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Early induced protein 1 (*PrELIP1*) and other photosynthetic, stress and epigenetic regulation genes are involved in *Pinus radiata* D. don UV-B radiation response

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The continuous atmospheric and environmental deterioration is likely to increase, among others, the influx of ultraviolet B (UV-B) radiation. The plants have photoprotective responses, which are complex mechanisms involving different physiological responses, to avoid the damages caused by this radiation that may lead to plant death. We have studied the adaptive responses to UV-B in *Pinus radiata*, given the importance of this species in conifer forests and reforestation programs. We analyzed the photosynthetic activity, pigments content, and gene expression of candidate genes related to photosynthesis, stress and gene regulation in needles exposed to UV-B during a 96 h time course. The results reveal a clear increase of pigments under UV-B stress while photosynthetic activity decreased. The expression levels of the studied genes drastically changed after UV-B exposure, were stress related genes were upregulated while photosynthesis (RBCA and RBCS) and epigenetic regulation were downregulated (MSI1, CSDP2, SHM4). The novel gene PrELIP1, fully sequenced for this work, was upregulated and expressed mainly in the palisade parenchyma of needles. This gene has conserved domains related to the dissipation of the UV-B radiation that give to this protein a key role during photoprotection response of the needles in *Pinus radiata*.

Introduction

Forests are important players in ecosystems, providing food and raw material production for processing industries. The employment in reforestation of fast growing species, like *Pinus radiata* D. don allows a significant increase both in forest productivity and CO_2 uptake. So both natural and plantation forests are a significant part of the global carbon cycle. Tree growth rate and CO_2 uptake ultimately depend upon the photosynthetic activity reached by the trees.

Interestingly, some of these evergreen forests appear to be photosynthetically inactive for entire seasons. Studies of net ecosystem exchange of carbon dioxide have shown that subalpine forests and boreal forests decease carbon uptake throughout the winter (Zarter et al. 2006). The trees, however, remain green and continue to absorb light energy that is not being used to

Abbreviations – Cab, chlorophyll a/b-binding; ELIP, early light-induced protein; FISH, fluorescence in situ hybridization; PBS, phosphate buffered saline; ROS, reactive oxygen species; UV-B, ultraviolet-B.

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drive photosynthesis throughout the winter. Under such conditions, wherein trees are exposed to high intensities of excess light, photoprotective mechanisms seem to be especially necessary to prevent massive photobleaching of chlorophyll and loss of the forest canopy (Tausz et al. 2001, Zarter et al. 2006).

Ultraviolet-B (UV-B) radiation (280–320 nm) is an integral component of sunlight. UV-B radiation can cause damage to macromolecules, including DNA and proteins, and generate reactive oxygen species (ROS; Brosché and Strid 2003, Brown et al. 2005). UV-B radiation affects the growth, development, reproduction and survival of many organisms. Therefore any further increases of ambient UV-B exposure resulting from climate change is of concern as it may have a significant impact on natural and agricultural and forestry ecosystems. Hence, it is important to understand how plants protect themselves against the potentially damaging effects of UV-B in order to apply this knowledge to improve forestry production.

Photoprotection response is a very complex process that leads not only to physiological changes in the pigment content (Long et al. 1994, Tausz et al. 2001), but also changes at transcriptomic level (Brosché and Strid 2003, Maruta et al. 2010). The effects of UV-B radiation involve differential gene expression stimulating the expression of several genes characteristic of stress, defense and wound responses (Brosché and Strid 2003, Cloix and Jenkins 2008, Kusamo et al. 2011). Other genes related to photosynthesis are silenced (Albert et al. 2011). The induction of specific light stress proteins, like early light-induced proteins (ELIPs) can be considered to be part of these protective responses (Rossini et al. 2006, Zarter et al. 2006). Higher plant ELIPs are nuclear-encoded, light stress-induced proteins located in thylakoid membranes and related to light-harvesting chlorophyll a/b-binding (Cab) proteins. The accumulation of ELIPs under light stress conditions is correlated with the photoinactivation of PSII, degradation of the D_1-protein of PSII reaction center and changes in the level of pigments (Adamska 1997). The accumulation of ELIPs in the thylakoids is strictly controlled by the pigment content, especially by chlorophylls (Casazza et al. 2005, Rossini et al. 2006). Despite the great relevance of this family of genes in stress response in species like *Arabidopsis*, no study to date has been performed for describing its sequence and expression in *P. radiata*.

Although many plant responses to UV radiation have been reported mostly in *Arabidopsis* (Cloix and Jenkins 2008, Lang-Mladek et al. 2010, Kusamo et al. 2011), complete mechanistic details of the majority of these responses have not been elucidated. Epigenetic mechanisms can play an important role in this UV sensing, because they have ability to alter rapidly and reversibly being a key to the flexibility of plant responses to the environment (Mirouze and Paszkowski 2011). Knowing these mechanisms is particularly interesting in forestry species with high economical interest like *P. radiata*.

This article presents physiological and gene expression studies on candidate genes related to stress, photosynthesis and epigenetic codes, in *P. radiata* plantlets undergoing UV-B stress. The physiological tests include pigment content and photosynthetic activity, while the gene expression experiments used reverse transcription quantitative polymerase chain reaction (RT-qPCR) and fluorescence in situ hybridization (FISH). Analysis of putative *ELIP1* gene is especially interesting, given its proven implication in UV-B response in other species (Zarter et al. 2006, Cloix and Jenkins 2008).

Materials and methods

Plant material and growth conditions

P. radiata plantlets originated from selected seeds belonging to the NEIKER upgrading program (NEIKER Tecnalia, Pais Vasco, Spain) were grown in peat : vermiculite (3:1) supplemented with slow release fertilizers (3.5 g L^{-1}, Basacote Plus 6 *M*, Compo) for 5–6 months under controlled environment greenhouse conditions. Plantlets of similar appearance were subjected to the photoperiod condition showed in Fig. 1 for a duration of 4 days. UV-B radiation was applied using a UV lamp PHILIPS TUV 36 W G36T8 (250–350 nm). Eight plants were used per treatment. Recovery ability of plants was assayed by transference during 1 month to control photoperiodic conditions after UV-B exposure. Three experimental replicas were assayed.

Apical needles were collected from control plants without UV-B exposure and UV-B plants exposed for durations of 24, 48, 72 and 96 h, as well as needles from recovered plantlets, frozen in liquid nitrogen and stored at 80°C until RT-qPCR and pigments analyses. For photosynthetic analysis, apical needles were excised at the same time points listed above and used immediately for photosynthetic activity testing. For FISH analysis, fresh needles from control plants and plants exposed to

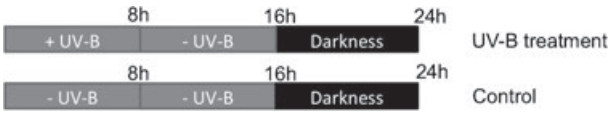

Fig. 1. Time line of UV-B treatment and controls.

UV-B for 48 h were fixed overnight (see section FISH analysis).

Measurements of photosynthetic activity

To evaluate photosynthetic damage, chlorophyll fluorescence induction kinetics was measured at room temperature on detached needles using either an imaging/pulse-amplitude modulation fluorimeter (OS1-FL, Opti-Sciences, Hudson, USA). Apical needles from eight plants per treatment were measured in each experimental replicate. Needles were pre-adapted in the dark for 20 min and then exposed to a saturating 1 s light flash. The minimal fluorescence (F_0) in the absence of actinic light and maximal fluorescence (F_m) after a saturating light flash were measured and the variable fluorescence ($F_v = F_m - F_0$) was calculated as described by Butler and Kitajima (1975). The photochemical yield of open PSII reaction centers, commonly known as the relative variable fluorescence, was calculated as F_v/F_m. This value reflects the maximal efficiency of PSII that is measured in dark-adapted tissues. The effective quantum yield of PSII photochemistry (PSII yield) was calculated as described by Maxwell and Johnson (2000): $\Delta F/F_m = ([F_m' - F_t]/F_m')$, where F_m' represented the maximal fluorescence in the light and F_t was the steady-state fluorescence yield in the light measured immediately before the saturating light flash. The PSII yield represents the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry.

Needle pigment content

For extraction and quantification of needle pigment content in all samples were collected in the three experimental replicates, using the protocol described by Sims and Gamon (2002).

Briefly, for chlorophyll/carotenoid measurement, 100 mg of needles were ground in 2 ml cold acetone/Tris buffer solution (80:20 vol : vol, pH = 7.8), centrifuged to remove particulates, and the supernatant was diluted to a final volume of 6 ml with additional acetone/Tris buffer. The absorbance of the extract solutions were measured to 663, 537, 647 and 470 nm and chlorophyll/carotenoid content calculated using the following equations:

$$\begin{aligned}\text{Chla} &= 0.01373 \times \text{A663nm} - 0.000897 \times \text{A537nm} \\ &\quad - 0.003046 \times \text{A647nm} \\ \text{Chlb} &= 0.02405 \times \text{A647nm} - 0.004305 \times \text{A537nm} \\ &\quad - 0.005507 \times \text{A663nm} \\ \text{Carotenoid} &= \{\text{A470} - [17.1 \times (\text{Chla} + \text{Chlb}) - 9.479 \\ &\quad \times \text{anthocyanins}]\}/119.26\end{aligned}$$

Anthocyanin quantifications of 100 mg needle samples were extracted with the same procedure as that for the chlorophyll measurements except that cold methanol/HCl/water (90 : 1 : 1, vol : vol : vol) was used in place of acetone/Tris buffer. The extract solutions were measured at 529 and 650 nm, and anthocyanin content calculated using the following equation:

$$\text{Anthocyanins} = \text{A529nm} - 0.288 \times \text{A650nm}$$

Candidate gene selection for gene expression analysis

Selection of candidate genes for RT-qPCR studies was performed based on the fact that the excess of UV-B light involves differential regulatory mechanisms in the plant (light and heat stress, oxidative stress, protein damage and gene regulation). Among a small number of *P. radiata* gene sequences available in public databases related to heat and oxidative stress (*EARLY LIGHT INDUCIBLE PROTEIN 1, ELIP1; ASCORBATE PEROXIDASE, APX; TAU CLASS GLUTATHIONE S-TRANSFERASE, GSTU; HEAT SHOCK PROTEIN 70, HSP70),* photosynthesis (*CARBONIC ANHYDRASE, CA; INORGANIC PYROPHOSPHATASE, iPyr; MALATE DEHYDROGENASE, MdH; RUBISCO ACTIVASE, RBCA; RUBISCO SMALL SUBUNIT, RBCS*) and epigenetic regulation (*COLD SHOCK DOMAIN PROTEIN2, CSADP2; MULTICOPY SUPRESSOR OF IRA, MSI1; S-ADENOSYLMETHIONINE SYNTETHASE 4, SHM4*) were considered for analysis based in the work of Valledor et al. (2010a). We have also amplified a sequence similar to a putative *ELIP1* gene in *Arabidopsis* to further study its implication in UV-B stress response in *P. radiata* given its key role in this process in *Arabidopsis*.

Quantitative RT-PCR expression analyses

Candidate genes were evaluated by RT-qPCR in needle samples taken from UV-B stressed, non-stressed and recovered plantlets.

RNA was isolated from 75 mg of frozen sample according to Chang et al. (1993). Resulting pellets were purified with RNeasy Clean Up (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) following the manufacturer's instructions. RNA integrity was tested in denaturing agarose gels. One microgram of total RNA from each sample and Superscript III first strand synthesis kit (Invitrogen, Carlsbad, California, USA) were used for first strand cDNA synthesis previous to qPCR. QPCR was performed as followed: 10 ng cDNA, 5 p*M* of each primer and Perfecta SYBR Green (Quanta Biosciences, Gaithersburg MD, USA) were mixed and amplified in ABI

7900HT system. Three measurements for each transcript and biological situation were analyzed using Prism software (Applied Biosystems, Carlsbad, California, USA). Relative quantifications were performed for all genes and *ACTIN* and *UBIQUITIN* (*UBI*) were used as loading controls. Primer sequences are in Table S1.

Amplification of 5′ and 3′ cDNA ends of *PrELIP1*

Available sequences for *PrELIP1* (dbEST accession GO096318) were amplified toward the 5′ and 3′ end employing RML-RACE kit (Ambion, Carlsbad, California, USA) according to manufacturer instructions. Primer sequences are in Table S2 (*PrELIP1* sequence available in Figure S1).

Phylogenetic and structural analyses of PrELIP1

The coding region of PrELIP1 was translated and compared with homologous proteins of *Brassica rapa* (GeneBank accession: AAR11456), *Arabidopsis thaliana* (gene model: AT3G22840.1), *Populus tremula* (GeneBank accession: XP_002312552), *Glycine max* (GeneBank accession: AAC16403), *Picea sitchensis* (GeneBank accession: ABK24316) and *Cupressus sempervirens* (ACI87755). Phylogenetic analysis was performed using the Jukes–Cantor genetic distance model and a tree was built using UPGMA method employing GENEIOUS 4.8 software for Mac (Biomatters Headquarters, Auckland, New Zealand). Distances tables are available in Table S3.

Protein structure was predicted employing ROSETTA V 3.3 homology modeling tool available at the server Robetta (http://robetta.bakerlab.org/). *PrELIP1*, *AtELIP1* (AT3G22840.1), *PsELIP* (ABK24316) and Arabidopsis Cab protein (AT1G29930.1) sequences were used. After modeling molecules were visualized using the latest available version software JMOL (JMOL: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/)

FISH of *PrELIP1*

PrELIP1 gene expression was evaluated in various needle tissues exposed to UV-B light, using FISH in combination with cryomicrotome sectioning under a confocal microscope. The following protocol described by Tirichine et al. (2009) was performed with several modifications. Fresh needles from control plants and from plants exposed to UV-B for 48 h were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.3 (Meijón et al. 2010). Tissues were sectioned to a 30–40 mm thickness using a cryomicrotome CH 1510-1 (Leica Instruments, Wetzlar, Germany). In the pretreatment to permeate the tissues the sections were incubated with 1 × PBS containing 0.5% triton X-100 for 10 min. Slides were washed in 1× PBS for 5 min to remove the detergent. Then, the sections were rendered permeable by incubating them for 2 h at 25°C in 2% cellulose in PBS and denatured in 0.2 N HCl for 30 min. The slides were rinsed with sterile water for 5 min before prehybridization. Next, the slide was rinsed in 2× SSC (3M NaCl, 0.3M Sodium Citrate, pH 7) for 2 min at RT and covered with approximately 100 µl prehybridization mixture HB50 (50% deionized formamide, 2× SSC, 50 m*M* phosphate, pH 7.0) (approximately 100 µl). The sample was incubated in a moist chamber at 37°C for 2 h. To make a fluorescent probe, PCR was performed with *PrELIP1* primers (Table S1) and cDNA template from control needles in the presence of 10× fluorochrome mixture (1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 0.65 m*M* dTTP, 0.35 m*M* ChromaTide Cy5-dUTP Amersham). After checking for the presence of probe in an agarose gel, the probe mix was prepared as follows: 10 µl of 20% dextran sulfate, 8 µl HB50 and 2 µl of probe. Then the probe mix was denatured at 100°C for 5 min and transferred immediately to ice. Finally the sample was denatured at 75°C for 5 min, and any excess fluid was drained from the denatured slide, and 20 µl of denatured *PrELIP1* probe was added to the sample. The hybridization was performed in a moist chamber at 58°C overnight. Then the sample was washed: 1 wash in 2× SSC for 15 min at 40°C; 1 wash in 1× SSC for 10 min at 40°C; 1 wash in 0.5× SSC for 10 min at RT and 1 wash in sterile water for 2 min at RT. Finally, the slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Fluka, St Louis, USA) 1 µg ml^{-1} for 10 min. Fluorescence was visualized using a confocal microscope (Leica TCS-SP2-AOBS, Wetzlar, Germany) connected to a work station and the images were processed with the Leica Confocal Software v2.5.

Statistical analysis

The data were analyzed using SIGMASTAT 3.1 software. Photosynthetic activity and pigment content were analyzed via one-way ANOVA (treatment and time) followed by the Holm–Sidak method.

For RT-qPCR, one-way ANOVA was performed. Subsequently, the Holm–Sidak test was used in all cases with the multiple comparison procedure. The significance level was set at $\alpha = 0.05$ for all tests.

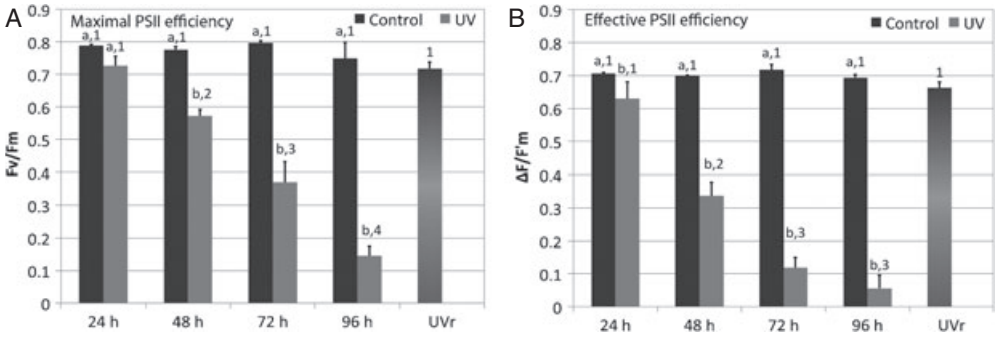

Fig. 2. Photosynthetic activity of controls, plants exposed to UV-B for 24, 48, 72, 96 h and plants recovered after 24 h exposure to UV-B (UVr): (A) maximal PSII efficiency (F_v/F_m) and (B) effective PSII efficiency ($\Delta F/F_m$). Different letters indicate significant differences between treatments at each time point. Different numbers indicate significant differences among times for each treatment (UVr plants were statistically analyzed as control plants). ANOVA test; *P*value < 0.05; n = 24. Bars indicate SD.

Results

Measurement of photosynthetic activity

UV-B radiation affected the photosynthetic activity of plants as observed by a decrease of F_v/F_m and $\Delta F/F_m$ with increasing exposure to UV-B radiation (Fig. 2). In the first 24 h, the UV-B plants show a decrease in photosynthetic activity; however, they do not reach values statistically different from control plants nor show F_v/F_m values below 0.6 which are typically associated with stress. After 48 h, the decrease of photosynthetic activity in the plants exposed to UV-B light is more pronounced and showing clear stress signals (Figure S2) with both F_v/F_m and $\Delta F/F_m$ values below 0.6 and statistically different from controls.

On the other hand, the UV-B plants recovered from 24 h UV-B exposure when transferred to normal photoperiodic conditions for 1 month showed values of F_v/F_m and $\Delta F/F_m$ not statistical different to control plants. The plants exposed to UV-B for more than 24 h could not recover.

Needle pigment content

Higher levels of all pigments were usually observed in the plants exposed to UV-B, with chlorophylls a and b, and anthocyanins showing striking statistically significant differences compared to control plants (Fig. 3). In contrast, all pigments analyzed in the recovered plants (UVr) always showed higher pigment content levels than the controls, but these differences were not statistically significant. Chlorophyll a and b reached elevated values in UV-B exposed plants, although both chlorophylls decreased at 72 h, presenting similar values to controls (Fig. 3A, B).

Carotenoids also showed higher levels in the plants treated with UV-B light, but in this case, the differences with the unexposed control were not significant and again a decrease in content was noted at 72 h (Fig. 3C). Anthocyanin content in UV-B exposed plants increased significantly when compared with the control, particularly at 72 and 96 h (Fig. 3D).

Quantitative RT-PCR expression analyses

The expression levels of genes associated with photosynthesis activity were also evaluated in RT-PCR products. The UV-B light exposure caused a clear decrease in the expression of genes related to photosynthesis (*CA*, MdH, *RBCA*, *RBCS;* Fig. 4A, C–E), except in *iPyr* (Fig. 4B). Conversely, in the recovery plants, these genes showed the highest levels of gene expression, except again *iPyr*.

A significant increase of gene expression, starting at 48 h of UV-B exposure, was also observed in all of genes related to stress, although the gene expression profile was different for each gene. The heat stress gene, *HSP70* (Fig. 4F), showed a gradual increase in its expression level, peaking in the recovered plants (UVr). Genes associated with oxidative stress, *GSTU* (Fig. 4G) and *APX* (Fig. 4H) both presented a similar expression peak at 72 h of UV-B exposure and a decline in mRNA levels between 72 and 96 h. Recovered plants showed similar mRNA levels to non-exposed control plants. *PrELIP1* expression (Fig. 4I) was especially interesting in UV-B plants irradiated 72 h or more, for the mRNA levels dropped drastically to reach values even lower than controls. The recovered plants showed levels slightly above control yet not significantly differences.

Finally, noteworthy epigenetic code associated genes included: SHM4, which relates to DNA methylation, MSI which is involved in the activation of imprinted genes, and CSDP2 which promotes DNA melting thus activating transcription. All these genes showed significant changes in mRNA levels. The specific expression profiles of

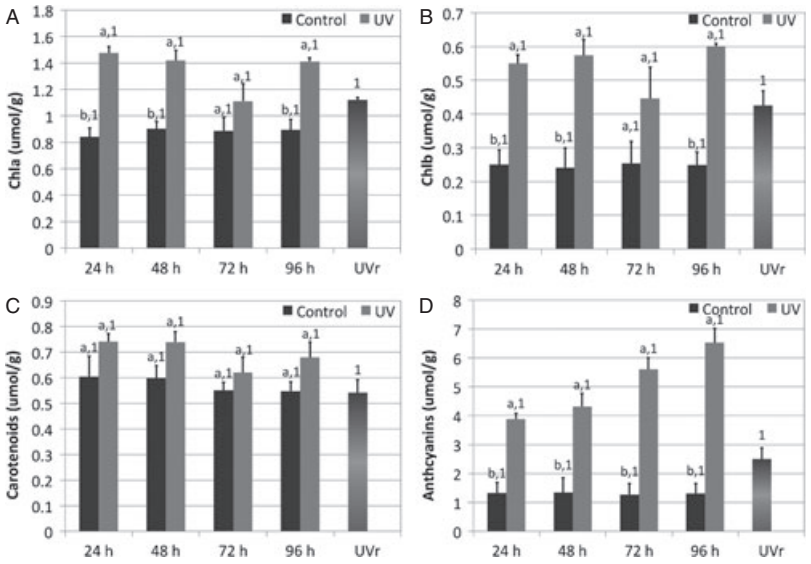

Fig. 3. Needle pigment content of control plants, plants exposed to UV-B for 24, 48, 72, 96 h and plants recovered after 24 h exposure to UV-B (UVr): (A) Chlorophyll a (Chla); (B) chlorophyll b (Chlb); (C) carotenoids and (D) anthocyanins. Different letters indicate significant differences between treatments within each time. Different numbers indicate significant differences among times within each treatment (UVr plants were statistically analyzed as control plants). ANOVA test; *P*value < 0.05; $n = 3$. Bars indicate SD.

these genes during UV-B radiation are summarized in Fig. 4J–L.

Phylogenetic analysis of *PrELIP1*

Given the key role of *ELIP1* in photoprotection of other species, the full-length cDNA in *P. radiata* were obtained and phylogenetic analysis performed to compare its similarity to other species. The *P. radiata ELIP1* ortholog to the *Arabidopsis SHR* gene was identified from EST clones in *P. radiata* cDNA libraries (Valledor et al. 2010a). By using 5′- and 3′-RACE, a full-length cDNA of 1 kb was identified (Figure S1). The cDNA contained a predicted protein sequence of 192 amino acids. ClustalW-based comparison of the amino acid sequence with previously described proteins revealed the presence of very conserved domains characteristic of ELIP proteins in all species analyzed (Figure S3). A phylogenetic analysis was performed to determine evolutionary relationships of *ELIP1* between other species and an unrooted neighbor-joining cladogram is presented (Fig. 5, Table S3). Evolutionary divergence was found between angiosperm and gymnosperms species, however, the genetic distance among the species analyzed for *ELIP1* sequence never exceed 0.25 units.

The structure of PrELIP (Fig. 6A) is similar to the ELIP proteins in other species (Fig. 6B, C). PrELIP has three transmembrane domains. The I and III a-helices, have a high homology with the corresponding helices of Cab proteins (Fig. 6D), and share chlorophyll-binding sites.

Fluorescent in situ hybridization

The function of the ELIP family proteins relates to the acclimation of sun exposure in other species, and a possible role in sustained photoprotection could be also considered in conifer needles. FISH method was applied to localize the *PrELIP1* expression in specific needle tissue of plantlets exposed to UV-B for 48 h (time of maximal expression detected by RT-qPCR) compared with control plants.

The needle structural organization consists in a central vascular cylinder (xylem and phloem) surrounded by a perivascular and outer spongy parenchyma layers. The two outermost cell layers correspond to palisade parenchyma which is covered by an epidermis layer (Valledor et al. 2010b). Needles exposed to UV-B show a strong signal of *PrELIP1* expression in palisade parenchyma (Fig. 7). Conversely, the central vascular cylinder, perivascular and spongy parenchyma layers showed a low *PrELIP1* signal. A very low signal was seen in palisade parenchyma and *PrELIP1* expression was not at all detected in the tissues surrounding the vascular cylinder of the control plants (Fig. 8).

Discussion

Increased UV-B radiation levels have been recently predicted as a consequence of the climate change. There has been much speculation about how increased UV

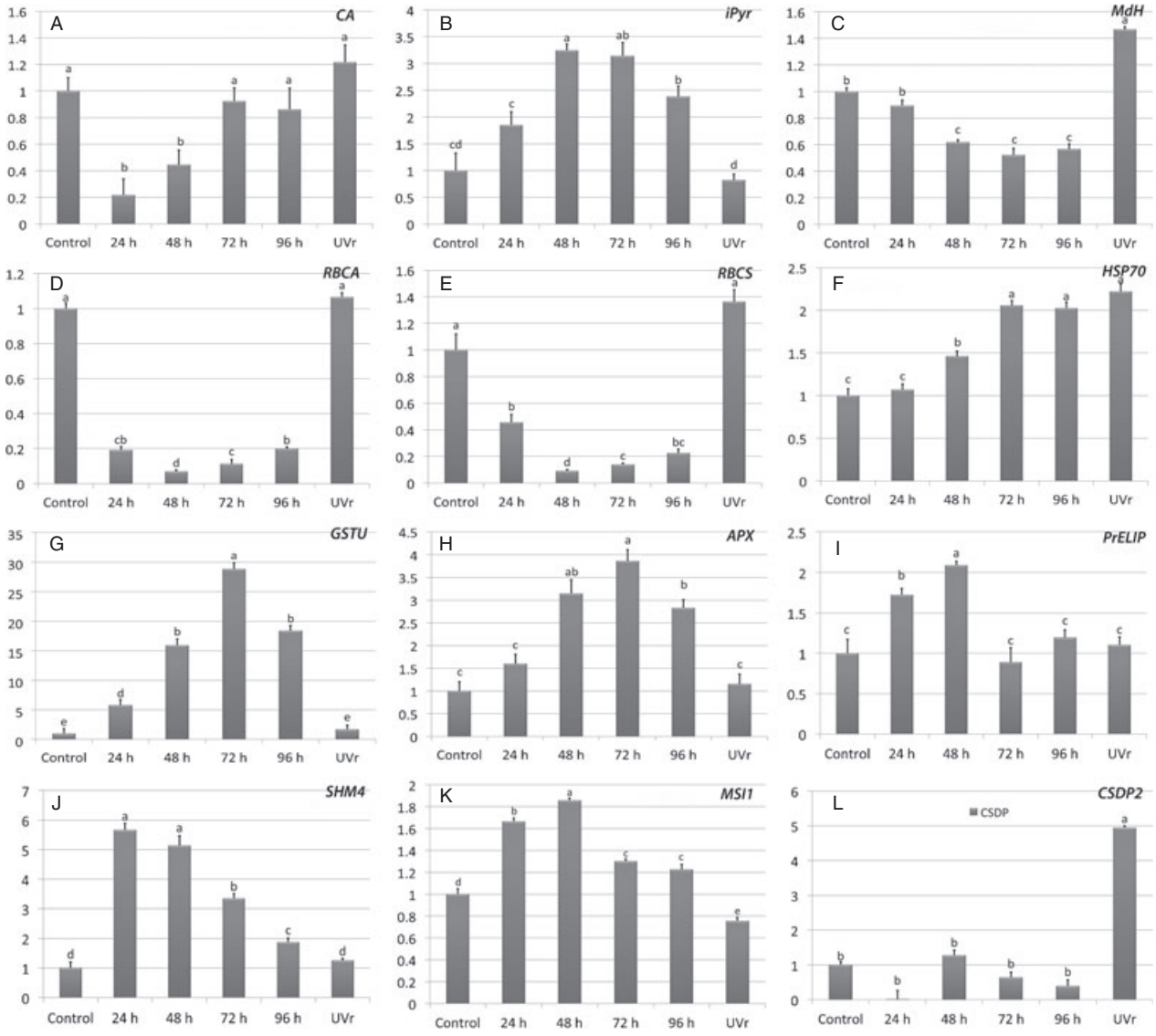

Fig. 4. mRNA expression levels obtained by RT-qPCR of needle samples from control plants and plants exposed to UV-B for 24, 48, 72, 96 h and plant recovered after 24 h exposure to Uv-B (Uvr), of indicated genes related to photosynthesis and stress: (A) *CARBONIC ANHYDRASE (CA);* (B) *INORGANIC PYROPHOSPHATASE (iPry)*; (C) *MALATE DEHYDROGENASE* (*MdH*); (D) *RUBISCO ACTIVASE (RBCA);* (E) *RUBISCO SMALL SUBUNIT* (*RBCS*); (F) *HEAT SHOCK PROTEIN 70 (HSP70*); (G) *TAU CLASS GLUTATHIONE S-TRANSFERASE* (*GSTU*); (H) *ASCORBATE PEROXIDASE (APX);* (I) *EARLY LIGHT INDUCIBLE PROTEIN 1 (PrELIP1);* (J) *S-ADENOSYLMETHIONINE SYNTETHASE 4 (SHMS4)*; (K) *MULTICOPY SUPRESSOR OF IRA1 (MSI1*) and (L) *COLD SHOCK DOMAIN PROTEIN2 (CSDP2).* Expression values were normalized and expressed as fold differences compared with *ACTIN* and *UBIQUITIN* constitutive gene expression. Different letters indicate significant differences between mRNA expression levels for each gene ANOVA test; P value < 0.05; $n = 3$. Bars indicate SD.

radiation exposure will affect plant development, but as yet, there are no definitive answers especially regarding forestry species. Forestry research is very scarce despite the importance of these species not only economically but also ecologically.

UV-B exposure has diverse effects on plants. It can damage macromolecules such as DNA and proteins, and cause the production of ROS that have the potential to inhibit cellular activities (Jenkins and Brown 2007). As a result, UV-B radiation may impair photosynthesis, membrane transport and other physiological processes, leading to tissue necrosis (Jansen et al. 1998). However, UV-B exposure can also act as a regulatory signal that affects morphogenesis and controls UV-protective responses (Brosché and Strid 2003, Jenkins and Brown 2007). In this context, there still are a number of issues

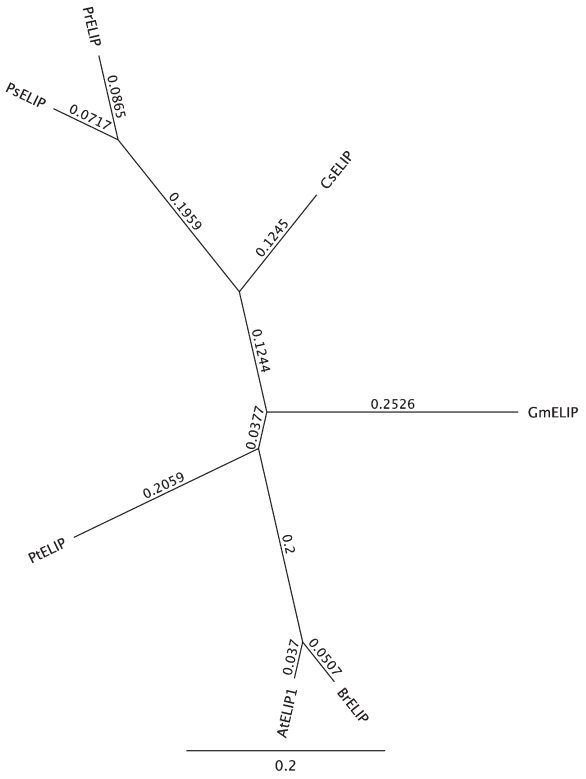

Fig. 5. Phylogenetic analysis of *ELIP* genes from different plant species. *Pinus radiata* (*PrELIP*), *Picea sitchensis* (*PsELIP*), *Cupressus sempervirens* (*CsELIP*), *Glycine max* (*CmELIP*), *Populus tremula* (PtELIP), *Arabidopsis thaliana* (*AtELIP*) and *Brassica rapa* (*BrELIP*).

of which little is known and which future research needs to address.

Through this work we can observe that *P. radiata* shows a great resistance to UV-B stress compared with other conifer species (Zarter et al. 2006). It was necessary to expose the *P. radiata* plantlets for at least 48 h of UV-B light to appreciate a decrease in photosynthetic activity. The maximal conversion efficiency of absorbed sunlight into PSII photochemistry, measured as F_v/F_m, was only significantly lower after 48 h of UV-B exposure (Fig. 2A) coinciding this time with the fastest increase of anthocyanins. Pigments are integrally related to the physiological function of needles in the conifer and provide interesting information about the health and physiology status of trees (Tausz et al. 2001). In this research, the plantlets exposed to UV-B light demonstrated an increase in all of the analyzed needle pigments, especially in anthocyanins after 48 h of exposure (Fig. 3). Pigments (chlorophylls, carotenoids and specially anthocyanins) help dissipate excess energy thus preventing damage to the photosynthetic system (Demmig-Adams and Adams 1996) and protect leaves from excess light (including UV). Anthocyanins may also serve as scavengers of reactive oxygen intermediates (Sherwin and Farrant 1998).

Numerous studies have shown that the effects of UV-B radiation involve the differential expression of a wide range of genes (Brown et al. 2005, Cloix and Jenkins 2008, Wolf et al. 2010). It is therefore important to understand the mechanisms of plant UV-B perception and signal transduction and how these processes lead to the regulation of gene activity.

Needles are the major photosynthetic organ of the coniferous trees. The expression of candidate genes relating to photosynthetic machinery were also evaluated in needles after UV-B exposure. The RBCA and RBCS are necessary enzymes for the RuBisCO activation mechanisms and consequently positive regulators of photosynthesis (Sage et al. 2008). The RT-qPCR data showed a significant decrease in the expression of these genes in the plants exposed to UV-B, especially after 48 h radiation. Similarly, ionic potential modulators of RuBisCO were also under expressed in needles exposed to UV-B light. These downregulated modulators include CA, which is implied in CO_2 capture, liberation and pH regulation, and MdH which is connected with NADPH photoactivation. The recovery plants had the most elevated levels of transcription. The energy production gene, iPyr, stimulates photosynthesis by hydrolyzing inorganic pyrophosphate as an alternative to an ATP energy donor for various energy requiring reactions (Serrano et al. 2007). iPyr is overexpressed, reaching maximal mRNA levels after 48 h of UV-B exposure. This overexpression must be related to the high incident radiation on plants as a result of exposure to UV-B. The photosynthetic machinery of the plant cannot effectively use this excess radiation. Thus, the roles of pigments and stress protection proteins seem to be essential in photoprotective response. This is corroborated by the high levels of *HSP70, APX* and *GSTU* expression and pigment content levels found after 48 h of UV-B light (Fig. 4F–H).

The imbalance between the rate of energy excitation arrival at the photosynthetic centers and the rate of the assimilation capability in these environmental situations results in an over excitation of the photosystems. This condition favors the formation of highly ROS that may produce photo-oxidative damages to the photosynthetic machinery components (pigments, proteins and nucleic acids). Plants have protective mechanisms against photo-oxidative damage, such as decreased light absorption, removal of excess excitation energy inside the photosystems, scavenging of ROS and up and downregulation of photosynthesis-related genes (Demmig-Adams and Adams 1996). ELIP is a protein family encoded by nuclear genes, synthesized in the cytoplasm and then

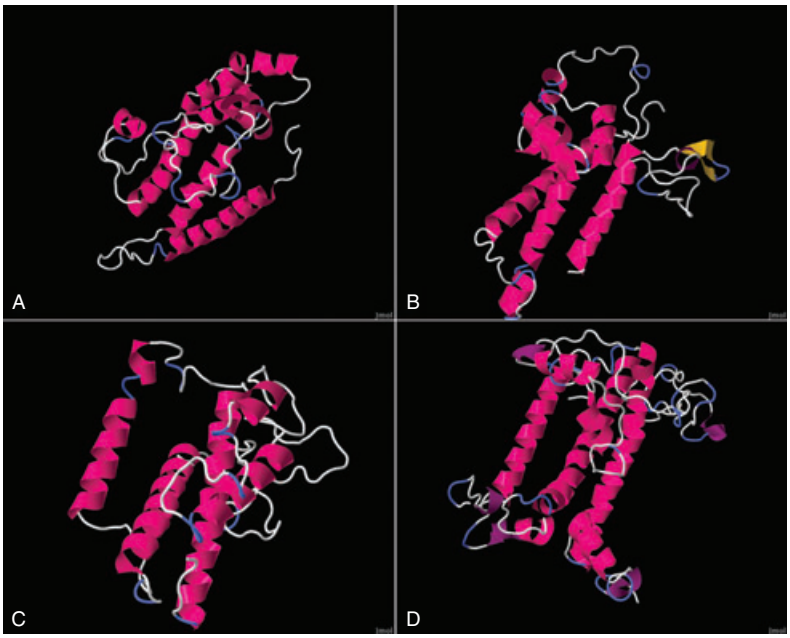

Fig. 6. Modeled 3D structure of different ELIP and chlorophyll-binding protein (Chlb). The three transmembrane helices are maintained across evolution: (A) *Pinus radiata* (PrELIP1); (B) *Picea sitchensis* PsELIP; (C) *Arabidopsis thaliana* (AtELIP1) and (D) *Arabidopsis thaliana* (AtChlb).

imported into the chloroplast to insert in the thylakoid membrane of plants exposed to stress (Adamska 2001, Hutin et al. 2002). The similarities found between the structures of the different ELIP proteins that have been modeled (Fig. 6), showing a highly conserved structure with the I and III a-helices sharing a high homology with the corresponding helices of Cab proteins. Furthermore, the comparison of the modeled structures and the amino acid sequences between ELIPs and Cabs (Fig. 6) suggests that ELIPs contain four putative chlorophyll-binding residues in the helices I and III structures which are maintained throughout evolution. Spectroscopic analysis of ELIP isolated from pea (*Pisum sativum)* leaves indicates the presence of chlorophyll a and lutein, with a high chlorophyll to lutein ratio. These data allow the hypothesis that ELIPs are more probably involved in energy dissipation than in light harvesting (Rossini et al. 2006). In this data, a rapid increase of *ELIP1* expression in the first hours under UV-B exposition (Fig. 4I) is supported by a strong FISH signal of *PrELIP1* mRNA located mainly in the needle palisade parenchyma (Fig. 7). Interestingly, after the first 48 h, *PrELIP1* expression levels decrease to values similar to control plants (Fig. 8A). This suggests a critical timing in the physiology of needles between 24 and 48 h under UV-B stress, after which it was not possible to recover plants. This 24–48 h time period seems long enough to permit damage to all of the protein repair mechanisms, which maintain the activity of most of the molecular mechanisms related to damage prevention. This hinders any protection against UV-B radiation and results in finally in cell death.

It has been demonstrated that the protein repair mechanisms are more stable and also upregulated under radiation in resistant organisms like *D. radiodurans*, making this organism more resistant to UV-B than other species (Daly 2009). These facts suggest that *PrELIP1* must provide an important role in the photoprotection of *P. radiata*. In *Arabidopsis* also have been observed that the accumulation of Elip1 transcripts and proteins increased almost linearly with increasing light intensities and correlated with the degree of photoinactivation and photodamage of PSII reaction centers (Heddad et al. 2006). In addition, the phylogenetic analysis showed that the sequence of *PrELIP1* is highly conserved between different species indicating again the relevance of this protection system for plants. Furthermore, in *Rhododendron catawbiense* (Peng et al. 2008) *ELIPs* expression constitutes an adaptive response to high radiation and winter cold playing a key function in the protection of photosynthetic.

Plants have developed sophisticated responses to adapt to heterogeneous environments. The epigenetic code response is needed for transducing environmental signals to altering gene expression, genomic architecture and defense (Grant-Downton and Dickinson 2006, Valledor et al. 2007). Epigenetic code is a gene expression regulatory mechanism that facilitates the necessary plasticity for plant survival, because it is quick

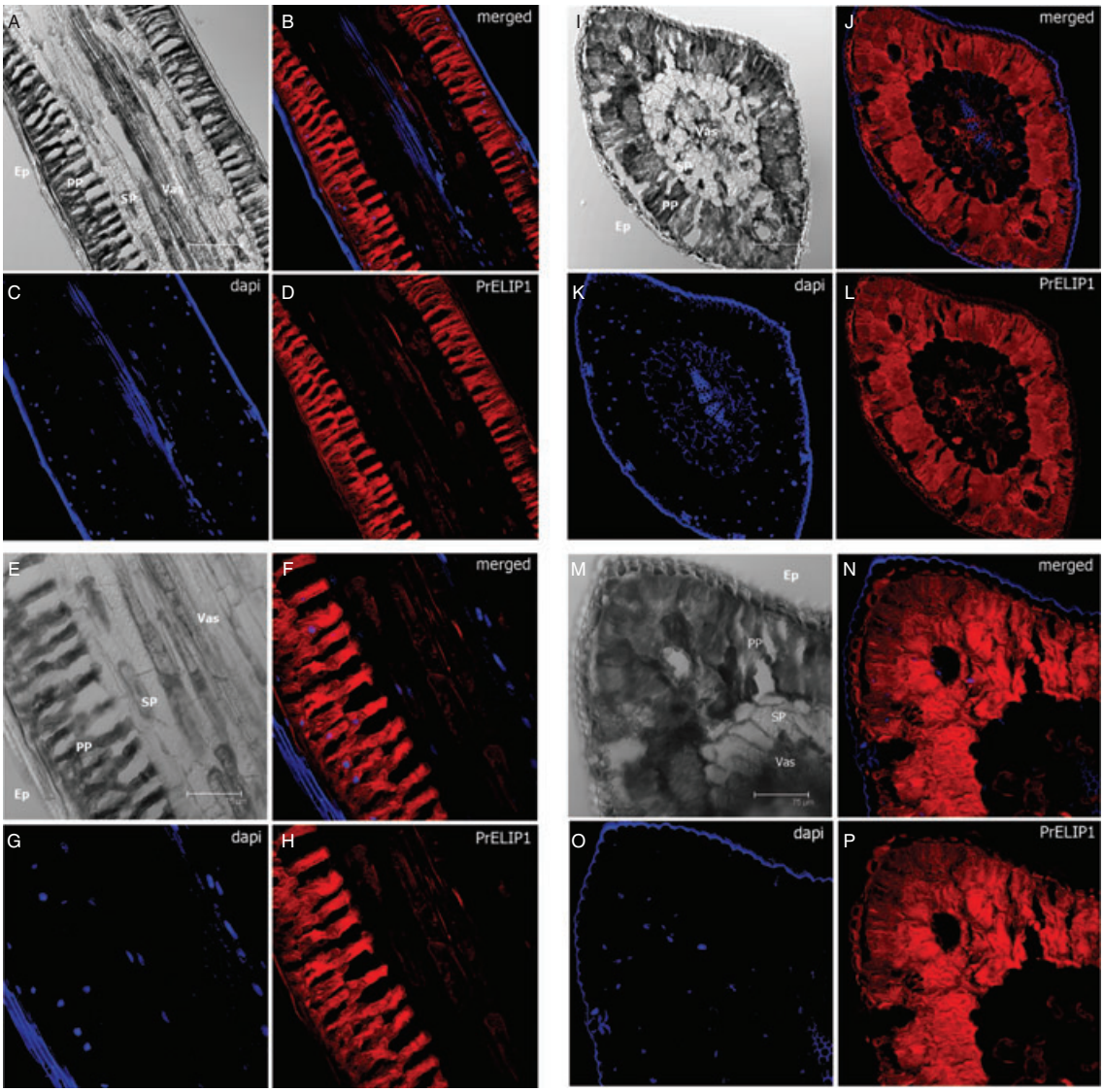

Fig. 7. FISH localization of *ELIP* (*PrELIP1)* mRNA in needles of *Pinus radiata* exposed to UV-B light for 48 h: (A) Differential interference contrast (DIC) in longitudinal axe (20×); (B) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals); (C) DAPI labeling; (D) *PrELIP1* mRNA labeling; (E) DIC in longitudinal axe (40×); (F) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals); (G) DAPI labeling; (H) *PrELIP1* mRNA labeling; (I) DIC in transversal axe (20×); (J) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals) (20×); (K) DAPI labeling; (L) *ELIP1* mRNA labeling; (M) DIC in transversal axe (40×); (N) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals) (40×); (O) DAPI labeling and (P) *PrELIP1* mRNA labeling. Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; Vas, vascular cylinder.

in response and reversible. Three different candidate genes related to epigenetic regulation were selected to evaluate the involvement of epigenetic changes in protective response to UV-B stress in *P. radiata*. *MSI1* was recently described as a Retinoblastoma-binding protein, related to the activation of imprinted genes in *Arabidopsis* through the transcriptional silencing of *MET1* (Jullien et al. 2008). Its expression level was significantly higher in the plants exposed to UV-B, and especially elevated in plants treated 48 h with UV-B light. This could be associated with the fact stressed needles activate the expression of genes involved in photoprotective responses, like *PrELIP1*. On the other hand, *CSDP2* encodes a glycine-rich protein that binds nucleic acids and promotes DNA melting, activating transcription. Recent discoveries point to the fact that *CSDP2* have an important role promoting growth under stress conditions (Park et al. 2009), a fact that may explain the high expression level of the transcript found in the recovery plants with high growth and new needle

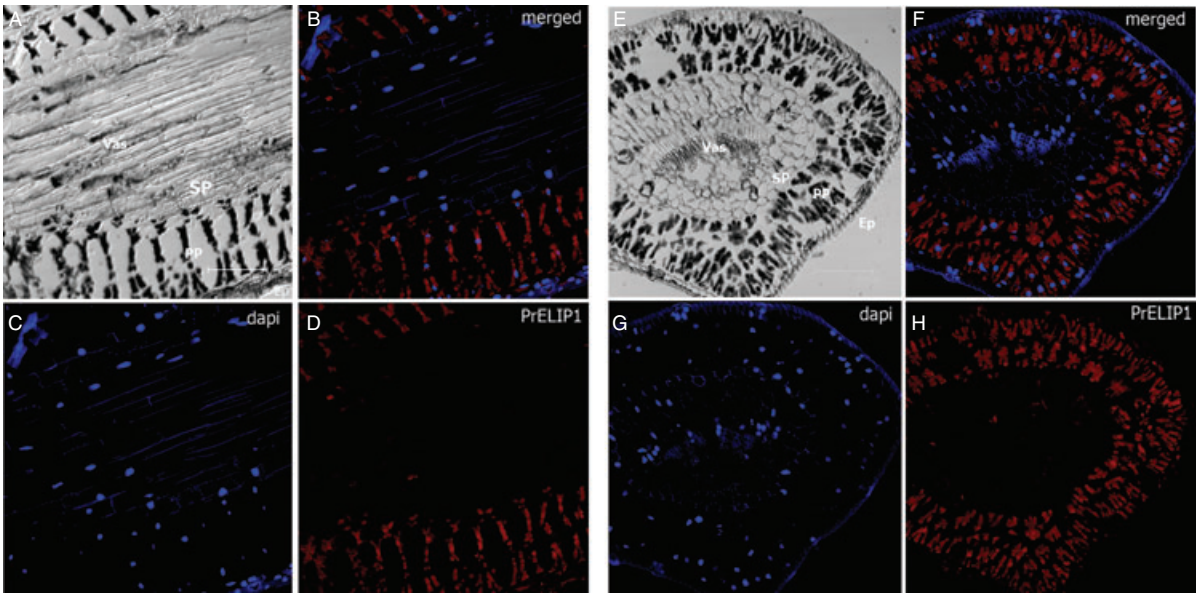

Fig. 8. FISH localization of *ELIP* (*PrELIP1)* mRNA in control needles of *Pinus radiata*. (A) Differential interference contrast (DIC) in longitudinal axe (20×); (B) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals); (C) DAPI labeling; (D) *PrELIP1* mRNA labeling; (I) DIC in transversal axe (20×); (J) DAPI (blue signals) superposition and *PrELIP1* mRNA (red signals) (20×); (K) DAPI labeling and (L) *PrELIP1* mRNA labeling. Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; Vas, vascular cylinder.

development. Finally, SHM4 is a stress-related enzyme which is related to the defense of photorespiratory impairment caused by an excess of light (McClung et al. 2000). SHM4 also serves predominantly to supply the cell with AdoMet, the substrate for various cellular methylation reactions (Martinov et al. 2000) such as DNA methylation (Ulrey et al. 2005), one of the best characterized epigenetic mechanisms. These functions maybe explain its extremely high levels of expression in the first 24 h under UV-B exposure and its subsequent decrease when the plant is too damaged to recover after UV-B exposure. Photoprotection response is a very complex process that leads not only to physiological changes, as the pigment content, but also changes at transcriptomic level. In *Arabidopsis* has been shown that the effects of UV-B involve clear differential gene expression stimulating the expression of several genes characteristic of stress, defense and wound responses (Cloix and Jenkins 2008) and silencing other related to photosynthesis (Albert et al. 2011) and these responses seem to be regulated largely by epigenetic code (Cloix and Jenkins 2008).

This research may ultimately advance knowledge about the physiological and transcriptional effects of UV-B on *P. radiata*, while conducting the first study of the *PrELIP1* gene sequence, its high expression levels in the palisade parenchyma of the *P. radiata* needle under the UV-B photoprotection response. Further studies are being carried out to understand the mechanisms of UV-B perception and signal transduction. Future research also will address how the epigenetic mechanisms regulate the plant response to UV-B radiation by changing the expression of specific genes and activating acclimatization mechanisms, particularly in conifer. This knowledge can be critical to generating biotechnological tools which allow trees to adapt in modulating climates.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *Pinus radiata ELIP1* sequence in 5′ → 3′ direction.

Fig. S2. Pictures of plants exposed to UV-B light for 24, 48, 72, 96 h, and plant recovered after 24 h exposure to UV-B.

Fig. S3. ClustalW-based comparison of ELIP amino acid sequence with previously described proteins.

Table S1. List of employed primers for RT-qPCR.

Table S2. List of employed primers for RACE 5′ and 3′ *ELIP1*.

Table S3. Distance matrix.

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