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Original Paper

AINTEGUMENTA negatively regulates age-dependent leaf senescence downstream of AUXIN RESPONSE FACTOR 2 in *Arabidopsis thaliana*

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Abstract Leaf senescence is the final stage of leaf development and is regulated by many internal and external cues. As a repressor of auxin signaling, AUXIN RESPONSE FACTOR 2 (ARF2) is involved in control of several developmental processes, but its functional mechanism on how to positively regulate leaf senescence has not been clearly defined. Here, we examined AINTEGUMENTA (ANT), a member of the AP2/ERF transcription factor family, and found that ANT played an important role in extending leaf longevity. The loss-of-function *ant-1* mutant showed premature leaf senescence, whereas overexpression of *ANT* led to a delay in leaf senescence. Genetic analysis revealed that loss of *ANT* repressed the delayed leaf senescence phenotype in *arf2-5* mutant. Taken together, our results suggest that ANT is involved in regulation of leaf senescence downstream of ARF2.

Key words: ANT, Arabidopsis, ARF2, leaf senescence.

Leaf senescence occurs at the final stage of leaf life history accompanied by cell death (Lim et al. 2007; Pennell and Lamb 1997). During leaf senescence, dramatic changes occur in cell structure and metabolism, including the degradation of chloroplasts, mitochondria and nuclei, and the catabolism of macromolecules, such as nucleic acids, proteins and lipids (Lim et al. 2007; Ulker et al. 2007). As an integral part of plant development, leaf senescence is fundamentally controlled by developmental age. However, it is also affected by multiple internal and external signals that are integrated into age information. Leaf senescence can be induced by pathogen infection, shading, limited nutrients, temperature stresses, and oxidative stress (Hopkins et al. 2007; Lim et al. 2007; Zhou et al. 2011). Phytohormones, such as auxin, cytokinin, ethylene, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid, play important roles in senescence (Buchanan-Wollaston et al. 2005; Gan and Amasino 1997).

In Arabidopsis, transcriptome analysis of senescent leaves showed that up to 2,500 genes were expressed in senescent leaves and hundreds of senescence-associated genes (SAGs) were distinctively up-regulated during leaf senescence (He et al. 2001; van der Graaff et al. 2006). Recent genetic studies on these senescence-associated genes have provided numerous insights into molecular events and their roles during leaf senescence, thereby revealing an extremely complex molecular network including a great number of regulatory factors (Guo 2013; Li et al. 2012). The characterized regulatory factors involved in leaf senescence regulation include transcriptional factors, signaling receptors and components of hormones and regulators of metabolism (Lim et al. 2007). However, the vast majority of the SAGs don't present a notable effect on plant senescence (Li et al. 2012).

Early studies indicate that auxin acts as a suppressor of leaf senescence by repressing transcription of some genes associated with senescence (Noh and Amasino 1999; Tucker et al. 2002). AUXIN RESPONSE FACTOR 2 (ARF2), a member of the auxin response factor (ARF) family, represses auxin responsive gene expression (Tiwari et al. 2003). The works of Lim and Ellis clearly indicated that ARF2 is a major player in the auxin-mediated control of leaf longevity (Ellis et al. 2005; Lim et al. 2010). *arf2* mutant plants exhibited delays in all senescence parameters examined, including chlorophyll content, the photochemical efficiency of photosystem II, membrane ion leakage and the expression of senescence associated genes (Lim et al. 2010). However, the underlying functional mechanism of ARF2 induced leaf senescence remains to be elucidated.

Loss of *ARF2* function prolongs expression of *AINTEGUMENTA* (*ANT*) (Schruff et al. 2006). ANT, a member of the AP2/ERF transcription factor family, contains a DNA-binding domain and the conserved

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intervening linker region. Loss of function of *ANT* leads to the reduce of the size of all lateral shoot organs by decreasing cell number, and overexpression of *ANT* results in the enlarged organs due to more cells in the leaves and larger cells in petals, stamen and carpels (Krizek 1999; Mizukami and Fischer 2000). ANT promotes organ growth by maintaining meristem competence and increasing cell numbers. *ANT* is expressed primarily in young actively dividing tissues of a plant, and low expression is observed by real time RT-PCR in mature leaves but not by RNA gel blot analysis, suggests that the expression of *ANT* is age-dependent (Klucher et al. 1996; Nole-Wilson et al. 2005). It is very interesting to study the relationship of ANT and ARF2 in the age-dependent leaf senescence.

In order to explore the importance of ANT in age-dependent leaf senescence, we examined the senescence parameters of the loss-of-function mutant *ant-1* and gain-of-function *35S-ANT* transgenic plants. Overexpression of *ANT* caused delayed leaf senescence and premature leaf senescence was detected in the rosette leaf of *ant-1*. Genetic analysis showed that loss of function of *ANT* blocked the delayed leaf senescence of *arf2-5* plants, indicating that ARF2 dependent ANT to positively regulates leaf senescence. Collectively, our results illustrated that ANT acts downstream of ARF2 to regulate leaf senescence.

Materials and methods

Plant materials

Arabidopsis ecotype Columbia Col-0 (WT), *ant-1* and *arf2-5* (Salk_041472) were used in this study. The seeds were sterilized and then plated on 1/2 MS medium containing 1% sucrose and 0.6% agar. After vernalization at 4°C in darkness for 2 days, the plate was then transferred to a culture room at 22±1°C with illumination of 80–90 μmol m^{-2} s^{-1} with a 16-h light/8-h dark photoperiod. The 7-day old seedlings after germination were planted in soil for further growth.

Plasmid constructs

The 1668-bp *ANT* coding sequence was amplified by reverse transcription polymerase chain reaction (RT-PCR) and cloned into pVIP96 for generation of the *35S-ANT* construct (Leu et al. 1995). The *35S-ANT* transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation (Zhang et al. 2006). More then 20 independently *35S-ANT* transgenic lines were generated and three T3 homozygous lines with a single T-DNA insertion were used for detailed analyses.

Gene expression analysis

Total RNA was isolated with a guanidine thiocyanate extraction buffer, and the reverse-transcribed PCR (RT-PCR) was performed to monitor the expression of *ANT* and *SAG* genes as described previously (Feng et al. 2011). Real-time quantitative RT-PCR (qRT-PCR) was carried out using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA) with the SYBR® Premix ExTM Taq II kit (Takara Biotechnology, Dalian, China). *ACTIN2* was used as an internal control. The primers used were as follows: for *ANT*, 5′-AAG CAC GGA TTG GTA GAG TCG-3′ and 5′-GCA TTT GTG CCA CGG AAC TTA-3′; *SAG12*, 5′-CGG TTT CTG TTG ACT GGA-3′ and 5′-AGC TGT TGT TCT GAC AAA GA-3′; *SEN4*, 5′-ATC GGC TTG TTC TTT GGA-3′ and 5′-GAC AAA GAG CAA CAA TTC CA-3′; *WRKY6*, 5′-TGG TTA TGG TTT CCC TCG-3′ and 5′-GTC AAT GGA GAA AAT ATG GC-3′; *ACTIN2*, 5′-GCT CCT CTT AAC CCA AAG GC-3′ and 5′-CAC ACC ATC ACC AGA ATC CAG C-3′. The primers used for semi-quantitative RT-PCR: *GAPC*, 5′-TGG TCG TTT GGT TGC TAG AGT-3′ and 5′-AAG GTC GGA CTT GTA TTC GTG-3′; *ANT*, 5′-GAT TGG TAG AGT CGC TGG-3′ and 5′-GTT GGA ACC ACC TTC CAC AA-3′; *ARF2*, 5′-GAA TTG CAC TTG GCC GTT C-3′ and 5′-TGA TGC AGA CTT GGC GTC-3′.

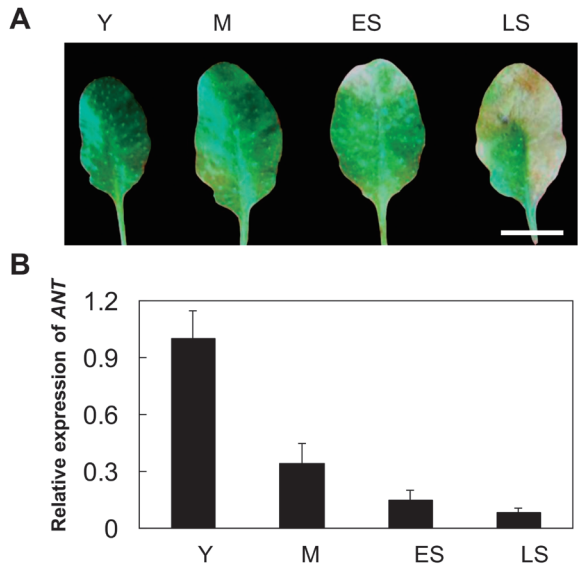

Figure 1. *ANT* is an age-dependent expressive gene. (A) The leaves at four different developmental stages. Y, young leaves of 10 days after emergence (DAE); M, fully expanded mature leaves of 17 DAE; ES, early senescent leaves of 26 DAE; LS, late senescent leaves of 32 DAE. Bar, 1 cm. (B) qRT-PCR analysis of transcript levels of *ANT* in the leaves at different developmental stages. Three biological replicates were performed. Error bars represent SD.

Age-dependent leaf senescence analysis

Age-dependent leaf senescence was assayed as described by Woo et al. (2001). The leaves used at each sampling time point were excised from at least three separate plants. Chlorophyll was extracted from leaves and measured according to the protocol of Grbic and Bleecker (Grbic and Bleecker, 1995).

Results

The expression of ANT is age-dependent

Previous study shows that *ANT* is expressed primarily

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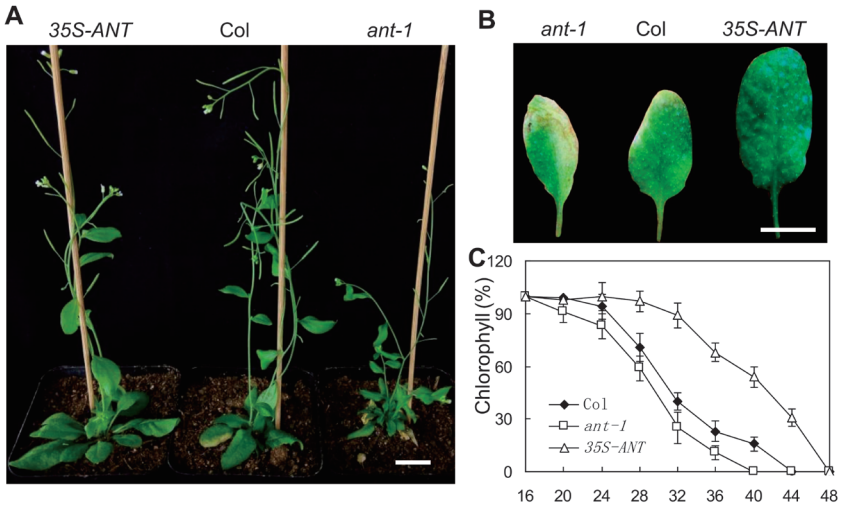

Figure 2. ANT plays a negative role in the age-dependent senescence. (A)The 35-d old plants of *ant-1*, Col and *35S-ANT*. Bar, 1 cm. (B) The fourth rosette leaves of 30-d old plants of *ant-1*, Col and *35S-ANT*. Bar, 1 cm. (C) Chlorophyll content of the fourth rosette leaves of *ant-1*, Col and *35S-ANT*. DAE, days after emergence. Three biological replicates were performed. Error bars represent SD.

in young tissues and is low-expression in mature rosette leaf. In order to test and verify the age-dependent expression of *ANT* in plants, its expression was examined in different development stage of the fourth rosette leaf by quantitative RT-PCR (qRT-PCR). As shown in Figure 1, the highest expressive level of *ANT* was detected in the fourth rosette leaf at the 10 days after emergence (DAE), and the expression was obviously decreased in the mature fourth rosette leaf at the 17 days after emergence. The expressive level was further decreased in the leaf that started to turn yellow at the leaf age of 26 days after emergence. These results indicated that the expression of *ANT* was decreased with age, suggesting that *ANT* is an age-dependent expressive gene.

ANT negatively regulates age-dependent leaf senescence

To explore the role of ANT in the leaf senescence, the loss-of-function mutant of *ant-1* and the gain-of-function of *35S-ANT* transgenic plants were used for analysis. Compared with wild-type plants, *ant-1* plants showed classic smaller organs and flowered 5 days earlier, whereas the *35S-ANT* transgenic plants had enlarged leaves and flowers and flowered a week later (Figure 2A and Figure S1). Next, the senescence symptoms of *ant-1* and *35S-ANT* transgenic plants were examined during age-dependent senescence. The emergence time and growth rate of the fourth rosette leaves were almost identical in wild-type plants, *ant-1* mutant and *35S-ANT* transgenic plants. In our culture condition, the wild-type leaves started to turn yellow at the leaf age of 26 days after emergence (DAE) and finally showed signs of necrosis at 41 DAE. By contrast, *ant-1* mutant leaves started to turn yellow at 21 DAE, and the *35S-ANT* transgenic plants started to turn yellow till at 33 DAE (Figure 2B). They finally showed signs of necrosis after 36 DAE and 47 DAE, respectively.

The preferential breakdown of chlorophyll during chloroplast degradation makes the leaves turn yellow, so chlorophyll content was measured to evaluate the symptom of age-dependent senescence. Consistent with the premature or delayed senescence, in wild-type plants the chlorophyll contents began to decline after 24 DAE, but the same chlorophyll loss took place at 20 DAE for *ant-1* mutant and 32 DAE for *35S-ANT* transgenic plants. By the age of 32 d, the chlorophyll content was reduced to 40% of that of 15 DAE leaves (mature green stage) in the wild-type plant, and in *ant-1* mutant the chlorophyll content was reduced to 25%. By contrast, losses in *35S-ANT* transgenic plants were slower, with 54% of its chlorophyll remaining at 40 DAE (Figure 2C). These results suggest that ANT plays a role in resistance to age-dependent senescence.

To further confirm the negative role of ANT in regulating leaf senescence, the expression of several *SAGs* was examined in the mutant and overexpressing plants of *ANT*. As a widely used molecular marker of leaf senescence, *SAG12* is specifically expressed in the senescing leaves (Noh and Amasino 1999; Pontier et al. 1999). Compared with in wild-type, the induction of *SAG12* was in advance in *ant-1* mutant but delayed in *35S-ANT* transgenic plants (Figure 3A). At the leaf age of 30 DAE, the increased expressions of SEN4 (Park et al. 1998) and WRKY6 (Robatzek and Somssich 2001) were detected in *ant-1* mutant, and the down-regulated expressions of them were observed in *35S-ANT* transgenic plants (Figure 3B and C).

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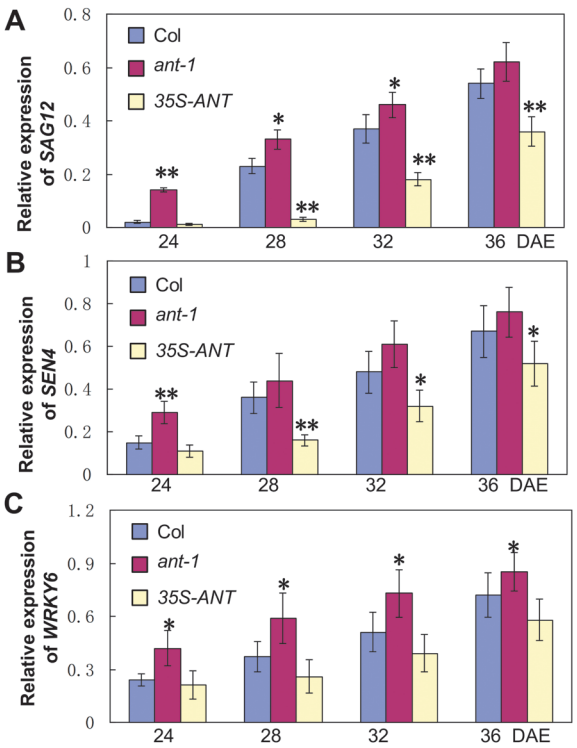

Figure 3. Expression of *SAGs* in *ant-1*, Col and *35S-ANT* plants. qRT-PCR analysis of transcript levels of *SAG12*(A), *SEN4*(B) and *WRKY6*(C) in the leaves of *ant-1*, Col and *35S-ANT* at different developmental stages. DAE, days after emergence. Three biological replicates were performed. Error bars represent SD. Student's *t*-test: $^{**}p<0.01$; $^{*}p<0.05$.

ANT acts downstream of ARF2 to regulate leaf senescence

AUXIN RESPONSE FACTOR 2 (ARF2) is a member of transcription factor family, and it binds to auxin-responsive elements in the promoters of auxin-regulated genes (Ellis et al. 2005; Liscum and Reed 2002). Previous work on *arf2* mutant plants found that the mutant plants have the enlarged seeds, stems and cotyledons and the phenotypes are similar to those obtained from the *35S-ANT* transgenic plants (Schruff et al. 2006). What's more important is that the expression of *ANT* is prolonged in leaves and stems of *arf2* mutants (Schruff et al. 2006). Given that ARF2 plays a major role in regulating auxin-mediated leaf longevity, we surmised that ANT may be controlled by ARF2 and act downstream of it to regulate leaf senescence. To test this hypothesis, we first confirmed the prolonged expression of *ANT* in the *arf2-5* mutants by quantitative RT-PCR (qRT-PCR). As the results shown in the Figure 4B, higher expressive level of *ANT* was observed in the 24-d old *arf2-5* mutants then in wild-type plants, just as reported by Schruff et al. The expressive level of *ARF2* in the *ant-1* plants and in the wild-type plants is no change (Figure S1C). Next, we crossed *arf2-5* mutants with heterozygous *ANT/ant-1* and obtained the double mutants in the F2 progeny. The *ant-1* mutation dramatically blocked leaf enlargement and also the delayed leaf senescence in *arf2-5* mutants. The fourth rosette leaf of *arf2-5/ant-*

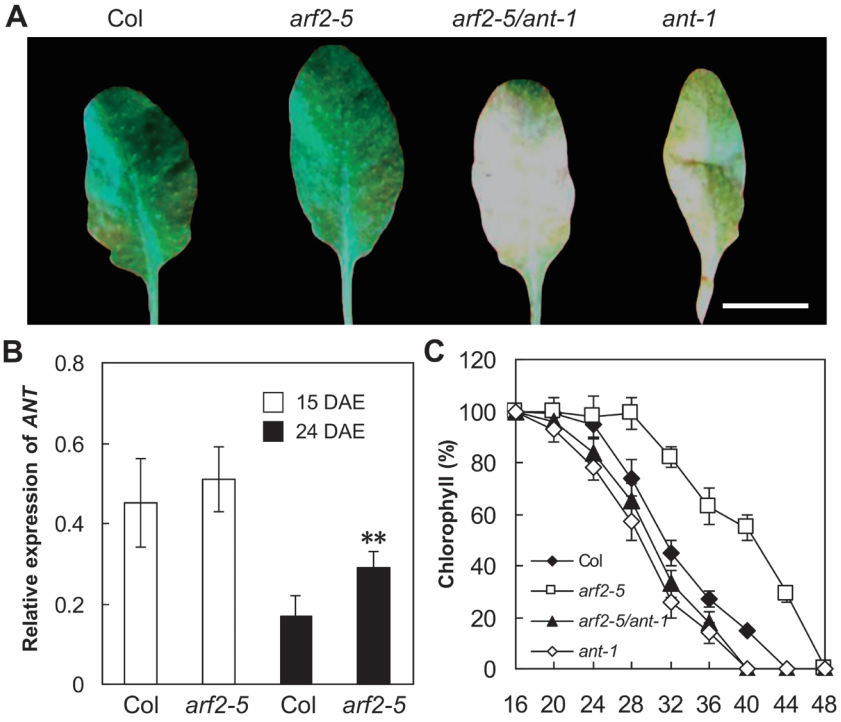

Figure 4. ANT acts downstream of ARF2 to regulate age-dependent leaf senescence. (A)The fourth rosette leaves of 30-d old plants of Col, *arf2-5*, *arf2-5/ant-1* and *ant-1*. Bar, 1 cm. (B) qRT-PCR analysis of transcript levels of *ANT* in wild-type and the *arf2-5* leaves at the 15 DAE and 24 DAE. DAE, days after emergence. (C) Chlorophyll content of the fourth rosette leaves of Col, *arf2-5*, *arf2-5/ant-1* and *ant-1*. DAE, days after emergence. Three biological replicates were performed. Error bars represent SD.

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1 double mutants turned yellow at the age of 23 DAE compared with 34 DAE in *arf2-5* mutants and 21 DAE in *ant-1* mutation (Figure 4 and S2). Thus, our findings indicate that loss of function of *ANT* block the delayed leaf senescence of *arf2-5* mutants to a great extent and hence ANT functions downstream of ARF2 to regulate age-dependent leaf senescence.

Discussion

Leaf senescence is the final stage of leaf development and promotes nutrient relocation from leaves to reproducing seeds. Dramatic changes at the cellular, tissue, organ and organism levels occur during leaf senescence under the control of a highly complex gene regulatory network (Buchanan-Wollaston et al. 2005; Lim et al. 2007). The complex nature of leaf senescence requests many regulatory factors to finely tune the initiation and progression of senescence. The positive factors must exist to trigger and promote the leaf senescence. In other hand, the negative factors are also essential to prevent prematurely senescence (Guo 2013; Lim et al. 2007). A number of these positive factors have been characterized during the last decades, but only few of negative factors are reported, such as microRNA164 (miR164) (Kim et al. 2009; Li et al. 2013). *ANT* is an age-dependent expressive gene that is expressed primarily in young tissues and is low-expression in mature rosette leaf. The loss-of-function and gain-of-function of *ANT* result in premature and delayed leaf senescence, respectively, indicating that ANT acts as a negative factor to prevent premature senescence. Together with the knowledge about the important roles of ANT on the plant development regulation, we propose that ANT is an actor in the developmental age initiated the senescence program.

Leaf senescence is affected by the environmental cues and the internal factors including phytohormones and reproductive development as well as developmental age (Lim et al. 2007). Auxin plays a negative role in leaf senescence by repressing transcription of some genes associated with senescence (Noh and Amasino 1999; Tucker et al. 2002). As a member of the auxin response factor (ARF) family, ARF2 also takes part in the auxin-mediated control of leaf longevity by repressing auxin responsive gene expression (Ellis et al. 2005; Lim et al. 2010; Tiwari et al. 2003). However, the underlying functional mechanism of ARF2 induced leaf senescence remains to be elucidated. Our study shows clearly that the prolonged expression of *ANT* was detected in the *arf2-5* mutant, and the loss-of-function of *ANT* blocked the delayed leaf senescence phenotype of *arf2-5* mutant, suggesting that ANT acts downstream of ARF2 to negatively regulate leaf senescence.

During plant development, the organ primordium initially consists entirely of cells undergoing coordinated division and expansion. Along with the organ growth, distal cells cease cell division and enter a phase of postmitotic expansion (Powell and Lenhard 2012). The transition from proliferation to expansion has often been described as an 'arrest front' moving from the tip towards the base of the leaf (White 2006). By coincidence, senescence symptoms usually start from the tip and outer edge of a rosette leaf at a given age (Guo and Gan 2006). It suggests that exit of cell growth may affect the timing of leaf senescence. In plant, ANT plays a role in maintaining meristem competence, and loss of function of *ANT* leads to prematurely exit of cell growth that might trigger the occurrence of senescence. Whereas, overexpression of *ANT* results in the postponed cease of cell growth and also the delayed senescence.

In Arabidopsis, AP2/ERF proteins make up one of the largest transcription factor families. ANT and AINTEGUMENTA-like (AIL) belong to the AP2 subfamily that consists of 18 members (Riechmann et al. 2000) and have redundant roles in specification of meristematic or division-competent states (Nole-Wilson et al. 2005). For example, ectopic expression of *AIL5* in wild type plants produces larger floral organs just like that in the transgenic plant overexpressing *ANT* (Nole-Wilson et al. 2005). The incomplete block of the delayed leaf senescence of *arf2-5* by *ant-1* might be due to the redundancy of ANT and AILs. ARF2 might act on the other members of AP2 subfamily to positively regulate leaf senescence.

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