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Isolation of cDNA Clones for Genes That Are Expressed during Leaf Senescence in *Brassica napus*

Identification of a Gene Encoding a Senescence-Specific Metallothionein-Like Protein

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cDNA clones representing genes that are expressed during leaf senescence in *Brassica napus* were identified by differential screening of a cDNA library made from RNA isolated from leaves at different stages of senescence. The expression of these genes at different stages of leaf development was examined by northern blot analysis, and several different patterns of expression were observed. One of the clones, LSC54, represented a gene that is expressed at high levels during leaf senescence. Analysis of this gene indicated strong expression in flowers as well as in senescing leaves. DNA sequence analysis of the LSC54 cDNA indicated a similarity between the deduced amino acid sequence and several metallothionein-like proteins previously identified in plants.

Leaf senescence is a key developmental step in the life cycle of an annual plant, since it is the time during which material built up by the plant during its growth phase is mobilized into the developing seed to prepare for the next generation. Leaf senescence is a period of massive mobilization of nitrogen, carbon, and minerals from the mature leaf to other parts of the plant. The process appears to be a highly regulated, ordered series of events involving breakdown of leaf proteins, loss of photosynthetic capability, disintegration of chloroplasts, loss of Chl, and export of metabolites (Nooden, 1988). In annual plants, which include most agricultural crops, mobilizable nutrients from the entire plant are eventually stored in the developed seeds. A considerable percentage of nitrogen for seed filling is derived from vegetative plant parts; therefore, remobilization from senescing leaves is critical for the nutrient budget in seed crops (Feller and Keist, 1986).

Although there are extensive physiological and biochemical data on leaf senescence, the molecular events that induce, and that are involved with, the process are not clearly understood. There is good evidence that leaf senescence is regulated as a controlled developmental process and that the expression of specific genes is induced. It has been shown that transcription and translation are required for senescence to proceed and that senescence itself is an energy-requiring process (Thomas and Stoddart, 1982; Davies and Grierson, 1989). In vitro translation experiments using RNA from senescing leaves have demonstrated the production of specific proteins during senescence (Thomas et al., 1992). Many enzymes are likely to be involved in the senescence process including proteases, nucleases, and other degradative enzymes as well as enzymes involved in chloroplast dismantling and Chl breakdown. Genes showing induced expression during senescence are likely to include those encoding these degradative enzymes as well as proteins that are required for the regulation of the process. For example, the expression of genes coding for enzymes of the glyoxylic acid pathway, which is involved with lipid breakdown, is induced during senescence (Gut and Matile, 1988), and some of the genes that show increased expression during fruit ripening in tomato, including an ethylene-biosynthesis gene, are also active during leaf senescence (Davies and Grierson, 1989). However, definitive identification of the key proteins that are involved in the senescence process has been difficult to achieve, partly due to the high background level of degradative enzymes that is already present in the plant cell (Huffaker, 1990).

The isolation of cDNA clones representing genes that show induced expression during leaf senescence should help to identify the proteins that are involved in the process and to facilitate the study of their mode of action. Analysis of the regulation of these genes will be of fundamental importance in the identification of the mechanisms that are involved in the induction of leaf senescence by developmental and environmental signals.

Brassica napus was chosen as the plant system in which to study leaf senescence for several reasons. The leaves of *B. napus* undergo senescence in an ordered fashion, from the base of the plant to the apex, which allows the senescent stage of each leaf from a plant to be estimated relatively reproducibly. *B. napus* is taxonomically related to *Arabidopsis*, in which parallel studies aimed at identifying senescence mutants are being carried out. *B. napus* is an important agronomic crop, and it can be transformed with foreign DNA using *Agrobacterium* transformation. Therefore, once senescence-related genes are identified, it may be possible to manipulate the rate of senescence and estimate the effects on seed yield and forage quality.

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Abbreviation: Cab, Chl *a*/*b*-binding protein.

In this paper, the cloning and preliminary analysis of several cDNA clones representing genes showing induced expression during leaf senescence in *B. napus* are described. Further characterization of one of these cDNAs has indicated a sequence similarity to a metallothionein-like protein. Possible functions of this protein in leaf senescence are discussed.

MATERIALS AND METHODS

Plant Material

Field-grown, healthy plants of *Brassica napus* cv Falcon were used as a source of material for RNA isolation. Leaves were removed from plants and immediately frozen in liquid nitrogen.

RNA Isolation

RNA was isolated from leaves at several stages of development. Developmental stages were assessed according to the proportion of yellowing the leaf had undergone. Stages S1, S2, S3, and S4 represent leaves at senescence stages showing approximately 0 to 5%, 5 to 25%, 25 to 50%, and 50 to 100% yellowing, respectively. Green leaf RNA, stage G4, was isolated from mature green leaves from the same plants from which the senescing leaves were collected. Green leaf RNA was also isolated from young plants from green leaves at several stages of expansion, G1, G2, and G3, representing leaves at approximately 50% expanded, 75% expanded, and fully expanded. RNA was also isolated from roots, flowers, small pods, and mature pods.

The RNA isolation method was similar to that described in Ainsworth et al. (1993). Five grams of frozen plant material was ground in liquid nitrogen with a precooled pestle and mortar. The frozen powder was transferred to a 50-mL tube containing 15 mL of extraction buffer (100 mM Tris-HCl, pH 9.0, 200 mM NaCl, 1% Sarcosyl, 20 mM EDTA) and 10 mL of phenol. The frozen powder was dispersed by grinding with a Polytron for 30 s. After centrifugation to remove insoluble material, the aqueous phase was extracted once with phenol:chloroform (50:50) and twice with chloroform. The solution was adjusted to 2 M LiCl with 8 M LiCl solution, and the RNA was precipitated overnight at 4°C. The RNA was pelleted by centrifugation (10,000*g* for 10 min), washed twice in 2 M LiCl by resuspension and centrifugation (10,000*g* for 10 min), and dissolved in TE (10 mM TrisCl, pH 8.0, 1 mM EDTA). After centrifugation to remove any insoluble material, the RNA was ethanol precipitated and dissolved in water.

In Vitro Translation

In vitro translation was carried out with 10 μg of total RNA using a rabbit reticulocyte in vitro translation system (Amersham) incorporating [^{35}S]Met (Amersham). Labeled proteins were separated on 10% polyacrylamide gels (Laemmli, 1970) and visualized by fluorography using ENHANCE (NEN, Dupont) and Kodak XAR-5 film.

mRNA Isolation and cDNA Library Construction

Polyadenylated mRNAs were isolated from total RNA by a single round of oligo(dT)-cellulose chromatography as described by Sambrook et al. (1989). Five micrograms of mRNA was used as a template for cDNA synthesis using a kit (Pharmacia) and processed according to the manufacturer's instructions. cDNA was cloned into λ ZAPII (Stratagene, La Jolla, CA). Phage DNA was packaged using Gigapack Plus (Stratagene) and plated using XL1Blue (Stratagene) as a plating bacterial strain.

Differential Screening

For differential screening, 10,000 to 20,000 plaque-forming units were plated with XL1Blue onto 20 × 20 cm plates (Nunc, Roskilde, Denmark). The plaques were lifted in duplicate onto Hybond N+ membranes (Amersham), denatured on a pad soaked with 0.5 M NaOH/1.5 M NaCl, neutralized with 0.5 M Tris-HCl, pH 7.2/1.5 M NaCl, rinsed in 2× SSC, dried, and fixed by UV irradiation. Filters were prehybridized in 0.6 M NaCl, 20 mM Pipes, pH 6.8, 4 mM EDTA, 0.2% gelatin, 0.2% PVP, 0.2% Ficoll 400, 1% SDS, containing 500 μg/mL denatured (autoclaved) salmon sperm DNA, for 4 h at 65°C. Differential probes were made using 2 μg of mRNA purified from total RNA isolated from green or senescing leaves. Labeled cDNA was synthesized from this mRNA using random primers in a DNA oligo-labeling kit (Pharmacia). In a 50-μL reaction, 2 μg of mRNA (denatured at 65°C for 5 min) was mixed with 10 μL of 5× random primer buffer, 100 units of avian myeloblastosis virus reverse transcriptase, 1 unit of RNAsin (Promega), KCl to give a final concentration of 50 mM and 50 μCi [^{32}P]dCTP, and this was incubated at 37°C for 2 h. Unincorporated nucleotides were removed by spin dialysis through Sepharose CL-6B (Pharmacia). Prehybridized filters were hybridized with the denatured labeled probes in the buffer described above for 16 h at 65°C. Hybridized filters were washed twice in 2× SSC, 0.5% SDS (15 min) and once in 0.2× SSC, 0.5% SDS and exposed to x-ray film.

Plasmid DNA Isolation

Bluescript clones carrying cDNA inserts were excised from the selected λ ZAP clones using helper phage as described in the manufacturer's instructions and selected on ampicillin. Plasmid DNA was purified by a miniprep method (Sambrook et al., 1989) and digested with *Eco*RI. The cDNA fragments were gel purified and extracted from agarose using QIAEX (Qiagen, Chatsworth, CA). DNA fragments released from the vector were labeled with [^{32}P]dCTP using an oligo-labeling kit (Pharmacia) and used in hybridization with northern blots.

Northern Blotting and Hybridization

Total RNA (10 μg/track) was separated on 1.2% agarose/formaldehyde gels and blotted to Hybond N+ membrane (Amersham) using 0.05 M NaOH. Blots were hybridized to labeled probes using prehybridization and hybridization conditions as described above for plaque screening.

Sequencing and DNA Sequence Analysis

Plasmid sequencing was carried out using [^{35}S]dATP as described by Murphy and Ward (1989). The sequence was compiled and analyzed using the DNASTAR package (DNASTAR, Madison, WI).

Primer Extension

A primer homologous to DNA close to the 5′ end of the cDNA with sequence CACCACATTTGCAACCGGAA was synthesized to use in primer extension studies. The primer (10 pmol) was 5′-end labeled with [γ-^{32}P]ATP (10 pmol) using T4 polynucleotide kinase (10 units) in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 0.1 mM EDTA. The reaction was incubated at 37°C for 45 min and then precipitated with ethanol in the presence of 0.3 M Na acetate and 5% glycogen. The labeled primer was pelleted by centrifugation, resuspended in 50 μL of TE. In a 10-μL reaction, 10 μg of total RNA was mixed with 1 μL of labeled primer (400,000 cpm) and 0.5 μL of RNAsin in 2 mM Tris-HCl, pH 8.0, 250 mM KCl, 0.2 mM EDTA. The reaction was set up in triplicate and incubated at 37, 45, or 55°C for 5 h with occasional centrifugation. The extension reaction was carried out by adding to each annealing reaction 0.5 μL (10 units) of SuperScript Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and 23 μL of a reaction mixture containing 10 mM $MgCl_2$, 5 mM DTT, 20 mM TrisCl, pH 8.3, and 33 mM each of dATP, dCTP, dGTP, and TTP and incubating at 37°C for 45 min. The products were precipitated with ethanol and centrifuged, and the pellet was dried under vacuum. The pellet was dissolved in 3 μL of NaOH (0.1 M) and incubated at 37°C for 10 min, and then 9 μL of formamide dye (98% formamide, 1 mM EDTA, 0.3% xylene cyanol, 0.3% bromphenol blue) was added. Samples were denatured at 95°C for 2 min and loaded onto a 6% sequencing gel.

RESULTS

In Vitro Translation

Total RNA isolated from green leaves (stage G4) and leaves at two stages of senescence, S1 (0–5% yellow) and S4 (50–100% yellow), was translated in vitro and the products were analyzed by one-dimensional PAGE (Fig. 1). The majority of protein bands were common to all developmental stages. However, several distinct changes in protein banding patterns were visible between the proteins produced from G4 RNA and from S1 RNA, and even more differences were apparent in the S4 RNA track. The level of transcript for several prominent proteins that are synthesized from the G4 RNA falls as senescence progresses, as is shown by the reduced abundance of these products in the S1 track and the even more reduced abundance in the S4 track. For example, strong bands at 44 and 41 kD are reduced in level in the S1 track and appear to be absent in the S4 track, and proteins at 40, 37, and 34 kD become reduced in abundance. Other proteins appear to increase in level during senescence. A protein of 54 kD is abundant in the S1 RNA track but not in the S4 track. A 55-kD protein becomes visible in the S1 RNA track and is also present as an S4 product, where an additional band at 57 kD is visible. A new band at 39 kD and two at around 33 kD are also evident in the S4 track.

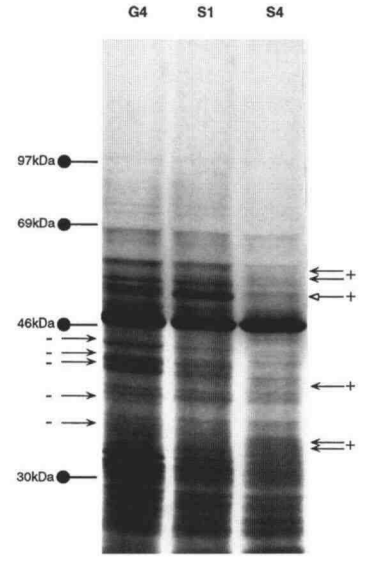

Figure 1. Proteins produced by in vitro translation of RNA isolated from leaves at different stages of development. RNA isolated from mature green (G4), early senescing (S1), and late senescing (S4) leaves was translated in a rabbit reticulocyte translation system and the proteins were labeled by incorporation of [^{35}S]Met. Labeled proteins were separated on an SDS 10% acrylamide gel. Protein sizes and arrows indicating protein bands that decrease (–) and increase (+) in intensity as senescence progresses are shown. The single open arrow shows the position of the 54-kD protein that is expressed strongly at the S1 stage.

It is obvious that induced expression of specific mRNA transcripts takes place during the senescence process and novel proteins are synthesized. Several differences were visible in the one-dimensional gel system used, and it is likely that many more differences would be resolved in a two-dimensional gel analysis. The results from this analysis indicate that it should be possible to isolate cDNA clones for senescence-specific genes by differential screening of a cDNA library made from RNA isolated from senescing leaves.

Library Construction and Screening

cDNA was synthesized using mRNA purified from a mixture of total RNAs isolated from senescence stages S2 to S4 and was cloned, after the addition of *Eco*RI/*Not*I adapters, into the *Eco*RI site of λ ZAPII. A library consisting of 7×10^5 independent clones was constructed. Clones were plated on 20 × 20 cm plates at a density of 10,000 to 20,000 plaque-forming units per plate. Duplicate lifts onto membranes were taken from each plate. One membrane was hybridized with ^{32}P-labeled first-strand cDNA synthesized from the same mRNA that was used for library construction (S2–S4), and the other was hybridized to a probe made from mRNA from mature green leaves (G4). Altogether, approximately 100,000 clones from the library were differentially screened to identify clones that showed increased expression in senescing leaves.

The first screening resulted in the isolation of 800 clones that hybridized to the probe made from the senescing leaf RNA and not to the green leaf probe. These clones were subjected to a second round of differential screening; individual plaques picked from the first round were eluted in 500 μL of phage buffer, and then 1 μL of eluted phage was spotted onto a top layer containing plating bacteria on an agar plate. The phage were grown overnight and then lifted to duplicate filter discs and hybridized as above. After this second screen, approximately 100 clones were selected for further analysis.

Identification of Senescence-Specific Clones

Bluescript derivatives of the cDNAs were excised from each λ ZAP clone that was selected after the second screening. Inserts from the clones obtained were purified, labeled with [^{32}P]dCTP, and used to hybridize to northern blots carrying two RNA samples, one from mature green leaves (G4) and the other from the same RNA mixture used to make the cDNA library (S2–S4). From the 100 clones originally selected, clones for genes showing a range of expression levels and patterns were seen. After the northern blot analysis, between 10 and 20 clones were identified for genes that were expressed in senescing leaves and showed very little or no expression in green leaves. These clones were subjected to further analysis. Several clones representing genes that were expressed strongly in senescing leaves but also showed fairly high levels of expression in green leaves were identified but not analyzed further.

Expression during Leaf Development

Clones for genes that showed differential expression in the initial northern analysis were examined further to determine the patterns of gene expression in leaves at different stages of development from young green to fully senescent. Northern blots carrying six different total RNAs (G1, G2, and G3 [young green leaves at different stages of expansion] and S1, S2, and S4 [senescing leaves at different stages of yellowing]) were hybridized with the cDNA inserts from the putative senescence-specific clones. The transcript sizes of the genes represented by these cDNAs were estimated from these northern blots.

A range of expression patterns was evident (Fig. 2). The expression of the photosynthesis-specific protein Cab, which is expressed in developing green leaves, was also assessed on the same northern blots as a control, using a Cab-1 gene from wheat (Lamppa et al., 1985). The Cab genes were expressed at high levels only in the green leaves, with little mRNA being present in the senescing leaves. By analysis of their expression patterns at different stages of leaf development, the cDNA clones could be divided into three classes.

One class of gene, which included clones LSC54 and LSC94, showed very little expression at any stage of green leaf development but was expressed strongly at all stages of senescence. The gene represented by LSC94 (0.65 kb) was expressed most strongly at the first senescence stage, whereas LSC54 (0.45 kb) showed increased expression as senescence progressed, with a very high level of expression in the final senescence stage.

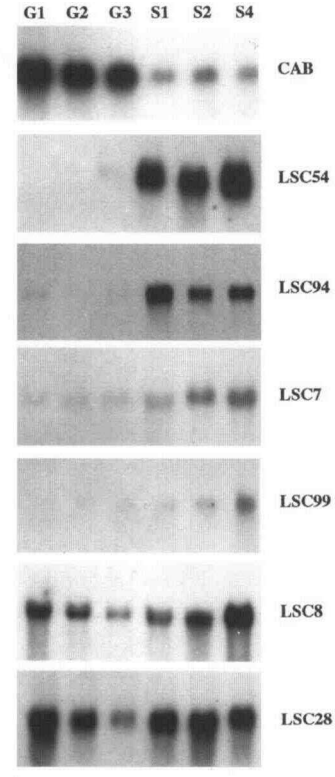

Figure 2. mRNA expression during leaf development. Northern blots carrying RNA isolated from leaves at six different stages of development were hybridized with ^{32}P-labeled inserts from the different cDNA clones. G1, G2, and G3 refer to RNA isolated from green leaves from young plants at approximately 50, 75, and 100% expansion, respectively. S1, S2, and S4 refer to RNA isolated from senescing leaves showing 0 to 5, 5 to 25, and 50 to 100% yellowing, respectively. Ten micrograms of total RNA was loaded in each track. Each northern blot was hybridized with a different cDNA insert and results obtained with six of these are shown as examples. As a control, the wheat Cab-1 gene probe was hybridized to the same northern blots.

Another class of gene, represented by LSC7 and LSC99, was expressed at very low levels throughout leaf development, and the level of mRNA increased during senescence. LSC7 (1.5 kb) mRNA levels increased in the S2 stage and LSC99 (1.3 kb) levels increased only in the final, S4 stage of senescence.

The third class of gene, when tested in the original northern analysis, was expressed at very low levels in mature green leaves (G4, data not shown), and these genes were thought to be senescence specific. After further experiments it was discovered that expression of these genes, which included LSC8 (1.1 kb) and LSC28 (0.9 kb), appeared not to be solely senescence related. Analysis of the expression of these genes in green leaves at different stages of development showed that they were expressed in young leaves, and that the level of expression decreased as the leaves matured to a fairly low level at stage G3 and then increased again as senescence progressed.

These cDNA clones are being characterized further to attempt to identify their possible functions in leaf senescence.

Use of the DNA sequences of the clones and identification of related sequences in the sequence data bases may be a useful method for identification of the gene function. One of these clones, LSC54, has been characterized by complete sequence analysis and further expression analysis.

Tissue-Specific Expression of LSC54

The levels of expression of the LSC54 gene in RNA isolated from different organs and from leaves at different stages of senescence is shown in Figure 3. High levels of expression were seen in the senescing leaves, with the highest level of expression in the last senescence stage. Very little expression was observed in the green leaf RNA (G4) or the young and mature pods, and no expression was detectable in the roots. A high level of expression was observed in the flower RNA. The flowers used for the RNA isolation were not obviously undergoing any petal senescence, but it is possible that some of the material was starting to senesce. Alternatively, the expression of LSC54 in flowers may not be senescence related.

Sequence Analysis of LSC54

Double-stranded sequence was obtained for the 422-bp insert of the LSC54 clone, using M13 forward and reverse primers and SK and KS primers. Sequencing of two *Eco*RI-*Pst*I subclones from the 3′ and 5′ ends of the gene (270 and 145 bp, respectively) using M13 forward and reverse primers gave the complementary strand (Fig. 4).

Primer extension analysis was carried out using RNA from senescing leaves and a primer homologous to DNA close to the 5′ end of the LSC54 cDNA. Annealing of the RNA and primer was carried out at three different temperatures. A strong band was produced when the annealing temperature was 37 or 45°C and a much weaker band was produced when annealing was at 55°C (Fig. 5). The band obtained from the primer-extension experiments co-migrated with a base 25 nucleotides from the *Eco*RI cloning site, which flanks the cDNA, indicating that the first base of the transcribed mRNA is situated 39 bases upstream from the 5′ end of the sequenced cDNA in the clone LSC54. The cDNA is therefore almost full length.

The DNA sequence flanking the proposed translation start

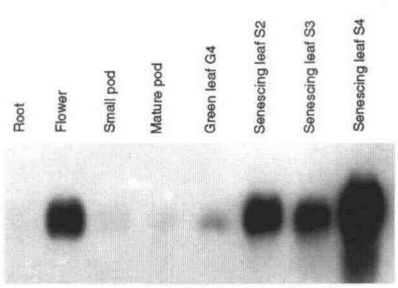

Figure 3. Northern analysis of LSC54 mRNA expression in other organs of the plant. RNA from root, flower, young and old pods, and leaves at different developmental stages was probed with the labeled insert from LSC54. Ten micrograms of RNA was loaded in each track.

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CAAACTACAACTTTAAATCAAAGAGAAGAGAAGCAATGGCAGGTTCTAAC 50
                                  Met Ala Gly Ser Asn
TGTGGATGTGGTTCCGGTTGCAAATGTGGTGACTCTTGCAGTTGCGAGAA 100
 Cys Gly Cys Gly Ser Gly Cys Lys Cys Gly Asp Ser Cys Ser Cys Glu Lys
GAACTACAACACGGAGTGCGACAGCTGCAGCTGCGGTTCAAACTGTAGCT 150
 Asn Tyr Asn Thr Glu Cys Asp Ser Cys Ser Cys Gly Ser Asn Cys Ser
GTGGGGACAGCTGCAGCTGTTAATTGTGGTGTAAATCACATGTACGCAGG 200
Cys Gly Asp Ser Cys Ser Cys  •
AAAACTGGTGGAAATTATGTGTTTGGTGTTGAGAGATGTGCGTGTGTGTT 250
TGAAAATATTTGGCATAATATGTTGCGTAATAACTCTCTTAACCTTGAAT 300
TTTCCTGCTTCTATGTGTGGATTGTGTGATTGTGTAATGTTTTCATTTGT 350
AACTTAAGAAAACAAAAAAAAAAAATATATATATATTGACTTTTGTGATT 400
GTTAAGATTTGTCTCAAAAAAA 422
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Figure 4. DNA and deduced amino acid sequence of LSC54. The DNA and deduced protein sequence of the 422-bp insert in LSC54 is shown. The DNA homologous to the synthesized primer used for the primer extension experiments and the *Pst*I sites used in subcloning are underlined.

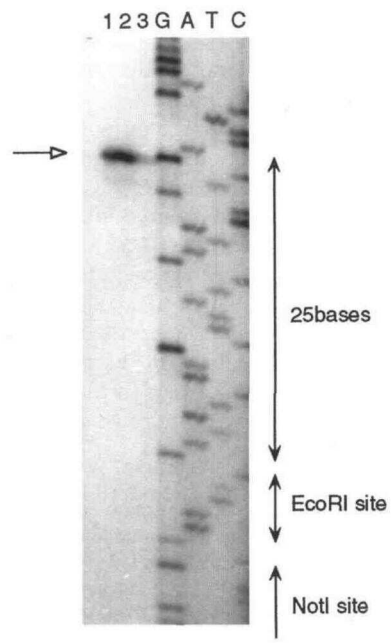

Figure 5. Primer extension analysis of the LSC54 transcript. Ten micrograms of total RNA from senescing leaves (stage S2) was hybridized with end-labeled primer for 5 h at three different temperatures. Lanes 1, 2, and 3 show the product obtained when incubation was at 37, 45, and 55°C, respectively. A sequencing reaction using the same primer with DNA of LSC54 was carried out and run in parallel on the same gel (G, A, T, and C represent the termination dideoxynucleotide in each track). The sequence shows part of the *Not*I site and the *Eco*RI site in the adapters that were used to clone the cDNA. The primer extension band is 25 bases upstream of the last base of the *Eco*RI site. The *Eco*RI and *Not*I sites constitute 14 bases (GAATTCGCGGCCGC). Therefore, the 5′ end of the mRNA is 39 bases upstream of the 5′ end of the cDNA cloned in LSC54.

codon (GCA **ATG** GC) corresponds closely with the consensus sequence that surrounds plant translation start sites (Cavaner and Ray, 1991) and should allow efficient translation initiation. The bases at positions −3 and +4, especially, have been shown by point-mutation analysis to be critical for an efficient translation start (Kozak, 1986). There are no other ATG sequences in the upstream cDNA in the LSC54 clone, and it is unlikely that the translation start would be closer than 39 bases to the 5′ end of the mRNA in the region that is missing from the cDNA clone. Therefore, it is likely that this ATG is the true translation start site.

The protein sequence deduced from the DNA sequence indicates a small protein of 45 amino acids (4.4 kD) as the likely product from the cDNA (Fig. 4). The majority of the amino acids in the protein are polar (29/45), and of these 13 are Cys residues. The isoelectric point of the protein is low (3.9). When the sequence of the protein was examined, a distinctive pattern of Cys residues throughout the protein was noticeable. The Cys residues fall into two domains; in each there are three Cys-X-Cys motifs where X is usually a Ser (4/6, with Gly [1] and Lys [1]).

The deduced protein sequence was used in a homology search with sequences in the translated GenBank/EMBL data base. Several metallothionein-like protein sequences that have been identified in plants were extracted from the data base in this search. These proteins have similarities in their sequence, especially in relation to the pattern of Cys residues that has been shown to occur in mammalian metallothionein proteins. Alignment of these proteins with the LSC54 protein indicates the common regions in the proteins (Fig. 6). The two polar domains, which include the Cys residues, at the N and C terminals of the proteins are highly conserved among all the proteins analyzed. There is less similarity in the intervening sequence between these domains, although two similar regions can be seen in most of the proteins except LSC54. The LSC54 protein sequence shows equal sequence similarity to the other proteins in the two Cys-rich regions but is different from all the others in that it has a very short intervening sequence between the two domains. The LSC54 protein has only 10 amino acid residues between the two domains, whereas the other proteins have between 40 and 45 residues in this region.

DISCUSSION

The molecular events that take place in a leaf during senescence are not well understood. The isolation and characterization of genes that show increased expression in the senescence process will help considerably in the elucidation of the enzymic events that are taking place during senescence, and the analysis of the regulation of these genes will help to identify the controlling factors for senescence. The results reported in this paper represent initial stages in a program aimed at identifying and characterizing senescence-related genes in *B. napus*.

Natural senescence in a plant does not proceed in a synchronous fashion; even within a single leaf, different areas of the leaf senesce at different rates. Therefore, it is not easy to identify characteristic growth stages to measure the progression of senescence. In attempts to overcome this problem, many of the physiological studies on leaf senescence have been carried out using artificial methods to induce senescence, such as detached leaf or dark induction. Recently, cDNA clones for genes showing increased expression in detached leaves incubated in the dark were identified by differential screening (Becker and Apel, 1993). Analysis of these clones showed that, of the three clones identified, only one represented a gene that was expressed in naturally senescing leaves; the others, therefore, were likely stress induced and not senescence related. This result indicates the possible confusion that might result when using artificial induction methods to identify senescence-related genes and shows the importance of using naturally senescing material, as was used in this study, as a source for senescence-specific mRNAs.

Few senescence-related genes have been identified in plants. Davies and Grierson (1989) showed that some of the genes previously identified as having induced expression in

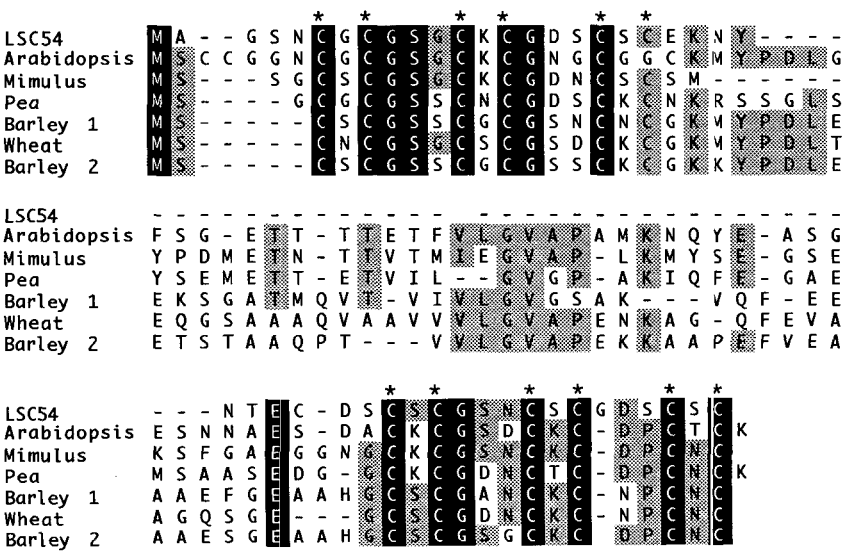

Figure 6. Alignment of the derived amino acid sequence of the LSC54 clone with metallothionein-like proteins from plants. Black boxes indicate that the sequence is identical in all cases, and shaded boxes show where the majority of genes (four or more) have the same amino acid residues. Asterisks (*) indicate the common Cys residues. Metallothionein-like proteins were identified by a homology search of a translated EMBL/GenBank data base. Sources: *Arabidopsis*, Takahashi (GenBank accession X62818); *Mimulus*, de Miranda et al. (1990); pea, Evans et al. (1990); barley 1, Okumura et al. (1991); wheat, GenBank accession WHTWALI1A_1; barley 2, GenBank accession S53707_1.

ripening tomato fruit were also induced in senescing leaves. One of these clones was shown to code for ethylene-forming enzyme. Expression of the antisense of this gene in transgenic tomato resulted in an increase in the lifetime of the leaves before senescence started, indicating an involvement of ethylene in the regulation of leaf senescence as well as in tomato fruit ripening (Picton et al., 1993).

Genes encoding enzymes involved in the breakdown and mobilization of cellular components such as protein, lipid, and nucleic acid might be expected to show increased expression during leaf senescence. Genes for enzymes that are involved in the glyoxylate pathway, e.g. malate synthase and isocitrate lyase, show induced expression during senescence (Gut and Matile, 1988; Graham et al., 1992). The role of this pathway in senescence is probably in the mobilization and recycling of the lipid components of the leaf. Several senescence-related cDNA clones have been identified in *Arabidopsis*; sequence analysis of one of these showed similarity to Cys proteinase proteins (Hensel et al., 1993). Also, a senescence-associated RNase has been identified in *Arabidopsis* (Taylor et al., 1993).

The different patterns of expression obtained with the cDNA clones from *B. napus* indicate that several modes of regulation may function in the expression of the proteins involved in the senescence process. Some genes, such as LSC54 and LSC94, are induced strongly at the start of senescence. This activation is likely to be at the transcriptional level and is presumably regulated by some senescence-specific signal.

In all the northern hybridization experiments the same amount of total RNA, whether it was isolated from green or senescing leaves, was used per lane on the gels. The level of total RNA decreases as senescence proceeds, with yields of RNA from the S4-stage isolations being 10-fold less than the green leaf and S1 isolations. Therefore, if the copy number of an mRNA per cell was maintained during senescence the signal obtained by northern analysis should increase 10-fold. The in vitro translation experiments showed that the relative proportions of the majority of the mRNAs remains the same, whatever the source material used, indicating a consistent degradation pattern for these mRNAs. In the northern analysis, genes represented by clones such as LSC7 and LSC99 showed an increase in mRNA levels as senescence proceeded. Expression of these genes may not be specifically induced, but their mRNAs may not be degraded at the same rate as the bulk of the mRNA population. These genes may code for enzymes that have important functions in the senescence process.

The third class of cDNA clones that was identified in this study represents genes that are expressed early in leaf development and have levels of mRNA that fall considerably in mature green leaves and then increase again during leaf senescence. Several of the clones from the original northern analysis fell into this category, including LSC8 and LSC28. Expression of these genes is probably actively induced during senescence, since the levels of expression seen cannot be explained by mRNA stability. It is possible that expression of these genes is induced in cells that are undergoing active metabolic functions and showing a high level of respiratory activity. In young leaf tissue the cells are expanding rapidly and conducting a high level of protein synthesis, and in senescing tissue metabolic activity again increases, a burst of protein synthesis occurs, and respiratory activity is high (Thomas and Stoddart, 1982; Thomas et al., 1992). In mature leaves, protein synthesis is at a very low level and metabolic activity is confined to the synthesis of fixed carbon by photosynthesis.

Further analysis of these senescence-related genes will be carried out to identify their functions and their mode of regulation. This analysis, together with similar characterization of other senescence-related cDNAs, is essential to help in the elucidation of senescence-related events.

The protein sequence of the senescence-specific clone LSC54 showed a distinctive pattern of Cys residues, and some homology to metallothionein-like proteins that have been identified in other plants was found after comparison of the sequence with sequences in the translated GenBank/EMBL data base. Metallothionein proteins are distinguished by the Cys residue motifs that allow binding of heavy metal ions. Several metallothionein-like proteins have been identified in plants (reviewed by Robinson et al., 1993). These have been identified by homology with mammalian metallothioneins only and there is little evidence for their function.

Metallothioneins that have been identified in animal systems are composed of around 60 amino acids and contain 20 Cys residues that bind metal ions in two clusters. The Cys residues have invariant positions and are distributed throughout the protein (Kagi, 1991). In contrast, metallothionein-like proteins that have been identified in plants differ in that the Cys residues are situated in two domains separated by an intervening sequence (Fig. 6). The distribution of Cys residues in the deduced protein of LSC54 is similar to these plant metallothionein-like proteins, but the central core region is considerably smaller. This small intervening sequence together with the overall small size of the protein may place LSC54 in a different class of metal-binding proteins from those not yet identified in plants.

In mammalian cells, expression of metallothionein genes is induced in response to heavy-metal treatment, and these proteins probably function as a detoxifying mechanism (Kagi, 1991). Some evidence that the plant metallothionein-like proteins may have a similar function comes from experiments with transgenic *Arabidopsis* in which plants that constitutively express the pea metallothionein-like protein accumulate more copper than control plants (Evans et al., 1992). The metallothionein-like proteins from both pea and maize were identified as root-specific cDNAs in differential screening experiments (de Miranda et al., 1990; Evans et al., 1990). The tissue-expression experiments showed that LSC54 transcripts were not detectable in the roots of *B. napus*.

Increased expression of plant metallothionein-like genes during leaf senescence has not previously been reported, and possible functions of this type of protein in this process are not clear. It is possible that during leaf senescence the release of metal ions from the protein breakdown that occurs is sufficient to warrant a detoxification role. Alternatively, these metal ions may form a valuable resource for the future development of the plant, so the presence of a metal-binding protein may have a function in the storage and possibly the transport of metal ions. A regulatory role has been postulated

for mammalian metallothionein genes in the control of zinc ions for transcriptional and translational processes (Kagi, 1991). There is some evidence that metallothioneins may act in mammalian systems to protect DNA from oxidative damage caused by free radicals (Chubatsu and Meneghini, 1993). Leaf senescence is an oxidative process; breakdown of Chl and membranes causes an increase in free radical production. Therefore, the presence of the LSC54 protein may protect the nuclear DNA from damage, thus allowing expression of senescence-specific genes that are required for the process to take place. Currently, there is no evidence that the protein coded by LSC54 has any such function. Further experiments involving expression of the antisense of the gene in transgenic plants and analysis of metal ion levels during leaf senescence may help to identify its role in the senesence process.

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