV-GFP, *N. benthamiana* plants pretreated with water after TMV-GFP infection (at 3 dpi); α-MMC + TMV-GFP, *N. benthamiana* plants pretreated with 0.5 mg/ml α-MMC obtained from the *E. coli* prokaryotic expression system after TMV-GFP infection (at 3 dpi). Bars represent mean and *SD* of values obtained from three biological replicates. Significant differences ($p < .05$) are denoted by different lowercase letters

### 2.3 | Application of α-MMC reduced the accumulation of ROS during TMV-GFP infection of *N. benthamiana* plants

ROS production is correlated with plant cell death and increased susceptibility to pathogenic infection (Overmyer *et al*., 2003; Mittler *et al*., 2004; Zhu *et al*., 2015). Therefore, we first determined the

levels of $O_2^{\bullet-}$ and $H_2O_2$ by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining, respectively, of α-MMC-treated plants after TMV-GFP infection (the TMV-GFP-inoculated leaves were sampled). Slight staining in areas of NBT and DAB occurred in α-MMC-treated plants and water-treated plants (CK) in the absence of TMV-GFP infection (Figure 2a). $O_2^{\bullet-}$ and $H_2O_2$ accumulation was significantly enhanced in α-MMC-treated plants and control plants during TMV-GFP infection (Figure 2a). However, in the presence of TMV-GFP, α-MMC-treated plants showed fewer stained areas and a less intense staining than control plants, indicating that α-MMC-treated *N. benthamiana* plants accumulated less ROS (Figure 2a). To investigate the $H_2O_2$ and $O_2^{\bullet-}$ contents more precisely, a sensitive quantitative Amplex red hydrogen peroxide/peroxidase assay kit was used to measure the $H_2O_2$ content, and the hydroxylamine oxygenation reaction method was used to examine the $O_2^{\bullet-}$ content. The results indicated that the $H_2O_2$ and $O_2^{\bullet-}$ contents were significantly reduced in α-MMC-treated plants compared with control plants after TMV-GFP inoculation (Figure 2b,c). Taken together, these results suggest that application of α-MMC on *N. benthamiana* plants reduces the accumulation of ROS during TMV-GFP infection.

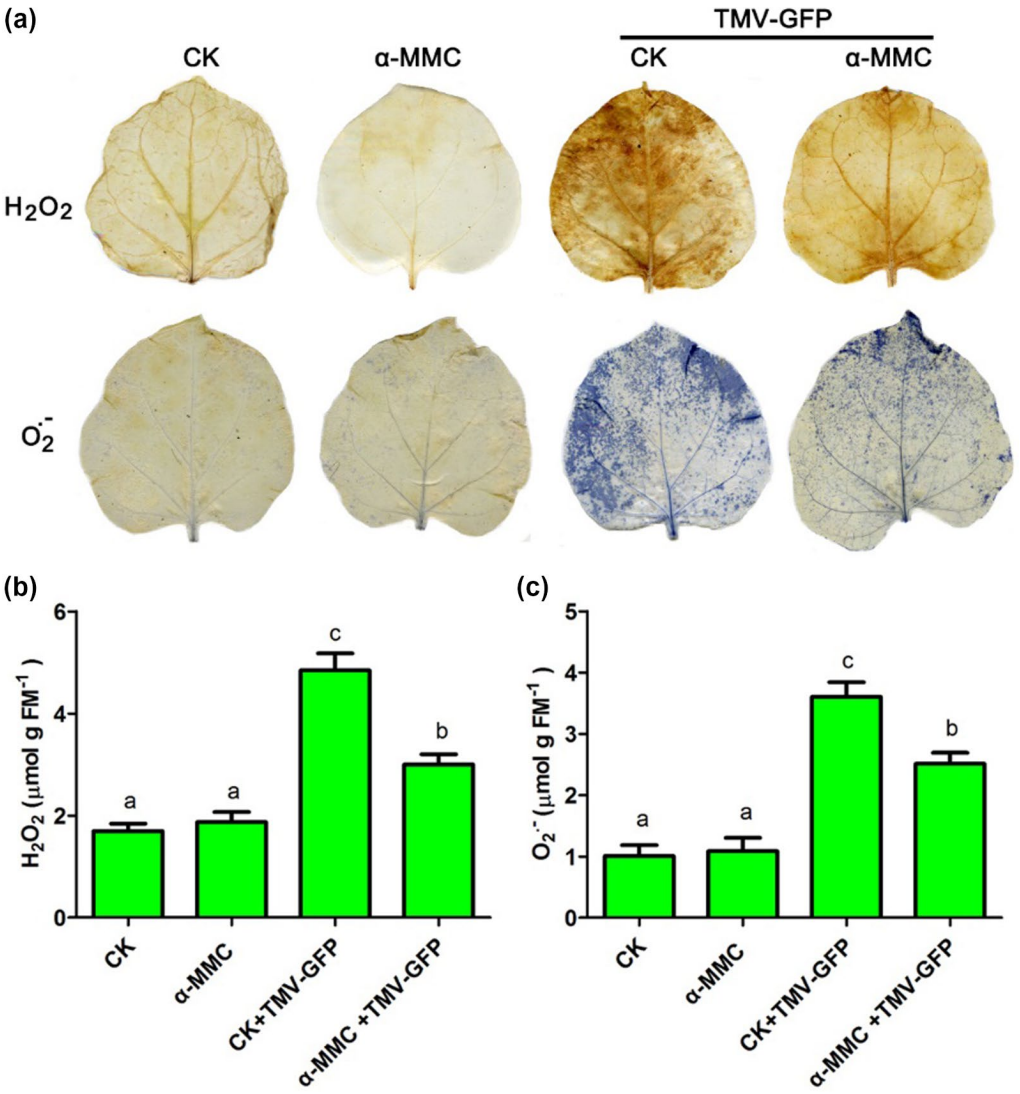


**FIGURE 2** The levels of reactive oxygen species (ROS) were measured in α-MMC-treated *Nicotiana benthamiana* plants infected with TMV-green fluorescent protein (GFP) (at 3 days postinoculation, dpi). The TMV-GFP-inoculated leaves were collected. (a) The levels of $O_2^{\bullet-}$ and $H_2O_2$ were determined by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining of α-MMC-treated plants after TMV-GFP infection. (b) $H_2O_2$ content was measured using a sensitive quantitative Amplex red hydrogen peroxide/peroxidase assay kit. (c) $O_2^{\bullet-}$ content was examined by the hydroxylamine oxygenation reaction method. CK, *N. benthamiana* plants pretreated with only water; α-MMC, *N. benthamiana* plants pretreated with only 0.5 mg/ml α-MMC obtained from the *Escherichia coli* prokaryotic expression system; CK + TMV-GFP, *N. benthamiana* plants pretreated with water after TMV-GFP infection (at 3 dpi); α-MMC + TMV-GFP, *N. benthamiana* plants pretreated with 0.5 mg/ml α-MMC obtained from an *E. coli* prokaryotic expression system after TMV-GFP infection (at 3 dpi). Bars represent mean and *SD* of values obtained from three biological replicates. Significant differences ($p < .05$) are denoted by different lowercase letters

## 2.4 | Application of α-MMC on *N. benthamiana* plants enhanced systemic resistance to TMV

To further investigate the positive role of α-MMC in *N. benthamiana* resistance to TMV infection, *N. benthamiana* plants were pretreated on their primary leaves with water and α-MMC, and then inoculated with TMV-GFP on the upper leaves (Figure 3). TMV accumulation was evaluated by direct observation of GFP fluorescence (Figure 3a), as well as by quantitative reverse transcription PCR (RT-qPCR) and western blotting analysis of viral replication at 3 and 5 dpi. (Figure 3b,c). There was a significant decrease in GFP fluorescence in the upper leaves after α-MMC treatment of the lower primary leaves relative to the control plants (CK) (Figure 3a). This conclusion is consistent with the RT-qPCR and western blotting analysis of TMV accumulation. As shown in Figure 3b, the RT-qPCR results suggested thata loweramount of TMV accumulation was detected in the leaves of the α-MMC-treated plants compared with control plants. Furthermore, western blotting analysis suggested that the levels of TMV movement protein were also significantly reduced in α-MMC-treated plants in comparison with controls (Figure 3c). These results indicate that the application of α-MMC in *N. benthamiana* plants enhances systemic resistance to TMV infection.

## 2.5 | Identification of transgenic *N. benthamiana* lines

The gene encoding α-MMC was cloned into the pCAMBIA1301 vector under the control of the ubiquitin (*Ubi*) promoter and used to transform *N. benthamiana* leaves. Kanamycin-resistant and PCR-positive transgenic *N. benthamiana* plants constitutively expressing α-MMC were selected. Independent $T_2$ transgenic lines grown in greenhouse conditions were screened by PCR analysis. Total RNA was isolated from positive lines as well as from the nontransformed wild type (WT). Semiquantitative RT-PCR analysis indicated that the levels of α-MMC were significantly increased in the OE-2 and OE-5 lines (Figure S4a). However, we did not detect the expression of α-MMC in the nontransformed WT (Figure S4a). This conclusion is consistent with the RT-qPCR and western blotting analyses of the levels of α-MMC in the OE-2 and OE-5 lines. As shown in Figure S4b, RT-qPCR results demonstrated that the expression of α-MMC was significantly enhanced in the OE-2 and OE-5 lines. Western blotting analysis also suggested that the α-MMC protein was detected in the OE-2 and OE-5 lines (Figure S4c). Therefore, the OE-2 and OE-5 lines were selected to further study the role of α-MMC in resistance against TMV infection.

## 2.6 | Overexpression of α-MMC reduced oxidative damage during TMV-GFP infection

Next, MDA content and electrolyte leakage were determined in the leaves of *α-MMC*-overexpressing plants (the OE-2 and OE-5 lines) and in WT plants inoculated with TMV-GFP at 3 dpi (Figure 4). There was no obvious difference in MDA content or electrolyte leakage between the *α-MMC*-overexpressing plants (OE-2

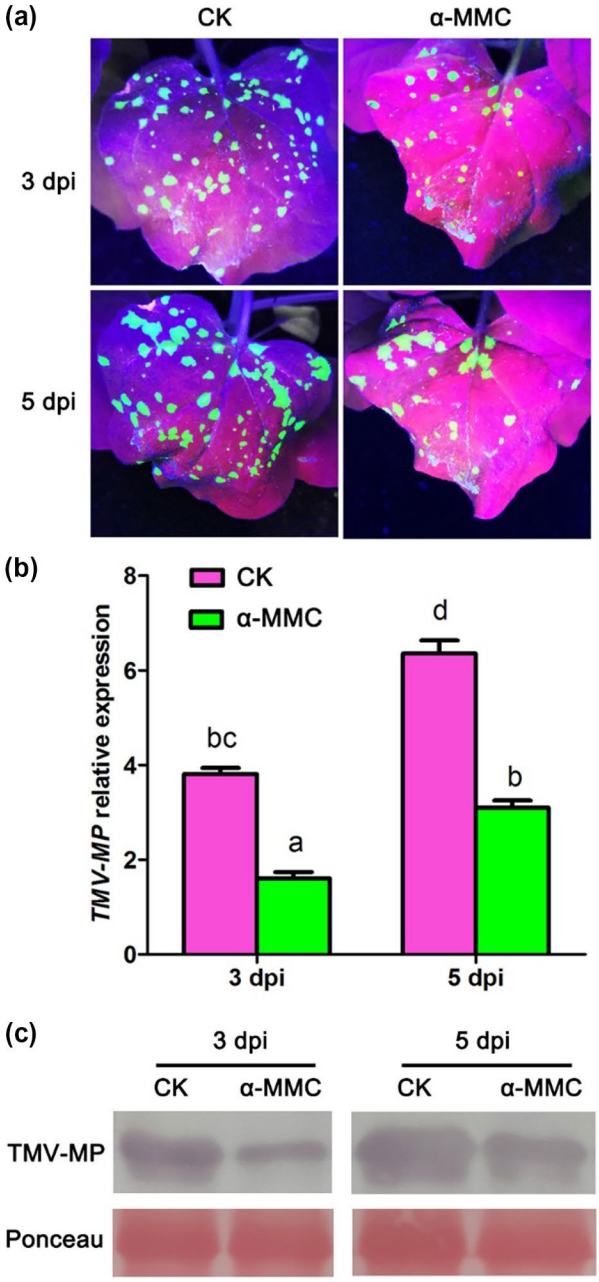


**FIGURE 3** Application of α-MMC in *Nicotiana benthamiana* plants enhanced systemic resistance to TMV-green fluorescent protein (GFP) infection. Plants were pretreated on their primary leaves with water and α-MMC, and 3 days later inoculated with TMV-GFP on the upper leaves. (a) Analysis of GFP fluorescence in water- and α-MMC-treated *N. benthamiana* plants. GFP fluorescence was photographed from water- and α-MMC-treated *N. benthamiana* plants after TMV-GFP infection at 3 and 5 days postinoculation (dpi). (b) Quantitative reverse transcription PCR analysis of TMV replication levels in water- and α-MMC-treated *N. benthamiana* plants after TMV-GFP infection at 3 and 5 dpi. Bars represent mean and *SD* of values obtained from three biological replicates. Different lowercase letters indicate significant differences ($p < .05$). (c) Western blotting analysis of movement protein (MP) accumulation of TMV in water- and α-MMC-treated *N. benthamiana* plants after TMV-GFP infection at 3 and 5 dpi. RuBisCO proteins were used as loading controls and were stained by Ponceau S. CK, *N. benthamiana* plants pretreated with water after TMV-GFP infection at 3 and 5 dpi; α-MMC, *N. benthamiana* plants pretreated with 0.5 mg/ml α-MMC obtained from an *Escherichia coli* prokaryotic expression system after TMV-GFP infection at 3 and 5 dpi

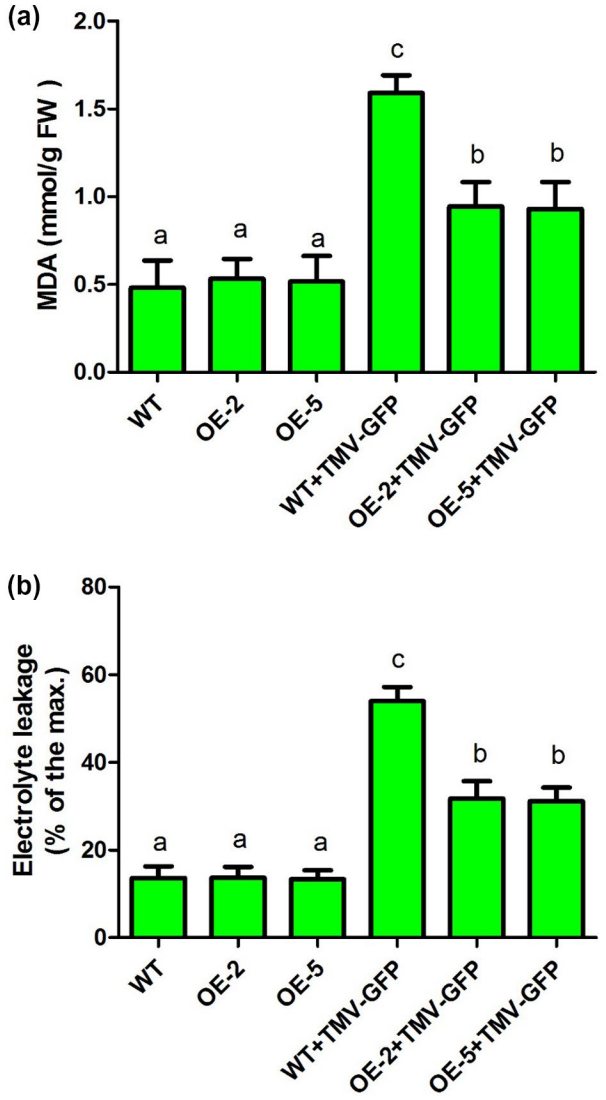


**FIGURE 4** Malondialdehyde (MDA) content (a) and electrolyte leakage (b) were measured in leaves of wild-type and the transgenic lines OE-2 and OE-5 inoculated with TMV-green fluorescent protein (GFP) (at 3 days postinoculation, dpi). WT, nontransformed wild-type *Nicotiana benthamiana* plants inoculated with 0.02 M phosphate-buffered saline (PBS); OE-2, *α-MMC*-transgenic *N. benthamiana* line 2 inoculated with 0.02 M PBS; OE-5, *α-MMC*-transgenic *N. benthamiana* line 5 inoculated with 0.02 M PBS; WT + TMV-GFP, leaves of nontransformed WT *N. benthamiana* plants inoculated with TMV-GFP (at 3 dpi); OE-2 + TMV-GFP, leaves of *α-MMC*-transgenic *N. benthamiana* line 2 inoculated with TMV-GFP (at 3 dpi); OE-5 + TMV-GFP, leaves of *α-MMC*-transgenic *N. benthamiana* line 5 inoculated with TMV-GFP (at 3 dpi). Bars represent mean and *SD* of values obtained from three biological replicates. Significant differences ($p < .05$) are denoted by different lowercase letters

and OE-5 lines) and the WT in the absence of TMV-GFP infection (Figure 4). Interestingly, MDA content was significantly decreased in the *α-MMC*-overexpressing plants compared with the WT after TMV-GFP infection (Figure 4a). These results indicated that overexpression of α-MMC in the *N. benthamiana* plants reduced lipid peroxidation during TMV-GFP infection. Furthermore, the *α-MMC*-overexpressing plants had a lower level of leakage than the WT after TMV-GFP inoculation (Figure 4b). These results suggested that the cytomembranes of *α-MMC*-overexpressing plants suffered less oxidative damage after TMV-GFP inoculation. Taken together, our results suggest that overexpression of α-MMC in *N. benthamiana* plants alleviates TMV-induced oxidative damage.

## 2.7 | Overexpression of α-MMC reduced the accumulation of ROS under TMV-GFP infection

Next, the levels of $O_2^{\bullet-}$ and $H_2O_2$ were determined by histochemical staining with NBT and DAB, respectively, of leaves from *α-MMC*-overexpressing plants (OE-2 and OE-5 lines) and WT plants in the absence or the presence of TMV-GFP infection. No obvious differences in the staining areas of NBT and DAB were observed in the *α-MMC*-overexpressing plants and WT without TMV-GFP (Figure 5a). The levels of $O_2^{\bullet-}$ and $H_2O_2$ were significantly increased in the *α-MMC*-overexpressing plants and WT during TMV-GFP infection (Figure 5a). However, the *α-MMC*-overexpressing plants showed fewer stained areas and less intense staining than the WT after TMV-GFP infection (Figure 5a). These results indicate that *α-MMC*-overexpressing plants accumulate less ROS. Their $H_2O_2$ and $O_2^{\bullet-}$ contents were further analysed. Our results suggested that the levels of $H_2O_2$ and $O_2^{\bullet-}$ were significantly decreased in the *α-MMC*-overexpressing plants compared with that of WT plants after TMV-GFP infection (Figure 5b,c). Overall, these results suggest that overexpression of α-MMC in *N. benthamiana* plants significantly reduces the accumulation of ROS during TMV-GFP infection.

## 2.8 | *N. benthamiana* lines overexpressing α-MMC showed enhanced resistance to TMV

*α-MMC*-overexpressing plants (OE-2 and OE-5 lines) were inoculated with TMV-GFP and monitored for viral replication and spread for at least 1 week. Our results indicated that there was a significant decline in GFP fluorescence in the *α-MMC*-overexpressing plants compared with WT (Figure 6a). Furthermore, the RT-qPCR results suggested that the *α-MMC*-overexpressing plants had lower TMV accumulation than the WT did (Figure 6b). These results were also confirmed by western blotting analysis, indicating that the level of TMV movement protein accumulation was also significantly reduced in the *α-MMC*-overexpressing plants in comparison with WT (Figure 6c). Therefore, these results suggest that overexpression of α-MMC in *N. benthamiana* plants increases resistance to TMV infection.

## 2.9 | The expression of ROS scavenging-related genes was up-regulated in *N. benthamiana* lines overexpressing α-MMC

The results related to ROS accumulation and content suggested that α-MMC may be involved in the regulation of ROS homeostasis.

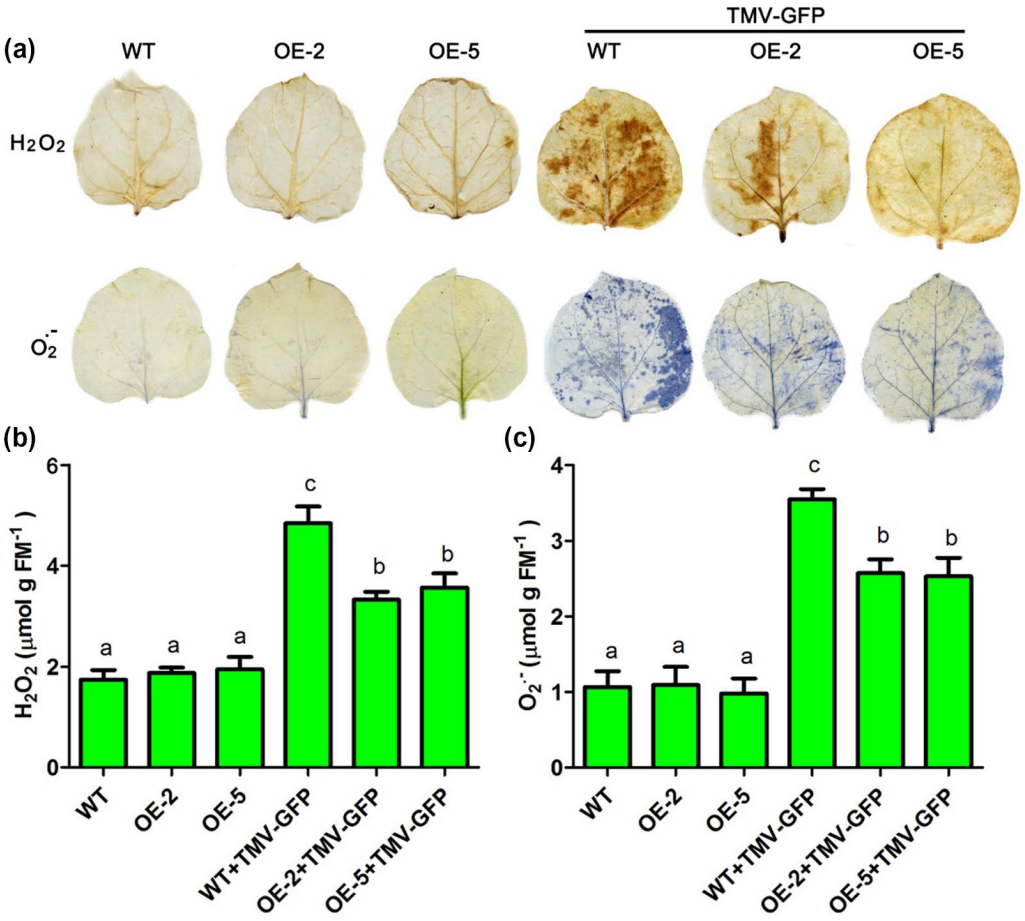


**FIGURE 5** The levels of reactive oxygen species (ROS) were measured in leaves of wild-type and transgenic lines OE-2 and OE-5 inoculated with TMV-green fluorescent protein (GFP) (at 3 days postinoculation, dpi). (a) The levels of $O_2^{\bullet-}$ and $H_2O_2$ were determined by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining of WT and transgenic lines OE-2 and OE-5 after TMV-GFP infection. (b) $H_2O_2$ content was measured using a sensitive quantitative Amplex red hydrogen peroxide/peroxidase assay kit. (c) $O_2^{\bullet-}$ content was examined by the hydroxylamine oxygenation reaction method. WT, nontransformed wild-type *Nicotiana benthamiana* plants inoculated with 0.02 M phosphate-buffered saline (PBS); OE-2, *α-MMC*-transgenic *N. benthamiana* line 2 inoculated with 0.02 M PBS; OE-5, *α-MMC*-transgenic *N. benthamiana* line 5 inoculated with 0.02 M PBS; WT + TMV-GFP, leaves of nontransformed WT *N. benthamiana* plants inoculated with TMV-GFP (at 3 dpi); OE-2 + TMV-GFP, leaves of *α-MMC*-transgenic *N. benthamiana* line 2 inoculated with TMV-GFP (at 3 dpi); OE-5 + TMV-GFP, leaves of *α-MMC*-transgenic *N. benthamiana* line 5 inoculated with TMV-GFP (at 3 dpi). Bars represent mean and *SD* of values obtained from three biological replicates. Significant differences ($p < .05$) are denoted by different lowercase letters

ROS-scavenging enzymes play important roles in ROS cellular homeostasis under normal and stressful conditions. Increasing evidence indicates that catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) are the three major types of ROS-scavenging enzymes (Apel and Hirt, 2004; Das and Roychoudhury, 2014). Therefore, we investigated the expression levels of 13 ROS-scavenging enzymes genes encoding CAT, SOD, or APX in the *α-MMC*-overexpressing plants (OE-2 and OE-5 lines) and WT using RT-qPCR (Figures 7 and 8). The results indicated that the two genes encoding CAT, *NbCAT1* and *NbCAT2*, were up-regulated in the OE-2 and OE-5 lines compared with WT (Figure 7a,b). The expression level of *NbCAT3* was also significantly increased in the OE-5 line in comparison with the basal expression levels (Figure 7c). Next, the gene encoding SOD, *NbFeSOD*, was found to be significantly enhanced in the OE-2 and OE-5 lines compared with WT (Figure 7d). The expression levels of *NbMnSOD* and *NbCu/Zn-SOD* were significantly increased in the OE-2 and OE-5 lines compared with WT, respectively (Figure 7e,f). Finally, the expression of the five genes encoding APX, *NbAPX1*, *NbAPX3*, *NbAPX5*, *NbAPX6*, and *NbAPX7*, were significantly up-regulated in the OE-2 and OE-5 lines compared with WT (Figure 8a,c,e,f,g). The expression of *NbAPX2* was statistically higher in OE-5 plants than in WT or OE-2 plants (Figure 8b). There were no obvious differences in the expression of *NbAPX4* between the *α-MMC*-overexpressing plants and WT (Figure 8d). Taken together, these results suggest that *α-MMC* may regulate the expression of a large number of ROS-regulating genes, and overexpressing *α-MMC* could trigger the induction of a series of ROS-scavenging genes to cope positively with oxidative stress induced by viral infection.

## 3 | DISCUSSION

Ribosomal inactivating proteins (RIPs) have been proven to confer resistance against bacteria, fungi, viruses, and insects in vitro (Zhu *et al.*, 2018). For example, an RIP isolated and purified from

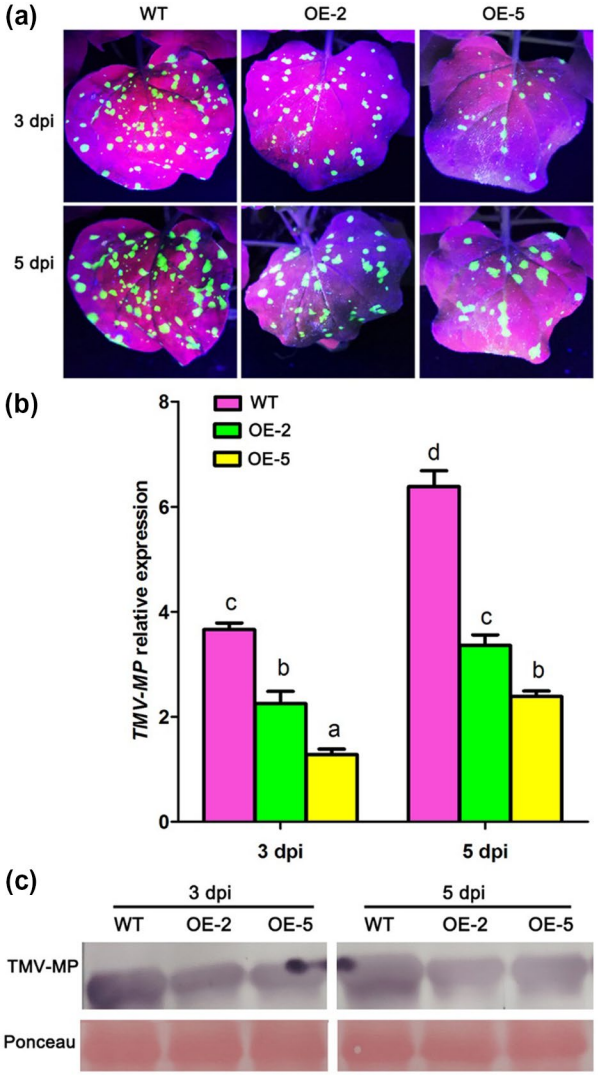


**FIGURE 6** Overexpression of α-MMC in *Nicotiana benthamiana* plants increased resistance to TMV infection. (a) Analysis of green fluorescent protein (GFP) fluorescence in leaves of TMV-GFP-inoculated wild-type (WT) and transgenic lines OE-2 and OE-5. GFP fluorescence was photographed from WT and transgenic lines OE-2 and OE-5 after TMV-GFP infection at 3 and 5 days postinoculation (dpi). (b) Quantitative reverse transcription PCR analysis of TMV replication levels in WT and transgenic lines OE-2 and OE-5 after TMV-GFP infection at 3 and 5 dpi. Bars represent mean and *SD* of values obtained from three biological replicates. Different lowercase letters indicate significant differences ($p < .05$). (c) Western blotting analysis of movement protein accumulation of TMV in WT and transgenic lines OE-2 and OE-5 after TMV-GFP infection at 3 and 5 dpi. RuBisCO proteins were used as loading controls and were stained by Ponceau S. WT, nontransformed WT *N. benthamiana* plants inoculated with TMV-GFP at 3 and 5 dpi; OE-2, *α-MMC*-transgenic *N. benthamiana* line 2 inoculated with TMV-GFP at 3 and 5 dpi; OE-5, *α-MMC*-transgenic *N. benthamiana* line 5 inoculated with TMV-GFP at 3 and 5 dpi

*Mirabilis jalapa* leaves showed significant antibacterial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis* (Rumiyati *et al*., 2014). A soluble recombinant α-MMC protein obtained from the *E. coli* prokaryotic expression system had significant antifungal activity against several fungal pathogens in vitro (Wang *et al*., 2012). Exogenous application of pokeweed antiviral protein (PAP), a type I RIP, to *N. benthamiana* plants enhanced their resistance to TMV infection (Zhu *et al*., 2016). Furthermore, an artificial diet supplemented with type I RIPs reduced the fecundity and survival of insect pests, such as *Anticarsia gemmatalis* and *Spodoptera frugiperda* (Bertholdo-Vargas *et al*., 2009). In recent years, increasing evidence has suggested that RIPs play important roles in plant defences against bacteria, fungi, viruses, and insects in vivo (Kaur *et al*., 2011). Overexpression of curcin 2, a newly identified RIP, in transgenic tobacco plants increased their resistance against *R. solani* (Huang *et al*., 2008). Transgenic plants expressing barley antifungal gene chitinase and RIP demonstrated antifungal activity against corynespora leaf spot fungal disease (Chopra and Saini, 2014). Overexpression of cassin, a new RIP isolated from *Cassia occidentalis*, in tobacco plants enhanced their resistance to TMV infection (Ruan *et al*., 2007).

Transgenic technologies have also been used to investigate the role of α-MMC in plant defences against fungal pathogens. For example, studies have shown that transgenic rice plants expressing α-MMC display enhanced resistance to rice blast (Qian *et al*., 2014). However, studies of the role of α-MMC in plant defence against virus infection using transgenic technologies have rarely been reported. Furthermore, the role of α-MMC in plant defence against virus attack and the mechanisms involved remain unclear. Therefore, in this study, we first focused on developing a prokaryotic expression system for producing α-MMC protein and investigated the role of α-MMC protein obtained from the *E. coli* prokaryotic expression system in plant defence against TMV infection. Furthermore, we also used transgenic technologies by overexpressing α-MMC in *N. benthamiana* to study the role of α-MMC in plant defence against TMV and the mechanisms involved.

It is time-consuming and laborious to purify α-MMC protein directly from plants. A recombinant DNA technology was used to produce the recombinant proteins in a microbial system. The structure and function of proteins are often studied after using prokaryotic expression techniques. The usual prokaryotic expression host organism is *E. coli* and its modified strains, such as Rosetta, because it has lots of advantages, such as easy expression induction, a simple structure, and high protein yield (Chen, 2012; Rosano and Ceccarelli, 2014). Therefore, a soluble recombinant His-tagged α-MMC protein was successfully induced and purified in an *E. coli* prokaryotic expression system (Figures S1–S3). Next, we investigated the role of the recombinant His-tagged α-MMC protein obtained from *E. coli* in plant defence against TMV infection. Oxidative damage induced by pathogen infection could adversely affect the cytomembrane. MDA content and electrolyte leakage have been considered as indicators for membrane lipid peroxidation and the penetrability of the cytomembrane (May *et al*., 1996; Zoeller *et al*., 2012; Zhu *et al*., 2016). Oxidative stress (i.e., the rapid and massive accumulation of ROS in infected tissues) is produced in some compatible virus–host plant interactions (Hernández *et al*., 2004, 2006, 2016; Diaz-Vivancos *et al*., 2006). An accumulation of ROS was noticed in pea plants in response to plum pox virus (PPV) infection (Diaz-Vivancos *et al*., 2008). Oxidative stress parameters such as protein oxidation,

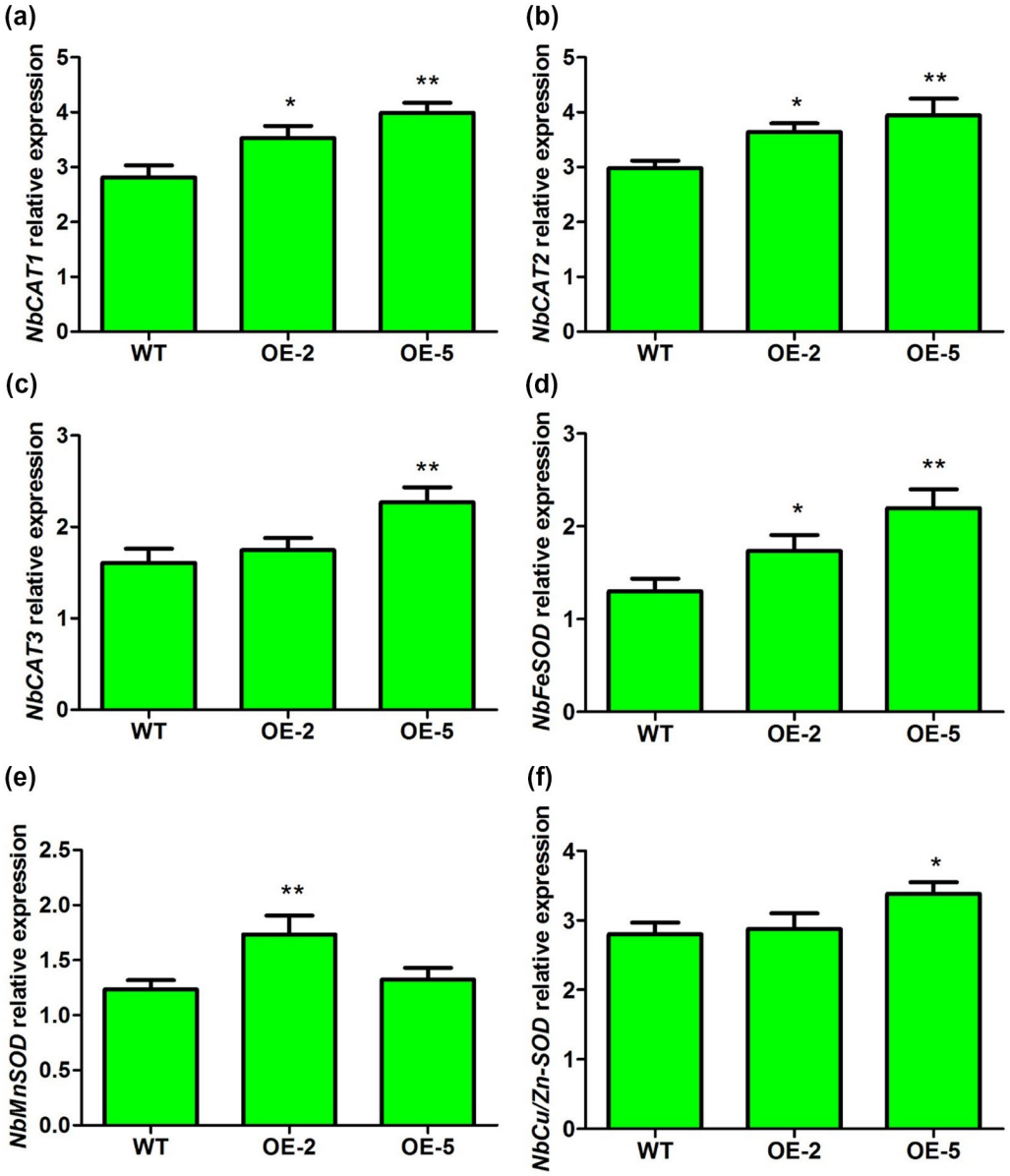


**FIGURE 7** Quantitative reverse transcription PCR analysis of the expression of catalase (CAT) and superoxide dismutase (SOD) genes in wild-type (WT) and transgenic lines OE-2 and OE-5. WT, nontransformed WT *Nicotiana benthamiana* plants; OE-2 and OE-5, *α-MMC*-transgenic *N. benthamiana* lines. Bars represent mean and *SD* of values obtained from three biological replicates. Asterisks represent significant difference determined by Student's *t* test (*$p < .05$; **$p < .01$)

lipid peroxidation, and electrolyte leakage were increased in different PPV-susceptible peach plants and apricot plants (Hernández *et al*., 2004, 2006; Diaz-Vivancos *et al*., 2006; Clemente-Moreno *et al*., 2015). Oxidative stress parameters ($H_2O_2$ content, lipid peroxidation, and protein oxidation) were also studied in the compatible hosts bell pepper and tomato during TMV and tomato mosaic virus (ToMV) infections (Madhusudhan *et al*., 2009; Hernández *et al*., 2016). Accumulation of higher amounts of $H_2O_2$ and, accordingly, higher levels of protein oxidation and lipid peroxidation occurred during these plant–virus interactions (Madhusudhan *et al*., 2009; Hernández *et al*., 2016). Our results showed that the level of leakage and MDA content were significantly reduced in α-MMC-treated plants compared with control plants after TMV-GFP inoculation (Figure 1). Therefore, these results indicated that α-MMC treatment alleviated TMV-induced oxidative damage and that α-MMC plays a positive role in *N. benthamiana* resistance to TMV infection. Furthermore, RT-qPCR and western blotting results suggested that application of α-MMC on *N. benthamiana* plants significantly reduced TMV replication (Figure 3). Overall, these results indicate that application of α-MMC increases systemic resistance to TMV infection.

Plant responses to pathogen invasion are associated with the generation of ion fluxes, the induction of kinase cascades, nitric oxide (NO), accumulation of ROS, and increased expression of genes encoding pathogenesis-related (PR) proteins (Kumar and Klessig, 2003; Baxter *et al*., 2014; Künstler *et al*., 2016). Various stresses may result in the overaccumulation of ROS, which can cause damage to plants (Mittler,

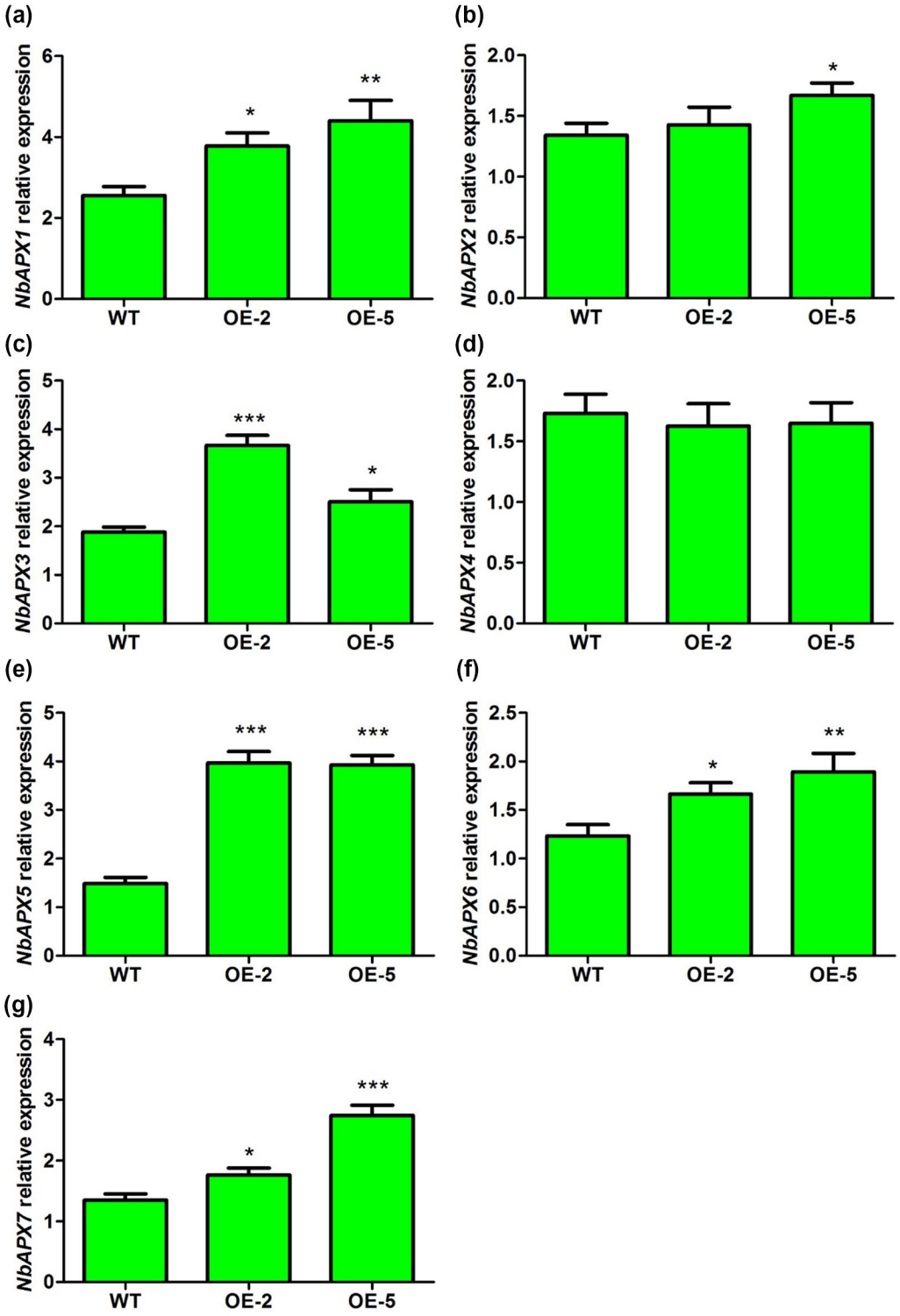


**FIGURE 8** Quantitative reverse transcription PCR analysis of the expression of ascorbate peroxidase (APX) genes in wild-type (WT) and transgenic lines OE-2 and OE-5. WT, nontransformed WT *Nicotiana benthamiana* plants; OE-2 and OE-5, $\alpha$-*MMC*-transgenic *N. benthamiana* lines. Bars represent mean and *SD* of values obtained from three biological replicates. Asterisks represent significant differences determined by Student's *t* test (*$p < .05$; **$p < .01$; ***$p < .001$)

2002). For example, high levels of ROS in plants result in cell death/limitation of invading pathogens due to their high toxicity (Yoshioka *et al*., 2003; Choi *et al*., 2007; Hernández *et al*., 2016). ROS have been considered to be nothing but harmful by-products for a long time. On the other hand, more recent studies have suggested that ROS also function as central signalling molecules during plant defence responses (Miller *et al*., 2009; Ge *et al*., 2015). In fact, low levels of ROS could increase tolerance during plant defence responses against various types

of stresses (Orozco-Cardenas *et al*., 2001; Baxter *et al*., 2014; Xu *et al*., 2014). To use ROS as signalling molecules, ROS must be maintained at nontoxic levels through a delicate balancing act between production, involving ROS-producing enzymes and the unavoidable production of ROS during basic cellular processes, and ROS-scavenging pathways (Mittler *et al*., 2004). ROS-scavenging enzymes, including SOD, CAT, GST, GPX, and enzymes related to the ascorbate-glutathione cycle, play important roles in normal cellular ROS homeostasis. Our previous studies indicated that PAP may improve plant resistance against TMV infection through regulating the levels of ROS (Zhu *et al*., 2016). A previous study suggested that α-MMC could induce an ROS burst in response to CMV infection in *M. charantia* (Yang *et al*., 2016). The present data revealed that α-MMC is a positive regulator of virus resistance and ROS scavenging processes in response to TMV infection in *N. benthamiana* plants. The ROS, electrolyte leakage, and MDA content that accumulated in the leaves of *α-MMC*-overexpressing plants and α-MMC-treated plants were significantly lower than that in the control plants (Figures 2 and 5). This suggests that the enhanced viral resistance of the *α-MMC*-overexpressing plants and α-MMC-treated plants may be due to a stronger capability of scavenging ROS during TMV infection to maintain a lower degree of membrane lipid peroxidation. ROS are considered to play an important role in plant disease resistance. Initial, very rapid, and transient increases in ROS levels can be detected in both incompatible and compatible plant–pathogen interactions (Hernández *et al*., 2016). Several studies have suggested that an early ROS burst, followed by suppression of ROS, is a requirement for successful resistance of plants to TMV and other viruses (Fodor *et al*., 1997; Abbink *et al*., 2002; Király *et al*., 2008; Balasubramaniam *et al*., 2014; Shang *et al*., 2019). In fact, overexpression of α-MMC led to the up-regulation of many ROS-scavenging genes (Figures 7 and 8) in transgenic plants. Taken together, overexpressing α-MMC can increase viral resistance by increasing the cell membrane stability and maintaining redox homeostasis. Therefore, it is proposed that α-MMC plays a positive role in *N. benthamiana* resistance to TMV infection by controlling the expression of downstream genes involved in the ROS scavenging pathway. These antioxidant-encoding genes may be promising candidates for genetic engineering to generate crops with enhanced resistance against virus infection.

In conclusion, α-MMC confers resistance to virus infection by modulating ROS homeostasis through controlling the expression of ROS-associated genes. Our findings thus reveal a new crosstalk mechanism between α-MMC and ROS during virus infection and provide a framework to understand the molecular mechanisms involved in the role of α-MMC in plant defence against virus pathogens.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Plant growth conditions

*N. benthamiana* was used as a transformation recipient in this study. The wild-type *N. benthamiana* and *α-MMC*-transgenic *N. benthamiana* plants were planted in a greenhouse with a 16 hr light/8 hr dark cycle (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25°C. Seedlings 6–7 weeks old were used in this study.

### 4.2 | Prokaryotic expression and purification of α-MMC

Prokaryotic expression and purification of the α-MMC protein from *E. coli* were conducted as described previously (Ma *et al*., 2020). First, the *α-MMC* gene was cloned from bitter gourd seeds by RT-PCR. Then, the gene was ligated into the prokaryotic expression vector pET-28a (+) and introduced into the *E. coli* Rosetta strain. A soluble recombinant protein with a molecular weight of approximately 29.7 kDa was successfully induced at 37°C and a final concentration of 1 mM IPTG. Finally, the α-MMC recombinant protein was purified by Ni-NTA resin affinity chromatography.

### 4.3 | Preparation of polyclonal antibodies

New Zealand white rabbits were first immunized subcutaneously with 1 mg/ml purified α-MMC proteins in the same volume of Freund's complete adjuvant. Further details of the antibody preparation have been described previously (Zhu *et al*., 2012; Ma *et al*., 2020).

### 4.4 | Virus inoculation, GFP imaging, and α-MMC treatments

TMV-GFP was maintained in an aqueous suspension of 0.02 M phosphate-buffered saline (PBS) at 4°C. The inoculation with TMV-GFP was performed as described previously (Zhu *et al*., 2016). *N. benthamiana* plants were treated at one site on the primary (lower) leaves with water and 0.5 mg/ml α-MMC for 3 days, then inoculated with TMV-GFP on the secondary (upper) leaves. PBS rubbed onto the leaves was used as a control. GFP fluorescence was photographed under UV light using a B-100AP longwave-UV lamp (Ultra-Violet Products) (Zhu *et al*., 2016).

### 4.5 | Measurements of electrolyte leakage and malondialdehyde content

Electrolyte leakage and malondialdehyde (MDA) content measurements were performed as described previously (Zhu *et al*., 2016). *N. benthamiana* leaves were cut into approximately 1-cm pieces and placed in deionized water at room temperature. First, the conductivity ($C_1$) was measured by a conductivity meter after 60 min at room temperature. Then, the *N. benthamiana* leaves were incubated in a boiling water bath for 15 min to achieve 100% electrolyte leakage ($C_2$). Finally, the percentage electrolyte leakage was calculated

according to the formula ($C_1/C_2$) × 100. Next, the MDA content was determined by the thiobarbituric acid (TBA) reaction. The MDA content was measured using an MDA assay kit (Solarbio).

### 4.6 | Histochemical detection and measurements of $H_2O_2$ and $O_2^{\bullet-}$

The level of $H_2O_2$ was determined using DAB following the method reported previously (Zhu *et al*., 2013). In brief, *N. benthamiana* leaves were excised at the base with a razorblade and soaked in 2 mg/ml DAB solution for 8 hr under a vacuum. The leaves were then destained in boiling ethanol (95%) for 15 min. Endogenous concentrations of $H_2O_2$ were determined according to previous methods (Zhang *et al*., 2012; Zhu *et al*., 2015). The $H_2O_2$ production was measured using a hydrogen peroxide ($H_2O_2$) assay kit (Solarbio). The accumulation level of $O_2^{\bullet-}$ in the tobacco leaves was observed using NBT as described previously (Zhu *et al*., 2013). *N. benthamiana* leaves were excised at the base with a razorblade and soaked in NBT (0.5 mg/ml) solution for 2 hr under a vacuum. The leaves were then destained in boiling ethanol (95%) for 15 min. The content of $O_2^{\bullet-}$ in the *N. benthamiana* leaves was determined according to a method previously described by Wang and Luo (1990). The $O_2^{\bullet-}$ production was measured using a superoxide anion assay kit (Solarbio).

### 4.7 | Plasmid construction and plant transformation

The plasmid pCAMBIA1301-α-MMC was kindly provided by Professor Yi Ding (Wuhan University, Wuhan, China). This vector is driven by the ubiquitin (*Ubi*) promoter and carries the kanamycin resistance (Kan) selectable marker in plants. The plasmid pCAMBIA1301-α-MMC was transformed into *Agrobacterium tumefaciens* EHA105 by electroporation. *α-MMC* transgenic *N. benthamiana* plants were generated by leaf disc transformation through an agrobacterium-mediated method, as previously described (Zhang *et al*., 2017). Transgenic plants were screened on Murashige & Skoog (MS) medium supplemented with 30 μg/ml kanamycin or hygromycin for 7–10 days. The survivors were transferred into the soil for propagation. Transgenic $T_0$ plants were selected, transferred into a greenhouse, and maintained up to the $T_2$ generation, which were used for further analysis.

### 4.8 | Molecular analysis of *α-MMC*-transgenic *N. benthamiana* lines

Total RNA was isolated from positive lines as well as from the non-transformed WT *N. benthamiana* plants. Overexpression of *α-MMC* in the transgenic plants was confirmed by RT-PCR using specific primers (Table S1). Semiquantitative RT-PCR was first performed to detect the expression levels of the *α-MMC* gene using 1 μg of total RNA isolated from WT *N. benthamiana* and *α-MMC*-transgenic *N. benthamiana* plants (Zhu *et al*., 2014). First-strand cDNA was carried out using M-MLV reverse transcriptase (Takara). Equal loading for amplification of each cDNA was determined by the *actin* PCR product.

### 4.9 | Quantitative real-time PCR analysis

To further assay the expression levels of the α-momorcharin gene, *TMV-MP*, and the ROS-scavenging genes in *N. benthamiana* plants, quantitative reverse transcription PCR analysis was performed. All of the RT-qPCR primers are listed in Table S1. Relative quantitation of the target gene expression level was performed using the comparative $C_t$ (threshold cycle) method (Zhu *et al*., 2016). Three technical replicates were performed for each experiment. Amplification of the *actin* gene was used as an internal control.

### 4.10 | Protein extraction and western blotting

Proteins were extracted from *N. benthamiana* leaves with extraction buffer (50 mM Tris-Cl, pH 6.8, 5% mercaptoethanol, 10% glycerol, 4% SDS, 4 M urea) in an ice bath. Protein samples were quantified by the Bradford method (Bradford, 1976). Western blotting was carried out according to the protocol described by Zhu *et al*. (2013). In brief, protein samples were electrophoresed on 12% polyacrylamide gels and transferred onto nitrocellulose membranes (PALL Life Sciences). Then, the membranes were blocked with 5% skim milk followed by α-MMC protein or TMV movement protein (TMV-MP) bands being detected using a rabbit polyclonal antiserum raised against α-MMC or TMV-MP as the primary antibody, respectively, and an anti-rabbit IgG conjugated to alkaline phosphatase (Sangon Biotech) as the secondary antibody. Immunoreactive proteins were visualized using 5-bromo-4-chloro-3-indolyl phosphate/NBT.

### 4.11 | Statistical analysis

The values are presented as mean ± *SD* of at least three replicates. Significant differences were analysed by two-tailed Student's *t* test between two groups and by one-way analysis of variance followed by Tukey test between multiple groups.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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

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