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Molecular Characterization of Free Radicals Decomposing Genes on Plant Developmental Stages

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***Abstract*—**Biochemical and molecular analysis of some antioxidant enzyme genes revealed different level of gene expression on oilseed (*Brassica napus*). For molecular and biochemical analysis, leaf tissues were harvested from plants at eight different developmental stages, from young to senescence. The levels of total protein and chlorophyll were increased during maturity stages of plant, while these were decreased during the last stages of plant growth. Structural analysis (nucleotide and deduced amino acid sequence, and phylogenic tree) of a complementary DNA revealed a high level of similarity for a family of Catalase genes. The expression of the gene encoded by different Catalase isoforms was assessed during different plant growth phase. No significant difference between samples was observed, when Catalase activity was statistically analyzed at different developmental stages. EST analysis exhibited different transcripts levels for a number of other relevant antioxidant genes (different isoforms of SOD and glutathione). The high level of transcription of these genes at senescence stages was indicated that these genes are senescence-induced genes.

***Keywords*—**Biochemical analysis, Oilseed, Expression pattern, Growth phases.

I. 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. Characterisation 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 $O_2^{\cdot -}$ in the plant cell. Activity of this family of enzymes is known to be linked with catalase activity, since SOD converts $O_2^{\cdot -}$ into H_2O_2 and catalase degrades H_2O_2 [2], [5], [13].

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 characterised in 1901 [27]. Catalase was one of the earliest enzymes isolated in a state of high purity [37]. No multicellular organism has been found that does not possess at least some catalase activity [36]. Catalase, together with SOD and hydroperoxidases, act as a defense system to detoxify $O_2^{\cdot -}$ and hydroperoxides [11], [13], [23]. Different isoforms of catalase, encoded by genes which are differentially regulated, have been identified during the life cycle of a plant [39], [5], [33]. 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 [21], [11], [25], [33].

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 [32], [5], [28], [33], [23]. 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 [7]. To investigate the role of the antioxidant genes, different clones were used and showed various transcript levels at different developmental stages.

II. 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 pBluescript 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 characterise protein structure, open reading frames (ORF) [26] 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 characterise different developmental stages of the

leaves on a plant. The leaf to be tested was placed between two LEDs to irradiate with 650 nm wavelengths for red and 940 nm wavelengths 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 1-12 h. 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 [22]:

$Chl\ a = 12.5A_{663} - 2.55A_{646}$

$Chl\ b = 18.29A_{646} - 4.58A_{663}$

A Bio-Rad protein assay kit was used to determine protein concentration [6]. 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 [3] 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 [29]. 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). Averages 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 [4] 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 visualise. Electrophoresis was carried out in 1 × MOPS buffer at 100 volts for 2-3 h. The RNA in the control track was visualised 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 were carried out with the protocol as described by [4]. 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 pattern 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 [8], 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).

III. Results and Discussion

Sequence analysis of the cDNA, LSC650, fragment revealed a cDNA of 1732 bp in length which 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 nucleotide sequence. The protein encoded 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. 1 a and b) were revealed a high level of similarity for LSC650 cDNA (90.3%) to the AtCat3 gene from *Arabidopsis*. There was a lower similarity to the other catalase genes. It is likely

that the LSC650 gene in *B. napus* may encode the homologous catalase to AtCat3 in *Arabidopsis* [20].

Catalase activity was measured in excised leaves that had been stored under different conditions before the protein extraction (Fig. 2). The immediate frozen of the collected leaves was chosen for protein extraction in all further experiments.

Two important traits, chlorophyll and protein levels in leaves were analyzed to characterize the progress of senescence (Table 1 and Fig. 3). The results obtained showed that the level of chlorophyll and total protein rises during maturity, and then drops with the progress of senescence as has been shown previously [7], [30]. No significant difference between samples was observed, when catalase activity was statistically analyzed (Table I, the last column, Fig. 3). The accumulation of catalase during senescence has been documented in some plants such as *Brassica napus* [7], *Iris* flowers [5], Arabidopsis [24], and carnations [42].

Analysis of expression of EST genes was exhibited different transcripts levels. Fig. 4 a, b and c shows a northern blot that indicates the expression pattern of the LSC650, Cat3, and Cat1 genes in *B. napus* during leaf development. 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. The strongly increasing level of transcript detected during senescence shows that the Cat3 isoform is a senescence-enhanced 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).

Northern blot carrying *B. napus* RNA during leaf development was hybridized with different radiolabelled probe of the EST clones (Fig. 5). Accumulation of AOS is the main signal causing the induction of SOD, which converts $O_2^{\cdot -}$ to H_2O_2. In this study, the abundance of three different SOD transcripts significantly increased during senescence in *B. napus* (Fig. 5 a, b, and c), suggesting that the accumulation of $O_2^{\cdot -}$ may occur during senescence. 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 [12]. The increase in transcript abundance of different enzymes involved in glutathione metabolism seen in this study (Fig. 5 d, e, and f) may be to protect enzymes and DNA, which still have a role in the progress of senescence, from oxidative damage. Expression of the LSC54 gene, a metallothionein gene, which was identified by differential screening of a cDNA library [8], 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 [41], [35].

The result of this study indicates that all studied genes have a role in the progression of leaf senescence, and may act as antioxidants in plant cells [20], [5], [24], [31]. 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 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*.

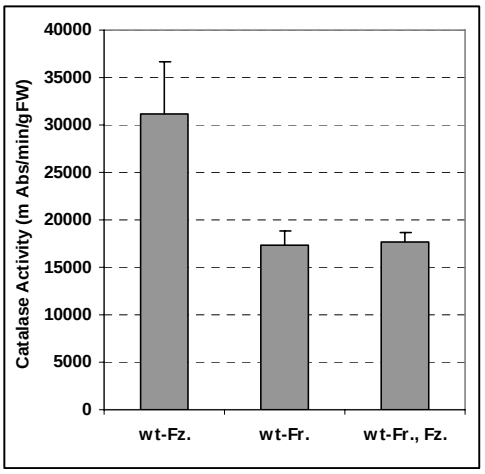


Fig. 2 Analysis of catalase activity in leaves kept in different storage conditions before extraction

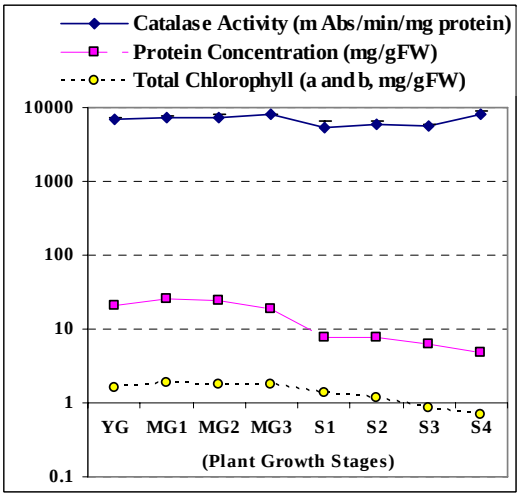
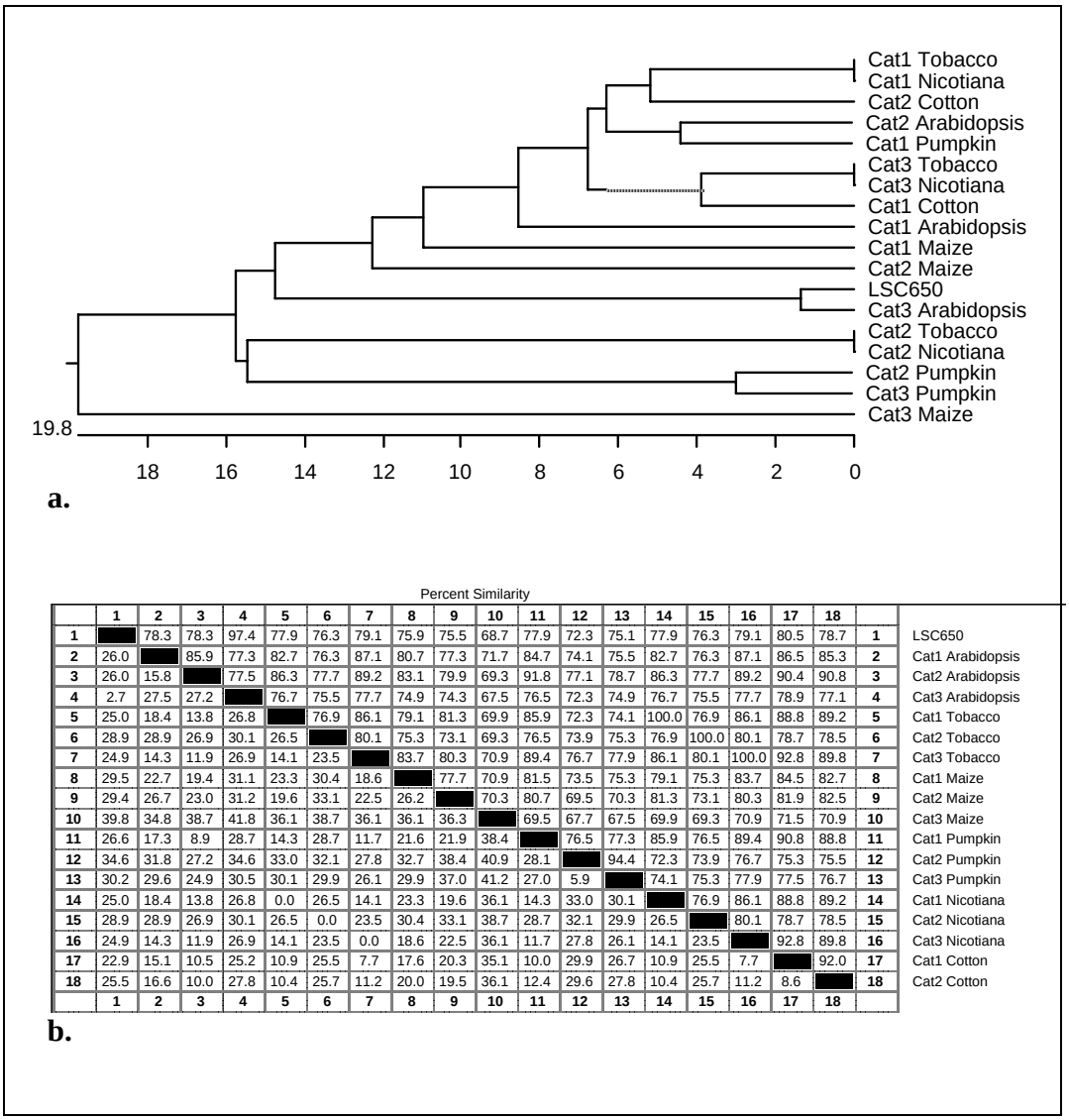


Fig. 3 Biochemical analysis of B. napus for total chlorophyll, protein, and catalase. This graph shows the variation of total chlorophyll, total protein, and catalase activities during leaf development. The data in Table I was used to draw this graph.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		78.3	78.3	97.4	77.9	76.3	79.1	75.9	75.5	68.7	77.9	72.3	75.1	77.9	76.3	79.1	80.5	78.7	1	LSC650
2	26.0		85.9	77.3	82.7	76.3	87.1	80.7	77.3	71.7	84.7	74.1	75.5	82.7	76.3	87.1	86.5	85.3	2	Cat1 Arabidopsis
3	26.0	15.8		77.5	86.3	77.7	89.2	83.1	79.9	69.3	91.8	77.1	78.7	86.3	77.7	89.2	90.4	90.8	3	Cat2 Arabidopsis
4	2.7	27.5	27.2		76.7	75.5	77.7	74.9	74.3	67.5	76.5	72.3	74.9	76.7	75.5	77.7	78.9	77.1	4	Cat3 Arabidopsis
5	25.0	18.4	13.8	26.8		76.9	86.1	79.1	81.3	69.9	85.9	72.3	74.1	100.0	76.9	86.1	88.8	89.2	5	Cat1 Tobacco
6	28.9	28.9	26.9	30.1	26.5		80.1	75.3	73.1	69.3	76.5	73.9	75.3	76.9	100.0	80.1	78.7	78.5	6	Cat2 Tobacco
7	24.9	14.3	11.9	26.9	14.1	23.5		83.7	80.3	70.9	89.4	76.7	77.9	86.1	80.1	100.0	92.8	89.8	7	Cat3 Tobacco
8	29.5	22.7	19.4	31.1	23.3	30.4	18.6		77.7	70.9	81.5	73.5	75.3	79.1	75.3	83.7	84.5	82.7	8	Cat1 Maize
9	29.4	26.7	23.0	31.2	19.6	33.1	22.5	26.2		70.3	80.7	69.5	70.3	81.3	73.1	80.3	81.9	82.5	9	Cat2 Maize
10	39.8	34.8	38.7	41.8	36.1	38.7	36.1	36.1	36.3		69.5	67.7	67.5	69.9	69.3	70.9	71.5	70.9	10	Cat3 Maize
11	26.6	17.3	8.9	28.7	14.3	28.7	11.7	21.6	21.9	38.4		76.5	77.3	85.9	76.5	89.4	90.8	88.8	11	Cat1 Pumpkin
12	34.6	31.8	27.2	34.6	33.0	32.1	27.8	32.7	38.4	40.9	28.1		94.4	72.3	73.9	76.7	75.3	75.5	12	Cat2 Pumpkin
13	30.2	29.6	24.9	30.5	30.1	29.9	26.1	29.9	37.0	41.2	27.0	5.9		74.1	75.3	77.9	77.5	76.7	13	Cat3 Pumpkin
14	25.0	18.4	13.8	26.8	0.0	26.5	14.1	23.3	19.6	36.1	14.3	33.0	30.1		76.9	86.1	88.8	89.2	14	Cat1 Nicotiana
15	28.9	28.9	26.9	30.1	26.5	0.0	23.5	30.4	33.1	38.7	28.7	32.1	29.9	26.5		80.1	78.7	78.5	15	Cat2 Nicotiana
16	24.9	14.3	11.9	26.9	14.1	23.5	0.0	18.6	22.5	36.1	11.7	27.8	26.1	14.1	23.5		92.8	89.8	16	Cat3 Nicotiana
17	22.9	15.1	10.5	25.2	10.9	25.5	7.7	17.6	20.3	35.1	10.0	29.9	26.7	10.9	25.5	7.7		92.0	17	Cat1 Cotton
18	25.5	16.6	10.0	27.8	10.4	25.7	11.2	20.0	19.5	36.1	12.4	29.6	27.8	10.4	25.7	11.2	8.6		18	Cat2 Cotton
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		

Fig. 1 Phylogenetic tree and sequence distances of plant catalase proteins.

a. This figure shows the phylogenetic relationships of plant catalases generated from the multiple alignment raised by a MegAlign computer program. **b.** Sequence distances of the proteins. The percentage sequence similarity and divergence between strands. Both figures were drawn using the computer program MegAlign, DNASTAR inc. with the Clustal method, PAM250 residue weight table. The database accession numbers of the sequences used are as follows: Cat1-*Arabidopsis*: U43340 [18], Cat2(A)-*Arabidopsis*: X94447 [10], Cat3-*Arabidopsis*: U43147 [17], Cat1-Tobacco: Z36975 [39], Cat2-Tobacco: Z36976 [39], Cat3-Tobacco: Z36977 [39], Cat1-Maize: P18122 [34], Cat2-Maize: X54819 [19], Cat3-Maize: L05934 [1], Cat1-Pumpkin: D55645 [14], Cat2-Pumpkin: D55646 [15], Cat3-Pumpkin: D55647 [16], Cat1-Nicotiana: Z36975 [40], Cat2-Nicotiana: Z36976 [40], Cat3-Nicotiana: Z36977 [40], Cat1-Cotton: X52135 [38], Cat2-Cotton: X56675 [38].

TABLE I
BIOCHEMICAL ANALYSIS OF *B. NAPUS* FOR TOTAL CHLOROPHYLL, PROTEIN, AND CATALASE. THIS TABLE SHOWS THE LEVELS OF TOTAL CHLOROPHYLL, TOTAL PROTEIN, AND CATALASE ACTIVITY DURING LEAF DEVELOPMENT OF *B. NAPUS* FROM YOUNG GREEN TO SENESCENCE

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 Error
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

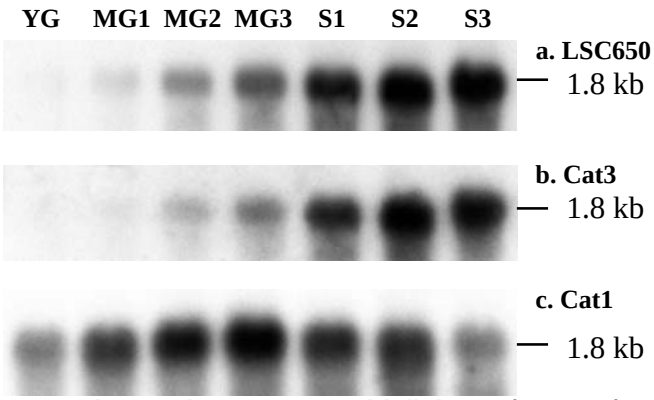


Fig. 4 Differential expression of LSC650 and two catalase genes is shown during different developmental phases. The lanes are corresponded to total RNA isolated from *B. napus* leaves of different developmental stages. Each blot was hybridised using labelled DNA fragments from the following clones: a. LSC650; b. catalase 3 (174P23T7, H36568, from EST clones); and c. catalase 1 (178B10T7, H36340, from EST clones).

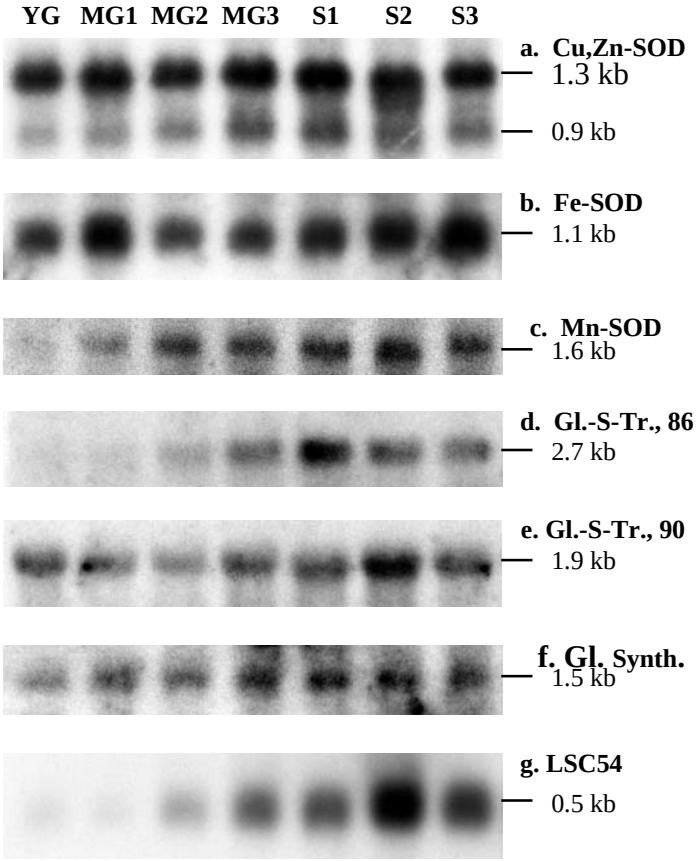


Fig. 5 Northern blot analysis was done at different developmental stages of *B. napus*. Fractionated RNA was hybridised with radiolabelled EST fragments as follows: **a:** Cu,Zn-SOD (110P7T7, T42186). **b:** Fe-SOD (113C21T7, T42379). **c:** Mn-SOD (123N9T7, T44258). **d:** glutathione-S-transferase AtpM24.1 (86H5T7, T21463). **e:** glutathione-S-transferase ERD11 (90L23T7, T21164). **f:** glutathione synthase (190E21T7, R90601). **g:** LSC54 clone [8].

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