Plant Physiol. (1996) 112: 705–715

Novel Molecular Markers for Late Phases of the Growth Cycle of *Arabidopsis thaliana* Cell-Suspension Cultures Are Expressed during Organ Senescence[1]

Didier Callard, Michèle Axelos, and Laurent Mazzolini*

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, Unité Mixte de Recherche no. 215 du Centre National de la Recherche Scientifique et de l'Institut National de la Recherche Agronomique, F-31326 Castanet-Tolosan, France

In an *Arabidopsis thaliana* T87-C3 cell-suspension culture, entry into the growth-arrest phase is rapidly followed by a loss of cell viability. Three cDNA clones, *SRG1*, *SRG2*, and *SRG3*, corresponding to genes with transcripts that accumulate during these late phases, were isolated by the mRNA differential display method. Amino acid sequence analysis shows that the putative SRG1 protein is a new member of the Fe(II)/ascorbate oxidase superfamily, and that *SRG2* codes for a protein with significant homology to β-glucosidases. Significantly, all three *SRG* genes are expressed in senescing organs of Arabidopsis plants. Two previously characterized genes, *SAG2* and *SAG4*, induced during natural senescence in Arabidopsis, were also found to be expressed in cell-suspension cultures and have expression kinetics similar to those observed for the *SRG1* gene. Taken together these findings suggest that certain molecular events are common to both plant senescence and growth arrest in Arabidopsis cell suspensions. Both internucleosomal cleavage of nDNA and an apparent compaction of chromatin, two characteristic features of programmed cell death in animal cells, have been observed in Arabidopsis cell cultures at a stage corresponding to loss of cell viability.

Cultured cells are now universally recognized as appropriate model systems with which to investigate the molecular responses of plants to stimuli that affect growth, such as nutrient availability (e.g. Roitsch et al., 1995) and plant growth factor requirements (e.g. Teyssendier de la Serve et al., 1985; Dominov et al., 1992; Lee and Chen, 1993). For example, limitations in phosphate concentration (Kodama et al., 1990; Köck et al., 1995; Malboobi and Lefebvre, 1995), nitrogen supply (Kodama et al., 1990), carbon source (Kodama et al., 1990; Sheu et al., 1994; Hsieh et al., 1995; Tseng et al., 1995), or hormone depletion (Crowell and Amasino, 1991) result in modifications in gene expression, leading to changes in the cellular mRNA pattern.

After nutrient exhaustion, certain cell cultures undergo rapid growth arrest, followed by a dramatic loss of viability. We are currently studying these late phases of the cell culture growth cycle, which exhibit a number of morphological symptoms similar to those that occur in senescing plant tissues, which have recently been described in sugar-starved, cultured rice cells (Chen et al., 1994).

As a first step toward characterizing the molecular events associated with growth arrest and viability loss, we have attempted to select genes with a relative expression that is enhanced at the end of the growth phase of the cell culture. For this purpose, we chose an *Arabidopsis thaliana* cell line (Axelos et al., 1992) with a particularly short growth-arrest period. Transcripts that accumulated at the end of the growth phase of the culture were characterized by the mRNA differential display method (Liang and Pardee, 1992). Three cDNA clones were isolated and the expression patterns of the corresponding genes were analyzed both throughout the cell culture growth cycle and in normal and senescing Arabidopsis plant tissues. These studies and reciprocal experiments performed with genes already identified as markers of plant senescence (Hensel et al., 1993; Taylor et al., 1993) suggest interesting molecular parallels between growth arrest in cell cultures and the classical senescence of plant tissues. Finally, loss of cell culture viability was found to be accompanied by internucleosomal cleavage of nDNA, suggesting a possible analogy with programmed cell death in animal cells.

MATERIALS AND METHODS

Plant Material and Chlorophyll Measurements

Arabidopsis thaliana (ecotype C24) plants were grown at 22°C under white light at a fluency of 230 $\mu E\ s^{-1}\ m^{-2}$ with a 9-h-day/15-h-night photoperiod. The *A. thaliana* T87-C3 cells were cultured under continuous illumination in 1-L Erlenmeyer flasks containing 300 mL of culture medium consisting of either JPL medium prepared according to Axelos et al. (1992) or Gamborg B5 medium (Flow Laboratories, McLean, VA), 20 g L^{-1} Suc, and 2.5 μM 2,4-D. After 12 d, the cells were transferred into fresh medium at a density of 14 g fresh cells L^{-1}. The chlorophyll content of the cells was determined photometrically using the method of Vernon (1960).

[1] This work was supported by the Centre National de la Recherche Scientifique (Unité Mixte de Recherche no. 5), by the Institut National de la Recherche Agronomique, and by the Groupement de Recherches et d'Etudes sur pes Génomes of the Ministère de l'Enseignement Supérieur et de la Recherche (M.E.S.R). D.C. holds a grant from the M.E.S.R.

* Corresponding author; e-mail mazzo@toulouse.inra.fr; fax 33–61–28–50–61.

Nucleic Acid Extractions

Organs were excised from 4- to 6-week-old plants. Total RNA was extracted from the Arabidopsis cells and from the plant organs with acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). RNA concentrations were determined on the basis of optical density measurements at 260 nm. Poly(A)$^+$ RNA was prepared from total RNA extracts by adsorption onto oligo-$(dT)_{25}$-coupled paramagnetic beads (Dynabeads, Dynal, Oslo, Norway). Total DNA was isolated by the method of Dellaporta et al. (1983), followed by a treatment with heat-treated (DNase-free) RNase A (Sigma). Standard techniques of DNA and RNA manipulation were as described by Sambrook et al. (1989).

mRNA Differential Display

Differential display analyses were performed according to the original procedure of Liang and Pardee (1992), with the modifications described by Callard et al. (1994). Thirty primer combinations were tested. Three of these, for which strongly regulated sequences could be detected (Fig. 1), were also used for the isolation of the corresponding cDNAs. The purification of the display products and their cloning in the vector (pBluescript, Stratagene), as well as the elimination of the contaminating cDNA clones, were performed following procedures previously reported (Callard et al., 1994).

Screening of the cDNA Library

Each of the displayed cDNAs, cloned into the vector, was amplified by PCR with the primer combination used in the display reaction and under the reamplification conditions described by Liang and Pardee (1992). The amplification products were then gel-purified and labeled with [α-^{32}P]dCTP by the oligolabeling procedure. For the initial screening step, each of the labeled probes was hybridized against about 3×10^5 plaque-forming units of a cDNA library from Arabidopsis cultured cells constructed in λ ZAP II (Stratagene) (Regad et al., 1993). Phage plaques were plated on membranes (Hybond-C-extra, Amersham) according to the manufacturer's instructions. Prehybridization, hybridization, and posthybridization washings were performed according to Sambrook et al. (1989).

DNA Sequencing and Database Homology Searches

In vivo excision of the λ ZAP clones was performed as described in the manufacturer's (Stratagene) protocol. Nucleotide sequencing of the λ cDNA clones were carried out on the corresponding double-stranded vector plasmids by the method of Sanger et al. (1977) using the Sequenase enzyme (version 2.0, United States Biochemical). All sequences were determined on both strands. To obtain the complete nucleotide sequence of clone *SRG1* (for senescence-related genes), a series of subclones containing nested deletions created by progressive digestion with exonuclease III was generated (Erase-A-Base kit, Promega). Homology searches against databases were performed using the BLAST computer program (Altschul et al., 1990) of the National Center for Biotechnology Information. Multiple amino acid sequence alignments and determination of the percentages of homology were made using the PILEUP and GAP programs, respectively, of the Wisconsin Genetics Computer Group software package (Devereux et al., 1984).

Transcript Analysis

For northern blot analyses, total RNA was denatured with glyoxal and DMSO and size-fractionated by electrophoresis on horizontal 1% (w/v) agarose gels. The RNA was then transferred to a nylon membrane (Hybond-N$^+$, Amersham) by vacuum-blotting, and hybridized to ^{32}P-labeled DNA probes according to a previously described procedure (Regad et al., 1993). The *RNS2* cDNA clone was kindly provided by Dr. Pamela J. Green (Michigan State University, East Lansing). The *SAG2* and *SAG4* cDNA clones were the generous gift of Dr. Anthony B. Bleecker (University of Wisconsin, Madison). The inserts of these clones and of the three *SRG* λ clones were amplified by PCR with the universal primers M13 reverse and M13(-20). The PCR products were gel-purified and then labeled with [α-^{32}P]dCTP using the oligolabeling procedure to generate probes for the different northern experiments. The *EF-1*α probe was a ^{32}P-labeled *Bgl*I/*Bgl*II fragment (404 bp), located in the 3′ coding region of the Arabidopsis translation elongation factor 1α gene *A1* (Axelos et al., 1989).

Microscopy

Arabidopsis cells were prepared for microscopic analysis according to a previously described procedure (Traas et al., 1992) with minor modifications. Cells were fixed by adding 0.5 mL of a solution containing 4% (w/v) formaldehyde (freshly made from paraformaldehyde) in PEM buffer (100 mM Pipes, pH 6.9, 10 mM EGTA, and 10 mM $MgSO_4$) to 0.5 mL of culture. After 30 min, the cells were washed three times in PEM and finally resuspended in PEM containing 0.2% (w/v) Triton X-100 (Sigma) and 1 μg mL^{-1} of the DNA-specific dye 4′,6-diamidino-2-phenylindole. The cells were then mounted and viewed with epifluorescence optics (Axiophot microscope, Zeiss).

RESULTS

Isolation of Clones Coding for mRNAs Differentially Expressed in Arabidopsis Cell-Suspension Cultures

We used the differential display technique to compare the mRNA populations of growth-arrested cells with those of cells resuming active proliferation. For this purpose, RNA was isolated either from cells arrested for growth following a 12-d culture period (0-h sample) or from cells collected 16 and 40 h after transfer of the arrested culture into fresh culture medium (16- and 40-h samples). A selection of display profiles obtained by using three primer combinations is presented in Figure 1. The patterns and intensities of bands for the three RNA samples (0, 16, and 40 h) were similar, with the exception of a small number of differentially displayed bands. Several of these bands appeared stronger in profiles corresponding to cells that had

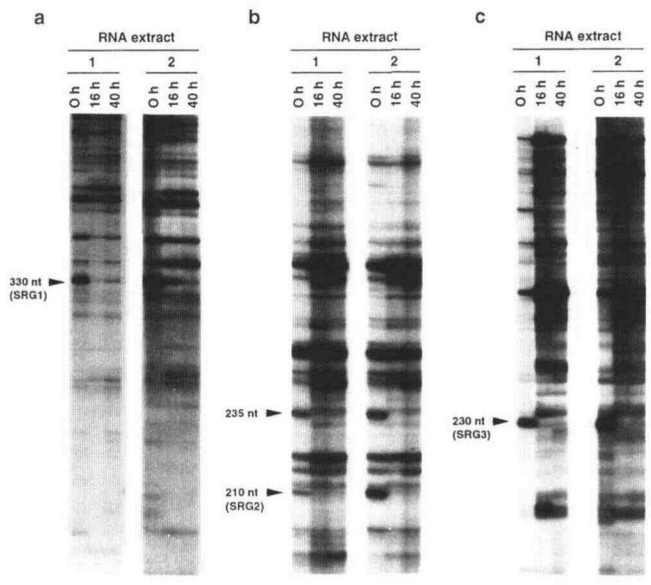

Figure 1. Differential display of Arabidopsis cell mRNAs. Poly(A)$^+$ RNA from 12-d-old cells (0-h extracts) and cells collected 16 and 40 h after subculture in fresh medium were reverse-transcribed and PCR-amplified with different primer sets: 5′T11GC + oligo1 (5′CGTCCTGCAA) (a); oligoX (5′A(C/T)(C/T)TC(C/T)-TC(A/G)TA(C/T)TT) + oligo2 (5′GTGACGTAGG) (b); and 5′T11GC + oligo3 (5′CTGCTGATGT) (c). The PCR products were resolved on a 6% (w/v) polyacrylamide sequencing gel and visualized by autoradiography. For each display experiment mRNA was extracted from cells grown either in JPL (extract 1) or in Gamborg (extract 2) medium. The estimated size of each differentially amplified band is indicated, along with the name in parentheses of the corresponding λ cDNA clone isolated by library screening.

resumed growth (16 and 40 h) and, conversely, a number of strongly enhanced PCR bands were present in growth-arrested cell samples (0 h). For four of these latter bands, indicated by arrows in Figure 1, a clear and reproducible amplification pattern was obtained from RNA extracts prepared independently from cell cultures maintained either in JPL (extract 1) or in Gamborg (extract 2) medium. These experiments suggest that nondividing cells express a set of mRNAs that are poorly represented or totally absent in cycling cells of young cultures.

The products of the PCR reaction were then isolated to obtain full-length cDNA clones corresponding to these differentially amplified molecules. Three of these were cloned into a plasmid vector and the unrelated contaminating cDNA molecules, a possible source of false-positives, were eliminated using a previously described method (Callard et al., 1994). Each insert sequence was then used as a hybridization probe to screen an Arabidopsis cultured cell cDNA library (Regad et al., 1993) constructed in a λ phage vector. This led to the isolation of three groups of λ clones, unrelated in sequence, and subsequently referred to as *SRG1*, *SRG2*, and *SRG3* (see "Discussion" for nomenclature). In each case, the nucleotide sequences of the cloned PCR products were found to be identical to that of the cDNA insert of the corresponding λ clones (data not shown), confirming that the cDNAs selected by library screening actually corresponded to the original differential display products and not to related, cross-hybridizing sequences.

Expression of the *SRG* Genes during Cell Culture

Recombinant plasmids were rescued from each of the selected phages, and inserts were then used as northern hybridization probes to total RNA extracted from cells during the first 15 h after transfer of a 12-d-old cell inoculum into fresh medium (Fig. 2). The *SRG1* and *SRG2* cDNA probes hybridized to unique mRNAs of 1.5 and 2.1 kb, respectively. The *SRG3* probe hybridized to a differentially expressed mRNA (*SRG3a* mRNA, 1.5-kb estimated size), and a second constitutively expressed transcript of smaller size (*SRG3b*, 0.8 kb). Since the *SRG3* λ clone contains an insert of 1.6 kb, which is comparable in size to the *SRG3a* mRNA, this mRNA corresponds to the differentially regulated gene of interest, referred to as *SRG3a* in the following experiments. As shown in Figure 2, the relative transcript levels of the *SRG* genes decreased upon transfer of cells into fresh medium. This decrease was extremely rapid for *SRG2* and *SRG3a* and more gradual for *SRG1*. In contrast, the transcript levels of the Arabidopsis translation elongation factor 1α gene (*EF-1α*, 1.8-kb mRNA), used as a hybridization control, increased significantly as cell growth resumed.

A typical growth curve of the Arabidopsis cell culture is presented in Figure 3a. After transfer into the fresh culture medium, the cells grew exponentially without any apparent lag. After 6 d, the proliferative activity declined. Cells reached a maximal density after 12 d. Suc was the primary factor for growth limitation in our culture conditions (re-

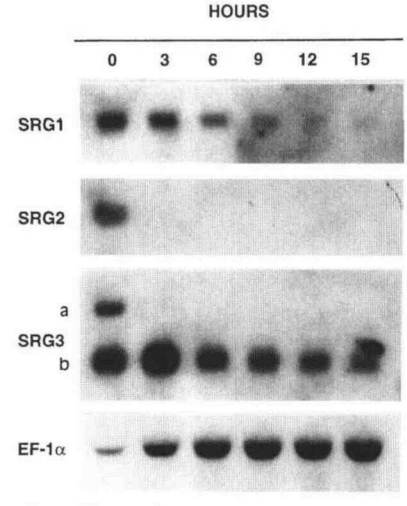

Figure 2. Northern blot analysis of *SRG* gene expression in Arabidopsis cells after transfer into fresh culture medium. Ten micrograms of total RNA isolated from 12-d-old growth-arrested cells (0-h extract) and from the same cells transferred to Gamborg medium and harvested after 3, 6, 9, 12, and 15 h was subjected to denaturing agarose gel electrophoresis, blotted, and sequentially hybridized with radioactively labeled inserts of the *SRG1*, *SRG2*, and *SRG3* λ clones and with a radioactive probe corresponding to the 3′ coding region of the Arabidopsis translation elongation factor 1α (*EF-1α*) gene. In all cases between 0.3 and 0.4 mg of total RNA was extracted per gram of fresh cells. SRG3a and SRG3b, See text.

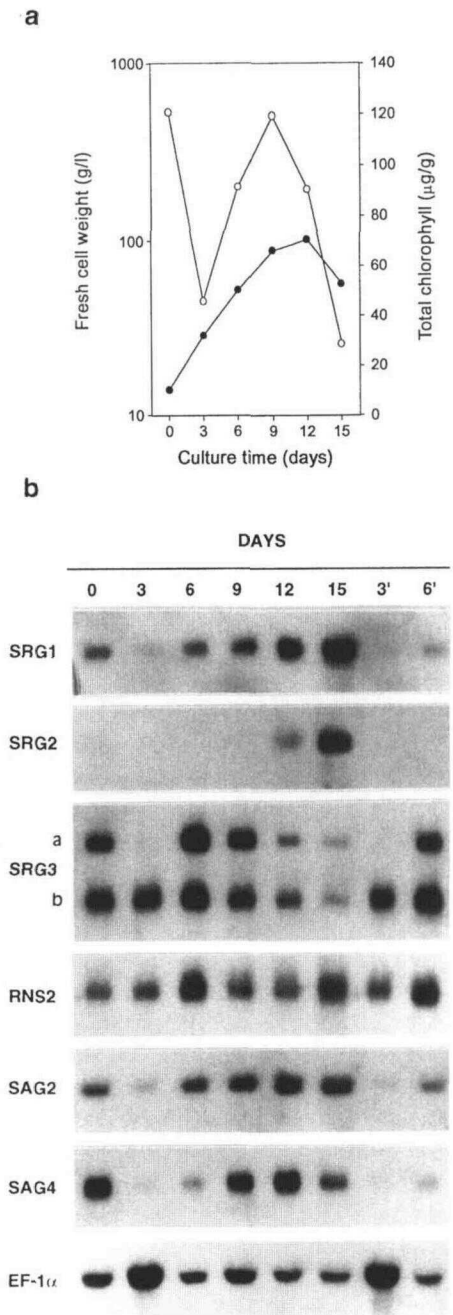

Figure 3. Analysis of *SRG* gene expression as a function of the phase of the Arabidopsis cell-suspension culture. a, Growth curve of the T87-C3 cell culture. Fresh cell weight values (●), expressed in grams per liter of medium, are plotted on a log scale. The chlorophyll content of the cells (○) is expressed in micrograms of chlorophyll per gram of fresh cells. b, Northern blot analysis of *SRG* gene transcript levels. Ten micrograms of total RNA extracted from 0-, 3-, 6-, 9-, 12-, and 15-d-old cells, as well as from the 12-d-old cells subcultured a second time and grown for 3 and 6 d (3′ and 6′ time points), was subjected to northern blotting and hybridized sequentially to the *SRG* cDNA probes and to probes corresponding to Arabidopsis senescence marker genes *RNS2*, *SAG2*, and *SAG4*. As a control the northern blot was also hybridized to the Arabidopsis *EF-1α* probe. Amounts of extracted RNA per gram of fresh cells: 0, 0.36 mg; 3, 1.05 mg; 6, 0.71 mg; 9, 0.43 mg; 12, 0.27 mg; 15, 0.06 mg; 3′, 0.91 mg; and 6′, 0.64 mg.

sults not shown). As estimated by fluorescein diacetate staining, nearly all of the cells were still viable at this stage and were able to resume multiplication without a lag after transfer into fresh medium. Cell viability started to decline within 24 h following growth arrest, and was significantly lower 2 d later (after approximately 15 d of culture). During this final phase a gradual decrease in fresh cell weight was observed, indicative of partial cell lysis.

We also monitored the cell chlorophyll content of the culture (Fig. 3a) and observed a sharp decrease soon after transfer to fresh medium, resulting from an arrest of chlorophyll synthesis associated with a metabolic switch of the cells toward Suc assimilation. The chlorophyll concentration in the cells then increased progressively to reach a maximal value after 9 d, and then decreased rapidly during the final phase of the culture.

The expression of the *SRG* genes throughout all phases of cell culture was studied by northern analysis (Fig. 3b) using equivalent amounts of total RNA extracted from cells collected at the different time points on the growth curve shown in Figure 3a. In young cultures the three *SRG* genes showed a marked reduction in relative transcript level (3- and 3′-d time points); however, the pattern of mRNA accumulation was different for each gene at later stages. *SRG1* relative transcript levels gradually increased toward the end of exponential growth, and were maximal for 15-d-old cells. The *SRG2* mRNA level was also maximal at this late stage, whereas this transcript could barely be detected in growing cells (3–9 d). In contrast, a maximal accumulation of *SRG3a* mRNA was observed between 6 and 9 d and subsequently decreased at the arrest of cell proliferation. The *SRG3b* transcript level was high and weakly modulated in growing cells. However, as for *SRG3a*, there was a progressive decline in mRNA level in late phases of the culture. The transcript level of *EF-1α*, used as a control, was maximal in freshly subcultured cells (3 d) and remained approximately constant at other time points of the growth curve.

These findings indicate that major modifications to mRNA content occur at the stage at which culture conditions become limiting for cell growth. This phenomenon is associated with a decrease in cell RNA content (see legend of Fig. 3), which presumably correlates with a reduction in cell metabolic activity. A similar situation also exists in plants during tissue and organ senescence. To investigate a possible relationship at the molecular level between late phases of cell culture and whole plant senescence, we examined the expression patterns of several previously characterized senescence-associated Arabidopsis genes. The *RNS2* gene, coding for an S-like RNase, is preferentially expressed in senescing tissues (Taylor et al., 1993), whereas the *SAG2* and *SAG4* genes are highly expressed during rosette-leaf senescence (Hensel et al., 1993; Grbić and Bleecker, 1995). We found that *RNS2* exhibited an irregular expression pattern with a nearly constant transcript level throughout the culture cycle (Fig. 3b). In contrast, the two *SAG* gene expression profiles resembled that of the *SRG1* gene, with a progressive increase in relative mRNA level reaching a maximal value after 12 d of culture,

and a sharp decrease after transfer of the cells into fresh medium (3- and 3'-d time points).

Expression of the *SRG* Genes in Arabidopsis Plants

As illustrated in Figure 4, *SRG* genes are poorly expressed in whole plant tissues, so low levels of *SRG1* transcripts are detected in roots and flowers. The expression of *RNS2*, introduced as a control in this experiment, correlated well with that previously reported (Taylor et al., 1993), with a higher transcript level in flowers compared with leaves and stems, and a very weak signal in roots.

To determine whether there is preferential *SRG* mRNA accumulation during natural plant senescence, we evaluated transcript levels during the senescence of Arabidopsis rosette leaves. This process is associated with a loss of chlorophyll, thus providing a reliable indicator of the extent of senescence in these organs. Six independent pools of leaves corresponding to progressive stages of yellowing were prepared from flowering Arabidopsis plants, and *SRG* transcript levels were then determined by northern analysis. We found that the relative mRNA levels of all three genes increase significantly during senescence (Fig. 5a). The *SRG1* transcript level is highest for leaves with extensive but not complete (75%, visual estimation) yellowing (pool 5), whereas for *SRG2* and *SRG3a* mRNA accumulates to a maximal level in completely chlorotic leaves (pool 6). For the three previously characterized Arabidopsis genes *RNS2*, *SAG2*, and *SAG4* a gradual increase in relative transcript levels can be correlated with the extent of chlorosis. *EF-1α* transcript levels were approximately constant between different leaf pools, with a decrease in pool 6 corresponding to completely chlorotic leaves.

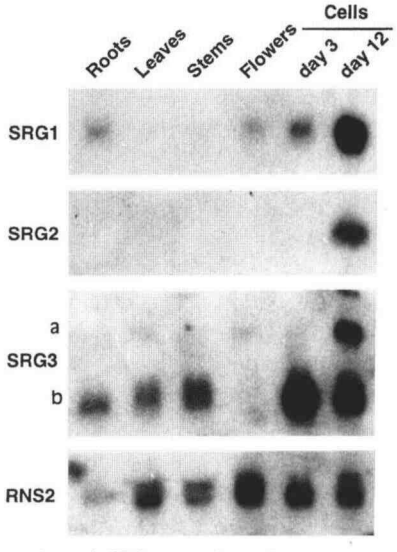

Figure 4. Expression of *SRG* genes in various organs of Arabidopsis plants. Fifteen micrograms of total RNA isolated from roots, fully expanded rosette leaves, inflorescence stems, and developing flowers collected at stage 13 according to Smyth et al. (1990) was hybridized to the *SRG* and *RNS2* cDNA probes following northern blotting. As a hybridization control, 15 μg of total RNA extracted from 3- and 12-d-old cell-suspension cultures was also loaded on the gel.

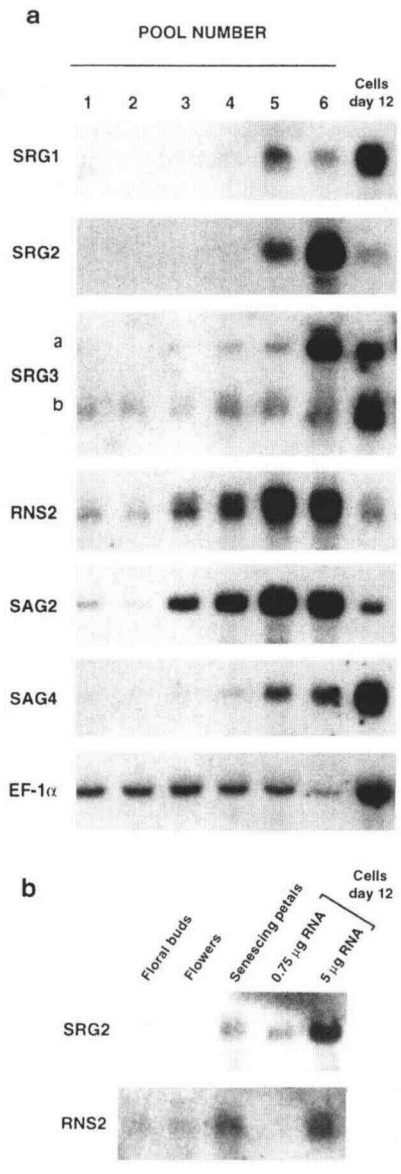

Figure 5. Expression of the *SRG* genes in senescing tissues of the whole plant. a, Northern analysis of *SRG* transcript levels in senescing rosette leaves. Fifteen micrograms of total RNA extracted from six independent pools of leaves was northern-blotted and hybridized against the *SRG*, *RNS2*, *SAG*, and *EF-1α* cDNA probes. Pool numbers correspond to: 1, young leaves; 2, fully expanded, green leaves; 3, 4, and 5, leaves with an increasing degree of yellowing (25, 50, and 75% estimated, respectively); and 6, chlorotic and withered leaves with apparent necrotic lesions. Fifteen micrograms of total RNA from a 12-d-old cell-suspension culture was loaded on the gel as a control. Amounts of extracted RNA per gram of leaf tissue were: 1, 0.27 mg; 2, 0.28 mg; 3, 0.18 mg; 4, 0.15 mg; 5, 0.10 mg; and 6, 0.07 mg. b, *SRG2* gene expression in senescing petals. Total RNA (0.75 μg) extracted from floral buds, developing flowers collected at stage 13 (Smyth et al., 1990), and senescing, withered petals (stage 16) was subjected to northern blotting and hybridized to the *SRG2* and *RNS2* cDNA probes. As positive hybridization controls 0.75 and 5 μg of total RNA extracted from a 12-d-old cell culture were loaded on the gel.

The induction of petal senescence following fertilization is a second, well characterized, developmentally controlled senescence process. As shown in Figure 5b, a significant level of *SRG2* transcript was found in the RNA extract obtained from senescing petals. Transcripts corresponding to *SRG1* and *SRG3a* could not be detected (data not shown), probably due to the limiting amount of available tissue and, hence, the relatively small quantity of RNA (0.75 μg) used in the northern analysis.

Sequence Analysis of the *SRG* Genes

To investigate the nature of the proteins encoded by *SRG1*, *SRG2*, and *SRG3a* we determined the complete nucleotide sequence of the *SRG1* cDNA and partial 5′ and 3′ sequences of the *SRG2* and *SRG3* cDNAs. Based on the estimated size of the *SRG1* mRNA (1.5 kb), a λ phage clone containing a possibly full-length cDNA insert 1396 nucleotides long was selected and sequenced. An open reading frame spanning 1074 nucleotides was identified within this cDNA, coding for a polypeptide of 358 amino acid residues with a calculated molecular mass of 41,039 D and a pI of 5.2. Comparison of the putative SRG1 protein sequence with sequences available in the databases revealed significant homology (around 55% similarity) with proteins of the Fe(II)/ascorbate oxidase superfamily (McGarvey et al., 1992). Figure 6 shows the optimal alignment of the SRG1 protein sequence with representative plant Fe(II)/ascorbate oxidase sequences. Twelve amino acids, known to be highly conserved among all members of the Fe(II)/ascorbate oxidase superfamily and localized within five conserved motifs (Britsch et al., 1993), were also present in the SRG1 protein. Likewise, a putative Leu zipper region, previously identified in this superfamily (Britsch et al., 1993), was also found in SRG1 (Fig. 6, underlined). A multiple alignment of the known protein sequences of the Fe(II)/ascorbate oxidase superfamily and SRG1 (results not shown) revealed a clustering of the proteins into distinct groups according to their ascribed functions; however, SRG1 could not be clearly assigned to any particular group on this basis.

Both the 5′ and 3′ regions of the *SRG2* cDNA were found to contain an open reading frame coding for a protein highly homologous to β-glycosidases belonging to glycosyl hydrolase family 1 (Henrissat, 1991). A homology search in databases using the BLASTX computer program (Altschul et al., 1990) showed that sequences producing high-scoring segment pairs were, in decreasing order: the β-glucosidases, the phospho-β-galactosidases, the thio-β-glucosidases, the phospho-β-glucosidases, and the β-galactosidases. An alignment of the NH_2- and COOH-terminal regions of the putative SRG2 protein with comparable sequences of a representative population of β-glycosyl hydrolase members of the family is shown in Figure 7. One of the signature patterns of this family is present in the NH_2-terminal region of SRG2 (Fig. 7a). The proteins sharing the highest degree of homology with the SRG2 sequence were plant β-glucosidases with 48 to 61% and 55 to 78% identity in the N- and C-terminal regions, respectively.

Finally, the deduced amino acid sequence of SRG3a does not share any significant homology with sequences deposited in the databases. The putative function of the corresponding gene was not investigated further.

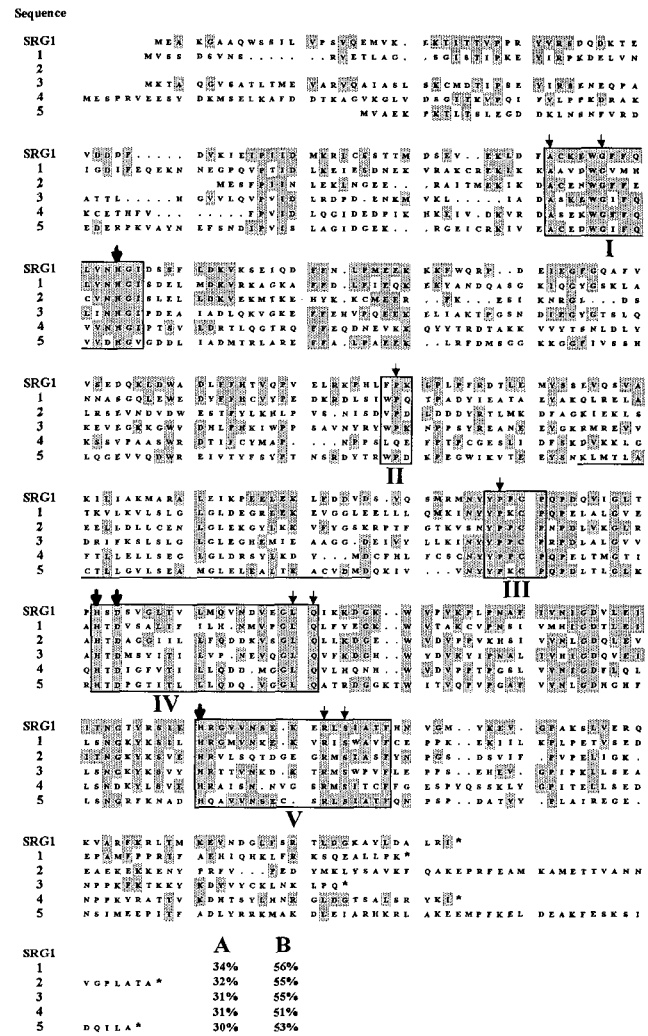

Figure 6. Alignment of SRG1 protein sequence with several members of the Fe(II)/ascorbate oxidase superfamily. The sequences presented are: SRG1, deduced SRG1 protein sequence; 1, anthocyanidin hydroxylase from *Malus* sp. (accession no. X71360); 2, ethylene-forming enzyme from *A. thaliana* (accession no. X66719); 3, flavonol synthase from *Petunia hybrida* (accession no. Z22543); 4, ethylene-responsive fruit-ripening gene *E8* from *Lycopersicon esculentum* (accession no. X13437); and 5, flavanone 3β-hydroxylase from *Dianthus caryophyllus* (accession no. X72592). Identical amino acid residues are shaded. Gaps are indicated by dots. Conserved motifs I to V are boxed. Amino acids highly conserved in the superfamily are indicated by arrows, with thick arrows showing the four residues potentially involved in iron binding. The Leu-rich region is underlined. Values at the end of the sequences are the percentages of identity (A) and similarity (B).

nDNA Fragmentation and Chromatin Compaction during Loss of Cell Viability in the Arabidopsis Cell-Suspension Culture

The rapid loss of viability of the T87-C3 cells after the arrest of multiplication led us to question whether this cell

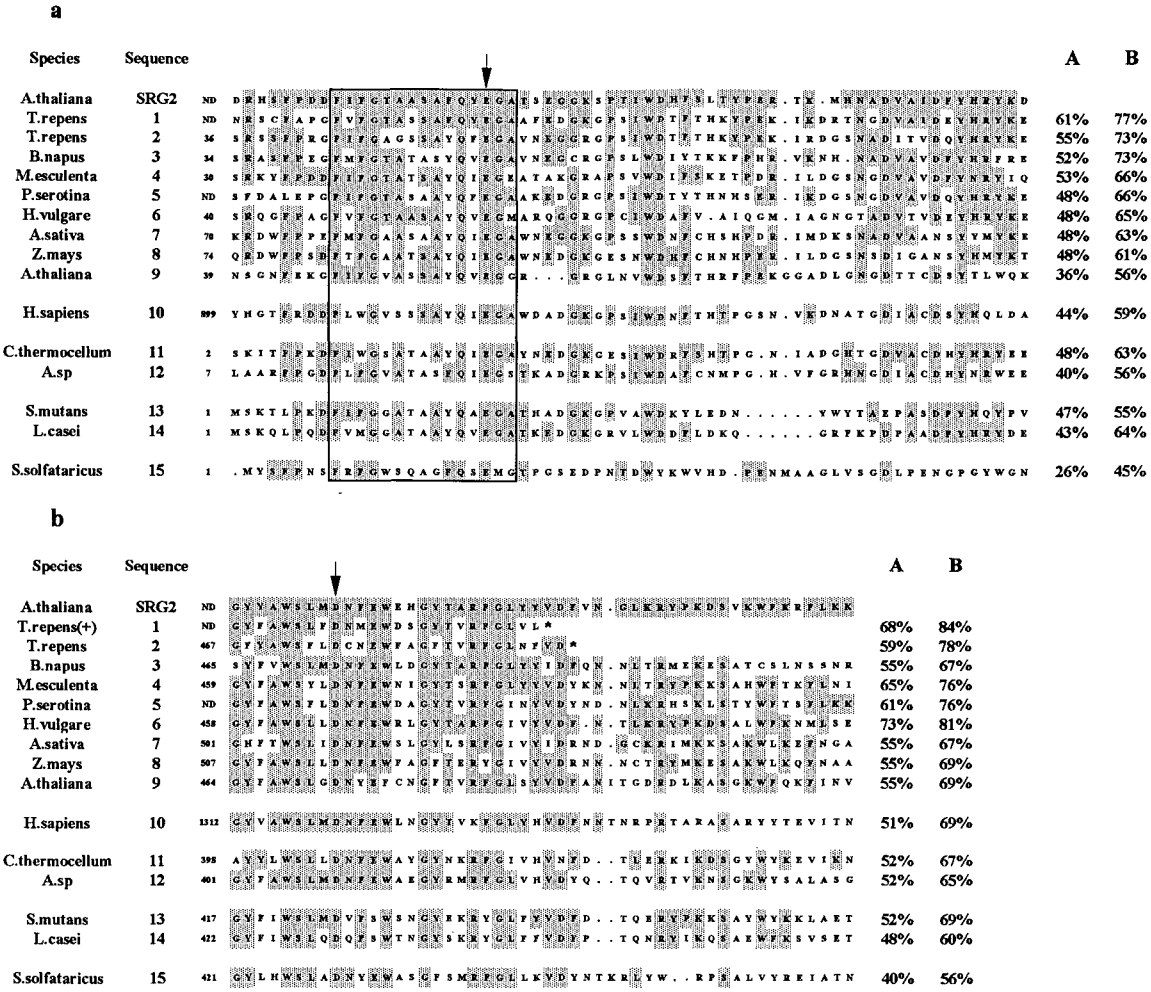

Figure 7. Multiple amino acid sequence alignments of the NH_2 (a) and COOH (b) terminal regions of SRG2 with proteins of the β-glycosyl hydrolase family 1. SRG2, Deduced amino acid sequence of SRG2; 1, cyanogenic β-glucosidase from *Trifolium repens* (accession no. X56733); 2, noncyanogenic β-glucosidase from *Trifolium repens* (accession no. X56734); 3, zeatin-*O*-glucoside-degrading β-glucosidase from *Brassica napus* (accession no. X82577); 4, cyanogenic β-glucosidase from *Manihot esculenta* (accession no. S35175); 5, amygdalin hydrolase from *Prunus serotina* (accession no. U26025); 6, β-glucosidase from *Hordeum vulgare* (accession no. L41869); 7, avenacosidase from *Avena sativa* (accession no. X78433); 8, β-glucosidase from *Zea mays* (accession no. X74217); 9, thioglucosidase from *A. thaliana* (accession no. L11454); 10, region III of the lactase/phlorizin hydrolase precursor from *Homo sapiens* (accession no. X07994); 11, β-D-glucosidase (bglA) from *Clostridium thermocellum* (accession no. X60268); 12, β-glucosidase from *Agrobacterium* sp. (accession no. M19033); 13, phospho-β-galactosidase from *Streptococcus mutans* (accession no. L18993); 14, β-D-galactoside galactohydrolase from *Lactobacillus casei* (accession no. M20151); and 15, β-D-galactosidase from *Sulfolobus solfataricus* (accession no. M34696). Alignments were maximized by introducing gaps, denoted by a dot. Identical amino acids are shaded. The glycosyl hydrolase family 1 motif is boxed. The consensus pattern of the motif as defined by the MOTIF program of the GCG software package (Devereux et al., 1984) is: Fx(FYWM)(GSTA)x(GSTA)x(GSTA)2(FYN)(NQ)xEx(GSTA). Strictly conserved amino acid residues potentially involved in the catalytic function of the enzymes (Gräbnitz et al., 1991) are indicated by arrows. Stop codons are indicated by stars. The number to the left of each sequence refers to the position of the first amino acid in the original protein. ND, Position not determined. Figures on the right side refer to percentages of identity (A) and similarity (B). (+), No significant homology was found in the COOH-terminal segment of the published sequence. However, by introducing a frameshift into the nucleotide sequence, we were able to identify a protein sequence, shown in the figure, with homology to SRG2 and the other β-glycosidases.

degeneration was accompanied by particular cytological changes or modifications to chromatin structure. We focused on alterations commonly associated with "programmed cell death" in animal cells (Raff, 1992; Vaux, 1993), including nuclear shrinking, an early event resulting from chromatin compaction, and nDNA fragmentation into nucleosomes.

The nuclear morphologies of actively growing (6-d-old), arrested (12-d-old), or low-viability (15-d-old) Arabidopsis cells were studied using 4′,6-diamidino-2-phenylindole staining coupled with fluorescence microscopy (Fig. 8). Nuclei from 6-d-old cells were characterized by diffuse staining and a large nucleolus (Fig. 8, a and b). Irregularly stained granular nuclei, presumably resulting from local-

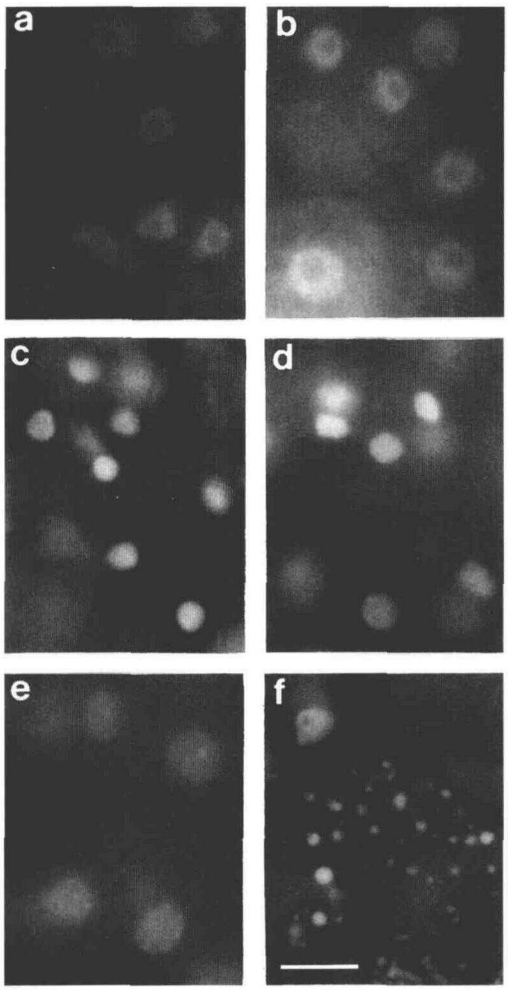

Figure 8. Cytological analysis of nuclei from Arabidopsis cultured cells. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole, as described in "Materials and Methods." a and b, Six-day-old actively growing cells; c, d, and f, 15-d-old nonviable cells; e, 12-d-old growth-arrested cells. Each photograph was obtained from an independent cell culture. The magnification is identical for all the photographs. The size bar corresponds to 10 μm.

ized chromatin condensation, were present in 12-d-old cells (Fig. 8e), but were also occasionally observed in preparations from 6-d-old cells. Since localized compactions of chromatin in dense heterochromic regions (chromocenters) have previously been observed in the interphase nuclei of Arabidopsis (Maluszynska and Heslop-Harrison, 1991), the exact nature of these granular structures needs to be determined. On the other hand, most of the nuclei observed in 15-d-old cells appeared to be of small size, with a slightly increased fluorescence intensity and usually no visible nucleolus (Fig. 8, c and d). In certain 15-d-old cells, nuclei appeared fragmented into highly fluorescent "chromatin granules" (Fig. 8f). Taken overall, these initial cytological studies indicate that the arrest of proliferative activity and subsequent cell death in the Arabidopsis culture are associated with a significant alteration of nuclear morphology, which could result from general or localized condensation of chromatin.

Analysis of the DNA content of Arabidopsis cells in suspension cultures showed that DNA fragmentation into nucleosomes occurred 15 d after subculturing. As shown in Figure 9, a ladder of fragments calculated to be multiples of 160 bp was reproducibly detected in 15-d-old cells (lanes 4–6). This characteristic internucleosomal DNA cleavage was not detected in cells harvested at an earlier time when the cells were still viable (lane 3, 6-d-old cells). Furthermore, neither heat-killing of the cells nor lysis by repeated freezing/thawing treatments resulted in nucleosomal ladders in the corresponding DNA extracts (lanes 1 and 2), suggesting that the DNA ladder fragmentation is not simply a nonspecific result of cell lysis, but results from specific cell-mediated degradation events.

DISCUSSION

When growth-arrested Arabidopsis cell-suspension cultures are subcultured into fresh medium, a qualitative change of the mRNA profile can be visualized using the differential display approach (Fig. 1). In addition to the appearance of a number of new mRNA species, we have also observed the disappearence of a small group of mRNAs present at high levels in the nondividing culture. In this article we report the identification and cloning of three cDNAs, *SRG1*, *SRG2*, and *SRG3*, which correspond to mRNAs of this latter group. It is interesting that the expression profiles of these genes differ throughout the culture growth cycle, as shown in Figure 3b. For *SRG1* and *SRG2* relative transcript levels were maximal in growth-arrested cells, whereas *SRG3a* mRNA levels peaked during the late growth phase. It is important to point out that, despite significant variations in total cell RNA contents throughout the culture cycle (Fig. 3b) and during organ senescence (Fig. 5a, see below), equivalent amounts of RNA were loaded for northern analysis. Such experiments can provide information only about the relative levels of

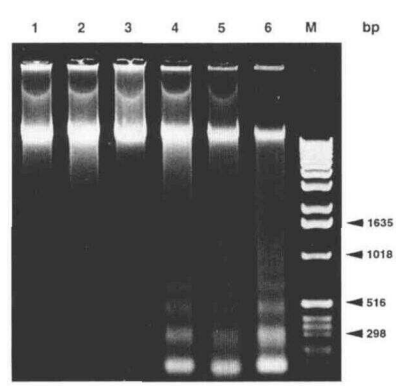

Figure 9. Agarose gel analysis of DNA fragmentation in cultured Arabidopsis cells. Total DNA was extracted from the cells and electrophoresed in a 1.2% (w/v) agarose gel, followed by staining with ethidium bromide. Lane M, 1-kb ladder DNA molecular size markers from BRL. Sizes, in bp, are indicated on the right. Lanes 1 to 3, DNA purified from 6-d-old cells; lane 1, cells heated to 56°C for 30 min; lane 2, cells subjected to three rounds of freezing (−195°C) and thawing (37°C); lane 3, untreated cells; lanes 4 to 6, DNA extracted from 15-d-old cells obtained from three independent cell cultures.

mRNAs resulting from a combination of transcript synthesis and degradation. However, it is clear that de novo gene transcription must be a major contributing factor in those cases in which certain *SRG* mRNAs accumulate to a highly significant level in both senescing tissues and during late phases of the cell-growth cycle. Differences in the *SRG* mRNA profiles means either that these genes are not coordinately regulated at the transcriptional level, or that there are differences in the mechanisms of posttranscriptional regulation (e.g. mRNA stability).

Northern analyses have shown that *SRG1*, *SRG2*, and *SRG3a* mRNA accumulation is specifically associated with senescence-related processes in the intact Arabidopsis plant, with maximal relative transcript levels detected at late stages of leaf senescence. These results suggest that the *SRG* genes represent a new class of markers for plant senescence. The *SAG2* and *SAG4* genes, previously known to be specifically expressed during leaf senescence (Hensel et al., 1993; Grbic and Bleecker, 1995), were also found to be expressed in Arabidopsis cell cultures with kinetics of transcript accumulation similar to that observed for *SRG1*. It would appear that, in terms of gene expression, growth arrest and entry into the stationary phase in Arabidopsis cell-suspension cultures have certain features in common with the classically described senescence processes of plants. In this context, it has already been reported that cultured cells (such as those from pear fruit) can furnish useful model systems in the study of some aspects of plant senescence (e.g. Lelièvre et al., 1987). More recently, senescence processes have also been described in cultured rice cells, in which Suc starvation leads to an increase in specific α-amylases, correlating to an increase in vacuolar autophagy and accompanied by a degradation of cytoplasmic structures, including plastids (Chen et al., 1994).

Following the transfer of viable, growth-arrested cells into fresh medium, dramatic reductions in mRNA levels were observed for *SRG1*, *SRG2*, and *SRG3a* (Fig. 2). It is interesting that the same phenomenon was observed for the senescence-associated genes *SAG2* and *SAG4*, transcript levels of which also decreased rapidly within 15 h of subculturing (data not shown). Hensel et al. (1993) hypothesized that the *SAG2* and *SAG4* genes might be subject to repression by specific metabolites. This could indeed be the case during subculturing, and suggests that the Arabidopsis cell-suspension culture might represent a useful biological system for metabolic repression studies.

The Arabidopsis S-like RNase-coding gene *RNS2*, which is preferentially expressed during both leaf and petal senescence (Taylor et al., 1993; Bariola et al., 1994), was also expressed in nondividing cell cultures. However, the relative transcript level of this gene was modulated in a complex fashion throughout the cell-growth cycle, with a significant level of mRNA present in cells even after transfer into fresh medium (Fig. 3). *RNS2* also shows a fairly high basal level of expression in the absence of senescence induction in whole plant tissues, as shown by Bariola et al. (1994) and by the results presented in this article (see Figs. 4 and 5).

The *SRG1*-encoded protein is a new member of the Fe(II)/ascorbate oxidase superfamily. This widely divergent family of structurally related proteins includes enzymes performing various functions in plants, such as the biosynthesis of flavonoid compounds, the production of ethylene, and the biosynthesis of alkaloids and hormones (for discussions, see Britsch et al., 1993; Tang et al., 1993; Xu et al., 1995). Unfortunately, the precise role of SRG1 could not be deduced by amino acid sequence comparison.

Sequence analysis of the putative polypeptide encoded by the *SRG2* gene provides strong evidence that this protein is a member of glycosyl hydrolase family 1 (Henrissat, 1991) and is most probably a β-glucosidase. This protein family is known to catalyze the hydrolysis of the β-D-glycosidic bond in a variety of natural β-glycoside conjugates, and their biological function is directly related to the nature of the cleaved conjugates (see Poulton, 1990; Brzobohatý et al., 1993; Leah et al., 1995). The precise function of the Arabidopsis SRG2 β-glucosidase could not be determined on the basis of the extent of sequence homology. As a rule, the glycosyl hydrolase activities are found associated with the ripening and senescence of plant tissues (e.g. Fisher and Bennett, 1991). A number of reports have described the expression of glycosyl hydrolases in nutrient-starved cell cultures, e.g. α-amylases from rice (Chen et al., 1994; Sheu et al., 1994), β-glucosidase from *Brassica* (Malboobi and Lefebvre, 1995), or *N*-acetyl-β-glucosaminidase from *Silene* (Lhernoud et al., 1994). It is well known that certain hydrolytic activities may be generated by the release of the corresponding enzymes from various cellular storage compartments. Given the *SRG2* mRNA profile, it is clear that de novo transcription of this gene is necessary for generating enzyme activity, both in stationary-phase cultures and in senescing leaves.

In Arabidopsis T87-C3 cell cultures, the transition from growth arrest to loss of cell viability is relatively rapid. We show that this rapid death can be correlated with internucleosomal cleavage of nDNA, as well as with an apparent compaction of the chromatin (Figs. 8 and 9). In animals such events have been correlated with a process of active cell death, a suicide program leading to the elimination of unwanted cells (Raff, 1992; Vaux, 1993). Increasing data suggest that related cell-death programs may operate in plants. Indeed, DNA fragmentation in plant tracheids has recently been reported (Mittler and Lam, 1995). The hypersensitive response of plant tissues to a variety of pathogens also shares striking similarities with programmed cell death in animals (Mittler and Lam, 1996). The recent isolation of plant cDNA clones coding for homologs of the DAD-1 protein, which is able to rescue animal cells from programmed cell death (Apte et al., 1995; Sugimoto et al., 1995), also suggests that certain molecular mechanisms of animal apoptosis might be conserved in plants. The results presented in this paper raise the exciting possibility that Arabidopsis cell-suspension cultures may provide model systems for studying molecular features of both organ senescence and programmed cell death in plants.

ACKNOWLEDGMENTS

We are grateful to Anthony B. Bleecker and Vojislava Grbić (University of Wisconsin, Madison) for the gift of the *SAG2* and

SAG4 cDNA clones. We are also indebted to Pamela J. Green and Pauline Bariola (Michigan State University, East Lansing) for providing the *RNS2* cDNA clone. We gratefully acknowledge David Barker for advice and for critical reading of the manuscript. We thank Jérôme Gouzy for active contribution to the computer work, and Dominique Douilhac for excellent assistance in cell suspension maintenance.

Received March 26, 1996; accepted June 30, 1996.
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The EMBL/GenBank/DDBJ accession numbers for the nucleotide sequences reported in this article are X79052 (*SRG1*), X82623 (*SRG2*: NH_2-terminal coding region), X82624 (*SRG2*: COOH-terminal coding region), and X98376 (*SRG3*: mRNA 5′-terminal region).

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Apte SS, Mattei MG, Seldin MF, Olsen BR (1995) The highly conserved defender against the death 1 (DAD1) gene maps to human chromosome 14q11–q12 and mouse chromosome 14 and has plant and nematode homologs. FEBS Lett **363:** 304–306

Axelos M, Bardet C, Liboz T, Le Van Thai A, Curie C, Lescure B (1989) The gene family encoding the *Arabidopsis thaliana* translation elongation factor EF-1α: molecular cloning, characterization and expression. Mol Gen Genet **219:** 106–112

Axelos M, Curie C, Mazzolini L, Bardet C, Lescure B (1992) A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiol Biochem **30:** 123–128

Bariola PA, Howard CJ, Taylor CB, Verburg MT, Jaglan VD, Green PJ (1994) The *Arabidopsis* ribonuclease gene *RNS1* is tightly controlled in response to phosphate limitation. Plant J **6:** 673–685

Britsch L, Dedio J, Saedler H, Forkmann G (1993) Molecular characterization of flavanone 3β-hydroxylases. Consensus sequence, comparison with related enzymes and the role of conserved histidine residues. Eur J Biochem **217:** 745–754

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science **262:** 1051–1054

Callard D, Lescure B, Mazzolini L (1994) A method for the elimination of false positives generated by the mRNA differential display technique. BioTechniques **16:** 1096–1103

Chen M-H, Liu L-F, Chen Y-R, Wu H-K, Yu S-M (1994) Expression of α-amylases, carbohydrate metabolism, and autophagy in cultured rice cells is coordinately regulated by sugar nutrient. Plant J **6:** 625–636

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Crowell DN, Amasino RM (1991) Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. Plant Physiol **95:** 711–715

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4:** 451–461

Fisher RL, Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. Annu Rev Plant Physiol **42:** 675–703

Gräbnitz F, Seiss M, Rücknagel KP, Staudenbauer WL (1991) Structure of the β-glucosidase gene bglA of *Clostridium thermocellum*. Sequence analysis reveals a superfamily of cellulases and β-glycosidases including human lactase/phlorizin hydrolase. Eur J Biochem **200:** 301–309

Grbić V, Bleecker AB (1995) Ethylene regulates the timing of leaf senescence in *Arabidopsis*. Plant J **8:** 595–602

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J **280:** 309–316

Hensel LL, Grbic V, Baumgarten DA, Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. Plant Cell **5:** 553–564

Hsieh H-M, Liu W-K, Huang PC (1995) A novel stress-inducible metallothionein-like gene from rice. Plant Mol Biol **28:** 381–389

Köck M, Löffler A, Abel S, Glund K (1995) cDNA structure and regulatory properties of a family of starvation-induced ribonucleases from tomato. Plant Mol Biol **27:** 477–485

Kodama H, Ando S, Komamine A (1990) Detection of mRNAs correlated with proliferation of cells in suspension cultures of *Catharanthus roseus*. Physiol Plant **79:** 319–326

Leah R, Kigel J, Svendsen I, Mundy J (1995) Biochemical and molecular characterization of a barley seed β-glucosidase. J Biol Chem **270:** 15789–15797

Lee SP, Chen THH (1993) Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss) suspension culture. Plant Physiol **101:** 1089–1096

Lelièvre J-M, Balagué C, Pech J-C, Meyer Y (1987) Protein synthesis associated with quiescence and senescence in auxin-starved pear cells. Plant Physiol **85:** 400–406

Lhernoud S, Karamanos Y, Priem B, Morvan H (1994) Carbon starvation increases endoglycosidase activities and production of "unconjugated *N*-glycans" in *Silene alba* cell-suspension cultures. Plant Physiol **106:** 779–784

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967–971

Malboobi MA, Lefebvre DD (1995) Isolation of cDNA clones of genes with altered expression levels in phosphate-starved *Brassica nigra* suspension cells. Plant Mol Biol **28:** 859–870

Maluszynska J, Heslop-Harrison JS (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J **1:** 159–166

McGarvey DJ, Sirevåg R, Christoffersen RE (1992) Ripening-related gene from avocado fruit. Plant Physiol **98:** 554–559

Mittler R, Lam E (1995) *In situ* detection of nDNA fragmentation during the differentiation of tracheary elements in higher plants. Plant Physiol **108:** 489–493

Mittler R, Lam E (1996) Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. Trends Microbiol **4:** 10–15

Poulton JE (1990) Cyanogenesis in plants. Plant Physiol **94:** 401–405

Raff MC (1992) Social controls on cell survival and cell death. Nature **356:** 397–400

Regad F, Bardet C, Tremousaygue D, Moisan A, Lescure B, Axelos M (1993) cDNA cloning and expression of an *Arabidopsis* GTP-binding protein of the ARF family. FEBS Lett **316:** 133–136

Roitsch T, Bittner M, Godt DE (1995) Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol **108:** 285–294

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Sheu J-J, Jan S-P, Lee H-T, Yu S-M (1994) Control of transcription and mRNA turnover as mechanisms of metabolic repression of α-amylase gene expression. Plant J **5:** 655–664

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in *Arabidopsis*. Plant Cell **2:** 755–767

Sugimoto A, Hozak RR, Nakashima T, Nishimoto T, Rothman JH (1995) *dad-1*, an endogenous programmed cell death sup-

pressor in *Caenorhabditis elegans* and vertebrates. EMBO J **14:** 4434–4441

Tang X, Wang H, Brandt AS, Woodson WR (1993) Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. Plant Mol Biol **23:** 1151–1164

Taylor CB, Bariola PA, delCardayré SB, Raines RT, Green PJ (1993) RNS2: a senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. Proc Natl Acad Sci USA **90:** 5118–5122

Teyssendier de la Serve B, Axelos M, Péaud-Lenoël C (1985) Cytokinins modulate the expression of genes encoding the protein of the light-harvesting chlorophyll a/b complex. Plant Mol Biol **5:** 155–163

Traas JA, Beven AF, Doonan JH, Cordewener J, Shaw PJ (1992) Cell-cycle-dependent changes in labelling of specific phosphoproteins by the monoclonal antibody MPM-2 in plant cells. Plant J **2:** 723–732

Tseng T-C, Tsai T-H, Lue M-Y, Lee H (1995) Identification of sucrose-regulated genes in cultured rice cells using mRNA differential display. Gene **161:** 179–182

Vaux DL (1993) Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci USA **90:** 786–789

Vernon LP (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal Chem **32:** 1144–1150

Xu Y-L, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA **92:** 6640–6644