Journal of Plant Biology, September 2004, 47(3) : 244-253

A Study of Cotyledon Senescence in Cucumber (*Cucumis sativus* L.) Based on Expressed Sequence Tags and Gene Expression

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Cucumber cotyledons provide an excellent experimental system in which to investigate developmental changes in gene expression, from the early phase of heterotrophism through phototrophic growth to senescence. A cDNA library was prepared from the final stage of senescing cucumber cotyledons (>95% yellow) for studying the genes responsible for lipid mobilization during germination and senescence. This library had produced numerous senescence-associated clones in a previous study. Here, a total of 365 cDNA clones and their expression levels were examined via semi-quantitative RT-PCR. Up-regulation of expression was detected for several known and unknown genes. These results were used to investigate the possible functions for senescence-related genes during cotyledon development.

Keywords: cotyledon, cucumber, EST, RT-PCR, senescence

Senescence in plants is the final developmental stage controlled by programmed gene sequences. Although this process is age-dependent, both endogenous and exogenous factors can greatly influence its onset. For example, nutrients, phytohormones, water, light, temperature, and stresses can lead to the early start of senescence in tissues and organs (Smart, 1994; Gan and Amasino, 1997). However, in this highly controlled sequence of biochemically and physiologically degenerative events, nutrients are recycled from senescing cells to other parts of the plants, such as the meristems, young leaves, developing flowers, or storage tissues (Thomas and Stoddart, 1980; Smart, 1994; Nooden et al., 1997). Because senescence is developmentally programmed and ultimately results in cell death, this stage has been proposed to qualify as a *bone fide* occurrence of programmed cell death (PCD) (Gan and Amasino, 1997; Nooden et al., 1997).

Senescence has been studied in many plant species (Graham et al., 1992; Smart 1994; Gan and Amasino, 1997; Nam, 1997; Delorme et al., 2000). One such approach has been to identify senescence-associated genes (SAG) or cDNA clones. In recent research, several clones have been isolated from a cDNA library of senescing cucumber cotyledons to investigate oil-seed germination and cotyledon development (Graham et al., 1992; Kim et al., 1997). Differential screening has led, first, to the identification of three glyoxylate cycle-related enzymes -- isocitrate lyase (ICL), malate synthase (MS), and glyoxysomal malate dehydrogenase (gMDH) -- which encode cDNA sequences. A second result has been the isolation of a key enzyme for the gluconeogenesis phospho*enol*pyruvate carboxykinase (PEPCK)-encoding cDNA clone. Finally, senescence-specific SE71 and SE74 cDNA clones have been isolated by screening the cucumber cotyledon senescence library (Kim and Smith, 1994). As a result, researchers have identified SE71 cDNA as an SPF1-type DNA binding protein that encodes sequences formerly characterized in sweet potato (Ishiguro and Nakamura, 1994; Kim et al., 1997). SE74 has been confirmed as a matrix metalloproteinase (MMP) encoding cDNA that is tightly controlled in the last stage of cotyledon and leaf senescence (Delorme et al., 2000). The cucumber *CsMMP* gene is now known to be strongly expressed late in senescence, even in drying cotyledons and leaves.

In many plants, lipids are the major carbon reserve that is stored either in the endosperm tissue surrounding the embryo or in the cotyledons. Upon germination, such seedlings show a distinctive metabolic conversion of fat to carbohydrates. Glyoxysomes are involved in storage-lipid conversion into sugars during this stage. They participate in reactions of the glyoxylate cycle, a pathway responsible for the net conversion of two molecules of acetyl coenzyme A (acetyl-CoA) into succinate. In germinating oil-seed plants, the gluconeogenic pathway begins with the hydrolysis of stored lipids. Fatty acids are liberated

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from triacylglycerols in the lipid bodies, then oxidized via glyoxysomal β-oxidation to produce acetyl-CoA, the first input into the glyoxylate cycle. Acetyl-CoA is metabolized within the glyoxysome. Succinate moves out from there and enters the mitochondrion, where it is further oxidized to oxaloacetate by enzymes of the tricarboxylic acid (TCA) cycle. The last step in the gluconeogenic pathway happens in the cytosol, where oxaloacetate from the mitochondrion is decarboxylated to form phospho*enol*pyruvate (PEP), which is finally converted to hexose via reverse glycolysis (Beevers, 1961).

Massive lipid mobilization was previously thought to operate primarily during the period after germination, before photosynthetic competence was established in the seedling. However, senescence also includes the utilization and metabolism of fatty acids associated with gluconeogenesis. Membrane deterioration is an early and characteristic feature of senescence that results in increased permeability, loss of ionic gradients, and decreased activities of key membrane-associated enzymes, including the ion pumps. Major alterations occur in the molecular organization of the lipid bilayer in senescing membranes, particularly the plasmalemma and microsomal membranes (Brown et al., 1991). The loss of chloroplast structural and functional integrity is a dominant feature of leaf senescence. Possible sources of acyl residues are the chloroplastic glycolipids, which represent the most abundant group of foliar lipids. The breakdown of chloroplasts in senescent leaves is associated with the metabolism of large quantities of acyl residues that are produced upon the hydrolysis of thylakoidal glycolipids (Gut and Matile, 1988, 1989). Genes for lipid mobilization, e.g., *ICL, MS, gMDH,* and *PEPCK,* are also active during cotyledon senescence (Kim and Smith, 1994). It is then that the cotyledons become yellow and begin to die.

Many SAGs have been studied in *Arabidopsis thaliana* (Lohman et al., 1994), maize (Smart et al., 1995), tomato (John et al., 1997), barley (Kleber-Janke and Krupinska, 1997), *Brassica napus* (Buchanan-Wollaston and Ainsworth, 1997), and *Populus tremula* (Bhalerao et al., 2003).

The report here describes recent research on cucumber cotyledon senescence.

MATERIALS AND METHODS

Plant Material

Seeds of cucumber (*Cucumis sativus* L.) were obtained from Heunong Jong Myo (Korea), then imbibed in sterile water at 4°C for 12 h and sown in wet vermiculite. The resultant plants were maintained in a growth chamber (Lab-Line Biotronette) at 25°C and 70% humidity, under continuous illumination. Cotyledons were collected for total RNA isolation from fully matured seedlings (15 d after seed imbibition) and at three stages of senescence (early, mid-, and late) as defined by their chlorophyll contents (see Delorme et al., 2000). All samples were immediately frozen in liquid nitrogen.

Other Biological Material

Cells of *Escherichia coli* strain XL-1/Blue (Stratagene, USA) were used as the host bacteria for propagation of the recombinant phage and *in vivo* excision of the Lambda Zap II cloning vector.

cDNA Library, Phage Propagation, and *in Vivo* Excision

Construction of the cucumber cDNA library has been described by Kim et al. (1997). After this library was titred, phage was propagated in Petri dishes on LB agar, at a density of approximately 100 pfu per 10 cm diameter of plate. Plaques were cored with a sterile Pasteur pipette and were transferred into microcentrifuge tubes containing 500 µl of SM buffer and 20 µl chloroform. The tubes were then vortexed and incubated at 4°C for 12 h to release phage particles. After centrifugation, the phage particle suspension was used for *in vivo* excision of the phagemid. These excisions were carried out according to the manufacturers instructions for the ExAssist helper phage (Stratagene) to the Lambda Zap II vector for the pBlueScript plasmid vector. Individual colonies appearing on the agar plates were streaked onto fresh LB agar containing the appropriate amount of X-Gal/IPTG, ampicillin, and tetracycline for blue/white selection (Sambrook et al., 1989). Only white colonies were chosen for further analysis.

Phagemid DNA Isolation and DNA Sequencing

Selected white colonies were inoculated into LB broth containing ampicillin, and were incubated overnight in a shaking incubator at 37°C. Phagemid DNA was purified using a Plasmid DNA purification kit (Bioneer, Korea). To identify insert DNA, phagemid DNA was treated by restriction enzymes *Eco*RI+*Xho*I (Roche, Germany), and the reaction products were

electrophoresed in agarose gel. For the expressed sequence tag analysis, insert cDNA sequencing was carried out at the Macrogen (Korea) with an *ABI PRISM BigDye* Terminator Cycler Sequencing kit (Perkin-Elmer, USA). The 5-end of the cDNA sequence was primed by either T3 (5-ATTAACCCTCACTAAAG-3) or the reverse primer (5-AACAGCTATGACCATG-3) for nucleotide sequencing.

Sequence Analysis

A general analysis and homology search of the sequenced DNA was conducted by the BLASTX algorithm (Altschul et al., 1990). Comparative analyses of the translated amino acid sequences were further evaluated with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Total RNA Isolation

An RNA easy Plant Mini Kit (Qiagen GmBH, Germany) was used to extract total RNA from cucumber cotyledon samples treated with DNase I (Qiagen GmBH). A pair of cotyledons was used from each developmental stage for individual extraction procedures. A final elution of total RNA was carried out in equal volumes (100 μl) of DEPC-treated sterile H_2O. We estimated the concentration of total RNA at 260 nm with a UV spectrophotometer (Sun-II Science, Korea), on the basis of single cotyledons.

Semi-Quantitative RT-PCR for Selected Clones

Clones selected via sequence analysis were chosen for semi-quantitative RT-PCR to examine their levels of gene expression. For first-strand cDNA synthesis, poly(A)$^+$ RNAs were primed by the oligo dT_{18} primer, using an RT-Premix kit (Bioneer). An equal volume (10 μl) of total RNA solution was used from each developmental stage for the reverse transcription reaction. PCR was conducted on the first-strand cDNA, using clone-specific DNA primers. Equal volumes (0.25 μl) of first-strand cDNA from each developmental stage were used in those reactions. PCR involved an initial heat treatment for 2 min at 95°C, followed by 25 cycles, each comprising 20 s of denaturation at 95°C, 30 s annealing at primer-specific Tm+2.0~2.5°C, and 20~40 s of primer extension at 72°C, all followed by a final extension for 7 min. Equal amounts (10 μl) of RT-PCR products were fractionated on a 1.2% (w/v) agarose gel in TBE buffer. The primers were designed using the cDNA sequence on both ends of the DNA sequences. All primers were synthesized by Bioneer. As a marker for constitutive expression, mRNA of *actin 2* (*Actin*; GenBank accession no. AY338231) was amplified with internal DNA sequence primers. All semi-quantitative RT-PCRs were performed 2~4 times from two separate sets of total RNA extracts. Afterward, clones showing consistent results were selected from the repeated RT-PCR procedure.

RESULTS AND DISCUSSION

ESTs from Senescing Cucumber Cotyledons

More than 500 cDNA clones were selected from the senescing cucumber cDNA library. A total of 365 cDNA clones possessed >300 bp of cDNA insert. The insert DNA containing the pBlueScript plasmid was used for template DNA of the 5' region. All 365 ESTs were produced by 5' to 3' single-direction sequencing. Open reading frames from the cDNA sequences were placed in the automatic translation and searching program of NCBI Blast. From this search, we were able to identify several interesting sequences from the cDNA clones (Table 1).

Semi-Quantitative RT-PCR for Selected Clones

The amount of total RNA reached a peak in the green-mature cotyledons, but decreased sharply during their senescence (Fig. 1). Based on this observation, we followed the changes in total RNA contents to study gene expression levels with semi-quantitative RT-PCR. We expected to find differences in relative amounts of mRNA for specific genes at the various developmental stages. Therefore, we adopted the cucumber *ICL* gene as a positive control of expression in senescence (Fig. 2A). Because its transcript levels showed similar patterns to what had been revealed in our previous northern blot analysis (Kim and Smith, 1994), we were able to demonstrate the validity of our semi-quantitative RT-PCR in the current study.

The *actin 2* gene from the *Phaseolus acutifolius* ACT2 cDNA sequence (GenBank Accession no. AY338231) was used to design our PCR primers and served as another control for evaluating relative changes in corresponding mRNA contents (Fig. 2B). Both the reverse and the forward primers share 92% homology with other plant *actin 2* gene sequences. The *actin 2* mRNA levels were well aligned with changes in total RNA amounts during cotyledon development (Fig. 1).

Table 1. Selected senescence-associated cDNA clones from cucumber cotyledons, and their amino acid sequence homology with other known genes or cDNA.

EST#	Possible Identity	Id (%)*	L/C**	Source (Accession No.)
Sen 060	Cytochrome P450 monooxygenase	67	104/153	*Zea mays* (AF465265)
Sen 066	Metallothionein-like protein type 3	56	37/65	*Musa acuminate* (Q40256)
Sen 083	Protein kinase (ATMRK1)	56	25/37	*Arabidopsis thaliana* (AAM63482)
Sen 087	Polyubiquitin	100	190/190	*Antirrhinum majus* (S25164)
Sen 090	Quinone oxidoreductase-like protein	65	130/199	*Arabidopsis thaliana* (NM_115504)
Sen 103	DEAD/DEAH box RNA helicase	78	64/82	*Saccharomyces cerevisiae* (NP_012485)
Sen 150	Uridine monophosphate kinase	73	76/103	*Arabidopsis thaliana* (AF375431)
Sen 153	Cytosolic superoxide dismutase	90	132/152	*Ipomoea batatas* (Q07796)
Sen 158	MMP21.2-like protein	62	18/29	*Arabidopsis thaliana* (BAB01942)
Sen 193	Lipoxygenase chloroplast presursor	67	21/31	*Hordeum vulgare* (CAD45187)
Sen 219	9-cis-epoxycarotenoid dioxygenase 2	80	81/101	*Pisum sativum* (BAC10550)
Sen 281	Matrix metalloproteinase	100	134/134	*Cucumis sativus* (CAB76364)
Sen 338	Osmotic stress-induced proline oxidase	63	136/214	*Arabidopsis thaliana* (NP_189701)
Sen 1-9	GA protein (light regulated *lir1*)	58	47/80	*Pisum sativum* (T06776)
Sen 3-5	Cysteine sythase	90	110/120	*Citrullus vulgaris* (D28777)
Sen 3-7	Phosphoglycerate kinase A (PGK A)	27	18/65	*Trypanosoma brucei brucei* (P08892)
Sen 8-5	ABC-transporter membrane protein	89	68/76	*Arabidopsis thaliana* (NP_192386)
Sen11-1	Serine carboxypeptidase precursor	76	80/105	*Gossypium hirsutum* (AAL67992)
Sen11-6	Peroxidase (ATP2a)	78	174/223	*Arabidopsis thaliana* (NP_181250)
Sen14-7	Protein phosphatase 2C (PP2C)	76	128/168	*Arabidopsis thaliana* (NP_564165)
Sen16-8	Aminopeptidase N (?)	41	12/29	*Oryctolagus cuniculus* (CAA35873)

*Id, the percentage of amino acid sequence identity in a continuous region of a particular length (overlap).
**amino acid numbers which length compared.

Although most of the RT-PCRs produced excellent results (Fig. 2 and 3), those for six clones (Sen 65, 90, 103, 150, 3-7, and 16-8) were unacceptable for publication from the independently repeated PCR. Therefore, they are not part of our final discussion.

Senescence-Associated Genes

Cytochrome P450-dependent monoxygenase (Sen 060) is part of a large group of heme-containing enzymes, most of which catalyze NADPH- and O_2-dependent hydroxylation reactions. These cytochrome P450s mediate a wide range of oxidative reactions involved in the biosynthesis of plant secondary metabolites, including the phenylpropanoids and phytoalexins. For example, two of them catalyze reactions in the phenylpropanoid pathway -- *trans*-cinnamate-4-hydroxylase (*t*-C4H), encoded by *CYP73*, and ferulate-5-hydroxylase (F5H), which is encoded by *CYP83*. The products of the phenylpropanoid pathway are critical to plant survival. Transcripts encoding CYP73A9 of pea are inducible by wounding (Frank et al., 1996). Another cytochrome P450-encoding plant gene (*CYP76C2*) is preferentially expressed during the hypersensitive response (HR) provoked by bacterial pathogens in *Arabidopsis*. Likewise, *CYP76C2* gene expression is associated with various processes leading to cell death, such as leaf senescence and other stresses (Godiard et al., 1998). Therefore, if plant senescence is considered a developmental stress, we should expect up-regulation of some gene activity as a final defense mechanism. In fact, a cytochrome P450 cDNA clone that shows up-regulated expression has been isolated from broccoli florets 3 d after their harvest (Page et al., 2001). In the current study, the cucumber cytochrome P450 gene was found to be up-regulated in the middle stage of natural senescence (Fig. 2C).

Metallothionein (Sen 066) is an abundant protein in senescing plant organs. Expression of its gene has been reported in *Brassica napus* leaves, post-harvest florets of broccoli (*Brassica oleracea*), and leaves of senescing sweet potato (*Imopoea batatas*) (Buchanan-Wollaston, 1994; Huang et al., 2001; Page et al., 2001). The metallothionein gene is also strongly expressed in mature *B. napus* flowers. One massive EST from autumn leaves of *Populus tremula* has revealed that

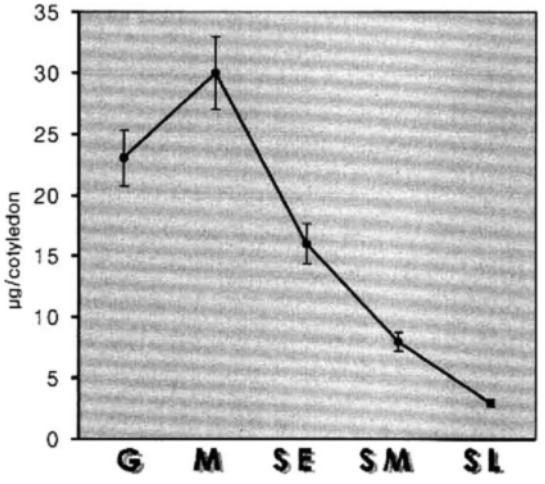

Figure 1. Total RNA levels during development of cucumber cotyledons, from germination to senescence. Three pairs of cotyledons were used for extraction of total RNA at each stage. Total RNA was dissolved in equal volumes (100 μl) of DEPC-treated H_2O for each pair, then an equal volume (50 μl) of total RNA solution was used to examine concentration at 260 nm. G, day-3 germinating cotyledon after seed imbibition; M, dark green and matured from 15 d after germination; SE, early stage of senescence (≤50% yellow) at day 28; SM, mid-stage of senescence (~70% yellow) at day 33; SL, late stage of senescence (>95% yellow) at day 38.

transcript of metallothionein is one of the most abundant sequences, along with those for light-inducible proteins and cysteine proteases (Bhalerao et al., 2003). Therefore, this major protein is likely to function in scavenging and detoxifying reactive oxygen molecules during the final stages of development in many organs, e.g., the leaf, floret, flower, and cotyledons. In our study, the relative amount of metallothionein mRNA reached a maximum during early senescence, then gradually declined to the end stage in the cucumber cotyledons (Fig. 2D).

Complete homology within the polyubiquitin sequences (Sen 087) in the plant kingdom may explain its importance in cell development. Polyubiquitin-encoding cDNA sequences have been reported from dark-incubated, senescing *Arabidopsis* leaves (Park et al., 1998). Its biomolecular functioning has not been confirmed in plant senescence, but this protein is involved in apoptotic regulation and recognition signals in animal cells (Fliermann et al., 2003; Lee and Peter, 2003). Thus, plant polyubiquitin may play a role in similar biological processes for senescence and PCD. Our examination showed that the cucumber polyubiquitin gene was expressed, without significant changes in transcript level, throughout cotyledon development (Fig. 2E). We found multiple forms of polyubiquitin sequences that were amplified in various lengths of cDNA from RT-PCR, even at an annealing temperature of Tm+5°C. Moreover, the expected length of polyubiquitin cDNA in the 560-bp band increased in the later stages.

Leaf senescence is strongly associated with increased oxidative damage to macromolecules by reactive oxygen species (ROS). Both the ROS level and their damage products increase during senescence in many plants (Ye et al., 2000). These changes are due to a sharp decline in the level (up to 5-fold) of certain antioxidant enzymes such as ascorbate peroxidase and catalase. We found such a response to be associated with an increase in superoxide dismutase (Sen 153 and Fig. 2F) in the chloroplasts. Its enzyme-mediated lipid peroxidation products were produced via lipoxygenases (Sen 193 and Fig. 2H). Surprisingly, however, peroxidase activity has been shown to rebound by the induction of antioxidant defenses (up to 5-fold) resulting in a decline in lipid peroxidation and the onset of visible signs of leaf senescence as the reproductive stage and seed-set progress (Ye et al., 2000). Thus, ROS increases are associated with further floral development due to programmed declines in certain peroxidase activity, all leading to the oxidative activation of lipoxygenase and subsequent lipid peroxidation. However, symptoms of this fluctuation in activity during the later stages of leaf development are correlated with intrinsic cytokinin concentrations and increases in levels of abscisic acid (Chaloupkova and Smart, 1994).

The decrease in cytokinin levels within an organ may be an initial step in leaf senescence that triggers intercellular self-destruction by ROS, as is observed with *in vitro* cell culturing. For example, levels of a thiol protease (P21) homologous SAG2 increase in *Petunia* calli subcultures produced on low-cytokinin media. Likewise, differential expression of the anionic peroxidase (P17) gene is highly correlated with the degree of leaf senescence as well as the age of the calli (Tournaire et al., 1996). Therefore, antioxidant enzyme genes, such as those for peroxidase, are associated with an early stage of senescence that may be due, in part, to a reduction in total cytokinin levels in the target organ (Sen 11-6 and Fig. 2L). According to our RT-PCR, cytosolic peroxidase gene(s) were strongly expressed in green to yellow cotyledons, but exhibited some decline in later stages (Fig. 2F).

As mentioned earlier, the *Cs1-MMP* gene (Sen 281 and Fig. 2I) encodes a pre-pro-enzyme with the hallmark motifs of the metzincin superfamily of zinc metalloproteinases, especially the matrix metalloproteinase

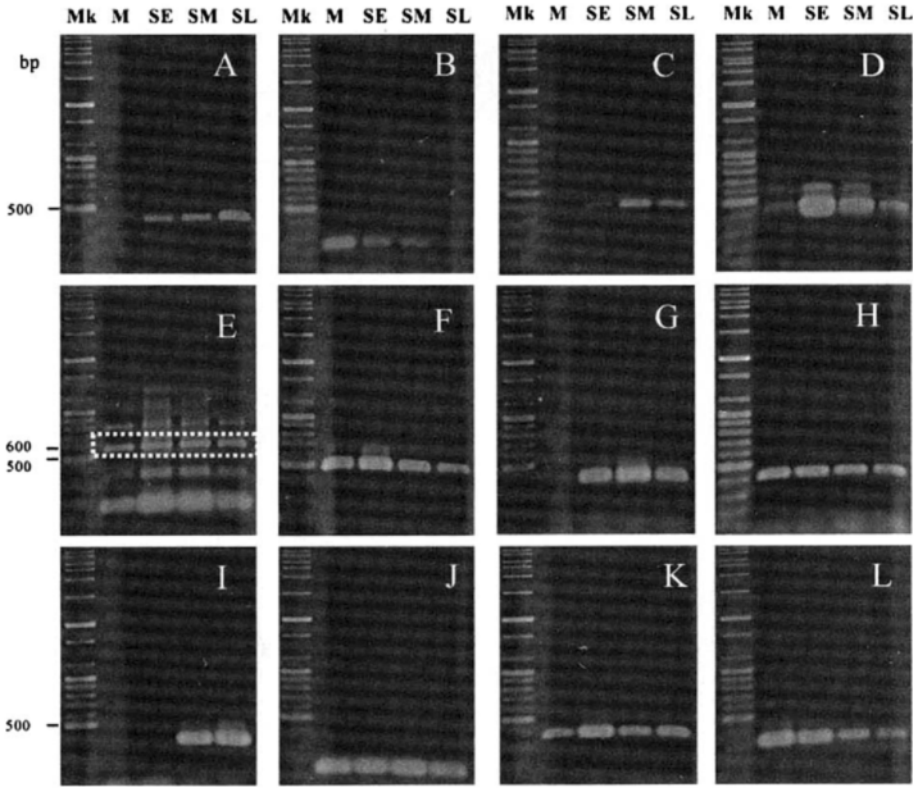

Figure 2. Semi-quantitative RT-PCR for selected cucumber cDNA clones. First-strand cDNA synthesis was performed on per-cotyledon basis of total RNA: 3.0 μg for Stage M (mature), 1.6 μg for Stage SE (early senescence), 0.8 μg for Stage SM (mid), and 0.3 μg for Stage SL (late). Equals volumes (0.25 μl each) of first-strand cDNAs from four developmental stages were used for PCR with clone-specific primers (see Table 2). Lanes from left to right: Bioneer's 100 bp plus DNA size marker (Mk), dark green and matured from 15 d after germination (Stage M), early stage of senescence (≤50% yellow) at Day 28 (Stage SE), mid-stage of senescence (~70% yellow) at Day 33 (Stage SM), and late stage of senescence (>95% yellow) at Day 38 (Stage SL). **A**, *ICL*; **B**, *Actin 2*; **C**, Sen 60; **D**, Sen 66; **E**, Sen 87; **F**, Sen 153; **G**, Sen 158; **H**, Sen 193; **I**, Sen 281; **J**, Sen 3-5; **K**, Sen 8-5; **L**, Sen 11-6.

family. These enzymes are found in various organisms, from bacteria to vertebrate species (Basset et al., 1990; Lepage and Gache, 1990). Metalloproteinase has also been identified in higher plants (Graham et al., 1991; McGeehan et al., 1992). Its vast functions include protein turnover, the action of tetanus and botulism toxins, embryogenesis development, and processes of cancer and arthritis. However, the role of the Cs1-MMP enzyme is unclear in plant cell senescence. We found that the *Cs1-MMP* gene was expressed *de novo* at the end stage, prior to the appearance of DNA laddering in cucumber cotyledon leaf discs and male flowers. Because the steady-state level of *Cs1-MMP* mRNA peaks late in senescence and the pro-enzyme must undergo maturation and activation, protease is probably not involved in nutrient remobilization during senescence, but may be involved in PCD (Delorme et al., 2000). Here, the first isolated cucumber MMP was remarkably active late in senescence. Two different lengths of Cs1-MMP encoding cDNA clones were isolated in this EST, which reflected the abundance of corresponding transcripts. Again, our RT-PCR revealed that its strongest expression was in the late stage (Fig. 2I). Furthermore, we identified a novel MMP21.2 encoding a cDNA clone (Sen 158 and Fig. 2G), whose corresponding gene expression was detected at the beginning of visible senescence (Fig. 2G). Therefore, we can categorize this as a novel class of MMPs in plants.

Lipoxygenases (LOXs) catalyze the hydroperoxidation of specific unsaturated fatty acids, and are widely distributed in plants and animals. It is widely accepted that plant LOXs have various physiological roles essential to plant life. Expression of *lox-1*, which corresponds to the LOX-1 enzyme in cucumber, is highly coordinated with that of a typical glyoxysomal enzyme, ICL, during the post-germinative growth of cotyledons. In contrast, such coordination between *lox-1* and *ICL* has not been reported during *in vitro* senescence of the cucumber cotyledon (Matsui et al., 1999).

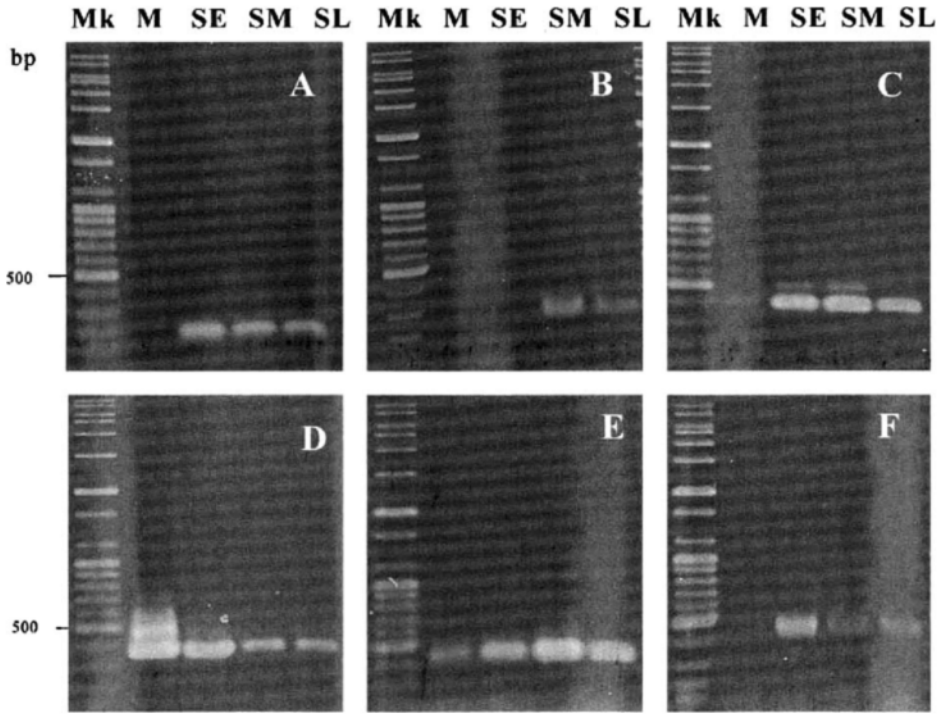

Figure 3. RT-PCR for novel SAGs from cucumber, performed as described in Fig. 2. Lanes from left to right: Bioneer's 100 bp plus DNA size marker (Mk), dark green and matured from 15 d after germination (Stage M), early stage of senescence (≤50% yellow) at Day 28 (Stage SE), mid-stage of senescence (~70% yellow) at Day 33 (Stage SM), and late stage of senescence (>95% yellow) at Day 38 (Stage SL). **A**, Sen 83; **B**, Sen 219; **C**, Sen 338; **D**, Sen 1-9; **E**, Sen 11-1; **F**, Sen 14-7.

Therefore, *lox-1* expression is under strict developmental regulation in parallel with that of the glyoxysomal genes, but this coordination is evident only in post-germinative cotyledons. It is clear that LOX-1 plays an important role in lipid mobilization during germination. However, only a single LOX2 cDNA clone has been isolated at the onset of leaf senescence in *Arabidopsis*, a species in which the *lox* gene is active at the final stage (Hinderhofer and Zentgraf, 2001).

At least four *LOX* genes have been identified in *Arabidopsis*. Although the nucleic acid sequence of *LOX1* diverges from that of *LOX2*, both *LOX3* (AJ249794) and *LOX4* (AJ302042) share high homology. These four genes behave rather differently in senescing *Arabidopsis* leaves (He et al., 2002), with the cytoplasmic form of *LOX1* being strongly up-regulated but the plastidial stroma-localizing *LOX2* sharply down-regulated. Interestingly, other plastidial forms of the *LOX3* and *LOX4* genes are up-regulated as leaf senescence progresses. This activity is likely responsible for the increase in jasmonic acid (JA) production from a specific unsaturated fatty acid, linolenic acid; JA itself promotes organ senescence at the onset of that stage. That result, however, may conflict with those reported previously for LOX2 (Hinderhofer and Zentgraf, 2001).

Here, we isolated a putative cucumber LOX2 cDNA clone (Sen 193) that exhibited relatively high homology for LOX2 sequences from barley (67%) and *Arabidopsis* (62%). Our cucumber *LOX2* gene was active, without significant change in transcript, in green to yellow cotyledons (Fig. 2H). Therefore, we believe this gene may have some role in senescing tissues for lipid mobilization, although we suggest further analysis of possible functioning for the *LOX2* gene in plant senescence.

Although we did not tag any cysteine protease-related clones in this study, a putative cysteine synthase gene encoding cDNA (Sen 3-5 and Fig. 2J) was isolated, which suggest that the amino acid cysteine may have a special role in mediating cell metabolism during senescence. Cysteine synthesis characteristically occurs in the plastids, mitochondria, and cytosol. Hell et al. (2002) have focused on its biosynthesis and have determined that reduced sulfur is channeled from cysteine into many sulfur-containing compounds in food and feed. Furthermore, cysteine biosynthesis is part of the regulatory network that mediates between inorganic sulfur supply and the demand for reduced sulfur during plant growth and in response to environmental changes. Our RT-PCR revealed high expression levels for the cucumber cys-

teine synthase gene in comparison to the activity of the *actin 2* gene (Fig. 2J).

The ATP binding cassette (ABC transporter membrane protein; Sen 8-5 and Fig. 2K) belongs to the largest protein family, a ubiquitous and diverse group of proteins, most of which mediate transport across biological membranes. Members can be found in bacteria, fungi, plants, and animals. Although over 129 plant ABC transporter protein sequences are known, study of those plant proteins is only just beginning (Sanchez-Fernandez et al., 2001). Their biological functions are as ATP-dependent pump and ion channel regulators in mammalian and microbial systems. An ABC transporter in *Arabidopsis* has manifested both glutathione-conjugate and chlorophyll catabolite transport activities. Although plant ABC transporters are implicated in detoxification processes, they are not limited to this role but also participate in chlorophyll biosynthesis, stomatal movement, and ion fluxes. Therefore, they may play a central role in plant growth and developmental processes (Martinoia et al., 2002). In addition, peroxisomal β-oxidation of the long chain fatty acids from *Arabidopsis* mutant *pxa1* has been suggested as a requirement for transporter proteins (Zolman et al., 2001).

Glyoxysomes or similar structures in the peroxisome (i.e., gerontosomes) may be essential for the uptake of degraded internal membrane debris, in the form of fatty acids, as a means for retrieving a final energy supply. For example, in yeasts and humans, long chain fatty acids are transported into the peroxisomes via ATP-containing ATPases in the peroxisomal membrane. Nevertheless, this type of mechanism has not been identified in any plant species. Therefore, we can only speculate that a peroxisomal ABC transporter protein might function significantly in plant senescence as part of various signal paths, e.g., hormone (auxin) biosynthesis and the detoxification process. Up-regulation of an ABC transporter gene has been detected during post-harvest senescence in broccoli (Page et al., 2001). Likewise, our study also revealed increments in transcript for an ABC transporter membrane protein-encoding gene, beginning from the early stage of cotyledon senescence and then reaching a stable level until the last stage (Fig. 2K).

Novel Senescence-Related Genes from Cucumber

The mitochondria may be important in conveying intracellular stress signals to the nucleus, thereby leading to alterations in gene expression. Using the mRNA differential display technique, Maxwell et al. (2002)

Table 2. Necleotide sequences of oilgonucleotides used in RT-PCRs from cucumber.

Oligonucleotides Name	Nucleotide sequences
Sen 60F	5'-GCAAG TGTGC TTAGT CGTAA GCTTG-3'
Sen 60R	5'-CCATG CTGAG GTTGA GTAGT TAAA-3'
Sen 66F	5'-CATTA ACAAG CTATC TGTTT CCCTT-3'
Sen 66R	5'-ATTCA TAACA TACCT CTTGC TAAG-3'
Sen 83F	5'-CAATG TTGCA AAGTT TTATG GAGCA-3'
Sen 83R	5'-TTAGG GTTCC ACCTG GAAGA TACT-3'
Sen 87F	5'-ATTAC AACAT CCAGA AGGAG TCCAC-3'
Sen 87R	5'-ATCAT TCAAA GACAA TCACC AACA-3
Sen 90F	5'-AATGG AAGCG CTGGT TTGCA GGAAG-3'
Sen 90R	5'-AACAT GCGAG GTCCC GAACG CAAC-3'
Sen 103F	5'-CAGAA CTAAT GTCCC TGCCA TTCTT-3'
Sen 103R	5'-CCCGA TCACG CATGT AATGT ACTT-3'
Sen 150F	5'-CACTG ATACA GCTGC GGCTC TTCGA-3'
Sen 150R	5'-CATAG CAAAC GCTCC CAATT CTGGC-3'
Sen 153F	5'-TCTTG GAAGT AGTGA GGGTG TTAGT-3'
Sen 153R	5'-ACTTG GGTTC AATGC TTCAA CAGT-3'
Sen 158F	5'-AACAT GAGTT CTGCT CAACC TGCAG-3'
Sen 158R	5'-CATCA GCAAG AAATT TCAAA CACCC-3'
Sen 193F	5'-AGTTC TCCGT GGAGG AAAGG AATT-3'
Sen 193R	5'-AACCT CAGCA CTGAT AAGCT CCAA-3'
Sen 219F	5'-TCTTA TCTTG CTCTA GCAGA GCCA-3'
Sen 219R	5'-CACAG TAGCT TCAAG CTCCA GAGT-3'
Sen 281F	5'-CCCTC TTCCC TAATC CAATT ACAT-3'
Sen 281R	5'-ATCTT GAACT CCACA TCTAG GCAT-3'
Sen 338F	5'-ACAAA GAGGC TTCCA AGTTG AAGG-3'
Sen 338R	5'-ATATC CACAG GTCCA AATGG CATG-3'
Sen 1-9F	5'-CCCAG CTCAA CACTT TCCAG TAAT-3'
Sen 1-9R	5'-CAGGT TCACA GAACT CTCCA CCAA-3'
Sen 3-5F	5'-CGCAT GAGTA AAGCA AAGAA AG-3'
Sen 3-5R	5'-AGAAA TTAGA AGCCA TTCAG GG-3'
Sen 3-7F	5'-GATGG AACAC CTTAC TCCAA CT-3'
Sen 3-7R	5'-TACGA GCCAG AAATC CCTAC AA-3'
Sen 8-5F	5'-CAGCA GAGAG GAATA GACTA TGAG-3'
Sen 8-5R	5'-TATGA ACAAG TCTGT CTCCC AGAC-3'
Sen 11-1F	5'-AATCA GTAAG AAGTG CGCTC GGAG-3'
Sen 11-1R	5'-TTTAC CCTGC ATCCA ACTCT GCAG-3'
Sen 11-6F	5'-AAGGG GCCAG TTGCA ACTGA ATTA-3'
Sen 11-6R	5'-AGCAA CGATG TCAGC ACAAG AAAC-3'
Sen 14-7F	5'-CTGTT TGCTG AATTT TGGAA GTGG-3'
Sen 14-7R	5'-CCAAA ACTCT TCCTC TTTCA GGAT-3'
Sen 16-8F	5'-TAAGG CAAAA GGCAA AAGGG TG-3'
Sen 16-8R	5'-ATTGG AACAA TGACA CGTGG AG-3'
ICL-R	5'-GAAGT TGCAG AGGTT CAGGC ATGGT-3'
ICL-F	5'-CACGT CCTTG TTCGT TCGAT ATACA-3'
Actin2-R*	5'-CCACT GAGGA CAATG TTACC ATAG-3'
Actin2-F*	5'-CTTGA CTATG AGCAA GAACT CGAG-3'

*Sequences obtained from *Phaseolus acutifolius* ACT2 cDNA (GenBank Accession no. AY338231).

have demonstrated that six out of seven isolated cDNAs show distinct similarities with genes known to be induced by processes involving senescence and pathogen attack. In our study, signal transduction may have been mediated within cell compartments by a series of phosphate cascades through protein kinase (Sen 83 and Fig. 3A) and protein phosphatase (Sen 14-7 and Fig. 3F), the former beginning gene expression in the early stage of senescence up to the end of final cotyledon development. A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase 2 showed transcript accumulation in yellowing cotyledons (Sen 219 and Fig. 3B). These results indicate that the cDNA encodes a 9-cis-epoxycarotenoid dioxygenase 2, and that its products have a key role in the synthesis of ABA under drought stress during the last stage of cotyledon senescence.

Osmotic stress-induced proline oxidase (Sen 338 and Fig. 3C) is another enzyme for ABA synthesis during the maturation of plant organs (Qin and Zeevaart, 2002). According to our RT-PCR results, osmotic stress was a significant stimulus in development, especially during cotyledon senescence. A gibberellin-stimulated gene (Sen 1-9 and Fig. 3D), which was strongly repressed at the beginning stage, is also responsive to light regulation for synthesis of MGDG (Yamaryo et al., 2003). Thus, our observation may reflect changes in endogenous GA levels in senescing organs. However, in preliminary experiments, a *GA-protein*-like gene also exhibited a dark response in detached cucumber cotyledons (data not shown). Therefore, further investigation is needed into the behavior of this gene.

Finally, the serine carboxypeptidase gene (Sen 11-1 and Fig. 3E) may be another candidate as a senescence-related gene. Its transcript increased gradually from the green to yellow cotyledons. A number of other putative SAG cDNA clones from our study, however, had no obvious senescence-related functioning while many others still remain to be identified. From these primary outcomes, however, we will continue to elucidate the precise biological roles of such genes during cucumber development, utilizing specific approaches such as anti-sense experimentation, proteomics, and functional genomics research.

ACKNOWLEDGEMENT

This work was supported in part by a Chungbuk National University Research Grant in 2004.

Received June 8, 2004; accepted July 6, 2004.

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