Expression analysis of genes related to oxidative protection during senescence in *Brassica napus*

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Abstract
Leaf senescence is the sequence of events leading to cellular disassembly and mobilization of released materials that cause accumulation of reactive oxygen species. DNA sequencing of one of senescence-isolated cDNA, LSC650, showed a high level of similarity with a catalase gene of Arabidopsis. Transcript level of this isoform was highly increased during senescence. The enzyme activity of catalase was also assayed during different developmental stages in Brassica napus and showed a high level of activity during last part of leaf senescence. Both kinetic assay and northern analysis exhibit that catalase has the potential to play a significant role in the cell defense against produced hydrogen peroxide as part of the macromolecules breakdown. Gene expression pattern was characterized by mRNA hybridization with several antioxidant cDNA clones. Transcript levels of discussed genes were markedly increased during senescence stages. We conclude that the major part of the mRNA changes observed during senescence stages of leaves is connected with leaf senescence, whereas free radical-related transcripts appear to protect cells from oxidative damage.
Keywords: *Active oxygen species, Free radicals, Transcription, Northern analysis, Leaf senescence, Brassica napus.*

INTRODUCTION

Senescence is an important part of the life span of a plant in which many genes are involved in a series of events necessary for the next generation. For this purpose, some of the senescence-enhanced genes are expressed at early stages of plant senescence and some of them at the later stages. Some of these genes are responsible for the degradation of macromolecules and cause accumulation of active oxygen species (AOS) that have to be decomposed by the products of other genes. Characterization of genes encoding these detoxifying enzymes may help to determine their function and elucidate the processes occurring during senescence. Superoxide dismutase (SOD) is an enzyme responsible for removal of O_2^- in the plant cell. Activity of this family of enzymes is known to be linked with catalase activity, since SOD converts O_2^- into H_2O_2 and catalase degrades H_2O_2 (Adam *et al.,* 1995; Bailly *et al.*, 2001; Demiral and Turkan, 2005).

Catalase is an enzyme presenting in all aerobic cells which catalyses the dismutation of H_2O_2 to water and oxygen. This enzyme was first characterized in 1901 (Loew, 1901). Catalase was one of the earliest enzymes isolated in a state of high purity (Sumner and Dounce, 1937) No multicellular organism has been found that does not possess at least some catalase activity (Scandalios, 1987). Catalase, together with SOD and hydroperoxidases, act as a defense system to detoxify O_2^- and hydroperoxides (Dalal *et al*., 2001; Demiral and Turkan 2005; Jebara *et al.,*2005). Different isoforms of catalase, encoded by genes which are differentially regulated, have been identified during the life cycle of a plant (Willekens *et al.,* 1994; Bailly *et al.,* 2001 and Parida *et al.,* 2004). Different isoforms of catalase have been identified during seed germination, photorespiration in mature organs, fatty acid degradation and senescence. Lipid and protein

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degradation during senescence causes accumulation of AOS, especially H_2O_2 that is detoxified by glyoxysomal catalase. To date, many different isoforms of catalase have been found in different organelles, not only in plants, but also in animals and other organisms (Esaka *et al.,* 1997; Diaz *et al.*, 2001; Lortz and Tiedge, 2003). The enzymatic activity of catalase is to detoxify H_2O_2 and expression of the genes encoding catalase is induced by different stresses. The production of catalase can enhance the resistance of a cell to oxidative damage, and according to results so far, its absence could result in cell death by necrosis (Haddad *et al.,* 1999; Dalal and Chopra, 2001; Kim *et al.,* 2004 and Parida *et al.,* 2004).

Different isoforms of SOD were detected in the different developmental stages of plants. Some of these isoforms were tissue specific and expressed during senescence, but not during maturity. The role of these isozymes has been reported to protect cells from oxidative damage (Ogawa *et al.,* 1997; Bailly *et al.*, 2001; Lortz and Tiedge, 2003; Parida *et al.,* 2004 and Jebara *et al.,* 2005). In plants, analysis of the SOD isozyme pattern revealed five major forms of SOD in leaf tissues; three of these were characterized as Cu,Zn-SOD isozymes and the other two were Mn-SOD forms (Kwiatowski and Kaniuga, 1984; Buonaurio *et al.,* 1987 and Scandalios, 1993). Pastori and del Rio (1994 and 1997) studied the activated oxygen metabolism of peroxisomes in natural and dark-induced senescent leaves of pea. The activity of different SOD enzymes was analysed in peroxisomes and glyoxysomes of senescent leaves and three different SOD isozymes were detected. Mn-SOD activity was increased in the peroxisomes and two new Cu,Zn-SOD were detected in these organelles, one of which was present in the peroxisomes and the other in the glyoxysomes.

The plants used in this research project were *Brassica napus* cv. Westar. For molecular and biochemical analysis, leaf tissues were harvested from plants at eight different developmental stages, from young to senescence. LSC650 cDNA, one of the genes analysed in this research project, was isolated from a subtractive hybridisation cDNA library (Buchanan-Wollaston and Ainsworth, 1997). To investigate the role of the antioxidant genes, different clones were used and showed various transcript levels at different developmental stages.

MATERIALS AND METHODS

Sequencing was carried out by a sequencer system (ABI 310, Perkin Elmer). The full length cDNA clone, LSC650, was used as a template. Sequencing was performed by primer walking, starting with the SK and KS primers of Bluescript plasmid as forward and reverse primers, respectively. The sequence of each 3′ end was then used to identify the sequence for the next primers and this was continued until both DNA strands were sequenced. Three hundred ng template DNA was used with 4 µl terminator ready reaction mix and 9 pmol primer in a total volume of 10 µl. Using 25 cycles of sequencing amplification (96°C for 10 sec, 50°C for 5 sec, and 60°C for 2 min), the DNA fragment was amplified and then precipitated with ethanol. The pellet was resuspended in the template suppression reagent and analysed by the sequencer. An ABI "Sequence Analysis" package was used to analyse the sequences. To characterize protein structure, open reading frames (ORF) (Lewin, 1997) were predicted by the EditSeq computer program.

For biochemical and molecular analysis, leaf samples were harvested at different developmental stages including young leaf stage (Y), mature green leaf stages with no sign of leaf chlorosis (MG1, MG2 and MG3), senescent stages with increasing yellowing of leaves (S1, S2, S3, and S4). Using a Minolta Chlorophyll Meter SPAD-502, SPAD readings were used to characterize different developmental stages of the leaves on a plant. The leaf to be tested was placed between two LEDs to irradiate with 650 nm wavelength for red and 940 nm wavelength for infrared lights. The intensity ratio of transferred light through the leaf is converted to an electrical signal, and then converted to a number. Leaf chlorophyll absorbs in the red wavelength. Hence, the ratio of a reading gives an estimate of chlorophyll accumulation. A detail of developmental stages is as follow:

Y	Young plants, no sign of flowering, 22 days from germination, 20 cm high, average SPAD 35.
MG1	Plants with 40 cm high, 2nd leaf from bolt, average SPAD 45.
MG2	Start of flowering, 70 cm high, 2nd leaf from bolt, average SPAD 41.
MG3	Same plant as MG2, 4th leaf from bolt, average SPAD 37.
S1	Leaf from plant with opened flowers, signs of silique development, 90 cm high, about 10% chlorosis, average SPAD 35.
S2	Leaf from plant with many green siliques developed, about 30-40% chlorosis, average SPAD 30.
S3	Leaf from plant with some mature siliques, about 60-70% chlorosis, average SPAD 24.
S4	Leaf from plant with almost half mature siliques, about 75-80% chlorosis, average SPAD 13.

For the chlorophyll assay, 200 µl of homogenised tissue used for protein extraction was mixed with 800 µl acetone and incubated at -20°C for 1-12h. Using a Spectrophotometer (SP8-100 UV/VIS, Pye Unicam), the supernatant of the centrifuged mixture was assayed against a blank (80% acetone) at A_{663} nm and A_{646} nm. Amounts (mg/ml) of chlorophyll a and chlorophyll b (Chl a and Chl b, respectively) were determined according to the equations of Hill *et al.* (1985):

$$\text{Chl a} = 12.5A_{663} - 2.55A_{646}$$
$$\text{Chl b} = 18.29A_{646} - 4.58A_{663}$$

A Bio-Rad protein assay kit was used to determine protein concentration (Bradford, 1976). Bovine serum albumin (5.32-105 µg) was used to prepare a standard curve. Protein samples were mixed with 1 ml of diluted dye reagent and left at room temperature for 5 min. The standard curve and sample readings were determined by a UV spectrophotometry at A_{595} nm. The protein concentration of the samples was estimated by using the standard curve.

Catalase activity was estimated by measuring the initial rate of disappearance of H_2O_2 by the spectrophotometric method of Aebi (1984) with minor modification. The 1 ml reaction mixture consisted of 1-25 µl of enzyme extract, depending on the linearised activity, 0.25 ml of 100 mM potassium phosphate pH 7, 0.25 ml of 70 mM H_2O_2, and 0.5 ml water. Catalase activity was assayed by measuring the decrease in absorbance of H_2O_2 at 240 nm (Luck, 1965). Assays were conducted at 25°C for 3 min and enzyme activity was expressed as ΔA_{240}/ min/ mg protein or converted as ΔA_{240}/ min/ gFw (gram fresh weight). An average of three independent samples were used to estimate the catalase activity, while one of these extracts was selected at random to use for other parts of the experiment. Data were exposed to analysis of variance using the ANOVA procedure of SAS (Statistical Analysis System) package.

RNA was isolated from plant tissue according to Ainsworth *et al.* (1995) at different developmental stages. 1.2 g of agarose was dissolved in 72 ml of distilled water, for a 100 ml agarose gel. The dissolved agarose was cooled to 65°C. Then, 10 ml 10 × MOPS buffer (23 mM MOPS, 6 mM EDTA, 50 mM sodium acetate, pH 7) and 17.85 ml formaldehyde solution were added, mixed by vigorous swirling and poured immediately into a gel former. Ten µg aliquots of total RNA were denatured by adding 16.5 µl of RNA loading dye (0.6 × MOPS, 8.5 % (w/v) deionised formaldehyde, 6% glycerol, 0.12 mM EDTA, 0.03% (w/v) Ficoll 400, 0.03% (w/v) bromophenol blue) at 65°C for 2 min. Denatured RNA was immediately chilled on ice before being loaded onto the agarose gel. Concentrations of RNA were equilibrated to give the same amounts in all tracks. A denatured RNA sample containing 1.3% ethidium bromide (EtBr) (10 mg/ml) was loaded in a single track of gel as a control to visualize. Electrophoresis was carried out in 1 × MOPS buffer at 100 volts for 2-3h. The RNA in the control track was visualized with the UV transilluminator. Excess parts of the gel that did not carry RNA were trimmed off. RNA was transferred onto a Biotrans+ (ICN Biomedicals inc.) nylon membrane with 0.05 M NaOH as the transfer solution. The membrane was then backed on the UV transilluminator for 2 min. Fractionated RNA was probed with relevant ^{32}P-labelled cDNA. Northern blotting, probe labelling, and hybridisation was carried out with the protocol as described by Ainsworth *et al.* (1995). Northern blot analysis was set up for all growth stages except the last stage, S4, which extracted RNA was degraded.

Different EST (Expressed Sequence Tag) clones (*Arabidopsis* from Biological Resource Centre at Ohio State) were used to measure the transcript levels of a number of other genes in *B. napus*. EST clones, representing genes that could have a role in oxidative protection, were used as probes for northern analysis, using extracted RNA from *B. napus* leaves. In addition the expression patterns shown by other genes that could have a role in oxidative protection, for example the LSC54 metallothionein gene, which was identified by differential screening of a cDNA library (Buchanan-Wollaston, 1994), was also examined. Ten µg of total RNA, extracted from *B. napus* leaves at different phases of development, were used for analysis of each expression pattern (as described above).

RESULTS

To investigate the role of the gene represented by the LSC650 cDNA, the complete DNA sequence was determined. The partial sequence analysis of this cDNA had revealed that LSC650 may be a catalase gene (Buchanan-Wollaston and Ainsworth, 1997). The nucleotide and deduced amino acid sequence of the LSC650 cDNA are shown in figure 1. Map positions were determined for some of the restriction enzymes. Sequence analysis revealed that the cDNA of 1732 bp in length contained an open reading frame of 1476 nucleotides encoding a polypeptide of 492 amino acid residues, followed by 215 nucleotides of untranslated sequence. A stop codon (TAG) was located at position 1517 in the cDNA. The protein encod-

ed by the LSC650 cDNA was predicted to have a molecular mass of 56.8 kDa with an isoelectric point of 7.75. The phylogenic tree analysis and sequence similarity (Fig. 2a and b) revealed a high level of similarity between LSC650 cDNA and the AtCat3 gene from *Arabidopsis* (90.3%). There was a lower similarity to the other catalase genes.

Biochemical analysis revealed that the levels of total chlorophyll (a+b) and protein increased from YG to MG1 and then steadily decreased from the MG3 stage through senescence (the results are shown in Table 1 and Fig. 3). The protein levels showed a sharper decrease between MG3 to S1, on the initiation of senescence. These reductions continued steadily to the last stage of senescence (S4). Catalase activity was detected in the all developmental stages. No significant difference between samples was observed, when catalase activity was statistically analysed (Table 1, the last calumn). A slightly higher activity was exhibited during maturity than in senescence, except S4 stage.

Northern blot analysis was used to visualize the expression pattern of the LSC650 gene in *B. napus* during leaf development (Fig. 4a and b). A 1.8 kb transcript was detected by the 1732 bp LSC650 cDNA insert probe. The expression of the LSC650 gene steadily increased from the MG1 stage to the S2 stage and then it remained constant in the S3 stage, where the leaf tissue showed the highest degree of yellowing.

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TAAACGTTATATTTGGGGATCATCAAAGCTTCTTCACCACCATGGATCCTTACAAGTACCGTCCCTCGAGCGCGTACAACGCTCCGTTTTACACCACAAA  100
                                        M  D  P  Y  K  Y  R  P  S  S  A  Y  N  A  P  F  Y  T  T  N
CGGCGGTGCTCCGGTCTCGAACAACATCTCCTCCCTCACCATCGGAGAAAGAGGTCCGGTTCTTCTTGAGGACTACCATCTGATCGAGAAGGTTGCTAAC  200
 G  G  A  P  V  S  N  N  I  S  S  L  T  I  G  E  R  G  P  V  L  L  E  D  Y  H  L  I  E  K  V  A  N
TTCACCAGGGAGAGGATTCCTGAGAGAGTGGTTCATGCCAGAGGGATCAGTGCTAAGGGCTTCTTTGAGGTCACCCATGACATCTCCAACCTCACTTGTG  300
 F  T  R  E  R  I  P  E  R  V  V  H  A  R  G  I  S  A  K  G  F  F  E  V  T  H  D  I  S  N  L  T  C
CTGATTTCCTCAGAGCCCCTGGTGTTCAGACTCCGGTCATCGTCCGTTTCTCCACTGTCGTTCACGAGCGTGCCAGCCCTGAAACCATGAGAGACATCCG  400
A  D  F  L  R  A  P  G  V  Q  T  P  V  I  V  R  F  S  T  V  V  H  E  R  A  S  P  E  T  M  R  D  I  R
TGGCTTCGCCGTCAAGTTTTACACCAGAGAGGGGAACTTTGATCTCGTTGGAAACAACACTCCGGTGTTCTTCATCCGCGACGGGATTCAGTTCCCGGAT  500
  G  F  A  V  K  F  Y  T  R  E  G  N  F  D  L  V  G  N  N  T  P  V  F  F  I  R  D  G  I  Q  F  P  D
GTTGTCCACGCCCTGAAGCCGAACCCGAAAACAAATATCCAGGAGTACTGGAGGATACTGGACTACATGTCCCACTTACCAGAGAGTCTCCTCACATGGT  600
 V  V  H  A  L  K  P  N  P  K  T  N  I  Q  E  Y  W  R  I  L  D  Y  M  S  H  L  P  E  S  L  L  T  W
GCTGGATGTTTGATGACGTCGGTATCCCACAAGACTACAGGCACATGGAAGGTTTCGGCGTCCACACCTACACTCTAGTCTCCAAATCCGGAAAGGTTCT  700
C  W  M  F  D  D  V  G  I  P  O  D  Y  R  H  M  E  G  F  G  V  H  T  Y  T  L  V  S  K  S  G  K  V  L
CTTCGTGAAGTTCCACTGGAAACCAACTTGTGGGATCAAGAATCTCACTGATGAAGAGGCTAAGGTAGTTGGAGGAGCCAATCACAGCCACGCGACTAAG  800
  F  V  K  F  H  W  K  P  T  C  G  I  K  N  L  T  D  E  E  A  K  V  V  G  G  A  N  H  S  H  A  T  K
GATCTTCACGATGCTATTGCGTCTGGTAACTACCCTGAGTGGAAGCTTTTTATTCAGACAATGGATCCTGCGGATGAGGATAAGTTTGACTTCGACCCGC  900
 D  L  H  D  A  I  A  S  G  N  Y  P  E  W  K  L  F  I  Q  T  M  D  P  A  D  E  D  K  F  D  F  D  P
TTGATGTGACCAAGATCTGGCCTGAGGATATCTTGCCTCTGCAGCCTGTTGGTCGGTTGGTTCTGAACAGGACTATTGATAACTTCTTTAATGAGACTGA  1000
L  D  V  T  K  I  W  P  E  D  I  L  P  L  O  P  V  G  R  L  V  L  N  R  T  I  D  N  F  F  N  E  T  E
GCAGCTTGCTTTCAACCCTGGTCTTGTGGTTCCTGGGATCTACTACTCGGATGACAAGCTGCTTCAGTGTAGGATCTTTGCGTATGGTGACACTCAGAGA  1100
  Q  L  A  F  N  P  G  L  V  V  P  G  I  Y  Y  S  D  D  K  L  L  Q  C  R  I  F  A  Y  G  D  T  Q  R
CATCGTCTTGGACCGAACTATCTGCAGCTTCCGGTTAATGCTCCCAAATGTGCTCACCACAACAATCACCATGAAGGGTTTATGAACTTCATGCACAGAG  1200
 H  R  L  G  P  N  Y  L  Q  L  P  V  N  A  P  K  C  A  H  H  N  N  H  H  E  G  F  M  N  F  M  H  R
ATGAGGAGATCAATTACTACCCATCAAAGTTTGATCCTGTCCGGTGCGCAGAGAAAGTTCCTATCCCTAACAAATCATACACTGGAATCAGAACAAAGTG  1300
D  E  E  I  N  Y  Y  P  S  K  F  D  P  V  R  C  A  E  K  V  P  I  P  N  K  S  Y  T  G  I  R  T  K  C
CATCATCAAGAAGGAGAACAACTTCAAGCAGCCAGGAGACAGATACAGATCATGGGCACCAGACAGGCAAGACAGGTTTGTGAAGAGATGGGTTGAGATA  1400
  I  I  K  K  E  N  N  F  K  Q  P  G  D  R  Y  R  S  W  A  P  D  R  Q  D  R  F  V  K  R  W  V  E  I
CTGTCAGAGCCGCGTCTGACTCATGAGATCCGCAGCATCTGGATCTCTTACTGGTCTCAGGCTGATCGATCACTGGGACAGAAACTAGCAAGCCGTCTCA  1500
 L  S  E  P  R  L  T  H  E  I  R  S  I  W  I  S  Y  W  S  Q  A  D  R  S  L  G  Q  K  L  A  S  R  L
ACGTGAGGCCAAGCATCTAGGGAGACCAATCTCTTGTCATCGGAAAGGTACAAATCATATGATACTCTCCATCATCACTTAAAATTCCTCAAGAAGATTA  1600
N  V  R  P  S  I
                                                  ↓
TGTTGTTGTTGTTGCTGTTTCGTGTCTGTATAATAAAAATGATGCTTTGTAATCCTTTTAAACCAAATGTTTCCTTGGTATTTTACTTGTCAAACTCTAC  1700
                  ↓
TCTTTGTAATCATCTCTTCATAATAAAAATCA   1732
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Figure 1. Nucleotide and deduced amino acid sequence of the cDNA, LSC650. The cDNA was sequenced and then the deduced amino acid sequence was identified by finding the longest open reading frame in the EditSeq sequence analysis program.

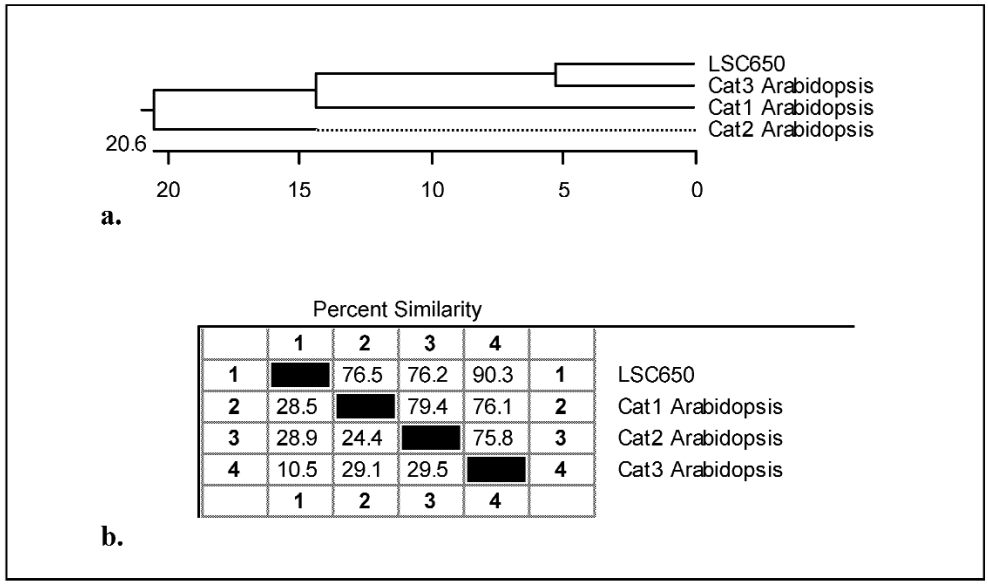

Figure 2. Phylogenetic tree and sequence distances of the DNA sequences comparing LSC650 with the *Arabidopsis* catalases. Figures show the phylogenetic relationships (a) and percentage sequence similarity and divergence between DNA (b) of *Arabidopsis* catalases (Cat1, Cat 2, and Cat 3) and LSC650 generated from the multiple alignment.

Table 1. Biochemical analysis of *B. napus* for total chlorophyll, protein, and catalase.

No.	Plant Developmental Stage	SPAD Chlorophyll Meter	Total Chlorophyll (a and b, mg/gFW)	Protein Concentration (mg/gFW)	Catalase Activity (m Abs/min/mg protein) ± Standard Errore
1	YG	34.87	1.574	20.85	7034±225
2	MG1	45.17	1.873	25.38	7410±377
3	MG2	41.73	1.791	24.86	7349±666
4	MG3	37.33	1.792	18.78	7989±181
5	S1	35.33	1.367	7.896	5240±1346
6	S2	29.93	1.163	7.617	5967±459
7	S3	23.97	0.841	6.351	5730±333
8	S4	13	0.686	4.809	8073±886

Gene expression of this isoform of catalase was undetectable in young leaves. The expression pattern of the gene represented by Cat3 (Fig. 4c) exhibited a steady increase from maturity (M2) to the final stage of senescence, while no transcript was detectable in young green (YG) and early mature leaves (MG1). The expression of the gene encoded by the Cat1 cDNA (Fig. 4d) was assessed during different stages of development in *B. napus* as described above. A 1.8 kb transcript was detected on the northern blot. Transcript abundance increased gradually from young green stage (YG) to final stage of maturity (MG3), and then, with the onset of senescence it steadily decreased to the last stage of development (S3).

EST analysis exhibited different transcripts levels. A northern blot carrying *B. napus* RNA was hybridised with a radiolabelled probe of the Cu,Zn-superoxide dismutase (Cu,Zn-SOD) cDNA (Fig. 5a). Two transcripts with size of 1.3 kb and 0.9 kb were detected, indicating the presence of two different isoforms. The northern blot of Cu,Zn-SOD indicated that transcription of this gene occurred throughout leaf development, with the transcript level of the larger band being stronger than that of the smaller. The intensity of the smaller transcript was low in young and mature leaves (YG, MG1 and MG2), and increased in MG3 and senescence phases (S1 and S2). However, the gene giving rise to the larger band exhibited a high level of

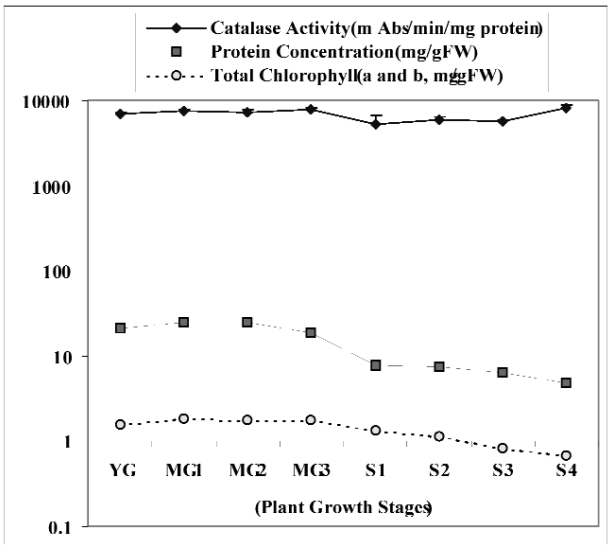

Figure 3. The variation of total chlorophyll, total protein, and catalase activities during leaf development revealed by biochemical analysis for total chlorophyll, protein, and catalase of *B. napus*.

transcription through all developmental stages with a gradual increase in final phases of development (MG3, S1 and S2). Figure 5b shows the expression of an Fe-SOD gene in *B. napus*, detected as described above. A 1.1 kb transcript was detected and sized using RNA size markers. The northern blot analysis showed that gene expression differed during the leaf developmental stages. The transcription of the Fe-SOD gene increased a little from young (YG) to the first stage of maturity (MG1) and then gradually from MG2 to the last stage of senescence (S3). Except for a drop in the MG2 stage, this increased expression appeared to coincide with the progress of senescence, indicating that the Fe-SOD may be a senescence-induced gene. As showing in figure 5c , the expression pattern of Mn-SOD gradually increased throughout development, reaching the highest level in mid senescence (S2). The increasing abundance of Mn-SOD coincided with the progress of senescence. This isoform was not abundant in young green tissue (YG), therefore it is unlikely to play any role in this tissue. There was no evidence for the expression of the 2.7 kb glutathione-S-transferase (86H5T7) gene in young tissue (YG, MG1), but the other developmental stages showed increased levels of this transcript (Fig. 5d). The level of expression increased from the second stage of maturity (MG2) to the first stage of senescence (S1), but with the progress of senescence, it gradually decreased. The highest transcription level of this gene was detected at the initiation of senescence (S1). Although, there was low level of transcript of another isoform of glutathione-S-transferase (90L23T7) during early development (YG and MG1), the level increased with the progress of senescence (Fig. 5e). First, the level decreased from young green to maturity (YG to MG2), then it increased during senescence to senescence stage 2 (S2), and then decreased again during the last phase of senescence. The strong expression of this gene during senescence indicated that it is a senescence-induced gene, although transcript levels were low. The transcription of the 1.5 kb of glutathione synthase gene (190E21T7) in young and mature tissues (YG, MG1, and MG2) obviously differs from that in senescing tissues (Fig. 5f). The highest level of this gene transcript at senescence stages indicating that this gene also is a senescence-induced gene. As shown in Fig. 5g, the 422 bp LSC54 cDNA insert detected a 0.5 kb transcript. The transcription was weakly visible at Y and MG1 stages, and was obvious-

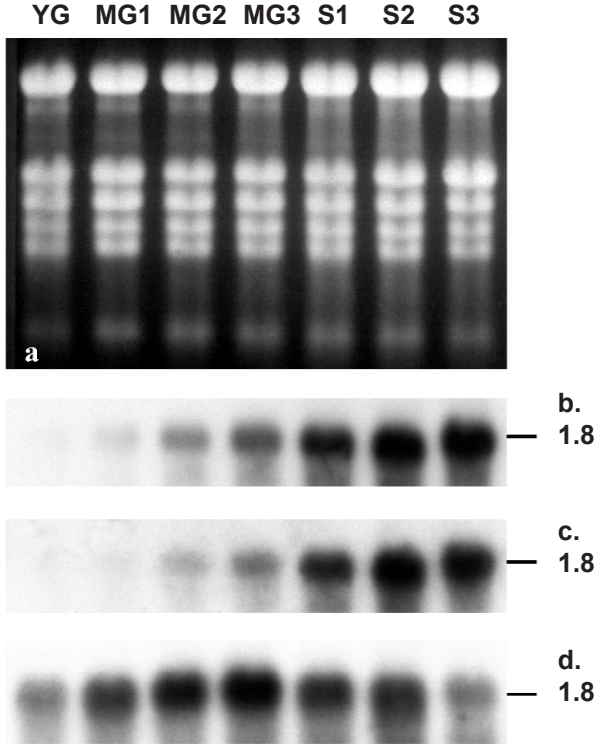

Figure 4. Differential expression of LSC650 and two catalase genes is shown during different developmental phases. The lanes are corresponded to total RNA isolated from *B. napus* leaves of different developmental stages. Ten µg aliquots of total RNA were loaded in each track. These were, then, equilibrated with eyes to give the same amounts in all tracks (**a**). The same amounts of each sample were used (without EtBr) for hybridisation gel. A denatured RNA sample containing 1.3% ethidium bromide (EtBr) (10 mg/ml) was loaded in a single track of gel as a control to visualise. Each blot was hybridised using labelled DNA fragments from the following clones: **b**. LSC650; **c**. catalase 3 (174P23T7, H36568, from EST clones); and **d**. catalase 1 (178B10T7, H36340, from EST clones).

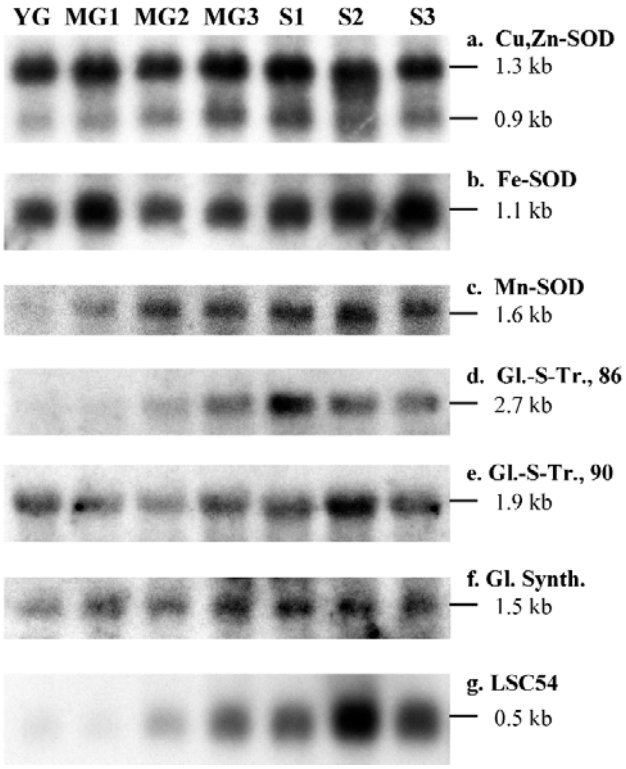

Figure 5. Northern blot analysis was done at different developmental stages of *B. napus*. Fractionated RNA was hybridised with radiolabelled EST fragments as follows: **a:** Cu,Zn-SOD (110P7T7, T42186). **b:** Fe-SOD (113C21T7, T42379). **c:** Mn-SOD (123N9T7, T44258). **d:** glutathione-S-transferase AtpM24.1 (86H5T7, T21463). **e:** glutathione-S-transferase ERD11 (90L23T7, T21164). **f:** glutathione synthase (190E21T7, R90601). **g:** LSC54 clone (Buchanan-Wollaston, 1994).

ly detectable in the MG2 stage. A steady increase of transcript abundance was visible from MG2 to the S3 stage. The highest level of expression was detected in tissue from the middle period of senescence (S2).

DISCUSSION

The LSC650 cDNA sequencing revealed no poly A tail in the sequence; while two putative polyadenylation sites sharing the motifs, AATAA and ATAA, with the consensus sequence of AATAAA (Birnsteil *et al.,* 1985) were detected at 111 and 201 base pairs downstream of the stop codon. The nucleotide sequence GTGTT which is required for efficient polyadenylation (Joshi, 1987) was not found in the LSC650 sequence. Among fifteen ORFs within the sequence, the first one extended through most of the cDNA and probably represents the LSC650 protein (Haddad *et al.,* 2000). Sequence comparison with other catalase genes indicated that the LSC650 cDNA represented a catalase gene. Phylogenetic analysis located the LSC650 gene sequence pair distances in a group with the Cat3 gene of *Arabidopsis*. It is likely that the LSC650 gene in *B. napus* may encode the homologous catalase to AtCat3 in *Arabidopsis* (Haddad *et al.,* 1999).

Two important traits, chlorophyll and protein levels in leaves were analysed to characterize the progress of senescence (Fig. 3). To analyse plant senescence, it is essential to recognize and characterize the different development phases of plant growth. Once the developmental stages are identified, it is possible to analyse selected plant characteristics to distinguish the differences that occur between the phases (Buchanan-Wollaston, 1997; Ghosh *et al.,* 2001and Ohe *et al.,* 2005).

Induction of catabolism that occurs during senescence, i.e. lipid and protein degradation, could enhance the levels of active oxygen species in the plant cell. The increased level of transcript detected during senescence shows that the Cat3 isoform is a senescence-enhanced the gene. The expression pattern of this gene coincides with the expression of LSC650 in *B. napus*, which is not surprising considering the strong structural similarity between both genes. Based on the expression pattern, the function of the LSC650 catalase is likely to be senescence-related in *B. napus*, but it could also be involved in a stress response (not tested). The gene represented by LSC650 is expressed at a much higher level during senescence than in mature leaves. The expression pattern is different from that reported by Buchanan-Wollaston (1997) where the transcription of LSC650 was highest in the last stage of senescence in the Falcon variety of *B. napus*. The abundance of senescence-related LSC650 catalase may prevent the accumulation of H_2O_2 during this stage of plant development. As shown in figure 4, the catalase gene, Cat1, is expressed during maturity and this steadily declined with the progress of senescence. It seems that with the decrease of Cat1 expression, the expression of Cat3 is increased. In the same experiment, a similar result was obtained for a different isoforms of SOD during different developmental stages. Therefore the total activity of these enzymes may not show much change during the different developmental stages in *B. napus.*

Northern blot analysis also displayed different transcript levels for different SOD genes. Accumulation of AOS is the main signal causing the induction of SOD, which converts O_2^- to H_2O_2. Accumulation of SOD enzyme decreased in some plants as senescence progresses (Dhindsa *et al.,* 1981 and 1982), but it was shown to significantly rise in other plants (Pastori and del Rio, 1994 and 1997; Bailly *et al.*, 2001and Tewari

et al., 2004). In this study, the abundance of three different SOD transcripts significantly increased during senescence in *B. napus* (Fig. 5a, b, and c), suggesting that the accumulation of O_2^- may occur during senescence. The H_2O_2 produced in the young and mature plant is scavenged by some isoforms of catalase in the peroxisomes, while other isoforms may have a role to scavenge the H_2O_2 produced through fatty acid degradation in the glyoxysomes (Mohr and Schopfer, 1995). SOD is the only enzyme which is known to convert O_2^- to H_2O_2. Therefore, the increase in SOD activity during senescence, in parallel with catalase increases, may serve to protect the plant cell from oxidative damage. As it was expected, the expression of SOD genes was synchronised with catalase activity. Since the activity of some SOD enzymes increased during senescence, they may be characterized as senescence enhanced enzymes.

Glutathione, which acts as an antioxidant, is widely believed to protect the cell against oxidative stress by maintaining cellular redox potential. It may react directly with AOS, protect protein thiol groups, form mixed disulfides with proteins, or be involved in enzymatic detoxification of H_2O_2 (De Kok and Stulen, 1993). The increase in transcript abundance of different enzymes involved in glutathione metabolism which has been observed in this study (Fig. 5d, e, and f) may be to protect enzymes and DNA, which still have a role in the progress of senescence, from oxidative damage. Metallothioneins consist of a family of proteins characterized by the presence of abundant cysteine (Cys) residues which are organised into two clusters and bind transition metals coordinately with strong affinity (Robinson *et al.,* 1993). Metallothioneins may be involved in metal storage and detoxification, control of cellular metabolism, protection from free radical toxicity, and in the UV response (Karin, 1985), but their functions have not been clearly defined as yet (Zenk, 1996). Expression of the LSC54 gene increased with the progress of senescence (Fig. 5 g) and this gene may be involved in the detoxification of heavy metal ions which are released during degradation of proteins during senescence. Stress can also cause increased expression of metallothionein-like protein genes (Zenk, 1996 and Brkljacic *et al.,* 2004).

The result of this study indicates that all the studied genes have a role in the progression of leaf senescence, and may act as antioxidants in plant cells (Haddad *et al.,* 1999, Bailly *et al.*, 2001; Parida *et al.,* 2004 and Jebara *et al.,* 2005). As senescence is the period of catabolism of macromolecules such as lipid and protein, this may cause the accumulation of AOS. The enzymes investigated in this study play a principle role to scavenge AOS from the plant cell and therefore assure cell survival until migration of the last micromolecules to other parts of the plant has been achieved. Catalase activity was detected at high levels during senescence and in mature leaves. The highest specific activity of catalase was detected in the last part of senescence (Fig. 3 S4). These results were complementary to northern blot analysis of LSC650 that showed increased transcript levels during senescence. As it is clear from catalase assay, this enzyme is abundant in all developmental stages. It seems with the decline of activity of one isoform, another isoform replaces it, for instance Cat1 is expressed during maturity, while Cat3, LSC650 in this study, is expressed during senescence. All together, the northern blot analysis of LSC650 and biochemical analysis presented in this study, indicate the importance of this catalase isoform during senescence in *B. napus* plant.

Catalase activity in seedlings and flower developmental phases of *B. napus* was not examined in this study. Further work could focus on these developmental stages to provide a picture of catalase activity throughout the life cycle of *B. napus*.

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