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A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence

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Abstract

We have characterized the structure and expression of a senescence-associated gene (*sen1*) of *Arabidopsis thaliana*. The protein-coding region of the gene consists of 5 exons encoding 182 amino acids. The encoded peptide shows noticeable similarity to the bacterial sulfide dehydrogenase and 81% identity to the peptide encoded by the radish *din1* gene. The 5′-upstream region contains sequence motifs resembling the heat-shock- and ABA-responsive elements and the TCA motif conserved among stress-inducible genes. Examination of the expression patterns of the *sen1* gene under various senescing conditions along with measurements of photochemical efficiency and of chlorophyll content revealed that the *sen1* gene expression is associated with *Arabidopsis* leaf senescence. During the normal growth phase, the gene is strongly induced in leaves at 25 days after germination when inflorescence stems are 2–3 cm high, and then the mRNA level is maintained at a comparable level in naturally senescing leaves. In addition, dark-induced senescence of detached leaves or of leaves *in planta* resulted in a high-level induction of the gene. Expression of the *sen1* gene was also strongly induced in leaves subjected to senescence by 0.1 mM abscisic acid or 1 mM ethephon treatment. The induced expression of the gene by dark treatment was not significantly repressed by treatment with 0.1 mM cytokinin or 50 mM $CaCl_2$ which delayed loss of chlorophyll but not that of photochemical efficiency.

Introduction

Monocarpic plants end their life span with senescence, a final phase of plant development prior to death. In the senescence phase, the metabolism of plants dramatically changes and the materials built up during the growth phase are mobilized to and stored in the developing seeds [27]. Thus, the

The nucleotide sequence of the cDNA clone and the genomic clone reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers U26945 and U26944, respectively.

senescence program of plants appears to contribute to their fitness by increasing the number and/or survival of their progeny. Senescence is now regarded an evolutionarily acquired and a highly regulated developmental strategy [27, 39] rather than a simple passive degenerative process.

One of the most notable and important changes that occur during the whole plant senescence of a monocarpic plant is leaf senescence. A markedly visible sign of leaf senescence is the gradual loss of chlorophyll and concomitant yellowing, which is due both to preferential breakdown of chlorophyll (Chl) as compared to other pigments and to the synthesis of new compounds such as anthocyanins and phenolics [12]. The metabolic changes during leaf senescence include hydrolysis of macromolecules such as proteins, nucleic acids and lipids, loss of photochemical capability, loss of Chl, loss of chloroplast integrity, and massive transport of nutrients to other parts of the plant [8, 27, 42].

Although leaf senescence mostly involves degenerative processes that result in degradation of the subcellular components, there is much evidence the leaf senescence is an ordered sequence of events under genetic control. The existence of genetic variations in symptoms of leaf senescence is an indication that leaf senescence is a genetically programmed event [11, 32, 38]. In addition, several recent reports indicate that leaf senescence involves the production of specific proteins [19, 39] and the induction of expression of specific genes [1–3, 7, 10, 14, 16, 19, 22, 23, 36]. Further detailed analysis of the regulation of gene expression along with the functional identification of senescence-associated genes will be required to understand the mechanism of induction of the genes during leaf senescence and the mechanism of genetic regulation of the leaf senescence process.

Arabidopsis, a monocarpic plant with a short life cycle and readily distinguishable developmental stages, is well suited for study of leaf senescence as well as whole plant senescence, due to the amenability of this organism to genetic and molecular analysis [24]. A previous study on leaf senescence in *Arabidopsis* has shown that the progression of leaf senescence appears to be readily predictable in *Arabidopsis* [16]. In addition, several genes induced during *Arabidopsis* leaf senescence have been isolated [14, 16, 22]. While these previous results describe mostly the developmental and age-related senescence of *Arabidopsis* leaves, it is known that the senescence of plant tissues is also highly influenced by environmental factors and hormones [23, 27, 39]. However, there has been little study on senescence response of *Arabidopsis* to the environmental factors and hormones. Examination of the senescence responses and regulation of senescence-associated genes to the environmental and hormonal stimuli in *Arabidopsis* will be necessary to further understand the mechanism of leaf senescence in *Arabidopsis*.

We are trying to understand the regulatory mechanism of plant leaf senescence by genetic and molecular approaches, taking advantage of *Arabidopsis* as a model plant. As an initial part of our efforts toward this goal, we have attempted to examine the expression pattern of a senescence-associated gene of *Arabidopsis* that is related to the previously isolated radish *din1* gene [1]. The radish *din1* gene was originally isolated from dark-treated radish cotyledons and was shown to be associated with dark-induced senescence of radish cotyledons. However, no further correlation of this gene with regard to senescence has been analyzed. Since the *din1* gene shows a high level of induction upon senescence of radish cotyledons, we expected the corresponding *Arabidopsis* gene may be a good candidate to study the mechanism of gene regulation during leaf senescence in *Arabidopsis*.

Here, we report the characterization of a cDNA and of a genomic clone of a senescence-associated gene of *Arabidopsis thaliana* and the examination of the expression pattern of this gene under various senescence-affecting conditions. Our data provide a comprehensive analysis of expression of a senescence-associated gene under various senescence-affecting conditions.

Materials and methods

Plant and other materials

Seeds of *Arabidopsis thaliana* ecotype Col-0 were originally obtained from the AIS seed stock center (Frankfurt, Germany). Plants were grown on a compound soil mixture of vermiculite/peat moss/perlite (1:1:1) in a growth chamber (Vision Scientific, Korea) with a light cycle of 16 h light/8 h dark and a temperature cycle of 23 °C day/18 °C night.

Ethephon, N^6-benzyladenosine (BA) and abscisic acid (ABA) as mixed isomers were purchased from Sigma. The *Arabidopsis cab2* gene was provided by J. Chory.

The detached leaves for treatment with several senescence-affecting factors were obtained by cutting at the approximately middle part of the petioles of the third or fourth foliar leaves with a sharp scalpel to minimize wound effect [29]. The detached leaves were floated on deionized water containing appropriate chemicals in 24-well Petri dishes with the abaxial side up. In this situation, the end (the cut side) of the petioles was contacted by the solutions and it is not likely the leaves were experiencing dehydration response. For dark treatment or for control in chemical treatment experiments, the detached leaves were floated on deionized water only. Ethephon solution was prepared as described previously [21].

Measurement of Chl content and Chl fluorescence

The Chl content of leaf pieces was measured as described [17] after thorough extraction of Chl in 80% (v/v) ethanol. Chl fluorescence of foliar leaves was measured after dark adaptation of the leaves for 15 min at room temperature by using a portable plant efficiency analyzer (Hansatech Instruments, England). The maximum yield of fluorescence (F_m) was obtained with a saturation beam from an array of 6 light-emitting diodes (1600 $\mu mol/m^2 \cdot s$). The maximum variable fluorescence (F_v) was obtained by subtracting the initial Chl fluorescence (F_0) from F_m. The ratio of F_v/F_m was used to show the potential quantum yield of photochemical reactions of PSII (referred to as photochemical efficiency hereafter [31]).

Isolation of plant DNA and RNA

Total cellular DNA was isolated from the whole aerial part of *Arabidopsis* at 25 days after germination (DAG), according to the method described by Sambrook *et al.* [33]. Total cellular RNA was isolated by the method of Nagy *et al.* [26]. Poly(A)$^+$ RNA was prepared from total cellular RNA with poly(A)$^+$ mRNA quick purification kit (Stratagene) according to the instructions of the manufacturer.

Construction and screening of a cDNA library

Five μg of poly(A)$^+$ RNA was isolated from detached leaves exposed to darkness for 48 h and was used for cDNA library construction. Double stranded cDNA was synthesized with a cDNA synthesis kit (Pharmacia) according to the instructions of the manufacturer. The cDNA was inserted into the λZAPII vector (Stratagene) using a cDNA cloning kit after ligating an *Eco*RI-*Not*I adapter to the cDNA and then was introduced into XL-1 Blue cells (Stratagene).

About 2×10^4 primary recombinant phages were screened using the radish *din1* cDNA clone [1] as a probe. The screening was performed by the standard plaque lift method [33], except that the hybridization and washing of the membranes were done according to Church and Gilbert [6]. The positive clones were converted to plasmid clones by *in vivo* excision using a helper phage (Stratagene).

Screening of a genomic library

A genomic DNA library of *Arabidopsis thaliana* ecotype La-0 (constructed by D. Voytas and kindly provided by H. Goodman) was used to isolate genomic clones. About 1×10^5 recombi-

nant phages were screened using cDNA clone (pSEN1) as a probe. Screening was performed as described for the cDNA library screening above. Phage DNA was isolated according to a standard method [33] using DE52 (Sigma). After restriction mapping of the inserted genomic DNA fragments of 3 positive clones and identification of the restriction fragments corresponding to the cDNA probe by Southern hybridization, a 4.8 kb *Hin*dIII fragment was subcloned into the plasmid vector pUC18 and the resulting clone was named pSEN1-G1.

RNA blot analysis

Thirty μg of total cellular RNA was size-fractionated by electrophoresis on a 1.2% formaldehyde-agarose gel and transferred onto nylon membranes (ICN Biomedicals) according to standard methods [33]. The membranes were then prehybridized in a solution of 7% (w/v) SDS, 0.5 M sodium phosphate pH 7.2, 1 mM EDTA, 1% (w/v) BSA at 65 °C and hybridized in a same solution to the labeled probe [6]. After hybridization, the filters were washed in 2 × SSC, 1% SDS at room temperature for 30 min and in 0.1 × SSC, 0.1% SDS at 42 °C for 30 min twice.

Genomic Southern blot analysis

Three μg of total cellular DNA was digested with *Hin*dIII, *Bgl*II, or *Dra*I and was size-fractionated on a 0.7% agarose gel. The DNA was transferred onto nylon membranes (ICN Biomedicals) with a vacuum blotting apparatus (Pharmacia-LKB, Model 2016) and cross-linked to the membrane by UV treatment (1.2 $\mu J/cm^2$; Stratalinker 1800, Stratagene). Hybridization and washing of the membranes were performed as described above for northern blot analysis.

Nucleotide sequencing

To sequence the cDNA insert of the pSEN1 clone, the cDNA insert (ca. 0.7 kb in size) was subcloned into pBluescript SK(−) vector as 0.38 and 0.32 kb fragments exploiting an internal *Eco*RI site. The two subclones were subjected to automated sequencing using the *Taq* dye primer cycling sequencing kit (ABI) and the ABI 373A automated sequencer.

Serial deletion clones of the 4.8 kb genomic clone, pSEN1-G1, which contains ca. 3.7 kb of the 5′-upstream sequence of the *sen1* gene, were made from the 5′ end of the clone using the Erase-A-Base kit (Promega). One of the deletion clones, named pSEN1-G3, was found to contain ca. 1.3 kb of the upstream sequence. This clone was sequenced after a nested set of deletion subclones was made from both ends using the Erase-A-Base system (Promega). Double-stranded plasmid sequencing was carried out partly by a standard dideoxy chain-termination procedure using Sequenase version 2.0 (United States Biochemicals) and partly by an automated sequencer as described above.

Mapping of the transcription start site by primer extension

An oligonucleotide primer (5′-AGAGGTGGAGAAATAGC-3′) complementary to the coding strand sequence between 103 and 119 bp from the *sen1* gene was used as a primer. Total cellular RNA was isolated from detached leaves that had been incubated in darkness for 2 days on deionized water to induce the transcription of the *sen1* gene and was used in the primer extension experiment. Forty μg of the RNA was coprecipitated with 2.0 pmol (10^5 cpm) of the primer labeled with polynucleotide kinase (Boehringer Mannheim) and was redissolved in 20 μl of water. Annealing of the primer to the RNA template proceeded at 37 °C for 16 h. After primer extension according to a standard procedure [33], the reaction mixture was resolved by electrophoresis on a 6% polyacrylamide sequencing gel. A sequencing reaction mixture of the genomic clone, pSEN1-G1, primed with the same oligonucleotide was resolved alongside the extension mixture as a size standard.

Results

Isolation of an Arabidopsis *cDNA clone, pSEN1, related to the radish* din1 *gene*

To examine first if the genome of *Arabidopsis* contains any sequence related to the radish *din1* gene, we first performed a Southern blot analysis of *Arabidopsis* genomic DNA using the *din1* gene as a probe. The result in Fig. 1A shows that the *Arabidopsis* genome contains most likely a single copy of a gene highly related to the radish *din1* gene. To isolate an *Arabidopsis* cDNA clone corresponding to the radish *din1* clone, we prepared an *Arabidopsis* cDNA library using the poly(A)$^+$ RNA extracted from the detached leaves that had been kept in darkness for 48 h with an assumption that this treatment would induce the expression of the corresponding *Arabidopsis* gene and thus increase the probability of isolating the desired clone. By probing this *Arabidopsis* cDNA library with the *din1* cDNA clone, we were able to isolate 24 positive cDNA clones from ca. 2×10^4 primary, unamplified recombinants. Both ends of 12 clones out of the 24 clones were sequenced and all the clones were found to have overlapping sequences with similarity to the sequence of the radish *din1* clone. The cDNA clone with the largest insert appeared to contain a DNA fragment with the entire coding region, when compared to the *din1* sequence. We designated this clone pSEN1 as a cDNA clone of a senescence-associated gene of *Arabidopsis* which we named *sen1*.

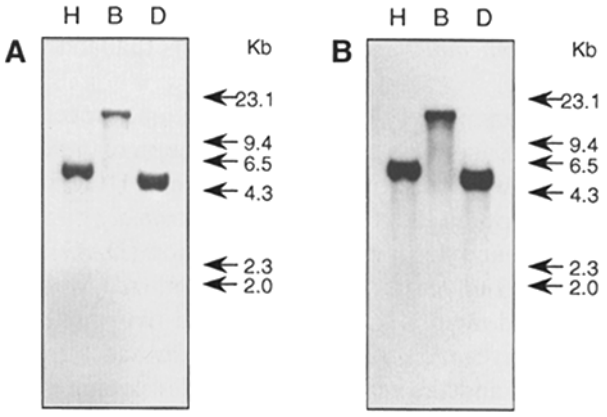

Fig. 1. Genomic Southern blot analysis of the *sen1* gene. Total cellular DNA of *Arabidopsis* (ecotype Col-0) was digested with *Hin*dIII (H), *Bgl*II (B), or *Dra*I (D). The filters were hybridized with either the radish *din1* gene probe (A) or with the pSEN1 clone (B). The positions of size markers are indicated.

The nucleotide sequence analysis revealed that the clone pSEN1 contains 34 bp of 5′-untranslated sequence, 546 bp of an open reading frame encoding 182 amino acids, and 120 bp of 3′-untranslated sequence (GenBank accession number U26945). A sequence comparison shows that the sequence of the pSEN1 clone is very similar to that of the radish *din1* gene with 78% and 81% identities at the nucleotide and the amino acid level, respectively. The amino acid sequence similarity between the products of the two genes is somewhat less in the amino terminal residues (70% identity in the amino terminal 70 residues) compared to the rest of the amino acids that show 92% identity. This fact is consistent with the observation that the DIN1 is a chloroplast targeted protein (A. Watanabe, unpublished results). The chloroplast targeting signal sequences do not have strict requirement for the conservation of their primary structure and are mostly found in the amino terminal regions. We were not able to find any other genes closely related to the pSEN1 clone by similarity search against the Genbank database, except the radish *din1* and the *Arabidopsis* EST sequences related to the *din1* gene. However, segments of the deduced peptide showed partial but noticeable similarity to the deduced peptide sequence of the bacterial *sud* [20], *glpE* [5] and *pspE* gene (GenBank accession number U42543) (Fig. 2).

Identification of the sen1 *gene as a single-copy gene in the genome of* Arabidopsis thaliana

DNA gel blot hybridization was performed to determine the copy number of the *sen1*-related genes in the genome of *Arabidopsis thaliana*. As shown in Fig. 1B, the pSEN1 cDNA hybridized to only one restriction fragment, when the ge-

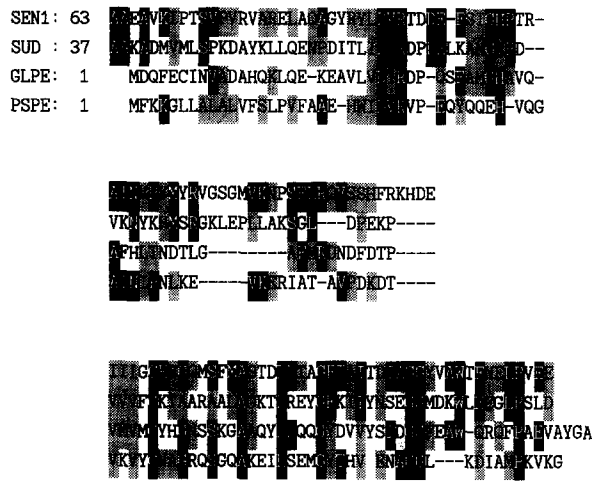

Fig. 2. Comparison of the amino acid sequence of the peptide encoded by the *Arabidopsis sen1* gene with that of related peptides. The amino acid residues of the peptide encoded by the *sen1* gene that are similar (hatched box) or identical (black box) to the amino acid residues of the peptide encoded by the bacterial *sud* [20], *glpE* [5] and *pspE* (GenBank accession number U42543) are indicated. Structurally similar amino acids are grouped according to Hanks *et al.* [15] into nonpolar chain R group (M, L, I, V, and C), aromatic or ring-containing group (F, Y, W, and H), small R groups with near neutral polarity (A, G, S, T, and P), acidic and uncharged polar group (D, E, N, and Q), and basic R group (K, R, and H).

nomic DNA was digested with *Hin*dIII, *Bgl*II, or *Dra*I. This result indicates that most likely there exists only a single copy of the *sen1* gene in the haploid genome of *Arabidopsis*. The pattern was the same, when an identical blot was probed with the radish *din1* clone (Fig. 1A). This result suggests that the pSEN1 clone we have isolated is the *Arabidopsis* counterpart of the radish *din1* gene. The result also indicates that the transcript we detected with the pSEN1 clone in northern blot analysis (shown below) is solely derived from the *sen1* gene and is not a mixture of transcripts from a gene family.

Genomic structure of the sen1 *gene*

The nucleotide sequence of one of the genomic clones of the *sen1* gene, pSEN1-G3 (see Materials and methods for creation of the clone) was determined to examine the genomic structure of the *sen1* gene. This genomic sequence (see GenBank accession number U26944 for the full sequence of pSEN1-G3) includes the entire protein coding sequence corresponding to the open reading frame of the cDNA clone, 51 bp of 5′-untranslated sequence, and 136 bp of 3′-untranslated sequence. The coding region is interrupted by 4 introns that are less than or equal to 100 bp in length. The range of the intron sizes is typical of *Arabidopsis*. The protein coding sequence consists of five exons that encode 50, 66, 24, 18, and 24 amino acid residues. Comparison of the genomic sequence with the cDNA sequence revealed a discrepancy in two nucleotides within the coding region, which results in two different amino acid residues. In addition, the 3′-untranslated region of the genomic clone contains eight additional nucleotides, when compared to that of the cDNA clone. This discrepancy is most likely due to the fact that different *Arabidopsis* ecotypes were used in constructing the cDNA and the genomic library. The transcription start site determined by primer extension analysis was the nucleotide A located 51 bp upstream of the translation initiation codon (data not shown). Thus the cDNA we isolated lacked 17 bp of the 5′-untranslated sequence.

Highly regulated expression of the sen1 *gene during developmental stages of* Arabidopsis thaliana

To examine developmental regulation, especially senescence stage-associated regulation of the *sen1* gene, we measured the abundance of the *sen1* transcript in the rosette leaves of *Arabidopsis* at 7, 14, 25 and 45 days after germination (DAG). At 7 DAG under our growth condition (see Materials and methods), *Arabidopsis* had two small foliar leaves. At 14 DAG, *Arabidopsis* had 6 foliar leaves and the seventh leaf just developing. The visible sign of the emergence of the floral inflorescence was observed at ca. 21 DAG. This stage was followed by a rapid growth of the floral inflorescence stem. At 25 DAG, the primary inflorescence had grown to a height of ca. 2–3 cm and

the first flower was opening. There was no visible sign of senescence on any foliar leaves at this stage yet. At 45 DAG, almost an entire area of the third foliar leaf turned yellow and all the other foliar leaves were also senescing.

The relative transcript levels of the *sen1* gene in the total cellular RNA isolated from all the foliar leaves at the various stages are shown in Fig. 3. At 7 and 14 DAG, transcripts of the *sen1* gene were not readily detectable. However, at 25 DAG, the transcription level of the *sen1* gene increased dramatically and remained at a similar level at 45 DAG.

To find a correlation between the expression of the *sen1* gene and leaf senescence, we have measured three parameters related to the function or biogenesis of chloroplasts: Chl content, photochemical efficiency (F_v/F_m, see Materials and methods), and expression of the *cab* gene. Chloroplasts are one of the earliest cellular components affected during leaf senescence [27] and thus the parameters related to the chloroplast disintegration such as loss of Chl and a reduced photosynthetic rate have been widely used as indicators of leaf senescence. The photochemical efficiency has not been widely adopted as a parameter for leaf senescence in plants. However, the photochemical efficiency (F_v/F_m) is affected by the integrity of PSII and thus can be a parameter for functional leaf senescence (see Discussion). The *cab* gene expression has been shown to decrease during senescence of *Arabidopsis* [16, 22]. However, measurement of any single parameter may be misled in following the progression of senescence [27] and we have consistently used all these three parameters to monitor leaf senescence. The third and fourth foliar leaves were chosen for measurements of the senescence parameters, since under our growth condition these leaves showed most consistent senescence patterns in accordance with the developmental stages of *Arabidopsis*.

The data in Fig. 3 show that, although *cab* gene expression is somewhat decreased, neither the photochemical efficiency nor the Chl content of the leaves changed appreciably at 25 DAG, when compared to that at 14 DAG. This result is consistent with our visual examination as mentioned above. However, the levels of the photochemical efficiency and Chl content at 45 DAG decreased to 40% and 41% of those at 14 DAG, respectively.

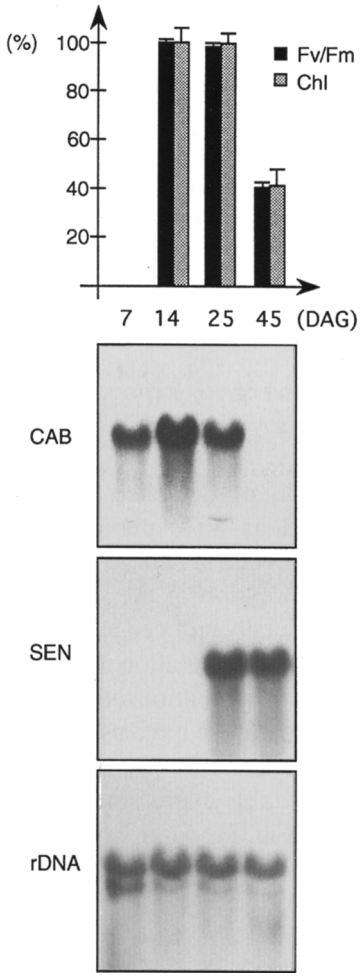

Fig. 3. Developmental regulation of the *sen1* gene. Thirty μg of total cellular RNA isolated from foliar leaves at 7 DAG, 14 DAG, 25 DAG, and 45 DAG was size-fractionated on a 1.2% formaldehyde-agarose gel. The blots were hybridized with the pSEN1 clone (SEN) or the *cab2* gene (CAB). The amount of RNA loaded in each lane is represented by the amount of 18S ribosomal RNA shown at the bottom (rDNA). The photochemical efficiency and Chl content of the 3rd or 4th foliar leaf at each time points are shown at the top as percent values of these of the leaves at 14 DAG. The data were obtained from 6 to 12 samples. The vertical bars denote standard deviations.

The results taken together show that the ex-

pression of the *sen1* gene is associated with the senescence symptoms developed at 45 DAG. This expression pattern is in clear contrast to that of the *cab* gene, which is not detectable at this stage (Fig. 3). However, the distinctive induction of the *sen1* gene at 25 DAG may be associated with the development of the inflorescence stem rather than with the progression of leaf senescence.

Induction of sen1 *gene expression during dark-induced senescence of leaves*

Dark incubation of detached leaves has been commonly used as an experimental system to study leaf senescence *in vitro* [37]. To investigate whether the expression of the *sen1* gene is associated with artificially induced leaf senescence, we have examined accumulation of the *sen1* transcript upon dark incubation of detached leaves. For this experiment, we have used the third foliar leaves of plants at 14 DAG, since at this stage there is no detectable expression of the *sen1* gene in the leaves (Fig. 3). First, the progression of senescence of the detached leaf during dark incubation was examined by measuring the photochemical efficiency and Chl content. In this experiment, the expression of the *cab* gene would not be an appropriate senescence parameter. Examination of the *cab* gene was included as a control for the dark treatment, since the expression of the *cab* gene is negatively regulated by dark treatment. As shown in Fig. 4A, a significant decrease in both photochemical efficiency and Chl content are observed after 3 days in darkness. After 5 days in darkness, both of the values dropped below 20% of those before transfer to darkness. The expression of the *sen1* gene was strongly induced after 1 day in darkness and the relative transcription level of *sen1* was maintained at a comparable level until 5 days in darkness. In contrast, the expression of the *cab* gene became undetectable after 2 days in darkness. This result shows that the linkage between the expression of the *sen1* gene and overt signs of senescence can be demonstrated at 3 days already. The expression pattern of the *sen1* gene associated with dark-induced senescence of leaves was further confirmed by transferring whole plants, instead of detached leaves, at 14 DAG to darkness and by measuring the level of transcription in the foliar leaves. As shown in Fig. 4B, the photochemical efficiency and Chl content of the leaves of plants transferred to darkness for 5 days fall to 7% and

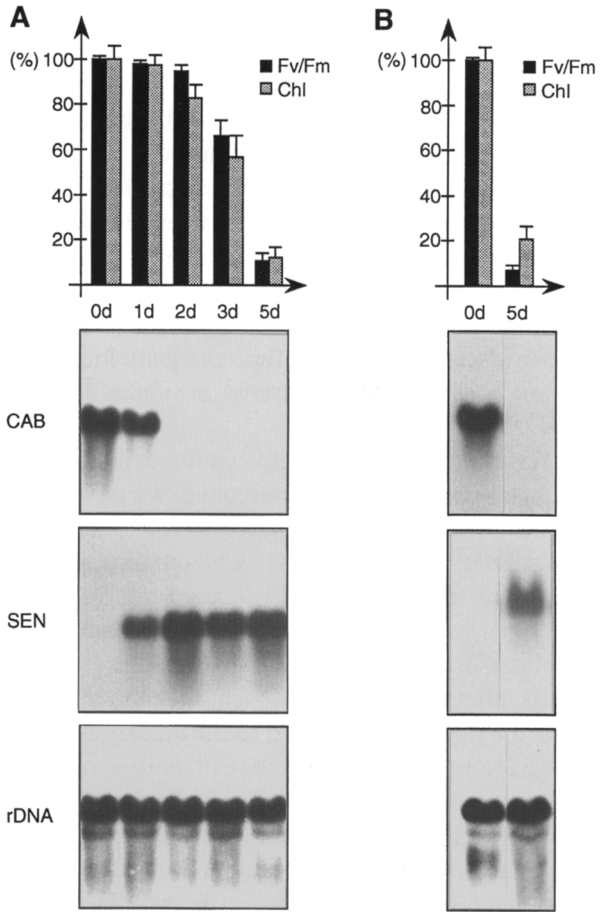

Fig. 4. Expression of the *sen1* gene during dark-induced senescence. Total cellular RNA of foliar leaves was isolated after incubation of detached leaves (panel A) or whole plants (panel B) in darkness for the indicated number of days. The photochemical efficiencies and Chl contents are shown at the top and are expressed as percent values of those of the leaves at 14 DAG without dark-treatment. The data were obtained from 6 to 12 samples. The vertical bars denote standard deviations. The blots were probed with either the *Arabidopsis cab2* gene (CAB) or pSEN1 clone (SEN). The amount of RNA loaded in each lane is represented by the amount of 18S ribosomal RNA shown at the bottom (rDNA).

21%, respectively, of the values before transfer to darkness. Again, after 5 days in darkness, the transcription level of the *sen1* gene became much higher. This result again shows that the expression of the *sen1* gene is associated with the dark-induced leaf senescence. However, it should be noted that, as we have observed upon examination of the expression of the *sen1* gene during the developmental stages, the expression of the *sen1* gene is induced (after 1 day) before any obvious signs of senescence symptoms develop and is then maintained during the senescence period.

Induction of sen1 *gene expression upon artificially induced leaf senescence by abscisic acid and ethephon*

We examined the expression of the *sen1* gene after artificially inducing leaf senescence using two senescence-promoting agents, abscisic acid (ABA) [39] and ethephon [27].

When a detached leaf of *Arabidopsis* at 14 DAG was incubated in 1 mM ethephon for 12 h or in 0.1 mM ABA for 2 days under continuous light, the leaf turned completely yellow, but the leaf tissue remained visually intact. Further incubation caused severe degeneration of the leaf tissue, so that it was not adequate for RNA extraction. In contrast, detached leaves incubated in light for 2 days without ethephon or ABA treatment did not show apparent symptoms of senescence such as yellowing. Detailed measurements revealed that the leaf incubated in light for 2 days retained 90% of the photochemical efficiency and 92% of the Chl content, when compared to the leaf before detachment (Fig. 5). Upon treatment with ethephon, the photochemical efficiency and the Chl content of the leaves dropped to 59% and 35% compared to the those of the leaves before detachment, respectively (Fig. 5). In addition, the expression of the *cab* gene was reduced significantly, compared to that of the leaves incubated in light for 2 days without chemical treatment. Upon treatment with ABA, the Chl content and the photochemical efficiency of the leaf tissue dropped to 27% and 41%, respectively, of the values in leaves before detachment. The expression of the *cab* gene was also significantly reduced under this condition. All these data indicated that the leaves became functionally senescent upon the chemical treatments. As shown in Fig. 5, the expression of the *sen1* gene was highly induced in the senescing leaves, whether senescence was induced by ethephon or ABA treatment. In con-

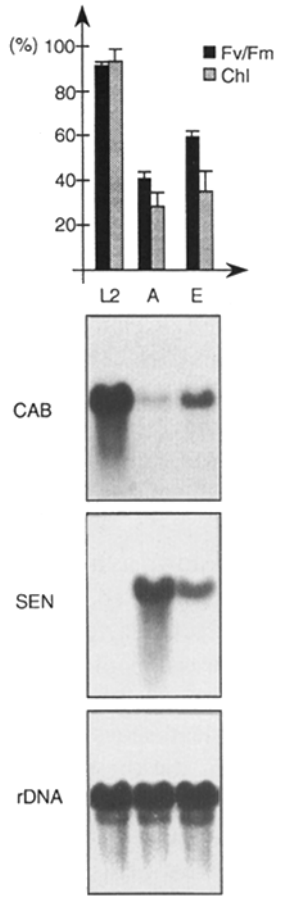

Fig. 5. Effects of ABA and ethylene on expression of the *sen1* gene. Total cellular RNA was isolated from the detached 3rd foliar leaf incubated in light only (L2) for 2 days, in 0.1 mM ABA (A) for 2 days, or in 1 mM ethephon (E) for 12 h. Photochemical efficiencies and Chl contents of the same leaves are shown at the top and are expressed as percent values of those of the leaves at 14 DAG with no chemical treatment. The data were obtained from 6 to 12 samples. The vertical bars denote standard deviations. The filters were probed either with the *Arabidopsis cab2* gene (CAB) or with the pSEN1 clone (SEN). The amount of RNA loaded in each lane is represented by the amount of 18S ribosomal RNA shown at the bottom (rDNA).

trast, the *sen1* gene was not induced in the detached leaves incubated for 2 days in light alone without the chemical treatment.

Effects of exogenous cytokinin or $CaCl_2$ on the senescence of detached leaves and on the expression of the sen1 *gene*

To further test the correlation between the *sen1* gene expression and the *Arabidopsis* leaf senescence, we examined the change of parameters of senescence symptoms and of expression of the *sen1* gene upon treatment with other chemicals that are known to delay senescence of *Arabidopsis* leaves.

Cytokinins are known as a primary senescence-retarding plant hormone [8, 34, 41]. The senescence-retarding effect of cytokinins could be mediated by the repression of senescence-induced genes. We first measured the effect of cytokinins on dark-induced leaf senescence by incubating detached leaves in darkness in the presence of 0.1 mM BA, a synthetic cytokinin. As shown in Fig. 6A, not all aspects of leaf senescence were restored by BA treatment. BA treatment was somewhat effective in delaying loss of Chl but not effective in delaying loss of photochemical efficiency as measured at 2 days after BA treatment. This became even more evident after 5 days. At this time point, the remaining Chl content and photochemical efficiency were 85% and 0%, respectively, with BA treatment and 16% and 10%, respectively, without BA treatment (Fig. 6A), when compared to the values in leaves before detachment (see Fig. 4A). The integrity of leaf tissue appeared more severely impaired after 5 days with BA treatment than that with dark treatment alone, when visually examined. Expression of the *cab* gene was also not restored by BA treatment (Fig. 6B) and this result is in contrast to what we have observed in $CaCl_2$ treatment (see below). Although cytokinins are generally considered as senescence-retarding plant hormone [8, 34, 41], the result we obtained here shows that BA did not prevent loss of all the aspects of the functional integrity of chloroplasts in our experimental scheme, but uncoupled the delay of Chl loss from the loss of photochemical efficiency.

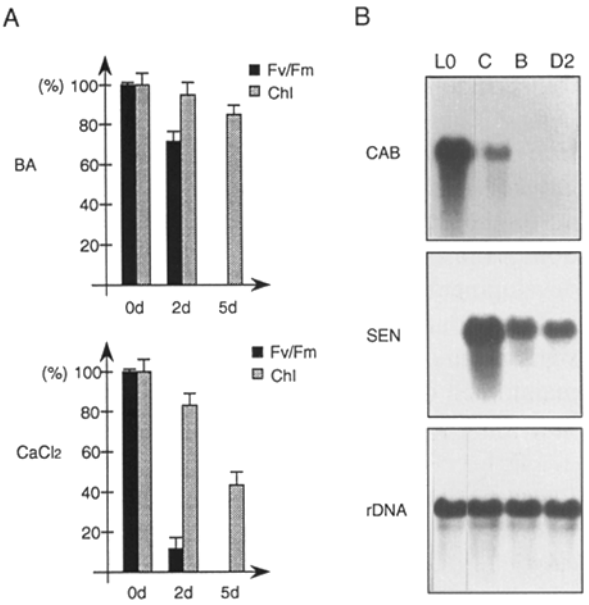

Fig. 6. Effects of BA and $CaCl_2$ on *Arabidopsis* leaf senescence and on expression of the *sen1* gene. A. The photochemical efficiencies and Chl contents of detached foliar leaves before any treatment (0 d) and after incubation in darkness for the indicated times in the presence of 50 mM $CaCl_2$ or 0.1 mM BA were measured and are expressed as percent values of those of the leaves at 14 DAG before transfer to dark. The data were obtained from 6 to 12 samples. The vertical bars denote standard deviations. B. Total cellular RNA was isolated from detached foliar leaves before any treatment (L0) and after incubation in darkness with 50 mM $CaCl_2$ (C), 0.1 mM BA (B), or with water only (D2) for 2 days. The filters were probed either with the *Arabidopsis cab2* gene (CAB) or with the pSEN1 clone (SEN). The amount of RNA loaded in each lane is represented by the amount of 18S ribosomal RNA shown at the bottom (rDNA).

Calcium ion has either a senescence-retarding or -promoting effect depending on experimental conditions [4, 30, 40]. We examined the effect of calcium ion on the expression of the *sen1* gene by incubating the detached leaves in 50 mM calcium chloride in darkness for 2 days. The concentration was chosen as a senescence-retarding condition after examining the effect of calcium ion on Chl loss of detached *Arabidopsis* leaves incubated in darkness for 5 days. The calcium concentrations of 1, 10, 25, 50 and 100 mM significantly delayed the loss of Chl (data not shown). How-

ever, higher calcium concentrations of 250 and 500 mM accelerated the loss of Chl in the detached leaves. Our result is consistent with the result obtained with the dark-incubated cabbage (*Brassica oleracea*) leaves [4]. They showed that the calcium ion concentration of 50 mM is effective in delaying the loss of Chl as well as protein loss and lipid degradation [4]. The calcium ion concentration of 50 mM was effective in delaying Chl loss of detached *Arabidopsis* leaves incubated in darkness for 5 days (46% of Chl retention with calcium ion and 16% Chl retention without calcium ion, Fig. 6A). However, calcium ion was not effective in retarding loss of photochemical efficiency but, instead, significantly accelerated the loss of photochemical efficiency after 2 days. The integrity of leaf tissue was affected more severely after 5 days with $CaCl_2$ than that with dark treatment alone, when visually examined. Interestingly, expression of the *cab* gene was retained at a significant level by 50 mM calcium ion (Fig. 6B).

The results of our examination of the expression of the *sen1* gene in detached *Arabidopsis* leaves incubated in darkness for 2 days in the presence of these chemicals are shown in Fig. 6B. BA did not significantly influence the expression of the *sen1* gene in our experimental scheme. Upon treatment with calcium ion, expression of the *sen1* gene was not repressed but rather significantly induced in 2 days, in comparison to detached leaves incubated in darkness without calcium ion.

Discussion

Senescence-associated genes are referred to as the genes of which mRNA increases in abundance during senescence [22]. Our results show that mRNA abundance of the *Arabidopsis sen1* gene increases in all the cases of leaf senescence we examined: the leaves at 45 DAG and the leaves subjected to senescence by dark, ABA, or ethephon treatment. Thus, the *sen1* gene can be considered one of senescence-associated genes in *Arabidopsis*. The observation that the *sen1* gene is induced by several senescence-promoting factors indicates that the gene functions at a common step of the leaf senescence pathway induced by these factors.

The cDNA sequence of the *Arabidopsis sen1* gene is highly similar to the radish *din1* gene and the *sen1* gene appears to be a single-copy gene in *Arabidopsis*. Thus, the *sen1* gene is likely to be an *Arabidopsis* counterpart of the radish *din1* gene. Some of the expression pattern of the *sen1* gene is also similar to that of the *din1* gene. Both genes are strongly induced by dark or ethylene treatment. However, the result of the BA treatment is different. The *din1* gene is slightly repressed but *sen1* gene is not appreciably changed upon BA treatment. The discrepancy may be due to the differences in the tissues used; the attached cotyledons were used for expression analysis of *din1* and the detached foliar leaves for that of *sen1*.

The previous study on the expression of the radish *din1* gene in relation to senescence is, however, limited. The radish cotyledons do not lose Chl during the first 24 h when incubated in darkness but than rapidly do so within subsequent 48 h incubation [19]. The *din1* gene is rapidly induced upon dark treatment and the level of the transcript remains high at least up to 48 h [1]. This is the only reported correlation between the *din1* expression and the cotyledon senescence. Here we have clearly shown that the *sen1* gene, a counterpart of the radish *din1* gene in *Arabidopsis*, is associated with *Arabidopsis* leaf senescence through parallel measurement of gene expression and progression of senescence upon several senescence-inducing conditions.

The previous studies on senescence of leaf tissue in *Arabidopsis* [16, 22] have provided an early foundation for molecular and genetic approaches towards dissection of the mechanism underlying leaf senescence in *Arabidopsis*. While these previous studies were focused only on the developmental and age-related senescence, here we have reported the influence of hormonal and environmental factors as well as that of development on *Arabidopsis* leaf senescence and on the regulation of a senescence-associated gene. Our study shows that the *Arabidopsis* leaf senescence can be induced by prolonged incubation in darkness and

by ABA or ethylene treatment. The senescence response of *Arabidopsis* to these factors is typical of that of many plant species [27]. Our results, thus, provide a further foundation for analyzing the mechanism of leaf senescence, using this genetically amenable plant. For example, the regulation of the previously isolated senescence-associated genes of *Arabidopsis* may be further examined using the conditions we reported here. This experiment may reveal the differential response of the genes to the several senescence-affecting factors. In addition, mutations that have altered senescence response to these factors may be isolated or, *vice versa*, senescence mutations may be analyzed for the response to these factors. In fact, we were able to isolate 6 delayed-senescence mutants of *Arabidopsis* by screening for the mutants that show delayed senescence response upon prolonged dark treatment (Oh *et al.*, unpublished data).

Progression of leaf senescence may be examined by looking at changes in Chl content, photosynthesis rate, RNA content, protein content, etc. [27]. In this experiment, we used three parameters to follow the progression of leaf senescence: Chl content, photochemical efficiency, and *cab* gene expression. During senescence of *Arabidopsis* leaves, whether induced naturally, by darkness, by ABA, or by ethephon, the change in Chl content and photochemical efficiency is correlated with the senescence event and either of these parameters can be used as indicator of senescence. In particular, our results show that photochemical efficiency, measured by a rather simple instrument (Materials and methods), can be a convenient indicator for the progression of leaf senescence in *Arabidopsis*. It can also be argued that the measurement of photochemical efficiency may actually be a better indicator than the assessment of photosynthetic rate, since the photochemical efficiency (F_v/F_m) represents the photosynthetic capacity and thus functional integrity of PSII. In contrast, the photosynthetic rate is influenced by sink demand [27] without a correlation to leaf senescence.

It is specially interesting that cytokinin did not prevent the loss of photochemical efficiency of the detached leaves subjected to senescence by dark treatment. Cytokinins are generally known as primary senescence-retarding hormones and were shown to delay the loss of Chl and/or proteins in senescing tissues of many plant species [8, 34, 41]. Our observation that the BA, a cytokinin, delays Chl loss of *Arabidopsis* leaves in consistent to the previous results. However, as far as we are aware, there are no previous reports that describe the effect of BA on photochemical efficiency. Our result suggests that cytokinins may block only parts of progression of senescence and that the role of cytokinins in leaf senescence of *Arabidopsis* and perhaps of other plants may need to be examined more carefully.

Ethylene is known to be a major hormonal factor in promoting senescence of plant organs [27, 39]. Senescence of the detached *Arabidopsis* leaves was highly accelerated by ethephon treatment as shown in Fig. 5. This senescence-promoting effect of ethephon was not due to the effect of the by-products generated during ethylene release [21], since the solution containing the by-products only did not cause loss of Chl content and photochemical efficiency within the same incubation time (data not shown). We have also found that 3 out of 6 delayed senescence mutants we have isolated (Oh *et al.*, unpublished data) are allelic to one of the ethylene-insensitive mutations, *ein2* [13]. These observations suggest that ethylene may be one of the major factors in *Arabidopsis* leaf senescence. Exogeneously added ABA also induces several senescence-associated symptoms in a variety of plant organs and ABA is considered as one of important senescence promoters [39]. Our results show that ABA readily induces all of the senescence symptoms we measured when applied to the detached *Arabidopsis* leaves. This result thus suggests that ABA may be a potent senescence promoter in progression of *Arabidopsis* leaf senescence. It would be revealing to examine if the ABA-insensitive or -deficient mutations of *Arabidopsis* may show altered leaf senescence.

The senescence parameters we used to examine the progression of the *Arabidopsis* leaf senescence change coordinatedly during the senes-

cence, whether it is caused developmentally or is induced by senescence promoting treatments. However, upon treatment with BA and $CaCl_2$, these parameters become uncoordinated (Fig. 6). BA treatment delayed the loss of Chl content, but not that of photochemical efficiency. Upon $CaCl_2$ treatment, the loss of Chl content is partially delayed, but the loss of photochemical efficiency is significantly accelerated. This result suggests that senescence symptoms may not necessarily be coupled to each other in *Arabidopsis* and further suggests that examination of a limited number of symptoms may be misleading in interpretation of senescence response.

Although we have clearly demonstrated in this report that the *sen1* gene is associated with leaf senescence, the regulation of the expression of the *sen1* gene may be complex. While induction of the *sen1* gene by several senescence-promoting factors could be mediated by a presumed senescence signal common to the senescence symptoms induced by these factors, the *sen1* gene may be, at least in part, directly regulated by the factors we used to influence the senescence progression. In this regard, the 5′-upstream sequence of the genomic clone shows a few interesting features that may play a role in regulation of the transcription of this gene (Fig. 7). The 5′-upstream sequence contains a TCA motif (TCATCTTCTT) at the nucleotide position −357 to −348 (TCATCTTCTC), which is highly conserved among genes induced by various stresses [9]. This sequence motif may be involved in the induction of the *sen1* gene under stresses that promote senescence of *Arabidopsis* leaves. A sequence element very similar to the consensus sequence of the heat-shock element (HSE) [28] is found in the upstream sequence. The sequence CTCGAATCGTCTAG

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CTTCCCCAAGCTCTGCACTTAGAGCGGTTACTACACCATACATAACAAATCCAATCACTG  -1150
GAAGAGGAACACCTGGCAAAATGTAAAAAAGGATTTTCATTGAGTTCAACTTACCTGCAA  -1090
TGCAATGTGTGGACAAACAACGAAGACAAAGCACTACATAGAGTGGAGTGAAGCATGCTA  -1030
TTTAACTCTAACAATTCTATGCATACTAAGTACCAAAGCTCCTAAATGATATAGCTTCAA  -970
GCAAAGATTCACAGAGACTATCACATGCGAGTATTTAATCATGCCTACTATAAAGGAGAG  -910
GAAAATTAGCTACAGATTTCAAACACTATAATCAATAAGGCGGTGGATAATACCGAAAAC  -850
GACGGCATAATCGCTGTTGAGGACATCACCGCAAGTGCCACCACCAATAGGGCAAAACGC  -790
ATCGGAGCCAGTGACCTTGAGGTAAGTCAAATACGCAGGTCTAACATCCCAATACCGCCG  -730
ATTCCGGTGTACCAGTTGTATGTCGAACTGTTACTAGTAGATACTTCACTTGTAGACGAT  -670
GAGGAGGAGGAAGACAGCGACGGTGCCGAATCCTCACCGTTTTCCGGCTCAGTTGAAGAA  -610
CATTTAATCGGAATCGCAGGAAATCTCCGATCAGAGACCTAATTCAGAACATCTGACTTT  -550
CAAATGACCGGTCACACACTACTGTCACTGAAGAAAACACATTGTCAGAATTTACCTCGA  -490
ATCGTCTAGGATAAGAAGTTACCGACGGAGGAGAAACTCACGGAAGCCGAAATGAAACTG  -430
ACAAGACGACACAGAAACAAACCTCGCCATCATCGTCGTCGAAGAATGCCTATTCCACCA  -370
TTGTTAACTTCATCATCTTCTCTCCTCGCTCCACTATCTCAGCCACTTGTTTCAATATTG  -310
CCTAAACGAGGCCCAAATACGATAATTTGGATCCATAATTAGTTTATCACAATTGGGCCT  -250
TACGGTCTTCTTATCCGTCCGAGTCAGCAAGTTATCCTCTTCAACACGACTCACGAGGCA  -190
CCGAGTTGGACTTATCATGATTCATTATCTACGCATTGTTTACATTTTGGAATCGACTTT  -130
GGTACATAATTGAATTTATCCATAGAAATGATAGAGACTCGAAGATCGGACATAAAACTT  -70
GCTGACGTGAGACCCATACCTTTGTGTCTGTATGTATTATATAAACGCTATATAACTAAG  -10
AGAAGAAGTATCTAAGTAGCATCAAAGCATTCTAAAAGAAGTTTCAAAGAAGAAGTGAAA  51
ATGGAAACCACTGCTTTTAACACAACATCACGAATTGGAAACTGGTCATCGGCTATTTCT  111
CCACCTCTACAAACAT                                              127
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Fig. 7. Nucleotide sequence of the genomic clone of the *sen1* gene. The nucleotide sequence of the promoter region and around the translational initiation site is presented here (refer to the GenBank accession number U26944 for the full sequence). Nucleotides are numbered from the transcription initiation site marked by an asterisk. A putative TATA box motif is underlined. The sequence motifs with similarities to the heat-shock element (from −494 to −481), to the ABA-responsive element (from −304 to −298, from −197 to −191 and from −67 to −60), to the TCA motif (from −357 to −348) and to the hydration-responsive element (from −470 to −462) are underlined. The sequence of the nucleotide primer used in primer extension analysis (from + 103 to + 119) is doubly underlined. The translation (+ 52) and transcriptional initiation sites (+ 1) are indicated by bold letters.

(−494 to −481) shows 90% identity to the HSE consensus sequence CT-GAA--TTC-AG. The presence of this element in the *sen1* gene and the fact that the radish *din1* gene is induced by heat-shock treatment [1] may suggest that the *sen1* gene can be regulated by heat-shock. The 5′ region also contains two repeats of the sequence ACGAGGC resembling the ABA-responsive element (ABRE [25, 35]), ACGTGGC, at nucleotide −304 to −298 and −197 to −19. An imperfect palindromic sequence between −67 and −60 (CTGACGTGAG) is also reminiscent of ABRE as well as of the TGA1a or 1b binding sequences [18]. The presence of these sequence elements suggests that the *sen1* gene may be directly regulated by ABA. The results of our RNA gel blot analysis in Fig. 5 may be, at least partly, attributed to induction by ABA. Finally, the sequence between −470 and −462 (TACCGACGG) is very similar to the dehydration-responsive element (TACCGACAT) found in the *Arabidopsis rd29A* promoter [43].

As shown in this report, expression of the *sen1* gene appears to be distinctively regulated by senescence-promoting hormones, during plant development, and in light-to-dark transition. In addition, the abundance of the *sen1* mRNA is relatively high when induced. We were able to find 24 positive cDNA clones in our initial screening for the *sen1* gene from the unamplified, primary cDNA library. This number represents 0.16% of the recombinant cDNAs. Thus, *sen1* appears to be a good model gene for the study of gene regulation during senescence. For the purpose of analyzing *cis*-acting regulatory elements of the *sen1* gene, we are now in the process of generating a deletion series of the promoter region fused to a reporter gene, *uidA*. More detailed expression studies of the *sen1* gene under several conditions affecting leaf senescence along with the identification of *cis*-acting elements should provide us with informations on regulation mechanism of the progression of leaf senescence. Our preliminary examination of the first emerging transgenic tobacco shoots transformed with a plasmid construct that bears the bacterial *uidA* gene fused to 726 bp of the 5′-upstream sequence of the *sen1* gene (Fig. 7) shows that the upstream element can lead to the dark- and ABA-induced expression of the *uidA* gene (data not shown).

Although there are now several senescence-associated genes identified in plants, only a few of them have known functions [22], including RNase [36], malate synthase [10], cystein proteinase [16, 22], and glutamine synthetase [17]. Like many other senescence-induced genes, the function of the *sen1* gene remains elusive at this moment. Although the deduced amino acid sequence of the *sen1* gene shows noticeable similarity to that of several bacterial genes (Fig. 2), the functions of most of these bacterial genes are also unknown. The only bacterial gene that encodes a protein with some similarity to the *sen1* product and has a known function is the sulphide dehydrogenase (*sud*) gene of *Wolinella succinogenes* [20]. This bacterial sulphide dehydrogenase catalyzed sulphide oxidation. However, this information does not allow us to readily recognize the function of the *sen1* gene, although we may speculate that the *sen1* gene is involved in a certain cellular redox reaction. It will require biochemical or transgenic approach to identify the function of this gene product.

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