INTRODUCTION

The effector-triggered immunity (ETI) response is triggered upon recognition of pathogen-derived effector molecules exported into the host cell or apoplast by plant resistance (R) proteins, usually located in the cytosol (Bent & Mackey, 2007). This recognition event results in a cascade of defence responses termed the effector-triggered immunity (ETI) response, including production of reactive...
oxygen species (ROS), a spike in calcium signalling, cell wall reinforcement, accumulation of various secondary metabolites, and in many cases a rapid localized cell death at the site of infection called a hypersensitive response (HR) (Balint-Kurti, 2019).

Most R-proteins are nucleotide-binding site, leucine-rich repeat (NLR) proteins where the C-terminal leucine-rich repeat (LRR) domain is fused with a central nucleotide-binding site (NBS) domain (Bent & Mackey, 2007). NLRs are further classified into two groups based on their N-terminal domains: CC-NB-LRRs (CNLs), which have coiled-coil (CC), and TIR-NB-LRRs (TNLs), which have toll interleukin 1 receptor (TIR), domains (Oojen et al., 2008). Another class of receptors, known as pattern recognition receptors (PRRs), that detect conserved microbial molecules known as microbe-associated molecular patterns (MAMPs), are generally receptor kinase proteins located at the cell surface (Newman et al., 2013). Detection of MAMPs triggers a response known as MAMP-triggered immunity (MTI), which includes many of the same responses as noted for ETI, although it is quantitatively lower and does not include an HR (Hamdoun et al., 2013). Recent studies suggest that the main role of ETI may be to potentiate the MTI response (Ngou et al., 2021; Yuan et al., 2021).

The Rp1 locus in maize is a complex locus carrying multiple, tandemly repeated, CNL paralogs (Hulbert, 1997). One paralog, Rp1-D, confers resistance to specific races of *Puccinia sorghi*, a causal agent of maize common rust. The Rp1-D21 gene is derived from an intragenic recombination between two paralogs, Rp1-D and Rp1-dp2, at this locus. It confers an autoactive phenotype in which ETI and HR are induced spontaneously without pathogen infection (Hu et al., 1996; Wang, Ji, et al., 2015). We have characterized the mode of action of Rp1-D21 (Negeri et al., 2013; Wang, Ji, et al., 2015). In particular, we have shown that the severity of the HR phenotype is modulated by genetic background and have used this feature to map modifiers of Rp1-D21 activity (Chintamanani et al., 2010; Olukolu et al., 2013, 2014). We have further identified several genes that affect Rp1-D21 function in various ways (He, Karre, et al., 2019; He, Kim, et al., 2019; Wang & Balint-Kurti, 2016; Wang, He, et al., 2015).

Because HR results in host cell death, inappropriate inactivation can be very costly for the plant. This is clear from the stunned phenotypes caused by Rp1-D21 and many other autoimmune mutants in which ETI and HR are triggered spontaneously (Bruggeman et al., 2015; Chintamanani et al., 2010). Consequently, HR is tightly regulated in plants by several transcriptional, translational, and post-translational mechanisms (Balint-Kurti, 2019). One of the most important of these is regulation of protein stability mediated by the ubiquitination pathway (Marino et al., 2012; Zhou & Zeng, 2017).

Ubiquitination is one of the major posttranslational modification (PTM) processes in eukaryotes (Khoury et al., 2011), and involves tagging of a small protein called ubiquitin to the target substrate. This process is mediated by E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin ligase) enzymes (Callis, 2014) and can be repeated so that chains of ubiquitin (polyubiquitin) are added to the target substrates. Polyubiquitinated proteins are then directed to the proteasome complex for degradation. While E1 and E2 enzymes are functionally critical, E3 ubiquitin ligases (henceforth E3s) are the key specificity components that specify which proteins are targeted for ubiquitination and subsequent degradation (Hershko & Ciechanover, 1998). Plant E3s can be monomeric, capable of binding an E2 and a substrate to facilitate ubiquitylation, without the involvement of other proteins, or they can be multimeric with the E3 itself acting as assembly points for a number of other proteins that together form a complex that facilitates the ubiquitination event. Monomeric E3s are broadly classified into three major subtypes: Really Interesting Genes (RING), Homologous to E6-AP Carboxyl Terminus (HECT), or U-box ligases (Hatakeyama & Nakayama, 2003; Shu & Yang, 2017). RING and U-box E3s facilitate the direct transfer of ubiquitin from the E2 to the lysine residue of the substrate protein, whereas HECT E3 ligases transfer the ubiquitin from the E2 to their HECT domain and then to the substrate (Chen & Hellmann, 2013). Multimeric E3s are usually based around cullin scaffold proteins that recruit RING-domain proteins that bind the E2s at their C-terminal ends, and adaptor proteins at their N-termini that recruit the substrates. Many of these adaptors carry F-box motifs, BTB/POZ domains, or WD40 domains (Chen & Hellmann, 2013; Furniss & Spoe, 2015).

Increasing experimental evidence suggests that E3 ligases have a critical role in modulating defence responses (Marino et al., 2012). Several E3 ligases are known to be involved in regulating levels of PRRs and R-proteins, thereby controlling amplitude and spread of the defence response (Duplan & Rivas, 2014). The rice RING E3 ubiquitin ligase XB3 is necessary for the accumulation of the receptor-like protein XA21 that confers resistance against *Xanthomonas oryzae pv. oryzae* (Wang et al., 2006). The Arabidopsis plant U-box 13 (PUB13) protein displays E3 ligase activity and negatively regulates MTI by targeting the PRRs FLS2 (Lu et al., 2011) and chitin receptor LYK5 proteins (Liao et al., 2017). The E3 PUB13 and its paralog PUB12 appear to direct the proteasomal degradation of the chitin receptor kinase CERK1 (Yamaguchi et al., 2017) after it is activated and as such may modulate the MAMP response. The *Arabidopsis* F-box motif E3 CPR1 targets and interacts with two NLRs, SNC1 and RPS2, and weakens downstream defence responses (Cheng et al., 2011; Gou et al., 2012). Two RING E3s, MUSE1 and MUSE2, interact with and control the accumulation of the NLRs SIKIC1, SIKIC2, and SIKIC3, which are redundantly required for the SNC1-mediated defence response (Dong et al., 2018). Apart from controlling PRR and NLR protein levels, E3 ligases also target proteins involved in downstream defence responses. AtPUB22 targets the exocyst complex subunit Exo70B2 protein, which is involved in vesicle tethering during exocytosis and is necessary for downstream MAPK signalling and ROS accumulation during the MAMP response (Stegmann et al., 2012). Grape EIRP1, a RING E3 ubiquitin ligase, controls protein levels of VVWRKY11, which is a negative regulator of defence response (Yu et al., 2013). The tomato E3 SINA1 is responsible for the degradation of SINA1, which is involved in the defence response (Miao et al., 2016). HR induced by the *Arabidopsis* RPM1 and RPS2 NLR proteins is positively regulated by two RING E3 ligases, RIN2 and RIN3; HR was decreased in RIN2 or RIN3 loss-of-function lines (Kawasaki et al., 2005). Similarly, in tobacco cells, silencing of the U-box E3 ligases CMPG1 reduced HR conferred by the tomato R proteins Pto.
and Cf-9 (González-Lamothe et al., 2006). In Arabidopsis, AtMIEL1 (MYB30 interacting E3 ligase 1), a RING-type E3, targets and directs the degradation of the AtMYB30 transcription factor, regulating downstream HR and defence responses (Marino et al., 2012).

Our previous mapping work used genome-wide association (GWA) mapping in the powerful maize nested association mapping (NAM) population (McMullen et al., 2009) to identify 44 loci associated with variation in the severity of the HR phenotype conferred by Rp1-D21 (Olukolu et al., 2014). The present study identifies ZmMIEL1 RING E3 as underlying the effect at one of these loci. It also shows that ZmMIEL1 interacts with and regulates the transcription factor ZmMYB83, which itself can modulate Rp1-D21-induced HR. We characterize the role of both proteins in modulating HR mediated by Rp1-D21 in maize.

2 RESULTS

2.1 GRMZM2G056270 encodes an Arabidopsis MIEL1 homolog

In a previous genome-wide association study we identified 44 loci associated with variation in the severity of HR conferred by Rp1-D21 (Olukolu et al., 2014). Because this study used the NAM population, a large multiparental mapping population capable of providing mapping precision at the single gene level (McMullen et al., 2009), we were able to identify candidate causal genes at each locus and have recently confirmed identities of four of them (He, Karre, et al., 2019; He, Kim, et al., 2019; Wang & Balint-Kurti, 2016; Wang, He, et al., 2015). A single nucleotide polymorphism (SNP) at 25,214,435 bp on chromosome 10 (B73 V2 genome; https://www.maizegdb.org/gbrowse/maize_v2) was one of the most highly associated of the 44 loci identified in the study based on p value. Six predicted genes are located within 100 kb of this SNP, among which four (GRMZM2G485875, GRMZM2G444675, GRMZM2G004427, and GRMZM2G516696) encode transposable elements. The remaining two genes, GRMZM2G303536 and GRMZM2G056270, encode a thioredoxin family protein and a RING E3 ligase protein, respectively (Figure 1a). GRMZM2G303536 is 36.45 kb distal and GRMZM2G056270 is 83.5 kb proximal of the SNP.

Because E3 ligases have been closely associated with the plant immune response and because, in particular, a homolog of GRMZM2G056270 has been implicated in control of HR in Arabidopsis (see below), we decided to focus on characterization of the possible role of GRMZM2G056270 in modulating HR. Domain analysis using the SMART domain search and PROSITE domain

**FIGURE 1** Identification and homology and phylogenetic analysis of ZmMIEL1. (a) An single nucleotide polymorphism at 25,214,435 bp on chromosome 10 (B73 maize genome v2, maizegdb.org) was previously associated with variation in Rp1-D21-induced hypersensitive response (Olukolu et al., 2014). The predicted genes in this region, including GRMZM2G056270, are indicated by arrows, which also indicate direction of transcription. (b) Predicted domains of the GRMZM2G056270 protein including zinc finger CHY, CTCHY, and RING2 domains. (c) Phylogenetic tree showing the GRMZM2G056270 protein and closely related proteins. The bootstrap values are indicated for each branch of the tree.
prediction tools identified three domains in the protein encoded by GRMZM2G056270 (Figure 1b); a zinc finger CHY domain (Prosite: PS51266), a zinc finger CTCHY domain (Prosite: PS51270), and a zinc finger RING2 domain (Prosite: PS50089). All three domains are believed to bind zinc, and in addition the RING domain is necessary for binding E2s (Metzger et al., 2014). The most closely related maize predicted protein, GRMZM2G062724, shares 29.57% similarity at the amino acid level (Figure S2) and lacks the CHY, CTCHY, and RING2 domains.

Phylogenetic analysis showed that the protein encoded by GRMZM2G056270 was closely related to the MYB30-INTERACTING E3 LIGASE 1 (MIEL1) proteins from Sorghum bicolor and Setaria italica (91.27% and 88.5% similar at the amino acid level, respectively; Figure S1), and that they cluster with AtMIEL1 (Figure 1c) from Arabidopsis, an E3 that regulates the protein levels of transcription factors like AtMYB30 and AtMYB96, regulating HR and abscisic acid (ABA) signalling (Lee & Seo, 2016; Marino et al., 2013).

GRMZM2G056270 shares 61.89% similarity with AtMIEL1 at the amino acid level. In particular the important cysteine and histidine amino acid residues that coordinate binding with zinc ions in the CHY, CTCHY, and RING2 domains (Borden et al., 1995; Gamsjaeger et al., 2007; Laity et al., 2001) are entirely conserved between GRMZM2G056270 and AtMIEL1 (Figure S3). Henceforth, we refer to GRMZM2G056270 as ZmMIEL1.

Because the ZmMIEL1 was identified using the NAM population, we wanted to see if there exists any functional polymorphism across NAM parental lines. We acquired all the protein sequences of the NAM parental ZmMIEL1 alleles from the maize genome database (https://www.maizegdb.org/) plus those from a few other lines, W22, Mo17, PH207, and EP1. Multiple sequence alignment was performed and a phylogenetic tree was built (Figures S4 and S5).Tx303, Mo17, W22, Mo17, PH207, and EP1. Multiple sequence alignment was performed and a phylogenetic tree was built (Figures S4 and S5). Tx303, Mo17, W22 ZmMIEL1 alleles were substantially different to the isogenic susceptible line without Rp1- D21 alone or Rp1-D21 coexpressed with β-glucuronidase (GUS), as determined both by visual assessment (Figure 2a) and by an ion leakage assay (Figure 2b). For this experiment we used the positive control ZmHCT1806, which we had previously shown to physically interact with Rp1-D21 and strongly suppress its activity (Wang, He, et al., 2015). As expected, expression of ZmMIEL1 alone did not cause cell death (Figure 2a,b). Immunoblot analysis showed that the levels of Rp1-D21 protein were relatively consistent when Rp1-D21 was expressed alone or coexpressed with ZmMIEL1, ZmHCT1806, or GUS (Figure 2c), suggesting that the effect of ZmMIEL1 on Rp1-D21-induced HR was not mediated by direct targeting of Rp1-D21 for degradation by ZmMIEL1. Microscope fluorescence imaging confirmed the expression of ZmMIEL1, ZmHCT1806, and GUS proteins in N. benthamiana because the expected fluorescence caused by their fluorescent tags was observed (Figure 2d). Additionally, localization studies suggested that ZmMIEL1 localized to the nucleus and cytosol (Figure 2e), similar to the subcellular localization pattern of AtMIEL1 (Marino et al., 2013).

To investigate whether ZmMIEL1 might perform a similar role in regulating HR induced by other NLR proteins, we used another antitargetive NLR, RPM1D505V, from Arabidopsis that also induced HR in N. benthamiana (Gao et al., 2011). Both visual observations and ion leakage assay showed that ZmMIEL1 suppressed HR induced by RPM1D505V to a similar extent that it suppressed Rp1-D21-induced HR (Figure S7a,b). Immunoblot analysis confirmed that RPM1D505V was expressed at similar levels when expressed alone or when coexpressed with GUS or with ZmMIEL1. ZmMIEL1 and GUS were tagged at the C-terminus with enhanced green fluorescent protein (eGFP) or enhanced yellow fluorescent protein (eYFP), respectively. Microscope fluorescence image analysis confirmed the expected fluorescence was detected (Figure S7c,d).

### 2.2 | ZmMIEL1 suppresses HR induced by autoactive NLRs in N. benthamiana

To determine whether ZmMIEL1 might play a role in regulating HR induced by Rp1-D21, we used an Agrobacterium tumefaciens-mediated transient expression assay that we have used in several other studies. We have previously shown that transient expression of Rp1-D21 in N. benthamiana conferred HR and that the effects of expressing other Rp1-D paralogs and various Rp1-D21 mutations in maize were reliably recapitulated in the N. benthamiana transient expression system (Wang, Ji, et al., 2015). We used this system to identify various modifiers of HR (He, Karre, et al., 2019; He, Kim, et al., 2019; Wang & Balint-Kurti, 2016; Wang, He, et al., 2015).

HR was suppressed when ZmMIEL1 was coexpressed with Rp1-D21 compared to the negative controls, expression of Rp1-D21 alone or Rp1-D21 coexpressed with β-glucuronidase (GUS), as determined both by visual assessment (Figure 2a) and by an ion leakage assay (Figure 2b). For this experiment we used the positive control ZmHCT1806, which we had previously shown to physically interact with Rp1-D21 and strongly suppress its activity (Wang, He, et al., 2015). As expected, expression of ZmMIEL1 alone did not cause cell death (Figure 2a,b). Immunoblot analysis showed that the levels of Rp1-D21 protein were relatively consistent when Rp1-D21 was expressed alone or coexpressed with ZmMIEL1, ZmHCT1806, or GUS (Figure 2c), suggesting that the effect of ZmMIEL1 on Rp1-D21-induced HR was not mediated by direct targeting of Rp1-D21 for degradation by ZmMIEL1. Microscope fluorescence imaging confirmed the expression of ZmMIEL1, ZmHCT1806, and GUS proteins in N. benthamiana because the expected fluorescence caused by their fluorescent tags was observed (Figure 2d). Additionally, localization studies suggested that ZmMIEL1 localized to the nucleus and cytosol (Figure 2e), similar to the subcellular localization pattern of AtMIEL1 (Marino et al., 2013).

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### 2.3 | MG132 attenuates ZmMIEL1 suppression of Rp1-D21-induced cell death

To determine if the effect of ZmMIEL1 on HR was mediated by the proteasome, we coexpressed Rp1-D21 and ZmMIEL1 in N. benthamiana and infiltrated either 50 µM of the proteasome inhibitor MG132 dissolved in dimethyl sulphoxide (DMSO) or DMSO alone as a control. MG132 caused an increase in cell death in regions where Rp1-D21 and ZmMIEL1 were coexpressed, compared to controls where only DMSO was infiltrated into the leaf (Figure 3a). Ion leakage analysis confirmed these results (Figure 3b), and immunoblot analysis with anti-HA antibody suggested Rp1-D21 protein was expressed successfully and protein levels remained unaltered with or without MG132 treatment (Figure 3c). These results suggest that the ZmMIEL1-mediated suppression of Rp1-D21-induced HR requires the proteasomal degradation pathway, but that Rp1-D21 itself is not targeted for degradation by ZmMIEL1.
2.4 | ZmMIEL1 displays autoubiquitination activity

Many RING domain E3s show autoubiquitination activity (Davydov et al., 2004; Yang & Xiaodan, 2003). To determine if ZmMIEL1 displayed autoubiquitination activity, in vitro ubiquitination assays were performed using human UBA1 (E1), UBE2D2 (E2), and HA-tagged ZmMIEL1 protein synthesized using a wheat germ cell protein translation system. When all necessary components—E1, E2, and ZmMIEL1—were present, a protein ladder was detected with an anti-ubiquitin antibody (lane 3, Figure 3d), indicating that ZmMIEL1 had undergone autoubiquitination. The observed autoubiquitination activity of ZmMIEL1 was dependent on E1 and E2 (lanes 1 and 2, Figure 3d). These results suggest that ZmMIEL1 is a functional E3 ubiquitin ligase.

2.5 | Silencing of ZmMIEL1 in maize enhances Rp1-D21-induced cell death

We wanted to confirm that ZmMIEL1 negatively regulated HR induced by Rp1-D21 in maize as we had observed in N. benthamiana. To accomplish this, we used a foxtail mosaic virus (FoMV)-based virus-induced gene silencing (VIGS) system (Mei et al., 2016) to suppress expression of ZmMIEL1 in B73 and B73:Rp1-D21 (B73 carrying Rp1-D21) plants. The pFoMV-ZmMIEL1 construct, which included a 253 bp insert fragment targeting the RING2 domain of ZmMIEL1, was used to suppress ZmMIEL1 expression. The pFoMV-v clone, carrying no insert, served as negative control. Seven-day-old B73 and B73:Rp1-D21 seedlings were infected with the two virus constructs
by particle bombardment, respectively. Two weeks after bombardment, B73:Rp1-D21 plants infected with pFoMV-ZmMIEL1 displayed stronger HR compared to B73:Rp1-D21 plants infected with pFoMV-v (Figure 4a,b). As expected, no HR was observed in B73 plants infected with either construct. Quantitative reverse transcription PCR (RT-qPCR) of lines infected with pFoMV-ZmMIEL1 and pFoMV-v showed that pFoMV-ZmMIEL1 infection suppressed the expression of ZmMIEL1 about 5-fold in B73:Rp1-D21 plants (Figure 4c). These results indicate that suppression of ZmMIEL1 expression in maize leads to more severe HR induced by Rp1-D21 and are consistent with the N. benthamiana transient studies both suggesting that ZmMIEL1 suppresses HR induced by Rp1-D21.

2.6 | ZmMYB83 plays a role in regulating Rp1-D21-mediated cell death

The Arabidopsis MYB transcription factor AtMYB30 is targeted for degradation by AtMIEL1 and is known to regulate the basal defence response and HR (Marino et al., 2013; Raffaele & Rivas, 2013; Raffaele et al., 2006, 2008; Vailleau et al., 2002). In a phylogenetic analysis (Figure S8) AtMYB30 grouped with three maize MYBs, the most highly related of which was ZmMYB83 with 55.8% homology at the amino acid level and 81.5% homology in the functionally critical Myb-type HTH (helix-turn-helix) DNA-binding domain. We therefore wanted to determine whether ZmMYB83 had any role in regulating Rp1-D21-induced HR. A 182 bp fragment from the 3′ end of the ZmMYB83 coding sequence was inserted in the antisense orientation in FoMV-v to obtain the pFoMV-ZmMYB83 infectious clone. Seven-day-old B73 and B73:Rp1-D21 maize seedlings were bombarded with pFoMV-ZmMYB83 and pFoMV-v (negative control), respectively. Two weeks after bombardment, B73:Rp1-D21 plants infected with pFoMV-ZmMYB83 showed substantially less HR and less associated chlorosis than B73:Rp1-D21 plants infected with pFoMV-v (Figures 5a and S9). As expected, no HR was observed in B73 plants infected with either construct. RT-qPCR demonstrated that B73:Rp1-D21 plants infected with pFoMV-ZmMYB83 accumulated 3.125-fold lower levels of ZmMYB83 transcripts compared to
B73:Rp1-D21 plants infected with pFoMV-v (Figure 5b). These results suggest that the presence of ZmMYB83 enhances the HR conferred by Rp1-D21.

Transient coexpression of ZmMYB83 with Rp1-D21 in N. benthamiana did not have any visible effect on HR compared to the negative controls, Rp1-D21 expressed alone or coexpressed with GUS (Figure 5c). Ion leakage data confirmed this lack of effect (Figure 5d). Immunoblots confirmed the expression of Rp1-D21, ZmMYB83, and GUS proteins (Figure 5e). Based on the maize VIGS data, the expression of ZmMYB83 in N. benthamiana might have been expected to enhance HR. The lack of such an effect may have been because its effect is dependent on its interaction with ZmMIEL1, which was not expressed in this experiment.

We therefore coexpressed Rp1-D21 with ZmMIEL1 and ZmMYB83 in N. benthamiana along with the appropriate controls (Figure 6a). Phenotypic data and ion leakage analysis demonstrated that Rp1-D21-mediated cell death was reduced when Rp1-D21 was coexpressed with either both ZmMYB83 and ZmMIEL1 or with ZmMIEL1 and GUS (Figure 6a,b). Intriguingly, the effect of ZmMIEL1 on HR appeared to be slightly reduced when it was coexpressed with ZmMYB83, though the effect was not statistically significant. Expression of ZmMYB83 with GUS had no effect on Rp1-D21-induced HR. So, while these data are inconclusive, they provide some support for the hypothesis that the enhancing effect of ZmMYB83 on HR induced by Rp1-D21 may be mediated through ZmMIEL1.

2.7 | ZmMIEL1 regulates levels of ZmMYB83 protein in maize and N. benthamiana

Previously Marino et al. (2013) showed that AtMYB30 protein levels were controlled by AtMIEL1 in Arabidopsis through the ubiquitination pathway. Lee et al. (2017) further showed that in mie1 mutant lines, the expression of AtMYB30 and AtMYB96 genes are both up-regulated about 10-fold. We wanted to explore if a similar pattern was observed in maize. Western blots showed that ZmMYB83 protein levels in N. benthamiana were substantially reduced when coexpressed with ZmMIEL1 with or without Rp1-D21, compared to coexpression with GUS and Rp1-D21. However, Rp1-D21 protein levels were similar between treatments (Figure 6c), in agreement with our previous data (Figures 2c, 3c, and 5e).
In maize, ZmMYB83 transcript levels increased in B73:Rp1-D21 plants in which ZmMIEL1 had been silenced (Figure 4c,d). To detect native ZmMYB83 protein, we used the plant-specific AtMYB75 polyclonal antibody that, according to the manufacturer’s notes, could recognize ZmMYB83 and other maize MYBs (Figure S10). Western blot analysis with an anti-MYB75 antibody was performed on extracts from the B73:Rp1-D21 plants infected with pFoMV-ZmMIEL1 in which ZmMIEL1 expression had already been demonstrated to be suppressed (Figure 4c) and with pFoMV-ZmMYB83 in which ZmMYB83 expression was shown to be suppressed (Figure 5b). The western blot identified a band at c.37 kDa, the predicted size of ZmMYB83. Accumulation of the presumed ZmMYB83 protein was suppressed in plants infected with pFoMV-ZmMYB83 compared to control plants infected with pFOMV-v, suggesting that this band was indeed ZmMYB83 (Figure 6d). We used the same AtMYB75 polyclonal antibody on an immunoblot carrying extracts from N. benthamiana leaf sectors transiently expressing Rp1-D21, ZmMYB83, and GUS or Rp1-D21, ZmMIEL1, and GUS. An additional band was noted in the Rp1-D21, ZmMYB83 plus GUS lane at the expected size for ZmMYB83, confirming that the AtMYB75 antibody recognized ZmMYB83 (Figure 6e). Combined, these results suggest that ZmMIEL1 may have a role in modulating levels of ZmMYB83 protein. Additional bands appeared on the blot, probably due to the detection of additional MYBs by the polyclonal antibody. At least two of these bands also varied in abundance between treatments. This may suggest a complex interplay between ZmMYBs and ZmMIEL1. In Arabidopsis, AtMIEL1 was shown to target at least two MYBs, AtMYB30 (Marino et al., 2013) and AtMYB96 (Lee & Seo, 2016).

2.8 | ZmMIEL1 physically interacts with ZmMYB83

In Arabidopsis, AtMIEL1 and AtMYB30 physically interact (Marino et al., 2013). To determine whether there was a similar physical
interaction between ZmMIEL1 and ZmMYB83, we performed co-immunoprecipitation (CoIP) and split enhanced yellow fluorescent protein bimolecular fluorescence complementation (BiFC) analyses. The CoIP analysis show that ZmMYB83 (tagged with 4 × Myc) coimmunoprecipitated with ZmMIEL1 (Figure 7a).

For the BiFC assay, ZmMIEL1 fused to the N-terminus of eYFP (nEYFP) and ZmMYB83 fused to the C-terminus of eYFP (cEYFP) were expressed in various combinations in N. benthamiana plants transgenically expressing nuclear localized histone protein (H2B) tagged with cyan fluorescent protein (CYP) (Martin et al., 2009). The yellow fluorescence observed when ZmMIEL1 and ZmMYB83 were coexpressed, and the lack of fluorescence observed in the negative controls (Figure 7b) suggested a physical interaction between ZmMYB83 and ZmMIEL1 occurred in the nucleus, similar to the results observed in Arabidopsis (Marino et al., 2013). The YFP signal outside the nucleus suggested that the interaction also occurred in the cytosol.

2.9 | ZmMIEL1 and ZmMYB83 regulate the expression of genes involved in fatty acid biosynthesis and cell death

In Arabidopsis, AtMYB30 is known to regulate HR through transcriptional activation of very long-chain fatty acid (VLCFA) biosynthetic genes (Raffaele et al., 2008). Because ZmMIEL1 can physically interact with ZmMYB83, we wanted to determine if transcription of VLCFA genes in maize is affected by these proteins. To investigate this, we looked at the expression levels of few VLCFA biosynthesis genes in plants infected with pFoMV-ZmMIEL1 and pFoMV-ZmMYB83 in which expression of ZmMIEL1 and ZmMYB83 has been suppressed by 5-fold and 3.125-fold, respectively (Figures 4c and 5b). The expression levels of the VLCFA biosynthesis genes KCS1 (3-ketoacyl-CoA synthase 1) homolog, fatty acid thioesterase 2, and two genes encoding fatty acid export proteins were substantially increased in pFoMV-ZmMIEL1-infected B73:Rp1-D21 plants (Figure 3b) compared to B73:Rp1-D21 plants infected with...
pFoMV-v. No increase, or a reduction, in transcript accumulation of these genes was observed in pFoMV-ZmMYB83-infected plants (Figure 8a). This was the expected pattern if ZmMYB83/ZmMIEL1 acted in an analogous fashion to AtMYB30/AtMIEL1 with respect to control of VLCFA gene expression. However, other VLCFA biosynthetic genes such as KCS1, KCS1 like, KCS2, and LTP2 (lipid transfer protein 2) did not follow the same expected pattern. In these cases, suppression of either ZmMIEL1 or ZmMYB83 resulted in increased expression levels compared to pFoMV-v plants (Figure 8b). Interestingly, the four genes that showed the expected pattern all carried a GTTTGTT AtMYB30 binding site (Li et al., 2009) in the 2 kb upstream of the start codon while none of the four genes showing the unexpected pattern carried such a binding site (Figure S11). All eight genes were highly expressed in pFoMV-v-infected B73:Rp1-D21 plants compared to pFoMV-v-infected B73 plants (Figure S12 and Table S2), suggesting that they were induced by HR.

3 DISCUSSION

ETI can have a substantial negative effect on normal plant growth and development, thus tight regulation of the ETI response is critical so that it is deployed only when necessary and is shut off as soon as possible. Regulation of HR in plants occurs at many levels, including transcription of NLR genes, inter- or intramolecular interactions between NLRs and other proteins, environmental conditions like temperature and light, genetic background effects, and regulation of NLR and associated proteins by PTM processes (Balint-Kurti, 2019).

Previously, Olukolu et al. (2014) identified 44 SNP loci that were associated with modulating HR caused by the autoactive NLR, Rp1-D21. Here we show that the causal gene at one of these loci on chromosome 10 is ZmMIEL1, a RING domain E3 ubiquitin ligase (Figure 1). We demonstrate that ZmMIEL1 suppresses Rp1-D21-induced HR in both N. benthamiana and maize, using transient expression in N. benthamiana and VIGS-mediated gene suppression in maize (Figures 2 and 4). We further showed that the effect of ZmMIEL1 is not specific to HR mediated by Rp1-D21 because HR mediated by the autoactive NLR RPM1D505V was affected in a similar way (Figure S5). This suppressive effect was dependent on the proteasomal degradation pathway as demonstrated by the effect of MG132 (Figure 3b). However, in neither case did ZmMIEL1 appear to affect the accumulation of the NLR. Therefore, we believe that the effect of ZmMIEL1 on HR is mediated through its effect on the abundance of one or more intermediate proteins.
The best candidate for this intermediate protein is ZmMYB83. In Arabidopsis, AtMIEL1 was shown to physically interact with the transcription factor AtMYB30 in the nucleus and ubiquitinate it, causing its degradation (Marino et al., 2013). Furthermore, AtMYB30 had been previously identified as a positive regulator of plant defence and the HR (Raffaele et al., 2008; Vailleau et al., 2002). AtMIEL1 was shown to attenuate these positive effects, presumably due to its effect on AtMYB30 protein levels. We were therefore interested to investigate the possible interaction of ZmMYB83, the closest maize homolog to AtMYB30, with ZmMIEL1 and its effect on Rp1-D21-mediated HR. We showed that suppressing expression of ZmMYB83 in maize suppressed the HR phenotype conferred by Rp1-D21 (Figure 5), and using CoIP and BiFC assays we showed that ZmMYB83 and ZmMIEL1 interacted in vitro and in vivo (Figure 7). We further demonstrated that in maize plants in which ZmMIEL1 expression was suppressed, ZmMYB83 protein levels appeared to increase (Figure 6d) and that expression of ZmMIEL1 in N. benthamiana reduced levels of coexpressed ZmMYB83 (Figure 6c). We also derived suggestive, though not statistically significant, evidence in N. benthamiana that expression of ZmMYB83 may decrease the level of suppression of Rp1-D21-induced HR conferred by ZmMIEL1, though ZmMYB83 had no effect on Rp1-D21-induced HR alone (Figures 5c and 6). We also showed that ZmMIEL1 appeared to regulate ZmMYB83 transcript levels (Figure 4c,d) just as they do in Arabidopsis (Lee et al., 2017). It is not clear from these data if the effect of ZmMIEL1 on levels of ZmMYB83 in maize is mediated at the transcriptional level or at the level of protein stability or both, but these findings were all consistent with the hypothesis that ZmMYB83 and ZmMIEL1 interact to regulate HR in maize in a fashion reminiscent of the interaction between AtMIEL1 and AtMYB30 in Arabidopsis.

VLCFAs play an important role in plant defence by acting as a barrier for pathogen invasion, breaking down to generate several defence signalling molecules and involvement in controlling apoptosis and programmed cell death in plants (Kachroo & Kachroo, 2009). In Arabidopsis, the exaggerated HR induced in AtMYB30-overexpressing lines was suppressed by the loss of function of the acyl-ACP thioesterase FATB, which causes severe defects in the supply of fatty acids for VLCFA biosynthesis (Raffaele et al., 2008). In our study of the maize transcriptional response to P. sorghi, several VLCFA biosynthetic genes differentially accumulated in near-isogenic lines differing for induction of the defence response mediated by Rp1-D (Kim et al., 2021). We found that the transcript levels of four VLCFA biosynthetic genes that carried AtMYB30-specific binding sites in their promoters were down-regulated in plants in which ZmMYB83 expression was suppressed and up-regulated in plants in which ZmMIEL1 expression was suppressed (Figure 8a), as would be expected if ZmMIEL1/ZmMYB30 acts in a similar fashion to AtMIEL1/AtMYB30. The expression of four other VLCFA biosynthetic genes that lacked AtMYB30 binding sites in their promoters did not conform to this expected pattern. In these cases, suppression of expression of either ZmMIEL1 or ZmMYB30 appeared to up-regulate gene expression. The mechanisms behind this induction are unclear.

Collectively, these data allow us to conclude that ZmMIEL1 and ZmMYB83 regulate HR induced by Rp1-D21 in opposite ways, ZmMIEL1 suppressing HR and ZmMYB83 enhancing it. Furthermore, the effect of ZmMIEL1 is dependent on the proteasomal pathway as would be expected for an E3 ligase but Rp1-D21 appears not be a
direct target of ZmMIEL1. Finally, we demonstrate the interaction of ZmMIEL1 and ZmMYB83, and provide evidence to suggest that the effect of ZmMIEL1 on HR is, at least partially, mediated through ZmMYB83. ZmMIEL1 mediating the degradation of ZmMYB83, in an analogous fashion to their Arabidopsis homologs AtMIEL1 and AtMYB30.

4 | EXPERIMENTAL PROCEDURES

4.1 | Protein domain search and phylogenetic analysis

Functional domains in the derived protein sequence of the GRMZM2G056270 gene (ZmMIEL1) were identified using the SMART domain analysis tool (Letunic et al., 2015) and PROSITE domain prediction tool (de Castro et al., 2006). Homologs from different plant species were identified using BLASTP (Rédei, 2008). The phylogenetic tree was constructed with the Phylogeny.fr software (Dereeper et al., 2008). The specific algorithms used for tree building were neighbour joining according to previous studies (Karre et al., 2017, 2019).

4.2 | Plant materials and growth conditions

N. benthamiana plants were grown in a growth chamber at 22 °C in a 16 hr light/8 hr dark cycle. Four-week-old plants were used for transient expression studies. The B73:Rp1-D21 maize (Zea mays) line was used for conducting FoMV-based induced gene silencing (VIGS) experiments. B73:Rp1-D21 is the commonly used maize line B73 into which Rp1-D21 has been introgressed by repeated back-crossing. Seed of this line is maintained by back-crossing to B73 so that the resulting seed segregates 1 to 1 for wild-type B73 and B73:Rp1-D21 orientation into pFoMV- v (empty vector) to generate the pFoMV-ZmMIEL1 and pFoMV-ZmMYB83 gene silencing constructs. The clones were confirmed by sequencing. Previously developed negative control (pFoMV-v) clone was also used for the experiment (Mei et al., 2016).

4.3 | RNA isolation, cDNA synthesis, and amplification of genes

Total RNA was isolated from 10 6 mm leaf punches with an RNeasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase I (Thermo Fisher Scientific) to remove contaminating DNA, according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA with RevertAi reverse transcriptase (Thermo Fisher Scientific) with oligo(dT) primers, according to the manufacturer’s protocol.

Total RNA was extracted from a B73 plant and used to synthesize cDNA of ZmMIEL1 and ZmMYB83 genes and to amplify partial open reading frames (ORFs) of ZmMIEL1 and ZmMYB83 to develop VIGS cassettes. Gene-specific primers for ZmMIEL1 and ZmMYB83 were used to amplify coding regions of the respective genes. To amplify VIGS fragments, gene-specific primers targeting the ORFs of ZmMIEL1 and ZmMYB83 were used. The details of all primers used in the present study are provided in Table S1.

4.4 | Plasmid construction

Primers used in this study are listed in Table S1. The constructs carrying Rp1-D21 and RPM1D505V cloned into the Gateway binary vector pGWBI4 (with a 3 × HA epitope tag in the C terminus) had been developed previously (Wang, He, et al., 2015; Wang, Ji, et al., 2015). ZmMIEL1 and ZmMYB83 genes were amplified from the cDNA and cloned into pDONR207 vector by Gateway BP reactions. After confirming the clones by sequencing, ZmMIEL1 and ZmMYB83 were subcloned into the Gateway binary vectors pGWBI41 (with an EYFP epitope tag at the C terminus) and pGWBI617 (with a 4 × Myc epitope tag at the C terminus) (Nakagawa et al., 2007), respectively, by Gateway LR reactions (Thermo Fisher Scientific).

To develop ZmMIEL1 and ZmMYB83 VIGS clones, B73 cDNA was used. PCR was performed using the primer pair listed in Table S1. The N-terminal region of the RING domain was targeted for transient silencing of ZmMIEL1. BLASTN analysis in MaizeGDB (https://www.maizegdb.org/) showed one significant hit localized to gene GRMZM2G052034, but sequence alignment with the VIGS fragment showed only 52% similarity, thereby ruling out plausible off-target silencing. For silencing of ZmMYB83, the N-terminal region of the ORF was used. BLASTN analysis in MaizeGDB (https://www.maizegdb.org/) showed no potential off-targets. PCR products were purified using Zymo DNA cleanup kit (Zymo Research), digested using XbaI and XhoI restriction enzymes, and purified using a silica column (DNA Clean & Concentrator-5; Zymo Research). The purified fragments were inserted in the antisense region of the ORF. PCR products were purified using Zymo DNA cleanup kit (Zymo Research), digested using XbaI and XhoI restriction enzymes, and purified using a silica column (DNA Clean & Concentrator-5; Zymo Research). The purified fragments were inserted in the antisense orientation into pFoMV-v (empty vector) to generate the pFoMV-ZmMIEL1 and pFoMV-ZmMYB83 gene silencing constructs. The clones were confirmed by sequencing. Previously developed negative control (pFoMV-v) clone was also used for the experiment (Mei et al., 2016).

4.5 | Agrobacterium-mediated transient expression in N. benthamiana

A. tumefaciens GV3101 (pMP90) transformed with binary vector constructs (Rp1-D21:3 × HA, ZmMIEL1:EYFP, ZmMYB83:4 × Myc, GUS:EGFP, and GUS:4 × Myc) was grown at 28 °C overnight in 50 ml of Luria-Bertini (LB) medium supplemented with appropriate antibiotics for 2 days. The culture was centrifuged at 2,800 × g for 30 min at 24 °C. The resulting pellet was resuspended in infiltration buffer and diluted to a final optical density of OD600 = 0.6 plus p19 (silencing suppressor) at OD600 = 0.3. EGFP, EYFP, 4 × Myc, and 3 × HA-tagged constructs were transiently coexpressed in N. benthamiana. The previously described detailed procedures for transient expression were followed (Wang, He, et al., 2015). Unless otherwise noted,
all experiments were repeated three times with each replicate consisting of six plants.

4.6 | Ion leakage measurement

Ion leakage was measured according to a previous study (Wang, Ji, et al., 2015). Five leaf discs (1.2 cm diameter) from different plants at 72 hr postinfiltration (hpi) were shaken for 3 hr at room temperature and the conductivity (C1) was measured by a conductivity meter (model 4403; Markson Science, Inc.). Subsequently, samples were boiled for 15 min and the total conductivity (C2) was measured again. The ion leakage was calculated using the C1:C2 ratio. Statistical analysis was performed using one-way analysis of variance (GraphPad PRISM).

4.7 | Protein analysis

For protein expression analysis, three 1.2 cm diameter leaf discs from different single plants were collected at 30 hpi. The samples were ground in liquid nitrogen and total protein was extracted in 500 µl of extraction buffer (20 mM Tris.HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% sodium dodecyl sulphate [SDS], 10 mM dithiothreitol [DTT], 40 µM MG132, and 1 x plant protein protease inhibitor mixture; Sigma-Aldrich). Forty micrograms of each sample was run on an SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and exposed with HA- (at 1:350, HA:HRP, cat. # 12,013,819,001; Roche), GFP- (at 1:3,000, GFP:HRP, cat. # A10260; Thermo Fisher Scientific), and Myc- (at 1:2,000, Myc:HRP, cat. # R951-25; Thermo Fisher Scientific) specific antibodies to detect expressed proteins. To detect ZmMYB83 (untagged) from maize silencing experiments, a polyclonal AtMYB75 antibody conjugated with HRP was used (at 1:2,000, cat. # MBS7114515; MyBiosource.com). The detailed procedures for protein analysis were described previously (Wang, He, et al., 2015). Phylogenetic trees were built using Geneious v. 8.1 software.

4.8 | FoMV-based VIGS in maize

Methods for biolistic inoculation of FoMV DNA constructs were carried out essentially as previously described (Mei et al., 2016). One-week-old B73:Rp1-D21 plants were subjected to biolistics using a biolistic PDS-1000/HE particle delivery system located in the NCSU Phytotron. Bombarded plants were transferred to 6-inch pots containing standard potting mix and grown for an additional 2 weeks using a 16 hr light/8 hr dark cycle with 26/22 °C day/night temperature in growth chambers at the NCSU Phytotron after which the top part of the fourth leaf was used for leaf scans and RNA samples were extracted from the bottom part. All experiments were repeated three times and each replicate consisted of at least 18 plants (at least 12 plants for silencing target genes, three plants for empty vector controls, and three plants for phytoene desaturase [PDS] control).

The lesion area was quantified using ImageJ software (Abramoff et al., 2004). Leaves from six plants per condition were used to quantify lesions. The normalized lesion area was calculated using a protocol suggested by Stewart et al. (2016), with some modifications. Total leaf area and green leaf area were measured for each leaf. The normalized lesion area was calculated by subtracting green leaf area from total leaf area and dividing by total leaf area. Statistical analysis was calculated using the Student’s t test (GraphPad PRISM).

4.9 | Gene expression analysis using RT-qPCR

RT-qPCR was carried out on a CFX384 real-time PCR detection system (Bio-Rad) with SsoAdvanced SYBR Green Master Mix (Bio-Rad), according to the manufacturer’s protocol. The primers used for RT-qPCR studies are described in Table S1. Gene expression was measured relative to the housekeeping gene ZmActin. The 2^(-ΔΔCt) (normalized to ZmActin expression and relative to a wild-type control) method was used to calculate relative gene expression (Livak & Schmittgen, 2001). Gene expression for each sample was assessed in three technical replicates. Statistical analysis was calculated using the Student’s t test (GraphPad PRISM).

4.10 | BiFC assay

For the BiFC assay, we used a split EYFP vector system. ZmMIEL1 was cloned into the Gateway DEST-nEYFP vector, and ZmMYB83 was cloned into the DEST-cEYFP vector by LR reactions. Agrobacterium-carrying constructs derived from EYFP-split vectors were mixed in equal quantities, and each construct was diluted to a final concentration of OD600 = 0.6. Helper plasmid p19 was used at OD600 = 0.3. Transgenic N. benthamiana plants harbouring nuclear localization protein H2B tagged with CFP (Martin et al., 2009) were used for these transient expression studies that were carried as described above. The EYFP signal and CFP signal was captured from the leaf sections obtained at 36 hpi. The YFP fluorescence was observed at 515 nm and CFP fluorescence was observed at 485 nm using a Zeiss LSM 710 confocal microscope at NC State University Cellular and Molecular Imaging Facility.

4.11 | Co-IP assay

Constructs carrying ZmMIEL1 tagged with EYFP at the C-terminus (ZmMIEL1: EYFP) and ZmMYB83 tagged with 4 x Myc at the C-terminus (ZmMYB83:4 x Myc) were coexpressed in N. benthamiana. For controls, ZmMIEL1:eYFP was coexpressed with or without GUS tagged with 4 x Myc at the C-terminus (GUS:4 x Myc). Samples were collected at 30 hpi. Total protein was extracted using native
extraction buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% SDS, 1% Triton X-100) and eluted using glycine elution buffer (200 mM glycine pH 2.5). GFP Trap agarose magnetic beads (Chromotek cat. # gta-10) were used to immunoprecipitate ZmMIEL1:EYFP. The immunoblots analyses were performed with GFP polyclonal antibody conjugated with HRP (Thermo Fisher Scientific; cat. # A10260) to detect ZmMIEL1:EYFP and Myc antibody conjugated with HRP (Thermo Fisher Scientific; cat. # R951-25) to detect ZmMYB83:4 × Myc and GUS:4 × Myc. The experiment was repeated twice.

4.12 | MG132 assay and autoubiquitination assay

Proteasome inhibition experiments were carried out with modifications of the previously described protocol (Matsushita et al., 2013). MG132 (50 μM) dissolved in DMSO was mixed with the different Agrobacterium carrying constructs Rp1-D21:3 × HA, ZmMIEL1:EYFP, and GUS: EGFP. Transient expression in N. benthamiana was carried out as described above. Samples for western blot analysis and ion leakage assay were collected at 30 and 72 hpi, respectively. DMSO alone was used for control experiments. This experiment was repeated twice.

An in vitro ubiquitination assay was performed as previously described (Takahashi et al., 2009; Zhao et al., 2012) with some modifications. Human UBA1/E1 and UBE2D2/ E2 (South Bay Bio; cat. # U-100At). The reaction mixture was incubated at 30 °C for 2 h. The reaction was performed in a total of 30 μl, including 1.5 μl of 20 × reaction buffer (1 M Tris pH 7.5, 40 mM ATP, 100 mM MgCl₂, 40 mM DTT), 50 ng E1, 200–500 ng E2, 2 μg ZmMIEL1, and 5 μg ubiquitin (BostonBiochem; cat. # U-100At). The reaction mixture was incubated at 30 °C for 90 min with agitation. The reaction was terminated by adding 30 μl of 2 × SDS sample buffer with β-mercaptoethanol and incubating at 95 °C for 5 min. The reaction products were separated with 8%–12% SDS-polyacrylamide gel and detected with plant ubiquitin polyclonal antibody (Thermo Fisher Scientific; cat. # A300-318A-M) or anti-HA antibody (conjugated with HRP, Roche; cat. # 2,013,819,001) by western blotting.

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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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