EXPRESSION OF EARLY LIGHT INDUCED PROTEIN IN GRAPEVINE AND PEA, UNDER DIFFERENT CONDITIONS AND ITS RELATION WITH PHOToinHIBITION

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Early light induced proteins (ELIPs) are a type of proteins which are expressed before than other chloroplast proteins in the presence of light. These proteins have been studied in a large number of annual species such as pea (Pisum sativum L.), barley (Hordeum vulgare L.) and Arabidopsis sp. In perennials plants the studies about ELIPs are very scarce. The possible photoprotective function of the ELIPs has motivated the interest in investigating the presence of this type of proteins in a perennial plant such as grapevine (Vitis vinifera L.) and if their characteristics differ from those found in annual plants. In this paper a comparative study was conducted on the ELIPs expression in grapevine and pea to investigate whether there are differences regarding to temperature and light intensity conditions necessary for maximum ELIP expression in each species and for studying in each case the relationship between ELIP expression and photoinhibition degree. The results of this study showed that the maximum ELIPs expression was reached from 25 ºC and 1000 µmol PAR m⁻² s⁻¹ in both species. Above these values the expression remained constant. Regarding the temperature and light intensity effect on the photoinhibition degree, it was observed that temperature produced inverse relation in grapevine but no relation with pea. On the other hand, the light intensity produced direct relation in both grapevine and pea. The light intensity effect on ELIP expression suggests that these proteins may have a photorepairing role of the photosynthetic system, but the effect of temperature on the ELIP expression in short-term stress may be associated rather to the optimum conditions for their synthesis.

Key words: Photoinhibition, ELIPs induction, abiotic stress, Vitis vinifera, Pisum sativum.

Photoinhibition can be defined as the loss of photosynthetic efficiency of plants exposed to a higher irradiation than considered normal for their development and survival (Maxwell and Johnson, 2000). If a high radiation is combined with other types of stresses, this phenomenon is accentuated (Hetherington et al., 1989). Under these conditions excessive reactive species accumulation is produced, allowing these molecules interact with proteins, lipids and pigments of the photosynthetic apparatus, damaging their structure and function (Apel and Hirt, 2004). Chlorophyll a can also be harmful to the plant because of its high oxidative capacity when it is synthesized or uncoupled from light harvesting complexes. To minimize damage from these reactive molecules, plants have different mechanisms of photoprotection (Niyogi, 1999; Osmond and Foster, 2004). In recent years, evidences of a new mechanism of protection against light stress have been found which would develop through the action of a class of chloroplast proteins induced transiently by light, early light induced proteins (ELIPs), whose first member was discovered in pea (Pisum sativum L.) by Meyer and Kloppstech in 1984. ELIPs have been included into the family of light-harvesting protein (LHCs) because they are structurally related with LHC II proteins, which correspond to the main component of this family (Montané and Kloppstech, 2000). Another feature in common with the LHCs is its ability to bind pigments demonstrating in vitro that ELIPs bind chlorophyll a and lutein (Adamska et al., 2001). The function of these proteins and their mechanism of action have not been proved yet, although there is evidence supporting a role of ELIPs as potential carriers of free chlorophyll a, or alternatively, as a stabilizing protein-chlorophyll complex of photosynthetic reaction centers, aided by the binding of the carotenoids and xanthophyll cycle pigments (Aro et al., 1993; Levy et al., 1993; Bruno and Wetzel, 2004).

ELIPs proteins have been studied in a large number of annual species such as pea, barley (Hordeum vulgare L.), Arabidopsis sp. and tomato (Lycopersicon esculentum Mill.) (Potter and Kloppstech, 1993; Adamska and Kloppstech, 1994; Hutin et al., 2003; Bruno and Wetzel, 2004). In all cases these proteins were induced by light alone or in combination with other stresses such as high and low temperatures, drought and salinity (Kloppstech et al., 1991; Adamska and Kloppstech, 1994; Zeng et

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al., 2002) and also by factors such as the stage of plant development, senescence (Binyamin et al., 2001; Norén et al., 2003), light quality (Sävenstrand et al., 2004) or hormonal action (Wiestra and Kloppstech, 2000). In perennial plants ELIPs studies are scarce (Bhalerao et al., 2003; Peng et al., 2008). A species that grows in high light radiation areas is grapevine (Vitis vinifera L.), therefore their leaves, particularly those exposed directly to sunlight, have a high photoinhibition risk (Bertamini et al., 2004; García de Cortázár et al., 2005). The finding of ELIP proteins in different species and their potential photoprotective function has motivated the interest of investigating the presence of these proteins in grapevine, and see if their characteristics differ from those found in annual plants. A protein expression like ELIP was recently discovered in young grapevine leaves using an anti-ELIP of pea antibody (Pinto et al., 2011). In this paper a comparative study between ELIP pea and ELIP grapevine was conducted to investigate whether there are differences regarding the conditions of temperature and intensity necessary for ELIP maximum expression in each species and to study in each case the relationship of the ELIP expression with the photoinhibition degree.

MATERIALS AND METHODS

Plant material
Grapevine plants cv. Sultanine were cultivated in 4 L black plastic bags containing humus and grown in greenhouse conditions (100 µmol PAR m⁻²; 25 ± 5 °C, with a photoperiod of 12:12 h). To obtain pea leaves cv. Perfected Freezer 400, seeds were germinated in paper moistened with 1% captan (N-(trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide), which were then transferred to a floating root hydroponic system, using the same green house conditions as used in grapevine plants.

Treatments
Detached mature pea leaves and young grape leaves, underwent different light and temperature conditions, for a constant time of 4 h. Leaves were kept floating in a bath with temperature control. The light was provided by a lamp with 1000-W halogen bulbs (Osram, München, Germany), whose incident intensity was modulated through the variation of the distance between the lamp and leaves. The light intensities were measured with a radiometer (185B, LI-COR, Lincoln, Nebraska, USA). Infrared radiation was blocked by a water filter located between the lamp and leaves. In the essay about the effect of temperature on ELIP expression the following temperatures were used: 10, 15, 20, 25, 30, and 35 °C at 1000 µmol PAR m⁻² s⁻¹. In the essay of the effect of light intensity on the ELIP expression, six levels of radiation were used: 100, 300, 700, 1000, 1500, and 2000 µmol PAR m⁻² s⁻¹ and the temperature was kept constant at 30 °C. The total number of treatments was 12. The leaf tissue samplings for the determination of protein expression were made at the end of each treatment and samples were collected in liquid nitrogen (-80 °C).

Extraction and detection of total protein
For protein extraction, the method described by Potter and Kloppstech (1993) was used with some modifications; 0.15 g leaves were macerated with 1.0 mL extraction buffer (21.7 mM Na₂CO₃, 2.0% SDS, 2 mM EDTA, 1 mM PMSF, 2.9 mM e-aminocaproic acid, 13.7% glycerol, 56 mM DTT, 0.1% bromophenol blue). Samples were incubated at 65 °C for 10 min in a water bath and centrifuged for 10 min at 10 000 g. The supernatants were transferred to new tubes and boiled for 5 min, then centrifuged again for 10 min at 10 000 g and the supernatants were transferred to new tubes which were stored at -80 °C for further analysis. The quantification of total proteins was made by the Bradford method (Bradford, 1976) using bovine albumin as standard.

Separation of proteins by electrophoresis in blotting conditions
Electrophoresis was carried out according to Laemmli (1970), using for the concentrate gel acrylamide/bis at 6% and for the separation gel acrylamide/bis at 16%. For the run, the electrophoresis system for minigels MiniProtein III, Biorad was used. Equal amounts of protein were loaded in each lane (25 µg), which were contained in an equal volume (25 µL). A pre-dyed standard molecular weight (See Blue II, Invitrogen, Carlsbad, California, USA) was used.

ELIPs Immunodetection
The proteins were transferred to a nitrocellulose membrane according to the Towbin et al. (1979) method. Then transferred proteins were stained by incubation the membrane for 5 min with 5% Ponceau Red solution. After protein staining, the membrane was faded by incubating in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.7 mM Na₂HPO₄, 1.8 mM KH₂PO₄), and after being blocked with 5% skim milk solution in PBST buffer for 1 h. Incubation with the primary antibody of pea anti-ELIPs obtained from goat in a 1:1000 dilution (diluted in same blocking solution) was proceed during all night at 4 °C. After to wash with PBST buffer, the membrane was incubated for 1 h at room temperature with a goat anti-IgG conjugated with alkaline phosphatase enzyme at a 1:20 000 dilution. This antibody was chosen after testing anti-ELIPs antibodies of different species using the technique of electric immune-microscopy being this result further corroborated by immunodetection. After to wash with PBST buffer, bands were revealed with the enzyme substrate 5-bromo-4-chloro-3-indoly phosphate p-toluidine/nitroblue-tetrazolium chloride in TMN buffer pH 9.5 (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂ × 2H₂O 5) (Harlow and Lane, 1988). The bands analysis
obtained by immunodetection was performed through SCAN-IT program. The intensity of each band was expressed as percentage, considering 100% as the average of four replicates in which the maximum band intensity was obtained.

**Determination of photoinhibition**
The photoinhibition degree was determined by fluorescence parameter Fv/Fm. This parameter is a measure of maximum photochemical efficiency of Photosystem II and its magnitude is related inversely with the photoinhibition degree (Maxwell and Johnson, 2000). To measure this parameter a portable no modulated fluorometer (PEA, Hansatech Instruments, King’s Lynn, Norfolk, UK) was used. This measurement was performed in samples treated at the beginning and end of each treatment.

**Data analysis and statistical model**
To analyze temperature effect on the different variables (ELIP proteins expression, fluorescence parameters) in the grapevine and pea species, and the effect of intensity on the same previous variables in both species a completely randomized design with four replicates with factorial structure of treatments was used. The experimental unit was one leaf and an ANOVA was carried out in each case (MINITAB Statistical Software, release 13.32 for WINDOWS; Minitab Inc, State College, Pennsylvania, USA). To verify the normality assumption, the Anderson-Darling test was used and the homogeneity assumption of variance was verified using the Bartlett test. Hypotheses of interest were compared using a significance level of 5%. Multiple comparisons of means were performed using the Tukey test. To analyze the correlation between the photoinhibition degree and the ELIP expression in both species, the same statistical software was used (MINITAB) and regression analyses were done using EXCEL.

**RESULTS AND DISCUSSION**

**Relationship between the developmental stage of leaves and the ELIP expression**
The developmental stage with the highest ELIP expression in both species was determined. According to previous results on grapevine (Pinto et al., 2011), the highest expression of ELIP in grapevine was found in young leaves 5-d old, irradiated with light intensity of 1000 µmol PAR m⁻² s⁻¹ at 30 °C. In mature leaves the expression was negligible under these same conditions. However, ELIP expression studies in pea has not been done so far using different stages of development and the expression studies conducted under conditions of high light stress have been performed only on mature leaves (Adamska et al., 1992). In order to investigate if there is ELIP expression in young pea leaves under high light conditions as it is in grapevine and if it is greater or less than the one produced in mature leaves, both young and mature leaves of pea and grapevine underwent at high and low light during 4 h at room temperature (Figure 1). It can be noted that, based on the obtained results, the ELIP expression level was high in young grapevine leaves as expected but almost nil in mature leaves, however, ELIP expression in pea was low in young leaves and high in mature leaves. This indicates that the state of development at which the highest expression of ELIP is found depends of the species. It was not found expression in any of the cases studied at low light intensity. From these results, it was decided to use young grapevine leaves and mature pea leaves to characterize ELIPs expression, as these were the states of development in which the highest expression was obtained under conditions of high light stress in each species.

**Effect of temperature on ELIP expression and the degree of photoinhibition in grapevine and pea leaves**
The effect of temperature on the ELIP expression level in young grapevine leaves was recently studied (Pinto et al., 2011) using a range between 10 to 40 °C. I that work was observed ELIP expression in grapevine leaves reached a peak to 30 °C decreasing over this temperature. To compare the effect of temperature on the ELIP expression level between both grapevine and pea leaves, detached leaves were exposed at different temperatures (10 to 35 °C) and high light intensity (1000 µmol PAR m⁻² s⁻¹) during 4 h. In Figures 2A and 2B the ELIP expression bands can be observed in function of the temperature for each species, obtained by Western blot technique. ELIP expression of each species (Figure 2), obtained from the quantification of the expression bands from four replications, shows that ELIP protein in both species decreased their relative expression as the temperature dropped and both proteins followed a pattern of expression in function of temperature almost identical. Pea leaves at 40 °C showed dehydration and presented foliar necrosis and since ELIP expression level in grapevine leaves severely decayed at 40 °C (Pinto et al., 2011), this temperature was ruled out from this study. These same tests were conducted at low light (100 µmol PAR m⁻² s⁻¹), obtaining no ELIP expression in young pea leaves under high light conditions as it is in grapevine and if it is greater or less than the one produced in mature leaves, both young and mature leaves of pea and grapevine underwent at high and low light during 4 h at room temperature (Figure 1). It can be noted that, based on the obtained results, the ELIP expression level was high in young grapevine leaves as expected but almost nil in mature leaves, however, ELIP expression in pea was low in young leaves and high in mature leaves. This indicates that the state of development at which the highest expression of ELIP is found depends of the species. It was not found expression in any of the cases studied at low light intensity. From these results, it was decided to use young grapevine leaves and mature pea leaves to characterize ELIPs expression, as these were the states of development in which the highest expression was obtained under conditions of high light stress in each species.
Early light induced proteins (ELIP) expression in young and mature grapevine and pea leaves under different temperatures in high light conditions (1000 µmol PAR m⁻² s⁻¹) during 4 h. A) Western blot using grapevine leaves. B) Western blot using pea leaves. Equal loading was checked by Ponceau Red staining. C) Graph showing the ELIPs expression in function of temperature in grapevine and pea leaves. Data are means of four replicates.

The fact that high light radiation and low temperature produce a lower ELIP expression may seem surprising, since this combination is known for accentuating photoinhibition (Somersalo and Krause, 1989; Huner et al., 1993), and because ELIP proteins have been associated with a possible photoprotective role.

However the behavior of the ELIP pea had already been studied at different temperatures but using a range of lower temperatures (between 0 and 20 °C) and a shorter time (2 h) (Adamska and Kloppstech, 1994), obtaining a similar result and observing that ELIP expression decreases linearly with temperature. The explanation for this decrease was found by these same authors when observing that low temperatures affected protein synthesis and that this increase was proportional to the increase of temperature. The wider range of temperatures used in this work, which also included high temperatures, allowed to prove that ELIPs expression in both grapevine and pea, reach a peak at 25 °C, since above this temperature no significant differences in the ELIP expression level were found. The different temperature obtaining the maximum ELIP expression level between this work and the previous study (Pinto et al., 2011) can be explained because of a better control of temperature in the present work. In order to study the impact of temperature on photoinhibition, the maximum photosynthetic efficiency (Fv/Fm) was measured in grapevine and pea leaves before and after being subjected to different temperatures in the presence of high light radiation (1000 µmol PAR m⁻² s⁻¹). From these values the percentage of photoinhibition was defined as the percentage of variation of Fv/Fm between its initial and final value. Fv/Fm and percentages values of photoinhibition are shown in Figures 3A and 3B, respectively. As expected, Fv/Fm (Figure 3A) decreased at low temperatures in both species, and therefore the percentage of photoinhibition increased in this temperature range (Figure 3B), since low temperatures combined with high radiation causes photoinhibition (Somersalo and Krause, 1989; Huner et al., 1993). But unlike of what was observed with the ELIP expression, in this case indeed differences were found between species being the lower percentage of photoinhibition in pea leaves at low temperatures. This indicates that pea leaves are more resistant to the combination of low temperatures and high light radiation that young grapevine leaves, which agrees with the conditions of growth of each leaf type (Hetherington et al., 1989; García de Cortázar, 2005).

The photoinhibition percentage in grapevine leaves at not stressful temperatures (25 to 30 °C), reached its minimum value and was similar to the values obtained from pea leaves, but at 35 °C increased again in both species.
species, being slightly higher in pea leaves. The fact that high light and high temperatures increases photoinhibition is expected since these conditions inhibit the photosynthetic efficiency in short time essays (Al-Khatib and Paulsen, 1989; Yin et al., 2010). It was observed when analyzing the relationship between ELIPs expression and photoinhibition percentage in both pea and grapevine species, there is a low ratio between these two variables under conditions previously described. This was confirmed by regression analysis (Figure 4). These results indicate photoinhibition degree caused by low or high temperatures had no effect on the induction of ELIP expression. As mentioned before, in the case of low temperatures, this result could be caused by the effect of temperature on protein synthesis in general (Adamska and Kloppstech, 1994). In fact, different studies have found relation between low temperature, photoinhibition and ELIP expression (Montané et al., 1997; Hutin et al., 2003) but these works used long time essays (acclimation conditions), therefore the effect of photoinhibition as one of the main factor in the induction of ELIPs cannot be ruled out. In the other hand, the photoinhibition because of high temperatures and high light was not relation with ELIP expression level because this parameter had not significant differences between 25 and 35 °C. No essays to analyze ELIP expression in long time conditions have been done using high light and high temperatures; hence more studies are necessary to know if ELIP expression is induced in these conditions, since plants are more tolerant to photoinhibition under high temperatures but in acclimation conditions (Karim et al., 2003).

**Effect of light intensity on the ELIP expression and the degree of photoinhibition**

When studying the effect of light intensity on the ELIP expression in both grapevine and pea leaves (Figure 5), it was observed that in young grapevine leaves light intensity affected linearly and directly the ELIP expression, until it reached 1000 µmol PAR m⁻² s⁻¹, after of which, the expression remained constant up to 2000 µmol PAR m⁻² s⁻¹. This fact contrasts with what was observed in pea leaves, where low light intensities have little effect on ELIP expression and only from 1000 µmol PAR m⁻² s⁻¹ an important jump is produced in the expression, which remained more or less constant up to 2000 µmol PAR m⁻² s⁻¹. These results indicate that the relative ELIP expression is less sensitive at low light intensities in pea leaves than in grapevine leaves, but at high light intensities this sensibility is high in both pea and grapevine leaves and the ELIP expression does not present significant differences between them. The behavior found for pea agrees with what was described previously (Adamska et al., 1992). These results also explain the lack of differences between species in the temperature essay versus ELIP expression, since in these essays a high light intensity (1000 µmol PAR m⁻² s⁻¹) was used, in which differences in the ELIP expression between grapevine and pea were not produced. The chosen temperature for the light intensity essays (30 °C) was an intermediate value within the temperatures
range in which the highest expression was obtained in both species (25-35 °C) in order to not cause unnecessary additional stress to the leaves, especially in cases where extreme light intensities were used.

When analyzing the effect of light intensity on Fv/Fm parameter and the photoinhibition percentage (Figure 6), it can be observed that in both grapevine and pea leaves the photoinhibition increase with the light intensity which it is agree with the literature (Aro et al., 1993; Niyogi, 1999). Although these values do not explain the higher ELIP expression at low light intensities in grapevine leaves, by having no significant differences in the photoinhibition degree between these species when varying light intensity, it can be said that in both grapevine and pea leaves are not required low temperatures and high light intensities to experience a significant photoinhibition degree as well as ELIP expression, as it does in Arabidopsis or barley (Montané et al., 1997; Hutin et al., 2003), which supports the idea that ELIPs are mainly induced in more sensitive species to photoinhibitory conditions or in plants supporting more extreme photoinhibitory conditions, indicating a participation in a photoprotection mechanism or in the photosynthetic apparatus repairing. Due to ELIP expression occurs when the photoinhibition has already been installed, it is most likely that the role of the ELIPs is related to repair mechanisms rather with those of protection or prevention.

Correlation analysis between photoinhibition percentage and ELIP expression in grapevine and pea leaves under different light intensities, showed that in both grapevine and pea leaves there was a high direct linear correlation and with a high significance degree, although this correlation was higher for pea. The regression analysis carried out for each species confirmed these results (Figure 7). These results along with those obtained by other species (Montané et al., 1999; Hutin et al., 2003) reinforces the idea that the photoinhibition degree, causes the activation of ELIP expression, when its synthesis is not limited by some factor such as temperature.

**CONCLUSIONS**

From these results, it can be concluded that ELIP expression is dependent of the leaves developmental stage but at the same time this is conditioned by the species. On the other hand, this expression is dependent of temperature, reaching its maximum over 25 °C and almost nil at low temperatures (10 °C) in short term stress. As far as the light intensity on ELIP expression it can be concluded that even though the expression is dependent of light intensity, this dependency varies with the species, being the expression in grapevine sensitive to a wide range of light intensities while expression in pea responds only to high light intensities (over 1000 µmol PAR m⁻² s⁻¹). In both species, the highest expression is reached at 1000 µmol PAR m⁻² s⁻¹. As far as the relationship between the degree of photoinhibition and ELIP expression it can be stated that exists a highly positive correlation.
correlation both in grapevine and pea when this expression is not limited by suboptimal conditions for protein synthesis (e.g., low temperatures). This fact supports the thesis that ELIP could be participating in repairing mechanisms of the photosynthetic apparatus rather than in photoprotection mechanisms.

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Expresión de una proteína inducida tempranamente por luz en vid y arveja bajo diferentes condiciones y su relación con la fotoinhibición. Las proteínas tempranamente inducidas por luz (ELIP) se expresan antes que otras proteínas del cloroplasto en presencia de luz. Estas proteínas han sido estudiadas en un gran número de especies anuales tales como arveja (Pisum sativum L.), cebada (Hordeum vulgare L.) y Arabidopsis sp. En plantas perennes los estudios sobre las ELIPs son muy escasos. La posible función fotoprotectora de las ELIPs ha motivado interés en investigar la presencia de este tipo de proteínas en una planta perenne como la vid (Vitis vinifera L.) y determinar si sus características difieren de aquellas encontradas en plantas anuales. En este trabajo se realizó un estudio comparativo sobre la expresión de ELIP en vid y arveja, para investigar si existen diferencias en las condiciones de temperatura e iluminación necesarias para la expresión máxima de ELIP en cada especie y para estudiar, en cada caso, la relación entre la expresión de ELIP y el grado de fotoinhibición. Los resultados de este estudio mostraron que la máxima expresión de ELIP se alcanzó a partir de los 25 °C y 1000 µmol PAR m⁻² s⁻¹ en ambas especies. Sobre estos valores la expresión permaneció constante. En cuanto al efecto de la temperatura y la intensidad de luz sobre el grado de fotoinhibición, la temperatura produjo una relación inversa en vid pero no hubo relación con arveja. Por otra parte, la intensidad de luz tuvo una relación directa en ambas especies. El efecto de la intensidad de luz sobre la expresión de ELIP sugiere que estas proteínas pueden tener un papel en fotoreparación del sistema fotosintético. El efecto de la temperatura sobre la expresión de ELIP, en ensayos a corto plazo puede estar asociado a las condiciones óptimas para su síntesis.

Palabras clave: Fotoinhibición, inducción de ELIPs, estrés abiótico, Vitis vinifera, Pisum sativum.

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