# Cloning and Characterization of a Receptor-Like Protein Kinase Gene Associated with Senescence

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Senescence-associated genes are up-regulated during plant senescence and many have been implicated in encoding enzymes involved in the metabolism of senescing tissues. Using the differential display technique, we identified a SAG in bean (*Phaseolus vulgaris)* leaf that was exclusively expressed during senescence and was designated senescence-associated receptor-like kinase (SARK). The deduced SARK polypeptide consists of a signal peptide, a leucine-rich repeat in the extracellular region, a single membrane-spanning domain, and the characteristic serine/threonine protein kinase domain. The mRNA level for SARK increased prior to the loss of chlorophyll and the decrease of chlorophyll *a/b*-binding protein mRNA. Detached mature bean leaves, which senesce at an accelerated rate compared with leaves on intact plants, showed a similar temporal pattern of SARK message accumulation. Light and cytokinin, which delayed the initiation of leaf senescence, also delayed SARK gene expression; in contrast, darkness and ethylene, which accelerated senescence, advanced the initial appearance of the SARK transcript. SARK protein accumulation exhibited a temporal pattern similar to that of its mRNA. A possible role for SARK in the regulation of leaf senescence was considered.

Leaf senescence is the last stage of development, during which cells undergo a major transition from carbon assimilation and other anabolic reactions to a catabolic pattern that results in cell dysfunction, structural disintegration and, eventually, cell death (Thimann, 1980; Smart, 1994). The catabolic pattern of the senescing organ involves chlorophyll (Chl) degradation and chloroplast breakdown (Gepstein, 1988). There is also proteolysis and nucleic acid hydrolysis leading to the redistribution of nitrogen and phosphorus from the degraded products to developing organs (Smart, 1994; Buchanan-Wollaston, 1997; Gan and Amasino, 1997).

To better understand the mechanisms that regulate cell death, several treatments have been developed that affect leaf senescence. For example, leaf detachment causes an accelerated yellowing and protein disappearance, which resembles that in attached leaves (Thimann, 1980). Darkness also hastens leaf yellowing in many plant species, in attached leaves and during artificially induced senescence of detached leaves (Becker and Apel, 1993; Kleber-Janke and Krupinska, 1997; Weaver et al., 1998). Furthermore, exogenous applications of ethylene usually advance and cytokinins usually delay the appearance of senescence symptoms (Gepstein and Thimann, 1981; van Staden et al., 1988; Weaver et al., 1998). The regulation of leaf senescence by endogenous levels of these hormones also has been demonstrated in genetically modified plants. Thus ethylene-resistant mutants of Arabidopsis or ethylene-deficient transgenic tomato plants exhibited delayed leaf senescence (Picton et al., 1993; Grbic and Bleecker, 1995; John et al., 1995) and transgenic plants oversynthesizing cytokinin during the senescence phase retarded tobacco leaf yellowing (Gan and Amasino, 1995).

The biochemical and molecular studies of leaf aging have provided evidence that leaf senescence is an active, genetically programmed process (Nooden et al., 1997). Senescence-related genes have been identified and classified according to their pattern of expression during senescence (Smart, 1994; Buchanan-Wollaston, 1997). Most of the genes involved in the process of carbon assimilation are down-regulated during leaf senescence, including those involved in photosynthesis (Gepstein, 1988; Bate et al., 1991; Hensel, et al., 1993; Jiang, et al., 1993; Smart, 1994; Buchanan-Wollaston, 1997). Other genes, designated senescence-associated genes (SAGs), are up-regulated during senescence and most of their deduced products indicate an association with senescence processes (Bleecker and Patterson, 1997; Buchanan-Wollaston, 1997; Lohman et al., 1994; Smart, 1994; Nam, 1997; Nooden et al., 1997; Quirino et al., 2000). These proteins may be enzymes that hydrolyze various macromolecules (proteins, nucleic acids, and lipids), that are involved in their mobilization, or that detoxify degradation products (Lohman et al., 1994; Bleecker and Patterson, 1997; Buchanan-Wollaston, 1997; Buchanan-Wollaston and Ainsworth, 1997; Gan and Amasino, 1997; John et al., 1997; Nooden et al., 1997; Park et al., 1998; Weaver et al., 1998; Perez-Amador et al., 2000). The gene-expression profile of artificially induced senescence in detached leaves has been compared with the natural senescence of intact leaves, and many, but not all identified senescence genes exhibit similar patterns of expression (Becker and Apel, 1993; Kleber-Janke and Krupinska, 1997; Weaver

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et al., 1998). In additional, induction of certain defense-related genes has been reported to occur during leaf senescence (Hanfrey et al., 1996; Quirino et al., 1999). Ozone-induced leaf senescence also has been found to involve expression of genes associated with natural leaf senescence (Miller et al., 1999).

Similar to other known developmental programs in plants, the senescence program may also express components of signal transduction pathways; the cell perceives a signal(s), and triggers the transduction cascade by mediating the induction, or increasing the levels of certain proteins encoded by SAGs. This eventually leads to the biochemical, physiological, and morphological changes of senescence (Smart, 1994; Gan and Amasino., 1995; Buchanan-Wollaston, 1997; Nam, 1997; Nooden et al., 1997). Recent studies on signal transduction pathways involved in plant developmental processes indicate similarities to known counterparts in animal and yeast systems (Braun and Walker, 1996). One of the well-characterized signal transduction pathways involved in developmental and biochemical processes is a cascade of processes acting via protein phosphorylation and dephosphorylation (Stone and Walker, 1995; Braun and Walker, 1996).

In the present report we describe the identification of a gene that is exclusively expressed during senescence. The initiation of its expression occurs at later stages of leaf maturation, but appears immediately prior to some symptoms of senescence. Based on DNA and amino acid sequences, the predicted structure indicates that the gene encodes a receptor-like protein kinase that has similarities to other kinase receptors associated with signal transduction pathways of various developmental and cellular processes (Walker, 1994; Becraft, 1998).

## RESULTS

### Isolation and Sequence Analysis of a Senescence-Related cDNA Clone

Differential display analysis carried out in bean (*Phaseolus vulgaris*) revealed many gene transcripts in expanded green and/or partially yellowing primary leaves (15 and 40 d post-germination, respectively). Several of the cDNA bands representing gene transcripts in the mature leaves disappeared or decreased in the senescing leaves, whereas others increased or were exclusive to the latter. One of the bands that was specifically detected in senescing leaves was used as a probe for confirmation of the differential display results and for further characterization. Comparative northern-blot hybridization between RNA extracted from leaves 15 and 40 d post-germination revealed a distinct band of about 3.1 kb that was solely detectable in the senescing leaf extract.

The senescence-related 3′-cDNA fragment was used as a probe to screen a cDNA library prepared from poly(A)$^+$ RNA of senescing primary bean leaves. A total of 10 putative cDNA clones were selected and analyzed for sequence homology. All selected clones contained identical sequence at the 3′-untranslated region. Therefore, the clone with the longest insert corresponding to the predicted size of the full-length cDNA (3,044 bp) was used for further DNA sequencing. There is open reading frame of 2,715 bp, 151 bp of the 5′-untranslated region and 178 bp of the 3′-untranslated region. The open reading frame encodes a predicted 904-amino acid protein with a calculated molecular mass of 100.9 kD. A stop codon is found upstream of the initiation ATG codon indicating that the entire translated region was identified. The calculated size of the cDNA clone fits that of the senescence-associated transcript estimated from the northern-blot analysis.

A sequence comparison with available data at the amino acid level indicated the existence of four prominent domains (Fig. 1): (a) a protein kinase domain at the carboxy-terminal (amino acids 578–876); (b) a hydrophobic domain in the middle constituting a putative transmembrane domain (amino acids 504–528); (c) an extracellular domain (amino acids 1–503); and (d) a hydrophobic domain resembling a signal peptide at the N-terminal (amino acids 1–20). The existence of these four main regions is characteristic of a protein kinase-like receptor (Becraft, 1998). Thus the gene was designated "SARK" for "senescence-associated receptor-like protein kinase." As shown in Figure 1, the carboxy-terminal region in particular exhibits a higher degree of amino acid homology with known Ser/Thr protein kinases consisting of all 11 conserved subdomains among protein kinases (Hanks and Quinn, 1991). The amino acid sequences in subdomain VIb (DVKSSN) and VIII (GTPGYLDPD) of the kinase region of the SARK protein are consistent with the consensus sequence of Ser/Thr kinases (DLKXXN and G(T/S) XX(Y/F) XAPE, respectively).

The external segment of the predicted SARK protein includes a Leu-rich repeat (LRR) region that is found in certain groups of receptors. The LRR consensus sequences are usually tandemly repeated and occur in different forms interspersed with intervals and insertions (Deeken and Kaldenhoff, 1997; Lease et al., 1998). The deduced SARK protein contains a region with 3.5 repeats of the L-X-L-4X-L-16X motif (Fig. 1). Although overall SARK shares only 46% amino acid sequence similarity with the light-repressible receptor protein kinase (Lrrpk) of Arabidopsis (accession no. X97774), it exhibits an identical LRR motif (Deeken and Kaldenhoff, 1997). A hydropathy plot (Fig. 1B) is consistent with the suggestion that the product of the SARK gene serves as a receptor consisting of two highly hydrophobic regions, a putative signal peptide and a single membrane-spanning region (Weinstein et al., 1982; von Heijne, 1990).

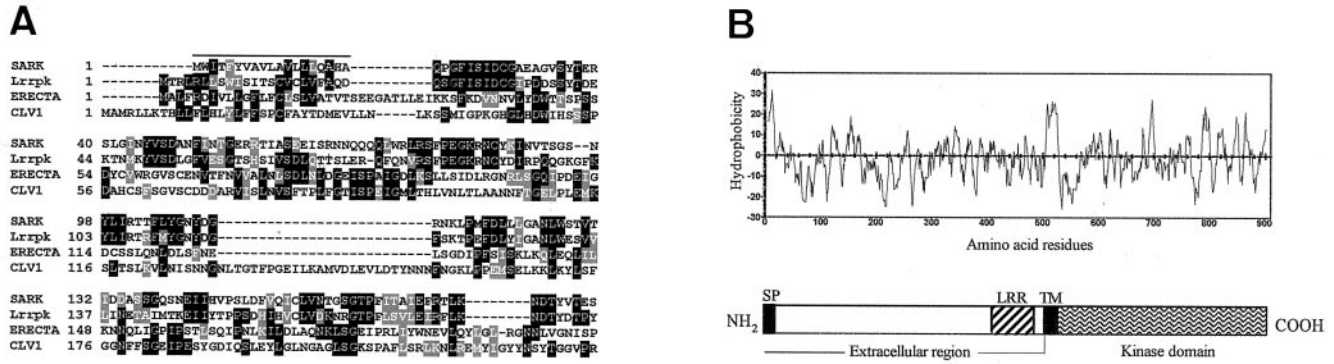


**Figure 1.** Amino acid sequence of the SARK protein and multiple alignment with related polypeptides. A, Amino acid sequence of SARK and multiple alignment. The deduced amino acid sequence of the complete SARK cDNA was aligned (multiple alignment by CLUSTAL W program) with sequences obtained from public databases and refer to the following: Lrrpk (accession no. AC X97774), ERECTA (accession no. AAC49302), and CLV1 (accession no. AAB58929). Gaps represented by dashes were introduced to produce the best match among the four species. The black boxes indicate identical residues and gray boxes indicate conservative substitutions. Solid lines indicate a putative signal peptide and transmembrane region and dashed line denotes the LRR region. Roman numerals indicate the 11 characteristic subdomains of protein kinases. Asterisks indicate invarient amino acids (Hanks and Quinn, 1991). The nucleotide sequence of SARK has been deposited in GenBank (accession no. AF285172) B, Hydrophobicity plot deduced from the SARK amino acid sequence, and analyzed by the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982). SP, Signal peptide; LRR, Leu-rich region; TM, transmembrane domain.

### Southern Blot of the SARK Gene Family

To consider the complexity of the SARK gene family, a cDNA probe corresponding to the extracellular domain of the SARK gene was prepared for Southern-blot analysis performed under high stringency conditions. As shown in Figure 2, several hybridized fragments resulted in each of the various digestion treatments. This suggests that the SARK gene is a member of a gene family.

### Temporal Pattern of SARK Gene Expression during Leaf Development and Senescence

During aging of the primary leaf, the level of the mRNA for the photosynthetic gene Chl *a/b*-binding

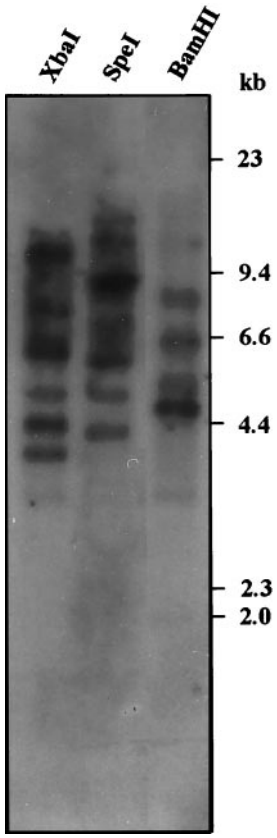


**Figure 2.** Southern-blot analysis of bean genomic DNA using a 5′-cDNA (1,076 bp) as a probe. DNA was digested by the restriction enzymes indicated above each lane. The size ladder is indicated on the right.

protein (CAB) decreased after 20 d post-germination (Fig. 3). In contrast the SARK transcript was detected at 20 d post-germination, which is also prior to the drop in levels of Chl (data not shown). From 20 d post-germination onwards, the steady-state levels of SARK mRNA increased, peaking at 40 d and then declining.

When various leaves at different developmental stages were compared, SARK mRNA was not restricted to primary leaves, but was negatively correlated with Chl content (Fig. 3B). The highest abundance was found in the oldest leaf at the bottom of the plant and decreased according to the leaf's position along the stem. In young leaves at the top of the plant, no SARK message was detected.

### Localization of the SARK Transcript

To see if SARK mRNA is restricted to leaves, RNA was extracted from other organs of the bean plant and analyzed on a northern blot (Fig. 4). As a reference, Figure 4, lane 1 represents the SARK mRNA in an aging leaf. It appeared that this mRNA was also present in the epicotyl and increased in older tissue (Fig. 4, lanes 2 and 3). Although the signal is very weak, SARK mRNA may also be present in flowers 1 to 2 d before their abscission (Fig. 4, lane 5), but was not detectable in unopened flower buds (Fig. 4, lane 4). The SARK transcript was also present in roots (Fig. 4, lane 6).

### Effect of Plant Growth Regulators on SARK Transcript Levels

The plant growth regulators cytokinin and ethylene, which may participate in the control of leaf senescence, were tested for their regulatory role in SARK gene expression. Addition of the cytokinin benzyladenine (BA) to detached leaves in the dark delayed Chl disappearance and the appearance of

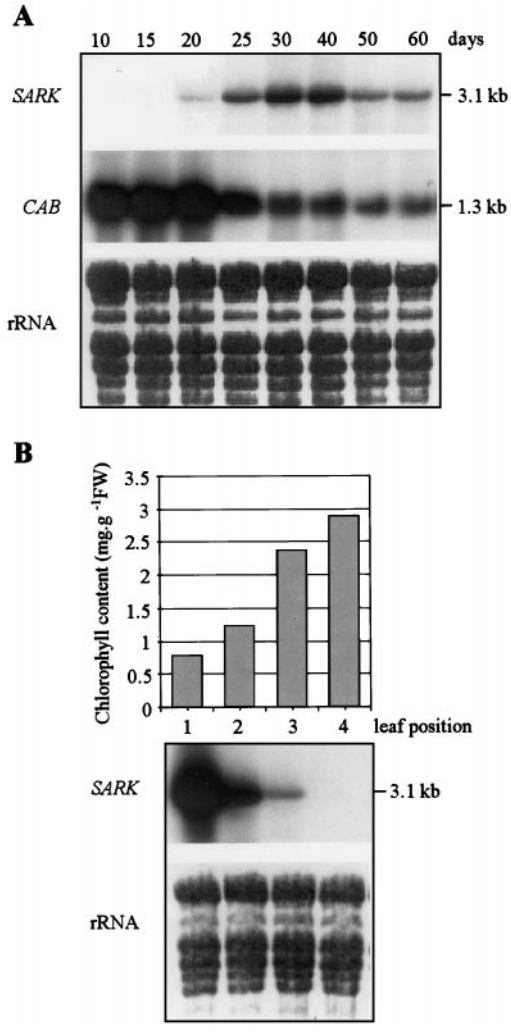


**Figure 3.** Changes in abundance of SARK mRNA in attached bean leaves during development and senescence. A, SARK mRNA in the primary leaf (top) compared with CAB mRNA (middle). The rRNA (bottom) is used as a loading control. B, Chl content of leaves according to their position on the stem (top), levels of SARK mRNA (middle), and rRNA loading control (bottom). Leaves are numbered from the bottom of the plant to the top. The membrane was probed with the radiolabeled SARK cDNA clone and rRNA was stained with methylene blue.

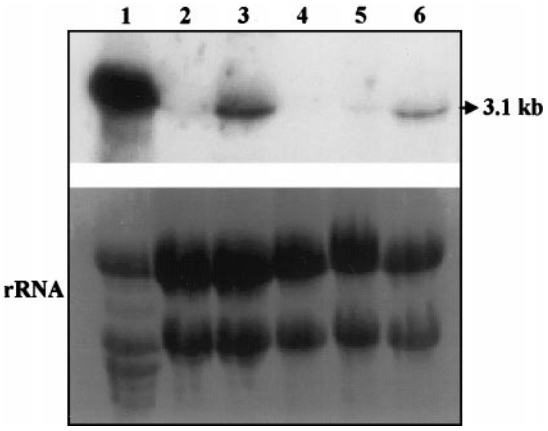


**Figure 4.** Northern blot showing SARK mRNA levels in various organs. Epicotyls of bean plants 15 d post-germination (lane 2) or 40 d post-germination (lane 3), flowers of unopened buds (lane 4) or 1 to 2 d before abscission (lane 5), and from the root (lane 6). Lane 1 indicates SARK mRNA in a senescing leaf.

SARK message (Fig. 5A). When 1-amino-cyclopropane-1-carboxylic acid (ACC), the immediate precursor of the senescence-promoting hormone ethylene, was added to a detached leaf incubated in the light, the SARK message appeared as early as 24 h after leaf detachment. In the control leaf no detectable band was detected until 48 h after leaf excision (Fig. 5B). The temporal pattern of SARK mRNA exhibited faster transient expression in the cytokinin and ACC-treated leaves as compared with the water treated leaf (Fig. 5, A and B). Similar to the known accelerating effect on leaf senescence, darkness also advanced the initiation of SARK appearance. As shown in Figure 5, SARK mRNA, which was undetectable in non-senescing leaves (zero time of incubation), appeared 24 h after leaf excision in the dark (Fig. 5A, water control). However, when the detached leaves were incubated in the light, the initiation of SARK expression was postponed for an additional 24 h; it was evident only after 48 h of incubation (Fig. 5B, water control)

### Confirmation of SARK Expression at the Protein Level in Senescing Leaves

To determine if levels of SARK mRNA are relevant for senescence, the amounts of SARK protein must also be measured. This was done by western-blot analysis of intact leaves using polyclonal antibodies raised against the extracellular portion of the recombinant SARK protein that had been expressed in *Escherichia coli*. As indicated in Figure 6A, the SARK protein is absent during leaf development and maturation, but similar to the trends for its mRNA, SARK protein was initially detected before Chl breakdown became apparent (20 d post-germination), and then increased during natural senescence. A similar temporal profile of SARK protein expression and that of SARK mRNA was also established in detached leaves (Fig. 6B).

## DISCUSSION

A successful general approach employed by several research groups to elucidate the genetic nature

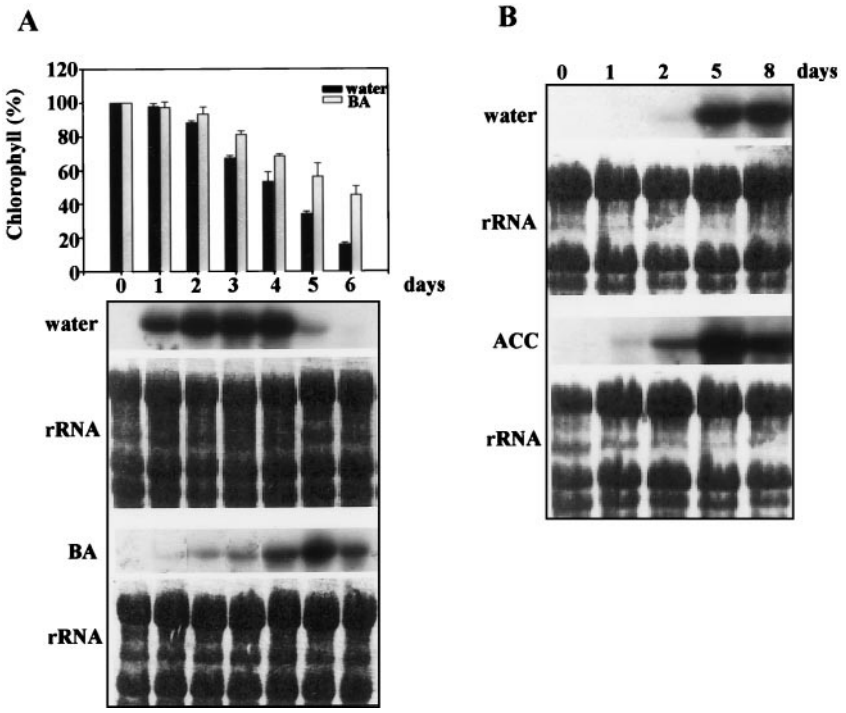


**Figure 5.** Effect of leaf detachment and plant hormones on the temporal pattern of SARK mRNA expression. A, Amounts of Chl in leaves (top) after treatment with water (black bars) or 0.1 mM BA (shaded bars) compared with SARK mRNA levels without or with BA. All treatments were in the dark. B, Analysis of SARK mRNA levels after incubation of detached leaves on water or 1 mM ACC. The membrane was probed with the radiolabeled SARK cDNA clone and rRNA was stained with methylene blue.

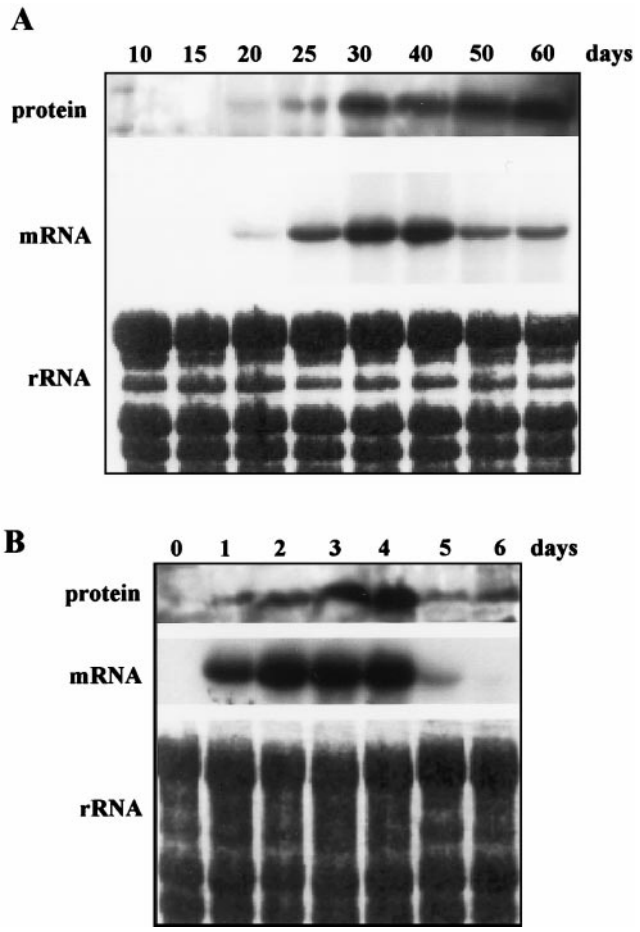


**Figure 6.** Comparison of the expression profile of SARK protein to that of SARK mRNA A, Timecourse of SARK protein as determined by a western blot and of SARK mRNA. The material was primary leaves attached to the plant. Numbers indicate days post-germination. B, Timecourse of SARK protein as determined by a western blot and SARK mRNA. The material was detached leaves incubated in the dark. Numbers indicate days post-germination. Fifteen micrograms of RNA was loaded onto each lane for the RNA-blot analysis and 100 $\mu$g of total protein was used for each sample for the western-blot analysis. Bottom sections of A and B, Methylene blue-stained blots showing relative amounts of rRNA in each lane.

and metabolic processes of plant senescence has yielded an identification of SAGs and predictions of their products' functions (Smart, 1994; Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nooden et al., 1997). Although the mRNAs of the SAGs have been described, the kinetics of proteins encoded by these SAGs has rarely been presented. In Arabidopsis many of the SAG mRNAs appear to increase only after decreases in CAB mRNA, and they are involved in the degradation of cellular components related to the mobilization characteristic of senescing tissues (Hensel et al., 1993; Weaver et al., 1998). The function of SAGs that appear before the mRNA for CAB begins to decrease, i.e. ones that might have a regulatory function, have not been identified.

Genes encoding proteins involved in signal transduction of senescence have not yet been identified, probably due in part to their low transcript abundance relative to transcripts of genes that participate in the metabolic processes themselves (Buchanan-Wollaston, 1997; Nam, 1997; Nooden et al., 1997). The mRNA differential display approach, which includes PCR steps, allows the identification of nonabundant transcripts in general (Liang and Pardee, 1992), and in this study, of a senescence-related protein kinase we designate SARK (Fig. 1). The gene encoding the SARK mRNA has homology to known protein kinase receptors that are key components in the perception of extracellular messages and in the subsequent phosphorylation cascade that results in the initiation of new developmental programs (Park et al., 1998). The SARK mRNA and its protein product appear before the detection of Chl breakdown and the decrease in CAB mRNA.

The southern blot revealed multiple hybridization bands when probed with the 5′-cDNA that was complementary to the more specific extracellular domain (Fig. 2). To establish that the isolated SARK cDNA clone is the family member corresponding to the SARK, the SARK promoter was isolated. An analysis of its regulatory activity in tobacco plants using $\beta$-glucuronidase staining indicated a similar temporal pattern to that of the SARK gene (A. Rosner, T. Hajouj, and S. Gepstein, unpublished data).

Although the mechanisms of the signal transduction cascades in plants are still unclear, sequence comparisons of cloned genes have been very useful for predicting the structure and putative function of kinase cascade components (Lease et al., 1998). The plant counterparts of membrane-related protein kinase activity (RLKs) show structural similarity to animal polypeptide growth factor receptors that contain an extracellular ligand-binding domain, a single membrane-spanning region, and a conserved cytoplasmic domain with protein kinase activity. Most of the equivalent animal protein kinase receptors are Tyr kinases, whereas in plants, most of the identified kinase receptors, including SARK, belong to the family of Ser/Thr protein kinases. Based on the structural similarity of their extracellular region RLKs are classified into three main categories: the S domain class, the LRR class, and the group that carries epidermal growth factor-like repeats (Braun and Walker, 1996). A novel class of receptor kinases containing a taumatin-like domain is related to plant defense proteins (Wang et al., 1996).

From sequence analysis of SARK cDNA, the SARK protein is predicted to contain a putative LRR motif with a different number and arrangement of repeats relative to most LRRs. Among the growing list of plant receptor-like kinases containing the LRR motif in their extracellular segment, the following three examples may be relevant: (a) the ERECTA gene product, which plays an important role in controlling

the shape of organs originating from the shoot apical meristem (Torii et al., 1996); (b) the CLAVATA protein that acts as a mediator of positional information to direct differentiation during shoot formation (Clark et al., 1997); and (c) PRK1, a receptor-like kinase that plays an essential role in the signal transduction pathway associated with pollen development (Mu et al., 1994). Perhaps the SARK gene will eventually be added to this list of regulatory genes involved in developmental processes.

LRR motif is found in various forms in plants with diverse functions and cellular localizations. Proteins containing this motif are thought to be involved in protein-to-protein interactions and the specificity of these interactions is hypothesized to be determined by the composition of the variable amino acids in the consensus core of the LRRs (Kobe and Deisenhofer, 1994). Due to its specific features the LRR motif in various receptor kinases is assumed to constitute a binding domain to a putative peptide ligand, but ligand binding by receptor kinases in plants has not been experimentally demonstrated (Becraft, 1998). The putative ligand for SARK, as for all other identified LRR-containing plant kinase receptors, has yet to be identified.

Several of the senescence up-regulated genes have been identified as defense-related genes and partial overlap between the genetic pathways of senescence and of pathogen-induced cell death has been suggested (Quirino et al., 2000).

One of the known defense-related genes is the Pto, a LRR-containing Ser/Thr protein kinase receptor. Because SARK and Pto genes encode protein kinases, the two genes may be compared. The Pto does not contain the membrane-spanning domain and appears to function as an intracellular receptor for the signal molecule (Sessa et al., 1998). Moreover, although SARK and Pto encode LRR-containing proteins, the SARK's LRR motif is clearly different from that of Pto. It is apparent that structural differences rule out the possibility of SARK mediating the senescence program through the Pto defense-related genetic pathway.

The SARK LRR motif shows high similarity to that of the Lrrpk in Arabidopsis (Deeken and Kaldenhoff, 1997), which may imply that both genes belong to the same family. However, the Arabidopsis Lrrpk gene is expressed exclusively in the dark and in the roots, a pattern different from that of SARK (Figs. 4 and 5), suggesting that the gene products have different roles. Moreover, we also could not detect any induction of the SARK gene in the cotyledons of bean seedlings in the dark (data not shown).

Although there are common biochemical pathways in the senescence systems of detached and attached leaves, different genes may participate in the two systems (Becker and Apel, 1993; Weaver et al., 1998). This may be due to the fact that various stress factors, such as wounding or darkness, cause stress responses that visually mimic the senescence syndrome, but the resultant expression of the stress-induced genes may not participate in the natural senescence syndrome (Park et al., 1998; Weaver et al., 1998). Indeed the functions of some of the identified SAGs are related primarily to protecting cells from stress (Becker and Apel, 1993; Nooden et al., 1997; Weaver et al., 1998).

One may ask, therefore, if SARK is more closely related to a stress response than to senescence. It is difficult to determine whether a SAG expressed under natural conditions is associated with the senescence process or with any related stress response. However, in cases where a senescence-related gene like SARK is expressed in intact leaves prior to senescence-related biochemical changes, it is possible that this gene is more directly associated with the senescence program than with the stress response that may be caused by senescence. Because the SARK gene is also activated in detached leaves (Figs. 5 and 6), its putative role in senescence is reinforced.

A further relationship between SARK and senescence is suggested by the application of various plant hormones (Fig. 5). Cytokinins that are known as anti-senescence factors in various plant organs (van Staden et al., 1988) are also considered to play a major role in delaying whole plant senescence. (Gan and Amasino, 1995). Thus exogenously applied BA to detached bean leaf delayed the senescence-related Chl decrease and the initiation of SARK expression in detached bean leaf in the dark (Fig. 5A). Similar to natural leaf senescence, the appearance of SARK in this treatment is also visualized prior to the decrease in Chl levels. ACC, the immediate precursor of ethylene (Yang and Hoffman, 1984) that advances various plant organ senescence, also accelerated Chl breakdown and the appearance of SARK mRNA in detached bean leaves (Fig. 5B). The fact that ACC had a promotive effect on the initiation of SARK expression is consistent with the notion that once the leaf reaches its mature stage, ethylene accelerates the same parameters of leaf senescence (Grbic and Bleecker, 1995; Weaver et al., 1998).

In summary we have cloned a gene from bean leaves for a putative SARK whose mRNA and protein product appear before decreases in Chl level and in the level of the mRNA for CAB. Furthermore, SARK mRNA is detectable earlier when senescence is accelerated by detaching the leaf, by incubating leaves in the dark, and by ACC applications; when senescence is retarded by cytokinin, appearance of the SARK mRNA is delayed. The putative function of the SARK gene, coupled with the fact that its transcript and protein product appear prior to parameters associated with senescence suggests that SARK expression may regulate some pathways of the senescence program.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Bean (*Phaseolus vulgaris* cv Bulgarian) plants were grown in soil-containing pots in the greenhouse under controlled environmental conditions with a 16-h photoperiod at 25°C ± 2°C. Primary leaves of different ages were harvested for Chl, protein, and RNA analyses. For studies of detached leaf senescence, 18-mm diameter leaf discs were cut from fully expanded primary leaves 15 d postgermination. The tissue was placed in Petri dishes on filter paper (Whatman, Clifton, NJ), soaked with distilled water or the desired solutions, and placed under continuous fluorescent light or in the dark. For localization experiments, 3-cm epicotyl segments including the cotyledonary node were cut from 15-d (young) or 40-d (old) plants. Young flowers were from unopened buds, whereas old flowers were harvested 1 to 2 d before abscission. Roots were removed from 15-d-old plants. Chl measurements were conducted according to Moran (1982).

### RNA Isolation and Gel-Blot Analysis

Total RNA was isolated according to the method of Puissant and Houdebine (1990). For gel-blot analysis, 15 $\mu$g of total RNA was separated on a 1.2% (w/v) formaldehyde-agarose gel and transferred to a nylon membrane. Blots were hybridized with specific cDNA probes labeled by the random primer method according to Sambrook et al. (1989).

### Differential Display Reverse-Transcription PCR

The differential display method was performed essentially as described originally by Liang and Pardee (1992) with several modifications. mRNA (0.1 $\mu$g) extracted from leaves of various ages was used as a template for reverse transcription in the presence of 20 $\mu$M dNTP and 1 $\mu$M T11-MN3′ primers (where M stands for a mixture of G, A, and C, and N stands for A, G, C, and T) using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The PCR amplification of cDNA was performed using the same anchor primer and an arbitrary 10-base primer for 40 cycles. The reaction conditions were as follows: 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s, followed by extension at 72°C for 5 min. Amplified cDNA was separated on a 6% (w/v) polyacrylamide sequencing gel and bands of interest were eluted and reamplified under the same conditions.

### Construction and Screening of a cDNA Library

Poly(A)$^+$ RNA extracted from senescing bean leaves was used to construct a cDNA library in λ- ZAP II (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The senescence-related cDNA generated by differential display was used as a probe for screening 300,000 plaque forming units of the amplified cDNA library from senescing leaves. Plaque lifts were made onto Nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and were hybridized overnight at 42°C in the hybridization buffer (2 × 1, 4-piperazine-diethanesuulfonic acid buffer, 50% [v/v] deionized formamide, 0.5% [w/v] SDS, and 100 $\mu$g/mL of salmon sperm DNA). Following washing for three times for 30 min each in 0.1% (w/v) SSC and 0.1% (w/v) SDS at 65°C, hybridization was visualized by autoradiography for 2 d on film (Xomat, Kodak, Rochester, NY).

### Genomic Southern-Blot Analysis

Genomic DNA was isolated from bean leaves as described by Dellaporta et al. (1983). DNA (15 $\mu$g) was digested with various restriction enzymes and subsequently separated on a 0.8% (w/v) agarose gel. DNA transfer and hybridization were done according to Sambrook et al. (1989).

### Expression of the SARK Gene in *Escherichia coli* and Production of Polyclonal Antisera

The SARK clone isolated from the cDNA library was digested with the restriction enzymes *Sal*I and *Hin*dIII. The resultant 691-bp fragment encoding the extracellular portion of the SARK protein (amino acids 78–308) was ligated to the pQE30 vector (Qiagen, Valencia, CA) after digesting with SamI and *Sal*I and filling the *Hin*dIII restriction sites in the insert and the SamI restriction site in the vector. The recombinant protein containing a 6×-His tag was expressed in *E. coli* and purified using the QIA expression kit (Qiagen). The expressed protein was then mixed with Laemmli buffer, separated by SDS-PAGE, transferred to nitrocellulose, and the recombinant protein band was sliced from the membrane. The solublized band was used for immunizing rabbits (Shurtz-Swirsky and Gepstein, 1985). Western-blot analysis of protein extracts prepared from leaves of various ages was performed essentially as described previously (Shurtz-Swirsky and Gepstein, 1985). The presence of the specific SARK protein bands in leaf extracts could be detected only by enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibody as described in the kit manufacturer's instructions (Pierce, Rockford, IL).

### DNA Sequence Analysis

The SARK clone was sequenced by the biological services at the Weizmann Institute (Rehovot, Israel). Database searches and comparison with published sequences were performed using the BLAST and BLASTX algorithms (Altschul et al., 1990).

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