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Dynamics of heat-shock induced DNA damage and repair in senescent tobacco plants

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Abstract

Oxidative stress plays an important role in plant ageing and in response to different stresses. Oxidative DNA damage, unless repaired, may have detrimental consequences and increase genetic instability. Therefore, we determined the role of heat-shock induced oxidative stress on induction and repair of DNA damage in relation to oxidative stress tolerance in senescent tobacco plants. One-month-old (young) and three-month-old (senescent) plants were exposed to 42 °C for 2 and 4 h and left to recover at 26 °C for 24 and 72 h. The progression of senescence was characterized by the lower soluble protein and malondialdehyde content compared to young plants. Immediately after the heat shock, an increase in lipid peroxidation and guaiacol peroxidase activity, as well as DNA damage measured by the Comet assay were induced to higher extent in the young plants than in the senescent ones compared to their respective controls. Moreover, after 24-h recovery, the DNA damage further increased in the young plants whereas tendency of DNA repair was observed in the senescent plants. Upon 72-h recovery, no significant differences were noticed in all parameters studied (regardless of plant age) compared to the controls. The random amplified polymorphic DNA (RAPD) analysis confirmed genetic stability of the tobacco plants during the heat-shock exposures as well as the subsequent recovery periods.

Additional key words: Comet assay, DNA polymorphism, oxidative stress, RAPD, temperature stress.

Introduction

Climate change in the last years resulted in global surface temperature increase of 0.2 °C per decade (Hansen *et al.* 2006). Most plants suffer after exposure to the temperature higher or lower than their optimum. A threshold temperature, above which detectable reduction in growth and changes in metabolic activities begin, varies for different plant species and genotypes within species (Wahid *et al.* 2007). Generally, rise in temperature of 10 - 15 °C above the ambient is considered as the heat shock.

Many different abiotic stresses, high temperature included, are known to disrupt metabolic balance of the cell by increasing content of reactive oxygen species (ROS). ROS (singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical) are highly reactive and can alter cellular homeostasis by causing protein denaturation, lipid peroxidation, and DNA lesion induction (Dat *et al.* 2000, Cadet *et al.* 2010). Since leaf senescence in many plant species is accompanied by an increased production of free radicals too, the process of leaf ageing itself is likely to be associated with plant stress response (Abarca *et al.* 2001).

Plants usually respond to oxidative stress by triggering the antioxidant defense mechanisms. Among the antioxidant enzymes, the induction of specific peroxidase isoenzymes has been recognized as a biomarker of various environmental stresses (Yoshida 2003). Moreover, peroxidases in plants have been

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Abbreviations: BSA - bovine serum albumin; EMS - ethyl methanesulfonate; MDA - malondialdehyde; GPX - guaiacol peroxidase; LMP - low melting point; NMP - normal melting point; PAGE - polyacrylamide gel electrophoresis; PCR - polymerase chain reaction; PVP - polyvinylpyrrolidone; RAPD - random amplified polymorphic DNA; ROS - reactive oxygen species; TAE buffer - Tris-acetate + EDTA buffer; TBA - 2-thiobarbituric acid; TBARS - 2-thiobarbituric acid reactive metabolites; TCA - trichloroacetic acid; tDNA - tail DNA.
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suggested to be involved in defense mechanism against high as well as low temperatures (Chaitanya *et al.* 2002, Gülen *et al.* 2008).

Abiotic stress does not only involve changes in plant physiology but also wide genomic changes (Madlung and Comai 2004), therefore here we focused on genomic, rather than physiological responses to stressful conditions. To detect genetic stability of temperature stressed plants, two frequently used techniques have been applied. The Comet assay allows the evaluation of DNA integrity at the resolution of a single cell without any need for cell progression into mitosis. The random amplified polymorphic DNA (RAPD) has been successfully applied to certify the genetic stability with additional advantage of possible application to different species without prior knowledge on nucleotide sequence.

Despite a lot of information dealing with heat stress in plants, the information of possible DNA damage and repair upon high temperature exposure is scarce. Therefore the aim of this study was to establish cross-talk between the possible genotoxic effect of temperature-induced oxidative stress in leaves of *Nicotiana tabacum* and the process of plant ageing, *i.e.*, senescence itself.

Materials and methods

Plants and stresses: Seeds of *Nicotiana tabacum* L. var. *xanthi* were kindly provided by Dr. T. Gichner, Institute of Experimental Botany, Prague, Czech Republic. Plants were clonally propagated from nodal explant in 350 dm^3 Erlenmeyer flasks for one or three months. Cultivation period of three months was shown to be the maximum period of time for *in vitro* plant growth after which the depletion of culture media would lead to death of tobacco plants. Growth conditions as well as culture media were described elsewhere (Gichner *et al.* 1999). In all experiments, the 5th leaf was used.

One-month-old (young) and 3-month-old (senescent) plants were incubated in a waterbath at 42 °C for 2 or 4 h and analyzed immediately as well as after 24 h and 72 h of recovery time. Control plants and those designated for recovery were kept in a plant growth chamber under a 16 h-photoperiod, irradiance of 90 μmol m^{-2} s^{-1}, and temperature of 26 ± 2 °C.

Lipid peroxidation was determined according to the modified method of Heath and Packer (1968). Fresh leaves (50 mg) were homogenized in 1 cm^3 of 0.25 % (m/v) 2-thiobarbituric acid (TBA) made in 10 % (m/v) trichloroacetic acid (TCA). After heating at 95 °C for 30 min, the samples were cooled in an ice bath and centrifuged at 10 000 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm using UV/VIS spectrophotometer *UV4-200* (*ATI Unicam*, Cambridge, UK), and readings were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The content of lipid peroxides was expressed as total 2-thiobarbituric acid reactive metabolites (TBARS), mainly malon-dialdehyde (MDA), using a coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Soluble protein content and guaiacol peroxidase assay: Fresh leaf tissue (500 mg) was homogenized by a mortar and pestle in 1.5 cm^3 of 50 mM potassium phosphate buffer (pH 7.0). The insoluble polyvinyl-pyrrolidone (PVP; 50 mg) was added to the tissue samples prior to grinding. The homogenates were centrifuged at 20 000 *g* and 4 °C for 15 min. The supernatant was collected and centrifuged again at 20 000 *g* and 4 °C for 60 min. The supernatant was collected again and protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

Guaiacol peroxidase activity (GPX, EC 1.11.1.7) was measured at 25 °C by monitoring the increase in absorbance at 470 nm due to the formation of tetra-guaiacol (molar absorbance coefficient of 26.6 mM^{-1} cm^{-1}). The test solution was prepared after Chance and Maehly (1955) and contained 50 mM potassium phosphate (pH 7.0), 18 mM guaiacol, 5 mM H_2O_2, and the extract (0.05 cm^3) in a total volume of 1 cm^3. In the control solution, the crude extract was replaced by the extraction buffer.

To separate GPX isoenzymes, the tissue extracts were analyzed electrophoretically in native conditions using 10 % (m/v) polyacrylamide vertical slab gels with the buffer system of Laemmli (1970). For GPX detection, the gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) for 30 min, then incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM guaiacol and 4 mM H_2O_2 until brown bands appeared (Chance and Maehly 1955).

The Comet assay: The procedure was carried out according to Gichner *et al.* (1999). In brief, leaf nuclei were mechanically isolated in 400 mM Tris-HCl (pH 7.5) at 4 °C, mixed with equal amounts (0.5 cm^3) of low melting point agarose (LMP, *Sigma*; 1 %, m/v, in PBS), preheated at 42 °C, spread onto partially frosted microscope slides precoated with 1 % normal melting point (NMP) agarose in distilled water, covered with a cover slip and left on ice for solidification of agarose. Prior to electrophoresis, the cover slip was removed and the slides were placed in a horizontal electrophoresis tank filled with freshly prepared chilled electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13) for 10 min to unwind DNA. Following denaturation, electrophoresis was done in the same buffer at 0.8 V cm^{-1} and 300 mA for 20 min. After the electrophoresis, the slides were gently rinsed three times with neutralization buffer (400 mM Tris-HCl buffer, pH 7.5) for 5 min and finally briefly dipped in distilled water.

For analysis, the slides were stained with ethidium

bromide (20 μg cm^{-3}) for 5 min. For each slide, 150 randomly chosen nuclei per each treatment were analyzed using a fluorescent microscope (*Zeiss Axioplane*, Jana, Germany) equipped with an excitation filter *BP* 520/09 nm and a barrier filter of 610 nm. A computerized image analysis system (*Komet version 5*, *Kinetic Imaging*, Liverpool, UK) was employed to measure the percentage of tail DNA (% tDNA) as the primary measure of DNA damage.

DNA isolation and RAPD procedure: DNA extraction was performed by grinding fresh leaf tissue (0.12 g) in liquid nitrogen and subsequently by homogenizing with 1.3 cm^3 of extraction buffer (100 mM Tris-HCl, 700 mM NaCl, and 50 mM EDTA, pH 8.0). Following incubation at 65 °C for 15 min with occasional inversion of the reaction tubes, the samples were cooled down to room temperature. In the next step, 0.65 cm^3 of chloroform: isoamylalcohol (24:1, v/v) mixture was added and the tubes were gently shaken for 5 min. After centrifugation at 16 000 *g*, the aqueous supernatant was collected and digested with RNAase A at 37 °C for 10 min. The DNA was precipitated by ice-cold isopropanol for 10 min and centrifuged at 16 000 *g* for 10 min. The DNA pellet was washed with ethanol, dried, and dissolved in distilled water. The integrity of extracted genomic DNA was checked by electrophoresis in 0.7 % (m/v) agarose gel using the λ-phage DNA as a molecular mass marker. The DNA content was determined spectrophotometrically and by comparison with known concentration of λ-phage DNA. The purity of the DNA isolate showed a 260/280 nm ratio of ~1.8 which indicates DNA of good quality.

The conditions of RAPD were set according to Atienzar *et al.* (2002). Briefly, the reaction volume of 0.025 cm^3 included 2 μM 10-mer primers, 0.33 mM dNTPs, 5.11 mM $MgCl_2$, 5 or 20 ng of genomic DNA, 2.8 units of *Thermus aquaticus* (Taq) DNA polymerase (*Fermentas*, Vilnius, Lithuania), 1× reaction buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08 % (v/v) *Nonidet P40*). Thermal cycling parameters consisted of a first cycle at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 74 °C for 1 min. A final extension at 74 °C for 10 min was performed. Primers used in this study were CG-rich decamer oligonucleotides obtained from *Sigma* (Steinheim, Germany). Out of 40 initially screened, 6 primers were chosen for the rest of the experiments. Their sequences are as follows: OPA7 (GAAACGGGTG), OPA8 (GTGACGTAGG), OPA14 (TCTGTGCTGG), OPA15 (TTCCGAACCC), OPA16 (AGCCAGCGAA), and OPA17 (GACCGCTTGT). Amplification products were resolved on 2 % (m/v) agarose gels in 1 % (m/v) TAE buffer and stained with 0.2 μg cm^{-3} ethidium bromide for not less than 1 h. The size of PCR product was estimated by comparison with *DirectStep* DNA ladder (*Sigma*). The image of each gel was captured using a *Kodak DC 40* digital camera (*Eastman Kodak*, New York, NY, USA) and DNA profiles were analyzed using *Kodak Digital Science 1D* image analysis software. All PCR reactions were done in duplicate for each of the primers used and each included a negative control reaction with no DNA template. To test the sensitivity of RAPD assay in detection of DNA damage, plants used for positive control were incubated at 52 and 54 °C for 1 h and immediately used for analysis. A mutagen ethyl methanesulfonate (EMS) was also used for induction of positive control. Fully developed leaves were detached from young plants and immersed for 24 h in water solution containing 10 and 20 mM EMS, and then used for further analysis.

Statistical analysis: Data were compared by analysis of variance (*ANOVA*) using *Statistica v.7.1* (*StatSoft*, Tulsa, OK, USA) software package and differences between corresponding controls and exposure treatments were considered as statistically significant at $P < 0.05$. Each data point is the average of three replicates obtained from two different experiments.

Results

Lipid peroxidation measured as MDA content increased with the heat shock duration in both the young and senescent plants (Fig. 1*A*). Increased MDA content at 42 °C was recorded in both the groups of the plants already after 2 h, whereas the highest content (3.7 times higher in the young and 2.9 times higher in the senescent plants compared to their respective controls) after 4 h. After 24 h of recovery, the MDA content showed the tendency of decrease whereas after 72 h, the MDA content was similar to the control level in both the groups of plants.

In the senescent leaves, there was no significant change in total protein content during the whole experiment (Fig. 1*B*). On the contrary, in the young leaves, the total protein content significantly increased after exposure to 42 °C lasting 2 or 4 h as well as after the 24-h recovery period. However, after the 72-h recovery, no significant difference was observed compared to the control. The young plants had the higher basal protein content compared to the senescent ones.

The senescent plants had approximately 4 times higher GPX activity than the young plants. The highest increase in the total GPX activity in the young plants was observed in the 4 h-treated samples after the 24-h recovery. The senescent plants also showed the highest GPX activity after the 24-h recovery of the 2 h treated samples. After the 72-h recovery, the GPX activity in both the plant types was similar to the control (Fig. 1*C*).

The pattern of specific isoenzymes was visualized on gels after equal amounts of isolated proteins were loaded onto a native PAGE gel (Fig. 2). The staining GPX isoenzymes revealed 11 isoforms in total. In the young

control leaves, only bands GPX1, 5, 7, and 9 were present, whereas in the senescent control leaves, all eleven isoforms were detected. Substantial difference in the GPX pattern was observed among the young plants where additional bands appeared with the prolonged heat treatment in comparison to the control; in the 2 h treated plants, appearance of isoforms GPX6, 8, 10, and 11 was noticed, whereas in the 4 h treated plants, additional GPX2 and 3 bands appeared. Isoforms GPX5-11 had a stronger staining intensity in the treated senescent leaves than in the young ones exposed to the heat shock which is in agreement with spectrophotometrically measured

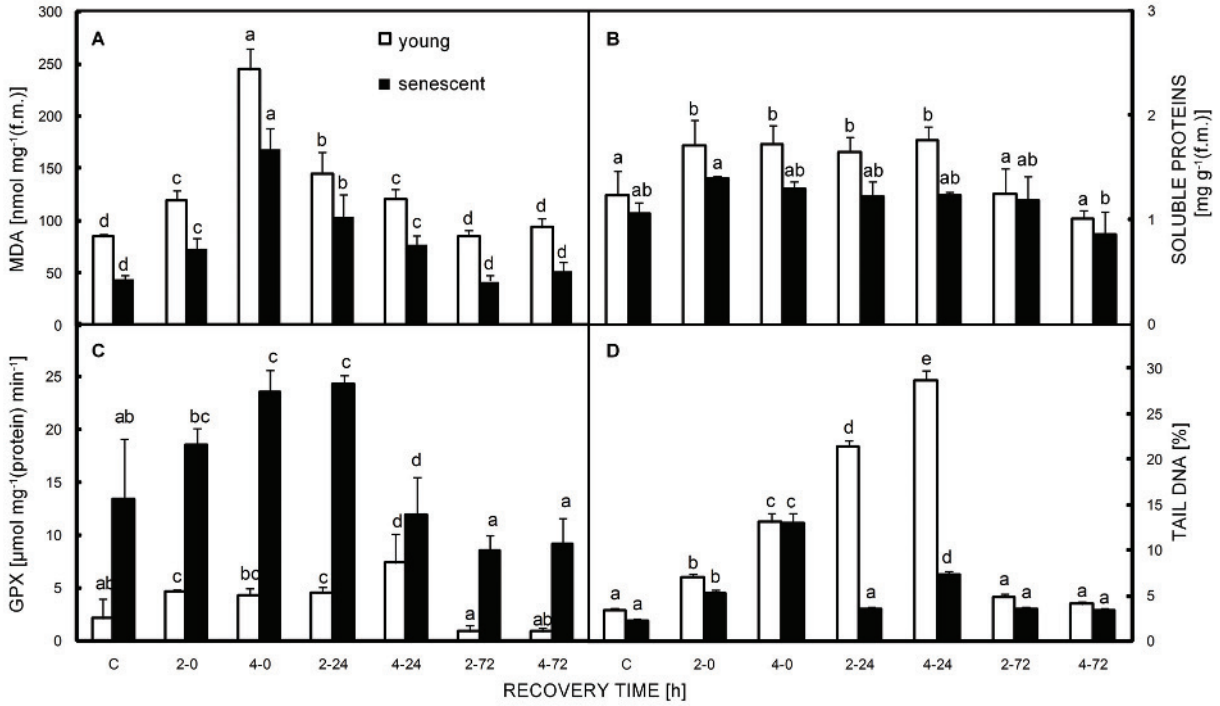

Fig. 1. The MDA content (*A*), total soluble protein content (*B*), guaiacol peroxidase activity (GPX; *C*), DNA damage as percentage of tail DNA (*D*) in *N. tabacum* young and senescent plants under control conditions (C), immediately after heat-shock treatment period of 2 h (2-0) or 4 h (4-0), after 24-h recovery of 2 h (2-24) and 4 h (4-24) treated plants, and after 72-h recovery of 2 h (2-72) and 4 h (4-72) treated plants. Means ± SE, $n = 6$. *Bars* with different letters are significantly different at $P < 0.05$.

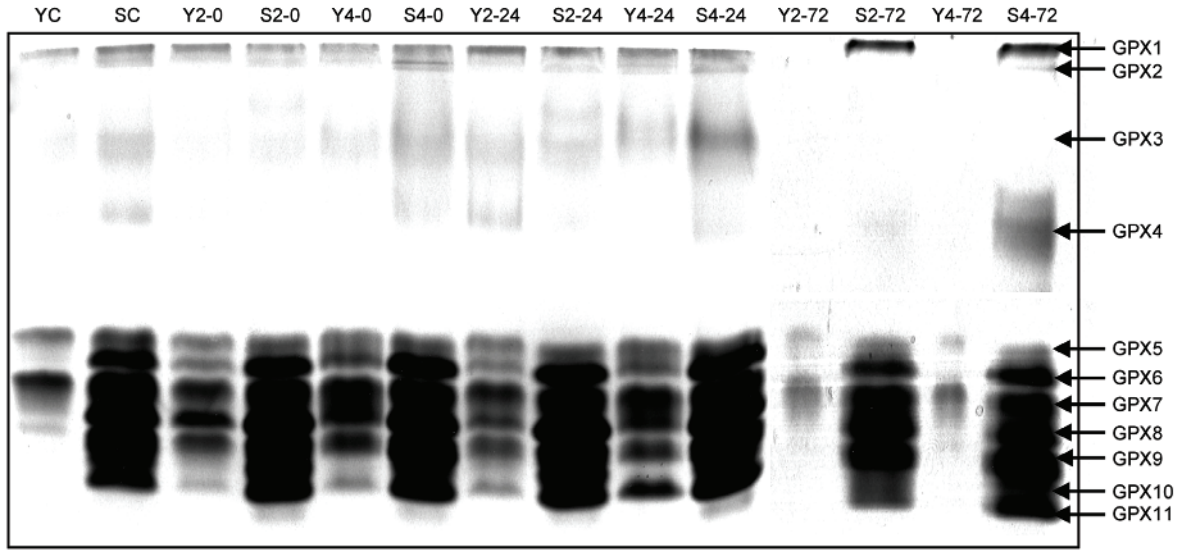

Fig. 2. The electrophoretic pattern of GPX isoenzymes in young (Y) and senescent (S) *N. tabacum* plants under control conditions (C), immediately after the heat-shock treatment period of 2 h (2-0) or 4 h (4-0), after 24-h recovery period of 2 h (2-24) and 4 h (4-24) treated plants, and after 72-h recovery of 2 h (2-72) and 4 h (4-72) treated plants.

Table 1. The number of bands in the control treatment (C) and molecular sizes [bp] of appearing (+) and disappearing (–) bands for different primers in leaf tissues of the tobacco plants exposed to EMS and the heat-shock treatment (n = 6; ND - no change in RAPD profiles compared to the control).

Primer	C		EMS 10 mM	20 mM	Temperature 52 °C	54 °C
OPA 7	19	(+)	922, 747, 274	602, 273	580, 569	ND
		(-)	2378, 1965, 1762, 592, 588	2378, 1965, 1762, 592, 588	2254, 2197, 1793, 1404, 821, 590, 563,527	2254, 2197,1793, 1404, 821, 767, 563, 527, 482
OPA 8	16	(+)	ND	ND	ND	510
		(-)	2487, 1419, 1000, 393	2487, 1419, 1157, 1337, 1000, 762	1052	1534, 1477, 1409, 1218, 1052, 744, 469
OPA 14	15	(+)	408	406	ND	ND
		(-)	1913	1913, 1826, 1488, 1366, 622, 447, 300	1938, 1560, 590, 434, 298	1938, 1855, 1560, 1446, 877, 590, 434, 298
OPA 15	22	(+)	ND	ND	ND	ND
		(-)	2527, 2041, 1756, 1506, 1407, 384	2527, 2041, 1756	2000, 1554, 1472	2000, 1772, 1554, 1472
OPA 16	21	(+)	833, 326	827	ND	ND
		(-)	2743, 2284, 1767, 1552, 1454	3622, 2743, 2284, 1767, 1552, 1454, 1262, 747	3606, 2732, 2268, 1782, 1606, 1513, 1347, 1119, 742, 596	3606, 2732, 2268, 1891, 1782, 1606, 1513, 1347, 1119, 1057, 800, 596
OPA 17	18	(+)	ND	ND	ND	ND
		(-)	ND	ND	477, 422	1430, 496, 422
Total	111		6 (+); 21 (-)	4 (+); 29 (-)	2 (+); 29 (-)	1 (+); 43 (-)

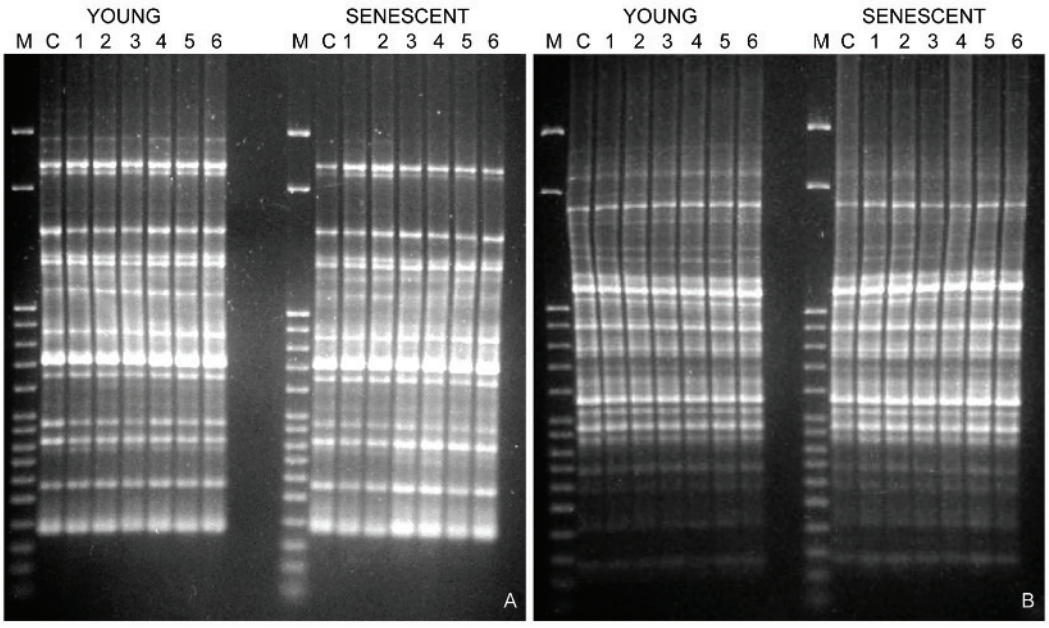

Fig. 3. The RAPD pattern of *N. tabacum* young and senescent plants (n = 6) generated using primers OPA 8 (*A*) and OPA 16 (*B*) under control conditions (C), immediately after the heat-shock treatment for 2 h (*lane* 1) or 4 h (*lane* 2), the 24-h recovery period of 2 h (*lane* 3), and 4 h (*lane* 4) treated plants, as well as the 72-h recovery period of 2 h (*lane* 5) and 4 h (*lane* 6) treated plants. M - DNA molecular size markers (3000, 2000, 1000, 900, 800, 700, 600, 500, 450, 400, 350, 300, 250, 200, 150, 100, and 50 bp, from top to bottom).

peroxidase activities. Following the recovery period, the GPX activity was slightly lower in the young plants than in the old ones. The GPX pattern of the 4 h treated young leaves after the 24-h recovery was devoid of isoform GPX4, whereas the isoenzyme GPX10 had a strong staining intensity (Fig. 2). After the 72-h recovery, the

peroxidase pattern of both 2 h as well as 4 h treated young plants was similar to the control pattern. On the other hand, in the senescent plants, a GPX3 isoform was missing after 72-h recovery in both the 2 h and 4 h treated plants whereas the 2 h treated senescent plant were also devoid of a GPX4 isoform (Fig. 2).

The Comet analysis shows significant increase in percentage of tDNA after the 2-h and 4-h exposure of both the young and senescent plants to 42 °C in comparison to the control plants (Fig. 1*D*). After a recovery period of 24 h, DNA damage was repaired only in the heat-treated senescent plants. Moreover, in the 2 h treated senescent plants, DNA damage was repaired completely as there was no significant difference in percentage of tDNA compared to the control plants. On the contrary, in the young plants regardless of the temperature treatment a further increase of DNA damage was noticed. After the 72-h recovery period, complete repair was observed regardless of the age of the treated plants.

In this study, we used the RAPD analysis in the temperature-stressed young and senescent tobacco plants. Since this method uses randomly primed sequences which do not necessarily coincide with the DNA loci sensitive to DNA damage, selection of primers has been done based on a polymorphism detected in plants after a heat shock of 52 and 54 °C for 1 h and in EMS treated plants (Table 1). Using 6 different primers, a total of 111 bands were obtained in the control. The molecular masses of amplified bands ranged from 500 to 2500 bp depending on the primer used (Table 1). Majority of modifications for different primers compared to the control was disappeared bands of molecular masses greater than 1000 kb.

On the contrary, the selection of the same primers used for experimental purpose did not reveal any polymorphism either in the young or in the senescent plants assessed immediately after the exposure to 42 °C for 2 or 4 h and subsequent 72-h recovery. Moreover, there was no difference in the band patterns due to senescence itself. All the studied plants, both the young and the senescent, displayed the same band pattern as the control (Fig. 3).

Discussion

High temperature is stressful and leads to altered gene expression and eventually to cell death (Chen *et al.* 1999, Luo *et al*. 2013). The molecular events involved in temperature stress response are poorly defined. Research is mainly focused on long-term acclimatization and acquiring thermotolerance but far less on the molecular events involving genomic stability after a heat shock. Therefore, the purpose of this study was to investigate the influence of a short-term heat exposure on induction of DNA damage and repair with the respect to the senescence-associated changes in tobacco plants.

Leaf senescence is an oxidative process induced in an age-dependent manner whose regulation is genetically controlled but also induced by various environmental stimuli (Prochazkova *et al.* 2001, Yoshida 2003). It has been reported that decline in antioxidative enzyme activities and increased production of ROS, especially hydrogen peroxide, mediate plant ageing (Ma *et al.* 2008) as well as heat stress response (Almeselmani *et al.* 2006).

Lipid peroxidation, as indicated by MDA content, has already been used as a measure of the extent of oxidative damage after heat stress in orchid and lily (Ali *et al.* 2005, Yin *et al.* 2008). In our case, although the young plants had two times higher basal MDA content in comparison to the senescent plants, they both responded in a similar way to the heat stress as a function of exposure time. The greater extent of oxidative damage indicated that the young plants were more sensitive to the heat stress than the senescent ones, but upon the 72-h recovery period, both plant types recovered from oxidative stress without senescence progression. Therefore, even if senescence is a process leading to programmed cell death (Abarca *et al.* 2001), senescing leaves are capable to withstand heat stress and even develop resistance against other stresses (Behera *et al.* 2003).

Since the process of senescence includes hydrolysis of all relevant biological macromolecules (Lim *et al.* 2007), the reduction in the basal protein content in the senescent tobacco leaves compared to the young ones was in agreement with other studies (Abarca *et al.* 2001, Ohe *et al.* 2005). Further, during the heat shock and recovery, the soluble protein content in the senescent plants was similar to the control but increasing protein content was noticed immediately after the heat treatment as well as after the 24-h recovery period in the young plants. Upregulation as well as *de novo* protein synthesis are known to be associated with a plant response to various stresses (Timperio *et al.* 2008). In the case of high temperature stress, protein denaturation and inhibition of constitutive protein synthesis (Gülen and Eris 2004) as well as increased synthesis of heat shock proteins (Park and Hong 2002, Tao *et al.* 2012) are prevailing. The long half-life of these protective proteins after an induced heat treatment plays an important role in repair and prevention of cellular damage (Derocher *et al.* 1991), therefore the significant increase in the soluble protein content in the younger plants might also be due to induction of heat shock proteins.

In this study, the GPX activity was used as a general, nonspecific indicator of stressful conditions in the plants. The GPX activity increased significantly in response to the heat shock in the young plants and to a lesser extent in the senescent plants suggesting its possible role in their tolerance and reestablishment of cellular homeostasis during the 72-h recovery period. This is in agreement with other studies reporting increased GPX activities in response to heavy metals, high irradiance, or heat stress

(Gechev *et al.* 2003, Gorinova *et al.* 2007, Gong *et al.* 2012). The role of GPX in scavenging ROS during high temperature was suggested in many studies (Chaitanya *et al.* 2002, Yin *et al.* 2008, Asthir *et al.* 2012). Thus, a constitutively higher baseline activity of GPX might contribute to the less oxidative stress in the senescent plants. Moreover, the presence of multiple GPX isoforms in the senescent leaves compared to the young ones throughout the whole experiment might contribute to age-related differences in plant tolerance during and after the heat treatment. Consistent with all parameters measured in our study, the tobacco plants of both the ages showed to endure and recover completely from heat treatment after the 72-h recovery period.

DNA damage as biomarker for genotoxicity has been detected in the both young and senescent plants exposed to high temperature. Although DNA damage has occurred to a greater extent in the young plants compared to the senescent ones, both groups displayed similar pattern of induced damage as a function of exposure time. Since oxidative damage was confirmed by enhanced MDA content immediately after heat treatment, we assume that oxidative DNA damage resulted in a significant DNA migration. This is well in agreement with other studies that applied Comet assay reveals oxidative or heat-induced DNA damage in tobacco plants (Gichner *et al.* 2006, Mancini *et al.* 2006).

On the other hand, after 24 h recovery period, there was still a significant increase in % tDNA for both the 2 h and 4 h treated young plants, whereas decrease in % tDNA was observed in the senescent ones. After 72-h recovery period, in the plants of both ages, DNA repair pathways were activated, therefore, no DNA damage was observed. The fact that non-replicating tobacco leaf cells possess fully functional DNA repair enzymes is already known from study of Gichner *et al.* (2000). Moreover, oxidative damage regarding DNA includes both altered bases and damaged sugar residues (Roldán-Arjona and Ariza 2009, Cadet *et al*. 2010), which are usually repaired by both nucleotide and base excision-repair processes (Tuteja *et al*. 2009). Therefore incision breaks that occur during excision repair and apurinic/apyrimidinic sites, known as AP sites, which include baseless sugar, due to alkali condition of the Comet assay are also resolved as DNA strand breaks. This suggests that accumulation of DNA strand breaks as repair intermediates may be responsible for apparent increase in % tDNA during 24-h recovery in the young plants.

Further, as the Comet assay is not indicative of the quality of the DNA repair (Rousset *et al.* 2000) and detects only subset of DNA lesion, we have conducted the RAPD analysis to examine stabile changes in DNA template which include mutations or structural DNA distortion that may affect the annealing of the primers or interfere with the Taq polymerase leading to alteration in the amplified band pattern (Atienzar and Jha 2006). In a preliminary study we have tested 40 different primers for assessing DNA polymorphism in young plants, both control ones and those recovered for 24 h from 4-h heat treatment (data not shown) because in this treatment the Comet assay displayed the highest DNA damage. As a result no changes in DNA polymorphism were observed (data not shown). To eliminate the fact that the choice of primers causes some limitation in DNA amplification in a sense that they do not anneal to the part of genome potentially affected by the temperature treatment, we have tested the susceptibility of tobacco plant genome to be damage-prone by using two types of positive controls: EMS, known alkylating agent, that does not involve oxidative DNA damage (Šrut *et al.* 2013), and high temperature treatment (52 and 54 °C), which involves oxidative stress that can generate necrotic DNA damage (Gichner *et al.* 2000.). Out of 40, only 6 of them showed decreased genetic stability in dose-specific manner, except for primer A 17 in EMS treatment. Consequently, the same 6 primers were further used to monitor induction and repair of DNA damage in the both young and senescent plants during and after the heat shock treatment, but no DNA polymorphism was detected. Moreover, banding pattern in the both young and senescent plants immediately after heat shock treatment as well as during 72 h recovery period was the same as in the control plants. Therefore, it is possible that the uniformity in banding pattern between the control and the treated samples might have involved efficient DNA repair so that induced DNA lesions were successfully repaired. These findings are well in agreement with the results of biochemical parameters measured in this study, which have also suggested the plant potential to recover from the heat shock induced oxidative stress.

We have also taken into account some criticism in terms of the reproducibility of RAPD results due to the presence of intrapopulation DNA polymorphisms common to *in vivo* systems. Therefore, in our study plants were clonally propagated in order to ensure genetically homogenous population (Martins *et al.* 2004, Peredo *et al.* 2009). As the results, the uniformity of RAPD banding patterns among young and senescent control tobacco plants has shown that culture conditions as well as micropropagation procedures derived regenerated plantlets devoid of genetic variability during the process of senescence.

The stress response in tobacco plants of different ages, exposed to heat-shock at 42 °C and subsequently left to recover during 72 h, has shown that DNA damage occurred to a greater extent in the young plants compared to the senescent ones as evident from lipid peroxidation indicating oxidative damage. Although both plants displayed similar patterns of stress recovery, the senescent leaves were apparently better equipped with DNA repair function, because DNA damage was partially repaired after 24 h of recovery period. Since DNA was completely repaired after 72 h, and RAPD analysis confirmed DNA integrity, the present findings confirmed that tobacco plants regardless of their ages were able to counter heat stress in preventing genotoxicity of heat shock exposure.

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