INTRODUCTION

To defend against pathogen infection, plants have evolved a sophisticated immune system (Jones & Dangl, 2006). Plants employ intracellular immune receptors to directly or indirectly recognize pathogen-secreted virulence factors (effectors) during infection (Jones et al., 2016; Wang et al., 2020). Most intracellular receptors belong to the superfamily of nucleotide-binding, leucine-rich repeat (NLR) proteins, which contain an N-terminal coiled-coil (CC) or toll/interleukin-1 receptor (TIR) domain, a conserved central nucleotide-binding (NB) domain, and a variable leucine-rich repeat (LRR) domain at the C-terminus (van der Burgh & Joosten, 2019; Wu et al., 2017). NLRs that contain the CC domain or the TIR domain are referred to as CC-NLRs (CNLs) or TIR-NLRs (TNLs), respectively (Bonardi et al., 2012; Meyers et al., 2003). Expression of the TIR domain or the CC domain alone can induce an autoactive hypersensitive response (auto-HR) in plants and they are therefore considered to be the signalling domains for the induction of cell death and initiation of downstream immune responses in many NLRs (Collier et al., 2011; Maekawa et al., 2011; Swiderski et al., 2009; Wróblewski et al., 2018). NLRs are usually maintained in an autoinhibited state in the absence of pathogens, and they are activated upon effector recognition, which triggers a series of cellular defence responses (Collier & Moffett, 2009; Sun et al., 2020), including a rapid influx of calcium ions from external stores, a burst of reactive oxygen species (ROS), reprogramming of defence-related genes expression, callose deposition in the cell wall, and a rapid localized cell death termed the hypersensitive response (HR) at the sites of attempted infection (Dangl et al., 2013; Dodds & Rathjen, 2010; Walters, 2015). Both TNLs and CNLs have been identified in dicotyledons, TNLs sometimes in

Abstract

Most plant intracellular immune receptors belong to nucleotide-binding, leucine-rich repeat (NLR) proteins. The recognition between NLRs and their corresponding pathogen effectors often triggers a hypersensitive response (HR) at the pathogen infection sites. The nicotinate N-methyltransferase (NANMT) is responsible for the conversion of nicotinate to trigonelline in plants. However, the role of NANMT in plant defence response is unknown. In this study, we demonstrated that the maize ZmNANMT, but not its close homolog ZmCOMT, an enzyme in the lignin biosynthesis pathway, suppresses the HR mediated by the autoactive NLR protein Rp1-D21 and its N-terminal coiled-coil signalling domain (CCD21). ZmNANMT, but not ZmCOMT, interacts with CCD21, and they form a complex with HCT1806 and CCoAOMT2, two key enzymes in lignin biosynthesis, which can also suppress the autoactive HR mediated by Rp1-D21. ZmNANMT is mainly localized in the cytoplasm and nucleus, and either localization is important for suppressing the HR phenotype. These results lay the foundation for further elucidating the molecular mechanism of NANMTs in plant disease resistance.

Keywords

disease resistance, hypersensitive response, maize, NANMT, plant innate immunity
more abundance than CNLs, while only CNLs have been reported in monocots (Shao et al., 2016; Wróblewski et al., 2018). Therefore, CNLs provide the core repertoire of receptors mediating intracellular immunity in monocots, and understanding the molecular mechanisms of the transition between the inhibited and activated states of CNLs is important for disease resistance breeding of monocotyledonous crops (Wang et al., 2020; Wróblewski et al., 2018).

The maize R protein Rp1-D is a typical CNL and confers resistance to maize common rust caused by specific races of the fungus *Puccinia sorghi* (Collins et al., 1999; Hu et al., 1996). The encoding gene *Rp1-D* is located at the *Rpt1* locus, in which multiple, tandemly repeated, CNL paralogs have been identified, including *Rp1-D* and eight other gene homologs: *Rp1-dp1* to *Rp1-dp8* (Collins et al., 1999; Smith et al., 2004). These paralogs often mispair in meiosis and recombine unequally to create new haplotypes carrying novel gene combinations. One of them was named *Rp1-D21*, which was derived from the combination of the 5′ end of *Rp1-dp2* and the 3′ end of *Rp1-D* (Smith et al., 2010; Sun et al., 2001). The haplotype carrying *Rp1-D21* exhibits a spontaneous necrotic lesion mimic phenotype in the absence of pathogen infection. Transient expression of *Rp1-D21* or its CC domain (*CCD21*) alone in *Nicotiana benthamiana* triggers an auto-HR (Smith et al., 2010; Wang, Ji, et al., 2015). The *Rp1-D21*-mediated auto-HR phenotype is affected by temperature, light, developmental stage, and genetic background (Negeri et al., 2013; Olukolu et al., 2014). Functional analyses showed that *CCoAOMT2*, mentioned above, caffeic acid O-methyltransferase (*CCoAOMT2*), has been proved to contribute to plant resistance to maize common rust caused by specific races of the fungus *Puccinia sorghi* (Collins et al., 1999; Hu et al., 1996). The encoding gene (*CCoAOMT2*) in switchgrass caused a lesion mimic phenotype in *Nicotiana benthamiana* that was derived from the combination of the 5′ end of *Rp1-dp2* and the 3′ end of *Rp1-D* (Smith et al., 2010; Sun et al., 2001). The haplotype carrying *Rp1-D21* exhibits a spontaneous necrotic lesion mimic phenotype in the absence of pathogen infection. Transient expression of *Rp1-D21* or its CC domain (*CCD21*) alone in *Nicotiana benthamiana* triggers an auto-HR (Smith et al., 2010; Wang, Ji, et al., 2015). From a nested association mapping and genome-wide association study (NAM-GWAS), the genes encoding homologs in the lignin biosynthesis pathway, hydroxycinnamoyltransferases (*HCT1806* and *HCT4918*) and caffeoyl-CoA O-methyltransferase (*CCoAOMT2*), have been identified to be associated with variation in *Rp1-D21*-mediated auto-HR (Olukolu et al., 2014). Functional analyses showed that *CCoAOMT2*, *HCT1806*, and *HCT4918* associate with *CCD21* and suppress *Rp1-D21*-mediated auto-HR (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015).

In plants, lignin itself and some key enzymes involved in the lignin biosynthesis pathway have been reported to play important roles in the defence response to pathogens. Apart from the HCTs and *CCoAOMT2* mentioned above, caffeic acid O-methyltransferase (*COMT*) is another key enzyme involved in the lignin biosynthesis pathway. We revealed the important role of *CCoAOMT2* in maintaining the net balance of cellular NAD levels in all organisms (Gossmann et al., 2012; Wang & Pichersky, 2007). In plants, excessive accumulation of NA is toxic to plant cells; hence, NA is maintained at an appropriate concentration by converting NA into different derivatives (Li et al., 2015; Wang & Pichersky, 2007). Various conjugates of NA have been detected in plants, including glycosylation or methylation at the N-position or carboxyl group of NA, which are catalysed by glycosyltransferases or methyltransferases, respectively (Li et al., 2015, 2017). Trigonelline (Tg), also known as N-methyl nicotinate, is an alkaloid derived from NA methylation at the N-position catalysed by S-adenosyl-l-methionine-dependent N-methyltransferases and has been widely detected in various plants (Ashihara et al., 2012; Li et al., 2017; Mizuno et al., 2014). Tg is likely to function as a plant growth regulator in various biological processes (Minorsky, 2002; Zhou et al., 2012). In *Arabidopsis thaliana*, the nicotinate N-methyltransferase 1 (*AtNANMT1*, *At3g53140*) was identified to be responsible for the conversion from NA to Tg and some NANMT homologs from different plant species have also been proved to have the activity by catalysing NA to Tg (Li et al., 2017). The closest homolog of *AtNANMT1* in *Arabidopsis* is *AtCOMT1*, which is a key enzyme in the lignin biosynthesis pathway (Goujon et al., 2003).

In maize, the brown midrib mutant *bm3* is caused by a mutation in *ZmCOMT* (*Zm00001D049541*) which has been proved to be involved in lignin biosynthesis (Vignols et al., 1995). *Zm00001D051870* was previously identified as a homolog of *ZmCOMT* (*Vélez-Bermúdez et al., 2015*). Here we found that it shows higher sequence similarity to *AtNANMT1* than *AtCOMT1*, and thus is named *ZmNANMT*. *COMT* has been reported to play important roles in disease resistance in different plants (Liu et al., 2017; Wang, Zhu, et al., 2018). However, whether NAMT is involved in plant disease resistance, especially in the NLR-mediated defence response, is unknown. In this study, we investigated the roles of both *ZmCOMT* and *ZmNANMT* in *Rp1-D21*-mediated auto-HR through transient coexpression in *N. benthamiana*. We found that *ZmNANMT*, but not *ZmCOMT*, suppressed *Rp1-D21*-mediated auto-HR. We further showed that *ZmNANMT*, but not *ZmCOMT*, interacted with *CCD21* and also formed a complex with *HCT* and *CCoAOMT2*. We revealed the important role of *ZmNANMT* as a novel NLR regulator in the modulation of *Rp1-D21*-mediated defence response.

## RESULTS

### 2.1 The effect of the key enzyme homologs in lignin biosynthesis on *Rp1-D21*-mediated auto-HR

From our previous RNA-Seq analysis, we found that most genes encoding key enzymes involved in the lignin biosynthesis pathway were up-regulated in maize lines carrying *Rp1-D21* compared to wild type in both B73 × H95 and Mo17 × H95 backgrounds (Table 1). We have shown that maize *HCT1806*, *HCT4918*, and *CCoAOMT2* suppress *Rp1-D21*-mediated auto-HR when they are transiently coexpressed.
| Gene name                  | Accession number | Chromosomal position | FC in B73 × H95 isogenic lines | FC in Mo17 × H95 isogenic lines | Rp1-D21-mediated HR phenotype | Reference                  |
|---------------------------|------------------|----------------------|--------------------------------|--------------------------------|-------------------------------|-----------------------------|
| ZmCOMT (bm3)              | Zm00001d049541   | Chr4: 33,817,769–33,820,095 | No read                       | No read                       | No effect                     | In this study               |
| ZmNAMMT                   | Zm00001d051870   | Chr4: 172,950,400–172,953,761 | 1.6                            | 1.9                            | Suppressed                    | In this study               |
| HCT1806                   | Zm00001d027946   | Chr1: 18,086,164–18,087,981 | 296.1                          | 223.7                          | Suppressed                    | Wang, He, et al. (2015)    |
| HCT4918                   | Zm00001d027948   | Chr1: 18,147,157–18,148,937 | 1,115.0                        | 568.5                          | Suppressed                    | In this study               |
| HCT0436                   | Zm00001d030542   | Chr1: 142,569,190–142,571,156 | 23.1                           | 46.0                           | Weakly suppressed             | In this study               |
| HCT7251                   | Zm00001d037073   | Chr6: 111,482,487–111,483,803 | 178.0                          | 158.8                          | No effect                     | In this study               |
| HCT584                    | Zm00001d017186   | Chr5: 188,273,104–188,277,915 | 6.1                            | 4.5                            | No effect                     | In this study               |
| HCT8083                   | Zm00001d03129    | Chr2: 33,327,793–33,333,386  | 4.4                            | 2.8                            | No effect                     | In this study               |
| CCoAOMT1                  | Zm00001d036293   | Chr6: 82,193,275–82,194,916  | 1.0                            | 0.7                            | No effect                     | Wang & Balint-Kurti (2016) |
| CCoAOMT2                  | Zm00001d045206   | Chr9: 16,076,158–16,081,626 | 2.2                            | 1.7                            | Suppressed                    | In this study               |
| PAL homologs              |                  |                      |                                |                                |                               |                             |
| Zm00001d030315            |                  | Chr2: 29,467,931–29,470,598 | 4.3                            | 13.5                           | No effect                     | In this study               |
| Zm00001d017276            |                  | Chr5: 191,474,696–191,476,810 | 10.8                           | 13.9                           | No effect                     | In this study               |
| Zm00001d017274            |                  | Chr5: 191,418,711–191,422,345 | 4.5                            | 2.2                            | No effect                     | In this study               |
| Zm00001d051163            |                  | Chr5: 146,793,740–146,798,809 | 17.6                           | 10.4                           | No effect                     | In this study               |
| CCR homolog               | Zm00001d009666   | Chr8: 75,117,321–75,122,209 | 6.6                            | 6.9                            | No effect                     | In this study               |
| Zm00001d032152            |                  | Chr1: 214,573,739–214,579,407 | 10.9                           | 5.3                            | Weakly suppressed             | In this study               |
| Zm00001d011438            |                  | Chr8: 150,482,983–150,484,456 | 35.0                           | 17.6                           | No effect                     | In this study               |
| CAD homologs              | Zm00001d015618   | Chr5: 101,493,553–101,498,009 | 7.6                            | 3.4                            | No effect                     | In this study               |
| Zm00001d045043            |                  | Chr9: 10,913,773–10,917,850  | 3.6                            | 5.3                            | No effect                     | In this study               |
| C4H homolog               | Zm00001d009858   | Chr8: 85,446,321–85,449,522  | 6.1                            | 6.2                            | No effect                     | In this study               |
| 4Cl homologs              | Zm00001d015459   | Chr5: 91,459,227–91,463,857  | 8.6                            | 5.6                            | No effect                     | In this study               |
| Zm00001d032103            |                  | Chr1: 213,126,776–213,129,233 | 13.1                           | 10.5                           | No effect                     | In this study               |
| C3H homolog               | Zm00001d038555   | Chr6: 159,789,930–159,794,329 | 16.4                           | 6.9                            | No effect                     | In this study               |
| MTHFR2 (bm2)              | Zm00001d034602   | Chr1: 297,605,177–297,611,407 | 4.3                            | 4.8                            | No effect                     | Zhu et al. (2020)          |

*The fold change (FC) of the transcript levels of maize genes in Rp1-D21 compared to the corresponding wild type. bm, brown midrib.*
with Rp1-D21 in *N. benthamiana* (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015). To investigate whether other enzyme homologs in the lignin biosynthesis pathway act in Rp1-D21-mediated auto-HR, we cloned most of their encoding genes and coexpressed them with Rp1-D21 in *N. benthamiana*. Of 16 proteins tested in this study, 14 of them showed no obvious effect on Rp1-D21-mediated auto-HR, including ZmCOMT, MTHFR2, and one CAD homolog (Zm00001d015618) (Table 1). The maize brown midrib mutants *bm1*, *bm2*, and *bm3* cause mutations in these three proteins, respectively (Halpin et al., 1998; Tang et al., 2014; Vignols et al., 1995). One CCR homolog (Zm00001d032152) showed a weak suppression effect on Rp1-D21-mediated auto-HR and only Zm00001D051870, a close homolog of ZmCOMT, suppressed Rp1-D21-mediated auto-HR (Table 1).

### 2.2 Phylogenetic analysis of COMT and NANMT homologs in plants

To determine the relationship between ZmCOMT and its homolog (ZM00001D051870), the amino acid sequences of ZmCOMT and
ZM00001D051870 were used as queries in a BLAST search against the plant genomics resource database (Phytozome, https://phytozome.jgi.doe.gov/pz/portal.html). Homologous sequences from different plant species were used for phylogenetic analysis. The phylogenetic tree was separated into two groups, the COMT group and the NAC group, and each group was further divided into two clades containing COMT homologs and NAC homologs from dicots and monocots, respectively (Figure 1). ZmCOMT was in the same clade as AtCOMT1, which was confirmed to have COMT activity (Li et al., 2017). ZM00001D051870 was in the same clade as AtNAC1, which was confirmed to have NAC activity (Li et al., 2017), therefore ZM00001D051870 was named ZmNANMT hereafter.

2.3 | ZmNANMT, but not ZmCOMT, suppresses Rp1-D21-mediated auto-HR in N. benthamiana

To further examine the function of ZmCOMT and ZmNANMT in Rp1-D21-mediated auto-HR, we used the Agrobacterium-mediated transient expression system in N. benthamiana, a widely used method for investigating the function of genes involved in auto-HR (Hamel et al., 2016; Qi et al., 2012; Slootweg et al., 2018; Wang, Ji, et al., 2015; Wang, Grubb, et al., 2018). Rp1-D21 was fused with a C-terminal 3× hemagglutinin (HA) tag (Rp1-D21:HA), while ZmNANMT and ZmCOMT were fused with a C-terminal enhanced green fluorescent protein (EGFP) tag (ZmNANMT:EGFP and ZmCOMT:EGFP). β-glucuronidase (GUS):EGFP and HCT1806:EGFP were used as negative and positive controls, respectively. When GUS:EGFP and Rp1-D21:HA were transiently coexpressed in N. benthamiana, an obvious auto-HR was observed at 3 days postinfiltration (dpi), while no obvious auto-HR was observed in leaves co-infiltrated with HCT1806:EGFP and Rp1-D21:HA (Figure 2a), which is consistent with our previous reports (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015). When ZmNANMT:EGFP or ZmCOMT:EGFP was transiently coexpressed with Rp1-D21:HA in N. benthamiana, ZmNANMT:EGFP, but not ZmCOMT:EGFP, suppressed Rp1-D21-mediated auto-HR (Figure 2a). To verify the visual observation results, we further measured the levels of ion leakage conductivity. Similar to HCT1806:EGFP, ZmNANMT:EGFP significantly reduced ion leakage levels compared to the GUS:EGFP control or ZmCOMT:EGFP when coexpressed with Rp1-D21:HA (Figure 2b). Western blot results
showed that coexpression of ZmNANMT:EGFP or ZmCOMT:EGFP did not affect the protein accumulation of Rp1-D21 compared to coexpression of GUS:EGFP control, and all the EGFP-tagged proteins were expressed at substantial levels (Figure 2c).

2.4 | ZmNANMT is not a general cell death suppressor

To determine whether ZmNANMT:EGFP could inhibit auto-HR mediated by other NLRs, we coexpressed it with two CNLs, barley MLA10(D502V) and Arabidopsis RPM1(D505V), that confer an auto-HR when transiently expressed in N. benthamiana (Bai et al., 2012; Gao et al., 2011). Consistent with our previous results (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015), GUS:EGFP and HCT1806:EGFP had no obvious suppression roles on either MLA10(D502V) or RPM1(D505V)-mediated auto-HR when transiently coexpressed in N. benthamiana leaves (Figure S1a,b). Similarly, neither ZmNANMT:EGFP nor ZmCOMT:EGFP suppressed the auto-HR mediated by either CNL protein (Figure S1a,b).

INF1 is a cell death elicitor secreted from Phytophthora infestans and Bax belongs to a death-promoting protein of the Bcl-2 family from mouse. INF1 and Bax can trigger cell death when transiently expressed in N. benthamiana (Kamoun et al., 1998; Lacomme & Santa Cruz, 1999) and therefore were used as representatives to determine whether ZmNANMT:EGFP could suppress cell death mediated by other proteins. When coexpressed with INF1 or Bax, ZmNANMT:EGFP and ZmCOMT:EGFP showed no obvious suppression on either protein-mediated cell death (Figure S1c,d). These results indicate that ZmNANMT might not be a general cell death suppressor.

2.5 | The putative enzymatic activity of ZmNANMT might not be required for suppressing Rp1-D21-mediated auto-HR

In Arabidopsis, AtNANMT1 has NA N-methyltransferase activity, catalysing the conversion of NA to Tg. Mutation of the catalytic residue Thr at position 264 in AtNANMT1 to the corresponding His in AtCOMT to generate AtNANMT1(T264H) caused the loss of NANMT enzyme activity (Li et al., 2017). We found that the catalytic residue Thr is conserved among different plant NANMT proteins, including ZmNANMT(T278); the corresponding residue in ZmCOMT is His (Figure S2a). To investigate the effect of the predicted catalytic residue
in ZmNA\textsubscript{MT} on suppressing Rp1-D21-mediated auto-HR, we created the corresponding mutation to generate ZmNA\textsubscript{MT}(T278H) and fused it with a C-terminal EGFP tag (ZmNA\textsubscript{MT}[T278H]:EGFP). When coexpressed with Rp1-D21 in \textit{N. benthamiana}, ZmNA\textsubscript{MT}(T278H):EGFP still suppressed Rp1-D21-mediated auto-HR, which was similar to the suppression effect caused by ZmNA\textsubscript{MT}:EGFP (Figure 3a,b). Western blot results showed that Rp1-D21:HA and all EGFP-tagged proteins were expressed at substantial levels (Figure 3c).

ZmNA\textsubscript{MT} was predicted to contain three amino acid residues (N34, Y129, and H133) in the active site, which were also conserved among different plant NAMTs (Figure S2b). The protein structure of ZmNA\textsubscript{MT} was modelled using the SWISS-MODEL platform (https://swissmodel.expasy.org/interactive) based on the structure of \textit{Sorghum bicolor} COMT (SbCOMT, PDB no. 4PGH) (Green et al., 2014), which showed 35.5% sequence identity with ZmNA\textsubscript{MT} (Figure S2c). The results suggest that ZmNA\textsubscript{MT} might form a homodimer with two activity centres (Figure S2d). Each activity centre contained the three predicted substrate-binding sites (N34, Y129, and H133) and the predicted activity site (T278), which were close in space. Among these four sites, Y129, H133, and T278 belonged to chain A of the dimer while N34 belonged to chain B. To further investigate which region is required for inhibiting Rp1-D21-mediated auto-HR, ZmNA\textsubscript{MT} was truncated into two segments: the 251 amino acids at the N-terminus (ZmNA\textsubscript{MT}-N251) and the 123 amino acids at the C-terminus (ZmNA\textsubscript{MT}-C123). ZmNA\textsubscript{MT}-N251 contains three predicted substrate-binding sites (N34, Y129, and H133) while ZmNA\textsubscript{MT}-C123 contains the predicted enzyme catalytic site (T278). When ZmNA\textsubscript{MT}-N251 and ZmNA\textsubscript{MT}-C123 were coexpressed with Rp1-D21, ZmNA\textsubscript{MT}-C123 suppressed Rp1-D21-mediated auto-HR to the same level as full-length ZmNA\textsubscript{MT}, but ZmNA\textsubscript{MT}-N251 showed
a partial suppression effect (Figure S3a,b). Western blot results showed that Rp1-D21:HA and all EGFP-tagged proteins were expressed at substantial and broadly comparable levels (Figure S3c).

2.6 ZmNANMT suppresses CC$_{D21}$-mediated auto-HR and interacts with CC$_{D21}$

Rp1-D21 contains three domains: the CC domain (CC$_{D21}$), the NB domain, and the LRR domain. When they were fused with C-terminal EGFP tags and transiently expressed in N. benthamiana, only CC$_{D21}$:EGFP conferred an auto-HR phenotype (Wang, Ji, et al., 2015). To determine the function of ZmNANMT and ZmCOMT in CC$_{D21}$-mediated auto-HR, we transiently coexpressed them with CC$_{D21}$:EGFP in N. benthamiana. The results showed that ZmNANMT, but not ZmCOMT, suppressed CC$_{D21}$-mediated auto-HR (Figure 4a,b). All proteins were expressed at substantial levels (Figure 4c).

To investigate whether ZmNANMT can interact with CC$_{D21}$, we performed coimmunoprecipitation (Co-IP) in N. benthamiana using EGFP-tagged proteins and CC$_{D21}$:Myc (with a 4×c-Myc tag fused at the C-terminal of CC$_{D21}$). The result showed that ZmNANMT, similar to the HCT1806 control, interacted with CC$_{D21}$, while ZmCOMT and GUS did not interact with CC$_{D21}$ (Figure 4d).

When ZmNANMT-N$_{251}$ and ZmNANMT-C$_{123}$ were coexpressed with CC$_{D21}$:EGFP, ZmNANMT-C$_{123}$ suppressed CC$_{D21}$-mediated auto-HR to the same level as full-length ZmNANMT, but ZmNANMT-N$_{251}$ showed a partial suppression effect (Figure S4a,b).

Western blot results showed that all EGFP-tagged proteins were expressed at substantial levels (Figure S4c).

2.7 ZmNANMT interacts with HCT1806 and CCoAOMT2

In our previous studies (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015), HCT1806 and CCoAOMT2 interacted with CC$_{D21}$ and suppressed CC$_{D21}$- and Rp1-D21-mediated auto-HR. To determine whether ZmNANMT or ZmCOMT can interact with HCT1806 or CCoAOMT2, we tested their interactions via yeast two-hybrid (Y2H) assays. The results showed that ZmNANMT, but not ZmCOMT, interacted with both HCT1806 and CCoAOMT2 in yeast (Figure 5). Maize CCoAOMT1 has high sequence similarity to CCoAOMT2 but has no suppression role on Rp1-D21-mediated auto-HR (Wang & Balint-Kurti, 2016). We found that neither ZmNANMT nor ZmCOMT interacted with CCoAOMT1 in yeast (Figure S5). All proteins transformed into yeast were substantially expressed (Figure S6).

2.8 ZmNANMT, HCT1806, CCoAOMT2, and CC$_{D21}$ form complexes

To test whether ZmNANMT, HCT1806, CCoAOMT2, and CC$_{D21}$ could form complexes, a Co-IP assay was performed by transiently
coexpressed EGFP-tagged ZmNANMT, ZmCOMT or GUS, HA-tagged HCT1806, and Myc-tagged CCoAMOT2 and CC\textsubscript{D21} in \textit{N. benthamiana}. The results showed that ZmNANMT, but not ZmCOMT or GUS, interacted strongly with all of the three proteins CC\textsubscript{D21}, CCoAMOT2, and HCT1806 (Figure 6), indicating that they can form a complex(es).
2.9 | Either the cytoplasmic or the nuclear localization of ZmNA\textsuperscript{N}MT is important for suppressing auto-HR

To determine the subcellular localization of ZmNA\textsuperscript{N}MT:EGFP or ZmCOMT:EGFP, they were transiently expressed in a transgenic \textit{N. benthamiana} line carrying the stably expressed nuclear marker H2B:red fluorescent protein (RFP) (Martin et al., 2009). It showed that ZmNA\textsuperscript{N}MT:EGFP, ZmCOMT:EGFP, and GUS:EGFP were all mainly located in the cytoplasm and nucleus (Figure S7).

Both Rp1-D21 and CC\textsubscript{D21} are located in the cytoplasm and nucleus, and nucleocytoplasm trafficking is required for auto-HR induction (Wang & Balint-Kurti, 2015). To investigate whether the suppression of ZmNA\textsuperscript{N}MT on CC\textsubscript{D21}-mediated auto-HR is due to

![Image of subcellular localization experiments](image-url)
a change in the subcellular localization of \( \text{CC}_{221} \). We coexpressed \( \text{ZmNaNMT-EGFP} \) and \( \text{CC}_{221}:\text{RFP} \) in \( \text{N. benthamiana} \). Consistent with our previous results (Wang & Balint-Kurti, 2015; Zhu et al., 2020), when transiently coexpressed with GUS:EGFP in \( \text{N. benthamiana} \) \( \text{CC}_{221}:\text{RFP} \) was mainly located in cytoplasm and nucleus (Figure S8). When transiently coexpressed with \( \text{ZmNaNMT-EGFP} \) or \( \text{ZmCOMT:EGFP} \) in \( \text{N. benthamiana} \) \( \text{CC}_{221}:\text{RFP} \) was also mainly distributed in the cytoplasm and nucleus (Figure S8). The results indicated that the \( \text{ZmNaNMT} \) suppression of \( \text{CC}_{221} \)-mediated auto-\( \text{HR} \) was not caused by affecting the \( \text{CC}_{221} \) subcellular localization.

To determine whether the cytoplasmic or the nuclear localization of \( \text{ZmNaNMT} \) is important for suppression of \( \text{CC}_{221} \)-mediated auto-\( \text{HR} \), we added a nuclear localization signal (NLS), nuclear export signal (NES), or their corresponding mutants nls or nes (that differ from NLS and NES by one and three amino acids, respectively), to the C-terminus of \( \text{ZmNaNMT} \) and then fused them with EGFP tag to generate four proteins, \( \text{ZmNaNMT-NLS:EGFP}, \text{ZmNaNMT-nls:EGFP}, \text{ZmNaNMT-nes:EGFP}, \text{and ZmNaNMT-nes:EGFP} \). When transiently expressed in \( \text{N. benthamiana} \), \( \text{ZmNaNMT-NLS:EGFP} \) and \( \text{ZmNaNMT-nes:EGFP} \) were predominantly located in the nucleus and cytoplasm, respectively, while \( \text{ZmNaNMT-nls:EGFP} \) and \( \text{ZmNaNMT-nes:EGFP} \) were still localized in both the cytoplasm and nucleus, which was similar to the localization of \( \text{ZmNaNMT-EGFP} \) (Figure 7a). When transiently coexpressed with \( \text{CC}_{221}:\text{EGFP} \) in \( \text{N. benthamiana} \), both \( \text{ZmNaNMT-NLS:EGFP} \) and \( \text{ZmNaNMT-NES:EGFP} \) suppressed \( \text{CC}_{221} \)-mediated auto-\( \text{HR} \) (Figure 7b). Similarly, \( \text{ZmNaNMT-nls:EGFP} \) and \( \text{ZmNaNMT-nes:EGFP} \) also suppressed \( \text{CC}_{221} \)-mediated auto-\( \text{HR} \) (Figure 7b). Western blotting results showed that all EGFP-tagged proteins were expressed at substantial levels (Figure 7c). These results indicate that both the nuclear and cytoplasmic localizations of \( \text{ZmNaNMT} \) play important roles in suppression \( \text{Rp1-D21} \)-mediated auto-\( \text{HR} \).

3 | DISCUSSION

3.1 | \( \text{ZmNaNMT} \) might act as a novel NLR regulator in plant defence response

The lignin biosynthesis pathway plays important roles in plant disease resistance. There are at least 10 key enzymes involved in the lignin biosynthesis pathway in plants (Vanholme et al., 2008); however, little is known about their roles in NLR-mediated defence response. We have shown that the maize HCT and CCoAOMT homologs, HCT1806, HCT4918, and CCoAOMT2, suppress the NLR protein \( \text{Rp1-D21} \)-mediated auto-\( \text{HR} \) when they were transiently coexpressed with \( \text{Rp1-D21} \) in \( \text{N. benthamiana} \) (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015). However, here we found that none of the genes encoding homologs of other tested enzymes in the lignin biosynthesis pathway had an obvious effect on \( \text{Rp1-D21} \)-mediated auto-\( \text{HR} \), even though they were significantly up-regulated in maize lines carrying \( \text{Rp1-D21} \) compared to the wild type (Table 1). Intriguingly, \( \text{ZmNaNMT} \), but not its close homolog \( \text{ZmCOMT} \), suppressed \( \text{Rp1-D21} \)-mediated auto-\( \text{HR} \), and even the gene encoding \( \text{ZmNaNMT} \) was only slightly up-regulated in \( \text{Rp1-D21} \) (Table 1). As a key enzyme in the lignin biosynthesis pathway, COMT has been shown to play important roles in plant disease resistance (Liu et al., 2017; Wang, Zhu, et al., 2018). These data suggest that the role of COMT in disease resistance and NLR-mediated auto-\( \text{HR} \) might be uncoupled. Interestingly, \( \text{AtCOMT1} \) and some of its homologs from different plants also showed weak \( \text{NANMT} \) activity, while \( \text{NANMT} \) homologs from different plants did show COMT activity. Phylogenetic and enzymatic analyses suggest that \( \text{NANMT} \) is probably derived from the duplication and subfunctionalization of an ancestral COMT gene during plant evolution (Li et al., 2017).

\( \text{Tg} \) is known to play important functions in plants and animals and it is believed to be derived from NA methylation catalysed by an \( \text{N-methyltransferase} \) (Minorsky, 2002; Upmeier et al., 1988; Zhou et al., 2012). However, there was no direct genetic and molecular evidence for the genes responsible for this catalytic reaction until \( \text{AtNANMT} \) was identified in \( \text{Arabidopsis} \) (Li et al., 2017). \( \text{NANMT} \) has been reported to participate in \( \text{Tg} \) synthesis in \( \text{Arabidopsis} \); however, there are no functional reports on other biological processes, including plant defence response. Many NLR regulators modulate the activity of NLRs through protein interaction in plants. Seven major classes of NLR regulators have been recently summarized, including transcription factors, E3 ligases, molecular chaperones, and kinases (Sun et al., 2020). In this study, we determined that \( \text{ZmNaNMT} \), a homolog of \( \text{AtNANMT1} \) in maize, acted as a novel class of NLR regulator to negatively regulate the NLR protein \( \text{Rp1-D21} \)-mediated auto-\( \text{HR} \).
3.2 | NANMT enzyme activity might not be required for suppression of Rp1-D21-mediated auto-HR

In AtNANMT1, the Thr residue at position 264 is important for its catalytic activity and the mutation of AtNANMT1T264H failed to convert NA to Tg (Li et al., 2017). We performed the corresponding mutation in ZmNANMT to generate ZmNANMT(T278H) and found it had a similar suppressive effect with ZmNANMT on Rp1-D21-mediated auto-HR (Figure 3a,b). Furthermore, when ZmNANMT was divided into the N-terminal and C-terminal parts, which contained the methyl donor-binding sites and the catalytic site, respectively, both ZmNANMT-N255 and ZmNANMT-C123 suppressed Rp1-D21-mediated auto-HR even though the suppression effect of ZmNANMT-N255 was weaker than that of ZmNANMT-C123 and the full-length protein (Figure S3). These results suggest that the enzymatic activity of ZmNANMT is not required for its function in suppressing Rp1-D21-mediated auto-HR. Interestingly, one of the breakthrough discoveries recently in the NLR field was that the TIR domains of TNLs act as NAD⁺-cleaving enzymes to promote cell death (Horsefield et al., 2019; Wan et al., 2019). The TIR domains cleave NAD⁺ to produce nicotinamide, ADP-ribose (ADPR), and a variant of cyclic ADPR (v-cADPR), and the latter two can trigger calcium influx to the cytoplasm, which is important for NLR function (Wan et al., 2019; Wu et al., 1997). NAD⁺ can be converted to NA with nicotinamide as intermediate in plants. NANMT is a key enzyme by converting NA into Tg (Li et al., 2017). Here we showed that NANMT acts as a regulator in CNL Rp1-D21-mediated auto-HR. However, the molecular mechanism of how NANMT functions is not clear. Unlike the TIR domain, no enzymatic activity has been demonstrated for the CC domain. According to cryoelectron microscopy examination of the structure of the CNL protein ZAR1 (HOPZ ACTIVATED RESISTANCE 1) resistosome, the CC domain of ZAR1 can form a funnel-like structure and insert into the plasma membrane to form a pore that might allow calcium influx to trigger auto-HR (Wang, Hu, et al., 2019; Wang, Wang, et al., 2019). It seems that calcium influx/efflux across the plasma membrane might be a convergent event for the signalling pathways mediated by CNLs and TNLs (Saur et al., 2020; Sun et al., 2020). It will be interesting to investigate whether NANMT can affect calcium homeostasis and modulate NLR activity.

3.3 | Both the cytoplasmic and nuclear localizations of ZmNANMT are important for auto-HR suppression

The subcellular localization results showed that both ZmCOMT and ZmNANMT are located in the cytoplasm and nucleus (Figure S7), indicating that the different suppression effect on Rp1-D21-mediated auto-HR is not due to their differential subcellular localizations. We previously reported that both Rp1-D21 and CC_D21 are located in the nucleus and cytoplasm, and that nucleocyttoplasmic trafficking plays an important role in auto-HR induction (Wang & Balint-Kurti, 2015). We have recently shown that two type I maize metacaspases (ZmMCs), ZmMC1 and ZmMC2, interact with CC_D21, and change its subcellular localization to suppress auto-HR (Luan et al., 2020). When ZmNANMT was coexpressed with CC_D21 in N. benthamiana, it did not cause the redistribution of CC_D21 in plant cells (Figure S8). When ZmNANMT was moved to the cytoplasm or nucleus by adding NES or NLS, it still suppressed CC_D21-mediated auto-HR (Figure 7). These results indicate that both the cytoplasmic and nuclear localization of ZmNANMT are important for suppression auto-HR and blocking Rp1-D21 anywhere in the nucleus or cytoplasm might destroy the nucleocyttoplasmic trafficking and its ability to induce auto-HR.

3.4 | ZmNANMT may cooperate with other NLR suppressors to trap effectors in Rp1-mediated immune response

NLR proteins can recognize pathogen effectors directly or indirectly. Several models have been proposed to explain how NLR receptors recognize different pathogen effectors, including the decoy model. According to this model, some plant proteins are employed as decoys by an NLR to selectively recognize different pathogen effectors (Cesari, 2018; Moffett, 2016). An NLR receptor can even interact with several host proteins to indirectly recognize multiple pathogen effectors from different pathogens (Sun et al., 2020), while direct recognition only allows NLRs to recognize one or a limited number of effectors. In addition, in the process of evolution, effectors can easily escape the direct recognition of receptors. Therefore, compared with direct recognition, indirect recognition through decoys by NLR receptors can provide more effective options for the plant immune system and increase the fitness costs of pathogens to avoid being recognized (Cesari, 2018). In Arabidopsis, the NLR protein ZAR1 is a typical example that can interact with and use several kinases or pseudokinases as decoys to perceive a few effectors from different pathogens (Baudin et al., 2017; Lafllamme et al., 2020; Lewis et al., 2013; Martel et al., 2020; Seto et al., 2017; Wang, Roux, et al., 2015). Among them, ZAR1 and a pseudokinase RK51 form a complex to recognize the uridylylated receptor-like cytoplasmic kinase PBL2 (PBL2 UMP) catalysed by Xanthomonas campestris effector AvrAC (Wang, Roux, et al., 2015). One of the most important recent discoveries is that the structure of the ZAR1-RK51-PBL2 UMP complex was characterized by cryoelectron microscopy and it showed that the activated complex forms a wheel-like pentameric “resistosome,” which is required for the immune response (Wang, Hu, et al., 2019; Wang, Wang, et al., 2019).

In maize, HCT1806, CCaOMT2, and ZmNANMT suppressed Rp1-D21-mediated auto-HR and functioned as negative regulators in plant defence response. Rp1-D confers disease resistance to common rust caused by the fungus P. sorghi (Collins et al., 1999). Due to the lack of the corresponding effectors of Rp1-D from P. sorghi, we do not know whether these suppressors can act as decoys to trap effectors. From our Co-IP and Y2H assays, we showed that...
ZmNANMT, HCT1806, and CCoAOMT2 interacted with CC\textsubscript{D21} (Figure 4) (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015), and all three suppressors can interact with each other (Figure 5) and form a complex(es) with CC\textsubscript{D21} (Figure 6). The structure of the N-terminal CC domain of ZAR1 is similar to those of the CC domains of the potato CNL Rx and the wheat CNL Sr33, suggesting structural and functional conservation of the CC domains of CNLs across plant lineages (Saur et al., 2020). Similar to the CC domain of ZAR1, CC\textsubscript{D21} can also trigger auto-HR and interact with several different NLR regulators. By analogy with the ZAR1 resistosome mentioned above, we speculate that Rp1-D21 might form a similar resistosome, which might include ZmNANMT and other regulator proteins (e.g., HCT1806 and CCoAOMT2) to recognize unknown pathogen effector(s) for activation.

In summary, we have demonstrated that ZmNANMT acts as a novel negative regulator to suppress the NLR protein Rp1-D21-mediated auto-HR. To our knowledge, it has not been reported previously in any species that NANMTs can play a role in the plant defence response. Although most data were derived from the ectopic system using Agrobacterium-mediated transient over-expression in \textit{N. benthamiana}, we believe it can largely reflect the situation in the endogenous maize system for the reasons mentioned previously (Luan et al., 2020; Wang, Ji, et al., 2015). We hope to verify these results when maize genetic materials are available in the future. Hopefully, our novel findings will lay the foundation for elucidating the molecular mechanism of NANMTs in plant innate immunity.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material and growth conditions

\textit{N. benthamiana} wild-type and H2B-RFP transgenic plants were grown in the greenhouse under conditions of 24 °C with a cycle of 16 hr light and 8 hr dark. Maize (\textit{Zea mays}) line B73 was grown in a plant growth chamber under conditions of 24 °C with a cycle of 12 hr light and 12 hr dark.

4.2 | Sequence alignment and phylogenetic analysis

The homologs of COMT and NANMT from different plant species were downloaded in FASTA format, and then aligned by ClustalW (www.ebi.ac.uk). The phylogenetic tree was constructed using MEGA v. 6.0 software applying neighbour-joining (NJ) algorithms with 1,000 bootstrap replicates (Tamura et al., 2013).

4.3 | Genes cloning and plasmid construction

RNA was extracted from B73 seedlings at the four-leaf stage using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription (RT) first-strand cDNA synthesis was performed using TransScript First-Strand cDNA Synthesis SuperMix (Transgene). The coding sequences (CDS) of ZmNANMT and ZmCOMT were amplified from the cDNA above and cloned into the Gateway Entry vector pENTR/D-TOPO (Invitrogen). The sequences of NLS, NES, nls, and nes were separately added to the C-terminus of ZmANAMT CDS by PCR (Wang & Balint-Kurti, 2015). Overlap extension PCR was performed to generate ZmNANMT CDS containing the site-directed mutation of ZmNANMT(T278H). All of the PCR products were cloned into the pENTR/D-TOPO vector. The primers used in PCR are listed in Table S1. After verification by sequencing, different target sequences carried in the pENTR/D-TOPO vectors were transferred into pSITEII-N1-EGFP vector (Martin et al., 2009) by LR recombination reaction following the manufacturer's instructions (Invitrogen) and used for transient expression in \textit{N. benthamiana}. Rp1-D21:HA, GUS:EGFP, HCT1806:EGFP, CC\textsubscript{D21}:EGFP, CC\textsubscript{D21}:Myc, and HCT1806:HA were generated in our previous studies (Wang & Balint-Kurti, 2016; Wang, He, et al., 2015). CCoAOMT2:Myc was generated by transferring CCoAOMT2 carried in the entry vector into the pGWB617 vector by LR reaction.

For construction of Y2H vectors, the target genes were amplified using primers with restriction enzyme sites (Table S1) by PCR. The PCR products and pGBK7T and pGADT7 vectors (Clontech) were digested by the corresponding restriction enzymes and then ligated using DNA T4 ligase (TransGen).

4.4 | Transient expression in \textit{N. benthamiana}

\textit{Agrobacterium tumefaciens} GV3101 (pMP90) was used for transformation of different vector constructs. The detailed procedures of \textit{Agrobacterium}-mediated transient expression in \textit{N. benthamiana} were according to the previous study (Wang, Ji, et al., 2015). Briefly, the agrobacteria were separately cultured using L-broth medium followed by resuspension and mixing according to the experiment requirements using resuspension buffer (10 mM MES pH5.6, 10 mM MgCl\textsubscript{2}, 200 μM acetosyringone). After being placed at room temperature for 3 hr, the solution was infiltrated into the abaxial side of 4-week-old \textit{N. benthamiana} leaves using a syringe with needle removed. At least 20 individual leaves were infiltrated by each construct, and the HR phenotype is illustrated with one representative leaf for each experiment. After infiltration, the plants were placed under the same growing conditions for auto-HR phenotype observation or protein extraction.

4.5 | Ion leakage measurement

The infiltrated region of \textit{N. benthamiana} leaves was sampled using a hole puncher (1.2 cm diameter) and used for ion leakage measurement. At least 12 leaf discs were sampled from different leaves for each protein and every three leaf discs were put in a 10-ml polypropylene tube containing 4 ml of sterile water. After shaking for 3 hr at
room temperature, the conductivity of different samples was measured individually using a conductivity meter (METTLER TOLEDO) and the values were recorded as C1. Subsequently, samples were boiled for 15 min and naturally cooled to room temperature. The conductivity was measured again as C2, which represented the total conductivity. The ion leakage was calculated as the C1/C2 ratio and the mean was used for final analysis (Wang & Balint-Kurti, 2015).

### 4.6 Protein extraction and analysis

Protein extraction from *N. benthamiana* leaves, protein expression, and Co-IP assays were performed according to previous studies (Wang, Ji, et al., 2015; Zhu et al., 2020) with minor modifications. Briefly, for protein expression detection, three leaf discs (each 1.2 cm diameter, total about 50 mg) from different leaves infiltrated by agrobacteria were collected at 30 hr postinfiltration (hpi). Total protein was extracted in 150 µl of western blotting buffer (20 mM Tris.HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 10 mM diethiothreitol [DTT], 40 µM MG132, and 1 x plant protein protease inhibitor mixture [Sigma-Aldrich]). Subsequently, total protein was loaded to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by western blotting. The target proteins were detected by immunoblot (IB) with α-HA (cat. # H6908; Sigma) or α-EGFP (cat. # A01704S; GenScript) antibodies at a 1:5,000 dilution. For the Co-IP assay, three whole leaves infiltrated by agrobacteria were collected at 48 hpi and 0.6 g of leaf samples was used for total protein extraction in 1.8 ml of Co-IP buffer (50 mM HEPES pH 7.5, 50 mM NaCl, 10 mM EDTA, 0.5% Triton 100, 4 mM DTT, 40 µM MG132, and 1 x plant protein protease inhibitor mixture). Subsequently, total protein was used for Co-IP assay using the kit containing anti-GFP microbeads (cat. # 130–091–125; Miltenyi Biotec) and the detailed procedures were performed following the manufacturer’s instructions and our previous studies (Wang, Ji, et al., 2015; Zhu et al., 2020). The target proteins were detected by IB with α-HA, α-EGFP, or α-Myc (cat. # A00704S; GenScript) antibodies at 1:5,000 dilution.

### 4.7 Yeast two-hybrid assay

*Saccharomyces cerevisiae* Y2HGold (Clontech) was used for the Y2H assay. pGBK T7- and pGADT7-derived constructs were transformed into Y2HGold and the Y2H experiment was performed according to the protocols provided by the manufacturer. DDO (minimal synthetic defined base with added double dropout supplement – Leu/-Trp) culture plate was used for positive transformation screening and QDO (SD base with added quadruple dropout supplement – Ade/-His/-Leu/-Trp)/Xα-Gal culture plate was used for protein interaction verification. Vectors pGBK T7-53 and pGADT7-T (Clontech) were used as positive control because the murine p53 protein (53) can interact with SV40 large T-antigen (T) in yeast, while pGBK T7-Lam (Clontech) and pGADT7-T were used as negative control because human lamin C protein (Lam) cannot interact with T in yeast.

### 4.8 Confocal microscopy

The abaxial sides of *N. benthamiana* leaves infiltrated by agrobacteria were used for fluorescence observation by a confocal microscope (LSM 880; Carl Zeiss) at 48 hpi. EGFP fluorescence was excited at 488 nm and observed between 495 and 550 nm. RFP was excited at 561 nm and observed between 580 and 675 nm.

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### CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential 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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