Running Title: Light stress and antioxidant defences in pea.

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Role of hydrogen peroxide and the redox state of ascorbate in the induction of antioxidant enzymes in pea leaves under excess light stress.

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Abstract

In this work we used two different pea cultivars: JI281 is a semidomesticated land race of pea from Ethiopia whereas JI399 is a typical domesticated pea variety. Exposure of pea leaves to excess light (EL) for 1 h caused a reversible photoinhibition of photosynthesis as showed by changes in F_v/F_m . Although little difference existed between the two pea genotypes with respect to photoinhibition, after 60 min of EL the decline in F_v/F_m was higher in JI281 than in JI399 leaves. As a consequence of EL, H_2O_2 increased in both pea cultivars, whereas lipid peroxidation and protein oxidation slightly increased, although differenced between cultivars were minimal. The redox state of ascorbate shifted towards its oxidized form under EL stress in both cultivars. Transcript levels of genes coding antioxidant enzymes varied upon EL in both cultivars, but the response was more pronounced in JI399. The induction observed during EL was maintained or increased after the stress period, as occurred for *cytGR* and *chlMDHAR*. GR protein accumulation and activity correlated to the transcript accumulation in JI399, but not in JI288. In this work, a possible role for H_2O_2 and redox status of ascorbate in the photooxidative stress signalling is discussed.

KEY-WORDS: antioxidant enzymes; ascorbate, excess light stress; gene expression; hydrogen peroxide; photoxidative stress; photoinhibition, *Pisum sativum*

Abbreviations: AOS, activated oxygen species; ASC-GSH cycle, ascorbate-glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; EL, excess light; GR,; glutathione reductase; MDHAR, monodehydroascorbate reductase; SOD superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

INTRODUCTION

In nature, plants respond to sudden and sustained fluctuations in light quality and quantity, partly via their chloroplasts' redox transducing mechanisms. In turn, these induce marked modulations of chloroplast components, ultimately leading to acclimation to environmental cues (Anderson *et al.*, 1995; Karpinski *et al.*, 1999). Thus, chloroplasts are not only energy transducers, but also are primary redox sensors of environmental conditions (Anderson *et al.*, 1995, Hunner *et al.*, 1996).

All plants subjected to excessive irradiance are photoinhibited with a loss of PSII function (Osmond, 1994). The principal site of photoinhibition is D1 reaction-centre protein of PS II, and D1 protein turnover is crucial for PSII function (Foyer and Noctor 2000). Singlet oxygen ($^1\text{O}_2$) causes D1 protein degradation and loss of PSII function under photoinhibitory conditions (Keren *et al.*, 1995). However, it has been demonstrated that the photodegradation of the D1 protein and Rubisco was prevented in the presence of antioxidant compounds (Landgraf *et al.*, 1997).

Photoinhibition *in vivo* can be viewed as the capacity of plants to adjust photosynthetically to prevailing environmental conditions, rather than as a process of damage to plants (Anderson *et al.*, 1995; Hunner *et al.*, 1996).

Prolonged exposure to the conditions that cause excess excitation energy can result in an increase in the generation of activated oxygen species (AOS), such as $^1\text{O}_2$, superoxide

radicals ($O_2^{\cdot-}$) and hydrogen peroxyde (H_2O_2) (Karpinski *et al.*, 1997, 1999; Yoshimura *et al.*, 2000). This can damage the ultrastructure and function of chloroplasts and photosynthetic pigments, leading to leaf chlorosis (Asada 1999; Foyer *et al.*, 1994).

Plants contain a complex antioxidant system to detoxify AOS that includes carotenoids, ascorbate, glutathione, tocopherols and anthocyanin pigments (Neill *et al.* 2002; Del Río *et al.* 2002), and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases, and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle; Foyer and Halliwell 1976): ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2).

ASC is a major metabolite in plants and, in association with other components of the antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis, and a range of biotic and abiotic stresses (Smirnoff, 1996). ASC protects the chloroplasts from photoinhibition (Asada 1999) and suppresses the accumulation of photoproducted H_2O_2 in the stroma by acting as the electron donor of APX to protect components of the Calvin cycle from inactivation (Asada 1999). It has been reported also that a decreased level of ASC could act as a metabolic signal leading to increased GSH synthesis in both animals (Meister, 1992) and plants (May and Leaver, 1993).

There are reports on the changes in activity and expression of antioxidant enzymes in response to high-light stress (Gillham and Dodge, 1987; Foyer *et al.*, 1989; Karpinski *et al.*, 1997; Yoshimura *et al.*, 2000) but the results vary according to plant materials and treatment conditions employed. In *Arabidopsis* plants, EL stress induced CAT1 and two cytosolic APX genes (*APX1* and *APX2*) (Karpinski *et al.*, 1997, 1999). This APX induction by EL could be diminished by infiltrating leaves with catalase, suggesting that H_2O_2 (but no $O_2^{\cdot-}$) seems to be involved in the EL-induced APX expression. However, H_2O_2 alone did not induce the

expression of APX (Karpinski *et al.*, 1999). Cytosolic APX activity and transcript abundance increased remarkably in response to high-light treatment in spinach plants, and H₂O₂ levels increased in parallel with cytosolic APX transcript levels, suggesting that the response of spinach cytosolic APX to oxidative stress caused by high light may be mediated by the transient accumulation of H₂O₂ (Yoshimura *et al.*, 2000). In addition to H₂O₂, it has been suggested that redox changes in electron transport through the plastoquinone pool in chloroplasts could be essential for cytosolic APX induction under EL stress (Karpinski *et al.*, 1997), and that H₂O₂ could act as a systemic messenger (Karpinski *et al.*, 1999). Moreover, GSH levels or redox status of glutathione could have a regulatory impact on this signalling pathway (Karpinski *et al.*, 1997). However, in this paper no data about the role of ascorbate in EL stress were given.

The ascorbate-deficient *Arabidopsis vtc-1* showed higher levels of peroxidase activity and cytosolic APX activity and transcripts than the wild-type (Veljovic-Jovanovic *et al.*, 2001). These authors suggested that ascorbate deficiency may trigger alarm signals that increase antioxidant activity specifically in the cytosol. As mentioned above, in wild-type *Arabidopsis*, EL is required to overcome the stabilising effects of ascorbate and to induce cytosolic APX expression (Karpinski *et al.*, 1997). In contrast, in the *vtc-1* mutant, where there is a constitutive ascorbate deficiency, the cytosolic APX appears to be “turned up” even during growth at low light (Veljovic-Jovanovic *et al.*, 2001). These authors suggested that in species where ascorbate contents change significantly with growth irradiance, ascorbate concentration is likely to play a part in the intracellular coordination of the antioxidative system in response to changing light intensity (Veljovic-Jovanovic *et al.*, 2001).

Jl281 is a semidomesticated land race of pea from Ethiopia whereas Jl399 is a typical domesticated garden pea variety (Ellis *et al.*, 1992), that showed some physiological differences regarding plant growth. The main difference between the two pea cultivars was

that cultivar JI281 showed a greater shoot length (2.5-fold) and fresh weight (20%) than JI399 (data not shown)

In this work, we investigate the effect of EL on PSII efficiency in pea leaves, and the possible contribution of AOS to the photoinhibition process. We measured lipid peroxidation, protein oxidation, reduced and oxidized ASC and H₂O₂ levels, as well as the levels of mRNAs encoding crucial enzymes of AOS metabolism. The possible relationship between H₂O₂, ascorbate levels and gene expression under EL stress is discussed.

MATERIALS AND METHODS

Plant material and growth and stress conditions

Pisum sativum cv JI-281 and cv JI-399 seedlings, individually planted in pots, were grown in a controlled growth chamber (18-h photoperiod, $200 \pm 25 \mu\text{mol}$ of photons $\text{m}^{-2} \text{sec}^{-1}$ photosynthetically active photon flux density (PPFD), a temperature of $15 \pm 1.5 \text{ }^{\circ}\text{C}$, and relative humidity of $75 \pm 5\%$) (Ellis *et al.*, 1992).

Detached pea leaves were placed in a petri dish with distilled water, and were exposed to a white light pulse for $2000 \mu\text{mol}$ of photons $\text{m}^{-2} \text{sec}^{-1}$ up to 1 hour. 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. After this period, leaves were returned to normal growth conditions. Samples were collected 2h and 24h after the stress period. In all the experiments, control leaves were taken at the same time as the treated samples but without the EL treatment. All samples were frozen in liquid N₂ and stored at -70°C .

Measurement of chlorophyll fluorescence

The photosystem II efficiency parameter F_v/F_m (the ratio of variable to maximal fluorescence reference), F_o and F_m were measured using a portable Plant Efficiency Analyser

(PEA) machine on 15 min dark-adapted pea leaves at 90% light saturation, according to the manufacturers' instructions (Hansatech Instrument, King's Lynn, Norfolk, UK).

Lipid peroxidation

Leaf samples were extracted at 4°C using a mortar and pestle, in 50 mM potassium phosphate buffer, pH 7.0 (0.1 g/ 1 ml buffer). Extracts were centrifuged at 15,000 x *g* (10 min) and lipid peroxidation (MDA plus 4-hydroxyalkenals) was determined in the supernatant at 586 nm, using the colorimetric assay for lipid peroxidation LPO-586 (Bioxytech[®], Oxis International, Inc., Portland, OR, USA) according instruction manual.

Protein oxidation

Protein oxidation, given as carbonyl protein (CO-protein) content was measured by reaction with 2,4-dinitrophenylhydrazine as described by Levine *et al.* (1990). Samples were homogenized in 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and complete protease inhibitor cocktail (Boehringer Mannheim). Aliquots (0.2 ml) of extract were reacted with 500 µl of 10 mM 2,4-dinitrophenylhydrazine or 2M HCl (control) for 1 h. The protein was precipitated with 20% TCA (w/v) and washed with ethanol:ethyl acetate (1:1) three times. The final protein pellets were dissolved in 6 M guanidine, pH 2.3, and the absorbance was determined at 360 nm. The carbonyl content was calculated using the extinction coefficient of hydrazone (22000 M⁻¹ cm⁻¹).

H₂O₂ and Ascorbate contents

Leaves (0.25 g) were homogenized, at 4°C in a mortar and pestle, in 1 ml 5% metaphosphoric acid. After 5 min centrifugation at 15000 x *g*, the supernatant was collected. For ascorbate determination, the supernatant was passed through a Sepak column and filtered (0.22 µm filter; Millipore) prior to analysis by HPLC (Dionex) as described elsewhere

(Jimenez *et al.*, 2002). Total ascorbate was determined by incubating samples with 1 mM DTT (final concentration), and the DHA concentration was estimated from the difference between total ascorbate and reduced ASC.

For H₂O₂ measurements, supernatants were passed twice through a Dowex basic anion exchange resin (Sigma) to remove pigments. Quantification of H₂O₂ was carried out by chemiluminescence as described by Warm and Laties (1982). The H₂O₂ concentration was measured with and without an internal H₂O₂ standard before and after incubation with catalase (25 Units, Sigma). The external standard of H₂O₂ was prepared in the range 0.1-1 µM.

GR activity of crude extracts and Western blot analyses

GR activity was assayed by measuring the GSSG-dependent oxidation of NADPH, as described in Hernández *et al.* (2001).

Protein samples were separated by native 10% PAGE followed by western blotting using antisera raised against pea GOR1 (Stevens *et al.*, 1997).

Northern blotting

Total RNA from pea leaves was extracted as previously described (Creissen and Mullineaux, 1995). Total RNA was enriched in Poly A⁺RNA by using oligo d(T) cellulose spin columns (Pharmacia) according to the manufacturer's instructions. Poly A⁺ enriched RNA (3-5 µg) was denatured, separated electrophoretically and transferred onto a nitrocellulose membrane as described in Hernández *et al.* (2000). Equivalent loading of Poly A⁺ enriched RNA for each time point was checked on gels stained with ethidium bromide, as some ribosomal RNA was present in the enriched RNA. Northern blots were visualized by autoradiography after hybridization with ³²P-labelled DNA probes. Hybridization was carried out in 0.3 M sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 7% SDS, and 1% BSA at 65°C (homologous probes) or 55°C (heterologous probes). Washing was carried out at

65°C in 0.1 XSSC for homologous probes and either 1XSSC or 2XSSC for heterologous probes. Northern blots were repeated twice and representative blots are shown.

Probes used were: cytosolic and chloroplastic Cu,Zn-SODs from *Nicotiana plumbaginifolia* (Bowler *et al.*, 1989; Van Camp *et al.*, 1990), cytosolic GR from pea (*GOR 2*, Stevens *et al.*, 1997), chloroplastic GR from pea (*GOR 1*, Creissen *et al.*, 1992), CAT from tobacco (Willekens *et al.*, 1994), PHGPX from pea (Mullineaux *et al.*, 1998), chloroplastic MDAR 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 visualized on a BAS 1000 Phosphorimager (Fuji Photofilm Co., Kanagawa, Japan). Scanning values were calculated by BASIS software (Fuji Photofilm Co.) installed on the BAS 1000.

Protein was measured according to Bradford (1976).

Statistical analysis

Comparisons among means were made using the Duncan's multiple range test, calculated at $P < 0.05$. Statistical procedures were carried out with the software package SPSS 10.0 for Windows. Control leaves had been detached for the same length of time as treated leaves, and all data were expressed in relation to their control leaves. Once results had been corrected in relation to their control values, statistical analyses were conducted relative to pre-stress controls (time 0).

RESULTS

Effect of excess light on photosynthetic parameters

In pea leaves, a significant decrease in F_v/F_m was observed after 15 min of EL (2000 μmol of photons $\text{m}^{-2} \text{sec}^{-1}$). However, after 1 h of irradiation, the decline in F_v/F_m was higher in JI281 than in JI399 plants (Fig 1A). After 2 h of poststress, the F_v/F_m values of treated leaves from both cultivars had not recovered and photoinhibition continued, but after 24 h of

the poststress period F_v/F_m had reached control values (Fig 1A). Changes in both F_o and F_m contributed to changes in F_v/F_m , being the changes more important in JI281 than in JI399 leaves (data not shown).

In control leaves, taken at the same time as the treated samples but without EL treatment, no important changes in F_v/F_m was observed and values was kept above 0.75 (data not shown).

The results presented here clearly indicate that under EL conditions, a reversible photoinhibition of photosynthesis in pea chloroplasts is produced.

Protein oxidation, lipid peroxidation and H_2O_2 levels

In control leaves, taken at the same time as treated leaves, a 14-16% increase in CO-protein was observed at 30 min and 25 h after excision, respectively (see legend to Figure 1). However, in JI399 control leaves, no important changes in CO-protein were observed. Regarding lipid peroxidation, a 15-17% increase was observed in JI281 leaves at 15 min, 60 min and 25 h after excision. However, in JI399 leaves, the increase in lipid peroxidation occurred only 25 h after excision (around 30%) (see legend to Figure 1). CO-proteins and lipid peroxidation contents were measured to determine the level of oxidative cellular injury caused by EL stress. In JI281 detached pea leaves, the CO-protein contents slightly increased during the stress period, although the increase was only significant after 30 min of stress (up to 17%) (Fig 1B). In contrast, in JI399 cultivar, the changes in CO-proteins were not significant (Fig 1B).

The two pea cultivars showed a similar pattern of lipid peroxidation, which exhibited a clear tendency to increase during the light stress period. However, the increases were only significant in JI281 pea leaves at 60 min of EL, but no differences between cultivars occurred. In the poststress period, lipid peroxidation data reached control values (Fig 1C).

In the control pea leaves from JI399, the foliar levels of H_2O_2 had not changed significantly 60 min after excision. In contrast, 3 h and 25 h after excision (corresponding to 2 and 24 h of poststress in treated leaves), the H_2O_2 levels had increased 25% and 38%, respectively (see legend to Figure 1). A similar pattern was seen for JI281 control leaves, but the increase in H_2O_2 was greater (50% after 24 h of the recovery period).

During EL, H_2O_2 values were higher than their respective controls and differences were significant for both cultivars. In JI281, H_2O_2 content reached a maximum after 15 min of treatment (63% increase) and remained raised after 1 h of EL (43% increase) (Fig 1D), and had returned to the control level after 2h and 24h of the recovery period. However, in JI399, the H_2O_2 content gradually increased during EL, reaching a maximum later than in JI281, at 45 min of light stress (68% increase). However, after 60 min of stress H_2O_2 values were similar to their control leaves. During the recovery period, the H_2O_2 levels returned to control values (Fig 1D).

Ascorbate levels

No important changes in reduced ASC were observed for control JI281 leaves. However, in JI399, an increase of up to 35% in reduced ASC was produced 45 min after detachment, declining to a 10% increase 25 h after excision (see legend to Fig 2). Leaf excision also affected DHA levels. Thus, in JI281, a 15% increase was observed 60 min after detachment, but no changes were observed 3 or 25 h after excision (equivalent to 2 and 24 h poststress, respectively, in treated leaves). More important changes were observed in JI399 leaves. An increase of 41% was produced 30 min after excision, the increase being about 2.5-fold 25 h after excision (see legend to Fig 2).

The leaf ASC pool behaved differently in the two cultivars. Thus, the fluctuation of the reduced ASC content was moderate in JI281. However, in cultivar JI399 the reduced ASC content was significantly decreased, to 20%, after 45 and 60 min of EL and during the

recovery period (Fig 2A). In addition, a significant accumulation of DHA was observed in JI281 plants during the stress period (up by 86% after 60 min of EL stress), increasing 2.4 fold after 24h of the poststress period (Fig 2B). In JI399 plants, DHA had accumulated to a lesser extent than in JI281 plants after 45-60 min of stress (by 29% and 47%, respectively) and after 24 h poststress (by 23%) (Fig 2B). These changes resulted in a increase in the ratio $[DHA/(Asc + DHA)]$ during EL and after the recovery period (Fig 2C).

Antioxidant enzyme expression

In JI399, the expression of all of the antioxidant enzyme mRNAs increased during the EL treatment with a maximum after 60 min of EL stress (between 2- and 7-fold increase) (Table 1). This tendency was maintained after the poststress period (*chlGR*, *PHGPX*, *cytAPX*, *stAPX*) or increased further [*cytGR* (12-fold) and *chlMDHAR* (14-fold)]. However, *catalase* and *chlCu,Zn-SOD* fell close to the control levels during this period (Table 1).

In JI281, the expression of all antioxidant enzymes also increased during EL, the maximum levels being at 45 min. Chloroplastic *GR*, *CAT*, *PHGPX*, *cytAPX*, *cytCuZn-SOD*, and *chlMDHAR* had increased by between 1.5- and 3-fold after 45 min of EL stress. These increases were maintained, or increased further, after 24h of the recovery period, as was the case for *cytGR* and *chlMDHAR*, which increased 7- and 11-fold respectively. However, transcript levels for *cytGR* mRNA and for *chlGR* and *stAPX* mRNAs were halved after 30 min and 60 min of EL, respectively (Table 1).

GR protein levels and GR activity

The level of chloroplastic/mitochondrial GR (GOR1) protein was assayed in detached leaves after 1 h of EL stress and 24 h after of the recovery period. In both cultivars, the amount of the immunologically-detectable GR increased in leaves treated for 1 h with EL (2- to 3-fold). There was also an increase (approx 5-fold) in GOR1 protein after 24h of the recovery period

(Fig 3A). However, the GR activity in crude extracts was significantly decreased in JI281 after 1 h of EL, although the activity level had returned to control values after 24h of the recovery period (Fig 3B). In contrast, in JI399 the activity significantly increased, by 27%, in treated leaves during 1 h of EL, but after 24h of the poststress period the GR activity was similar to control values (Fig 3B).

DISCUSSION

Short-term exposure of pea plants to EL caused a significant photoinhibition, although F_v/F_m values had recovered after 24h of the poststress period. The variation of F_v/F_m , after 60 min of stress period, indicates that cultivar JI281 was more affected than JI399.

These results indicate that EL caused a reversible photoinhibition of photosynthesis in pea chloroplasts. A similar response in F_v/F_m has been described in spinach and *Arabidopsis* plants subjected to EL stress (Yoshimura *et al.*, 2000; Karpinski *et al.*, 1997).

Short-term exposure to EL also increased H_2O_2 accumulation, lipid peroxidation, protein oxidation, and oxidized ascorbate in both cultivars. Although the increases in lipid peroxidation and CO-protein levels were significant only in JI281, differences between cultivars were minimal.

Photosynthetic electron transport in chloroplasts is inevitably accompanied by the reduction of O_2 to O_2^- on the reducing site of PS I and any perturbation in the photosynthetic capacity results in the formation of AOS, which probably originated from PSI, ferredoxin, or excited chlorophyll, as described by Asada (1999). A wide range of environmental stresses such as drought, salinity, excess light and low temperature lead to enhanced production of AOS, and much of the injury to plants due to stress is associated with oxidative damage at the cellular level (Alscher *et al.*, 1997, Karpinski *et al.*, 1997; Broetto *et al.*, 2002; Hernández *et al.*, 2003a, 2003b; Foyer and Noctor 2003).

All mRNAs from genes encoding antioxidant enzymes accumulated in both cultivars during stress, although to different extents. Thus, the maximum value was higher in JI399, compared to JI281 leaves. The higher induction of *cytCuZn-SOD*, *cytAPX*, *stAPX* and *chlCuZn-SOD* mRNAs in JI399 pea leaves could contribute to minimize the damage produced by O_2^- and H_2O_2 in chloroplasts and cytosol during the EL stress and the poststress periods. In JI281 pea leaves, the lack of induction of *stAPX* and *chlCu,Zn-SOD* mRNAs after 1 h of EL, could diminished the capacity for scavenging O_2^- and H_2O_2 in the chloroplasts, in a situation in which the AOS production could be stimulated, increasing the risk of oxidative damage. This is in agreement with the less-pronounced variation in all parameters measured in JI399 plants, in relation to JI281 (see previous section).

In JI399 leaves, the progressive accumulation of *cytAPX* and *stAPX* transcripts correlated with the increase in the foliar H_2O_2 content during the EL period, suggesting a possible role of H_2O_2 in APX induction, as has been described previously (Karpinski *et al.*, 1999; Morita *et al.*, 1999; Yoshimura *et al.*, 2000). It seems likely that the induction of *cytAPX* and *stAPX* in JI399 leaves during an early stage of oxidative stress plays an important role in minimizing photooxidative damage. H_2O_2 is a membrane-permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Levine *et al.*, 1994; Foyer *et al.*, 1997, Karpinski *et al.*, 1999; Neill *et al.*, 2002), and an H_2O_2 elevation of 20 to 40% seems to be sufficient for inducing *cytAPX* (Morita *et al.*, 1999; Karpinski *et al.*, 1999; Yoshimura *et al.*, 2000). In CAT-deficient tobacco, *cytAPX* mRNA and APX activity increased following accumulation of H_2O_2 under high-light conditions (Willekens *et al.*, 1997), emphasizing the role of H_2O_2 in APX induction under light stress. In JI399 leaves, besides *cytAPX* and *stAPX*, increased H_2O_2 levels also correlated accumulation of the other transcripts encoding for antioxidant enzymes studied, suggesting also a role for H_2O_2 in their induction during EL period.

However, in leaves of JI281 pea plants, the accumulation of *cytAPX* and *stAPX* transcripts, as well as the others mRNAs analysed, did not correlate with that of H_2O_2 , which had accumulated 15 min after EL. It has been reported that a high level of thylakoidal APX suppresses the photoinhibition under photooxidative stress, providing tolerance to photooxidative damage imposed by AOS and preventing SOD inactivation by photooxidative stress (Yabuta et al., 2002). These data agrees with our results in pea plants. In JI399 plants, *stAPX* were gradually increased during EL treatment, and a protective effect on the chlCuZn-SOD isozyme could be produced during stress. However, in JI281 plants, where *stAPX* decreased near 50% after 60 min of EL stress, *chlCuZn-SOD* transcripts also showed a 25% decrease.

In our study, the lack of changes in lipid peroxidation in JI399 leaves correlated with the higher *PHGPX* expression levels found, both during the stress and poststress periods, compared to JI281 leaves. PHGPX reduces lipid hydroperoxides to their corresponding alcohol using GSH as the electron donor (Ursini *et al.*, 1995) and this enzyme has been shown to be localized in the chloroplast stroma (Mullineaux *et al.*, 1998). *PHGPX* mRNA increases in tissues of several plant species undergoing stress, including salt-stress, heavy metal poisoning, and infection by viral or bacterial pathogens (Criqui *et al.*, 1992; Levine *et al.*, 1994; Hernández *et al.*, 2000).

In JI399 plants, the decrease in reduced ASC during the stress period (45 and 60 min of stress) also paralleled the induction of genes encoding antioxidant enzymes, including *cytAPX* and *stAPX* as well as *cytGR* and *chlGR*. Recently, in the *vtc-1* mutant of *Arabidopsis*, which is deficient in ASC biosynthesis, Veljovic-Jovanovic *et al.* (2001) reported differential regulation of *APX* transcript abundance linked to changes in ASC concentration that was independent of redox changes, which is in agreement with our results in the JI399 cultivar. Moreover, it is suggested that ASC deficiency may trigger alarm signals that lead to an increase in antioxidant activity, specifically in the cytosol (Veljovic-Jovanovic *et al.*, 2001).

In the present study, we have not measured DHAR expression nor activity in pea leaves but the induction observed in *chlMDHAR* in both cultivars suggests that, in chloroplasts, ASC could be regenerated via MDHAR under EL stress. In wheat plants, DHAR activity declined during the early stages of seedling growth under a high-light regime and this implies that the regeneration of ASC for scavenging H₂O₂ is catalyzed mostly by MDHAR and not by DHAR (Mishra *et al.*, 1995). These results are agreement with our results. The accumulation of DHA under EL stress has been described also in spinach plants, for which ASC levels were unchanged, resulting in a decrease in the redox status of ascorbate (Yoshimura *et al.*, 2000). However, in this case, after 48 h recovery, the DHA levels had fallen to almost the same values as in the control plants. These data differed from those of the pea leaves, for which DHA levels remained raised after 24 h recovery (JI399) or even increased (JI281).

Literature regarding stress revival is scarce, and only a few studies have been carried out. In pea plants recovered from drought, an increase in cytosolic APX and CuZn-SOD transcript abundance occurred (Mittler and Zilinskas 1994). In other experiments, Stevens *et al* (1997) reported that increased *cytGR* mRNA levels were also induced in the poststress periods after drought or chilling stresses, although foliar GR activity did not change significantly. In pea plants, short-term NaCl-stress produced reversible effects on leaf water relations, growth, water content and SOD and APX activities (Hernández and Almansa, 2002). However, during the first hours of both stress and recovery, an increase in lipid peroxidation occurred, suggesting the involvement of an oxidative stress during these periods (Hernández *et al.*, 2002). During the first hours of recovery, the induction of SOD, APX and GR cannot prevent lipid peroxidation, and probably the change of culture conditions (elimination of NaCl from the hydroponic culture) is perceived by plants as a hypo-osmotic stress situation (Hernández and Almansa, 2002). In the present work, after 2 h of the recovery period, photoinhibition continued, whereas protein and lipid oxidation and H₂O₂ levels

returned to control values. This decrease in H_2O_2 during poststress may be related to the observed increase in the antioxidant enzymes mRNAs. During recovery of pea plants from salt and drought stress, an oxidative stress during the first hours of recovery was suggested (Mittler and Zilinskas 1994; Hernández and Almansa 2002). In contrast, in pea leaves recovering from EL stress, it seems that no oxidative stress is produced. Conversely, and in our experimental conditions, only during EL is an oxidative stress produced, according to the data for H_2O_2 and protein and lipid oxidation.

The strong induction of *cytGR* mRNAs was correlated with a protein accumulation, but not with a higher GR activity but GR activity increased only in JI399 leaves treated for 60 min with EL, indicating a higher capacity in GSH recycling than JI281 leaves. A similar induction of *cytGR* has been described in *Arabidopsis* plants during recovery of photooxidative stress (Karpinski et al., 1997). This suggests that the poststress increase in *cytGR* transcript levels could be a common feature of different plant species in response to different environmental stresses (Karpinski *et al.*, 1997). These authors suggest that GSH levels or the redox status of glutathione could be involved in rapid signalling from the chloroplasts to the nucleus during EL stress (Karpinski *et al.*, 1997). Although we did not measure GSH levels, it is clear that glutathione is an important redox agent in chloroplasts and could influence this signal transduction pathway during photooxidative stress in pea leaves. Recently, it has been suggested that H_2O_2 -induced chilling tolerance in mung bean plants might be mediated by an elevation of GSH contents (Yu et al., 2002, 2003).

Very little difference existed between the two pea genotypes regarding photoinhibition. However, after 1 h of EL stress, the decrease in F_v/F_m was larger in JI281 than in JI399 leaves. It is interesting to highlight the lack of correlation between H_2O_2 levels and photoinhibition, at least in JI281 leaves, because the highest H_2O_2 increase occurred after 15 min of EL stress (63% increase) whereas the decrease in F_v/F_m was no more than 15%. However, after 60 min of EL stress the F_v/F_m decrease was higher (40%) with a lower H_2O_2 increase (43%). This

response differed to that found in JI399 leaves, where the progressive increase in H_2O_2 during EL stress were correlated with the progressive drop in F_v/F_m . These results could suggest that, in JI281 leaves, other factors, besides H_2O_2 , are involved in the photoinhibition response during EL stress. As mentioned above, JI281 seems to have a lower capacity for scavenging of O_2^- and H_2O_2 in the chloroplasts, and this situation could favour the generation of the extremely dangerous $\cdot\text{OH}$ radicals, which could also be involved in the oxidative-stress response observed.

The data obtained about the expression of genes encoding antioxidant enzymes suggest that EL could alter AOS levels, both in chloroplasts and in cytosol of pea plants. The induction of cytosolic antioxidant enzymes could provide an alternative protection when the chloroplastic defense systems are compromised under light stress conditions (Karpiski *et al.*, 1997). The cytosol could be an important compartment in the plant's response to oxidative stress, as has been described previously for the response to paraquat, SO_2 , and NaCl exposure (Alscher *et al.*, 1997; Hernández *et al* 2000).

In conclusion, it seems that EL produces an increase in AOS as well as changes in the ascorbate redox status, and both could act by regulating the accumulation of mRNAs encoding antioxidant enzymes. However, field and/or greenhouse experiments would be necessary to confirm these data, as a different EL regime and time of exposure could modify this finding.

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Legend to Figures

Fig 1.- Effect of EL on F_v/F_m ratios (A), protein oxidation (A), lipid peroxidation (B) and H_2O_2 levels (C) in pea leaves.

(A) Detached pea leaves were exposed to excess light ($2000 \mu\text{mol}$ of photons $\text{m}^{-2} \text{sec}^{-1}$) for up to 1 h. Measurements were made at 0, 15, 30, 45, and 60 min in EL and 2 and 24 h after the EL treatment. (B) The CO-protein values ($\text{nmol mg}^{-1} \text{prot}$) in control leaves, taken at the same time as the treated samples but without EL treatment, at 0, 15, 30, 45, and 60 min after excision and at 3 and 25 h of excision were: 1.72, 1.77, 1.96, 1.89, 1.82, 1.80, 2.0 (JI281) and 1.67, 1.55, 1.57, 1.60, 1.63, 1.64, 1.79 (JI399). (C) The lipid peroxidation values ($\text{nmol g}^{-1} \text{FW}$) in control leaves at 0, 15, 30, 45, and 60 min after excision and at 3 and 25 h of excision were: 34.32, 39.55, 36.12, 34.03, 38.82, 35.22, 40.23 (JI281) and 37.99, 37.35, 37.68, 37.63, 37.62, 37.86, 49.79 (JI399). (D) The H_2O_2 contents ($\text{nmol g}^{-1} \text{FW}$) in control leaves at 0, 15, 30, 45, and 60 min, and 3 and 25 h of excision were: 626, 568, 685, 677, 685, 791, 944 (JI281) and 609, 620, 669, 647, 652, 763, 842 (JI399), respectively. Means with different letters are significantly different according to Duncan's multiple range test ($P=0.05$).

Fig. 2.- Effect of EL on the reduced ascorbate (A), DHA (B) contents and on the redox status of ascorbate from pea leaves. Measurements were made at the same points as given in Fig. 1

(A) The reduced ascorbate values ($\mu\text{mol g}^{-1} \text{FW}$) in control leaves at 0, 15, 30, 45, and 60 min after excision, and at 3 and 25 h of excision were: 8.47, 8.20, 7.85, 8.38, 8.25, 8.12, 8.11 (JI281) and 7.5, 8.4, 8.67, 10.12, 9.10, 8.86, 8.25 (JI399), respectively. (B) The oxidized ascorbate values (DHA) ($\mu\text{mol g}^{-1} \text{FW}$) in control leaves at 0, 15, 30, 45, and 60 min, and at 3 and 25 h of excision were: 0.74, 0.79, 0.65, 0.62, 0.85, 0.79, 0.68 (JI281) and 0.75, 0.90, 1.06, 0.84, 0.97, 1.24 and 1.98 (JI399), respectively. Data are the mean $\pm$ SE of at least three different experiments. Means with different letters are significantly different according to Duncan's multiple range test ($P=0.05$).

Fig 3.- Effect of EL on GR protein levels determined by immunoblot analysis (A) and on total GR activity in pea leaves (B). Measurements were made at 60 min in EL and after 24 h poststress. The crude homogenates of pea leaves (50 μ g of protein) were subjected to native PAGE and immunoblotting using polyclonal antibodies as described in “Materials and Methods”. GR activities ($\text{nmol min}^{-1} \text{mg}^{-1} \text{prot}$) in control leaves at 0 and 60 min, and at 25 h of excision were: 41.55, 39.34, 37.78 (JI281) and 36.63, 37.23, 37.77 (JI399), respectively. Means with different letters are significantly different according to Duncan’s multiple range test ($P= 0.05$).

Table 1. Transcript levels, relative to time zero of genes encoding antioxidant enzymes, from detached pea leaves exposed to excess light (EL) and returned to low light for 24 h (poststress). Data for a representative Northern blot is shown.

Probe	JI-281 pea plants						JI-399 pea plants							
	EL (min)					Poststress (h)		EL (min)					Poststress (h)	
	0	15	30	45	60	2	24	0	15	30	45	60	2	24
chlMDHAR	1 ^a	1.71	1.64	2.74	1.33	4.66	11.5	1	0.96	1.73	2.27	4.67	6.0	14.3
cytGR	1	0.9	0.4	1.64	1.13	2.5	7.2	1	0.75	1.34	1.92	3.71	5.6	11.8
chlGR	1	1.15	1.03	2.33	0.53	0.94	1.76	1	0.77	1.11	1.64	2.43	1.95	2.25
CAT1	1	1.17	1.04	2.6	1.4	1.97	2.1	1	0.77	1.35	1.5	3	2.4	1.5
PHGPX	1	1.01	0.96	1.9	0.75	1.4	2.05	1	1.07	1.63	2.05	2.88	3.3	3.04
cytAPX	1	0.9	0.85	3.1	1.7	2.9	3.5	1	1.2	2.1	3.1	6.9	6.4	4.98
stAPX	1	0.78	0.71	1.56	0.56	1.03	1.92	1	1.99	2.19	3.48	4.81	5.15	5.96
cytCuZnSOD	1	0.90	1.01	2.02	1.20	1.44	2.25	1	1.21	1.93	1.73	2.14	2.01	3.19
chlCuZnSOD	1	0.83	0.76	1.50	0.75	0.94	1.31	1	0.73	1.19	1.21	2.24	1.36	0.80

Fig. 1

Fig 2.

Fig 3