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Expression Analysis of Two Senescence Involved Genes in *Brassica napus* and *Arabidopsis thaliana*

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ABSTRACT

Expression analysis was carried out to characterise the level and time of expression of two senescence related cDNAs, LSC650 and LSC54, in *Brassica napus* and *Arabidopsis thaliana*. The extent of DNA sequence similarity showed that the LSC650 gene may encode the catalase homologous to Cat3 in *Arabidopsis thaliana* the role of which is to scavenge H_2O_2. Also, LSC54 gene encodes a metallothionein protein that may detoxify metal ions in plant cells. Plant leaves were characterised at different developmental stages by biochemical analysis, including chlorophyll and protein assays. Northern analysis revealed strong levels of LSC650 and LSC54 expression in senescent leaves and lower levels in mature green leaves, but very weak or no expression in young leaves in *B. napus*. The transcription of genes, LSC650 and LSC54, was almost at the same level with few changes between maturity and senescence in leaves of *Arabidopsis* at different plant growth phases, being due to a possible unknown stress. The results indicate that both genes studied may act as antioxidants, and have a role in scavenging active oxygen species (AOS) caused by catabolism of macromolecules during senescence.

Keywords: Active oxygen species (AOS), *Arabidopsis thaliana*, *Brassica napus*, Catalase, Metallothionein-like protein, Senescence.

INTRODUCTION

Senescence is an important part of the life span of a plant in which many genes are involved in carrying out a series of events to provide for the next generation. Some of these genes are responsible for the degradation of macromolecules and cause accumulation of active oxygen species (AOS) that have to be degraded by the products of the other genes.

Lipid and protein 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 [4, 13, 14, 15, 34, 36]. The enzymatic activity of catalase is to detoxify H_2O_2 and expression of the genes encoding catalase is induced by different stresses [38]. 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 [4, 16, 32, 37]. As reported recently, darkness and natural nodule senescence affect antioxidant enzyme activity. Rapid leaf senescence increased lipid peroxidation but loss of catalase activity in *Lilium* [3]. This is also suggested by a recent report that continuous darkness significantly decrease the catalase activity in lupin plants [19].

Metallothioneins are proteins involved in the binding and detoxification of heavy met-

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als in cells [28]. On the other hand, some metallothioneins may play a direct role in cellular defence against oxidative stress by functioning as an antioxidant [18, 27, 33, 35]. These enzymes may be involved in sequestering free copper ions in the cytoplasm and delivering copper to post-Golgi vesicles. Therefore, these may have a role in copper salvage in senescing leaf tissue [21], or protect plant cell from oxidative damage during senescence [6, 8]. It seems that the role of these enzymes may be clearer in animals than in plants. Different cDNAs, which encode metallothionein, were isolated from cDNA libraries. Analysis of these genes has revealed different expression patterns during plant growth [6, 7, 8, 11, 12, 16].

Northern analysis is a method by which details of gene expression can be detected and can show the level and time of expression of a gene in different tissues. In this study, northern analysis was used to determine the expression profile of the LSC650 and LSC54 genes during senescence. Expression analysis of these genes during senescence may be an indication that protection from oxidative damage is important in this phase of plant development.

MATERIAL AND METHODS

The plants used in this research project were *Brassica napus* cv. Westar and *Arabidopsis thaliana* accession Columbia. Seeds of *B. napus* were sown in a soil mix consisting of four parts peat-based compost (4 parts Irish moss peat, 1 part grit (6mm + cinders), lime to PH 6.5, nutrients) containing macro elements and one part vermiculite. The *Arabidopsis* seed was sown on the surface of the mixture of four parts commercial peat compost containing macro nutrients (Levington F2 mix), one part vermiculite and one part fine sand. The air temperature was maintained at a continuous 21±4°C for both *Arabidopsis* and *B. napus* plants. Plants in the glasshouse were grown under a long day 16 h photoperiod.

The first senescence-enhanced cDNA clone, LSC650, was isolated from a subtractive cDNA library [8]. The DNA sequence of this gene, when compared with other catalase genes, identified its role in the plant cell [16, 17]. The second cDNA clone, LSC54, was identified by differential screening of a cDNA library and was sequence analysed [6].

To extract RNA, both *B. napus* and *Arabidopsis* plants were characterised at different developmental stages. *B. napus* leaves were characterised at different growth phases including young leaves (Y), a range of mature green leaf stages with no sign of leaf chlorosis (MG1, MG2 and MG3), senescent stages with increasing yellowing of leaves (S1, S2, and S3), as follows:

Y Leaves from very young plants, no sign of flowering, 22 days from germination, 20 cm high, average SPAD 32.

MG1 40 cm high, 2nd leaf from bolt, average SPAD 42.

MG2 Start of flowering, 70 cm high, 2nd leaf from bolt, average SPAD 39.

MG3 Same plant as MG2, 4th leaf from bolt, average SPAD 36.

S1 Leaf from plant with opened flowers, signs of silique development, 90 cm high, about 10% chlorosis, average SPAD 32.

S2 Leaf from plant with many green siliques developed, about 30-40% chlorosis, average SPAD 22.

S3 Leaf from plant with some mature siliques, about 60-70% chlorosis, average SPAD 15.

Also, *Arabidopsis* leaves were characterised at different developmental stages from very young to those on late flowering plants, as detailed below:

VY Very young, no bolting.

Y Young plant, bolting just starting.

VEF Very early flowering, about 4 cm flower stem.

EF Early flowering, about 8 cm flower stem, about 10% yellow leaves.

MF Middle flowering, about 18 cm flower stem, about 30% yellow leaves.

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LF Late flowering, about 25 cm flower stem, most of the flowers open, few siliques, about 50% yellow leaves.

To confirm the developmental phases, the leaves were analysed by chlorophyll and Bradford protein assays. For the chlorophyll assay, 200 µl of homogenised tissue 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 Hill *et al*. [20]:

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

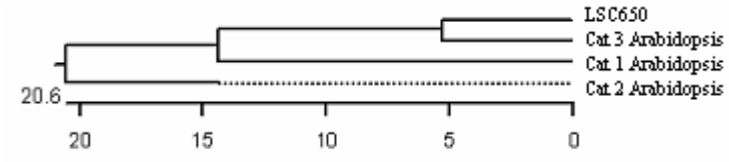
Chl b = $18.29A_{646}$ - 4.58 A_{663}

A Bio-Rad protein assay kit was used to determine protein concentration [5]. Bovine serum albumin (5.32-105 µg) was employed to prepare a standard curve. Protein samples were mixed with 1 ml of diluted dye reagent. The reaction mixture was mixed by pipetting and left at room temperature for 5 minutes. 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.

Total RNA was isolated from plant tissue [1] at different developmental stages. All leaf samples were harvested from glasshouse grown plants and immediately frozen at -70°C prior to RNA extraction. Northern blotting, probe labelling, and hybridisation were carried out with the same protocol as described by Ainsworth *et al*. [1, 2]. The membrane carrying total RNA of each plant was hybridised with radiolabelled LSC650 and LSC54 cDNA fragments, as probe.

RESULTS

The extent of DNA sequence similarity between the three catalases of *Arabidopsis* (AtCat1, AtCat2, and AtCat3) and the *B. napus* gene LSC650 was identified by phylogenic tree analysis and sequence similarity (Figure 1 a and b). This result re-



a.

Percent Similarity

	1	2	3	4		
1		76.5	76.2	90.3	1	LSC 650
2	28.5		79.4	76.1	2	Cat 1 Arabidopsis
3	28.9	24.4		75.8	3	Cat 2 Arabidopsis
4	10.5	29.1	29.5		4	Cat 3 Arabidopsis
	1	2	3	4		

b.

Figure 1. Phylogenetic tree and sequence distances comparing the DNA sequences of LSC650 with the *Arabidopsis* catalases. Figures show the phylogenetic relationships (**a**) and percentage sequence similarity and divergence between DNA (**b**) of *Arabidopsis* catalases [14, 13, 9], (Cat1, Cat 2, and Cat 3, respectively) and LSC650 generated from the multiple alignment. Both figures were drawn using the computer program MegAlign, DNASTAR inc. with the Clustal method, PAM250 residue weight table.

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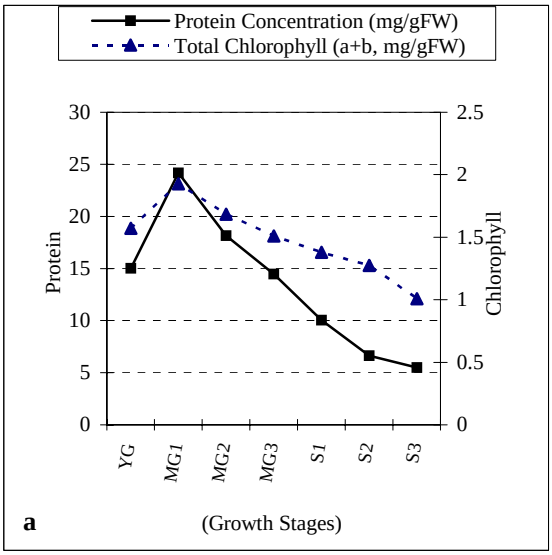
vealed a high level of similarity, at the DNA level (90.3%), for LSC650 to the AtCat3 gene from *Arabidopsis*. There was a lower similarity to the other catalase gene (76%). Therefore, it is likely that the LSC650 gene in *B. napus* may encode the homologous catalase to AtCat3 in *Arabidopsis* [17].

Biochemical analysis of different tissues confirmed progress of plant growth, and, therefore different developmental stages in both plants (Figure 2 a and b). Levels of total chlorophyll (a+b) and protein increased from YG to MG1 and then steadily decreased from the MG3 stage through senescence in *B. napus* (Figure 2 a). In *Arabidopsis* (Figure 2 b), the protein levels showed a



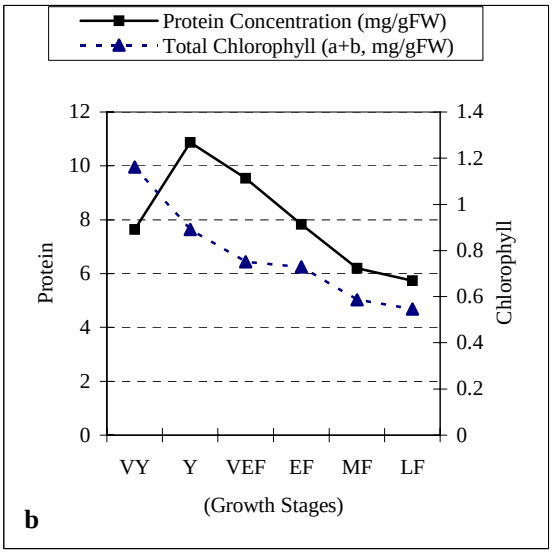


Figure 2. Biochemical analysis of *B. napus* and *Arabidopsis* plants. Figures show total biochemical analysis of various tissues of wild-type *B. napus* (**a**) and *Arabidopsis* (**b**) plants during different developmental stages from young green to senescence. Total RNA extracted from these samples was used to analyse the expression of the LSC650 and LSC54 genes (YG: young green, MG: mature green, S: senescence, VY: very young, Y: young, VEF: very early flowering, EF: early flowering, MF: middle flowering, LF: late flowering).

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sharp rose from VY to Y, then slowly reduced to the last stage of the plant growth; while the level of the total chlorophyll (a+b) steadily decreased from the VY stage to the LF stage.

Concentrations of RNA were equilibrated to give the same amounts in all tracks (Figure 3 a and d). Figure 3 b shows a northern blot that indicates the expression pattern of the LSC650 gene in *B. napus* during leaf development. A 1.8 kb transcript was detected by the probe, the 1732 bp LSC650 cDNA insert. 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. Gene expression of this isoform of catalase was undetectable in young leaves. The gene represented by LSC650 is expressed at a much higher level during senescence than it is in mature leaves, which indicates that the encoded protein is likely to have a senescence related function. This expression pattern is different from that reported by Buchanan-Wollaston [8] where the transcription of LSC650 was highest in the last stage of senescence in Falcon variety of *B. napus*.

In *Arabidopsis*, the LSC650 cDNA fragment (1732 bp) detected a 1.8 kb transcript on the northern blot (Figure 3 e) and showed the same level of expression of the gene during all phases of development. The transcription of a gene declined a little in the last stage of development (LF). It seems that this isoform of catalase (LSC650) is not a

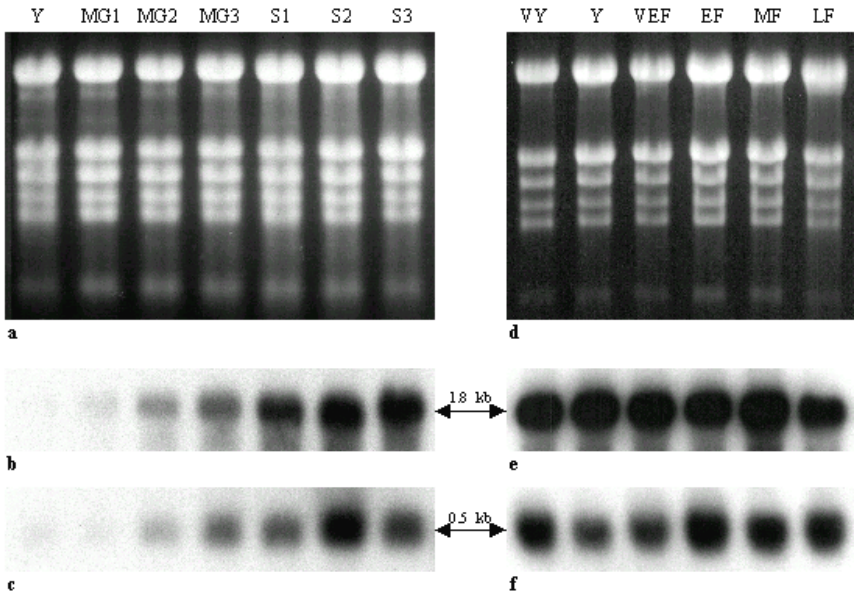

Figure 3. Fractionated RNA (approximately 10 μg in each track) on denaturing formaldehyde agarose gels and northern blot analysis for *B. napus* and *Arabidopsis* plants.

a. Total RNA was extracted from *B. napus* plants during different developmental stages, and fractionated on a 1.2% denaturing formaldehyde agarose gel. RNA was detected using EtBr staining.
b and **c.** The same fractionated RNA (containing no EtBr) blotted to nylon membrane and hybridised with radiolabelled LSC650 and LSC54 fragments, respectively.
d. Total RNA extracted from leaves of *Arabidopsis* plants at different developmental stages was fractionated as described for **a**.
e. and **f.** Fractionated RNA from **d** (containing no EtBr in the loading dye) was used for northern blot analysis of LSC650 and LSC54, respectively.

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senescence-related gene in *Arabidopsis*.

A northern blot carrying total RNA extracted from leaves of *B. napus*, as described above, was hybridised with a radiolabelled cDNA fragment of LSC54, a metallothionein gene (Figure 3 c). The 422 bp LSC54 cDNA insert detected a 0.5 kb transcript, measured using an RNA size marker. The transcript for the gene represented by LSC54 was weakly visible at Y and MG1 stages, and was obviously 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).

A northern blot was carried out using total RNA isolated from *Arabidopsis* leaves at different developmental stages and was hybridised with a radiolabelled LSC54 cDNA fragment (Figure 3 f). A transcript size of 0.5 kb was detected and sized using RNA size markers. The expression pattern of the gene represented by LSC54 (Figure 3 f) showed that transcript levels varied during different developmental stages. The transcript of the gene fell from VY to Y, then, it rose gently during the later stages. The hybridisation signal was the weakest at the young (Y) stage, increased a little in VEF, and was stronger in the other stages with highest levels detected in the last three stages of development. The EF stage presents the highest level of transcript, which is comparable to the S2 stage of *B. napus* (with 10% and 35% chlorosis, respectively). If during the last 3 stages (EF, MF and LF) senescence is underway, then it can be concluded that LSC54 is a senescence-induced gene in *Arabidopsis*. From the northern analysis above, the gene expression of LSC54 appears to be similar to that found in the northern analysis of this gene in the Westar variety of *B. napus* except the gene is expressed in the very young tissue.

DISCUSSION

The accumulation of catalase during senescence has been previously documented in some plants such as *B. napus* [8], wheat and rye [22], *Nicotiana plumbaginifolia* [36], pea [29], *Lilium* [3], and lupin [19]. Based on the expression studies carried out in *B. napus* (Figure 3 b), 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 enzymatic function of catalase is to degrade H_2O_2. The abundance of senescence-related LSC650 catalase may prevent the accumulation of H_2O_2 during this stage of plant development.

Higher plants contain several different catalase enzymes, which are encoded by a small gene family. Differential expression of catalase genes in these plants results in different levels of the various catalase enzymes during development. In this study, it has also been shown that the catalase gene is differentially expressed during development in *B. napus* (Figure 3 b). The expression pattern of LSC650 in *Arabidopsis* (Figure 3 e), showed that the gene is expressed at the same level at all stages of leaf development. Three isoforms of catalase, AtCat1, AtCat2, and AtCat3, are expressed in *Arabidopsis* leaves [14]. It is not clear whether each gene is co-expressed within a single cell type, and also whether the leaves investigated in this study were at a senescence stage. So, with respect to the high percentage of similarity between LSC650 and Cat3 of *Arabidopsis* (Figure 1), it is likely that the LSC650 probe detected the Cat3 of *Arabidopsis* in the northern blot with *Arabidopsis* RNA (Figure 3 e). The promoter of AtCat3 was active from the early stage of development in *Arabidopsis* plants used in this study. It is possible that stresses experienced in the glasshouse-such as pathogen, drought etc., could cause accumulation of AOS, therefore inducing expression of Cat3 in young leaves of these *Arabidopsis* plants. Also, as AtCat3 has a consensus peroxisomal targeting signal [14], it may not be senescence related in *Arabidopsis*.

The catalase multigene family in *Arabidopsis* includes three genes encoding indi-

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vidual subunits that associate to form at least six isozymes [14]. Based on the amino acid structure, these six isozymes were encoded by three genes, AtCat1, AtCat2, and AtCat3. For AtCat1, a cytosolic or mitochondrial role was suggested [14], for AtCat2 a glyoxysomal role [9], and for AtCat3 a peroxisomal role [13]. All three isoforms have been cloned and produced a transcript of the size predicted by the cDNA (AtCat1 2 kb; AtCat2 and AtCat3 1.8 kb each). AtCat1 mRNA is most abundant in bolts, with nearly half of the expression in the leaf and one-third in the root. Expression of this gene is detectable at a very low level in flowers, cauline leaves, and siliques. AtCat2 mRNA is most abundant in leaves, about half as abundant in cauline leaves and bolts, and of very low abundance in siliques, flowers, and roots. AtCat3 mRNA is highly abundant in bolts, moderately abundant in leaves and cauline leaves, but of low abundance in roots, siliques, and flowers. AtCat2 and AtCat3 were characterised as peroxisomal enzymes [14].

Metallothioneins consist of a family of proteins characterised by the presence of abundant cysteine (Cys) residues, which are organised into two clusters and bind transition metals coordinately with strong affinity [31]. Metallothioneins may be involved in metal storage and detoxification, control of cellular metabolism, protection from free radical toxicity, and in the UV response [23], but their functions have not been clearly defined as yet [39]. Expression of the LSC54 gene increased with the progress of senescence (Figure 3 c) and this gene may be involved in the detoxification of heavy metal ions which are released during degradation of proteins during senescence. Unlike the expression pattern seen in *B. napus*, this gene was expressed at all stages of plant development in *Arabidopsis* (Figure 3 f) although it obviously increased during senescence. Stress can also cause increased expression of metallothionein-like protein genes, which increases the heavy metal tolerance of plant cell [30, 39]. It is possible that *Arabidopsis* plants, which were chosen to extract the RNA for experiments, had been under some kind of unknown stress.

The result of this study indicates that all the genes studied may have a role in the progress of leaf senescence, and may act as antioxidants in plant cells [4, 10, 24, 25, 26]. As senescence is the period of catabolism of macromolecules such as lipid and protein, this may cause the accumulation of AOS. Both enzymes (investigated in this research project) play a principal role in scavenging 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 and metallothionein activities in seedlings and flowers of *B. napus* and *Arabidopsis* were not examined in this study. Further work could focus on these developmental stages to provide a picture of gene expression and also enzyme activity throughout the life cycle of both *B. napus* and *Arabidopsis*.

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تجزیه بیان دو ژن عامل پیري در کلزا و اربیدوپسیس

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چکیده

زمان و میزان بیان دو ژن (cDNA) جدا شده از مرحله پیري برگ کلزا ، به نام هاي LSC650 و LSC54 ، در گیاهان کلزا و اربیدوپسیس بررسي شدند . تشابه بالاي ساختمان ژن LSC650 با ژن کاتالیز (Cat3) گیاه اربیدوپسیس نشان داد که این ژن همردیف ژن Cat3 بوده و مسئول پاکسازي پراکسید هیدروژن از محیط سلولي مي باشد . ژن LSC54 نیز مسئولیت پاکسازي یون‌هاي فلزي از محیط سلولي را بر عهده دارد. جهت تعیین دقیق مراحل مختلف رشد گیاه ، میزان کلروفیل و پروتئین برگ گیاهان تعیین گردید. میزان بالایي از بیان ژن در برگ‌هاي پیر کلزا براي هر دو ژن مشاهده شد. ولي این بیان در برگ‌هاي بالغ کمتر بوده و در برگ هاي جوان تقریباً تظاهري مشاهده نشد. در برگ هاي گیاه اربیدوپسیس بیان ژن‌ها در مراحل مختلف رشد تقریباً یکسان بود، که این نتیجه مي‌تواند احتمالاً ناشي از نوعي استرس محیطي باشد. مجموعه این نتایج نشان داد که هر دو ژن، بعنوان ضد اکسنده، میتوانند در پاکسازي رادیکال هاي

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فعال اکسیژن ، که بر اثر تجزیه مولکولهاي درشت در زمان پیري گیاه بوجود مي‌آیند ، نقش داشته باشند .

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