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Genes involved in leaf senescence and regulation of their expression

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Abstract

We have isolated more than a dozen cDNA clones corresponding to genes that were expressed in *Arabidopsis* leaves when they were kept in the dark. The nucleotide sequence analysis showed that some of the clones encoded proteins with significant homology to β-glucosidase (*din2*), branched-chain α-keto acid dehydrogenase subunit E1β (*din3*), and another subunit E2 (*din4*), yeast RAD23 (*din5*), asparagine synthetase (*din6*), pre-mRNA splicing factor SRp35 (*din7*), phosphomannose isomerase (*din9*), seed imbibition protein (*din10*), and 2-oxoacid-dependent oxidase (*din11*). Accumulation of transcripts from *din3*, *4*, *6* and *10* occurred rapidly after the plants were transferred to darkness. Transcripts from *din2*, *9*, and *11* could be detected only after 24 h of dark treatment. Inhibition of photo-synthesis by DCMU strongly induced the accumulation of transcripts from those genes, and application of sucrose to detached leaves suppressed the accumulation both in the dark and by DCMU. These observations indicate that expression of the genes is caused by sugar starvation resulted from the cessation of photosynthesis. We further showed that *din2*-encoded protein also accumulated in senescing leaves. Given these results, possible roles of *din* genes in leaves in the dark and senescing leaves are discussed.

Light is an essential component in development, growth, and metabolism of higher plants and the plants employ flexible mechanism to adapt themselves to various light conditions (Frankland and Taylorson, 1983) [15, 36]. Light has been shown to control the expression of photoregulated genes via photoreceptor such as phytochrome. Light-induced gene expression has been well characterized in several plant species [30, 3, 31, 16]. Light also regulates gene expression in a negative fashion. However, only a few negatively regulated genes have been identified such as protochlorophyllide reductase [22] and asparagine synthetase [35, 20].

Light also significantly affects the expression of metabolite-regulated genes. For example, carbohydrate starvation has been shown to induce a dramatic increase in enzymatic activities related to catabolism of fatty acids and amino acids (Genix et al., 1990) [4, 10, 14, 1]. Many reports also described the induction of various genes by sugar starvation in cultured cells (Yu et al., 1991) [12, 26], root tips [4, 6, 7], and protoplast [29]. However, the relationship between sugar starvation and dark-induced gene expression is apparent only in a few cases [20, 21, 8].

Exposure to darkness is one of the most potent stimuli that accelerates leaf senescence. In many plant species, dark treatment causes degradation of chlorophyll and protein similar to what occurs in senescing leaves [23, 9]. With regard to gene expression, transcripts from several genes accumulated in senescing leaves as well as in dark-treated leaves [17, 2, 24, 19, 25, 37, 11]. These findings suggested that certain molecular events are common to the response to darkness and the naturally progressing senescence in leaves.

Identification of genes that are specifically expressed in the leaves in the dark is an important approach to understand the adaptation of plants to unfavorable light conditions. Isolation of as many as dark-inducible (*din*) genes is necessarily to study the molecular events occurring in the leaves in the dark. Here we used RNA isolated from 6, 24 and 72 h-dark treated leaves for differential display reverse transcription PCR (RT-PCR). We have identified more than a dozen cDNA clones for the *din* genes. We also found the expression of the genes in senescing leaves. We found that application of sucrose to leaves repressed dark-induced expression of the genes, indi-

Nucleotide sequences data will appear in the EMBL and GenBank Nucleotide Sequence Databases

cating that sugar is a key factor that modulates expression of the *din* genes.

Materials and Methods

Plant material

Arabidopsis thaliana L. Heynh (ecotype Columbia) plants were grown at 23°C under continuous light illumination (60 μmol m^{-2} s^{-1}). Young plants grown for 3 weeks were transferred to dark for various periods of time. Cut leaves were fed with sugars as described previously [11]. Naturally senescing leaves were collected from approximately 60-70 d-old plants grown under the photoperiod of 16/8 h light/dark. Fully expanded green leaves and other three groups of leaves were collected according to the stage of yellowing [11].

Differential display RT- PCR

Three-week-grown plants were exposed to darkness either for 6, 24 or 72 h. Leaves were harvested from each dark-treated plant and from control plants. Differential display RT-PCR analysis was carried out as previously described [11]. The PCR products were gel-purified and cloned in pGEM-T vector (Stratagene). The cDNA inserts of these plasmids were used as probes for northern analysis.

DNA sequencing

DNA sequence was determined by the dideoxynucleotide chain termination method [28]. DNA sequences were analyzed by a GENETYX program (Software Development Corp., Tokyo) and sequence homology was searched by the BLAST Network Service (National Center for Biotechnology Information, NCBI).

RNA isolation and northern analysis

RNA isolation, electrophoresis and blotting were performed according to standard method [27]. pSEN1 clone, carrying an insert of cDNA for *sen1* gene, was a kind gift from Dr. H. G. Nam (Pohang University of Science and Technology, Korea).

Screening of cDNA library

λ-Zap II cDNA library constructed from leaves of 24 h-dark treated plants [11] was screened with the partial cDNA fragments as probes. Isolation of full-length cDNA clones from the library was carried out according to manufacturer's instruction (Stratagene).

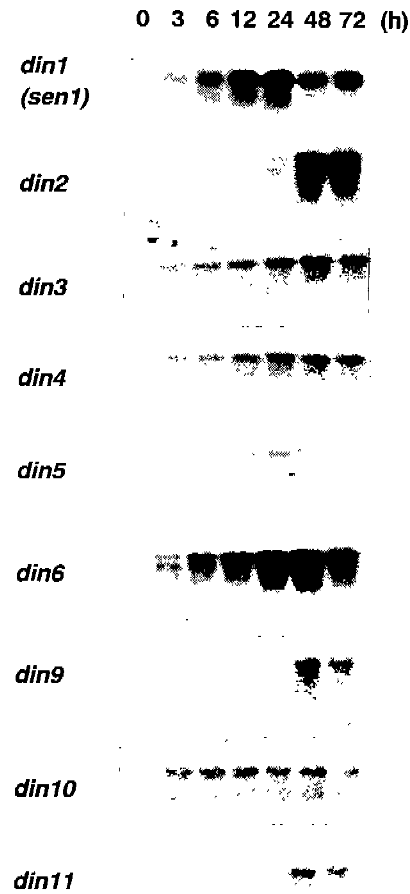

Figure 1. Time course analysis of dark-induced expression of din genes. Total RNA was isolated form leaves kept in darkness for 0, 3, 6, 12, 24, 48, or 72 h. Ten g of total RNA was loaded in each lane. Membranes were hybridized with 32P-labeled inserts from the different cDNAs, and with 25S rDNA as a control of relative loadings of RNA in each lane.

Results and Discussion

Isolation of cDNA clones for dark-inducible genes

We used the differential display RT-PCR technique to obtain cDNA fragments corresponding to dark-inducible genes from *Arabidopsis*. In this study, we have compared cDNAs derived from leaves of light-grown plants and from leaves of plants that were placed in the dark either for 6, 24, and 72 h. In the course of differential display RT-PCR, PCR bands which appeared specific to dark-treated samples were identified by ethidium bromide staining. We confirmed that these PCR products are actually corre-

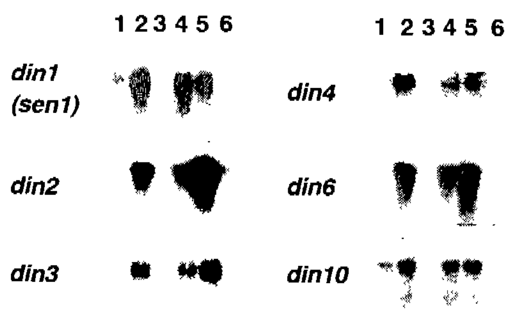

Figure 2. Effects of sucrose and DCMU on the expression of din genes. Detached leaves were incubated with different media for 48 h either under continuous light illumination (lanes 1, 5, and 6) or in darkness (lanes 2, 3, and 4). Lanes 1 and 2, water with no addition of sugar; lane 3, 3% (w/v) sucrose; lane 4, 3% (w/v) mannitol; lane 5, 10 M DCMU; lane 6, 10 M DCMU plus 3% (w/v) sucrose. Total RNA (20 g) from each sample was analyzed by northern blot hybridization.

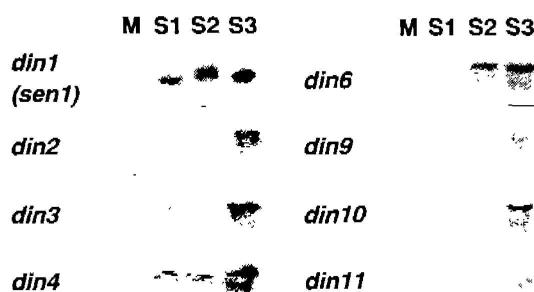

Figure 3. Accumulation of transcripts from din genes in senescing leaves. Total RNA was isolated from mature green leaves (lane, M) and from leaves with an increasing degree of yellowing: approximately 25, 50, and 75 % of leaf area have become yellow (lanes, S2, S2, and S3, respectively). Ten g of total RNA was loaded in each lane and was analyzed by northern bolt hybridization.

sponded to transcripts accumulating during dark treatment by northern blot analysis (data not shown). As a result, we obtained several cDNA fragments corresponding to dark-inducible genes. cDNA fragments of *din10* were obtained from 6-h dark-treated sample, *din2* to *din6* were obtained from 24-h sample, and *din9* and *11* were from 72-h sample, respectively (Table I).

Deduced protein sequences of these cDNA fragments showed significant similarity to sequences within database; β-glucosidase (*din2*), branched-chain α-ketoacid dehydorgenase E1β subunit (*din3*) and E2 subunit (*din4*), yeast RAD23 protein (*din5*), asparagine synthetase (*din6*), pre-mRNA splicing factor SRp35 (*din7*), phosphomannose isomerase (*din9*), seed imbibition protein (*din10*), and 2-oxoacid-dependent oxidase (*din11*).

Time course analysis of dark-induced expression of din genes

The accumulation of transcripts for *din1*, *3*, *4*, *6* and *10* was detected as early as 3 h after the beginning of dark treatment. The transcript levels of these *din* genes reached maximum after 24-48 h of dark treatment (Figure 1). On the other hand, the transcripts for *din2*, *9* and *11* was detected only after 24 h of dark treatment. The levels of these transcripts continued to increase up to 72 h of treatment (Figure 1).

We also examined the expression of *sen1*, which was previously characterized as a senescence-associated *Arabidopsis* gene [24]. The *sen1* is an *Arabidopsis* homologue of *din1*, which was originally isolated from dark-treated cotyledon of radish [2]. Transcripts from the *sen1* gene began to accumulate rapidly after the onset of dark treatment, the profile of which resembled those of *din3*, *4*, *6* and *10* genes (Figure 1).

The *din* genes can be classified into two distinct groups according to the pattern of dark-induced expression. The first group includes "early-induced genes" such as *sen1*, *din3*, *4*, *6* and *10*. The transcripts of these genes increased immediately at an early stage of dark treatment. *Din6* and *7* may belong to this group, but their response to darkness was not very strong like other genes. Their expression would occur in natural diurnal cycle (i.e. in the night). The second group includes "late-induced genes" such as *din2*, *9* and *11*, whose expression was induced after 24 h of dark treatment. The dark-induced expression of these late-induced genes would not occur during a natural light/dark cycle. They would rather be required for plants to survive prolonged unfavorable light conditions such as shading.

Suppression of dark-induced expression of din genes by sugar

The photosynthetic activity declines during dark treatment, resulting in decrease in cellular sugar levels [18, 33]. Such alteration in the level of carbohydrate has been demonstrated to affect the expression of dark-induced genes, such as ASN1 [20], *sen1* [8], *din3* and *din4* [11]. It can be considered that the decrease in cellular sugars may be one of potent signals to enhance the expression of the din genes. To test this possibility, we examined the effect of application of sucrose and a photosynthesis inhibitor, DCMU, on the transcripts levels of the *din* genes (Figure 2). The expression of all the genes examined were induced even under illumination of white light, when leaves were treated with 10 μM DCMU for 48 h. On the

other hand, supplement of 3% of sucrose to detached leaves reduced the transcripts levels of din genes in leaves kept in the dark for 48 h. These results suggested that the expression of a variety of *din* genes were regulated in a common manner, that is, suppressed when sugar level is high and expressed when it is low.

Accumulation of transcripts from din genes in senescing leaves

Dark treatment has been used to artificially induce leaf senescence. Leaf senescence, as the final period of leaf development, is the series of complex biochemical and physiological events [34, 23]. It is well established that leaf senescence is a highly regulated process requiring specific gene expression (Smart, 1994) [5].

We performed Northern blot analysis to examine a correlation between din genes and leaf senescence. Figure 3 showed that transcripts from din gene were abundant in senescing rosette leaves, but expression patterns showed slightly differences among din genes. Transcripts from *din6* and *sen1* began to accumulate at the first stage of senescence (stage S1). Transcripts from *din2*, *9* and *11* were detected clearly only at the last stage of senescence (stage S3). The similarities of expression patterns of *din* genes between dark-induced senescence and natural senescence suggested that some molecular events occurred parallel in a same manner in these different systems.

Senescence-associated gene expression may occur in part due to the decline in cellular sugar levels caused by cessation of photosynthesis [13]. Observations begin to accumulate to evidence that expression of several senescence-associated genes is regulated in part by sugar levels [8, 11 and this study]. Sugar starvation may act as one of a potent signal to induce the expression of din genes in senescing leaves, as well as in dark-treated leaves.

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