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PoCHL P expression pattern in *Posidonia oceanica* is related to critical light conditions

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ABSTRACT: In past decades, meadows of *Posidonia oceanica* (L) Delile have declined extensively in the Mediterranean basin mainly due to disturbances associated with reduced light availability. To investigate the molecular mechanisms of photoacclimation in this seagrass, we selected the *CHL P* gene as a marker of plant response to light availability because it is involved in the chlorophyll-tocopherols biosynthetic pathways. After characterizing the *PoCHL P* gene, its expression pattern was analyzed in leaves of plants growing in 2 distinct meadows that were previously categorized as disturbed and preserved. Plants were sampled during different seasons and light availability was monitored in the selected meadows over a 1 yr period. *PoCHL P* expression was also analyzed in leaves of plants that were exposed to controlled light conditions in aquaria. We found that *PoCHL P* was expressed differentially with respect to leaf developmental stage and that its expression pattern changed rapidly under light conditions far below the compensation value established for *P. oceanica*. Hence, the recognition of this gene as a reasonable marker of critical light conditions provides an important step towards the functional characterization of stress-related genes in seagrasses.

KEY WORDS: *PoCHL P* expression · *Posidonia oceanica* · Light availability

INTRODUCTION

Prolonged human activities in coastal areas of the Mediterranean basin have significantly influenced the quality of the marine environment and its hydrodynamic regime (Short & Wyllie-Echeverria 1996, Cancemi et al. 2003). One of the most relevant indicators of habitat degradation in this region is water turbidity, which threatens the survival of submersed plant species by reducing light availability (Marbà et al. 1996, Short & Wyllie-Echeverria 1996, Acunto et al. 2006). Unquestionably, light is a critical ecophysiological factor for plants. It not only influences resource synthesis through photosynthesis, but also modulates hormonal levels (Davies 1995) and signalling cascades (Sweere et al. 2001, Hutchison & Kieber 2002, Monte et al. 2007), which in turn regulate plant growth and development. Thus, the ability to elicit specific responses in relation to light quantity and quality represents a fundamental adaptation of plants to their native environment. Understandably, plants grown under prolonged light deprivation often exhibit decreased metabolic activity, predictably leading to premature senescence (Weaver & Amasino 2001, Lin & Wu 2004). Notably, light attenuation is a key factor affecting the growth and depth distribution of different seagrasses (Dennison 1987, Duarte 1991).

Posidonia oceanica (L) Delile is the most widespread and ecologically important seagrass of coastal Mediterranean ecosystems (Jeudy de Grissac & Boudouresque 1985, Pergent-Martini et al. 1994, Alcoverro et al. 1997). Due to its sensitivity to environmental perturbations, *P. oceanica* is widely recognized as a bioindicator of sea quality in this region (Pergent et al. 1995, Ferrat et al. 2003, Ruiz & Romero 2003, Tranchina et al. 2005, Lafabrie et al. 2007, Montefalcone 2009). The *P. oceanica* meadows have

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declined significantly throughout the Mediterranean basin over the past 2 decades (Marbà et al. 1996, Short & Wyllie-Echeverria 1996). In this context, we sought to obtain specific insights to help us understand how this particular seagrass responds to altered light availability. In the present work, we identified the *GERANYLGERANYL REDUCTASE* (*CHL P*) gene in *P. oceanica*, which encodes a multifunctional chloroplastic enzyme involved in both chlorophyll (chl) and tocopherol (TP) biosynthesis (Keller et al. 1998). This gene functions in vegetative growth and stress-defense mechanisms, mainly by protecting plastid membranes against oxidative stress through TP action (Kamal-Eldin & Appelqvist 1996). Accordingly, the *CHL P* gene is a reasonable candidate marker for studying light-stress responses of *P. oceanica* at the molecular level.

To date, *CHL P* has been studied in red algae (Bollivar et al. 1994), cyanobacteria (Addlesee et al. 1996) and in some terrestrial dicots, where its role in stress responses has been emphasized (Keller et al. 1998, Giannino et al. 2004, Bruno et al. 2009a). However, in monocots, information on *CHL P* orthologs exists mainly as a result of sequencing projects (*Zea mays*, GenBank: NM_001155910, and *Oryza sativa*, GenBank: Q6Z2T6) or direct database submission (*Triticum aestivum*, GenBank: DQ139268) (Alexandrov et al. 2009, International Rice Genome Sequencing Project 2005). To date, the function of *CHL P* has not been examined in any monocot, including all of the seagrasses, although a limited number of genes was generally investigated in the latter (Giordani et al. 2000, Cozza et al. 2006, Cozza & Pangaro 2009). In addition, a partial expressed sequence tag (EST) database of *P. oceanica* reporting 1219 tentative unigenes has recently been released (Wissler et al. 2009).

To investigate the putative role of the *PoCHL P* gene (*Posidonia Oceanica CHL P*) in stress response, we studied its expression pattern in *P. oceanica* growing in 2 distinct meadows. These meadows were previously classified as preserved and disturbed based on crucial ecological descriptors (Rende et al. 2005, Acunto et al. 2006), hormonal parameters (Cozza et al. 2004, Bruno et al. 2009b) and proteomic data (Mazzuca et al. 2009). Because the disturbed site was characterized by higher water turbidity (Rende et al. 2005, Acunto et al. 2006), the present study monitored light conditions in the 2 meadows over a 1 yr period. Expression of the *PoCHL P* gene was analyzed in leaves of plants that were sampled during different seasons as well as in those grown in aquaria under selected light conditions. In this way, we tested the extent to which *PoCHL P* was expressed differentially in relation to leaf development and light availability.

MATERIALS AND METHODS

Field sites. Plant material was collected in 2 *Posidonia oceanica* meadows located 3 km apart along the western coast of Calabria (Italy): a disturbed meadow (DM) (39° 48′ 45.71″ N, 15° 47′ 53.74″ E), and a preserved one (PM) (39° 54′ 28.83″ N, 15° 46′ 28.38″ E) (Acunto et al. 2006). From June 2005 to May 2006, light (lx) and temperature (°C) parameters were recorded every 30 min using a data logger (Hobo Pendant, Onset Computer Corporation) with a measurement range of 0 to 320 klx. The sensors were anchored in each meadow at 10 m depth. For light analysis, at each season (summer, autumn, winter and spring), specific time intervals per day (T) were considered: T-I (from 5:00/7:00–11:00 h), T-II (11:00–15:00 h) coincident with sampling time, and T-III (15:00–18:00/20:00 h). For the comparison between preserved and disturbed sites, statistical analysis was performed for each season plus T-time intervals using 1-way ANOVA. A post-hoc Neuman-Keul's test was used when the p-value was significant (<0.05).

Sampling of plant material. PM and DM plants were sampled at a depth of 10 m during different seasons (i.e. summer: June 2005; autumn: November 2005; spring: April 2006), which reflected different growth rates and metabolic states of *P. oceanica* (Ott 1979, Pirc 1985, Pirc 1986, Zupo et al. 1997). The winter season was excluded because it is a metabolic resting period for this plant species. Excised shoots, leaves and rhizomes were frozen in liquid nitrogen or fixed for *in situ* hybridization. Leaves were categorized as young (YL), intermediate (IL) or adult (AL) according to Giraud (1977), but only the first 2 cm of apical portions were used. Before fixation or freezing, epiphytes were gently removed from leaves using a sterile razor blade.

Aquarium experiments. Subsets of PM and DM plants (n = 12 for each sample) collected in April 2006 were transferred into 2 aquaria containing 100 l of seawater (pH 7.8, 15.5 ± 0.5°C [SE]) and provided with a water flow-through system and aeration throughout the course of the experiments. Plants were acclimated for 3 d under light conditions recorded at the T-II interval of the week before sampling. Thereafter, PM (n = 6) and DM (n = 6) plants were positioned side by side in each aquarium and exposed to either (mean ± SE) 3.37 ± 0.5 (L1) or 1.7 ± 0.4 µmol m^{-2} s^{-1} (L2), corresponding to the T-II mean values recorded in June in the PM and DM meadows, respectively. An incandescent lamp (15 W) was positioned at an adequate distance to achieve the desired conditions and was constantly monitored using the Hobo Pendant data logger. The selected light conditions were applied from 11:00 to 15:00 h, coincident with the T-II interval. For the remaining periods, the aquaria were exposed to ambi-

ent room light conditions, which ranged from 1 to 0 µmol m^{-2} s^{-1} (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m415p061_supp.pdf). After treatment, plant material (shoot apices, leaves, rhizomes and roots) was excised and frozen immediately.

Light data conversion. Photometric units that were provided by the Hobo sensors (lx) were converted into radiometric units (µmol m^{-2} s^{-1}) using the conversion factors (18 and 20 for daylight and incandescent light sources, respectively) previously reported by McCree (1981). Importantly, McCree's conversion is based on measurements performed in air. Thus, measurements taken under water at 10 m depth are approximate and relative rather than absolute, whereas values estimated for the aquaria are fairly close to the absolute.

Total RNA extraction and reverse transcription. Total RNA was extracted from samples following the procedure of Doyle & Doyle (1990) with some modifications. All solutions were prepared with RNase-free distilled water. Tissues (300 mg) were ground in liquid nitrogen while adding 0.1 g PVP-40 (polyvinylpyrrolidone, Sigma) directly to the mortar. One ml of freshly prepared extraction buffer, i.e. 200 mM Tris/HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA, 3 % hexadecyltrimethylammonium bromide (CTAB, w/v) and thereafter β-mercaptoethanol (final concentration 1.3 %), were added to the samples. After 30 min incubation at 60°C, 1 volume of chloroform/isoamyl alcohol (49:1) was added. After centrifugation at 6500 rpm for 15 min, the supernatant was recovered and precipitated overnight with isopropanol at –20°C. After centrifugation at 13 000 rpm for 15 min and washing with 0.2 M sodium acetate/70 % ethanol for 1 h at 4°C, RNA was resuspended in RNase-free water and treated with 30 U of DNase I (Roche) for 15 min at 37°C. The quality and quantity of RNA was verified using a spectrophotometer (NanoDrop ND-1000) and the integrity was checked electrophoretically on 0.8 % agarose gels. Total RNA (1 µg) from each sample was reverse transcribed with the SuperScript III reverse transcriptase and oligo dT(22) according to the manufacturer's instructions (Invitrogen).

Isolation and sequence analysis of cDNA and genomic clones. Leaf cDNA was amplified by PCR using the primers FW1 (5′-GTG CTG GCG GTN GAY GAY AG-3′) and BW1 (5′-GCG GAC GGG CTT NAG NAG-3′), which were selected within the region of highest conservation between tobacco and *Arabidopsis*. The resulting fragment was 911 bp long and homologous to the *CHL P* of the above mentioned plants. The 3′ region was identified by the 3′ RACE according to the manufacturer's instructions (Invitrogen) using a provided primer and the primer FW2 (5′-CAA GGC CTA GAA GGT TGC TG-3′). The 5′ region was isolated using primer BW5R (5′-ACT CTC CGG TCG ATG ATG TC-3′) and kit anchor primers (Invitrogen). Introns were identified by amplifying leaf genomic DNA (gDNA) with the following primers: FW1/BW1 and FW3 (5′-GGC CAG TAA GCC TTG TCA AC-3′)/BW3 (5′-GAG ACA TCG TCC TTG GGA GA-3′) (see Fig. 2A). Exon-intron boundaries were annotated by aligning the obtained genomic sequences with the full-length cDNA and comparing the results using 2 web programs (Plant Gene Data Bank server, www.plantgdb.org/cgi-bin/GeneSeqer/index.cgi, and ClustalW, www.ebi.ac.uk/clustalw/).

PCR components were 500 ng of gDNA or 200 ng of cDNA, 1 mM of each primer, 0.5 mM of dNTP, 2.5 U Taq DNA polymerase (GoTaq, Promega), 1/5 of 5X Taq buffer, and 7.5 mM of $MgCl_2$, in a final volume of 50 µl.

Thermocycling conditions used for gDNA PCR, cDNA and RACE-PCR experiments had a common initial cycle of 94°C for 3 min. The subsequent 35 amplification cycles were performed at 94°C for 40 s, 60°C for 60 s and 72°C for 90 s for gDNA, and at 94°C for 40 s, 55°C for 30 s, and 72°C for 30 s for cDNA. A final extension step at 72°C for 5 min was a common feature of both amplification thermal profiles. Multiple clones of all PCR fragments were cloned into the pGEM-T-Easy vector system (Promega) and sequenced by the Genelab ENEA service (Rome, Italy). Sequencing of overlapping fragments did not identify any differences or the presence of single nucleotide polymorphisms (SNPs).

Southern blot. gDNA was extracted from leaves following the Doyle & Doyle (1990) protocol. Southern blots and hybridizations were performed as described in Bruno et al. (2009a). Briefly, 10 µg of gDNA was restricted overnight at 37°C with *Sph*I and *Spe*I endonucleases (Promega), which do not cut in the probe (see Fig. 2A), in a 20 µl final volume. Digested DNA was precipitated, resuspended and electrophoresed for 20 h at 45 V on 0.8 % agarose gel and vacuum blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech). Digoxigenin (DIG)-labeled probe 2 (see Fig. 2A) was obtained after PCR amplification using 10 ng of gDNA, DIG-dNTPs (Roche) and primers FWS (5′-TGC GCT TGT TAC CAG AAC AC-3′) and BWS (5′-GCT GCG AAG TAG ATG CCT TC-3′), according to the manufacturer's instructions (see Fig. 2B). Prehybridization (3 h at 52°C) and hybridization (overnight at 52°C) were carried out in 20X SSC (3M NaCl, 300 mM sodium citrate), 0.01 % sarkosyl (10 %), and 0.1 % blocking reagent (2.5 % prepared in maleic acid buffer, pH 7.5) in a hybridizer (HB-2D, Techne). Detection was performed with anti-DIG-AP (Roche), nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) following the manufacturer's instructions (Roche). Hybridization bands were visualized by keeping the membrane in the dark for 2 h (blocked with Tris-ethylenediaminetetraacetic acid, TE) and then computer scanned (UMAX SPEED II).

***In situ* hybridization.** Excised tissues were fixed, dehydrated, embedded in paraffin and cut into 8 µm sections. Hybridizations were performed according to the procedure of Cañas et al. (1994), using the probe 1 spanning the 1162–1483 stretch which was cloned after PCR amplification with FW4 (5′-ACA AGG TGC TCG ATG TGT TG-3′) and BW4 (5′-CCC AGA TTC CCC TCT ACA GC-3′) primers. DIG-labeled RNA sense and anti-sense probes were obtained as described in Bruno et al. (2009a). Immunological detection was as reported by Chiappetta et al. (2009). Transcript accumulation was visualized as a violet/brown staining.

Semi-quantitative RT-PCR analysis. Primers used for *PoCHL P*, 223 bp were SeQFW (5′-ATC TCC GCC AAG TCC TTG TA-3′) and SeQBW (5′-ACG ATG TCT CCC CTT ACG TG-3′); for *P. oceanica 18S rRNA* (AY491942), 363 bp (used to check for equal amounts of cDNA template): 18S-FW1 (5′-CAT GGC CGT TCT TAG TTG GT-3′) and 18S-BW2 (5′-GTA CAA AGG GCA GGG ACG TA-3′); for the Rubisco large subunit gene (*rbcL*, U80719), 485 bp: R-FW (5′-ATC TTG GCA GCA TTC CGA GTA-3′) and R-BW (5′-AGC CCA CCA CGC AGA CAT T-3′). PCR trials were performed at distinct cycles to assess the variation in transcript abundance before signal saturation, at fixed primer pair and reaction parameters. Amplification products were sequenced to avoid artifacts. PCR reactions were conducted in a 50 µl total volume containing 3 µl of cDNA (other PCR components as given above).

PCR conditions for *PoCHL P* were 94°C for 3 min, 30 cycles of 94°C for 50 s, 58°C for 40 s, 72°C for 30 s, and 72°C for 5 min; for the *18S rRNA* gene, 18 cycles were used; for *rbcL* PCR, conditions differed in the number of cycles (30), annealing temperature (55°C for 40 s) and extension time (72°C for 35 s).

Fifteen µl were electrophoresed in a 1% agarose gel; each signal was determined using quantitative optical densitometry (OD ± SD) based on the pixel intensity/area of each PCR product as described in Chiappetta et al. (2009). Three replicate analyses were performed and density differences were evaluated using Tukey's test.

Chlorophyll pigments extraction. Experimental procedures followed Bruno et al. (2009a). Three replicate extractions were performed and for each replicate, 6 measurements were carried out on each sample. Statistical analysis was performed using 1-way ANOVA followed by Neuman-Keul's post-hoc test.

RESULTS

Meadow light conditions

Light data recorded in the 2 meadows were analyzed using the mean values of compensation ($\bar{I}_c$ = 7.8 ± 1.8 µmol m^{-2} s^{-1}) and saturating ($\bar{I}_k$ = 73.3 ± 16.1 µmol m^{-2} s^{-1}) irradiance, which respectively represent the minimum light available for survival and light conditions for maximum photosynthetic performance estimated for *Posidonia oceanica* (Lee et al. 2007). Specifically, lux based measurements were converted into photosynthetic photon flux density (PPFD, µmol m^{-2} s^{-1}) according to McCree (1981). The monthly mean values of PPFD for February and April to May were significantly lower, and always below the $\bar{I}_c$ value, for DM than for PM (Fig. 1A–C). These results are consistent with the greater number of days during which light values were recorded to be below the $\bar{I}_c$ value in DM (483 d) than in PM (232 d), with significant differences ($p < 0.001$) being detected in February, April and May (Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m415p061_supp.pdf). In Fig. 1D, the mean PPFD values recorded during the week before the sampling are highlighted. At the T-II interval, which is coincident with the sampling time, light availability was significantly higher in PM compared to DM for all samples. However, values for both meadows were below the $\bar{I}_c$ in June and were higher than the $\bar{I}_k$ limit values in November. In April, values were above and below the $\bar{I}_c$ limit for PM and DM, respectively.

Temperature did not differ significantly between the 2 meadows during the entire observation period (Fig. S2 in Supplement 1 at www.int-res.com/articles/suppl/m415p061_supp.pdf).

PoCHL P features and genomic organization

The *PoCHL P* gene (GenBank accession GQ421690) was 2515 nucleotides (nt) long, including an open reading frame (ORF) of 1392 nt and a canonical polyadenylation signal in the 3′UTR region (Fig. 2A). The deduced protein comprised 464 amino acids having a molecular mass of 51.3 kDa (estimated using BioEdit software). The PoCHL P protein exhibited (1) a transit peptide (tp) for cytoplasm-to-chloroplast import, identified using the ChloroP 1.1 prediction server (www.cbs.dtu.dk/services/ChloroP/), (2) 2 signature sequences conserved among CHL P proteins encoding for an enzyme with monoxygenase activity (Atta-Asafo-Adjei et al. 1993), and (3) the GXGXXG motif representing the binding site for the NADPH nucleotide moiety (Addlesee & Hunter 1999).

The PoCHL P protein exhibited highest similarity to the CHL P protein in *Arabidopsis thaliana* (92%) followed by those in *Nicotiana tabacum* (90%), *Glycine max* (89%), *Oryza sativa* (86%), *Triticum aestivum* (85%), and *Synechocystis* PCC 6803 (73%) as evaluated using the BioEdit program (Hall 1999).

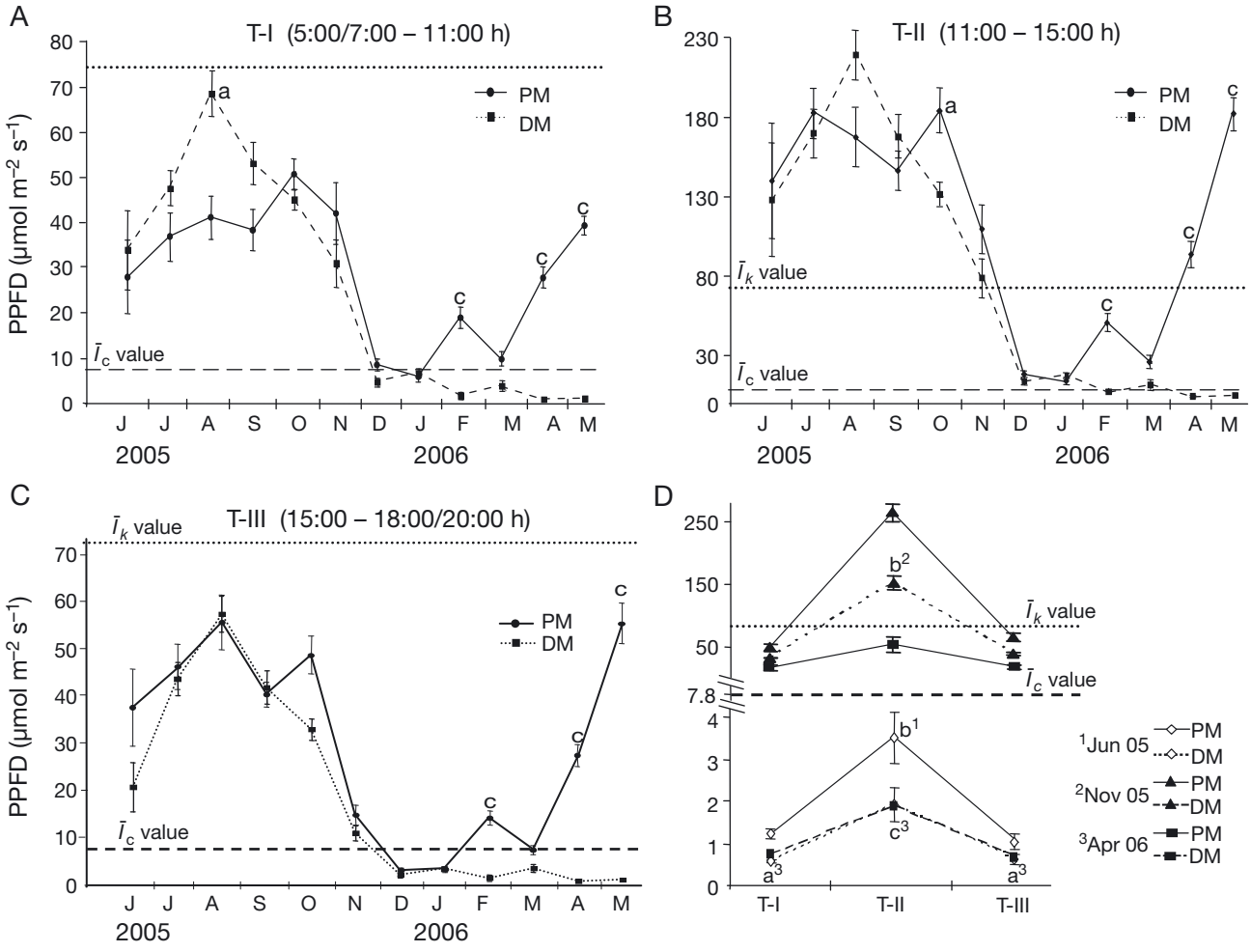

Fig. 1. (A–C) Monthly and (D) weekly mean photosynthetic photon flux density (PPFD) values (µmol m^{-2} s^{-1} ± SEM) recorded at specific time intervals (T) in preserved (PM) and disturbed (DM) *Posidonia oceanica* meadows. In (A–C), irradiance compensation mean value ($\bar{I}_c$ = 7.8 ± 1.8 µmol m^{-2} s^{-1}) (horizontal dashed line), and saturating irradiance mean value ($\bar{I}_k$ = 73.3 ± 16.1 µmol m^{-2} s^{-1}) (dotted line), estimated for *P. oceanica* (Lee et al. 2007). In (D), the reported values are related to the week before *P. oceanica* shoot sampling. Statistical analysis: 1-way ANOVA followed by post-hoc Neuman-Keul's test when p-value was significant (<0.05); a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$. Superscript numbers indicate significance related to comparisons of specific months

Southern blot analysis (Fig. 2A) produced a single band, thus indicating a single copy of *PoCHL P* in the *P. oceanica* genome. This observation is consistent with the absence of SNPs in the overlapping fragments (Fig. 2B).

Similar to the *CHL P* in *Arabidopsis* (but unlike other reported orthologs), *PoCHL P* exhibited 2 introns that both harbor the GT/AG editing motif; moreover, the position of the shorter intron was conserved.

PoCHL P tissue-specific expression

Using RT-PCR, *PoCHL P* expression was detected in shoot apices, leaves and faintly in roots, whereas transcripts were not detected in rhizomes notwithstanding the high number (i.e. 35) of PCR cycles used (Fig. 3).

Based on these results, *in situ* hybridizations (ISH) were performed using only shoot apices and leaves at different developmental stages. Gene expression was detected faintly in the shoot apical meristem, but was more intense in leaf primordia and developing leaflets (Fig. 4A). In fully developed leaves of all ages, *PoCHL P* transcripts accumulated primarily in the epidermal cells, especially along the blade margin (Fig. 4D); the signal in mesophyll tissue was faint and scattered (Fig. 4B,C,E). The phloem vascular bundles were also characterized by a marked signal (Fig. 4B,C,E).

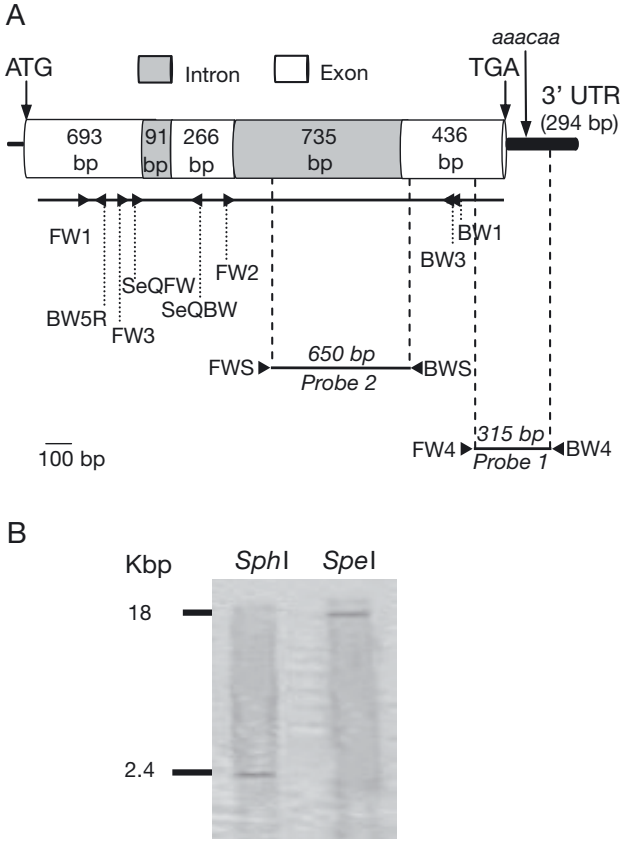

Fig. 2. *Posidonia oceanica*. *PoCHL P* gene features. (A) Scheme of the exon/intron organization; numbers inside indicate the length of base pairs (bp). Start and stop codons, polyadenylation signals and untranslated region (UTR) are also indicated. Relative position of probes (black bars), forward (FW) and backward (BW) directed primers (arrows) are shown. (B) Southern blot analysis: genomic DNA digested with *Sph*I and *Spe*I restriction endonucleases and hybridized with digoxigenin-labeled probe 2. Molecular weights of co-migrating DNA markers are in kilo base pairs (Kbp)

PoCHL P expression pattern in leaves

Natural environmental conditions

PoCHL P expression was evaluated using semi-quantitative RT-PCR in leaves (at different developmental stages) taken from plants sampled from PM and DM during June 2005, November 2005 and April 2006 (Fig. 5).

In PM plants, *PoCHL P* expression was higher in YL and IL compared to AL regardless of the period analyzed (Fig. 5). A comparable pattern was observed in DM plants sampled in November 2005, while an opposite trend was detected in DM plants sampled in June 2005 and April 2006 where gene expression was weaker in YL and IL as compared to AL (Fig. 5).

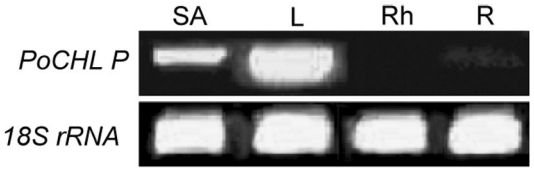

Fig. 3. *Posidonia oceanica*. *PoCHL P* gene expression in shoot apices (SA), leaves (L), rhizomes (Rh) and roots (R) estimated by 35 cycles of RT-PCR. Expression of the *18S rRNA* gene is also reported

Controlled light conditions

PoCHL P expression was evaluated in plants grown under controlled light conditions in aquaria. Plants sampled in April were chosen for this experiment because of their active physiological state and high growth rate. Two light treatments were applied: L1 (mean ± SE, PPFD = 3.37 ± 0.5 µmol m^{-2} s^{-1}) and L2 (PPFD = 1.7 ± 0.4 µmol m^{-2} s^{-1}), which corresponded to PPFD values at which differences in gene expression between plants from the 2 meadows had been previously detected (i.e. June 2005 sampling).

For PM plants, the switch from environmental to L1 conditions did not modify *PoCHL P* expression in the leaves, whereas the pattern of gene expression under L2 conditions was quite comparable to that observed under environmentally disturbed conditions (DM plants) (Fig. 5). In contrast, exposure of DM plants to L1 conditions affected their *PoCHL P* expression pattern, yielding results similar to those of PM plants although transcription in YL still appeared to be down regulated (Fig. 5). Under L2 conditions, *PoCHL P* expression in DM plants was almost unchanged, with the exception of a higher expression level in IL compared to IL expression in environmentally disturbed conditions.

In both the field and aquarium experiments, the expression pattern of the *rbcL* gene (a marker of photosynthetic activity) overlapped with that observed for *PoCHL P*.

Leaf chlorophyll content

Total chl content generally increased during leaf development, with the lowest values being detected in YL (Table 1). Furthermore, for naturally growing plants, the total chl content was higher in YL of DM vs. those of PM in June 2005 and April 2006; a reduced chl content was observed in IL of DM vs. those of PM plants in November 2005 (Table 1A).

In aquarium experiments, chl content increased in the leaves of DM plants exposed to L2 conditions

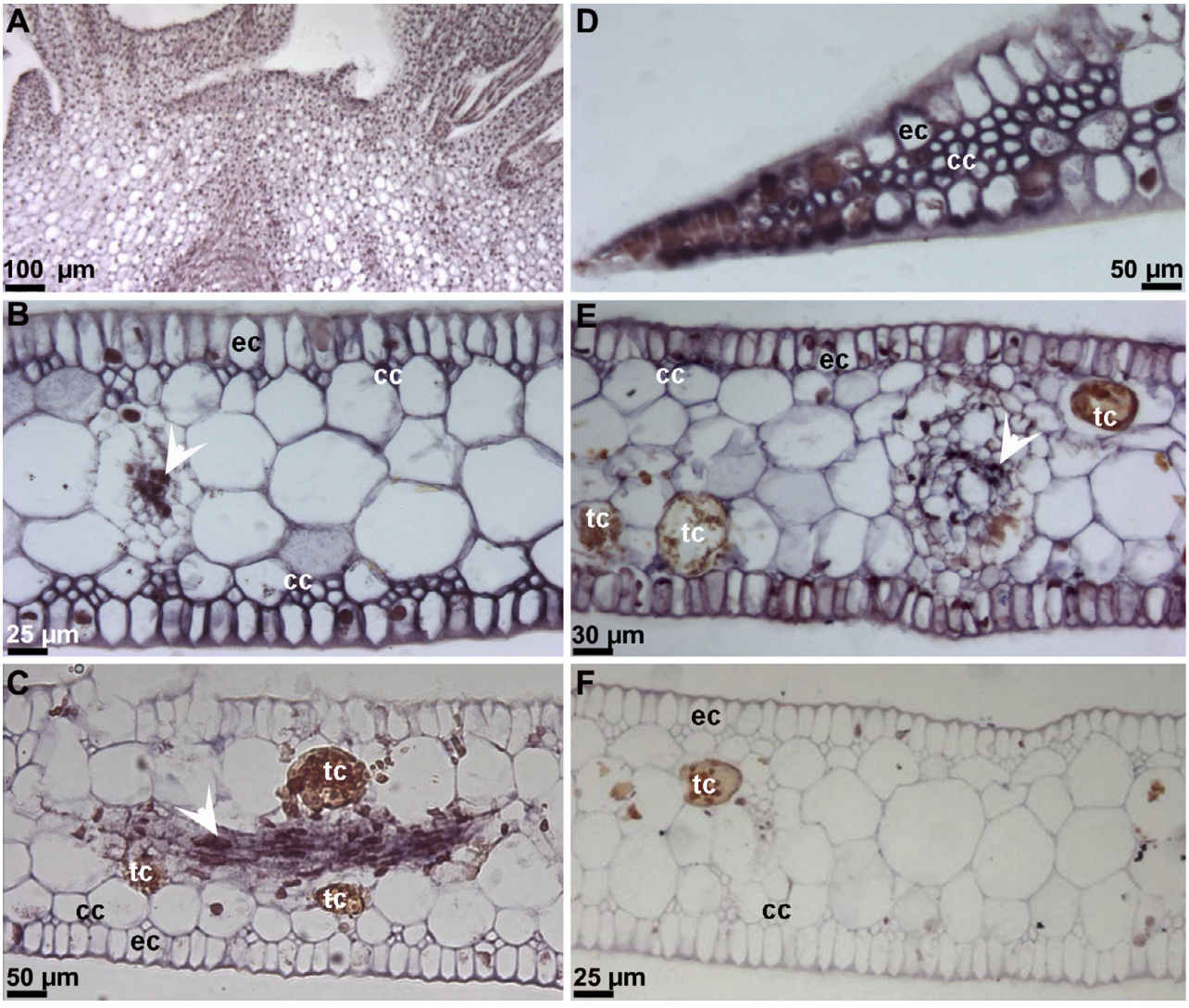

Fig. 4. *Posidonia oceanica*. *In situ* localization of *PoCHL P* mRNAs (violet signal) on (A) shoot apex longitudinal section and (B–F) leaf cross sections; (B–D) young leaf, (E–F) intermediate leaf, (A–E) samples treated with anti-sense probe. (F) Leaf treated with sense probe as negative control. ec: epidermal cells, cc: collenchyma cells, tc: tannin cells, white arrowhead: phloematic portion of vascular bundles

regardless of their developmental stage (Table 1B). However, in both naturally growing and aquarium plants, the chl *a*/*b* ratio remained relatively constant, ranging from 1.81 ± 0.06 to 2.82 ± 0.09 (Table S2 in Supplement 1 at www.int-res.com/articles/suppl/m415p061_supp.pdf).

DISCUSSION

Here, we demonstrate that the *PoCHL P* gene of *Posidonia oceanica* is expressed differentially with respect to both endogenous developmental cues and stressful light conditions, in a manner comparable to that found in terrestrial plants (Giannino et al. 2004, Bruno et al. 2009a). Indeed, this report represents the first functional characterization of a light-stress related gene in any seagrass analyzed under different environmental conditions.

The *CHL P* gene encodes a protein with a multifunctional metabolic role that is closely associated with chloroplast differentiation and protection (Keller et al. 1998). In *Posidonia oceanica*, *PoCHL P* was expressed primarily in the leaves and transcripts accumulated mainly in the epidermal layer. This observation is consistent with the anatomy of *Posidonia* leaves in which the epidermal cells harbor numerous chloroplasts in contrast to the mesophyll cells; the latter contain few chloroplasts and exhibit large vacuoles that are often entirely filled with phenolics (Mariani Colombo et al.

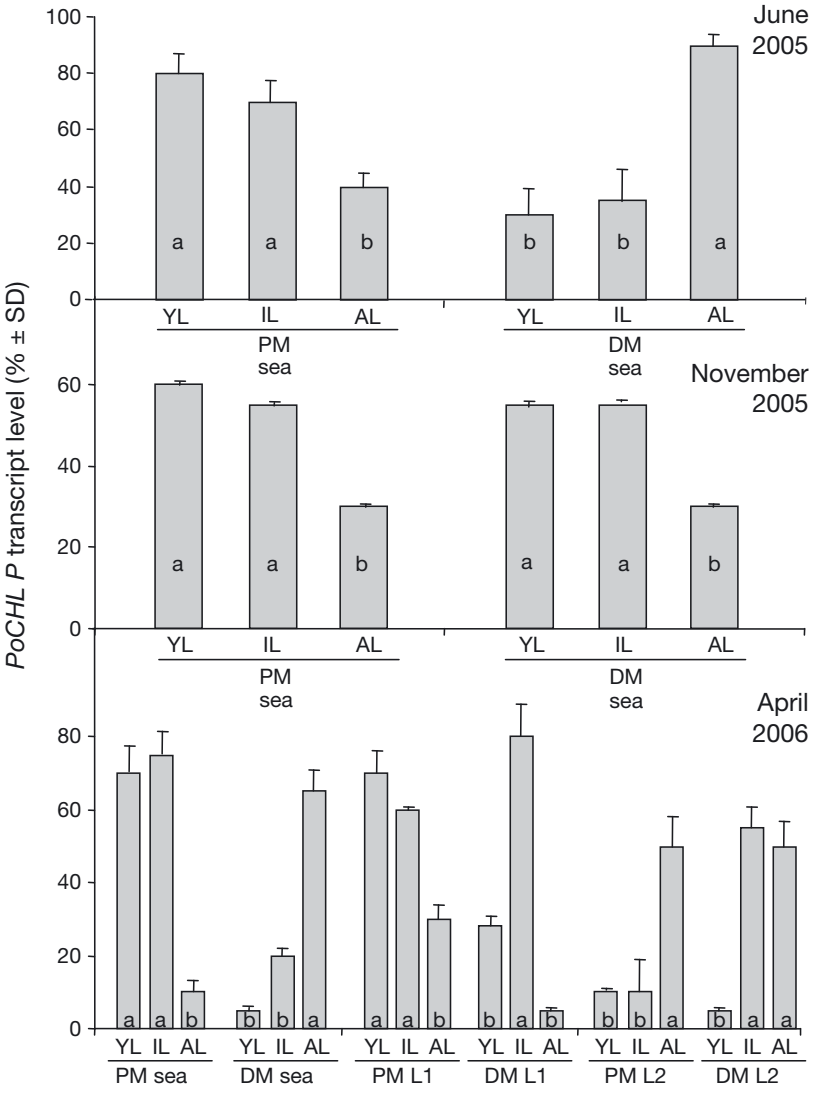

Fig. 5. *Posidonia oceanica*. Relative levels of *PoCHL P* transcripts, normalized with the *18S rRNA* gene, in plants sampled from preserved (PM) and disturbed meadows (DM) at different seasons and grown in the natural environment or in aquaria under controlled light conditions (L1 = 1.7 ± 0.4 µmol m^{-2} s^{-1} and L2 = 3.37 ± 0.5 µmol m^{-2} s^{-1}). Relative levels of *PoCHL P* transcripts were estimated by quantitative densitometry of the PCR products resolved by electrophoresis. All columns marked with the same letter are not significantly different at p = 0.05 (Tukey's test). YL: young leaf, IL: intermediate leaf, AL: adult leaf

As reported in terrestrial plants (Giannino et al. 2004, Bruno et al. 2009a), our results showed that *PoCHL P* is also expressed differentially in leaves with respect to their developmental stage. However, the pattern of gene expression did not overlap between plants growing in different meadows that were previously categorized as preserved (PM) and disturbed (DM) sites for *Posidonia oceanica* based on several relevant fitness parameters (Cozza et al. 2004, Rende et al. 2005, Acunto et al. 2006, Bruno et al. 2009b). Indeed, in plants from preserved sites, *PoCHL P* expression was higher in fast-growing YL and IL than in AL — an observation that is consistent with the pattern described for pluriannual vs. deciduous leaves of terrestrial plants (Bruno et al. 2009a). In contrast, plants from disturbed sites exhibited such a pattern only in the autumn sampling. Instead, a down regulation of gene expression was detected in YL and IL of DM vs. those of PM plants during late spring and early summer. At the same time, AL, which are characterized by a slow growth rate, exhibited higher gene expression in DM compared to PM plants. Consistent with photoadaptive responses to modified light conditions reported elsewhere (e.g. Tomasko 1993), a different modulation of chl content in relation to developmental stage was also observed in PM vs. DM plants, together with a chl *a*/*b* ratio that is analogous to that reported by Pirc (1986) for *P. oceanica*. All these results suggest different photosynthetic competence in PM vs. DM plants.

The expression of the *rbcL* gene in *Posidonia oceanica* leaves varied with environmental conditions and consistently overlapped with that of *PoCHL P*. Thus, it is reasonable, at least in this case, for *PoCHL P* expression to provide an indirect link to photosynthetic activity. In this context, the down regulation of both these genes in YL and IL of DM plants is consistent with the observed reduction in leaf growth rate that was previously recorded in DM (Rende et al. 2005, Acunto et al. 2006, Rende et al. 2006). Notably, the monthly mean irradiance recorded in the DM site for 3 consecutive months during spring (a period that is coincident with carbohydrate storage in the rhizome; Pirc 1985, Alcoverro et al. 2001) was lower than the $\tilde{I}_c$, which represents

1983, Cozza et al. 2004). Notably, *PoCHL P* transcripts were also detected in the leaf phloem tissue — a finding that has not been previously reported (Giannino et al. 2004, Bruno et al. 2009a). Because the *CHL P* gene functions in the biosynthesis of TP and chl, its expression in the phloem could be linked to a proposed role of TP in regulating the translocation of photoassimilates through the modulation of symplastic continuity between the bundle sheath and vascular parenchyma (Hofius et al. 2004, Abbasi et al. 2007, Maeda & DellaPenna 2007).

Table 1. *Posidonia oceanica*. Total chlorophyll content in leaves sampled from preserved (PM) and disturbed meadows (DM) (A) at different periods and (B) after 1 wk of aquarium treatment at L1 and L2. YL: young leaf, IL: intermediate leaf, AL: adult leaf. Statistical analysis: 1-way ANOVA followed by Neuman Keul's post-hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Sampling period and site	Total chl (mg g^{-1} fresh weight ± SEM) YL	IL	AL
(A) Meadows (Sea)			
June 2005			
PM	0.054 ± 0.01	0.581 ± 0.03	0.644 ± 0.02
DM	0.314 ± 0.03*	0.513 ± 0.03	0.516 ± 0.02
November 2005			
PM	0.087 ± 0.01	0.754 ± 0.02	0.978 ± 0.04
DM	0.201 ± 0.02	0.442 ± 0.01*	0.723 ± 0.04
April 2006			
PM	0.155 ± 0.06	0.681 ± 0.01	0.839 ± 0.01
DM	0.413 ± 0.01***	0.643 ± 0.03	0.716 ± 0.03
(B) Aquaria			
April 2006			
PM-L1	0.071 ± 0.01	0.646 ± 0.01	0.681 ± 0.04
PM-L2	0.385 ± 0.05***	0.509 ± 0.07	0.641 ± 0.01
DM-L1	0.234 ± 0.06	0.301 ± 0.03	0.692 ± 0.02
DM-L2	0.368 ± 0.01*	0.450 ± 0.04**	1.242 ± 0.05***

minimal light requirements to sustain optimal growth of *P. oceanica* (Lee et al. 2007). Resource depletion associated with such prolonged periods of low irradiance ($<\bar{I}_c$) could possibly account for reduced growth rates in DM (Alcoverro et al. 2001). However, a high level of *rbcL* and *PoCHL P* expression was detected in AL of DM vs. those of PM plants. Given the subsidiary role of *CHL P* in both chl and TP biosynthesis, the similar levels of chl in AL of PM and DM plants prompt us to speculate that the high level of *PoCHL P* expression in these leaves may be related to TP requirement as an adaptive response to shield plants from both oxidative and aging processes (Weaver & Amasino 2001, Havaux et al. 2005). Oxidative and aging processes could precociously occur in AL of PM plants due to the absence of adequate levels of cytokinins (Cozza et al. 2004), which are known to play a key role in preventing leaf senescence. Moreover, TP might be required to sustain resource translocation (Hofius et al. 2004) at a critical moment in the plant life cycle. This is suggested by our evidence of localized gene activity in vascular tissue as well as by the changes in the pattern of *PoCHL P* expression that were observed in the leaves of DM plants at very critical light conditions for plant growth (mean ± SE, 1.7 ± 0.4 µmol m^{-2} s^{-1}). However, because we did not experimentally investigate the fluxes through these pathways, further investigations are necessary to tightly relate gene expression with the overall metabolic state of plants.

Our results also demonstrated that temperature did not modulate *PoCHL P* transcriptional activity within the thermal range normally experienced in this marine environment. Indeed, the pattern of *PoCHL P* expression in DM plants sampled in April and June was quite comparable despite the observed variation in temperature (15.5 to 22.8°C). Interestingly, PM plants grown in an aquarium for 1 wk at 1.7 ± 0.4 µmol m^{-2} s^{-1} exhibited a pattern comparable to that recorded in DM plants under corresponding natural light conditions independent of temperature. Thus, critical light conditions over periods of <1 wk can switch the pattern of gene expression in leaves. Notably, a reversion to the PM pattern was obtained in aquarium grown DM plants at 3.7 ± 0.5 µmol m^{-2} s^{-1}, although partial gene expression in YL leaves indicated that the reversion was incomplete.

In conclusion, our results showed that the pattern of *CHL P* expression in *Posidonia oceanica* is modulated by light conditions and is related to both the differentiation and metabolic status of the leaves. This finding is consistent with other reports indicating that seagrasses can reconfigure their physiology in relation to the photo-acclimation process (Neverauskas 1988, Ruiz & Romero 2001). In this context, it should be noted that our light data was derived from lux measurements. However, this is not critically important, as the relevant light values recorded in this work are close to, or are below the $\bar{I}_c$ and substantially lower than the $\bar{I}_k$. Hence, we have interpreted the laboratory and field data in relation to relative as opposed to absolute differences in light intensity. Notably, the narrow window of irradiance values (3.7 to 1.7 µmol m^{-2} s^{-1}), which can induce changes in *PoCHL P* expression, indicates that this gene is a reasonable molecular marker for evaluating the extent of stress imposed by low light levels in *P. oceanica*. Hence, this study of the *PoCHL P* gene provides some novel insights into the development and application of molecular environmental indicators for seagrasses, and in particular, the management of *P. oceanica* ecosystems. However, the rapid alteration of gene activity in response to changes in irradiance makes *PoCHL P* expression an unsuitable marker for meadow-monitoring activities, which seek to provide information over longer time scales.

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