A Soybean Dual-Specificity Kinase, GmSARK, and Its Arabidopsis Homolog, AtSARK, Regulate Leaf Senescence through Synergistic Actions of Auxin and Ethylene

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As the last stage of leaf development, senescence is a fine-tuned process regulated by interplays of multiple signaling pathways. We have previously identified soybean (Glycine max) SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE (SARK), a leucine-rich repeat-receptor-like protein kinase from soybean, as a positive regulator of leaf senescence. Here, we report the elucidation of the molecular mechanism of GmSARK-mediated leaf senescence, especially its specific roles in senescence-inducing hormonal pathways. A glucocorticoid-inducible transcription system was used to produce transgenic Arabidopsis (Arabidopsis thaliana) plants for inducible overexpression of GmSARK, which led to early leaf senescence, chloroplast destruction, and abnormal flower morphology in Arabidopsis. Transcript analyses of the GmSARK-overexpressing plants revealed a multitude of changes in phytohormone synthesis and signaling, specifically the repression of cytokinin functions and the induction of auxin and ethylene pathways. Inhibition of either auxin action or ethylene biosynthesis alleviated the senescence induced by GmSARK. Consistently, mutation of either AUXIN RESISTANT1 or ETHERYLENE INSENSITIVE2 completely reversed the GmSARK-induced senescence. We further identified a homolog of GmSARK with a similar expression pattern in Arabidopsis and named it AtSARK. Inducible overexpression of AtSARK caused precocious senescence and abnormal floral organ development nearly identical to the GmSARK-overexpressing plants, whereas a T-DNA insertion mutant of AtSARK showed significantly delayed senescence. A kinase assay on recombinant catalytic domains of GmSARK and AtSARK revealed that these two leucine-rich repeat-receptor-like protein kinases autophosphorylate on both serine/threonine and tyrosine residues. We inferred that the SARK-mediated pathway may be a widespread mechanism in regulating leaf senescence.

Leaf senescence is a highly organized process providing a mechanism for the mobilization of nutrients from aging leaves to support the development of younger organs or seeds. The onset and process of leaf senescence are influenced by various internal signals and environmental factors (Lim et al., 2007a). Modifications of the leaf senescence process directly affect agricultural traits of crop plants, including biomass, seed yield, seed protein composition, and abiotic stress resistance (Zhang et al., 1987; Guimé et al., 1990; Gan and Amasino, 1995; Rivero et al., 2007).

A recent high-resolution temporal profiling of transcripts reveals a distinct chronology of metabolic processes and signaling pathways during leaf senescence (Breeze et al., 2011). The expression levels of many regulatory genes change greatly during this process (Lin and Wu, 2004; van der Graaff et al., 2006). Among them, several transcription factors, including NAC1, AtNAC2, AtNAP, WRKY6, WRKY53, RAV1, and CBF2, have been demonstrated to play important roles in the regulation of leaf senescence (Robatzek and Somssich, 2002; Miao et al., 2004; Guo and Gan, 2006; Kim et al., 2009; Sharabi-Schwager et al., 2010). Despite these impressive advances in the field, the nature of the plant developmental age, the transduction pathway of the so-called senescence signal, and the mechanisms of action of and the interactions between exogenous and endogenous regulatory factors during this process have not been fully defined.

The phytohormones play a critical role in leaf senescence; they might regulate leaf senescence by coordinating the responses to environmental cues with those induced by developmental signals. Among them, cytokinin is considered to be a senescence-delaying hormone. Specific expression of the IPT gene, which encodes the rate-limiting enzyme in cytokinin biosyn-
thesis, can significantly delay leaf senescence in *P. sagittata* (Gan and Amasino, 1995). One of the three known cytokinin receptors, AHK3, has been demonstrated to function in the cytokinin-mediated delay of leaf senescence; and the phosphorelay of AHK3 to ARR2, a type B Arabidopsis (*Arabidopsis thaliana*) cytokinin response regulator, is essential for controlling leaf longevity (Kim et al., 2006). Although there is evidence that AHK3 and ARR2 are negative regulators of leaf senescence, the specific molecular mechanisms involved in their regulation of this process remain unknown.

Auxin has also been implicated in the regulation of leaf senescence (Sexton and Roberts, 1982). Measurement of endogenous auxin levels demonstrated that although total indole-3-acetic acid (IAA; free IAA plus amide- and ester-linked conjugates of IAA) content drops during Arabidopsis leaf senescence, the level of free IAA in a senescent leaf is 2-fold higher than that of a nonsenescent fully expanded leaf (Quirino et al., 1999). Transcriptome studies also revealed enhanced expression levels of the key enzymes in auxin biosynthesis during age-dependent leaf senescence (van der Hoorn et al., 2001). The unique structure of RLKs makes them critical components of plant development and signal transmission pathways that respond to environmental factors (Walker, 1994; Torii, 2004; Johnson and Ingram, 2005). A common structural element of many plant RLKs is an extracellular leucine-rich repeat (LRR) domain that is generally thought to mediate ligand discrimination (Kobe and Kajava, 2001; Kinoshita et al., 2005). Examples include CLAVATA1, which regulates the morphogenesis of the apical meristem of Arabidopsis (Clark et al., 1997), and CLAVATA3, which controls floral organ abscission (Jinn et al., 2000), and BR11 and BAK1, both of which are involved in brassinosteroid signaling (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002). In addition to their involvement in other developmental stages, LRR-2RLKs are also involved in the regulation of leaf senescence. For example, transcripts of an Arabidopsis LRR-RLK, SIRK, and a bean (*Phaseolus vulgaris*) LRR-RLK, *PvSARK*, were all found to be accumulated in great quantity in senescent leaves (Hajouj et al., 2000; Robatzek and Somssich, 2002). However, the detailed functions of these LRR-RLKs in leaf senescence and the mechanisms and factors involved in the signal transductions mediated by these RLKs have not been definitively addressed.

We have previously demonstrated that a typical LRR-RLK gene, soybean (*Glycine max*) *SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE* (*GmSARK*), plays an important role in the regulation of soybean leaf senescence (Li et al., 2006). RNA interference (RNAi)-mediated knockdown of *GmSARK* expression in soybean can significantly delay senescence, while both the transgenic soybean and the transgenic Arabidopsis that overexpress the *GmSARK* gene exhibit a stepped-up progression of leaf senescence and premature death. *GmSARK*-RNAi transgenic soybeans, flowers exhibit never-open petals and abnormal “curvistigma,” so that the plants cannot perform successful pollination and are sterile (Li et al., 2006). Therefore, no lines of either *GmSARK*-RNAi or 35S:*GmSARK* could be maintained. Besides, the low transformation efficiency of soybean and the limited mutant lines and genomic resources in soybean are also disadvantages to the detailed analysis of the functions of *GmSARK*. To deal with these problems, we took advantage of a glucocorticoid-mediated transcriptional induction system (GGV system; GGV is the abbreviation of a glucocorticoid-mediated transcriptional induction system consisting of the DNA-binding domain of the yeast transcription factor GAL4, the trans-activating domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor; Aoyama and Chua, 1997) to...
ectopically express GmSARK in the model plant Arabidopsis. Extensive analyses on GmSARK-mediated leaf senescence revealed the coordinated roles of cytokinin, auxin, and ethylene in the regulation of this process. Furthermore, a functional GmSARK homologous LRR-RLK gene, which we called AtSARK, was identified in Arabidopsis. Both of these two LRR-RLKs were demonstrated to possess dual specificity and to auto-phosphorylate in vitro on both Ser/Thr and Tyr residues. The data presented in this paper suggest that the two SARK genes regulate leaf senescence through synergistic actions of auxin and ethylene and that the SARK-mediated leaf senescence pathways may be widespread among higher plants.

RESULTS

Inducible Overexpression of GmSARK Causes Precocious Senescence and Abnormal Flower Development in Transgenic Arabidopsis Plants

Inducible GmSARK expression would enable rescue of the lethal phenotype of the 35S:GmSARK plants and thus allow study of the role of GmSARK throughout plant development. For this, we constructed the GVG:GmSARK fusion gene and produced transgenic Arabidopsis plants. The homozygous GVG:GUS transgenic plants were taken as the transformation control. Seeds of four independent homozygous GVG:GmSARK transgenic lines, S9, S23, S33, and S48, and of the GVG:GUS control line G28 were sown on a 0.5× Murashige and Skoog (MS) semisolid plate containing either 10 μM dexamethasone (DEX; the induction plate) or its solvent ethyl alcohol only (the mock plate). It was found that the G28 transgenic seedlings expressing inducible GUS showed normal growth and development on the induction plate (Fig. 1Ab). All S lines were indistinguishable from the G28 control in the absence of DEX (Fig. 1Aa); however, although the S9, S23, and S33 seeds germinated and their hypocotyls elongated in the presence of DEX, their cotyledons did not stretch open and the seedlings died soon after (Fig. 1Ab). The S48 line showed no significant difference in growth and development compared with the G28 control (Fig. 1Aa). Semiquantitative reverse transcription (RT)-PCR analysis showed that the 24-h DEX treatment induced high expression levels of GmSARK in lines S9, S23, and S33, whereas the transcript of GmSARK was undetectable in the S48 and G28 seedlings (Fig. 1Ac), indicating that the expression of GmSARK was silenced in line S48. S23 was selected as a typical line expressing inducible GmSARK for further study in subsequent experiments.

The time course of DEX-induced GmSARK expression in the S23 seedlings was analyzed. It was found that the inducible GmSARK expression could be detected after 2 h of DEX treatment, peaked at 24 h, and gradually decreased during 72 h. Upon DEX treatment, expression of the leaf senescence marker gene SAG12 was induced within 2 h in the S23 seedlings and gradually increased along with the extension of DEX treatment time (Fig. 1B).

To study the effects of GmSARK overexpression on the development of Arabidopsis seedlings, 4-d-old S23 seedlings and their G28 transgenic control were vertically grown on either the induction or the mock plate for 96 h. The DEX-treated S23 seedlings not only showed a growth-inhibiting and precocious leaf senescence phenotype but also displayed obviously short and curved roots (Fig. 1C).

In addition to young seedlings, the effects of the inducible overexpression of GmSARK were also studied in adult S23 plants. The 20-d-old S23 seedlings were sprayed once a day with either 30 μM DEX or mock solution three times. Four days later, the DEX-sprayed plants showed an obvious senescence phenotype. Their juvenile rosette leaves turned yellow (Fig. 1Da). The senescence symptoms were further enhanced along with the length of time after the DEX treatment (Supplemental Fig. S1). The chlorophyll content in the fifth and sixth rosette leaves was greatly decreased 9 d after the DEX treatment (Fig. 1Db). The image taken 14 d later showed that the plants overexpressing GmSARK exhibited dwarfism. The main shoot elongation in Arabidopsis was greatly suppressed, and no lateral branches were produced (Fig. 1De). Closer observation revealed that the GmSARK-overexpressing flowers had stunted petals and protruding gynoecium, and their stamen filaments never elongated to position the anthers above the stigmatic papillae (Fig. 1Dg), as their mock control did for normal pollination. All these are morphological characteristics of mutants with enhanced ethylene responses (Kieber et al., 1993; Alonso et al., 1999; Hall and Bleecker, 2003; Qu et al., 2007; An et al., 2010).

To rule out the side effects of prolonged DEX treatment, both seedlings and adult plants of the GVG:GUS control line G28 were treated with DEX. It was found that neither the G28 seedlings grown on the induction plate containing 10 μM DEX for 96 h nor the 20-d-old G28 adult plants sprayed with 30 μM DEX for 3 d and grown for an additional 14 d showed phenotypes distinguishable from their mock controls (Supplemental Fig. S2).

Quantitative RT-PCR analysis revealed that overexpressing GmSARK induced the expression of critical senescence-related transcription factors, including NAC1, NAC2, WRKY6, and AtNAP, in Arabidopsis seedlings. As shown in Figure 2A, the expression of NAC1 and WRKY6 was increased after 2 h of DEX treatment, while that of NAC2 and AtNAP was induced
Figure 1. DEX-induced overexpression of GmSARK causes precocious senescence and abnormal flower development in transgenic Arabidopsis plants. A, Seeds of four independent GVG:GmSARK lines (S9, S23, S33, and S48) and a GVG:GUS transformation control line (G28) were germinated on 0.5× MS medium for 5 d in the absence (mock; a) or presence (b) of 10 μM DEX (+DEX). Panel c shows the determination of GmSARK transcript levels in the GVG:GmSARK lines S9, S23, S33, and S48 by semiquantitative RT-PCR analysis. Four-day-old transgenic seedlings were incubated with either 10 μM DEX (+) or its mock solution (−) for 24 h. The TIP41-like gene was used as an internal control in RT-PCR. Three biological replicates with at least three technical repeats were done. For details, see “Materials and Methods.” B, Comparative analyses of the time-course expression profiles of GmSARK and SAG12 genes in a typical GVG:GmSARK line, S23, upon DEX treatment. Four-day-old transgenic Arabidopsis seedlings were incubated on a 10 μM DEX-containing plate for 0, 2, 6, 24, 48, or 72 h. The accumulation of GmSARK and SAG12 transcripts was monitored by quantitative RT-PCR analysis, with the expression of the TIP41-like gene used as an internal control. Three biological replicates with at least three technical repeats were done. Error bars represent SD. C, Four-day-old GVG:GUS (line G28) and GVG:GmSARK (line S23) transgenic Arabidopsis seedlings were grown on vertical plates containing either 10 μM DEX (+DEX) or its mock solution (mock) for an additional 96 h. D, Twenty-day-old GVG:GmSARK transgenic Arabidopsis plants (line S23) were daily sprayed with either mock solution (mock) or 30 μM DEX (+DEX) for 3 d. Photographs were taken at 4 d (a) and 14 d (c). Chlorophyll content of the fifth and sixth leaves of the mock- or DEX-treated S23 plants was determined 9 d after DEX/mock treatment (b). Values represent means of three independent replications (n = 5). Photographs were also taken of flowers from S23 plants sprayed with either mock solution (mock; d and f) or 30 μM DEX (+DEX; e and g). FW, Fresh weight. [See online article for color version of this figure.]
in 24 h. The expression levels of these transcription factors were persistently increased with the extension of DEX treatment time. The induced expression of these senescence-related transcription factors and SAG12 was also observed in 24 h in the rosette leaves of 20-d-old adult S23 plants that were sprayed with 30 μM DEX (Fig. 2B). Among them, similar to what was observed in the young S23 seedlings, the NAC1 transcript accumulated most abundantly. No significant transcript accumulation of these senescence marker genes were detected in the DEX-treated G28 plants.

**GmSARK Overexpression Destroys the Structure of Chloroplasts and Consequently Results in Feedback Promotion of the Activity of the GmSARK Promoter**

To characterize the cellular events caused by the inducible expression of the GmSARK gene, mesophyll cells from DEX-treated GVG:GmSARK transgenic Arabidopsis seedlings were examined by electron microscopy. Ultrastructural morphology analysis revealed a much simpler organized inner membrane system and accumulations of huge starch grains in GmSARK-overexpressing chloroplasts. The amounts of thylakoid and membrane stacking per chloroplast and the layers per granal stack were greatly decreased (Fig. 3Ab).

Quantitative RT-PCR was used to detect the expression levels of genes encoding key enzymes involved in chlorophyll metabolism and chloroplast functions in the DEX-treated GVG:GmSARK transgenic seedlings (Fig. 3B). The results showed a gradual decrease of the expression level of GTR1, the gene encoding glutamyl-tRNA reductase, a key enzyme of chlorophyll biosynthesis, and a significant increase of the expression level of ACD1, which encodes pheide α oxygenase, a key enzyme of chlorophyll breakdown. DEX treatment also resulted in greatly decreased expression of the...
photosynthetic genes \( RbcL \) and \( RbcS \). The expression levels of several critical components of the chloroplast protein transport machinery, including \( Alb3 \), \( cpTatC \), \( Thf1 \), and \( AtcpSecA \), were all successively reduced with continuous DEX treatment (data not shown). The expression level of \( SIG5 \), which is induced under adverse conditions to protect plants from stresses by enhancing repair of the PSII reaction center, was continuously increased with the extension of DEX treatment. Similar changes in the expression levels of the above-mentioned marker genes were found in rosette leaves of adult \( GV\text{G:GmSARK} \) plants that were sprayed with 30 \( \mu M \) DEX (Fig. 3C). These results, consistent with the ultrastructural morphology analysis of the transgenic chloroplasts (Fig. 3Ab), suggested that \( GmSARK \) overexpression promoted the disintegration of chlorophyll and caused deficiencies in the structures and functions of chloroplasts.

We further studied the activity of the \( GmSARK \) promoter in three mutants involving \( AtcpSecA \), \( AtTic20 \), and \( IspF \), all of which exhibited severe defects in both the structure and function of chloroplasts. \( AtcpSecA \) encodes the ATPase subunit of the chloroplast Sec translocation machinery of the thylakoid lumen (Liu et al., 2010); \( AtTic20 \) encodes a component of the import machinery of the inner envelope membrane...
(Chen et al., 2002); and IspF encodes a key enzyme in the nonmevalonate pathway of plastid isoprenoid biosynthesis (Hsieh and Goodman, 2006). Plants carrying loss-of-function mutations in these genes showed albino-lethal phenotypes (Fig. 4B, b–d), due to the developmental defects present in the chloroplasts, including the deficiency of normal thylakoid lamellar structure in their interior (Chen et al., 2002; Hsieh and Goodman, 2006; Liu et al., 2010). The GUS reporter system was used to determine the features of the GmSARK promoter in Arabidopsis. Figure 4A shows that the expression level of the GmSARK:GUS transgene in rosette leaves gradually increased with increasing leaf age. When the GmSARK:GUS cassette was transferred into the seca, ispf, and tic20 mutants by crossing, as shown in Figure 4Be, histochemical GUS staining revealed much darker blue colors in the seca/GmSARK:GUS, ispf/GmSARK:GUS, and tic20/GmSARK:GUS transgenic seedlings than in wild type/GmSARK:GUS. The GUS fluorescence activity assay confirmed the significant increases in the expression of GmSARK:GUS in rosette leaves in all three mutant backgrounds (Fig. 4C). These results suggested a positive feedback loop in which the expression of GmSARK was reinforced by the disintegration of the structure and function of chloroplasts resulting from its expression, to further facilitate the senescence process during the last stage of leaf development.

Overexpression of GmSARK Reduces the Accumulation and Function of Cytokinin in Transgenic Arabidopsis Plants

We have previously found that the exogenous application of 6-benzyl aminopurine (6-BA) effectively retards the senescence-induced accumulation of GmSARK transcripts in soybean (Li et al., 2006). Consistent with this observation, exogenous application of 5 μM 6-BA significantly inhibited the activity of the GmSARK promoter in GmSARK:GUS transgenic Arabidopsis seedlings (Fig. 5A). Cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12) catalyzes the irreversible degradation of cytokinins and in many plant species is responsible for the reduction of active cytokinin (Mok and Mok, 2001). The Arabidopsis CKX gene family has seven members (CKX1–CKX7; Schmulling et al., 2003). Overexpression of different members (CKX1–CKX6) of this family resulted in cytokinin-deficient phenotypes in Arabidopsis (Werner et al., 2003). Quantitative RT-PCR showed that the transcript levels of CKX3 and CKX6 were most highly induced upon DEX treatment (Fig. 5B; CKX6 data not shown). However, the expression levels of IPT3, which encodes the key enzyme (isopenetyltransferase) of cytokinin biosynthesis, AHK3, which encodes the cytokinin receptor functioning in the delay of leaf senescence, the type A ARR5 and ARR6, which have been commonly used as markers for cytokinin responses (Cui et al., 2010), and At2g18300 and At1gl03850 (GRXS13, named recently), which encode two cytokinin-responsive genes, were all significantly reduced (Fig. 5B; ARR5 data not shown). Similar changes in the expression profiles of these marker genes were revealed in adult GV GmSARK plants sprayed with DEX (Fig. 5C). It was interesting that the changes in expression levels of most of the cytokinin-related genes happened rapidly, within 2 h after DEX treatment.

Overexpression of GmSARK Enhances Auxin Responses in Transgenic Arabidopsis Plants

GUS histochemical staining demonstrated that exogenous application of 1 μM IAA significantly enhanced the expression of GmSARK:GUS in transgenic
Arabidopsis seedlings (Fig. 6A). It is interesting that the exogenous auxin also altered the distribution of GmSARK promoter activity, with the staining blue color appearing clearly in the root elongation zone, where no GUS activity was detected in the untreated control (Fig. 6Ab). In addition, exogenous 1 μM IAA treatment for 96 h resulted in a much higher density of lateral roots in the DEX-treated GVG:GmSARK seedlings.
lings than in the GVG:GUS control (Fig. 6B), indicating supersensitivity of the GmSARK-overexpressing seedlings to exogenous auxin. We further used a synthetic auxin-inducible promoter, DR5, to detect auxin accumulation and distribution in the GmSARK-overexpressing seedlings. In the wild-type background, DR5:GUS signals were detected mainly in root tips. However, when transferred into the GmSARK-overexpressing background, they occurred not only at the root tip but also in the root elongation region, with more intense blue colors at both sites (Fig. 6C). These results indicated that the overexpression of GmSARK might exert an influence on auxin actions not only by increasing its content/response but also by modifying its transport and/or distribution in roots of the transgenic seedlings.

Quantitative RT-PCR was used to determine the expression levels of genes involved in auxin synthesis, signal transduction, and responses in the GmSARK-overexpressing seedlings. Upon DEX treatment, the transcripts of two auxin synthesis-related genes, CYP79B2 (encoding cytochrome P450 enzyme, which catalyzes the conversion of Trp to indole-3-acetaldoxime) and TSA1 (encoding the enzyme that catalyzes the conversion of indole-3-glycerolphosphate to indole, the penultimate reaction in the biosynthesis of Trp), and an auxin-responsive gene, GH3.5, were significantly increased, and their expression continued to increase with the extension of DEX treatment time. We also measured the expression level of a repressor of auxin signaling, ARF2, in the GmSARK-overexpressing seedlings, where a lower level of ARF2 was revealed (Fig. 6D). Similar changes in expression profiles of these marker genes were shown in adult GVG:GmSARK plants sprayed with DEX (Fig. 6E).

Overexpression of GmSARK Induces the Biosynthesis of and Responses to Ethylene in Transgenic Arabidopsis Plants

The gas hormone ethylene has been suggested to play a critical role in the regulation of leaf senescence. Exogenous application of 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene, significantly improved the activities of the GmSARK promoter in Arabidopsis seedlings (Fig. 7A). As described above, the morphological phenotypes of flower organs implied enhanced ethylene responses in the GmSARK-overexpressing plants; therefore, we first examined the gene expression levels of the ACC synthase (ACS) family in transgenic plants by quantitative RT-PCR. Among the nine authentic ACS genes in Arabidopsis (Yamagami et al., 2003), only seven members, ACS2, -4, -5, -6, -7, -9, and -11, could be shown to be expressed in the 5-d-old Arabidopsis seedlings; expression of the other two members, ACS1 and ACS8, at this developmental stage was undetectable in our experimental system (Fig. 7B). In the GVG:GmSARK transgenic seedlings at the same developmental stage, DEX treatment for 24 h significantly promoted the expression of ACS4, ACS6, ACS7, and ACS9 but greatly inhibited the expression of ACS2, ACS5, and ACS11, resulting in an apparent increase of active, especially ACS7-containing, ACS dimers (Supplemental Table S1; for details, see “Discussion”).

We further investigated the effects of GmSARK on the expression of four classical ethylene-responsive genes, ERF1, ERF2, ERF4, and AtEBP (Ohme-Takagi and Shinshi, 1995; Büttner and Singh, 1997). The expression of ERF4 and AtEBP was rapidly increased with 2 h of DEX treatment and then kept at steadily higher levels with the extension of DEX treatment. The transcript accumulation of ERF1 and ERF2 was greatly enhanced at 24 h after DEX treatment in GVG:GmSARK seedlings (Fig. 7C). As expected, 24 h of spraying of DEX caused significant increases in the expression levels of these four genes in adult GVG:GmSARK plants (Fig. 7D).

Both Auxin and Ethylene Act as Positive Regulators of GmSARK-Induced Precocious Senescence, and Auxin Functions Upstream of Ethylene

In order to gain further insights into the roles of auxin and ethylene in GmSARK-mediated leaf senescence, we performed exogenous hormone treatment and drug inhibition experiments. As shown in Figure 8, the senescence phenotypes caused by GmSARK overexpression could be effectively attenuated by exogenous application of 10 μM 6-BA (Fig. 8B), 5 μM p-chlorophenoxyisobutyric acid (PCIB; an auxin antagonist; Fig. 8C), or 5 μM aminooxyacetic acid (AVG; an inhibitor of ethylene biosynthesis; Fig. 8D). PCIB and AVG showed stronger effects than 6-BA, and cotreatment of seedlings with these three compounds did not exhibit a significant synergistic effect (Fig. 8E).

We further found that exogenous IAA effectively inhibited the senescence-delay function of 6-BA (Fig. 8F) but had no effect on the inhibitory function of AVG (Fig. 8G). When DEX was combined with 10 μM ACC, neither 6-BA nor PCIB could exert its effects on the GmSARK-induced seedling senescence (Fig. 8, H and I); however, when PCIB and 6-BA were applied together, exogenous ACC was ineffective (Fig. 8J). The results of chlorophyll assays of transgenic seedlings receiving multiple drug treatments were in accordance with the above-mentioned phenotypic observations (Fig. 8K). Besides, quantitative RT-PCR analysis showed that the exogenous 6-BA, PCIB, or AVG treatment effectively inhibited GmSARK-induced expression of the senescence-related marker genes mentioned previously (data not shown).

To further verify the results obtained from the exogenous hormone and inhibitor treatments, we crossed a typical GVG:GmSARK transgenic line, S23, with either the auxin influx transporter mutant aux1-7 or the ethylene-insensitive mutant ein2-1. The homozygous lines produced from the crosses above were identified and used to determine the effects of the absence of proper auxin transport or ethylene signal-
ing on GmSARK functions. Semiquantitative RT-PCR analysis proved that neither aux1-7 nor ein2-1 mutation affected the DEX-induced accumulation of GmSARK transcript in the transgenic Arabidopsis seedlings (Fig. 9Aa). As described previously, when treated with 10 μM DEX for 120 h, the GVG:GmSARK seedlings e-

**Figure 6.** DEX-induced overexpression of GmSARK changes auxin sensitivity, distribution, and responses in Arabidopsis. A, Effects of exogenous IAA on GUS activity in GmSARK:GUS transgenic Arabidopsis seedlings. GUS activities were determined by histochemical staining in 7-d-old GmSARK:GUS seedlings treated with (+IAA) or without (ck) 1 μM IAA for 24 h (a). Panel b shows a closeup image of roots in the transgenic seedlings treated with (+IAA) or without (ck) IAA for 12 h. B, Overexpression of GmSARK enhanced auxin sensitivities in Arabidopsis seedlings. a, Four-day-old GVG:GUS (line G28) and GVG:GmSARK (line S23) transgenic seedlings grown on vertical plates containing 10 μM DEX and 1 μM IAA for 96 h. b, Magnification of the roots of the seedlings in a, showing that the lateral root density of S23 was significantly higher than that of the G28 control. C, Expression profile of DR5:GUS in the roots of 5-d-old wild-type (WT/DR5:GUS) and GmSARK-overexpressing (GVG:GmSARK/DR5:GUS) seedlings. Four-day-old GVG:GmSARK (line S23) and GVG:GUS (line G28) seedlings were incubated with 10 μM DEX for 0, 2, 6, 24, or 48 h. The transcript accumulation of the marker genes was monitored by quantitative RT-PCR, with the expression of the TIP41-like gene used as an internal control. Three biological replicates with at least three technical repeats were done. Error bars represent SD. E, Overexpression of GmSARK changes the expression of auxin-related marker genes in adult Arabidopsis plants. Twenty-day-old S23 and G28 transgenic plants were sprayed with either mock solution (mock) or 30 μM DEX (+DEX) for 24 h before the fifth and sixth leaves were harvested. The transcript accumulation of the marker genes was determined by quantitative RT-PCR, with the expression of the TIP41-like gene used as an internal control. Three biological replicates with at least three technical repeats were done for each gene. Error bars represent SD. [See online article for color version of this figure.]

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hibited significantly early senescence; however, both the aux1-7/GVG:GmSARK and ein2-1/GVG:GmSARK seedlings developed normally, resembling the mock-treated or GVG:GUS control seedlings, and did not show any precocious leaf senescence phenotypes (Fig. 9Ab). Similarly, the GmSARK-induced precocious senescence was also suppressed in the DEX-sprayed aux1-7/GVG:GmSARK and ein2-1/GVG:GmSARK adult plants. They possessed leaves that were comparable in size to the mock control (Fig. 9Ba), and their flower development was also indistinguishable from that of the mock plants (Fig. 9Bb); the reduction of chlorophyll content that resulted from GmSARK overexpression was also recovered in the two mutant backgrounds (Fig. 9Bc).

The GmSARK-induced increases in expression levels of several senescence- or hormone-related marker genes used in the above described experiments, such as SAG12, NAC1, WRKY6, GH3.5, ERF1, and ERF2, were also effectively suppressed by the AUXIN RESISTANT1 (AUX1) and EIN2 mutations (Fig. 10). These results are consistent with the observations from the above inhibitor experiments, supporting that the absence of action of auxin or ethylene would destroy the functions of GmSARK and, further, that both auxin and ethylene act as positive regulators of GmSARK-induced precocious senescence. However, as reported previously (Noodén, 1988a; Grbić and Bleeker, 1995), exogenous application of 10 μM ACC, 1 μM IAA, or a combination of ACC and IAA, over a period of 120 h, was insufficient to initiate leaf senescence in wild-type Arabidopsis seedlings (Supplemental Fig. S4), suggesting cooperation between GmSARK and these phytohormones.

Figure 7. DEX-induced overexpression of GmSARK promotes the expression of genes involved in ethylene synthesis and responses in Arabidopsis. A, Effects of exogenous ACC on GUS activity in GmSARK:GUS transgenic Arabidopsis seedlings. GUS activities were determined by histochemical staining in 7-d-old GmSARK:GUS seedlings treated with (+ACC) or without (ck) 10 μM ACC for 24 h. B, Quantitative RT-PCR analysis of the expression levels of ACS genes in GmSARK-overexpressing seedlings. Four-day-old transgenic plants were treated with either mock solution (mock) or 10 μM DEX (DEX) for 24 h. C, Analysis of time-course expression profiles of several ethylene-responsive genes in DEX-induced GVG:GmSARK seedlings. Four-day-old GVG:GmSARK (line S23) and GVG:GUS (line G28) seedlings were incubated with 10 μM DEX for 0, 2, 6, 24, or 48 h. The transcript accumulation of the ethylene-responsive genes was monitored by quantitative RT-PCR, with the expression of the TIP41-like gene used as an internal control. Three biological replicates with at least three technical repeats were done. Error bars represent SD. D, Overexpression of GmSARK enhances the expression of the ethylene-responsive genes in adult Arabidopsis plants. Twenty-day-old S23 and G28 transgenic plants were sprayed with either mock solution (mock) or 30 μM DEX (+DEX) for 24 h before the fifth and sixth leaves were harvested. The transcript accumulation of the marker genes was determined by quantitative RT-PCR, with the expression of the TIP41-like gene used as an internal control. Three biological replicates with at least three technical repeats were done for each gene. Error bars represent SD. [See online article for color version of this figure.]
Identification of a GmSARK Homologous Gene in Arabidopsis

The importance of GmSARK in mediating leaf senescence engaged us in a search for a functional homolog of GmSARK in the model plant Arabidopsis. Since GmSARK was originally identified as one of the highly induced LRR-RLK genes during leaf senescence (Li et al., 2006), and accelerated senescence was indeed observed in the GVG:GmSARK transgenic Arabidopsis lines following DEX treatment, we postulated that a functional homolog of GmSARK may be identified from the most highly induced Arabidopsis LRR-RLKs in the GVG:GmSARK transgenic lines following DEX treatment. When transcript changes of LRR-RLKs in the GmSARK-overexpressing seedlings were analyzed, one gene (At4g30520) did emerge with the highest induction level at all DEX treatment time points (Supplemental Fig. S5A). Both quantitative RT-PCR and promoter-reporter analysis supported that the expression level of this LRR-RLK gene in rosette leaves was also gradually increased with increasing leaf age (Supplemental Fig. S5, B and C). Thus, we called this gene AtSARK, following the designation of GmSARK.

Sequence analysis suggested that the 649-amino acid product of AtSARK is a typical LRR-RLK. The SignalP 3.0 Server program (Bendtsen et al., 2004) revealed a signal peptide at its N terminus, with the most likely cleavage site located between amino acid residues 30 and 31 (Thr-Thr). A strong transmembrane helix from amino acid 274 to 296 was predicted by Tmpred (Hofmann and Stoffel, 1993). The Scansite program (Obenauer et al., 2003) revealed the presence of five LRR motifs from amino acid 34 to 192 in the extracellular region and a typical protein kinase domain from amino acid 303 to 571 in the cytoplasmic region (Supplemental Fig. S5D). Homology analysis showed that AtSARK is 20.02% and 15.13% identical to GmSARK and PvSARK, with higher homology in the extracellular domain (PvSARK and GmSARK) or the kinase domain (GmSARK and AtSARK), respectively (Supplemental Fig. S6).

As performed for the study of GmSARK, a GV:AtSARK cassette was introduced into Arabidopsis to

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Figure 8. Blocking either auxin- or ethylene-related functions effectively prevents GmSARK-induced early leaf senescence in Arabidopsis. A to J, Phenotypic characterizations of GmSARK-overexpressing seedlings treated with exogenous phytohormones or chemical inhibitors. Four-day-old GVG:GUS (line G28) and GVG:GmSARK (line S23) transgenic Arabidopsis seedlings were grown vertically on 0.5×MS medium containing 10 μM DEX and combinations of phytohormones or inhibitors for 120 h. K, Determination of chlorophyll content in GmSARK-overexpressing seedlings treated with exogenous phytohormones or chemical inhibitors. Shoots of 4-d-old G28 and S23 seedlings treated with 10 μM DEX and combinations of the indicated phytohormones or inhibitors (GmSARK-ox) was normalized to it. Values represent means of three independent replications (n = 60). [See online article for color version of this figure.]
characterize the functions of the AtSARK gene. Seeds of five independent GVG:AtSARK transgenic lines, AtS1, AtS5, AtS20, AtS22, and AtS35, and of the GVG:GUS-transformed control strain G28 were sown on either DEX-containing or mock plates. It was found that all AtS lines were indistinguishable from the G28 control on the mock plate (Supplemental Fig. S7Aa); however, when grown on the DEX plate, lines AtS5, AtS20, AtS22, and AtS35 exhibited seedling lethality (Supplemental Fig. S7Ab). The expression levels of the leaf age-related senescence marker gene SAG12 and senescence-related transcription factors NAC1, NAC2, AtNAP, and WRKY6 were all greatly promoted (Supplemental Fig. S8). Moreover, DEX spraying caused great growth inhibition (Fig. 11Ba) and a stepped-up progression of leaf senescence in adult AtS20 plants (Fig. 11Bb). The chlorophyll content in AtSARK-overexpressing plants was also much lower than in the control plants (Fig. 11Bc). The DEX-sprayed AtS20 plants also developed abnormal flowers with stunted petals, short stamens, and protruding gynoecium, as the GmSARK-overexpressing seedlings did (Fig. 11Bd). As shown in Supplemental Figure S9, exogenous application of either 5 μM PCIB or 5 μM AVG could effectively restore the senescence phenotypes caused by AtSARK overexpression in the transgenic seedlings.

SARKs Promote Leaf Senescence through Auxin and Ethylene

Figure 9. Mutations in AUX1 and EIN2 effectively prevent GmSARK-induced premature leaf senescence and abnormal flower development in Arabidopsis. Aa, Determination of GmSARK transcript levels in GVG:GUS (line G28), GVG:GmSARK (line S23), aux1-7/GVG:GmSARK (aux1-7/S23), and ein2-1/GVG:GmSARK (ein2-1/S23) transgenic seedlings by semiquantitative RT-PCR analysis. Four-day-old transgenic seedlings were treated with either 10 μM DEX (+) or the mock solution (−) for 24 h. The TIP41-like gene was used as an internal control in RT-PCR. Ab, Four-day-old GVG:GUS (line G28), GVG:GmSARK (line S23), aux1-7/GVG:GmSARK, and ein2-1/GVG:GmSARK transgenic seedlings grown on vertical plates containing either 10 μM DEX (+DEX) or its solution (mock) for an additional 120 h. B, Twenty-day-old GVG:GUS (line G28) GVG:GmSARK (line S23), aux1-7/GVG:GmSARK, and ein2-1/GVG:GmSARK transgenic plants were daily sprayed with either mock solution (mock) or 30 μM DEX (+DEX) for 3 d. The rosettes (a) were photographed at an additional 9 d, and the flowers (b) were photographed at an additional 14 d. Chlorophyll content of the fifth and sixth leaves of the plants mentioned above was determined at an additional 9 d (c). Values represent means of three independent replications (n = 6). FW, Fresh weight. [See online article for color version of this figure.]

Characterize the functions of the AtSARK gene. Seeds of five independent GVG:AtSARK transgenic lines, AtS1, AtS5, AtS20, AtS22, and AtS35, and of the GVG:GUS-transformed control strain G28 were sown on either DEX-containing or mock plates. It was found that all AtS lines were indistinguishable from the G28 control on the mock plate (Supplemental Fig. S7Aa); however, when grown on the DEX plate, lines AtS5, AtS20, AtS22, and AtS35 exhibited seedling lethality (Supplemental Fig. S7Ab). The AtS1 line, in which the expression of AtSARK was silenced, as revealed by quantitative RT-PCR (Supplemental Fig. S7B), showed no significant difference in growth and development compared with the G28 control group. In subsequent experiments, AtS20 was selected as a typical line for further study of AtSARK overexpression.

Similar to what was observed in the GmSARK-overexpressing seedlings, AtS20 seedlings cultured vertically on the DEX plate exhibited both precocious senescence and the curved-roots phenotypes (Fig. 11A). The expression levels of the leaf age-related senescence marker gene SAG12 and senescence-related transcription factors NAC1, NAC2, AtNAP, and WRKY6 were all greatly promoted (Supplemental Fig. S8). Moreover, DEX spraying caused great growth inhibition (Fig. 11Ba) and a stepped-up progression of leaf senescence in adult AtS20 plants (Fig. 11Bb). The chlorophyll content in AtSARK-overexpressing plants was also much lower than in the control plants (Fig. 11Bc). The DEX-sprayed AtS20 plants also developed abnormal flowers with stunted petals, short stamens, and protruding gynoecium, as the GmSARK-overexpressing seedlings did (Fig. 11Bd). As shown in Supplemental Figure S9, exogenous application of either 5 μM PCIB or 5 μM AVG could effectively restore the senescence phenotypes caused by AtSARK overexpression in the transgenic seedlings.

The AtSARK gene consists of 11 exons. A Salk T-DNA line, SALK_111290, which has a T-DNA insertion in the 10th exon (Fig. 12A), was obtained from the
Arabidopsis Biological Resource Center at Ohio State University. The absence of accumulation of full-length AtSARK transcripts in homozygous SALK_111290, named sark-1 in our studies, was confirmed by semi-quantitative RT-PCR analysis of 32-d-old seedlings (data not shown). When phenotypic changes in growth and development between the sark-1 mutant and wild-type seedlings were compared, obvious delays in chlorophyll degradation and leaf senescence were observed in the sark-1 mutant (Fig. 12, B–D). To gain a better view of the function of AtSARK in leaf senescence, the development process of the fourth leaves of the sark-1 mutant and the wild type was compared over a period of time. The sark-1 mutant exhibited prolonged leaf longevity in comparison with the wild type (Fig. 12E). While wild-type leaves started to turn yellow at the leaf age of 20 d after emergence, the sark-1 mutant leaves started to turn yellow at 24 d after emergence (i.e. 4 d later than wild-type leaves). In addition, the bolting time and flowering time of sark-1 plants were nearly 2 d later than in the wild-type controls. The sark-1 seedlings also produced smaller rosettes and fewer leaves than did the wild-type plants (Supplemental Table S2).

Both GmSARK and AtSARK Encode Functional Ser/Thr and Tyr Dual-Specificity Protein Kinase

To determine biochemical characteristics of GmSARK and AtSARK, their cytoplasmic kinase domains (KD) were fused with glutathione S-transferase (GST) and expressed in Escherichia coli. The GST-GmSARK-KD
and GST-AtSARK-KD fusion proteins were purified by affinity chromatography using a Glutathione Sepharose 4B column and tested for autophosphorylation activity by western blotting with either anti-phospho-Ser or anti-phospho-Tyr antibody. Both GST-GmSARK-KD and GST-AtSARK-KD possessed functional kinase activities and could autophosphorylate on both Thr and Tyr residues in vitro (Fig. 13), indicating that both GmSARK and AtSARK are dual-specificity protein kinases.

DISCUSSION

We have previously demonstrated, using GmSARK-RNAi and 35S:GmSARK transgenic plants, that GmSARK plays a critical role in the regulation of soybean leaf senescence (Li et al., 2006). In this study, a glucocorticoid-regulated transcriptional induction system (Aoyama and Chua, 1997) that allows controllable overexpression of a target gene at any desirable stage was employed to analyze the detailed functions of GmSARK as well as its Arabidopsis homologous gene, AtSARK, in leaf senescence. We found that exogenous DEX-induced GmSARK overexpression resulted in early leaf senescence in the GVG:GmSARK transgenic Arabidopsis plants, and similar phenotypes were also observed in the AtSARK-overexpressing plants (Figs. 1 and 11). These results confirmed our previous observations and suggested that the mechanism of GmSARK-mediated leaf senescence may be widespread among higher plants.

Both developmental signals and environmental cues regulate leaf senescence. In the GVG:GmSARK and GVG:AtSARK transgenic plants, the accumulation of SAG12 transcript, which is taken as a molecular marker of developmental senescence (Noh and Amasino, 1999), occurred at quite early times after exogenous DEX treatment (Fig. 1B; Supplemental Fig. S8B). The expression levels of several critical senescence-related transcriptional factors, NAC1, NAC2, AtNAP, and WRKY6, were all greatly enhanced, and the sequence of changes in their expression is similar to their gene expression mode in the natural senescence process (Robatzek and Somssich, 2002; Guo and Gan, 2006; Kim et al., 2009). These results are in accordance with the observations that promoter activities of the two LRR-RLK genes were up-regulated during natural leaf senescence (Fig. 4A; Supplemental Fig. S5), suggesting that the functions of GmSARK and AtSARK are closely correlated with the natural leaf senescence process controlled by developmental signaling.

During leaf senescence, the most prominent change in cell structure is the breakdown of the chloroplast,
which has been widely used as a biomarker for leaf senescence (Thomson and Plat-Aloia, 1987; Otegui et al., 2005; Pruzinska et al., 2005; Prins et al., 2008). However, no definite conclusions have been reached as to whether and how the disintegration of chloroplasts affects the process of leaf senescence. Ultrastructural morphology analysis revealed much simpler inner membrane systems in chloroplasts of mesophyll cells from the GmSARK-overexpressing leaves. The number of thylakoids was greatly reduced, and large starch grains accumulated in the transgenic chloroplasts (Fig. 3Ab). On the other hand, defects in chloroplast structure and function, caused by mutations in either the key component of the chloroplast protein-sorting machines, AtcpSecA or AtTic20, or the key enzyme of plastid isoprenoid biosynthesis, IspF, significantly promoted the activity of the GmSARK promoter (Fig. 4). Taken together, these results implied the existence of a positive feedback loop involved in the regulation of GmSARK-mediated leaf senescence. We postulated that, during the last stage of leaf development, the so-called senescence signal(s) initiated the senescence process by inducing the expression of GmSARK or a GmSARK-like gene (e.g. AtSARK in Arabidopsis); the high expression of SARK gene(s) resulted in disintegration of the structure and function of chloroplasts, which, in turn, reinforced the expression of SARK(s) to further facilitate the senescence process to enter an irreversible stage.

Cytokinin is best known as a senescence-delaying hormone. The reduction of cytokinin levels has been observed during the leaf senescence process in many plant species, including soybean (Singh et al., 1988), tobacco (Singh et al., 1992), and sunflower (Helianthus annuus; Skene, 1975). Although cytokinin deficiency does not appear to trigger leaf senescence in soybean, a decline in foliar cytokinin level may sensitize the soybean leaf to the action of the developmental senescence signal (Nooden, 1988b). We have previously shown that the expression of GmSARK is down-regulated by exogenous cytokinin (Li et al., 2006). In this study, we found that the promoter activity of GmSARK was significantly increased with the onset of leaf senescence but greatly decreased by treatment with exogenous cytokinin (Figs. 4A and 5A). These results tempted us to postulate that during leaf development,
enhanced auxin sensitivity and senescence (van der Graaff et al., 2006). The evidently genes are up-regulated during age-dependent leaf senescence (van der Graaff et al., 2006), the precise role of auxin in leaf senescence was unclear (Lim et al., 2007a). Quantitative RT-PCR analysis revealed an up-regulation of the expression of auxin-related genes changed greatly during leaf senescence (van der Graaff et al., 2006). Quantitative RT-PCR expression during Arabidopsis natural senescence published DNA chip data showing elevated ACS expression during Arabidopsis natural senescence (van der Graaff et al., 2006). Quantitative RT-PCR analysis revealed much enhanced expression of the ethylene-responsive factors in the GmSARK-overexpressing plants (Fig. 7, C and D), suggesting that the high expression of GmSARK not only enhanced the synthesis of ethylene but also promoted its signal responses.

Ethylene exerts profound effects on floral organ morphology. Many Arabidopsis mutants displaying enhanced ethylene responses, such as ctr1-1, ctr1-3 ers1-9 double mutant, ein2-2:GFP, and ein3 ebf1 ebf2/35S:EIL1:GFP (OE/tn) plants, have been reported to produce flowers with stunted petals and protruding gynoecia, whereas the ethylene-insensitive mutants or plants showing normal ethylene responses displayed normal flower morphology (Kieber et al., 1993; Alonso et al., 1999; Hall and Bleeker, 2003; Qu et al., 2007; An et al., 2010). It is interesting to find that both GmSARK- and AtSARK-overexpressing flowers exhibited similar morphological characteristics to those of constitutive ethylene response mutants (Figs. 1D, e and g, and 11Bd), strongly supporting the above conclusions of enhanced ethylene responses during SARKs-induced senescence.

Ethylene and auxin have a long history of reported interactions both at the physiological and molecular

![Figure 13](http://www.plantphysiol.org/content/157/6/2147/F13.large.jpg)

**Figure 13.** Both GmSARK and AtSARK autophosphorylate on Thr and Tyr residues in vitro. Autophosphorylation activities of the recombinant GmSARK (A) and AtSARK (B) kinase domains were detected by western blotting (WB) using either anti-phospho-Thr (p Thr Ab) or anti-phospho-Tyr (p Y Ab) antibody. Equivalent protein loading was visualized by Ponceau S staining. [See online article for color version of this figure.]
levels (Pitts et al., 1998; Rahman et al., 2002; Swarup et al., 2002; Stepanova et al., 2005, 2007). There also exist synergistic regulations in the biosynthesis of the two hormones (Jones and Kende, 1979; Stepanova et al., 2005; Swarup et al., 2007). The observations that exogenous application of PCIB and AVG could effectively inhibit \textit{GmSARK} or \textit{AtSARK}-induced precocious senescence in Arabidopsis suggested that auxin and ethylene are directly involved in the regulation of \textit{SARK}s-mediated leaf senescence. The involvement of auxin and ethylene in the positive regulation of leaf senescence was further confirmed by the facts that both \textit{AUX1} and \textit{EIN2} mutations almost completely recovered the \textit{SARK}s-induced symptoms in Arabidopsis, including the dwarf and early-senescence phenotypes, the short and curved roots, the reduced rosette size, and abnormal flower development (Fig. 9), as well as the higher expression levels of the typical senescence marker genes (Fig. 10). The fact that exogenous application of IAA or ACC alone could not induce senescence in Arabidopsis seedlings (Supplemental Fig. S4) indicated that the high expression of \textit{SARK}s and the \textit{SARK}s-mediated developmental signal(s) were necessary for the actions of the two hormones in senescence promotion. It is interesting to find that the senescence-delay effect of PCIB was effectively inhibited by exogenous ACC, while the exogenous IAA had no effect on the ability of AVG to restore the precocious leaf senescence induced by \textit{GmSARK} (Fig. 8, G and I). These observations tempted us to postulate that ethylene works downstream of auxin in the regulation of \textit{SARK}s-mediated senescence.

We also found that exogenous ACC could inhibit the effect of either 6-BA or PCIB on \textit{GmSARK}-induced senescence; however, when PCIB and 6-BA were applied to the \textit{GmSARK}-overexpressing seedlings together, exogenous ACC exhibited no effects (Fig. 8, H–J). These results indicated that ethylene could promote leaf senescence only at a specific developmental stage that meets at least three prerequisites: (1) \textit{SARK}-mediated development/senescence signal transduction; (2) the decrease of cytokinin content and/or responses; and (3) the increase of auxin level and/or responses resulted from the senescence signaling. We hypothesize that the balance among cytokinin, auxin, and ethylene plays important roles in senescence initiation and the senescence process. This helps explain why exogenous application of IAA or ACC alone could not induce senescence in young Arabidopsis seedlings.

The \textit{AUX1} gene encodes an auxin influx transporter in Arabidopsis roots (Maher and Martindale, 1980; Marchant et al., 1999). The \textit{aux1}-7 mutation in this gene confers both auxin and ethylene resistance (Pickett et al., 1990). In addition to its role as a central component of the ethylene signal transduction pathway (Alonso et al., 1999; An et al., 2010), \textit{EIN2} also functions as a cross-link node in ethylene and other phytohormone signaling pathways (Ghassemian et al., 2000; Wang et al., 2007). We found that the \textit{ein2}-1 seedlings also exhibited insensitivity to exogenous IAA (data not shown). Thus, it is unsurprising to find that both \textit{aux1}-7 and \textit{ein2}-1 mutations could recover the precocious senescence symptoms caused by \textit{SARK} overexpression in transgenic Arabidopsis seedlings.

It is notable that the expression level of \textit{ARF2}, which has been suggested to function as a repressor of auxin signaling, was reduced in \textit{GmSARK}-overexpressing seedlings (Fig. 6, D and E). Although this result is in accordance with our observations that \textit{GmSARK} overexpression could promote the responses to auxin, it seems contrary to the previous studies reporting that the \textit{arf2} mutants show a delayed-senescence phenotype, thus indicating that enhanced auxin level can delay leaf senescence (Ellis et al., 2005; Lim et al., 2010). We cannot definitively explain this contradiction based on current data. It has been suggested that \textit{ARF2} regulates leaf senescence and floral organ abscission independently of the ethylene and cytokinin pathways (Ellis et al., 2005). It is possible that \textit{GmSARK} and \textit{ARF2} regulate leaf senescence by different molecular mechanisms. In \textit{GmSARK}-mediated senescence, auxin functions downstream of cytokinin and works together, at least with ethylene, to regulate leaf development.

Based on the above analyses and results, we propose that \textit{GmSARK} and functional \textit{GmSARK} homologous genes, such as \textit{AtSARK} in Arabidopsis, play critical roles in the initiation and progress of leaf senescence. In young leaves, developmental signals and high cytokinin levels repress the expression of \textit{SARK} genes. During natural leaf senescence, because of the age-dependent reduction of cytokinin content, the expression of \textit{SARK} genes is released to enhance the synthesis of and responses to auxin and ethylene and possibly other pathways, thereby accelerating senescence. The acceleration of senescence and the resulting destruction of chloroplasts cause feedback promotion of the expression of \textit{SARK} genes, and the increased expression of \textit{SARK}s further inhibits the functions of cytokinin to form a feed-forward loop to regulate the progress of leaf senescence.

Leaf senescence in \textit{sark}-1, a T-DNA knockout mutant of \textit{AtSARK}, was significantly delayed (Fig. 12). As observed in many senescence-delayed mutants, such as \textit{arf1}, \textit{arf2}, heterozygous \textit{ORE7/ore7-1D}, and \textit{etr1-1} (Grbic and Bleecker, 1995; Ellis et al., 2005; Lim et al., 2007b), the \textit{sark}-1 plants flowered later than their wild-type control (Supplemental Table S2). This raises the question of whether the delay in flowering causes a delay in leaf senescence in the \textit{sark}-1 mutant. In many monocarpic species, such as soybean, leaf longevity is controlled by the reproductive structures. Arabidopsis, however, has been reported to show a disconnection between the reproductive structures and the lives of individual leaves (Hensel et al., 1993; Noorden and Penney, 2001). Neither male- and female-sterile mutations nor surgical removal of the stems with inflorescences (bolts) at various stages could significantly
increase the longevity of individual rosette leaves (Noodén and Penney, 2001). In co-2 and fai, representatives of the two distinct groups of late-flowering mutants in Arabidopsis, the longevity of individual leaves is not changed (Hensel et al., 1993). The best way to address the concern about differences in flowering is to examine senescence in individual leaves, such as the fourth leaf in Arabidopsis (Hensel et al., 1993; Yang et al., 2011). The observations that senescence in the fourth leaf was significantly delayed in the sark-1 mutant (Fig. 12E) and that the leaf senescence-delayed phenotype of sark-1 (a 4-d delay) was much more pronounced than the flowering-delayed phenotype (a 2-d delay) suggested that the senescence-delayed phenotype of sark-1 is not directly linked to its late-flowering phenotype.

As mentioned above, leaf senescence in soybean is governed by the reproductive structures. Our previous observations that the GmSARK-RNAi transgenic soybean plants exhibit abnormal development of flower organs confirm the existence of such correlative controls in soybean (Li et al., 2006). In this study, however, similar phenotypes were not observed in the sark-1 mutant, supporting that leaf senescence in Arabidopsis is not linked with the development of the reproductive structures in the same way as it is in soybean. The phenotypic differences in flower development between the GmsARK-RNAi transgenic soybean and the sark-1 Arabidopsis mutant might reflect the differences between soybean and Arabidopsis in the correlative controls of leaf senescence and reproductive development at the whole plant level.

Animals contain a large family of receptor Tyr kinases, but no classical protein Tyr kinase has hitherto been cloned from plants (de la Fuente van Bentem and Hirt, 2009). Instead, characterizations of protein kinases with both Ser/Thr and Tyr specificities, which are named dual-specificity protein kinases (Lindberg et al., 1992), were reported in several plants, including soybean (Feng et al., 1993), petunia (Petunia inflata; Mu et al., 1994), tobacco (Sessa et al., 1996; Cho et al., 2001), peanut (Arachis hypogaea; Rudrabhatla and Rajasekharan, 2002, 2003), rice (Oryza sativa; Gu et al., 2005), tomato (Solanum_lycopersicum; Mayrose et al., 2004), and Arabidopsis ( Hirayama and Oka, 1992; Ali et al., 1994; Tregear et al., 1996). A recent work also documented that several functionally known Arabidopsis receptor-like kinases, including HAESA (Jinn et al., 2000), BKK1 (He et al., 2007), and BRI1 and BAK1 (Wang et al., 2008), appeared to be dual-specificity protein kinases (Oh et al., 2009). Although an increasing number of dual-specificity protein kinases have been documented, data supporting the existence of such kinases involved in the regulation of leaf senescence are scarce. To the best of our knowledge, BKK1/BAK7, a component of the BR signaling pathway (He et al., 2007), is the only known example. Down-regulation of BAK7 expression by RNAi resulted in severe growth retardation and early senescence in the transgenic plants (Jeong et al., 2010). In this study, the results that GmSARK and AtSARK could autophosphorylate in vitro on both Ser/Thr and Tyr residues defined them as dual-specificity protein kinases. It has been suggested that Tyr phosphorylation is as extensive in plants as in animals (de la Fuente van Bentem and Hirt, 2009). Our results provide further evidence for the critical roles of dual-specificity protein kinases in regulating leaf senescence and reproductive growth in plants. Identification of the autophosphorylation sites, the ligands, and the substrates of GmSARK and AtSARK will help elucidate the molecular mechanisms controlling leaf development in higher plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 was used for all experiments. The T-DNA insertion lines used in this study were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003).

The Arabidopsis seeds were surface sterilized in a solution of 25% bleach plus 0.01% Triton X-100 for 10 min and washed with sterilized water five times. Following 3 d of stratification in the dark at 4°C, these seeds were germinated and grown on vertical plates (0.5× MS medium containing 0.8% agar and 1% Suc, pH 5.7), supplemented with or without a variety of antibiotics and chemical reagents) at 20°C ± 1°C with cycles of 16 h of light and 8 h of darkness to produce Arabidopsis seedlings. The 10-d-old seedlings were transferred into soil and grown under the same conditions for further experiments and seed production.

For the activity analysis of the SARK promoters, GmSARK::GUS and AtSARK::GUS transgenic seedlings were germinated and grown on vertical selection plates (0.5× MS medium containing 30 mg L⁻¹ hygromycin [Roche], 0.8% agar, plus 1% Suc, pH 5.7). The 7-d-old transgenic GmSARK::GUS seedlings were subjected to different hormone treatments. Alternatively, both the GmSARK::GUS and AtSARK::GUS transgenic adult plants exhibiting a 1-cm visible inflorescence shoot were sampled for histochemical GUS staining assay. To characterize the activities of the GmSARK promoter in mutants with defects in chloroplast function, 21-d-old homozygous “albino” seca/GmSARK::GUS, ispf/GmSARK::GUS, and tic20/GmSARK::GUS seedlings and green wild type/GmSARK::GUS seedlings were sampled.

Glucocorticoid treatments were performed as described by Aoyama and Chua (1997). For the assessment of the effects of SARK genes on seed germination, GVG::GmSARK or GVG::AtSARK transgenic seeds and their corresponding GVG::GUS control seeds were germinated and grown horizontally on 0.5× MS plates containing either 10 μM DEX (Sigma; DEX plate) or its solvent, 0.01% Tween 20 (mock plate), for 5 to 7 d before the images were recorded. To investigate the functions of the two SARK genes, 4-d-old GVG::GmSARK or GVG::AtSARK transgenic seedlings and their corresponding GVG::GUS transformation controls were transferred from the basal medium to the DEX plate or mock plate. Where indicated, the plates were supplemented with different hormones or chemicals. The plates were kept under the growth conditions mentioned above for 96 to 120 h. The images were recorded with a scanner (Epson 1260) at the indicated time points. The seedlings were sampled and subjected to transmission electron microscopy examination after 120 h of treatment.

For phenotypic analyses of the adult plants, 20-d-old soil-grown GVG::GmSARK or GVG::AtSARK transgenic plants were daily sprayed with either 30 μM DEX or its mock solution containing 0.01% Tween 20 three times. The phenotypes were recorded with a camera (Canon PowerShot G10) at the indicated time points. The camera was also used to record the phenotypes of sark-1 mutants. Morphological changes in flowers of GVG::GmSARK or GVG::AtSARK transgenic plants were examined using a microscope (Leica M165 FC) and photographed with a Leica DFC420 C camera.

For the chlorophyll content assay, the shoots of 4-d-old GVG::GUS and GVG::GmSARK seedlings incubated with 10 μM DEX or combinations of the indicated phytohormones or inhibitors for an additional 120 h were harvested. Alternatively, the fifth and sixth leaves of DEX-sprayed adult plants were sampled at the indicated time points for the determination of chlorophyll content.

In order to detect the expression levels of the described marker genes, 4-d-old GVG::GmSARK or GVG::AtSARK transgenic seedlings were transferred from
the basal medium to fresh horizontal DEX plates. After 0 to 72 h of incubation, the seedlings were sampled and the expression levels of the marker genes were detected. Meanwhile, 20-d-old transgenic plants were sprayed with DEX or its mock solution for 24 h, and then the fifth and sixth leaves were harvested and the expression levels of the marker genes were detected.

Identification of T-DNA Insertion Mutants

As suggested by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimer2.html), a PCR-based method was used to identify homozygous T-DNA insertion mutants. The T-DNA left border primer LBi1.3 and gene-specific primers tic20-LP and tic20-RP for tic20-1 (SALK_009676) and sark-1-LP and sark-1-RP for sark-1 (SALK_111290) were used in the PCR. The identifications of homozygous seca (SALK_063371) and ispf (SALK_002470) mutants were performed as described by Liu et al. (2010) and Hsieh and Goodman (2006). All mutant plants used in this study were homozygous for the T-DNA insertions. The primers presented in this paper are listed in Supplemental Table S3.

Constructs, Plant Transformation, and Crossing

To construct the GmSARK-GUS and AISARK-GUS fusion genes, a 1,801-bp DNA fragment covering the 5′ flanking region of the GmSARK gene (GenBank accession no. AY687391) and a 1,688-bp promoter fragment of the AISARK gene (A4tg30250) were amplified from soybean (Glycine max) and Arabidopsis (Arabidopsis thaliana) genomic DNA by PCR, respectively. The pairs of primers used in the PCR were Gm-P1 and Gm-P2 for GmSARK and At-P1 and At-P2 for AISARK. These DNA fragments were then inserted into the binary vector pCAMBIA1301 to create the recombinant transcription units GmSARK-GUS and AISARK-GUS, respectively. The transcripts were terminated with a NOS terminator.

For the construction of GVG::GmSARK, GVG::AISARK, and GVG::GUS units, a 1,812-bp DNA fragment of the GUS gene was first amplified by PCR from the binary vector pBI121 using the primer pair GUS-1 and GUS-2. This DNA fragment was inserted into a binary vector pTA7002 (Aoyama and Chua, 1997) to create a recombinant transcription unit GmSARK-GUS and AISARK-GUS, respectively. The transcripts were terminated with a NOS terminator.

Histochemical GUS staining, fluorometric GUS activity assay, chlorophyll content determination, and transmission electron microscopy examination were done as described previously (Liu et al., 2010). Chlorophyll content was spectrophotometrically measured as described (Arnon, 1949). At least three independent samples were examined to get the typical results shown in this paper.

RNA isolation and RT-PCR analysis of gene expression

RNA extraction, cDNA synthesis, and RT-PCR analysis were performed as described previously (Liu et al., 2010). For semiquantitative RT-PCR analysis, gene-specific primers GmSARK-1 and GmSARK-2 for GmSARK and At-1 and At-2 for AISARK were used to amplify the transcripts of GmSARK and AISARK, respectively. As an internal control, a fragment of TIP41-like, one of the recommended new quantitative RT-PCR standards (Czechowski et al., 2005; Udvardi et al., 2008), was amplified under the same conditions with the primers TIP-F and TIP-R. Three independent repeats were done to give the typical results shown here. All primers used in quantitative RT-PCR analysis are listed in Supplemental Table S3. TIP41-like was used as an internal control. All reactions were done at least in triplicate.

Recombinant protein purification and in vitro kinase assay

The GST-GmSARK-KD and GST-AISARK-KD fusion proteins were expressed in E. coli Rosetta 2 (DE3) pLysS and purified by affinity chromatography using a Glutathione Sepharose 4B column (GE Healthcare) according to the manufacturer’s recommendations. Autophosphorylation reactions were performed by incubating 1.5 μg of the purified GST-kinase fusion proteins in a 15-μL reaction buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 10 mM MnCl2, and 1 mM diithiothreitol at 28°C for 1 h in the absence (control) or presence of 1 μg/mL 32P ATP. The reactions were terminated by the addition of 2× SDS sample buffer. Western blotting was performed to detect the in vitro autophosphorylation activities of the purified proteins by either anti-phospho-Thr or anti-phospho-Tyr antibody (Invitrogen) as described previously (Chen and Guan, 1994). Fumonisin S staining of the final blot was used to confirm that equivalent amounts of GST fusion proteins were used in the assay. Data shown are representative of at least three independent experiments.

Sequence data from this article can be found in the Arabidopsis Genome Initiative (AGI) genome database under the following accession numbers: GmSARK (AY687391), NAC1 (At1g56030), NAC2 (At1g504410), AINAP (At1g69490), WRKY6 (At1g26300), SAG12 (At1g58390), SGS (At1g24120), GTR1 (At1g85820), AC1T (At1g34480), Rhl (AtCC00490.1), Rhs (At1g67090), AHK3 (At1g27230), ARR6 (At5g62902), IPT3 (At5g53110), CKX3 (At5g6970).
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Overexpression of GmSARK causes precocious senescence in adult Arabidopsis plants.

Supplemental Figure S2. DEX treatment has no effect on the growth and development of GVG:GUS transgenic plants.

Supplemental Figure S3. Effects of different concentrations of hormones or chemical inhibitors on the growth and development of GmSARK-overexpressing seedlings.

Supplemental Figure S4. Neither IAA nor ACC, or a combination of both, initiates leaf senescence in Arabidopsis.

Supplemental Figure S5. Expression analyses of the AtSARK gene and structural illustration of the AtSARK protein.

Supplemental Figure S6. Sequence alignment of GmSARK, AtSARK, and PvSARK.

Supplemental Figure S7. DEX-induced overexpression of AtSARK results in seedling lethality.

Supplemental Figure S8. DEX-induced overexpression of AtSARK enhances the expression of several senescence-related marker genes in Arabidopsis.

Supplemental Figure S9. Exogenous treatment of PCIB or AVG effectively prevents AtSARK-induced early senescence in Arabidopsis.

Supplemental Table S1. DEX-induced overexpression of GmSARK changes the relative ratio between active and inactive ACS isoforms in transgenic Arabidopsis plants.

Supplemental Table S2. Differences in growth parameters between the wild type and sark-T.

Supplemental Table S3. Primers developed for the construction of plant transformation vectors, RT-PCR, and genotyping of T-DNA insertion lines.

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GRXS13 (At1g30850), AT2g18300, CYP79B2 (At4g39950), TASA (At1g56400), GH3.5 (At4g27750), ARF2 (At5g53200), ACS2 (At1g03480), ACS4 (At2g28510), ACS5 (At1g56800), ACS6 (At4g12180), ACS7 (At1g26200), ACS9 (At1g87000), ACS11 (At1g08040), ERF1 (At3g23240), ERF2 (At1g67220), AIEBP (At3g16770), ERF4 (At3g15210), AIsARK (At4g30520), and TIP41-like (At4g34270).
SARKs Promote Leaf Senescence through Auxin and Ethylene