RTP1 encodes a novel endoplasmic reticulum (ER)-localized protein in *Arabidopsis* and negatively regulates resistance against biotrophic pathogens

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Summary

- Oomycete pathogens cause serious damage to a wide spectrum of plants. Although host pathogen recognition via pathogen effectors and cognate plant resistance proteins is well established, the genetic basis of host factors that mediate plant susceptibility to oomycete pathogens is relatively unexplored.
- Here, we report on *RTP1*, a nodulin-related *MtN21* family gene in *Arabidopsis* that mediates susceptibility to *Phytophthora parasitica*.
- *RTP1* was identified by screening a T-DNA insertion mutant population and encoded an endoplasmic reticulum (ER)-localized protein. Overexpression of *RTP1* rendered *Arabidopsis* more susceptible, whereas RNA silencing of *RTP1* led to enhanced resistance to *P. parasitica*. Moreover, an *RTP1* mutant, *rtp1-1*, displayed localized cell death, increased reactive oxygen species (ROS) production and accelerated *PR1* expression, compared to the wild-type Col-0, in response to *P. parasitica* infection. *rtp1-1* showed a similar disease response to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, including increased disease resistance, cell death and ROS production. Furthermore, *rpt1-1* exhibited resistance to the fungal pathogen *Golovinomyces cichoracearum*, but not to the necrotrophic pathogen *Botrytis cinerea*.
- Taken together, these results suggest that *RTP1* negatively regulates plant resistance to biotrophic pathogens, possibly by regulating ROS production, cell death progression and *PR1* expression.

Introduction

Oomycetes, particularly the *Phytophthora* species, infect hundreds of different plant species, including many crops causing a number of serious agriculturally relevant diseases. These infections include potato late blight mediated by *P. infestans* (Nowicki *et al.*, 2012), *Phytophthora* root and stem rot by *P. sojae* (Tyler *et al.*, 2006), and sudden oak death caused by *P. ramorum* (Grunwald *et al.*, 2012). Due to their fungus-like morphology, oomycetes were originally classified as fungi, until evolutionary analysis clustered them into a separate kingdom, Stramenopila (Van de Peer & De Wachter, 1997). Current studies on the interactions between *Phytophthora* and plants focus primarily on the recognition specificities between pathogen effectors and cognate host resistance proteins (Pais *et al.*, 2013). Since the first oomycete avirulence gene, *Avr1b*, was cloned from *P. sojae* and shown to be specifically recognised by the corresponding resistance gene *Rps1b* (Shan *et al.*, 2004), many other effector-resistance gene pairs have been reported, such as *PiAvrblb1-RB* (Song *et al.*, 2003; Champouret *et al.*, 2009) and *PiAvr3a-R3a* (Armstrong *et al.*, 2005; Huang *et al.*, 2005). The dual function of AVR3a during the *P. infestans*–host interaction has been well characterized: AVR3a can repress INF1 elicitor induced cell death by stabilizing the plant E3 ligase, CMPG1 (Bos *et al.*, 2010). However, in resistant plants, AVR3a can be recognised by R3a activating *R* gene-mediated hypersensitive cell death (Armstrong *et al.*, 2005; Bos *et al.*, 2006). Following the recent availability of genome sequences corresponding to a variety of *Phytophthora* pathogens and subsequent bioinformatic analysis, hundreds of candidate effector genes have now been predicted based on common features such as small protein size, the presence of secretion signals, and the possession of an RXLR domain (Tyler *et al.*, 2006; Haas *et al.*, 2009), including Avirulence Homolog (*Avh*) genes (Jiang *et al.*, 2008). Avh proteins are known to be capable of inhibiting cell death triggered by effectors and PAMP elicitors (Wang *et al.*, 2011a). Recently, a RXLR effector of *P. parasitica*, Penetration-Specific Effector 1 (PSE1), was shown to promote pathogen infection by modulating auxin accumulation during penetration

progression (Kebdani *et al.*, 2010; Evangelisti *et al.*, 2013). However, little is known about the host factors that participate in compatible plant–*Phytophthora* interactions.

We have taken advantage of the compatible interaction between *P. parasitica* and the model plant *Arabidopsis* (Wang *et al.*, 2011b) to help uncover the genetic and molecular basis of plant susceptibility to oomycete pathogens. We screened a collection of T-DNA insertion *Arabidopsis* mutants for increased resistance to *P. parasitica* infection. This led to the identification of a resistant mutant, 574-34. Determination of T-DNA insertion sites led to the identification of *RTP1* (*Resistance to Phytophthora parasitica 1*), which has sequence similarities to the *Medicago truncatula* NODULIN 21 (*MtN21*) homologue. *MtN21* was originally shown to be induced during nodulation of *M. truncatula*, hence the name 'nodulin' (Gamas *et al.*, 1996). Although it is commonly accepted that *Arabidopsis* cannot form nodules, many genes have been predicted to be *MtN21* family members in this model plant species (Denance *et al.*, 2014). However, the function of *MtN21* genes remains largely unexplored.

We show that downregulation of *RTP1* leads to increased resistance in *Arabidopsis* to infection by the oomycete pathogen *P. parasitica*, biotrophic bacterial pathogen *Pseudomonas syringae* and fungal pathogen *Golovinomyces cichoracearum*, but not to the necrotrophic fungal pathogen *Botrytis cinerea*. Furthermore, *rtp1-1* plants displayed localized cell death at infection sites, rapid accumulation of reactive oxygen species (ROS) and increased *PR1* expression, all of which may lead to increased disease resistance.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis* T-DNA mutant collection was generated and kindly provided by Dr Jianru Zuo (Zuo *et al.*, 2000). The *RTP1* (Genbank accession number AT1G70260) T-DNA insertion line was obtained from the Arabidopsis Biological Resource Center (ABRC): *rtp1-1* (SALK_094320). The T-DNA insertion homozygous mutants were confirmed by PCR using the primers *rtp1-1*-LP, *rtp1-1*-RP and LBb1.3 (Supporting Information Table S1). Seeds were surface-sterilized and planted as described (Wang *et al.*, 2011b). Treated seeds were sown on half-strength Murashige and Skoog (1/2 MS) plates and subsequently cold stratified for 2 d. The plates were then kept in a growth chamber under 12-h photoperiod conditions at 22°C. For whole-seeding inoculation, 2-wk-old plants were used, and for the other experiments 10-d-old seedlings were transferred to the pot and grown in the same conditions to 4 wk.

Plasmid constructs and plant transformation

In order to create the expression cassette, the coding region of *RTP1* was fused at the C terminus with the 3 × FLAG sequence and inserted into the *Eco*RI and *Bam*HI sites of pKannibal (Wesley *et al.*, 2001), then inserted into the binary vector pART27 (Gleave, 1992) at the *Not* I site.

To create the RNA silencing transgenic plants, a 600-bp fragment (26–625 bp) was chosen with consideration of minimum off-target effects. The fragment was amplified using the primers RTP1Ri-F and RTP1Ri-R (Table S1), then cloned into pKannibal vector between the *Xho*I–*Eco*RI sites in sense orientation and the *Cla*I–*Xba*I sites in antisense orientation. Finally, the construct was subcloned into the binary vector pART27.

In order to make the Promoter^{RTP1}::*GUS* reporter fusion, a 2708-bp promoter fragment of *RTP1* was amplified from Col-0 genomic DNA using the primers pRTP1-F and pRTP1-R (Table S1) and inserted into the binary vector pMDC162 (Curtis & Grossniklaus, 2003) at the *Pst* I site. The final construct (p*RTP1*::*GUS*) was verified by sequencing.

The constructs were transformed into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation using the floral dipping method (Zhang *et al.*, 2006). The generated transformants were confirmed by both antibiotic resistance and genotyping. Two independent T3 homozygous lines with single insertion were used for the further experiments.

Pathogen infection assays

The maintenance and zoospores production of *Phytophthora parasitica* strain Pp016 have been described elsewhere (Wang *et al.*, 2011b). The concentration of zoospores used in this paper is 1×10^5 zoospores ml^{-1} unless otherwise specified. For detached leaf inoculation, *c.* 20 leaves of each line were wounded by toothpicks and drop-inoculated. Sterile water was used as a control. For gene expression analysis by real-time reverse transcription polymerase chain reaction (RT-PCR) the leaves were not wounded. For the quantitation of infection, genomic DNA was extracted by the CTAB method (Llorente *et al.*, 2010) from three independent biological samples each containing five inoculated leaves (diameter 1 cm) at the indicated time point. Specific primers for *P. parasitica UBC* (*PpUBC*) were used to monitor the level of pathogen colonization and *AtUBC9* was used to indicate plant biomass. Results were presented as a proportion between pathogen and plant genomic DNA to reveal the extent of infection.

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 was cultured in low salt Luria Bertani medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl, pH 7.0) with appropriate antibiotics and grown overnight at 28°C. Bacteria from the liquid culture were harvested and washed twice, then resuspended in 10 mM $MgCl_2$ and adjusted to the concentration needed. The final concentration of 1×10^5 CFU ml^{-1} was used for bacterial growth assays and the plants were grown under high humidity after inoculation. Bacterial suspension at 1×10^7 CFU ml^{-1} was used for cell death analysis and the plants were kept at low humidity after infiltration. At least 10 leaves from five plants were used for each inoculation experiment. For ROS production experiments, bacterial cells were suspended in sterile water and the final concentration of 5×10^7 CFU ml^{-1} was used. The experiments were repeated three times.

The fungus *Golovinomyces cichoracearum* (*Gc*) UCSC1 was grown on Col-0 to maintain constant aggressiveness. Methods of plant inoculation were the same as reported (Yu *et al.*, 2013); *c.*

10 plants from each line were inoculated. The number of conidiospores per colony was counted under a microscope after trypan blue staining at 6 d post-inoculation (dpi). At least 25 colonies were counted for each genotype in each experiment. And the phenotype was photographed at 10 dpi. The experiments were repeated three times.

Microscopic characterization

In order to determine the progress of infection or cell death, inoculated leaves were stained with trypan blue solution (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water and 10 mg of trypan blue) by boiling for 2 min. After cooling to room temperature, the samples were destained with 2.5 g ml^{-1} chloral hydrate solution. The samples were rinsed with water and visualized under an Olympus (Tokyo, Japan) microscope.

Whole seedlings of 2-wk-old plants were dip-inoculated with *P. parasitica* zoospores or placed in the bacterial solution without wounding, then stained in the DAB solution (1 mg ml^{-1}) for 8 h or NBT solution (0.5 mg ml^{-1} in PBS) for 3 h in dark at the indicated time point and then cleared with ethanol for 1 d. *c.* 10 plants were used from each line, and experiments were repeated three times.

Subcellular localization

The full-length coding region of *RTP1* was translationally fused with GFP and inserted into the plant expression vector pART27 under control of a 35S promoter. The generated construct, free GFP and ER-rk (ABRC stock number CD3-959) constructs (Nelson *et al.*, 2007) were individually transformed into *A. tumefaciens*. The *Agrobacterium* cultures carrying individual constructs were harvested and suspended in infiltration media (10 mM $MgCl_2$, 10 mM MES, pH 5.6, plus 200 mM acetosyringone) and adjusted to a final concentration of OD_{600} 0.1 before being used for infiltration into 4- to 6-wk-old tobacco plants.

For transient expression in *Arabidopsis* protoplasts, the protoplasts were prepared and transformed following a previously described method (Yoo *et al.*, 2007). Ten micrograms of constructs were used for each transformation.

Photographs were taken 2 d post infiltration or 16 h post transformation under Leica SP5 (Wetzlar, Germany) confocal microscopy. The excitation laser wavelength is 488 nm for GFP and 543 nm for mCherry, emission collection is 500–540 nm for GFP and 600–680 nm for mCherry.

Gene expression analysis

Leaves from 4-wk-old *Arabidopsis* seedlings were collected with or without inoculation and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Northern blot hybridization to detect small interfering RNAs was performed as described previously (Zhang *et al.*, 2011), with 10 μg of total RNA being loaded for each sample and the 600-bp fragment of *RTP1* used as the RNA silencing target labeled by ^{32}P-dCTP for use as the probe. For the RT-PCR analysis, 1 μg of total RNA was used to synthesize the first strand cDNA using M-MLV Reverse Transcriptase (TaKaRa, Dalian, China). Quantitative analysis of gene expression was carried out using SYBR Premix Ex Taq™ II (TaKaRa) on iCycler IQ5 (BioRad). Primer pairs used are listed in Table S1. Mean values and standard deviations were obtained from three biological replicates.

Statistical analyses

Statistical analysis of data was performed based on Student's *t*-test between samples from two genotypes and based on a one-way ANOVA for samples from multiple genotypes.

Results

Identification of *rtp1*

We screened a collection of 12 000 independent T-DNA insertion mutants of *Arabidopsis* for resistance to the broad host range oomycete pathogen *Phytophthora parasitica*. One of the identified mutants, 574-34, showed stable resistance and was chosen for further study (Fig. S1a). The T-DNA insertion sites of 574-34 were determined by TAIL-PCR and subsequent sequencing. The results indicated that 574-34 carries two T-DNA insertion sites, in the promoter regions of two separate genes, one encoding a hypothetical protein and the other a Nodulin MtN21 family protein. We named the nodulin-related gene as *RTP1* (*Resistance to Phytophthora parasitica 1*) and examined an additional independent T-DNA insertion line (*rtp1-1*) obtained from the Arabidopsis Biological Resource Centre (ABRC) (Fig. S1b). Like that in 574-34, the T-DNA insertion site in *rtp1-1* is located in the promoter region (Fig. S1b). Typically, *P. parasitica* infection causes water-soaked lesions on *Arabidopsis* Col-0 (Wang *et al.*, 2011b). By contrast, upon infection with *P. parasitica*, *rtp1-1* water-soaked lesion expansion was absent (Fig. 1a). Furthermore, almost no GFP-expressing hyphae colonized *rtp1-1* leaves, following infection with stable GFP-expressing *P. parasitica* transformant. Conversely, wild-type (WT) plants showed heavy colonization (Fig. 1b). Real-time RT-PCR analysis showed that *RTP1* transcript levels were reduced by 70% in *rtp1-1* in comparison to WT Col-0 (Fig. 1c), indicating that the T-DNA insertion within the promoter region had disrupted *RTP1* transcription. Moreover, pathogen colonization was greatly reduced and delayed in *rtp1-1* plants (Fig. 1d). These results implied that *rtp1-1*, like 574-34, was resistant to *P. parasitica*, suggesting that *RTP1* plays a key function in the development of the disease phenotype of 574-34. Based on these results, we suspected that *RTP1* might play important role(s) as a negative regulator of *P. parasitica* infection in *Arabidopsis*.

rtp1-1 exhibits root resistance to *Phytophthora parasitica* infection

Phytophthora parasitica is a typical soil-borne pathogen and normally affects the roots and basal stems of the host plants (Csinos,

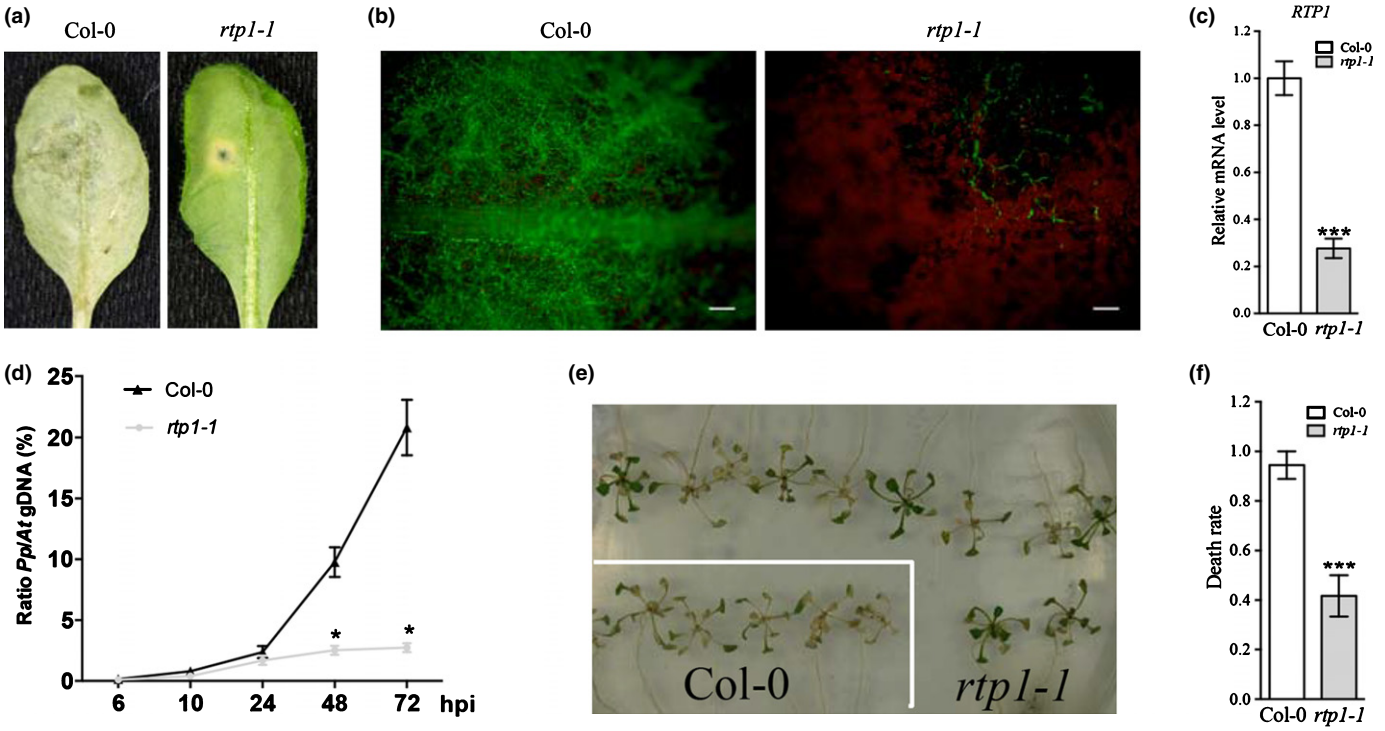

Fig. 1 The *Arabidopsis rpt1* mutants are resistant to *Phytophthora parasitica*. (a) Detached leaves of *rtp1-1* showed resistance to infection by *P. parasitica* zoospores, 3 d post-inoculation (dpi). (b) Resistant phenotype of *rtp1-1* against GFP expressing *P. parasitica* infection, 2 dpi. Bars, 200 μm. (c) *RTP1* expression by real-time RT-PCR analysis. *UBC9* was used as the internal control. Error bars indicate ± SE of three biological replicates. (d) Pathogen biomass analysis by real-time RT-PCR. Error bars indicate ± SE of three biological replicates each containing five leaves. hpi, hours post-inoculation. (e) Symptoms of *rtp1-1* against root infection by *P. parasitica* zoospores, 10 dpi. (f) The ratios of death plants in (e) were analysed. Error bars indicate ± SE of three biological replicates. *, $P < 0.05$; ***, $P < 0.001$.

1999; Attard *et al.*, 2010). To examine whether *RTP1* is also involved in root susceptibility, 2-wk-old seedlings were dip-inoculated with *P. parasitica* zoospores and incubated at 20°C for 10 d (Fig. 1e). Over 94% of WT Col-0 seedlings were heavily infected and colonized with *P. parasitica* at 10 dpi, whereas more than half of *rtp1-1* plants remained healthy at the same time point (Fig. 1f). Thus, suggesting that *RTP1* might play an important role in root defence against *P. parasitica*.

Expression profile of *RTP1*

In order to further determine the expression pattern of *RTP1*, a 2.7-kb *RTP1* promoter fragment was cloned and fused to the β-glucuronidase reporter gene. The resulting construct (p*RTP1::GUS*) was transformed into *Arabidopsis*. Two transgenic lines with single transgene insertion were obtained and homozygous plants were used to examine GUS activities in different organs and development stages. The results showed that p*RTP1::GUS* was highly expressed in roots and hypocotyls and moderately in cotyledon and true leaves of seedlings (Fig. 2a–f). p*RTP1::GUS* was also highly expressed in individual rosette leaves of 4-wk-old plants (Fig. 2g). Moreover, p*RTP1::GUS* expression was also detectable in flowers (Fig. 2h), especially in the stigma and anthers (Fig. 2i).

RTP1-GFP is localized in the ER

Protein subcellular localization typically reflects biological function and most members of the MtN21 family were predicted to be localized in the membrane system (Denance *et al.*, 2014). In order to confirm localization, *RTP1* was fused to the N-terminus of green fluorescent protein (GFP) driven by the 35S promoter. The *RTP1-GFP* construct was co-transformed into tobacco leaves together with an ER organelle marker (Nelson *et al.*, 2007). Similar to the mCherry-labelled ER marker, the RTP1-GFP signal was observed in the ER network around the nucleus, whereas free GFP, as a control, was detected throughout the cell including the nucleus (Fig. 3a). Western blot analysis confirmed the absence of RTP1-GFP cleavage (Fig. 3b). A RTP1-GFP localization distinct from free GFP, which is distributed throughout the cell, was also observed in *Arabidopsis* protoplasts (Fig. 3c). Collectively, these results suggest that *RTP1* encodes an ER-localized protein.

RTP1 negatively regulates defence responses against *Phytophthora parasitica* challenge

In order to further explore the loss of *RTP1* function, we performed genetic complementation experiments in the *rtp1-1* background and *RTP1* overexpression and RNA silencing

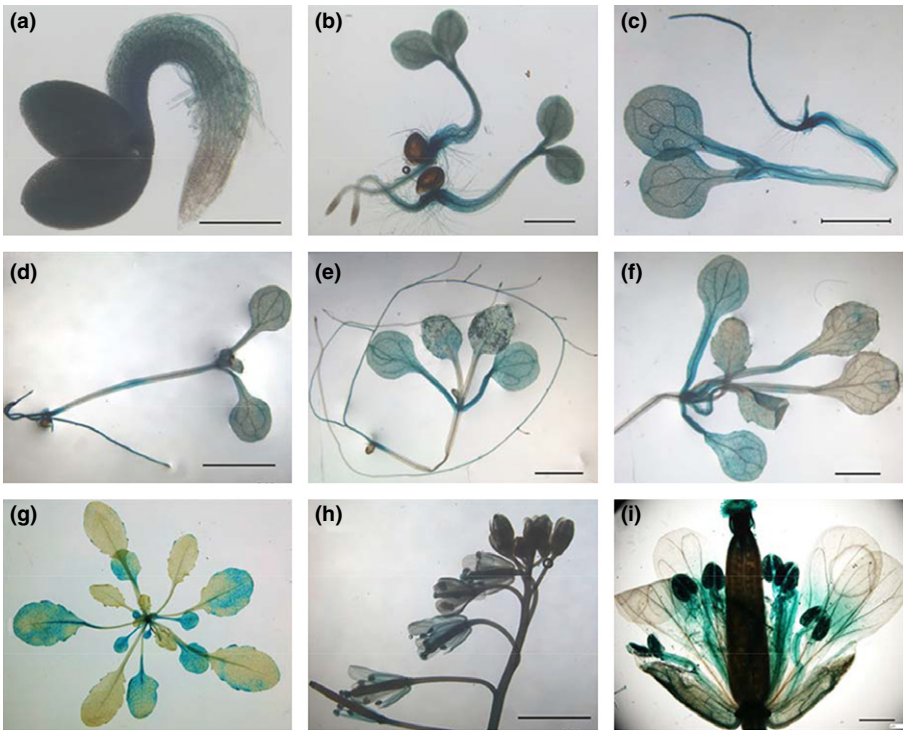

Fig. 2 Expression pattern of *RTP1*. p*RTP1::GUS* plants were stained at different growth stage. (a) Mature embryo imbibed for 24 h. Bar, 200 μm. (b) 2-d-old seedlings. Bar, 500 μm. (c) 5-d-old seedling. Bar, 1 mm. (d) 7-d-old seedling. Bar, 2 mm. (e) 10-d-old seedling. Bar, 2 mm. (f) 2-wk-old plant. (g) 4-wk-old plant. (h) Inflorescence. Bar, 2 mm. (i) Flower. Bar, 500 μm.

experiments in the WT Col-0 background. These transgenic plants showed no obvious changes in growth or morphology compared to WT Col-0 plants (Fig. 4a). The expected *RTP1* expression levels in the complementation (CM-c, CM-e), overexpression (OE-c, OE-e) and knockdown (KD-1, KD-10) lines were confirmed by real-time RT-PCR (Fig. 4b). Furthermore, expression of the RTP1 protein in the complementation and overexpression plants was confirmed by Western blot using anti-FLAG polyclonal antibodies (Fig. 4c). Successful silencing of *RTP1* in knockdown lines was correlated with the accumulation of homologous siRNAs detected by northern blot hybridization (Fig. 4d). Subsequently, confirmed homozygous transgenic plants were used to analyse the contribution of *RTP1* to *P. parasitica* infection.

Pathogenicity assays showed that *P. parasitica* colonized more rapidly in both the complemented and the overexpression lines relative to WT Col-0 (Fig. 4e). This implies that overexpression of *RTP1* increased disease susceptibility to *P. parasitica* and overexpression of *RTP1* in *rtp1-1* reversed the resistant phenotype of *rtp1-1* to attempted *P. parasitica* infection. Consistently, the *RTP1* knockdown lines exhibited restricted pathogen colonization (Fig. 4e), indicative of increased resistance to *P. parasitica* infection. These results support the notion that *RTP1* might function as a negative regulator in *Arabidopsis* resistance against *P. parasitica* infection.

rtp1-1 plants exhibit restricted cell death in response to *Phytophthora parasitica*

In order to investigate possible mechanisms underlying the resistance of *rtp1-1* plants to *P. parasitica* infection, we performed cytological characterization of the infection process. The inoculated leaves were stained with trypan blue and examined by microscopy. The staining pattern for the Col-0 plants was similar to that described previously (Wang *et al.*, 2011b). Briefly, germinated cysts with appressoria were notable on the leaf surface of Col-0 at 3 h post-inoculation (hpi); intercellular hyphae formed within 6 hpi and invasive hypha extended at 10 hpi (Fig. 5a). For the *rtp1-1* plants, no obvious differences were observed compared to Col-0 at 3 hpi, but at 6 hpi frequent cell death occurred at the penetration sites and the germinated cysts were visible beyond the dead cell (Fig. 5a). No obvious hyphal extension was visible at 10 hpi (Fig. 5a) or even at 48 hpi (Fig. 1b). These results suggest that *rtp1-1* plants exhibit restricted cell death at *P. parasitica* infection sites, which may lead to constrained pathogen colonization.

Accelerated ROS accumulation in *rtp1-1* plants in response to *P. parasitica*

Plant cells undergoing cell death usually accumulate ROS, notably superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Grant & Loake, 2000; Apel & Hirt, 2004; Skelly & Loake, 2013). Generation of O_2^- and H_2O_2 in inoculated seedlings was analysed by Nitroblue tetrazolium (NBT) staining and 3,3′-diaminobenzidine- tetrahydrochloride (DAB) staining, respectively. No obvious difference in NBT staining was observed among Col-0, *rtp1-1* and the complemented lines at 0 hpi (Fig. 5b) and all the mock treated time points (Fig. S2a). However, NBT staining was stronger in *rtp1-1* plants than in Col-0 and the complemented lines at 0.5 hpi and 1.0 hpi, although the staining was finally cleared in all plants at 3 hpi (Fig. 5b). These

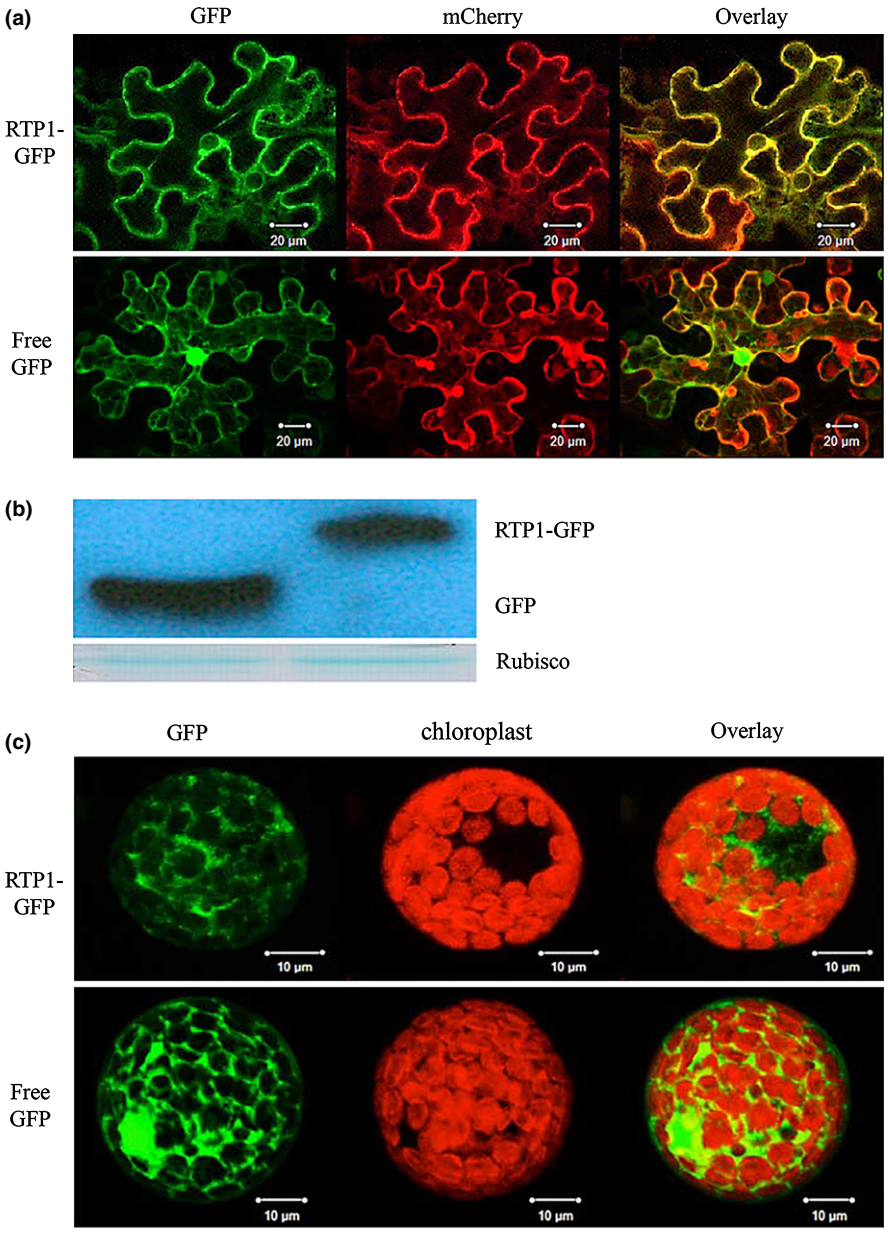

Fig. 3 Subcellular localization of RTP1-GFP. The *RTP1-GFP* or free *GFP* construct was transiently expressed in tobacco leaves or *Arabidopsis* protoplasts and the fluorescence was observed under a confocal microscopy. (a) RTP1:GFP fusion protein localizes to the endoplasmic reticulum (ER) in tobacco epidermal cells. Free GFP served as control. ER marker is in red and GFP fluorescence is in green. (b) Western blot analysis to detect the protein intact showed in (a), Coomassie brilliant blue stained rubisco served as loading control. (c) Localization of RTP1-GFP and free GFP in *Arabidopsis* protoplasts. The free GFP signal was observed throughout the cytoplasm, whereas RTP1-GFP signal was not, even in the nucleus. GFP fluorescence is in green and auto-fluorescence from chloroplasts is in red.

results imply that O_2^- accumulated more rapidly in *rtp1-1* plants relative to Col-0 and the complemented lines. Similarly, no obvious differences in DAB staining were visible among these lines at 0 hpi (Fig. 5c) including all the mock treated time points (Fig. S2b). By contrast, at 0.5 hpi and 1 hpi stronger DAB staining was observed in the *rtp1-1* plants (Fig. 5c). These results suggest that the ROS burst appears to be more rapidly engaged in *rtp1-1* plants than in Col-0.

PR1 transcripts accumulate faster in *rtp1-1* plants

PR1 and *PDF1.2* are key marker genes for salicylic acid (SA) and jasmonic acid (JA), respectively (Uknes *et al.*, 1993; Yun *et al.*, 2003). Previous research has shown that *Phytophthora* infection induces the expression of *PR1* and *PDF1.2* (Attard *et al.*, 2010; Wang *et al.*, 2013). Strong induction of *PR1* was observed following *P. parasitica* infection in both Col-0 and *rtp1-1* plants. However, *PR1* was activated more rapidly and strongly in *rtp1-1* plants (Fig. 6). Interestingly, *PR1* transcript accumulation peaked in Col-0 at 24 hpi following full *P. parasitica* colonization. Conversely, a similar *PR1* transcript level was reached at 6 hpi in *rtp1-1* when cell death occurred (Fig. 5b). In contrast to *PR1*, *PDF1.2* was downregulated to similar levels in both Col-0 and *rtp1-1* plants (Fig. 6). These data suggest that resistance of *rtp1-1* plants to *P. parasitica* infection might be a result of accelerated activation of SA responsive genes such as *PR1*.

rtp1-1 plants display enhanced basal defence against *Pst* DC3000

In order to explore whether *RTP1* is involved in plant immunity against other pathogens, *Pst* DC3000 was employed to uncover a

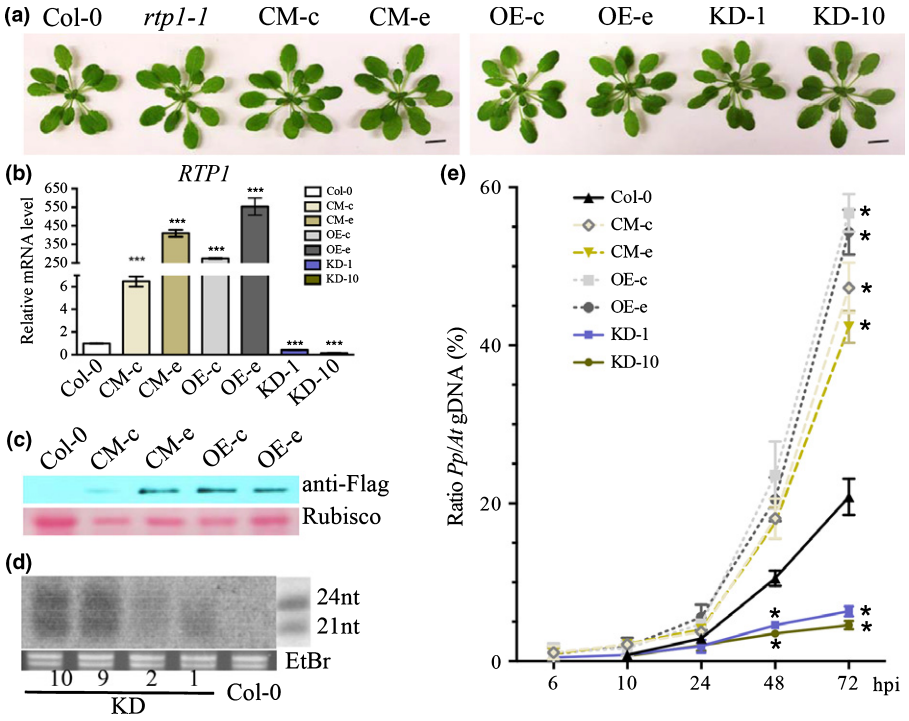

Fig. 4 Infection of *Arabidopsis* transformants altered with *RTP1* expression levels. (a) Phenotype of 4-wk-old *RTP1* transgenic plants. (b) *RTP1* expression level in Col-0, complementation (CM-c, CM-e), overexpression (OE-c, OE-e) and knockdown (KD-1, KD-10) lines. Error bars indicate ± SE of three biological replicates. (c) Western blot analysis of FLAG-RTP1 in complementation and overexpression lines. (d) Detection of siRNAs by Northern blotting in *RTP1* RNA silencing plants. (e) Quantitative analyses colonization of above transgenic lines by the pathogen. Error bars indicate ± SE of three biological replicates each containing five leaves. hpi, hours post-inoculation; CM, complemention lines; OE, overexpression lines; KD, knockdown lines. *, $P < 0.05$. ***, $P < 0.001$.

possible role for *RTP1* in the basal defence system. *rtp1-1*, transgenic complementation and WT Col-0 plants were inoculated with the bacterial pathogen *Pst* DC3000. At a concentration inoculum of 10^5 CFU ml^{-1}, a reduced titre of bacteria was observed in *rtp1-1* relative to Col-0 or the complemention line at 3 dpi (Fig. 7a), suggesting that the *rtp1-1* mutation enhanced basal defence. Moreover, infiltration with a higher inoculum, 10^7 CFU ml^{-1}, induced dramatic desiccation necrosis in *rtp1-1* plants at 1 dpi but not in the WT Col-0 line (Fig. 7b). Furthermore, trypan blue staining also implied increased cell death in *rtp1-1* leaves (Fig. 7b). To investigate if *RTP1* expression is modulated in response to pathogens, RT-PCR was also employed to monitor *RTP1* expression. This analysis showed that *RTP1* expression was induced by *Pst* DC3000, especially at the early stage of infection but then decreased to a basal level at 6 hpi (Fig. 7c).

We performed NBT staining to assess ROS accumulation in the early stage of *Pst* DC3000 infection. Similar to the response to *P. parasitica*, no difference was evident among the different plant lines at 0 hpi. However, more O_2^- accumulated in *rtp1-1* plants by 0.5 hpi, followed by a reduction to basal levels by 3 hpi (Fig. S2c). Taken together, these results suggest that *RTP1* might delay pathogen-triggered cell death development and ROS production following the engagement of basal defence.

rtp1-1 plants show enhanced *R* gene-dependent defence against *Pst* DC3000

In order to examine whether *RTP1* functions in *R* gene-dependent defence, the bacterial pathogen *Pst* DC3000 carrying the avirulence gene *AvrRpm1* was used to infect Col-0, *rtp1-1* and the associated transgenic plants. When infiltrated at 10^5 CFU ml^{-1}, a significant reduction in bacterial growth was observed in *rtp1-1* in comparison to Col-0 (Fig. 7d,e). Infiltration with 10^7 CFU ml^{-1} triggered leaf necrosis in *rtp1-1* plants within 1 dpi, whereas at this time no visible necrosis was observed in Col-0 and the *RTP1* complementation lines (Fig. 7f). Notably, *RTP1* was downregulated by *Pst* DC3000 infection in Col-0, with only 16.5% of transcripts remaining at 3 hpi, whereas at 12 hpi *RTP1* transcript levels were undetectable (Fig. 7g). In addition, NBT staining showed that ROS production in response to this avirulent bacterial pathogen followed a similar pattern to that in response to a virulent *Pst* strain (Fig. S2c,d).

To determine if the HR phenotype of *rtp1-1* was specific to AvrRpm1, we utilised a second *Pst* DC3000 strain carrying a different avirulence gene, *AvrRps4*. Again, more rapid HR cell death was also observed for this avirulent strain (Fig. S3a,b). Taken together, these data suggest that *RTP1* also plays an important role in *R* gene-dependent responses by negatively regulating the kinetics of cell death development.

rtp1-1 plants are resistant to the biotrophic fungal pathogen *Golovinomyces cichoracearum*

Phytophthora parasitica is a hemibiotrophic oomycete pathogen and *Pst* a biotrophic bacterial pathogen. To investigate whether *RTP1* functions in plant defence against a biotrophic fungal pathogen, we inoculated Col-0, *rtp1-1* and *RTP1*-complemented plants with conidia of the powdery mildew pathogen, *G. cichoracearum*. Trypan blue staining was employed to monitor the development of hyphae and conidiophores (asexual reproductive structures) at 6 dpi. Both the WT Col-0 and the

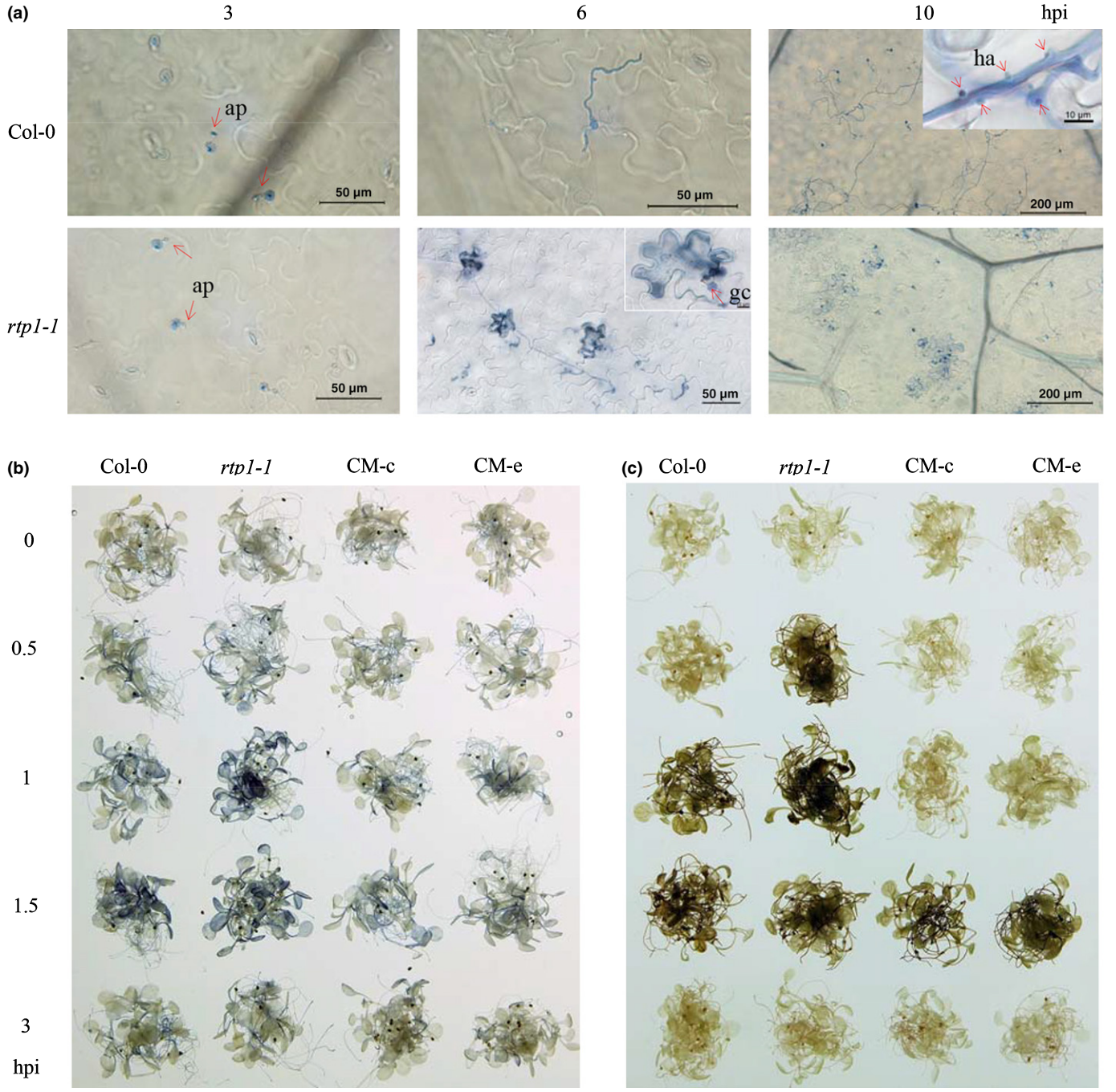

Fig. 5 Cytological analysis of Col-0 and *rtp1-1* plants infected with *Phytophthora parasitica*. (a) Infected leaves of Col-0 and *rtp1-1* were stained with trypan blue to indicate pathogen colonization and cell death. (b) Detection of superoxide by NBT staining. (c) Detection of hydrogen peroxide (H_2O_2) by DAB staining. Whole seedlings of 10-d-old plants were dip-inoculated with *P. parasitica* zoospores without wounding, then stained in the DAB solution for 8 h or NBT solution for 3 h in dark at indicated time points; *c*. 10 plants were used for each line and experiments were repeated three times. hpi, hours post-inoculation; ap, appressoria; ha, haustoria-like structure; gc, germinated cyst.

complemented lines were susceptible, with abundant fungal hyphae and conidiophores produced on the leaves. By contrast, reduced hyphae and conidiophore development was observed in *rtp1-1* leaves (Fig. 8a,b). Consistent with the results of trypan blue staining, the WT and complemented plants supported significantly more fungal growth than the *rtp1-1* line at 10 dpi (Fig. 8c).

RTP1 does not affect infection by the necrotrophic pathogen *Botrytis cinerea*

In order to examine whether *RTP1* functions in plant defence against necrotrophic pathogens, we inoculated plant leaves with *B. cinerea* mycelia plugs and measured the diameter of lesions at 36 hpi (Methods S1). The results showed that both Col-0 and

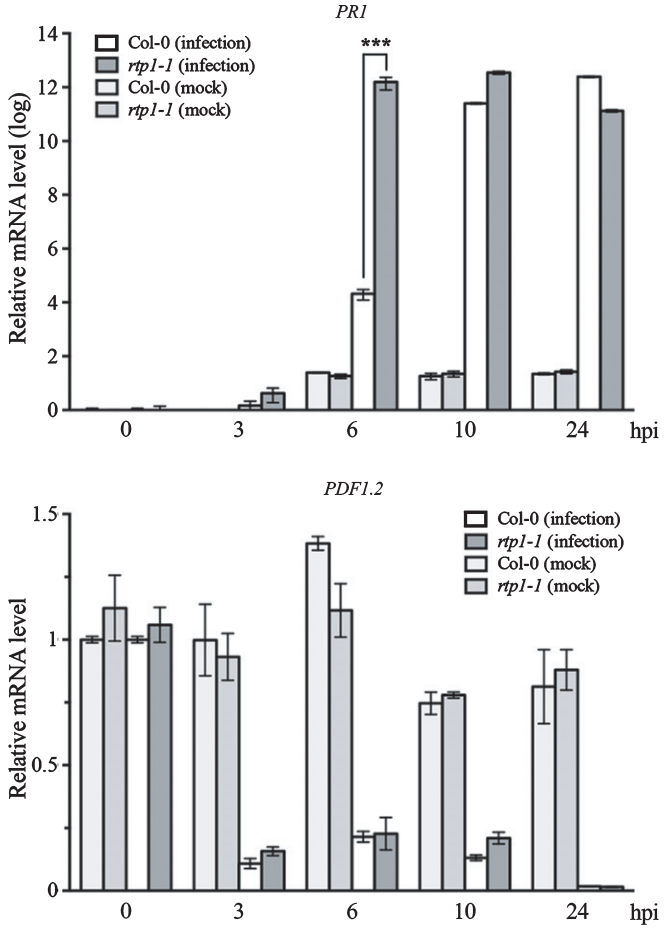

Fig. 6 Real-time RT-PCR analysis of expression of *PR1* and *PDF1.2* in response to *Phytophthora parasitica* infection. *UBC9* was used as the internal control. The gene expression level in Col-0 was arbitrarily set as 1. Error bars indicate ± SE of three biological replicates. hpi, hours post-inoculation. ***, $P < 0.001$.

rtp1-1 plants were fully susceptible, with no obvious phenotypic difference between them (Fig. S4a,b), suggesting that *RTP1* may not be required for plant responses to necrotrophic fungal pathogens.

Discussion

We have generated evidence suggesting that the *Arabidopsis RTP1* gene functions as a negative regulator of disease resistance to multiple pathogens, including *Phytophthora parasitica*, *Pst* DC3000 (virulence and avirulence) and *Golovinomyces cichoracearum*.

RTP1 belongs to the *MtN21* family and encodes an ER localized protein

Evolutionally, *RTP1* is similar to the nodulin related genes of the *MtN21* family in *Medicago truncatula*. This family comprises *c.* 40 members in *Arabidopsis*, but only two of them have been described, namely *SIAR1* (*Siliques Are Red 1, UMAMIT18*) and *WAT1* (*Walls Are Thin 1*, *UMAMIT5*). *SIAR1* is involved in plant development and abiotic resistance (Ladwig *et al.*, 2012), whereas *WAT1*/*UMAMIT5* has been implicated in both plant immunity and development (Ranocha *et al.*, 2010; Denance *et al.*, 2013). In particular, *SIAR1* has an important function in amino acid homeostasis as a bidirectional amino acid transporter, especially in developing *Arabidopsis* siliques (Ladwig *et al.*, 2012). *WAT1* encodes a tonoplast-localized vacuolar auxin transporter and is essential for secondary cell wall formation in fibres (Ranocha *et al.*, 2010, 2013). Furthermore, *wat1* mutants showed enhanced resistance to various pathogens and changing the balance between salicylic acid (SA) and auxin abolished this resistance (Denance *et al.*, 2013), indicating that *WAT1* plays important roles in plant defence. Here, we characterized *RTP1*, a new *MtN21* family member which might function as a negative regulator in the plant immune response to *Phytophthora*. Importantly, these findings may help our understanding of how plant factors might function in the plant–*Phytophthora* compatible interaction.

Most members of the MtN21 family are predicted to act as transporters and are localized to the membrane system (Denance *et al.*, 2014). Bioinformatic predictions showed the presence of eight to ten transmembrane domains in RTP1. Consistent with this, we showed that RTP1 is localized in the endoplasmic reticulum (ER) membrane system. The ER is responsible for quality control (ERQC) of transmembrane proteins, such as their modification, folding and secretion (Inada & Ueda, 2014). Furthermore, several ER-localized proteins have been identified to function in plant defence. For example, silencing of *Nicotiana benthamiana* Calreticulin 3a, an ERQC chaperone, lead to compromised reactive oxygen species (ROS) production and reduced resistance to *P. infestans* infection (Matsukawa *et al.*, 2013).

rtp1-1 plants display broad spectrum resistance to biotrophic but not necrotrophic pathogens

Our data suggest that *rtp1-1* is resistant not only to *P. parasitica*, but also to *Pst* DC3000 and *G. cichoracearum*, all of which are biotrophic pathogens. However, *rtp1-1* plants were not found to be resistant to the necrotrophic pathogen *B. cinerea*.

Plants have developed different defence systems against infections by biotrophic and necrotrophic pathogens. Generally, SA signalling is essential to resist infection from biotrophic pathogens, whereas jasmonic acid/ethylene (JA/ET) signalling is necessary for inhibiting infections from necrotrophic pathogens (Dodds & Rathjen, 2010). Interestingly, several reports have shown that both SA and JA/ET signalling are required to resist infection by *Phytophthora* pathogens in *Arabidopsis* and *N. benthamiana* (Attard *et al.*, 2010; Shibata *et al.*, 2010; Wang *et al.*, 2013). Analysis of the expression of *PR1* and *PDF1.2*, marker genes for SA and JA/ET, respectively, showed that *PR1* was upregulated quickly but *PDF1.2* was downregulated in Col-0 in response to *P. parasitica*. This result is consistent with the published data on root responses to *P. parasitica*. However, *PR1* accumulated more rapidly in *rtp1-1* than in Col-0 in response to infection, whereas no difference was found for the *PDF1.2*

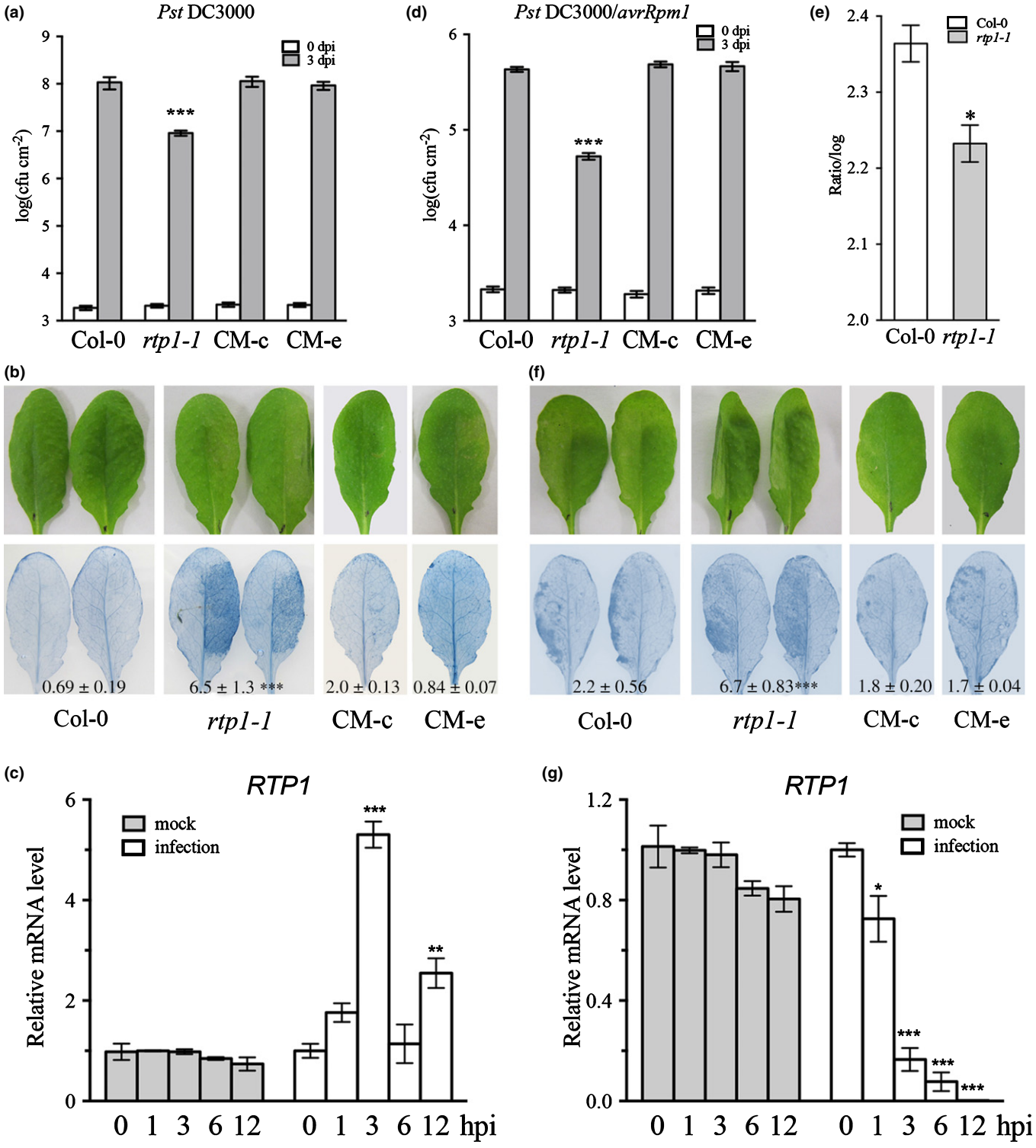

Fig. 7 *rpt1-1* is resistant to bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Quantification of bacterial growth in plants following infiltration of virulent *Pst* DC3000 (a) or avirulent *Pst* DC3000 carrying *avrRpm1* (d) (1×10^5 CFU ml^{-1}). Error bars indicate ± SE of seven biological replicates. The difference of the reduced titre of avirulent and virulent bacteria was compared between Col-0 and *rtp1-1* (e). Symptoms (upper row) and trypan blue staining (lower row) of leaves infiltrated with high density *Pst* DC3000 (b) or *Pst* DC3000 carrying *avrRpm1* (f) at 1 d post-inoculation (1×10^7 CFU ml^{-1}). The cell death development was measured by relative intensity of trypan blue staining using ImageJ (v1.50a) and the numbers represent mean ± SD. ***, $P < 0.001$. Real-time RT-PCR analysis of *RTP1* expression in response to infection by *Pst* DC3000 (c) or *Pst* DC3000 carrying *avrRpm1* (g). *UBC9* was used as the internal control. Error bars indicate ± SE of three biological replicates each containing five leaves. hpi, hours post-inoculation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

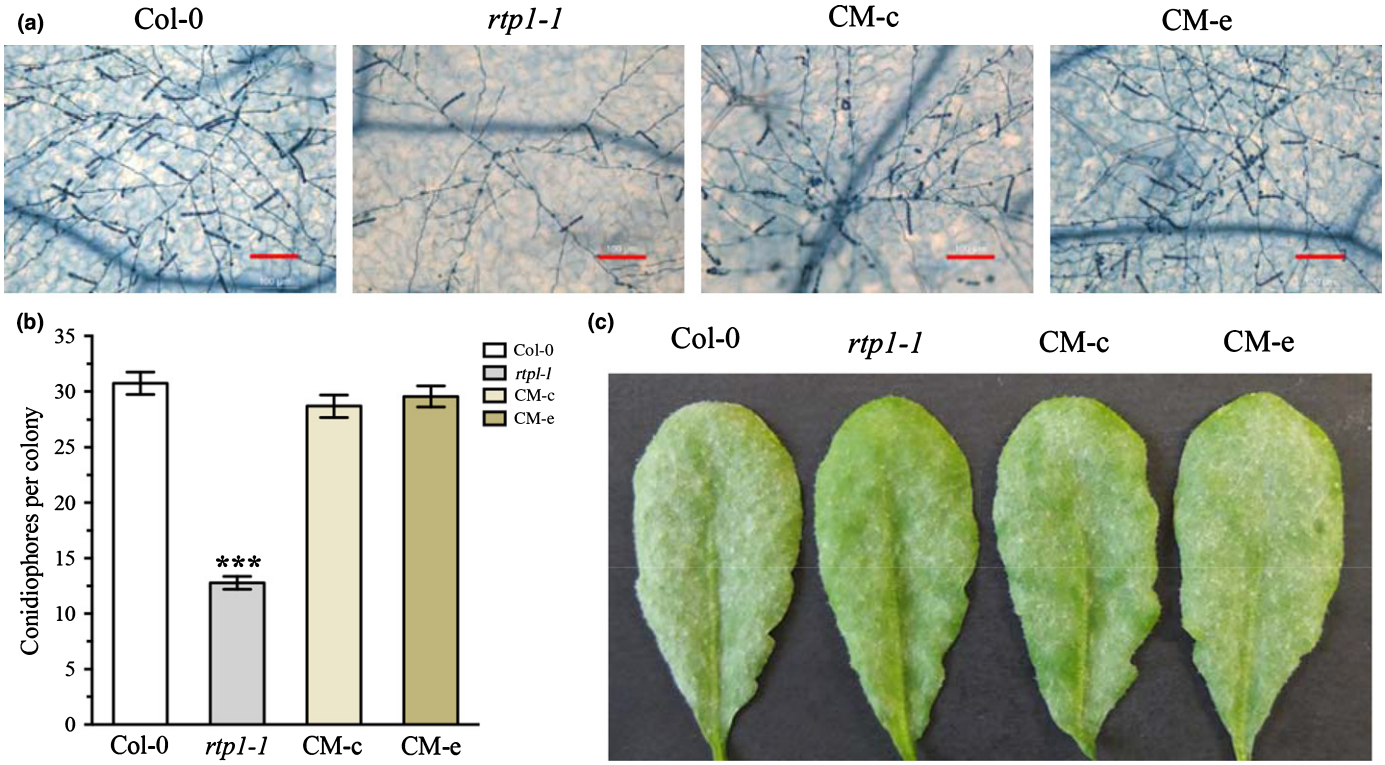

Fig. 8 *rtp1-1* is resistant to the powdery mildew fungal pathogen *Golovinomyces cichoracearum*. (a) Trypan blue-staining of infected leaves at 6 d post-inoculation (dpi). Bars, 100 μm. (b) Quantification of fungal growth in plants as determined by the number of conidiophores per colony at 6 dpi. Error bars represent ± SE. (c) Infected leaves at 10 dpi. ***, $P < 0.001$.

expression, suggesting that *RTP1* may affect SA signalling but not the JA signalling network. Consistent with this notion, *rtp1-1*, Col-0 and transgenic *rtp1-1* complemented lines showed no difference in response to infection by the necrotrophic fungal pathogen *B. cinerea*, suggesting that *RTP1* is not involved in plant defence against necrotrophic pathogens.

RTP1 negatively regulate ROS and cell death in plant immunity

Most *Phytophthora* species, including *P. parasitica*, are hemibiotrophic pathogens, thus these pathogens have a distinct developmental phase, usually during early infection, in which nutrients are acquired from living host cells.

In order to limit infection by biotrophic pathogens, plants have evolved cell death, a process that can deprive pathogens access to a nutrient supply (Coll *et al.*, 2011). As one of the earliest reactions during plant disease responses, rapid production of ROS is important for resistance to the biotrophic pathogens because it helps drive cell death development (Coll *et al.*, 2011). The emerging data suggest that the levels and timing of ROS production are important determinants of cell death in incompatible *P. parasitica*–tobacco interactions and correlate with compatible *P. parasitica* proliferation in susceptible plants (Wi *et al.*, 2012). NADPH oxidases have been uncovered as essential enzymes for ROS production in plant defence (Torres *et al.*, 2002; Yoshioka *et al.*, 2003). NADPH oxidase knock-down or knock-out plants showed increased susceptibility to oomycetes (Shibata *et al.*, 2010). Recently, it has been shown that resistance in the root of *Arabidopsis* against *P. parasitica* requires an NADPH oxidase-mediated oxidative burst (Larroque *et al.*, 2013). These findings indicate that ROS is important for plant resistance to *Phytophthora* pathogens. Our data suggest that *rtp1-1* plants exhibit both root and leaf resistance to *P. parasitica* and correspondingly, O_2^- and H_2O_2 accumulated more rapidly in *rtp1-1* plants than in a wild-type (WT) Col-0 line during the early infection phase. In addition, cell death occurred at the infection sites in *rtp1-1* plants following *P. parasitica* infection but not in WT Col-0 plants, where the pathogen could complete its infection cycle. Taken together, these results suggest that both the timing of the oxidative burst and cell death development are important for resistance of *Arabidopsis* against *P. parasitica* and further that *RTP1* plays a key role in these processes.

It has been hypothesized that NADPH oxidase function could lead to SA accumulation and in this context, the expression of PR proteins have been shown to be partly dependent on the ROS accumulation in response to *P. parasitica* in tobacco (Wi *et al.*, 2012). Furthermore, *PR1* has been shown to play an important role in plant defence against *P. capsici* (Wang *et al.*, 2013). In addition, *PR1* also participates in root resistance of *Arabidopsis* against *P. parasitica* (Attard *et al.*, 2010). Our findings imply that *PR1* expression was rapidly induced during the early infection period in *rtp1-1* plants which suggests that this gene might be involved in leaf resistance against *P. parasitica* infection.

Compared to Col-0, *rtp1-1* plants showed increased ROS accumulation, accelerated cell death and potentiated *PR1* gene expression in response to *P. parasitica* infection, suggesting that *RTP1* might function as a negative regulator of cell death and *PR1* gene expression at the early stage of attempted infection.

Further, *rtp1-1* plants showed more rapid accumulation of ROS and increased cell death in response to infection by *Pst* DC3000. This further suggests that *RTP1* negatively regulates ROS and cell death, and may play a role in plant resistance to biotrophic pathogens. Moreover, *RTP1* was downregulated post *Pst* DC3000/*AvrRpm1* inoculation, which is consistent with previous reports (Bricchi *et al.*, 2012), but upregulated by virulent *Pst* DC3000. It is therefore possible that *RTP1* might act as a negative regulator of plant immunity or be targeted by pathogen effectors to interfere with the defence response.

In conclusion, our study identified a novel gene, *RTP1*, which might negatively regulate resistance to a broad range of biotrophic pathogens but not necrotrophs. The increased resistance of *rtp1-1* plants to *P. parasitica* and *Pst* DC3000 is associated with rapid production of ROS and cell death at the infection sites. Furthermore, *PR1* gene expression is induced more strongly and rapidly in *rtp1-1* plants than in Col-0. Collectively, these results suggested that *RTP1* plays a role in plant defence against biotrophic pathogens possibly by regulating ROS production, cell death and *PR1* expression.

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Author contributions

W.S. and Q.P. designed the research. Q.P., B.C., F.D. and J.Q. performed the experiments. Q.P., B.C., G.J.L. and W.S. analysed the data. Q.P., B.C. and W.S. wrote the manuscript with contributions from all authors.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Identification of 574-34 resistant to *P. parasitica* infection.

Fig. S2 Promoted ROS accumulation in the *rtp1-1* mutant after inoculation with *Pst* DC3000.

Fig. S3 *rtp1-1* displays more rapid cell death in response to *Pst* DC3000 carrying *avrRps4*.

Fig. S4 Infection of mutant *rtp1-1* by the necrotrophic fungal pathogen *Botrytis cinerea*.

Table S1 List of primers used in this study for genotyping, vector construction and real-time RT-PCR

Methods S1 *Botrytis cinerea* infection assay.

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