III. Isolation of a Nearly Full-Length cDNA Clone of din1 and Characterization of Encoded Protein

<INTRODUCTION>

The genes corresponding to clone-1 and 5 showed very similar properties, that is, the time course of accumulation of their transcripts after exposure to darkness, the response to ethylene, heat stress and BA, and the molecular mass of encoded polypeptides. As the process of constructing the cDNA library included a step of digestion of cDNA with Sau3AI, there is a possibility that insert sequences of clone-1 and 5 are derived from different regions of mRNA of a unique sequence. In addition, genes corresponding to these two clones are of particular interest, because they responded to the various stimuli which affect the progress of senescence. I, therefore, decided to isolate nearly full-length cDNA clones complementary to these two sequences from another cDNA library constructed with a plasmid vector which can directly be used in the experiments of in vitro transcription. As will be described in the RESULTS section, a cDNA clone with expected properties was successfully isolated, and I named as pRDI-1. I found from sequence analysis that three clones, clone-1, 5 and pRDI-1 were derived from a unique gene, and named the gene as din1 after its dark-inducible nature. I refer to the gene corresponding

this sequence as din1 and to its protein product as din1 protein in this thesis. pRDI-1 contained an ORF of 549 nucleotides. In order to characterize din1 protein, I determined nucleotide sequence of pRDI-1 and prepared antisera against din1 protein.

<MATERIALS AND METHODS>

1. Plant Material and Preparation of RNA

Radish seedlings were grown for 14 days as described in Chapter II. Poly(A)$^+$RNA was prepared from the radish cotyledons of the seedlings that were placed in darkness for 24 h as described in Chapter II.

2. cDNA Library for Isolation of Full Length cDNA

ds cDNA was synthesized according to the method of Gubler and Hoffmann (1983) with a cDNA synthesis kit purchased from Boehringer Mannheim.

About 1.6μg of ds cDNA was synthesized from 4.0 μg of poly(A)$^+$RNA. cDNA was methylated at 37^{o}C for 15 min with 10 units of BamHI methylase (Takara) in cDNA synthesis solution supplemented with 100 mM KCl, 10 mM EDTA, and 80μM S-adenosyl methionin. After extraction with phenol/chloroform (1:1) and chloroform, the cDNA was precipitated with ethanol. About 0.5 μg of the cDNA was ligated with 0.05 μg of BamHI linker (Takara), precipitated with ethanol, and digested with 100 units of BamHI in 100 μl of reaction mixture at 30^{o}C for 3 h. The cDNA was separated from free linker fragments by gel filtration through a Sephadex G-75 column with TE buffer.

Vector plasmid, pBS+, was digested with BamHI and

dephosphorylated with bacterial alkaline phosphatase (Takara). The pooled cDNA was precipitated with 1 μg of the digested vector DNA in 70% ethanol and ligation was carried out. Transformation of JM103 cells was performed according to the method of Hanahan (1985).

3. Selection of Nearly Full-Length cDNA Clones Corresponding to Clone-1 Insert

Candidate clones for full-length cDNA of clone-1 insert sequence were selected by hybridization with a DNA probe of clone-1 sequence. Hybridization was carried out as described for Hybridization with Immobilized RNA in Chapter II.

Plasmid DNA was prepared by alkaline lysis method (Maniatis et al., 1982) from colonies indicating hybridization signals with clone-1 probe, and digested with EcoRI and HindIII. Size of insert cDNA was estimated by electrophoresis on 1% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) (Maniatis et al., 1982). Twenty colonies were selected to contain plasmids with insert fragments larger than 700 bp.

4. *In Vitro* Transcription Translation Assay

As pBS+ has promoters for T3 and T7 RNA polymerase at both ends of multiple cloning site, both strands of the insert cDNA

can be transcribed into RNA separately by T3 or T7 RNA polymerase. Plasmid DNA was purified on CsCl gradients, and digested with EcoRI for T3 RNA polymerase (Stratagene, Madison, WI, USA) or with HindIII for T7 RNA polymerase (Stratagene). In vitro transcription was carried out with 50 units of T3 or T7 RNA polymerase and 5 μg of the digested DNA as a template in 25 μl of reaction mixture; 0.4 mM ATP, UTP, CTP, 0.05 mM GTP, 0.4 mM $m^7G(5')ppp(5')G$ (New England BioLabs, Bevery, MA, USA), 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM DTT, and 1 unit/μl human placental ribonuclease inhibitor (Amersham). After incubation at 37^{o}C for 30 min, GTP was added at 0.4 mM and incubated for another hour. Synthesized RNA was extracted with phenol-chloroform (1:1) then with chloroform, and precipitated with ethanol.

The synthesized RNA was translated into polypeptides and analyzed by SDS-PAGE as described in the section of Hybrid-Select Translation in Chapter II.

5. Sequence Analysis of cDNA clones

i) Determination of Nucleotide Sequence of pRDI-1

A series of truncated clones was prepared by the method of Henikoff (1984) and Yanish-Perron et al. (1985) with a Deletion Kit for Kilo-Sequence (Takara).

Two series of truncated clones were prepared from pRDI-1 after digestion at the multiple cloning site. One was deleted

from T3 promoter side and the other was from T7 promoter side. Plasmid DNA of respective clone was prepared by alkaline lysis method (Maniatis et al., 1982). About 2 μg of plasmid DNA in 50 μl of TE buffer was treated with 5 ng of RNase A at 37 ℃ for 15 min and placed on ice for at least 30 min after addition of 30 μl of 20% polyethyleneglycol, 2.5 M NaCl. The precipitated DNA was dissolved in 20 μl of H_2O, added with 5 μl of 1 N NaOH and incubated at room temperature for 5 min. Then the mixture was added with 8 μl of 3 M sodium acetate pH 5.2 and 90 μl of ethanol. Sequence reaction was performed with the precipitated DNA as a template by a sequencing reaction kit (Pharmacia LKB) which involves [α-^{35}S]dCTP and dideoxynucleotide chain termination method.

ii) Determination of Nucleotide Sequence of Clone-1 and 5

Insert DNA sequence of clone-1 and 5 was determined from both ends. ss M13 phage DNA was prepared according to Messing (1983), and the plus strand sequence was determined with M13 M1 primer (Takara). RF DNA of the clones were prepared as described in the previous section, and the minus strand sequence was determined with M13 RV primer (Takara).

iii) Computer Analysis of Nucleotide Sequence

The structure of the nucleotide sequence was analyzed by a computer program, GENETYX (Software Development Corp.). Homology search was carried out in GenBank and NBRF data base.

IEDAS's programs, SEQFN and SEQFP, were operated on a computer MicroVAXII at Center for Gene Research, Nagoya University.

6. Preparation of Antisera against the Polypeptide Encoded by Cloned cDNA Sequence

i) Preparation of an Antigen from Fusion Protein with Protein A.

pRDI-1 DNA, and a plasmid DNA, pRIT2T (Pharmacia LKB), were digested with PstI and SmaI. Insert sequence of pRDI-1 was separated from the vector DNA by electrophoresis on a 1% agarose gel in TBE buffer, and extracted electrophoretically from gel pieces with an Extraphor electric concentrator (Pharmacia LKB). The insert sequence was ligated with the pRIT2T DNA so that coding sequence of pRDI-1 was fused at the 3' end of Protein A gene of the vetor in the same coding frame.

E. coli N 4830-1 (Pharmacia LKB) was transformed with the plasmid bearing the fusion gene and grown on a plate containing ampicillin (50μg/ml). A single colony was isolated from the transformants, and cells were grown overnight at 30^{o}C in LB medium containing ampicillin. The culture was transferred to a fresh medium of 100-fold volume. After incubation at 30^{o}C for 6 h, the culture was mixed with a equal volume of the same medium heated at 54^{o}C and continued to be incubated at 42^{o}C for another 90 min. The cells were cooled on ice and harvested by centrifugation at 5000g for 5 min at 4^{o}C. The pellet was

suspended in 1/10 volume of ice cold TST buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). The suspension was added with 1/100 volume of 20% SDS and boiled for 5 min. After cooled on ice, the suspension was added with 1/100 volume of 200 mM PMSF and centrifuged at 20000g for 20 min at 4°C. Fusion protein was purified from the supernatant with a IgG-Sepharose 6FF column (Pharmacia) by affinity of protein A to IgG.

ii) Preparation of Antigens from Chemically Synthesized oligopeptides

Oligopeptides, NSNFRWRKVTTGRANVAAEA and AGYKHLDVRTPDEF-SIGHPS, were chemically synthesized to correspond to two hydrophilic regions of amino acid sequence predicted from the cDNA sequence by Multiple Peptide System (San Diego, CA, USA). The polypeptides were coupled to KLH (Calbiochem, La Jolla, CA, USA) with glutaraldehyde according to the method of Sayre et al. (1986). Five mg of the hemocyanin protein was mixed with 5 mg of mixture of the synthesized polypeptides in 2 ml of 0.1 M potassium phosphate buffer pH 7.5. One ml of 20 mM glutaraldehyde was added dropwise at room temperature. The mixture was then dialyzed against 2 l of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, overnight at 4°C.

iii) Immunization of Rabbits and Preparation of Antisera

One ml of the antigen solutions (1 mg/ml) was separately

mixed and emulsified with an equal volume of Freund's complete adjuvant (DIFCO, Detroit, Michigan, USA) and the mixture was injected into female rabbits. Half milligram of the antigen emulsified with incomplete adjuvant was injected as boosters after 3 and 4 weeks. Blood was collected from the rabbits after 5 weeks and incubated at room temperature for 1 h, then centrifuged at 5000g for 10 min. The supernatant was stored at -20^{o}C and used as an antiserum.

7. Immunochemical Analysis

i) Western Blotting

Polypeptides were separated by SDS-PAGE and electrophoretically transferred to a Millipore GV nylon membrane using Semi-Dry Electroblotter (Sartorius, Gottingen, Germany). Antigenic proteins on the membrane were immunochemically detected according to Blake (1984) with antisera specified in the experiments as primary antibodies and goat anti-rabbit IgG coupled with alkaline phosphatase as secondary antibodies.

ii) Immunochemical Analysis of din1 Protein Synthesized in vitro and in vivo

Cotyledons placed in the dark for 6 h were painted with ^{35}S-methionine in 50 mM MES-KOH pH 7.5, 0.1% Tween 20 on the both surface and kept in darkness for 6 or 18 h. Four

cotyledons were homogenized with 0.5 ml of 50 mM Tris-HCl pH 7.5 on ice. The homogenate was added with SDS at 1% (w/v) and boiled for 3 min, then centrifuged at 10000g for 5 min at 4^0C. The antiserum against the Protein A-*din*1 protein was added to the supernatant. For the detection of antigenic polypeptides in cell-free translation products, poly(A)$^+$RNA from cotyledons at 24 h of dark treatment was translated in a wheat germ system as described in Chapter II. The reaction mixture was boiled in 1 % (w/v) SDS, added with Triton X-100 at 2% (w/v), and the antiserum was added to the mixture. The resultant antigen-antibody complex was collected by binding to Protein-A Sepharose, eluted with SDS-sample buffer as described by Watanabe and Price (1982), and analyzed by SDS-PAGE and fluorography as described by Minami and Watanabe (1984).

<RESULTS>

1. Full-Length cDNA Clone for din1 Gene

I prepared another cDNA library for isolation of full-length cDNA clones. This library was constructed on a plasmid vector pBS+ (Fig.3-1) by insertion of the synthesized cDNAs at BamHI site. The library was screened by hybridization with the insert sequence of clone-1. Sixty clones survived two consecutive screenings and were examined for the size of insert DNA. Twenty clones that had cDNA sequences larger than 700 bp were selected and asked whether they had entire coding region by *in vitro* transcription and translation assay. Both strands of cDNA was transcribed by T7 and T3 RNA polymerase and translated in a wheat germ cell-free system. Nine clones produced the same polypeptides of 23 kDa (Fig.3-2) which is the same size as the product from mRNA selected by hybridization with clone-1 sequence (Fig.2-9). These observations indicate that these clones contained a cDNA sequence which covers the entire coding region of the original mRNA. One of the clones was named as pRDI-1 and used in further experiments.

The nucleotide sequence of the insert DNA of pRDI-1 was determined and shown in Fig.3-3. pRDI-1 contained an insert sequence of 699 bp. The sequences of clone-1 and 5 was also determined, and the results indicates that the sequence of pRDI-1 covers the sequences of the two original clones of

Fig.3-1. Structure of pBS plasmid vector. T3 and T7 with arrows indicate promoters for respective RNA polymerase and the direction of transcription.

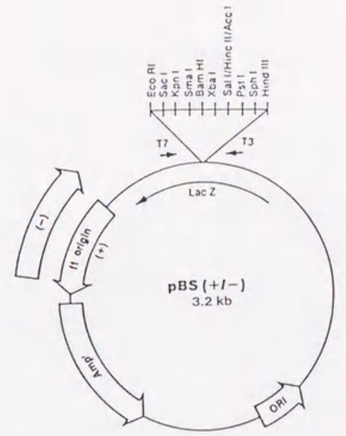


Fig.3-2. Translation products from RNA transcribed from cDNAs. Twenty pBS+ clones survived two cycles of hybridization screening with a probe of clone-1 sequence and selection by size of insert cDNA. Insert cDNAs of the clones were transcribed by T7 or T3 RNA polymerase, translated in a wheat germ cell-free system and separated on 13% acrylamide gel. The results of 6 clones, of which numbers are shown on the lanes, are presented. Lanes designated with 3 and 7 show in vitro translation products from RNA transcribed by T3 and T7 RNA polymerase, respectively. The arrow indicates translation product specific to transcribed RNA.

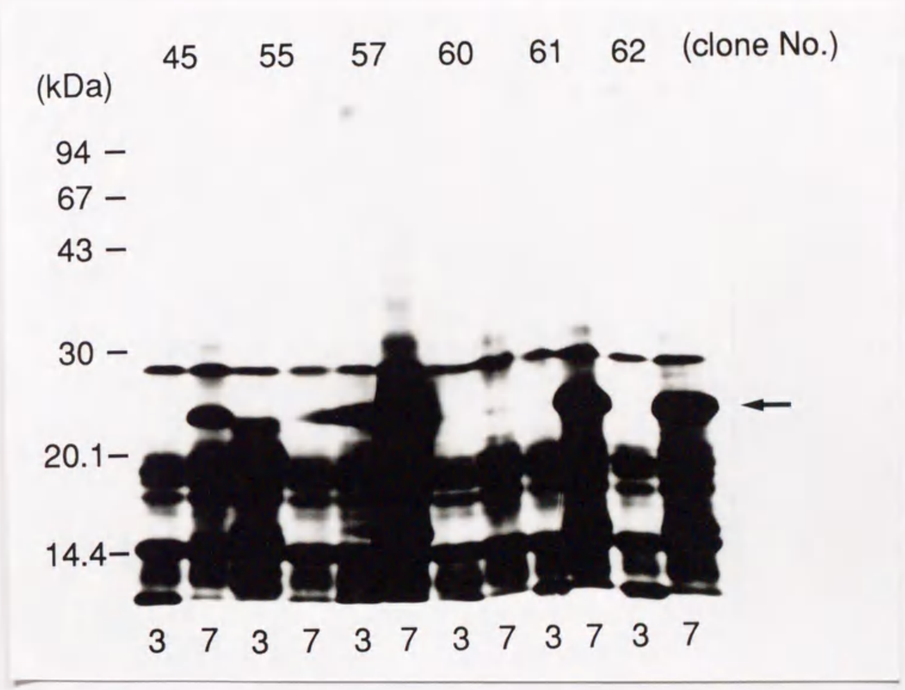


Fig.3-3. Nucleotide sequence and deduced amino acid sequence of pRDI-1. Amino acid sequence is translated from the first ATG codon. A possible poly(A) signal is underlined. Charged amino acid residues were marked by "+" or "-". Regions overlapping with clone-1 and 5 are marked with arrows at both ends of respective clones.

```
1   AAAAAATGGAATCTACTTTAAACACAACCGCACGAATCGGGAGCTGGTCATCGTTTATA
        M  E  S  T  L  N  T  T  A  R  I  G  S  W  S  S  F  I
                                   +

60  TCTCCACCTCTACAAGTGTGTGAATCTTTCAAGTGGAAGCTACCAAAGGCAACAAGAAGA
    S  P  P  L  Q  V  C  E  S  F  K  W  K  L  P  K  A  T  R  R
                                +     +        +        +  +

                  ┌→clone-1
120 GTCGTCAGTGTTGCAGATCGTCAGAACTCAAACTTCCGATGGAGGAAAGTAACAACAGGC
    V  V  S  V  A  D  R  Q  N  S  N  F  R  W  R  K  V  T  T  G
                   +                    +     +  +

180 AGAGCAAATGTTGCGGCAGAGGCAGCAGCTAGAGTTCCGACATCAGTACCGGTGAGAGTT
    R  A  N  V  A  A  E  A  A  A  R  V  P  T  S  V  P  V  R  V
    +                                +                       +

240 GCGCGTGAGCTAGCTCAAGCAGGATACAAACATCTTGACGTCAGGACACCGGACGAGTTC
    A  R  E  L  A  Q  A  G  Y  K  H  L  D  V  R  T  P  D  E  F
          +  -              +  +        -     +           -  -

300 AGTATCGGACATCCGTCTAGAGCTATTAACGTGCCTTACATGTACAGAGTCGGGTCAGGA
    S  I  G  H  P  S  R  A  I  N  V  P  Y  M  Y  R  V  G  S  G
             +        +                          +

360 ATGGTTAAGAACCCGAGTTTTCTAAGGCAGGTATCGTCTCATTTCAGGAAACACGACGAG
    M  V  K  N  P  S  F  L  R  Q  V  S  S  H  F  R  K  H  D  E
          +              +                 +  +  +  -  -

420 ATCATCATCGGTTGTGAGAGCGGAGAAAGATCTCTCATGGCTTCCACTGAACTTCTCACT
    I  I  I  G  C  E  S  G  E  R  S  L  M  A  S  T  E  L  L  T
                   -     -  +                    -

                        ┌→clone-5
480 GCTGGCTTCACCGGGGTTACAGACATTGCTGGAGGATACGTTCCCTGGACAGAGAATGAA
    A  G  F  T  G  V  T  D  I  A  G  G  Y  V  P  W  T  E  N  E
                         -                          -     -

540 CTACCAGTAGAAGAGTGAAAACAAAAAAAAAACATGGACCTATCCGTTTGTAAATTTGCAA
    L  P  V  E  E  *                                          A
             -  -

600 ATAAATAATTGTATATGTTTCAACACAAGTGTTTGTGTATGTCAATGGCACTACCAAATG

                  clone-5←┐clone-1←┐
660 ATATAAGTTTCATTTGGAACAAAACAAAAAAAAAAAAAAA
```

partial length, clone-1 and 5 (Fig.3-3). By this observation, it was confirmed that these two original clones were in fact derived from mRNA of a unique gene. I named the gene as din1 after its dark-inducible nature.

It was also found that the 3'-terminal end of the insert of clone-1 and both ends of that of clone-5 lack Sau3AI site sequences. This observation may suggest that some irregular reaction had occurred during ligation of BamHI-digested M13 RF DNA and Sau3AI-digested cDNA fragments. pRDI-1 contained a poly(A) stretch of 15 nucleotides at the 3' terminal. The sequence of pRDI-1 contained an ORF of 549 bp. A typical poly(A) signal was found at 42 nucleotides down stream of the stop codon.

The polypeptide coded for by the ORF consists of 183 amino acids and its molecular weight is calculated to be 20,280. As described in the former and the present chapter, molecular mass of din1 protein was estimated to be about 23 kDa by the SDS-PAGE of the translation products from RNA hybrid-selected by clone-1 and 5 and from RNA transcribed from pRDI-1. The estimation is in a reasonable range of the calculated molecular mass. The translation product from RNA transcribed from pRDI-1 plasmid DNA perfectly comigrated with that of mRNA hybrid-selected by the original clones. These observations suggest that the insert of pRDI-1 is most likely to contain a full coding region of din1, and that the ORF of 549 nucleotides codes for the din1 protein, though the upstream sequence from

the first ATG was very short and did not contain in-frame stop codons.

Amino acid sequence which was deduced from the nucleotide sequence of the ORF indicates that it codes for a very hydrophilic polypeptide and that nearly a quarter of the total amino acid residues are charged, N-terminal being relatively rich in basic amino acids, and C-terminal in acidic amino acids.

Homologous sequence was searched in GenBank and NBRF data base with SEQFN and SEQFP, homology search program of IDEAS, on a MicroVAXII computer system. Homology at moderate level (about 50%) was found in nucleotide sequences between pRDI-1 and cDNAs for tobacco PR proteins, PR1a, PR1b and PR1c (Cornelissen et al., 1986) (Fig.3-4). However, the amino acid sequences of din1 protein shows rather a low homology (20%) with those of the PR proteins. Partial homology was also found with ndh1 protein (a homologue of NADH-ubiquinone oxidoreductase of tobacco chloroplast), tobacco chloroplast RNA polymerase β chain, cutinase of Fusarium solani, and actin 7 protein of fruit fly (Fig.3-5), but it does not seem to be significant.

2. Immunochemical Analysis of din1 Protein

Antisera were raised in rabbits against two differently prepared antigens of din1 protein. One of them is a protein

A

```
din    --AAAAAATG GAA----TCT ACTTTAAACA ---------- --CAACCGCA CGAAT--CGG GAGCTGGTCA TCGTTTATA- ---TCTC-CA CCTCTACAAG
PR1b   GTTCAAAATA AAACATTTCT CCTATAGTCA TGGGATTTTT TCTCTTTTCA CAAATGCCCT CATTTTTTC- TTGTCTCTAC ACTTCTCTTA TTCCTAATAA

       TGTGTGAATC TT-TCAAG-T GGAAGCTACC A--AAGGCAA CAAGAAGAGT CGT----CAG -TGTTGCAGA T-----CGTC AGAACTCAAA CTTCCGATGG
       TATCTCACTC TTCTCATGCC CAAAACTCTC AACAAGACTA TTTGGATGCC CATAACACAG CTCGTGCAGA TGTAGGCGTG GAACCATTAA CTTGGGACAA

       AGGAAAGTAA CAAC--AGGC AGAGCA-AAT GTTGCGGCAG AGGCAGCAG- CT-AGAGTTC CGACATCAGT ACCGGTGAGA GTTGCGCG-T GAGCTAGCTC
       CGG--GGTAG CAGCCTATGC ACAAAATTAT GTTTCACAAT TGGCTGCAGA CTGCAACCTC GTACAT---T CTCATGGCCA ATACGGCGAA AACCTAGCTC

       AAGCAGGATA CAAACATCTT GACGTCAG-G ACACCGGACG AGTTCAGTAT CG--GACATC CGTCTAGAGC TATTAACGTG CCT----TAC ATGT--ACAG
       AGGGAAG-TG GCGATTTTAT GACGGCTGCT AAGGCCGTCG AGATGTGGGT CGATGAGAAA C----AGTAC TATGACCATG ACTCAAATAC TTGTGCACAA

       AGTCGGGTCA GGAATGGTTA AGA-ACCC-- GAGTTTT--- CTAAGGCAGG TATCGTCTCA TTT--CAGG- --AAACACGA CGAGATCATC ATCGGTTGTG
       GGACAGGT-- -GTGTGGACA CTATACTCAG GTGGTTTGGC GTAACTCGGT TCGTGTTGGA TGTGCTAGGG TTAAGTGCAA C-AATGGAGG ATATGTTGTC

       AGAGCGGAGA AAGAT-CTC- ---TCATGGC TTCCACTGAA CTTCTCACTG CTGG--CTTC ACCGGGGTTA CA-GACATTG CTGGAGGATA CGTTCCCTGG
       TCTTGCAACT ATGATCCTCC AGGTAATGTC ATAGGCCAAA GTCCATACTA ATTGAAATGA ATGTCCATTT CACGTTATAT ATGTATG-GA CTTCTGCTTG

       ACAGAGAATG AACTACCAGT AGAAGAGTGA AAACAAAAAA AAACATGGAC CTATCCGTTT GTAA-ATTTG CAA----ATA AATAATTGTA TATGTTTCAA
       ATATA-TATA AACAAC---T TAAATAATTG CACTAAAAAG CAACTTATAG TCAAAAGTAT ATAATATTTG TAATCCTCTG AAGAACTGGA TCTGTAAAAA

       -CACAAGTGT TTGTGTATGT CAATGGCACT A--CCAAATG ATATAAGTTT CAT-TTGGA- ---ACAAAAC AAAAAA---- --AAAAAAAA A
       GTCCAAGTG- --GTCTTAAT TAAGGGGGGG AGGATATATG AATTCAGCTT GATGTATGAT CTGATATTAT TATGAACTCT TTAGTACTCT T
```

B

```
din    MESTLNTTAR IGSWSSFISP PLQVCESFKW KLPKATRRVV SVADRQNS-N FRWRKVTTGR ANVAAEAAAR VPTSVPVRVA RELAQAGYKH LDVRTPDEFS
PR1b   MGFFL----- ---FSQMPSF FL-----VST LLLFLIISHS SHA--QNSQQ DYLDAHNTAR ADVGVE---- -PLTWDNGVA -AYAQNYVSQ LAADCNLVHS

       IG-HPSRAIN VPYMYRVGSG MVKNPSFLRQ VSSHFRKHDE IIIGCESGER SLMASTELLT AGFTGVTDIA GGYV-----P WTENELPVEE
       HGQYGENLAQ GSGDFMTAAK AVEMWVDEKQ YYDH--DSNT CAQGQVCGHY TQVVWRNSVR VGCARVKCNN GGYVVSCNYD PPGNVIGQSP Y
```

Fig.3-4. Comparison of din1 and PR1b. A: Nucleotide sequences of insert sequence of pRDI-1 and clone 69 of PR1b were compared using a GENETYX program MAXMH. Identical bases are marked with "*". Arrows indicate start and end points of coding region. B: Amino acid sequences of din1 and PR1b were compared using the same GENETYX program MAXMH. "*" indicates identical amino acid residue. ":" indicates conservative amino acid replacement grouped according to Dayhoff (1978) as follows: C; S,T,P,A,G; N,D,E,Q; H,R,K; M,I,L,V; F,W,Y.

Fig.3-5. Proteins having sequence homology with din1 protein. Amino acid sequences having homology with din1 protein were searched for in GenBank and NBRF with SEQFP homology search program of IDEAS. Parts of amino acid sequences of din1 (upper sequence) and selected proteins (lower sequence) are aligned with best match. 1, ndh1 protein; 2, RNA polymerase beta chain of tobacco chloroplast; 3, Cutinase of Fusarium solani; 4, actin 7 protein of fruit fly.

1

```
      10        20        30        40        50
ESTLNTTARIGSWSSFISPPLQVCESFKWKLPKATRRVVSVADRQNSNFR-W
: :       :     :    :  ::     ::   ::  :          :
ENTISSMCVINISTVFLISSLAECERLPFDLPRSRRRISSRVSNRIFRYQIW
     180       190       200       210       220
HOMOLOGY SCORE = -85   PERCENT MATCH  =  25.0%   (  13 /  52 )
```

2

```
      10        20
MESTLNTTARIGSWSSFISPPLQVCE
    :   :::: : :  ::     :
LIGSLAIHARIGHWGSLESPFYEISE
   440       450
HOMOLOGY SCORE = -58   PERCENT MATCH  =  38.5%   (  10 /  26 )
```

3

```
      80        90       100       110           120       130
PTSVPVRVARELAQAGYKHLDVRTPDEFSIGHPSRAINVPYMY-RVGS--G-MVK-NPSFLRQVS
 :                :   : :    :       :   : :    :      ::
ATASALPTSNPAQELEARQLGRTTRDDLINGNSASCRDVIFIYARGSTETGNLGTLGPSIASNLE
      20        30        40        50        60        70

      140       150       160       170
SHFRKHDEIIIGCESGERSLMASTELL-TAGFTGVTDIAGGY
: : :    : :     :       :             :
SAFGKDGVWIQGVGGAYRATLGDNALPRGTSSAAIREMLGLF
  80        90       100       110
HOMOLOGY SCORE = -65   PERCENT MATCH  =  17.8%   (  19 / 107 )
```

4

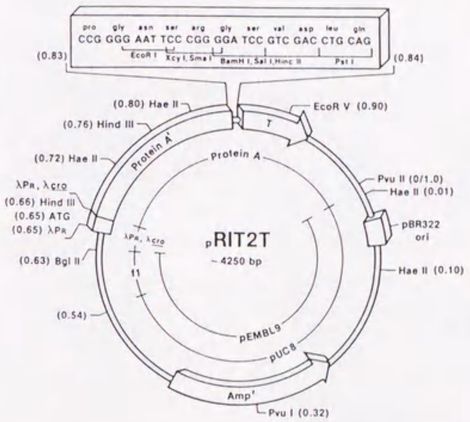
```
 20        30        40        50        60        70        80
ISPPLQVCESFKWKLPKATRRVVSVADRQNSNFRWRKVTTGRANVAAEAAAR-VPTSVPVRVA-R
 :      :    :: :  :    : :       :  : :       : :          :
VSHTVPIYEGYA--LPHAILRL-DLAGRDLTDY-LMKILTERGYSFTTTAEREIVRDIKEKLAYV
         170          180       190       200       210      219

         90        100       110       120       130       140
ELA-QAGYKHLDVRTPDEFSIGHP-SRAINVPY-MYRVGSGMVKNPSFLRQVSSHFRKHD-EIII
 :             : :   :    :       :       ::::   :          :
ALDFEQEMQTAASSSALEKSYELPDGQVITIGNERFRCPEALFQ-PSFLGMESAGIHETTYNSIM
        230       240       250       260       270       280

      150       160
GCESGERSLMASTELLTAGFTGVTDIAG
 :    :        :  : :    ::
KCDVDIRKDLYGNVVLSGGTTMFPGIAD
    290       300       310
HOMOLOGY SCORE = -88   PERCENT MATCH  =  20.9%   (  33 / 158 )
```

produced by E. coli cells. The coding sequence of pRDI-1 was fused in frame with a sequence of the affinity tail of Protein A and inserted in an expression vector, pRIT2T (Fig.3-6). The recombinant plasmid was introduced into E. coli N4830-1 and expressed in the cells. The fusion protein was purified from the cell extracts by affinity column chromatography. The molecular mass of the purified protein was estimated to be about 50 kDa, which is larger than that of the affinity tail by 20 kDa (Fig.3-7). The difference in the molecular mass coincides with that of din1 protein and indicates that the two sequences were correctly ligated and expressed. An antiserum raised against the purified din1- Protein A fusion protein specifically precipitated a polypeptide of 23 kDa in the in vitro translation products from poly(A)$^+$RNA (Fig.3-8). This polypeptide was found only in those from RNA of dark-treated cotyledons but not in those of light-grown cotyledons.

Two oligopeptides of 20 amino acid residues were chemically synthesized to correspond to two separated hydrophilic regions of the deduced amino acid sequence (Fig.3-9). These oligopeptides were conjugated with KLH by glutaldehydrate method and used as antigens to raise antiserum. This antiserum also recognized a translation product of 23 kDa only from poly(A)$^+$RNA of senescing cotyledons, but its titer appeared to be lower than the former antiserum. Therefore, the former was used in the subsequent experiments.

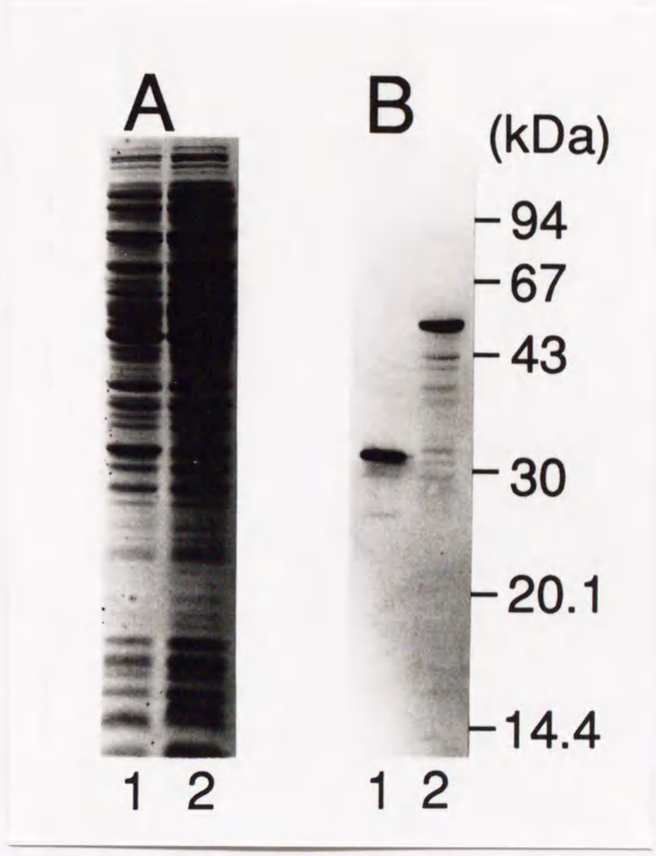
I tried to examine protein product of din1 in cotyledonary

Fig.3-6. Structure of pRIT2T expression vector.



map unit	origin
0-0.52	pUC8 (631-2686/1-234)
0-0.63	pEMBL9
0.54-0.63	phage f1 (6407-6041)
0.63-0.66	λPR, 5'-cro gene (12 codons)
0.66-0.83	Protein A (358-1103)
0.83-0.84	pUC9 Polylinker
0.84-0.93	Protein A (1550-1920)
0.93-1.0	Protein A 3'-noncoding

Fig.3-7. Chimeric protein of din1 and Protein A expressed in E. coli. E. coli N4830-1 cells containing pRIT2T or pRIT2T ligated with pRDI-1 insert sequence were grown, and the expression of the Protein A gene and din1-Protein A fusion gene on the plasmids was induced by incubation at 42°C. A; extracts of cells containing the Protein A gene (lane 1) and of cells containing the fusion gene (lane 2) were separated by SDS-PAGE and stained by Coomassie Brilliant Blue R. B; extracts of cells containing the Protein A gene (lane 1) and of cells containing the fusion gene (lane 2) were separated by SDS-PAGE and transferred to Millipore nylon membrane. Protein A (lane 1) and din1-Protein A fusion protein (lane 2) was detected on the membrane by reaction with rabbit IgG, then with alkaline-phosphatase conjugated anti-rabbit-IgG.



1 2 3 4

Fig.3-8. Immunoprecipitation of din1 protein from in vitro translation products. Poly(A)$^+$RNA was translated in a wheat germ cell-free system containing ^{35}S-methionine. din1 protein was precipitated by the antiserum raised against din1-Protein A fusion protein, electrophoresed on SDS-polyacrylamide gel and fluorographed. Lane 1, total translation products from poly(A)$^+$RNA prepared from light-grown radish cotyledons. Lane 2, those from poly(A)$^+$RNA prepared from dark-treated cotyledons. Lane 3, precipitates by control serum from those from poly(A)$^+$RNA of dark-treated cotyledons. Lane 4, precipitates with the anti-din1 - Protein A fusion protein antiserum from those from poly(A)$^+$RNA of dark-treated cotyledons.

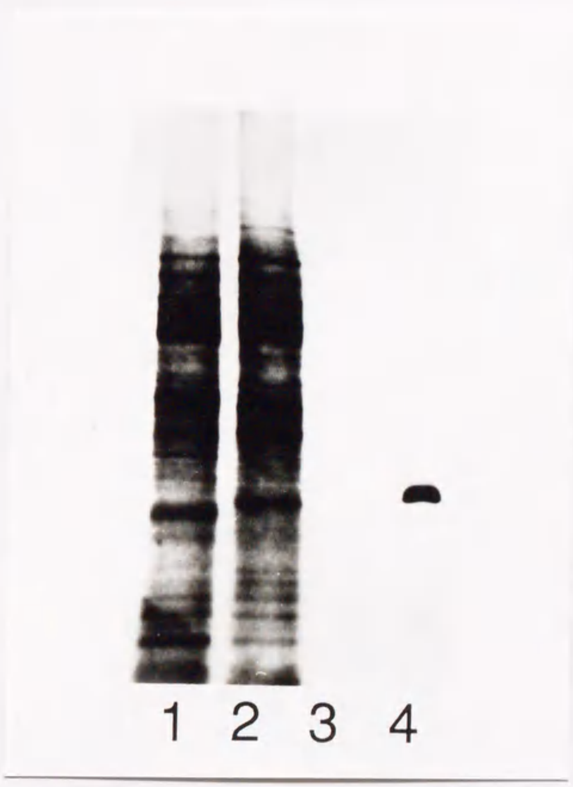
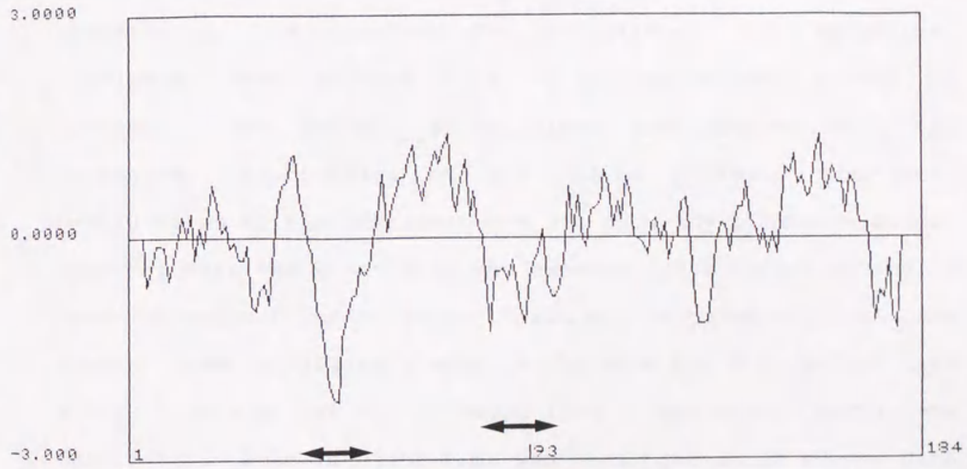


Fig.3-9. Hydropathy plot of predicted amino acid sequence of din1 protein. Oligopeptides of hydrophilic regions marked with arrows were chemically synthesized and used for immunization of rabbits.



cells by Western-blotting analysis using the anti-din1-Protein A fusion protein antiserum. Cotyledons kept in darkness for 24 h were homogenized, and proteins were separated on SDS-polyacrylamide gel, then blotted on a membrane. The antiserum could not detect antigenic polypeptides in the blots. The failure in detection may be due to the low titer of the serum. Therefore, I decided to label the presumptive gene product by radioisotope to increase the sensitivity of detection. Cotyledons were painted with ^{35}S-methionine and placed in darkness. The extract of the discs was treated with the antiserum. Fluorograms of SDS-PAGE of proteins that were precipitated by the antiserum did not show any prominent bands. However, when the X-ray film was exposed for a longer period, a band of polypeptide of about 17 kDa was observed only in the sample from cotyledons placed in the dark for 6 h before and after labeling, but not in those from light-grown cotyledons (Fig.3-10). When labeling time was prolonged to 18 h, the band disappeared. These results may suggest that din1 protein is synthesized temporarily and does not accumulate in the cells. The half life of the mRNA for din1 protein was estimated to be between 3 and 4 hours (Fig.3-11). The short half life of the mRNA also suggests that din1 protein is likely to turn over rapidly in the cotyledonary cells.

Fig.3-10. Immunoprecipitation of proteins synthesized in radish cotyledons. Cotyledons placed in darkness for 6 h were painted with ^{35}S-methionine and kept in the dark for another 6 h (lane 2 and 6) or for 18 h (lane 3). Cotyledons of light-grown seedlings were painted with ^{35}S-methionine and kept in the light at 25°C for 6 h (lane 1 and 5) or at 35°C for 2 h (lane 4). The cotyledons were homogenized and centrifuged at 10000g for 5 min. The antigenic polypeptides were precipitated from the supernatants with the antiserum raised against din1-Protein A fusion protein (lane 1, 2, 3, and 4) and with control serum (lane 5, 6), then electrophoresed on SDS-polyacrylamide gel and fluorographed.

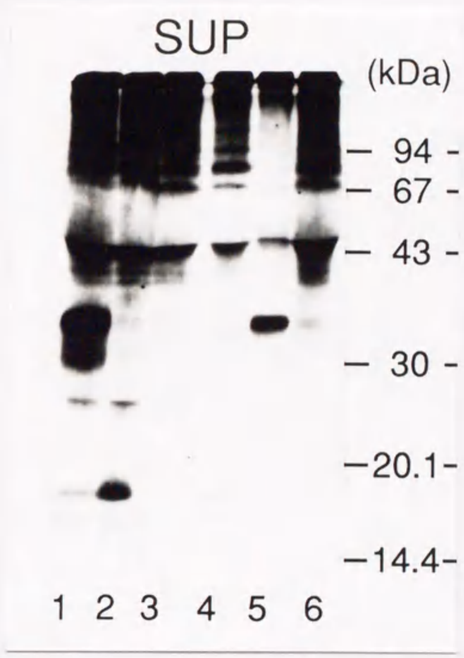
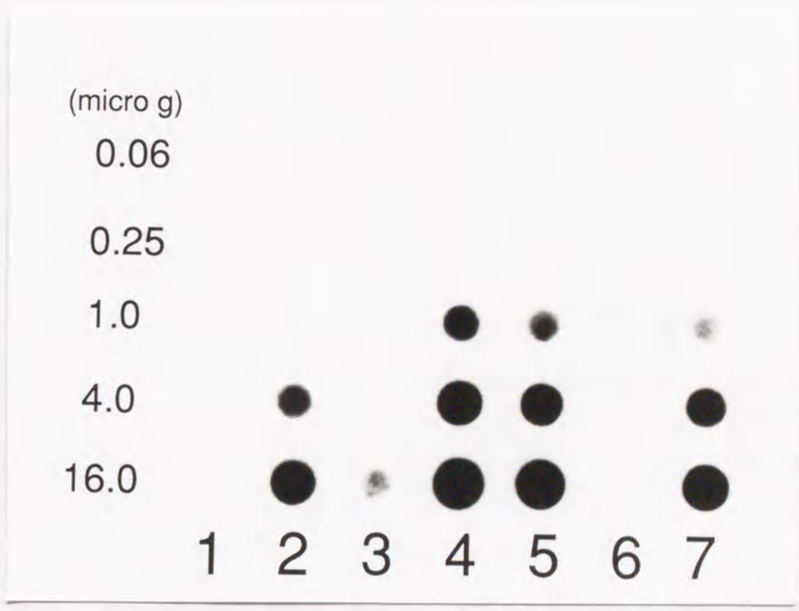


Fig.3-11. Effects of reillumination on the accumulation level of transcripts from din1 gene. Total cellular high-molecular-weight RNA was prepared from cotyledons exposed various light conditions, serialy diluted and blotted on a nylon membrane. The accumulation level of din1 transcripts was examined by hybridization with a probe of clone-1 sequence. Lane 1, cotyledonary RNA of light-grown seedlings; lane 2, cotyledonary RNA of those placed in the dark for 6h; lane 3, cotyledonary RNA of those placed in the dark for 6 h then in the light for 18 h; lane 4, cotyledoary RNA of those placed in the dark for 24 h; lane 5, cotyledonary RNA of those placed in the dark for 12 h; lane 6, cotyledonary RNA of those placed in the dark for 12 h then in the light for 24 h; lane 7, cotyledonary RNA of those placed in the dark for 36 h.



<DISCUSSION>

As shown in Chapter II, transcripts complementary to clone-1 and 5 showed a number of similar properties, such as the size, molecular mass of coding polypeptides, and in responses to various stimuli. While sequencing the inserts of clone-1 and 5, I was successful in isolating a clone with nearly full-length cDNA (pRDI-1) from new library using clone-1 insert as a probe. This clone was sequenced, and the comparison of the sequences revealed that clone-1 and 5 originate from unique mRNA transcribed from the same gene, din1.

The largest ORF contained in pRDI-1 was 549 bp, and other ORFs which can be found in different reading frames were much smaller. A translation product from RNA transcribed in vitro from pRDI-1 completely comigrated on SDS-polyacrylamide gel with that from RNA selected from poly(A)$^+$RNA of dark-treated cotyledons by clone-1 and 5 phage DNA. The ORF coded for a polypeptide of 183 amino acid. The calculated molecular mass is slightly smaller than that predicted by SDS-PAGE of translation product from poly(A)$^+$RNA, but it is in an allowable range of difference due to the methods. These results indicate that pRDI-1 contains the full coding sequence of din1. Start site of transcription is determined in Chapter V to be at 48 bp upstream from the initiation codon of the ORF. This datum shows that there is no other ATG sequence between the start

site of transcription and the initiation codon. These observations confirm the correctness of the ORF as the protein-coding sequence of din1.

The antiserum precipitated a polypeptide of 23 kDa from translation products of total poly(A)$^+$RNA. This 23-kDa polypeptide corresponds to the polypeptide produced from RNA selected by clone-1 and 5 and from RNA transcribed from pRDI-1. These results also support the idea that the ORF of pRDI-1 actually codes for a protein and guarantee the accuracy of the determined nucleotide sequence.

Amino acid sequence deduced from the nucleotide sequence suggests that din1 protein is a hydrophilic protein, though it has some stretch of hydrophobic regions close to near N- and C-terminal. The amino acid sequence does not contain information to specify subcellular localization of din1 protein. The nucleotide sequence of the ORF was found to be moderately homologous (about 50%) to cDNA sequences of a subfamily of pathogenesis-related proteins, PR1a PR1b and PR1c. These PR proteins have been reported to be present in aged leaves in flowering tobacco plants (Fraser, 1981). Therefore, the PR proteins may have some role in leaf senescence, and may be a member of senescence-associated genes. Homology in amino acid sequence between din1 protein and the PR proteins was low. This observation suggests that the functions of din1 protein and the PR proteins are divergent. The data base search also found partial homology with other proteins, but the score was

too low to suggest the function of din1 protein.

As din1 gene was quickly activated by heat shock, it may belong to the gene family of heat shock proteins. However, sequence comparison did not show any homology with known heat shock proteins. Okubara et al. has recently reported a cDNA clone for dark-inducible mRNA in Lemna plants (1989), but pRDI-1 did not have any similarity to its sequence either. Cloning of cDNA for senescence-associated genes was reported for carnation flower petals, but none of them shows resemblance with din1 gene as they are expressed only in flower organ and sizes of either transcripts or translation products from these genes are distinct from those of din1.

The antisera detected an in vitro translation product from din1, but failed to precipitate effectively antigenic proteins from extract of cotyledonary cells. It can not be excluded that the polypeptide of 17 kDa precipitated by the antisera is an experimental artifact because background noise was very high, especially in region for high-molecular-weight polypeptides. There are several possible reasons for this ineffectiveness of the antisera; i) the affinity of the antibodies to the antigen was not high enough, ii) in spite of the accumulation of din1 mRNA, it was not translated in vivo and other signal(s) is necessary to activate the translation, iii) din1 protein was degraded very rapidly and did not accumulate in the cotyledonary cells. The last case may be most probable as the antiserum detected a polypeptide of 17 kDa

which is likely a degradation product of din1 protein and din1 mRNA has a short half life which may be an indication of unstable nature of the protein. Whatever the reason is, it was very disappointing that the antisera have provided only negative data on the occurrence of din1 protein in cotyledonary cells. Localization of din1 protein in the cell was expected to give us information on the function of the protein.