INTRODUCTION

Ribonucleases (RNases) are RNA-degrading enzymes that are widespread among many different organisms and function in various cellular processes, mostly via RNA catabolism. Among them, the transferase-type RNases are a family of enzymes that catalyse the cleavage of single-stranded RNA through a 2',3'-cyclic phosphate intermediate, producing mono- or oligonucleotides (Deshpande and Shankar, 2002). These RNases have been classified based on their distribution, pH, and base specificity into three different families:

1. **Tomato T2 ribonuclease LE is involved in the response to pathogens**

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Abstract

T2 ribonucleases (RNases) are RNA-degrading enzymes that function in various cellular processes, mostly via RNA metabolism. T2 RNase-encoding genes have been identified in various organisms, from bacteria to mammals, and are most diverse in plants. The existence of T2 RNase genes in almost every organism suggests an important biological function that has been conserved through evolution. In plants, T2 RNases are suggested to be involved in phosphate scavenging and recycling, and are implicated in defence responses to pathogens. We investigated the function of the tomato T2 RNase LE, known to be induced by phosphate deficiency and wounding. The possible involvement of LE in pathogen responses was examined. Expression analysis showed LE induction during fungal infection and by stimuli known to be associated with pathogen inoculation, including oxalic acid and hydrogen peroxide. Analysis of LE-suppressed transgenic tomato lines revealed higher susceptibility to oxalic acid, a cell death-inducing factor, compared to the wild type. This elevated sensitivity of LE-suppressed lines was evidenced by visual signs of necrosis, and increased ion leakage and reactive oxygen species levels, indicating acceleration of cell death. Challenge of the LE-suppressed lines with the necrotrophic pathogen Botrytis cinerea resulted in accelerated development of disease symptoms compared to the wild type, associated with suppressed expression of pathogenesis-related marker genes. The results suggest a role for plant endogenous T2 RNases in antifungal activity.

KEYWORDS
Botrytis cinerea, pathogenesis, RNase LE, T2 ribonuclease, tomato

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RNase A, RNase T1, and RNase T2 (Luhtala and Parker, 2010). RNase T2 family members are widely distributed, have been identified in various organisms, and are most diverse in plants (MacIntosh et al., 2010; MacIntosh, 2011; Ramanaukas and Igić, 2017). Most of the RNase T2 family proteins are either secreted or targeted to cellular compartments of the secretory pathway, such as the endoplasmic reticulum, vacuoles or lysosomes and act at acidic pH with nonspecificity in vitro for base cleavage (Irie, 1999; Deshpande and Shankar, 2002; MacIntosh, 2011). The T2 S-locus RNases (S-RNases) are involved in the gametophytic self-incompatibility process responsible for prevention of self-pollination observed in many flowering plants (McCleure et al., 1989). Incompatibility results from the cytotoxic activity of S-RNase, whereas compatible pollen tubes evade this enzyme (McCleure et al., 2011).

Despite being structurally conserved, T2 RNase family members are thought to perform a variety of functions in different organisms. In nonplant organisms, T2 RNases are involved in diverse processes, including oxidative stress response, regulation of programmed cell death (PCD)/apoptosis, and antitumorigenic and antiangiogenic functions (Smirnoff et al., 2006; Schwartz et al., 2007; Luhtala and Parker, 2010; Wang et al., 2014; Caputa et al., 2016; Roggiani et al., 2019).

In plants, T2 RNases, such as tomato LX and LE, and Arabidopsis RNS1 and RNS2, are highly induced during phosphate starvation and senescence, suggesting their involvement in phosphate scavenging and RNA recycling (Löffler et al., 1992; Taylor et al., 1993; Bariola et al., 1994; Dodds et al., 1996; Lers et al., 1998; Liang et al., 2002; Hillwig et al., 2011). In carnivorous plants, high and constitutive expression of T2 RNases in digestive organs may be related to phosphate scavenging from the prey as part of the carnivory function (Nishimura et al., 2013).

The induction of T2 RNases during defined developmental processes known to involve cell death, such as endosperm development, xylem differentiation, and senescence, in Arabidopsis and tomato indicates the possible involvement of these enzymes in processes implicated in PCD (Taylor et al., 1993; Lers et al., 1998; Lehmann et al., 2001). A delay in leaf senescence and abscission in RNase LX-suppressed tomato lines suggested a regulatory role in these processes (Lers et al., 2006).

Plant T2 RNases have been implicated in defense responses associated with pathogenic attack or wounding based mostly on gene-expression data (Deshpande and Shankar, 2002; MacIntosh, 2011). T2 RNases identified in Petunia hybrida nectar have been suggested as functioning as antimicrobial defence agents (Hillwig et al., 2010). Induction of T2 RNase-encoding genes or activities has been documented in response to different bacterial, fungal or viral pathogens (Hugot et al., 2002; Hayashi et al., 2003; MacIntosh et al., 2010; MacIntosh, 2011). The responsiveness of plant T2 RNases to wounding also suggests their involvement in defence-related functions, and they have been proposed as antimicrobial enzymes that inhibit pathogen colonization in wounds used as an entry site (Kurata et al., 2002; LeBrasseur et al., 2002; MacIntosh, 2011). The tobacco gene encoding the T2 RNase NE was induced in leaves in response to inoculation with the pathogen Phytophthora parasitica (Galiana et al., 1997). Purified NE protein inhibited hyphal elongation in P. parasitica zoospores and in Fusarium oxysporum conidia in vitro, suggesting its involvement in tobacco pathogen-defence mechanisms (Hugot et al., 2002). While these observations support a possible role of T2 RNases in pathogen defence, no studies are available that link in vivo changes in endogenous expression of plant T2 RNase and defence response.

The tomato (Solanum lycopersicum) T2 RNase LE is a secreted protein that is expressed during phosphate starvation and has been detected in vacuoles of cultured tomato cells (Nürnberger et al., 1990; Löffler et al., 1992). Expression of the LE gene is highly induced by wounding, suggesting its involvement in defence against pathogens (Lers et al., 1998). While information, mainly regarding expression of T2 RNase-encoding genes, has suggested possible involvement in plant pathogen-defence responses, it is correlative and there is no direct evidence for an in vivo role. Therefore, experiments were performed to examine the hypothesis that LE is involved in vivo in the pathogen-defence response of tomato plants. RNA interference (RNAi)-transgenic lines were developed in which LE expression was significantly suppressed, and their sensitivity to the pathogen Botrytis cinerea was examined. The results, included in this report, support the involvement of the T2 RNase LE in pathogen defence in tomato leaves.

2 | RESULTS

2.1 | Expression analysis of T2 RNase LE

To determine LE’s possible involvement in pathogen defence, the response of the encoding gene to inoculation with the pathogen B. cinerea was examined. Detached tomato leaves were inoculated with a spore suspension of the fungus and LE transcript levels were measured at different times after inoculation by quantitative reverse transcription PCR (RT-qPCR) analysis. Following B. cinerea infection, significant activation of LE expression was observed. LE transcript level was induced about 18-fold after 24 hr compared to its initial level, and further increased to about 40-fold 72 hr after inoculation (Figure 1a). The similar activation of LE transcript in control and B. cinerea-inoculated leaves at the 24-hr time point (Figure 1a) was possibly due to the mechanical stimuli involved in the inoculation treatment, which were present in the control tissue as well, and represented the wound-like response of the LE gene. In addition, a certain low level of induction may have resulted from leaf detachment. Oxalic acid (OA) seems to be one of the determinants of pathogenicity in phytopathogenic fungi such as B. cinerea and Sclerotinia sclerotiorum (Lehner et al., 2008; Williams et al., 2011). LE expression was measured following treatment of the tomato leaves with 10 mM OA. Significant LE transcript induction, more than 10-fold compared to the initial level, was measured 3 hr after the treatment (Figure 1b). This induction was significantly higher than that measured in control leaves, but decreased at the 12- and 24-hr time points (Figure 1b). OA has been found to induce an increase in reactive oxygen species (ROS) levels in plants during compatible interactions with pathogens (Kim et al., 2008; Williams et al., 2011). Thus, treatment of leaves
with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was examined for its consequences on LE expression. To check the signalling effect, we used 10 mM H\textsubscript{2}O\textsubscript{2}. LE expression levels were significantly different between the treated and control samples at 12 and 24 hr post-treatment (hpt) (Figure 1c). In both OA and H\textsubscript{2}O\textsubscript{2} controls (Figure 1b,c) LE expression was not induced as observed for the 24 hr control of fungal treatment (Figure 1a) because both did not involve mechanical stimuli and were sprayed with the agents. Nitric oxide is involved in defence signalling (Bellin et al., 2013). To examine its effect on LE expression, sodium nitroprusside (SNP) treatment was used. There were no significant changes in LE transcript levels at different times after this treatment in either control or treated samples (Figure S1).

2.2 | OA sensitivity is enhanced in LE-suppressed transgenic tomato plants

To study the possible involvement of the RNase LE in biotic defence, transgenic plants were generated as described in Section 4 using an RNAi approach. To check the silencing effects on LE gene expression, leaves of wild-type and LE-suppressed transgenic lines were wounded for activation of endogenous LE expression (Lers et al., 1998). Measurement of LE transcript level 24 hr after wounding revealed very significant inhibition of LE transcript in the transgenic lines, about 15- to 20-fold lower than that measured in the wild type (Figure 2). Similar degrees of suppression were measured in the three LE-suppressed lines. The consequences of suppression of LE gene expression on wound-induced ribonuclease activity in leaves was examined. Leaves of wild-type and LE-suppressed lines were wounded and proteins extracted after 3 hr were used to treat total intact plant RNA. Reduced RNA degradation was observed in reactions that included LE-suppressed protein extracts compared to control wild-type enzyme extracts indicative of reduced wound-induced ribonuclease activity in the transgenic lines (Figure S2).

The effect of LE suppression on the plant’s response to OA was examined by floating leaf discs from transgenic and wild-type lines on a solution of 10 mM OA. Distinct differences in phenotype were observed at 72 hpt (Figure 3). Discs from the LE-suppressed lines displayed more intense chlorosis than the wild type (Figure 3a), which was reflected in reduced chlorophyll levels measured in the three LE-suppressed lines compared to that measured in the wild type (Figure 3b). In the control water treatment, no differences were observed between LE-suppressed and wild-type discs and all maintained green-coloured tissue (Figure 3a). No differences could be measured...
in chlorophyll levels between the wild-type and the LE-suppressed lines (Figure 3b). As a control, the effect of malic acid solution in the same pH 5.0 was examined. Only a mild effect on chlorophyll loss was observed, which was the same for both wild-type and LE-suppressed lines (Figure 3b). The extent of damage to the leaf tissue due to cell death induced by OA was examined by measuring ion leakage levels 72 hr after exposure to OA. Significant differences were observed, with ion leakage values in the three LE-suppressed lines increasing to between 65% and 75% compared to about 40% ion leakage in the wild-type leaf tissue (Figure 3c). The LE-suppressed line 7.4 seemed to exhibit somewhat higher relative ion leakage values compared to the other lines but it was not significant. In the control, water-treated, leaf discs, no significant differences in ion leakage values were measured between mutants and the wild type (Figure 3c). Also the control malic acid treatment did not result in enhanced ion leakage in the LE-suppressed lines, as OA did, and resulted in ion leakage levels similar to that measured for the OA-treated wild type, resulting probably from the pH effect (Figure 3c).

OA has also been reported to induce an increase in ROS levels in plant cells, correlated to PCD (Kim et al., 2008). After 72 hr treatment with 10 mM OA, ROS levels were compared between the LE-suppressed lines and the wild type by 3,3′-diaminobenzidine (DAB) staining. H₂O₂ accumulation in the leaves of the three LE-suppressed transgenic lines was considerably higher than in the wild type (Figure 4a). In control leaf discs subjected to 72 hr water treatment, no significant differences in ROS levels could be detected (Figure 4a). Quantitative analysis of cell death in the tomato wild-type and LE-suppressed leaf discs was measured using

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** Expression of LE gene is suppressed in the three independent transgenic plants compared to the wild type. Detached leaves from three LE-suppressed RNA interference (RNAi) lines (1.9, 2.9, 7.4) and the wild type (WT) were wounded uniformly with a Pasteur pipette tip and kept under humid conditions at room temperature. Total RNA was isolated 24 hr after treatment and LE transcript level was measured by quantitative reverse transcription PCR analysis. Relative expression levels are shown. Error bars represent standard error of the mean (n = 3). Different letters denote significant difference (p < .05, analysis of variance). Actin gene expression served as an internal control.

![Figure 3](https://example.com/figure3.png)

**FIGURE 3** LE-suppressed lines exhibit enhanced sensitivity to oxalic acid (OA) treatment. Leaf discs were sampled from same-position leaves of 6-week-old plants (wild type [WT] and the three independent LE RNA interference lines [1.9, 2.9, 7.4]) and floated on water, 10 mM OA solution (pH 5.0) or 10 mM malic acid (MA) solution (pH 5.0). Samples were kept under 16 hr light/8 hr dark at 25 °C. After 72 hr of treatment, photographs were taken (a) and analyses were performed for measurements of total chlorophyll content (b) and relative ion leakage (c), using 10 leaf discs for each line in each replicate. Error bars represent standard error of the mean (n = 3). Different letters denote significant difference (p < .05, analysis of variance). The experiment was repeated three times with similar results.
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Evans blue staining 72 hr after OA exposure (Figure 4b). Significantly higher (by about 40%) cell-death values were measured in the three LE-suppressed lines compared to the wild type. No significant differences in cell death were measured in the control water-treated discs (Figure 4b).

FIGURE 4  Oxalic acid (OA)-induced reactive oxygen species accumulation and cell death are accelerated in the LE-suppressed lines compared to the wild type (WT). The effects of OA (10 mM, pH 5.0) treatment on H$_2$O$_2$ and cell death were examined in leaf discs of the LE-suppressed (1.9, 2.9, 7.4) and WT plants 72 hr after treatment initiation. (a) In situ detection of H$_2$O$_2$ accumulation in leaf discs treated with water or OA. H$_2$O$_2$ production was visualized by 3,3′-diaminobenzidine (DAB) staining. (b) Cell death occurrence in leaf discs, determined by Evans blue staining. Spectrophotometric measurements at an absorbance of 600 nm were expressed as relative values, with 1 corresponding to the highest measured value. For each measurement, 10 leaf discs were used for each line in each replicate. Error bars represent standard error of the mean (n = 3). Different letters denote significant difference (p < .05, analysis of variance). The experiment was repeated three times with similar results.

Evans blue staining 72 hr after OA exposure (Figure 4b). Significantly higher (by about 40%) cell-death values were measured in the three LE-suppressed lines compared to the wild type. No significant differences in cell death were measured in the control water-treated discs (Figure 4b).

2.3 | Sensitivity to B. cinerea is enhanced in LE-suppressed transgenic tomato plants

To investigate the involvement of LE in the response to fungal pathogens, transgenic LE-suppressed lines and wild-type plants were
compared for their response to \(B.\) cinerea. Detached leaves from wild-type and transgenic lines were inoculated with \(B.\) cinerea spore suspension and the development of fungus-induced symptoms was followed. Disease symptoms were observed in the wild-type leaves 3 days after inoculation, whereas significantly larger necrotic lesions were observed in the LE-suppressed transgenic leaves (Figure 5a). The average decay diameter was taken as an indicator of susceptibility in each group and measured 3 days after inoculation. All three transgenic lines showed significantly larger decay diameters, more than 2-fold higher than that measured for the wild type (Figure 5c). Ion leakage was also measured 3 days after inoculation as an indicator of cell death, and the measured values were found to be significantly higher in all three LE-suppressed lines compared to the wild type (Figure 5d). Following 5 days of \(B.\) cinerea development on the leaves, severe necrosis was observed and the fungus-infected tissue covered 70%-80% of the surface area of the leaves in LE-suppressed lines, whereas fungal spread was much more limited in the wild-type leaves (Figure 5b). In addition, in all three LE-suppressed lines, significant chlorosis was evident in the leaf tissue that was not killed by the fungi, while the respective tissue of the wild-type leaves tended to retain a greener colour (Figure 5a). These results indicate that silencing of LE causes reduced resistance or increased susceptibility to \(B.\) cinerea disease in tomato.

### 2.4 Expression of defence-related genes is attenuated in LE-suppressed lines

The consequence of LE suppression on the expression of genes known to be associated with the pathogen response was examined. Leaves of LE-suppressed lines and wild-type plants were inoculated with \(B.\) cinerea spore suspension or treated with control buffer, and the expression of pathogen defence response genes was measured 72 hr post-inoculation by RT-qPCR. The pathogenesis-related (PR) genes were pathogenesis related 1a (PR1a), basic chitinase (chitinase), 1-aminocyclopropane-1-carboxylic acid synthase-2 (ACS2) and phenylalanine ammonia-lyase-1 (PAL1). Expression of all four genes was found to be induced in the wild type 72 hr after inoculation with \(B.\) cinerea, as expected (Figure 6). However, 72 hr after pathogen treatment, the expression of PR1a, chitinase, ACS2, and PAL1 was inhibited by about 50% in the LE-suppressed lines compared to the induction in their levels in the wild type (Figure 6). No difference in the transcript levels of the four PR genes could be measured between the wild-type and LE-suppressed lines in the control leaves not subjected to pathogen inoculation.

### 3 DISCUSSION

The tomato T2 RNase gene LE expression was found to be regulated by \(B.\) cinerea infection, as well as by the pathogen-related stress factors OA and \(H_2O_2\). In this functional study, LE-suppressed tomato plants were observed to display higher susceptibility to fungal pathogenicity. The LE-suppressed lines were more sensitive to OA, known to be involved with fungal pathogenicity, as indicated by increased ROS levels and cell death. Moreover, the LE-suppressed transgenic lines exhibited accelerated development of disease symptoms following \(B.\) cinerea inoculation compared to wild-type plants. T2 RNase involvement in plant defence has been suggested (Luhtala...
and Parker, 2010; Maclntosh et al., 2010; Maclntosh, 2011), but no direct evidence was reported for the in vivo involvement of a plant endogenous T2 RNase in antifungal activity. The results of our study support an in vivo requirement for tomato LE T2 RNase as part of a defence mechanism against the pathogenic fungus B. cinerea.

3.1 LE-expression response to pathogens and related agents

The observed induction of LE expression in response to inoculation with B. cinerea spores supports LE involvement in the plant’s defence response against the pathogen. Transient induction of LE expression also peaked 24 hpt in the control, and then declined (Figure 1a). This transient induction probably resulted from the physical contact associated with application of the buffer solution. The LE gene is extremely sensitive to wounding (Lers et al., 1998), which can induce its expression to very high levels, 1,500 to 3,000-fold in 3 to 24 hr, respectively (Figure S3). Thus, treatments that physically challenge the tissue can also result in significant transient induction of LE. In contrast to the control, expression of LE continued to be significantly induced in the pathogen-inoculated leaves, indicating responsiveness to the pathogen itself (Figure 1a). OA was associated in previous studies with pathogenicity of phytopathogenic fungi, including B. cinerea (Han et al., 2007; Lehner et al., 2008; Williams et al., 2011). We have observed induction of LE in OA-treated leaves, as early as 3 hpt (Figure 1b). The OA concentration used in these experiments, which induced LE expression, were similar to those (up to 10 mM) measured previously in fungal-infected tissues (Bateman and Beer, 1965; Marciano et al., 1983). The observation that H2O2 can also induce LE gene expression during the first day of treatment also suggests that early activation of LE is part of the pathogen-defence mechanism. OA has been found to induce increased ROS levels in the plant during compatible interactions with pathogens (Kim et al., 2008; Temme and Tuzdysnki, 2009).

3.2 LE expression in the wound response

LE regulation of expression suggests involvement in the wound response (Lers et al., 1998; Groß et al., 2004). This wound response of LE is local, and has been shown to be independent of jasmonic acid (JA) and systemin, suggesting that it is not involved in the specific wounding-induced herbivore-defence response (Sun et al., 2011). The expression of LE in phloem cells (Kock et al., 2004) could also be related to its involvement in defence responses against phloem-feeding insects. LE expression is activated by abscisic acid (ABA), and does not respond to other hormonal treatments such as ethylene, JA, and salicylic acid (Lers et al., 1998; Groß et al., 2004). We were unable to measure any induction of LE expression during SNP treatment, which releases nitric oxide, a crucial regulatory molecule associated with ROS in plant disease resistance (Asai and Yoshioka, 2009). The lack of LE responsiveness to these different plant hormones, whereas it is induced by ABA, wounding, fungal infection, OA, and H2O2, suggests a possible association of LE regulation with a unique signalling pathway. The Arabidopsis T2 RNase RNS1, which bears high protein sequence similarity to LE, may be its functional homolog. Expression of RNS1 is induced by ABA and wounding, and ABA is necessary to produce the full wound response (Hillwig et al., 2008). LE and RNS1 may respond to a similar regulatory circuit; however, whereas RNS1 expression is induced systemically by wounding (LeBrasseur et al., 2002), LE responds only locally (Lers et al., 1998; Groß et al., 2004).

3.3 Possible LE function in pathogen response

It is not yet clear how suppressed LE expression resulted in the observed increased susceptibility to B. cinerea (Figure 5). One possibility is a cytotoxic effect of LE on the pathogen, which limits its development following spore attachment and germination on an intact leaf or at the wound sites that are more vulnerable to invasion of opportunistic pathogens. Secretory RNases have been proposed to act as antimicrobial enzymes against pathogens (Galiana et al., 1997; LeBrasseur et al., 2002; Hiroshi and Ehara, 2005). A study in tobacco revealed that exogenous application of the T2 RNase NE in the extracellular space of tobacco leaves inhibits fungal development (Hugot et al., 2002). However, in planta effective concentrations of the exogenously added NE protein were significantly higher than in vitro-effective concentrations and the estimated concentrations of tobacco NE in the leaves on pathogen infection (Hugot et al., 2002). It was proposed that NE acts directly against pathogens that are present in the apoplastic compartment of the plant cell. This antimicrobial effect of NE was found to be dependent on its RNase activity (Hugot et al., 2002).

Possibly relevant to such putative cytotoxic activity is the function of T2 S-RNases in the mechanism of gametophytic self-incompatibility in a few plant families, preventing self-pollination by selective inhibition of pollen tube growth. This function was found to be mediated by a cytotoxic effect of the S-RNases, which was dependent on the RNase activity (McClure et al., 1989, 2011; Clarke and Newbiggin, 1993; Huang et al., 1994). The hypothesis was raised that the cytotoxic activity of an ancient flower RNase involved in pathogen-defence mechanisms was recruited during the development of the S-RNases’ function in the gametophytic self-incompatibility mechanism (Hiscock et al., 1996; Nasrallah, 2005). Interestingly, some PR proteins in the PR4 and PR10 families, involved in plant resistance against pathogenic fungi, exhibit RNase activity, suggesting its requirement for their pathogen-defence function (Filipenko et al., 2013). For the peanut AhPR10, it was shown that RNase activity is required for in vitro antifungal activity (Chadha and Das, 2006). In nonplant organisms, T2 RNases have also been described to function as cytotoxic agents, which can be both catalysis-dependent and catalysis-independent (Luhtala and Parker, 2010).
Thus, an attractive hypothesis for LE function in pathogen defence may include its direct interaction with the pathogen, exerting a cytotoxic effect that might be dependent on its catalytic activity. However, some of our observations suggest a possible indirect function as well. LE expression responded to OA treatment (Figure 1b) and even more significantly, LE suppression resulted in higher sensitivity to OA, exhibited by increased cell death and ROS accumulation compared to wild-type plants (Figures 3 and 4). Thus, suppression of LE modified the cellular aspects that are relevant to pathogen sensitivity even before exposure to the pathogen itself. The observed suppression in expression of genes associated with pathogen defence in the LE-suppressed mutants following inoculation with B. cinerea spores (Figure 6) also supports a possible consequence for the plant’s pathogen-defence system.

OA plays a role as a fungal elicitor that can induce plant defence responses at low concentrations, but at high concentrations can cause serious damage by inducing cell death (Lehner et al., 2008). This strategy can be used to stop the development of biotrophic pathogens, but not for necrotrophic fungus–plant interactions in which host cell death is advantageous for the pathogen. In addition, OA can also interfere with host defence pathways by suppressing the oxidative burst in plant cells (Cessna et al., 2000; Heller and Witt-Geiges, 2013). Though the exact mechanism is not known, it has been shown that OA can induce genetically controlled PCD, which requires the activation of gene expression associated with apoptosis-like features (Errakhi et al., 2018). If LE is involved in tRNA cleavage, its down-regulation could be related to the regulation of defence-related gene expression, observed to be affected in the LE-suppressed lines. OA has been reported to induce ROS levels in the plant, which correlate with PCD occurrence, and when ROS induction is inhibited, cell death induced by OA does not occur (Kim et al., 2008). T2 RNases have been shown to be involved with PCD/apoptosis and oxidative stress in a variety of different organisms. The tomato LX and Arabidopsis RNS3 are highly associated with PCD (Lehmann et al., 2001; Bar-Dror et al., 2011; Olvera-Carrillo et al., 2015). The single human T2 RNase RNASET2 was found to be involved in oxidative stress-mediated cell death and apoptosis (Wang et al., 2014; Caputa et al., 2016). The yeast T2 RNase Rny1 can induce cell death during oxidative stress in a process that is independent of its catalytic activity (Thompson and Parker, 2009). Rny1 was reported to be responsible for tRNA cleavage in yeast in response to oxidative stress. Interestingly, the Arabidopsis T2 RNases RNS1 and RNS3 have been recently reported to be responsible for the tRNA-cleavage function (Megel et al., 2019). In plants, tRNA-derived fragments have been suggested in recent years to be involved in fine-tuning of gene expression during abiotic and biotic stresses (Asha and Soniya, 2016; Sablok et al., 2017; Park and Kim, 2018; Soprano et al., 2018). If LE is involved in tRNA cleavage, its down-regulation may affect pathogen susceptibility via modulation of gene expression.

Overall, the observations from our study demonstrate, for the first time, in vivo involvement of a T2 RNase in the plant defence response against B. cinerea, mediated by either a cytotoxic effect of the RNase or its involvement in cellular mechanisms affecting pathogen-defence responses. Further studies are required to understand the functional role of LE in plant defence against pathogens.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant and fungal material

Tomato (S. lycopersicum) plants, cv. Moneymaker, were used in this study. Seeds were germinated on perlite at 28 °C in the dark for 3 days and then transferred to the greenhouse under a controlled temperature of 25 °C and natural daylight conditions. About a week later, fully developed seedlings were transferred to pots filled with artificial soil (Green 77, Even-Ari Ltd). B. cinerea strain B05.10 was cultured on potato dextrose agar (PDA; BD Difco) and plates were maintained at 25 °C.

4.2 | Treatments

Leaves at the same position on the stem from 6-week-old plants were used for the different treatments. For B. cinerea infection, detached leaves were kept in trays on filter paper saturated with sterile double-distilled water and covered with plastic wrap to maintain humidity. Each tomato leaf was inoculated with 7 µl of inoculation solution containing 10^5 spores/ml in 10 mM glucose and 10 mM KH₂PO₄ (pH 5.0). To facilitate fungal infection, inoculation sites were slightly pressed with the tip of a 1-ml pipette tip. In the control, inoculation solution without spores was used in a similar manner. Trays were transferred to a 25 °C room in the dark. For chemical treatments, leaves were sprayed with 10 mM oxalic acid (OA) pH 5.0, 10 mM H₂O₂ or 100 mM sodium nitroprusside (SNP) solutions. Experiments for leaf treatments were performed under a 16 hr light/8 hr dark regime, at 25 °C. Treatment with sterile water served as the control. Samples were collected from different treatments at the specified time points, quickly frozen in liquid nitrogen, and stored at −80 °C until use. For OA and malic acid (MA) treatment of leaf discs, discs were obtained using a cork borer (8 mm diameter) from same-position leaves of 6-week-old plants. Discs were floated on 10 mM OA (pH 5.0) or 10 mM MA (pH 5.0) solution or sterile water and incubated under 16 hr light/8 hr dark conditions at 25 °C. Plates were routinely observed for 3 days. Photographs were taken 72 hr after treatment.

4.3 | Generation of RNAi-transgenic plants

A specific 400-bp fragment of the LE gene, from positions 132 to 531 on the LE gene (Solyc05g007950) coding sequence, was
used to construct the RNAi vector. Primer pair P1 (forward 5'-GTGGCCGCGGCAAACAGTTGCGCGTGTCATCTGTA-3' and reverse 5'-CTACCTTTAAATGAGCAGCTTAAAGATTGCCCTCA-3'), with built-in Acsl and Sswd sites) and P2 (forward 5'-GTTCTAGAC AACAGTTGCGCGTGTCATCTGTA-3' and reverse 5'-GTGGAT CCTGACAGGTCTTTAAGATTGCCCTCA-3', with built-in XbaI and BamHI sites) were used to generate the two identical 400-bp fragments to be cloned in the sense and antisense orientations. These two fragments were inserted into vector pGSA1285, separated by the CHSA intron, under the control of the CaMV 35S promoter (Figure S4). The constructed p1285-LEi vector was mobilized into Agrobacterium tumefaciens EHA105 using the freeze–thaw method. Plant transformation was performed using the cotyledons of tomato cv. Moneymaker as described previously (McCormick, 1991). Transformants were raised on kanamycin, and antibiotic-resistant T0 plants were analysed by PCR with primer pair P3 (forward 5'-CAACAGTTGCGCGTGTCATCTGTA-3' and reverse 5'-GTGCAATTTGAGCAGGTTGTCATCCA-3') and P4 (nptII forward 5'-GAGGCCTATTGAGGCTACTGTGA-3' and reverse 5'-TCCATTTAAATGAGCAGCTTAAAGATTGCCCTCA-3') to verify the presence of the chimeric gene. T3 homozygous lines were used for the experiments.

4.4 | RNase activity measurement

Detached leaves of wild-type and RNAi lines were wounded with a Pasteur pipette tip and kept under humid conditions for 3 hr at room temperature. Tissue samples were disrupted under liquid nitrogen and 150 mg of each sample was suspended in 1 ml of Tris- HCL buffer (pH 7.0). Extract samples were centrifuged at 8,000 × g for 10 min at 4 °C. The supernatant including tissue soluble proteins was used for the RNase enzymatic assay after determination of protein concentration following the Bradford method. Protein extracts (0.4 µg) were incubated at room temperature with 50 µl of 50 mM Tris-HCL (pH 7.0) containing 2 µg purified plant RNA. Reactions were stopped after 5 or 30 min of incubation by adding RNA loading dye and heating at 70 °C for 10 min. Treated RNA samples were fractionated on agarose gel and RNA degradation was evaluated following separation.

4.5 | Quantitative reverse transcription PCR

Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and reverse-transcribed with the Maxima cDNA Synthesis Kit (Thermo Scientific). RT-qPCR was performed with a StepOne Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) and gene-specific primers. The expression data were analysed by ΔΔCt (Livak and Schmittgen, 2001). For each sample, three independent biological replicates were used.

Expression level of LE in the wild type and the three independent transgenic lines was measured following induction of LE expression by wounding of detached leaves, as described previously (Lers et al., 1998), followed by RT-qPCR analysis. Primer pair P5 (LE forward 5'-ACCAGTGCGTAACAGTCA-3' and LE reverse 5'-GTGCTGTGGCACATTTCCCTCA-3') was used for LE expression analysis. Actin gene expression served as the internal expression control, reported previously (Vossen et al., 2010). For measuring the expression of defence-related genes following fungal leaf infection, tissue outside the necrotic lesion area was used and gene expression was measured by RT-qPCR analyses using primer pairs as indicated in Table S1.

4.6 | Ion leakage measurement

For conductivity measurements, indicative of ion leakage from the cells, leaf discs sampled from same-position leaves were subjected to 24, 48, or 72 hr of treatment with OA or water as a control. Discs were then washed once with distilled water and transferred to 50-ml Falcon tubes containing 25 ml distilled water for overnight incubation at room temperature. Each tube held 10 discs, and three replicates were used for each line. The change in the ion conductivity of the water was measured using a conductivity meter (Eutech CON 700, Thermo Scientific). Percentage of electrolyte leakage was calculated relative to the total value of electrolytes released from the discs following full disintegration of the tissues by autoclaving of the sample.

4.7 | Chlorophyll measurements, cell death quantification, and detection of H2O2

The total chlorophyll content of the leaf discs was measured according to the method described (Hiscox and Israelstam, 1979). Cell death was quantified by measuring the accumulation of Evans blue, which specifically stains dead cells, following a previously described method (Baker and Mock, 1994). In brief, treated leaf disks were submerged in a solution of 0.25% (wt/vol) Evans blue in water (Sigma-Aldrich) for 30 min, then gentle vacuum pressure was applied for 15 min. The stained leaf discs were washed with deionized water to remove excess and unbound dye. Discs were ground in a 1% (wt/vol) sodium dodecyl sulphate solution and the extracts were centrifuged at 12,000 × g for 5 min. The supernatant was collected and absorbance was measured at 600 nm.

In situ H2O2 accumulation was detected by DAB staining. Leaf discs were treated with 10 mM OA solution or sterile water as a control for 72 hr, then stained in 1 mg/ml DAB solution (pH 3.8) overnight in the dark. Samples were boiled in absolute ethanol for 10 min and then transferred to Whatman filter paper, pre-soaked with 60% glycerol. Images were captured on a white background.

4.8 | Pathogenicity assays

Detached leaves were collected and fungal inoculation was performed as described in Section 4.2. Around 30 same-position leaves,
from 5–10 different plants, were used for each line. Infection was evaluated by measuring the decay diameters at 72 hpt and the averages were plotted on a graph. Photographs were taken at 3 and 5 days after inoculation. Ion leakage was measured as described in Section 4.6 by taking at least five infected leaves from each line at 72 hpt.

4.9 | Stress-related gene expression

Same-position leaves were used from a few different plants of each line. Fungal and control treatments were performed as described in Section 4.2. Samples were collected 72 hpi from three independent experiments, quick-frozen in liquid nitrogen, and stored at −80 °C. In this study, we analysed the expression levels of the PR genes PR1a (Solyc09g007010), chitinase (Solyc10g055810), ACS2 (Solyc01g095080), and PAL1 (Solyc09g007890) using gene-specific primers (Table S1). The nucleotide sequences of the PR1a, ACS2, PAL1, and actin primers for RT-qPCR analysis were as previously reported (Kamioyoshihara et al., 2010; Buxdorf et al., 2014; Gonorazky et al., 2016). The primers for chitinase were designed in this study.

4.10 | Statistical analysis

Statistical analyses were carried out using SIGMASTAT v. 11.0. Statistical differences were assessed by Student’s t test and analysis of variance (Duncan’s multiple range test method).

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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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