Antioxidant enzymes induction in pea plants under high irradiance

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Abbreviations: AOS - Activated oxygen species; APX - ascorbate peroxidase; ASC-GSH cycle - ascorbate-glutathione cycle; CAT – catalase; CuZn-SOD – copper,zinc-containing superoxide dismutase; DHAR – dehydroascorbate reductase; HI – high irradiance; GR - glutathione reductase; GPX - glutathione peroxidase; H_2O_2 - hydrogen peroxide; MDHAR - monodehydroascorbate reductase; SOD - superoxide dismutase; $O_2^{\cdot-}$ - superoxide radicals; PHGPX – phospholipid hydroperoxide glutathione peroxidase

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Abstract

Exposure of pea plants to high irradiance (HI) for 60 min caused a reversible photo inhibition of photosynthesis as shown by changes in variable to maximum fluorescence ratio (F_v/F_m). A significant decline in F_m was observed in leaves from both pea cultivars subjected to HI, the decrease being higher in JI281 than in JI399 plants. The values recovered during the post-stress period in both cultivars. In both cultivars, under HI increased F_0, but in cultivar JI399 initial values were recovered during the post-stress period.

The expression of antioxidant enzyme genes was higher in JI399 than in JI281, both in control and stressed plants. In JI281, after 60 min of HI, an induction of the transcripts of *CAT, chlMDHAR, cytAPX* and *cytCu,Zn-SOD* was observed, whereas there were a slight increase in *PHGPX, stAPX* and *chlCu,Zn-SOD* mRNAs. After 24 h of the recovery period, the induction of some transcripts was not maintained (*CAT, cytAPX* and *cytCu,Zn-SOD*), whereas the induction of others was maintained (*PHGPX* and *chlCu,ZnSOD*) or even increased (*cytGR, stAPX* and *chlMDHAR*).

In JI399, *CAT* and *cytAPX* were increased strongly after 60 min of HI, and slight increases were observed in *cytGR, chlGR and chlMDHAR*. In the poststress period the expression of *stAPX*, *cytGR* and *chlMDHAR* was even slightly higher than after 60 min of HI, however, expression of *CAT*, *cytAPX*, *cytCu,ZnSOD*, *chlCu,ZnSOD* and *chlGR* was decreased.

Additional keywords: antioxidants; gene expression; photoxidative stress; *Pisum sativum*

Introduction

High irradiance (HI) exposure is one of the most common causes of oxidative stress in plants (Dat *et al*. 2000). Under excess of irradiation, enzymatic processes for CO_2 fixation become rate-limiting and, as a result, photosynthesis produces more NADPH and ATP than necessary. This accumulation of redox and energy equivalents will decrease the plastoquinone pool and/or inhibit the water-splitting complex, inevitably leading to PS 2 inactivation, the so-called photoinhibition (Anderson *et al*. 1997; Karpinski *et al*. 1997, 1999).

There are reports on the changes in activity and expression of antioxidant enzymes in response to HI stress (Gillham and Dodge 1987; Foyer *et al.* 1989; Karpinski *et al.* 1997; Yoshimura *et al.*, 2000; Hernández *et al*. 2004) but the results vary according to plant materials and treatment conditions employed.

The protection of the plants against AOS produced in excess during adverse environmental conditions is achieved by means of different strategies and, in particular, by partial suppression of its production and scavenging of the AOS already produced (Murgia *et al*. 2004). During HI stress, free radicals are formed in PS 2, which are harmful to the photosynthetic apparatus (Aro *et al*. 1993). Partial suppression of AOS production in the chloroplasts during HI stress is achieved by degradation of the D1 protein of PS 2 leading to its inactivation (Anderson *et al*. 1997; Karpinski *et al*. 1997, 1999).

Plants contain a complex antioxidant system to detoxify AOS that includes carotenoids, ascorbate, glutathione, tocopherols, anthocyanin pigments and enzymes such as superoxide dismutase, catalase, glutathione peroxidase, peroxidases and the enzymes involved in the ascorbate-glutathione cycle (Foyer and Halliwell 1976): ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase.

In this work, we investigate the effect of EL on PS 2 efficiency in pea leaves, as well as on the levels of mRNAs encoding crucial enzymes of AOS metabolism.

Materials and methods

Plants, growth and treatments: *Pisum sativum*, cvs. JI281 and JI399, seedlings, individually planted in pots, were grown in a controlled-environment growth chamber (18-h photoperiod, irradiance of 200 ± 25 μmol (photon) m^{-2} s^{-1}, temperature of 15 ± 1.5 ºC and relative humidity of 75 ± 5%) (Ellis et al., 1992). JI281 is a semi-domesticated land race of pea from Ethiopia whereas JI399 is a typical domesticated garden pea cultivar (Ellis *et al.* 1992).

Experiments were performed with 21-day-old pea plants exposed to HI exposure for up to 60 min (4000 μmol m^{-2} s^{-1}). To eliminate heat effects and to disperse light evenly, light was reflected by a mirror and directed through a frosted-glass filter filled

with cold water. Control plants were exposed to 200 ± 25 μmol m^{-2} s^{-1}. After this period, some light-stressed plants were returned for 24 h to normal growth conditions (re-exposed to 200 ± 25 μmol of photons m^{-2} sec^{-1}) before sampling (poststress period). All leaves were frozen in liquid N_2 and stored at -70ºC until use.

Measurement of chlorophyll fluorescence: The photosystem 2 efficiency parameter F_v/F_m (the ratio of variable to maximal fluorescence reference), F_0 (minimal chlorophyll fluorescence) and F_m (maximal chlorophyll fluorescence) were measured using a portable Plant Efficiency Analyser (PEA) machine (Hansatech Instrument, King`s Lynn, Norfolk, UK), on 10 min dark-adapted leaves at 90 % light saturation, according to the manufacturers' instructions.

Northen blotting: Total RNA from leaves was extracted as previously described (Creissen and Mullineaux, 1995). Poly A^+-mRNA was purified by chromatography, using oligo d(T) cellulose spin columns (Amersham Biosciences, UK) according to the manufacturer's instructions. Poly A^+-mRNA (3-5 μg) was denatured, separated electrophoretically and transferred onto a nitrocellulose membrane as described in Hernández *et al* (2000). The loading of an equivalent amount of Poly A^+-mRNA for each time point was checked on gels stained with ethidium bromide and they showed equal intensities (data not shown). Northern blots were visualised by autoradiography after hybridisation with ^{32}P-labelled DNA probes. Hybridisation was carried out in 0.3 M sodium phosphate buffer pH 7.2, containing 1 mM EDTA, 7% SDS and 1% BSA, at 65ºC (homologous probes) or 55ºC (heterologous probes). Washing was with 0.1 X SSC (65ºC) for homologous probes and with1 X SSC or 2 X SSC for heterologous probes. Northern blots were performed twice and representative blots are shown.

The specific probes used were: cytosolic and chloroplastic Cu,Zn-SOD from *Nicotiana plumbaginifolia* (Bowler *et al*. 1989; Van Camp *et al*. 1990), cytosolic cytGR (*GOR 2*, Stevens *et al*. 1997) and chloroplastic GR (*GOR 1,* Creissen and Mullineaux 1995), catalase from tobacco (Willekens *et al*. 1994), *PHGPX* (Mullineaux *et al*. 1998), chlMDAR from Arabidopsis (Genbank accession number T04550), cytosolic APX from pea (Santos *et al*. 1996) and stromal APX from spinach (Ishikawa *et al*. 1995).

The filters were exposed to x-ray film and/or were visualised on a BAS 1000 Phosphoimager analyser (Fuji Photofilm Co., Kanagawa, Japan). Scanning values were calculated by BASIS software (Fuji Photofilm Co.) installed on the BAS 1000.

Statistics: Comparisons among means were made using Duncan's multiple range test, calculated at $P<0.05$. Statistical procedures were carried out with the software package SPSS 11.0 for Windows.

Results

Exposure of 3-week-old pea plants at HI (4000 μmol m^{-2} s^{-1}) for 60 min caused a significant photoinhibition in both cultivars as indicated by the decline in the PS 2 efficiency parameter F_v/F_m (Fig. 1A). After 24 h at 200 μmol m^{-2} s^{-1}, the efficiency of PS 2 had recovered as shown by the increase in F_v/F_m (Fig. 1A) to see values of the control plants. In both cultivars, HI caused an increase in F_0. In JI281, F_0 partially recovered after 24 h, but still was increased in relation to time zero. However, in JI399, F_0 values had reached control values after 24 h (Fig. 1B). A significant decline in F_m was observed in leaves from both cultivars subjected to HI, the decrease being greater in JI281 than in JI399 plants. F_m values recovered during the post-stress period in both cultivars.

The expression levels of antioxidant enzyme genes was higher in cultivar JI399 than in JI281, both in control and in stressed plants (Fig.1). In cultivar JI281, after 1 h of HI, an induction of the transcripts of *CAT, chlMDHAR, cytAPX* and *cytCu,Zn-SOD* was observed, whereas there were a slight increases in *PHGPX, stAPX* and *chlCu,Zn-SOD* mRNAs. After 24 h of the recovery, some transcripts were not maintained (*CAT, cytAPX* and *cytCuZn-SOD*), whereas others were maintained (*PHGPX* and *chlCu,ZnSOD*) or even increased (*cytGR, stAPX* and *chlMDHAR*) (Fig.1, Table 1).

In cultivar JI399, *CAT* and *cytAPX* were increased after 1 h of HI, and slight increases were observed in *cytGR, chlGR and chlMDHAR* (Fig. 1, Table 1). After 24 h recovery, the expression of *CAT, stAPX, cytGR* and *chlMDHAR* was slightly higher than after 60 min of HI (Fig. 1, Table 1). However, contents of *cytAPX, cytCu,ZnSOD, chlCu,ZnSOD* and *chlGR* transcripts were lower when compared to those exhibited in control plants (time zero) and after 1 h of HI stress (Fig. 1, Table 1).

Discussion

Short-term exposure of pea plants to HI caused a significant photoinhibition, although F_v/F_m values had recovered after 24 h of the post-stress period. A similar response in photosynthesis has been described in spinach and *Arabidopsis* plants as well as in detached pea leaves subjected to HI stress (Yoshimura *et al*. 2000; Karpinski *et al*. 1997; Hernández *et al*. 2004). The increase in F_0 during HI has been described also in *Arabidopsis* plants as well as in detached pea leaves (Karpinski *et al*. 1997; Hernández *et al*. 2004). Elevated F_0 (zero fluorescence) has been considered as reflecting thylakoid membrane disturbance and photoinhibitory damage, and decreases in F_v/F_m and F_m during HI indicate that photoinhibition of photosynthesis ocurred (Krause and Weiss 1991; Balachandran and Osmond 1994; Karpinski *et al*. 1997; Bertamini and Nedunchezhian 2004).

mRNAs from some genes encoding antioxidant enzymes accumulated in both cultivars during HI, although to different extents. At time zero, as well as in stressed and recovered plants, expression of all mRNAs was higher in cultivar JI399 than in JI281. This could contribute to a more efficient response to the AOS that may be generated in chloroplasts and cytosol during the HI and post-stress periods. However, after HI, the increases observed for *chlMDHAR, cytCuZnSOD* and *cytAPX* were higher in JI281 than in JI399 plants, which could be due to the lower AOS generation in JI399 compared to JI281 after HI, as shown previously in detached leaves from the same cultivars (Hernández *et al.* 2004).

The strong induction of *CAT*, *cytAPX* and *cytCu,ZnSOD* in JI281 indicates that HI could have increased $O_2^{\cdot -}$ and H_2O_2 levels in the cytosol. In the same way, the induction of *CAT* and *cytAPX* in JI399 could indicate also an increased H_2O_2 generation in the cytosolic compartment. An increase in H_2O_2 has been observed also in detached leaves from these pea cultivars after 60 min of HI, being higher in JI281 than in JI399 (Hernández *et al.* 2004).

On the other hand, the higher expression of *chlMDHAR*, *chlGR*, *chlPHGPX*, *stAPX* and *chlCu,Zn-SOD* observed in JI399 under HI, could suggest a higher protection against AOS in chloroplasts from this pea cultivar. Probably, the greater alterations in F_0 and F_m observed in JI281 could be related with the lower expression of all antioxidant enzymes analysed.

In detached pea leaves and in *Arabidopsis* plants, photoinhibition was correlated with an increase in H_2O_2 (Karpinski *et al.* 1997; Hernández *et al.* 2004). A similar response has been observed in spinach, where a transient increase in H_2O_2 levels was observed after 1 h of HI, correlated with a drop in F_v/F_m values (Yoshimura *et al.* 2000). So, the decrease in F_v/F_m and F_m, as well as the increase in F_0, observed in pea plants subjected to HI could also be due to an increase in AOS in their chloroplasts. In bean plants, continuous irradiation induced premature senescence caused by enhanced production of AOS (Procházkova and Wilhelmová 2004).

The increase in *cytGR* transcripts observed in pea leaves after 24 h post-stress has been previously described in *Arabidopsis* plants and in detached pea leaves (Karpinski *et al.* 1997; Hernández *et al.* 2004), and a similar increase in *chlMDHAR* has been described also in detached pea leaves recovered from HI (Hernández *et al.* 2004). In wheat, DHAR activity declined during the early stages of seedling growth under a high-light regime and this implies that regeneration of ASC for scavenging of H_2O_2 is catalysed mostly by MDHAR and not by DHAR (Mishra *et al.* 1995). In the present study, we have not measured DHAR activity or expression, but the induction of *chlMDHAR* and the slight decrease in *chlGR*, in both pea cultivars, suggest that in chloroplasts, ASC seems to be regenerated mostly *via* MDHAR. Conversely, the increase in *cytGR* in recovered plants indicates that ASC could be regenerated mainly *via* GSH in the cytosolic compartment.

It has been suggested that in the induction of transcripts encoding for antioxidant enzymes under HI, both H_2O_2 and the redox status of ascorbate could be involved (Hernández *et al*., 2004). However, in *Arabidopsis* plants, a role for the redox state of glutathione and the plastoquinone pool also has been proposed (Karpinski *et al*. 1997). Therefore, in the present study, and although no measurements of H_2O_2, ascorbate or glutathione levels have been carried out, the induction of transcripts encoding for antioxidant enzymes in response to HI could also have been mediated by AOS or by the alteration in the redox state of ascorbate and/or glutathione.

The data obtained about the expression of genes encoding antioxidant enzymes suggest that HI could alter AOS levels, both in the chloroplasts and cytosol of pea leaves. The induction of cytosolic antioxidant enzymes could provide an alternative protection when the chloroplastic defence systems are compromised under light stress conditions (Karpiski *et al.* 1997; Hernández *et al.* 2004). Support for this was reported by Yoshimura *et al*. (2000) who found that stress conditions, including HI, drought,

salinity and methyl viologen treatments, which enhanced AOS in chloroplasts, also induces the cytosolic scavenging system.

In conclusion, results indicated that HI caused a reversible photoinhibition of photosynthesis in pea chloroplasts, and that changes in both F_0 and F_m contributed to the decrease in F_v/F_m, the changes being more important in JI281 than in JI399 leaves. Also, it seems that HI produces an increase in AOS that could regulate the accumulation of mRNAs encoding antioxidant enzymes. These data also suggest that pea cultivar JI399 seems to be relatively more tolerant than JI281 to HI, at least partly, due to its higher expression levels of antioxidant enzymes.

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Table 1. Transcript levels relative to the time 0 of genes coding antioxidant enzymes from attached pea leaves exposed to EL. A representative Northern blot is shown.

[a]Values were obtained after the scanning of RNA gel blot hybridization with cDNA probes. Value at time 0 is set to 1.

	JI-281		JI-399	
Probe	**HI**	**Post-stress**	**HI**	**Post-stress**
chlMDHAR	2.04	3.33	1.4	1.64
GOR 2	1.35	2.22	1.21	1.50
GOR 1	0.96	0.81	1.2	0.7
CAT1	2	1.3	2.24	1.15
PHGPX	1.71	1.93	1.04	0.8
cytAPX	4.16	1.4	2.03	0.73
stAPX	1.4	3.15	0.5	1.4
chlCuZnSOD	1.53	1.33	0.97	0.60
cytCuZnSOD	3.36	1.95	0.8	0.62

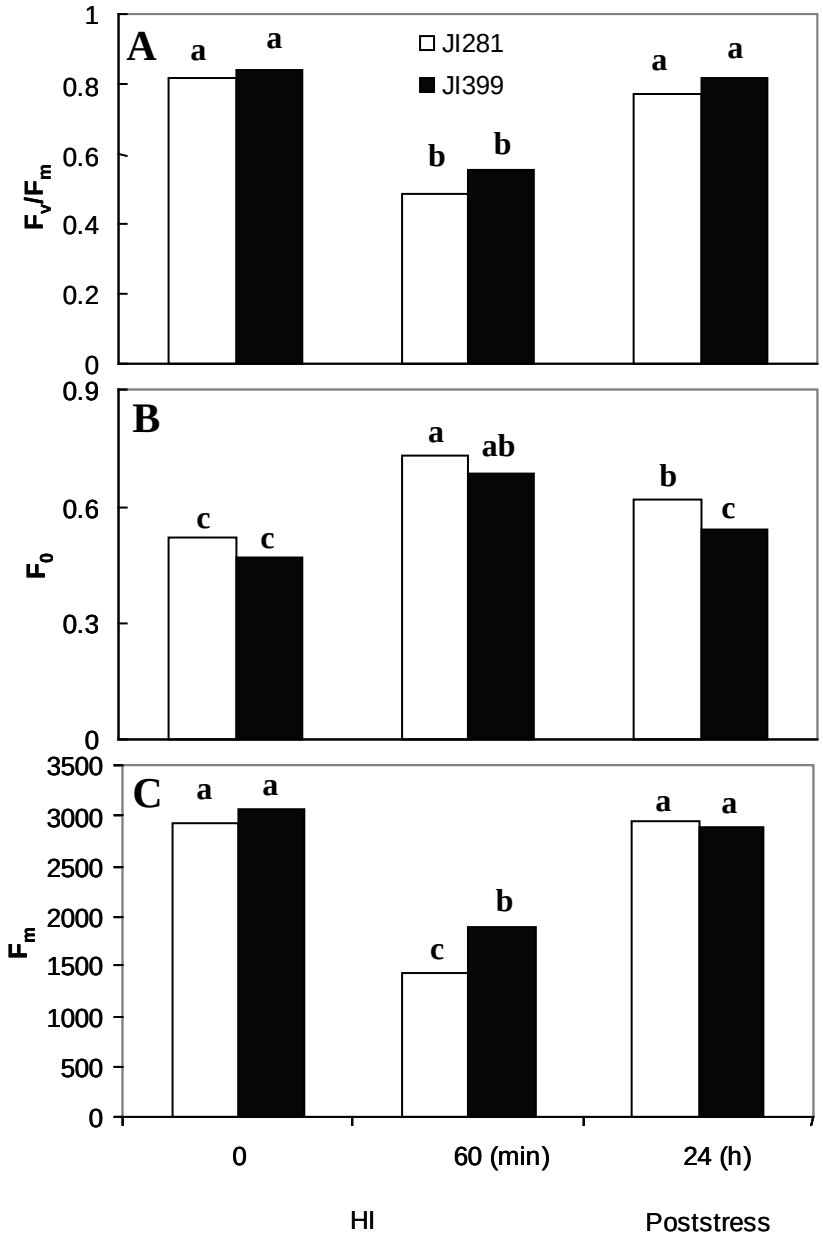

Fig 1.- Effect of HI on F_v/F_m ratios (A), F_0 (B) and F_m (C). The F_v/F_m ratio, F_0 and F_m were measured in three individual plants obtained from three independent experiments (n=9). The seedlings were grown at irradiance 200 μmol m^{-2} s^{-1}. In the middle of the photoperiod, 3-week-old plants were exposed to HI (4000 μmol m^{-2} s^{-1}) for 60 min and returned to low irradiance. Means with different letters are significantly different according to Duncan's multiple range test (P= 0.05).

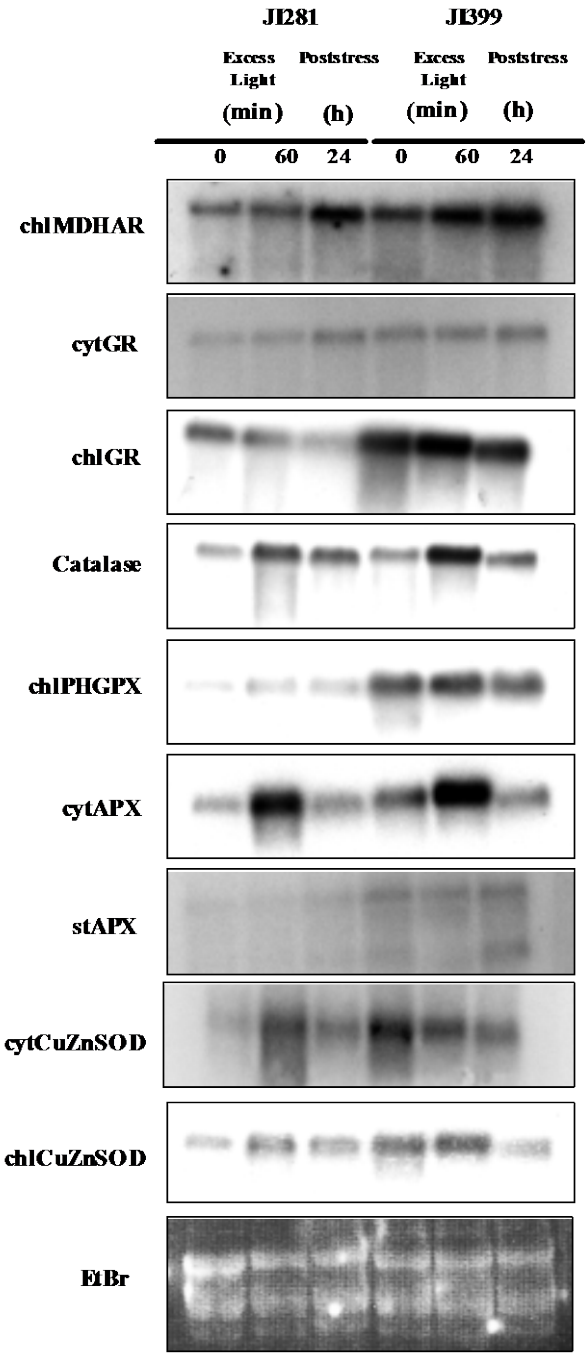

Fig 2.- Northern blot hybridisation analysis of poly (A+) RNA from pea plants exposed to HI. Names of probes used are indicated on the left. A representative Northern blot is shown.