AHK3-Mediated Cytokinin Signaling Is Required for the Delayed Leaf Senescence Induced by SSPP

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Abstract: Leaf senescence is a highly-programmed developmental process regulated by an array of multiple signaling pathways. Our group previously reported that overexpression of the protein phosphatase-encoding gene SSPP led to delayed leaf senescence and significantly enhanced cytokinin responses. However, it is still unclear how the delayed leaf senescence phenotype is associated with the enhanced cytokinin responses. In this study, we introduced a cytokinin receptor AHK3 knockout into the 35S:SSPP background. The phenotypic analysis of double mutant revealed that AHK3 loss-of-function reversed the delayed leaf senescence induced by SSPP. Moreover, we found the hypersensitivity of 35S:SSPP to exogenous cytokinin treatment disappeared due to the introduction of AHK3 knockout. Collectively, our results demonstrated that AHK3-mediated cytokinin signaling is required for the delayed leaf senescence caused by SSPP overexpression and the detailed mechanism remains to be further elucidated.

Keywords: cytokinin signaling; AHK3; leaf senescence; SSPP

1. Introduction

Senescence represents the final stage of leaf development, featured by degradation and recycling of nutrients in senescent leaves to actively growing organs [1]. It is not only critical for a plant’s fitness and nutrient relocation, but also crucial for crop yield and quality in agricultural perspectives [2]. As a process highly regulated by genetic programming, both the initiation and progression of leaf senescence occur in response to environmental factors and endogenous signals such as plant hormones [3]. As one of the ancient plant hormones [4], cytokinin has long been widely acknowledged as a negative regulator of leaf senescence [5]. In Arabidopsis, cytokinin is perceived by three different receptors, namely AHK2, AHK3, and AHK4/CRE1/WOL [6–8]. Among the three receptors, AHK3 plays a major role in leaf senescence regulation through a specific phosphorylation of the B-type response regulator ARR2 [9]. Cytokinin-mediated leaf longevity also involves the cytokinin response factors [10,11], and the downstream extracellular invertase that may function in source-to-sink nutrient mobilization [12].

We previously found that overexpression of a PP2C type protein phosphatase-encoding gene SSPP led to significantly delayed leaf senescence and enhanced cytokinin responses in Arabidopsis [13]. However, it remains largely unknown how SSPP-mediated delayed leaf senescence signaling is associated with cytokinin signaling. Therefore, we obtained ahk3-3/35S:SSPP hybrid Arabidopsis, and found that knockout of AHK3 reversed the delayed leaf senescence caused by SSPP overexpression. The hypersensitivity of 35S:SSPP to exogenous cytokinin treatment is lost in ahk3-3/35S:SSPP. These results together indicate that AHK3-mediated cytokinin signaling is required for the delayed leaf senescence induced by SSPP overexpression.
2. Results and Discussion

In order to gain a better understanding of the molecular mechanism underlying SSPP-mediated delayed leaf senescence, we generated hybrid Arabidopsis by introducing cytokinin receptor AHK3 knockout [14] into 35S:SSPP background (SSPPox). The genotyping of the hybrid Arabidopsis was confirmed by semi-quantitative RT-PCR using the Tip41-like gene as an internal control (Figure S1). Phenotypic analysis assay was performed with reciprocal comparisons of ahk3-3/SSPPox, SSPPox, ahk3-3 mutants and the wild-type (WT) control (Figure 1). In line with our previous report [13], the leaves of SSPP overexpressing Arabidopsis exhibited significantly delayed senescence in comparison to the ones of the WT. Conversely, the leaves of the ahk3-3 mutant showed precocious senescence. Of particular note, the hybrid Arabidopsis ahk3-3/SSPPox displayed accelerated leaf senescence which was similar to the ahk3-3 mutant but reversed the late senescence of SSPPox at 59 days after emergence (DAE) (Figure 1A). We also compared the rosette diameter, plant height, bolting time, and flowering period of all the different plants (Figure S2). Compared to the WT, SSPPox showed smaller rosettes, reduced plant height, delayed bolting time as well as flowering time, which is consistent with our previous findings [13]. However, no differences were observed among ahk3-3 and ahk3-3/SSPPox.

![Figure 1. Loss of AHK3 reversed the delayed leaf senescence mediated by SSPP. (A) The 59-day-old ahk3-3, 35S:SSPP(SSPPox), ahk3-3/SSPPox and wild-type (WT) control Arabidopsis were photographed. (B) chlorophyll contents in the sixth leaf of ahk3-3, SSPPox, ahk3-3/SSPPox, and wild-type control Arabidopsis were determined at 30, 35, 40 and 45 days after emergence (DAE). FW, fresh weight. Three biological replicates with at least three technical repeats were done. Error bars represent standard errors (SE). Asterisks indicate significant differences with the wild-type at each time point (α = 0.05).](image)

To get a quantitative comparison in leaf senescence, we measured the chlorophyll contents in the sixth leaf of all the above-mentioned transgenic plants and WT from 30 to 45 DAE. The results showed that contents of chlorophyll were significantly increased in the leaves of SSPPox while they were declined in the leaves of the ahk3-3 mutant at all stages in comparison to those in the WT (Figure 1B). In the leaves of ahk3-3/SSPP-ox, chlorophyll contents were lower than in WT and SSPPox, but were at similar levels to those in the ahk3-3 mutant (Figure 1B). This demonstrates that the hybrid Arabidopsis showed similar senescence phenotype to the ahk3-3 mutant.

Consistent with chlorophyll contents, expressions of senescence-associated marker genes including SAG12 [15], SAG113 [16], NAP [17], WRKY6 [18], and NAC2 [19] were remarkably inhibited in the leaves of SSPPox but were significantly enhanced in the leaves of ahk3-3 mutant and the hybrid Arabidopsis ahk3-3/SSPPox (Figure 2). These results indicated that knockout of AHK3 reversed the delayed leaf senescence in SSPP overexpressing Arabidopsis.
AHK3 with were transferred to vertical half-strength MS plates containing either mock solution or 5 μM 6-BA and kept growing for another six days. Upon mock treatment, the SSPPox mutant and the hybrid Arabidopsis ahk3-3/SSPPox seedlings showed retarded root growth compared to the WT as reported previously [13]. By contrast, the root development of the ahk3-3 mutant and hybrid Arabidopsis ahk3-3/SSPPox did not show any significant difference with WT (Figure 3A,B). Under 6-BA treatment, SSPP overexpressing seedlings exhibited significantly inhibited root growth compared with WT (Figure 3A,B). As expected, the hybrid Arabidopsis ahk3-3/SSPPox displayed reduced sensitivity to 6-BA application similar to the ahk3-3 mutant (Figure 3A,B). We also compared the inhibitory rates of root growth in all the above-mentioned transgenic seedlings and WT at six days after 6-BA treatment compared to the mock treatment. It was found that the inhibitory rate was significantly higher in SSPPox while was remarkably reduced in both the ahk3-3 mutant and the hybrid Arabidopsis ahk3-3/SSPPox in comparison to that in WT (Figure 3C). These results indicated that hypersensitivity of SSPP overexpressing Arabidopsis to exogenous cytokinin application is linked with AHK3. Taken together, the SSPP-induced delayed leaf senescence and enhanced cytokinin responses are closely linked, and its hypersensitivity to cytokinin is associated with AHK3-mediated cytokinin signaling.
wild-type phosphotransmitter proteins, which are then translocated to the nucleus and phosphorylate specifically the type-B response regulator ARR2. ARR2 then activates the expressions of downstream target genes such as SAG12, SAG113, NAP, WRKY6, and NAC2 (Figure 2), which collectively demonstrate that loss of AHK3 leads to accelerated leaf senescence.

The molecular mechanisms underlying the cross-talk between SSPP-mediated delayed leaf senescence signaling and AHK3-mediated cytokinin responses will be a subject for further exploration. Upon the binding of cytokinin, AHK3 was reported to phosphorylate the downstream phosphotransmitter proteins, which are then translocated to the nucleus and phosphorylate specifically the type-B response regulator ARR2. ARR2 then activates the expressions of downstream target genes and induce a range of physiological processes and cellular responses in the cytoplasm and the organelle during dark-induced leaf senescence [9]. However, the mechanism underlying the role of AHK3 during natural leaf senescence needs to be further studied. Recent cell biological and biochemical evidences demonstrated that AHK3 was localized in the endoplasmic reticulum (ER) [21]. Our previous research found that the protein phosphatase SSPP was localized in the cytoplasm [13]. Moreover, SSPP overexpression resulted in increased expressions of several cytokinin-responsive markers genes such as type-A response regulators ARR5 and ARR6. In addition, the SSPP loss-of-function mutant named sspp-1 exhibited no significant differences in growth and development when compared to wild-type Arabidopsis. As this study demonstrated that AHK3-mediated cytokinin response is required

Figure 3. Loss of AHK3 diminished the hypersensitivity of SSPP-overexpressing Arabidopsis to exogenous cytokinin treatment. (A) Four-day-old WT, ahk3-3, SSPPox, and ahk3-3SSPPox transgenic seedlings were grown on vertical plates containing either 6 µM 6-BA or a mock solution for additional 6 days and photographed. (B) Comparison of relative root length of WT, ahk3-3, SSPPox, and ahk3-3SSPPox transgenic seedlings under either mock or 6-BA treatment every day for a total period of six days. (C) Comparison of growth inhibition rates of main root growth of WT, ahk3-3, SSPPox, and ahk3-3SSPPox at 6 days after treatment (DAT). Data shown are the typical results of three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) (α = 0.05). Error bars represent SE.

The essential role of AHK3 as the major contributor in regulating leaf longevity was firstly reported by Kim et al. [9] based on the senescence analysis assays of dark-induced excised leaves. But this study claimed that the loss-of-function mutant ahk3 did not show a significantly early senescence phenotype during age-dependent senescence. Conversely, a recent study from Danilova et al. [20] argued that the ahk3 single mutant displayed a slightly accelerated vegetative growth. In this study, we carried out a thorough phenotypic analysis, and compared the chlorophyll contents as well as the transcript levels of several senescence-associated marker genes (SAGs) in the ahk3-3 mutant and the wild-type control plants. From these integrated analyses, we found the ahk3-3 mutant not only showed declined chlorophyll contents (Figure 1B) but also significantly up-regulated expressions of five critical SAGs such as SAG12, SAG113, NAP, WRKY6, and NAC2 (Figure 2), which collectively demonstrate that loss of AHK3 leads to accelerated leaf senescence.
in SSPP-induced delayed leaf senescence, it would be interesting to further investigate whether SSPP could directly interact with or dephosphorylate AHK3, or whether SSPP can interact with other components of the cytokinin signal transduction pathway mediated by AHK3. In addition, our group found that SSPP negatively regulates leaf senescence by directly interacting with and suppressing the leucine-rich repeat receptor-like protein kinase (LRR-RLK) SARK [13], which works as a positive regulator of leaf senescence [22]. Transcript analysis of SARK-overexpressing plants revealed a wide range of changes in phytohormone synthesis and signaling including a strong repression of cytokinin functions [22]. Therefore, it would be also interesting to investigate whether there are cross-talks between AHK3 and SARK, and whether the dephosphorylation of SSPP is SARK-specific. The current study laid foundation for further understanding towards the role of cytokinin in leaf senescence and the detailed signal transduction pathways of the delayed leaf senescence process mediated by SSPP overexpression.

3. Materials and Methods

3.1. Plant Material and Growth Condition

*Arabidopsis thaliana* (Columbia-0 ecotype) was used in this study. The loss-of-function *ahk3-3* mutant seeds [14] were provided by Professor Shuhua Yang from China Agricultural University. Seeds were surface sterilized in 10% (v/v) sodium hypochlorite (Tianjin Chemicals, 559, Tianjin, China) for 2 min, washed at least 10 times with sterilized water, and germinated on one half-strength Murashige Skoog (MS) medium (Duchefa Biochemie, M0222, Haarlem, the Netherlands) containing 0.8% (w/v) agar (Solarbio, A8190, Beijing, China), pH5.7, 1% (w/v) Suc (Jiangtian Chemicals, 11411, Nantong, China), supplemented with or without antibiotics, stratified at 4 °C for 2 days in the dark, and then grown in plant growth chamber at 22/19 °C with cycles of 16 h light and 8 h darkness under 100 to 150 µmol m⁻²·s⁻¹ light intensity. The 10-day-old seedlings were then transferred to soil and grown under the same conditions for further experiments.

3.2. Generation of Hybrid Arabidopsis

The hybrid *Arabidopsis ahk3-3/SSPPox* was generated by crossing 35S:SSPP with the *ahk3-3* mutant, which served as the male parent and female parent, respectively. Homozygous plants were identified by segregation analysis and PCR-based genotyping among the F3 progeny. All the primers used were listed in Table S1.

3.3. Measurements of Chlorophyll Contents

The chlorophyll contents in mesophyll cells were spectrophotometrically measured as described in Arnon (1949) [23].

3.4. RNA Extraction and RT-PCR Analysis of Gene Expression

RNA extraction, cDNA synthesis, and RT-PCR analysis were performed as described previously in Liu et al. [24]. The real-time RT-PCR analyses were performed on an iQ5 (Bio-Rad, Hercules, CA, USA) machine using a SYBR Green reagent (Takara, Berkeley, CA, USA) with gene-specific primers (Table S1). The relative expression levels were calculated as described previously [25]. At least three independent replicates were performed to give typical results shown here.

3.5. Cytokinin Response Assay

Five-day-old 35S:SSPP(SSPPox), *ahk3-3*, and *ahk3-3/SSPPox* transgenic *Arabidopsis* seedlings and the wild-type control were transferred from basal half-strength MS medium to fresh induction plates containing 5 µM 6-BA (6-Benzylaminopurine, Sigma, B3408, Darmstadt, Germany) or a mock solution. During an additional growth period of indicated durations, the root lengths were measured using
magnified images via ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/download.html). Each treatment was replicated three times.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1422-0067/20/8/2043/s1, Figure S1: The genotyping of the hybrid Arabidopsis ahk3-3SSPPox was confirmed by semi-quantitative RT-PCR. Figure S2: Reciprocal comparisons between ahk3-3SSPPox, ahk3-3, SSPPox, and WT on rosette diameters, plant height, bolting time, and flowering time. Table S1: Primers used in this study.

**Author Contributions:** N.N.W. conceived and designed the study, supervised the experiments, and compiled and finalized the article. Y.W., X.Z. and Y.C. performed the experiments. N.N.W., Y.M., Y.W. and D.W. analyzed the data. Y.M., L.L. and N.N.W. drafted and revised the manuscript. All authors read and approved the final manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| DAE          | Days After Emergence |
| DAT          | Days After Treatment |
| LRR-RLK      | Leucine-Rich Repeat Receptor-Like Protein Kinase |

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