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Coordinate Expression of Senescence-associated Genes in Both Cotyledon and Petal Development of Cucumber (*Cucumis sativus* L.)

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Abstract: We investigated expression of cucumber senescence-associated genes (SAGs) from developing cotyledons and flower petals. Several cucumber SAGs have been reported in earlier reports. This is an extension of the previous findings. Semi-quantitative RT-PCR revealed that most of the cucumber SAG transcripts were consistently produced until the organ senescence. These results imply that many cucumber senescence-related genes are still active during the final development stage, playing some executive biological roles, possibly in remobilization of nutrients to the other parts of tissues or organs. These results were used to search for possible functions of senescence-related genes during organ development.

Key words: Cotyledon, cucumber, petal, RT-PCR, senescence, starvation

Senescence can be easily observed in the manner of cotyledon yellowing and petal wilting in developing cucumber plant. Furthermore, senescence takes place at every stage of natural plant organ development and is the final developmental stage controlled by planned gene expression. Cotyledon senescence is a noticeable example in young cucumber plants. Similarly, flower petal senescence shows principally short life of developmental pattern as a part of reproductive organ that displays complete pathways of programmed cell death (PCD) by rapid and synchronous pattern. This process is age-dependent, and both endogenous and exogenous factors can significantly influence its onset. The death of petal cells is preceded by a loss of membrane permeability, due in part to an increase in the levels of reactive oxygen species (ROS) that are in turn related to up-regulation of oxidative enzymes and a decrease in the activity of certain protective enzymes. The senescence process also consists of a loss of proteins caused by activation of various proteinases, a loss of nucleic acids as nucleases are activated, and enzyme-mediated alterations of carbohydrate polymers (Rubinstein, 2000). However, in this precisely controlled sequence of biochemically and physiologically degenerative events, nutrients are recycled from senescing tissues 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). Since an organ's senescence is developmentally programmed, this stage is a clear example of PCD in plant development (Gan and Amasino, 1997). In the final stage of organ development, senescence leads to cell death. Two distinct types of cell death can be described in plants. The first, of which the hypersensitive response to invading micro-organisms is an example, is limited to a relatively low number of cells and requires a short time between external stimulus and death. In the hypersensitive response, rapid cell death is required as the dead cells pose a barrier to the invading organism. The second type, of which leaf and petal senescence are examples, is characterized by export of valuable materials and takes considerably more time.

Intra-cellular lipid mobilization was previously thought to operate primarily during the germination period, before photosynthetic competence is established in oil-seed plants. However, senescence also includes the utilization and metabolism of fatty acids associated with gluconeogenesis. Furthermore, degradation of proteins, polysaccharides and nucleic acids results in mobilization of sugars and nitrogenous compounds, before visible senescence takes place. Massive mobilization of degraded small molecules is common to all senescing organs. There are three possible signals for the molecular mobilization: maturation, starvation

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and sugar accumulation (Doorn, 2004). Therefore, the organ senescence may occur due to sugar starvation or sugar accumulation. A hypothesis that sugar starvation is the direct cause of leaf senescence (Thimann et al., 1977) has been proposed. In a way, sugar starvation may be involved in leaf senescence because application of sugars to cut flowers generally delays visible senescence. A role for sugar starvation is also suggested by the similarities between starvation-induced changes in cell physiology and those observed before cell death during senescence (Quirino et al., 2000; Lam et al., 2001). However, high sugar concentrations in petals show the first visible senescence symptoms (Masclaux et al., 2000; Yoshida, 2003). Therefore, the role of sugars should be further studied, if any, in petal and cotyledon senescence. These lines of experimental evidence indicate that metabolic status may be a vital signal for leaf and petal senescence in plant system.

This research paper discusses the possible role of selected cucumber genes in cotyledon and petal senescence. The discussion is mostly relevant to petal senescence but also draws some parallels with cotyledon and leaf senescence and may have a bearing to senescence-related cell death in general.

MATERIALS AND METHODS

Plant material, growth and organ collection

Seeds of cucumber (*Cucumis sativus* L. cv. Cheong Nak Hap) were obtained from Heung Nong 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 (130 μmol m^{-2} s^{-1}). Cotyledons were collected for total RNA isolation at three stages of senescence (early, mid-, and late) as defined by their chlorophyll contents (Delorme et al., 2000). Cucumber florets were obtained from growing cucumber plants. Only male flowers were collected 12 to 15 weeks after the seed imbibition from cucumber plants for total RNA isolation. Flower petal development was also divided into three senescence stages, early (mature flower bud, stage I), mid (opened petal, stage II) and late (wilting petal, stage III). The stage I florets were collected around 24 h before the petal opening. The stage II flowers were collected from cucumber plant after 24 h of petal opening. The stage III flowers were detached from cucumber plant after 48 h of petal opening which usually showed visible signs of petal wilting. All samples were immediately frozen in liquid nitrogen and stored at -70°C freezer for total RNA preparations.

Total RNA preparation and quantification

An RNAeasy Plant Mini Kit (Qiagen GmBH) was used to extract total RNA from cucumber cotyledon and petal samples treated with DNase I (Qiagen GmBH). Three pairs of each cotyledon and petal were used from classified developmental stages 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 amount of total RNA at 260 nm on the basis of single cotyledon and petal.

Reverse transcription (RT) reaction and PCR for cucumber SAGs

RT-PCR was used to examine their levels of gene expression during organ development. For the first-strand cDNA synthesis (RT), poly$(A)^+$ RNAs were primed by the oligo dT_{18} primer, using an RT-Premix kit following manufacturer's instruction (Bioneer, Korea). Basic RT-PCR was performed as described in previous report (Kim, 2004). Equal volume (10 μl) of RT-PCR products were fractionated on a 1.2% (w/v) agarose gel in 0.5X TBE buffer. The primers were designed using the cDNA sequences in the appropriate region of the cDNA. The primers were synthesized from the Bioneer Co. (Daejeon, Korea). The primer sequences are Sen 60F 5'-GCAAG TGTGC TTAGT CGTAA GCTTG-3', Sen 60R 5'-CCATG CTGAG GTTGA GTAGT TAAA-3', Sen 87F 5'-ATTAC AACAT CCAGA AGGAG TCCAC-3', Sen 87R 5'-ATCAT TCAAA GACAA TCACC AACA-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 TGC AG-3' Sen 158R 5'-CATCA GCAAG AAATT TCAAA CACCC-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'. As a control of constitutive expression gene, mRNA of *actin 2* (*actin*; GenBank accession no. AY338231) was amplified with internal DNA sequence primers: actin2-R 5'-CCACT GAGGA CAATG TTACC ATAG-3', actin2-F 5'-CTTGA CTATG AGCAA GAACT CGAG-3'. All semi-quantitative RT-PCRs were performed three times from three separate sets of total RNA extracts. Genes showing consistent results were chosen from the repeated RT-PCR procedure and one of the best gel images is presented in this report.

RESULTS AND DISCUSSION

Changes of total RNA level and gene expression of cucumber genes

The quantity of total RNA reached a peak in the green-mature cotyledons (stage I) and in the fully opened flower petal (stage II), but decreased sharply during their senescence (Fig. 1). The rapid decrease of total RNA levels

corresponded well with the sharp decrease of chlorophyll contents in cotyledons during development as we have presented in earlier report (Delorme et al., 2000). However, the decline of total RNA level in senescing petal is almost sudden in the wilting cucumber flower. The contents of total RNA are extremely low (≥2.0 μg per petal) in the wilting petal due to shrinking of house keeping genes including *actin*. According to this primary observation, we could anticipate that *actin 2* gene expression must be falling to ground level at the end stage of development. Based on this observation, we followed the changes in total RNA contents to study gene expression levels by 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* (isocitrate lyase) gene as a positive control of gene expression in senescence, because its transcript levels showed similar patterns to what had been revealed in our previous northern blot analysis (Kim and Smith, 1994). Again, we were able to demonstrate the validity of our semi-quantitative RT-PCR in the previous study (Kim, 2004). The *actin 2* gene (*Phaseolus acutifolius*, GenBank Accession no. AY338231) DNA sequence was used as a control for evaluating relative changes in corresponding mRNA contents (Fig. 2). The primers share 92% homology with other plant *actin 2* gene sequences. The *actin 2* mRNA levels corresponded well with the changes in total RNA amounts during cotyledon and petal development (Figs. 1 and 2).

Cucumber SAGs expression in cotyledons and petals and expected biological function

We have obtained about 400 of cDNA clones from senescing cDNA library of cucumber cotyledons and analyzed sequence similarity by web supporting GenBank databases via Blast search from NCBI (Altschul, et al., 1990). We were able to identify several SAGs in cucumber including novel genes such as MMP 2.21. Moreover, the cucumber SAGs expression levels were examined by RT-PCR technique in the senescing cucumber cotyledons. From the

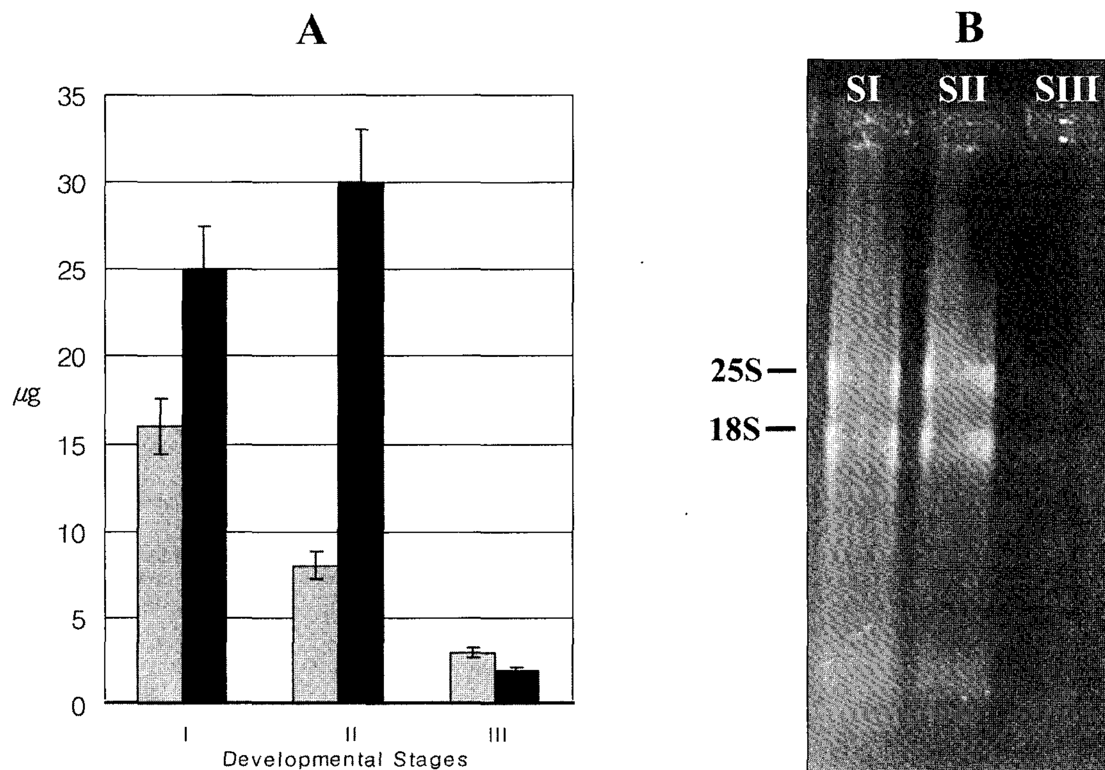

Fig. 1. Developmental changes of total RNA contents during senescence of cucumber cotyledons and flower petals. A, Total RNA contents in a single cucumber cotyledon (grey bars) and flower petal (black bars) during senescence. Three pairs of cotyledons and three individual flower petals were used for extraction of total RNA at each stage. Total RNA was dissolved in equal volumes (100 μl) of DEPC-treated H_2O for each extraction, then an equal volume (50 μl) of total RNA solution was used to examine concentration at 260 nm. Stage I, early stage of senescence (≤ 50% yellow) at Day 21 ~ 28; stage II, mid-stage of senescence (~70% yellow) at Day 28 ~ 33; stage III, late stage of senescence (>95% yellow) at Day 35 ~ 40 for cotyledon. Flower petals were collected by the developmental turnover from the beginning of the flower bud formation then classified as stages I, II and III as shown in Figure 2. B, Electrophoretically fractionated total RNA of developing flower petals on a 0.5X TBE agarose gel (1.2%) stained with ethidium bromide. One fifth of each total RNA extracts from a single petal were loaded in a well and 25S/18S indicates major rRNA bands.

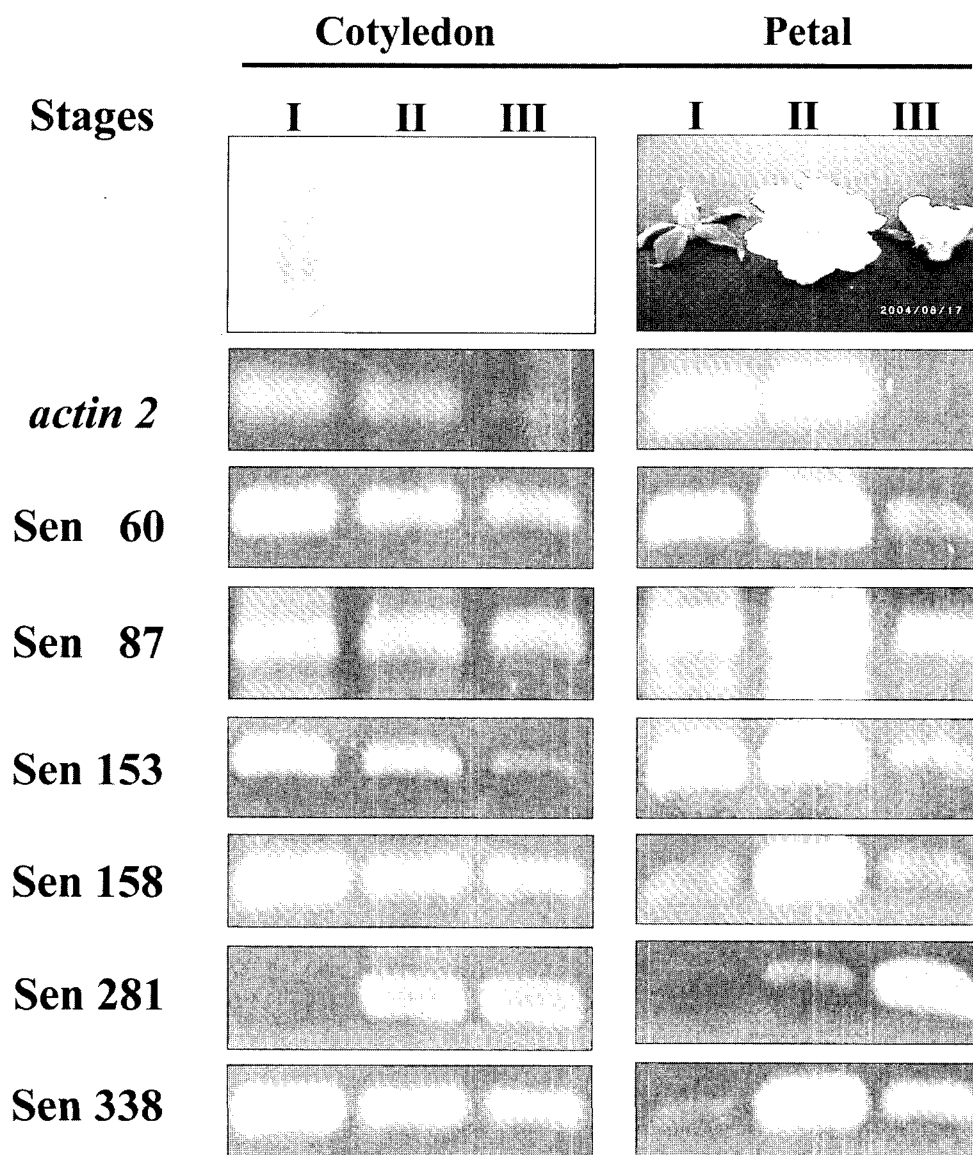

Fig. 2. Agarose gel electrophoresis images of semi-quantitative RT-PCR products for the senescence-associated cucumber genes. First-strand cDNA synthesis was performed on per-cotyledon [1.6 μg for stage I (early senescence), 0.8 μg for stage II (mid), and 0.3 μg for stage III (late)] and per-petal [2.5 μg for stage I (early senescence), 3.0 μg for stage II (mid), and 0.2 μg for stage III (late)] basis of total RNA: Equal volumes (0.25 μl each) of first-strand cDNAs from each developmental stage was used for PCR with gene-specific primers. Lanes from left to right, senescence stage I, II and III. Genes are *actin 2* (from *Phaseolus acutifolius*) as constitutive expression control; Sen 60 (cytochrome P450-dependent monoxygenase); Sen 87 (polyubiquitin); Sen 153 (cytosolic superoxide dismutase); Sen 158 (matrix metalloproteinase 21.2); Sen 281 (*Cs1-MMP*); and Sen 338 (osmotic stress-induced proline oxidase).

previous outcomes, we wanted to see the cucumber SAGs are truly involved in the senescence of other parts of the plant such as flower petal. Using the same approach in flower petals, we identified a few clones that are coordinately expressed, in part or as a whole, in both cotyledons and petals senescence (Table 1).

Firstly, hundreds of cytochrome P450-dependent monoxygenase (Sen 60) has been isolated from various organism including bacteria, animals and plants. However, relatively a small number of P450s has been cloned from plants. The P450s mediate a wide range of oxidative reactions involved in the biosynthesis of secondary metabolites, including the phenylpropanoids and phytoalexins. Two P450s catalyze reactions of general phenylpropanoid pathway in plants: cinnamate-4-hydroxylase (C4H) and ferulate-5-hydroxylase (F5H). The products of this pathway are critical to plant survival. Furthemore, flavonoids and hydroxycinnamic acid esters as well as lignin are crucial components for plant cells. The cytochrome P450-encoding plant gene *CYP76C2* is preferentially expressed during the hypersensitive response

Table 1. Cucumber senescence-associated cDNA clones from senescing cucumber cotyledon and their amino acids sequence identity with other known genes or cDNA from various organisms

Clone#	Enzyme identity	Id* (/)**	Score	e-value	Source organisms
060	Cytochrome P450 monooxygenase	67 (104/153)	214	2e-54	*Zea mays* (AF465265)
087	Polyubiquitin	100 (190/190)	370	e-101	*Antirrhinum majus* (S25164)
153	Superoxide dismutase	90 (137/152)	286	3e-76	*Ipomoea batatas* (Q07796)
158	MMP21.2-like protein	62 (18/29)	44	3e-04	*Arabidopsis thaliana* (BAB01942)
281	Matrix metalloproteinase	100 (134/134)	211	2e-54	*Cucumis sativus* (CAB76364)
338	Proline oxidase	65 (140/214)	279	2e-74	*Arabidopsis thaliana* (NP_189701)

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

(HR) provoked by bacterial pathogens in *Arabidopsis*. The *CYP76C2* gene expression is also associated with various processes leading to cell death, such as leaf senescence and other stresses (Godiard et al., 1998). We have shown gene expression of cytochrome P450-dependent monoxygenase in senescing cucumber cotyledon in a previous report (Kim, 2004). Cucumber P450 shares 67% of amino acid sequence homology with maize. Therefore, if plant senescence is considered as developmental stress, we would 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 in cucumber petal and sustain its gene activity until the end of petal development (Fig. 2). However, we need to further carry out the study to identify its biological function in cucumber plants.

Secondly, polyubiquitin (Sen 87) has been shown distinct pattern of RT-PCR in cucumber cotyledon development in the previous report (Kim, 2004). Cucumber petal also showed a similar pattern with multiple forms of PCR band in this study (data not shown). Cell removal through programmed cell death is an evolutionarily conserved phenomenon found from embryological development to immune homeostasis in animal systems. The relationship between ubiquitin and apoptosis is only beginning to be defined. In the past few years, several reports of polyubiquitin conjugation of key pro-and anti-apoptotic molecules have characterized ubiquitin as an essential regulatory modification targeting proteins for proteosomal degradation. In recent research, the relationship between monoubiquitin and polyubiquitin conjugation to apoptotic molecules has revealed an additional level of diversity to the role of ubiquitin in apoptotic regulation beyond degradation. Polyubiquitin serves as a recognition signal in *retro translocation* of proteins across the ER membrane back into the cytosol, where they are degraded by the proteasome. Although many of the apoptotic signaling pathways have been elucidated, the relationship between ubiquitin and apoptosis is only beginning to be defined. Furthermore, absolute homology within the polyubiquitin sequences in the plant kingdom may explain its importance in plant senescence like animal system. Polyubiquitin-encoding cDNA sequences have been reported from dark-incubated, senescing *Arabidopsis* leaves (Park et al., 1998). Its biomolecular functions in plant senescence has not been confirmed, but this protein is involved in apoptotic regulation and recognition signals in animal cells (Flierman 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 and petal senescence (Fig. 2). Therefore, it can be presumed that polyubiquitin may be functional in both cotyledon and petal senescence in cucumber development, particularly· for organ's senescence.

Thirdly, leaf senescence is strongly associated with increased oxidative damage to macromolecules by 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 of certain antioxidant enzymes such as ascorbate peroxidase and catalase. We have shown such a response to be associated with an increase in superoxide dismutase (Sen 153) in the chloroplasts (Kim, 2004). Its enzyme-mediated lipid peroxidation products are produced via lipoxygenases. However, peroxidase activity has been shown to rebound by the induction of antioxidant defenses resulting in a decline in lipid peroxidation and the onset of visible signs of leaf senescence as the reproductive stage (Ye et al., 2000). Thus, ROS increases are associated with further floral development due to programmed declines in certain peroxidase activity, leading to 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). Here, the expression level of cucumber superoxide dismutase gene or genes are relatively active in the middle of senescence process in both cotyledon and petal (Fig. 2). Again, we need to do genetic and biochemical studies to elucidate the exact function of these genes in cucumber senescence.

In fourth, the matrix metalloproteinase enzymes are found in most of cellular organisms from bacteria to vertebrate species (Basset et al., 1990; Lepage and Gache, 1990). The metalloproteinase gene has also been identified in cucumber as *Cs1-MMP* gene (Delorme et al., 2000). The *Cs1-MMP* gene (Sen 281) is expressed strongly at the very late stage of cotyledon senescence (Kim, 2004). Its various functions include these in 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 until today. 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 (Delorme et al., 2000). Again, our RT-PCR revealed that its strongest expression was in the late stage of cotyledon and petal senescence (Fig. 2). As a related class of metalloproteinase, we reported a novel MMP21.2 encoding a cDNA clone (Kim, 2004), whose corresponding gene (Sen 158) expression was detected at the beginning of visible senescence and the final development of cotyledon (Fig. 2). But the *MMP21.2* gene is most active in the fully open petal. Although slight differences were found from the two MMPs gene expression, they can be classified as strong candidates as the senescence associated genes in cucumber.

Finally, cellular water reserves are the critical factor for plant cell survival. The developing leaves experience osmotic stress throughout senescence. Osmotic stress will induce expression of many stress related genes. Osmotic stress-induced proline oxidase (Sen 338) is an enzyme for ABA synthesis during maturation of plant organs (Qin and Zeevaart, 2002). According to our RT-PCR results, osmotic stress is a significant stimulus during development, especially organ senescence. In particular, we can presume that flower petal may encounter severe water stress at the time of petal opening during unique flower development: e.g. rapid cell expansion. Therefore, strong action of osmotic stress responsible genes can be expected in the final stage of organ senescence as a part of defense mechanism. From these extended outcomes in petal senescence, 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 broad transcriptomic study in the near future.

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