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Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins

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Abstract

Genes that are expressed during leaf senescence in *Brassica napus* were identified by the isolation of representative cDNA clones. DNA sequence and deduced protein sequence from two senescence-related cDNAs, LSC94 and LSC222, representing genes that are expressed early in leaf senescence before any yellowing of the leaves is visible, showed similarities to genes for pathogenesis-related (PR) proteins: a PR-1a-like protein and a class IV chitinase, respectively. The LSC94 and LSC222 genes showed differential regulation with respect to each other; an increase in expression was detected at different times during development of healthy leaves. Expression of both genes was induced by salicylic acid treatment. These findings suggest that some PR genes, as well as being induced by pathogen infection, may have alternative functions during plant development, for example in the process of leaf senescence.

Introduction

Leaf senescence is an important developmental stage in the life cycle of a plant during which the cells of the leaf undergo many physiological changes, ultimately resulting in the death of the leaf. Before death, the material that has been built up by the leaf during its growth phase is broken down and mobilised into the developing organs of the plant, such as younger leaves, developing flowers, pods and seeds. Leaf senescence appears to be a highly regulated, ordered series of events involving breakdown of leaf proteins, loss of photosynthetic capability, disintegration of chloroplasts, loss of chlorophyll and export of metabolites [32].

Although there are extensive physiological and biochemical data on leaf senescence, few of the molecular events that are involved with the process have been elucidated. It has been shown that leaf senescence is a controlled developmental process requiring expression of specific genes [39, 10]. Many enzymes are likely to be involved including proteases, nucleases and other degradative enzymes as well as enzymes involved in chloroplast dismantling and chlorophyll breakdown. However, identification of the key enzymes that are involved in the senescence process has been

The nucleotide sequence data reported will appear in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession number U21849 (LSC94, PR1a-like) and U21848 (LSC222, chitinase).

hard to achieve, partly due to the high background level of degradative enzymes already present in the plant cell [23].

From biochemical studies it is possible to postulate the types of genes which may be expressed during leaf senescence (reviewed in [37]) but few have been isolated or shown to have a specific role in senescence. For example, increased synthesis of enzymes that are involved in the degradation of the cellular components, such as protein, chlorophyll and lipids might be expected to involve changes in gene expression. Genes for senescence-specific proteases have been identified in *Arabidopsis* [21, 27], maize [38] and *Brassica napus* (Fife and Buchanan-Wollaston, unpublished). The level of the enzymes isocitrate lyase and malate synthase, involved in the glyoxylate cycle, which would be induced to aid lipid degradation, have been shown to increase during senescence of barley leaf segments [17] and enhanced expression of the gene for malate synthase occurs during senescence in cucumber cotyledons and leaves [14]. The level of a cytosolic glutamine synthetase increases during senescence in rice and *Arabidopsis*, probably to allow mobilisation of N compounds that are released during macromolecule breakdown [24, 4]. In *B. napus*, a senescence-related gene that codes for a metallothionein-like protein has been identified which may play a role in detoxification or act to store and mobilise metal ions released through protein breakdown [6]. However, genes encoding proteins for many of the functions that are known to occur during senescence, such as chlorophyll breakdown, or genes that are involved in regulation of the senescence process have yet to be identified. Studies on fruit ripening in tomato have allowed the identification of the gene for ACC oxidase which catalyses the last step in the biosynthesis of ethylene [19]. Tomato plants expressing antisense of this gene show alterations in the regulation of fruit ripening and the onset of leaf senescence is delayed [33]. This indicated that ethylene is involved in the induction of leaf senescence but is not the only signal required for the initiation of the process.

The isolation and characterisation of cDNA clones representing genes that show induced expression during leaf senescence should help to identify more of the proteins that are involved in the process. 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. The construction and differential screening of a cDNA library from RNA isolated from senescing leaves has been described previously [6]. The isolation of two cDNA clones, LSC54 and LSC94, which represent genes that were expressed early in leaf senescence, was reported. Sequence analysis of one of these senescence-related cDNA clones, LSC54, indicated that it represented a gene for a metallothionein-like protein. Further screening of the cDNA library has since identified further senescence-specific genes that are expressed early in senescence. One of these has been designated LSC222. In this paper we describe the analysis of the two cDNA clones LSC94 and LSC222 which are similar in sequence to two genes that code for pathogenesis-related (PR) protein (a PR-1a protein and a chitinase). Possible reasons for the induced expression of these genes during leaf senescence are discussed.

Materials and methods

Plant material

Brassica napus cv. Falcon plants were used as a source of material for RNA isolation. Seeds were germinated and plants grown in a greenhouse until the four-leaf stage at which time they were vernalised for 6 weeks at 4 °C and then grown under 16 h photoperiod in a growth room with a day temperature of 22 °C, and a night temperature of 16 °C. Leaves were removed from plants, samples taken for chlorophyll assays and the leaves were then immediately frozen in liquid nitrogen. Leaves were also removed from young plants before vernalisation.

Salicylic acid treatment. Plants were grown from seed for 8 weeks in a growth chamber under the

above conditions but without vernalisation. Plants were sprayed with a solution of 50 mM salicylic acid [44] and, after 48 h, fully expanded leaves were harvested and frozen in liquid nitrogen.

Flowers, roots and siliques. These tissues were harvested from healthy field-grown plants and frozen in liquid nitrogen.

RNA isolation and northern analysis

RNA isolation, electrophoresis and blotting were carried out as previously described [1, 6]. Prehybridisation and hybridisation were in 0.25 M sodium phosphate buffer pH 7.2, 7% (w/v) SDS at 65 °C. The method for labelling of the probe DNA and the washing conditions were carried out as previously described.

DNA isolation and Southern analysis

For the isolation of DNA from leaves, a method adapted from that of Dellaporta [12] was used. 5 g fresh weight of healthy leaves was harvested, snap frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The frozen powder was transferred to a tube containing 15 ml of a buffer containing 100 mM TrisCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol. 1.2 ml of 20% (w/v) SDS was added, mixed thoroughly by vigorous shaking, and the samples incubated at 65 °C for 10 min. 5 ml of 5 M potassium acetate was added, and the samples incubated on ice for 20 min. After centrifugation at 10000 × *g* for 20 min the supernatant was filtered through Miracloth and the DNA precipitated with isopropanol. The DNA was spooled out with a flamed pasteur pipette. DNA pellets were dissolved in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA), treated with RNAse, phenol/chloroform-extracted and reprecipitated with ethanol. Southern blotting and hybridisation was carried out as described [1].

Chlorophyll assays

Leaf discs (10 mm diameter) were excised in triplicate from different areas of each leaf, were pooled and ground with a Polytron homogeniser in 1 ml 80% acetone. Extracts were diluted tenfold in 100% acetone and the A_{645} and A_{663} were measured. Chlorophyll *a* and *b* concentrations were calculated by the equations of Hill *et al.* [22]:

Chl *a* (μg/ml) = 12.5 A_{663} − 2.55 A_{646};
Chl *b* (μg/ml) = 18.29 A_{646} − 4.58 A_{663}.

DNA sequencing and exonuclease III deletion

DNA sequencing was carried out as previously described [6]. LSC94 was sequenced using M13 forward and reverse primers on the original clone. Two subclones were constructed by digesting the *Eco*R1-digested cDNA insert with *Sau*3A and cloning the two fragments into pUC19. An internal primer was synthesised for use in sequencing across the junction of the two subclones. LSC222 was sequenced by the construction of a nested deletion set from both ends of the cDNA using Exonuclease III (Kit from Pharmacia). The deleted DNA was sequenced with the M13 forward and reverse primers. DNA sequence was assembled and analysed using the DNASTAR sequence analysis package. Database searches were carried out using the BLAST Network Service (National Center for Biotechnology Information, NCBI).

Results

Identification of senescence stages in Brassica napus

In order to examine the expression of genes involved in leaf senescence by northern analysis it was important to identify leaves at different stages of development for the isolation of RNA. A number of *B. napus* plants at the same stage of development (in flower, with the first siliques de-

veloping), carrying leaves at different stages of senescence, were selected. Leaves from eight plants were numbered from the base of the plant and the chlorophyll content of each leaf was measured. Four different pools of leaves were identified according to chlorophyll content and position on the plant (Table 1). A fifth pool of leaves, a little earlier in development, was isolated from plants on which the flowers were just opening. These leaves were designated as mature green leaves (MG1). The uppermost leaves on the more advanced plants had the same chlorophyll levels as the mature green leaves and were designated MG2. The next leaves down the plant from MG2 showed a slight reduction in chlorophyll level (85%) and were designated as first senescence stage SS1. Stages SS2 and SS3 were from leaves still lower down the plant with average chlorophyll levels of 55% and 35% respectively. Only SS3 leaves showed visible yellowing.

Total RNA was isolated from each pool of leaves. Yields of RNA were similar (200–240 μg/g) from all pools except SS3 where a dramatically lower yield (80 μg/g) was obtained (Table 1). This indicated that general RNA degradation had commenced in the SS3 leaves. RNA was also isolated from fully expanded green leaves from a young, immature plant (YG) that had not been vernalised.

Table 1. Chlorophyll and RNA levels in the five leaf pools selected.

Leaf pool	Chlorophyll level (μg/ml)	% Chlorophyll level	RNA yield (μg/g tissue)
MG1	36.1	100	220
MG2	35.2	98	240
S1	30.2	85	200
S2	20.4	55	220
S3	12.4	35	80

Chlorophyll *a* and *b* concentrations were calculated as described in the Materials and methods and combined to give the total chlorophyll concentration, each of which is a mean of the samples taken from 6–8 leaves in each pool. RNA yield is the average yield obtained from two preparations.

Expression of LSC94 and LSC222 during leaf development

In a previous report [6], differential screening of a cDNA library from senescing leaves was described. Several clones representing genes showing increased levels of expression in senescence were identified including LSC54, the sequence of which showed similarity to a metallothionein-like protein, and LSC94, which was not characterised in detail. Further differential screening of the cDNA library resulted in the isolation of a number of cDNA clones including LSC222.

In the previous report, it was shown that expression of the genes represented by LSC54 and LSC94 was induced at some time between the young, fully expanded green leaf stage and the stage designated as the first stage of senescence (showing the first signs of yellowing). A similar pattern of expression was seen with LSC222 (unpublished). In the experiments described here, several intermediate developmental stages have been identified by analysing growth characteristics and by the measurement of chlorophyll levels, and the northern analysis has been extended to include these stages. The expression of the genes represented by LSC94 and LSC222 at early stages of leaf senescence, i.e., before any yellowing was visible, was analysed and compared to the expression shown by LSC54 (Fig. 1). The transcript size of each gene was estimated from the northern blots using RNA size markers; the LSC 94 gene had a transcript size of about 650 bp while that of the LSC222 gene was about 1.0 kb.

Levels of total RNA were stable in all tissues tested except for the sample SS3 indicating that general RNA degradation did not start until this stage. In the northern analysis the same amount of RNA was used on each track regardless of the total amount of RNA in the tissue. The level of transcript detected therefore, represented the proportion of the specific message to the total RNA present. The increased level of transcripts detected by northern analysis in RNA from leaves at earlier stages of senescence (MG2, SS1 and SS2) is likely to be due to increased transcription of the senescence related genes since the levels of

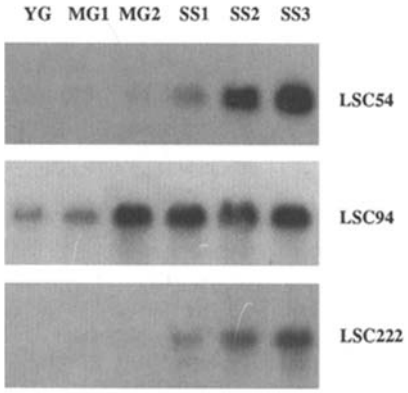

Fig. 1. mRNA expression during leaf development. Northern blots carrying RNA isolated from leaves at 6 different stages of development were hybridised with ^{32}P-labelled inserts from the different cDNA clones, LSC54, LSC94 and LSC222. YG refers to RNA isolated from fully expanded green leaves from young plants, MG1 refers to RNA isolated from mature green leaves isolated from plants which had just started flowering, MG2 refers to RNA isolated from mature green leaves on plants that had just started silique development, SS1, SS2 and SS3 refer to RNA isolated from senescing leaves showing 80%, 55% and 35% of green leaf chlorophyll levels, respectively. Only the leaves from which the SS3 RNA was isolated showed visible yellowing or reduced total RNA levels. 10 μg of total RNA was loaded in each track.

total RNA were constant in the sampled leaves. The increase seen in SS3 however, could be due to higher stability of the specific mRNA transcript relative to the total RNA.

Although previous studies of these genes had indicated that they were all expressed at the first visible stage of senescence (i.e. the first sign of yellowing, equivilent to stage SS3 in this analysis), this more extensive analysis showed that this expression was initiated at different times (Fig. 1). Transcripts from the gene represented by LSC54 are undetectable in YG and MG1 leaves, are very low in the MG2 leaves, are clearly visible in stage SS1 (the first stage in which chlorophyll degradation has commenced) and increase in level with each further stage of senescence. The gene represented by LSC94 showed expression at low levels in green leaves from an immature plant (YG), and in mature green leaves, MG1, from plants which had only just started flowering. However, the expression level increased dramatically in the mature green leaves, MG2, from the more developed plants, were flowering was well advanced and silique development had started. The high level of expression of this gene was maintained in the leaves at further stages of senescence, SS1, SS2, and SS3. The gene represented by LSC222 showed a similar pattern of expression to that seen with LSC54; very low expression is detectable in the MG1 and MG2 leaves, expression increased significantly in the SS1 leaves and then increased further in the more senescent leaves.

Therefore, although all three cDNAs represent genes that are expressed early in senescence before any chlorophyll loss is visible, these genes are differentially expressed. In order to predict the possible functions of these genes in leaf senescence, the cDNA clones LSC94 and LSC222 were analysed by DNA sequencing.

DNA sequence analysis of LSC94 and LSC222

The two cDNA clones LSC94 and LSC222 were sequenced fully on both strands.

LSC94. The longest open reading frame in the 631 bp sequence of LSC94 was of 423 bp which could encode 161 amino acids, resulting in a protein of 22.4 kDa (GenBank accession number U21849). Primer extension analysis of this cDNA (not shown) indicated that the mRNA start was about 30 bp upstream of the first base on the cDNA sequence. Therefore, the cDNA is almost full-length and it is likely that the first ATG of the cDNA is the start of translation. Analysis of the protein structure of the derived protein sequence indicated an N-terminal hydrophobic region of 25 amino acids. The protein that would be obtained after cleavage of this putative transit peptide is neutral with an isoelectric point of 6.7 and would have a molecular mass of 14.8 kDa.

Scanning the EMBL and GenBank databases with both the DNA sequence and the deduced protein sequence revealed a number of sequences with significant similarity. The DNA and protein sequence comparisons showed that the LSC94 gene has the closest similarity to an *Arabidopsis*

gene (ATHRPRP1A [40]) the two genes showing 80% sequence identity in the mature proteins (Fig. 2). Therefore, LSC94 represents a gene that codes for a protein with considerable sequence similarity to the pathogenesis-related protein, PR-1, that has been identified in many plants including tobacco, tomato, barley, maize and *Arabidopsis* (Fig. 2). Comparison of the protein sequence of other mature PR-1 proteins indicated that the identity between proteins from different sources is 50–60%. Another PR-1 gene has been isolated from *Arabidopsis* (ATHPR1 [39]) but this showed only about 50% similarity to both the LSC94 protein and the other *Arabidopsis* PR-1 protein.

LSC222. The longest open reading frame in the 985 bp sequence of LSC222 would encode 267 amino acids giving a protein of about 28.5 kDa (GenBank accession number U21848). There was no ATG initiation codon in the frame of the longest open reading fame, indicating that the cDNA was probably not full length and lacked a short region encoding the N-terminus of the protein.

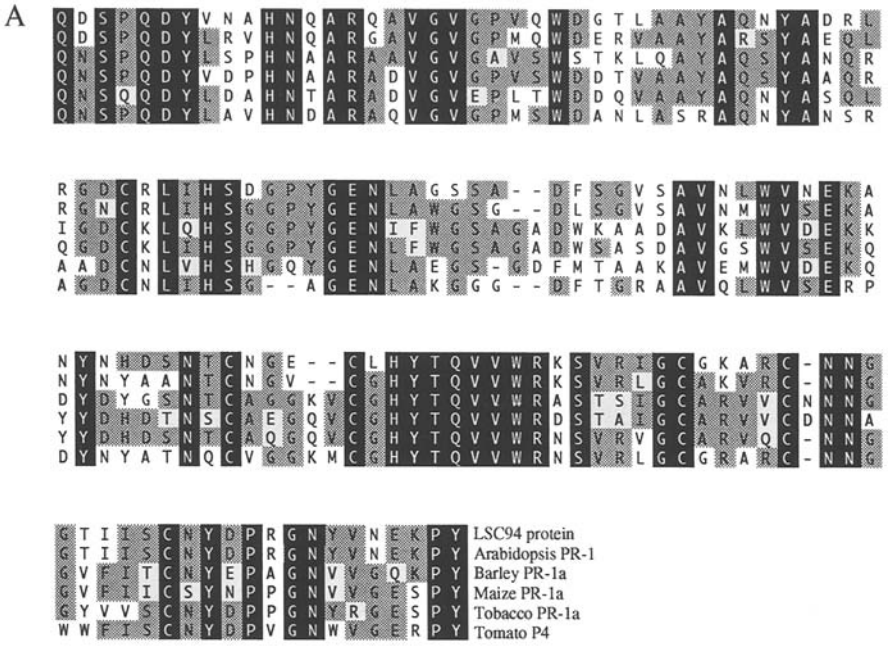

Fig. 2. Comparison of the derived amino acid sequence of the mature LSC94 protein with PR-1a genes from plants. Deduced transit peptides have been removed from the protein sequences before alignment. A. Multiple alignment of the sequences. Black boxes indicate where the sequence is identical in all cases, shaded boxes indicate where the majority of the genes show the same sequence. B. Sequence similarities expressed as percent similarity, calculated using the formula: similarity = 100 × the sum of the number of matches/([length – gap residues] – [gap residues]). Sources: *Arabidopsis* PR-1 ([40] accession number P33154), barley PR-1a ([5] accession number S37166), maize PR-1a ([15] accession number A33155), tobacco PR-1a (Pfitzner, U.M.; accession number M36691) and tomato P4 ([41] accession number Q04108).

Analysis of the deduced protein indicated that this protein, also, had an N-terminal hydrophobic region. The protein that would be obtained after removal of this hydrophobic region has a molecular mass of 26.7 kDa and is an acidic protein with an isoelectric point of 4.98.

The mature protein sequence of LSC222 is similar to several plant genes that encode Class

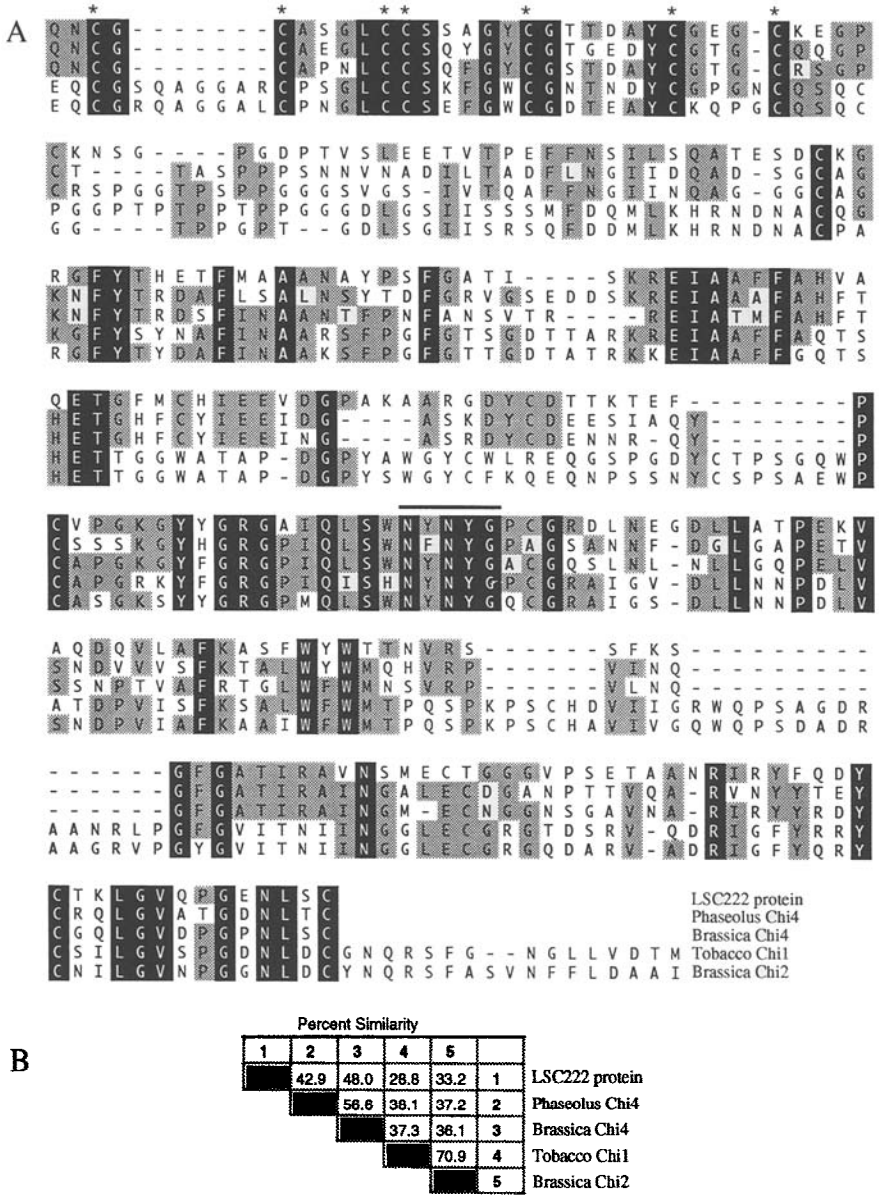

Fig. 3. Comparison of the derived amino acid sequence of the mature LS222 protein with class I and class IV chitinase genes from plants. A. Multiple alignment of the sequences. Black boxes indicate where the sequence is identical in all cases, shaded boxes indicate where the majority of the genes show the same sequence. The conserved cysteine residues that are involved in chitin binding are shown by an asterisk and the proposed active site sequence NYNYG is overlined [43]. B. Sequence similarities expressed as percentage of similarity. Sources: Class I chitinases – *Phaseolus* Chi4 ([29], accession number P27054), *Brassica* Chi4 ([34] accession number Q06209). Class IV chitinases – Tobacco Chi1 ([36] accession number P08252), *Brassica* Chi2 ([18], accession number Q09023).

I and Class IV chitinases (Fig. 3). Plant chitinases have been classified into several groups according to their structure [2, 8]. Mature class I chitinases have a cysteine-rich N-terminal region and a vacuolar C-terminal targeting signal. The N-terminal cysteine-rich region is believed to be a chitin-binding domain and is also found in other proteins, such as wheat germ agglutinin, hevein, and wound-induced proteins. The active site has been tentatively located to the common NYNYG motif [43]. Class IV chitinases are very similar to class I except that they are smaller, due to four internal deletions, and they lack the C-terminal vacuolar sequence. Class IV chitinases are probably secreted since they have no vacuole-targeting signal. The LSC 222 protein shows a high degree of similarity to the other chitinases over the N-terminal domain and the catalytic domain, and contains the NYNYG motif. The protein is more closely related to the chitinases that have been classified as Class IV since it has no C-terminal targeting sequence and is of similar size to a Class IV chitinase.

Two chitinase genes have been identified previously in *Brassica napus*. One of these, Chi4, is a Class IV enzyme but the mature protein from this gene shows only 48% identity to the deduced mature protein from LSC222 [34]. The other *Brassica* chitinase that has been cloned, Chi2, is a Class 1 chitinase and shows only around 35% identity to both Chi4 and LSC222 [18]. A Class 1 chitinase that has been cloned from *Arabidopsis* is very similar in sequence to the *Brassica* gene Chi2. Therefore, LSC222 represents a chitinase that has not previously been identified in *Brassica* or *Arabidopsis*. The difference in DNA sequences between LSC222 and the other two *B. napus* chitinases is such that these genes would not have been detected under the stringent conditions in which the northern analysis described here was carried out.

Tissue-specific expression of LSC94 and LSC222

The levels of expression of the genes represented by LSC94 and LSC222 in other organs of *B. napus* plants were analysed by northern analysis of RNA isolated from flowers, roots and siliques (Fig. 4A). The gene represented by LSC94 was expressed in siliques but very little expression was detectable in flowers and roots. The gene represented by LSC222 was expressed in flowers and siliques but almost no hybridisation was seen to RNA isolated from roots. In both cases, the genes represented by these two cDNA clones showed the highest levels of expression in senescing leaves (SS2).

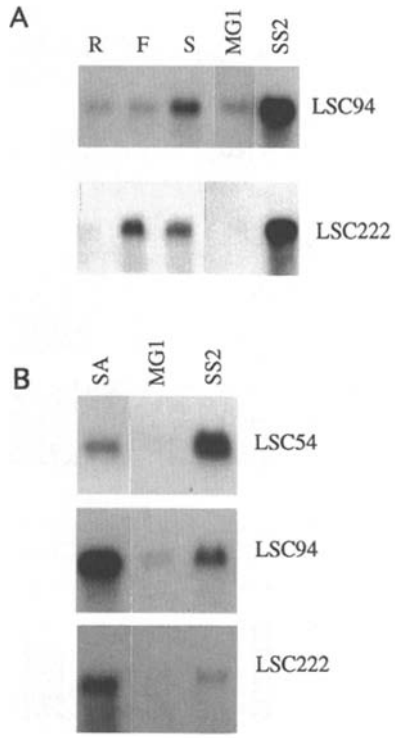

Fig. 4. A. Tissue-specific gene expression. Northern blots carrying RNA isolated from different parts of the plant were hybridised with ^{32}P-labelled inserts from the different cDNA clones LSC94 and LSC222. 10 μg of total RNA was loaded in each track. R, roots; F, flowers; S, siliques; MG1, green leaves; SS2, leaves at senescence stage 2. B. Salicylic acid treatment. Northern blots carrying RNA isolated from leaves treated with salicylic acid were hybridised with ^{32}P-labelled inserts from the different cDNA clones LSC54, LSC94 and LSC222. RNA from green and naturally senescing leaves was also analysed on the same northern blots. 10 μg of total RNA was loaded in each track except for the SA track where 2 μg of total RNA was loaded. SA, salicylic acid; MG1, green leaves; SS2, leaves at senescence stage 2.

Gene expression induced by salicylic acid

Since the two genes under study, LSC94 and LSC222, appeared to have similarity to pathogenesis-related (PR) protein, RNA was also isolated from tissue that had undergone treatment that would be expected to induce PR genes. Expression of PR genes is induced when a plant is attacked by a pathogen. Salicylic acid (SA) is synthesised in the plant in response to pathogen infection and is required for the induced expression of at least some of these genes [44]. RNA was isolated from plants that had been sprayed with salicylic acid.

Expression of the genes represented by the senescence-related cDNA clones LSC54, LSC94 and LSC222 in leaves that had undergone this treatment was analysed by northern hybridisation (Fig. 4B). All three genes showed increased levels of expression after salicylic acid treatment. LSC94 and LSC222 showed considerably higher levels of gene expression than that seen during senescence (five times less RNA from leaves after salicylic acid treatment was loaded on the northern blots than was RNA from senescing leaves). The slight increase in expression of the gene represented by LSC54 after salicylic acid treatment may indicate that some senescence processes are induced by the salicylic acid treatment. However, increased expression of other senescence-related genes such as a cysteine protease was not detected (data not shown).

Genomic organisation of genes represented by LSC94 and LSC222

In order to investigate the organisation of the LSC94 and LSC222 genes in the *B. napus* genome, genomic Southern blots were hybridised at high stringency with labelled probes derived from the cDNA inserts from the two clones LSC94 and LSC222. Hybridisation to *B. napus* DNA digested with either *Eco*RI or *Bam*HI (Fig. 5) showed a relatively simple pattern of hybridising bands indicating that both cDNAs represented genes that were present in one or two copies per genome and were not part of complex gene families.

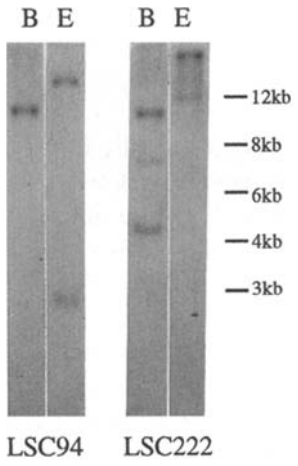

Fig. 5. Southern analysis. Southern blots carrying *B. napus* DNA digested with either *Bam*HI (B) or *Eco*RI (E) were hybridised with labelled cDNA inserts from the clones LSC94 and LSC54. 10 μg of DNA was loaded in each track.

Discussion

In this paper we report the analysis of two genes, represented by cDNA clones LSC94 and LSC222, that show considerably increased levels of expression in early stages of leaf senescence in healthy *B. napus* plants. These genes have sequence similarity to genes that show induced expression in plants in response to environmental and physiological stresses, first identified as being induced after pathogen attack.

By measuring chlorophyll levels and by visual assessment of the developmental stages of the plant, it has been possible to identify stages of leaf development and to analyse the time of expression of senescence-related genes in more detail than was reported previously. Measurement of the chlorophyll levels in a leaf is one way to assess the stage of senescence that the leaf has reached, although senescence must be initiated and other related processes may occur before chlorophyll breakdown can be measured. Chlorophyll degradation can be detected well before yellowing of the leaves becomes visible and sev-

eral developmental stages were identified by measuring chlorophyll levels in leaves from different parts of the plant and from plants of different ages.

The genes represented by the two senescence related cDNA clones LSC94 and LSC222 are not expressed at the same time in leaf development. Expression of the LSC94 gene was detectable at low levels in both the immature green leaves and the mature green leaves, MG1, from a plant that had just started flowering. However, expression increased considerably at the next stage, MG2, in leaves showing the same chlorophyll level as MG1 but isolated from a more mature flowering plant that was starting to set siliques. Therefore, it appears that the expression of the gene represented by LSC94 is induced before any detectable chlorophyll loss occurs and is presumably triggered by a developmental process such as flowering or silique development. In contrast, increased expression of the genes represented by LSC222 and LSC54 was not detected until the first senescence stage SS1, where the first indication of chlorophyll loss was also detectable. Although LSC94 and LSC222 both appear to be similar to PR genes, they are differentially controlled during development (they show differential expression during leaf development and LSC222, only, shows expression in flowers). The differential expression of these two genes indicates that they are not necessarily under the control of the same regulatory events.

The PR proteins can be classified into several groups (reviewed in [26]). When the existence of these proteins was first discovered, it was thought that their function was to protect the plant against pathogen attack. However, the appearance of many PR proteins in situations where pathogen invasion is not evident has given some cause to doubt the sole function postulated for these genes. Several PR proteins have been found to be developmentally regulated. For example, chitinases have been detected in the aleurone layer of developing barley seeds [25], are highly abundant in pistils of potato [45] and have an important function in early embryo development of carrot [11]. In addition, the PR-1 genes have been shown to be under developmental regulation; induced expression has been reported in the sepals of developing flowers in tobacco [28] and in the leaves of healthy flowering tobacco plants [13]. A recent analysis of the promoter of a PR-1a gene in tobacco indicated that expression of the gene was induced in the lower leaves of tobacco plants [16]. The data presented here indicate that, in *B. napus*, a PR-1a gene and a gene for a Class IV acidic chitinase are induced at different times during leaf development and become active in senescing leaves that are otherwise healthy and not infected by pathogens.

At this time it is not clear whether the two genes identified in this study are specific to senescence or whether their expression would also be induced in response to pathogen attack. The expression of many PR proteins is induced by salicylic acid and this compound has been implicated as a component in the transduction of a signal from the site of infection to the rest of the plant [20, 44, 46]. Expression of the genes represented by the two senescence related cDNA clones LSC94 and LSC222 was induced in plants treated with salicylic acid, indicating that these two genes were, in part, under the same regulation as other PR proteins.

Genomic Southern analysis of these two genes indicated that they are both single copy genes although the possibility exists that several genes could be clustered on the large DNA fragments. Since *B. napus* is an allotetraploid, carrying one genome from *B. campestris* and one from *B. oleracea*, the presence of two copies of each gene would be expected. Therefore, it is likely that both genes are present at only one copy per genome. The isolation of a PR1-like gene from *B. napus* has not previously been reported but LSC94 is probably the homologous gene to ATHRPRP1A [40] the *Arabidopsis* PR-1 gene that has 80% protein sequence similarity to LSC94. Two chitinase genes from *B. napus* (chi2 and chi4) have been characterised previously [18, 34]; both show less than 50% identity in protein sequence to the LSC222 protein. Under the hybridisation conditions used here, transcripts from these genes were not detected. This is also indicated by the lack of

expression seen when hybridising LSC222 to root RNA; the *B. napus* chi2 gene shows strong constitutive expression in the roots [18]. Therefore, LSC222 represents a chitinase gene that has not been previously identified in *B. napus*.

The reason for the induction of the expression of these genes during leaf senescence is not known. It is possible that the presence of the PR proteins in these situations reflects the vulnerability of the tissues and performs an ancillary function as part of the defence system against invading pathogens. It is conceivable that this is the function of the LSC94 and LSC222 proteins in senescing leaves, which, with the relatively high concentration breakdown products present and a diminished physical structure, are extremely susceptible to pathogen attack. An alternative explanation is that these genes play fundamental roles in this developmental process.

Chitinases catalyse the hydrolysis of chitin, a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues. Immunological studies have revealed the presence of GlcNAc residues in secondary cell walls of plants, probably in the form of glycolipids [3]. It is possible that chitinase has a role in the processes involved with cell wall breakdown. Alternatively, it has been postulated that the action of plant growth regulators may be mediated via oligosaccharins released from plant cell walls by hydrolytic enzymes [9] thus, chitinases may be involved in signal transduction pathways for developmental events. Most chitinase genes have been identified by sequence similarity to genes described previously and in very few cases have the proteins encoded been demonstrated as having chitinase activity [30]. It is conceivable that the LSC222 protein, which has not been shown to have chitinase activity, may encode a protein with hydrolytic properties similar to those of chitinases, but which is active against endogenous plant substrates rather than chitin.

The enzymatic activity or function of PR-1 proteins has not been identified and their role in protection against pathogens is not clear. The induction of senescence may be a response to pathogen attack; the chlorosis of leaves that often occurs around the sites of pathogen invasion would support this hypothesis. Some invading pathogens, such as rust and mildew, synthesise cytokinins, resulting in the so called 'green islands' surrounding sites of infection [7, 35]. Since cytokinins are known to delay leaf senescence in many species [42], this action by the pathogen may be a precaution to inhibit the plant's response to invasion. This would imply that that the natural response of the plant may be to induce senescence in the infected area. The mobilisation of cellular components out of the leaf, away from the site of infection, may help the plant to restrict the growth of the invading pathogen. Therefore, the expression of some of the PR proteins (e.g. PR-1a) could be concerned with the induction and control of leaf senescence and only indirectly with defense against pathogens.

The experiments reported in this paper provide a further indication that the role of the so-called pathogenesis-related proteins may not necessarily be restricted to plant defence. It is clear that there is correlation between leaf senescence and the pathogenesis-related response. Further experiments will analyse the expression of these senescence-related genes in *B. napus* tissue infected with fungal or bacterial pathogens (*Peronospera* and *Xanthomonas*) to investigate the involvement of these genes in pathogen response. The analysis of senescence in transgenic plants expressing antisense to the PR-1a gene and to the chitinase encoded by LSC222 will help to identify and clarify the role that these genes play in the senescence process.

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