Calmodulin 1 Regulates Senescence and ABA Response in Arabidopsis

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Cellular calcium acts as a second messenger and regulates diverse developmental events and stress responses. Cytosolic calcium has long been considered as an important regulator of senescence, however, the role of Ca\(^{2+}\) in plant senescence has remained elusive. Here we show that the Calmodulin 1 (CaM1) gene, which encodes Ca\(^{2+}\)-binding protein calmodulin 1, positively regulates leaf senescence in Arabidopsis. Yellowing of leaves, accumulation of reactive oxygen species (ROS), and expression of the senescence-associated gene 12 (SAG12) were significantly enhanced in CaM1 overexpression plants. In contrast, abscisic acid (ABA)-triggered ROS production and stomatal closure were reduced in amiRNA-CaM1 plants. We found a positive-feedback regulation loop among three signaling components, CaM1, RPK1, and RbohF, which physically associate with each other. RPK1 positively regulates the expression of the CaM1 gene, and the CaM1 protein, in turn, up-regulates RbohF gene expression. Interestingly, the expression of CaM1 was down-regulated in rbohD, rbohF, and rbohD/F mutants. We show that CaM1 positively regulates ROS production, leaf senescence, and ABA response in Arabidopsis.

Keywords: calmodulin 1, NADPH oxidase, reactive oxygen species, RPK1, senescence

INTRODUCTION

Leaf senescence is the terminal stage of leaf development and is genetically programmed. Apparent morphological changes involved in leaf senescence include the yellowing of leaves caused by the degradation of chlorophyll, followed by reduction in photosynthesis and protein synthesis. During senescence, the metabolism and structure of leaf cells continuously change to effectively utilize plant nutrients for the developing parts of the plant, including young leaves, seeds, and fruits (Lim et al., 2007).

Calcium is a universal second messenger that exerts an allosteric effect on many enzymes and proteins in various cellular responses. In plants, calcium signaling is evoked by endogenous and environmental cues, such as drought, salt or osmotic stresses, temperature, light, and plant hormones (Dodd et al., 2010; Steinhorst and Kudla, 2014; Edel and Kudla, 2016; Ranty et al., 2016). Ca\(^{2+}\) ions appear to play an important role in plant senescence as well. For instance, exogenously supplied Ca\(^{2+}\) delays the senescence of a detached leaf (Poovaiah and Leopold, 1973), and the Ca\(^{2+}\) ionophore A23187 rescues MeJA-mediated leaf senescence (Chou and Kao, 1992). Moreover,

Abbreviations: ABA, abscisic acid; CaM1, calmodulin 1; DAB, dianminobenzidine; H\(_2\)DCF-DA, dichlorofluorescein diacetate; H\(_2\)O\(_2\), hydrogen peroxide; MeJA, methyl jasmonate; NBT, nitroblue tetrazolium; NO, nitric oxide; RbohF, reactive burst oxidase homolog F; RPK1, receptor-like potein kinase 1; SAG12, senescence-associated gene 12.
Ca\textsuperscript{2+}-mediated (NO production negatively regulates the expression of senescence-associated genes and production of H\textsubscript{2}O\textsubscript{2} during the initiation of leaf senescence (Ma et al., 2010). Calmodulin (CaM), a small Ca\textsuperscript{2+}-binding protein, is one of the major calcium sensor proteins conserved in eukaryotes (Chin and Means, 2000; McCormack et al., 2005). Ca\textsuperscript{2+} binding to CaMs causes a conformational change in the protein structure, thereby modifying its interaction with various target proteins, which leads to the transduction of Ca\textsuperscript{2+} signals (Zielinski, 1998, 2002). In Arabidopsis, seven genes encode four CaM isoforms: CaM1/CaM4, CaM2/CaM3/CaM5, CaM6, and CaM7 (McCormack et al., 2005). Four amino acid substitutions differentiate CaM7 from CaM2/CaM3/CaM5, and one amino acid substitutions differentiate CaM7 from CaM2/CaM3/CaM5, and CaM6. CaM7 is a transcriptional regulator that directly interacts with the promoters of several light-inducible genes, contributing to the regulation of photomorphogenesis (Kushwaha et al., 2008).

Receptor-like Protein Kinase 1 (RPK1) localizes to the plasma membrane and plays an important role in embryo development, plant growth, ABA signaling, and stress responses (Hong et al., 1997; Osakabe et al., 2005, 2010; Hodin et al., 2007; Hodin and Tax, 2008). Additionally, RPK1 positively regulates leaf aging (Lee et al., 2011). The RbohF, an NADPH oxidase, produces reactive oxidation species (ROS) in response to biotic and abiotic stresses in Arabidopsis (Torres et al., 2002; Kwak et al., 2003; Joo et al., 2005; Desikan et al., 2006; Zhang et al., 2009). The presence of EF-hand Ca\textsuperscript{2+} binding motifs on NADPH oxidases has suggested a regulatory effect of Ca\textsuperscript{2+} on the enzymatic activity (Torres et al., 1998, 2002), which was further supported by the finding that Ca\textsuperscript{2+} and phosphorylation synergistically activate RbohD NADPH oxidase (Ogasawara et al., 2008). Furthermore, it has been recently shown that RbohF is responsible for RPK1-mediated ROS production, senescence-associated gene expression, and age-induced cell death (Koo et al., 2017). Several lines of evidence have suggested that cytosolic Ca\textsuperscript{2+} acts as a regulator of senescence in plant, but how senescence is regulated by cytosolic Ca\textsuperscript{2+} remains elusive. Here, we report that Arabidopsis CaM1 plays a positive role in RPK1-mediated leaf senescence and ABA response. Senescence phenotypes, including leaf yellowing, ROS accumulation, and expression of the SAG12 were drastically promoted in CaM1-overexpressing plants, whereas plants expressing amiRNA-CaM1 showed no visible senescence phenotype, which is likely to be due to functional redundancy in the calmodulin gene family. Expression analyses revealed that CaM1 is positively regulated by RPK1, RbohF by CaM1, and CaM1 by RbohD and RbohF, suggesting a positive-feedback loop among the three signaling components at the transcriptional level.

RESULTS

**CaM1 Gene Expression Is Associated With Aging**

To identify calmodulin genes that are involved in plant senescence, in silico analysis using publicly available microarray data was conducted and showed that the expression of Arabidopsis CaM genes can be divided into two groups during leaf senescence: age-dependent increase (CaM1, CaM3, and CaM4) and age-dependent decrease (CaM2, CaM5, and CaM7) (no data was available for CaM6; Figure 1A) (Schmid et al., 2005). This implies that CaM1, CaM3, and CaM4 probably play a positive role in Arabidopsis leaf senescence. To verify the microarray data, we examined age-dependent expression pattern of the CaM genes: The fourth rosette leaves of Arabidopsis plants were collected every 3 days starting with 13-day-old plants and transcript levels of the CaM1, CaM2, and CaM4 genes were analyzed using quantitative RT-PCR (Figure 1B). We found that CaM1 and CaM4 were indeed up-regulated in the older leaves compared with the younger leaves. The expression pattern was similar to that of the leaf senescence marker, SAG12 whose expression gradually increased from 13 days after sowing in an age-dependent manner (Figure 1C) (Woo et al., 2001). By contrast, the expression of CaM2 was down-regulated (Figure 1B).

In silico analysis showed that the expression level of CaM1 was similar to that of CaM4 in most of the tissues, except for reproductive organs (Supplementary Figures S1A,B), which was confirmed by qRT-PCR analyses (Supplementary Figure S1C). CaM1 is highly expressed in pollen whereas CaM4 is highly expressed in dry seeds (Supplementary Figures S1A,B). To verify the expression pattern of CaM1, at least 10 independent pCaM1::GUS transgenic lines were subjected to GUS histochemical analysis. Data revealed that CaM1 was highly expressed in vascular bundles of cotyledons, roots, and both rosette and cauline leaves (Figures 2A–E). CaM1 was also detected in reproductive tissues, including flowers and siliques (Figures 2F–K). Dissection of stained flowers revealed that CaM1 was expressed in sepals, petals, carpels and filaments, but not in anthers (Figures 21–K). Interestingly, CaM1 was highly expressed in guard cells (Figure 2B), suggesting a role of CaM1 in stomatal development and/or movement.

**Leaf Senescence Is Accelerated in CaM1-Overexpressing Plants**

Previously, we have shown that CaM4 plays a role in RPK1-mediated leaf senescence in Arabidopsis (Koo et al., 2017). Thus, CaM1 was chosen to investigate its role in plant senescence. As a first step, transgenic plants expressing 35S::CaM1-GFP were generated. Western blot analyses using anti-GFP antibody showed enhanced accumulation of CaM1 in the transgenic plants (Figure 3A). Leaves of three independent 35S::CaM1-GFP transgenic lines turned yellow earlier than the WT (Figures 3B,C). No other growth abnormalities including seed size and mass were observed in the 35S::CaM1-GFP transgenic lines compared with the WT (Supplementary Figure S2). It is known that the production of ROS is induced during senescence in both animals and plants (Lim et al., 2007; Vigneron and Vousden, 2010). Thus, we monitored the accumulation of H\textsubscript{2}O\textsubscript{2} and superoxide, respectively in the leaves of WT and 35S::CaM1-GFP transgenic lines at different developmental stages using DAB and NBT staining. We found that ROS accumulation...
FIGURE 1 | Expression profiles of CaM family members in leaves during senescence. (A) Expression of the CaM genes in rosette leaves, including leaves undergoing senescence 35 days after sowing. Data corresponding to three arrays for each data point were obtained from Genevestigator (https://genevestigator.com/gv/). (B) qRT-PCR data showing the expression of CaM1, CaM2, and CaM4 genes in the fourth rosette leaves at the indicated time points. (C) qRT-PCR analysis of SAG12 expression in the fourth rosette leaves of WT plants. The inset shows SAG12 expression until 22 days after sowing (DAS). Expression levels of genes were normalized to Actin2. Error bars represent means ± SEM of three independent experiments.

FIGURE 2 | Visualization of CaM1 expression in Arabidopsis transgenic plants harboring a CaM1pro-GUS construct. Histochemical GUS staining images of seedlings (A,B), roots (C), cauline leaves (D), rosette leaves (E), siliques (F), flowers (G,H), petals (I), anthers (J), and sepals (K) are shown. Arrowheads indicate GUS staining in the guard cells of leaf (B) and in filaments (J). Scale bar, 1 cm.
was detected earlier in the transgenic plants than in WT (Figures 3D–F). Higher accumulation of H$_2$O$_2$ and superoxide was detected at 16 and 19 days, respectively, in the transgenic plant leaves after sowing (Figures 3E,F).

To further examine the role of CaM1 in leaf senescence, we generated transgenic plants in which the expression of CaM1 was knocked down using an artificial microRNA targeting CaM1 (amiRNA-CaM1). In the amiRNA-CaM1 transgenic lines, the transcript levels of CaM2, CaM3, and CaM4 were not significantly changed, whereas CaM1 transcript level was significantly down-regulated compared with the WT (Figures 4A,B). This result implies that the amiRNA-CaM1 specifically down-regulates CaM1 transcripts, allowing us to examine cellular responses that CaM1 particularly mediates.

qRT-PCR analyses showed that early leaf senescence phenotype of the 35S::CaM1-GFP transgenic lines was strongly correlated with the drastically enhanced expression of senescence markers SAG12, SIRK, ATG2, and ATG5 compared with the WT (Figure 4C and Supplementary Figure S3A). This suggests that CaM1 overexpression results in various senescence-associated changes, including yellowing of leaves, enhanced production of H$_2$O$_2$ and superoxide, and up-regulation of the senescence marker genes. In contrast, no altered senescence phenotypes were observed in the amiRNA-CaM1 transgenic lines, and the expression level of SAG12, SIRK, ATG2, and ATG5 in the amiRNA-CaM1 transgenic lines was comparable with that of the WT (Figure 4D and Supplementary Figure S3B). cam4 knockout mutants displayed no altered senescence phenotype.
FIGURE 4 | The expression of senescence marker genes is accelerated in the 3SS::CaM1-GFP transgenic lines. (A,B) Expression analysis of CaM genes in T3 homozygous 3SS::amiRNA-CaM1 lines by RT-PCR (A) and qRT-PCR (B). (C,D) qRT-PCR analysis of senescence-related genes in 3SS::CaM1-GFP transgenic lines (C) and 3SS::amiRNA-CaM1 plants (D). Expression levels of the genes were normalized to Actin2. Error bars represent means ± SEM of three independent experiments. Statistical analysis was performed using heteroscedastic t-test (**p < 0.01).

(Supplementary Figures S3C,D). This result suggests that there is functional redundancy between CaM1 and CaM4 and that CaM1/CaM4 functions as a positive regulator of age-dependent leaf senescence.

**CaM1 Positively Regulates ABA-Induced ROS Production**

We found that the production of H$_2$O$_2$ and superoxide was increased in the 3SS::CaM1-GFP transgenic plants during leaf senescence (Figure 3C). Since ABA is also known to induce ROS production (Pei et al., 2000; Kwak et al., 2003) as well as leaf senescence (Zhao et al., 2016), we examined whether CaM1 is linked to ABA-mediated ROS production. The expression of CaM1 was induced within 30 min of ABA treatment, reaching a peak at 3 h (Figure 5A). We also determined ROS levels in leaves of 3-week-old plants before and after the ABA treatment using DAB and NBT staining. The steady-state H$_2$O$_2$ level in 3SS::CaM1-GFP plants was higher than in WT plants prior to the ABA treatment (Figure 5B). Although ABA...
FIGURE 5 | CaM1 positively regulates ABA-induced ROS production. (A) qRT-PCR analysis reveals that the expression of CaM1 is induced 0.5 h after ABA treatment (50 µM). Actin2 was used as an internal control. (B,C) Quantitative analyses of ROS staining in the fourth rosette leaves of 3-week-old plants expressing 35S::CaM1-GFP (B) and 35S::amiRNA-CaM1 (C). Leaves were treated with 100 µM ABA for 1 h and stained with DAB and NBT to visualize accumulation of H$_2$O$_2$ and superoxide, respectively. Leaves without ABA treatment (mock) were used as a control. Error bars represent means ± SEM of two independent experiments. Each data point represents 10 leaves. Statistical analysis was performed using heteroscedastic t-test (*p < 0.05; **p < 0.01).

induced H$_2$O$_2$ and superoxide production in both WT and the 35S::CaM1-GFP transgenic plants, we found a higher production of H$_2$O$_2$ and superoxide in the 35S::CaM1-GFP transgenic plants (Figure 5B). By contrast, ABA-induced ROS production was reduced in amiRNA-CaM1 transgenic lines (Figure 5C). These results imply that CaM1 positively regulates ABA-induced ROS production.

Stomatal aperture is one of the targets of ABA-mediated signaling in plants. Furthermore, ROS have been shown to act as a positive regulator of ABA signaling in guard cells (Pei et al., 2000; Kwak et al., 2003). Thus, we investigated whether CaM1 functions in guard cell ABA response. We determined ROS levels in guard cells of the fourth rosette leaves harvested from 25-day-old plants before and after ABA.
FIGURE 6 | CaM1 mediates ABA-induced ROS production in guard cells and stomatal closure. (A,B) ABA-induced ROS accumulation in guard cells of 35S::CaM1-GFP plants (A) and of the 35S::amiRNA-CaM1 transgenic plants (B). ROS levels were measured 30 min after ABA treatment (50 µM) using 2′,7′-dichlorofluorescein diacetate. Error bars represent means ± SEM of three independent experiments (n = 50). (C,D) ABA (5 µM), CaCl₂ (2 mM), and H₂O₂ (100 µM)-induced stomatal closure in 35S::CaM1-GFP plants (C) and amiRNA-CaM1 plants (D) (n = 100). (E) Germination rate of WT (Ler) and transgenic amiRNA-CaM1 L2 and L9 seeds were measured after 7 days of incubation on MS medium containing 0, 0.5, 1, or 2 µM ABA without sucrose. Error bars represent means ± SEM of three independent experiments. Statistical analysis was performed using heteroscedastic t-test (*p < 0.05; **p < 0.01).

**treatment. H₂O₂ mainly detects H₂O₂ and can be easily quantitated in guard cells (Kwak et al., 2003). The steady-state ROS level in 35S::CaM1-GFP guard cells was higher than in WT guard cells (Figure 6A). Although no significant differences were detected between WT and the 35S::CaM1-GFP transgenic lines after ABA treatment (Figure 6A), it is likely to be due to the saturation of DCF fluorescence in the cells analyzed. By contrast, ABA failed to trigger ROS production in guard cells of amiRNA-CaM1 transgenic plants (Figure 6B). Overexpression of CaM1 enhanced ABA-induced stomatal closure, whereas down-regulation of CaM1 inhibited ABA-induced stomatal closure (Figures 6C,D). These results imply that CaM1 positively regulates ABA-induced ROS production in guard cells, thereby modulating stomatal movements.

Calcium and H₂O₂ are second messengers that induce stomatal closure (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003). In the CaM1 overexpression plants, calcium- and H₂O₂-induced stomatal closure was enhanced (Figure 6C). In contrast, down-regulation of CaM1 reduced the calcium- and H₂O₂-induced stomatal closure (Figure 6D), implying that CaM1 functions in calcium- and H₂O₂-mediated stomatal closure. More importantly, even though there is functional redundancy between CaM1 and CaM4 in leaf senescence (Figure 4 and Supplementary Figure S3) and ABA-mediated seed germination.
CaM1 appears to play a unique role in the regulation of ROS production in guard cells.

Positive-Feedback Regulation Among RPK1, CaM1, and RbohF

Peptide sequence alignments of CaM proteins revealed a high level of sequence similarity (Supplementary Figure S4). Phylogenetic analysis indicated that CaM1 is closest to CaM4 (Supplementary Figure S5). Although the sequence similarity between CaM1 and CaM4 coding sequences was 89.56% at the nucleotide level, the amino acid sequences encoded by these genes were identical (Supplementary Figure S4). We have previously shown that CaM4 physically interacts with RPK1 and RbohF (Koo et al., 2017). To test whether CaM1 also acts with RPK1 and RbohF and to investigate potential regulation among RPK1, CaM1, and RbohF at the transcriptional level, we examined the expression of CaM1 in rpk1-5 null mutants and iRPK1 transgenic plants in which the expression of RPK1 is under the control of ecodyose-inducible promoter (Lee et al., 2011). In rpk1-5, the expression of CaM1 was significantly down-regulated in leaves of 25-day-old plants (Figure 7A; p < 0.01). By contrast, RPK1 expression was notably up-regulated in the iRPK1 plants when treated with methoxyfenozide, the chemical inducer of the promoter (Padidam, 2003) (Figure 7B). These results indicate that the expression of CaM1 is positively regulated by RPK1. However, no significant changes in the expression of RPK1 were detected in the 35S::CaM1-GFP or 35S::amiRNA-CaM1 transgenic lines (data not shown), suggesting that RPK1 is not transcriptionally regulated by CaM1.

In addition, we found that the expression of RbohF was up-regulated in the 25-day-old 35S::CaM1-GFP transgenic plants, whereas the expression of RbohD was not altered (Figure 7C). Interestingly, the expression of CaM1 was down-regulated in rbohD, rbohF, and rbohD/F double mutants (Figure 7D). Taken together, these data suggest a positive-feedback loop among the CaM1, RbohF, and RPK1 genes at the transcriptional level (Figure 7E).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

T-DNA insertional line GK-309E09 (cam4, AT1G66410) was obtained from the Arabidopsis Biological Resource Center. Seeds of wild type (WT) Arabidopsis, cam4, and
**Constructs and Plant Transformation**

Artificial microRNA of CaM1 (amiRNA-CaM1) was designed by WMD3\(^\dagger\). Primers, including attB1-oligo A, attB2-oligo B, CaM1-1-miR-s, CaM1-1-miR-a, CaM1-III-miR’s, and CaM1-IV miR’\(a\) were used to PCR amplify on pRS300 (Schwab et al., 2006). The PCR product, amiRNA-CaM1 was first cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo (12535-035, Invitrogen) and then into the pMDC32 vector using Gateway LR cloning (Gateway\(^\text{TM}\) LR Clonase\(^\text{II}\) Enzyme mix, 11791-100, Invitrogen). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2, and the coding region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB. The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003). The promoter region of CaM1 was amplified by pair of primers CaM1p-attB1 and CaM1p-attB2. The PCR products were cloned into the Entry vector pDONR\(^\text{TM}\)/Zeo and then into the pMDC162 vector for pCaM1::GUS and pMDC43 for 35S::CaM1::GFP (Clough and Bent, 2003).

**RT-PCR Analysis**

Total RNA was extracted from WT and transgenic lines using the TRIzol reagent (15596-026, Invitrogen) and subsequently used for cDNA synthesis using RevertAid Reverse Transcriptase kit (#EP0441, Thermo). The primer pairs CaM1-qs and CaM2-qa, CaM2-qs and CaM1-qa, CaM4-qs/-qa, RPK1-qs/-qa, RP1-qs/-qa, SAG12-qs/-qa, SIRK-qs/-qa, ATG2-qs/-qa, ATG5-qs/-qa, RbohD-qs/-qa, and RbohF-qs/-qa were used to examine the expression of CaM1, CaM2, CaM4, RPK1, SAG12, SIRK, ATG2, ATG5, RbohD, and RbohF, respectively, using qPCR. Actin2 amplified using the primer pair Actin-1/-2 was used as an internal positive control.

For semi-quantitative RT-PCR, equal amounts of first-strand cDNAs were used as templates for PCR amplification. The primer pairs CaM1-s/-a, CaM2-s/-a, CaM3-s/-a, and CaM4-s/-a were used to analyze the expression pattern of CaM1, CaM2, CaM3, and CaM4, respectively, in the different amiRNA-CaM1 transgenic lines. The Actin2 gene was used as an internal control. The primer sequences used for qRT and RT-PCR analysis are provided in Table 2.
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Diaminobenzidine (DAB) and Nitroblue Tetrazolium (NBT) Staining

For all histochemical staining experiments, fourth rosette leaves were detached at different time points (13, 16, 19, 22, and 25 days) and treated with 100 µM ABA, 100 µM H$_2$O$_2$, or 2 mM CaCl$_2$ for one hour. DAB and NBT staining were performed as described (Lee et al., 2011). The leaves were submerged in NBT solution [1 mg/mL (NBT, N5514, Sigma), 10 mM Na$_2$S$_3$, and 10 mM potassium phosphate, pH 7.8] or DAB solution [1 mg/ml 3,3’-DAB, D12384, Sigma], pH 3.8] and stained for 30 min at room temperature. Samples were then boiled in 95% ethanol for 10 min and stored in 60% glycerol. After staining, all samples were mounted on slides and photographed using a digital camera (G12, Canon). To quantify DAB and NBT staining, the stained pixels were obtained from 6 to 12 leaves per genotype using the channels function in Adobe Photoshop and their intensity was measured using the TotalLab100 program (Nonlinear Dynamics).

Stomata Movement Assay

Stomatal assays were performed as described (Desikan et al., 2002). Briefly, epidermal strips were prepared from 3- to 4-week-old rosette leaves and floated abaxial side down in 10 mM MES buffer (pH 6.15) containing 20 mM KCl for 2.5–3 h under white light (95 E m$^{-2}$O$^{-2}$ or 2 mM CaCl$_2$) to open the stomata. Following this, 100 µM H$_2$O$_2$ or 2 mM CaCl$_2$ was added to the buffer and incubated for another 1–2 h to assay stomatal closure. Pictures of stomata were taken using a microscope equipped with a digital camera (Axiovert 200, Zeiss). The guard cell aperture was analyzed using Image J.

Seed Germination Assay

Dry seeds were collected and stored in a dehumidifier cabinet for at least 2 months before testing the germination capacity of seeds. Seeds were surface-sterilized using 20% commercial Clorox bleach for 15 min, and washed five times with sterile water. At least 100 sterilized seeds were plated on MS medium (MSP02-10LT, Caisson) containing 0.8% (w/v) Bacto Agar (Difco, BD) supplemented with different concentrations (0.5, 1, 2 µM) of ABA solution (A4906, Sigma-Aldrich) prepared in methanol. Seeds plated on MS media without ABA were used as control. Seeds were stratified in the dark at 4°C for 3 d and then transferred to the growth chamber maintained at 22°C with a 16 h light/8 h dark photoperiod. Germination was defined as the first sign of emergence of green cotyledons and scored daily for 7 days. The germination results were calculated based on at least three independent experiments.

Histochemical Staining

Positive transgenic plants were identified using GUS staining as described (Jefferson et al., 1987). Pictures were taken using a digital camera (G12, Canon) or microscope (STEMI SV6, Zeiss) equipped with a digital camera.

Phylogenetic and Alignment Analysis

Homolog sequences of Zea mays (gi|162463080, gi|162463001, gi|162462264, gi|162464382, gi|226490894, gi|226494641, gi|226507438, gi|226507713, gi|226958443, and gi|293334895), Glycine max (gi|35721559, gi|310563, gi|35721835, gi|35722047, gi|170072, gi|170076, and gi|170074), Chlamydomonas reinhardtii (gi|159490918 and gi|158280344), Acyrthosiphon pisum (gi|209870032), Triticum aestivum (gi|291246022), Brassica napus (gi|497992), B. juncea (gi|899058), B. oleracea [gi|374922809, gi|374922811, gi|374922813, and gi|374922807], Hevea brasiliensis (gi|313767030), Arabidopsis thaliana (CaM1 to CaM7), Medicago truncatula (gi|355509904 and gi|357477127), Vitis vinifera (VIT_18s0001g03880.101) and Oryza sativa [LOC_Os05g41200.1 (OsCaM9), LOC_Os07g48780 (OsCaM1-1), LOC_Os03g20370 (OsCaM1-2), LOC_Os01g16240 (OsCaM1-3), LOC_Os11g3980 (OsCLM2) and gi|17066588] were obtained from NCBI or ABRC (see footnote 2). Sequence alignments were generated using Clustal Omega. Alignments between different species were adjusted for the construction of phylogenetic tree. Neighbor-joining analyses were performed using MEGA5 (Tamura et al., 2011) with pair-wise deletion, Poisson correction set for the distance model and 1,000 bootstrap replicates.

Analysis of Seed Yield

Average seed mass was determined by weighing 1,000 dry seeds. The weight of at least five independent batches was measured. To analyze relative seed size, seed images were captured on an Epson V370 scanner with the supplied software, which were then analyzed by SmartGrain software (Tanabata et al., 2012). More than 200 seeds per plant were analyzed.

DISCUSSION

In this report, we investigated the function of the senescence-induced gene CaM1. Overexpression of CaM1 in WT plants enhanced several aspects of age-dependent senescence, including chlorophyll content, ROS production, and expression of senescence marker genes (Figures 3, 4). These results suggest that CaM1 acts as a positive regulator of age-dependent leaf senescence. Our previous study has shown that Ca$^{2+}$ induces the expression of the senescence marker gene SIRK via CaM4 (Koo et al., 2017). In this study, we found enhanced senescence phenotypes in CaM1-overexpressing plants (Figure 3). These two studies demonstrate that CaM1/CaM4 together with Ca$^{2+}$ positively regulate age-dependent leaf senescence. On the contrary, calcium signaling plays a negative role in MeJA- and NO-mediated leaf senescence (Chou and Kao, 1992; Ma et al., 2010). Thus, it is plausible that other members of the CaM gene family might function in the process of leaf senescence. Differences between the expression profiles of various CaM genes and antagonistic functions of calcium signaling in leaf senescence

Footnotes:

1. http://www.ncbi.nlm.nih.gov/
2. http://www.ebi.ac.uk/Tools/msa/clustalo/
suggest that CaM proteins fine-tune the balance of calcium signaling in leaf senescence.

**CaM1 Functions in RPK1-Mediated Leaf Senescence**

Receptor-like protein kinase 1 is a plasma membrane-localized receptor kinase (Osakabe et al., 2005) and plays multiple roles in various cellular signaling and developmental processes, including embryo development (Nodine et al., 2007; Nodine and Tax, 2008), plant growth, stomatal opening, stress response (Hong et al., 1997; Osakabe et al., 2005, 2010), and senescence (Lee et al., 2011; Koo et al., 2017). How RPK1 controls these diverse cellular processes may be partly explained by the proteins interacting with RPK1 in those processes. It has been suggested that calcium and CaM play a role in receptor kinase-mediated cellular processes (Oh et al., 2012). Previous studies have shown that RPK1 interacts and phosphorylates a serine residue on CaM4 (Koo et al., 2017). The expression of CaM1 and CaM4 was induced during leaf aging (Figure 5B), and knockdown of CaM1 or CaM4 did not affect leaf senescence (Figure 4 and Supplementary Figure S3). These data indicate that CaM1 and CaM4 are functionally redundant in RPK1-mediated leaf senescence. Ectopic expression of RPK1 in Arabidopsis at a young developmental stage did not result in senescence (Lee et al., 2011), and CaM1 overexpression did not result in detectable senescence phenotypes at early developmental stages (16-day-old plants; Figure 3D), suggesting that the RPK1–CaM1 module regulates senescence in conjunction with other senescence-related components.

**CaM1 Positively Regulates ROS Production**

Cellular ROS homeostasis and signaling are crucial parts of signaling networks in plants (Miller et al., 2009). Several lines of evidence show crosstalk between ROS and calcium signaling. For example, NADPH oxidase RHD2/RbohC produces ROS that facilitate pyrophosphatase-mediated activation of CaM2, CaM4, and CaM6 activates NAD kinase and phosphodiesterase with different kinetics in vitro (Liao et al., 1996; Reddy et al., 1999; Kohler and Neuhaus, 2000). CaM2, CaM4, and CaM6 activates NAD kinase and phosphodiesterase with different kinetics in vitro (Liao et al., 1996; Reddy et al., 1999), and CaM2 and CaM4 bind to cyclic nucleotide-gated ion channels and a kinesin-like motor protein with different affinities (Reddy et al., 1999; Kohler and Neuhaus, 2000). These findings suggest that CaMs may exert different functions through their binding targets that may be located in different cellular compartments.

ROS modulate activities of target proteins or expression of genes by changing the redox state in the cell (Schmidt and Schippers, 2015; Saxena et al., 2016). A recent study has shown that the ratio between superoxide and H2O2 in roots determines the cell fate of either differentiation or proliferation, in which NADPH oxidases appear to function (Tsukagoshi et al., 2010). It would be interesting to test whether the ratio between different kinds of ROS also plays a role in age-dependent cell death in leaves. Furthermore, identification of the direct cellular targets of ROS will further shed light on our understanding of ROS-mediated plant senescence.

**Diversified Functions of CaMs With High Level of Sequence Similarity**

Peptide sequence alignments of CaM proteins and phylogenetic analysis revealed a high level of sequence similarity among the family members (Supplementary Figures S4, S5). Among the seven genes encoding CaM in Arabidopsis, CaM7 is the least related genes with others (Supplementary Figure S4), but only one and four amino acid substitutions differentiate CaM7 from CaM2/CaM3/CaM5 and CaM1/CaM4, respectively. One possible explanation for the conservation of Arabidopsis CaM genes is that CaMs share similar functions. Alternatively, different CaM genes might have evolved distinct expression patterns or regulatory behaviors to ensure important cellular functions. Recent evidence supports the latter. An expression analysis using the publicly available data shows that spatial expression of six CaM genes is differentially regulated (Supplementary Figure S1), and biochemical analysis indicates functional non-redundancy in CaMs (Liao et al., 1996; Reddy et al., 1999; Kohler and Neuhaus, 2000). CaM2, CaM4, and CaM6 activates NAD kinase and phosphodiesterase with different kinetics in vitro (Liao et al., 1996; Reddy et al., 1999), and CaM2 and CaM4 bind to cyclic nucleotide-gated ion channels and a kinesin-like motor protein with different affinities (Reddy et al., 1999; Kohler and Neuhaus, 2000). These findings suggest that CaMs may exert different functions through their binding targets that may be located in different cellular compartments.

The expression analysis shows that CaM genes encoding identical proteins (CAM2/CAM3/CAM5 and CAM1/CAM4) share a similar expression pattern (Supplementary Figure S1), implying functional redundancy in the closest paralogs, as observed in the senescence phenotype of the amiRNA-CaM1 and cam4 plants (Figure 4). Nevertheless we identified a specific role for CAM1 in the ABA-mediated ROS production and stomatal closure (Figures 5, 6). Fine-tuning of gene expression in a cell type-specific or stimulus-specific manner and/or different post-transcriptional regulation between CAM1 and CAM4 could diversify their functions. Interestingly, potential miRNA target sites on CAM1 differ from them on CAM4 (Adai et al., 2005). Furthermore, various forms of splicing variants of CAM1 are distinct from those of CAM4. It would be of interest to figure out the molecular mechanism that renders the specificity of each CaM gene in plants.

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http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi

http://www.arabidopsis.org
AUTHOR CONTRIBUTIONS

JK and CD conceived the study and designed the experiments. CD and IL performed the experiments and analyzed the data with YL, HN, and JK. CD, YL, HN, and JK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00803/full#supplementary-material

FIGURE S1 | The expression pattern of CaM genes. (A) Expression of the CaM family genes in different tissues. Data were obtained from ePF-Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Log2-transformed read counts without normalization were used in the computation. (B) Relative expression of CaM1 compared to CaM4 (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The result indicates the expression of CaM1 and CaM4 are almost identical in most of the tissues, except CaM1 is highly expressed in pollen while CaM4 highly expressed in dry seeds. (C) qRT-PCR analysis of CaM1 and CaM4 in different plant tissue. Expression levels of the genes were normalized to Actin2. Error bars represent means ± SEM of three independent experiments.

FIGURE S2 | Overexpression of CaM1-GFP has no effect on seed size and mass. (A) Representative images of mature seeds of WT and 35S::CaM1-GFP transgenic plants. (B, C) The relative seed size (B) and weight of 1,000 seeds (C) were measured in WT and 35S::CaM1-GFP transgenic plants. Error bars represent means ± SEM (n = 6). Scale bars, 5 mm.

FIGURE S3 | Functional redundancy between CaM1 and CaM4 in age-dependent leaf senescence. (A, B) qRT-PCR analysis of senescence related genes in 35S::CaM1-GFP transgenic lines (A) and 35S::amiRNA-CaM1 plants (B). Expression levels of the genes were normalized to Actin2. Error bars represent means ± SEM of three independent experiments. (C) Senescence phenotypes of WT and cam4. (D) Survival rates of WT and cam4. Plants with third and fourth rosette leaves showing complete yellowing were counted in WT (n = 4) and cam4 (n = 8), in each experiment, 21–25 plants were analyzed. Statistical analysis was performed using heteroscedastic t-test (*p < 0.01). Scale bars, 1 cm.

FIGURE S4 | Protein sequence alignment of the calmodulin family. The EF-hand motifs of CaM4 were conserved across various homologs, including Zea mays (gi|162462264), Glycine max (gi|351721559), Chlamydomonas reinhardtii (gi|167411, gi|159009198), Acyrthosiphon pisum (gi|200870032), Trichum aestivum (gi|291246022), B. napus (gi|497992), B. juncea (gi|690058), B. oleracea (gi|374922807), Hevea brasiliensis (gi|313767030), Arabidopsis thaliana (CaM2 to CaM7), and Oryza sativa (gi|170665888). Black lines indicate EF-hand motifs. Red asterisks indicate phosphorylation sites.

FIGURE S5 | Phylogenetic analysis of the calmodulin family proteins.

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