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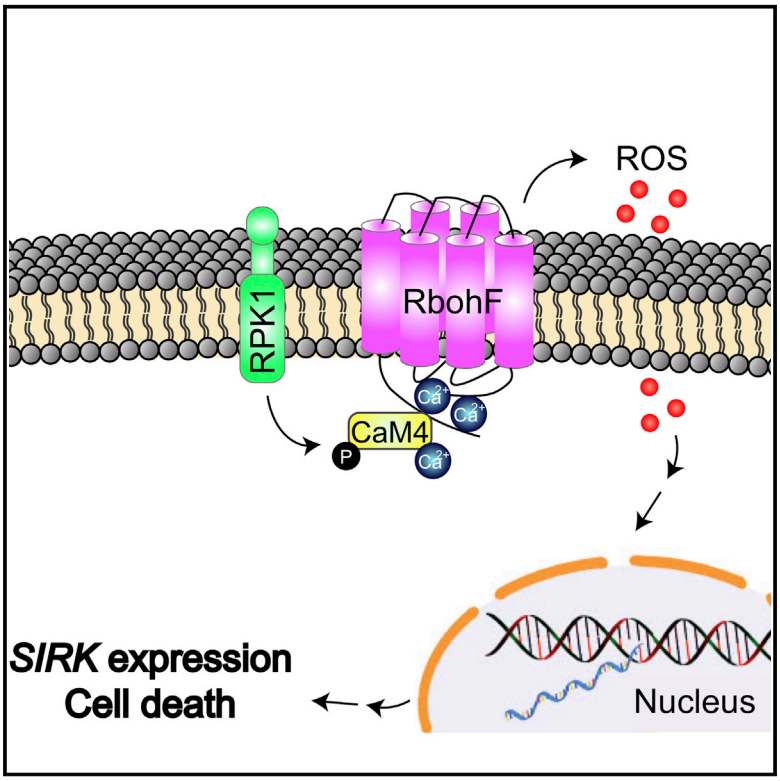
Graphical Abstract



Highlights

- RPK1 mediates the superoxide accumulation to regulate age-dependent cell death
- NADPH oxidase RbohF is responsible for the RPK1-triggered superoxide production
- Calmodulin 4 acts as a molecular link between RPK1 and RbohF

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In Brief

Senescence and cell death in plants are the final developmental stages that ensure a plant's survival and succession to the next generation. Koo et al. show that age-dependent cell death is controlled by the interplay between RPK1, CaM4, and RbohF via the accumulation of the superoxide.

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The Protein Trio RPK1–CaM4–RbohF Mediates Transient Superoxide Production to Trigger Age-Dependent Cell Death in *Arabidopsis*

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SUMMARY

Reactive oxygen species (ROS) are inevitable by-products of aerobic metabolic processes, causing non-specific oxidative damage and also acting as second messengers. Superoxide is a short-lived ROS that functions in various cellular responses, including aging and cell death. However, it is unclear as to how superoxide brings about age-dependent cell death and senescence. Here, we show that the accumulation and signaling of superoxide are mediated by three *Arabidopsis* proteins—RPK1, CaM4, and RbohF—which trigger subsequent cellular events leading to age-dependent cell death. We demonstrate that the NADPH oxidase RbohF is responsible for RPK1-mediated transient accumulation of superoxide, *SIRK* kinase induction, and cell death, all of which are positively regulated by CaM4. RPK1 physically interacts with and phosphorylates CaM4, which, in turn, interacts with RbohF. Overall, we demonstrate how the protein trio governs the superoxide accumulation and signaling at the cell surface to control senescence and cell death.

INTRODUCTION

Senescence and cell death in plants are the final developmental stages that ensure a plant's survival and succession to the next generation. Thus, senescence and cell death are tightly yet flexibly controlled at multiple levels for the plant to fine-tune the endogenous developmental programs in response to environmental cues. Molecular genetics and functional genomics approaches have elucidated genes and molecular mechanisms that elicit senescence processes leading to cell death (Woo et al., 2013). However, our understanding of how environmental cues are integrated into the senescence program is very limited. Receptor protein kinase 1 (RPK1), an upstream component of abscisic acid (ABA) signaling that modulates seed germination, stomatal regulation, and stress responses (Osakabe et al., 2005, 2010), acts as a positive regulator in age-dependent senescence, suggesting the role for RPK1 in dealing with both environmental and developmental cues (Lee et al., 2011). Nevertheless, the molecular mechanisms and downstream targets of RPK1 remain elusive.

Reactive oxygen species (ROS) act as second messengers in various cellular responses, possibly playing a role in fine-tuning the senescence program. JUB1 is one of the NAC transcription factors that are upregulated during senescence and play an important role in regulating senescence (Breeze et al., 2011). *JUB1* is induced by H_2O_2, lowers cellular H_2O_2 levels, and negatively regulates senescence (Wu et al., 2012). In contrast, another NAM, ATAF, and CUC (NAC) factor, NTL4, elevates ROS levels by promoting the expression of ROS biosynthetic genes during stress-induced senescence (Lee et al., 2012). The aforementioned studies indicate that ROS play a key role in plant senescence and programmed cell death by regulating transcriptional factors and gene expression. Therefore, many studies have been conducted to understand ROS-mediated cellular networks that govern senescence. However, functional redundancy in ROS-generating cellular machineries and a broad spectrum of ROS targets have hindered the deciphering of how the production of superoxide is controlled to specifically mediate plant senescence and cell death. Ten NADPH oxidases in *Arabidopsis*, for example, function in various cellular responses with overlapping functions yet play a unique role as well (Liu and He, 2016), indicating that the regulatory mechanism for each NADPH oxidase contributes to the specific function. However, it remains largely unknown how the functional specificity of NADPH oxidases is attained. Here, we show how three signal transduction proteins—RPK1, CaM4, and RbohF—cooperate to regulate the transient accumulation of superoxide at the cell surface, which subsequently controls the expression of downstream genes and age-dependent cell death.

RESULTS

RPK1 Mediates the Accumulation of Superoxide at the Cell Surface to Regulate Age-Dependent Cell Death

RPK1 acts as a positive regulator of age-dependent programmed cell death (PCD) in *Arabidopsis* (Lee et al., 2011; Osakabe et al., 2005). While investigating the genetic mechanisms of age-dependent PCD in plants, we confirmed that loss-of-function mutations in *RPK1* significantly suppressed PCD (Figures 1A and S1A). To examine the basis for the age-dependent PCD mediated by RPK1, we compared the accumulation of superoxide and H_2O_2 in leaves of wild-type and *rpk1* mutant plants, because the ROS accumulation has long been known to be associated with senescence and age-dependent PCD (Buchanan and Balmer, 2005; Lim et al., 2007). Superoxide and H_2O_2 were visualized using nitrobluetetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively (Carol et al., 2005; Torres et al., 2005). In wild-type leaves, NBT staining indicated that superoxide accumulation transiently increased, reaching its peak at 19 days (Figures 1A and S1A). By contrast, in *rpk1-3* and *rpk1-4* mutant leaves, the transient superoxide accumulation was almost completely abolished. Staining of leaves with DAB shows that the accumulation of H_2O_2 also peaked at 19 days in wild-type plants, and the *rpk1* mutations showed a slight reduction in H_2O_2 accumulation with a shift in the peak time to 22 days (Figures 1A and S1A). These results indicate that RPK1 is critical for superoxide accumulation. In turn, it may act as an initial signal for age-dependent PCD.

We then tested whether expression of *RPK1* is sufficient to trigger superoxide accumulation by inducing *RPK1* expression (*iRPK1*) or *RPK1-GFP* (*iRPK1-GFP*) under the control of an inducible promoter (Koo et al., 2004). Five days after treatment with the chemical inducer methoxyfenozide (MOF) (Padidam, 2003), the RPK1 protein level was highly increased in leaves of *iRPK1-GFP* plants (Figure S1B), which was followed by the enhanced accumulation of superoxide visualized with NBT and cell death, as well as elevated ion leakage (Figures 1B and S1C). It is interesting to note that RPK1 induction by MOF induced growth retardation, but not senescence, in young plants (Lee et al., 2011), suggesting developmental stage-specific roles of ROS. Since the *senescence-induced receptor-like kinase/FLG22-induced receptor-like kinase 1* (*SIRK/FRK1*) gene, which has been used as a senescence- and immune-response marker (Segonzac et al., 2012), has been shown to depend on *RPK1* (Lee et al., 2011), we examined the *SIRK* expression to quantify cellular response triggered by RPK1. *SIRK* expression was upregulated by *RPK1*, but not by exogenous H_2O_2, in two independent *iRPK1-GFP* lines (Figure 1C). Interestingly, an RNA blot analysis and qRT-PCR analyses revealed that *RPK1* was upregulated by xanthine and xanthine oxidase, which produce equimolar amounts of superoxide and H_2O_2, but not by H_2O_2 only (Figure S1D). In contrast, two ROS-responsive genes, *ZAT12* and *CML37* (Desikan et al., 2001), were upregulated by both xanthine/xanthine oxidase and H_2O_2 (Figure S1E). These results imply a superoxide-specific regulation of *RPK1* and the subsequent *SIRK* expression.

To investigate how RPK1 triggers superoxide accumulation at the cellular level, we analyzed the superoxide accumulation in *rpk1-3* and *iRPK1-GFP* plants using dihydroethidium (DHE), which is oxidized to fluorescent oxyethidium by superoxide (Fink et al., 2004). In wild-type plants, DHE staining exhibited a transient increase in superoxide accumulation at 19 days, primarily at the plasma membrane (Figure 1D). This was largely absent in *rpk1-3*, and an MOF treatment of *iRPK1-GFP* in plants triggered the accumulation of DHE-stained superoxide at the plasma membrane (Figure 1E). We then tested whether the superoxide accumulation at the plasma membrane was mediated by the kinase activity of RPK1. A mutant version of RPK1-GFP (*mRPK1-GFP*), having a partial deletion in the kinase domain, was not able to lead to the activation of the SIRK promoter (Figure 1F) and failed to produce DHE-stained superoxide at the plasma membrane (Figure 1G). A mutation in the kinase domain did not alter its expression and membrane localization (Figure 1G). These results indicate that the kinase activity of RPK1 is essential for both the generation of superoxide and the subsequent signaling events, including gene expression. Close examination of the localization pattern of RPK1-GFP uncovered the presence of numerous patches of GFP at the plasma membrane and, to some extent, in the cytoplasm (Figure S1F). The patches of RPK1-GFP fluorescence co-localized with the endocytic lipid marker FM4-64 (Figure S1F). Moreover, while the receptor-mediated endocytosis inhibitor tyrphostin A23 (Banbury et al., 2003) abolished MOF induction of *SIRK* expression, the inactive analog tyrphostin A51 did not (Figure S1G). This result indicates that endocytosis of RPK1 plays a pivotal role in RPK1-triggered *SIRK* expression.

NADPH Oxidase RbohF Is Required for *RPK1*-Mediated Superoxide Accumulation that Triggers *SIRK* Expression and PCD

We then asked what cellular mechanism is responsible for RPK1-triggered superoxide accumulation at the plasma membrane. Since cells possess several mechanisms by which superoxide is produced, we first used a pharmacological approach to address the question. NADPH oxidases are well-known enzymatic sources of superoxide at the plasma membrane that play a role in diverse cellular responses (Boisson-Dernier et al., 2013; Torres and Dangl, 2005). Thus, we tested whether NADPH oxidases are involved in RPK1-triggered superoxide accumulation and found that the NADPH oxidase inhibitor diphenylene iodonium (DPI) significantly inhibited the RPK1-triggered *SIRK* expression (Figure 2A). The *Arabidopsis* genome encodes 10 NADPH oxidases. To pinpoint which of the 10 NADPH oxidase genes is responsible for RPK1-mediated superoxide accumulation and signaling, we analyzed their expression patterns (Figure S2A). qRT-PCR analyses revealed that the expression levels of *RbohD* and *RobhF* highly increased in aging leaves, similarly to that of *RPK1* (Figure 2B). The expression pattern of *RbohD* and *RbohF* led us to the hypothesis that these genes may function in RPK1-mediated superoxide accumulation. To test the hypothesis, we examined the effect of the *rbohD* and *rbohF* null mutations on the RPK1-mediated induction of superoxide. Interestingly, superoxide accumulation visualized by staining with NBT was remarkably reduced in plants with the *rbohF* null mutation, *iRPK1-GFP/rbohF* plants, whereas it was detected earlier in plants with the *rbohD* mutation (Figure 2C). This result was

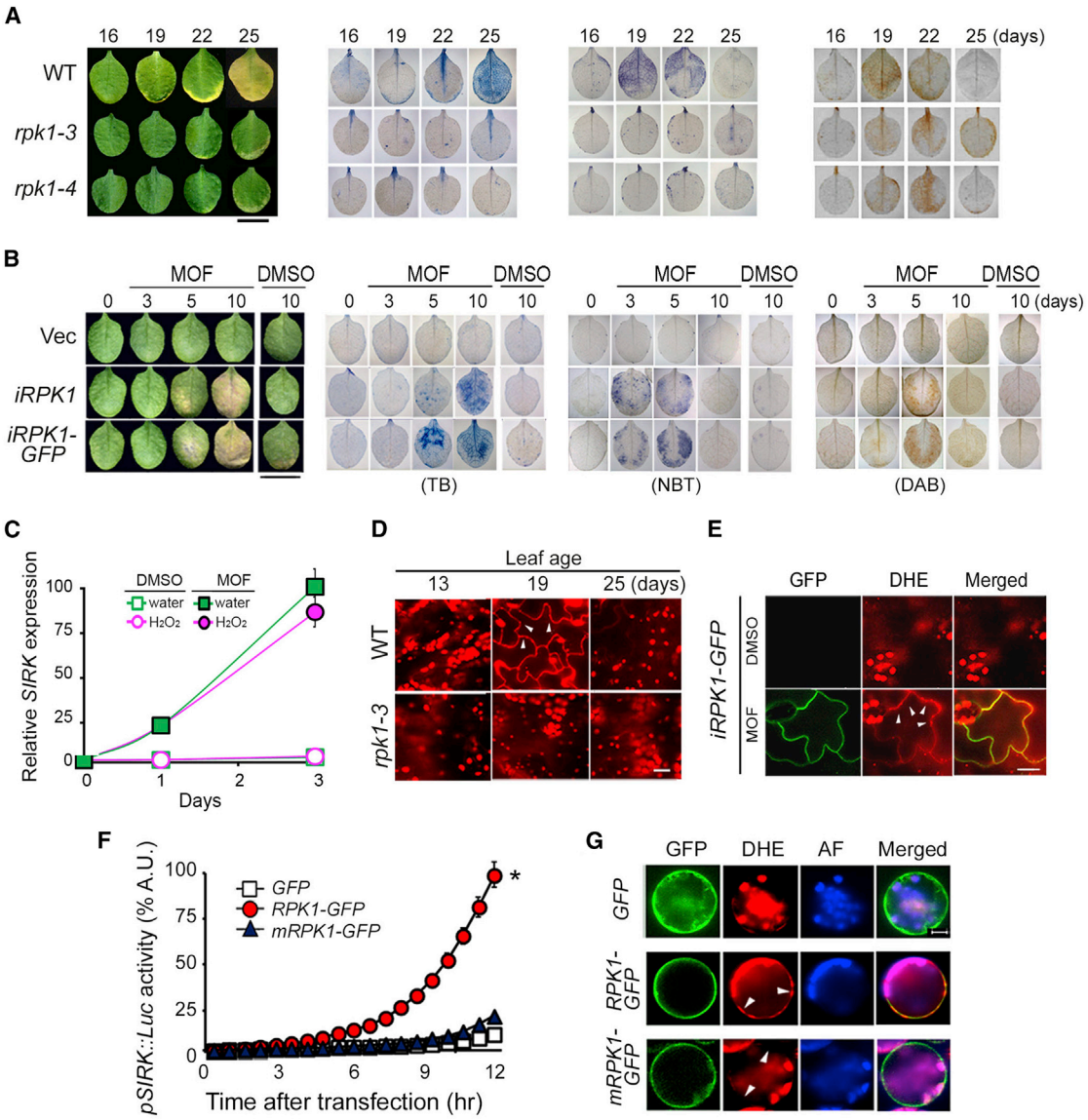

Figure 1. RPK1 Mediates a Transient Burst of Superoxide Accumulation during Aging at the Plasma Membrane

(A and B) Cell death and ROS accumulation during leaf aging in the *rpk1-3* and *rpk1-4* mutants (A), and the *iRPK1*, *iRPK1-GFP*, and vector-transformed control (Vec) plants (B). Three-week-old plants were treated with MOF or DMSO. The fourth leaves were stained with trypan blue (TB), nitrobluetetrazolium (NBT), and 3,3-diaminobenzidine (DAB) to visualize cell death, superoxide, and H_2O_2, respectively. Scale bars, 1 cm.

(C) qRT-PCR analysis of the *SIRK* expression in *iRPK1-GFP* transgenic plants. The total RNA was extracted from the fourth rosette leaves of *pSIRK-Luc/iRPK1-GFP* plants that were treated with 4 mM H_2O_2 or water together with DMSO or MOF. The expression levels of the *SIRK* normalized to *Actin2* are shown in comparison to its expression level in the MOF/water-treated plants. Error bars indicate SD of two independent experiments.

(D) DHE staining of superoxide at the plasma membrane in 13-, 19-, and 25-day-old wild-type and *rpk1-3* leaves. Note that the *rpk1-3* mutation impaired the superoxide staining at the plasma membrane of 19-day-old leaves (arrowheads). The bright red spots indicate chloroplast-derived auto-fluorescence.

(E) DHE staining of superoxide at the plasma membrane of leaf cells of *iRPK1-GFP* plants treated with MOF or DMSO. Arrowheads indicate the enhanced fluorescence at the plasma membrane in the MOF-treated plants, indicating superoxide accumulation.

(F) Activation of the *SIRK* promoter requires the RPK1 kinase activity. *rpk1-3* protoplasts were transfected with *RPK1-GFP*, *mRPK1-GFP*, or *GFP* (vector) along with the *pSIRK::Luc* construct (mean ± SD of n = 6 for each data point).

(G) A mutant RPK1 (mRPK1) lacking a kinase domain fails to generate superoxide stained with DHE. *rpk1-3* protoplasts were transfected with *CsV::RPK1-GFP*, *CsV::mRPK1-GFP*, or *CsV::GFP* (vector) and stained with DHE. Note the absence of DHE staining in *mRPK1-GFP* (arrowheads). *$p < 0.01$. AF, autofluorescence. Scale bars, 10 μm in (D)–(G).

See also Figure S1.

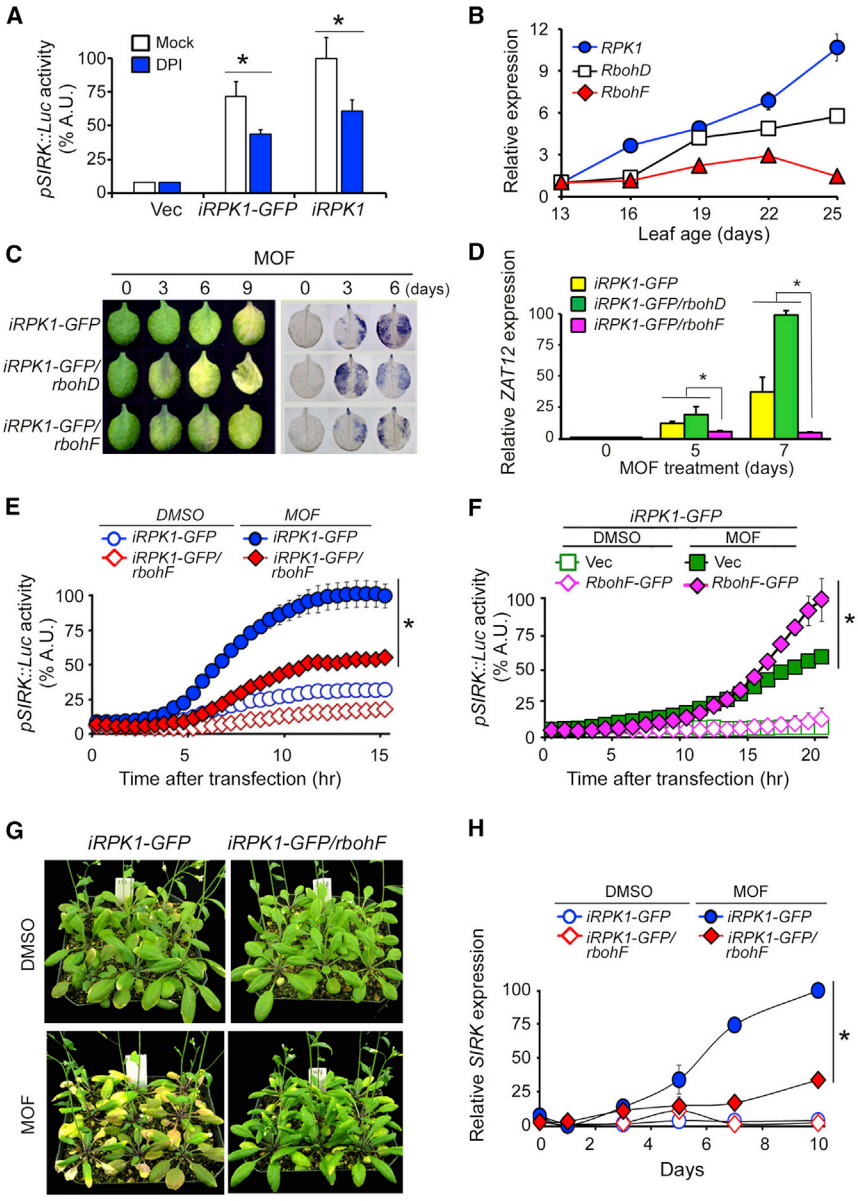

Figure 2. RbohF Is Responsible for *RPK1*-Mediated Superoxide Accumulation, *SIRK* Induction, and Programmed Cell Death

(A) DPI inhibits the *RPK1*-triggered *SIRK* expression. The *rpk1-3* protoplasts were transfected with *RPK1-GFP*, *RPK1*, or *CsV::GFP* (vec) along with the *pSIRK::Luc* construct (n = 4).

(B) qRT-PCR analysis shows that *RPK1*, *RbohD*, and *RbohF* are upregulated with leaf aging. Data are from three independent measurements. The expression levels of the genes normalized to *Actin2* are shown in comparison to the expression levels at day 13. Error bars are smaller than the symbols when not visible.

(C) NBT staining of superoxide in *rbohD* and *robhF* mutant plants expressing *iRPK1-GFP* indicates that RPK1-triggered superoxide generation is largely reduced in *rbohF* but not in *rbohD*.

(D) qRT-PCR analysis of the ROS-responsive gene *ZAT12* shows that *RPK1*-dependent expression of *ZAT12* is largely reduced in *rbohF* but not in *rbohD*. The expression levels of the *ZAT12* normalized to *Actin2* are shown in comparison to its expression level in the *iRPK1-GFP/rbohD* plants at day 7. Error bars indicate SEM of two independent experiments.

(E) The *RPK1*-triggered *SIRK* induction upon a MOF treatment is greatly reduced by the *rbohF* mutation (n = 6).

(F) The *iRPK1-GFP* protoplasts transfected with *RbohF* show an enhanced induction of *SIRK* expression upon a MOF treatment (n = 6).

(G) RPK1-induced senescence upon a MOF treatment is remarkably suppressed by the *rbohF* mutation.

(H) qRT-PCR analysis of *SIRK* expression in *iRPK1-GFP* and *iRPK1-GFP/rbohF* transgenic plants. Total RNA was extracted from the third and fourth rosette leaves of the *iRPK1-GFP* and *iRPK1-GFP/rbohF* plants (10 plants each) that were treated with water together with DMSO or MOF. The expression levels of *SIRK* normalized to *Actin2* are shown in comparison to its expression level in the MOF-treated *iRPK1-GFP* plants at day 10. Data are from two independent experiments (error bars indicate mean ± SEM).

Error bars in (A), (B), (E), and (F) indicate mean ± SD. *$p < 0.01$.

See also Figure S2.

consistent with the expression of ROS-responsive genes, *ZAT12* and *CML37* (Figures 2D and S2B) (Desikan et al., 2001). This suggests a distinctive role of RbohF in RPK1-mediated ROS accumulation and signaling pathways, which was further confirmed by the *SIRK* expression. The *rbohF* mutation highly suppressed the RPK1-induced *SIRK* expression (Figure 2E), and an overexpression of *RbohF* strongly enhanced the RPK1-induced *SIRK* expression (Figure 2F). Furthermore, RPK1-triggered PCD and senescence in *iRPK1-GFP* plants were drastically suppressed in the *iRPK1-GFP/rbohF* plants compared with the *iRPK1-GFP* plants (Figure 2G), consistent with the *SIRK* expression in the plants (Figure 2H). These results clearly indicate that RbohF plays a crucial role in RPK1-mediated superoxide accumulation and subsequent cellular events, including *SIRK* expression and PCD.

Phosphorylation of CaM4 Is Involved in the RPK1-Triggered Signaling

We next examined how RPK1 mediates superoxide accumulation through RbohF. NADPH oxidases are highly regulated by protein phosphorylation (Nühse et al., 2007; Sirichandra et al., 2009). For instance, the bacterial peptide flg22 is recognized by the FLS2/BAK1 complex, leading to activation of BIK1 protein kinase, which, in turn, phosphorylates RbohD (Kadota et al., 2014). RbohF is phosphorylated by the ABA-activated protein kinase OST1 (Sirichandra et al., 2009). Thus, we speculated that RPK1 may regulate the activities of RbohF by phosphorylation. However, *in vitro* and *in vivo* kinase assays showed no indication of RbohF phosphorylation by RPK1 (data not shown). To identify downstream targets of the phosphorylation, we analyzed publicly available age-dependent transcriptome data

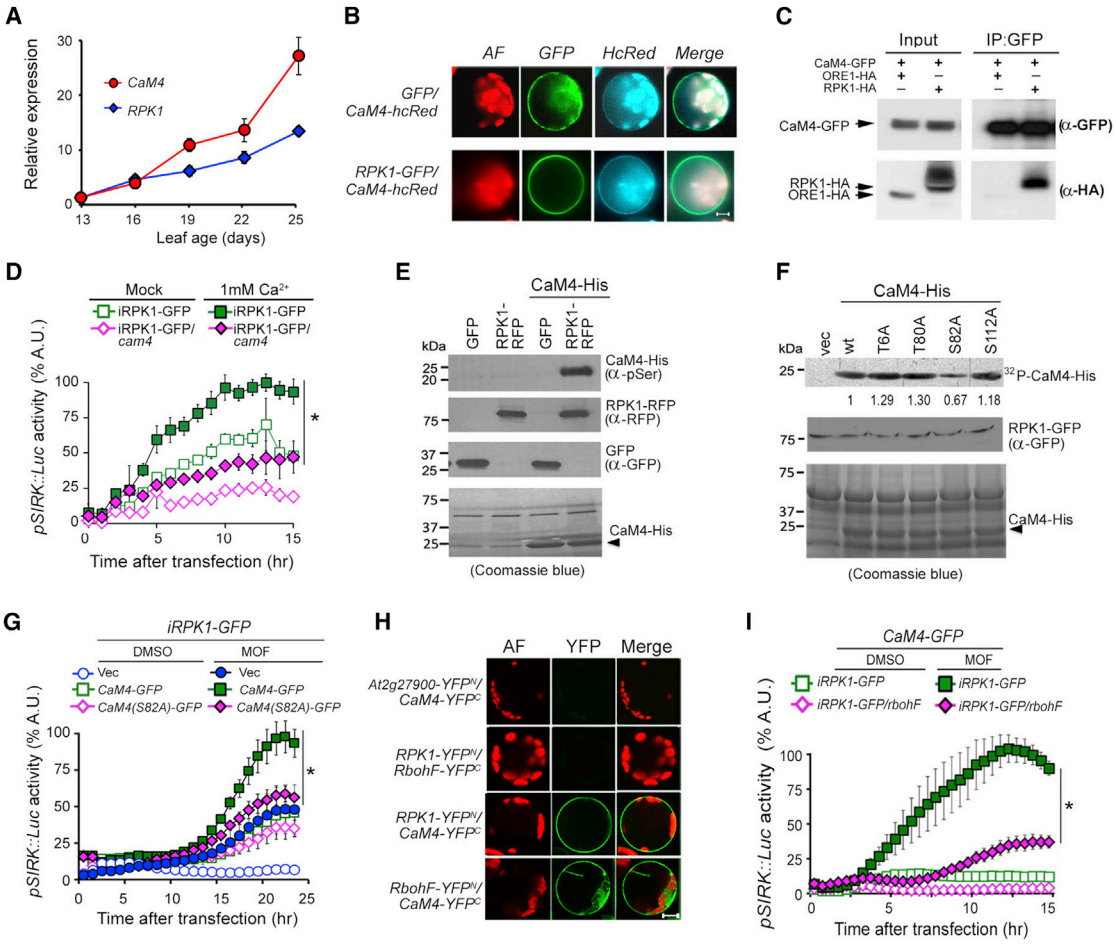

Figure 3. Phosphorylation of CaM4 Is Involved in RPK1-Triggered Signaling

(A) qRT-PCR analysis of *RPK1* and *CaM4* during leaf aging. Data were obtained from three independent experiments. The expression levels of the genes normalized to *Actin2* are shown in comparison to their expression levels at day 13. Error bars are smaller than symbols when they are not visible.

(B) A protein co-localization analysis shows that a fraction of CaM4 co-localizes with RPK1 at the plasma membrane. hcRED, far-red fluorescent protein; AF, autofluorescence.

(C) Co-immunoprecipitation shows a physical interaction between CaM4 and RPK1. Wild-type protoplast cells were co-transfected with CsV::CaM4-GFP and CsV::RPK1-HA or CsV::ORE1-HA (a negative control). Cells were incubated for 12 hr in the medium containing MG132 (10 μM). The expression of CaM4-GFP, RPK1-HA, and ORE1-HA was examined by an immunoblot analysis using anti-GFP or anti–HA antibody (input).

(D) Promotion of RPK1-triggered *SIRK* expression by extracellular Ca^{2+} (1 mM) was inhibited in *cam4* mutants (n = 3) upon MOF treatment.

(E) An *in vitro* kinase assay demonstrated that RPK1 phosphorylates CaM4. Proteins extracted from protoplasts expressing either *GFP* or *RPK1-RFP* constructs were incubated with CaM4-His. An immunoblot analysis with an anti-pSer antibody detected phosphorylated CaM4.

(F) An *in vitro* kinase assay showing an autoradiogram of ^{32}P-labeled CaM4 proteins and immunoblot detection of RPK1-GFP. Proteins extracted from protoplasts expressing *RPK1-GFP* were incubated with wild-type and mutant CaM4-His proteins (T6A, T80A, S82A, and S112A).

(G) *iRPK1-GFP* protoplasts expressing *CaM4-GFP* exhibit enhanced upregulation of *SIRK* expression upon a MOF treatment, and this enhancement was decreased in the mutant CaM4-S82A (n = 4).

(H) YFP fluorescence is reconstituted when CaM4-YFP^C was expressed with RPK1-YFP^N or RbohF-YFP^N. Note the absence of the YFP fluorescence in the pair of RPK1-YFP^N and RbohF-YFP^C or CaM4-YFP^C and At2g27900-YFP^N (negative control).

(I) The *rbohF* mutation diminishes the CaM4-mediated enhancement of the *RPK1*-induced *SIRK* expression. The *iRPK1-GFP* or *iRPK1-GFP/rbohF* protoplasts were transfected with *CsV::CaM4-GFP* and then treated with MOF or DMSO.

Data are from more than four independent measurements (n = 6). Error bars indicate mean ± SD. *$p < 0.01$. Scale bars, 10 μm.

See also Figure S3.

and protein-protein interaction studies (Arabidopsis Interactome Mapping Consortium, 2011; Popescu et al., 2007) and identified calmodulin 4 (CaM4) as a putative downstream target of RPK1. The *CaM4* transcript levels increased in aging (Figure 3A) and CaM4 co-localized with RPK1 at the plasma membrane (Figure 3B). The physical interaction between CaM4 and RPK1 was tested by an immunoprecipitation analysis, and we found that CaM4 and RPK1 physically interact with each other (Figure 3C). To investigate the physiological roles of CaM4 in the RPK1-mediated signaling pathway, RPK1-induced *SIRK*

expression was analyzed in *cam4* knockout mutants. As shown in Figure 3D, extracellular Ca^{2+} greatly enhanced the RPK1-mediated *SIRK* expression in both *iRPK1-GFP* and *iRPK1-GFP/cam4* plants. In addition, the *cam4* mutation significantly reduced the RPK1-medidated *SIRK* expression in both the absence and the presence of extracellular Ca^{2+} (Figure 3D). These results indicate that Ca^{2+}/CaM4 positively regulate the RPK1-mediated *SIRK* expression.

The phosphorylation of CaM4 proteins has been shown in previous phosphoproteomics studies (Engelsberger and Schulze, 2012; Nakagami et al., 2010), and we examined whether CaM4 is a substrate for RPK1. Figure 3E demonstrates that proteins extracted from protoplasts expressing *RPK1-RFP* can phosphorylate a recombinant CaM4 *in vitro*. Proteins extracted from protoplasts expressing *mRPK1-GFP* lacking the kinase domain did not phosphorylate CaM4-GFP *in vitro* (Figure S3A). The physical interaction between RPK1 and CaM4, as well as the *in vitro* kinase assay results, strongly suggests that RPK1 phosphorylates CaM4; however, we cannot exclude the possibility that RPK1 indirectly phosphorylates CaM4 via another protein kinase (or kinases). To determine the RPK1 phosphorylation site(s), we generated four different CaM4 point mutant proteins (T5A, T80A, S82A, and S112A) based on the previous phosphoproteomics studies (Engelsberger and Schulze, 2012; Nakagami et al., 2010). *In vitro* kinase assays showed that RPK1 specifically phosphorylates Ser82 on CaM4 (Figure 3F), suggesting a role for Ser82 phosphorylation in RPK1-mediated signaling. Indeed, we found that RPK1-induced *SIRK* expression was significantly decreased when Ser82 on CaM4 was replaced with Ala (Figure 3G).

Since split yellow fluorescent protein (YFP) assays showed that RPK1 did not physically interact with RbohF (Figure 3H), we hypothesized that CaM4, a downstream target of RPK1, functions as a regulatory molecule in the RPK1-mediated activation of RbohF. RbohF has five CaM-binding sites (Figure S3B), and the binding ability of CaM4 to RbohF was verified by split YFP assays as well as yeast two-hybrid assays (Figures 3H and S3C). The result implies a signaling cascade from RPK1 to RbohF via CaM4. In support of this notion, we found that the enhanced *SIRK* expression by CaM4 was less profound in *iRPK1/rbohF* plants (Figure 3I). These results demonstrate that RbohF is a key regulator of the *SIRK* expression mediated by RPK1 and that CaM4 plays critical roles in the signaling cascade.

DISCUSSION

Cellular ROS are crucial parts of the signaling networks that respond to various developmental and environmental stimuli and induce distinct cellular responses. Discoveries made in the past decade have unveiled that ROS production and signaling are modulated by complex mechanisms (Baxter et al., 2014; Yang et al., 2014), but it is still unclear how the spatiotemporal specificity of ROS signaling is achieved. One of the major obstacles in understanding spatiotemporal ROS signaling is the technical limitation in ROS detection. Despite the fact that ROS-staining dyes have drawbacks, such as non-specific reaction and potential artifacts (Woolley et al., 2013), NBT, DAB, 2′,7′-dichlorofluorescein, and DHE have been used to measure ROS accumulation in numerous studies (Carol et al., 2005; Torres et al., 2005; Tsukagoshi et al., 2010). Therefore, when using the ROS-staining dyes, data should be carefully interpreted. Furthermore, we cannot exclude the possibility that changes in peroxidase activity and interfering endogenous metabolites could affect ROS accumulation in plants.

Among 10 RBOHs in the *Arabidopsis* genome, RbohD has been shown to be mainly responsible for the ROS burst induced by biotic and abiotic stresses (Kadota et al., 2015; Miller et al., 2009). RbohF is also partly involved in the ROS production associated with bacterial infection (Chaouch et al., 2012; Morales et al., 2016), but its contribution was relatively less significant, and the specificity of the RbohF function was not clear. Recently, differential promoter activities between RbohD and RbohF have been reported (Morales et al., 2016), and unique functions of RbohF in plant roots have been reported in the regulation of vascular Na^+ concentration (Jiang et al., 2012) and Casparian strip formation in the root endodermis (Lee et al., 2013), but the regulatory mechanisms that differentially modulate RbohF compared to RbohD still remain ambiguous. In this context, it is interesting that RPK1 and CaM4 proteins specifically utilize RbohF to produce superoxide to regulate the *SIRK* expression and age-dependent cell death (Figures 2 and 3). Together with previous studies, our results suggest that NADPH oxidases have overlapping functions but also play unique roles, depending upon the regulatory mechanisms. Our results indicate the unique function of RbohF in age-dependent cell death and provide insights into the specific regulatory mechanisms of RbohF.

CaM is a highly conserved Ca^{2+}-binding protein present in all eukaryotic organisms and it plays a critical role in Ca^{2+}-modulated signaling processes as a calcium sensor (Zeng et al., 2015). Upon Ca^{2+} binding, CaM modulates the function of the target proteins by physically interacting with them. Moreover, growing evidence shows that post-translational modifications, such as the phosphorylation and methylation of CaM, play a role in the modulation of the distinct cellular processes by regulating the activity of CaM (Banerjee et al., 2013; Benaim and Villalobo, 2002). To understand the physiological roles of CaM4 and CaM4 phosphorylation, the regulatory mechanism of CaM4 on RbohF activity has to be further determined. In addition, investigating whether modulation of the enzyme activity by CaM is conserved in other NADPH oxidases will help us decipher sophisticated Ca^{2+} and ROS signaling networks. For instance, it is possible that each of the NADPH oxidases integrates the Ca^{2+} and phosphorylation signals differently, which may contribute to stimulus-specific cellular responses.

The identification of RPK1 as an upstream regulator of RbohF could provide a molecular link for ROS production and subsequent cellular responses. *RPK1* encodes a leucine-rich repeat receptor-like kinase that comprises the largest subfamily of transmembrane receptor-like kinases in plants, with over 200 members in the *Arabidopsis*, and regulates a wide variety of developmental and defense-related processes. *RPK1* was identified to be upregulated by ABA and abiotic stresses (Hong et al., 1997) and has been shown to function as a positive regulator of ABA signaling (Osakabe et al., 2005, 2010). Furthermore, RPK1 plays important roles in other developmental processes, including age-dependent cell death (Lee et al., 2011), embryonic

pattern formation (Nodine et al., 2007), shoot regeneration (Motte et al., 2014), and cotyledon development (Luichtl et al., 2013). It is uncertain whether ABA, via RPK1, is involved in these developmental processes, but RPK1 in senescence has been shown to depend on ABA (Lee et al., 2011). These studies indicate that RPK1 has multifunctional roles in development and stress response, but it remained unknown how RPK1 mediates such various cellular responses. Taken together, our results provide a detailed mechanistic view of RPK1-mediated age-dependent PCD and the lifespan of plants, which are governed by the protein trio RPK1, CaM4, and RbohF by modulating the transient accumulation of the superoxide. Considering that many developmental and stress responses are accompanied with ROS production, it will be interesting to determine whether the RPK1-CaM4-RbohF module is also used in those cellular processes involving RPK1.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

The isolation of *rpk1-3* and *rpk1-4* mutants (L*er* background) used in this study was described previously (Lee et al., 2011). The *cam4* mutant carrying a transfer DNA (T-DNA) insertion was obtained from The Nottingham Arabidopsis Stock Centre (NASC: N322182), and the T-DNA insertion was confirmed by PCR amplification with primers CaM4-F and GABI-LB (Table S2). *iRPK1/rbohD*, *iRPK1-GFP/rbohD*, *iRPK1/rbohF*, and *iRPK1-GFP/rbohF* plants were generated by crossing *rbohD* and *rbohF* (Kwak et al., 2003) to *iRPK1* and *iRPK1-GFP* transgenic plants (Lee et al., 2011), respectively. *pSIRK::Luc/iRPK1-GFP* plants were generated by crossing *pSIRK::Luc* to *iRPK1-GFP* transgenic plants. Homozygous plants were confirmed by PCR genotyping and antibiotic resistance. Plant growth conditions and preparation of leaf samples were performed as described previously (Lee et al., 2011).

Histochemical Analyses of Cell Death and ROS Accumulation

To visualize cell death, superoxide, and H_2O_2, the fourth leaves were stained with trypan blue (TB), NBT, and DAB, respectively. To measure the *SIRK* promoter activity, protoplasts from *rpk1-3* were transfected with *CsV::RPK1-GFP*, *CsV::mRPK1-GFP*, or *CsV::GFP*, respectively, along with the *pSIRK::Luc* construct. Protoplasts from wild-type plants expressing iRPK-GFP (*iRPK1-GFP*/Col-0) or *rbohF* expressing iRPK1-GFP (*iRPK1-GFP/rbohF*) were transfected with *CsV::GFP* or *CsV::AtrbohF-GFP* (Figure S3D) along with *pSIRK::Luc* to test the role of RbohF in superoxide accumulation and the SIRK induction regulated by RPK1.

The fourth rosette leaves were detached and stained with NBT, DAB, and lactophenol-trypan blue to visualize superoxide, H_2O_2 and dying cells, respectively, as described elsewhere (Torres et al., 2005). To quantify the DAB and NBT stains, the stained pixels were obtained from 6 to 12 leaves per phenotype using the channels function in Photoshop (Adobe), and the intensity was measured using the TotalLab120 program (Nonlinear Dynamics). In Figure S1A, each intensity value obtained from 16- to 22-day-old leaves was normalized by subtracting the mean value of 13-day-old leaves. Similarly, each stained value obtained from the MOF-treated samples was normalized with the mean value of DMSO-treated samples (Figure S1C). Statistical comparisons between L*er* and *rpk1* mutants or vector controls and *iRPK1* transgenic lines were performed using a one-factor ANOVA and pairwise Tukey's HSD (honestly significant difference) tests.

Fluorescence Microscopy

Production of RPK1-GFP and superoxide patches in the leaves and protoplasts was monitored after staining with DHE as previously described (Peshavariya et al., 2007). Briefly, the detached leaves of *rpk1* mutants or the MOF-treated detached leaves of *iRPK1-GFP*, *iRPK1-GFP/rbohD*, or *iRPK1-GFP/rbohF* plants were floated on a 3-mM MES solution containing 30 μM DHE (Sigma) for 15 min. To stain the protoplasts, transfected protoplasts were incubated for 8 hr to allow transgene expression and then stained with DHE for 15 min.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.077.

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AUTHOR CONTRIBUTIONS

H.G.N. and J.M.K. conceived the study and designed the experiments. J.C.K., I.C.L., C.D., H.K.C., Y.K., B.-K.P., H.K., I.H.L., S.H.C., S.J.P., and I.S.J. performed experiments and analyzed data with Y.L., H.G.N., and J.M.K.; J.C.K., C.D., Y.L., H.G.N., and J.M.K. wrote the manuscript. All authors contributed to the revision of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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