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The chloroplast protein RPH1 plays a role in the immune response of Arabidopsis to *Phytophthora brassicae*

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Summary

Plant immune responses to pathogens are often associated with enhanced production of reactive oxygen species (ROS), known as the oxidative burst, and with rapid hypersensitive host cell death (the hypersensitive response, HR) at sites of attempted infection. It is generally accepted that the oxidative burst acts as a promotive signal for HR, and that HR is highly correlated with efficient disease resistance. We have identified the Arabidopsis mutant *rph1* (*resistance to Phytophthora 1*), which is susceptible to the oomycete pathogen *Phytophthora brassicae* despite rapid induction of HR. The susceptibility of *rph1* was specific for *P. brassicae* and coincided with a reduced oxidative burst, a runaway cell-death response, and failure to properly activate the expression of defence-related genes. From these results, we conclude that, in the immune response to *P. brassicae*, (i) HR is not sufficient to stop the pathogen, (ii) HR initiation can occur in the absence of a major oxidative burst, (iii) the oxidative burst plays a role in limiting the spread of cell death, and (iv) RPH1 is a positive regulator of the *P. brassicae*-induced oxidative burst and enhanced expression of defence-related genes. Surprisingly, *RPH1* encodes an evolutionary highly conserved chloroplast protein, indicating a function of this organelle in activation of a subset of immune reactions in response to *P. brassicae*. The disease resistance-related role of RPH1 was not limited to the Arabidopsis model system. Silencing of the potato homolog *StRPH1* in a resistant potato cultivar caused susceptibility to the late blight pathogen *Phytophthora infestans*.

Keywords: *Phytophthora*, disease resistance, hypersensitive response, oxidative burst, defence gene expression.

Introduction

Plant disease resistance depends on the rapid activation of defence responses. The plant immune system is usually activated by pathogen-derived molecules whose presence is recognized by plasma membrane-localized or cytoplasmic receptor proteins (Chisholm *et al.*, 2006; Jones and Dangl, 2006). The initial recognition events ultimately lead to induction of many defence responses such as cell-wall strengthening, changes in gene expression patterns, and accumulation of anti-microbial compounds (Nimchuk *et al.*, 2003). While the recognition processes have been increasingly well described, we are far from understanding the network of complex downstream signalling events that lead to the activation of multiple defences (Nimchuk *et al.*, 2003; Bent and Mackey, 2007). One of the most prominent features of plant immunity is the rapid death of host cells at the site of infection, a process known as the hypersensitive response (HR). The HR is highly correlated with disease resistance, and may directly help to restrict the spread of pathogens (Van Breusegem and Dat, 2006; Mur *et al.*, 2008). Another hallmark of plant disease resistance is the oxidative burst, which consists of the rapid production of reactive oxygen species (ROS), such as superoxide and its dismutation product H_2O_2, at infection sites (Doke, 1983; Torres *et al.*, 2006). The enhanced level of ROS can directly kill some pathogens, contribute to cell-wall strengthening, and act as a signal for further defences (Torres *et al.*, 2006). There are many sources of ROS in plants, but it is generally accepted that the activity of plasma membrane-localized NADPH oxidases makes a major contribution to the pathogen-induced oxidative burst (Torres *et al.*, 2002; Yoshioka *et al.*,

2003; Torres and Dangl, 2005). The NADPH oxidases, also known as plant respiratory burst oxidase homologues (Rboh), are homologous to the mammalian gp91^{phox}subunit of the phagocyte oxidase (Torres and Dangl, 2005). The accumulation of ROS frequently coincides with the induction of hypersensitive cell death, and the consensus is that the oxidative burst is important for the initiation of cell death (Torres *et al.*, 2006; Van Breusegem and Dat, 2006).

We study immunity mechanisms of plants to the oomycete genus *Phytophthora* (Greek: plant destroyer), which includes devastating pathogens of many agricultural plants as exemplified by the potato late blight pathogen *Phytophthora infestans* (Fry, 2008). *Phytophthora* species also cause damage to native flora, as seen in the ongoing epidemic of sudden oak death in California. Despite its fungus-like hyphal growth, *Phytophthora* is not related to true fungi, but, together with diatoms and brown algae, belongs to the taxonomic kingdom of Stramenopila. Consequently, the ability of *Phytophthora* to infect plants has evolved independently from fungal pathogens. However, plant defence responses to *Phytophthora* are similar to the responses triggered by fungal pathogens. Resistance to *Phytophthora* has been shown to require recognition by cytoplasmic coiled-coil/nucleotide-binding site/leucine-rich repeat (CC-NBS-LRR) plant resistance proteins (Ballvora *et al.*, 2002), and is associated with an oxidative burst, HR and enhanced expression of defence-related genes (Doke, 1983; Able *et al.*, 2000; Torres and Dangl, 2005).

We have previously established a model pathosystem based on the interaction of *Arabidopsis thaliana* with *Phytophthora brassicae* (Roetschi *et al.*, 2001). Here we describe the identification of the loss-of-resistance mutant *rph1*, which, in response to *P. brassicae*, shows a strongly reduced oxidative burst and a deficiency in the induction of defence-related genes but a phenotypically normal HR. Because *RPH1* encodes a chloroplast protein, our results demonstrate an essential role of the chloroplast in activation of immune responses to *Phytophthora*.

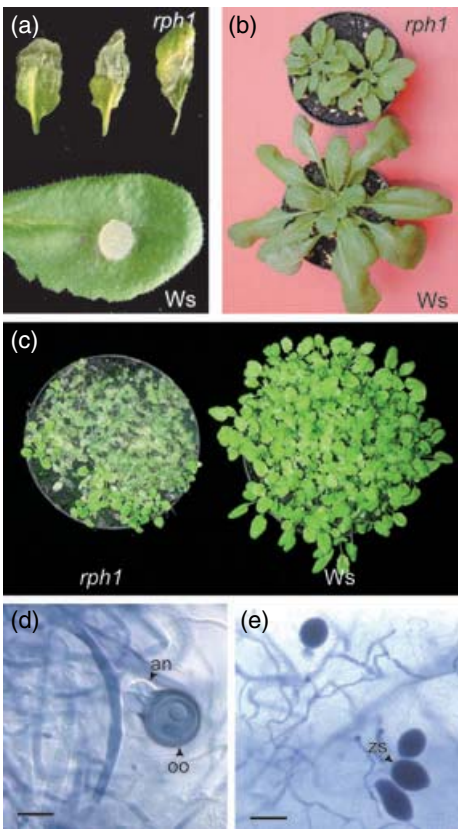

Figure 1. The Arabidopsis mutant *rph1* is susceptible to *Phytophthora brassicae*.
(a) In contrast to the wild-type accession Ws, the *rph1* mutant is highly susceptible to *P. brassicae*. Six-week-old plants were inoculated with agar plugs containing *P. brassicae* isolate HH, and photographed at 5 dpi.
(b) Comparison of the growth phenotype of 6-week-old Ws and *rph1*.
(c) Susceptibility of *rph1* in response to zoospore inoculation. Four-week-old Ws and *rph1* plants were spray-inoculated with a zoospore suspension of *P. brassicae* isolate D, and photographed at 7 dpi.
(d) Fertilization of an oogonium (oo) by an antheridium (an) to form an oospore in *rph1*.
(e) Formation of external zoosporangia (zs) in *rph1*.
Leaf samples were stained with lactophenol–trypan blue at 5 dpi. Scale bar = 20 μm.

Results

Identification and phenotype of the loss-of-resistance mutant rph1

To identify components of disease resistance against *Phytophthora*, a T-DNA-tagged population of the resistant *Arabidopsis thaliana* accession Wassilewskija (Ws) was screened for susceptibility by placing agar plugs containing *P. brassicae* on top of the leaves. The first susceptible mutant identified was named *rph1* (*resistance to Phytophthora 1*). In contrast to Ws, *rph1* leaves were quickly colonized by *P. brassicae*, resulting in large lesions within 3 dpi (days post-inoculation) that eventually spread through the entire leaf (Figure 1a). The *rph1* plants were also susceptible to inoculation with zoospores (Figure 1c), and *P. brassicae* completed its life cycle within 5 dpi by forming sexual oospores and asexual zoosporangia (Figure 1d,e). Tests with various isolates of *P. brassicae* [isolates HH, II, D (CBS179.89) and A (CBS212.82); Roetschi *et al.*, 2001] showed that disease resistance conferred by RPH1 was not isolate-specific, as all four isolates were virulent on *rph1*.

The *rph1* mutant was smaller than Ws but did not show obvious qualitative growth defects (Figure 1b). Microscopic analysis revealed that the size of epidermal cells and mesophyll cells was similar in *rph1* and Ws (data not shown). The content of chlorophyll (w/v) was reduced in *rph1* by about 20% relative to Ws. The pleiotropic phenotype suggested reduced fitness as a possible cause of suscepti-

bility of *rph1* to *P. brassicae*. In this case, *rph1* is expected to become susceptible to other pathogens. However, *rph1* showed wild-type disease resistance in response to (i) virulent and avirulent isolates of another oomycete pathogen, *Hyaloperonospora arabidopsis*, (ii) virulent and avirulent isolates of the bacterial pathogen *Pseudomonas syringae*, and (iii) the fungal pathogen *Botrytis cinerea* (Figure 2). The enhanced biomass of *B. cinerea* detected in *rph1* at 1 dpi might indicate a more rapid initial colonization; however, this did not result in increased biomass of *B. cinerea* or differences in lesion size at 3 dpi. *rph1* was also resistant to the non-host pathogen *Phytophthora infestans* (data not shown). The specific limitation of enhanced disease susceptibility of *rph1* to *P. brassicae* argued against reduced fitness as the primary cause of susceptibility.

In support of this conclusion, *rph1* showed specific deficiencies in the expression of established markers of immune and stress hormone signalling. In comparison to wild-type, *rph1* showed an attenuated induction of *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*) and *PAD4* (*PHYTOALEXIN DEFICIENT 4*), in response to *P. brassicae*. *EDS1* and *PAD4* encode important elements of disease resistance signalling (Figure 3a). Similarly, marker genes of stress hormone signalling such as *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*), *PDF1.2* encoding an anti-microbial defensin, *AVIRULENCE-INDUCED GENE 1* (*AIG1*; At1g33960) and the gene encoding tyrosine aminotransferase (At2g24850) were much less induced in *rph1* in comparison with the wild-type (Figures 3b and S1).

Identification of RPH1

The F_1 progeny of a back-cross of *rph1* with Ws were wild-type in size and resistant to *P. brassicae*. Among 107 selfed F_2 plants, 19 showed an *rph1* phenotype, suggesting that the *rph1* phenotype is determined by a monogenic recessive allele ($\chi^2 = 1.947$, $P > 0.15$). Southern blotting using T-DNA right and left border probes confirmed the tagging of *rph1* with a single T-DNA copy. The T-DNA insertion was localized to position -48 relative to the start codon in the promoter region of gene At2g48070. RNA blot analysis showed that At2g48070 was constitutively expressed in leaves of Ws, and the expression level was not affected by *P. brassicae* (Figure 4d). However, transcripts from the At2g48070 gene could not be detected in *rph1*, indicating that *rph1* encodes a null allele of At2g48070. Stable transformation of *rph1* with a genomic fragment with At2g48070 as the single predicted ORF resulted in wild-type expression (Figure 4d) and concomitantly complemented the mutant phenotype (Figure S2a,b). We concluded that the *rph1* phenotype is caused by a mutation in the 5′ UTR of gene At2g48070, which encodes a protein with a dual role in development and in immunity to *P. brassicae*.

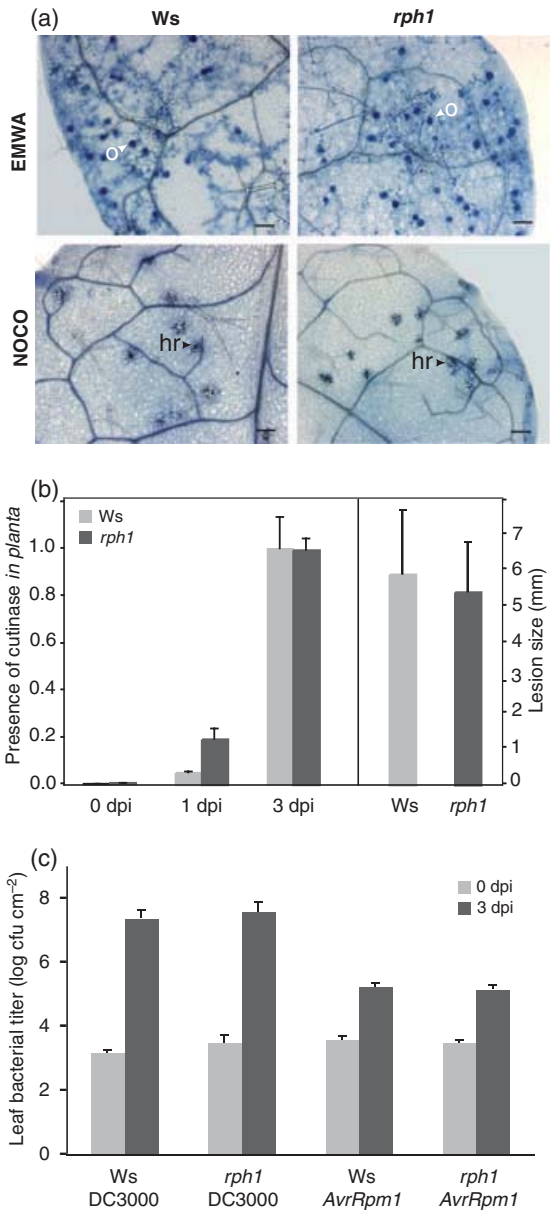

Figure 2. Disease resistance to other pathogens is not affected in *rph1*.
(a) Comparison of disease resistance of Ws and *rph1* to the avirulent isolate NOCO and the virulent isolate EMWA of the biotrophic oomycete pathogen *Hyaloperonospora arabidopsis*. Conidia (10^5 ml^{-1}) were sprayed onto 15-day-old seedlings, and the inoculated leaves were stained at 6 dpi with lactophenol–trypan blue. Resistance is exemplified by a hypersensitive response (hr). Susceptibility is recognized by the growth of intercellular hyphae and the production of oospores (o). Scale bar = 100 μm.
(b) Analysis of disease resistance to the necrotrophic fungal pathogen *Botrytis cinerea*. Leaves of 5-week-old plants were inoculated with 3 μl droplets of a conidial suspension (10^4 conidia ml^{-1}). Left: quantitative PCR analysis of the cutinase gene of *B. cinerea* (as an indicator of pathogen proliferation) normalized to the expression of *ACTIN2* of Arabidopsis. The difference observed at 1 dpi is at the border of statistical significance ($P = 0.069$; *t* test). Right: comparison of disease lesion size at 3 dpi.
(c) Disease resistance of Ws and *rph1* to the virulent isolate of *Pseudomonas syringae* pv. *tomato* DC3000 or the isogenic avirulent strain carrying the avirulence gene *avrRpm1*. Leaves of 4-week-old plants were injected with a bacterial suspension (10^5 cfu ml^{-1}), and the bacterial titre was determined at the time of inoculation and at 3 dpi.

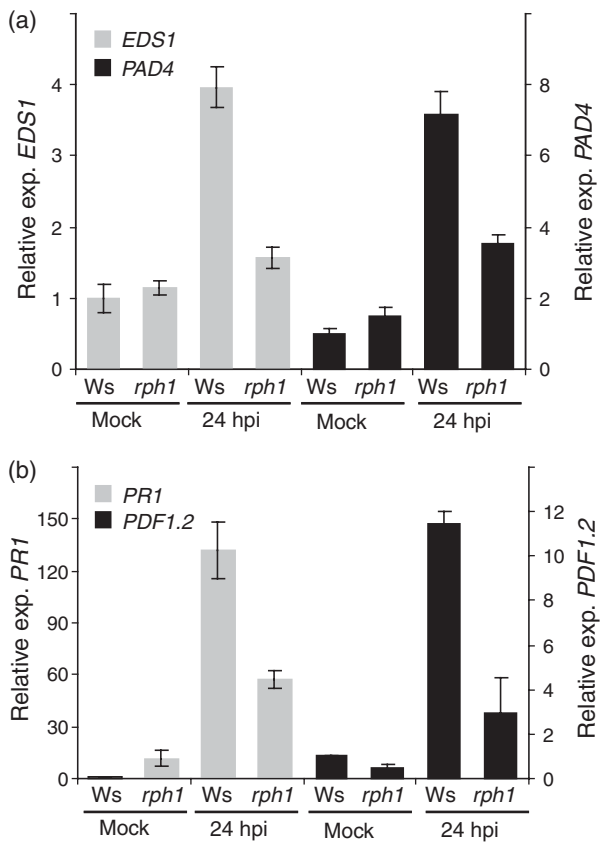

Figure 3. *rph1* has a deficiency in the expression of defence-related genes. Four-week-old Ws and *rph1* plants were spray-inoculated with a zoospore suspension of *P. brassicae* isolate D, and RNA was extracted at 24 hpi for quantitative RT-PCR with gene-specific primers for (a) *EDS1* and *PAD4* and (b) *PR1* and *PDF1.2*. Expression levels were normalized on the basis of transcript amounts of *AtActin2* (At3g18780), and relative expression was normalized to the expression in uninoculated Ws.

RPH1 is a single-copy nuclear gene encoding a small protein of 197 amino acids. Structure prediction indicated that RPH1 is an integral membrane protein with three putative transmembrane domains. RPH1 shares no similarities with functionally characterized proteins or defined functional domains. Highly similar RPH1 homologues are present in other plants (Figure 4a) but not in other kingdoms. Interestingly, all RPH1 proteins contain a putative N-terminal plastid transit peptide. Apart from the plastid transit peptide, AtRPH1 is more than 80% identical to RPH1 homologues of potato and rice. Transformation of *rph1* with the tomato homologue *LeRPH1* complemented all aspects of the mutant phenotype, suggesting that RPH1 homologues are functionally equivalent (Figure S2c,d). The high degree of sequence conservation extends to gymnosperms (*Pinus taeda*; 75% identity) and bryophytes (*Physcomitrella patens*; 71% identity). The region of similarity includes the complete mature RPH1 protein, indicating that the entire protein is under strong evolutionary selection. The presence of RPH1-like homologues in primitive unicellular algae (Figure S3) supports a function of RPH1-like proteins in early photosynthetic eukaryotes.

The subcellular localization of RPH1 was analysed using a GFP fusion to its C-terminus. The RPH1-GFP fusion protein accumulated in the chloroplasts of transiently transformed Arabidopsis protoplasts (Figure 4b), while GFP expressed alone accumulated in the cytoplasm (Figure 4c). Stable expression of the RPH1–GFP fusion protein in *rph1* complemented the mutant phenotype (Figure S2e,f), thus confirming its functionality. To confirm the plastidial localization, the putative signal peptide of RPH1 was replaced with the plastid targeting peptide of ferredoxin (FD). The constitutive expression of FD-RPH1 in *rph1* led to a reversion of the mutant phenotype to wild-type (Figure S2e,g). This did not occur with RPH1 lacking a signal peptide (Figure S2e,h).

Initiation of hypersensitive cell death is not affected in rph1

The effect of the *rph1* mutation on pathogenesis was analysed at the cellular level. The early steps of infection were similar in Ws and *rph1* plants. Zoospores of *P. brassicae* encysted, germinated and formed infection structures between 2 and 3 h post inoculation (hpi), prior to penetration between anticline walls of epidermal cells. In both genotypes, a few mesophyll cells below the sites of penetration typically showed an HR that became visible at around 6 hpi (Figure 5a,b). An earlier epidermal HR (4 hpi) was only observed in a few directly penetrated epidermal cells. The area of dead cells did not spread much further in leaves of Ws (Figure 5c), and hyphae remained confined to the zone of host cell death and lost their cytoplasm (Figure 5e). In contrast, the region of cell death continued to expand in *rph1*, and finally affected large areas of the leaf (Figure 5d). Growth of *P. brassicae* was unaffected by the cell-death response of *rph1*. Hyphae within the zone of cell death looked healthy and continued to proliferate (Figure 5f), later producing oospores and zoosporangia, and finally destroying the leaves. We concluded that (i) *rph1* is not fully impaired in the detection of *P. brassicae* as shown by the rapid initiation of an HR, (ii) *rph1* is an example of a 'death-no-defence'mutant, showing that hypersensitive cell death *per se* is not sufficient to stop the growth of *P. brassicae*, and (iii) essential immunity factors either downstream of or not related to hypersensitive cell death are missing in *rph1*.

Loss of RPH1 negatively affects the oxidative burst

The capacity of *rph1* to locally accumulate H_2O_2 in response to zoospore inoculation was tested using a histochemical assay based on staining with 3,3'-diaminobenzidine (DAB). In the wild-type, local H_2O_2 accumulation at typical penetration sites was first detected at 9 hpi in underlying mesophyll cells

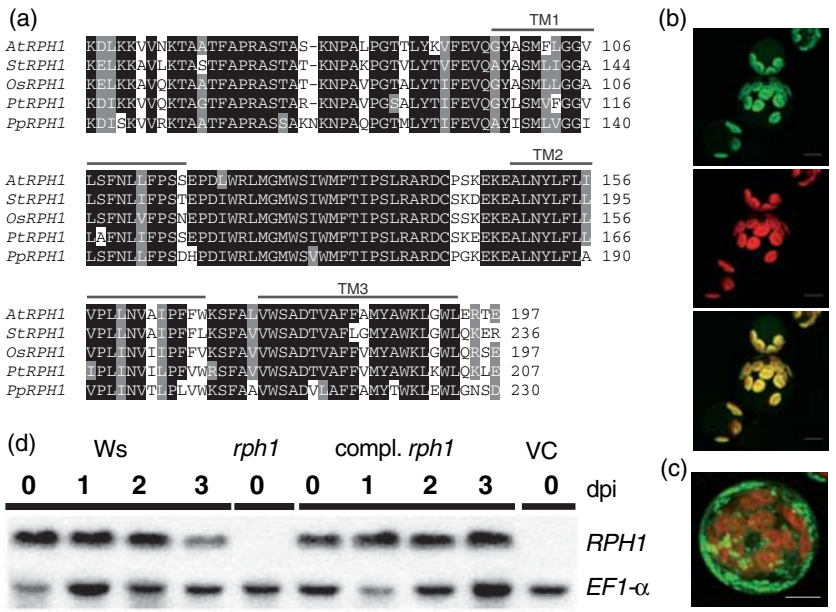

Figure 4. *RPH1* encodes a highly conserved plastid protein.
(a) Sequence alignment of *RPH1* homologues of various plant species: *AtRPH1* from Arabidopsis; *StRPH1* from potato; *OsRPH1* from rice; *PtRPH1* from *Pinus taeda*; *PpRPH1* from *Physcomitrella patens*. Predicted plastid transit peptides are omitted. Identical amino acids are highlighted in black and conserved amino acids in grey. Predicted transmembrane domains are indicated by a line above the sequence.
(b) Subcellular localization of an RPH1–GFP fusion protein transiently expressed in Arabidopsis leaf protoplasts. Size marker = 10 μm. Left: red chlorophyll autofluorescence; middle: green fluorescence emitted by GFP; right: overlay of green and red channels.
(c) Subcellular localization of GFP without a signal peptide as a control for (b).
(d) RNA blot analysis of *RPH1* transcript accumulation. Ws, *rph1*, *rph1* stably transformed with a genomic fragment containing *RPH1* (compl. *rph1*) and a vector control (VC) were inoculated with *P. brassicae* isolate HH and RNA was extracted at 0–3 dpi. *EF1-α* served as a loading control.

and persisted until 24 hpi (Figure 5g,i). In contrast, only very weak DAB staining was observed in inoculated *rph1* plants (Figure 5h,j). Hence, the susceptibility of *rph1* was associated with a deficiency in local H_2O_2 accumulation. The residual DAB staining observed in *rph1* appeared to originate mainly from the pathogen (Figure 5j). Intriguingly, a normal oxidative burst was observed in *rph1* upon wounding (data not shown) and in response to inoculation with an avirulent isolate of the bacterial pathogen *Pseudomonas syringae* (Figure 6a). This showed that *rph1* is not *per se* defective in stress-mediated ROS production, but specifically fails to activate this response in the interaction with *P. brassicae*.

To independently validate the ROS deficiency of *rph1*, expression of *AtGSTF6* (At1g02930), which serves as a marker of ROS accumulation (Levine *et al.*, 1994), was analysed in response to *P. brassicae* (Figure 6b). *AtGSTF6* transcripts started to accumulate at 6 hpi in Ws, and reached a transient maximum at 9 hpi. The induction of *AtGSTF6* expression in *rph1* was delayed and strongly reduced in comparison to Ws. The remaining induction may be explained by the fact that expression of *AtGSTF6* is regulated by additional pathogenesis-related signals (Wagner *et al.*, 2002). We conclude that RPH1 is a positive regulator of the oxidative burst and the activation of defence-related genes.

Reduced expression of NADPH oxidase D *in* rph1

The plastidial localization of RPH1 suggested chloroplasts as possible source of ROS in response to *P. brassicae*. However, *P. brassicae*-induced ROS accumulation was also observed in the dark, which excluded a direct link between photosynthetic electron transport and ROS accumulation. In plant responses to pathogens, ROS production is primarily catalysed by the activity of plasma membrane-localized NADPH oxidases (Rbohs) whose activity is regulated at the transcriptional and post-translational level (Yoshioka *et al.*, 2001; Simon-Plas *et al.*, 2002; Torres *et al.*, 2002). Changes in *RbohD* (At5g47910) transcript levels in response to *P. brassicae* were analysed as a marker of the oxidative burst (Figure 6b). A transient increase in *RbohD* transcripts was detected at 6 hpi in wild-type Ws, while only marginal changes in *RbohD* expression were observed in *rph1* within 28 hpi. Thus, the defect in *rph1* with respect to ROS production in response to *P. brassicae* correlated with a reduced accumulation of *RbohD* transcripts.

The contribution of RbohD to ROS accumulation in response to *P. brassicae* was tested using an *rbohD* mutant (Torres *et al.*, 2002) in the Col-0 genetic background. Similar to Ws, Col-0 reacted to zoospore inoculation with local accumulation of H_2O_2 in a few host cells in the vicinity of

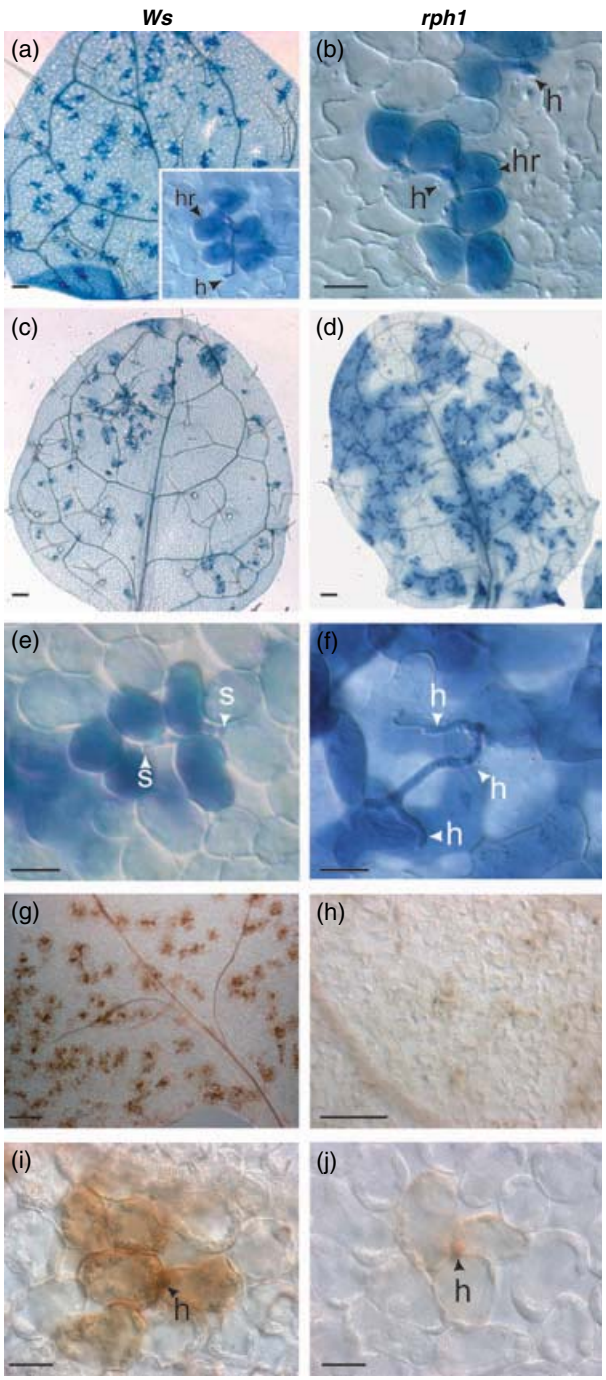

Figure 5. Loss of *RPH1* function blocks the oxidative burst but has no effect on the initiation of hypersensitive host cell death.
Ws and *rph1* plants were spray-inoculated with a zoospore suspension (1.5×10^5 zoospores ml^{-1}) of *P. brassicae* isolate D. Leaves were stained with lactophenol–trypan blue at various time points after inoculation (a–f) to visualize pathogen structures and dead host cells (both stained blue) and with DAB (g–j) for *in planta* H_2O_2 accumulation visualized as brown staining. Hr, hypersensitive response; h, hyphae; s, septa. Scale bars = 200 μm (a,c,d), 100 μm (g,h) or 20 μm (b,e,f,i,j).
(a) Ws at 6 hpi; (b) *rph1* at 6 hpi; (c) Ws at 24 hpi; (d) *rph1* at 24 hpi; (e) Ws at 24 hpi: cytoplasm-free hyphae with septa are labeled with arrowheads; (f) *rph1* at 24 hpi: healthy looking hyphae are labeled with arrowheads; (g) Ws at 9 hpi; (h) *rph1* at 9 hpi; (i) Ws at 14 hpi; (j) *rph1* at 14 hpi.

penetration sites, but no such accumulation was observed in the *rbohD* mutant (Figure 6c). The only dedectable H_2O_2 in *rbohD* was narrowly restricted to epidermal penetration sites, indicating that it might be of pathogen origin. We conclude that local H_2O_2 accumulation in response to *P. brassicae* is dependent on RbohD.

The *rbohD* mutant showed a spreading lesion phenotype but remained resistant to *P. brassicae*. Hence, the oxidative burst appears to control the spread of cell death but is not essential for disease resistance (data not shown). However, this conclusion must be viewed with caution because the tested *rbohD* mutant is in the genetic background of the Arabidopsis accession Col-0, which shows a higher degree of resistance to *P. brassicae* than Ws does. The possibility cannot be excluded that the effect of ROS deficiency on disease resistance is hidden in *rbohD*/Col by additional resistance responses that are absent in Ws.

RPH1 function is essential for immunity of potato to late blight

To test whether the potato homolog *StRPH1* is involved in resistance to the late blight pathogen *P. infestans*, the expression of *StRPH1* was silenced in the resistant potato cultivar Matilda. Constitutive expression of an *RPH1* hairpin construct led to down-regulation of *RPH1* expression (Figure 7a), which correlated with reduced plant size and loss of resistance to *P. infestans* (Figure 7b). *P. infestans* readily colonized the *RPH1*-silenced plant lines, leading to extensive tissue damage and the production of zoosporangia that became visible as white dust on the leaf surface. Pathogen proliferation was quantified using an isolate of *P. infestans* that constitutively expresses GFP as a visible marker. Ten days after inoculation, lines with silenced *StRPH1* expression contained up to 60 times more pathogen biomass than the resistant cultivar Matilda (Figure 7c). Hence, RPH1 plays a similar dual role in development and in disease resistance to *Phytophthora* in potato and Arabidopsis.

Discussion

RPH1 is a single-copy gene that encodes a small chloroplast protein with no known functional domains except putative transmembrane domains. The entire primary sequence of the mature protein is highly conserved in plants, suggesting a conserved plant-specific function. Loss of RPH1 in the Arabidopsis null mutant *rph1* as well as in *StRPH1*-silenced potato led to reduced plant growth and to susceptibility to *Phytophthora*. Although *rph1* showed a developmental phenotype, this was not responsible for the susceptibility to *P. brassicae*, as the resistance to other pathogens was not affected. In addition, loss of RPH1 led to specific deficiencies in defence-related reactions in response to *P. brassicae*. Our results identify RPH1 as a positive regulator of *Phytophthora*-

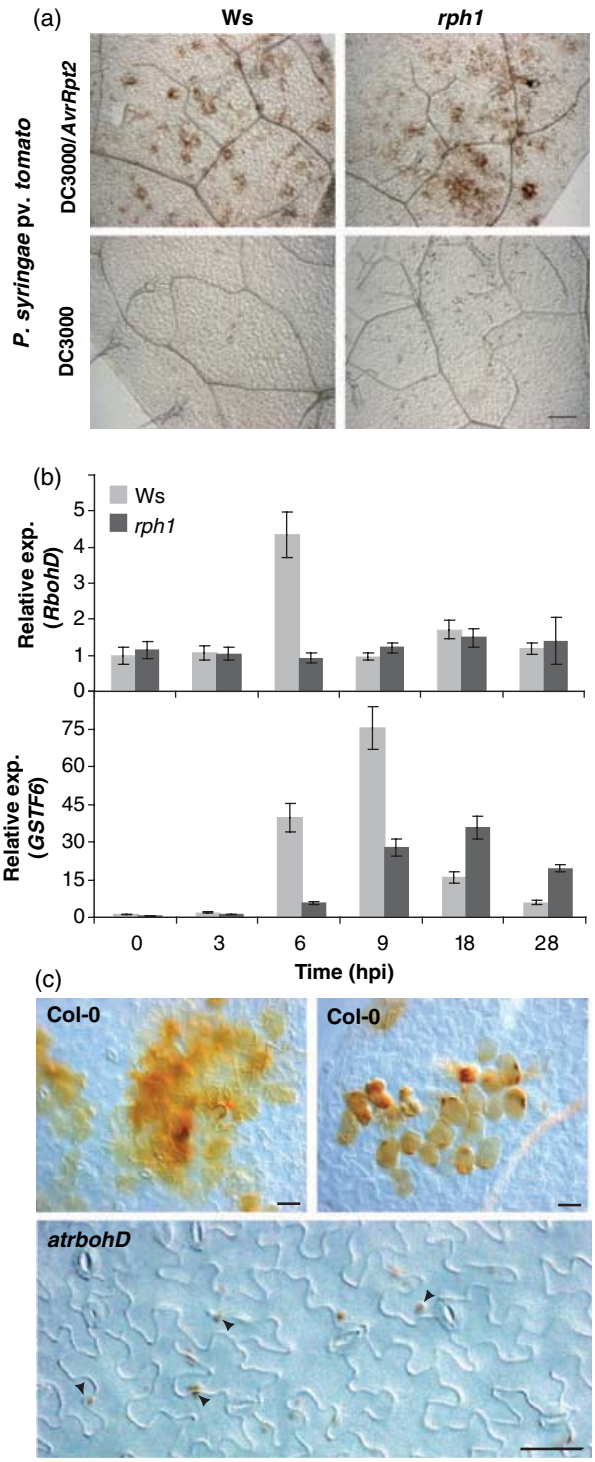

Figure 6. *P. brassicae*-induced ROS accumulation depends on *AtRbohD*, and the ROS deficiency of *rph1* is specific to the interaction with *P. brassicae* and is linked to the decreased expression of *RbohD* and *GSTF6*.
(a) Analysis of the oxidative burst in response to *Pseudomonas syringae*. Four-week-old Ws or *rph1* plants were dip-inoculated with the virulent isolate DC3000 of *P. syringae* pv. *tomato* or an avirulent isolate containing *avrRpt2*. DAB staining (5–10 hpi, brown) was used to visualize H_2O_2 production. Scale bar = 100 μm.
(b) Expression analysis of *AtRbohD* and *AtGSTF6*. Four-week-old Ws and *rph1* plants were spray-inoculated with a zoospore suspension of *P. brassicae* isolate D, and the expression of *AtRbohD* and *AtGSTF6* was analysed by quantitative RT-PCR. Expression levels were normalized on the basis of transcript amounts of *AtActin2* (At3g18780), and relative expression is normalized to expression of uninoculated Ws.
(c) Analysis of ROS accumulation in response to *P. brassicae* in Col-0 and a mutant with no functional RbohD (*trbohD*). Leaves of 2-week-old seedlings were inoculated with zoospores of *P. brassicae* isolate D and stained at 15 hpi with DAB for 5 h. Upper left: Col-0 in the focal plane of the epidermis with DAB staining of the underlying mesophyll cells. Upper right: Col-0 in the focal plane of the mesophyll. Lower: *rbohD* at the focal plane of the epidermis. DAB staining is restricted to penetration sites (some labelled by arrowheads). No additional DAB staining was detectable in epidermal or mesophyll cells. Scale bars = 30 μm.

specific disease resistance, and reveal a role for chloroplasts in the activation of immune responses to *Phytophthora*.

Efficient disease resistance is often associated with local host cell death. However, it is not always clear whether cell death is a prerequisite of disease resistance, and the importance of cell death varies in different plant–pathogen interactions (Mur *et al.*, 2008). Disease resistance can occur in the absence of an HR (Bendahmane *et al.*, 1999; Hennin *et al.*, 2002; Gassmann, 2005), and susceptibility can occur in the presence of an HR (Century *et al.*, 1995). The most prominent example is the Arabidopsis 'defence-no-death' mutant *dnd1* that does not react with an HR to avirulent strains of the bacterial pathogen *Pseudomonas syringae* but nonetheless remains resistant (Yu *et al.*, 1998). The *rph1* mutant has a complementary phenotype to *dnd1* as it is a 'death-no-defence' mutant, showing that hypersensitive cell death is not sufficient to stop *P. brassicae*.

Increased ROS production is one of the earliest physiological responses of plants to potential pathogens, and the resulting redox signalling is known to play a key role in the integration of plant defence reactions (Levine *et al.*, 1994; Torres *et al.*, 2006; Van Breusegem and Dat, 2006). In contrast to the HR, the oxidative burst in response to *P. brassicae* was compromised in *rph1*. Disease resistance to *P. brassicae* correlated with the oxidative burst. Similar conclusions have been drawn from analysis of the resistance of *Nicotiana bentamiana* and potato to *P. infestans* (Wu *et al.*, 1995; Yoshioka *et al.*, 2003). It remains to be shown whether enhanced ROS production directly inhibits *P. brassicae* and how ROS-controlled downstream events might contribute to resistance. The intricate relationship between oxidative burst and the initiation and control of HR is a matter of debate (Torres *et al.*, 2006). The oxidative burst often seems to precede the appearance of visible HR symptoms, and accumulation of ROS can result in host cell death, consistent with a role of ROS as a promotive signal in the initiation process (Levine *et al.*, 1994; Van Breusegem and Dat, 2006). However, in some cases, enhanced ROS accumulation and HR initiation are uncoupled (Century *et al.*, 1995; Glazener *et al.*, 1996; Yano *et al.*, 1999; Sasabe

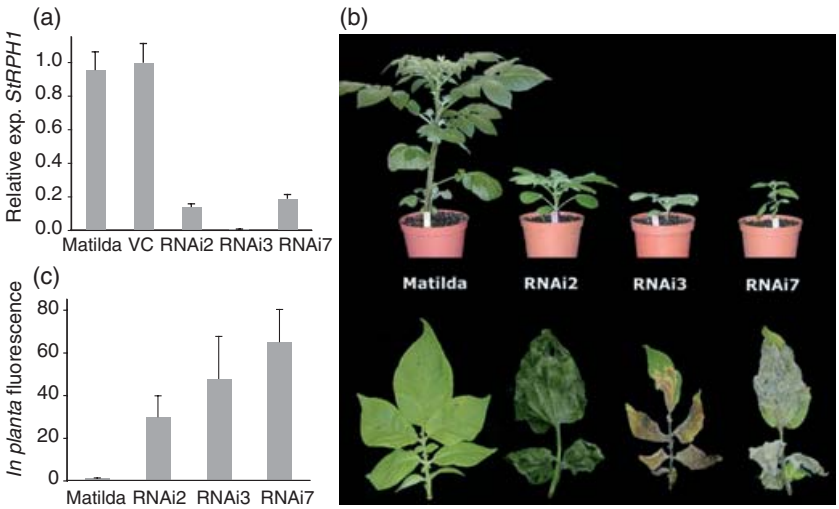

Figure 7. Silencing of *StRPH1* expression interferes with late blight resistance of potato.
StRPH1 expression was silenced in the resistant potato cultivar Matilda. The plants were 12 weeks old at the time of inoculation with 10 μl droplets of a zoospore suspension (2×10^5 spores ml^{-1}).
(a) Relative *RPH1* transcript accumulation determined by quantitative RT-PCR of Matilda, Matilda transformed with an empty vector control (VC), and three silenced *StRPH1* potato lines (RNAi2, RNAi3 and RNAi7). Relative *StRPH1* expression was normalized to that of the vector control.
(b) Effects of *RPH1* silencing on plant growth and disease resistance to *P. infestans* at 10 dpi.
(c) Analysis of GFP fluorescence in Matilda and three silenced potato lines at 10 days after inoculation with *P. infestans* constitutively expressing GFP as a visible marker. Relative GFP expression was normalized to that for uninoculated Matilda.

et al., 2000; Mur *et al.*, 2005). We found that HR formation in the Arabidopsis–*P. brassicae* pathosystem did not depend on a major oxidative burst. However, we cannot rule out the possibility that an early minor burst, possibly including the production of nitric oxide, plays a role in cell-death initiation. Our results are compatible with recent genetic evidence showing that H_2O_2 accumulation can negatively regulate cell-death propagation (Torres *et al.*, 2002, 2005). Lack of ROS accumulation in an Arabidopsis *rbohD* mutant in response to *B. cinerea* and an avirulent isolate of *P. syringae* led to uncontrolled expansion of cell death (Torres *et al.*, 2005) as observed in *rph1* in response to *P. brassicae*. *P. brassicae* is known to cause lesions in later stages of compatible interactions (Roetschi *et al.*, 2001). Thus, the spreading lesions observed in infected *rph1* might reflect enhanced symptomatic necrosis. This alternative explanation is less probable, because necrotic cell death is not observed before 3 dpi in other compatible interactions with *P. brassicae* but expanding cell death in *rph1* occurs within the first 24 hpi. However, it is possible that, as a hemibiotrophic pathogen, *P. brassicae* can take advantage of the spreading cell death.

The RPH1 sequence is highly conserved in plants, and RPH1-like proteins are present in various unicellular algae. This suggests that the original function of RPH1 was in chloroplast-related processes, and that its involvement in *Phytophthora*-specific disease resistance is a derived trait. Because the *rph1* mutant responded with a normal oxidative burst to other biotic and abiotic stresses, it is unlikely that RPH1 functions in the actual process of ROS production but rather controls this process in the interaction with *Phytophthora*.

In Arabidopsis, the plasma membrane-localized NADPH oxidase RbohD plays a central role in the pathogen-triggered generation of ROS (Torres *et al.*, 2002), and *RbohD* gene expression has been shown to reflect oxidative burst activity under biotic and abiotic stress conditions (Yoshioka *et al.*, 2001; Simon-Plas *et al.*, 2002; Torres *et al.*, 2002). The effect of the RPH1 mutation on NADPH oxidase enzyme activity was not tested, but we show that the *rph1* mutation negatively affected the transient accumulation of *RbohD* transcripts observed in wild-type plants in response to *P. brassicae*. In addition, ROS accumulation in resonse to *P. brassicae* was dependent on RbohD, as shown by the lack of H_2O_2 accumulation in an *rbohD* mutant. The reduced oxidative burst in *rph1* may thus be explained by the reduced accumulation of *RbohD* transcripts.

The *rph1* mutant is defective in the induction of *EDS1* and *PAD4*, genes whose products function as positive regulators of disease resistance (Wiermer *et al.*, 2005). In addition, stress hormone-controlled defence signalling is compromised in *rph1*, as indicated by the attenuated induction of salicylic acid- and jasmonic acid-regulated genes. Hence, RPH1 appears to be important for full induction of these defence-related genes in response to *P. brassicae*, but RPH1-independent mechanisms are also involved in the induction process. Although dedicated stress hormone mutants remained resistant (Roetschi *et al.*, 2001), and *eds1-1* and

pad4-5 mutants showed only partially compromised resistance to *P. brassicae* (data not shown), it is conceivable that the combined defects in defence gene expression contribute to the susceptibility of *rph1*. Susceptibility of *rph1* to *P. brasssicae* may be caused by the combined partial deficiency in salicylic acid and jasmonic acid signalling. Ws lines with a combined defect in salicylic acid and jasmonic acid signalling are required to test this possibility.

The lack of known functional domains makes it difficult to integrate RPH1 function into the general picture of disease resistance. RPH1 appears to function upstream of *RbohD* expression and ROS production, which are some of the earliest known defence reactions. There are two possible scenarios to explain our results. In the first scenario, a single recognition event first induces an HR and consequently ROS accumulation. Because RPH1 is predicted to function between the HR and regulation of ROS production, this model fails to explain the observed specificity of *rph1* susceptibility to *P. brassicae*, and does not take into account the fact that *rph1* reacts to avirulent bacteria with an HR and an oxidative burst. Alternatively, HR and ROS production may be initiated independently. This model is compatible with the specificity of *rph1* for susceptibility to *P. brassicae*, because RPH1 function is placed between the initial pathogen-specific recognition event and the activation of *RbohD*. Separate induction pathways for HR and the oxidative burst have been revealed in tobacco cell cultures in response to the cell death-inducing elicitin INF1 of *P. infestans* (Yano *et al.*, 1999; Sasabe *et al.*, 2000). In addition, our results suggest that enhanced defence gene expression in response to *P. brassicae* is not primarily linked to the hypersensitive cell-death response as reduced expression occurs despite a phenotypically normal HR. The connection between ROS deficiency and reduced expression of defence-related genes requires more detailed analysis.

RPH1 plays a *P. brassicae*-specific role early in the interaction close to the initial recognition event leading to enhanced ROS production. Our results are most compatible with the hypothesis that RPH1 functions in the recognition process as a target of *Phytophthora* effector(s), and this interaction may trigger activation of a subset of immune responses. The absence of RPH1 in the *rph1* mutant prevents interaction with the postulated pathogen effector, and as a result the oxidative burst and the expression of defence-related genes are not properly activated, as observed in *rph1*. *Phytophthora* species are known to produce many host-targeted effector proteins (Kamoun, 2006), and there is increasing evidence of chloroplast proteins functioning as targets of pathogen effectors (Jelenska *et al.*, 2007; Caplan *et al.*, 2008). Interestingly, photosynthesis is rapidly inhibited at infection sites in tobacco leaves challenged with *Phytophthora nicotianae* (Scharte *et al.*, 2005). The properties of RPH1 make it an interesting target candidate because the RPH1 sequence is highly conserved and interference with RPH1 function might weaken the plant as shown by the developmental phenotype of *rph1*. Identification of the postulated interacting *Phytophthora* effector is required to clarify this speculative hypothesis of RPH1 function.

Experimental procedures

Biological material

T-DNA insertional mutants generated in the *Arabidopsis thaliana* accession Wassilewskija (Ws; INRA Versailles lines) were obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). Arabidopsis was grown in Jiffy-7 peat pellets (Samen Mauser AG, http://www.samen-mauser.ch) or a mixture of soil and perlite (6:1) in a growth chamber with a 10/14 h day/night photoperiod at 19°C/17°C. Growth of *P. brassicae* isolates, plant inoculation and determination of disease resistance were performed as described previously (Roetschi *et al.*, 2001). For zoospore inoculation, 4-week-old plants were spray-inoculated with a zoospore suspension (1×10^5 spores ml^{-1}) at the start of the dark period. The inoculated plants were incubated at 100% humidity. Potato plants (*Solanum tuberosum*) were grown in a mixture of soil and perlite (6:1) in a growth chamber under a 12 h photoperiod with day/night temperatures of 23°C/19°C. The light intensity was 80–100 μE m^{-2} sec^{-1}. Cultivation of *P. infestans*, zoospore production, inoculation of potato and GFP-based disease quantification were performed as described previously (Si-Ammour *et al.*, 2003). Cultivation of *B. cinerea*, *H. arabidopsis* and *P. syringae*, and the respective disease resistance tests were performed as described previously (Zimmerli *et al.*, 2000, 2001). PCR quantification of the fungal cutinase gene using genomic DNA as a template was used as a measure of infection intensities for *B. cinerea*. All disease resistance tests were repeated at least twice.

Identification of RPH1 *and genetic complementation*

Genomic segments flanking the T-DNA insert of *rph1* were isolated by PCR walking (Riley *et al.*, 1990). Left and right border PCR products were ligated into pGEM-T Easy AT vector (Promega, http://www.promega.com/), and the sequence of the inserts was used to identify the map position of the T-DNA insertion. A 4.9 kb genomic fragment spanning the T-DNA insertion site of *rph1* was amplified using primers 5′-GACCTTACATAGGATAGCTGCAATAGCA-3′ and 5′-TTCTCCTCTGGTGATGCTAGCTC-3′, and the product was introduced into the pGEM-T Easy AT vector. A *Not*I fragment containing the genomic RPH1 sequence was filled-in using Klenow fragment, and inserted into *Sma*I-digested and phosphatase-treated binary vector pCAMBIA 1302 (http://www.cambia.org/daisy/cambia/585.html) to yield pCAM4.9. Using a tomato EST of *LeRPH1* (EST 471212; Clemson University Genomics Institute, Clemson, SC, USA) as a template, a full-length *LeRPH1* cDNA was PCR-amplified using primers 5′-CACCATGAATTTAGCTACTACAATGTCAGC-3′ and 5′-GCGAGCTCAAGAACACCACTGATCC-3′. The resulting 753 bp PCR product was first mobilized into the pENTR vector and then into the binary Gateway destination vector pBENDER (http://www2.mpiz-koeln.mpg.de/~weisshaa/BW-research/Vectors.html) to yield pBEN-*LeRPH1*. pCAM4.9 and pBEN-*LeRPH1* were introduced by electroporation into *Agrobacterium tumefaciens* strain GV3001 (Koncz and Schell, 1986), and *rph1* plants were transformed by the floral-dipping method (Clough and Bent, 1998).

Sequence analysis

The Clustal W (http://www.ebi.ac.uk/clustalw/) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html) programs were used for multiple sequence alignment. The plant membrane protein database ARAMEMNON was used for signal peptide and transmembrane domain predictions (http://aramemnon.botanik.uni-koeln.de/). The following sequences were used for the alignment shown in Figure 4(a): *AtRPH1*, Arabidodopsis At2g48070; *StRPH1* from potato (BM112240); *OsRPH1* from rice (*Oryza sativa*, AAT85080); *PtRPH1* from *Pinus taeda* (DR691592); *PpRPH1* from *Physcomitrella patens* (XP_001785690; PP_4616_C1, http://www.cosmoss.org/). Sequence information for RPH1 homologues is available at http://www.tigr.org/tdb/tgi/.

Subcellular localization of RPH1

The full-length *AtRPH1* cDNA was amplified using primers 5′-CACCATGAGTTGGTCTCTCTGCAGCAC-3′ and 5′-CTTGCTCTGTTCTTTCCAGCCATCC-3′ to produce an *RPH1* fragment that lacked a stop codon and allowed in-frame fusion with GFP. The PCR product was cloned into the pENTR/D-TOPO vector (Invitrogen, http://invitrogen.com) and then mobilized into the binary vector pMDC85 (Curtis and Grossniklaus, 2003) to yield an *RPH1::GFP* fusion construct. Protoplasts of Arabidopsis accession Ws were transiently transformed with the *RPH1::GFP* fusion construct and analysed by confocal microscopy (Bio-Rad MRC 1024 microscope, http://www.bio-rad.com/) after 24 h of dark incubation. Image analysis was performed using public-domain Image program (http://rsb.info.nih.gov/nih-image).

The putative plastid-targeting signal of RPH1 was replaced by the plastid-targeting signal of ferredoxin (FD) by a PCR-based method. Primers were designed to introduce complementary overlapping nucleotides (underlined) at the 3′ end of the *FD* signal sequence and the 5′ end of *RPH1*, respectively. The FD plastid-targeting signal was amplified from vector pFD-SAS (Mauch *et al.*, 2001) using primers 5′-CACCATGGCTTCCACTGCTCTCTCAAGC-3′ and 5′-CTAATCCGTCGACCTTGTATGTAGCCATGGCTG-3′. A 513 bp *RPH1* fragment lacking the targeting signal was amplified using primers 5′-CATACAAGGTCGACGGATTAGAACCCAAGGACGAC-3′ and 5′-CCCATCATGATTACGAGTAGTC-3′. The two resulting PCR products were joined together by an additional PCR step using the forward primer for *FD* and the reverse primer for *RPH1*. The final product was cloned into the pENTR/D-TOPO vector. The *FD::RPH1* insert was mobilized into Gateway binary vector pH2GW7 (http://www.psb.ugent.be/gateway/), and Arabidopsis was transformed as described above.

Analysis of gene expression

Total RNA was extracted using an RNeasy plant mini kit, including a treatment with RNase-free DNase I (Qiagen, http://www.qiagen.com/). PolyA$^+$ RNA (100 ng) purified using an Oligotex kit (Qiagen) was used for RNA blots. Blots were hybridized to ^{32}P-labelled cDNA probes of *RPH1* and *EF1-α*, respectively. For quantitative RT-PCR, total RNA (2 μg) was reverse-transcribed using an Omniscript RT kit (Qiagen), and triplicate samples were analysed using a Rotor-Gene 2000 apparatus (Corbett Research, http://www.corbettlifescience.com) using SYBR Green as the fluorescent reporter dye (SYBR Green PCR Master Mix, Applied Biosystems, http://www.appliedbiosystems.com/). The primers used for quantitative RT-PCR are listed in Table S1. Expression levels were normalized on the basis of transcript amounts of *AtActin2* (At3g18780) and *StTubulin2* (Z33402) for samples of Arabidopsis and potato, respectively, and are reported as mean values with standard deviations. The experiments were repeated with similar results at least once.

Cytological analysis

Lactophenol–trypan blue staining (Roetschi *et al.*, 2001) was used to visualize pathogen structures and dead host cells. *In planta* H_2O_2 production was revealed by staining with 3,3′-diaminobenzidine (DAB), which reacts in the presence of H_2O_2 and peroxidase to form a brownish precipitate (Thordal-Christensen *et al.*, 1997). Excised leaves were vacuum-infiltrated with a solution containing 1 mg ml^{-1} of DAB, and incubated under the conditions used for plant growth. The staining procedure was stopped by replacing the staining solution with a solution of ethanol:glycerol:acetic acid (3:1:1). The long staining time required for this method complicated determination of the kinetics of ROS accumulation. Leaves were stained for a 5 h period starting at various time points from 2–9 hpi (at 1 h intervals), or at 12, 16 or 24 hpi. Uninoculated control leaves did not show positive DAB staining. Control experiments showed that Ws and *rph1* contained similar amounts of peroxidase activity (data not shown). The experiments were repeated at least twice.

Silencing of StRPH1 *of potato*

The full-length *LeRPH1* cDNA was mobilized from p*LeRPH1* into the RNAi destination vector pK7GW1WG2(II) (http://www.plantgenetics.rug.ac.be/gateway/) using the Gateway system to yield p*LeRPH1*/RNAi. Potato cv. Matilda was transformed (Schneider *et al.*, 2002) using *in vitro*-grown clones obtained from the Swiss Federal Agronomy Station (RAC-Changins, Nyon, Switzerland) and *A. tumefaciens* strain GV3101 carrying p*LeRPH1*/RNAi. After selection in agar tubes containing 50 μg ml^{-1} kanamycin, the transformed plants were planted in soil and cultivated as described above.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Deficiency of *rph1* in transcript accumulation of defence-related genes.

Figure S2. Genetic complementation of the *rph1* phenotype.

Figure S3. Partial sequence alignment of selected RPH1 homologs of algae with RPH1 of Arabidopsis.

Table S1. List of primers used for quantitative RT-PCR.

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