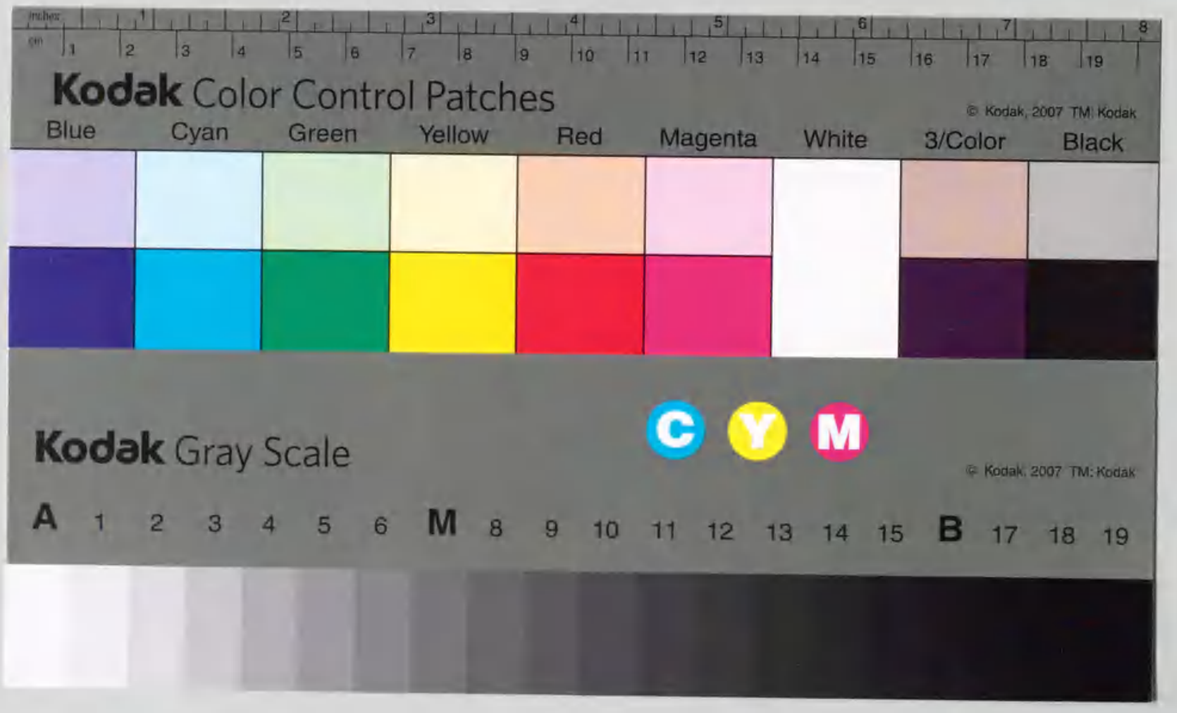
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(1)

CLONING AND CHARACTERIZATION OF A SENESCENCE-ASSOCIATED GENE, *din1*, OF RADISH (*Raphanus sativus* L.)

A DISSERTATION

Submitted to the Graduate School of Agriculture

Nagoya University

In partial fulfillment of the requirements for the degree of

DOCTOR OF AGRICULTURE

(Major: Biochemical Regulation)

by

YOSHITAKA AZUMI

Research Institute for Biochemical Regulation,

School of Agriculture, Nagoya University,

Nagoya, Japan

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CONTENTS

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BA	6-benzyladenine
βmGlc	methyl-β-D-glucoside
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine 5'-triphosphate
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
dCTP	deoxycytidine 5'-triphosphate
dGlc	2-deoxy-D-glucose
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
hs	heat shock
HSE	heat shock element
Ig	immunoglobulin
IPTG	isopropyl-β-D-thio-galactopyranoside
kDa	kilodalton(s)
KLH	keyhole limpet hemocyanin
MES	2-(N-morpholino)ethanesulfonic acid

3mGlc	3-o-methyl-D-glucose
mRNA	messenger ribonucleic acid
ORF	open reading frame
PAGE	polyacrylamidegel electrophoresis
PIPES	piperadine-N,N'-bis(2-ethane-sulfonic acid)
PMSF	phenylmethanesulfonyl fluoride hydrochloride
poly(A)$^+$RNA	polyadenylated ribonucleic acid
PR	pathogenesis-related
RF	replicative form
SDS	sodium dodecylsulfate
SSC	saline sodium citrate (0.15 M NaCl, 0.015 M sodium citrate)
ss	single-stranded
SSPE	saline sodium phosphate EDTA (0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA)
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
v	volume
w	weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactoside

SUMMARY

Senescence is the final process of life cycle of an organism or its organ that induces deterioration in cell structure and function and ultimately leads the cells to death. The yellowing and abscission of leaves is an example of plant senescence. This leaf senescence is important and indispensable for normal plant growth and is believed to be genetically programmed, but little is known about molecular mechanism of this process. I have directed my research to the elucidation of molecular mechanism of leaf senescence by studying the senescence process of radish cotyledons as a model system.

Radish seedlings were placed in the dark for induction of senescence, and poly(A)$^+$RNA was extracted from the senescing cotyledons. Double-stranded cDNA was synthesized from the poly(A)$^+$RNA and digested with Sau3AI. A library with the digested cDNA was constructed on an M13mp18 bacteriophage vector. Clones corresponding to genes which were activated by the dark treatment were selected by differential screening with two different poly(A)$^+$RNA probes, one of which was prepared from light-grown cotyledons, and the other was from those after dark treatment.

Four clones survived the screening, and two of them, clone-1 and 5 showed properties of particular interest. Transcripts corresponding to either of these clones began to

accumulate in cotyledons within 6 h after exposure to darkness and increased 100- to 150-fold at 24 h. Accumulation of the transcripts was also induced by such senescence-inducing treatments as exposure to ethylene and to heat stress. 6-Benzyladenine, which is known to delay the progress of senescence, suppressed the accumulation of the transcripts induced by dark treatment, though partially. These results strongly suggested that corresponding genes were expressed in relation to the progress of senescence.

Northern-blot hybridization indicated that clone-1 and 5 corresponded to transcripts of the same size of about 800 nucleotides. Translation products from mRNA selected by these clones co-migrated on SDS-PAGE and were estimated to be 23 kDa. These observations and responses of the genes for clone-1 and 5 to various stimuli as well suggested that these two clones were possibly derived from a same gene. The determination of nucleotide sequences indicated that inserts of these clones shared a common sequence and therefore were originated from a unique gene. This gene was named as *din*1 after *d*ark-*in*ducible gene *1*.

A cDNA clone which covered nearly full-length of *din*1 mRNA (pRDI-1) was isolated from another cDNA library constructed with a plasmid vector, pBS+. pRDI-1 clone contained an open reading frame of 549 nucleotide long, which coded for a hydrophilic polypeptide of 183 amino acid residues. Antisera were raised against *din*1 protein expressed in *E. coli* and

against chemically synthesized oligopeptides which covered two different portions of the predicted amino acid sequence. They detected a 23-kDa polypeptide in the *in vitro* translation products from total poly(A)$^+$RNA prepared from dark-treated cotyledons but not from light-grown tissues. These results proved the correctness of the deduced amino acid sequence and hence the nucleotide sequence of the cDNA. Nucleotide sequence of pRDI-1 showed a homology of about 50% with genes for the members of group 1 pathogenesis-related proteins of tobacco.

Illumination with red light, far-red light, or blue light had no effect on the induction of *din*1 by dark treatment. This observation suggested that phytochrome and blue-light receptor are not involved in its regulation. Inhibition of photosynthesis by 3-(3,4-dichlorophenyl)-1,1-dimethylurea or atrazine strongly induced the expression of the gene. Sucrose, fructose, and glucose also suppressed the induction of *din*1 by dark treatment. 3-o-methyl-D-glucose, which is an analog of glucose and is not metabolized in plant cells, was not able to suppress the induction. These results suggested that a metabolite(s) of glucose rather than the sugar itself is regulating the gene. Accumulation level of sugars in the cotyledonary cells was shown to decrease rapidly in darkness and the expression profile of the gene in the dark was inversely proportional to the level of sugars. This observation and the fact that supplied sugars suppressed the induction of the gene by dark treatment imply that the level of

the metabolites of glucose depends on the intracellular level of sugars at least to some extent.

Administration of glucose also suppressed the induction of din1 expression by exposure to ethylene and heat stress. As these stimuli may possibly lower sugar level in the cell by activating respiration, these stimuli may also regulate din1 changing the sugar level.

Radish genomic DNA library was constructed with EMBL3 lambda phage vector and a genomic clone of din1 (λGDI1) was isolated from the library. Nucleotide sequence of λGDI1 showed that din1 is composed of 5 exons and 4 introns. In the upstream region of din1, several sequences were found to have homology with heat shock element, with a part of regulatory sequence of potato patatin genes which are regulated by sucrose, and with a part of upstream sequence of a bean chitinase which is known to be regulated by ethylene. These findings may suggest that sugar level, ethylene and heat stress regulate the expression of din1 through distinctive pathway, and suppression by a high level sugar metabolites dominates induction pathway of other signals like ethylene and heat stress.

My research established the presence of a senescence-associated gene, din1, in radish and a biochemical signal for its expression during senescence of cotyledons. din1 was regulated by the level of sugar metabolites. The level largely depends on the activity of photosynthesis. I propose from the

results of my study a hypothetical model of regulatory system that controls the progress of senescence; when photosynthetic activity of leaves is lowered by some causes, the leaf cells sense it through the level of sugar metabolites and express senescence-associated genes like din1, and start senescing.

I. GENERAL INTRODUCTION

What Is Senescence ?

It is a piece of beautiful scenery that maple and ginkgo leaves turn to red and yellow and are falling down in a gentle autumn wind. This is the most poetic example of plant senescence. The leaves to fall degrade their cellular components in advance and prepare to die. This deteriorative process that finally leads the cell to death is called senescence. As a result of senescence, deciduous trees lose their leaves, and green grasses disappear from the ground in winter time. However, this does not mean either the death of trees or extinction of grasses. In spring, the trees shoot out sprouts on branches and fresh grasses grow again from their seeds. Senescence in plants has been thought to play important roles on this rejuvenation process by shedding off unnecessary parts and keeping nutrients in storage organs for rejuvenation.

These phenomena of plant senescence were classically classified into following four categories from a macroscopic view (Leopold, 1961) (Fig.1-1). 1) Overall senescence, where the complete plant dies leaving storage organs like seeds; 2) top senescence, where the above ground plant organs seasonally die off; 3) deciduous or leaf senescence, where foliage dies off; 4) progressive senescence, where older organs and tissues

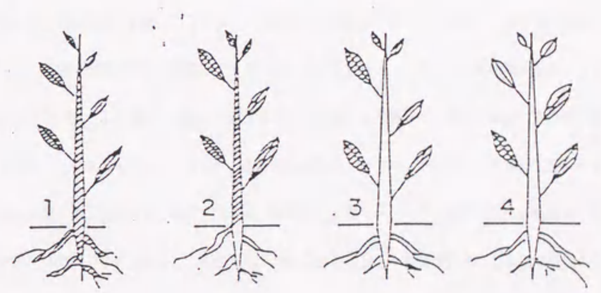
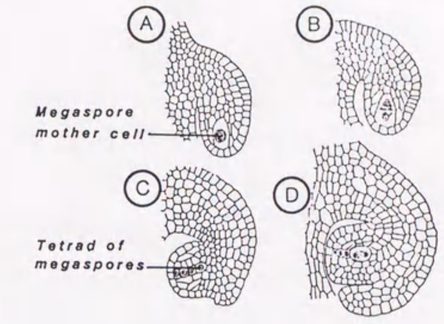


Fig.1-1. Senescence patterns according to Leopold (1961). 1) overall senescence; 2) top senescence; 3) deciduous senescence; 4) progressive senescence.

die off.

Including the examples described above, senescence occurs in plants at various levels, that is, in cells, in tissues, in organs and in whole plants. Following quotations are cited from Senescence and Aging in Plants (Nooden and Leopold, 1988): "Usually, three out of the four cells produced by meiosis during female gametophyte formation degenerate (Fig.1-2), and usually the surviving cell (megaspore) is that farthest from the micropylar end (Maheshwari, 1950; Johansen, 1950)." "Larger groups of cells (tissues) may degenerate and die. The cells surrounding the microsporocytes or pollen (tapetum, Fig.1-3), embryo sac (nucellus) or embryo (endosperm and nucellus, Fig.1-4) may disintegrate. Here, the constituents of the dying cells are thought to be redistributed to the sporogenous tissue or the embryo." "Senescence may be induced in almost any organ, most notably leaves (Leopold, 1961; Simon, 1967; Thimann, 1980), but also in roots (Head, 1973), root nodules (Sutton, 1983), flower parts (Halevy and Mayak, 1979, 1981; Mayak and Halevy, 1980), branches and shoots (Millington and Chaney, 1973), unfertilized overies, and developing fruits (Simons, 1973; Sweet, 1973; Stephenson, 1981)." "With the possible exception of plant clones, whole organisms have well-defined life spans; eventually, they degenerate and die, some slowly, some abruptly. In plants (or at least native, adapted plants), death is often induced by internally programmed changes and may even be a part of preparation for an adverse

Fig.1-2. Megasporogenesis and early development of the embryo sac of Anemone patens. (A) Young ovule with megaspore mother cell. (B) Completion of first meiotic division. (C) Linear tetrad of megaspores. (D) Three of the megaspores disintegrate, while one enlarges and begins mitotic divisions to form the embryo sac. [Reprinted from Haupt (1953).]



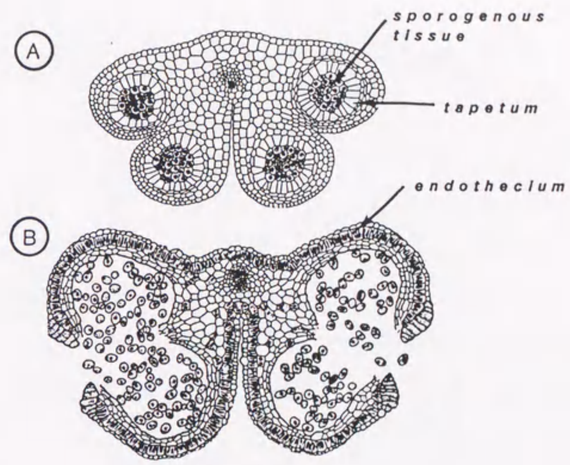
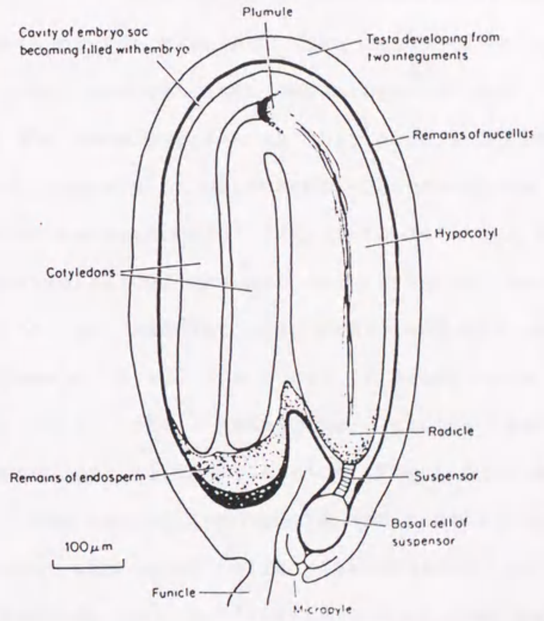


Fig.1-3. Cross section of lily (Lilium) anthers. (A) Young anther, with four microsporangia containing sporogenous tissue. Note the conspicuous tapetum surrounding the sporogenous tissue. (B) Mature anther with two pollen sacs containing pollen grains. The tapetum surrounding the sporogenous tissue has broken down, while the endothecium has developed bands of thickening. [Reprinted from Haupt (1953).]

Fig.1-4. An almost mature seed of Capsella bursa-pastoris sectioned longitudinally to show the orientation of the embryo. Note the disintegrated endosperm and nucellus. The suspensor cells usually senesce at or before this stage. [Reprinted from Bell and Woodcock (1983).]



season."

Among these variety of plant senescence, this thesis mainly concerns with progressive senescence of radish leaves. Cotyledons of this plant serve as an organ to supply nutrients to growing axis by degrading stored materials during early germination, then develop as a photosynthetic organ and help further growth of the plants. When leaves grow up, cotyledons end their role and get into senescence stage, which follows essentially the same process as that occurs in leaves.

The wide variety of phenomenal observations of senescence patterns makes senescence difficult to define. However, among the many physiological changes occurring in senescing cells, disintegration of cellular components and the cell itself is possibly common to all the types of senescence as a general phenomenon. All the senescing cells surrounding the microsporocyte or pollen (tapetum, Fig.1-3), the embryo sac (nucellus), the embryo (endosperm and nucellus, Fig.1-4), the flower petals and leaf cells disintegrate as a result of increased degradation activities, such as proteolysis and membrane deterioration.

This senescence-associated disintegration has been extensively studied with leaves. Disappearance of chlorophyll is one of the most prominent phenomena of leaf senescence, and the rate of chlorophyll degradation is usually considered to be a reliable measure of leaf senescence (Thomas and Stoddart, 1980). Chloroplasts degenerate synchronously (Wardley et al.,

1984) after their constituents like chlorophyll and Rubisco protein are hydrolyzed (Wittenbach, 1978; Hall et al., 1978; Peoples and Dalling, 1978). Thylakoid membranes deteriorate in the early stage, and chloroplast envelopes and plasma membranes do so in the later stage of senescence (Suttle and Kende, 1980; Harris and Arnott, 1973). In the senescing leaves, the content of RNA and protein decreases (Thomas and Stoddart, 1980). Synthesis of chloroplast rRNA is known to cease after leaves fully developed (Brady et al., 1971; Ness and Woolhouse, 1980). It was observed in various leaves that net protein synthesis lowers in the senescing tissues (Martin and Thimann, 1972; Brady and Tung, 1975; Roberts et al., 1987). Trials have been made to purify senescence-specific proteinase, but so far successful result has not been obtained. Several pathways for protein degradation in plants have been proposed, namely, compartmentation theory (Boller and Kende, 1979), ATP-dependent protease pathway (Waxman and Goldberg, 1985), and ubiquitin-dependent proteolysis (Vierstra et al., 1985). The first one is that the protein to be degraded and the proteolytic enzymes are segregated in the compartments. The second one is that the peptide hydrolase is only active when ATP is bound to the enzyme and degrade proteins. Vierstra et al. first detected ubiquitin in Avena sativa and suggested occurrence of a ubiquitin-dependent proteolytic pathway in plants. Proteins in senescing tissues may be degraded by increased activities of these pathways.

The observations described above are giving an impression that senescence is simply a process of degradation. However, senescence has been recognized from accumulated phenomenal observations as an indispensable process for plant normal growth and for the survival of an individual plant and its species. This concept implies the occurrence of senescence process brings benefits to the organism, and several putative roles have been imposed on senescence as follows:

1) The withering cells in a gametophyto and the disintegrating endosperm cells can nourish the surviving megaspore and embryo, respectively, by redistributing their constituents. Similarly, senescing cotyledons helps development of the plant. Plants take up mineral nutrients only from soil around their roots, thus readily available nutrients are very limited. Therefore, the redistribution from dying cells is the most important process of senescence (Thomas, 1978; Peoples et al., 1980).

2) The redistribution of nutrients contributes to the increase in total photosynthetic activity of a plant. After having fully developed, the old leaves lose photosynthetic capacity. In order to maximize total productivity under favorable conditions, plants let such parts of low productivity die off by senescence and help the growth of new leaves by nourishing the tissue with the nutrients from dying leaves.

3) Deciduous senescence, in which all leaves die off, is considered to be a preparation process for an adverse season. Senescence is also a means for plants to survive from or to

maintain their species through unfavorable conditions. Plants can not move and hide themselves from adverse environment. Instead, they shed off lowly viable parts by accelerating senescence of these parts to increase a possibility to survive the adverse environment.

4) Death of senescing part may prevent the infection of pathogens to living part.

These meanings of senescence in the life cycle of a plant indicate that the process is biologically very important and have attracted deep interest of many investigators. Physiological stimuli which affect the progress of senescence have been well documented as described in later part of this chapter.

Another implication of the concept is that the progress of senescence is regulated very systematically. Without effective regulation, degradation activity of senescence will be harmful for the cell. From some experimental evidences, it has been suggested that the progress is under the control of nuclear genes. Molecular-biological techniques have been introduced to study molecular events and its regulatory system of senescence and new information has been produced.

Stimuli Affecting The Progress of Senescence

A number of factors as follows are known to affect the progress of senescence:

1) Age of the cells is one of the major factors which determine the onset of senescence (Lloyd, 1980).

2) Light represses and the darkness promotes the progress of senescence presumably through a photoreceptor (Cuello et al., 1987) and photosynthesis (Udvardy, 1967).

3) Growth of a sink organ influences the senescence of source organ. For example, ripening of soybean seeds promotes foliar senescence (Lindoo and Nooden, 1977).

4) Ethylene treatment has been shown to result in increased activities of many hydrolytic enzymes (ATPase, acid phosphatase, α-amylase, catalase, chitinase, β-1,3-glucanase, pectinesterase and cellulase) (Abeles, 1972, 1973) and to enhances the loss of protein (Abeles, 1973), starch (Steffens, 1983), and chlorophyll (Gepstein and Thimann, 1981).

Cytokinin treatment increases chloroplast DNA, promote chloroplast protein synthesis, maintain pigment levels (Naito et al., 1978, 1979; Tsuji et al., 1979; Caers et al., 1985). It has been suggested that cytokinins exert their effect on chloroplast metabolism indirectly through action on the nucleus or cytoplasm (Thimann, 1977; Parthier, 1979).

ABA promotes chlorophyll loss (Lindoo and Nooden, 1978) and protein breakdown, and inhibits protein and nucleic acid synthesis (Beevers, 1968; Paranjothy and Wareing, 1971).

Gibberellin retards chlorophyll loss (Beevers, 1966; Aharoni and Richmond, 1978) and inhibits RNA and protein breakdown (Fletcher and Osborne, 1965; Goldthwaite and Laetsch,

1968).

There are many reports which studied the interaction of phytohormones in senescence. In nasturtium leaf discs, both gibberellic acid and cytokinin were able to retard senescence, cytokinin caused an increase in gibberellin-like activity (Chin and Beevers, 1970). This observation suggests that the cytokinin treatment could act on senescence affecting the level of gibberellin, but there is also evidence in other species that gibberellin and cytokinin may retard leaf senescence through independent actions (Back and Richmond, 1971). ABA treatments lowered cytokinin level in nasturtium leaves (Chin and Beevers, 1970), that would promote senescence. Conversely, cytokinin treatments lowered ABA level (Aharoni and Richmond, 1978), but as indicated above, ABA may also exert its effects independently of counteracting cytokinins as reported for other species. In detached rice leaves, BA promotes ethylene production and delays senescence at the same time (Kao and Yang, 1983). Cytokinin treatments appeared to decrease tissue sensitivity to ethylene (Eisinger, 1977; Mayak et al., 1977).

5) Free fatty acids promote senescence (Weaver, 1972). L-serine and cysteine are active in promoting senescence (Martin and Thimann, 1972). Sucrose is known to retard the progress of senescence (Nicols, 1973).

6) Stress is a promotive factor of senescence. Heat stress is known to accelerate yellowing of tobacco leaves (Engelbrecht and Mothes, 1964). Drought and flooding produce senescence

symptoms (Palag and Aspinall, 1981; Kozlowski, 1984). Mineral deficiency induces senescence-like response (Marschner, 1986).

Molecular-Biological Approaches to Leaf Senescence

Many physiological changes were observed in senescing leaves, such as decrease in the content of protein, RNA, and chlorophyll and deterioration of cellular and chloroplast membranes. These changes occur as a result of many enzymatic reactions. Enzymes involved in these reactions, therefore, should be activated or inactivated in senescing leaves, and the regulation of the enzymes possibly involves rearrangement of the pattern of gene expression. Enucleation of Elodea leaf cells by hypertonic treatment (Yoshida, 1961) and application of cycloheximide to oat leaves (Martin and Thimann, 1972) delayed the appearance of senescence symptoms. These observations suggest that the progress of senescence needs the expression of nuclear genes. In this thesis, I refer to a gene, of which expression is shown to correlate to the progress of senescence, as a senescence-associated gene.

Analysis of cell-free translation products of poly(A)$^+$RNA prepared from senescing tissues indicated that many genes are newly expressed on that process of wheat and oat leaves (Watanabe and Imaseki, 1982; Malik, 1987) and of carnation petals (Woodson, 1987). Kawakami and Watanabe (1988a, 1988b) also reported that a number of genes are activated in radish

cotyledons when placed in the dark to accelerate senescence. These studies strongly support the existence of senescence-associated genes. Next step was to isolate clones of these senescence-associated genes.

I started my research in 1985 to study senescence events occurring in radish cotyledons by isolating cDNA clones of senescence-associated genes. During conducting my research, cDNA clones of those genes were isolated from tomato fruits (Davies and Grierson, 1989) and from carnation petals (Lawton et al., 1989). The clones of the tomato genes was isolated as those of ripening-associated genes and expression of the genes were also detected in yellowing leaves. The expression of the carnation genes were shown to be specific to petal senescence. The identity of most of these genes are still unknown.

Clones of glutamine synthetase have been isolated in our laboratory and in others (Tingey et al., 1987). The content of protein and mRNA of cytosolic glutamine synthetase has been shown to increase during the process of senescence (Kawakami and Watanabe, 1989). This observation is consistent with a proposed scheme of metabolism in senescing leaves that proteins in senescing cells are hydrolyzed and the amino group of the hydrolysis products is transferred to glutamic acids or aspartic acid and translocated to storage organ or growing tissues (Simpson and Dalling, 1981). The glutamine synthetase transfers amino group of the hydrolysis products to glutamic acid to form glutamine and may help efficient redistribution of

nitrogen.

Objective and Strategy of My Study

At the time when I started my research, our laboratory was conducting research to reveal the process of senescence occurring radish cotyledons at molecular level. Molecular basis for events and regulation occurring in senescing tissues was mostly left to be elucidated yet. With regard to radish, as described earlier, abundance of mRNA from a set of genes (dark-inducible genes) was shown to increase in the cotyledons senescing in darkness as the result of analysis of cell-free translation products from poly(A)$^+$RNA (Kawakami and Watanabe, 1988a, 1988b). I assumed that the dark-inducible genes must include senescence-associated genes which have essential roles in the progress of leaf senescence. I was sure that the cloning of senescence-associated gene is indispensable to study senescence at molecular level and that I should start with cloning cDNAs for the dark-inducible genes and then select those of senescence-associated genes. For these reasons, I set ultimate goal of my research on the isolation and characterization of senescence-associated genes and expected that the result of this research would contribute to the elucidation of a part of molecular events and regulatory system of senescence.

The most fundamental problem involved in my study is what

is a senescence-associated gene and what character a senescence-associated gene should have. This problem is deeply related to the screening method that should be employed, because efficient screening is impossible without distinctive traits of the genes to be cloned. The only character of senescence-associated genes by which they can be distinguished from other genes is the specific expression of the genes in senescing tissue. The relationship between expression of the genes and the progress of senescence is the only advantage I can take. A number of known stimuli induce senescence and activate senescence-associated genes. However, it is hard to find a suitable stimuli to induce specifically the senescence-associated genes. Exposure to darkness, which promotes senescence, for example, may induce expression of not only senescence-associated genes but also other genes. Therefore, I adopted the following strategy of several steps to isolate of cDNA clones of senescence-associated genes; 1) induce senescence in cotyledons by exposing radish seedlings to darkness, 2) select cDNA clones for genes which are newly expressed in the cotyledons by differential screening, 3) examine the response of the genes to other stimuli which affect the progress of senescence. Genes truly associated with the progress of senescence should be expressed by treatments promoting progress of senescence and repressed by treatments retarding the progress.

Chapter II describes the process of cDNA cloning for dark-

inducible mRNA and characterization of the obtained clones. Chapter III involved the characterization of din1 gene product. For this purpose, a nearly full-length cDNA clone for din1 was isolated and nucleotide sequences resembling to the cloned cDNA is searched in data base. Immunochemical detection of the gene product was also tested. Chapter IV is devoted for the search for the biochemical signal which is released by the wide variety of stimuli and activates din1 gene. Chapter V focused on the isolation and characterization of genomic clones of din1 from radish.