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TOR inhibition primes immunity and pathogen resistance in tomato in a salicylic acid-dependent manner

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Abstract

All organisms need to sense and process information about the availability of nutrients, energy status, and environmental cues to determine the best time for growth and development. The conserved target of rapamycin (TOR) protein kinase has a central role in sensing and perceiving nutritional information. TOR connects environmental information about nutrient availability to developmental and metabolic processes to maintain cellular homeostasis. Under favourable energy conditions, TOR is activated and promotes anabolic processes such as cell division, while suppressing catabolic processes. Conversely, when nutrients are limited or environmental stresses are present, TOR is inactivated, and catabolic processes are promoted. Given the central role of TOR in regulating metabolism, several previous works have examined whether TOR is wired to plant defence. To date, the mechanisms by which TOR influences plant defence are not entirely clear. Here, we addressed this question by testing the effect of inhibiting TOR on immunity and pathogen resistance in tomato. Examining which hormonal defence pathways are influenced by TOR, we show that tomato immune responses and disease resistance to several pathogens increase on TOR inhibition, and that TOR inhibition-mediated resistance probably requires a functional salicylic acid, but not jasmonic acid, pathway. Our results support the notion that TOR is a master regulator of the development–defence switch in plants.

KEYWORDS
Botrytis, immunity, pathogenesis, tobacco mosaic virus, tomato, TOR, *Xanthomonas*

1 | INTRODUCTION

Plants constantly assess nutrient availability and energy status to ensure that they are aligned with growth processes. The "plant dilemma" (Herms & Mattson, 1992) describes the state in which resource restrictions dictate trade-offs between growth and defence. When plants are attacked by pathogens and activate their immune responses, the high energy demands of immunity result in the redirection of the plants' resources, and to associated growth arrest (Walters & Heil, 2007).

In all eukaryotes, nutrient and energy sensing is achieved mainly by the conserved target of rapamycin (TOR) protein kinase. TOR modulates developmental and metabolic processes by coordinating nutrient availability and energy status, as well as stresses and hormones (Fu et al., 2020; O'Leary et al., 2020; Xiong et al., 2013). TOR is found in two functionally and structurally distinct multiprotein complexes, TORC1 (TOR complex 1) and TORC2, but only TORC1, which contains TOR, RAPTOR, and LST8 (lethal with SEC13 protein 8), is found in plants (Dobrenel et al., 2016). When the energy

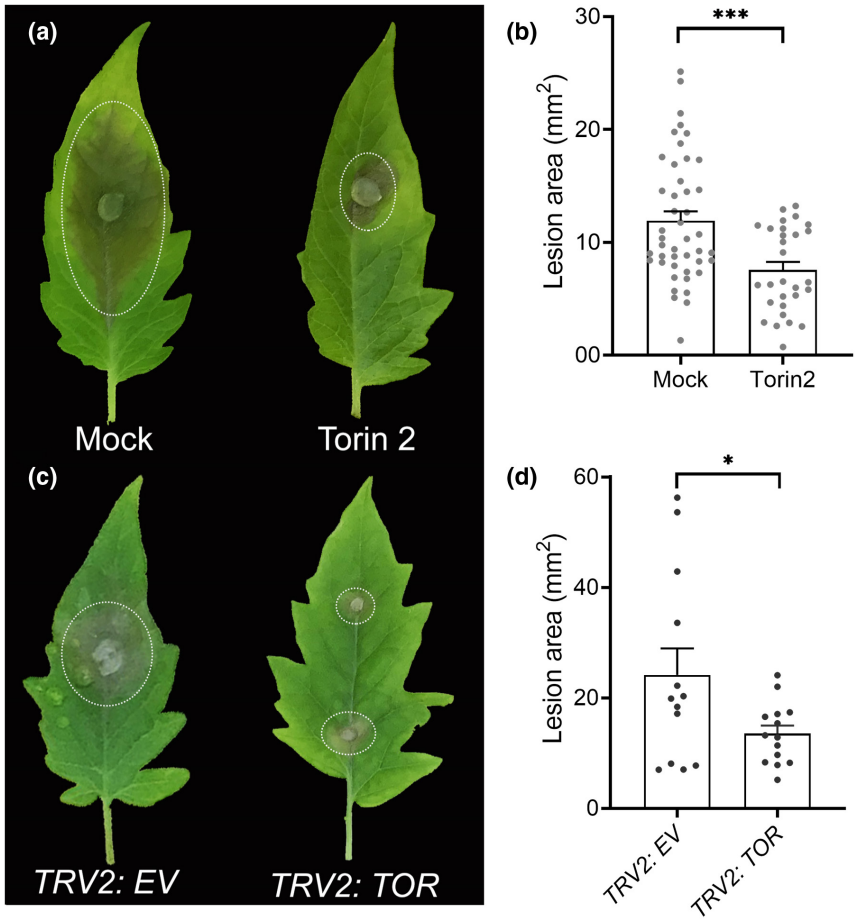

FIGURE 1 TOR inhibition promotes *Botrytis cinerea* disease resistance. Tomato cultivar M82 plants were mock treated (with 1:5000 dimethyl sulphoxide [DMSO] in double-distilled water), treated with 2 μM Torin2 (a and b), or *TOR*-silenced using the virus-induced gene silencing (VIGS) system (c and d). Plants were challenged with *B. cinerea* mycelia from a 72 h culture, 24 h after Torin2 treatment, or 4 weeks after VIGS on leaflets derived from leaves five to six. (b) The experiment was repeated four independent times, $n = 30$. Asterisks denote a statistically significant reduction in disease on TOR inhibition in a two-tailed *t* test with Welch's correction, $p < 0.0001$. (d) The experiment was repeated three independent times, $n = 14$. The asterisk denotes a statistically significant reduction in disease on *TOR* silencing when compared with empty vector silencing (EV) in a two-tailed *t* test with Welch's correction, $p = 0.038$

conditions are favourable, TOR is active and anabolic processes are promoted. Under environmental stresses or energy-deficient conditions, TOR is inhibited and catabolic processes such as autophagy are activated (Dobrenel et al., 2016; Saxton & Sabatini, 2017). TOR regulation of metabolism is mediated through phosphorylation of various key regulatory proteins (Valvezan & Manning, 2019) and regulation of the expression of genes that are related to rRNA and ribosomal proteins, ribosome biogenesis, and translation initiation and elongation processes (Pacheco et al., 2021).

Recent studies have suggested that reduced TOR activity promotes plant resistance to pathogens. For example, TOR was shown to inhibit defence-related transcription factors as well as jasmonic acid (JA) and salicylic acid (SA) signalling, and resistance to a variety of pathogens, including *Xanthomonas oryzae* (De Vleesschauwer et al., 2018). Conversely, pharmacological inhibition of TOR increased resistance to *Fusarium graminearum* in *Arabidopsis* (Aznar et al., 2018). Viruses can hijack TOR signalling to drive their replication (Schepetilnikov et al., 2011) and *TOR* silencing or TOR inhibition was shown to promote resistance against watermelon mosaic virus (WMV; Ouibrahim et al., 2015).

Suggested mechanisms for the role of TOR in plant defence include negative regulation of immunity by TOR at the transcriptional level (Meteignier et al., 2017) and immunity activation in TOR-deficient lines (De Vleesschauwer et al., 2018). Despite these suggestions, the molecular mechanism by which TOR affects disease response is still unknown. Understanding the mechanisms linking TOR to plant immunity could help decipher growth–defence trade-offs and limit yield loss to diseases.

Here, we attempted to elucidate the role of TOR in tomato immunity. Pharmacological or virus-induced inhibition of TOR promoted immunity signalling and pathogen resistance. Testing various pathogens and immunity parameters, our results indicate that manipulating the growth–defence switch via TOR inhibition can prime tomato immunity and disease resistance in an SA-dependent manner.

2 | RESULTS

2.1 | TOR inhibition promotes disease resistance

The ability of TOR inhibitors to inhibit plant growth in an efficient and TOR-specific manner was previously demonstrated (Li et al., 2017a; Montané & Menand, 2013; Schepetilnikov et al., 2013). Because the *tor* null mutation was shown to be embryo lethal in *Arabidopsis* (Menand et al., 2002), we explored the role of TOR in immunity by examining pathogenesis and plant immune responses using pharmacological inhibition of TOR and virus-induced gene silencing (VIGS)-based *TOR* silencing. To understand how inhibition of TOR affects the response of tomato to fungal infection, we used the fungus *Botrytis cinerea* (Bc; Figure 1). Wild-type (WT) tomato (*Solanum*

lycopersicum) cultivar M82 leaves were imbibed with Torin2 or the control dimethyl sulphoxide (DMSO; 1:5000 in water, identical to the DMSO concentration in the Torin2 samples) and infected with Bc 24 h later. Bc disease was assessed by measuring lesion size 5 days postinoculation (dpi). As can be seen in Figure 1a,b, TOR inhibition with Torin2 promotes Bc disease resistance. To address any possible secondary effect of Torin2 on Bc growth and confirm our results in another system, in a parallel set of experiments we silenced *TOR* expression in planta using VIGS. Control plants were mock-silenced with a control empty vector (EV). Three weeks after infiltration, we confirmed the down-regulation of *SlTOR* by reverse transcription-quantitative PCR (RT-qPCR), finding that the expression of the *SlTOR* gene was repressed in silenced plants by about 50% in comparison to that of control plants (Figure S1). The *TOR*-silenced plants were infected with Bc and disease was assessed as described. As with Torin2 treatment, the *TOR*-silenced plants displayed significantly reduced disease when compared with the control plants (Figure 1c,d). The results with Torin2 were also repeated with an additional TOR inhibitor, WYE-132 (Figure S2a,b).

TOR inhibitors such as rapamycin were originally developed as a therapeutic modality for human disease. Rapamycin's limited success as an anticancer drug led to the development of ATP competitive mTOR inhibitors, such as Torin2 (Xie et al., 2016; Zoncu et al., 2011), which is a specific chemical inhibitor of TOR, commonly used in plant research (Li et al., 2017a). Rapamycin was reported to have limited or no activity in different plant systems (reviewed in Montané & Menand, 2019). Rapamycin's lack of ability to inhibit the growth of different plant species (including *Arabidopsis*, cotton, tomato, and potato) is attributed to the low ability of the plant FKBP12 proteins to form a complex with rapamycin (reviewed in Montané & Menand, 2019). Bc and other fungal plant pathogens also have TOR proteins that are important in translational regulation (Meléndez et al., 2009). In Bc, TOR inhibition via RNA-mediated *BcTOR* silencing in transgenic plants was reported to decrease Bc virulence (Xiong et al., 2019). While not necessarily negative in the context of disease reduction for the plant, we wanted to decipher whether the disease reduction following *TOR* silencing was a result of plant immunity priming alone or also of direct *BcTOR* inhibition.

To examine whether treating tomato plants with Torin2 could decrease disease by direct inhibition of *BcTOR*, we treated plants with rapamycin, which has minimal effect in tomato (Xiong et al., 2016), and compared this treatment to Torin2 treatment, both in planta and in vitro. Bc was subcultured on potato dextrose agar (PDA) plates supplemented with Torin2 or rapamycin, and mycelial area growth was measured after 3 days (Figure S3). We observed a strong response to rapamycin, with Bc growth inhibited by more than 97% on 100 nM rapamycin and virtually 100% on 1 μM rapamycin (Figure S3a,d). In the presence of 2 μM Torin2, we observed a milder reduction of 20% in Bc growth (Figure S3a,d). In contrast, in planta, rapamycin had a very mild effect on Bc-induced disease, with lesion areas not significantly smaller than those achieved in the control (Figure S3b,e), while Torin2 reduced disease levels significantly by about 40%. These results suggest that the observed decrease in Bc-incited grey mould disease is a result of inhibition of plant TOR, and not significantly affected by the action of Torin2 on the Bc TOR protein.

To further confirm the specificity of Torin2 in tomato, we treated VIGS *TOR*-silenced plants with Torin2 and repeated the Bc disease assay. Disease reduction in the VIGS *TOR*-silenced plants was not augmented by Torin2 treatment (Figure S3c,f), indicating that Torin2 is probably specific to TOR in tomato and that the enhanced resistance is indeed a result of TOR inhibition in the plant. We continued to use the inhibitor Torin2 in subsequent assays.

2.2 | TOR inhibition enhances immunity

Given that TOR inhibition resulted in disease resistance in tomato (Figures 1, S2, and S3), we next examined whether TOR inhibition enhances immune responses. To address this, we tested activation of plant immune responses by measuring ethylene (ET) production, reactive oxygen species (ROS) accumulation, and ion leakage following application of Torin2. We compared the activation of plant immune responses by TOR inhibition to immune responses activation elicited by EIX, a fungal elicitor known to induce immune responses mostly via the jasmonic acid (JA)-mediated pathway (Gupta et al., 2020; Shoresh et al., 2005), and flg22, a bacterial-derived elicitor known to potentiate immune responses mostly via the SA pathway (Zipfel et al., 2004). As seen in Figure 2, both Torin2 and EIX treatments increased ET production (Figure 2a) and ion leakage (Figure 2b). Notably, co-treatment with Torin2 and EIX led to a further increase in ET production and ion leakage when compared with each treatment alone, which could indicate that the responses to Torin2 and EIX are mediated through different pathways. The increase in ET production on TOR inhibition is in accordance with previous findings where inhibition of TOR has been shown to increase expression of ET signalling and biosynthesis genes (Punzo et al., 2018). Flg22-induced ROS production was examined with and without Torin2 pretreatment. Torin2 was found to enhance flg22-mediated ROS production (Figure 2c,d). Once again, in a parallel set of experiments, we found that ROS accumulation and ion leakage were also induced in M82 VIGS *TOR*-silenced plants (Figure S4). The TOR inhibitors Torin2 and WYE-132 did not induce ROS production when applied on their own (Figure S5). These results confirm that TOR inhibition induces immune responses in tomato. Enhanced immune responses could explain the increased disease resistance.

2.3 | TOR inhibition-mediated immunity and disease resistance probably require SA-dependent signalling

We next asked whether TOR's function in disease and defence requires a specific hormonal pathway. To this end, we examined the ability of TOR inhibition to mediate disease resistance in tomato in the JA-insensitive *jai-1* mutant and the SA-deficient transgenic

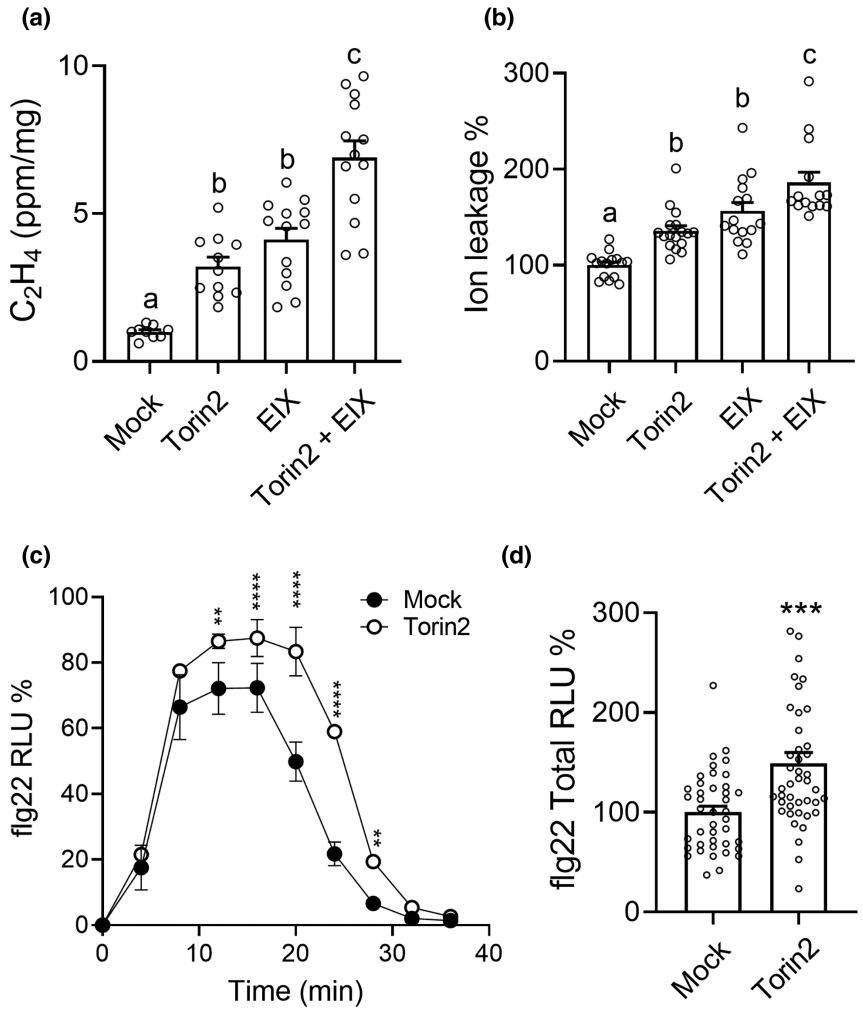

FIGURE 2 TOR inhibition increases immune responses. Tomato cultivar M82 plants were treated with 1:5000 dimethyl sulphoxide (DMSO) in double-distilled water (mock) or treated with 2 μM Torin2. Plants were challenged with the immunity elicitors EIX (1 μg/ml) (a and b) or flg22 (1 μM) (c and d) 24 h after Torin2 treatment. (a) Ethylene induction was measured using gas chromatography. (b) Conductivity of samples immersed in water for 40 h was measured. Average conductivity of the mock treatment was defined as 100%. (c and d) Reactive oxygen species (ROS) production was measured immediately after flg22 application every 4 min, using the horseradish peroxidase-luminol method, and expressed as relative luminescent units (RLU). For total RLU (d), average ROS production of the mock treatment was defined as 100%. Bars represent mean ± *SEM*, with all points shown. Experiments were repeated three or four independent times. (a) Different letters indicate statistically significant differences between samples in Welch's analysis of variance (ANOVA) with a Dunnett post hoc test, $n = 9$, $p = 0.0037$. (b) Different letters indicate statistically significant differences between samples in one-way ANOVA with a Tukey post hoc test, $n = 15$, $p = 0.038$. (c) Asterisks indicate a statistically significant increase from mock treatment in multiple *t* tests with Holm–Sidak correction. The experiment was repeated four times on at least five plants per experiment per treatment, $n = 40$, $^{****}p < 0.0001$, $^{**}p < 0.01$. (d) Asterisks indicate a statistically significant increase from mock treatment in a *t* test with Welch's correction, $n = 40$, $^{***}p < 0.001$

line *NahG*. Figure 3 shows that both the *jai-1* mutant and its background cultivar M82 responded to Torin2 with a strong reduction in lesion size, whereas Torin2 did not effect significant Bc disease reduction in *NahG*, despite doing so in its background cultivar Moneymaker (Figure 3a,b). Similar results were achieved using VIGS *TOR* silencing (Figure 3c,d). *jai-1* plants were more sensitive to Bc infection in comparison with their WT M82 background line, as expected (Figure 3a,c; AbuQamar et al., 2008; Gupta et al., 2020).

Because we observed that TOR inhibition and *TOR* silencing enhanced disease resistance in *jai-1* but not in *NahG*, we further tested the effect of TOR inhibition on immune responses in these mutants. Consistent with the Bc assay results, TOR inhibition with Torin2 (Figure 4) or silencing of *SlTOR* using VIGS (Figure S4) in the JA-insensitive mutant *jai-1* significantly increased ET production (Figure 4a), ion leakage (Figures 4b and S4a), and ROS production (Figures 4c,e and S4b,c). As expected, the *jai-1* mutant did not respond to EIX elicitation (Gupta et al., 2020). TOR inhibition with Torin2 or silencing of *SlTOR* using VIGS in the SA-deficient *NahG* did not result in a significant increase in ET production (Figure 4a) or ion leakage (Figures 4b and S4a), although *NahG* did respond to EIX. ROS production in response to flg22 was not enhanced on TOR inhibition in *NahG* (Figures 4d,f and S4d,e). The background cultivar Moneymaker displayed increased immune responses on TOR

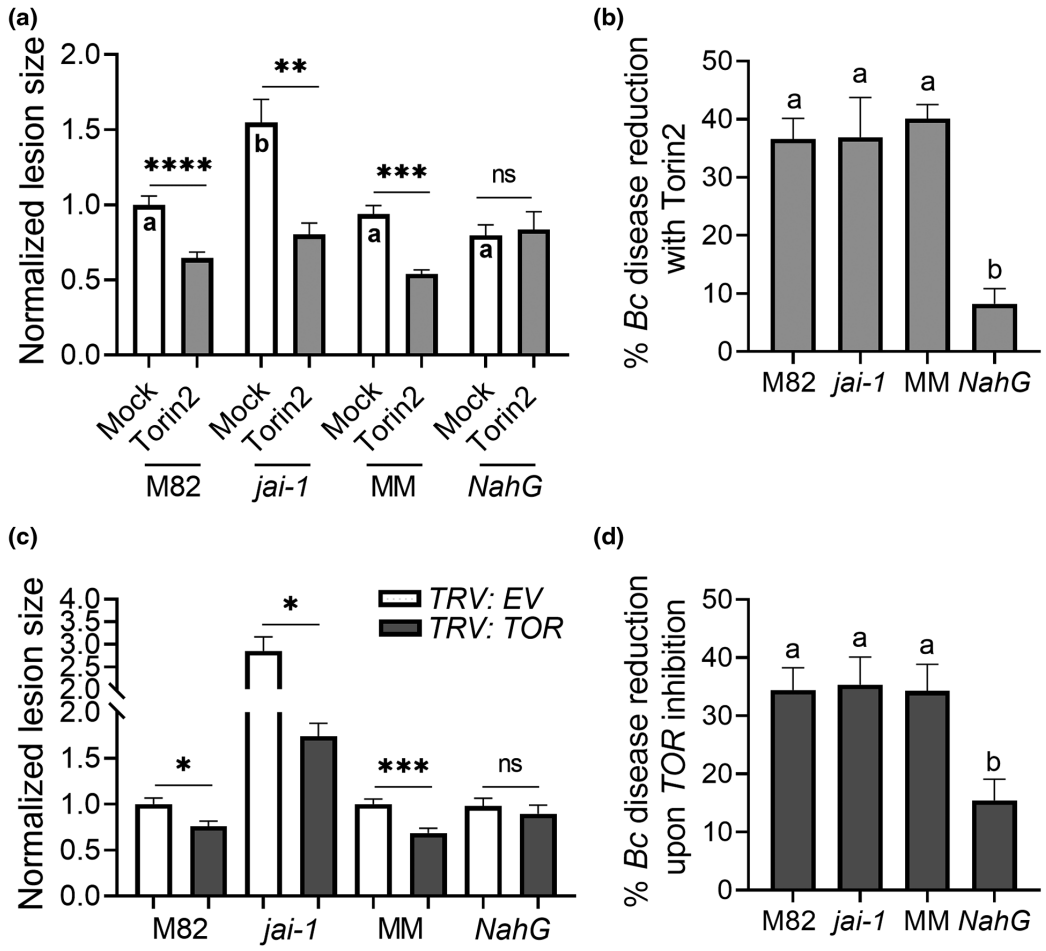

FIGURE 3 TOR inhibition-mediated disease resistance is salicylic acid (SA)-dependent. Tomato plants of the indicated genotypes (cultivar M82 and its mutant *jai-1*, cultivar Moneymaker [MM] and its transgenic line *NahG*) were treated with 2 μM Torin2 (a and b) or *TOR*-silenced using the virus-induced gene silencing (VIGS) system. Plants were challenged with *Botrytis cinerea* (Bc) mycelia from a 72 h culture, 24 h after Torin2 treatment, or 4 weeks after VIGS on leaflets derived from leaves five to six. (a and c) Normalized Bc necrotic lesion size. (b and d) Percentage of disease reduction following TOR inhibition in the different genotypes. Bars represent mean ± *SEM*. Experiments were repeated three or four independent times. (a) Asterisks indicate statistically significant disease reduction with Torin2 treatment and letters indicate statistically significant differences among samples in a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test, $n = 9$, $p = 0.021$ (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$; ns, not significant). (b) Different letters indicate statistically significant differences between samples in Welch's ANOVA with a Dunnett post hoc test, $n = 9$, $p < 0.006$. (c) Asterisks indicate statistically significant disease reduction on *TOR* silencing when compared with empty vector (EV) silencing in Welch's ANOVA with a Dunnett post hoc test, $n = 2\,4$, $p = 0.03$ (***$p < 0.001$, *$p < 0.05$; ns, not significant). (d) Different letters indicate statistically significant differences between samples in Welch's ANOVA with a Dunnett post hoc test, $n = 24$, $p = 0.012$

inhibition in all three assays. Because *jai-1* does not respond to EIX, and *NahG* did not respond to TOR inhibition, neither mutant displayed the additive effects observed in the combined treatment in the background WT lines.

The *NahG* line was previously reported to affect defence independently of SA in *Arabidopsis* (Heck et al., 2003; van Wees & Glazebrook, 2003). Therefore, to further investigate possible roles for SA in TOR inhibition-mediated immunity, we examined the relationship between TOR inhibition-mediated immunity and immunity effected by treatment with the SA analogue acibenzolar-*S*-methyl (ASM). Treatment with Torin2 or ASM, or both in combination, resulted in a similar increase in ROS burst levels in response to flg22 (Figure 5). Torin2 and ASM were not able to augment each other, indicating that they may promote immune responses via the same pathway.

Together, these results imply that immune priming due to TOR inhibition probably requires SA-dependent signalling.

2.4 | TOR inhibition primes defence

To better understand the effect of TOR down-regulation on defence during Bc infection, we next analysed the transcriptional response of various known defence genes to Torin2 treatment, alone and in combination with Bc infection. M82 leaves were treated with Torin2 or DMSO (mock, 1:5000 in double-distilled water) for 24 h, and then inoculated with Bc or mock inoculation. Total RNA was extracted 48 h after inoculation and gene expression was analysed by RT-qPCR.

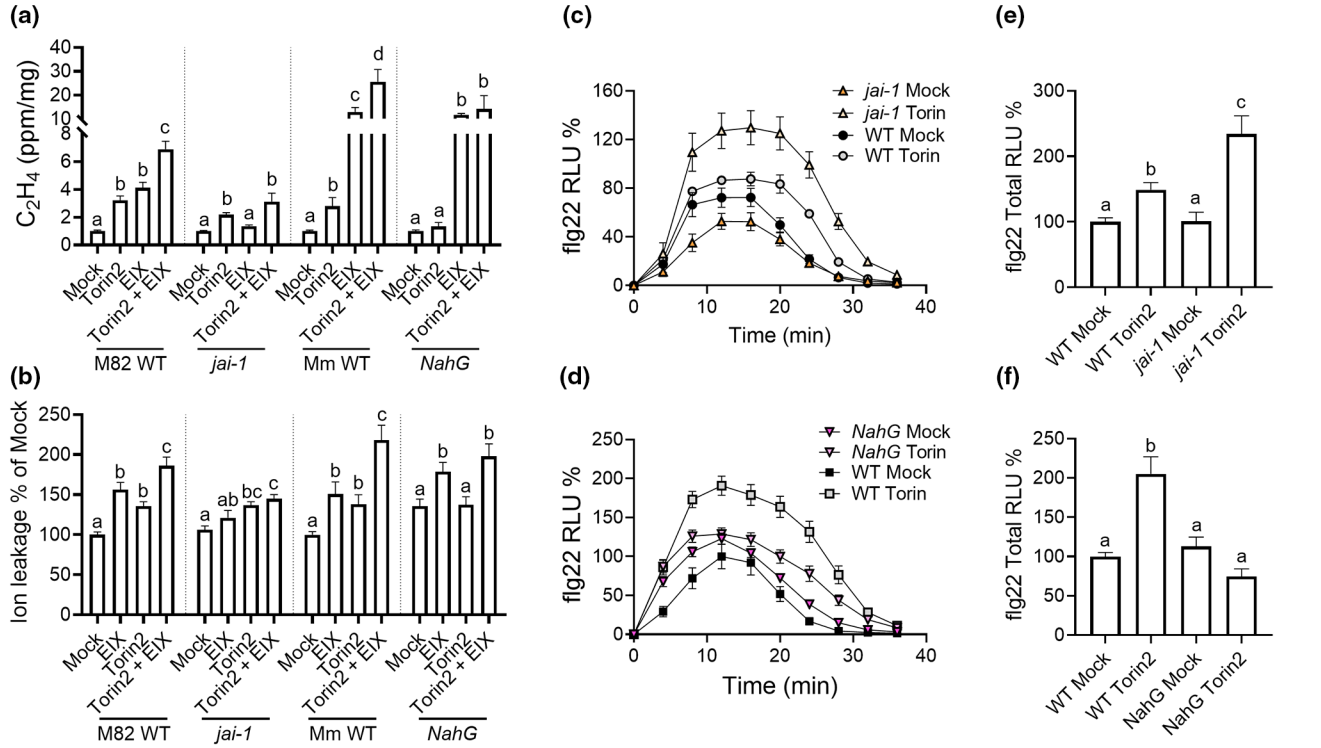

FIGURE 4 TOR inhibition-mediated increased immunity is salicylic acid (SA)-dependent. Tomato plants of the indicated genotypes. The SA-deficient line *NahG* and its wild-type (WT) background Moneymaker (Mm), and the jasmonic acid (JA)-insensitive mutant *jai-1* and its WT background M82 were treated with 1:5000 dimethyl sulphoxide (DMSO) in double-distilled water (mock) or treated with 2 μM Torin2. Plants were challenged with the immunity elicitors EIX (1 μg/ml) (a and b) or flg22 (1 μM) (c–f) 24 h after Torin2 treatment. (a) Ethylene induction was measured using gas chromatography. (b) The conductivity of samples immersed in water for 40 h was measured. Average conductivity of the mock treatment was defined as 100%. (c–f) Reactive oxygen species (ROS) production was measured immediately after flg22 application every 4 min, using the horseradish peroxidase-luminol method, and expressed as relative luminescent units (RLU). For total RLU (e and f), average ROS production of the mock treatment was defined as 100%. Bars represent mean ± *SEM*. Experiments were repeated three independent times on at least five plants per experiment per treatment, (c) $n = 40$, (d) $n = 24$. (a) Different letters indicate statistically significant differences between samples in Welch's analysis of variance (ANOVA) with a Dunnett post hoc test, $n = 9$, $p = 0.042$. (b) Different letters indicate statistically significant differences between samples in one-way ANOVA with a Tukey post hoc test, $n = 9$, $p = 0.04$. (e) Different letters indicate statistically significant differences between samples in Welch's ANOVA with a Dunnett post hoc test, $n = 40$, $p = 0.044$. (f) Different letters indicate statistically significant differences between samples in Welch''s ANOVA with a Dunnett post hoc test, $n = 24$, $p = 0.0037$

Torin2 treatment induced the expression of *pathogenesis-related proteins PR1a* (Solyc01g106620) and *PR-1b* (Solyc00g174340), *Pto-interacting 5* (*Pti-5*, Solyc02g077370), and *pathogen induced 1* (*Pi-1*, Solyc01g097270), all of which have been reported to be induced following pathogen exposure (Du et al., 2015; Meller Harel et al., 2014; Vega et al., 2015; Yang et al., 2015). *PR1a* is SA responsive and considered to be systemic acquired resistance (SAR)-related (López-Ráez et al., 2010; Martínez-Medina et al., 2013). *PR1b* is up-regulated by both SAR and induced systemic resistance (ISR) activation (Li et al., 2017b; Meller Harel et al., 2014). *Pti5* is ET responsive (Thara et al., 1999). *Pi-1* is JA responsive and considered to be a marker of ISR (Cui et al., 2019; Iberkleid et al., 2014). The expression levels of these genes displayed a 3- to 6-fold increase on Torin2 treatment (Figure 6). As expected, the examined defence genes were strongly up-regulated in response to Bc infection, 40- to 300-fold. Torin2 pretreatment followed by Bc infection did not further increase expression of the tested defence genes, suggesting that the modest increase observed with Torin2 treatment alone is sufficient to prime immunity and effect disease resistance on pathogen exposure.

2.5 | TOR inhibition promotes disease resistance against additional pathogen classes and in an additional solanaceous host

We proceeded to examine whether TOR inhibition mediates disease resistance to additional pathogens and in an additional solanaceous species. VIGS *TOR*-silenced plants were naturally infected with the early blight fungal pathogen *Alternaria alternata* by introducing the plants, 4 weeks after silencing, into a chamber containing *A. alternata*-infected plants. Disease symptoms were scored 2 weeks after exposure. *TOR* silencing resulted in a significant decrease in *A. alternata* symptoms (Figure 7a,b).

Previous studies have demonstrated that TOR down-regulation results in resistance to bacterial pathogens in *Arabidopsis* and rice (Meteignier et al., 2017; De Vleesschauwer et al., 2018). We therefore tested whether *TOR* silencing can improve tomato and *Nicotiana benthamiana* (Nb) responses to bacterial pathogens. *TOR*-silenced tomato and Nb plants were infected with *X. euvesicatoria* (Xcv) and

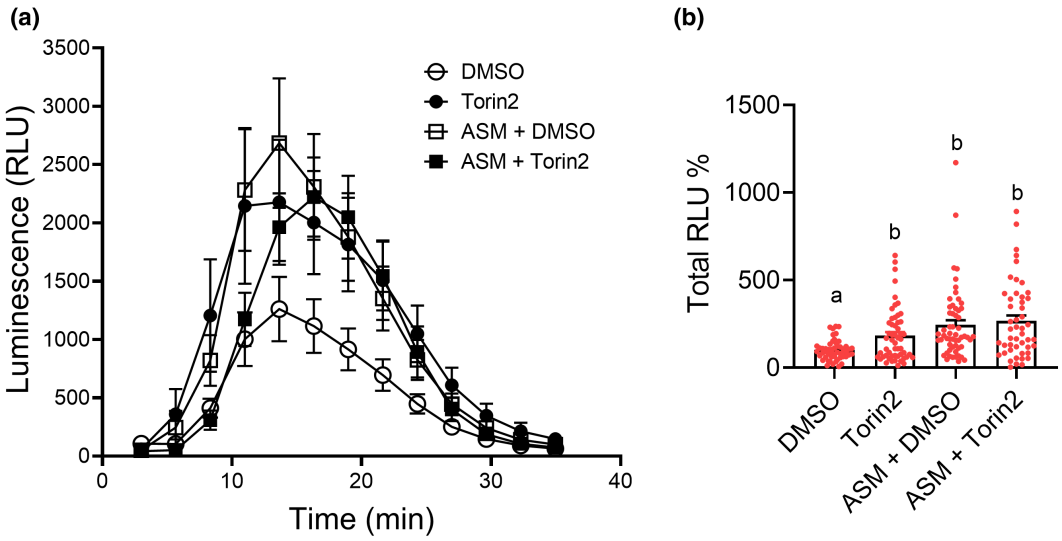

FIGURE 5 TOR inhibition and the salicylic acid (SA) analogue acibenzolar-*S*-methyl (ASM) generate a nonadditive increase in immunity. Tomato cultivar M82 plants were treated with 1:5000 dimethyl sulphoxide (DMSO, mock) or treated with 0.001% ASM, with or without the addition of 2 μM Torin2. Plants were challenged with flg22 (1 μM) 24 h after ASM and/or Torin2 treatment. Reactive oxygen species (ROS) production was measured immediately after flg22 application every 3 min, using the horseradish peroxidase-luminol method, and expressed as relative luminescent units (RLU, a). For total RLU (b), average ROS production of the mock treatement was defined as 100%. Bars represent mean ± *SEM*, with all points shown. Experiments were repeated four independent times. (b) Different letters indicate statistically significant differences between samples in a Kruskal–Wallis test with Dunn's post hoc test, $n = 48$, $p = 0.047$

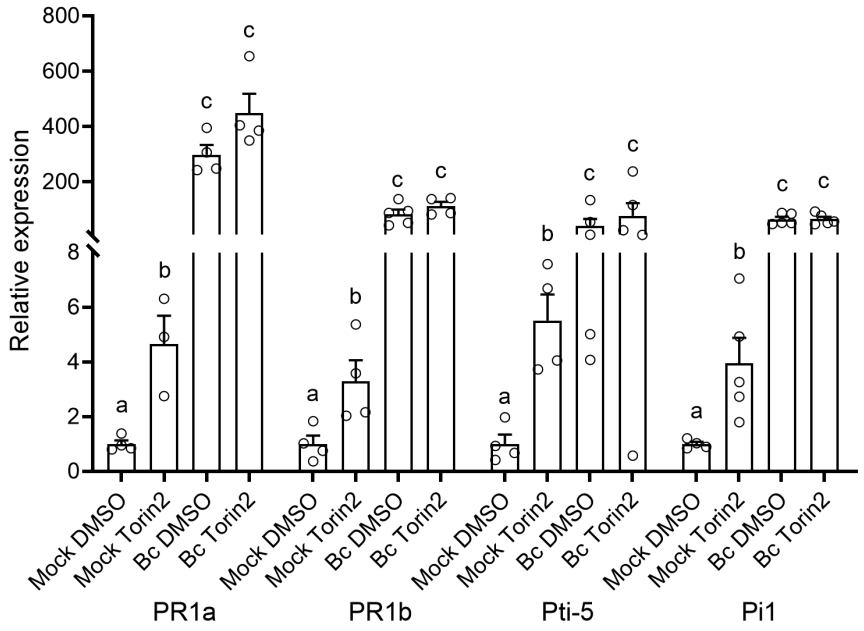

FIGURE 6 Defence genes are induced by TOR inhibition. Gene expression analysis of the indicated defence genes in indicated samples. Mock (1:5000 dimethyl sulphoxide [DMSO] in double-distilled water), Torin2 (2 μM) treatment, *Botrytis cinerea* (Bc) infection, and Bc infection combined with Torin2 treatment were measured by reverse transcription-quantitative PCR. Relative expression was calculated using the mean between the gene copy number obtained for three reference genes, *RPL8* (Solyc10g006580), *EXP* (Solyc07g025390), and *CYP* (Solyc01g111170), and normalized to the mock treatment. Analysis was conducted on four or five individual plants. Bars represent mean ± *SEM* with all points shown. Different letters indicate statistically significant differences between samples in Welch's *t* test comparing each gene, $p = 0.034$

pathogen titre in the plant was measured 4 days after inoculation by assessing colony-forming unit (cfu) count in infected tissues. *TOR* silencing resulted in a significant reduction in Xcv cfu count (Figure 7c,d).

It has been suggested that TOR inhibition can delay systemic infection by a number of plant viruses (reviewed in Schepetilnikov & Ryabova, 2018). We next tested whether *TOR*-silenced Nb plants possessed altered viral movement. *Agrobacterium tumefaciens*

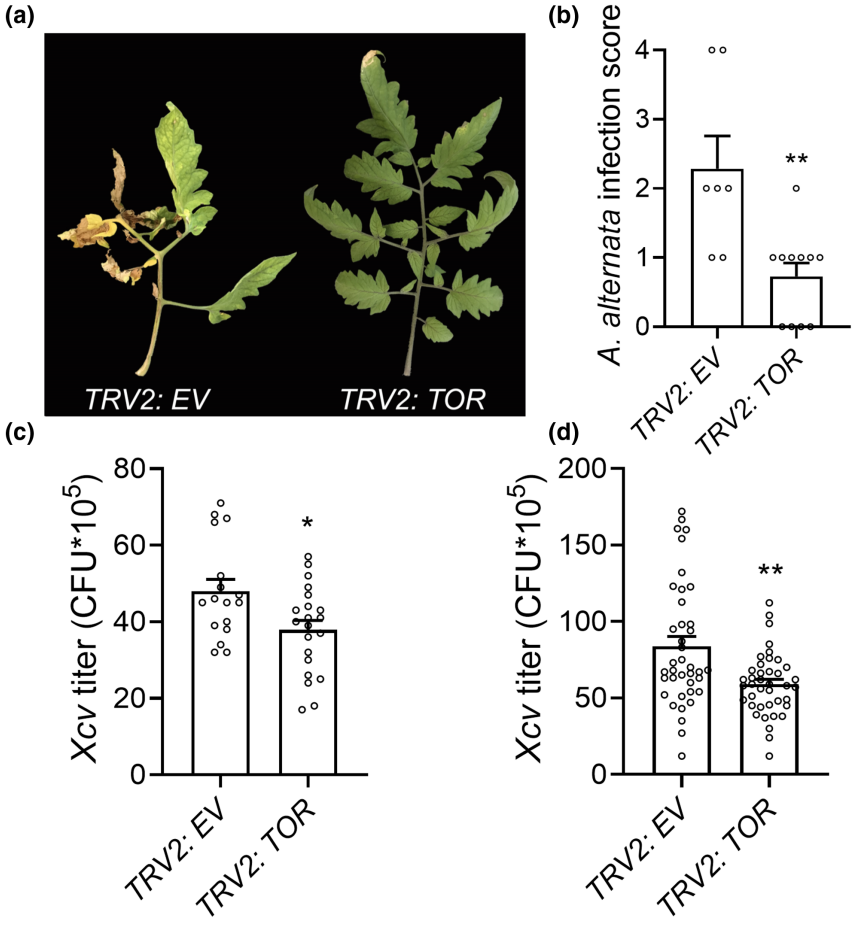

FIGURE 7 TOR inhibition promotes *Alternaria alternata* and *Xanthomonas euvesicatoria* disease resistance. Tomato cultivar M82 plants (a, b, and d) or *Nicotiana benthamiana* plants (c) were *TOR*-silenced using the virus-induced gene silencing (VIGS) system. Plants were naturally infected with *A. alternata* (a and b) or challenged with *X. euvesicatoria* (Xcv) (c and d) 4 weeks after VIGS. Bars represent mean ± *SEM*, with all points shown. At least seven individual plants were analysed. Asterisks denote statistical significance in a two-tailed *t* test, (a) $^{**}p < 0.01$, (b) $^{*}p < 0.05$

harbouring an infectious tobacco mosaic virus-green fluorescent protein (TMV-GFP) clone (Lindbo, 2007) was infiltrated into the fourth leaf of 4-week-old *TOR*-silenced and control-silenced Nb plants. Systemic infection was examined 7–10 days postinfection using in vivo imaging analysis of GFP (IVIS). IVIS scanning demonstrated the silenced Nb plants showed fewer and weaker fluorescent signals in systemic leaves when compared to control plants (Figure 8). Thus, *TOR* silencing also enhanced the resistance to a viral pathogen.

3 | DISCUSSION

In this work, we investigated the roles of TOR in tomato immunity. Several lines of evidence suggest that TOR plays an important role in plant immune responses and disease resistance. Silencing of *AtTOR* has been shown to activate a subset of defence-related genes and promote resistance against *Pseudomonas syringae* (Meteignier et al., 2018). Furthermore, TOR inhibition using AZD8055 promotes resistance against *Xanthomonas citri* (Soprano et al., 2018). Overexpression of *OsTOR* in rice enhances the susceptibility to several bacterial and fungal pathogens, whereas *TOR*-RNAi improves resistance against those pathogens (De Vleesschauwer et al., 2018). Interestingly, *OsTOR* overexpression resulted in increased susceptibility to only some, but not all, pathogens in a lifestyle-independent manner, with higher susceptibility to *Xanthomonas*, *Cochliobolus miyabeanus*, and *Rhizoctonia solani* reported. The authors suggested that the role of TOR in disease resistance is dependent on the specific characteristics of the host–pathogen interaction.

Here, silencing *TOR* expression or treatment with the specific TOR inhibitor Torin2 increased the resistance against Bc, *A. alternata*, and Xcv in tomato and *N. benthamiana* (Figures 1, 7 and S2). Silencing of *TOR* expression in *N. benthamiana* resulted in the accumulation of less TMV in systemic leaves, indicating that TOR activity is involved in TMV infection (Figure 8). In mature, source leaves, TOR restricts transport through the plasmodesmata and promotes sugar trafficking (Brunkard et al., 2020). The decreased rate of transport promotes sugar uptake in growing tissues, and could explain the reduced amount of systemic TMV-GFP in *TOR*-silenced plants.

Our results strengthen previous studies and support the notion that TOR is a negative regulator of the response to bacterial, fungal, and viral pathogens across different plant species.

We found that TOR inhibition can activate plant immune responses in tomato. Interestingly, Torin2 and the fungal elicitor EIX activated plant immunity to similar levels, whereas co-treatment with EIX and Torin2 had an additive effect on plant immune responses (Figures 2 and 4). This additive effect might indicate that activation of immunity by TOR inhibition is mediated through a different pathway than the response to EIX, which is known to be mediated by JA.

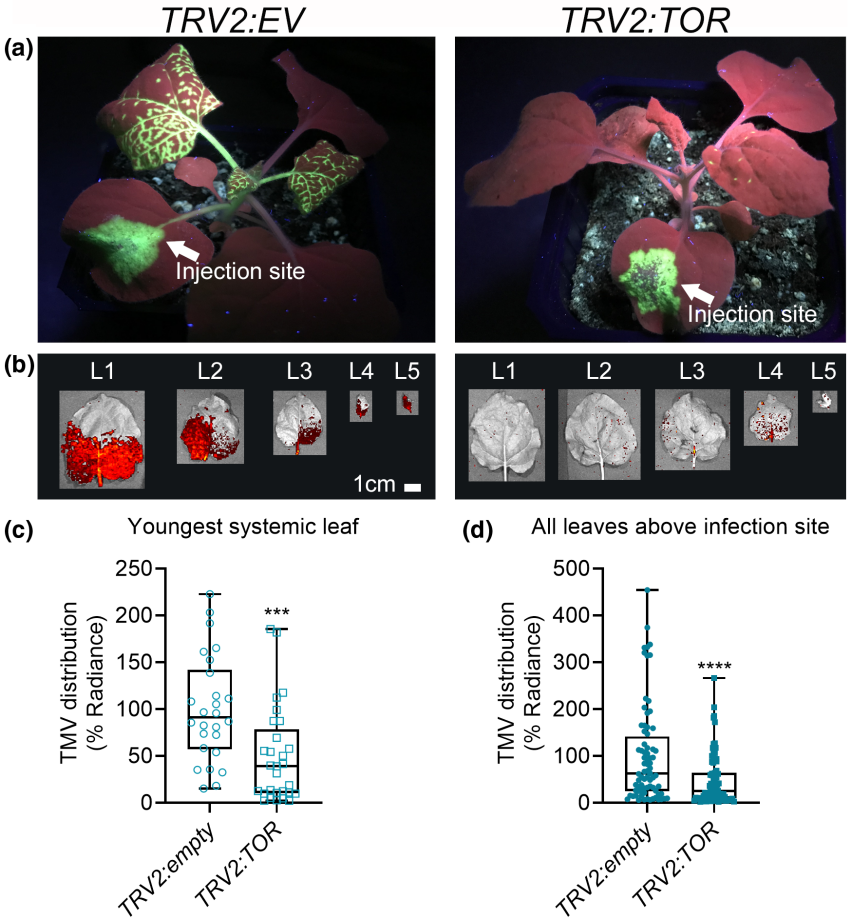

FIGURE 8 TOR inhibition promotes tobacco mosaic virus (TMV) disease resistance. *Nicotiana benthamiana* plants were *TOR*-silenced using the virus-induced gene silencing (VIGS) system. Plants were infected with TMV-GFP at a single injection site (indicated) on leaf four, 4 weeks after VIGS. Virus movement was assessed by measuring green fluorescent protein (GFP) radiance in all leaves above the injection site, 7 days postinoculation (a and b). In (b), L1 denotes the leaf immediately above the injection site, with leaves numbered successively upward until the youngest leaf (L5). (c) Average TMV distribution in the youngest system leaf (L5). Asterisks indicate a significant reduction in TMV observed in L5 in the Mann–Whitney *U* test, $n = 26$, $^{***}p < 0.001$. (d) Average total TMV distribution in all systemic leaves (L1–L5). Asterisks indicate a significant reduction in TMV observed in L1–L5 in a two-tailed *t* test with Welch's correction, $n = 75$, $^{****}p < 0.0001$. (c and d) Boxplots represent minimum to maximum values with inner quartile ranges (box), outer quartile ranges (whiskers), median (line in box), all points shown. The experiment was repeated three times

TOR inhibition resulted in increased ET production in steady state (Figure 2a). The ET signalling factor EIN2 (ethylene-insensitive protein 2) has been shown to be regulated by TOR (Fu et al., 2021). Furthermore, TOR can inhibit ET biosynthesis and ET-insensitive mutants were all resistant to TOR inhibition (Zhuo et al., 2020). This is consistent with our observation that TOR inhibition induced expression of ET-responsive genes (Figure 6) and suggests that ET-mediated defence, as well as additional ET-dependent processes, could be downstream to TOR regulation.

TOR inhibition was previously reported to trigger transcriptional changes in the expression of defence-related genes in *Arabidopsis* (Ren et al., 2013). Similarly, De Vleesschauwer et al. (2018) showed that Torin2 or rapamycin treatment increase the activation of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes in comparison to fungal and bacterial PAMP treatment alone. These reports are consistent with what we observed here in tomato (Figure 6). Interestingly, we observed that pretreatment with Torin2 prior to Bc inoculation did not result in further augmentation of expression of defence genes, indicating that the main activity of Torin2 is in priming plant defences, resulting in higher immune system activation in steady state.

Previous reports have suggested that TOR is involved in plant immunity via its interaction with the SA and JA signalling pathways. Inhibition of TOR by AZD8055 in cotton seedlings led to differential expression of JA biosynthetic and signalling genes, and to elevated JA levels, suggesting that TOR acts as a negative regulator of JA signalling (Song et al., 2017). TOR was also suggested to be a negative regulator of JA and SA signalling, as rapamycin treatment resulted in the up-regulation of JA- and SA-related genes in rice suspension cells infected with *Xanthomonas oryzae* (De Vleesschauwer et al., 2018).

To decipher the hormonal signalling pathways required for TOR inhibition-mediated defence, we used the JA-insensitive mutant *jai-1* and

the SA-deficient line *NahG*, as well as the SA analogue ASM. In *NahG Arabidopsis* plants, SA is converted to catechol. Reports have indicated that changes in the immune responses or disease resistance in *NahG* could stem from the effects of catechol and not changes in SA (Heck et al., 2003; van Wees & Glazebrook, 2003). Therefore, we used the SA analogue ASM to further investigate the role of SA in immune priming induced by TOR inhibition. We found that SA-dependent signalling is necessary to achieve TOR-mediated immunity and resistance to Bc in tomato (Figures 3–5 and S4). Inhibition of TOR might prime SA-dependent immunity, resulting in disease resistance and possibly influencing TMV infection, as SA is known to inhibit TMV (Chivasa et al., 1997). In our work, we did not observe a requirement for JA pathway signalling in TOR inhibition-mediated disease resistance or immune response activation; however, this is specific to tomato and the pathogens and elicitors we examined. We cannot rule out a requirement for JA in TOR inhibition-mediated disease resistance in additional systems, and the possibility that TOR can act downstream to JA in certain cases seems likely.

How does TOR inhibition increase resistance and what is the role of TOR in plant immune responses? The increased resistance observed in *TOR*-silenced and TOR-inhibited plants could be attributed to an "improved" plant state. TOR is known to be a negative regulator of autophagy (Mugume et al., 2020). In *Arabidopsis*, TOR negatively regulates autophagy under nutrient-rich conditions (Liu & Bassham, 2010; Pu et al., 2017). Autophagy supports the remobilization of nutrients in times of depletion. A higher autophagy rate can result in increased resources and, together with growth arrest, can lead to a faster ability to redirect energy to defence.

In summary, we show here that TOR inhibition activates immunity and reduces susceptibility to several pathogens in tomato in an SA-dependent manner. Our data support the notion that TOR probably functions as a negative regulator of plant immunity. Understanding how plants arrest their growth and activate immune responses is important to improve the management of biotic stresses and increase crop yield. Further research will be required to reveal the specific mechanism in which TOR regulates immunity, uncovering the candidate genes and pathways targeted by TOR. TOR signalling could be a good candidate pathway to be explored in the future, with partial inhibition of TOR possibly serving as a tool to improve crop productivity under biotic stresses.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material and growth conditions

Tomato (*S. lycopersicum*) cultivar M82 plants were used throughout this study. The JA-insensitive mutant *jai-1* is in the M82 background (Gupta et al., 2020; Li et al., 2002) and the decreased SA-line *NahG* is in the cv. Moneymaker background (Brading et al., 2000). All plants were grown in soil (Green Mix 443; Even-Ari Green) in a growth chamber set to long-day conditions (16/8 h light/dark) at 24°C. In other experiments, plants were grown in a greenhouse under natural day length conditions.

4.2 | Torin2, WYE-132, and rapamycin treatments

TOR inhibitors Torin2, WYE-132, and rapamycin (Sigma-Aldrich) were applied to detached tomato leaves by petiole feeding for 24 h prior to pathogen inoculation or measurement of immune responses. Stock solutions were prepared in DMSO (Sigma-Aldrich) and diluted to the desired concentration in water. Leaves treated with water containing a similar volume of DMSO constituted the mock treatment. In the case of Torin2, the stock of 10 mM (prepared in undiluted DMSO) was diluted 1:5000 and mock samples were treated with 1:5000 of DMSO.

4.3 | VIGS

VIGS in tomato and *N. benthamiana* was performed as previously described (Liu et al., 2002). A 360 bp fragment of the *SlTOR* gene was amplified using the forward primer 5′-GGTCTAGAATGGCTGCCAC CGTTCAGGCGATCCG-3′ and the reverse primer 5′-GGGGATCCTT CGCTGATGGTGACATCTAT-3′, and cloned into the *Xba*I and *Bam*HI sites of TRV RNA2 (pYL170) vector. The final construct, as well as an empty TRV RNA2 for control and a TRV RNA1 (pYL155), were introduced into *A. tumefaciens* GV3101::pMP90. TRV RNA1 was mixed at a ratio of 1:1 with RNA2 (either empty or TRV2:*TOR*) in infiltration buffer and infiltrated into tomato or *N. benthamiana* cotyledons. Fifth leaves of 6-week-old tomato plants were used in pathogenicity assays, RT-qPCR, and for measurement of immune responses.

4.4 | Pathogenesis assays

Bc pathogenicity assays were performed as previously described (Gupta et al.,2020). Briefly, Bc isolate Bcl16 was maintained on potato dextrose agar (PDA; Difco) plates at 22°C. Agar discs (0.4 cm diameter) were pierced from colony margins and used to inoculate detached leaves. Inoculated leaves were kept in a growth chamber at 22°C under long-day conditions. Necrotic lesion area was measured 4–5 days after inoculation using ImageJ. For RT-qPCR analysis, Bc spores were collected in 1 mg/ml glucose and 1 mg/ml K_2HPO_4, filtered through cheesecloth, and tomato leaves were then spray inoculated with the spore suspension. The mock treatment was plants sprayed with similar concentrations of glucose and K_2HPO_4. For *A. alternata* infection, 4-week-old *TOR*-silenced plants were placed in a chamber with plants naturally infected with *A. alternata* MB20-3. The introduced plants were randomly and equidistantly interspersed with the infected plants. *A. alternata* infection was graded on a 1–5 scale (1 = 10%, 2 = 25%, 3 = 50%, 4 = 75%, and 5 = 100% infected leaf tissue) following 2 weeks of cohabiting with infected plants. Bacterial infection was performed as described in Gupta et al. (2021). Xcv strain 91-118 (Roden et al., 2004) was grown in Luria Bertani broth with 100 mg/L rifampicin overnight at 28°C. Bacterial cultures were diluted in 10 mM $MgCl_2$ to a final concentration of 10^5 cfu/ml (OD_{600} = 0.0002). The fourth leaf of 4-week-old *TOR*-silenced and control *N. benthamiana* plants was then inoculated using a 1-ml needleless syringe. Negative

controls were inoculated with 10 mM $MgCl_2$ without bacteria. Nine days after inoculation, one leaf disc of 1 cm in diameter was collected and ground in 1 ml of 10 mM $MgCl_2$. Bacterial colonies were counted 2 days after plating of serial dilutions to determine bacterial cfu.

4.5 | Mycelia area growth assay

To assess the effect of TOR inhibitors on the growth of Bc, Torin2 and rapamycin were dissolved in DMSO and added to PDA at concentrations of 2 μM or 1 μM, and 100 nM, respectively. Bc mycelia from a fresh plate (0.5 cm diameter mycelial plugs) were placed in the centre of the plates. The plates were incubated at 22°C for 4–5 days and the area of the mycelial growth was measured using ImageJ.

4.6 | ET measurement

ET measurement was performed according to Leibman-Markus et al. (2017). Leaf discs (0.9 cm diameter) were harvested and washed in a 50-ml distilled water tube for 3 h. Five leaf discs were sealed in a 10-ml flask with 1 ml of buffer (adaxial surface down), with or without 1 μg/ml EIX, or with or without Torin2, overnight at room temperature, with agitation. ET production was measured using gas chromatography (Varian 3350; 501 Varian).

4.7 | Ion leakage (conductivity) measurement

Ion leakage (conductivity) measurement was performed according to Leibman-Markus et al. (2017). Leaf discs (0.9 cm diameter) were harvested and washed with distilled water for 3 hr in a 50-ml tube. For each sample, five discs were placed in a 10-flask with 1 ml of distilled water, with or without 1 μg/ml EIX, and with 2 μM Torin2 or DMSO, for 48 h with agitation. After incubation, 1.5 ml of distilled water was added to each sample and conductivity was measured using a conductivity meter (EUTECH instrument con510).

4.8 | Measurement of ROS generation

ROS measurement was carried out as previously described by Leibman-Markus et al. (2017). Leaf discs (0.5 cm diameter) were collected and each disc was incubated in 250 μl of distilled water in a 96-well plate (SPL Life Science) at room temperature with agitation. After 4 h, the water was removed and 50 μl of distilled water was added. Immediately before measurement, 100 μl of distilled water with or without 1 μM flg22 (Phytotech lab, product ID: P6622, amino acid sequence: QRLSTGSRINSAKDDAAGLQIA) was added. Light emission was measured using a luminometer (Turner BioSystem Veritas). For samples pretreated with the SA analogue ASM, plants were sprayed with 0.001% ASM (Bion; Syngenta) in water 24 h prior to tissue harvest.

4.9 | RNA extraction and RT-qPCR

Isolation of total RNA was performed according to the TRI reagent (Sigma-Aldrich) procedure, with DNase (ThermoFisher) treatment performed to remove genomic DNA. One microgram of RNA was used for cDNA synthesis using Maxima reverse transcriptase (ThermoFisher). qPCR was conducted with Power SYBR Green Mix (Life Technologies), using specific primers (Table S1), in a Rotor-Gene Q machine (Qiagen). Standard curves were achieved by dilutions of one cDNA sample. Relative expression was quantified by dividing the expression of the relevant gene by the geometric mean of the expression of three normalizers: ribosomal protein *RPL8* (Solyc10g006580), Cyclophillin *CYP* (Solyc01g111170), and EXPRESSED *EXP* (Solyc07g025390). All primer pairs had efficiencies in the range of 0.97–1.03 (Table S1).

4.10 | TMV-GFP movement and accumulation assay

To test whether *TOR* silencing can delay systemic infection of plant viruses, *A. tumefaciens* harbouring a TMV-GFP clone was infiltrated into the fourth leaf of 4-week-old *TOR*-silenced and control *N. benthamiana* plants as previously described by Hak and Spiegelman (2020). The clone was obtained from Dr Ziv Spiegelman, ARO. Seven days after infection, GFP fluorescence was measured using in vivo imaging analysis (IVIS Lumina LT, Perkin Elmer) equipped with a XFOV-24 lens and Living Image 4.3.1 software (Perkin Elmer) set (excitation/emission: 420/520 nm). The data from the optical luminescence image were displayed in pseudocolour representing intensity terms of radiance (photons·s^{-1}·cm^{-2}·steradian^{-1}) and calculated as average radiance per leaf.

4.11 | Statistical analysis

All data are presented as average ± *SEM*, or as boxplots showing minimum to maximum values, with the box representing inner quartile ranges and the whiskers representing outer quartile ranges. Data sets were analysed for normality using the Shapiro–Wilk test. Differences between two groups were analysed for statistical significance using a two-tailed *t* test, with Welch's correction for samples with unequal variances or with Holm–Sidak correction for multiple comparisons, where appropriate. Differences among three groups or more were analysed for statistical significance using one-way analysis of variance (ANOVA). Regular ANOVA was used for groups with equal variances and Welch's ANOVA for groups with unequal variances. When a significant result for a group in an ANOVA was returned, significance in differences between the means of different samples in the group were assessed using a post hoc test. Tukey's or Bonferroni's test were used for samples with equal variances and Dunnett's test was employed for samples with unequal variances. For non-Gaussian-distributed samples, the Mann–Whitney *U* test was used for analysing the differences between two samples, and Kruskal–Wallis ANOVA with Dunn's post

hoc test was used for analysing the diferences between three samples or more. All statistical analyses were conducted using Prism 8.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M.B. and I.M. conceived and designed the study. I.M., M.L.-M., R.G., A.A., and M.B. formulated the methodology and carried out the experiments. I.M., M.L.-M., R.G., and M.B. analysed the data. All authors contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its Supporting Information files. Raw data is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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