A Soybean Dual-Specificity Kinase, GmSARK, and Its Arabidopsis Homolog, AtSARK, Regulate Leaf Senescence through Synergistic Actions of Auxin and Ethylene[1][C][W][OA]

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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 seedlings 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 *ETHYLENE 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; Guiamét 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-

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thesis, can significantly delay leaf senescence in P_{SAG12}:*IPT* transgenic tobacco (*Nicotiana tabacum*; 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 Graaff et al., 2006). These results suggest that auxin may be a positive regulator of leaf senescence. However, some studies reported opposite conclusions; for example, a T-DNA insertion in *ARF2*, a repressor of auxin signaling, causes senescence delay in Arabidopsis rosette leaves (Ellis et al., 2005). And two allelic mutations, *ore14-1/arf2-10* and *ore14-2/arf2-11*, also cause significant delays in all senescence parameters in Arabidopsis (Lim et al., 2010). These data imply that auxin is involved in the negative regulation of leaf senescence. Taken together, the precise role of auxin in leaf senescence is unclear and requires further investigation.

A large body of evidence indicates that ethylene promotes leaf senescence. Ethylene specifically promotes the transcription of senescence-associated genes but represses the expression of photosynthetic genes (Grbić and Bleecker, 1995). Two ethylene-insensitive mutants, *etr1-1* and *ein2-1*, both show increased leaf longevity (John et al., 1995; Oh et al., 1997). Overexpression of a mutant form of the Arabidopsis ethylene receptor gene, *AtETR1-1*, not only aborted ethylene sensitivity but also delayed both the onset and the progression of leaf and flower senescence in transgenic tobacco (Yang et al., 2008). However, neither the ethylene constitutive response mutant *ctr1* (Kieber et al., 1993) nor wild-type Arabidopsis seedlings grown in the continuous presence of exogenous ethylene showed premature senescence (Grbić and Bleecker, 1995). Aided by the analysis of *old* mutants, Jing et al. (2002, 2005) suggested that ethylene does not directly regulate the onset of leaf senescence but acts to modulate the timing of leaf senescence. A recent study suggested that a critical component of ethylene signal transduction, *ETHYLENE INSENSITIVE2* (*EIN2*), plays an important role in the "trifurcate feed-forward pathway" for the regulation of Arabidopsis leaf senescence (Kim et al., 2009).

Receptor-like protein kinases (RLKs) are cell surface receptors that typically consist of an extracellular domain to perceive a specific signal, a transmembrane domain to anchor the protein within the membrane, and a cytoplasmic kinase domain to transduce the signal downstream via autophosphorylation, followed by further phosphorylation of specific substrates (Stone and Walker, 1995). The unique structure of RLKs makes them critical components of plant developmental 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), HAESA, which controls floral organ abscission (Jinn et al., 2000), and BRI1 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. In *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 (GVG system; GVG is the abbreviation of a chimeric transcription factor, 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 autophosphorylate 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. 1A). 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. 1Dc).

In addition to facilitating leaf senescence, *GmSARK* overexpression also affected the development of floral organs. As shown in Figure 1D, e and g, the 20-d-old *S23* seedlings sprayed with DEX produced flowers with severely altered morphology compared with the mock-treated control. These flowers were reduced in size and remained almost completely closed (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

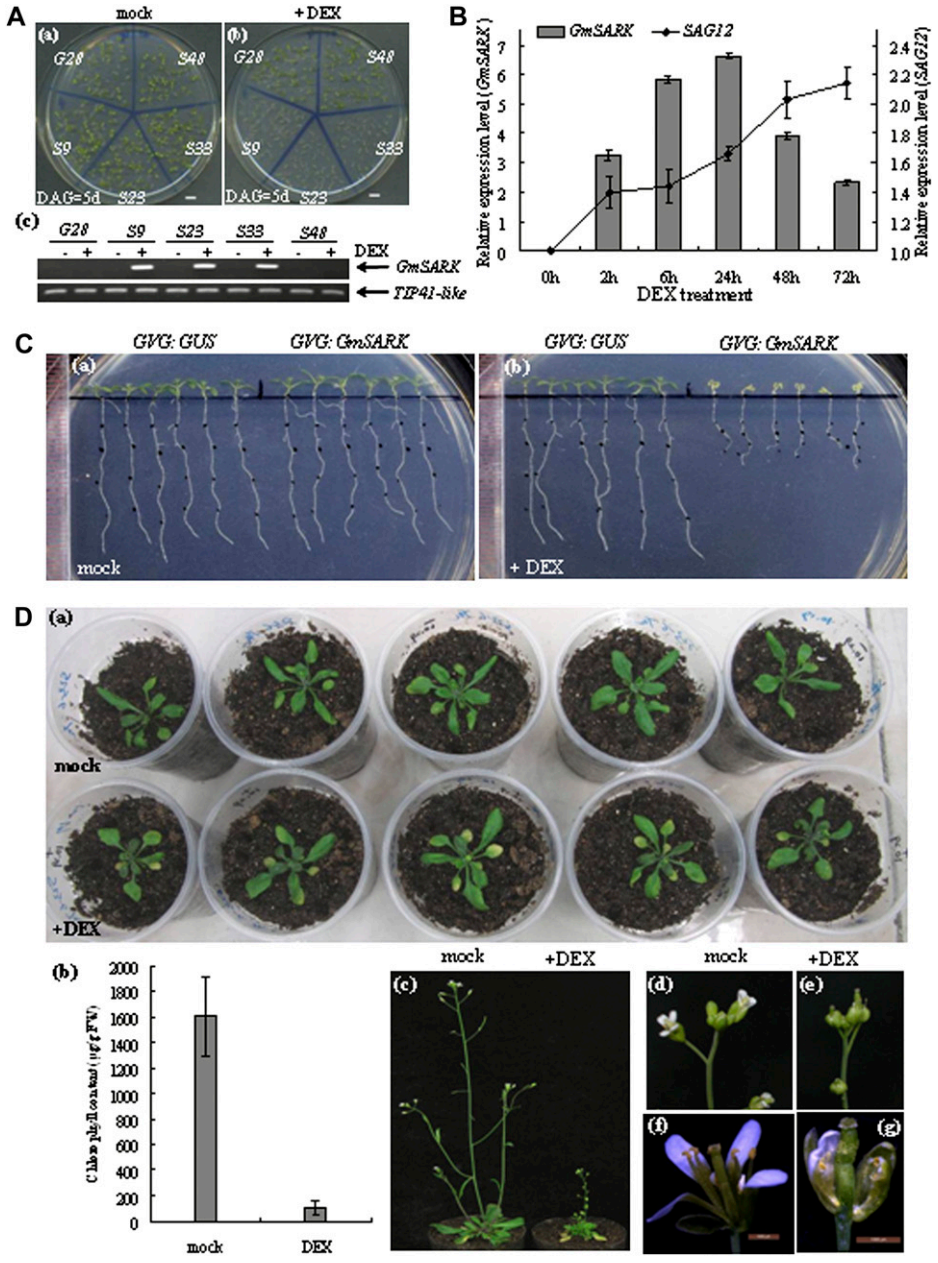

Figure 1. DEX-induced overexpression of *GmSARK* causes precocious senescence and abnormal flower development in transgenic Arabidopsis plants. A, Seeds of four independent *GVG*:*GmSARK* lines (*S9*, *S23*, *S33*, and *S48*) and a *GVG*:*GUS* transformation control line (*G28*) were germinated on 0.5× 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.]

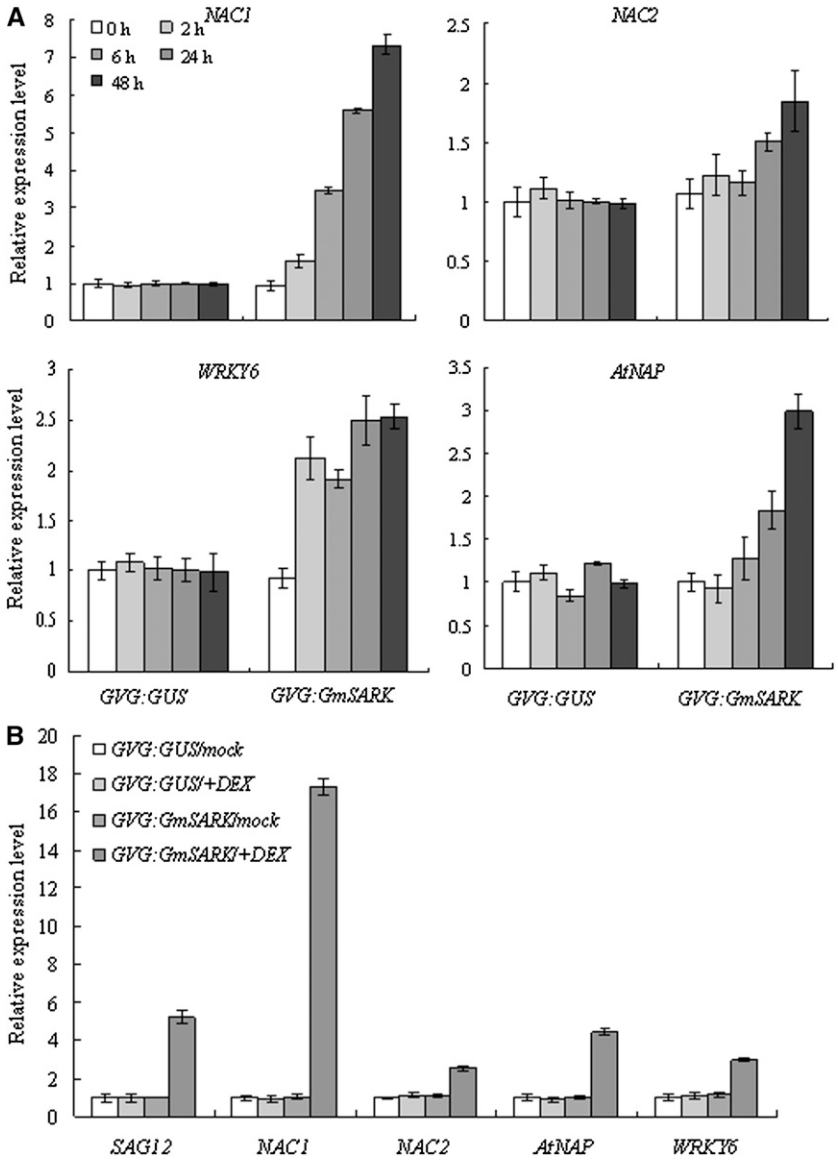

Figure 2. DEX-induced overexpression of *GmSARK* enhances the expression levels of critical senescence-related transcriptional factors in transgenic Arabidopsis plants. A, Analysis of time-course expression profiles of several senescence-related transcriptional factors in DEX-induced *GVG*:*GmSARK* seedlings. 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 senescence-related 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. B, Overexpression of *GmSARK* enhances the expression of senescence-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.

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 *a* oxygenase, a key enzyme of chlorophyll breakdown. DEX treatment also resulted in greatly decreased expression of the

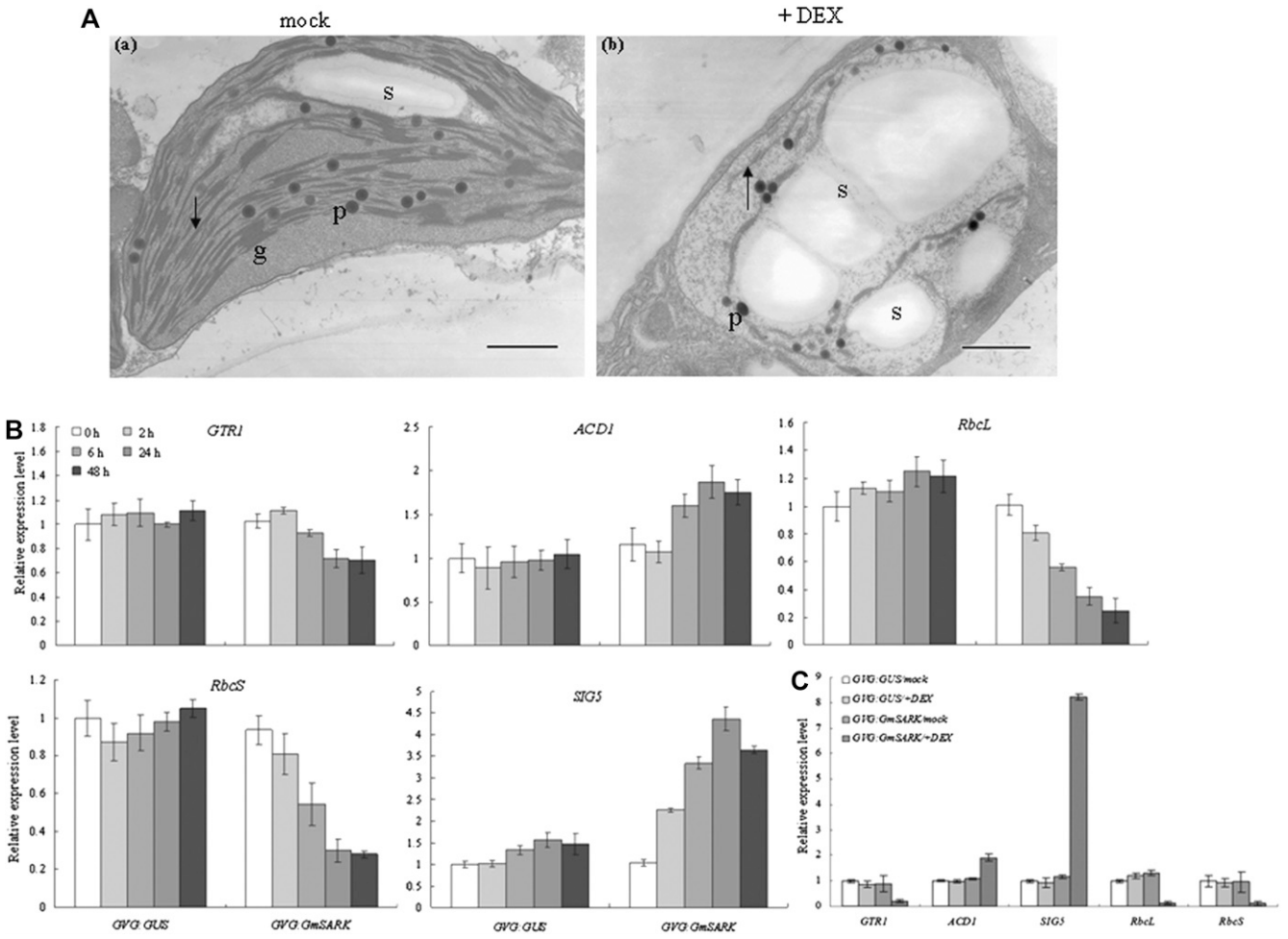

Figure 3. DEX-induced overexpression of *GmSARK* causes chloroplast dysfunction in transgenic Arabidopsis seedlings. A, Ultrastructural morphology of chloroplasts in mesophyll cells of *GVG:GmSARK* transgenic seedlings in the absence (mock; a) or presence (b) of 10 μM DEX (+DEX). g, Grana; p, plastoglobuli; s, starch. The arrows indicate the thylakoid. Bars = 1.0 μm. B, Analysis of time-course expression profiles of several genes involved in chlorophyll metabolism and chloroplast functions 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 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. C, Overexpression of *GmSARK* changes the expression of genes involved in chlorophyll metabolism and chloroplast functions 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.

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 *GVG:GmSARK* plants that were sprayed with 30 μM 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*; Schmülling 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 (isopentenyltransferase) of cytokinin biosynthesis, *AHK3*, which encodes the cytokinin receptor functioning in the delay of leaf senescence, the type A ARRs *ARR5* and *ARR6*, which have been commonly used as markers for cytokinin responses (Cui et al., 2010), and *At2g18300* and *At1g03850* (*GRXS13*, named recently), which encode two cytokinin-responsive genes, were

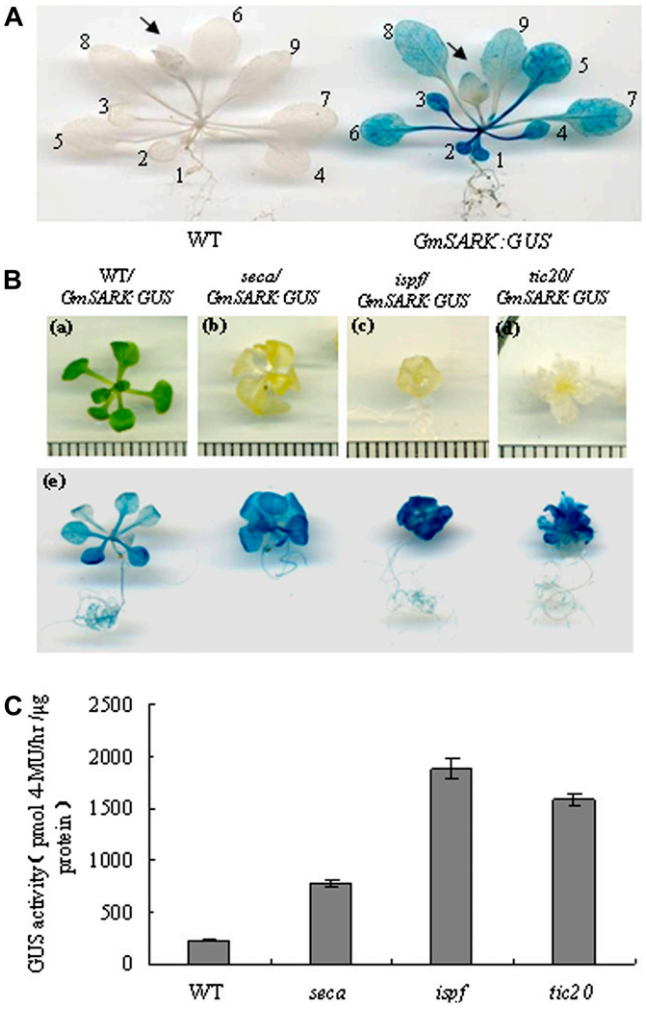

Figure 4. Defects in chloroplast structure and function increase the *GmSARK*:*GUS* activity in transgenic Arabidopsis plants. A, Expression of *GmSARK*:*GUS* in rosette leaves increased gradually with leaf aging. *GmSARK*:*GUS* transgenic Arabidopsis plants with a 1-cm-long inflorescence located at the center of the rosette were sampled for histochemical GUS staining. The numbers indicate the order of leaf emergence. B and C, *GmSARK*:*GUS* activities in 21-d-old *seca*, *ispf*, and *tic20* mutants and their wild-type control determined by histochemical staining (B) and fluorometric assay (C). 4-MU, 4-Methylumbelliferone; WT, wild type. [See online article for color version of this figure.]

all significantly reduced (Fig. 5B; *ARR5* data not shown). Similar changes in the expression profiles of these marker genes were revealed in adult *GVG*:*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

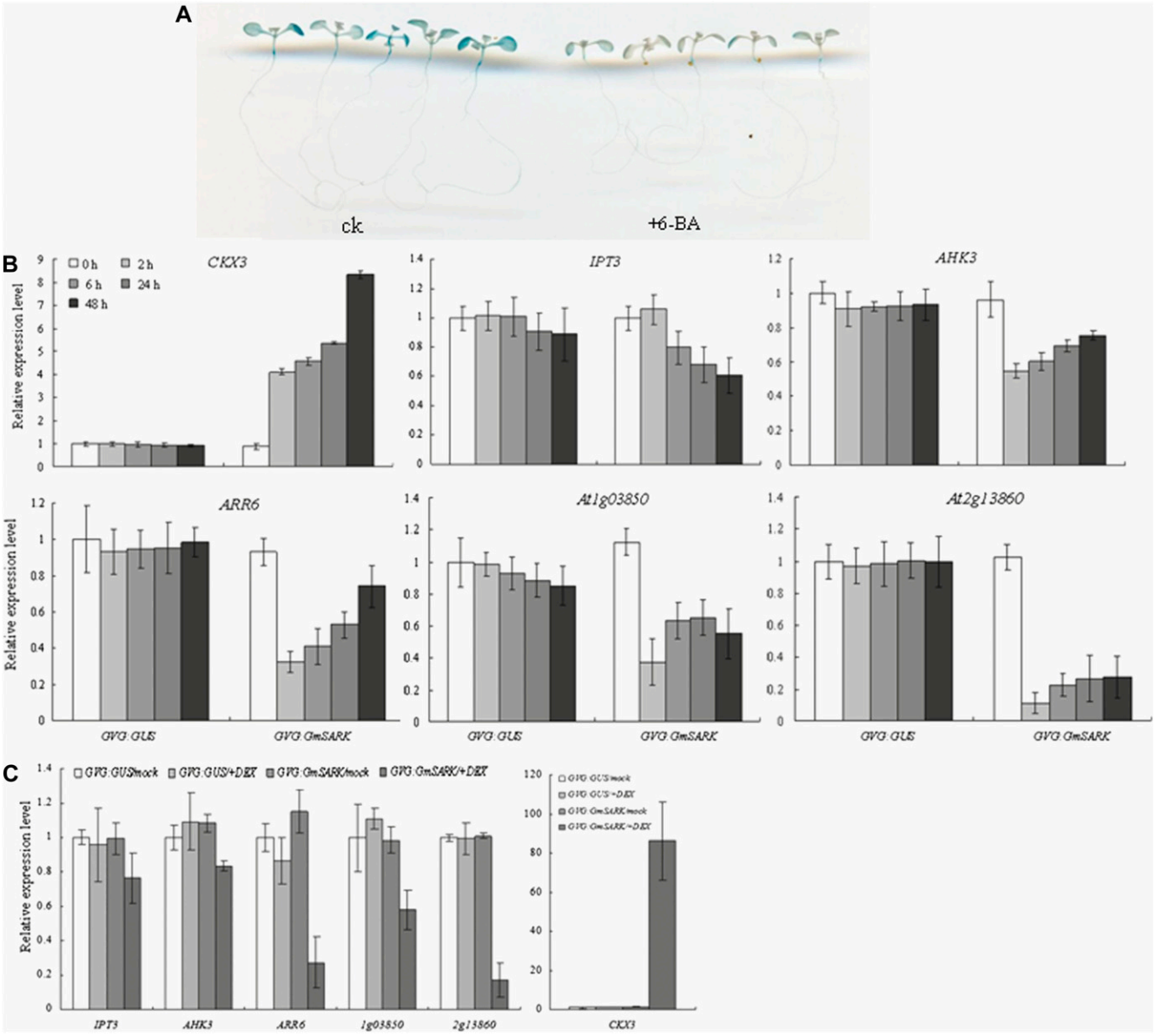

Figure 5. DEX-induced overexpression of *GmSARK* suppresses the expression of genes involved in cytokinin biosynthesis and responses and induces the expression of genes functioning in cytokinin degradation. A, Exogenous 6-BA inhibits *GmSARK*:*GUS* expression in Arabidopsis. GUS activity was determined in 7-d-old *GmSARK*:*GUS* transgenic Arabidopsis seedlings treated with (+6-BA) or without (ck) 5 μM 6-BA for 24 h. B, Analysis of time-course expression profiles of cytokinin-related marker 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 cytokinin-related markers 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. C, Overexpression of *GmSARK* changes the expression of cytokinin-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.]

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* seed-

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 aminoethoxyvinylglycine (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-

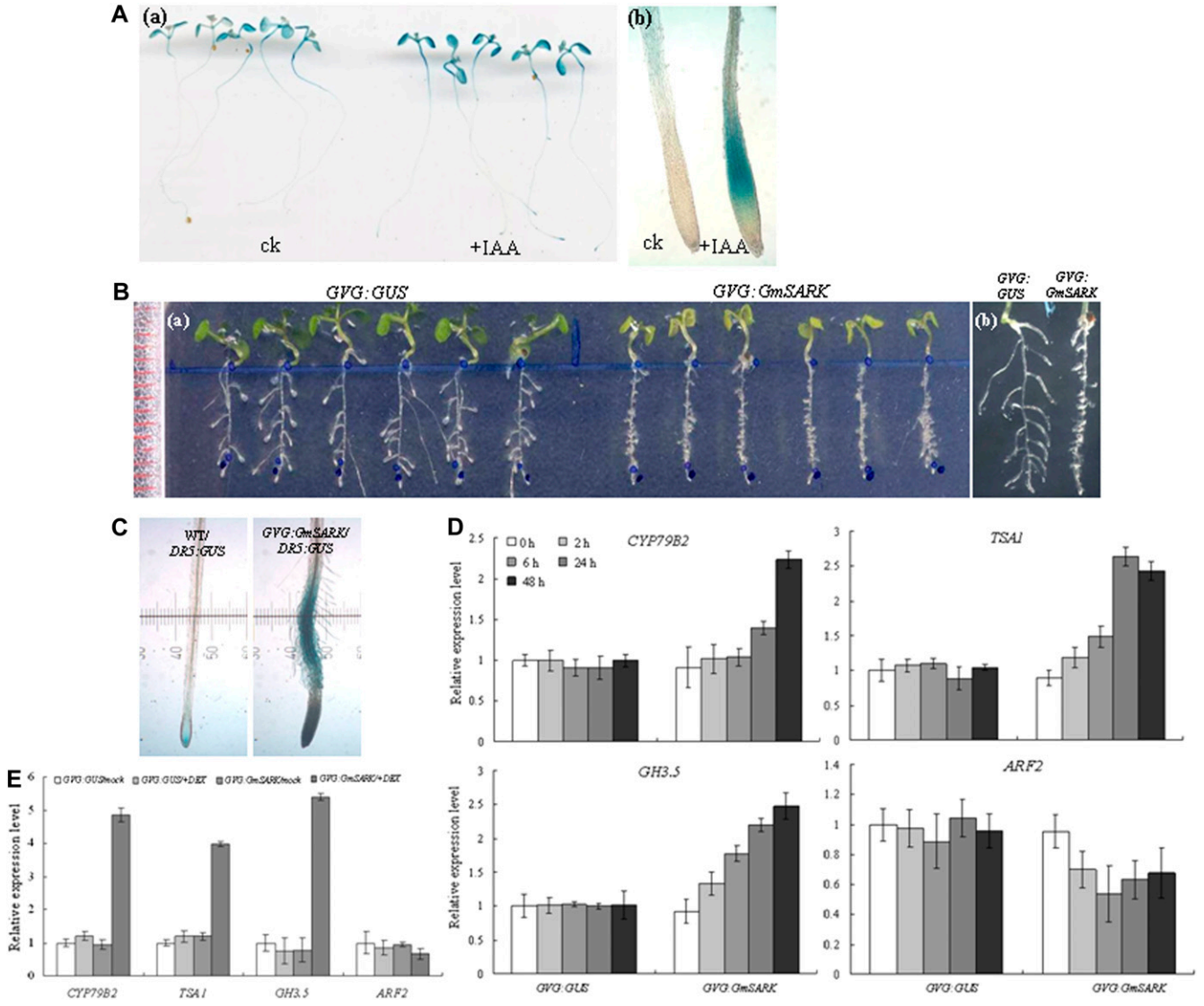

Figure 6. DEX-induced overexpression of *GmSARK* changes auxin sensitivity, distribution, and responses in Arabidopsis. A, Effects of exogenous IAA on GUS activity in *GmSARK*:*GUS* transgenic Arabidopsis seedlings. GUS activities were determined by histochemical staining in 7-d-old *GmSARK*:*GUS* seedlings 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. D, Analysis of time-course expression profiles of several auxin-related marker 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 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.]

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 ex-

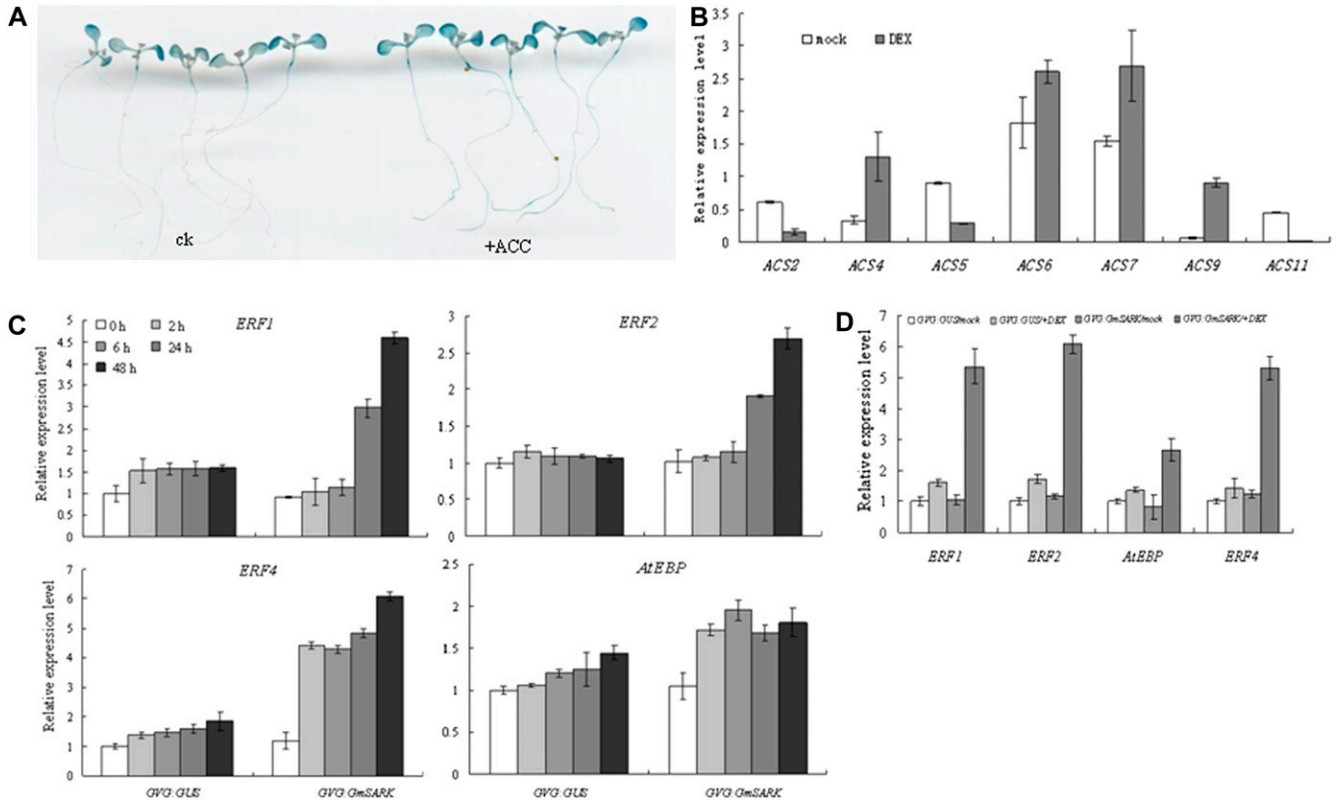

Figure 7. DEX-induced overexpression of *GmSARK* promotes the expression of genes involved in ethylene synthesis and responses in Arabidopsis. A, Effects of exogenous ACC on GUS activity in *GmSARK*:*GUS* transgenic Arabidopsis seedlings. GUS activities were determined by histochemical staining in 7-d-old *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.]

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 Bleecker, 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.

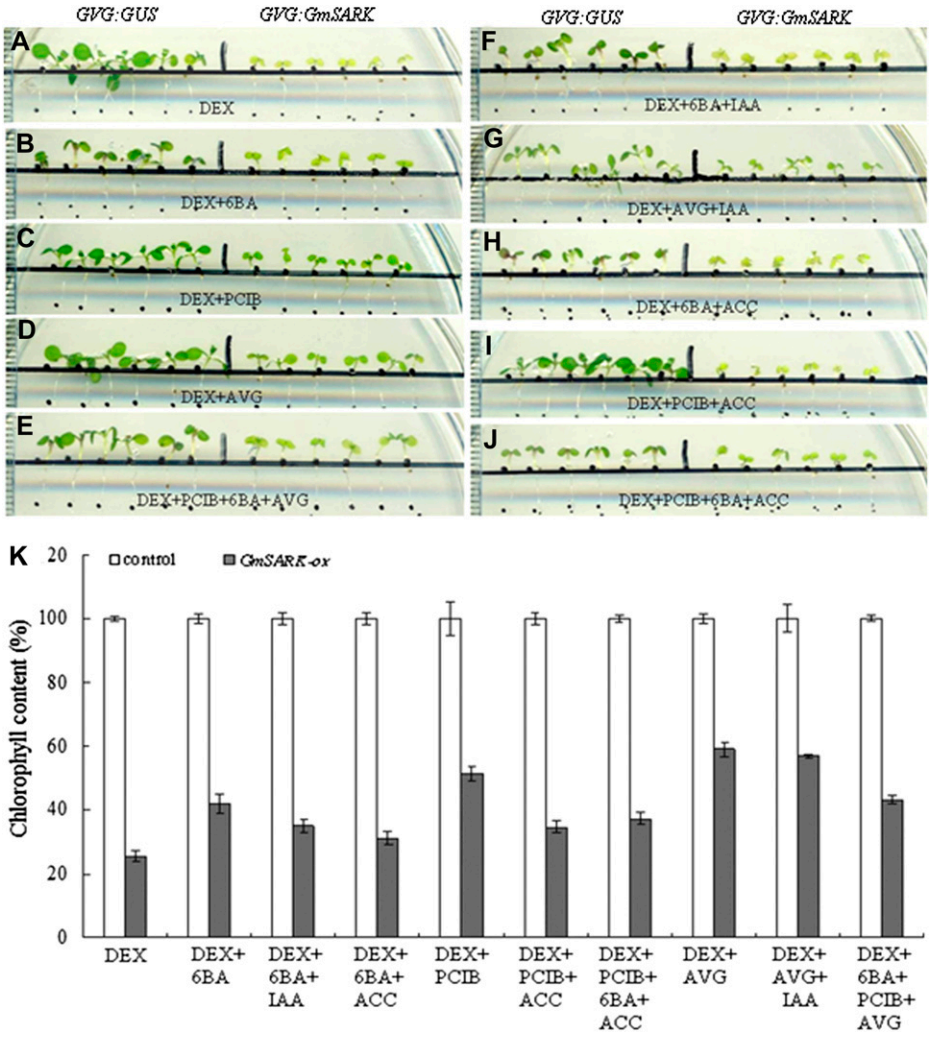

Figure 8. Blocking either auxin- or ethylene-related functions effectively prevents *GmSARK*-induced early leaf senescence in Arabidopsis. A to J, Phenotypic characterizations of *GmSARK*-overexpressing seedlings treated with exogenous phytohormones or chemical inhibitors. Four-day-old *GVG:GUS* (line *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 for an additional 120 h were harvested for chlorophyll content assay. The chlorophyll content in the *G28* seedlings in each treatment (control) was taken as 100%, and the chlorophyll content in the *S23* seedlings treated with the same combination of 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.]

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 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 *GVG:AtSARK* cassette was introduced into Arabidopsis to

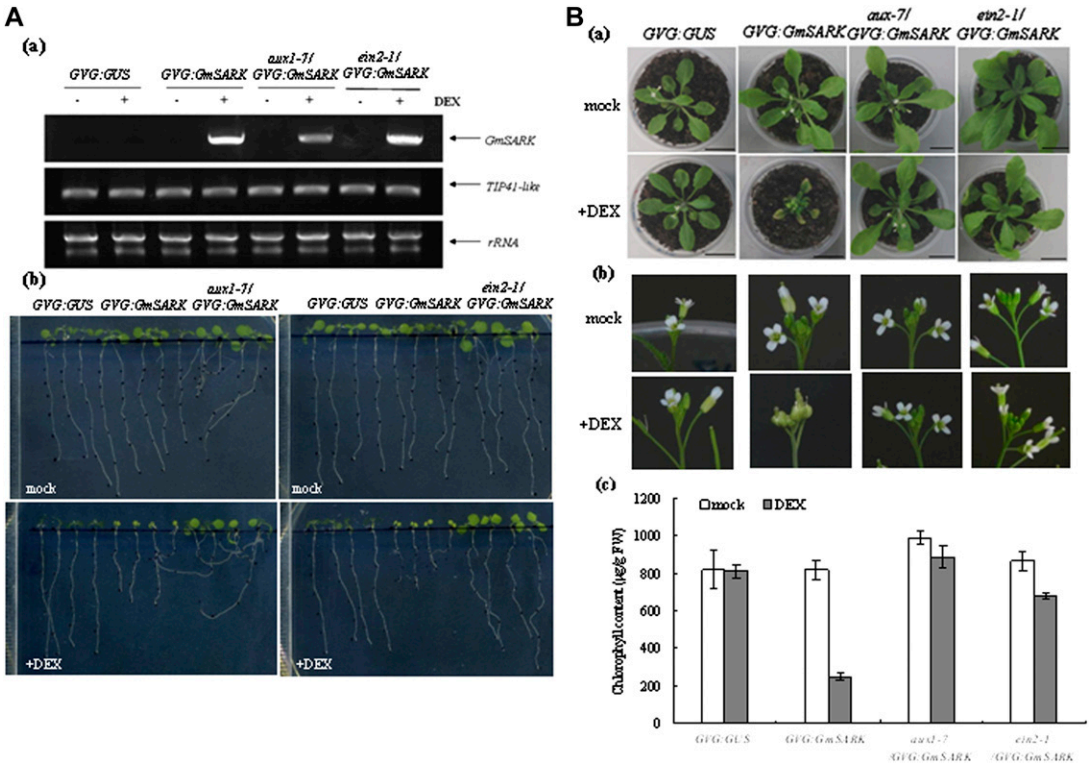

Figure 9. Mutations in *AUX1* and *EIN2* effectively prevent *GmSARK*-induced premature leaf senescence and abnormal flower development in Arabidopsis. Aa, Determination of *GmSARK* transcript levels in *GVG:GUS* (line *G28*), *GVG:GmSARK* (line *S23*), *aux1-7/GVG:GmSARK* (*aux1-7/S23*), and *ein2-1/GVG:GmSARK* (*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

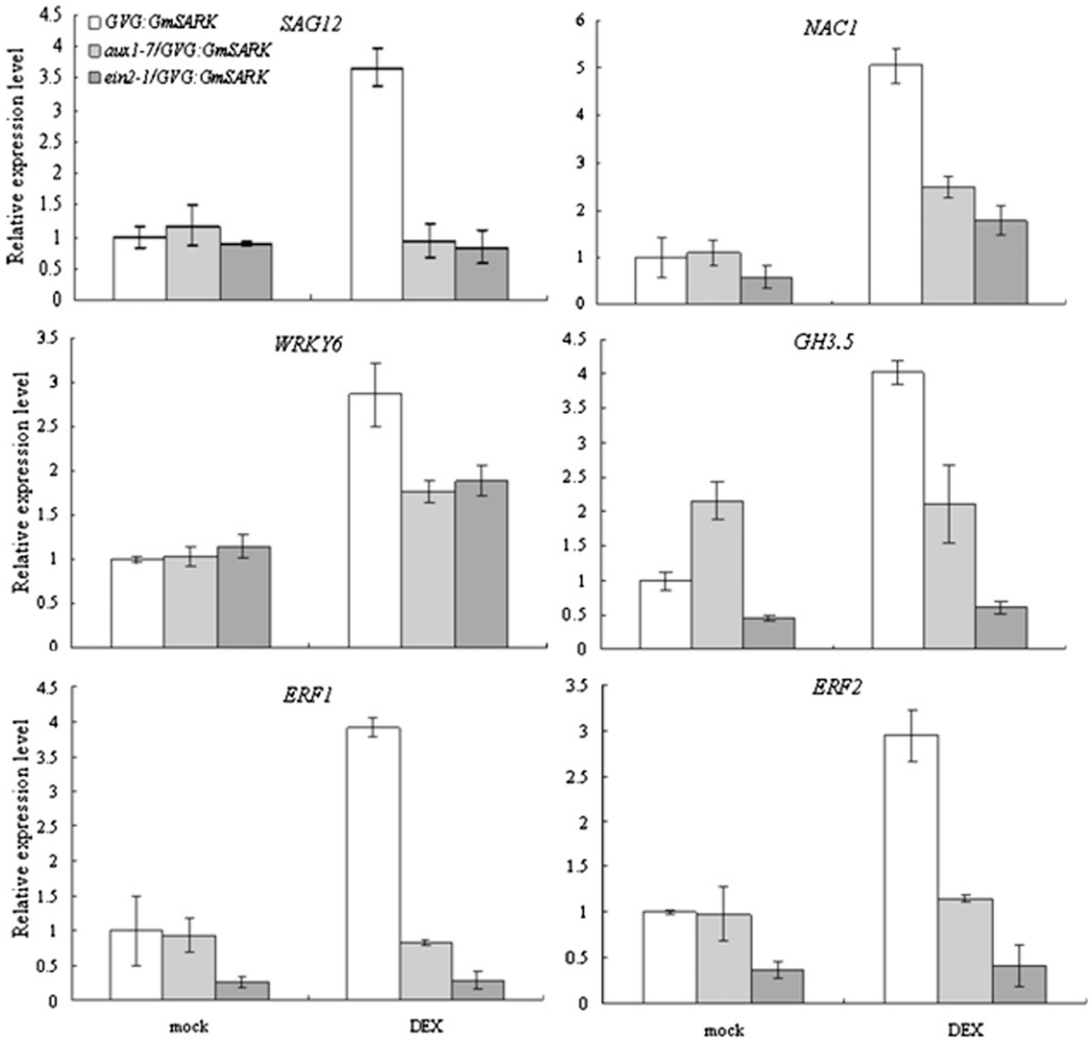

Figure 10. Mutations in *AUX1* and *EIN2* effectively reduce the *GmSARK*-induced expression of senescence- and hormone-related maker genes in Arabidopsis. Quantitative RT-PCR analysis is shown for the expression levels of *SAG12*, *NAC1*, *WRKY6*, *GH3.5*, *ERF1*, and *ERF2* in *GVG*:*GmSARK* (line *S23*), *aux1-7*/*GVG*:*GmSARK* (*aux1-7/S23*), and *ein2-1*/*GVG*:*GmSARK* (*ein2-1/S23*) seedlings. Four-day-old transgenic seedlings were treated with either 10 μM DEX (DEX) or the mock solution (mock) for 24 h. The *TIP41-like* gene was used as an internal control. Three biological replicates with at least three technical repeats were done for each gene. Error bars represent SD.

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

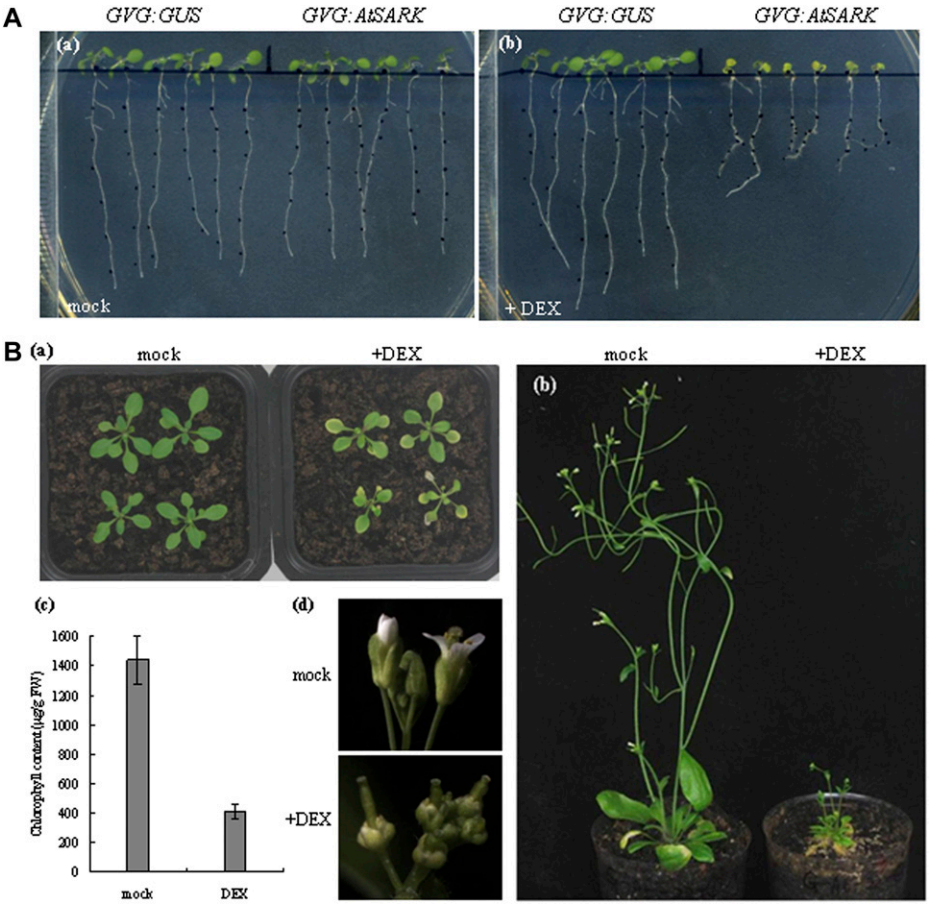

Figure 11. DEX-induced overexpression of *AtSARK* causes precocious senescence and abnormal flower development in Arabidopsis. A, Four-day-old *GVG:GUS* (line *G28*) and *GVG:AtSARK* (line *AtS20*) transgenic seedlings were grown on vertical plates containing either 10 μM DEX (+DEX) or its mock solution (mock) for an additional 120 h. B, **T**wenty-day-old *GVG:AtSARK* seedlings (line *AtS20*) were daily sprayed with either mock solution (mock) or 30 μM DEX (+DEX) for 3 d, and the photographs were taken at an additional 4 d (a) or 14 d (b). Chlorophyll contents of the fifth and sixth leaves of DEX-treated *AtS20* were determined at an additional 9 d (c). Values represent means of three independent replications (n = 5). FW, Fresh weight. Panel d shows photographs of flowers from the DEX-treated *AtS20* plants. [See online article for color version of this figure.]

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,

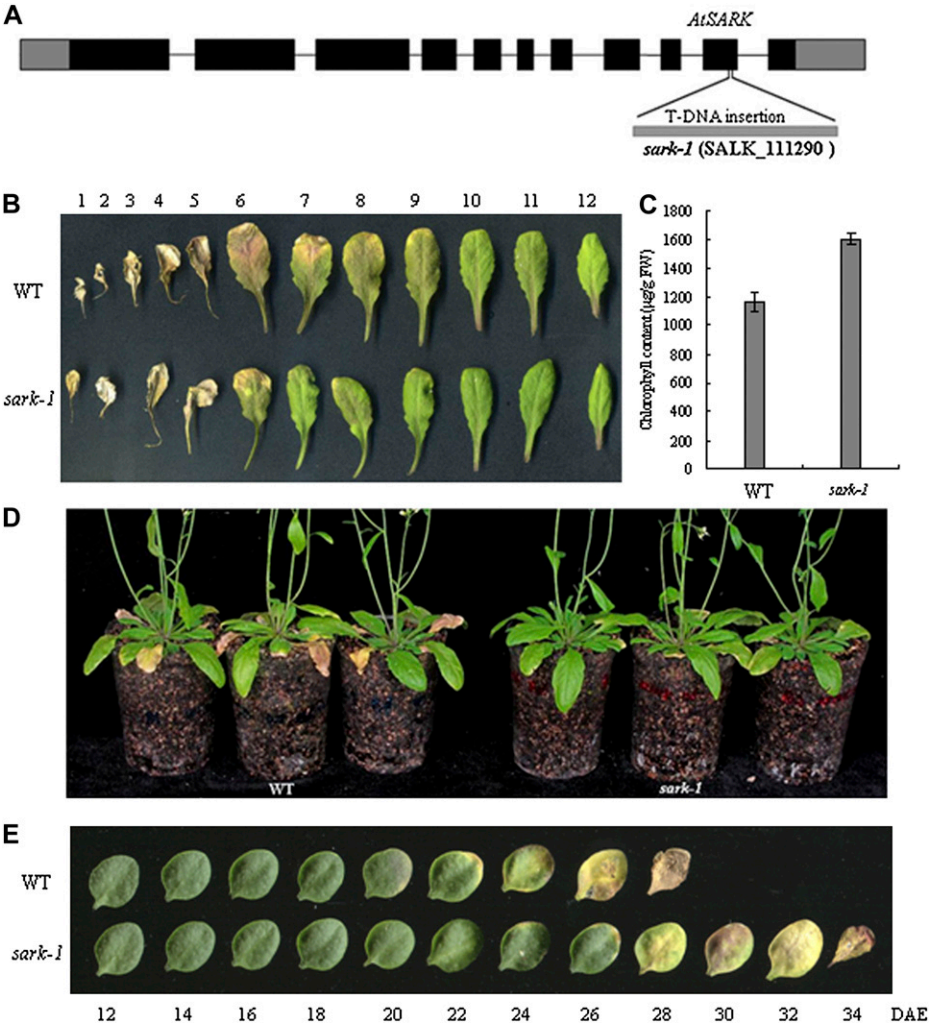

Figure 12. Leaf senescence is delayed in the *AtSARK* T-DNA insertion line *sark-1*. A, Gene structure of *AtSARK* and the T-DNA insertion site in *sark-1*. B, Leaves from 45-d-old wild-type (WT) and *sark-1* plants were laid out in order of emergence. Under long-day growth conditions in our laboratory, Arabidopsis (Columbia-0) plants typically produced 12 rosette leaves. C, Chlorophyll contents of the fifth and sixth leaves of 32-d-old wild-type and *sark-1* plants. Values represent means of three independent replications (n = 5). FW, Fresh weight. D, Leaf senescence was delayed in 41-d-old *sark-1* mutant plants. E, Age-dependent senescence phenotypes of wild-type and *sark-1* leaves. The fourth leaves from plants grown in soil under long-day growth conditions for up to 34 d were photographed. DAE, Days after emergence. [See online article for color version of this figure.]

which has been widely used as a biomarker for leaf senescence (Thomson and Plat-Aloia, 1987; Otegui et al., 2005; Pruzinská 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 (Noodén, 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,

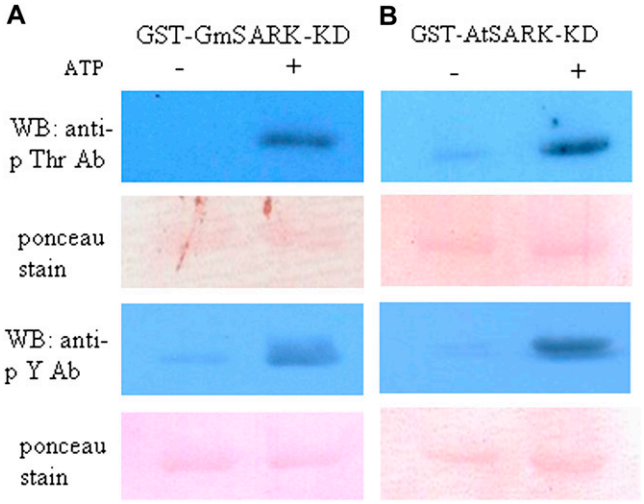

Figure 13. Both GmSARK and AtSARK autophosphorylate on Thr and Tyr residues in vitro. Autophosphorylation activities of the recombinant GmSARK (A) and AtSARK (B) kinase domains were detected by western blotting (WB) using either anti-phospho-Thr (p Thr Ab) or anti-phospho-Tyr (p Y Ab) antibody. Equivalent protein loading was visualized by Ponceau S staining. [See online article for color version of this figure.]

high cytokinin levels in young leaves repressed the expression of *GmSARK* and *GmSARK*-like genes, whereas in aged leaves, the inhibitory effect on the expression of *SARK(s)* was relieved, because of the age-dependent decline in foliar cytokinin level and the up-regulated expression of *SARK(s)* thus induced and/or promoted the leaf senescence process. The observations that both the biosynthesis and functions of cytokinin were reduced to a large extent in *GmSARK*-overexpressing plants (Fig. 5, B and C) suggested that once the senescence process began, the increase in *GmSARK* expression or the senescence process itself exerted a negative feedback effect on the functions of cytokinin.

Although the previous GeneChip analysis showed that the expression of auxin-related genes changed greatly during 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 synthesis and auxin-responsive genes and a down-regulation of the negative regulatory factors of its signaling in *GmSARK*-overexpressing plants (Fig. 6, D and E). These results are consistent with the previously reported microarray data that IAA biosynthetic genes are up-regulated during age-dependent leaf senescence (van der Graaff et al., 2006). The evidently enhanced auxin sensitivity and *DR5*:*GUS* activity by *GmSARK* overexpression (Fig. 6) further support the conclusion that overexpression of the *GmSARK* gene significantly promoted auxin synthesis and responses in Arabidopsis. Furthermore, in *GmSARK*-overexpressing seedlings, the localization of *DR5*:*GUS* activity in the root changed so that it was also highly apparent in the elongation zone (Fig. 6C), indicating that *GmSARK* overexpression affected not only the in vivo levels of auxin but also its distribution. This may be the cause of the abnormally curved roots in transgenic soybean and transgenic Arabidopsis that overexpress *GmSARK*.

In higher plants, the rate-limiting enzyme that controls the synthesis of ethylene is ACS. Seven members of the *ACS* family, *ACS2*, *-4*, *-5*, *-6*, *-7*, *-9*, and *-11*, were shown to be expressed in the DEX-untreated *GVG*:*GmSARK* seedlings (Fig. 7B). *ACS7*, together with *ACS6*, was shown to be the major expressed *ACS*. According to the results of Tsuchisaka et al. (2009), all the expressed ACS members formed 17 active and 10 inactive homodimers and heterodimers in plants (Supplemental Table S1). The relative ratio between active and inactive isozymes was changed by DEX-induced *GmSARK* overexpression, resulting in more active ACS homodimers and heterodimers accumulated (Supplemental Table S1). Among *ACS* members in Arabidopsis, the expression of *ACS7* is subjected to positive feedback regulation by ethylene (Tang et al., 2008). It is interesting to find that upon DEX treatment, the expression levels of *ACS* members that formed active homodimers (*ACS7*) or heterodimers (*ACS4*, *-6*, and *-9*) with ACS7 were all up-regulated, whereas those of the partners that formed inactive dimers (*ACS2*, *-5*, and *-11*) were down-regulated (Fig. 7B). Taken together, these results suggested that the DEX-induced overexpression of *GmSARK* might promote ethylene production in the transgenic Arabidopsis seedlings. This presumption was consistent with the 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*, *etr1-3 ers1-9* double mutant, *ein2-5*:CEND, and *ein3 ebf1 ebf2*/*35S*:*EIL1*:*GFP* (*OE/tm*) 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 Bleecker, 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

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 *GmSARK*- or *AtSARK*-induced precocious senescence in Arabidopsis suggested that auxin and ethylene are directly involved in the regulation of *SARKs*-mediated leaf senescence. The involvement of auxin and ethylene in the positive regulation of leaf senescence was further confirmed by the facts that both *AUX1* and *EIN2* mutations almost completely recovered the *SARKs*-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 *SARKs* and the *SARKs*-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 *GmSARK* (Fig. 8, G and I). These observations tempted us to postulate that ethylene works downstream of auxin in the regulation of *SARKs*-mediated senescence.

We also found that exogenous ACC could inhibit the effect of either 6-BA or PCIB on *GmSARK*-induced senescence; however, when PCIB and 6-BA were applied to the *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) *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 *AUX1* gene encodes an auxin influx transporter in Arabidopsis roots (Maher and Martindale, 1980; Marchant et al., 1999). The *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), *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 *ein2-1* seedlings also exhibited insensitivity to exogenous IAA (data not shown). Thus, it is unsurprising to find that both *aux1-7* and *ein2-1* mutations could recover the precocious senescence symptoms caused by *SARK* overexpression in transgenic Arabidopsis seedlings.

It is notable that the expression level of *ARF2*, which has been suggested to function as a repressor of auxin signaling, was reduced in *GmSARK*-overexpressing seedlings (Fig. 6, D and E). Although this result is in accordance with our observations that *GmSARK* overexpression could promote the responses to auxin, it seems contrary to the previous studies reporting that the *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 *ARF2* regulates leaf senescence and floral organ abscission independently of the ethylene and cytokinin pathways (Ellis et al., 2005). It is possible that *GmSARK* and *ARF2* regulate leaf senescence by different molecular mechanisms. In *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 *GmSARK* and functional *GmSARK* homologous genes, such as *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 *SARK* genes. During natural leaf senescence, because of the age-dependent reduction of cytokinin content, the expression of *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 *SARK* genes, and the increased expression of *SARKs* further inhibits the functions of cytokinin to form a feed-forward loop to regulate the progress of leaf senescence.

Leaf senescence in *sark-1*, a T-DNA knockout mutant of *AtSARK*, was significantly delayed (Fig. 12). As observed in many senescence-delayed mutants, such as *arf1*, *arf2*, heterozygous *ORE7/ore7-1D*, and *etr1-1* (Grbić and Bleecker, 1995; Ellis et al., 2005; Lim et al., 2007b), the *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 *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; Noodén 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 *fca*, 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 seeds 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 *GmSAKR:GUS* seedlings were subjected to different hormone treatments. Alternatively, both the *GmSAKR: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, ethyl alcohol (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 and 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 contents.

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/tdnaprimers.2.html), a PCR-based method was used to identify homozygous T-DNA insertion mutants. The T-DNA left border primer LBb1.3 and gene-specific primers *tic20*-LP and *tic20*-RP for *tic20-1* (SALK_039676) 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 indicated mutations. The primers presented in this paper are listed in Supplemental Table S3.

Constructs, Plant Transformation, and Crossing

To construct the *GmSARK*:*GUS* and *AtSARK*:*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 *AtSARK* gene (At4g30520) were amplified from soybean (*Glycine max*) and Arabidopsis genomic DNA by PCR, respectively. The pairs of primers used in the PCR were Gm-PC1 and Gm-PC2 for *GmSARK* and At-PC1 and At-PC2 for *AtSARK*. These DNA fragments were inserted into the binary vector pCAMBIA1301 to create the recombinant transcription units *GmSARK*:*GUS* and *AtSARK*:*GUS*, respectively. The transcripts were terminated with a *NOS* terminator.

For the construction of *GVG*:*GmSARK*, *GVG*:*AtSARK*, 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. The *GUS* fragment was inserted into a binary vector pTA7002 (Aoyama and Chua, 1997) to create a recombinant transcription unit, *GVG*:*GUS*. The 3,000- and 1,947-bp full-length cDNAs of *GmSARK* and *AtSARK* were amplified from the soybean and Arabidopsis cDNA by RT-PCR, respectively. The primer pairs used in the PCR were Gm-1 and Gm-2 for *GmSARK* and At-1 and At-2 for *AtSARK*. These DNA fragments were then inserted into the *GVG*:*GUS* plasmid by replacing the *GUS* fragment to create the recombinant transcription units *GVG*:*GmSARK* and *GVG*:*AtSARK*, respectively.

In the case of constructing the *GST-GmSARK-KD* and *GST-AtSARK-KD* recombinant transcription units, DNA fragments covering the kinase domains of GmSARK (residues 628–937; 930 bp) and AtSARK (residues 270–569; 900 bp) were amplified by PCR from the corresponding full-length cDNA using the following primer pairs: Gm-KD1 and Gm-KD2 for *GmSARK* and At-KD1 and At-KD2 for *AtSARK*. These kinase domain fragments were inserted into the vector pGEX-6P-1 to create the fusion genes *GST-GmSARK-KD* and *GST-AtSARK-KD*, respectively.

All primers used are listed in Supplemental Table S3. The authenticities of all constructs were confirmed by DNA sequencing.

The recombinant plasmids were then introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type Columbia-0 Arabidopsis plants using the floral dip method (Clough and Bent, 1998). Transformants were screened on 0.5× MS medium containing 30 mg L^{-1} hygromycin, and the resistant seedlings were transferred to soil. The PCR primers used to confirm the recombinant transgenes in transgenic plants are listed in Supplemental Table S3. Homozygous T3 plants were used for all experiments.

The *seca*/*GmSARK*:*GUS*, *ispf*/*GmSARK*:*GUS*, and *tic20*/*GmSARK*:*GUS* plants were obtained by crossing the *GmSARK*:*GUS* transgenic line with the corresponding heterozygous mutants *seca*/+, *ispf*/+, and *tic20*/+, respectively. The homozygous *DR5*:*GUS* line was crossed with the homozygous *GVG*:*GmSARK* line *S23* to produce *GVG*:*GmSARK*/*DR5*:*GUS* plants. Line *S23* was also used to cross with *aux1-7* and *ein2-1* mutants to produce *aux1-7*/*GVG*:*GmSARK* and *ein2-1*/*GVG*:*GmSARK* plants, respectively. Homozygous plants were identified by segregation analysis, comparison with the parental phenotypes and PCR-based genotyping in the F3 progeny.

Hormone and Chemical Treatments

In the promoter activity assay experiments, 7-d-old *GmSARK*:*GUS* transgenic seedlings were transferred to fresh horizontal 0.5× MS plates containing 5 μM 6-BA, 1 μM IAA, or 10 μM ACC. The treatments continued for 24 h before GUS activity assay. Seedlings grown on mock plates containing the corresponding solvents of the indicated hormones were used as controls. All these treatments were carried out under the same growth conditions as mentioned above.

For the functional analysis of *SARK* genes by treatment with hormone or drug inhibitor, a range of concentrations of 6-BA (from 0 to 10 μM), PCIB (from 0 to 10 μM), or AVG (from 0 to 15 μM) were tested. The *GVG*:*GmSARK* transgenic seeds and their corresponding *GVG*:*GUS* control were germinated and grown horizontally on DEX plates containing chemical inhibitors for 6 d. Exogenous 6-BA and PCIB at any concentration tested, or AVG at concentrations from 2 to 7 μM, were effective in alleviating the *GmSARK*-induced symptoms (Supplemental Fig. S3). The most effective concentrations were found to be 10 μM for 6-BA, 5 μM for PCIB, and 5 μM for AVG. Therefore, these concentrations were used in all experiments involving hormone and inhibitor treatments. The adequate concentrations for IAA and ACC were shown to be 1 and 10 μM, respectively. The 4-d-old *GVG*:*GmSARK* and *GVG*:*AtSARK* transgenic seedlings and their corresponding *GVG*:*GUS* control were transferred from basal medium plates to fresh vertical DEX or mock plates supplemented with or without the indicated combinations of 6-BA, PCIB, AVG, ACC, and IAA. The treatments continued for 120 h.

Histochemical GUS Staining, Fluorometric GUS Activity Assay, Chlorophyll Content Determination, and Transmission Electron Microscopy Examination

Histochemical GUS staining, fluorometric GUS activity assay, 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 give 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 done 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 *AtSARK* were used to amplify the transcripts of *GmSARK* and *AtSARK*, 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-AtSARK-KD fusion proteins were expressed in *Escherichia 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 $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol at 28°C for 1 h in the absence (control) or presence of 1 μg μL^{-1} 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). Ponceau 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 or GenBank/EMBL databases under the following accession numbers: *GmSARK* (AY687391), *NAC1* (At1g56010), *NAC2* (At5g04410), *AtNAP* (At1g69490), *WRKY6* (At1g62300), *SAG12* (At5g45890), *SIG5* (At5g24120), *GTR1* (AT1G58290), *ACD1* (AT3G44880), *RbcL* (AtCG00490.1), *RbcS* (At1G67090), *AHK3* (At1g27320), *ARR6* (AT5G62920), *IPT3* (At3g63110), *CKX3* (At5g56970),

GRXS13 (At1g03850), At2g18300, *CYP79B2* (At4g39950), *TSA1* (At3g54640), *GH3.5* (At4g27260), *ARF2* (At5g62000), *ACS2* (At1g01480), *ACS4* (At2g22810), *ACS5* (At5g65800), *ACS6* (At4g11280), *ACS7* (At4g26200), *ACS9* (At3g49700), *ACS11* (At4g08040), *ERF1* (At3g23240), *ERF2* (At5g47220), *AtEBP* (At3g16770), *ERF4* (At3g15210), *AtSARK* (At4g30520), and *TIP41-like* (AT4G34270).

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. Expressional 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 isozymes in transgenic Arabidopsis plants.

Supplemental Table S2. Differences in growth parameters between the wild type and *sark-1*.

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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