II. Isolation of cDNA Clones for Senescence-Associated Genes

<INTRODUCTION>

Cotyledons of dicotyledonous plants have two roles in the life cycle of a plant; one is as a storage organ that supplies materials necessary for growth during early stage of seed germination, and the other is as a photosynthetic organ to support early growth of the plant. When adult leaves have fully developed and had sufficient activity of photosynthesis, cotyledons complete their roles and enter the final phase of development, senescence. Radish seedlings grown under continuous light for 14 days have fully developed cotyledons, which can be induced to senesce by exposure to darkness. As synchronously senescing cotyledons thus can be collected in sufficient amounts and in a short period, they provide a good material for experiments of leaf senescence. Considering these advantages, I took up radish cotyledons as a model system for molecular biological study of leaf senescence.

Exposure to darkness is one of the most effective stimuli to induce green leaves to senesce. Prolonged light deficiency causes many biochemical changes in leaf cells, resulting in the alteration of gene expression and eventually in appearance of senescence syndromes. As a number of genes have been shown to be activated in radish cotyledons during the first 24 h in

darkness (Kawakami and Watanabe, 1988a), I decided to clone cDNAs corresponding to these dark-inducible genes as a first step for isolation of senescence-associated genes.

The progress of leaf senescence is influenced by a number of stimuli other than darkness. A gaseous phytohormone, ethylene is one of these stimuli, and is well-known to promote the whole process of leaf senescence. Its production is involved in many processes of plant development, such as seed germination, seedling growth, fruit ripening, and senescence of many organs as well (Abeles, 1973). Heat stress is another stimulus which accelerates senescence of aged leaves (Engelbrecht and Mothes, 1964). A group of a phytohormone, cytokinins, and in some cases gibberellic acids retard the progress of leaf senescence (Ray et al, 1983; Back and Richmond, 1969). The dark-inducible genes, of which cDNA clones were isolated in this study, were examined what responses they showed to these stimuli.

<MATERIALS AND METHODS>

1. Plant Materials

Radish (Raphanus sativus L. cv. Comet) seedlings were grown at 25°C on vermiculite beds under continuous light at about 5500 lux from white fluorescent lamps. Hoagland's solution was given as nutrients (Hoagland, 1933). On the 14th day of growth, whole plants on a bed were transferred to either of the following experimental conditions.

Dark Treatment: Seedlings were kept in complete darkness at 25°C. Cotyledons were harvested under dim green light after a defined period.

Exposure to Ethylene: Seedlings were transferred into an air-tight Plexiglas box in which ethylene was added at 10 μl/l, and kept under continuous light at 25°C for 24 h. As a control experiment, a bed was kept in a similar box containing a 5 ml solution of 0.25 M HgO in 2 M perchloric acid to absorb ethylene produced by the plants.

BA Treatment: Seedlings on a bed were sprayed with 100 μM BA in 0.2% (v/v) Tween 20 and kept in the dark at 25°C. Control seedlings were sprayed with 0.2% Tween 20 solution. Cotyledons were harvested after 24 h under dim-green light.

Heat Shock Treatment: Seedlings were transferred to a growth chamber at 35°C and kept for 2 h. Cotyledons were harvested immediately after the treatment.

2. Preparation of RNA

Total cellular RNA was prepared according to Kawakami and Watanabe (1988a). Cotyledons or cotyledon discs were homogenized with 2 to 3 volumes per weight of homogenizing buffer [7 M guanidine-HCl, 0.1 M 2-mercaptoethanol, 2% (w/v) sodium lauroylsarcosinate, 25 mM sodium citrate] containing a small amount of antifoam reagent, Shin-Etsu Silicone KM-72 (Shin-Etsu Kagaku, Tokyo) in a blender. The homogenate was extracted twice with phenol/chloroform (1:1) and then once with chloroform. Total RNA was precipitated by adding 0.6 volume of isopropanol and placing the mixture at -20^{o}C for at least 4 h. The total cellular RNA was dissolved in a small volume of H_2O and reextracted with phenol/chloroform and chloroform as before, and precipitated again by adding two volumes of ethanol. High-molecular-weight RNA was precipitated in a solution of 2 M LiCl at 0^{o}C.

Poly(A)$^{+}$RNA was purified from high-molecular-weight RNA by oligo(dT)cellulose column chromatography (Aviv and Leder, 1972).

3. Construction of Library of cDNA Digested with Sau3AI

ds cDNA was synthesized with a cDNA synthesis system purchased from Amersham International (Amarsham, UK), which

follows the method of Gubler and Hoffman (1983).

Five μg of poly(A)$^+$RNA was prepared from the cotyledons exposed to darkness for 24 h and used as a template. cDNA was recovered by ethanol precipitation and dissolved in TES buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl), and about 1.2 μg of ds cDNA products was recovered. The recovery was calculated from incorporation ratio of ^{32}P from [α-^{32}P]dCTP into synthesized cDNA. cDNA larger than 500 bp were separated by Sephadex G100 column chromatography with TES buffer, and precipitated with ethanol.

The cDNA was cloned into bacteriophage M13mp18 according to the method of Shirras and Northcote (1984) with a slight modification. The cDNA was digested with 20 units of Sau3AI (Takara Shuzo, Kyoto) in 50 μl of a reaction mixture at 37℃ for 3 h, and extracted with phenol/chloroform (1:1), then with chroloform, and precipitated by ethanol. The digested cDNA (25 ng) was ligated with 250 ng of M13 RF DNA that was digested with BamHI and dephosphorylated. E. coli strain JM103 cells were made competent by $CaCl_2$ treatment and transformed with the ligation mixture.

4. Differential Screening

M13 plaques were transferred successively to two sheets of Biodyne A nylon membrane (Pall BioSupport, New York, USA) according to the procedure described by Maniatis et al. (1982).

The membranes sheets were incubated at 42°C for 16 h in a prehybridization solution; 50% (v/v) deionized formamide, 5X SSPE, 5X Denhardt's solution (Denhardt, 1978), 0.2% (w/v) SDS, 100 μg/ml calf thymus DNA, 100 μg/ml yeast tRNA. One of the membranes was then incubated at 42°C for 60 h in 2 ml of the prehybridization solution containing 0.1 μg (10^8 cpm/μg) of poly(A)$^+$RNA probe prepared from cotyledons kept in the dark for 24 h. The other membrane was hybridized under the same conditions but with 0.1 μg (10^8 cpm/μg) of poly(A)$^+$RNA probe prepared from control plants. The membranes were washed four times in 2X SSC at room temperature, and twice in 0.1x SSC at 50°C. They were then exposed to Fuji RX X-ray film (Fuji Photo Film; Tokyo) with an intensifying screen (Hi-Screen B2, Fuji Photo Film) at -80°C.

Probes were prepared from 1 μg of poly(A)$^+$RNA by poly(A) polymerase reaction in 50 μl of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.4 M NaCl, 1 mM dithiothreitol, 5 μM ATP, 37 kBq/μl [α-^{32}P]ATP (111 TBq/mmole, Amersham), 0.02 unit/μl poly(A) polymerase from E. coli (Takara). The labeled RNA was purified by phenol/chloroform extraction and adsorption to Nensorb 20 (DuPont Co.).

5. Hybridization with Immobilized RNA

Dot-blot and Northern-blot hybridization were carried out according to Thomas (1983). One μg of glyoxalated poly(A)$^+$RNA

was electrophoresed on 1% (w/v) agarose gel at 2 volts/cm for 2.5 h and transferred to a Biodyne A membrane by capillary flow of 20X SSC solution. For dot-blot hybridization high-molecular-weight RNA was glyoxalated, diluted serially with 4X SSC, and applied to a nylon membrane as described Minami and Watanabe (1988). The cDNA insert was excised from clone-1 RF DNA at EcoRI and HindIII cites, electrophoretically separated on a low-melting agarose gel, and the gel was cut to isolate the band of insert DNA fragment. Hybridization probe was prepared from the excised agarose block by labeling with a Multiprime DNA Labeling System (Amersham) using [α-^{32}P]dCTP as a labeled nucleotide. Hybridization and autoradiography were carried out as described for the differential hybridization.

For quantitation of the autoradiographic data, X-ray film was scanned in a densitometer (Densitorol DMU-33C, Toyo Kagaku Sangyo; Tokyo) at 530 nm, and area under the peak was measured. The logarithms of the areas were plotted against the dilution values of RNA to find a linear portion of the curves. The multitude of increase in the content of specific transcripts was calculated from the linear portion, and expressed as the multiplicity of zero time value.

6. Selection of mRNA by Hybridization to cDNA

mRNA complementary to cloned cDNA sequence was selected by

hybridization with phage DNA of the clones according to the method of Bunemann and Westhoff (1983). As the cDNA clones were selected by poly(A)$^+$RNA probes, the phage DNA contains antistrand sequence and can be effectively used for hybrid-selection of specific mRNA. One mg of ss phage DNA was prepared according to Messing (1983), and coupled to 3 g of diazotized Sephacryl S-500 (Pharmacia LKB, Uppsala, Sweden). DNA-coupled Sephacryl beads were suspended in 1 ml of hybridization solution containing 50 μg of poly(A)$^+$RNA isolated from dark-treated radish cotyledons and incubated at 42^{o}C for 4 h with occasional shaking. The beads were washed four times at 42^{o}C with 50% (v/v) formamide, 2x SSC, 0.2% SDS, and four times with 50 mM Tris-HCl, 20 mM EDTA, pH 8.0 at 60^{o}C. RNA was eluted by suspending the beads in 1 ml of boiling water and incubating at 100^{o}C for 2 min. After collecting supernatant, the beads were extracted again with boiling water. The supernatants were combined and RNA was precipitated with ethanol after adding 50 μg of yeast tRNA. The RNA precipitates were collected by centrifugation, washed twice with 70% (v/v) ethanol and dissolved in H_2O.

7. Translation and SDS-PAGE

Poly(A)$^+$RNA was translated in a wheat germ cell-free system under conditions described by Watanabe and Price (1982). The translation products were denatured in a SDS sample buffer

(62.5 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol, 10% (w/v) glycerol, 0.5% (w/v) Bromophenol Blue) at 100^{o}C for 3 min.

The denatured samples were separated by electrophoresis on SDS-polyacrylamide gel (13% (w/v)) with Laemmli's buffer system (Laemmli, 1970) and visualized by fluorography as described by Minami and Watanabe (1984).

<RESULTS>

1. Isolation of cDNA Clones for "Dark-Inducible mRNA"

Radish seedlings were grown under continuous light for 14 days (Fig.2-1) and placed in darkness for 24 h for induction of senescence. Poly(A)$^+$RNA was prepared from cotyledons of the seedlings. As shown in Fig.2-2, ds cDNA was synthesized from the poly(A)$^+$RNA according to the method of Gubler and Hoffman (1983) and digested with Sau3AI. The cDNA fragments were ligated into BamHI site of M13mp18 RF DNA and a cDNA library was constructed by transforming bacterial strain JM103. This strategy simplifies construction of cDNA library and decreases background noise in differential screening by elimination of poly(A) tracts from cDNAs.

For differential screening, two RNA probes were prepared; one was end-labeled poly(A)$^+$RNA prepared from cotyledons of light-grown radish, and the other was that prepared from cotyledons of radish kept in the dark for 24 h. About 4,000 recombinant plaques were screened with the differential probes (Fig.2-3). All the possible clones were selected and tested again by differential screening with the same probes. Positive clones in the second screening were spread on plates and single plaques were isolated. Five plaques were picked up from respective clones and screened again (Fig.2-3). Four clones, clone-1, 5, 11, and 17, survived the three cycles of screening

Fig.2-1. Radish seedlings grown for 14 days (1) and 21 days (2) under continuous light at 25°C. At the 14th day of growth, primary and secondary leaves have developed and cotyledons are ready to senesce.

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Fig.2-2. A scheme of cloning strategy of dark-inducible mRNA sequences. ds-cDNA was synthesized from poly(A)$^+$RNA prepared from cotyledons of radish seedlings that were placed in darkness for 24 h. The ds-cDNA was completely digested with Sau3AI and ligated into RF DNA of bacteriophage M13mp18 which was digested with BamHI and dephosphorylated by calf intestinal alkaline phosphatase prior to ligation. A library was constructed by transforming E. coli strain JM103 with the ligated cDNA. Phage DNA in plaques was successively transferred to two sheets of nylon membranes. One of the membranes was probed with end-labeled poly(A)$^+$RNA from light-grown cotyledons and the other was with that from dark-treated cotyledons. Clones were selected to show stronger hybridization signals with the later probe than with the former.

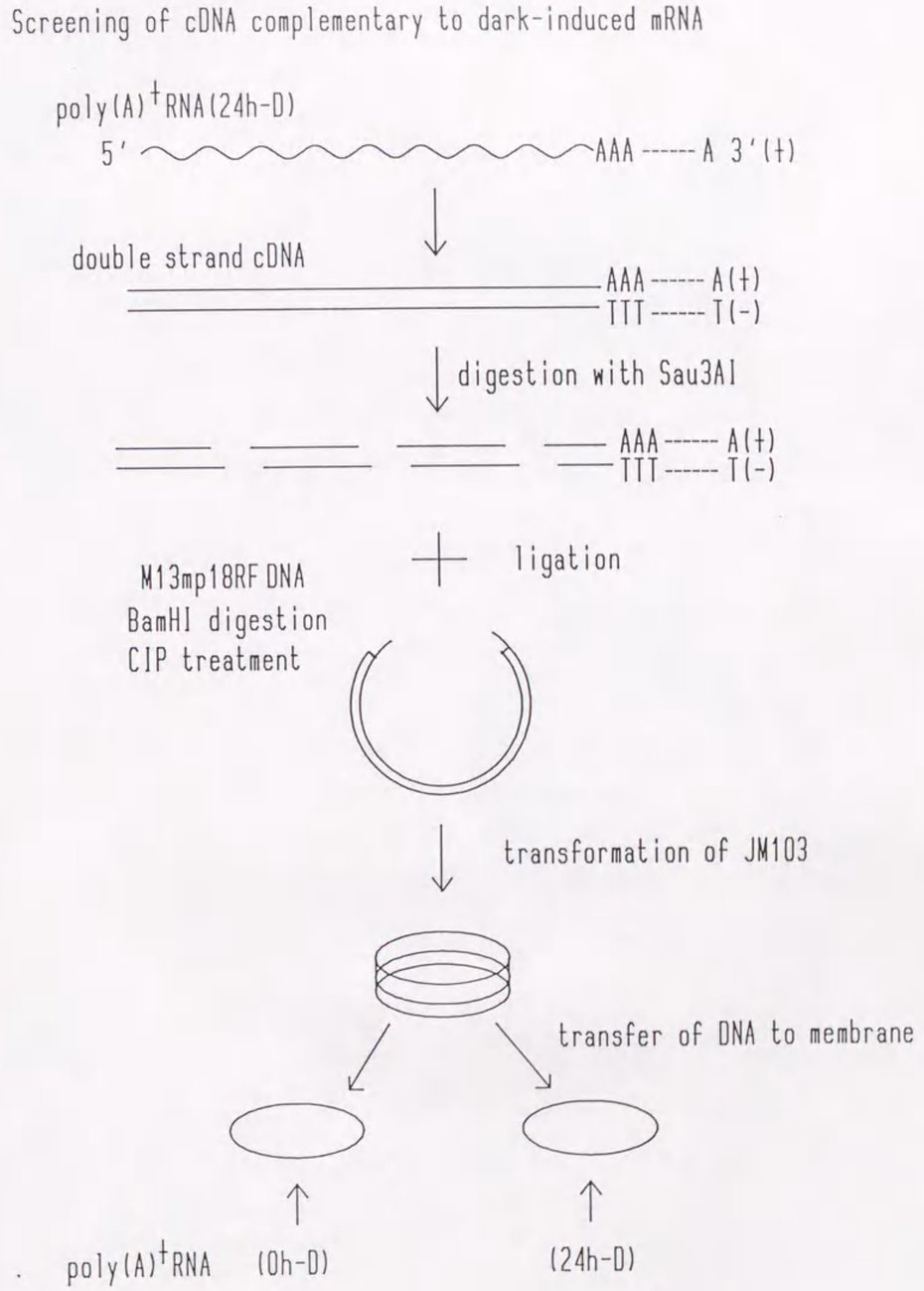
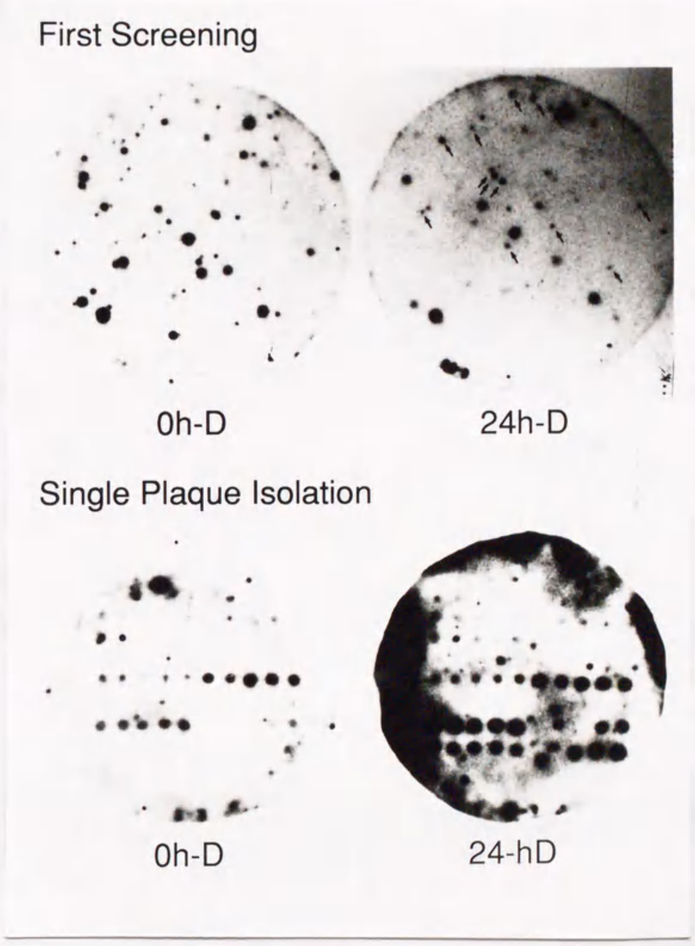


Fig.2-3. Autoradiography of nylon membranes hybridized with the differential probes, which are prepared by end-labeling of poly(A)$^+$RNA of light-grown cotyledons (0h-D) and dark-treated cotyledons (24h-D). Upper panel is a result of first screening. Clones marked with arrows are indicating positive signals. Lower panel shows a result of third screening, in which positive clones in the second screening were purified from others by single plaque isolation and five representatives of each clones were examined again by differential hybridization.



by showing stronger hybridization signals to the latter probe than to the former.

These four clones contained insert DNAs of about 570, 220, 750, and 350 bp, respectively. I examined changes in the accumulation level of transcripts corresponding to the respective clones quantitatively by dot-blot hybridization using the insert DNA sequences as probes (Fig.2-4). In the experiment with poly(A)$^+$RNA, clone-1, 5, 11, and 17 showed about 30-, 30-, 5-, and 10-fold increase in abundance of complementary transcripts, respectively, at 24 h of dark treatment. Accumulation of transcripts complementary to clone-1, 5, and 17 decreased at 48 h, though still being at a higher level than 0 h level. Another clone, clone-C, was also isolated from the library. This clone showed equally strong hybridization signals to both probes in the screening process. The dot-blot hybridization experiment indicated that steady state-level of transcripts corresponding to this clone was not affected by dark treatment (Fig.2-4).

2. Identification of Transcripts from Dark-Inducible Genes and Its Expression in Radish Seedlings

Northern-blot hybridization of poly(A)$^+$RNA showed that insert DNAs of clone-1, 5, 11, and 17 hybridized to RNAs of about 800, 800, 1400, and 800 nucleotide long, respectively (Fig.2-5). Clone-17 showed week hybridization with additional

Fig.2-4. Change in the accumulation level of transcripts complementary to cloned cDNA during dark treatment. Poly(A)$^+$RNA was prepared from cotyledons of light-grown seedlings (lane 1), from those placed in darkness for 24 h (lane 2), and from those placed in darkness for 48 h (lane 3). The poly(A)$^+$RNA was serially diluted, blotted on nylon membranes and hybridized with probes prepared from the cDNA clones of which numbers are indicated on panels.

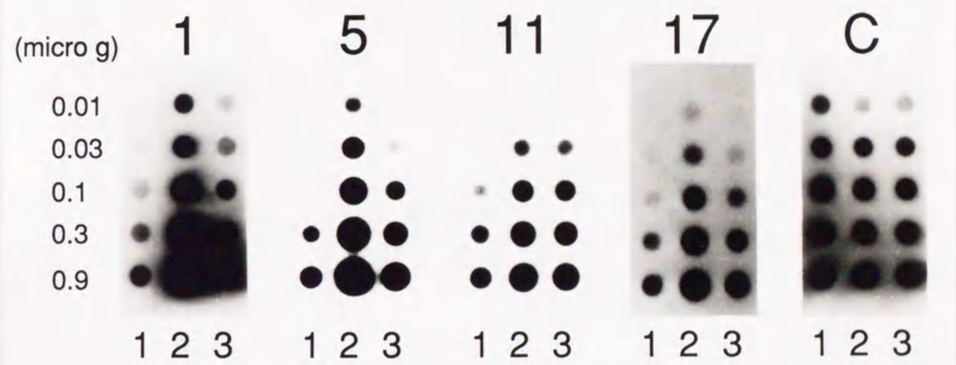
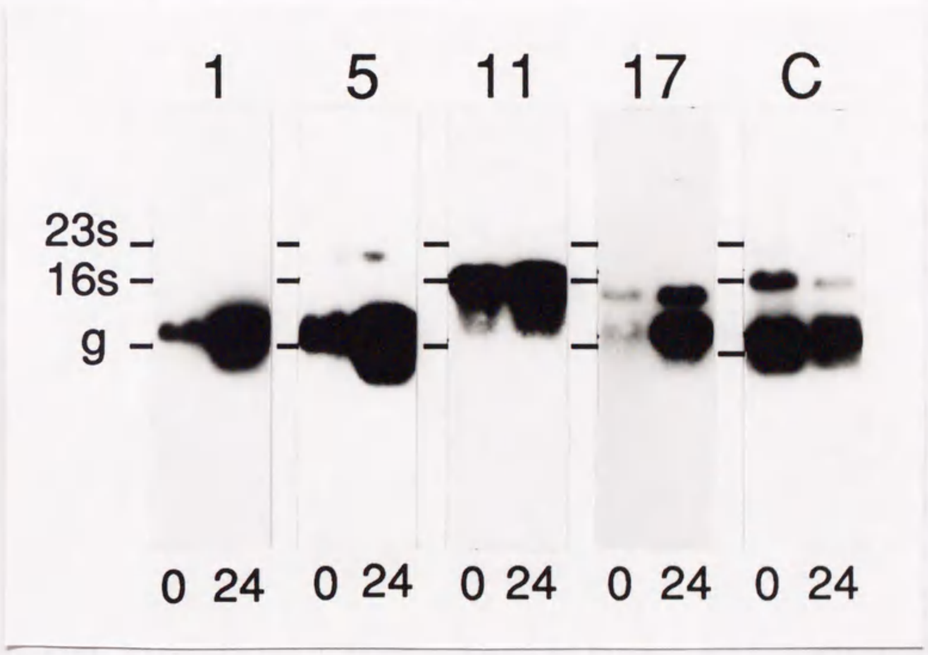


Fig.2-5. Size of transcripts complementary to cloned cDNA. Poly(A)$^+$RNA was prepared from cotyledons at 0 h and 24 h after dark treatment. One μg of poly(A)$^+$RNA was electrophoresed on 1.0 % agarose gel and then transferred to nylon membranes. E. coli rRNA (23s and 16s) and rabbit α and β globin mRNA (g) were run in parallel as size marker. Probes were prepared from cloned cDNA, the numbers of which are shown on the panels, and hybridized to the blots. Lane 0, RNA from light-grown radish cotyledons; lane 24, RNA from dark-treated radish cotyledon.



RNA of 1200 nucleotides. There may be another gene homologous to a gene corresponding to clone-17. As the signal of the additional band is so weak, its contribution to the data in further experiments should be negligible. Clone-C hybridized to RNA of 700 nucleotides, and also to a minor RNA species of 1400 nucleotides. The transcripts complementary to clone-1, 5, and 17 prominently increased in abundance after 24 h of dark treatment, and that to clone-11 showed only small accumulation over zero time value.

For the determination of precise kinetics of accumulation of the transcripts, dot-blot hybridization was done with total cotyledonary RNA (Fig.2-6). The accumulation of transcripts specific to clone-1 and 5 was detected clearly as early as 6 h, and reached a 140- and 120-fold level over that of 0 h, respectively, at 24 h. Then the abundance of the transcripts decreased gradually until 48 h, but it remained at a high level. Clone-17 showed a similar kinetics of accumulation of its specific transcripts, but the level increased only to a 10-fold level at its maximum. Clone-11 did not show appreciable change in the content of its specific transcripts in total RNA, though the clone passed the selection by differential hybridization and its transcript showed clear increase in the dot-blot experiments with poly(A)$^+$RNA. Dark treatment did not affect the level of transcripts complementary to clone-C throughout the experimental period as expected.

Accumulation of transcripts corresponding to these clones

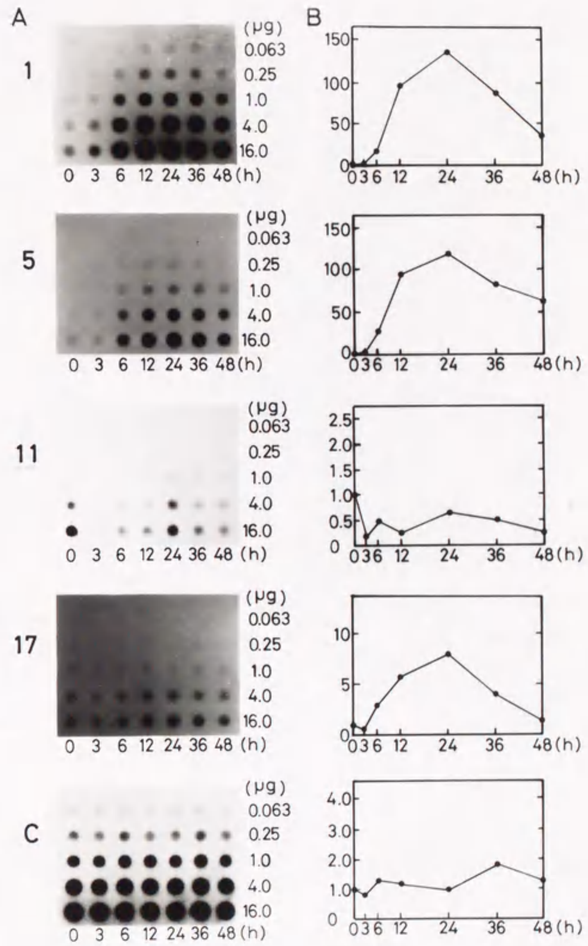


Fig.2-6. Change in the accumulation level of transcripts complementary to cloned cDNA during dark treatment. A: Radish seedlings were transferred to darkness on the 14th day of growth, and total cellular high-molecular-weight RNA was prepared from cotyledons at 0, 3, 6, 12, 24, 36, and 48 h during dark treatment. The total cellular RNA was serialy diluted, dotted on nylon membranes and hybridized with the probes prepared from the cDNA clones the numbers of which are shown on the panels. B: Hybridization signals on the X-ray films were quantified by scanning the films with a densitometer. Respective graphs indicate amounts of transcripts complementary to the clones relative to the level at 0 h.

were examined in different organs of the old seedlings (Fig.2-7). Poly(A)$^+$RNAs were prepared separately from apical buds, primary and secondary leaves, cotyledons, hypocotyls, and roots on the 18th day of growth, when cotyledons start senescing and begin to accumulate mRNA specific to clone-1 and 5. mRNA specific to clone-1, 5, and 17 accumulated only in cotyledons. A low level of mRNA specific to clone-11 was detected in all the organs examined. mRNA specific to clone-C was found in all the green tissues at a high level.

3. Response of the Dark-Inducible Genes to Various Stimuli

As dark treatment induces the cotyledons to senesce, the cDNA clones selected for the dark-inducible mRNA may include those which correspond to genes which are not only negatively regulated by light but also associate with the progress of senescence. In order to ask this question, I examined the level of transcripts complementary to the clones after application of various stimuli which are known to affect the progress of leaf senescence to the seedlings.

Radish seedlings at 14 days of growth were exposed to 10 μl/l of ethylene for 24 h. Cotyledons of the seedlings accumulated transcripts complementary to clone-1 and 5 even under light to about 20-fold higher level than that in control cotyledons (Fig.2-8). However, this treatment did not affect the level of transcripts specific to either clone-11, 17, or C.

Fig.2-7. Tissue-specific accumulation of transcripts complementary to the cloned cDNAs. Poly(A)$^+$RNA was prepared from apical buds (A), primary and secondary leaves (P), cotyledons (C), hypocotyls (H) and roots (R) of radish seedlings grown for 18 days under continuous light. Poly(A)$^+$RNA was blotted on nylon membranes and hybridized with the probes prepared from the clones of which numbers are indicated on the lanes. Lane C is a result with a probe of clone-C DNA.

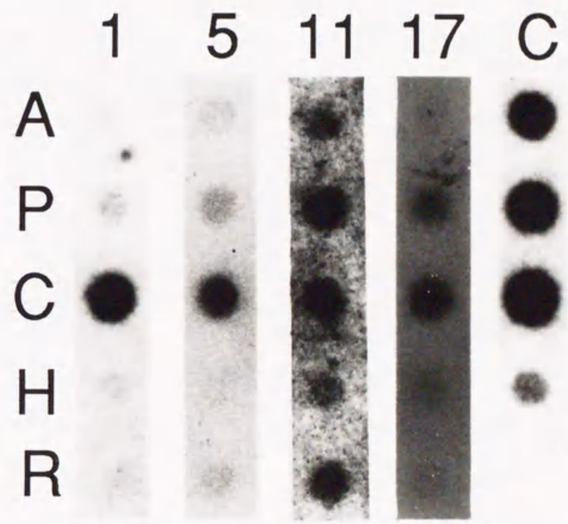
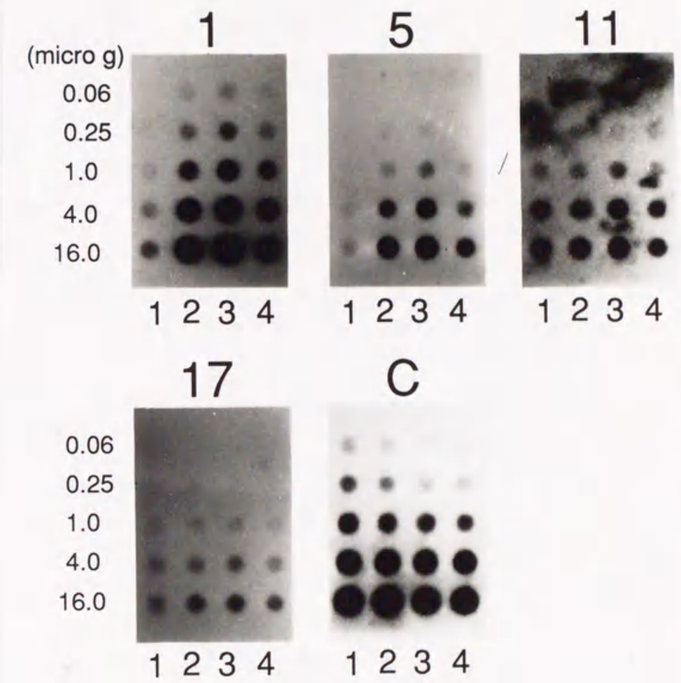


Fig.2-8. Effects of ethylene and BA on the accumulation of transcripts. Radish seedlings were placed in an air-tight Plexiglas box containing 10 μl/l ethylene (lane 2) or without ethylene (lane 1) under continuous light for 12 h. The seedlings were kept in the dark for 24 h after being sprayed with 100 μM BA in 0.2% (v/v) Tween 20 (lane 4), or with 0.2% Tween 20 without BA (lane 3). Total cellular RNA was prepared from cotyledons, blotted on nylon membranes, and hybridized to the probes prepared from the clones of which numbers are indicated on panels.



Another set of seedlings were sprayed with BA at 100 μM and placed in the dark. BA is a potent synthetic cytokinin, which is known to repress development of senescence symptoms. Accumulation of transcripts specific to either of the four clones induced by dark treatment were partially repressed (Fig.2-8). The gene for clone-C did not respond to BA treatment and was fully expressed in the dark.

Exposure to heat stress promotes progress of senescence. Seedlings were heated at 35^{o}C for 2 h under light. This treatment induced about 10-fold accumulation of transcripts specific to clone-1 and 5 (Fig.2-9). The level of transcripts specific to clone-17 or C was not affected by this treatment.

4. Polypeptides Encoded by the cDNA Sequences

Polypeptides encoded by the genes corresponding to the isolated clones were examined by in vitro translation of mRNA specifically selected by hybridization with the phage DNA of the isolated clones. The specific mRNA was selected from poly(A)$^{+}$RNA that was extracted from cotyledons at 24 h of dark treatment and translated in a wheat germ system. Fig.2-10 shows that distinct polypeptides were predominantly translated from the mRNA above background synthesis of those programmed by endogenous mRNA of the wheat germ extract. Polypeptides with a similar size of 23 kDa were translated from mRNAs selected by clone-1, 5, and 17. The polypeptide of 23 kDa was detected

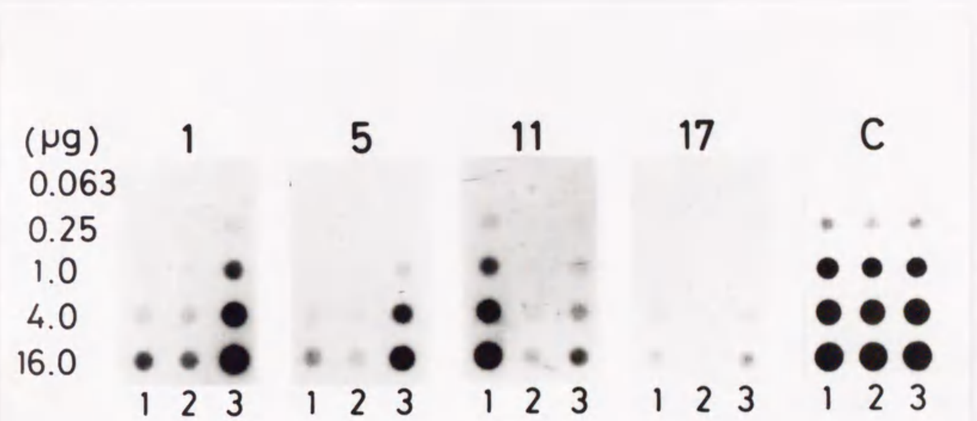
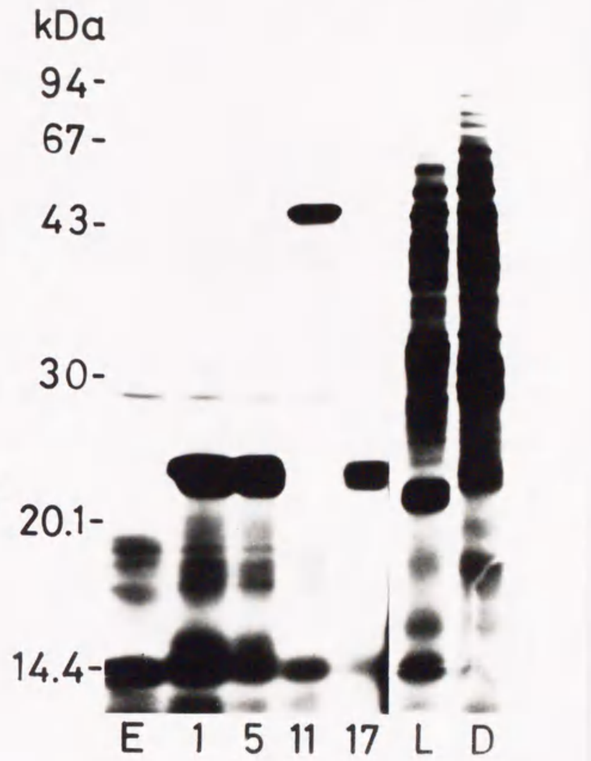


Fig.2-9. Accumulation of transcripts in response to heat stress. Radish seedlings of 15-day-old were kept at 35°C for 2 h, and total cellular RNA was extracted from the cotyledons immediately after the treatment. RNA was blotted on nylon membranes and hybridized with probes prepared from the cDNA clones of which numbers are indicated on the panels. Lane 1, RNA from cotyledons grown under light for 14 days; lane 2, RNA from those grown for 15 days under light; lane 3, RNA from those heat-stressed on 15th day of growth under light.

Fig.2-10. SDS-PAGE of translation products from mRNAs complementary to the cloned cDNAs. mRNA was selected from poly(A)$^+$RNA of dark-treated cotyledons by hybridization with phage DNA of respective clones. The selected mRNA was translated in a wheat germ system. The translation products were separated on 13% polyacrylamide gel electrophoresis. Lane 1, 5, 11 and 17 are translation products from mRNA selected by the DNA of clone-1, 5, 11 and 17, respectively. Lane L and D are translation products from poly(A)$^+$RNA prepared from light-grown cotyledons and those placed in darkness for 24 h, respectively. Lane E is translation products from endogenous mRNA of wheat germ.



only in the products from poly(A)$^+$RNA of dark-treated cotyledons but not in those from poly(A)$^+$RNA of light-grown cotyledons. A polypeptide of 45 kDa was translated from mRNA hybridized to clone-11. These observations proved that the cloned cDNA sequences were derived from mRNA of protein-coding genes.

<DISCUSSION>

When light-grown plants are exposed to darkness, they adapt themselves to the new condition by rearranging the pattern of gene expression. As a result, some leaves start senescing after ascertain duration of darkness. Kawakami and Watanabe (1988a) showed that radish cotyledons accumulated a set of new translatable mRNA when they were placed in the dark to accelerate senescence. Four cDNA clones for such dark-inducible mRNAs were selected from a cDNA library of radish cotyledonary mRNA by differential screening. Genes for these dark-inducible mRNAs were likely to include senescence-associated genes. Not all of these dark-inducible mRNAs could be associated with the progress of senescence, because the level of some of these mRNA species was simply down-regulated by light (Kawakami and Watanabe, 1988b). Therefore, further investigation was required for the selection of senescence-associated genes, and the dark-inducible genes were examined for their response to BA, ethylene, and heat stress. Among the four clones, clone-1 and 5 were of particular interest because the steady-state level of their corresponding mRNA responded to these stimuli as expected for senescence-associated genes.

Change in the mRNA level does not necessarily indicates the transcriptional activity of corresponding genes, but it is most likely that genes for clone-1 and 5 were quickly activated upon exposure to darkness. These mRNAs were accumulated to a

level higher than 100-fold over zero time value within 24 h. Genes for these mRNAs were activated not only by exposure to the darkness but also to ethylene and to heat shock, both of which are known to accelerate leaf senescence. The activation of the genes by the dark treatment was repressed by prior spray of BA, though partially. All of these observations consistently suggest that cDNA sequences of clone-1 and 5 are representatives of genes of which expression is coupled with the start of senescence.

Clone-17 was also specific to a dark-inducible mRNA and the size of corresponding transcript was very similar to those for the clone-1 and 5. It also resembles the latter two clones in respect to its encoding polypeptide. However, this clone differs from that of the latter two in the response of corresponding gene to other stimuli examined. The transcripts accumulated by the dark treatment but only to a 10-fold level, and the gene did not respond to ethylene. The gene corresponding to this clone is, therefore, simply down-regulated by light like those reported by Okubara et al. (1989), and may not be related to the progress of senescence.

Clone-11 was giving positive signals in the three cycles of selection by differential hybridization, but showed a complicated kinetics of accumulation of corresponding transcripts in total cellular RNA during the dark-induced senescence. This complexity may be caused by some accidental activation of the gene in light-grown cotyledons. Because I

could not determine what activated the gene in the control plants and the gene was not induced by exposure to ethylene, I abandoned this clone.

cDNA clones for senescence-associated genes have recently been isolated from carnation flower petals (Lawton et al., 1989) and from tomato fruits (Davies and Grierson, 1989). The carnation genes which are expressed when the petals senesce were classified into two groups by response to ethylene. The genes of the first class are closely regulated by ethylene, but the gene of second class was expressed prior to the increase in ethylene production. din1 has some similarities to the former in respect to expression in senescing tissue and to regulation by ethylene, but differs in organ specificity; the genes in carnation are expressed predominantly in the floral organ. Interestingly, the cDNA clones in tomato were isolated from ripening fruits and detected the expression of corresponding genes in senescing leaves. Senescence of leaves involves many events in common with fruits ripening, namely degradation of photosynthetic apparatus, degradation of starch and chlorophyll, stimulation of respiration, and so on (McGlasson et al., 1975; Roberts and Osborne, 1981). The tomato genes were also expressed in yellowing leaves, and the expression of some of them were prevented by ethylene antagonist silver thiosulphate. The authors suggested that these genes are regulated by endogenous level of ethylene. These observations of gene regulation by ethylene in radish cotyledons, carnation

petals and tomato leaves indicated that a part of ethylene actions on plant senescence is through the activation of specific genes.

Kawakami and Watanabe (1988a, 1988b) have classified the population of translatable mRNA into three groups according to the pattern of change in abundance during dark-induced senescence; the first group is for those which continuously increase, the second for those which decrease and are eliminated from the cells, and the third for those which increase but transiently and decrease in later stages. It was also shown that mRNA for cytoplasmic glutamine synthetase continuously increases in abundance in the later stage of senescence, and therefore, belongs to the first group (Kawakami and Watanabe, 1989). mRNA corresponding to clone-1 and 5 increases at an early stage of dark-induced senescence and is included in the third group of mRNA.

After all of these studies, I was confident that the expression of the genes corresponding to clone-1 and 5 was tightly correlated with the progress of senescence, and that these genes were senescence-associated genes. Therefore, I decided to focus on the properties of these genes.