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Identification of novel genes expressed in *Brassica napus* during leaf senescence and in response to oxidative stress

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Abstract

Senescence is a genetically regulated oxidative process that involves a general degradation of cellular structures and enzymes and the mobilization of the products of degradation to other parts of the plant. The cDNA-AFLP (cDNA-Amplified Fragment Length Polymorphism) analysis has been used under stringent PCR conditions afforded by ligation of adapters to restriction fragments, and the use of specific primer sets. It was of interest to analyse the proportion of senescence-enhanced genes in *Brassica napus* (CV Falcon) that do respond to different stress signals and to determine whether other known stress response genes are also expressed during leaf senescence or in response to treatments that increase ROS (Reactive Oxygen Species). The cDNA-AFLP technique uses the standard AFLP protocol on a cDNA template. Total RNA was extracted from mature leaves treated with Ascorbic Acid (As-A), silver nitrate ($AgNO_3$), UV-B irradiation, combined treatment (As-A/$AgN0_3$), (As-A/UV-B) as well as untreated control and senescence stage3 (S3). The first and double-stranded cDNAs were synthesised by PCR amplification. The result shows the expression pattern for 30 genes that were identified on the original cDNA-AFLP gel. Each gene that was identified as a band on the cDNA-AFLP gel and successfully cloned was characterised by both sequencing and expression analysis using hybridisation technique. The clone6 has high similarity to a DNA-binding protein that acts as a transcription factor and can be an important component of signalling pathways. This gene exhibits a senescence-enhanced expression and is highly induced by UV-B but not by $AgNO_3$. The northern hybridisation results for this gene in different organs showed high expression in flowers, pod and senescing leaves and no expression in green leaves and roots. The combined treatments had large effect on the expression of clone9. The expression pattern for this gene showed high transcript level in roots and flowers, low level in early senescing leaves and no expression in pod, mature leaves and late senescing leaves.

Keywords: *Brassica napus*; cDNA-AFLP; Gene expression; Oxidative stress.

Introduction

Most currently cloned Senescence Associated Genes (SAGs) were identified by differential cDNA cloning approaches, and little is known about them beyond the fact that their mRNA levels increase during natural senescence. The function of some of these genes can be inferred from the deduced amino acid sequences, but for others it is not the case. It is hoped that by studying the expression of various genes in response to stress hormones and senescence some insight into the relationships among those processes will be obtained. Becker and Apel (1993), for instance, have shown that of three genes differentially expressed in detached barley leaves left in the dark, only one was similarly upregulated during natural senescence, suggesting that the two processes are not identical. In a more comprehensive study, expression of a relatively large number of SAGs in response to age, dehydration, detachment, darkness, ABA, cytokinin, and ethylene have been examined (Weaver et al., 1998). They examined responses in leaves to address the question of whether a gene cloned as a SAG is in fact primarily a SAG, or whether it responds more strongly to one or several other factors, and only indirectly to age.

Several techniques such as differential screening and subtractive hybridisation have been applied to monitor alterations in gene expression that occur during senescence. In addition, differential display reverse transcription-polymerase chain reaction (DDRT-PCR) has been used. This method can generate a high proportion of false-positives (Jones and Harrower, 1998) and has other problems such as lack of sensitivity and difficulties with reproducibility (Zhang et al., 1998). The use of longer primers has reduced but not fully alleviated problems of false-positive bands (Martin and Pardee, 1999). Similar modifications have been employed to address sensitivity and reproducibility problems (Linskens et al., 1995). However, DDRT-PCR techniques are not very accurate in quantitatively profiling global levels of gene expression. To counteract the problems associated with differential display, cDNA-AFLP (cDNA-Amplified Fragment Length Polymorphism) analysis has been used under stringent PCR conditions afforded by ligation of adapters to restriction fragments, and the use of specific primer sets (Bachem et al., 1996). This method allows for a systematic survey of the organism's transcriptome through the use of selective fragment amplification (Shimkets et al., 1999).

Van der Biezen et al., (2000) applied the cDNA-amplified fragment length polymorphism (AFLP) technique to identify *Peronospora parasitica* genes expressed during infection in *Arabidopsis thaliana*. They used 228 primer combinations and obtained on average 70 amplification products per reaction. Sixty fragments (0.4%) were present only in samples from infected plants. Twenty-three of these cDNA fragments were excised from the gel and the DNA sequence determined. Similarly, Page et al., (2001) used cDNA-AFLP to identify senescence-enhanced genes in broccoli.

Here, in order to analyse the expression pattern of senescence associated as well as stress related genes in response to environmental factors that generate ROS in *Brassica napus*, a cDNA-AFLP analysis was performed.

Materials and Methods

Plant materials

Seeds of *Brassica napus* cv. Falcon were germinated and plants grown in a greenhouse until the four leaf stage at which time they 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 mature green, treated and senescing leaves (stage3: showing 30%-50% yellowing). Leaves were immediately frozen in liquid nitrogen and stored at -70 °C until use.

Chemical and UV-B treatments

A range of concentrations of each treatment was tested initially to identify the optimum level to use. Final experiments were carried out using 3mM silver nitrate ($AgNO_3$) and 30mM ascorbic acid (As-A). Spray treatments were applied until liquid dripped from the leaves. For the UV-B treatment the plants were given supplementary UV-B radiation for 6 hours from four UV lamps (Philips TL 12, 40 W). The fluence rate between 280-320 nm was 3.2 μmol m^{-2} s^{-1}. The UV lamps were covered with cellulose acetate sheets, which were changed daily, to exclude UV radiation below 320 nm. All treatments were carried out using fully expanded mature green leaves. For the combined treatments, the first treatment (As-A) was applied onto the plants followed 2 h later by the second treatments ($AgNO_3$ or UV-B). All samples were taken 48 h after the last treatment. All chemicals were obtained from Sigma-Aldrich. Each experiment was repeated at least three times, samples were taken at random from different parts of cabinets.

Purification of total RNA

Leaf material (5 g) was ground to a fine powder in liquid N_2. The frozen powder was transferred to a 10 ml phenol and 15 ml extraction buffer (100 mM Tris-HCl pH 9, 200 mM NaCl, 5 mM dithiothreitol, 1% (w/v) sarcosyl, 20 mM EDTA) and mixed gently. The mixture was centrifuged at 3,000 rpm for 10 min. The upper aqueous layer was extracted two or three times with an equal volume of chloroform, until it became clear. The solution was adjusted to 2 M LiCl with 8 M LiCl and left overnight at 4°C. Precipitated RNA was pelleted by centrifugation at 10,000 rpm, 4°C, for 10 min. The pellet was washed twice with 1.5 ml of 2 M LiCl and recentrifuged each time. To remove any insoluble material, the RNA was ethanol precipitated and dissolved in water. The RNA was separated on a 1.5% agarose gel containing 6% (v/v) formaldehyde in 2×MOPES buffer (23 mM MOPS, 6 mM EDTA, 50 mM sodium acetate, pH 7). Each gel was checked under UV light to ensure both RNA integrity and that equal amounts of RNA were loaded in each lane (data not shown).

cDNA synthesis and AFLP

First and second strand cDNA was synthesised from total RNA isolated from green and senescing leaves and leaves treated, using the SMART PCR cDNA Synthesis Kit (CLONTECH Laboratories, Palo Alto, CA). One microgram of total RNA was used as a template. The cDNA was digested with *Mse*I and *Msp*I and adapters (Life Technologies /Gibco-BRL, Cleveland) were ligated to the digested DNA. The cDNA was then used in AFLP reactions using a method based on the AFLP analysis system 1 (Life Technologies/ Gibco-BRL). Selective primers based on the *Mse*I adapter + 3 sequence were used with the smart PCR primer (end labelled with ^{32}P) to amplify a subset of cDNA fragments. Samples were denatured and separated on 6% (w/v) acrylamid gels and visualised by autoradiography. Excised bands were eluted in 100 μl of elusion buffer (0.5 ammonium

acetate, 10 mM magnesium acetate, 10 mM EDTA, pH 8, 0.1% (w/v) SDS) at 37 °C overnight. The eluted DNA was purified, and dissolved in 10 µl of T0.1E (10 mM TrisCl pH 8, 0.1 mM EDTA). The DNA was ligated into the pGEMT-vector (Promega, Madison, WI) and transferred into JM109 (Promega).

cDNA blot

PCR-amplified cDNA was used for virtual northern analysis (Suthern blot). cDNA synthesised as described above was used as the template. *Mse*I core primer and the SMART PCR primer, provided the cDNA for the cDNA blots (CLONTECH Laboratories, Smart PCR cDNA kit). 20 cycles (94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 30 sec) were used for amplification. DNA samples were separated on 1.5% agarose gels and blotted on to Hybond-N+ membrane (Amersham) using 0.4 M NaOH. Filters were hybridised with ^{32}P-labelled, PCR-amplified cDNA fragments as described above.

DNA sequence analysis

Sequencing was carried out using a DNA sequencing kit (Big Dye Terminator Cycle sequencing Ready Reaction Kit, PE-Applied Biosystems, Perkin-Elmer) with an ABI 377 DNA sequencer (PE-Applied Biosystems). Five hundred ng template DNA was mixed with 4 µl BIG DYE and 0.5 µl T7 primer (10 µg) in a total volume of 10 µl. Using 25 cycles of sequencing amplification (96 °C for 30 sec, 50 °C for 20 sec, 60 °C for 4 min), the reaction mixture was amplified and then 2 µl NaAC pH 5.2 and 50 µl 100% EtOH were added. Samples were vortexed, placed on ice for exactly 10 min and centrifuged at 13000rpm for 15 min. The supernatant was removed and the pellet washed with 100 µl 70% EtOH. Pellets were air dried and sequenced, using an ABI 377 DNA sequencer. Homology searches were done using BLASTN and BLASTX programs (Altschul et al., 1997) in the NCBI database.

Results and Discussion

Characterisation of cDNA-AFLP expression

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

Many cDNA clones have been identified using differential and subtractive hybridisation techniques. These clones represent genes that are induced by internal and external environmental factors, including the presence of heavy metals (Hsieh et al., 1995), salicylic acid and dark (Walter et al., 1996), heat shock (Genschik et al., 1994), nutrient starvation (Bariola et al., 1994), wounding (Garbarino et al., 1995) and senescence-affecting hormones (Oh et al., 1996). Other cDNA clones for mRNAs that increase in abundance during leaf senescence have been found in radish (Azumi and Watanabe, 1991), cucumber (Graham et al., 1992), *Arabidopsis* (Hensel et al., 1993), barley (Becker and Apel, 1993).

The specific advantages of cDNA-AFLP over the methods mentioned above, arise primarily from the stringency achieved by addition of universal anchors at each end of the cDNA-derived restriction fragments used as a substrate for the PCR. Also, cDNA-AFLP is an ideal method for the simultaneous visualisation of expression of the same gene in several RNA samples from different tissues, as it shows both good reproducibility and sensitivity and good correlation with Northern analysis (Bachem et al., 1996).

Total RNA was extracted from *Brassica napus* leaves as described in materials and methods. RNA samples were used for the cDNA synthesis. First and double-strand cDNA were synthesised by PCR amplification (Figure1). A range of cDNA fragments was seen, from 0.3-1.7 Kb in size, which indicates that the cDNA synthesis was effective. Various different expression patterns were detected and these are indicated on the Figure2. In a parallel experiment we found out a similar expression pattern for untreated control and ascorbic acid treated plants. This result since ascorbate is quit harmless for plant and naturally present in all subcellular compartments is expected.

Amplification products showing interesting expression patterns were excised from the gel, purified by spin chromatography, reamplified, cloned into the pGEMT-vector and transformed into JM109 cells. Colonies obtained in the transformation were picked and the inserted fragment amplified by colony PCR (data not shown). The fragments amplified from the colonies were sequenced as described in materials and methods. Homology searches were done using the BLAST program and the results are summarised in Table 1.

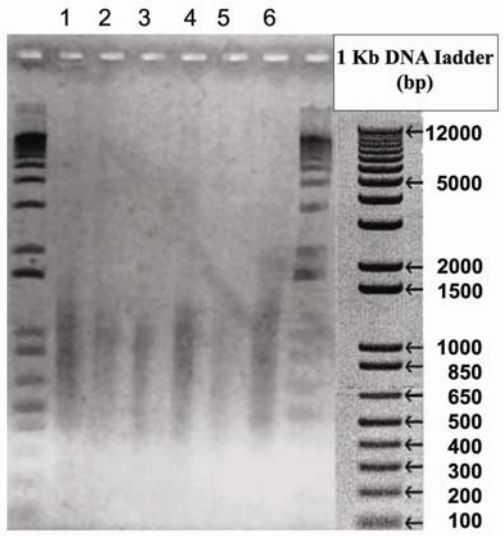

Figure 1. PCR amplification of double stranded cDNA made from RNA isolated from *Brassica napus* leaves after treatment and senescent (S3).
First and last track 1Kb DNA ladder
1-Ascorbic-Acid (As-A) 2-Senescence stage 3 (30-50% yellowing)
3-Silver nitrate (AgNO3) 4-UV-B Radiation (UV-B)
5-Combined treatment As-A / AgNO3 6-Combined treatment As-A / UV-B

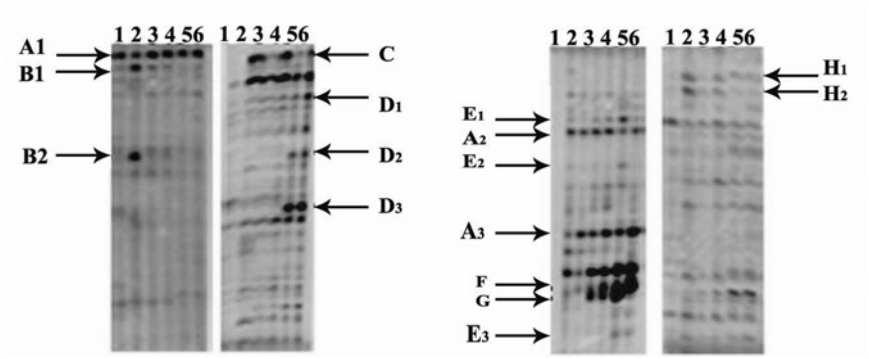

Figure 2. Selected bands on cDNA-AFLP gel. Letters indicate the bands that have been selected for cloninig Sample for each track explain as follow:

1-Ascorbic-Acid (As-A)
2-Senescence stage 3 (30-50% yellowing)
3-Silver nitrate (AgNO3)
4-UV-B Radiation (UV-B
5-Combined treatment As-A / AgNO3
6-Combined treatment As-A / UV-B

Table 1. Homologies of sequences of cDNA-AFLP fragments to sequences in the databases.

Number	cDNA-AFLP code	Accession number	Function	e-value
1	a1	At2g20290	Putative myosin heavy chain	$2e^{-141}$
2	a2	At2g03580	*Arabidopsis* root gene	e^{-83}
3	a3	Av541552	Unknown protein	$2e^{-101}$
4	b1	At5g15410	Nucleotide-gated chanel	$5e^{-81}$
5	b2	At5g17380	2-Hydroxyphytanoyl-COA lysase protein	$2e^{-38}$
6	c	At1g32640	Protein kinase (Putative bHLH transcription factor)	$462e^{-130}$
7	d1	At4g21810	Putative protein	$4e^{-101}$
8	d2	At1g01140	Serine theonine kinase	$6e^{-91}$
9	d3	At5g03190	Putative protein various	$9e^{-18}$
10	e1	At5g10470	TH65 protein	$3e^{-78}$
11	e2	At2g05530	Putative glycine rich protein	$2e^{-601}$
12	e3	At4g30890	Ubiqutin-specific protease	e^{-112}
13	f	Av53630	*Arabidopsis thaliana* gene	e^{-123}
14	g	At1g11230	Unknown protein	$2e^{-27}$
15	h1	At3g25180	Cytochrome P450	$9e^{-141}$
16	h2	At2g19760	Profilin1	$4e^{-107}$

Identification of novel genes expressed in senescence and in response to ROS

It was important to identify genes that might help to elucidate the events that are occurring when ascorbic acid protects the plant from the severe stress caused by the $AgNO_3$ and UV-B treatments. UV-B radiation is harmful to living organisms and numerous studies have demonstrated the detrimental effects of UV-B on plant growth, development and physiology (Teramora and Sullivan, 1994). In plants, UV-B exposure has been found to lead to increase in the generation of Reactive Oxygen Species (ROS) in particular, superoxide and hydrogen peroxide that have distinct roles in mediating gene expression (Dai et al., 1997). Silver nitrate has been used to induce heavy metal stress in plants (Wettlauffer et al., 1991) and excess concentrations of heavy metals have been shown to lead to increased levels of ROS and hence cause oxidative stress (Navabpour et al., 2003). Therefore, treatment of *Brassica napus* tissue, with silver nitrate solution or UV-B radiation is likely to cause an increase in ROS in the treated tissue. Ascorbate is an efficient quencher of a number of different ROS such as singlet oxygen, superoxide and hydrogen peroxide (Sturgeon et al., 1998). Using an optimised combination of standard protocols to identify, isolate, reamplify and clone individual bands, a number of transcripts related to $AgNO_3$, UV-B and combined treatments (As-A/$AgNO_3$ and As-A/UV-B) expression pattern were identified.

A number of transcripts which showed altered expression, after different chemical treatments were identified in this analysis. Sequence analysis of the 16 cDNA clones identified in the cDNA-AFLP experiment showed a range of possible functions. One gene (clone6) encodes a kinase, which, according to the database search, also contains a basic helix-loop-helix domain. Proteins with this domain are DNA-binding proteins which can act as a transcription factors. Also, the clone9 encodes a putative protein which may act as a transcription factor. These genes may have a regulatory role in controlling gene expression during stress responses and/or senescence. Two of the genes encode unknown proteins, and the other 12 encode various different enzymes (Table1).

Verification of band identity using cDNA-AFLP

One of the more difficult procedures in cDNA-AFLP is to verify that the band isolated and analysed further is the same as the one labelled and visualised in the original amplification. To examine the accuracy of the cDNA-AFLP process, virtual Northern analysis was carried out. The same cDNA that was prepared for the AFLP was digested with *Mse*1 and *MSP*1 adapters ligated. This was amplified and the products run on an agarose gel and blotted to nylon membrane. The amplified fragments of each gene were labelled with ^{32}P and hybridised to the membranes carrying the cDNA. The hybridisation experiments gave a similar expression pattern to that seen on the original AFLP gel (Figure 3). This showed that the correct gene had been cloned. Many of the cloned genes showed senescence enhanced expression and most of these also showed enhanced expression in response to the ROS treatments. Some genes showed a stronger expression in the tissues subjected to the combined treatments than the single treatments alone. These genes included clone9 encoding a putative protein, clone8 encoding serine thionine kinase and clone7 encoding a putative protein. The functions of these genes may be important to

protect the plant from the potential damage caused by the silver nitrate and UV-B irradiation.

To find out the expression pattern of the genes clone6 and clone9 in different organs in *Brassica napus*, the Northern hybridisation has been done. The results for clone6 encoding protein kinase (acting as a transcription factor) in different organs showed high expression in flower, pod and senescing leaves and no expression in green leaves and roots. The combined treatments had large effects on the expression of clone9 encoding a putative protein. The expression pattern for this gene showed high transcript levels in roots and flowers, low levels in early senescing leaves and no expression in pods, mature leaves and late senescing leaves (Figure 4).

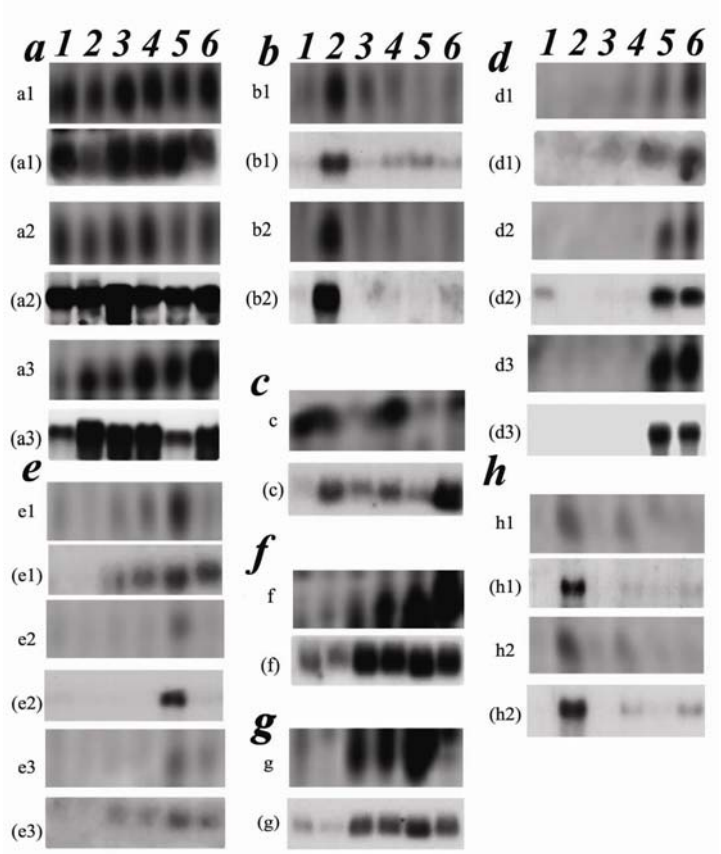

Figure 3. Comparison of gene expression pattern with cDNA-AFLP and virtual northen result (letter inside parentheses). sample for each track explain as follow:

1-Ascorbic-Acid (As-A) 2-Senescence stage 3 (30-50% yellowing)
3-Silver nitrate (AgNO3) 4-UV-B radiation (UV-B)
5-Combined treatment As-A / AgNO3 6-Combined treatment As-A / UV-B

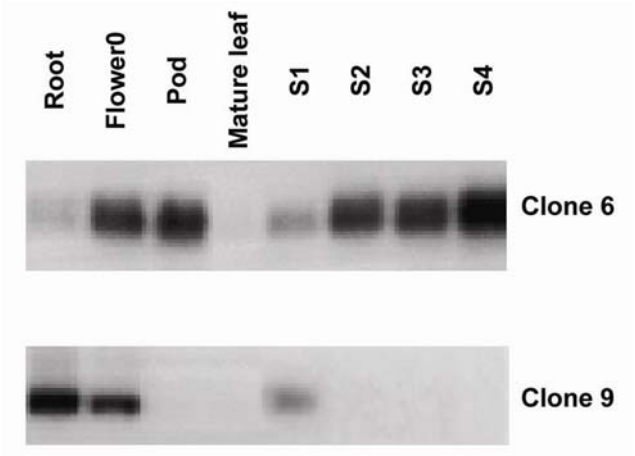

Figure 4. Northern hybridisation of clones no 6 and 9.RNA from root, flower, pod and leaves at different developmental stages was probed with the labeled insert from clones 6 and 9.
S1:10-25%, S2:25-40%, S3:40-55%, S4:55-70% yellowing. Ten micrograms of RNA was loaded in each track

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