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Age-Dependent Action of an ABA-Inducible Receptor Kinase, RPK1, as a Positive Regulator of Senescence in Arabidopsis Leaves

In Chul Lee[1], Suk Whan Hong[2], Sung Soo Whang[3], Pyung Ok Lim[4], Hong Gil Nam[1,5,*] and Ja Choon Koo[3,*]

[1]Division of Molecular Life Sciences, POSTECH, Pohang, Korea
[2]Division of Plant Science, Chonnam National University, Kwangju, Korea
[3]Division of Science Education, Chonbuk National University, Jeonju, Korea
[4]Department of Science Education, Jeju National University, Jeju, Korea
[5]The I-BIO graduate program, POSTECH, Pohang, Kyungbuk, Korea
*Corresponding authors: Hong Gil Nam, E-mail, nam@bric.postech.ac.kr; Fax, +82-54-279-5972; Ja Choon Koo, E-mail, jkoo@jbnu.ac.kr; Fax, +82-63-270-2781.
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Leaf senescence, which constitutes the final stage of leaf development, involves programmed cell death and is intricately regulated by various internal and environmental signals that are incorporated with age-related information. ABA plays diverse and important physiological roles in plants, and is involved in various developmental events and stress responses. ABA has long been regarded as a positive regulator of leaf senescence. However, the cellular mediators of ABA-induced senescence have not been identified. We sought to understand the ABA-induced senescence signaling process in Arabidopsis by examining the function of an ABA- and age-induced gene, *RPK1*, which encodes a membrane-bound, leucine-rich repeat-containing receptor kinase (receptor protein kinase 1). Loss-of-function mutants in *RPK1* were significantly delayed in age-dependent senescence. Furthermore, *rpk1* mutants exhibited reduced sensitivity to ABA-induced senescence but little change to jasmonic acid- or ethylene-induced senescence. RPK1 thus mediates ABA-induced leaf senescence as well as age-induced leaf senescence. Conditional overexpression of *RPK1* at the mature stage clearly accelerated senescence and cell death, whereas induction of *RPK1* at an early developmental stage retarded growth without triggering senescence symptoms. Therefore, RPK1 plays different roles at different stages of development. Consistently, exogenously applied ABA affected leaf senescence in old leaves but not in young leaves. The results, together, showed that membrane-bound RPK1 functions in ABA-dependent leaf senescence. Furthermore, the effect of ABA and ABA-inducible *RPK1* on leaf senescence is dependent on the age of the plant, which in part explains the mechanism of functional diversification of ABA action.

Keywords: ABA • Arabidopsis • Cell death • Leaf senescence • Receptor-like kinase.

Abbreviations: AAO1, Arabidopsis aldehyde oxidase 1; ACS2, ACC synthase2; CAB, Chl *a*/*b*-binding protein; AtNCED2, 9-*cis* epoxycarotenoid dioxygenase 2; AtPT2, phosphate transporter2; CsVMV, cassava vein mosaic virus; DAE, days after leaf emergence; GFP, green fluorescent protein; GST21, glutathione *S*-transferase 21; LRR, leucine-rich repeat; MeJA, methyl jasmonate; MOF, methoxyfenozide; RLK, receptor-like kinase; RPK1, receptor protein kinase 1; RT–PCR, reverse-transcription–PCR; SAG12, senescence-associated gene12; TB, trypan blue; SIRK, senescence-induced receptor-like kinase; WRKY6, WRKY transcription factor 6.

Introduction

Leaf senescence is a genetically programmed deteriorative process that ultimately leads to the death of an annual plant. During this process, leaf cells undergo dramatic changes in cellular metabolism and structure, which is regarded as a means to maximize the fitness of the whole plant by relocating and recycling nutrients. The typical phenotypic change that occurs during leaf senescence is leaf yellowing, which is due to the loss of Chl pigments during chloroplast degradation and hydrolysis of macromolecules (Bleecker and Patterson 1997, Noodén 2004, Lim et al. 2007). These hydrolyzed molecules migrate to developing parts of the plant, such as young leaves, developing seeds and fruits. Despite its degenerative nature, senescence is under the control of a genetically programmed sequence. Arabidopsis has been successfully utilized to reveal the molecular genetic mechanism of age-dependent

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senescence and cell death, since Arabidopsis leaves show a reliable and reproducible aging pattern with relatively simple sets of tissue and cell types (Noodén 2004, Lim et al. 2007). Several genetic loci that alter specific senescence symptoms and numerous genes involved in leaf senescence have been identified in Arabidopsis and a variety of crop plants (Woo et al. 2001, Buchanan-Wollaston et al. 2005, Otegui et al. 2005, Kim et al. 2006, Kim et al. 2009, Yoshimoto et al. 2009, Fischer-Kilbienski et al. 2010). Thus, leaf senescence has been regarded as an evolutionarily acquired process that maximizes the plant's fitness (Noodén 2004, Lim et al. 2007).

Although leaf senescence occurs in an age-dependent manner, its initiation and progression can be modulated by a variety of environmental cues and endogenous factors, including various plant hormones (Thomas and Stoddart 1980, Weaver et al. 1998, Dai et al. 1999, Quirino et al. 2000, Buchanan-Wollaston et al. 2005, Lim et al. 2007). For example, cytokinins function a senescence-retarding growth regulators in Arabidopsis and other plants (Gan and Amasino 1995). Recently, an Arabidopsis cytokinin receptor, AHK3, was found to be a major player in the control of cytokinin-mediated leaf longevity (Kim et al. 2006). Ethylene modulates the rate of leaf senescence, and some ethylene-insensitive mutants exhibit delayed leaf senescence symptoms (Grbić and Bleecker 1995, Chao et al. 1997, Oh et al. 1997, Jing et al. 2005). ABA has been regarded as a hormonal trigger of leaf senescence (Gepstein and Thimann 1980, Quiles et al. 1995), and exogenously applied ABA promotes leaf senescence. Endogenous ABA levels increase during leaf senescence in many plants, including tobacco (Even-Chen and Itai 1975), oat (Gepstein and Thimann 1980), rice (Philosoph-Hadas et al. 1993), maize (He et al. 2005) and Arabidopsis (Zhao et al. 2010). Moreover, expression profiles in senescing leaves of Arabidopsis indicate that a subset of genes implicated in the key step of ABA biosynthesis and signaling are up-regulated during age-dependent leaf senescence (Tan et al. 2003, Buchanan-Wollaston et al. 2005), suggesting the presence of a substantive mechanism that connects leaf senescence and ABA signaling. Thus, ABA is regarded as an important regulator of plant senescence. However, although numerous studies have identified ABA-defective and ABA-insensitive mutants (Leung and Giraudat 1998, Finkelstein et al. 2002, Ma et al. 2009, Park et al. 2009), our understanding of the mechanism underlying ABA-controlled senescence remains limited due to the lack of genetic evidence. This might be explained by the fact that ABA acts not only as a positive regulator of leaf senescence but also as a positive regulator of plant growth, depending on the developmental stage, the endogenous level of ABA or environmental conditions (Leung and Giraudat 1998, Shinozaki and Yamaguchi-Shinozaki 2000). In fact, ABA-defective mutants exhibited various levels of growth defects or pleiotropic phenotype during plant growth. It was noted that genes with pleiotropic functions are important for regulation of the senescence process (Jing et al. 2005, Lim et al. 2007). Here, we report that RPK1 (receptor protein kinase 1), a membrane-bound receptor kinase that contains a leucine-rich repeat (LRR), plays an important regulatory role in ABA-mediated and age-dependent leaf senescence, and that RPK1 undergoes functional switching to act as a positive regulator of senescence in an age-dependent manner.

Results

rpk1 mutants exhibit delayed age-dependent senescence symptoms

Senescence involves changes in the expression of a plethora of genes. In an attempt to understand the roles of senescence-associated genes in Arabidopsis, we sought to identify genes that are up-regulated at an early stage of leaf senescence using PCR-based subtractive hybridization. One of the genes identified during this effort was the *RPK1* gene (see **Supplementary Fig. S1B** for age-induced expression of *RPK1*), which encodes an LRR domain (**Fig. 1A**) at its N-terminus. This gene was previously reported to be an ABA-inducible gene (Hong et al. 1997), and was later found to be an upstream component of ABA signaling during seed germination, stomatal regulation and stress responses (Osakabe et al. 2005, Osakabe et al. 2010). We thus investigated a possible role for *RPK1* in age- and/or ABA-mediated leaf senescence.

To examine RPK1 function in age-dependent leaf senescence, we isolated two *rpk1* knock-out mutants, *rpk1-3* and *rpk1-4*, from transposon-mutagenized pools generated with a modified maize *Ds* element (Sundaresan et al. 1995). The *rpk1-3* and *rpk1-4* mutants have a *Ds* element inserted into the coding region of *RPK1* (**Fig. 1A**, **Supplementary Fig. S1A**), which abolishes *RPK1* expression (**Supplementary Fig. S1B**). We then examined various age-dependent senescence symptoms in these two knock-out mutants. Leaf yellowing, due to Chl loss, is a typical symptom of age-dependent senescence. Visual examination of whole plants showed that the mutant leaves remained green for a longer period than the wild-type leaves. While there was no noticeable alteration in the overall developmental process, including the timing of leaf emergence and growth (**Fig. 1B**), the *rpk1* mutants were slightly shorter than wild-type plants. The *rpk1* mutant leaves maintained their Chl pigments and architectural integrity for a longer period than the wild-type leaves (**Fig. 1C**). The *rpk1* mutant leaves started to show yellowing from the blade and tip at 29 days after leaf emergence (DAE), which is 8 d later than the wild-type leaves. Moreover, the progression of leaf yellowing and Chl loss occurred at a much slower rate in the mutant than in the wild type during aging (**Fig. 1D**). Since leaf senescence is accompanied by cell death, we examined the effect of the *rpk1* mutations on cell death by measuring membrane ion leakage (**Fig. 1E**) and trypan blue (TB) staining (**Fig. 1C**). TB staining (Koch and Slusarenko 1990) is widely used to detect dying cells in the fourth rosette leaf of plants. Both assays indicated that age-dependent cell death is reduced in *rpk1* mutant during leaf senescence. The down-regulation of the Chl *a*/*b*-binding protein gene, *CAB*, and the induction of a

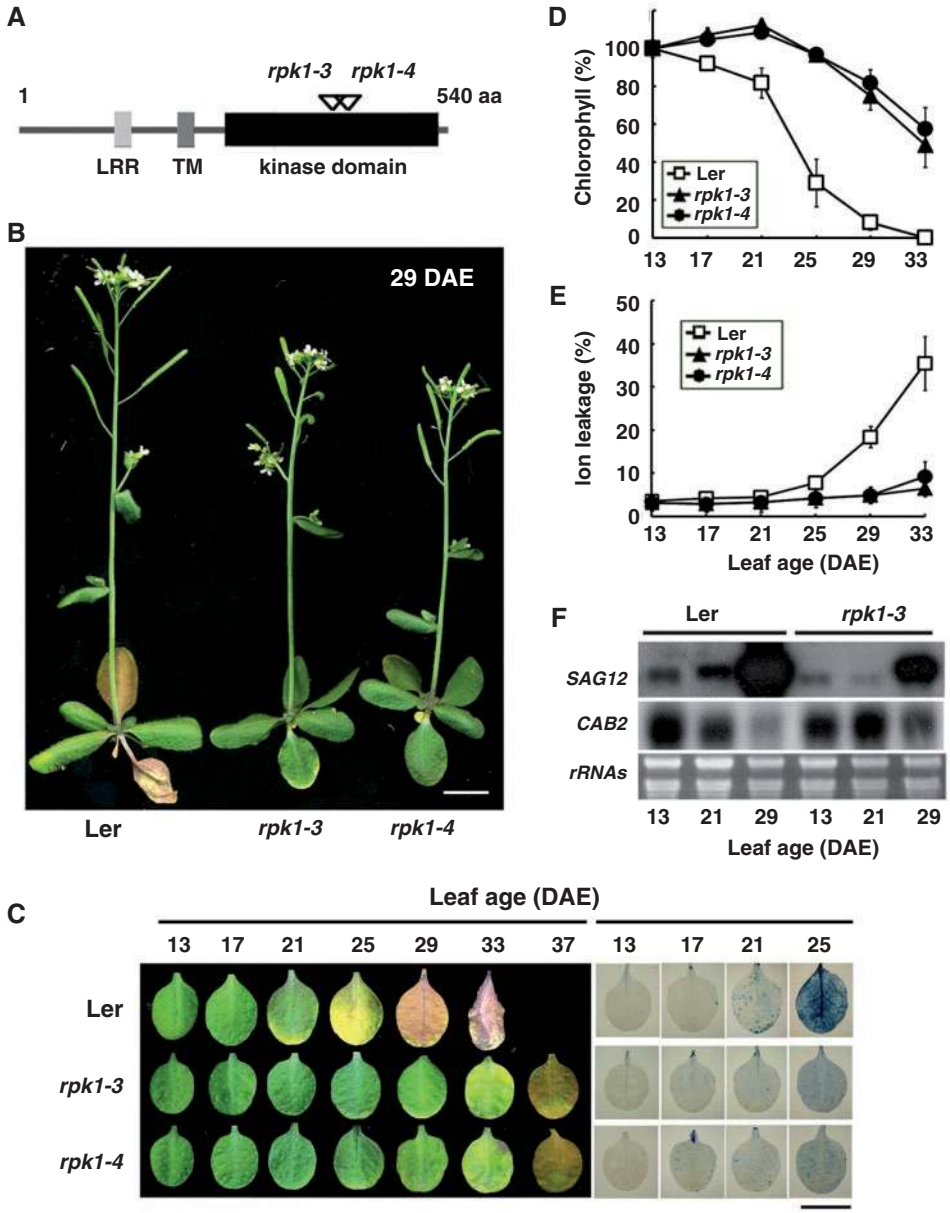

Fig. 1 Delay in age-dependent leaf senescence in the *rpk1* mutants. (A) Structure of RPK1. LRR, leucine-rich repeat domain; TM, transmembrane domain. The *rpk1-3* and *rpk1-4* mutants carry a *Ds* element insertion at the locations indicated by arrowheads. (B) Whole-plant phenotypes of the wild type (WT; L*er*) and *rpk1* mutants (*rpk1-3*, *rpk1-4*) at 29 DAE. (C) Age-dependent phenotype of single leaves. The fourth rosette leaves of WT and the *rpk1* mutants were examined at the indicated leaf ages. Cell death was visualized by trypan blue staining. Photographs show representative phenotypes at each time point. Chl content (D), membrane ion leakage (E) and age-dependent expression of the *SAG12* and *CAB2* genes (F) in the WT and *rpk1* mutant leaves at the indicated leaf ages. Error bars indicate the SD ($n = 8$). Scale bar = 1 cm.

senescence-associated gene, *SAG12*, is a molecular indication of age-dependent leaf senescence (Woo et al. 2001). The *rpk1-3* mutant leaves showed higher levels of *CAB* and lower levels of *SAG12* expression than wild-type leaves at the indicated ages (**Fig. 1F**). Thus, the *rpk1* mutation extends leaf longevity and reduces various aspects of senescence-associated symptoms, including cell death. RPK1 thus functions as a positive regulator of age-dependent leaf senescence and cell death.

RPK1 is a positive regulator of leaf senescence

To examine the effect of *RPK1* overexpression on leaf senescence and cell death, we generated *RPK1* overexpression lines that are driven by the constitutive cassava vein mosaic virus (CsVMV) promoter. As expected, some of the transgenic lines showed earlier leaf senescence; however, over half of the transgenic plants showed pleiotropic phenotypes with highly reduced growth (**Supplementary Fig. S2**). Since RPK1 was

also shown to function in other ABA responses, including stress responses (Osakabe et al. 2005, Osakabe et al. 2010), constitutive expression of *RPK1* throughout the plant's life probably masks the role of RPK1 at the later stage.

Thus, we generated transgenic lines that express *RPK1* and *RPK1-GFP* (green fluorescent protein) under the control of the ecdysone agonist-inducible promoter (Koo et al. 2004), which induces expression of the transgene upon soil drenching with the chemical inducer, methoxyfenozide (MOF) (**Supplementary Fig. S3**). Homozygous transgenic lines that reproducibly showed stable induction of *RPK1* and *RPK1-GFP* were established. The phenotypes of most transgenic lines did not differ noticeably from those of the wild type under non-induced conditions. However, when the expression of *RPK1* and *RPK1-GFP* was induced 3 weeks after germination, the leaves of these transgenic plants exhibited significantly earlier yellowing (**Fig. 2A**, **Supplementary Fig. S4**). The senescence symptoms were then examined at the single-leaf level. Loss of Chl and induction of *RPK1-GFP* transcripts were elevated in the fourth rosette leaves of these transgenic lines compared with those of control transgenic plants (**Fig. 2B, C**). Upon induction of *RPK1-GFP* expression, *SAG12* was up-regulated and *CAB* was down-regulated in the leaves of the transgenic lines (**Fig. 2B**). Membrane ion leakage analysis (**Fig. 2D**) and TB staining (**Fig. 2E**) indicated that the leaves of the transgenic plants contained more dying cells than the control plants 5 d after MOF treatment. These results support the conclusion that RPK1 is a positive regulator of age-dependent leaf senescence and cell death.

The *rpk1* mutants impair ABA-induced leaf senescence and cell death

RPK1 was previously reported to mediate various ABA responses, such as seed germination, stomatal opening and stress responses, and to function as an upstream component of the ABA signaling pathway (Osakabe et al. 2005, Osakabe et al. 2010). Furthermore, *RPK1* expression is induced by ABA (Hong et al. 1997, Osakabe et al. 2005). We thus tested if RPK1 is also involved in ABA-induced leaf senescence by comparing the senescence response of 12 DAE leaves of wild-type and *rpk1* mutants upon ABA treatment. After 5 d of ABA treatment, wild-type leaves lost most of their Chl content and exhibited

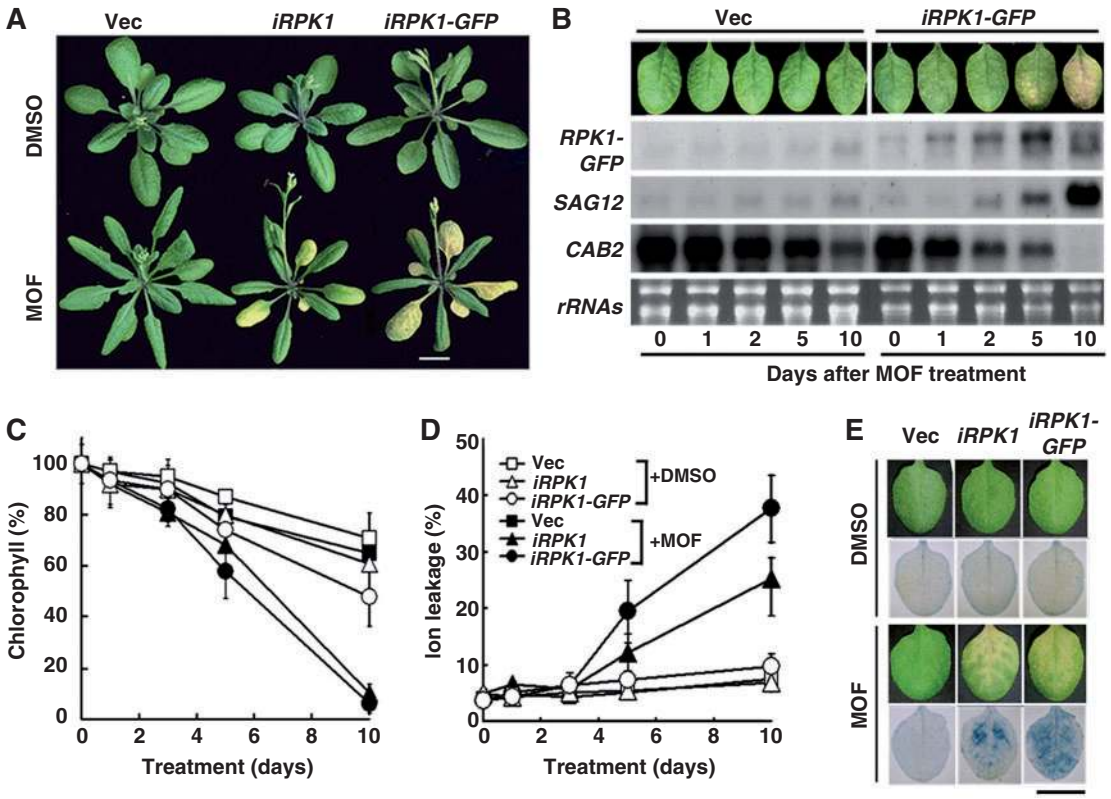

Fig. 2 Inducible *RPK1* and *RPK1-GFP* expression accelerated leaf senescence and cell death. (A) Whole plant phenotypes of the inducible *RPK1* (*iRPK1*) and *RPK1-GFP* (*iRPK1-GFP*) transgenic plants. Three-week-old plants were treated with MOF or dimethylsulfoxide (DMSO) as a control. Vec, transgenic plants transformed with empty vector and used as a control. The photograph was taken 10 d after the treatment. (B) The senescence response examined at the single-leaf level in *iRPK1-GFP* transgenic plants. The yellowing phenotype and altered expression of *SAG12* and *CAB2* in the fourth rosette leaves were examined on the indicated days after MOF treatment. Chl contents (C), membrane ion leakage (D) and trypan blue staining (E) of the fourth rosette leaves of inducible *RPK1* and *RPK1-GFP* transgenic plants were examined after treatment with MOF or DMSO. Photographs show representative phenotypes at each time point. Error bars indicate the SD; $n = 8$. Scale bar = 1 cm.

reduced cellular integrity. In contrast, the leaves of the *rpk1* mutant retained 60% of their Chl content and maintained their cellular integrity (**Fig. 3A, B**). Membrane ion leakage and TB staining also indicated that ABA-induced cell death was markedly reduced in the leaves of *rpk1* mutants (**Fig. 3A, C**). These results indicate that RPK1 mediates ABA-induced leaf senescence and cell death.

Two other plant hormones, methyl jasmonate (MeJA) and ethylene, are also considered to promote plant leaf senescence (Buchanan-Wollaston et al. 2005, Lim et al. 2007). Although *RPK1* expression is specifically induced by ABA, but not by MeJA and ethylene (Hong et al. 1997, Osakabe et al. 2005), we tested if RPK1 is also involved in the leaf senescence response to these hormones (**Fig. 4B, C, E**). As for ABA, the exogenous application of both MeJA and ethylene accelerated leaf senescence. However, in contrast to ABA, the leaves of *rpk1* mutants treated with MeJA or ethylene showed little effect on senescence responses compared with wild-type leaves. These results indicate that RPK1 specifically functions in the ABA-mediated senescence response.

Leaf senescence is also affected by environmental factors. For instance, starvation of leaves by incubation in darkness is a potent inducer of leaf senescence (Woo et al. 2001). The senescence symptoms of the leaves of *rpk1* mutants incubated in darkness did not differ from those of the wild type (**Fig. 4D, E**). Thus, RPK1 specifically mediates ABA-induced leaf senescence.

RPK1 controls expression of various senescence-associated and ABA-inducible genes

Age-dependent leaf senescence and cell death are associated with changes in the expression of a plethora of genes involved in metabolism and hormone signaling (Buchanan-Wollaston et al. 2005). To understand how RPK1 controls leaf senescence at the molecular level, we examined the genes that exhibited altered expression in response to the induction of *RPK1*. The initial candidate genes were selected based on previously published microarray data (Buchanan-Wollaston et al. 2005, Osakabe et al. 2005). The expression of these candidate genes was then examined by reverse transcription–PCR (RT–PCR) analysis in the fourth rosette leaves of the inducible *RPK1* and *RPK1-GFP* transgenic plants (**Fig. 5**). Genes up-regulated in response to the induction of *RPK1* included senescence-associated genes, such as *9-cis epoxycarotenoid dioxygenase 2* (*AtNCED2*), *ACC synthase 2* (*ACS2*), *glycosyl hydrolase*, *FAD-linked oxidoreductase*, *phosphate transporter2* (*AtPT2*), *sugar transporter*, *Arabidopsis aldehyde oxidase 1* (*AAO1*), *glutathione S-transferase 21* (*GST21*), *senescence-induced receptor-like kinase* (*SIRK*) and *WRKY transcription factor 6* (*WRKY6*).

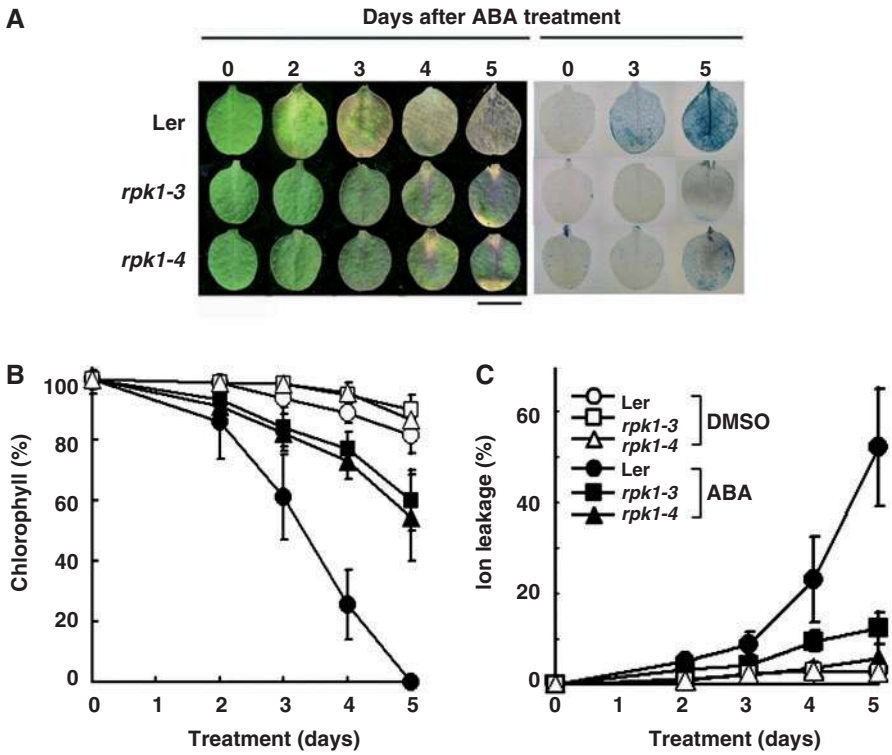

Fig. 3 Delay of ABA-induced leaf senescence and cell death in the *rpk1* mutants. The fourth rosette leaves of the wild type and *rpk1* mutants were detached at 12 DAE and incubated under continuous light in 50 μM ABA or dimethylsulfoxide (DMSO; control). (A) Delays in the ABA-induced senescence phenotype of the *rpk1* mutants. Dying cells were visualized by trypan blue staining at the indicated number of days after treatment. Changes in Chl content (B) and membrane ion leakage (C) were examined at the indicated time points. Error bars indicate the SD; $n = 8$. Scale bar = 1 cm.

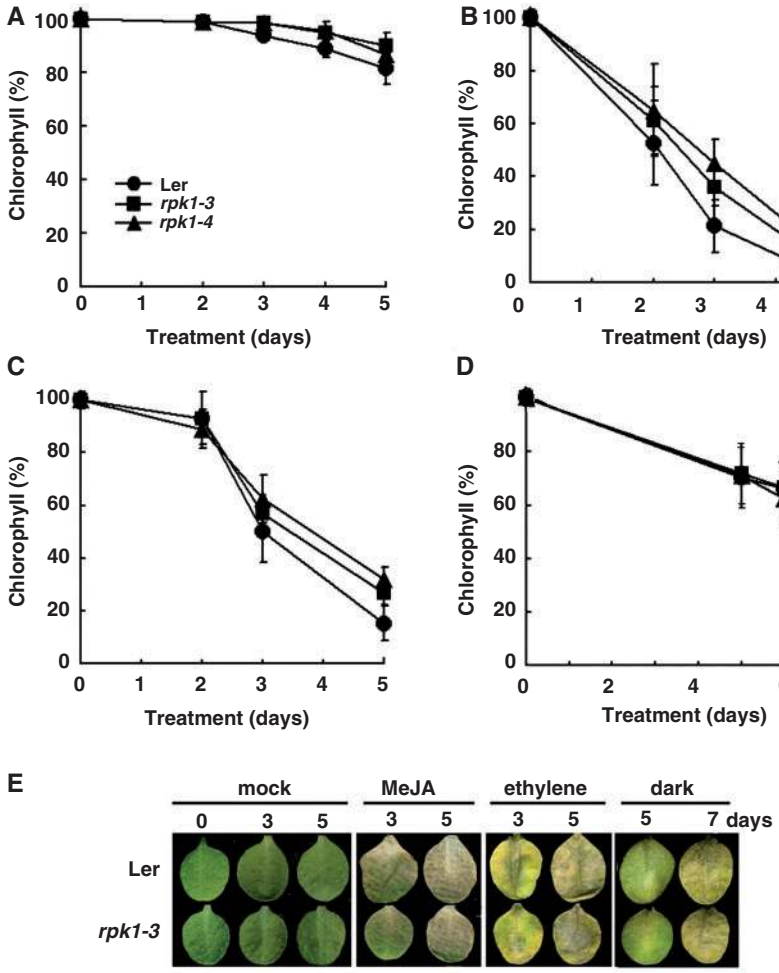

Fig. 4 Leaf senescence responses of the wild type and *rpk1* mutants to various senescence-accelerating factors. The fourth rosette leaves were detached at 12 DAE and incubated under continuous light in MES buffer (A), in MES buffer containing 50 μM methyl jasmonate (B) or 5 μM ethylene gas (C), or in darkness in MES buffer (D). The change in Chl content was examined on the indicated days after treatment. Error bars indicate the SD; $n = 8$. (E) Representative leaf images of the wild type and the *rpk1-3* mutant after treatment.

Among these, *ACS2*, *glycosyl hydrolase*, *FAD-linked oxidoreductase*, *AtPT2* and *sugar transporter* are also known to be ABA-responsive genes (Li et al. 2006). Moreover, seven of these 10 genes were expressed at a lower level in the fourth rosette leaves of *rpk1-3* and *rpk1-4* plants compared with those of the wild type during age-dependent leaf senescence (**Fig. 6**). These results confirmed that RPK1 positively regulates the age- and ABA-dependent senescence signaling pathway as an upstream regulatory component.

Induction of RPK1 expression in young plants results in growth retardation but not senescence

The senescence response assays in the inducible *RPK1* and *RPK1-GFP* transgenic plants presented in **Fig. 2** were performed by treating the transgenic plants with the inducer, MOF, after 3 weeks of germination. However, when MOF was applied to 2-week-old *RPK1* and *RPK1-GFP* transgenic plants, the response of the plants was clearly distinguishable from that of 3-week-old plants. Induction of *RPK1* in 2-week-old plants resulted in noticeable growth retardation 10 d after MOF treatment (**Fig. 7A**), but showed little evidence of senescence. For example, expression of the senescence-associated *SAG12* was not induced in the leaves of transgenic *RPK1-GFP* plants when the plants were treated with MOF for 10 d (**Fig. 7D**). Expression of *CAB* was only affected slightly by MOF treatment (**Fig. 7D**). Furthermore, membrane ion leakage and the TB assay showed little evidence of cell death in the leaves of the 2-week-old transgenic plants by 10 d of MOF treatment (**Fig. 7A, C**). The Chl content of the leaves of *RPK1* and *RPK1-GFP* transgenic plants was maintained at 50% of the level of untreated plants after 10 d of MOF treatment (**Fig. 7B**), whereas it was almost completely lost when 3-week-old transgenic plants were treated with MOF for 10 d (**Fig. 2C**). These responses were in sharp contrast to those observed in 3-week-old mature transgenic plants (**Fig. 2**). These result, in conjunction with previous reports on the role of RPK1 in seed germination and stomatal regulation of young

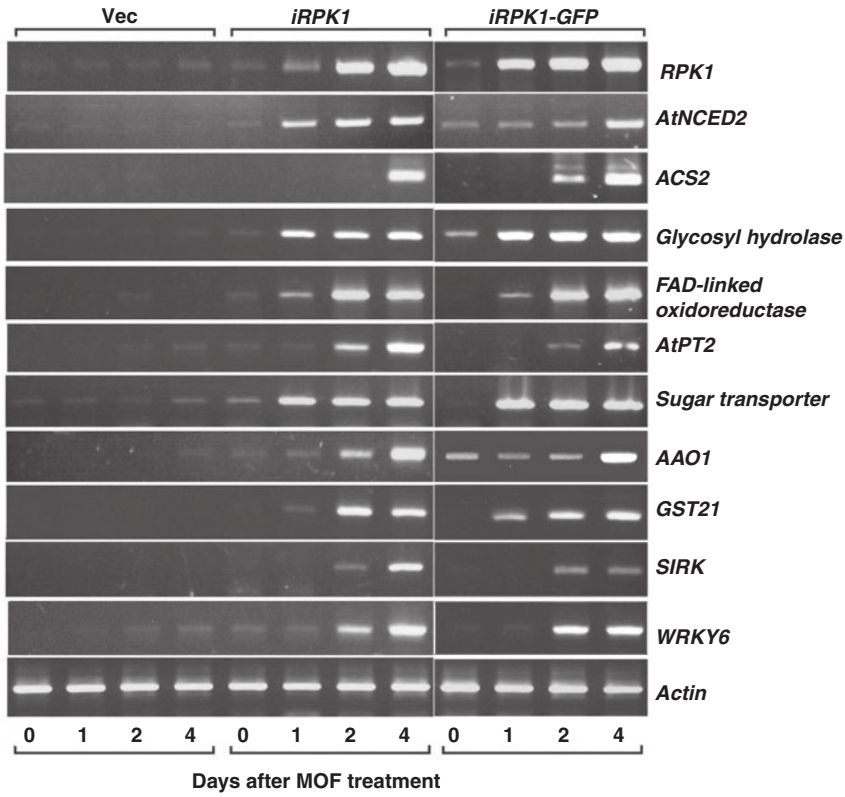

Fig. 5 Semi-quantitative RT–PCR analysis of senescence-inducible and ABA-responsive genes in the *RPK1* and *RPK1-GFP* inducible transgenic plants. The first-strand cDNAs were prepared using RNAs isolated from the fourth leaves of 3-week-old transgenic plants on the indicated days after MOF treatment. The PCR was optimized for specific amplification of genes (**Supplementary Table S1**). *AtNCED2* (At4g18350), *9-cis epoxycarotenoid dioxygenase 2*; *ACS2* (At1g01480), *ACC synthase 2*; *glycosyl hydrolase* (At1g02310); *FAD-linked oxidoreductase* (At2g34810); *AtPT2* (At2g38940), *phosphate transporter 2*; *sugar transporter* (At3g05400); *AAO1* (At5g20960), *Arabidopsis aldehyde oxidase 1*; *GST21* (AT2G29470), *glutathione S-transferase 21*; *SIRK* (At2g19190), *senescence-induced receptor-like kinase*; *WRKY6* (At1g62300), *WRKY transcription factor 6*; and *Actin* (At1g49240).

plants (Osakabe et al. 2005), indicate that RPK1 has different roles in plants of different ages. This also suggests that the function of RPK1 in leaf senescence and cell death is greatly enhanced in an age-dependent manner.

The effect of ABA on leaf senescence is age dependent

ABA has long been known to promote leaf senescence. However, ABA also plays highly diverse roles. How is ABA able to regulate the diverse functions from seed germination even to leaf senescence? RPK1 is ABA inducible. Furthermore, RPK1 functions differently, depending on the age of the plant, on the induction of *RPK1* expression. These observations led to us postulate that at least a part of the functional diversification of ABA as a promoter of senescence may depend on the age of the plant. We tested this hypothesis by examining the effect of exogenously applied ABA on the senescence and cell death of 4 and 12 DAE leaves. When treated with ABA for 5 d, 12 DAE leaves showed a much greater decline in Chl content than the 4 DAE leaves (**Fig. 8**). Furthermore, TB staining indicated that almost no detectable cell death occurred in the 4 DAE leaves, whereas the 12 DAE leaves showed substantial evidence of cell death. The differential effect of ABA on cell death in the leaves of different ages was further confirmed by the quantitative measurement of membrane ion leakage. The result confirmed that, among the diverse roles of ABA, ABA-induced leaf senescence and cell death preferentially occur in older leaves.

Discussion

In this report, we investigated the function of an ABA- and age-induced gene, *RPK1*. The *rpk1* mutants were delayed in several aspects of age-dependent senescence, such as Chl content, cell death and the expression of senescence marker genes (**Fig. 1**) during age-dependent and ABA-induced senescence (**Fig. 3**).

A signal from a membrane receptor kinase can mediate age-dependent senescence and cell death

The Arabidopsis genome contains >600 receptor-like kinases (RLKs) (Shiu and Bleecker 2001). Some of these genes were

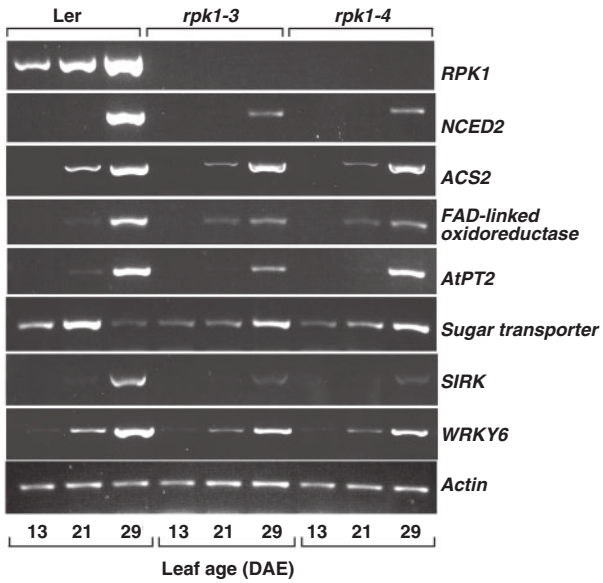

Fig. 6 Semi-quantitative RT–PCR analysis of senescence-inducible and ABA-responsive genes in the *rpk1-3* and *rpk1-4* mutants. The first-strand cDNAs were prepared using RNAs isolated from the fourth leaves at the indicated leaf age. The PCR was conducted as shown in **Fig. 5**. *Actin* was included as an internal control.

found to have a critical function in the perception of a wide range of signals; for instance, *CLAVATA1* (*CLV1*; Clark et al. 1997) is involved in shoot meristem development; *Brassinosteroid Insensitive1* (*BRI1*; Schumacher and Chory 2000) functions in brassinosteroid recognition; *FLAGELLIN-SENSITIVE 2* (*FLS2*; Gómez-Gómez and Boller 2000) perceives bacterial flagellin elicitor; and *S-locus receptor kinase* (*SRK*; McCubbin and Kao 2000) controls self-incompatibility.

RPK1 is a membrane-localized LRR RLK whose expression appears to be induced by ABA, drought, salt and cold stress (Hong et al. 1997). Gain-of-function and loss-of-function phenotypes of *RPK1* reveal its role as a positive regulator of ABA signaling, including seed germination, plant growth, stomatal cloure and abiotic stress (Osakabe et al. 2005, Osakabe et al. 2010). Furthermore, RPK1 together with TOADSTOOL2 (TOAD2) is redundantly required for the establishment of embryonic pattern formation in Arabidopsis (Nodine et al. 2007), suggesting its diverse roles in several aspects of plant development. In this study, we established that RPK1 can control senescence and cell death. Since RPK1 is a plasma membrane-localized receptor kinase, our finding shows that a signal arising at the membrane can mediate senescence and the accompanying cell death, and that phosphorylation at the membrane, a widely adopted

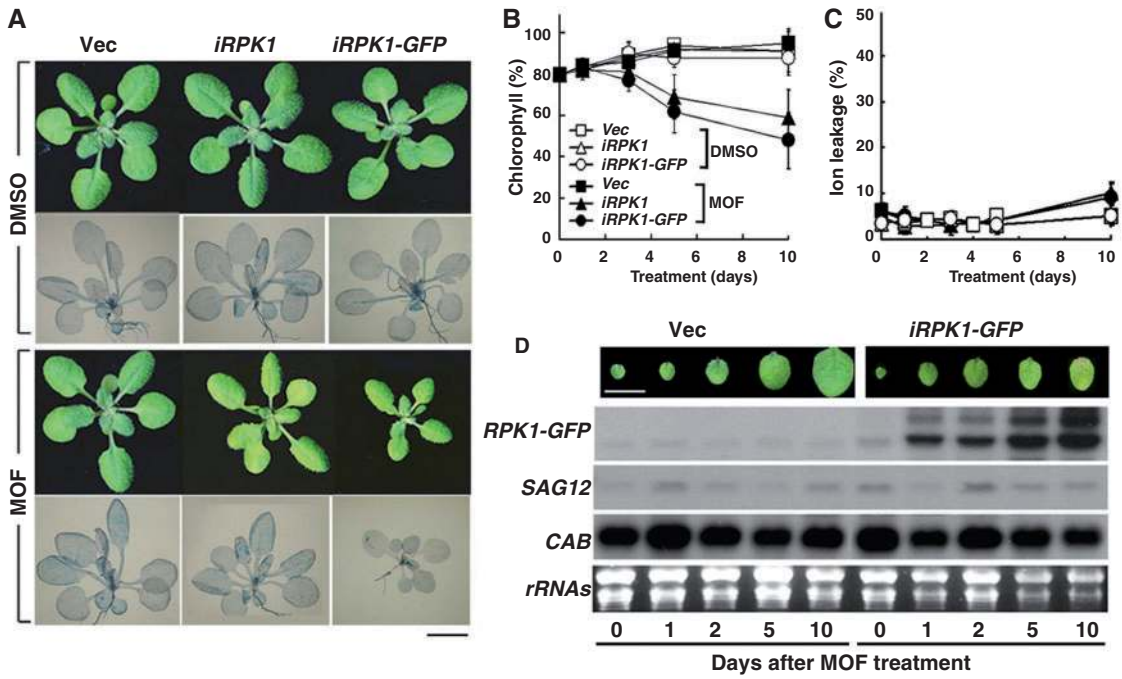

Fig. 7 Growth retardation following the induction of *RPK1* and *RPK1-GFP* expression in young plants. (A) Whole plant phenotypes of the inducible *RPK1* (*iRPK1*) and *RPK1-GFP* (*iRPK1-GFP*) transgenic plants. Vec, vector control. Two-week-old transgenic plants were treated with MOF or dimethylsulfoxide (DMSO). The photograph was taken 10 d after the treatment. Trypan blue staining showed no significant appearance of dying cells 10 d after induction of *RPK1* and *RPK1-GFP* expression. Measurement of Chl content (B) and membrane ion leakage (C) in the fourth rosette leaves of transgenic plants treated with MOF or DMSO. Error bars indicate the SD, $n = 8$. (D) The effect of RPK1 at the single-leaf level in *iRPK1-GFP* transgenic plants. No significant alterations in leaf yellowing and expression of *SAG12* and *CAB2* in the fourth rosette leaves were observed by 10 d after MOF treatment. Scale bar = 1 cm.

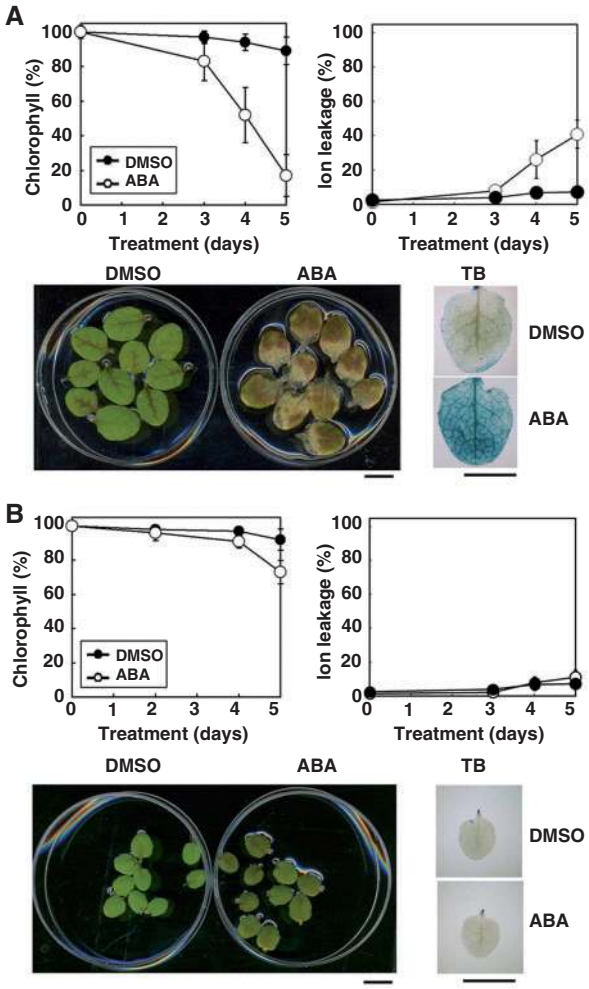

Fig. 8 Age-dependent differential effect of ABA on leaf senescence. The fourth rosette leaves of Arabidopsis (Ler) were carefully detached at 12 (A) and 4 (B) DAE and floated on MES buffer containing 50 μM ABA or dimethylsulfoxide (DMSO; control). Photos were taken after 5 days of treatment. TB, trypan blue staining. Error bars indicate the SD ($n = 10$). Scale bar = 1 cm.

biological regulatory mechanism, is an important factor in the regulation of plant senescence.

ABA-induced senescence is mediated by RPK1

ABA appears to have multiple functions in the plant. In addition to playing important roles in various aspects of plant development, including seed development, dormancy, germination and vegetative growth, ABA is also involved in the defense response to environmental stresses, such as drought, salinity, cold and pathogen infection (Leung and Giraudat 1998, Shinozaki and Yamaguchi-Shinozaki 2000, Finkelstein et al. 2002). ABA-mediated senescence is difficult to study because ABA affects such a wide variety of physiological events, including germination and seedling growth. The effects of ABA on later developmental stages, such as senescence, are thus masked by pleiotropic effects that occurred beforehand. This argument is also demonstrated by the pleiotropic effects that arise from inducing *RPK1* expression during the early development of the plant (**Supplementary Fig. S2**).

In this study, we demonstrated that RPK1 mediates ABA-induced senescence. The *rpk1* mutants exhibited greatly delayed progression of senescence in response to exogenously applied ABA compared with other hormones. This result, in conjunction with previous reports on the role of RPK1 in ABA signaling (Hong et al. 1997, Osakabe et al. 2005), strongly suggests that RPK1 is involved in ABA-mediated senescence signaling. This, in turn, suggests that ABA-induced senescence is not just a pleiotropic effect but a physiological event that is mediated by a specific signaling cascade.

The action of RPK1 on leaf senescence depends on age

RPK1 regulates diverse processes, including stomatal opening, plant growth, the stress response (Osakabe et al. 2005, Osakabe et al. 2010) and senescence. How does RPK1 control these diverse processes? Here, we demonstrated that the role of RPK1 varies with the age of the plant. Thus, RPK1 affects a variety of processes during the early development of the plant and regulates senescence at a later stage. How does RPK1 control senescence? Age-dependent up-regulation of this gene contributes to this control. However, up-regulation of *RPK1* expression is not solely responsible for RPK1-mediated senescence, since artificial up-regulation of *RPK1* at a young age did not result in senescence. Thus, RPK1 regulates senescence in conjunction with other age-related factors.

RPK1 regulates expression of senescence- and ABA-inducible genes

It was known that gene expression during age-dependent leaf senescence is under the control of a complex combination of hormonal pathways including ethylene, jasmonic acid, ABA and salicylic acid, and endogenous and exogenous signaling pathways (Buchanan-Wollaston et al. 2005). To understand RPK1 function in senescence signaling, we examined the expression patterns of typical senescence-associated genes in the leaves of *RPK1*-inducible transgenic plants (**Fig. 5**) and *rpk1* mutants (**Fig. 6**), respectively. As might be expected, most senescence-associated and ABA-inducible genes were greatly increased after induction of *RPK1*, whereas 70% of them showed significantly decreased expression in the *rpk1* mutants during age-dependent leaf senescence. Interestingly, the expression of genes possibly involved in hormone biosynthesis, such as *AtNCED2* for ABA biosynthesis (Tan et al. 2003) and *ACS2* for ethylene biosynthesis (Liang et al. 1992), appeared to be dependent on the function of RPK1. The expression of *ACS2* was previously reported to be induced by ABA treatment (Li et al. 2006). ABA transiently stimulates ethylene production in the mature leaf of citrus and tomato fruit (Riov et al. 1990). Moreover, previous studies suggested that ABA acts as an initiating agent of senescence, whereas ethylene exerts its effects at

a later senescence stage (Gepstrin and Thimann 1981). Taken together, these findings suggest that RPK1 functions as an important upstream regulator in the senescence pathway highly involved in ABA signaling. At the same time, it would be interesting to know whether RPK1 functions in ABA signaling through a positive feedback regulation of ABA biosynthesis and, more specifically, whether the transcriptional activation of *NCED2* could increase the endogenous ABA levels during age-dependent leaf senescence.

ABA exhibits age-dependent functional specification for senescence

The phytohormone ABA plays diverse roles in plants (Shinozaki and Yamaguchi-Shinozaki 2000, Ton et al. 2009). In this study, we showed that the role of ABA is differentially specified dependent on the developmental age of the plant. The effect of ABA on senescence is only clearly observed in older leaves. ABA-induced senescence is delayed in the *rpk1* mutant. Thus, at least some aspect of ABA-induced senescence is mediated by RPK1. However, ABA-induced senescence also requires other age-related factors, since the induction of *RPK1* expression is not sufficient to lead to senescence in young leaves.

It will be important to investigate which age-related factors are involved in RPK1- and ABA-induced senescence. Age-dependent functional specification was also reported for ethylene-induced senescence (Grbić and Bleecker 1995, Jing et al. 2005). Thus, age-dependent functional specification is a common method of functional diversification of these multifunctional hormones. It will be interesting to pinpoint which mechanisms link the actions of these hormones to the developmental age of the plant.

Materials and Methods

Plasmid constructs

To generate the *RPK1* overexpression vector, full-length *RPK1* or *RPK1-GFP* and *GFP* were placed between the CsVMV promoter and the *Nos* terminator in the CsV vector (Koo et al. 2004). To avoid the pleiotropic effects of constitutive *RPK1* expression, we employed an ecdysone agonist-inducible system (Koo et al. 2004) to limit *RPK1* and *RPK1-GFP* expression to the desired stages and conditions. Briefly, full-length *RPK1* in the pBluescript vector (Hong et al. 1997) was digested with *Sma*I and *Sal*I, and was then ligated into the same sites of the inducible vector, VGE/linker (Koo et al. 2004), to yield *iRPK1*. The eGFP-N3 (Clonetech) DNA was digested with *Sma*I and *Not*I, subjected to Klenow treatment and inserted into the blunt-ended *Xba*I site of *RPK1* to yield *iRPK1-GFP*. The resulting *RPK1-GFP* fusion gene was digested with *Sma*I and *Sal*I and ligated into the same site of the VGE/linker to yield VGE/RPK1–GFP. All constructs were confirmed by DNA sequencing.

Plant materials and growth conditions

Arabidopsis thaliana plants were grown in an environmentally controlled growth room at 22°C with a 16 h light/8 h dark cycle. The *rpk1-3* and *rpk1-4* mutants, which bear the gene-trap transposable *Ds* element, were screened from the Cold Spring Harbor Laboratory collection, and their insertions were confirmed by genomic PCR analysis using *RPK1*-specific primers, 5′-TGGTGGTCTCCACGACGAAGCTGCT-3′ and 5′-TGTATCA CAATCTAGAAGGCTGGATTCG-3′, together with *Ds*-specific primers, Ds5, 5′-ACGGTCGGGAAACTAGCTCTAC-3′ and Ds3, 5′-GGTTCCCGTCCGATTTCGACT-3′, according to standard protocols (Sundaresan et al. 1995, http://arabidopsis.info/imainfo.html). The *RPK1* overexpression lines, which were driven by the constitutively active *CsVMV* promoter, and transgenic plants expressing inducible *RPK1* (*iRPK1*) and *RPK1-GFP* (*iRPK1-GFP*) under the control of the ecdysone agonist-inducible promoter were generated by the floral dip method (Clough and Bent 1998). Homozygous *iRPK1* and *iRPK1-GFP* transgenic lines that reproducibly showed stable *RPK1* and *RPK1-GFP* induction and phenotypes and that were indistinguishable from the wild type in non-induced conditions were used in this study. To drive *RPK1* and *RPK1-GFP* expression, the inducible transgenic plants were treated once with 20 μM MOF by soil drenching 3 weeks after germination.

Artificially induced leaf senescence

To examine hormone-induced leaf senescence, the fourth rosette leaves of wild-type or mutant plants at 12 DAE were carefully detached and floated on 3 mM MES buffer (pH 5.8) that contained various concentrations of hormone, including 5 μM ethylene, 50 μM ABA and 50 μM MeJA. In the case of ethylene treatment, detached leaves were floated on MES buffer and incubated in a glass box containing ethylene gas. All chemical treatments were performed at 22°C under continuous lighting. For dark treatment, leaves were incubated in MES buffer at 22°C in darkness.

Measurement of chlorophyll (Chl) content, photochemical efficiency and ion leakage

Chl content and membrane ion leakage were measured as described previously (Kim et al. 2009).

Trypan blue (TB) staining

Lactophenol–TB staining was performed to visualize dying cells, similar to previous descriptions (Koch and Slusarenko 1990). Leaves were submerged in 0.05% lactophenol–TB solution [0.05% TB, 25% (w/v) lactic acid, 25% water-saturated phenol and 50% ethanol] at 37°C for 1 h. The samples were then washed in chloral hydrate solution (2.5 g ml^{-1}) to reduce the background.

RNA blot analysis

Total RNA was isolated from the fourth rosette leaves using Tri-Reagent (Molecular Research Center), according to the

manufacturer's instructions. For RNA blot analysis, 10 μg of RNA was denatured, separated on 1.5% agarose gels containing formaldehyde and transferred onto a Hybond-N membrane (Amersham Biosciences). The *RPK1*, *SAG12* and *CAB* DNA fragments were labeled with [^{32}P]dCTP by random priming, according to the manufacturer's instructions (Promega). The RNA blots were hybridized to their respective ^{32}P-labeled probes as described previously (Woo et al. 2001). After washing, the blots were exposed to X-ray film.

RT–PCR analysis

For RT–PCR analysis, first-strand cDNA was synthesized from 2 μg of RNA using the ImProm-II Reverse Transcription System, according to the manufacturer's protocol (Promega). The PCR primers and reaction conditions for amplification are given in **Supplementary Table S1**. All PCRs were performed for 20–30 cycles and were repeated twice.

Supplementary data

Supplementary data are available at PCP online.

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