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A Gene Encoding an Acyl Hydrolase Is Involved in Leaf Senescence in Arabidopsis

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***SAG101*, a leaf senescence-associated gene, was cloned from an Arabidopsis leaf senescence enhancer trap line and functionally characterized. Reporter gene and RNA gel blot analyses revealed that *SAG101* was not expressed until the onset of senescence in leaves. A recombinant SAG101 fusion protein overexpressed in *Escherichia coli* displayed acyl hydrolase activity. Antisense RNA interference in transgenic plants delayed the onset of leaf senescence for ~4 days. Chemically induced overexpression of *SAG101* caused precocious senescence in both attached and detached leaves of transgenic Arabidopsis plants. These data suggest that *SAG101* plays a significant role in leaf senescence.**

INTRODUCTION

Leaf senescence, encompassing a period from maturation to attrition, is a unique developmental process. Unlike other developmental processes, which are composed primarily of cell division, cell differentiation, and/or cell expansion, leaf senescence involves massive programmed cell death (Gan and Amasino, 1997). During senescence, leaves lose their photosynthetic and other anabolic capacity, and catabolism of chlorophylls, lipids, proteins, and nucleic acids predominates. The released nutrients are mobilized subsequently to growing leaves, developing seed, or storage tissues. Therefore, leaf senescence also is a nutrient-mining and -recycling process that is distinct from many other developmental programs (Noodén, 1988; Buchanan-Wollaston, 1997; Quirino et al., 2000).

The onset of leaf senescence can be regulated by an array of endogenous and external factors. Environmental cues such as drought stress, extreme temperature, nutrient deficiency, and pathogen infection readily trigger leaf senescence. Among the internal factors are reproduction and plant growth regulators such as abscisic acid, ethylene, jasmonic acid, and salicylic acid (Smart, 1994). In the absence of stress factors, leaf senescence is believed to be initiated by age (Hensel et al., 1993; Jiang et al., 1993).

Gene expression is required for senescence to progress because leaf senescence can be inhibited by enucleation and by inhibitors of RNA and protein biosynthesis (reviewed by Noodén, 1988). To date, ~100 genes whose transcript abundance is increased during leaf senescence have been isolated from a variety of plant species such as Arabidopsis, rapeseed, tomato, maize, rice, tobacco, potato, and bean (Buchanan-Wollaston, 1997; Quirino et al., 2000). These genes include those encoding degradative enzymes, such as proteinases, nucleases, lipases, and chlorophyllase, and those with products involved in nutrient recycling, such as glutamate synthase (reviewed by Gan and Amasino, 1997). Pathogenesis-related genes also have been isolated (Quirino et al., 2000). In addition, a senescence-induced receptor-like protein kinase was isolated recently (Hajouj et al., 2000). The identities of all of these genes are predicted by sequence comparison, except for those of a few genes such as RNases (Lers et al., 1998) whose enzymatic activities have been demonstrated biochemically, and the function of these senescence-associated genes (*SAG*s) in leaf senescence remains to be characterized genetically.

During leaf senescence, lipids are hydrolyzed and metabolized further. Membrane phospholipids in senescing leaves are thought to be hydrolyzed by lipid-degrading enzymes such as phospholipase D (PLD), phosphatidic acid phosphatase, lipolytic acyl hydrolase, and lipoxygenase (Thompson et al., 1998); chloroplast thylakoid lipids appear to be degraded initially by galactolipase and lipolytic acyl hydrolase (Woolhouse, 1984). It has been shown that both *PLD* transcription and enzymatic activity increase during castor bean leaf senescence (Ryu and Wang, 1995). A petal senescence-associated lipase gene has been isolated from carnation (Hong et al., 2000). Although the free linolenic acid that is released from membrane lipid hydrolysis may serve as the primary substrate for jasmonic acid biosynthesis (Creelman

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and Mullet, 1997), the vast majority of fatty acids released from membranes in senescing leaves are either oxidized to provide energy for the senescence process or converted to α-ketoglutarate via the glyoxylate cycle. α-Ketoglutarate can be converted into phloem-mobile sugars (through gluconeogenesis) or used for the mobilization of amino acids released during leaf protein degradation (Smart, 1994; Buchanan-Wollaston, 1997).

Here, we report the cloning and functional characterization of a *SAG* designated *SAG101* from an Arabidopsis enhancer trap line. The enhancer trap T-DNA inserted at the 3′ end of *SAG101*. SAG101 possessed acyl hydrolase activity when overexpressed in *Escherichia coli*. Antisense suppression of this gene led to a delayed leaf senescence phenotype, and inducible ectopic overexpression of *SAG101* caused premature senescence in planta and in detached leaves. These data suggest that *SAG101* plays an important role in leaf senescence.

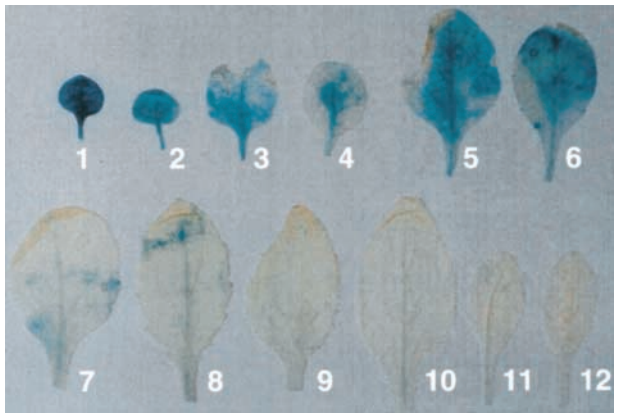

Figure 1. *GUS* Expression Pattern in Rosette Leaves of a 38-Day-Old Sel139 Plant.

The rosette leaves are numbered from bottom to top (cotyledons are excluded). At the time of GUS staining, leaves 1 through 5 had become yellow, leaf 8 had just turned yellow at its tip, leaves 9 and 10 were fully expanded but with no visible yellowing, and leaves 11 and 12 were expanding.

RESULTS

Identification and Genetic Analysis of Sel139, an Arabidopsis Leaf Senescence Enhancer Trap Line

By screening 1300 Arabidopsis enhancer trap lines, we identified 147 lines in which the reporter gene β-glucuronidase (*GUS*) was expressed in senescing leaves but not in nonsenescing leaves; we called these lines senescence enhancer trap lines (Sel) (He et al., 2001). One line, Sel139, which exhibited strong senescence-specific expression, as shown in Figure 1, was chosen for further characterization.

Genetic analyses were performed to determine the number of T-DNA insertions in Sel139. Sel139 was backcrossed to its parental nontransgenic plants (ecotype Columbia *glabrous1*), and the kanamycin-resistant (kan is the selection marker of the enhancer trap cassette; Campisi et al., 1999) progeny were allowed to self-pollinate to produce a segregating population. The selfed progeny segregated in a ratio of 3:1 (107 kanamycin-resistant plants to 34 kanamycin-sensitive plants), suggesting that Sel139 contains one T-DNA insertion. GUS staining of the progeny showed that the senescence-specific reporter expression cosegregated with the T-DNA insertion.

Molecular Cloning of *SAG101* from Sel139

A 1.0-kb genomic DNA flanking the right border of the T-DNA insertion in Sel139 was cloned using thermal asymmetric interlaced (TAIL) polymerase chain reaction (Liu et al., 1995). Sequence analysis revealed that part of this DNA fragment is identical to an Arabidopsis expressed sequence tag. We screened an Arabidopsis cDNA library that was made from mRNAs of senescing leaves (Gan, 1995) and obtained a 1.4-kb cDNA clone of this expressed sequence tag. RNA ligase–mediated rapid amplification of cDNA ends was used to obtain an additional 0.5 kb of the 5′ end of this cDNA. The full-length cDNA has a 1491-bp open reading frame, a 230-bp 5′ untranslated region, and a 186-bp 3′ untranslated region. We named this gene *SAG101*. The cDNA encodes a 497–amino acid protein with an apparent molecular mass of 58 kD, as shown in Figure 2B. Hydropathy analysis (MacVector 7.0; Oxford Molecular, Oxford, UK) revealed that the N-terminal region (amino acid residues 68 to 186) was hydrophobic (Figure 2C), suggesting that SAG101 may be a membrane-associated protein.

To determine the genomic structure and organization of *SAG101*, we cloned and sequenced a 7-kb genomic DNA. The *SAG101* cDNA was contained in a 3.7-kb genomic region. This genomic sequence was compared with the *SAG101* cDNA sequence. As shown in Figure 2A, *SAG101* consisted of three exons and two introns, and the first intron was in the 5′ untranslated region.

We performed both DNA gel blot analysis and an Arabidopsis genome sequence search. DNA gel blot analysis suggested that there was only one copy of *SAG101* in the Arabidopsis genome, even under reduced-stringency conditions (data not shown). The Arabidopsis genome sequence search also revealed that *SAG101* is a unique gene and that there are no homologs of this gene at the nucleotide level.

The Expression Pattern of *SAG101* Matches the Reporter Expression Pattern in Sel139

As shown in Figure 2A, the enhancer trap T-DNA in Sel139 inserted at 266 bp downstream from the poly(A) site of

SAG101. To determine if *SAG101* was the tagged gene of interest, we performed RNA gel blot analysis. RNA gel blot analysis of the steady state mRNA level of *SAG101* showed that *SAG101* was expressed in leaves at early, mid, and late senescence stages (visibly <25%, between 25 and 50%, and >50% yellowing, respectively); in contrast, there was little detectable *SAG101* transcript in young, expanding leaves or in fully expanded, nonsenescing leaves, as shown in Figure 3.

Despite the correlation between the expression pattern of the Sel139 *GUS* and *SAG101*, it was possible that a gene flanking the left border of the T-DNA might be responsible for the Sel139 *GUS* expression pattern. To evaluate this possibility, a 2.5-kb region of genomic DNA downstream of the T-DNA insertion was used as a probe to hybridize to the RNA gel blot described above. No detectable transcripts

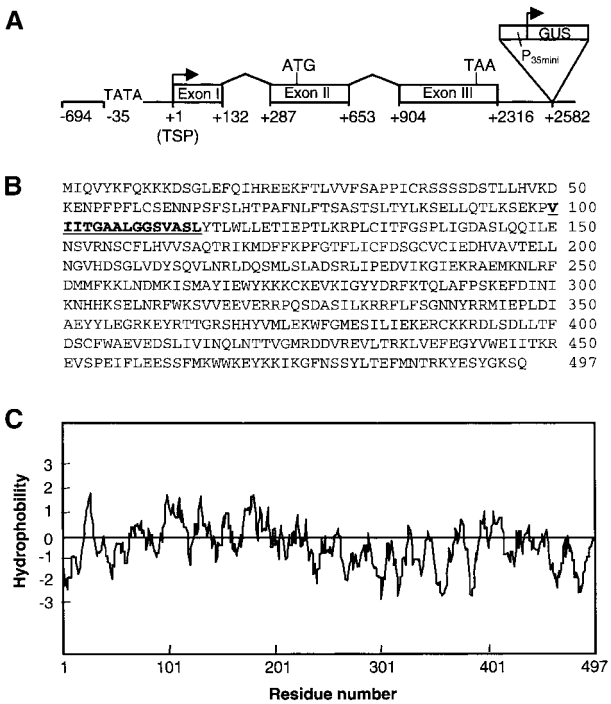

Figure 2. Gene Structure, Predicted Amino Acid Sequence, and Hydropathy Plot of SAG101.

(A) *SAG101* gene structure. ATG, translation start codon; P_{35mini}, 35S minimal promoter of *Cauliflower mosaic virus* (−60 region); TAA, translation stop codon; TATA, TATA box; TSP, transcriptional start point.

(B) Deduced amino acid sequence of SAG101. The conserved domain of lipolytic acyl hydrolase is shown in boldface and underlined (predicted by the CD-SEARCH program at the National Center for Biotechnology Information).

(C) Hydropathy plot of the predicted SAG101 protein. The plot was constructed according to Kyte and Doolittle (1982) with a window of 10 amino acid residues.

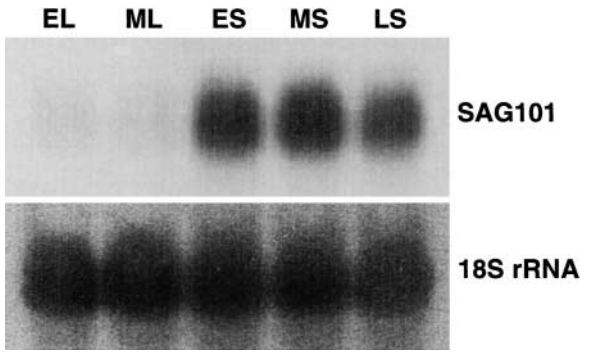

Figure 3. RNA Gel Blot Analysis of *SAG101* Expression during Leaf Development in Arabidopsis.

Approximately 15 μg of total RNA from each sample was loaded. Total RNAs were isolated from expanding leaves (EL; half the size of the fully expanded leaves), fully expanded mature leaves (ML), early-stage senescing leaves (ES; up to 25% of leaf area yellowing), mid-stage senescing leaves (MS; yellowing leaf area ranging from 25 to 50%), and late-stage senescing leaves (LS; >50% of leaf area yellowing).

were observed (data not shown). These data further suggested that *SAG101* is the endogenous gene corresponding to the *GUS* expression pattern in Sel139.

SAG101 Encodes an Acyl Hydrolase

The predicted SAG101 amino acid sequence was used to compare against the GenBank protein database using the BLASTP program. SAG101 exhibited 45% similarity (27% identity) to PAD4 (phytoalexin-deficient protein 4) across a region of 388 amino acid residues and 41% similarity (26% identity) to EDS1 (enhanced disease susceptibility 1) across a region of 456 amino acid residues. Both PAD4 and EDS1 were predicted to be triacylglycerol acyl hydrolases, and both genes were involved in salicylic acid–mediated defense against pathogens in Arabidopsis (Falk et al., 1999; Jirage et al., 1999).

In most of the known esterases and lipases (which are carboxylic ester hydrolases; EC 3.1.1), the motif GxSxG that contains the active site Ser residue is conserved (Tesch et al., 1996; Arpigny and Jaeger, 1999). However, there are some exceptions; for instance, an esterase (EstA) secreted by the plant pathogen *Streptomyces scabies*, which hydrolyzes ester bonds in suberin of plants, lacks the motif GxSxG (Raymer et al., 1990). The deduced SAG101 protein also lacks this motif, but it did possess a similar motif (GxSxA) in the N-terminal conserved region (Figure 2B).

To determine if SAG101 possessed acyl hydrolase activity, we overexpressed it as a maltose binding protein (MBP)::SAG101 fusion protein (Figure 4A) in the proteinase-deficient *E. coli* strain *ER2508* using the pMAL protein fusion system (New England Biolabs, Beverly, MA). More than

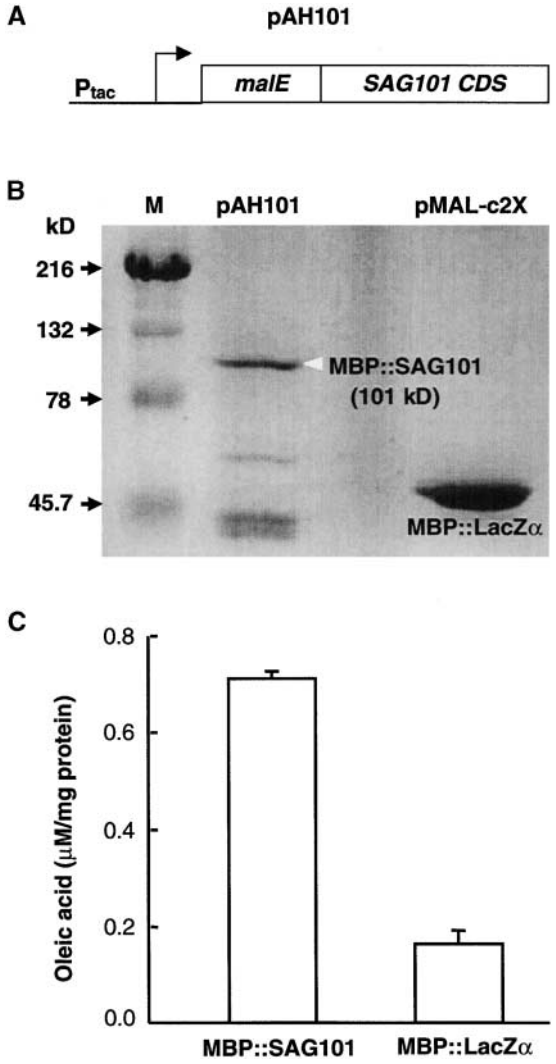

Figure 4. Overexpression and Activity Assay of the SAG101 Fusion Protein in *E. coli*.

(A) Scheme of the pAH101 construct used for the expression of the SAG101 fusion protein. CDS, coding sequence; *malE* encodes the MBP; P_{tac}, a strong bacterial hybrid *trp-lac* promoter containing the –35 region of the *trp* promoter fused to the –10 region of the *lac* UV5 promoter.

(B) SDS-PAGE analysis of the purified SAG101 fusion protein. LacZα, the α-complementation fragment of the *E. coli* β-galactosidase; M, molecular mass marker.

(C) Acyl hydrolase activity of the SAG101 fusion protein. Triolein was used as the substrate. The reaction proceeded for 2 hr, and four replications were made. Error bars indicate ±SE.

75% of the SAG101 recombinant proteins were insoluble. The fusion protein was purified by amylose column chromatography. As shown in Figure 4B, the purified fusion protein was ~101 kD on the silver-stained SDS–polyacrylmide gel, which was consistent with the expected size of the fusion protein (SAG101, 58.0 kD; MBP, 42.7 kD). The additional smaller bands on the gel are likely to be the degraded products of the fusion protein. The degradation of fusion proteins involving the pMAL protein fusion system is not unusual according to the manufacturer (New England Biolabs). SAG101 is likely to be a membrane-associated protein; membrane-associated proteins tend to be labile when expressed in *E. coli*.

The purified SAG101 fusion protein was assayed for acyl hydrolase activity using triolein (Sigma) as a substrate. The released oleic acids were quantified colorimetrically (Nixon and Chan, 1979). The purified MBP from *E. coli* harboring the empty vector pMAL-c2X served as a control. As shown in Figure 4C, the SAG101 fusion protein released approximately fourfold more oleic acids than did the control (MBP alone), suggesting that SAG101 possesses acyl hydrolase activity.

Antisense Suppression of *SAG101* Leads to a Delayed Leaf Senescence Phenotype

To investigate the potential role of *SAG101* in leaf senescence, we made an antisense construct (pAT101) in which a 333-bp fragment (+1086 to +1418) of *SAG101* cDNA was placed under the control of the 35S promoter of *Cauliflower mosaic virus* in the antisense orientation, as shown in Figure 5A. We examined 41 transgenic Arabidopsis lines. Eight lines and their progeny displayed a delayed leaf senescence phenotype, as shown in two examples (Figures 5B and 5C).

We analyzed the changes in the chlorophyll contents of leaves of the *SAG101* antisense transgenic lines and their parental nontransgenic plants, as shown in Figure 6A. The content of total chlorophyll in leaves of the wild-type plants started declining 3 days after their full expansion (Figure 6A), which is consistent with previous observations (Lohman et al., 1994; Grbic and Bleecker, 1995). In contrast, the chlorophyll content did not decrease until 6 to 7 days after full expansion in the *SAG101* antisense transgenic plants (Figure 6A). The delay was attributable primarily to a deferred onset (~3 to 4 days) of leaf senescence; the progression of senescence, once initiated, appeared to be unaffected compared with that in wild-type plants (Figure 6A).

We further investigated the expression of *SAG12* and *SAG13* in the *SAG101* antisense transgenic plants. *SAG12* and *SAG13* are two leaf senescence-specific genes in Arabidopsis (Gan, 1995) that have been used widely as molecular markers of leaf senescence (Weaver et al., 1998; Ludewig and Sonnewald, 2000; Morris et al., 2000; Hinderhofer and Zentgraf, 2001; Woo et al., 2001). As shown in Figure 6B, the *SAG12* message was readily detectable in leaves at 6 and 9 days after full expansion in wild-type and antisense plants, respectively. In an overexposed autoradiograph, the *SAG12* signal could be seen in leaves at 3 and 6 days after full expansion in wild-type and antisense plants, respectively (data not shown). Similarly, the *SAG13* transcripts ac-

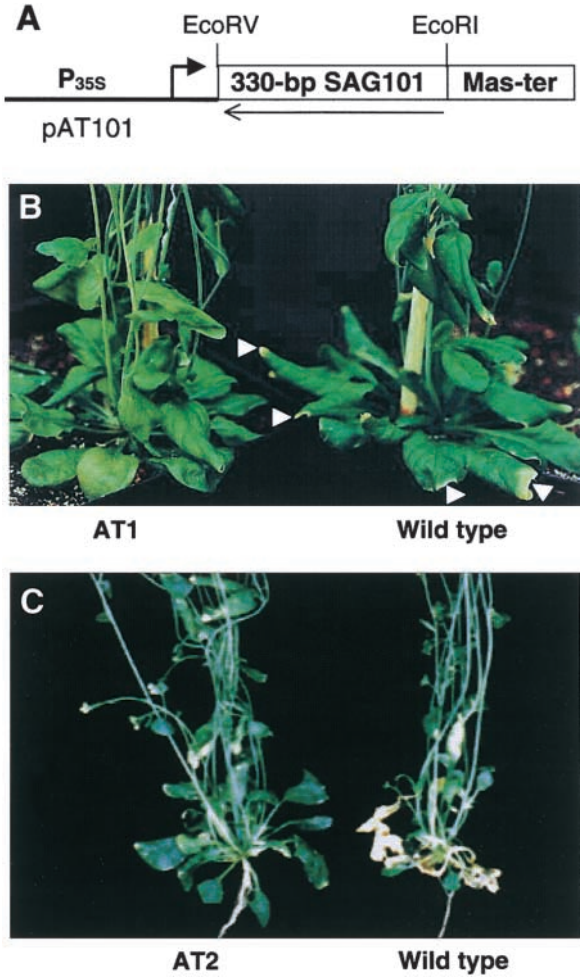

Figure 5. Phenotypes of *SAG101*-Suppressed Arabidopsis Lines.

(A) Scheme of the *SAG101* antisense construct pAT101. P_{35S}, 35S promoter of *Cauliflower mosaic virus*. Mas-ter, terminator of the manopine synthase gene.

(B) Delayed onset of leaf senescence in *SAG101*-suppressed line AT1. Arrowheads indicate the leaf tips of the wild-type plant that have already started senescing.

(C) Comparison of *SAG101* antisense line AT2 with an age-matched wild-type plant at the terminal stage of the life cycle.

cumulated in leaves at 3 and 6 days after full expansion in wild-type and antisense transgenic plants, respectively, and the abundance increased with the progression of leaf senescence (Figure 6C). These data confirmed the delay of leaf senescence in the antisense transgenic plants at the molecular level.

Next, we performed RNA gel blot analysis to determine the steady state levels of *SAG101* transcripts in antisense transgenic plants. We used 0.7-kb DNA of *SAG101* cDNA (+1419 to +2125) as a probe; this fragment did not overlap with the cDNA used for the antisense construct. As shown in Figure 7, the levels of *SAG101* mRNA in senescing leaves of both antisense lines (AT1 and AT2) were reduced to 40 and 30% of the levels in wild-type plants, respectively. Both lines displayed the delayed leaf senescence phenotype, as shown in Figure 5. In contrast, the level of *SAG101* transcripts in a pAT101-transformed line called N1 showed little change compared with that in wild-type plants; N1 plants did not exhibit any of the "mutant" phenotypes described above. The blots also were probed with *SAG13*; the expression level of *SAG13* served as a developmental marker of leaf senescence to show that the leaf samples were approximately at the same stage of senescence (Gan, 1995; Weaver et al., 1998). It should be noted that the expression of *SAG13* in Figure 7 should not be compared with that in Figure 6C, in which age-matched leaf samples were used.

Inducible Overexpression of *SAG101* Causes Premature Leaf Senescence

To further investigate the role of *SAG101* in leaf senescence, we performed gain-of-function experiments using the glucocorticoid-regulated transcriptional induction system pTA7001 (Aoyama and Chua, 1997) to direct *SAG101* expression in transgenic Arabidopsis; the related construct pIE101 is shown in Figure 8A. Fifteen transgenic lines were generated for the constructs pIE101 and its precursor vector pTA7001. To determine the effect of *SAG101* overexpression on leaf senescence in planta, transgenic plants (T2) were grown hydroponically. After 3.5 days of treatment with dexamethasone (DEX), a synthetic glucocorticoid, chlorophyll loss (or yellowing) in leaves of pIE101 transgenic plants became readily visible. After an additional 2.5 days, these leaves turned completely yellow (Figure 8B). In contrast, there was no obvious chlorophyll loss in leaves of pTA7001 vector–transformed lines after 6 days of treatment with DEX (Figure 8B). RNA gel blot analysis not only revealed the induction of *SAG101* in these completely yellowed leaves but also showed the expression of two senescence molecular marker genes (Figure 8C), indicating that the yellowing that results from the inducible overexpression of *SAG101* mimics the natural senescence program.

The senescence-promoting effect of *SAG101* overexpression also was assessed in detached leaves. As shown in Figure 9A, DEX treatment for 4 days induced precocious senescence in young leaves detached from pIE101 transgenic lines; in contrast, the same treatment failed to accelerate senescence in young leaves detached from transgenic plants harboring the pTA7001 vector. We further measured the ratios of variable to maximal chlorophyll fluorescence (F_v/F_m) in these leaves. F_v/F_m reflects the photochemical quantum efficiency of photosystem II as well as the photoreduction efficiency of the primary electron-accepting plastoquinone of photosystem II (Dai et al., 1999). As shown in Figure 9B,

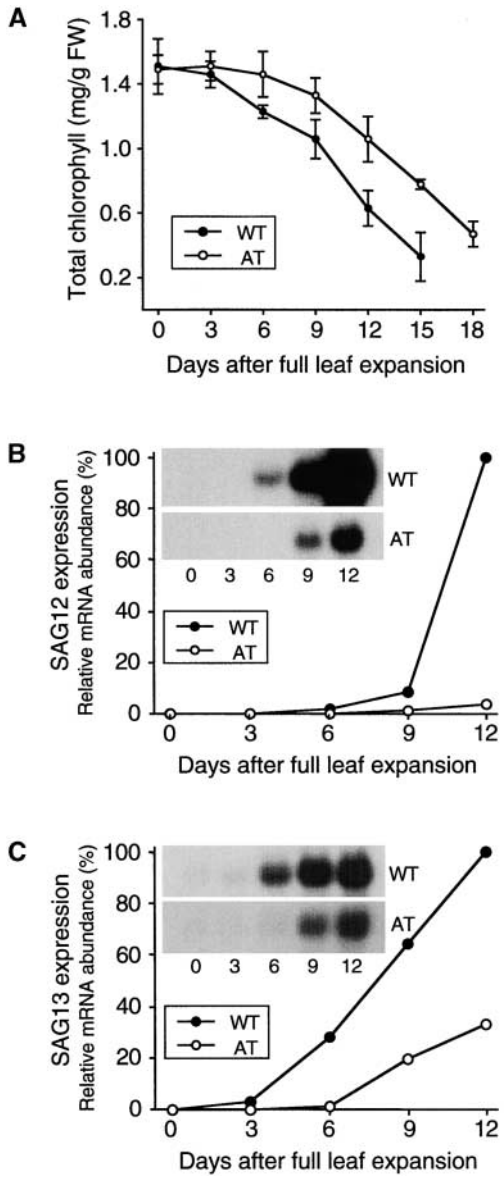

Figure 6. Chlorophyll Content and Expression of Two Senescence Marker Genes in Leaves of *SAG101*-Suppressed Arabidopsis Line AT1.

(A) Changes in chlorophyll content as a function of leaf age in AT1 and nontransgenic wild-type plants.

(B) Delayed expression of *SAG12* in AT1 leaves.

(C) Delayed expression of *SAG13* in AT1 leaves.

The fifth rosette leaves were used. Approximately 12 μg of total RNA was loaded in each lane. The blots were quantitated using an FLA-2000 phosphorimage system (Fuji Medical Systems USA, Stamford, CT). The mRNA abundance in a lane was adjusted based on the 18S rRNA amount in that lane and then normalized to the highest level, which was set at 100%. AT, AT1; FW, fresh weight; WT, wild type.

F_v/F_m values in detached pIE101 leaves treated with DEX for 4 days were very low, indicating that the overexpression of *SAG101* caused a sharp decline in photosynthetic activity, a characteristic of leaf senescence.

DISCUSSION

Leaf senescence is accompanied, and perhaps driven, by the activation of a subset of genes (e.g., *SAG*s) and the inactivation of another subset of genes that repress the senescence program. The maize homeobox gene *knotted1* (Ori et al., 1999) and the Arabidopsis F-box gene *ORE9* (Woo et al., 2001) have been shown to regulate the leaf senescence program. In this report, we have demonstrated that SAG101 possesses acyl hydrolase activity and that this gene plays an important role in leaf senescence.

Membrane deterioration is one of the early events during leaf senescence (Thompson et al., 1998). This deterioration is characterized by the progressive decline of the amount of phospholipid and the relative enrichment of free fatty acids and sterols in the membrane (Manoharan et al., 1990) that lead ultimately to the loss of membrane structural integrity and functional permeability (Thompson et al., 1998). It is believed that PLD, phosphatidic acid phosphatase, acyl hydrolase, and lipoxygenase are responsible for the degradation of membrane lipids (Thompson et al., 1998). To date, the significance of PLDα (Fan et al., 1997) and an acyl hydrolase (this report) in leaf senescence has been investigated.

In this report, we show that the fusion protein MBP:: SAG101 released oleic acids from triolein at a rate four times greater than did the MBP alone (Figure 4), suggesting that *SAG101* encodes an acyl hydrolase. This acyl hydrolase is likely to be membrane associated, because its N-terminal portion is largely hydrophobic (Figure 2C) and because the majority of the SAG101 recombinant protein overexpressed in *E. coli* is insoluble. However, additional cell biological experiments are needed to investigate the possible membrane association of this enzyme. The relatively low acyl hydrolytic activity may be attributable to the nonspecificity of SAG101 acyl hydrolase in terms of substrate or to the possibility that the substrate used for the enzymatic assay is not its natural substrate. A low enzymatic activity also was observed in an experiment involving a carnation petal senescence-associated acyl hydrolase: the carnation acyl hydrolase/MBP fusion protein was shown to release linoleic acids from trilinolein at a rate only two times that released by the MBP alone (Hong et al., 2000).

It has been reported that the application of lysophosphatidylethanolamine, an inhibitor of PLD, retarded leaf senescence in tomato (Ryu et al., 1997). Antisense suppression of *PLDα* also delayed abscisic acid– and ethylene-promoted senescence in detached Arabidopsis leaves, but it had no effect on natural leaf senescence (Fan et al., 1997). In contrast, transgenic Arabidopsis plants with suppressed *SAG101*

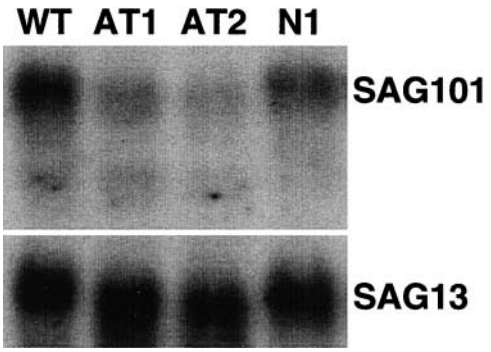

Figure 7. RNA Gel Blot Analysis of Steady State mRNA Levels of *SAG101* in Antisense Suppression Lines.

Total RNA was isolated from early-stage senescing leaves of wild-type plants (WT), antisense lines 1 and 2 (AT1 and AT2, respectively), and the N1 line that harbored the antisense construct but did not show any mutant phenotypes. Twelve micrograms of total RNA was loaded in each lane. The same blot also was hybridized with the *SAG13* probe. Comparable levels of *SAG13* in each lane indicate that the leaves were at the same senescence stage.

expression (Figure 7) exhibited an ~3- to 4-day delay in the timing of senescence onset (Figures 5 and 6). The antisense suppression is most likely *SAG101* specific, because this unique gene has no homologs in the Arabidopsis genome, as revealed by nucleotide sequence search and low-stringency hybridization. When the 330-bp nucleotide sequence of the *SAG101* cDNA that was used to make the antisense construct (pAT101; Figure 5A) was compared against the Arabidopsis genome sequence database using the default parameters of BLASTN, no matches were found except for its own genomic sequence. The involvement of *SAG101* in leaf senescence is further supported by the gain-of-function experiment. When this gene was expressed ectopically in young leaves using a chemically inducible gene expression system, these leaves were promoted to undergo premature senescence (Figures 8 and 9).

How does the SAG101 acyl hydrolase modulate leaf senescence? We propose that *SAG101* serves as a "facilitator" in membrane breakdown in senescing leaf cells. Although many lipolytic enzymes, such as PLD, phosphatidic acid phosphatase, and lipoxygenase, are expressed in nonsenescing young leaves in addition to an increased expression in senescing leaves (Fan et al., 1997; Thompson et al., 1998), these enzymes act on intact phospholipid bilayers poorly (Thompson et al., 1998). SAG101 acyl hydrolase expressed at the onset of leaf senescence (Figures 1 and 3) will attack on membrane phospholipids, and this initial attack and the accompanying release of free fatty acids will perturb the bilayer structure of the membrane, which facilitates other lipid-degrading enzymes to hydrolyze the membrane lipids because the perturbed phospholipid bilayers are better substrates for lipid-degrading enzymes than is unperturbed membrane (Thompson et al., 1998). The initial attack of membranes by the SAG101 acyl hydrolase marks the onset of leaf senescence. In the *SAG101* antisense plants, *SAG101* expression was reduced significantly; thus,

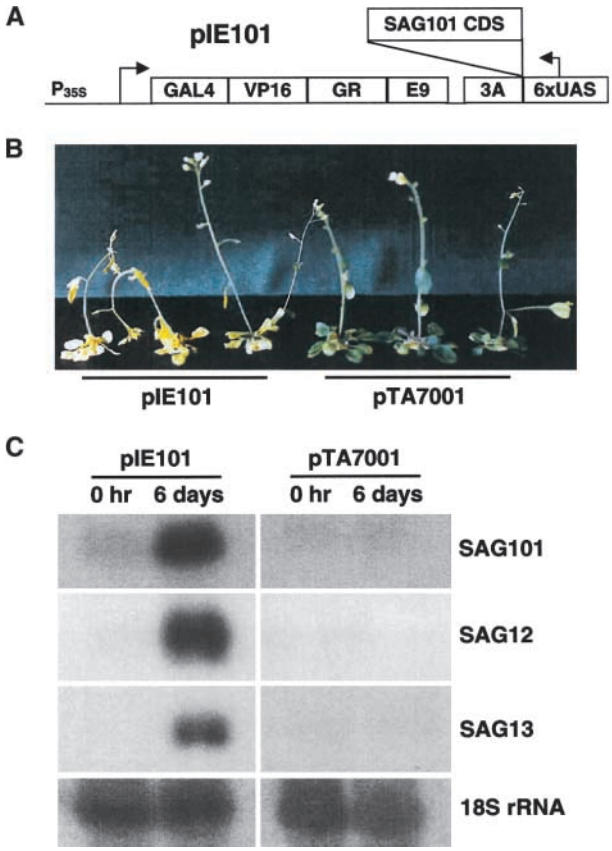

Figure 8. Effect of Inducible *SAG101* Overexpression on Leaf Senescence.

(A) Scheme of the inducible overexpression construct pIE101. E9, terminator of pea rbcS-E9; GAL4::VP16::GR, glucocorticoid-responsive chimeric transcription factor; P_{35S}, 35S promoter of *Cauliflower mosaic virus*; CDS, coding sequence; 6xUAS, six copies of the upstream activating sequence to which the yeast transcription factor GAL4 binds; 3A, terminator of pea rbcS-3A (Aoyama and Chua, 1997).

(B) Phenotypes of one of the *SAG101* overexpression lines (pIE101L3; T2 progeny) after continuous treatment with 15 μM DEX for 6 days under hydroponic conditions. A transgenic line harboring the empty vector pTA7001 (without the *SAG101* sequence) served as a control.

(C) Expression of *SAG101*, *SAG12*, and *SAG13* in leaves of the plants shown in **(B)**. Total RNA was isolated from leaves of the pIE101 plants or the control vector pTA7001 plants that had been treated with DEX for 0 hr or 6 days. Six micrograms of total RNA was loaded in each lane.

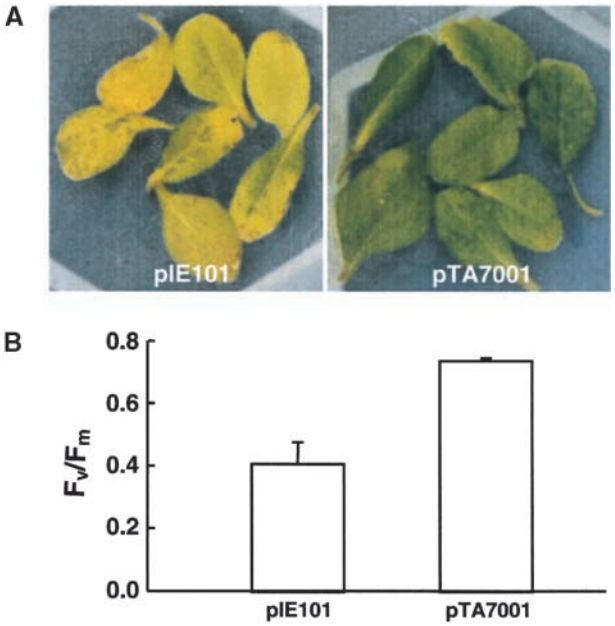

Figure 9. Effects of Inducible *SAG101* Overexpression on Senescence in Detached Arabidopsis Leaves.

(A) Precocious senescence in leaves detached from line pIE101L3 (T2 progeny). The young, nonsenescing leaves were treated with 30 μM DEX under darkness for 4 days.
(B) Chlorophyll fluorescence parameter F_v/F_m in the leaves shown in **(A)**.

the onset of leaf senescence was delayed. In contrast, in the overexpressors of *SAG101*, the precocious accumulation of *SAG101* led to an earlier onset of senescence in young leaves.

An alternative model is that SAG101 deesterifies membrane lipids to release fatty acids, including α–linolenic acid. The released α–linolenic acid is used subsequently to synthesize jasmonic acid and its derivatives (Creelman and Mullet, 1997). There are emerging lines of evidence that jasmonic acid may induce senescence (He and Gan, 2001; He et al., 2002). An acyl hydrolase has been proposed to be responsible for the release of α–linolenic acid from phosphatidic acid in the wound-induced accumulation of jasmonic acid (Wang et al., 2000).

METHODS

Arabidopsis and Growth Conditions

Arabidopsis thaliana ecotype Columbia *glabrous1* (Col-*gl1*) was used. The growth conditions were the same as those described previously by He et al. (2001) unless indicated otherwise. The *SAG101* antisense lines and their parental nontransgenic line Col-*gl1* were grown side by side under a 16-hr-light/8-hr-dark photoperiod unless indicated otherwise.

Cloning of *SAG101* Genomic DNA and cDNA

A 1.0-kb genomic sequence flanking the right border of the T-DNA insertion in Sel139 was amplified by thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR) (Liu et al., 1995) using AD3 and oligonucleotide 86 (Campisi et al., 1999). An Arabidopsis genomic library was screened for *SAG101* genomic sequence by a PCR-based strategy. Briefly, two primers, G158 (5′-TAGTTTCTAGTAAATTTCGTTACCAATTTCTTCCCG-3′) and G159 (5′-CGAGTCGTTCGATCATCCGTCTGTAATTGTTCC-3′), annealing to the known sequence of the 1.0-kb fragment, and two other primers, G156 (5′-GCCTAATACGACTCACTATAGGGAGCTCGAG-3′) and G157 (5′-GGCCATTTAGGTGACACTATAGAAGAGCTCGAG-3′), annealing to the right and left arms, respectively, of λGEM-11 (the library vector), were synthesized, and PCR reactions involving G156-G158, G156-G159, G157-G158, or G157-G159 were performed using the genomic library as the template. LA Takara Polymerase (PanVera, Madison, WI) was used. A 7.0-kb genomic fragment was amplified when the primer pair G156-G159 was used; this fragment was cloned subsequently into pGEM-T vector (Promega, Madison, WI) and sequenced. An Arabidopsis leaf senescence cDNA library was screened similarly, and a 1.4-kb cDNA was obtained. An additional 0.5 kb of the 5′ end of the cDNA was obtained by the 5′ RNA ligase–mediated rapid amplification of cDNA ends method according to the manufacturer's instructions (Ambion, Austin, TX). The full length of the *SAG101* cDNA is 1912 bp.

Plasmid Construction

To overexpress *SAG101* in *Escherichia coli*, the *SAG101* coding sequence (1491 bp) was amplified with a pair of primers, G188 (5′-GCGGATCCATGATCCGGGTCTACAAGTT-3′; the underlined section is an engineered BamHI site) and G189 (5′-CTTAAGCTTCCATTATTGTGACTTACCATA-3′; the underlined section is an engineered HindIII site) using Pfu polymerase (Stratagene, La Jolla, CA). The PCR product was digested with BamHI and HindIII and cloned into pMAL-c2X expression vector (New England Biolabs, Beverly, MA) to form pAH101. In this plasmid, *SAG101* was fused translationally with *malE* (which encodes the maltose binding protein [MBP]) to form a 100.7-kD MBP::SAG101 fusion protein.

The *SAG101* antisense plasmid pAT101 was constructed by placing the EcoRI-EcoRV fragment (333 bp; +1086 to +1418) of the *SAG101* cDNA between the 35S promoter of *Cauliflower mosaic virus* and the MAS terminator in the antisense orientation. The resulting P_{35S}::*SAG101*(RV-RI)::MAS chimeric gene then was cloned into the binary vector pPZP211 (Hajdukiewicz et al., 1994).

The inducible *SAG101*-overexpressing plasmid pIE101 was made by first PCR amplifying the *SAG101* coding sequence using Pfu polymerase (Stratagene) with primers G201 (5′-CGAACCGTCGACACGATCCGGGTCTACAAGT-3′; the underlined section is an engineered SalI site) and G202 (5′-CGCCTAGGTTATTGTGACTTACCATAACT-3′; the underlined section is an engineered AvrII site). The PCR product then was digested with AvrII and SalI and cloned into pTA7001 (Aoyama and Chua, 1997) at SpeI (compatible cohesive end with

AvrII) and XhoI (compatible cohesive end with SalI) sites. The *SAG101* coding region in this plasmid was sequenced.

Expression and Purification of the Recombinant SAG101 Fusion Protein in *E. coli*

The SAG101 fusion protein was overexpressed and purified from *E. coli* strain *ER2508* using the pMAL protein fusion and purification system according to the manufacturer's instructions (New England Biolabs). Briefly, 1 liter of culture was induced with 0.3 mM isopropyl-β-D-thiogalactoside (LabScientific, Livingston, NJ) for 2 hr; cells were harvested and broken using the French press method. The fusion protein was purified using amylose-mediated affinity chromatography and desalted using Micro Bio-Spin P-30 chromatography columns (Bio-Rad, Hercules, CA). The fusion protein was examined by SDS-PAGE according to Sambrook et al. (1989), and the gel was stained using the Silver Stain Plus kit (Bio-Rad).

Acyl Hydrolase Activity Assay of the SAG101 Fusion Protein

Acyl hydrolase activity was measured in vitro according to Lin and Huang (1983) and Hong et al. (2000). The 0.2-mL reaction mixture contained 0.1 mM Tris-HCl buffer, pH 7.6, 3.4 mM triolein (Sigma, St. Louis, MO), and 0.2 mg of the SAG101 fusion protein. After incubation at 28°C for 2 hr, the reaction mixture was extracted with chloroform:*n*-heptane:methanol (4:3:2 [v/v]). The released oleic acids were converted to copper soaps and were quantitated subsequently using the colorimetric method (Nixon and Chan, 1979). MBP alone was induced similarly, purified, and used as a control for the acyl hydrolase assay. Proteins were quantitated spectrometrically (OD_{280}). BSA was used as the standard.

DNA and RNA Gel Blot Analyses

Total RNA extraction from Arabidopsis leaves and RNA gel blot analysis were performed as described (He and Gan, 2001). Arabidopsis genomic DNA isolation and DNA gel blot analysis were performed according to Gan (1995) and He and Gan (2001). The hybridization was performed at 65°C (for normal stringency) or 50°C (for lower stringency).

Agrobacterium and Plant Transformation

The constructs described above in binary vectors were transferred into *Agrobacterium tumefaciens* strain ABI (pAT101) or LBA4404 (pIE101 and pTA7001) using the freeze–thaw method described by Xie et al. (2001). Briefly, ~2 to 5 μg of each of the DNA constructs was added to a 1.5-mL microcentrifuge tube containing 100 μL of competent ABI or LBA4404 cells. Upon mixing, the mixture was frozen in a dry ice–ethanol bath and then placed in a 37°C water bath for 5 min. YEP medium (100 μL; 10 g/L bacto-peptone, 10 g/L bacto yeast extracts, 5 g/L NaCl) was added, and the whole cells were plated on the YEP plate (10 g/L phytoagar) containing 100 mg/L spectinomycin (ABI) or 50 mg/L kanamycin (LBA4404). The Agrobacterium cells containing the various constructs then were used to transform Arabidopsis ecotype Col-*gl1* via vacuum infiltration (Bechtold et al., 1993). Transgenic plants were selected with 50 mg/L kanamycin (pAT101 transformants) or 25 mg/L hygromycin (pIE101 and pTA7001 transformants).

Glucocorticoid Treatments

The glucocorticoid treatments were performed as described by Aoyama and Chua (1997). Detached young rosette leaves were floated on 30 μM dexamethasone (Fluka Chemie AG, Buchs, Switzerland) under darkness at 23°C for 4 days. For in planta treatments, 16-day-old seedlings were allowed to grow hydroponically for 1 week and continued to grow hydroponically in the presence of 15 μM dexamethasone for an additional 6 days.

β-Glucuronidase Staining in Plants

Histochemical β-glucuronidase staining in Sel139 was performed as described previously (He et al., 2001).

Measurements of Chlorophyll Content and Fluorescence

Chlorophyll was extracted from the fifth rosette leaves and quantitated according to Lohman et al. (1994) and Grbic and Bleecker (1995). At least three leaves from separate plants at each sampling time were used. Total fluorescence in leaves was measured using a portable modulated chlorophyll fluorometer (model OS1-FL) according to the manufacturer's instructions (Opti-Sciences, Tyngsboro, MA). The variable and maximal fluorescence of each leaf was quantified directly using the fluorometer's module 9 program.

Accession Numbers

The GenBank accession numbers for the gene and proteins described in this article are AF239887 (*SAG101* genomic sequence), AF239888 (*SAG101* full-length cDNA sequence), AI995772 (Arabidopsis expressed sequence tag that matched with part of the genomic sequence flanking the right border of the T-DNA insertion in Sel139), AF188329 (PAD4, phytoalexin-deficient protein 4), and AF128407 (EDS1, enhanced disease susceptibility 1).

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