## V. Structure of din1 Gene

<INTRODUCTION>

Until now, a number of genomic DNA clones have been isolated from animals, plants, insects, and other organisms, and a large amount of information has been accumulated about the relationship of gene structures to the regulation of corresponding genes. In plant kingdom, structure of genes which respond to such environmental signals as light, temperature, pathogen infection and phytohormones have been extensively studied, and specific sequences for the response to these signals have been analyzed for some of these genes.

CH5B, an endochitinase gene of bean, is regulated by ethylene and its upstream DNA sequence was shown to contain regulatory elements for induction by ethylene (Broglie et al., 1989). Increased ethylene biosynthesis has been shown to accompany with fruit ripening, leaf senescence, wounding, and pathogenic attack (Toppan, 1982). In the case of bacterial or fungal attack, ethylene production has been shown to correlate with the induction of several proteins, hydroxyproline-rich glycoproteins, β-1,3-glucanse, chitinase, that presumably function in the defense response of plants. The chitinase catalyzes the hydrolysis of chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine. No endogenous substrate for this

enzyme is known to occur in higher plants, but chitin is a ubiquitous component of the cell wall of fungi (Abeles, 1970). For this reason, it has been suggested that chitinase functions as a defense against chitin-containing pathogens.

A consensus sequence that confer responsiveness to heat shock has been found in hs genes of various organisms, e.g. yeast, slime mold, amphibia, nematodes, mammals, and plants (Nover, 1987). The consensus HSE sequence has been originally deduced from Drosophila hs genes as a short, 14 bp palindromic sequence containing a symmetrical sequence of 10 bp, 5' CT-GAA--TTC-AG 3', which represents the binding site for the hs transcription factor. In plant kingdom soybean hs genes have been extensively analyzed, and 10-bp consensus sequences of HSE was defined within palindromes of 18 bp (5' G--T-TTC--GAA-A--C 3') (Schoffl et al., 1989). The sequence of soybean HSE partially overlaps with that of Drosophila HSE.

Up to 40% of the total soluble protein of potato tubers is represented by a family of immunologically related glycoproteins, that are named as patatin. The high content of patatin in potato tubers argues for its function as a storage protein. Patatin is encoded by a gene family of more than 10 members per haploid genome, which consists of class I and class II subfamilies. Class I patatin genes are expressed mainly in tubers and their transcription is inducible by a high concentration of sucrose. A repeated sequence was found in upstream region of Pat 21 patatin gene and was conserved among

class I members. This repeated sequence was, therefore, suggested to contain a sucrose-responsive element (Jefferson et al., 1990).

As already described in this thesis, din1 responds to various stimuli, such as light condition, ethylene, heat stress, and cytokinin. It has also been found in Chapter IV that the gene responds to light condition by sensing the accumulation level of sugars in the cotyledonary cell. I am interested in the structure of din1 gene, especially in the upstream region, because it may contain a clue to find the mechanism of response of the gene to these stimuli. Therefore, I have decided to isolate genomic clones of din1 gene and determine its nucleotide sequence. The sequence data will enable me to obtain some informations on the regulatory mechanism of the gene by comparison with sequences known to work as regulatory elements.

<MATERIALS AND METHODS>

1. Construction of Genomic Library

Radish seedlings were grown in the dark at 25°C and cotyledons were harvested after 7 days of growth. The cotyledons were frozen in liquid $N_2$, ground and lyophilized. Genomic DNA was extracted from the tissue powder according to the method of Rogers and Bendich (1988). After being purified by centrifugation on a CsCl gradient, DNA was partially digested with Sau3AI and separated by size by centrifugation on a 10-40% sucrose gradient (Ausubel et al., 1987). DNA ranging between 15-25 kbp was recovered, ligated with EMBL3 BamHI arms, and packaged in vitro using a Gigapack system (Stratagene). E. coli VCS 257 cells were infected with the phage, and a library of $10^6$ was obtained.

2. Analysis of Genomic Clone

Three positive clones were isolated from the library by hybridization with a probe prepared from clone-1 as described in Chapter II. As these clones showed an identical physical map in respect to restriction sites for HindIII and EcoRI, one of the clones, λGDI1 was used for the structure analysis.

Overlapping restriction fragments of λGDI1 were subcloned into M13 phage RF DNA for sequencing. Nucleotide sequence was determined by dideoxynucleotide chain termination method with an automated DNA sequencer (Applied Biosystems, Model 370A). The nucleotide sequence was analyzed by a computer program, GENETYX (Software Development Corp., Tokyo).

### 3. Determination of Transcription Initiation Site

Transcription start site was determined by primer extension method. Primer DNA (5'-CCCGATTCGTGCGGTTGTGT-3') which is complementary to sense-strand sequence between +65 and +84 relative to transcription start site was synthesized at Center for Gene Research, Nagoya University. The primer DNA (40 pmoles) was labeled with $^{32}P$ by T4 polynucleotide kinase (Takara). Poly(A)$^+$RNA was prepared from radish cotyledons that were kept in the dark for 24 h as described in Chapter II. Five μg of poly(A)$^+$RNA was mixed with 2 pmoles of the primer DNA in 6μl of $H_2O$, heated at 65°C for 10 min, and cooled to 4°C. The primer DNA was extended at 37°C for 30 min in 10μl of reaction mixture; 50 mM Tris-HCl pH 8.3, 8 mM $MgCl_2$, 10 mM DTT, 0.1 mM dATP, dCTP, dGTP, TTP, 20 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia). Sequencing reactions were carried out by dideoxynucleotide chain termination method with the same labeled primer and a M13

clone, pEX1 which contained XbaI-XbaI DNA fragment encompassing translation start codon (Fig.5-2). The primer extension mixture was electrophoresed in parallel with the sequencing reaction mixtures on the same sequencing gel.

<RESULTS>

I isolated three independent genomic clones, λGDI1, λGDI16, λGDI18, of which restriction maps overlapped each other (Fig.5-1). In addition, clone-1 probe detected only one band in a Southern hybridization experiment with HindIII digests of genomic DNA. It is most likely that din1 is a single copy gene. Therefore, a region covering din1 gene of λGDI1 was sequenced (Fig.5-2).

Comparison with the cDNA sequence showed that din1 consists of 5 exons and 4 introns. All the introns began with GT and ended with AG. Introns were all AT rich and ranging between 80 to 100 bp. Transcription start site was located at 48 bp upstream from translation start codon by primer extension method (Fig.5-3). A stop codon, UAA, was found in 5' non-translatable region at +13 bp from transcription start site. This stop codon is in the same reading frame of din1 gene. No ATG codon other than the translation start site was found in the 5' non-translatable region. TATA box-like sequence was found at -11 bp from the transcription start site (Fig.5-2 underline 5). CAT sequences were found at -73, -93, -102, but CAAT sequence was not found up to -243 bp (Fig.5-2). Polyadenylate tract found in the cDNA sequence was added at +1072 bp relative to transcription start site.

I examined the upstream sequence of din1 between +48

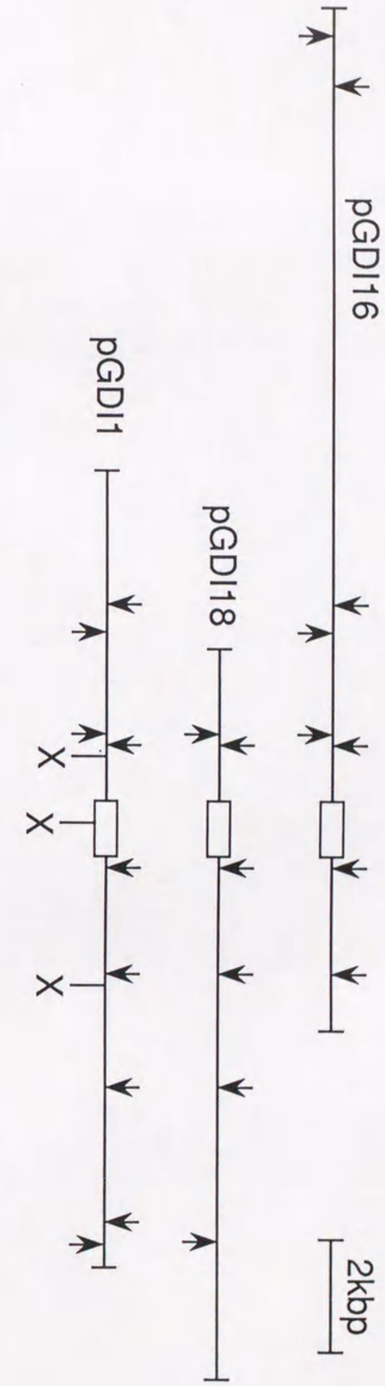


Fig.5-1 Restriction map of genomic clones. din1 gene region was shown by boxes. Upward arrows, downward arrows, and X indicate EcoRI, HindIII, and XbaI recognition sites, respectively.

Fig.5-2 Nucleotide sequence of din1 gene between -802 and +2124 relative to the transcription start site. Exon sequences are capitalized. Underlined sequences marked with numbers are putative TATA box and sequences having homology with known sequences (see text). Arrow head indicates the polyadenylation site. Underlined sequences marked with asterisks are XbaI sites used for cloning of pEX1 for primer extension assay.

```
                                                    tc tagactcagg agactataca tgaatcacac
-770 taaagcgatg tacagtacca aaaacaaccg cataatcact gttcaagaca tcgccacaag taccaccacc
-700 aacggggcaa aatgcatcgg agccagtaag tttaaggtag gttaaatacg cggtgtctaa tatcccgata
-630 ccgccgagtc cggtgaacca gttgtacgtc gaactgttaa tagatacttc acttgttgac gacgatgagg
-560 aggaggaaga cagcgacggc gccgaatcca caccgtcttc agtctcagac gacgagcatt tgatcgggaa
-490 tctccggtga gagacctgat taagaacata tgacttcaat taaccaaatc acactaccat cgcagaagag
-420 aatgaaacga gagttacctc gaaacgtctc ggataaagag aagacgaaga aggaggaagc tcacggaagc
-350 cgaaacgaaa ctgacaagac gacacaggga taaacctcgc catcatcatc gtcgtcgtcg aacgatcgct
-280 aatccactat tgtcaaaaaa gattccatcc attacaatat tgggcctaag gcccaagtaa tatttagagt
-210 tggggcctat taagaaacgt atagtagtat ctaatccgag tcagcacgtt atccatatta cgacttacga
-140 ggtaaccgac ttggacttat caagatccat tctccgcatg gcttacatgt tggaaccgga tagatcatgt
 -70 aaaactggct gacgtgtcgc tggttccata tcctttggct ttgtgtctgt gtatatatag ataacatgca

  +1 cgagaagaag tataactcaa agtatctacg aacacaagaa gaaaaaaaAT GGAATCTACT TTAAACACAA
 71 CCGCACGAAT CGGGAGCTGG TCATCGTTTA TATCTCCACC TCTACAAGTG TGTGAATCTT TCAAGTGGAA
141 GCTACCAAAG GCAACAAGAA GAGTCGTCAG TGTTGCAGAT CGTCAGAACT CAAACTTCCG ATGgttagct
211 aacgttttgg aatctctctt agcattagag gaaatgactt aagatttaag ttatttattc aattgttttt
281 ttttttgaag gagGAAAGTA ACAACAGGCA GAGCAAATGT TGCGGCAGAG GCAGCAGCTA GAGTTCCGAC
351 ATCAGTACCG GTGAGAGTTG CGCGTGACTA GCTCAAGCAG GATACAAACA TCTTGACGTC AGGACACCGG
421 ACGAGTTCAG TATCGGACAT CCGTCTAGAG CTATTAACGT GCCTTACATG TACAGAGTCG GGTCAGgtta
491 ttctttcaac cgacctttg gtttcctcta tataaaaaaa agttgaaact aaaccaaagc gtaaaaaatg
561 ttacagGAAT GGTTAAGAAC CCGAGTTTTC TAAGGCAGGT ATCGTCTCAT TTCAGGAAAC ACGACGAGAT
631 CATCATCgta agtttttaag ttttgattaa aatcttgagg aacagttatg aaaacaaact catattataa
701 aaccattttg aggcgtcttt atgaaacatt gcagGGTTGT GAGAGCGGAG AAAGATCTCT CATGGCTTCC
771 ACTGAACTTC TCACTGCTgt aagtgtgatt tatttatgtg atagtatcgg tcaaaacaga gaaagcggta
841 taatgctgcc ttgtgtgtat gtctacagGG CTTCACCGGG GTTACAGACA TTGCTGGAGG ATACGTTGCC
911 TGGACAGAGA ATGAACTACC AGTAGAAGAG tgaaaacaaa aaaaaacatg gacctatccg tttgtaaatt
981 tgcaaataaa taattgtata tgtttcaaca caagtgtttg tgtatgtcaa tggcactacc aaatgatata
1051 agtttcattt ggaacaaaac aatgataaca tattggcaca gtgtttctgt ggtgactcgg ttccatggat
1121 tcgaggggaa gagattaagc aaccgaatat tgtccctagc ctctgaaatt agcaatcagg aaaagaacaa
1191 taataatagg agggcctgac aataagaatc ggcctttcaa atttcaatcc taacacagca aagcttgtta
1261 gggcccatat tcgcttggtg tgcccaacta gccaactggt ctactgaaat aaaaatagat cagtgcggag
1331 agatgtacaa aactaaagaa aactacacct atactctctc ttaagagact taacacaagt aaaaaaagtg
1401 ccaacattcg caaagttaaa ggtagtatta agtttaatga ccacaataga aatgtgttat tattgaaata
1471 atagtgtatt tatttatatc aagaaaaaaa caagaatgga ggaatattac acagttgtca aaggaagtgt
1541 gacgacgtgg caaatagcaa tcagactatc aatgattggt cactgatatt ttttcctcca ggtactttat
1611 tctcttccac cagttcatgc ttgacgtttt tctgcgccca tgatgatcca ttgctagtat attaccctaa
1681 gaaaaataat acctctaccg gtcgatgagc ttaaaaaact aattgacgac cgtgagatac gaggtactaa
1751 tgatatgatt acaatcagag gatgacacgt ataagcaaga gataagccat tgtctcgtcg ggtacgttaa
1821 acccacctga gacgcctttt ttgttattca ttacatgata acttgttaaa ggtttcctta cattaacagc
1891 ctcacagtat caagtatatg caaagaagag ttaatatctt agtatctttt atttatggtt atatatatat
1961 atatatatat atactcaaag acaacgattc cactatttta atgaactgat ttcaaaataa caacgataaa
2031 tcgtagtgag aaattataaa atcgcgtaag tacacaagca aaaaggaact agtatgtttc taggaggata
2101 cgtgagatta aaaagagggg gcaa
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Fig.5-3 Determination of transcription start site by primer extension. Oligonucleotide complementary to a coding sequence between +65 and +84 was synthesized and used as a primer in extension reaction. The reaction mixture (lane 1 and 6; lane 6 contained 4 volumes of the reaction mixture of lane 1) was electrophoresed on a sequence gel with sequencing reaction mixtures (lane 2-5) which were obtained with the same primer and a M13 clone, pEX1. The allow indicates the full-size product of the extension reaction. Lane 2, C; lane 3, T; lane 4, A; lane 5, G. Nucleotide sequence shown on the right indicates that of sense strand.

and -802 to find homology with known sequences suggested to be required for response to sucrose (potato patatin gene), ethylene (bean chitinase gene) and heat stress (Drosophila and soybean heat shock genes).

Two short sequences of din1 between -388 and -377 (ATAAAGAGAAGA; Fig.5-2, underline 4) and between +36 and +48 (AAGAAGAAAAAAA; Fig.5-2, underline 6) were found to have similarities (10 bp out of 12 in the former and 11 bp out of 13 in the latter) to repeated sequences of potato patatin gene, PAT 21, between -175 and -164 (ATAAAGAATAGA) and between -172 and -160 (AAGAATAGAAAAA), respectively (Jefferson et al., 1990).

An upstream sequence of din1 between -456 and -443 (TTCAATTAACCAAA; Fig.5-2, underline 1) had similarity (12 bp out of 14) to a sequence of a bean chitinase gene (CH5B) between -453 and -440 (TTTAATTAACTAAA) (Broglie et al., 1989).

Ten bp of consensus sequences of Drosophila and soybean HSE have been defined to localize within palindromes of 14 bp (CT-GAA--TTC-AG) and 18 bp (G--T-TTC--GAA-A--C), respectively (Nover, 1987; Schoffl et al., 1989). The sequence of soybean HSE partially overlaps with that of Drosophila HSE. A din1 upstream sequence between -403 and -390 (CTCGAAACGTCTCG; Fig.5-2, underline 3) had similarity (8 bp out of 10) to Drosophila HSE. Another din1 sequence between -410 and -393 (GAGTTACCTCGAAACGTC; Fig.5-2, underline 2) had similarity (7 bp out of 10) to soybean HSE in consensus sequence and also in

flanking sequence, and overlapped with the *Drosophila* HSE-like sequence of *din*1.

<DISCUSSION>

Comparison of nucleotide sequence of genomic clone with cDNA sequence shows that the gene is a compact gene consisted of 5 exons and 4 short introns. The sequence surrounding transcription start site (TGCACGAGA) is not strictly consistent with a consensus sequence of the start site, CTCATCA (Joshi, 1987). A typical TATA box was found at -11 bp from transcription start site.

The upstream region of din1 contains sequences resembling to a part of upstream sequences of a patatin gene (PAT 21), a bean chitinase gene (CH5B) and hs genes of Drosophila and soybean. Two short sequences of din1 upstream region are similar to the PAT 21 repeated sequence. The repeated sequence present in PAT 21 is conserved among class I patatin genes and suggested to contain a sucrose responsive element (Jefferson et al., 1990). If these sequences are responsible for the regulation of din1, they have to work negatively in din1 expression in response to sugar level. CH5B is a gene known to be regulated by ethylene, and its upstream sequence between -575 and -422 has been suggested to contain an enhancer element(s) (Broglie et al., 1989). A 14-bp sequence of din1 was found to be very similar to a part of this region of CH4B. This sequence is worth examining to uncover regulation of these genes by ethylene. din1 upstream sequence between -410 and -

390 contains sequences homologous to *Drosophila* HSE and soybean HSE-like sequence. This region is very likely to function as a *cis*-element in the observed induction of *din*1 by heat stress.

Ethylene is known to stimulate respiration, therefore, may cause lowering of sugar level in the cotyledonary cells. Heat stress may also lower sugar level in the cells by increased energy consumption at higher temperature. The induction of *din*1 by ethylene and heat stress is shown to be suppressed by exogenous supply of sugar (Chapter IV). These observations may suggest that ethylene and heat stress induce *din*1 expression by lowering sugar level. However, if all the genes responsive to ethylene were activated when sugar level lowers, most of the defense-related genes such as those for phenylalanine ammonia lyase, chalcon synthetase, chitinase, etc. would be induced in the dark. All of the heat shock proteins would also be accumulated in the dark-treated cotyledons. The activation of these genes would bring a profound metabolic disorder to the cotyledonary cells. It is, therefore, very likely that the activation mechanism by these stimuli is independent from intracellular level of sugars. The fact that *din*1 contains sequences similar to elements which are known to respond to these stimuli supports this view. These possibilities need to be examined by further experimentation.

# VI. GENERAL DISCUSSION

There are many ways to investigate the phenomenon of senescence, such as the observation of ultrastructural changes in cellular membranes and organelles of the cells, the assay of activities of various enzymes which may associate with the progress of senescence, the analysis of rearrangement of gene expression and cloning of senescence-associated genes, the isolation of mutants which display abnormal senescence appearance, etc. These approaches have respective advantages and disadvantages. In the first two approaches, the results directly represent structural or physiological changes occurring in senescing tissues, such as decrease in the content of Rubisco protein, increase in ribonuclease and peptidase activity, and deterioration of plasma membranes. A number of senescence symptoms have been described in the literature with these approaches. Nevertheless, the process of senescence has not been fully understood. One of the reasons is that the physiological studies have not yet been extensive enough, and another is that the changes observed with physiological approaches are likely to be a result of the progress of senescence. Therefore, these approaches have not led us to the process that initiates senescence. Molecular-biological

techniques could help us to study such molecular events that remain to be examine. If we have a molecular probe for an appropriate marker gene, of which expression is associated with the progress of senescence, we would be able to study the structure of the gene and its protein product. The regulatory system of the genes could also be uncovered.

When I became involved in this research, many years had already passed since molecular-biological techniques had been established. However, at that time reports on molecular-biological analysis of plant physiology were still very few. One of the early reports on differential screening of cDNA clones was that by Shirras and Northcote (1984), describing that they successfully cloned cDNA sequences of wound-inducible genes of potato. Another one is that by Theologis et al. (1985), in which they isolated cDNA clones of auxin-inducible genes from pea. Enlightened by these studies, I started on my research to approach leaf senescence with molecular-biological techniques. As the work by Shirras and Northcote was particularly suggestive, I prepared first cDNA library following their method of construction of cDNA library. My first aim was to clone cDNAs for marker genes of senescence. The strategy for this purpose consisted of following steps:

1) Selection of cDNAs corresponding to dark-inducible genes from the cDNA library prepared from mRNA of radish cotyledons

by differential screening.

2) Examination of the dark-inducible genes if their expression is associated with the senescence of cotyledons by testing their response to ethylene, heat stress, and cytokinin.

As a result of experiments according to this strategy, a cDNA sequence was cloned. The differential screening which used poly(A)$^+$RNA from light-grown and dark-treated radish cotyledons successfully isolated cDNA clones for a dark-inducible gene, din1. Accumulation level of din1 mRNA was about 100-fold higher in dark-treated cotyledons than in those of light-grown seedlings. However, to be a senescence-associated gene, din1 had to stand further examination.

Besides technical problems, difficulties have been anticipated in the definition of a senescence-associated gene. This problem is inherent to the breadth and ambiguity of the definition of senescence itself. Strictly speaking, involvement of a gene in senescence must wait for identification of function of the gene product. This was not an easy task. In this research, function of din1 was left to be uncovered. However, the expression mode of din1 eloquently tell us that the gene is a senescence-associated gene. din1 was activated in response to such senescence-inducing stimuli as exposure to darkness, ethylene, and heat stress. In addition, the induction of din1 was suppressed, though

partially, by a senescence-retarding growth regulator, BA. These results indicated that the expression of din1 is tightly associated with the progress of senescence, and therefore, din1 may play an essential role in the process of leaf senescence.

It was disappointing that the anti-din1 protein antisera did not detect successfully the presence of the gene product in the cotyledonary cell. This failure suggested technical difficulties to find immunochemically cellular location of the protein. Finding of the location in a cell may have provided some clues to solve the problem of knowing the function of the gene. For example, if the protein localizes within a nucleus, the observation makes it more probable that the protein is a kind of regulatory factors of gene expression. If the protein localizes in cytoplasm, the protein may be an enzyme with activity to degrade cellular components.

A nearly full-length cDNA clone, pRDI-1, contained an ORF of 549 bp. The ORF encoded a hydrophilic polypeptide of 183 amino acids, the molecular mass of which was calculated to be 20,280 D. The polypeptide appeared to be very hydrophilic, and nearly a quarter of the total amino acid residues are charged; the N-terminal half of the sequence is relatively rich in basic amino acids, and the C-terminal half in acidic amino acids. The translation product in an in vitro system showed slightly higher molecular mass than the calculated value when estimated

by SDS-PAGE, probably because of its highly charged nature. Anti-oligopeptide antisera detected in translation products from total poly(A)$^+$RNA a polypeptide the size of which is the same as that of the translation products from the poly(A)$^+$RNA selected by clone-1 or RNA transcribed from pRDI-1. transcription start site was determined at 48 bp upstream from the initiation codon of the ORF. The nucleotide sequence of genomic clone does not contain other ATG codon between the transcription start site and the initiation codon. All of these results support the correctness of the ORF as the protein coding sequence of din1.

Comparison of cDNA sequence of din1 with those of other genes showed a medium homology with cDNA sequences of PR1a, PR1b and PR1c, a group of PR-proteins of tobacco. This group of PR protein are known to be expressed in senescing leaves as well as leaves infected by pathogens, but the functions of the gene products is not known (Fraser, 1981). The homology between din1 and the genes of PR1 group is not very high, but homologous region covered all the coding region. This may suggest that these genes have a common ancestor gene, and din1 became a gene having introns but the PR1 genes did not gain introns during evolution. PR-P and PR-Q proteins are reported to have endochitinase activity (Legrand et al., 1987) and PR-2, PR-N, and PR-O proteins are known to have 1,3-β-glucanase

activity (Kauffmann et al., 1987). It is of interest that amino acid sequence of din1 protein has homology, though very low, with that of cutinase, because all of these enzymes have activities to degrade extracellular components.

din1 mRNA begins to accumulate as early as 6 h after exposure to darkness. I have not measured the sugar level in radish leaves grown under natural condition, but it is very likely that sugars are exhausted at night and din1 gene is expressed every night. Even if this is the case, it does not devalue the relationship of this gene to leaf senescence. Physiological processes including senescence consist of many series of biochemical reactions. I would like to call a series of these biochemical reactions as a module in this thesis. When a leaf cell senesces, a set of modules are selected to operate. Selection of the modules may depend on the type and physiological conditions of the cell. Some of the modules may be specific to senescence, and others may operate also in other processes. These modules are all necessary for and related with senescence. Without any one of the modules senescence process will not proceed effectively. din1 gene can be regarded as a member of genes which are expressed at an early stage of dark-induced senescence and may also work in other processes.

Analysis of biochemical signals to induce din1 gene to

express resulted in surprising finding. Accumulation level of sugars is regulating the gene expression. Sucrose has been known to delay the progress of senescence of carnation flower (Nichols, 1973). The author suggested that the delay was due to the prevention of decrease in the materials for respiration by supplied sucrose. They did not discuss about the reason why sufficient supply of the materials for respiration delays the senescence. I would suggest a possible answer to this question. Supplied sucrose maintains sugar content at a level high enough to repress senescence-associated genes in carnation flower, that are regulated by sugar level similarly to din1. As a result, senescence is retarded.

Ethylene exerts influences on a variety of cellular events of plant life cycles. Ethylene induces senescence in leaves and petals and is known to increase respiration rate, which inevitably causes lowering of sugar level in the cell. din1 expression is induced by ethylene treatment and supplied sugar suppresses the induction. These observation propose a possible induction pathway of din1 by ethylene that ethylene increases respiration rate and lowers the sugar level, and as a result din1 is activated. As the proposed scheme, some of cellular events influenced by ethylene may be regulated by ethylene indirectly or through products of primary action of ethylene, while ethylene regulates other events directly by affecting

enzyme activity or gene expression. cDNA sequences of senescence-associated genes have been cloned from carnation flower petals (Lawton et al., 1989) and from tomato fruits (Davies and Grierson, 1989), and expression of these genes were shown to be correlated with ethylene production in the tissues. Intracellular signal for the regulation of these genes has not been investigated, but are of interest.

Supplied glucose suppressed the induction of din1 by exposure to darkness, ethylene, and heat stress. These observations suggest that accumulation level of sugar is a key factor of regulation of din1 expression. Intracellular level of sugar is physiologically very important and plays a central role, or at least one of central roles, in determining the fate of the cell. The level directly represents photosynthetic activity, which is the major function of leaf cells. Decrease in photosynthetic activity means that the time has come for the cells to senesce. Necessary systems are to be activated to break down structures of the cells to redistribute their constituents to young tissues to develop. For this process, the simplest system for monitoring photosynthetic activity would be a sensor of the level of sugar. This system and senescence process enable plants to maximize total productivity of photosynthesis by removing old leaves with low activity and helping new leaves with high activity to grow. The system

consequently contributes to the survival of an individual plant in natural environment. This would be one of the biological meaning of senescence.

I believe that a progress has been made in molecular-biological approach to leaf senescence in this research. Further study of the regulatory system and function of the senescence-associated genes will bring us new understanding of senescence. New methodology of molecular-genetical approach is now developing. Mutants which display abnormally slow senescence could be isolated. Analysis of these mutations will be a great help to further understanding of the mechanism of senescence. However, senescence is still largely a black box as ever. I am successful in getting important information and a hypothesis on the mechanism, though much work has been left to be carried out to determine the function of the gene product, to identify the regulatory factors of the genes for the elucidation of the whole system of the regulation of senescence.

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## LIST OF PUBLICATIONS

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