UNIVERSITA' DELLA CALABRIA

PhD thesis, Doctorate Research Program in Plant Biology
XIX Cycle (2003-2006)

Response to light conditions in *Posidonia oceanica* (L) Delile plants: some cytophysiological and molecular aspects

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Settore disciplinare scientifico: BIO/01

PhD thesis, Doctorate Research Program in Plant Biology
Ecology Department – University of Calabria
XIX Cycle (2003-2006) – Coordinator Prof. A.M. Innocenti)

...to my parents

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Aim of the work

My PhD work has been focused on the investigation of specific cytophysiological and molecular parameters dealing with the response of *Posidonia oceanica* plants to light conditions.

P. oceanica (L) Delile is a paleoendemic seagrass of Mediterranean basin which plays a major ecological role in marine ecosystem, supporting many relevant functions (Ott, 1980; Ghirardelli, 1981; Jeudy de Grissac and Boudouresque, 1985; Pergent-Martini et al., 1994; Alcoverro et al., 1997; Piazzi et al., 2004). Moreover, due to its sensitivity to disturbance factors, this seagrass is considered an effective bioindicator species of environmental quality (Pergent et al., 1995).

In the last two decades a wide range of factors, mostly related to human activities (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Invers et al., 2004; Rende et al., 2005, 2006), are causing a significant and widespread decline of *P. oceanica* meadows in the Mediterranean basin (Peres, 1984; Sheperd et al., 1989; Sanchez Lizsaso et al., 1990; Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Fonseca et al., 1998; Cozza et al., 2004).

In the context of monitoring projects, research has been till now focused mainly on ecological aspects (Pergent-Martini et al., 2005; Rende et al., 2005) and several markers of stress condition have been identified at level of meadow structure (Pergent-Martini, 1998; Ancora et al., 2004; Pergent-Martini et al., 2005; Rende et al., 2006). Further insights for the conservation of *P. oceanica* meadows, have been provided by studies on population genetic and phylogeography of this species (Procaccini and Mazzella, 1998; Procaccini et al., 2001; Micheli et al., 2005). However, the ever increasing levels of anthropic impact on marine environment has aroused much interest towards physiological and molecular parameters, as novel stress-related biomarkers which allow precocious screening of impaired features in this seagrass.

A limiting factor to the achievement of this goal is the very little knowledge on the molecular and physiological bases underlying the adaptative response of *P. oceanica* to marine environment. So far, a little number of genes has been identified in this monocot (Giordani et al., 2000; Maestrini et al., 2004) (Tab.1). To this concern it must be also mentioned that, only recently, about 3000 EST clones obtained by a subtractive cDNA

library have been sequenced (Migliaccio and Procaccini, 2006), but these sequences are not yet available.

Gene name	Gene activity/notes	Accession number	Year
PIP1A	plasma membrane protein[1]	AJ314584	2005
TIP1	tonoplast intrinsic protein[2]	AJ314583	2005
AQ1	partial cds for putative aquaporin	AJ289696	2005
AQ2	partial cds for putative aquaporin	AJ289695	2005
coIII	Cytochrome oxidase (mitochondrial subunit)	POC566361	2005
18S rRNA	full length sequence	AY491942	2003
RPS13	mitochondrial ribosomal protein S13	AJ458460	2003
microsatellite sequences		AF547650; -51; -52; -53; - 54; -55; -56 ; -57; AY152814	2003
DNA repetitive sequences		AJ404320; AJ404318	2001
DNA tandem repeat		Y15420	2000
Pomt family	putative metallothionein	AJ249602, -603, -604	2000
5.8S rRNA	full length sequence	AJ225091	1999
rbcL	rubisco large subunit, partial cds	U80719	1998

Tab.1 List of known genes in *Posidonia oceanica* (L) Delile provided by NCBI web site. ([1] from Maestrini et al., 2004; [2] from Giordani et al., 2000).

The present study aimed to enrich physiological and molecular knowledge on this relevant species taking into account some aspects related to metabolic response of *P. oceanica* plants to light conditions. To this concern, it is important to underline that in marine ecosystem light availability is a critical abiotic factor for plant ecophysiology, controlling photosynthetic process that is essential to autotrophy. Notably, the human influence on sea water quality and hydrodinamic regime is greatly related to the presence of suspended material along the water column (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Cozza et al., 2004; Rende et al., 2005). Due to its relationship with a reduced light availability, water turbidity is considered one of the main factors causing the regression of *P. oceanica* meadows (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Cozza et al., 2004; Rende et al., 2005).

On the basis of cytokinin role in light signaling cascade my work was firstly addressed to investigate the distribution of these phytohormones, central for plant development (Mok and Mok, 2001) in organs like shoot and rhizome which account for plant growth and resource storage, respectively. The intention was to verify the occurrence of specific differences in cytokinin distribution in relation to light regime. Secondly, I

analyzed the expression pattern of *PoCHL P* (*GERANYLGERANYL REDUCTASE*) as a candidate gene to investigate light response in plants, being engaged in the biosynthetic pathway of both chlorophylls and tocopherols (Benz et al., 1980; Soll et al., 1983; Soll, 1987; Keller et al., 1998).

In particular, the distribution of cytokinins and the expression pattern of *PoCHL P* were analyzed in plants of *P. oceanica* growing in two distinct meadows for which differences in light conditions were preliminarily assessed. It is worth remarking that, on the basis of phenological, lepidochronological (Rende et al., 2005, 2006) and cytophysiological features (Cozza et al., 2004), these two meadows have been classified as well preserved and disturbed site, respectively.

Introduction

1.1- General features of study system: *Posidonia oceanica* [L] Delile

Our study system is represented by marine phanerophyte *Posidonia oceanica* (L) Delile, whose systematic position is (Les et al., 1997):

Subphylum: *Angiospermae*
Class: *Monocotiledonae*
Subclass: *Alismatidae*
Order: *Alismatales*
Family: *Posidoniaceae*
Gender: *Posidonia*
Species: *Posidonia oceanica*

Although aquatic Angiosperms represent fewer than two percent of all flowering plant species and only a smaller fraction (0.02%) is represented by seagrasses, these latter are an important component of marine ecosystems (Les et al., 1997).

Marine Angiosperms are confined taxonomically to five monocotyledon families (Kuo and McComb, 1989; Philbrick, 1991), within the single subclass *Alismatidae*, and accounts only 60 species (Kuo and McComb, 1989; Philbrick, 1991) localized in tropical and temperate waters. A huge fraction (1/3) of these plants is still poorly investigated (Duarte, 1999). The remaining plants are known only at a descriptive and ecological level and only few species have been better examined: *Thalassia testudinum* and *Posidonia oceanica* firstly, *Zostera marina*, *Halodule wrightii* and *Zostera noltii* secondly (Duarte, 1999).

The colonization of marine habitats by Angiosperms represents a difficult transition for a group whose evolutionary history essentially reflects adaptation to terrestrial condition. There are different theories that can explain this event, but the most accredited (den Hartog, 1970) is based upon the morphological and distribution comparisons among actual species and fossils dated from Cretaceous to Mesozoic Era (about 70 millions of years ago). The migration of terrestrial Angiosperms, probably already adapted to terrestrial fresh water life (Les et al., 1997), became reliable through capability in tolerating brief submersions in salt water, surviving in submersed habitats and possessing an effective

anchorage system. Furthermore, the capability to use reproductive strategies water-related (hydrophilous pollination) was the main requisite to submersed life (Les et al., 1997). Initially, these Angiosperms colonized border coastal habitats. Subsequently, they progressively colonized submersed littoral areas through hydrophily dispersal (den Hartog, 1970), with the lower limit of colonization being imposed by light availability for the photosynthesis (Pirc, 1984; Duarte, 1991; Buia et al., 1992; Lorenti et al., 1993; Lorenti et al., 1995).

Although the ecology of seagrasses has been extensively studied, the systematic relationships remain inadequately investigated and their phylogeny is still debated. In fact the grouping of seagrass under a single subclass (*Alismatidae*) could lead to a monophyletic origin. Despite of this taxonomic position, Hartog's (1970) hypothesis based on a polyphyletic origin is supported by morphological studies (Dahlgren et al., 1985) and more recent molecular works (Les et al., 1993; Les and Haynes, 1995; Les et al., 1997). So, the current evolutionary opinion on marine Angiosperms proposes that three different ancestors evolved independently one from each other (Les et al., 1997).

As for as *Posidonia* genus is concerned, it exhibits a bipolar distribution, living exclusively in the Mediterranean basin and along south-eastern coasts of Australia. In particular, *P. oceanica* species is the predominant seagrass of the Mediterranean basin (den Hartog, 1970; Pasqualini et al., 1998), while it is completely absent from the Australian continent, where other eight different species exist: *P. australis*, *P. sinuosa*, *P. coriacea*, *P. ostenfeldi*, *P. angustifolia*, *P. robertsoniae*, *P. denhartogii*, *P. kirkmanii*.

P. oceanica is capable to live along a wide batimetric range, till 40 m in very clear waters (den Hartog, 1970; Ros et al., 1985) and forms extensive meadows made by individual living for centuries. It exhibits a basal root apparatus and a modified stem (rhizome) 4-6 mm thick, slightly flattened and partially inserted into the substrate. Rhizomes can grow horizontally (plagiotropic rhizome) or vertically (orthotropic rhizome) on the basis of environmental conditions. Plagiotropic rhizomes promote colonization of new substrate while orthotropic rhizomes develop vertically contrasting sedimentation and taking advantage of light availability. Roots are produced along ventral side of rhizome and promote further anchorage to the ground. Furthermore, the developing of orthotropic rhizomes determines an uprising of depths which originates the typical formation of *P. oceanica* meadows called with the French term: '*matte*'. A *matte* is constituted by a top area covered by *P. oceanica* shoots and by a basement presenting an intricate layer of plagiotropic and orthotropic rhizomes, roots of older plants and a very compact and caged

sediment. *Matte* formation is the result of a seed germination on a depth sequentially colonized by *Cystoseira* brown algae and then by a meadow of *Cymodocea nodosa* thus constituting the right habitat to seedling development (Boudouresque and Meinesz, 1982).

Similarly to terrestrial plants, lateral roots of *Posidonia* adsorb nutritive substances from the sediment that are then translocated to the leaves through vascular tissue. Furthermore, rhizome and root cells of the peripheral layers lignify gradually during development of these organs protecting from mechanical wounding.

The top side of the rhizome bears shoot apex that originates leaves showing whorled phyllotaxis. Normally, a *P. oceanica* shoot has 5-10 ribbon-shaped leaves with a rounded apical portion and of green colour. Average width of a leaf ranges between 7 and 11 mm, while length varies between 40 and 120 cm (Giraud, 1977). *P. oceanica* leaves support a continuous basal growth and it has been estimated that they can reach ages up to 300 days (Ott, 1980; Romero, 1989), which is a very long period of time if compared with other aquatic phanerogams. In every shoot of *P. oceanica* the external leaves are longer and older than the internal ones which present themselves shorter; moreover, the apical portion constitutes the older portion of the leaf lamina, being subjected to degenerative processes becoming visible with the appearance of a brown stain. Every leaf bears a basal portion delimitated by a concave line (named ligule) where older leaves detach. This basal portion isn't photosynthesizing and remains instead anchored to the rhizome becoming a thin scale lignified. Analysis of cyclic variation of these scales make possible to calculate plant age (and approximately the age of the meadow of sampling) through the study of lepidochronology (Pergent, 1990).

P. oceanica is a hermaphroditic seagrass (Les et al., 1997) capable to reproduce sexually by hydrophilous pollination of its flowers. Because of its monoecy, *P. oceanica* bears male and female flowers placed on a terminal stem to give an inflorescence composed by 3-4 spikelet and surrounded by two floral bracts. The reproductive period is during the months of September and October, where is possible to observe presence of inflorescence in shallow waters (Thelin and Boudouresque, 1985; Gambi et al., 1996; Balestri and Cinelli, 2003; Balestri, 2004). In November and December flowers are pollinated and after four months the fruit is completely developed (Buia and Mazzella, 1991), the mature fruit is floating and it presents a single non-quiescent seed that appears light green coloured. Once fruit tissue became degraded, the seed of *P. oceanica* reaches the bottom and germinates in few days (Caye and Meinesz, 1984). Notably this cycle verifies with a two month

retardation in plants living in a deeper batimetric range, as observed for some meadows of Ischia isle (Mazzella and Ott, 1984).

Although some reproductive events for *P. oceanica* in the Mediterranean sea have been reported (Thelin and Boudouresque, 1985; Buia and Mazzella, 1991; Semroud, 1993, Balestri and Cinelli, 2003; Balestri, 2004), seedling establishment rarely occurs (Piazzi et al., 1999; Balestri and Cinelli, 2003). Consequently, the main mechanism by which the species of the gender *Posidonia* colonizes a submersed area depends on the clonal reproduction accomplished through stolonization and recruitment of seedling patches from a meadow to another (Buia and Peirano, 1988; Gambi et al., 1996; Balestri et al, 1998; Hemminga and Duarte, 2000; Campbell, 2003; Di Carlo et al., 2005).

Annual growth pattern of *P. oceanica* has been long studied (Ott, 1980; Bay, 1984; Mazzella and Ott, 1984; Wittmann, 1984; Pirc, 1985; Alcoverro et al., 1995), it has an internal mechanism regulating this process (Ott, 1979) that in field study reaches a maximum in spring and a minimum in late-summer (Alcoverro et al., 1995). Leaf growth rate can vary with seasonal period, showing higher values during spring and slowing progressively in summer, when the covering of specialized colonizing epiphytes rises (Silverstein et al., 1986), light availability and temperature of the water increase (Buia et al., 1992). Growth rate of *P. oceanica* is very slow: in fact orthotropic rhizomes develop with 1 cm/year (Caye, 1982), while plagiotropic rhizomes develop with 3.5-7.5 cm/year (Caye, 1982; Meinesz and Levèfre, 1984). Moreover, from a plagiotropic rhizome can originate from one to six rhizomes every year.

Annual production of *P. oceanica* has been extensively studied (Ott, 1980; Bay, 1984; Pirc, 1985), it is mainly influenced by light and nutrients (Pirc, 1985; Pirc, 1986) while the seasonal production pattern is modulated by temperature. Biomass increase is quite high during winter up to spring despite to a lower temperature and irradiances (Ott, 1980; Bay, 1984, Pirc, 1984), moreover the role of the temperature on growth, like for other seagrasses (Walker and Cambridge, 1995), has been demonstrated for *P. oceanica* productivity (Buia and Mazzella, 1991; Zupo et al., 1997). Photosynthetic activity, instead, is mainly determined by the vegetative stage increases progressively from autumn to spring along with temperature increase, while during summer leaf senescence and wave motion causes detaching of older leaves decreasing drastically photosynthetic rates (Pirc, 1986). Furthermore, spring period is used by *P. oceanica* to store carbohydrates reserves in the rhizome in order to support the subsequently winter leaf growth (Pirc, 1985; Pirc, 1989),

and also to maintain a high leaf growth rate and develop a dense foliar canopy well before the maximum summer irradiance (Alcoverro et al., 1995).

P. oceanica genome has 20 chromosomes for diploid (2C) cells (Contadiopoulous and Verlaque, 1984; den Hartog et al., 1987) with a value of 6.2 pg (Koce et al., 2003). Although this value is greater respect to other Mediterranean phanerogams, it is markedly lower than the average value found for other Monocots (~11.5 pg) thus confirming a genome with a reduced size generally being conserved along evolutionary time in all the species of *Alismatidae* Subclass. To this concern it is probable that a slow evolutionary rate and the reduced diversity observed in the other hydrophytes could explain this phenomenon (Les et al., 1988; Koce et al., 2003).

1.2 – Role of *Posidonia oceanica* as bioindicator for marine environment

P. oceanica meadow is widely recognized as a key and unique ecosystem in the Mediterranean coastal zone, supporting many functions such as to be ecological substrate to a broad number of marine biocenosis (Alcoverro et al., 1997; Piazzi et al., 2004), contributing to water oxygenation and attenuating wave erosion through its long leaves (Jeudy de Grissac and Boudouresque, 1985). Moreover, *Posidonia* is one of the major sources of carbon primary production (~20 g carbon/m^2/d versus ~11 g carbon/m^2/d produced by coral reefs) (Ghirardelli, 1981; Pergent-Martini et al., 1994).

In spite of the importance of sexual reproduction in long-term dynamics of population, reproductive events for *P. oceanica* rarely occurs (Piazzi et al., 1999; Balestri and Cinelli, 2003) and clonal reproduction accomplished through stolonization and recruitment of seedling patches from a meadow to another (Gambi et al., 1996; Buia and Peirano, 1988; Balestri et al, 1998; Hemminga and Duarte, 2000; Campbell, 2003; Di Carlo et al., 2005), is the main mechanism by which *Posidonia* (genus) colonizes submersed area. This feature is clearly related to a genetic similarity and a consequent reduced gene flow between isolated meadows (Capiomont et al., 1996; Procaccini and Mazzella, 1998; Procaccini et al., 2001; Dalmazio et al., 2002), which in turn is tightly intertwined to the sensitivity of *Posidonia* plants to moderate-to-high levels of disturbance (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996) associated to abiotic and biotic stress factors , first of all the growing coastal anthropization. For all these reasons *P. oceanica* is considered an effective bioindicator of a stress condition in marine environment (Pergent et al., 1995).

In the last two decades, in the Mediterranean basin, *P. oceanica* meadows are widespread declining (Peres, 1984; Sheperd et al., 1989; Sanchez Lizsaso et al., 1990;

Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Fonseca et al., 1998; Rende et al., 2005) principally owing to the human influence on water quality and hydrodynamic regime that is related to the presence of suspended material along the water column (Marbà et al., 1996; Short and Wyllie-Echeverria, 1996; Cozza et al., 2004; Rende et al., 2005).

In this context, research has been pre-eminently focused on ecological aspects (Ancora et al., 2004; Rende et al., 2006) and several markers of stress condition have been identified at level of meadow structure (Pergent-Martini, 1998; Ancora et al., 2004; Pergent-Martini et al., 2005; Rende et al., 2006). To provide further insights for meadow conservation, population genetic and phylogeography of *Posidonia oceanica* have been also largely investigated (Procaccini and Mazzella, 1998; Procaccini et al., 2001; Micheli et al., 2005). By contrast, in spite of its relevant ecological role molecular and physiological bases underlying the adaptative response of *P. oceanica* to marine environment are not yet fully understood. So far, a little number of genes has been identified in this monocot (Maestrini et al., 2004; Giordani et al., 2000) (Table 1). Only recently, about 3000 EST clones obtained by a subtractive cDNA library have been sequenced (Migliaccio and Procaccini, 2006) although these sequences are not available. However, with ever increasing levels of anthropic impact on marine environment it is becoming even more urgent to identify new markers at cytophysiological and molecular level which allow a precocious monitoring of stressful conditions for this important seagrass.

Structure and function of cytokinins

2.1– Structural features of cytokinins

Cytokinins are plant hormones promoting cell division and differentiation. Since the discovery of the first synthetic cytokinin, kinetin, (Miller et al., 1955) the number of chemicals defined cytokinins has grown to include natural and synthetic compounds, adenine and phenylurea derivatives (Fig.1). Cytokinins were defined substances capable to stimulate cell division when provided in combination with auxin. They were also called "cell division factors" (Miller et al., 1955; Strnad, 1997; Mok and Mok, 2001). However, since its discovery cytokinins have been shown exert other effect on plant growth and development. They promote shoot organogenesis and lateral buds release (Werner et al., 2001; Werner et al., 2003), chloroplast development (Hutchison and Kieber, 2002), delay tissue senescence (Mok, 1994; Gan and Amasino, 1995). The natural occurring cytokinins are adenine derivatives and are classified by the configuration of N^6-side chain as isoprenic-derived or aromatic cytokinins (Strnad, 1997; Mok and Mok, 2001). Moreover it is largely known that also t-RNA molecules are a source of cytokinins, located at the anticodon arm (Swaminathan and Bock, 1977). Measurements of t-RNA breakdown indicated that it contribute up to 50% of *cis*-zeatin. However this event occur in all tissues while cytokinins production is localized in root, shoot meristems and immature seeds (Letham, 1994). Therefore, if t-RNAs contribute significantly to endogenous cytokinin level is an aspect not yet fully elucidated.

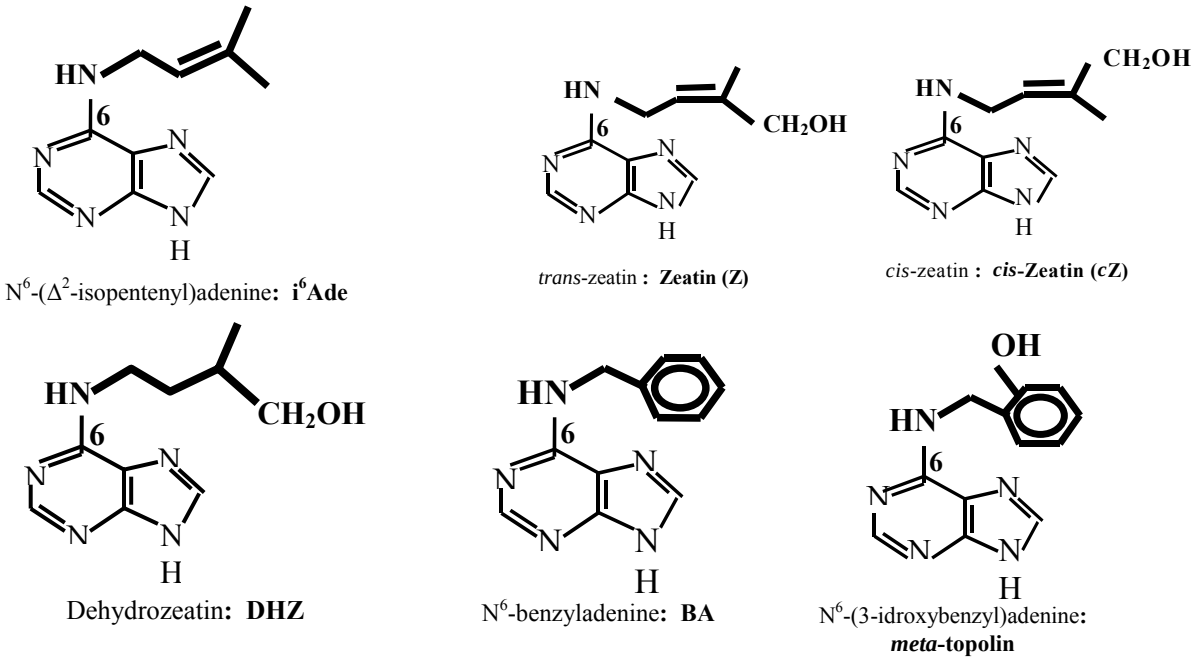

Fig.1 Natural occurring cytokinins.

Both isoprenoid and aromatic cytokinins are naturally occurring compounds, frequently found in some plant species (Strnad, 1997). Common natural isoprenoid cytokinins are N^6-(Δ^2-isopentenyl)-adenine (iP), *t*-Z, *cis*-zeatin (cZ) and dihydrozeatin (DZ) (Fig.1). There is a lot variation in cytokinin content, in relation to plant species, tissue and developmental stage. In *Arabidopsis* thaliana *t*-Z and iP represent the major source of this class of hormones, while in *Zea mays* and *Oryza sativa c*-Z is the predominant cytokinin (Veach et al., 2003). Cytokinin nucleobases, often referred as free cytokinins, exhibit higher biological activity and likely act binding specific receptors. Whereas, cytokinin conjugates with sugar like glucose or xylose, are considered less active or inactive molecules (Martin et al., 1999a, 1999b; Martin et al., 2001). Cytokinin receptors are encoded by a small gene family (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yonekura-Sakakibara et al., 2004). Furthermore, they display a specific spectrum of molecular binding and they are capable to discriminate cytokinins side chains and modification of adenine moiety (Inoue et al., 2001; Yamada et al., 2001; Yonekura-Sakakibara et al., 2004).

2.2– Cytokinin metabolism

The knowledge of cytokinin biosynthesis in plants is an active research field. In particular, in the last years some progresses in cytokinins knowledge were achieved by identifying of key genes encoding enzymes and proteins involved in critical steps of biosynthetic/catabolic pathway, translocation and signaling of this class of plants hormones (Higuchi et al., 2004; Sakakibara, 2006; Shani et al., 2006).

The key enzyme of this biochemical process, adenosine phosphate-isopentenyltransferase (IPT), was identified following the complete sequencing of *Arabidopsis thaliana* genome (The *Arabidopsis* genome initiative, 2000). The IPT constitutes a large family of nine genes in *A. thaliana* and it is characterized by tissue-specific expression and nitrogen induction (Takei et al., 2004b). Two members of this family are t-RNA IPT and catalyze the first step of cytokinin biosynthesis by binding dimethylallyl pyrophosphate (DMAPP) to adenosine monophosphate (AMP) (Kakimoto, 2001; Takei et al., 2001; Miyawaki et al., 2004). Metabolic pathway culminates with formation of free forms of *t*-Z, *c*-Z, iP and DZ. Afterwards, the adenine ring can be conjugated with sugars such as ribose and glucose. In particular glycosilation is performed by specific enzymes at N3, N7 and N9 positions of purine moiety, or at hydroxyl group of the side chains of *t*-Z, DZ and *c*-Z. Concerning N-glycosilation, this conjugation is an

irreversible event, while O-glycosilation is a reversible process, which is catalyzed by β-glucosidase (Brzobohaty et al., 1993).

Steady-state levels of active cytokinins in *planta* are determined by the rate of release of nucleobase from the conjugates and by metabolic degradation and inactivating of cytokinins. The irreversible degradation is operated by a cytokinin oxidase/dehydrogenase (CKX), which causes cleavage of the side chain (Galuszka et al., 2001; Schmülling, 2003). Moreover, phosphoribosylation of nucleobase by adenine phosphoribosyltransferase, that is a cytochrome P450 monoxygenase, also reduces the biological activity (Takei et al., 2004a). The release of nucleobases from conjugate cytokinins represents an important step to regulate the level of active cytokinins, but the genes and enzymes that catalyze this step have not been identified yet.

2.3– Cytokinins role in plants

Cytokinins play a crucial role in regulating proliferation and differentiation in plant cells. They exert many effects on different phases of plant growth and development such as chloroplast development (Hutchison and Kieber, 2002), delay tissue senescence (Mok, 1994; Gan and Amasino, 1995), regulate shoot-root balance (Werner et al., 2001; Werner et al., 2003), resources translocation (Mok and Mok, 2001), transduction of nutritional signals (Samuelson and Larsson, 1993; Takei et al., 2001; Sakakibara, 2005), increase in crop productivity (Ashikari et al., 2005) and also induce tumour formation (Akiyoshi et al., 1983).

Cytokinins are synthesized in non-photosynthetic tissues, such as root tips (Feldman, 1975), apical meristems (Koda and Okazawa, 1980) and immature seeds (Blackwell and Horgan, 1994) and act at various sites in the body plants. In fact they exert their control as long-distance messengers as well as local paracrine signals. Considering the effects exerted by cytokinins and their translocation pathway, these hormones could be proposed as factors, which mediate gene expression in plant cells (Burkle et al., 2003).

Despite the key role of cytokinins in many physiological events, their action at molecular and the whole plant level is still largely unknown. In addition the putative receptor/signal transduction genes, such as CKI1, ARR, and GCR1, proposed as cytokinin targets, include cell cycle genes and genes affecting shoot meristem formation (Jacqmard et al 1994; Kubo and Kakimoto, 2000; Sweere et al., 2001). The *knotted* homeobox genes family was also implicated in cytokinin action since transgenic plants overexpressing the *ipt* or *knotted 1-like* genes have similar phenotypes.

The candidate gene

*GERANYLGERANYL REDUCTASE (*CHL P*)*

3.1 - General features

GERANYLGERANYL REDUCTASE (CHL P) gene encodes for an enzyme involved in chlorophyll, tocopherols and phylloquinones pathways (Keller et al., 1998) (Fig.2). The name of the gene encoding for CHL P enzyme derives from the subunit P of the chlorophyll synthase enzymatic complex in prokaryotes (Bollivar et al., 1994). This gene has been characterized for the first time in *Rhodobacter capsulatus* (Bollivar et al., 1994), subsequently in the cyanobacterium *Synechocystis* sp. PCC 6803 (Addlesee et al., 1996) and in higher plants like *Arabidopsis thaliana* (Keller et al., 1998), *Glycine max*, and *Prunus persica* (Giannino et al., 2004).

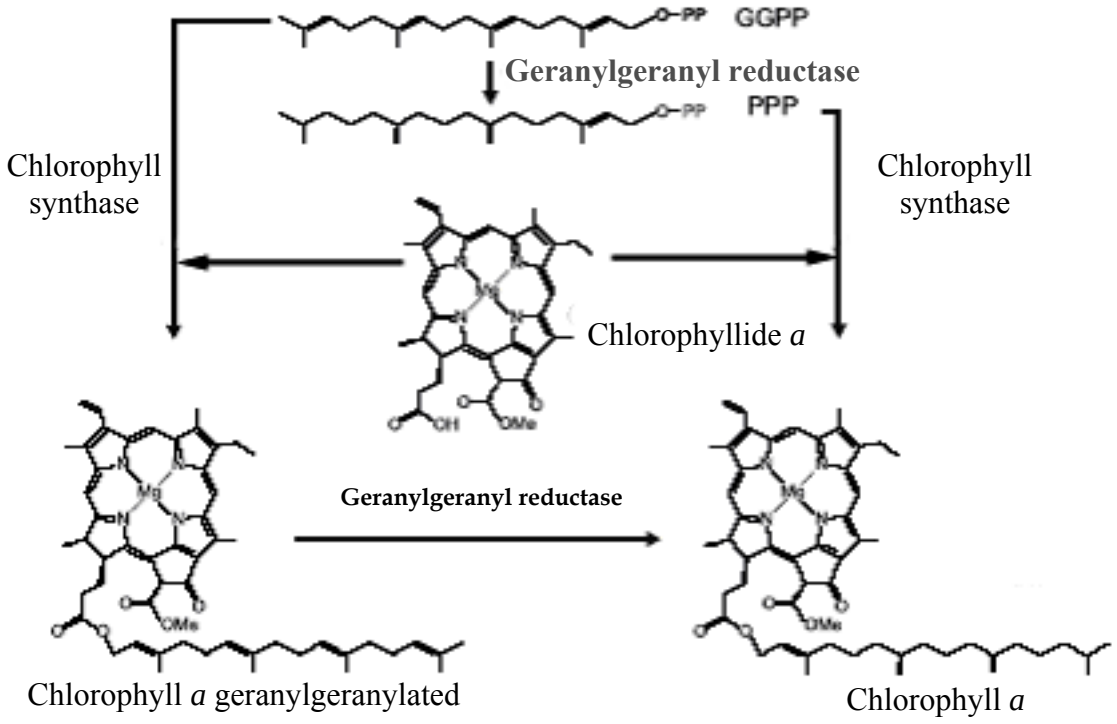

Fig.2 Geranylgeranyl reductase pathway (from Shpilyov et al., 2005, modified).

CHL P gene appears to be a single copy gene, with the exception of almond and cherry tree (Giannino et al., 2004), where two copies have been detected. The gene exhibits two introns in *Arabidopsis thaliana* (At1g74470, one of 107 bp and one of 87 bp), one intron in *Glycine max* (AF068686, 310 bp), and *Prunus persica* (AY230212, 633 bp) (Giannino et al., 2004). In all cases the position of the intron is conserved among these

species, while its dimension can be variable. In monocots plants, *CHL P* gene homologues have been identified in *Zea mays* (GenBank: AY104414) and *Oryza sativa* (GenBank: NM_187998) in the context of sequencing projects, and in *Triticum aestivum* (GenBank: DQ139268), through direct submission. Hence, in these cases non evidence for gene function is provided.

In the higher plants, CHL P aminoacidic sequence is constituted by an average number of 460 aminoacids and shows a signal sequence for transit peptide for chloroplasts translocation (Keller et al., 1998; Giannino et al., 2004), thus supporting the identification as a nuclear gene. On the contrary, in prokaryotes this signal sequence is totally absent (Bollivar et al., 1994; Addlesee et al., 1996). Downstream the transit peptide three signature sequences for an enzyme with flavin monoxygenase activity are present. The signatures were identified for the first time in mammals (Atta-Asafo-Adjei et al., 1993). Furthermore, a specific function was identified for signature nearer to N-termini of CHL P proteins, which motif is GXGXXG, forming a secondary structure βαβ (Wierenga et al., 1986) specific for the binding of ADP portion of NADPH or FAD cofactors (Atta-Asafo-Adjei et al., 1993, Addlesee and Hunter, 1999). For the remaining two signatures, instead, it isn't known a specific role (Addlesee and Hunter, 1999) (Fig.3).

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Arabidopsis thaliana           -RVAVIGGGPAGGAAAETLAQGGIETILIER--
Nicotiana tabacum              -RVAVVGGGPAGGAAAETLAKGGIETFLIER--
Glycine max                    -RVAVVGGGPAGGAAAETLAKGGVETFLIER--
Mesembryanthemum cristallinum  -RVAVIGGGPAGGSAAETLAKNGIETFLIER--
Synechocystis spp.             –RVAVVGGGPAGSSAAEILVKAGIETYLFER--
Oryza sativa                   -RVAVVGGGPAGGAAAEALAKGGVETVLIER--
Prunus persica                 -RVAVVGGGPAGGSAAETLAKGGIETFLIER--
Medicago truncatula            -RVAVIGGGPAGGAAAETLAKGGIETFLIER--
Consensus βαβ                   ** * * *  *          *   *  *
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Fig.3 Alignment of aminoacid sequences containing consensus βαβ for CHL P proteins. Accessions: *Arabidopsis thaliana* At1g74470**,** *Nicotiana tabacum* CAA07683, *Glycine max* AF068686, *Mesembryanthemum cristallinum* AF069318, *Synechocystis* PCC 6803 CAA66615, *Oryza sativa* XP_467759, *Prunus persica* AY230212, *Medicago truncatula* AAX63898.

Hydrophatic profiles calculated with predictive algorithms such as Kyte-Doolittle (Kyte and Doolittle, 1982) and Klein (Klein et al., 1985) don't predict the presence of transmembrane domains in CHL P proteins. Although not supported by experimental procedures, these results let to hypothesize that CHL P enzymes are not membrane bound but free to float into plastidial stroma and interact either with chlorophyll, tocopherols and phylloquinones pathways (Keller et al., 1998).

3.2 - Geranylgeranyl reductase role in chlorophyll biosynthetic pathway

Plant organisms are constantly in contact and communication with the surrounding habitat. Since plants are sessile organisms, they must respond correctly to the continuous environmental changes due to both biotic and abiotic factors. Light is the keystone abiotic factor interacting with plants. It is captured by plant photoreceptor able to discriminate different wavelengths and either converted to metabolic energy or used to monitor environment and trigger specific responses (Gyula et al., 2003; Kevei and Nagy, 2003; Whippo and Hangarter, 2003; Nagatani, 2004).

Light conversion to chemical energy (photosynthesis) is a process in which light energy Photosynthetic Active Radiation (PAR) is used for biochemical reactions. Specialized pigments (such as chlorophylls, carotenoids and xanthophylls) localized in thylakoid membranes of plastids capture light energy and many stromatic enzymes (primarily ribulose-1,6-diphosphate carboxylase oxigenase**,** rubisco) cooperate to convert it into ATP. Chlorophyll is the principal pigment related in photosynthesis and exists in two forms (*a* and *b*) which differs respectively for a methyl group and a formyl group in the tetrapyrrole ring II), respectively. Its molecule consists of two portions that are synthesized in different pathways before to be enzimatically attached:

1) pathway of tetrapyrrole in which the starting precursor 5-aminolevulinate leads to chlorophyllide *a* synthesis, a molecule bearing a tetrapyrrole ring with a magnesium atom in its centre (Oster et al., 1997; Rüdiger, 1997; Thomas, 1997; Grimm, 1998);

2) synthesis of an unsaturated fatty side chain (phytol) starting from isopentenyl pyrophosphate (IPP). The phytol side chain favours insertion of chlorophyll molecule into thylakoids membranes. Because of IPP is an isoprenic derivative, this pathway is referred as isoprenoid pathway.

Isoprenoid biosynthetic pathway can follow two alternative ways leading to IPP synthesis, that appear to act on the basis of chloroplasts differentiation state (Heintze et al., 1990):

1) a mevalonate dependent pathway (MVA);

2) a mevalonate non-dependent pathway (non-MVA), that is recently identified in plastids (Rohmer et al., 1993; Lange et al., 2000; Iijima et al., 2004) and is

considered a metabolism derived from ancestors prokaryotes of chloroplasts (Lichtenthaler et al., 1997). This pathway is addressed as glyceraldehyde-3-phosphate/pyruvate pathway (GAP/pyruvate).

MVA pathway involves two cellular compartments: cytoplasm and chloroplast. In the first, enzymatic reactions joining acetyl-CoA molecules leading to the formation of IPP which is subsequently translocated to chloroplast (Kreuz and Kleinig, 1984; Gray, 1987).

GAP/pyruvate pathway begins with the formation of deoxy-D-xylulose-5-phosphate (DXP) from pyruvate and D-glyceraldehyde-3-phosphate. DXP molecule is first reduced, then isomerised into 2-C-methyl-erythritol-4-phosphate (MEP) and conjugated with cytidyl-diphosphate to 4-(cytidine 5'-diphospho)-2-C-methyl-erythritol (CM). A specific kinase phosphorylates CM and CM synthase leading to the formation of 2-C-methyl-erythritol-2,4-cyclodiphosphate (MECPP). MECPP is converted into 4-hydroxy-3methylbutenyl-2-enyl diphosphate (HMBPP) and subsequently into IPP and its isomer dimethylallyl pyrophosphate (DMAPP) (Fig.4).

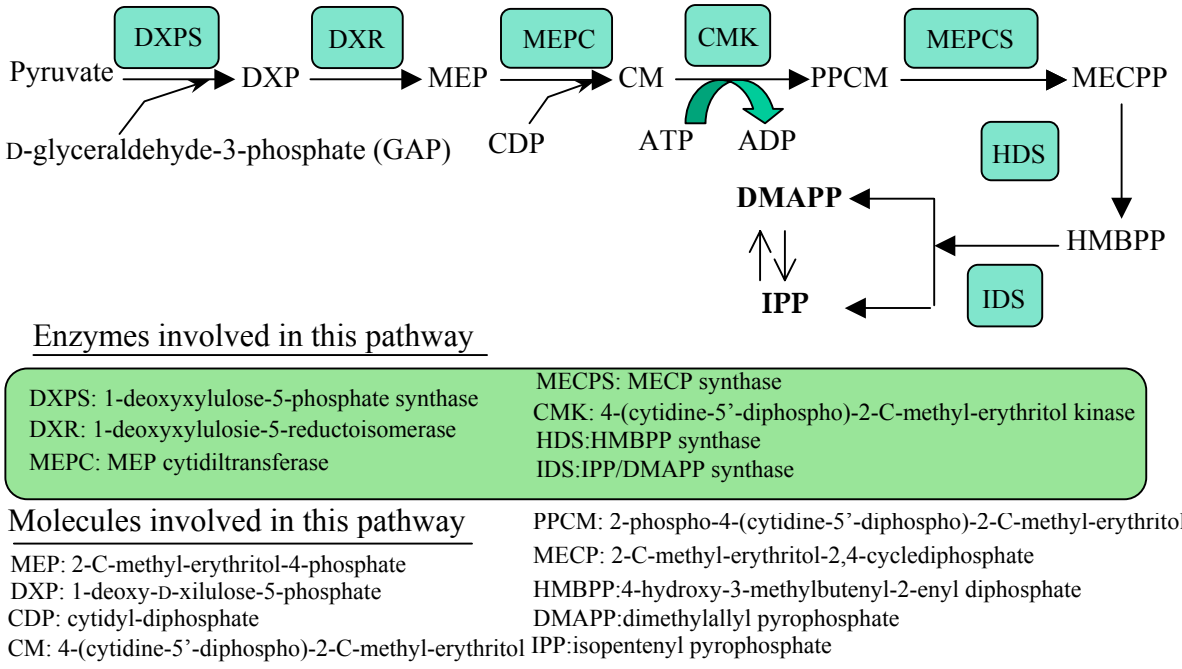


Fig.4 Pathway GAP/pyruvate, IPP plastidial biosynthetic pathway (from Iijima et al., 2004 modified).

Four IPP molecules are sequentially joined: *a)* the first IPP with its isomer DMAPP thus forming geranyl pyrophosphate (GPP), *b)* another IPP molecule is condensed with GPP to obtain farnesyl pyrophosphate (FPP) and *c)* one latter IPP molecule is joined to FPP to give the 20C polyunsaturated geranylgeranyl pyrophosphate (GGPP). GGPP can be either immediately esterified with chlorophyllide through chlorophyll synthase action to give

geranylgeranylated chlorophyllide ($Chlide_{GGPP}$), or converted into phytyl pyrophosphate (PhyPP) prior to be joined to mature chlorophyll (Bollivar et al., 1994) (Fig.2). The enzyme responsible of this reaction is geranylgeranyl reductase (CHL P) (EC 1.3.1), that in a NADPH dependent manner (Keller et al., 1998; Tanaka et al., 1999) reduces double bonds of GGPP at positions 6, 10 and 14 (Shpilyov et al., 2005), converting it into mature phytol chain that confers hydrophobicity to chlorophylls (Addlesee and Hunter, 1999).

In plants, in normal light exposure condition GGPP is reduced before condensation with chlorophyllide because PhyPP is the preferred substrate to the chlorophyll synthase (Soll et al., 1983; Keller et al., 1998). In etiolated plants and during the first stages of leaf differentiation, $Chlide_{GGPP}$ is firstly formed and later PhyPP synthesis occur (Wellburn, 1976; Schoch et al., 1977; Benz et al., 1980; Oster and Rüdiger, 1997).

3.3 - Geranylgeranyl reductase role in tocopherols biosynthetic pathway

Tocopherols are amphipathic compounds not bound to proteins that together with tocotrienols are produced by plant organisms. These molecules are generally classified as vitamin E (Fryer, 1992, Bramley et al., 2000, Munné-Bosch and Alegre, 2002a) and are important elements of human diet (Wang et al., 1993). Namely, tocopherols strongly contribute to food nutritive value (Cahoon et al., 2003), being involved in controlling cholesterols levels in blood serum (Parker et al., 1993; Theriault et al., 1999; Raederstorff et al., 2002) and growth inhibition of mammalian cancer (Nesaretnam et al., 1998). While tocopherols are principally localized in leaves respect to seeds (Bramley et al., 2000), tocotrienols are the main compound in seeds of dicots (Aitzetmüller, 1997) and of many monocots such as rice and corn. Although tocopherols and tocotrienols are powerful antioxidant compounds (Serbinova and Packer, 1994; Kamal-Eldin and Appelqvist, 1996; Packer et al., 2001), tocotrienols show best capability to limit lipid membrane peroxidation thus reducing free radicals (Suzuki et al., 1993).

Both tocopherols and tocotrienols have protective activity against oxidative substances, like oxygen singlet ($^1O_2$) and Reactive Oxygen Species (ROS) (Halliwell, 1981) which can damage chlorophylls and membrane compounds. ROS enclose superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl ($HO^{\bullet}$) and perhydroxyl ($O_2H^{\bullet}$) radicals (Halliwell, 1981), while oxygen singlet occurs following energy transfer to chlorophyll while it remains in a triplet excited state (Foote et al,. 1974; Matsushita et al., 1978; Logani and Davies, 1980). The protective activity of both tocopherols and tocotrienols ends with

the irreversible oxidization of these molecules that become no more useful to plant organism (Foote et al., 1974; Neely et al., 1988).

From a structural point of view (Fig.5), both tocopherols and tocotrienols possess a chromanolic polar ring joined to a 15C hydrocarbon chain isoprenic derivative (e.g. the pathway in which CHL P is involved) that confers hydropathicity and favours molecular interactions with acilic chains of fatty acid. Chromanol ring, that retains the antioxidative properties, is localized at the interface plastidial membrane/cytosol. Tocotrienols structure differs from that of tocopherols because of three *trans* double bonds along hydrocarbon chain, absent in tocopherols, and for the number and position of methyl groups inside the chromanol ring.

The first step of tocopherol pathway consists in the synthesis of isoprenic chain that will be joined to homogentisic acid, synthesized from *p*-hydroxyphenyl pyruvate by the enzyme *p*-hydroxyphenyl pyruvate dioxygenase (HPPD) (Tsegaye et al., 2002). Homogentisic acid is produced through shikimic acid pathway in which the precursor is the aminoacid tyrosine or the molecule of prephenate molecule (Lophukina et al., 2001). After the joining of isoprenic chain, the homogentisate is condensed with PhyPP produced by the CHL P enzyme (Soll and Schultz, 1981) and then converted into 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Savidge et al., 2002). The enzyme responsible of this reaction is named homogentisate phytyltransferase (HGPT) and is rate limiting for tocopherols pathway (Collakova and DellaPenna, 2003). Subsequently, MPBQ can follow two alternatives ways: a) conversion into δ-tocopherol (Porfirova et al., 2002) and then methylation to β-tocopherol, or b) methylation to α-tocopherol and then cyclization that gives γ-tocopherol (Shintani and DellaPenna, 1998; Shintani et al., 2002).

Biosynthetic pathway of tocotrienols uses the same enzymes of tocopherols pathway except for the first reaction, that is rate limiting and is catalysed by homogentisate geranylgeranyl transferase enzyme which produces 2-methyl-6-geranylgeranyl-benzoquinol (Soll and Schultz, 1979) (Fig.5).

Quantitatively, tocopherols are differentially present in plant tissues in relation to development of organs; for example quiescent seeds accumulate primarily γ-tocopherol, while α-tocopherol is predominant in leaves, thus indicating possible different roles for the two isomers (Shintani and DellaPenna, 1998; Bramley et al., 2000). Furthermore, α-tocopherol tends to accumulate during leaf senescence (Molina-Torres and Martinez, 1991; Tramontano et al., 1992) suggesting a protective action against the ongoing oxidative stressful condition (Munné-Bosch and Alegre, 2002b). Generally, tocopherols increase is a

marker response for biotic and abiotic stress derived form higher exposition to solar radiation, drought, salinity variation and low temperatures (Havaux et al., 2000; Lopukhina et al., 2001; Munné-Bosch and Alegre, 2002a; Sandorf and Hollander-Czytko, 2002).

Recently a secondary role has been proposed for tocopherols, related to cell signaling acting in the control of lipid hydroperoxidation and in the modulation of size exclusion limit of plasmodesmata between vascular bundles sheath and leaf parenchyma (Hofius et al., 2004). More precisely, it has been hypothesized that tocopherol-induced increase of lipid peroxidation products (hydroperoxides) can regulate jasmonic acid (JA) accumulation in leaves, indirectly influencing responses related to gene specific transcription (Munné-Bosch and Falk, 2004).

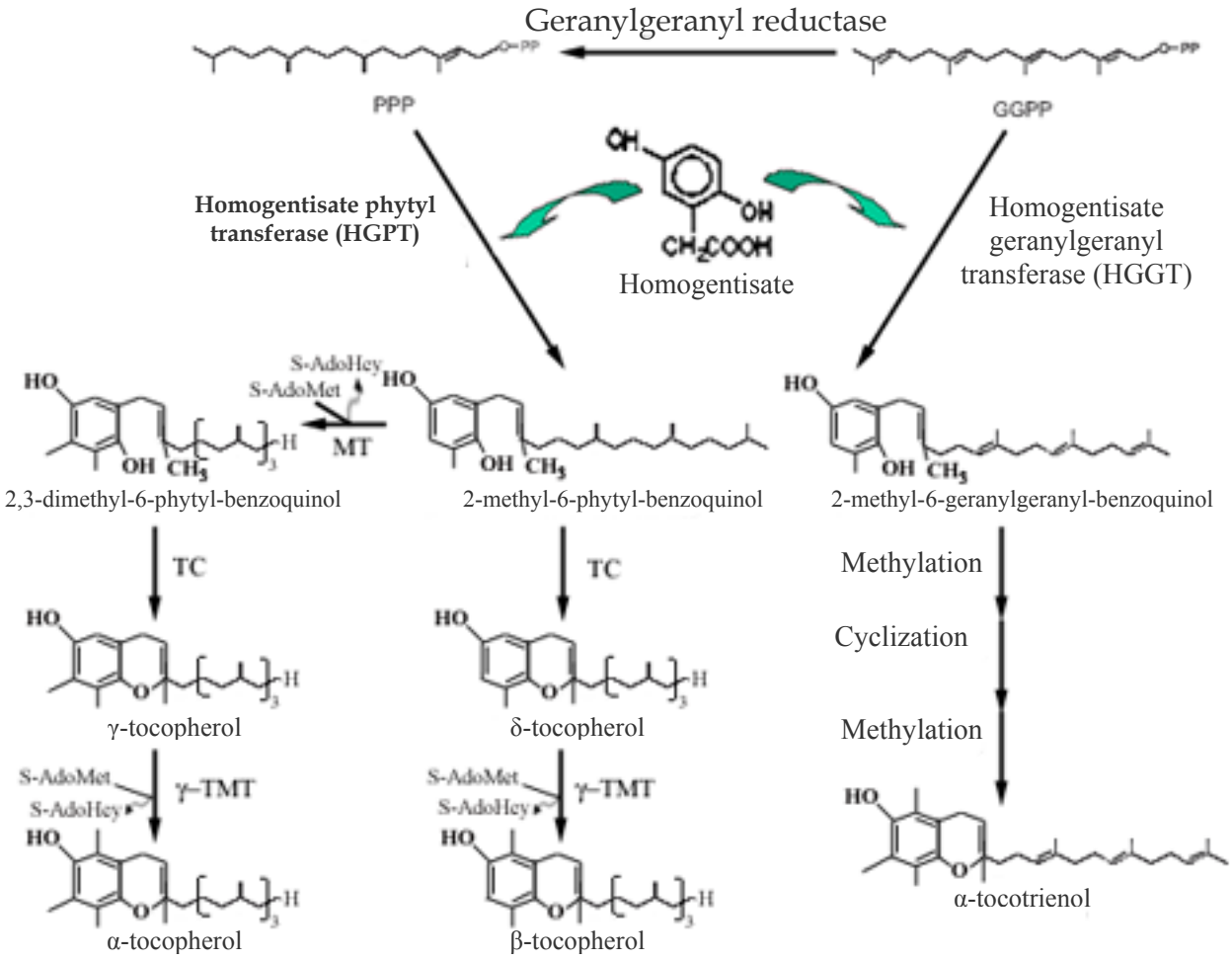


Fig.5. Biosynthetic pathway of tocopherols and tocotrienols. PPP: phytyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; S-AdoMet: S-adenosyl methionine; S-AdoHcy: S-adenosyl homocysteine; TC: Tocopherol cyclase; γ-TMT: γ-tocopherol methyltransferase.

Moreover, it has been observed a relationship between the strong reduction of tocopherols and plasmodesmata occlusion due to callose accumulation (Hofius et al., 2004), that blocks photoassimilates transport via phloem. Because tocopherols deactivate free radicals of lipid hydroperoxides, responsible of polyunsaturated fatty acids production (Munné-Bosch and Alegre, 2002b), it is tempting to speculate on a putative indirect influence of tocopherols deficiency on callose biosynthesis (Yamamoto et al., 2001).

3.4 - Expression pattern of geranylgeranyl reductase

Studies of organ specific gene expression of *CHL P* gene in peach showed that transcripts were present in leaves, in not disclosed floral bud, sepals, petals and stamens of open flower, in epicarp (skin) and mesocarp (pulp) of non mature fruits photosynthetically active (Giannino et al., 2004). A very faint signal was identifiable in roots only through RT-PCR (Giannino et al., 2004). In apical tips (e.g. apical shoot and very young leaves) transcripts were located in leaf primordia cells and along mesophyll when not completely differentiated (Giannino et al., 2004). In differentiated leaves *PpCHL P* (*Prunus persica CHL P*) signal was reported in palisade cells while was absent in vascular bundles (Giannino et al., 2004).

Furthermore transcriptional activity increases progressively during the development of photosynthetic organs, such as leaves, as well *CHL P* messengers in leaves were more abundant during daylight respect to night period where a steady state condition was observed (Giannino et al., 2004). Thus, according to key role in regulation of isoprenoid biosynthetic pathway (Lichtenthaler et al., 1997), *CHL P* gene expression appeared to be related to chloroplasts differentiation as well to the transformation into chromoplasts during fruit maturation (Keller et al., 1998).

In addition a clear relationship has been observed between *CHL P* expression and plant response with abiotic factor light (although it is still not understood if in a direct or indirect way); particularly to this issue, time-lagged experiments on cyanobacterium *Synechocystis* sp. PCC 6803 showed that *CHL P* is rapidly expressed after light exposure (Schmitt et al., 2004). On the contrary, both light stress conditions (Grasses et al., 2001) and leaf senescence increase transcripts abundance confirming the role of this gene addressed to synthesize membrane protecting compounds like tocopherols, tocotrienols and phylloquinones (Havaux et al., 2003).

CHL P expression appears negatively regulated during stress conditions such as cold exposure, mechanic damage to leaves or fungal pathogens infection (Giannino et al., 2004).

Silencing studies of *CHL P* transcripts showed the accumulation of geranylgeranylated chlorophyll *a* and *b* (Chl a_{gg} and Chl b_{gg}) other than α-tocotrienol, indicating the involvement of this enzyme in both pathways and sensibility to high light condition (Tanaka et al., 1999; Grasses et al., 2001; Havaux et al., 2003). Furthermore, in transgenic plants in which *CHL P* expression was silenced, it wasn't observed a specificity for the channeling of the little quantity of PhyPP produced towards the chlorophylls or tocopherols pathway, as well as the GGPP accumulation as a consequence of CHL P enzyme paucity. Nor was evidenced an upraise of carotenoids, showing the existence of a constant ratio between pigment fraction and binding proteins involved in stabilization of photosynthetic complexes (Plumley and Schmidt, 1995; Tanaka et al., 1999).

Parallely, *CHL P* gene inactivation in cyanobacterium *Synechocystis* sp. PCC 6803 showed both the increase of Chl a_{gg} and instability of photosystem I to operate in high light conditions (100 μmol photons m^{-2} s^{-1}), as a consequence of the reduced capability of chlorophyll molecule to insert itself in thylakoid membranes in association with constitutive elements of photosystem, but with not apparent changes in photosynthesis and growth in high or low light conditions (Shpilyov et al., 2005). This observation has been confirmed through rice mutants in which both photosystem stability and photoprotection were reduced (Shibata et al., 2004).

Results

1 – Monitoring of light conditions in *P. oceanica* meadows

Our investigations were carried out on plants of *P. oceanica* growing in two different meadows located along north-western coasts of Calabria, and referred as disturbed and preserved site (see Materials and Methods). Both these meadows have been characterized for their macrostructure (Rende et al., 2005) and according to Giraud (1977) they were classified as 'sparse meadow of III type' and 'dense meadow of type II', respectively. Furthermore, phenology, lepidochronology (Rende et al., 2005, 2006), and cytophysiology (Cozza et al., 2004) of these meadows have been also investigated.

In order to monitor light and temperature conditions, two sensors (Hobo® Pendant Temperature/Light Data Logger, Onset Computer Corporation) were located at 10 m depth in both meadows and left from June 2005 up to June 2006. Sensors were monthly cleaned to eliminate deposited particle material that could affect light measurements. The light sensors measured Temperature (°C) and light quantity (lux intensity) at time intervals of 30'. The photometric unit (lux) is defined as the density of the luminous flux incident at a point on a surface, where 1 lux = 1 lumen·m^{-2} = cd·sr·m^{-2}. Daily values obtained were grouped in three time intervals (07:30/10:30 – 10.30/15:30 – 15:30/19:30) on the basis of the diurnal changes of light intensity and mean values were compared through ANOVA followed by Neuman-Keul's *post-hoc* test (Fig.7).

Moreover, it was possible to convert lux values into Photosynthetic Photon Flux Density (PPFD), that is the number of photons in the 400-700 nm waveband incident per time unit per surface trait, by using the subsequent equation:

$$\text{Lux} = 683 \int_{400}^{700} y_\lambda \, W_\lambda \, d_\lambda$$

where y_λ is the luminosity coefficient of standard CIE (Commission Internationale de l'Eclairage) curve (with $y_\lambda = 1$ at 550 nm) and W_λ is the spectral irradiance (measure units are: W m^{-2} nm^{-1}) (Principle of Radiation Measures, available at www.licor.com). Considering a flat spectral distribution curve of light source over the 400-700 nm range (equal spectral irradiance over the 400-700 nm range), the approximation of this equation gives the subsequent conversion factor: 1 lux = 51.2 R, where R is expressed in µmol s^{-1} m^{-2} (Principle of Radiation Measures, available at www.licor.com). For this reason all PPFD values indicated are a conversion derived from illuminance data.

The statistical analysis of data retrieved from sampling performed in the first week of June 2005, showed a significant difference among the chosen time intervals. In particular, in both sites the highest light availability occurred in the intermediate time interval (11:00-15:00). In addition, a significant reduction ($p<0.01$) in light exposure characterized the disturbed site with respect to the preserved one, just in this time interval (Fig.6).

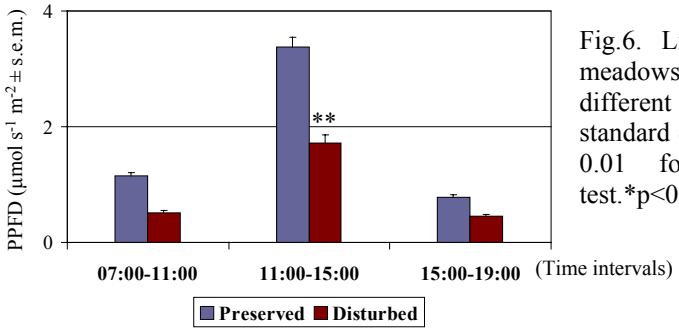


Fig.6. Light condition (PPFD) of *P. oceanica* meadows in preserved and disturbed site, at different time intervals. Error bars represent standard deviation. Statistical analysis: ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

On the basis of these results (Fig.6), data collected along one year were analyzed using the same rationale (Figs.7, 8 and 9). In all the three time intervals monthly average values of PPFD significantly differed in the two sites (Figs.7, 8 and 9) and a significant decrease in light availability was detected in the disturbed sites for a long period (about 5-6 months) (Figs.7, 8 and 9). Notably, the period of reduced light availability in disturbed sites coincided with winter months and the beginning of spring (Figs.7, 8 and 9).

An inverted trend was evidenced by statistical analysis in August and September 2005, but it must be noticed that it occurs only in the initial time interval (07:00/11:00),

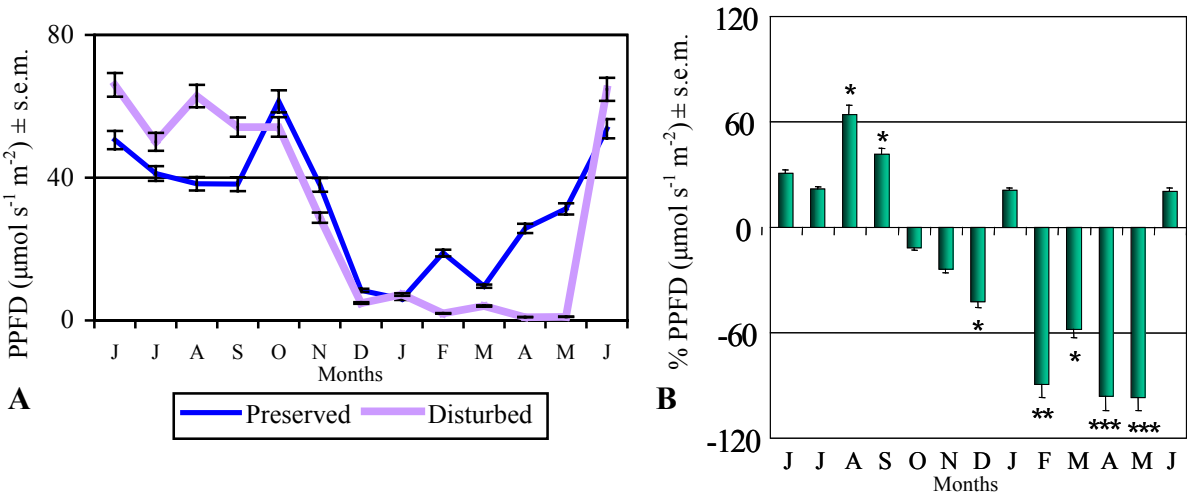


Fig.7 Light condition (PPFD) in preserved and disturbed sites at 07:00-11:00 time interval. **A.** average monthly values from June 2005 to June 2006. **B.** Disturbed vs preserved mean value ratio %. Error bars represent standard deviation. Statistical analysis: ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

characterized by a general low light availability and during a season in which development and growth of *P. oceanica* are commonly reduced (Alcoverro et al., 1995).

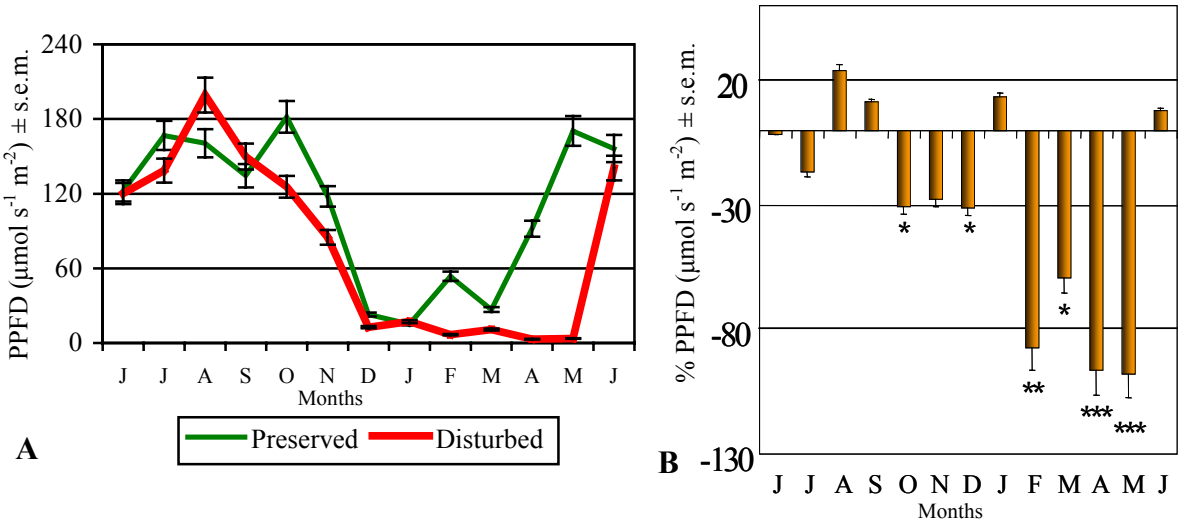


Fig.8 Light condition (PPFD) in preserved and disturbed sites at 11:00-15:00 time interval. **A.** average monthly values from June 2005 to June 2006. **B.** Disturbed vs preserved mean value ratio %. Error bars represent standard deviation. Statistical analysis: ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

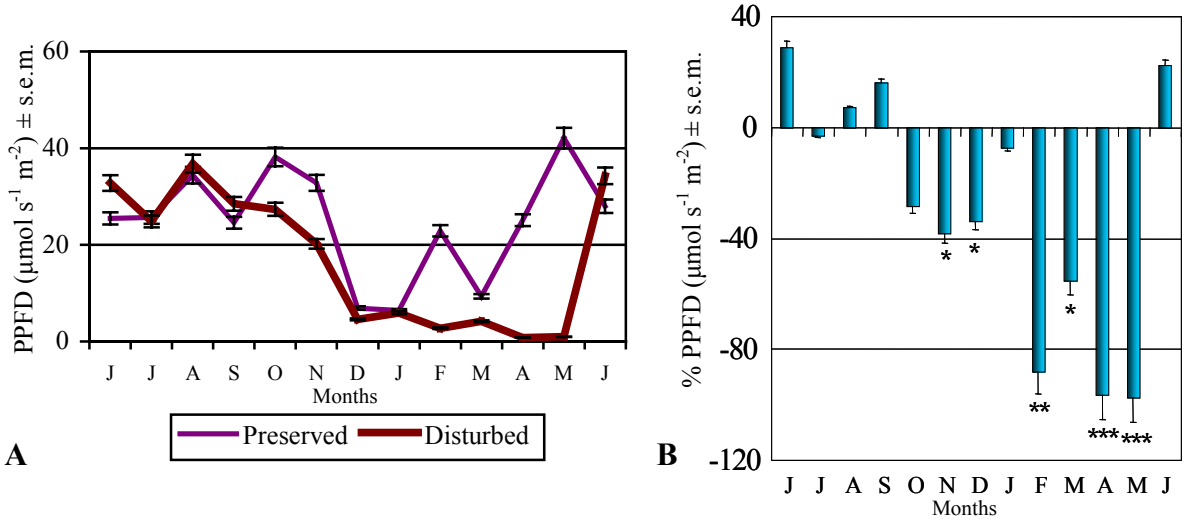


Fig.9 Light condition (PPFD) in preserved and disturbed sites at 15:00-19:00 time interval. **A.** average monthly values from June 2005 to June 2006. **B.** Disturbed vs preserved mean value ratio %. Error bars represent standard deviation. Statistical analysis: ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

Moreover, looking to the trend of daily values (Fig.10), it appeared clear that disturbed site needed more days to recover after a stressful-low-light condition as compared to preserved one (Fig.10).

Temperature parameter was also monitored and values didn't change significantly (Fig.11) as expected because of the short distance (3 Km) occurring between the two analysed sites.

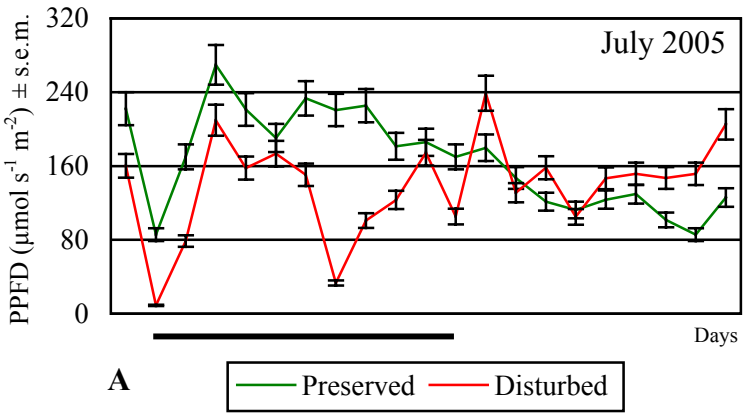
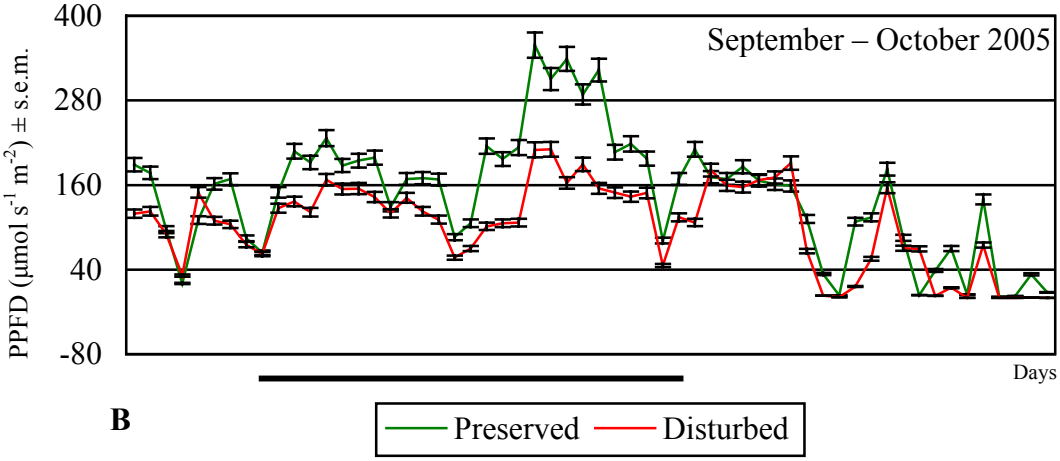


Fig.10 Daily values of light condition (PPFD) for preserved and disturbed site at 11:00-15:00 time interval. **A.** month of July 2005. **B.** months of October and November 2005. Error bars represent standard deviation. Black bars indicate the period during which the disturbed site restores a low-light stressful condition.



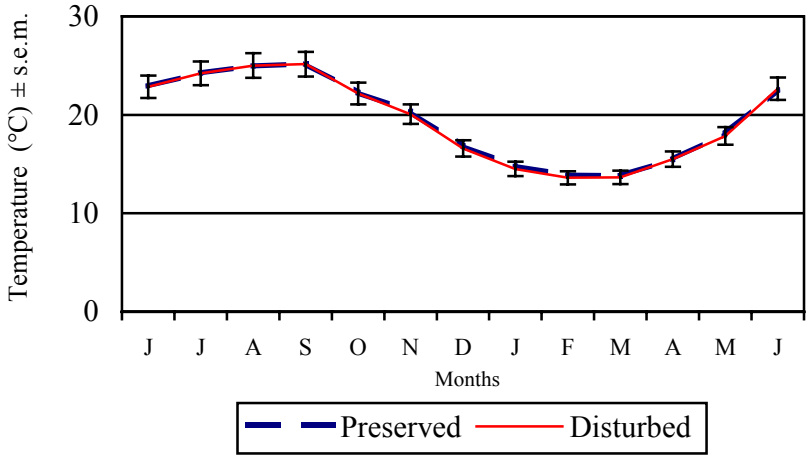


Fig.11 Monthly mean values of Temperature in preserved and disturbed site from June 2005 to June 2006. Error bars represent standard deviation.

2 – Cytophysiological analyses on P. oceanica plants

2.1- Histological analysis of rhizome

Before performing cytokinin localization, an accurate analysis of rhizome anatomy was accomplished. According to old literature data (Fig.12) (Albergoni et al., 1978), rhizome exhibited a polystelic structure characterized by the presence of two kinds of stele embedded in the parenchyma tissue: *i)* a central stele with numerous perixylematic vascular bundles surrounded by two concentric unicellular layers (i.e. endodermis and pericycle); *ii)* six, more rarely eight, minor steles, grouped three-four per side, which were located on the same plane of central stele orthogonal to leaf traces branching from the central stele. In this context, we may recall that such organization of vascular bundles in rhizome determinates parallel-veined feature of *Posidonia* leaves, with midvein deriving from central stele and peripheral veins originating from lateral ones.

In our samples a limited number of perixylematic vascular bundles, enclosing phloem cells, were present in central stele (Photo 1) which exhibited an atactostelic disposal. Similarly to central stele, lateral steles (Photos 1, 2 and 3) were surrounded by endodermis and pericycle which featured as previously described. However, lateral stele was constituted by a single perixylematic vascular bundle. Caspary band was clearly detectable on endodermis cells (Photo 2). Numerous bulks of fibers with lignified cell wall were arranged in unordered fashion in the parenchymatic tissue, providing mechanical support to the rhizome (Photos 1 and 3).

Thus, “haploendoleptostele” and “xestomeristele” for lateral steles and the entire rhizome structure were confirmed (Albergoni et al., 1978).

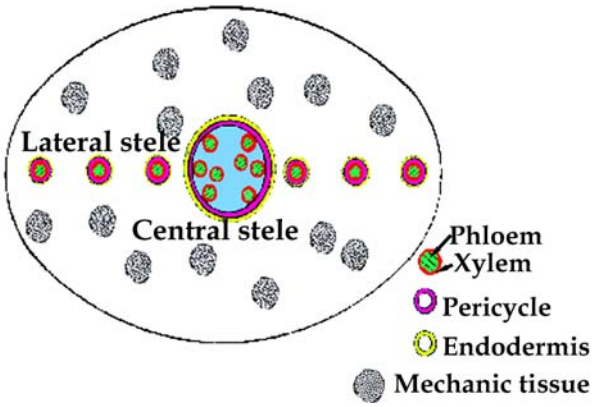


Fig.12 Schematic representation of rhizome cross section (modified from Albergoni et al., 1978).

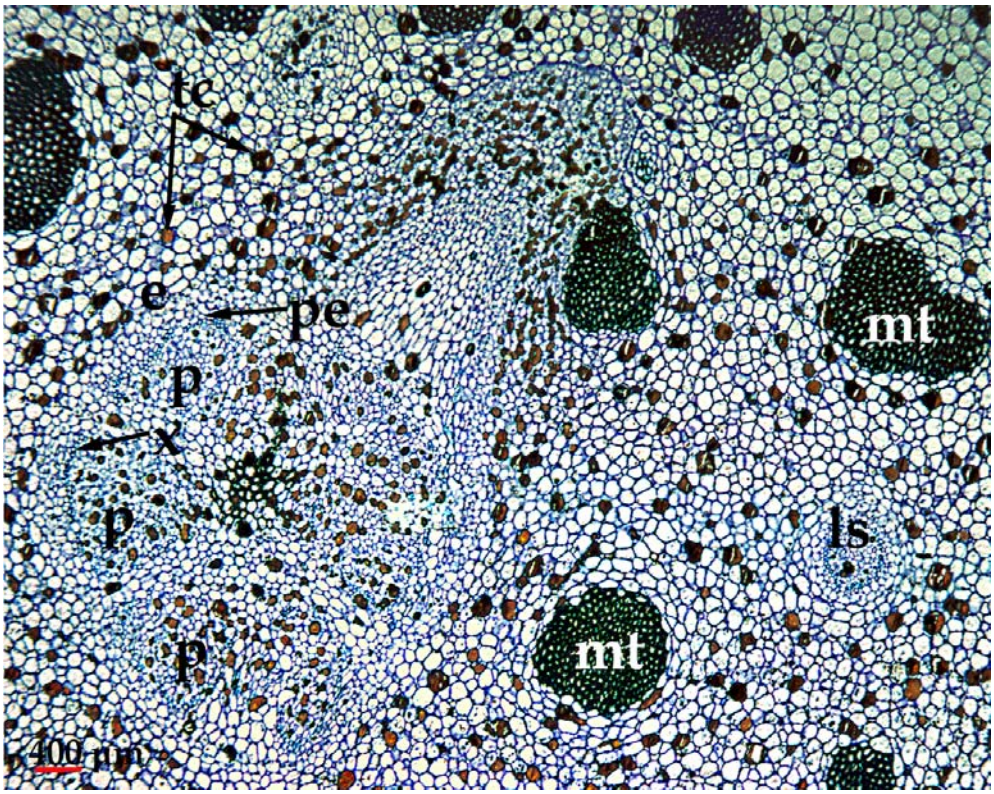


Photo 1

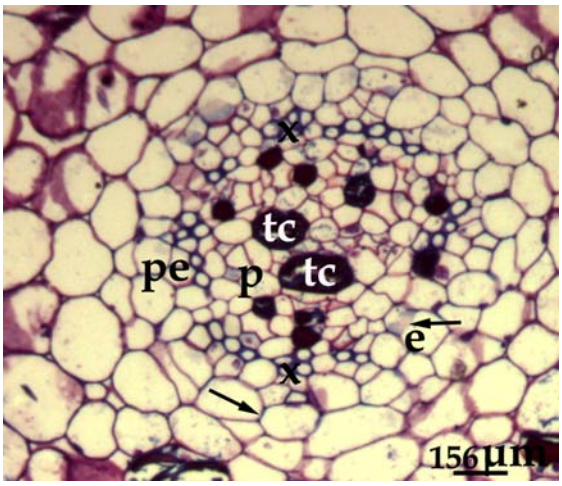


Photo 2

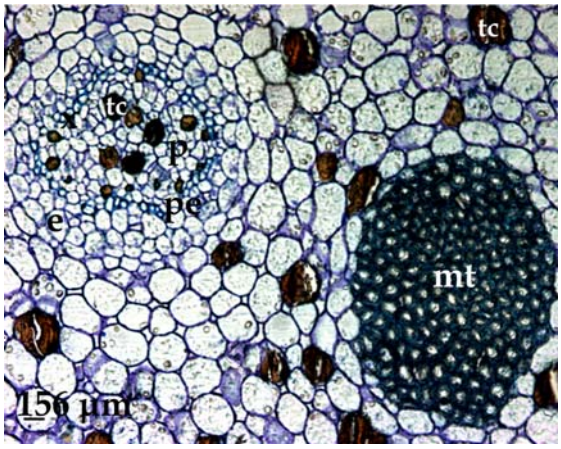


Photo 3

TABLE1. Cross section of *P. oceanica* rhizome stained with toluidine blue. Photo 1, rhizome section comprising both central and lateral stele; Photo 2 and Photo 3, particular of lateral stele. tc = tannin cells, e = endodermis, p = phloem, pe = pericycle, ls = lateral stele, mt = mechanic tissue, x = xylem. Arrows in photo 2 indicate Caspary band.

2.2 - Zeatin immunolocalization in the shoot and in the rhizome

Zeatin immunolocalization in rhizomes and shoots of *P. oceanica* was performed on plants sampled at two different periods and therefore characterized by different metabolic conditions: late spring (June 2005), a period in which photosynthesis and production usually occur at very high levels (Ott, 1980; Pirc, 1986); the end of winter (March 2006) when plant photosynthetic metabolism is not very efficient (Pirc, 1986). After immunodetection of zeatin on tissue sections, evidenced as a blue staining in the photos, uncalibrated measurements of optical density (OD) were carried out by image analysis and data were used for statistical analysis.

In the shoot apical meristem (SAM) of plants sampled in March from the preserved meadow, a weak zeatin immunoreaction was detected throughout the *corpus*, while a very strong signal localized along tunic layers (Photo 4). On the contrary, in shoots sampled in disturbed meadow a faint signal resulted uniformly spread all over the SAM (Photo 4). Percentage ratio of OD values (Fig.13A) demonstrated a significant reduction of zeatin level in the shoot of disturbed vs conserved sites.

In the SAM of plants sampled in June, zeatin immunoreaction was generally stronger as compared to that observed in March. This result is consistent with the maximum annual production exhibited by *P. oceanica* in this period (Alcoverro et al., 1995). Moreover, significant differences were once again detected when comparing shoots of plants collected in the two sites. In particular, a strong signal spread in the whole meristematic dome was detected in the SAM of samples from preserved meadow (Photo 6, Fig.13B). By contrast, in the SAM of samples from disturbed site the central zone (CZ) and rib meristem (RM) appeared almost devoid of zeatin (Photo 7 and Fig.13B). Furthermore, shoots sampled in preserved meadow appeared clearly more developed (Photo 6), as evidenced by their larger size, with respect to disturbed one (Photo 7).

A different picture was obtained for the rhizome. In particular, in rhizomes sampled in March from preserved site (Photo 8, 9 and 10), zeatin immunoreaction pre-eminently localized in xylematic elements of vascular bundles in both central (Photo 8, 9) and lateral steles (Photo 10). In the surrounding parenchyma cells (Photo 9 and 10), only a faint and diffuse signal was observed with an increasing gradient in the proximity of central and lateral steles. In rhizomes sampled in June (Photo 13-15) zeatin immunoreaction appeared stronger than in March but the distribution pattern was quite comparable. This picture was confirmed by statistical analysis of OD values which allowed us to assess that

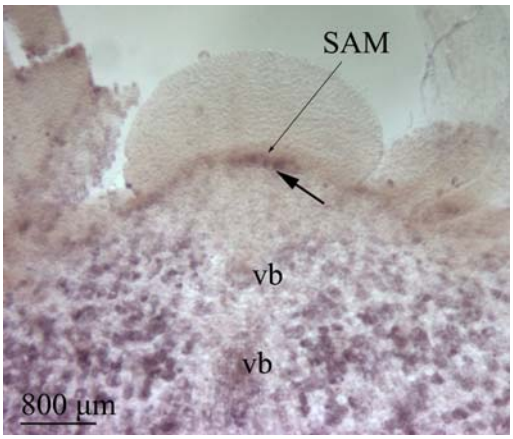


Photo 4

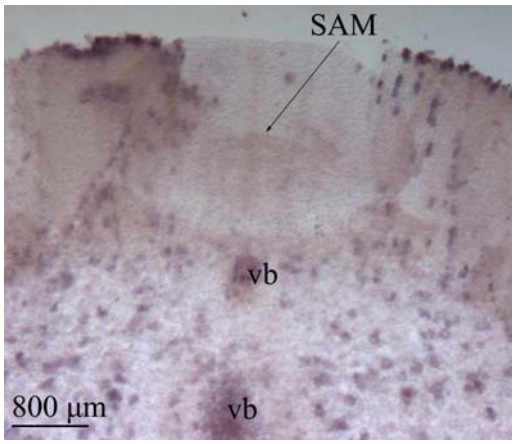


Photo 5

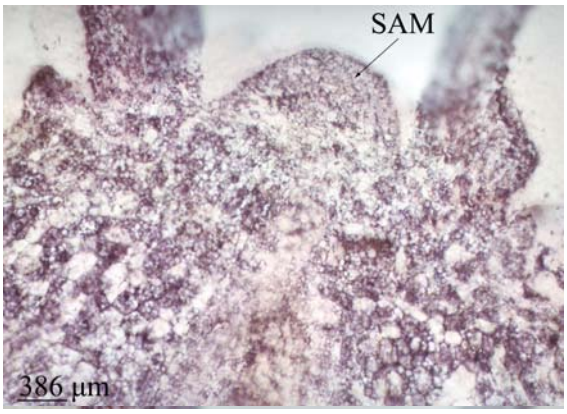


Photo 6

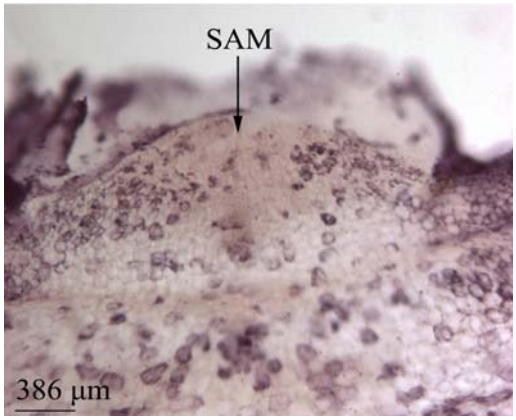


Photo 7

TABLE2. Zeatin immunolocalization in longitudinal sections of *P. oceanica* shoots. Photos 4, 5: shoots sampled in March; Photos 6, 7: shoots sampled in June; Photos 4, 6: preserved site; Photos 5, 7: disturbed site; Photo 4: black arrow indicates *tunica* layers.

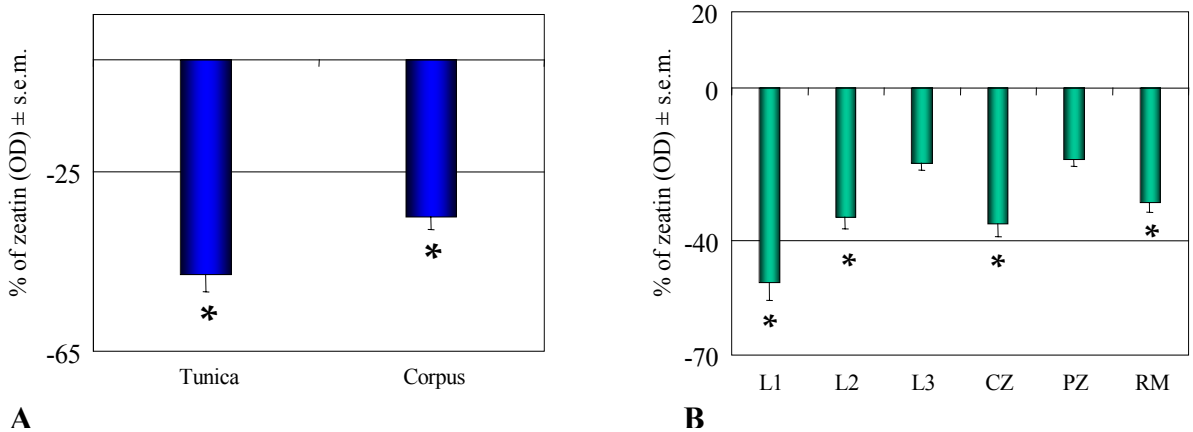


Fig.13 **A.** Comparison disturbed vs preserved in March; **B.** Comparison disturbed vs preserved in June. Statistical analysis (n=5): data are reported as ratio (%) of means value in disturbed vs preserved. CZ: central zone, L1, L2, L3: tunica layers, PZ: peripheral zone, RM: rib meristem, SAM: shoot apical meristem, vb: vascular bundles. ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

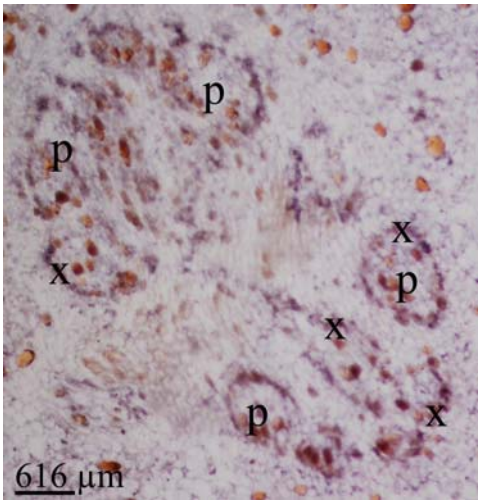
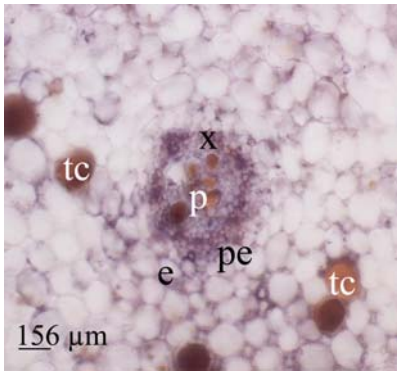


Photo 8

Photo 9



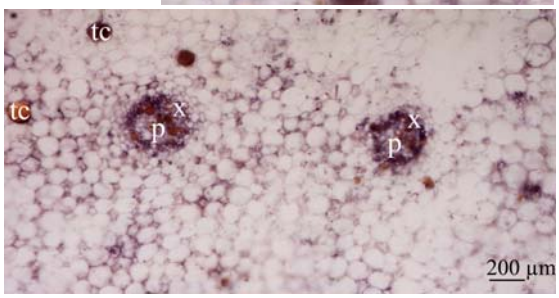


Photo 10

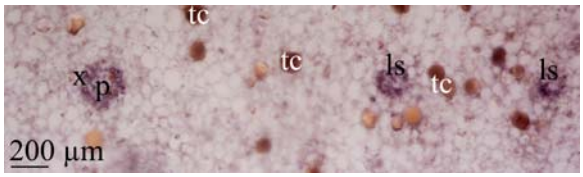


Photo 11

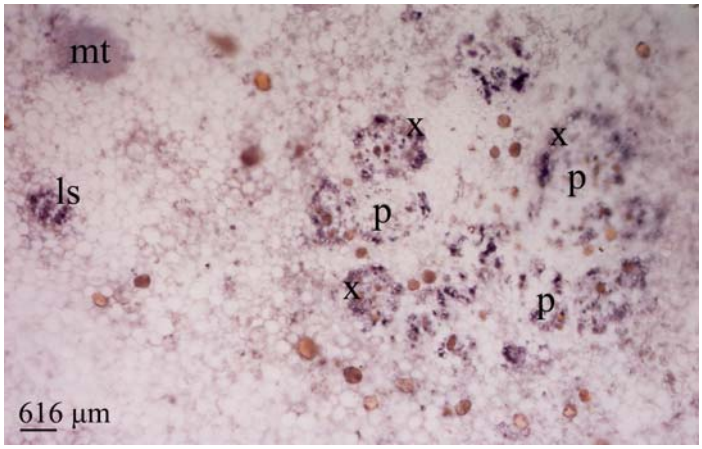


Photo 12

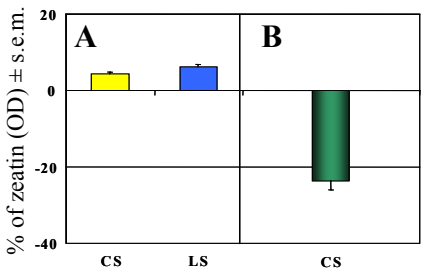


Fig.14. Zeatin immunolocalization on cross section of *P. oceanica* rhizomes. **A.** comparison for the March sampling; **B.** comparison for the June sampling. CS: central stele, LS: lateral stele, Statistical analysis (n=5): data are reported as ratio (%) of means value in disturbed vs preserved. ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

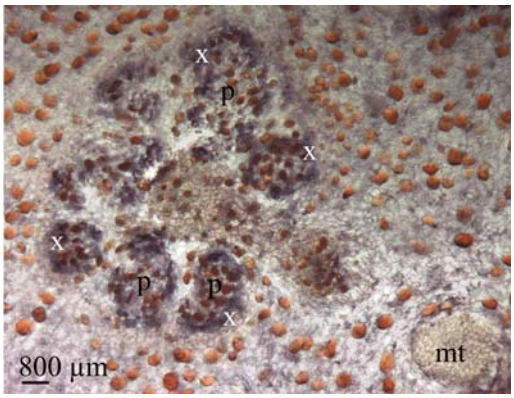


Photo 13

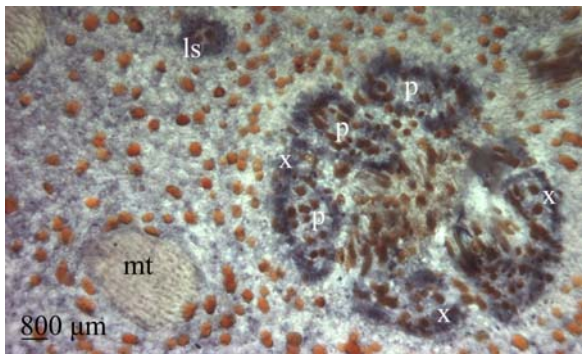


Photo 14

TABLE3. Zeatin immunolocalization on cross section of *P. oceanica* rhizomes. Photos 8-12: rhizome sampled in March; Photos 13,14: rhizome sampled in June. Photos 8-10, 13: preserved site; Photos 11, 12, 14: disturbed site. Photo 8,12,13,14: central stele; Photos 9,10,11: lateral stele. e: endodermis; ls: lateral stele, mt: mechanic tissue p: phloem; pe: pericycle; tc: tannin cells; x: xylem. Statistical analysis (n=7): data are reported as ratio (%) of means value in disturbed vs preserved. ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*p<0.05; **p<0.01; *** p<0.001.

no differences occurred in disturbed vs conserved meadows in both seasonal sampling period (Fig.14A and 14B).

On the contrary, for both disturbed and preserved site significant difference were detected when evaluating cytokinin levels as a function of sampling (Fig.15).

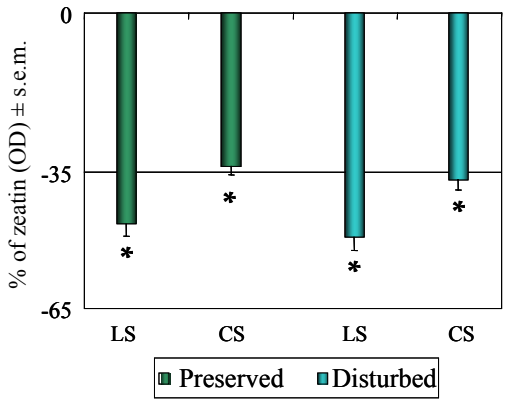


Fig.15 Data dealing with zeatin immunolocalization (OD) in rhizome are reported as March vs June means value ratio (%). LS: lateral stele, CS: central stele. Statistical analysis(n=5): data are reported as ratio of means value in. ANOVA α= 0.01 followed by *post-hoc* Neuman-Keul's test.*$p<0.05$; **$p<0.01$; *** $p<0.001$.

2.3- Total RNA detection by orange acridine staining

To evaluate transcriptional activity in *P. oceanica* organs, total RNA was localized in shoots and intermediate leaves, sampled in the well-preserved meadow in the period of high growth and productivity (June). At this aim acridine orange, a metachromatic fluorochrome that stains monomeric substrates in red (e.g. RNA) and polymeric substrates in green (e.g. DNA), was used.

In shoot apexes red staining appeared uniformly spread and very intense in the SAM, leaf primordia and along procambial strands as compared to differentiated cells (Photo 15). Furthermore, in the SAM the staining is mainly related to metabolically active histological domain, such as the peripheral zone (PZ), respect to "quiescent" central zone (CZ).

In leaves, acridine orange staining was more intense in epidermis, vascular bundles and collenchyma tissue with respect to leaf mesophyll (Photo 16 and 17). Such a feature is likely related to the vacuole differential development occurring in these cells, which actually confined cytoplasm and consequently red staining to cell periphery near cell wall.

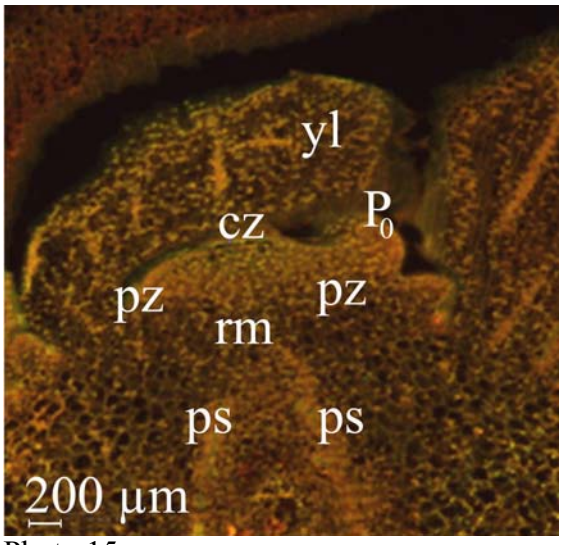


Photo 15

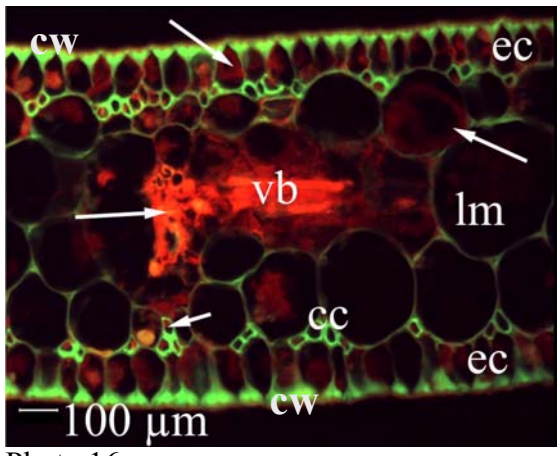


Photo 16

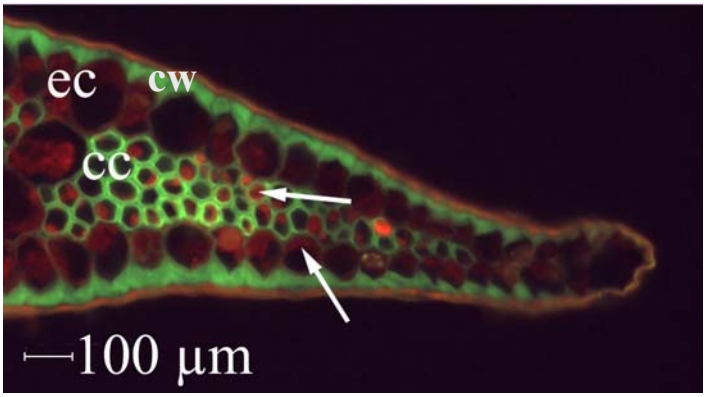


Photo 17

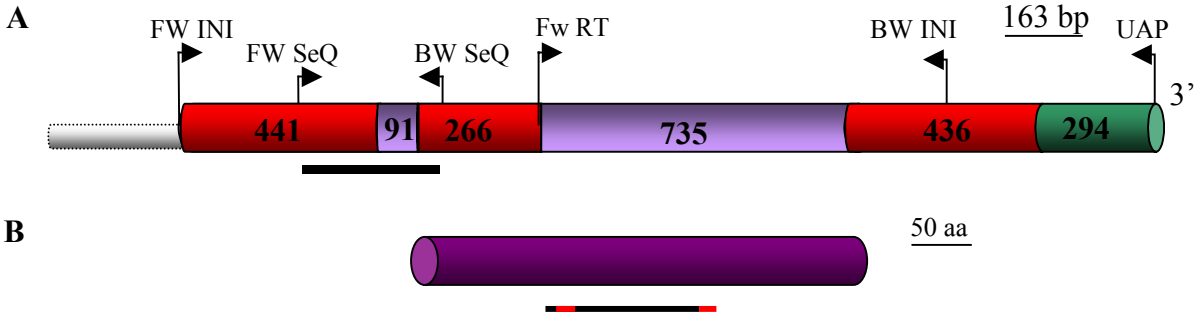
TABLE4 Acridine orange staining in shoots and leaves of *P. oceanica*. Signal is red-orange staining indicates RNA, green staining indicates DNA, intense green staining along cell walls is due to lignin self-florescence. **A.** longitudinal section of shoot; **B.** cross section of leaf middle part; **C.** cross section of leaf margin. cc: collenchyma cells, cw: cell wall, cz: central zone, ec: epidermal cells, lm: leaf mesophyll, P_0: leaf primordium, pz: peripheral zone, rm: rib meristem, vb: vascular bundles, yl: young leaf.

Molecular analyses on *P. oceanica* plants

3.1 - Identification and characterization of PoCHL P (GERANYGERANYL REDUCTASE) gene

CHL P gene in *P. oceanica* was isolated from cDNAs obtained from leaves by RT-PCR. Using the primers reported in 'material and methods' section a coding sequence with an overall length of 1437 bp was characterized. This coding sequence includes 294 bp of 3' untranslated region (UTR) while 5' end has been not yet identified (Fig.16A and Fig.17). In this partial sequence two introns, of 91 bp and 735 bp respectively, have been identified, and the splicing sites being AGGT/AGGA for the little intron and AGGT/AGGT for the bigger one. Moreover, the position of 91 bp intron, is conserved among the known *CHL P* genes.

The deduced partial protein displayed 380 aminoacids with an estimated molecular mass of ~42.6 kDa that was calculated by using BioEdit software (Hall, 1999). A monoxygenase domain, common to all known CHL P proteins, and spanning for PoCHL P from 119-248 aminoacid, showed the presence of two sequence signature whose biological function is still unknown (Addlesee and Hunter, 1999) (Fig.16B, 16C; Fig.18).



C

Arabidopsis thaliana	-EVDA**VIGADGA**N**S**RVA---KR**V**A**L**V**GDAAG**Y**V**TK--
Nicotiana tabacum	-EVDA**VIGADGA**N**S**RVA---DR**V**A**L**V**GDAAG**Y**V**TK--
Glycine max	-EVDA**VIGADGA**N**S**RVA---GR**V**A**L**V**GDAAG**Y**V**TK--
Mesembryanthemum cristallinum	-EVDA**VIGADGA**N**S**RVA---DR**V**A**L**V**GDAAG**Y**V**TK--
Synechocystis spp.	-KVDV**VIGADGA**N**S**RIA---GR**V**A**L**V**GDAAG**T**V**TK--
Oryza sativa	-EVDAIV**GADGA**N**S**RVA---GR**V**T**L**V**GDAAG**Y**V**TK--
Prunus persica	-EVDA**V**IGADGA**N**S**R**VA---GR**V**A**L**V**GDAAG**Y**V**TK--
Medicago truncatula	-EVDV**VIGADGA**N**S**RVA---GR**V**A**L**V**GDAAG**Y**V**TK--
Posidonia oceanica	-EVDA**VIGADGA**N**S**RVA---GR**V**A**LI**G**DAAG**Y**V**TK–
	116 238

Fig.16 **A.** *PoCHL P* genomic organization. Exons are indicated in red, introns are marked in violet, 3'UTR is showed in dark green. Clear grey indicate the sequence still unidentified. Numbers indicate dimension of sequence in base pairs. Arrows indicate the used primers. The black bar indicate the region used to synthesize the southern probe. **B.** Schematic diagram of PoCHL P protein, the black bar indicates monoxygenase signature, the red bars indicate the position of the monoxygenase signatures. **C.** Alignment of aminoacidic sequences in the region of the monoxygenase signature for CHL P proteins. Numbers indicate the signature position for *P. oceanica* protein. Accessions: *Arabidopsis thaliana* At1g74470**,** *Nicotiana tabacum* CAA07683, *Glycine max* AF068686, *Mesembryanthemum cristallinum* AF069318, *Synechocystis* PCC 6803 CAA66615, *Oryza sativa* XP_467759, *Prunus persica* AY230212, *Medicago truncatula* AAX63898.

>Genomic sequence (2'263 bp)

AAGATGGACAACTGCAAGCCCTGCGGCGGCGCGATCCCGCTCTGCATGGTGGGGGAGTTCGACCTCCCGCTCGACAT
CATCGACCGGAGAGTGACCAAGATGAAGATGATCTCGCCGTCCAACGTCGCCGTCGACATCGGGCGCACGCTGAAGC
CGCACGAGTACATCGGGATGTTGAGGAGGGAGGTCCTCGACGCGTACTTGAGGGACAGGGCTTCCTCTCTCGGCACC
ACCGTCATCAACGGTCTCTTCCTGAAAATGGACT*CTCCCAAGGACGATGTCTCCC*CTTACGTGCTCCACTACACGCA
CTACGATCGCTCCGGCCCCTCCGGGAAGCCGTCCACGCTCGAGGTCGATGCCGTCATCGGAGCCGACGGTGCCAACT
CCAGGGTCGCCAAGGCGATATCTGCCGGGGACTACGACTACGCCATTGCCTTCCAGGTAACCAACCAGATATGCTCC
GATTATGGTCTCGACGACGTCTTCTTCTTCTAGTTGTTGTTGGTTTGATACTGTTGTTTTGTGGGAATAGGAGAGGG
TTAAGATTCCGGATGACAAGATGGAGTACTACAAGGACTTGGCGGAGATGTATGTTGGGCAGGACGTGTCGCCGGAT
TTCTACGGGTGGGTGTTCCCCAAATGCGACCATGTTGCGGTTGGCACCGGCACCGTCACTCATAAGTCTGACATCAA
GAAGTTCCAGACGGCCACCAGGCTGAGGGCCAAGGACAAGATCGAGGGTGGCAAGATCATACGGGTTGAGGCTCACC
CCATTCCTGAGCACCCAAGGCCTAGAAGGTAACTACAACCTCTCTGGATCCTTGCTAGCATCAGTGGATTGTGTTTT
TGAAATAATGGTTGGTATCCGTTACAAGATGTGTGTACAATTCCAGGTGCATGTAGAATTCAAAACTTCACCTTGGT
AGTGCATCTAAAGGTGTCGCCTTTGATAAGGTGCAAGTTTTGCGCTTGTTACCAGAACACTCTGAATTCAGCTTGAG
TAGATTGTTAGTTTGTAGATTGTGCTTGGAATTGGTTAGAAGAAGTGTTAACGATTCCATTTACAAGCATAAGCACC
TAATGTGTTATTGTTTCTTCAAAATTTTCACCTTTGAATCTTTGATGTTTTATAAGCTTCTCATAGTGTGTTGACAA
TTTAGCTCGAGTAAAGCTTTTAGCTTGTACATTATGTTTATTATCAGTTAGTCGATCCTTTTAAACAGAAAATACCA
GTTAGAAGAAGCATAGAAGGGGGTCTATGGACAAACCGAAGCACTTAATGCGGTATTTCAATAGAATTTTAAACTGG
GTAGCCTATAAGCTTTTCAGAATGTGTCATCAACACTCTCATTTCAGCTCAAATAGTTTTTTAGGTTGTAAATTATC
AGTTAGAGGAAGTGTTGCGCAATTTGGTCCAAGCACCTCGTGTGTTGTTTCTTCAAAATCTCATCTTGAGTAGCTTA
TAAGCTTTTCAGATATGTTCTCAATCGCTATCGGTTTAAATTGATTTTAATATTTCACATGGCAATGCAGGTTGCTG
GGACGAGTTGCCCTGATCGGCGACGCAGCAGGGTACGTGACGAAGTGCTCCGGAGAAGGCATCTACTTCGCAGCGAA
GAGCGGGAGGATGTGTGCAGAGGCAATCGTGGAAGGGTCGGAGAATGAGAAAAAACTGGTGGACGAATCCGACCTCA
GGAAGTACCTGAAGAAGTTTGACAAGGCTTACTGGCCAACTTACAAGGTGCTCGATGTGTTGCAGAAGGTGTTCTAC
AGGTCCGACCCAGCAAGGGAGGCGTTCGTGGAGATGTGCGCCGACGAGTACGTGCAGAAGATGACATTCAACAGCTA
CCTTTACAAGAAGGTGGTGCCAGGGAACCCAGTCGACGACATCAAGCTTGCATTCAACACCATCGGCAGCCTCGTGA
GGGCCAATGCCATGCGTCGGGAGATGACCAAGCTCGTCGACTGAAAAGAAAAAACAAAGAACATCGAAACTCACAAA
GGCTATCTACTGGTGTAGAGCCTGTTTCAGCTGTAGAGGGGAATCTGGGATGGTGGCAACGATGGGAAAGAGATCAA
GATGTGGAAGTGGTATTTGAGTTCAGTTTCGATTCAGAAGACTGGGTAATGTTCCTCCTGTGAACAAACAGTATTTT
TCTATTCTTCTCTTTCACATTAAAGAGATAGAGAGTGATCACTTTTTTATTTATAAATCTCAAGAAATACAAGTTAT
AACCCACTACTTTTACAAAAAAAAAAAAAA

Fig.17 Genomic sequence of *PoCHL P* gene. In red are indicated introns, the stop codon TGA is underlined.

>PoCHL_P

KMDNCKPCGGAIPLCMVGEFDLPLDIIDRRVTKMKMISPSNVAVDIGRTLKPHEYIGMLRREVLDAYLRDRASSLGT
TVINGLFLKMDSPKDDVSPYVLHYTHYDRSGPSGKPSTLEVDA**VI**G**ADGA**NSRVAKAISAGDYDYAIAFQERVKIPD
DKMEYYKDLAEMYVGQDVSPDFYGWVFPKCDHVAVGTGTVTHKSDIKKFQTATRLRAKDKIEGGKIIRVEAHPIPEH
PRPRRLLGR**V**A**L**I**GDAA**GYVTKCSGEGIYFAAKSGRMCAEAIVEGSENEKKLVDESDLRKYLKKFDKAYWPTYKVLD
VLQKVFYRSDPAREAFVEMCADEYVQKMTFNSYLYKKVVPGNPVDDIKLAFNTIGSLVRANAMRREMTKLVD

Fig.18 Deduced protein by *PoCHL P* gene, colours indicate spliced exons. The underlined region indicates the two monoxygenase domains, while bold characters indicate the matching aminoacids respect to domains indicated also in Fig.16.

Similarity percentage, calculated taking into account monoxygenase domain, was very high with respect to plant kingdom, while was low respect to bacterial sequence (Tab.2).

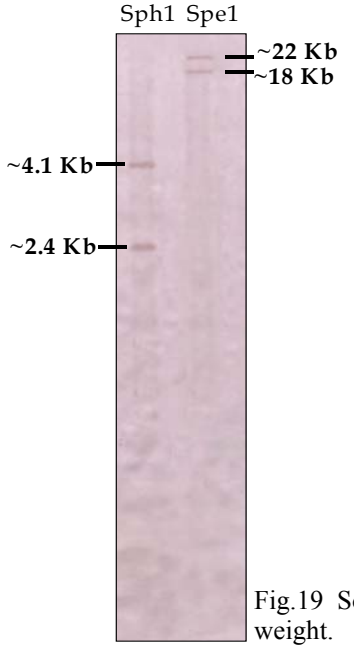


Fig.19 Southern blot for *PoCHL P* gene, the black bars indicate molecular weight.

Reign	Species	Similarity %	Identity %
Plants	*Glicine max*	91.1%	85,9%
Plants	*Mesembryanthemum cristallinum*	91.8%	83.9%
Plants	*Nicotiana tabacum*	91.5%	83.6%
Plants	*Arabidopsis thaliana*	91.1%	83.3%
Plants	*Medicago truncatula*	90.5%	83%
Plants	*Prunus persica*	90.5%	80%
Plants	*Triticum aestivum*	88.9%	78.5%
Plants	*Oryza sativa*	89.5%	77.2%
Cyanobacteria	*Synechocystis* spp. PCC 6803	81.7%	66.7%
Cyanobacteria	*Rhodobacter capsulatus*	51.4%	32.9%

Tab.2 Similarity and identity of *Posidonia* CHL P respect to known CHL P proteins in databank.

The position of introns was identified by aligning the cds sequence of 1437 bp with the genomic one. In order to predict correctly exon boundaries (Haas et al., 2002). the the AAT gene server (http://genome.cs.mtu.edu/aat/aat.html), the Plant Gene Data Bank server (http://www.plantgdb.org/PlantGDB-cgi/GeneSeqer/PlantGDBgs.cgi) and ClustalW (http://workbench.sdsc.edu) were used.

Southern blot (Fig.19) revealed the presence of two copies according to results obtained for some species of *Prunus* genus (Giannino et al., 2004). Phylogram for *PoCHL P* protein didn't identify a specific clustering, showing only that this gene from monocot plant groups with dicots ortholog genes (Fig.20), according to recent data deriving from analysis of an EST library for *P. oceanica* genes (Migliaccio and Procaccini, 2006).

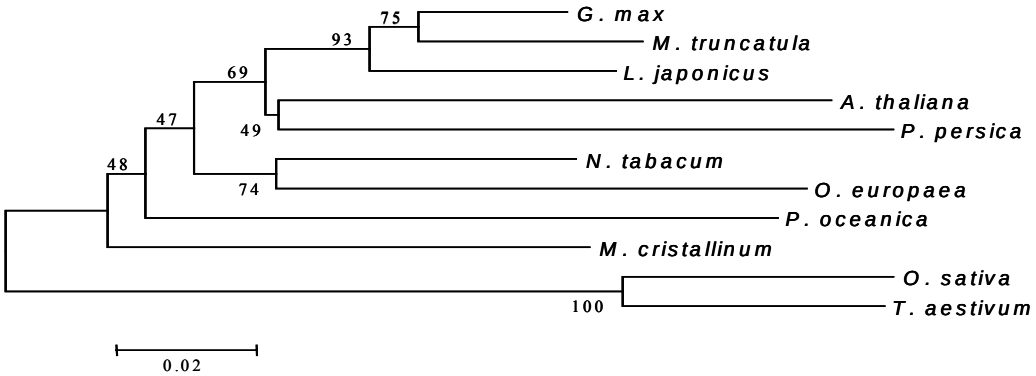


Fig.20. Phylogram was constructed by MEGA 3.1 software (Kumar et al., 2004). Numbers on the tree indicate bootstrap (1000). Accession numbers of protein examined: *Glycine max* (AF068686), *Medicago truncatula* (AAX63898), *Lotus japonicus* (AAY52460), *Arabidopsis thaliana* (At1g74470), *Prunus persica* (AY230212), *Nicotiana tabacum* (CAA07683), *Olea europaea* (DQ424963), *Mesembryanthemum cristallinum* (AF069318), *Oryza sativa* (NM_187998), *Triticum aestivum* (DQ139268).

3.2 - Organ-specific expression of PoCHL P

In order to evaluate which organs of *P. oceanica* express *PoCHL P*, 35 cycles of RT-PCR with primers SeQ (reported in material and methods session) were performed on cDNA synthesized from apical tips, leaves, rhizomes and roots (Fig.21).

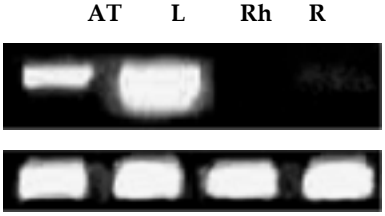


Fig.21 Organ-specific expression of *PoCHL P*. AT: apical tips, L: leaf, Rz: rhizome, R: root. Amplicon form SeQ primers was of 223 bp, while it was 363 bp for 18S primers.

PoCHL P transcripts were abundant in leaves, and present at lower extent also in apical tips. Moreover, a very low gene expression was detected in roots according to data in literature (Giannino et al., 2004). No *PoCHL P* transcripts were detected in rhizomes (Fig.21).

3.3 - *In situ* hybridisation (ISH)

Through *PoCHL P* transcripts were detected everywhere in the shoot (Photos 18, 19), appearing particularly abundant in SAM, leaf primordia and procambial strands. Notably, in leaves, epidermal cells resulted to be the primary site of transcripts accumulation (Photos 20-26). This result is consistent with the occurrence in *P. oceanica* of numerous differentiated chloroplasts in the cells of this tissue as compared to terrestrial plant (data not shown, see Cozza et al., 2004). A weak transcript accumulation was instead evidenced in mesophyll cells (Photos 20-26) which resulted greatly vacuolated and in some case very rich in phenolic inclusions (Cozza et al., 2004), mainly in adult leaves (Photo 23). Messengers were detected also in subepidermic collenchyma cells, and above in all phloematic portion of vascular bundles (Photos 20-26). Furthermore, a gradient in gene expression was detected along leaf, since transcripts accumulated at higher extent in the margins as compared to the mid-part of leaf (Photos 20, 21).

Notably, in spite of some differences in signal intensity, which was subsequently confirmed by relative RT-PCR analysis, the histological pattern of transcripts localization was almost the same regardless the sampling site (preserved vs disturbed) (Photos 20-26).

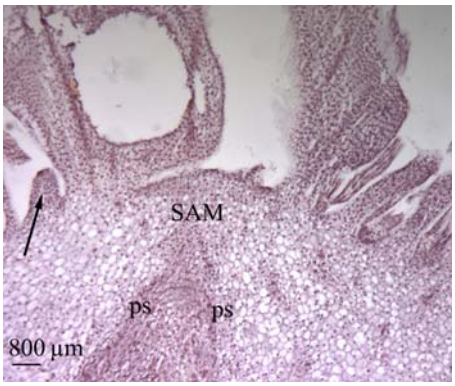
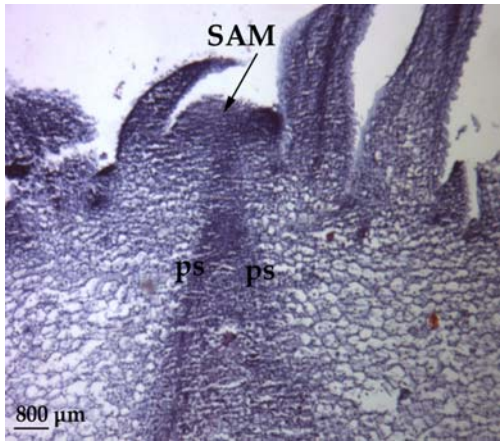


Photo 18



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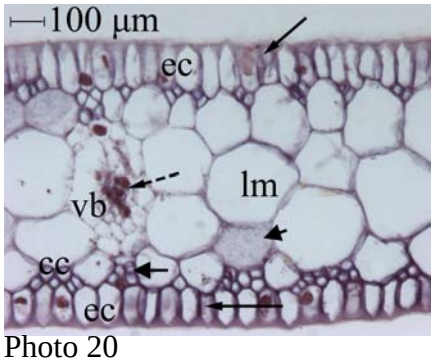


Photo 20

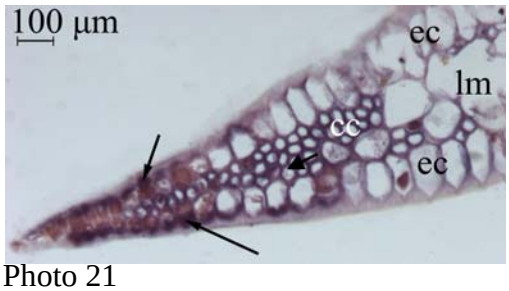


Photo 21

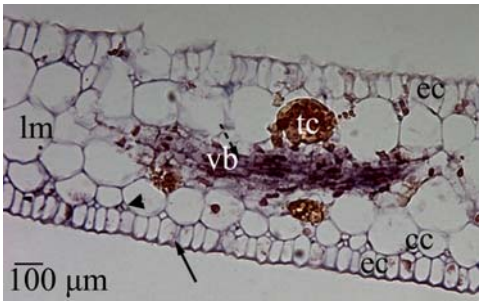


Photo 22

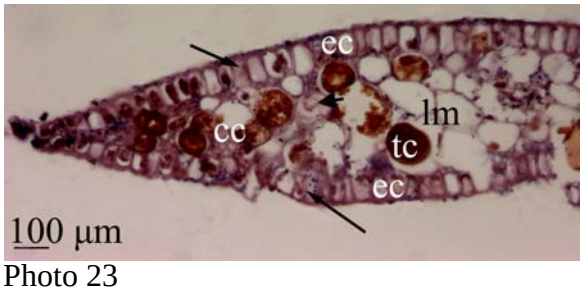


Photo 23

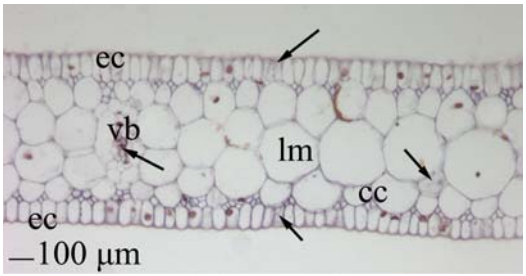


Photo 24

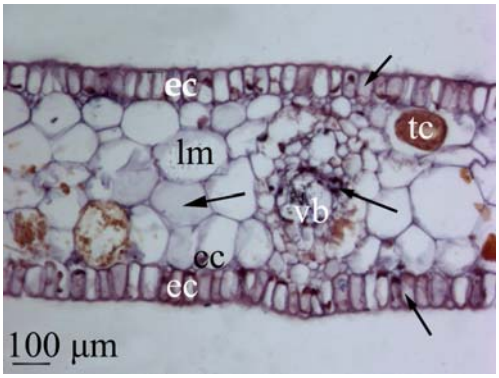


Photo 25

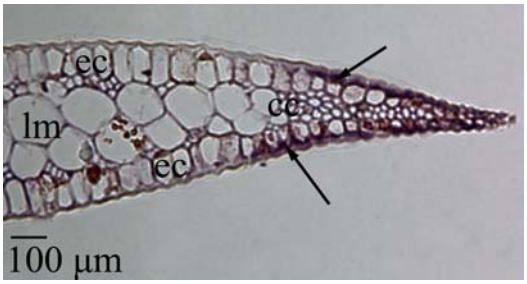


Photo 26

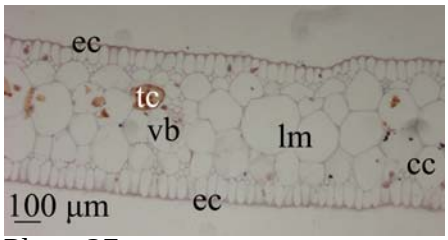


Photo 27

TABLE5 *In situ* hybridisation with antisense probe for *PoCHL P* gene (violet signal) on cross sections of *P. oceanica* leaves sampled in preserved (Photos 18-21, 25) and in disturbed (22-24) sites. Photo 20, young leaf; Photo 21, young leaf margin; Photo 22, intermediate leaf; Photo 23, adult leaf margin; Photo 24, young leaf; Photo 25, intermediate leaf; Photo 26, intermediate leaf margin; Photo 27, intermediate leaf, experimental control. ec = epidermal cells; cc = collenchyma cells; lm = leaf mesophyll; tc = tannin cells; vb = vascular bundles.

3.4 - Relative RT-PCR analysis

3.4a - Plants growing in marine environment

Semiquantitative RT-PCR analysis was performed on apical tips and young, intermediate and adult leaves. SeQ couple of primers spanning the 91 bp intron was used for *PoCHL P* in order to assess any possible contamination from genomic DNA. 18S primers designed upon *P. oceanica 18S* gene were also applied. Analysis was performed on plant material sampled in the disturbed and well-preserved meadows in different periods (June and November 2005, June 2006).

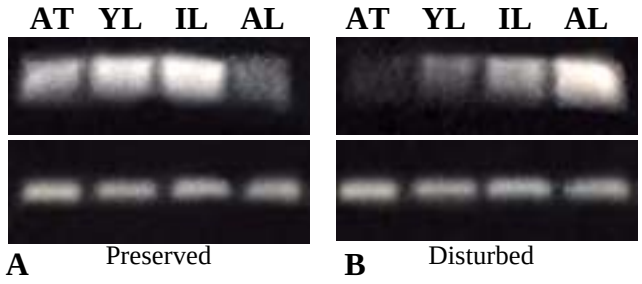


Fig.22 RT-PCR semiquantitative performed between the preserved (**A**) and disturbed (**B**) site respectively in June 2005. AT: apical tips, YL: young leaf, IL: intermediate leaf, AL: adult leaf. Amplicon form SeQ primers was of 223 bp, while it was 363 bp for 18S primers. 18S rRNA gene bank: AY491942.

In plants sampled in preserved site at June 2005 *PoCHL P* transcripts resulted to increase progressively from very young leaves of apical tips to intermediate leaves, whereas in adult leaves signal appeared reduced, indicating a steady state level of gene expression (Fig.22A).

In the disturbed site *PoCHL P* expression exhibited an opposite trend, appearing weak at early developmental stages and progressively increasing until the maximum level detected in the adult leaves (Fig.22B).

In biological replicates performed on November 2005 and June 2006 *PoCHL P* expression pattern was confirmed for both preserved and disturbed samples even if expression levels reflected plant metabolic state (Fig.23).

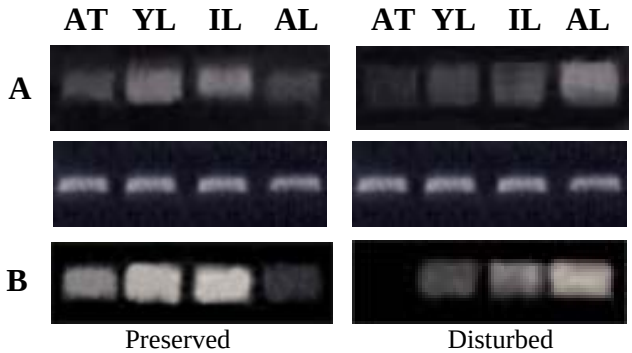


Fig.23 RT-PCR semiquantitative performed between the preserved and disturbed site respectively in November 2005 (**A**) and June 2006 (**B**). AT: apical tips, YL: young leaf, IL: intermediate leaf, AL: adult leaf. Amplicon form SeQ primers was of 223 bp, while it was 363 bp for 18S primers. 18S rRNA gene bank: AY491942.

3.4b - Plants grown in aquarium

In order to evaluate gene expression under controlled light conditions *P. oceanica* plants sampled in April were grown in aquarium as illustrated in "Material and method", setting the light at different values of PPFD as reported in Fig.24.

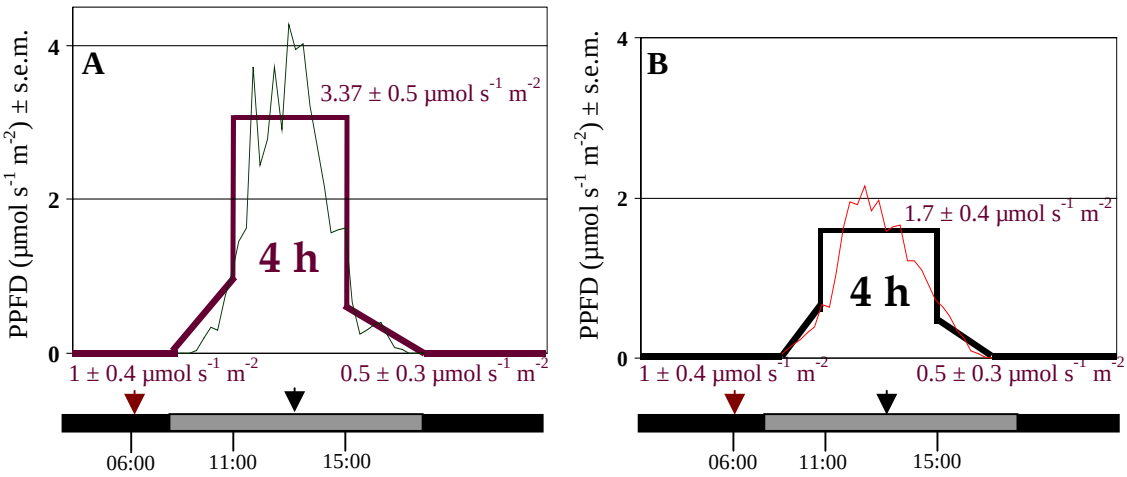


Fig.24 Light conditions settings for aquaria experiments. **A.** HL condition, **B.** LL condition. Values on the bottom of the graph indicate the light condition external to the 4h time interval at which was applied the HL(3.37± 0.5 µmol $s^{-1}m^{-2}$) and LL(1.7± 0.5 µmol $s^{-1}m^{-2}$) conditions. Black and red arrows correspond to the sampling moment. HL: high light, LL: low light.

The above light conditions, that will referred to as high (HL) and low (LL) light, regardless their intrinsic low value, were selected on the basis of the lowest values recorded in sea environment and for which differences in expression patterns of two sites were assessed. Temperature was instead set at the value found in sea environment at the sampling time (15.5 ± 0.5 °C).

Plants from preserved and disturbed sites were acclimated for one week at HL and LL, respectively. Thereafter, in order to proceed to a cross-check, plants from both meadows were grown side-to-side in the same aquaria exposed either to HL or LL for one week more and then sampled for RT-PCR analysis.

PoCHL P expression was evaluated in the leaf developmental stages previously considered through semiquantitative RT-PCR. Furthermore *RbcL* gene was used in order to mark photosynthetic activity.

In plants collected in the preserved site and exposed to HL the pattern of *PoCHL P* (Fig.25AP) did not change as compared to that of sea-growing plants (Fig.22A). By contrast, at LL condition (Fig.25BP) the pattern strongly differed, becoming tightly comparable to that recorded in disturbed marine environment (Fig.22B). Moreover, expression level indicated a more intense activity in intermediate and adult leaf of plants exposed to HL (Fig.25AP) and LL (Fig.25BP) condition, respectively.

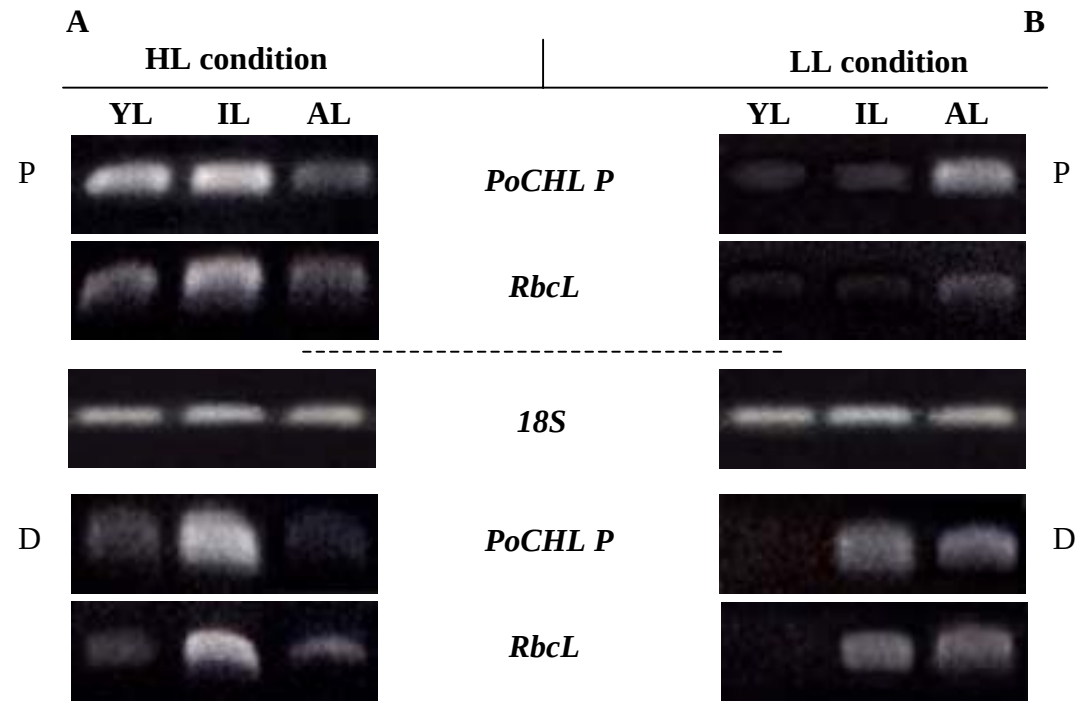


Fig.25 RT-PCR semiquantitative in plants sampled in preserved (P) and disturbed (D) site and grown at HL (3.37 ± 0.5 µmol s^{-1} m^{-2}) and LL (1.7 ± 0.4 µmol s^{-1} m^{-2}) in aquarium. AT: apical tips, YL: young leaf, IL: intermediate leaf, AL: adult leaf. Amplicon were 223 bp for *PoCHL P*, 485 bp for *RbcL* and 363 bp for 18S primers. Accessions were: 18S rRNA (AY491942), *RbcL* (U80719).

The behavior of plants sampled from disturbed site resulted to be more complex. As a general feature, it was confirmed that at HL (Fig. 25AD) and LL (Fig. 25BD) conditions adult leaves exhibited the lowest and the highest expression level, respectively. However, at LL condition the intermediate leaves sampled in disturbed site exhibited an expression level quite similar to that of adult ones (Fig. 25BD). Moreover, in young leaf gene expression was very low and completely absent at HL (Fig. 25AD) and LL (Fig. 25BD), respectively. As for as *RbcL* is concerned, it must be noticed that at HL condition the highest expression level was detected in intermediate leaves, whatever the sampling site was considered (Fig 25AP and AD). When LL condition was taken into account, the highest expression level of RbcL was instead observed in adult leaves (Fig.25BP and BD). However, in plants sampled from disturbed site a relevant level of expression was equally recorded in intermediate leaves (Fig.25BP andBD).

PoCHL P and *RbcL* expression was also monitored during dark period (sampling hour: 06:00 am). At the cycling used for semiquantitative experiment no expression was detected for both genes (data not shown). Expression bands were visualized by doubling cDNA quantity and cycles number, indicating a low expression levels as already reported (Giannino et al., 2004).

Finally, *PoCHL P* gene expression was monitored during the day (Fig.26), using only plants collected from preserved site and grown in aquarium, prepared as previously described, and maintained under room sunlight conditions.

Plant were acclimated for two weeks before sampling material at: 10:00, 12:30 and 16:30 during the day, referred to as I-, II- and III sampling. The average weekly values of light intensity recorded in the three samplings resulted to be respectively: 6.8 ± 2 µmol s^{-1} m^{-2}, 23.4 ± 3 µmol s^{-1} m^{-2} and 7.8 ± 1.7 µmol s^{-1} m^{-2}. Despite of the significant difference in light intensity, expression pattern of *PoCHL P* didn't vary significantly along the day (Fig.26). In addition it resulted similar to that observed for preserved meadow both in marine environment (Fig. 22A) and HL aquarium condition (Fig.25AP).

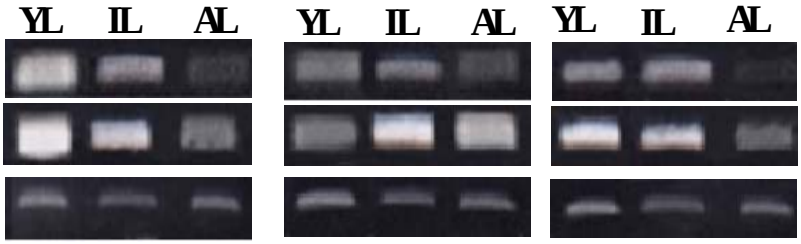
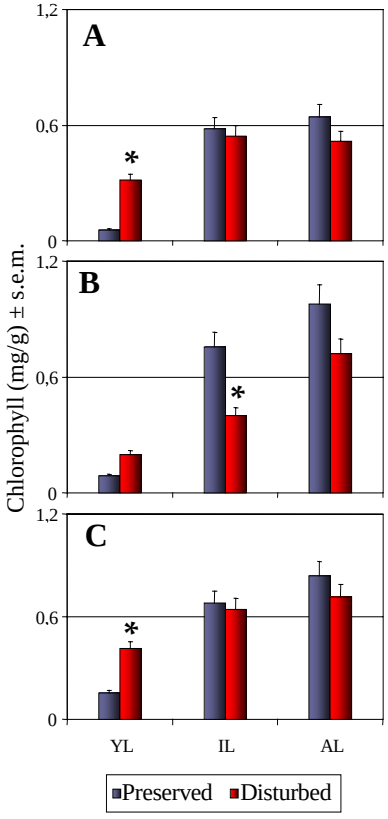


Fig.26 RT-PCR semiquantitative for daily variation of *PoCHL P* gene. Aquarium light conditions were 6.8 ± 2 µmol s^{-1} m^{-2} for I-TI, 23.4 ± 3 µmol s^{-1} m^{-2} for II-TI and 7.8 ± 1.7 µmol s^{-1} m^{-2} for III-TI. Legend: AT: apical tips, YL: young leaf, IL: intermediate leaf, AL: adult leaf.

4.1 – Total chlorophyll content evaluation

For all the thesis analyzed total chlorophylls content (mg/g) was evaluated. As a general feature, chlorophyll quantity differed in relation to leaf developmental stage, being lower in the young leaf and almost the same in intermediate and adult leaves (Figs.27, 28, 30).



Chlorophyll ratio

	Preserved *a*/*b*	Disturbed *a*/*b*
YL	1.94 ± 0.03	2.06 ± 0.01
IL	2.00 ± 0.04	2.14 ± 0.06
AL	1.96 ± 0.07	1.91 ± 0.05

	Preserved *a*/*b*	Disturbed *a*/*b*
YL	2.17 ± 0.06	2.17 ± 0.05
IL	2.09 ± 0.02	2.27 ± 0.09
AL	2.06 ± 0.05	1.87 ± 0.09

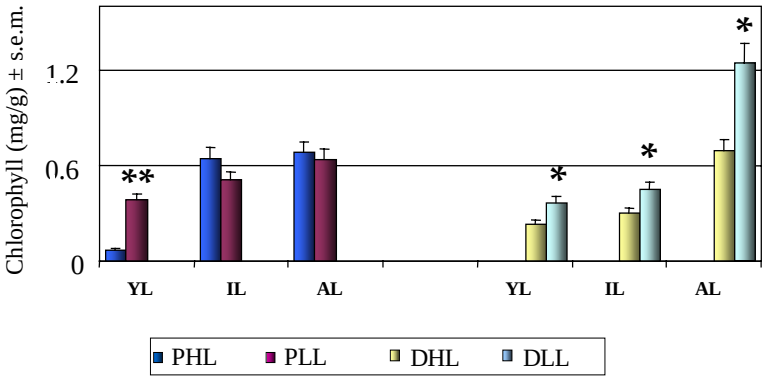
	Preserved *a*/*b*	Disturbed *a*/*b*
YL	1.93 ± 0.06	2.04 ± 0.08
IL	1.81 ± 0.10	2.15 ± 0.14
AL	1.91 ± 0.08	1.93 ± 0.05

Fig.27 Total chlorophyll content in leaves sampled at for June 2005 (**A**), November 2005 (**B**) and June 2006 (**C**) from preserved (blue) and disturbed (red columns) site. On the table is reported chlorophyll *a*/*b* ratio relative to the considered period. YL: young leaf, IL: intermediate leaf, AL: adult leaf. Statistical analysis (n=3): ANOVA followed by Neuman-Keul's *post-hoc* test.*p<0.05; **p<0.01; *** p<0.001.

In addition, chlorophyll *a*/*b* ratio exhibited a value (about 2) similar to that already reported (Pirc, 1986).

However, our results evidenced a significant increase of chlorophyll in young leaves of plants sampled from disturbed site both at June 2005 and 2006, as compared to the other developmental stages considered (Figs.27A and C). A different result was obtained in November (Fig.27B), when an evident reduction was observed in intermediate leaves of disturbed site. In this context, it must be underlined that at the sampling moment a high light availability was recorded in both sites (222 ± 30 µmol m^{-2} s^{-1} and 145.9 ± 17 µmol m^{-2} s^{-1}).

Aquarium session confirmed that chlorophyll content varied in relationship to leaf developmental stage and in PHL (Preserved High Light) and PLL (Preserved Low Light) plants the trend was similar to that identified in environmental conditions (Fig.28).



Chlorophyll ratio

	PHL*a*/*b*	PLL*a*/*b*
YL	2.12 ± 0.03	2.82 ± 0.09
IL	2.22 ± 0.07	2.25 ± 0.04
AL	2.11 ± 0.08	2.15 ± 0.06
	DHL*a*/*b*	DLL*a*/*b*
YL	2.07 ± 0.06	2.11 ± 0.04
IL	2.11 ± 0.09	2.14 ± 0.08
AL	2.24 ± 0.06	2.10 ± 0.12

Fig.28 Total chlorophyll content in leaves sampled from plant grown in aquarium. In the table (on the right) is reported chlorophyll *a*/*b* ratio. HL: high light aquarium, LL: low light aquarium. PHL: plants collected in preserved site and treated at HL, PLL: plants collected in preserved site and treated at LL, DHL: plants collected in disturbed site and treated at HL, DLL: plants collected in disturbed site and treated at LL. YL: young leaf, IL: intermediate leaf, AL: adult leaf. Statistical analysis (n=3): ANOVA followed by Neuman-Keul's *post-hoc* test.*p<0.05; **p<0.01; *** p<0.001.

A different condition was detected in disturbed plants where an increase of chlorophyll content was detected in DLL (Disturbed Low Light) vs DHL (Disturbed High Light) leaves, for all developmental stages (Fig.28). As for as chlorophyll *a*/*b* ratio is concerned, the most significant difference was observed in young leaves (Fig.28).

Chlorophylls data obtained for plants grown in aquarium were compared with those of sea-growing plants under corresponding light conditions (June) (Fig.29). No statistical variability was detected, with the exception of two cases, both related to plants collected in disturbed site and dealing with a reduction and increase of chlorophyll in DHL intermediate leaves and DLL adult leaves, respectively (Fig.29).

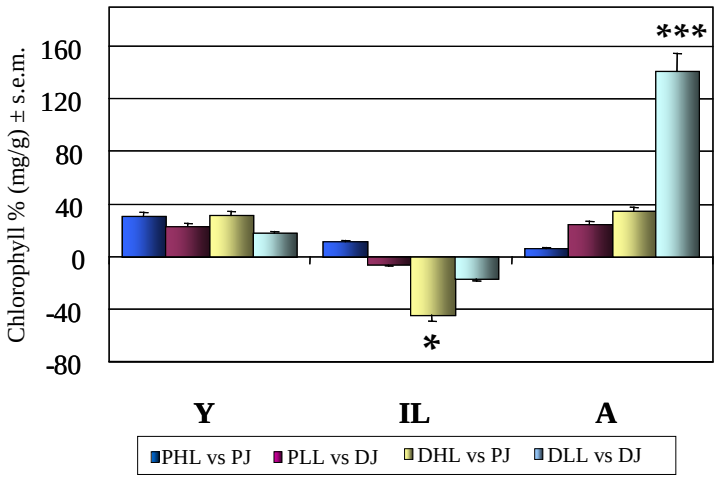
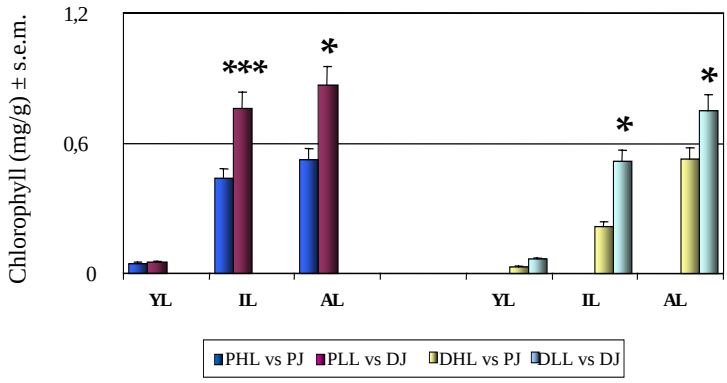


Fig.29 Aquarium sessions vs marine environment (June 2005): chlorophyll percentage ratio. PJ: preserved site in June; DJ: disturbed site in June; PHL: high light aquarium with plants sampled in preserved site, PLL: low light aquarium with plants sampled in preserved site, DHL: high light aquarium with plants sampled in disturbed site, DLL: low light aquarium with plants sampled in disturbed site. Statistical analysis: data are reported as ratio of mean value in aquarium treatments vs environmental conditions in June 2005. Statistical analysis (n=3): ANOVA followed by Neuman-Keul's *post-hoc* test. *p<0.05, **p<0.01, ***p<0.001 YL: young leaf, IL: intermediate leaf, AL: adult leaf.

At dark condition (*d)* a significant increase of chlorophylls content was detected in intermediate and adult leaf of both experimental groups treated at LL (Fig.30. Chlorophyll *a*/*b* ratio was as usually reported (Pirc, 1986) with the exception of a very low value in young leaves of *d*DLL plants and high level in *d*PHL and *d*PLL plants (Fig.30 and related table).



Chlorophyll ratio

	*d*PHL *a*/*b*	*d*PLL *a*/*b*
YL	2.54 ± 0.08	2.30 ± 0.09
IL	2.22 ± 0.06	2.25 ± 0.05
AL	2.24 ± 0.03	2.20 ± 0.08
	*d*DHL *a*/*b*	*d*DLL *a*/*b*
YL	2.41 ± 0.11	1.21 ± 0.09
IL	2.35 ± 0.07	2.10 ± 0.12
AL	2.21 ± 0.07	2.06 ± 0.07

Fig.30 Total chlorophyll content for aquarium experimental session at dark period (*d*). On the table is reported chlorophyll *a*/*b* ratio. *d*PHL: plants collected in preserved site and treated at HL at dark period, *d*PLL: plants collected in preserved site and treated at LL at dark period, *d*DHL: plants collected in disturbed site and treated at HL at dark period, *d*DLL: plants collected in disturbed site and treated at LL at dark period. YL: young leaf, IL: intermediate leaf, AL: adult leaf. Statistical analysis (n=3): ANOVA followed by Neuman-Keul's *post-hoc* test.*p<0.05; **p<0.01; *** p<0.001.

Moreover, in the transition from dark period to the light conditions set in aquarium (Fig.31A and B), it was observed a chlorophyll increase in all the leaves of DLL plants (Fig.31B), while in DHL plants an increase and a reduction of chlorophyll content was detected in young and in intermediate leaves, respectively.

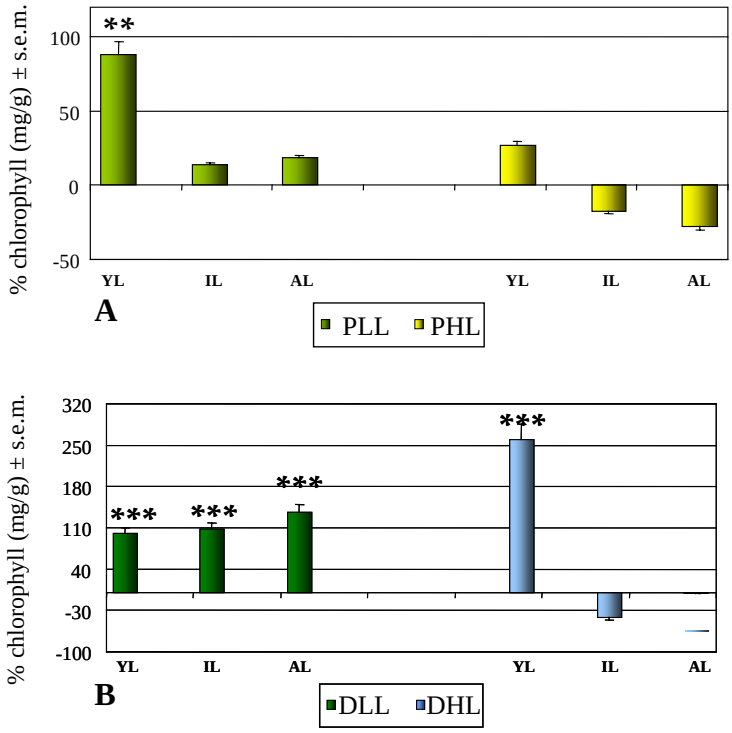


Fig.31 Comparison about chlorophyll content for experimental group when passing from dark period to set light conditions in aquarium session. **A.** PLL and PHL condition respectively. **B.** DLL and DHL condition respectively. YL: young leaf, IL: intermediate leaf, AL: adult leaf. Statistical analysis (n=3): data are reported as ratio of means value in diurnal experiment vs dark period. ANOVA followed by Neuman-Keul's *post-hoc* test.*p<0.05; **p<0.01; *** p<0.001.

Discussion

In the present work it was demonstrated that zeatin distribution, as well as the expression pattern of *PoCHL P* gene differed when analyzed in plants of *P. oceanica* growing in two distinct meadows, which on the basis of their macrostructure have been classified as preserved and disturbed sites, respectively (Cozza et al, 2004; Rende et al., 2005, 2006). In addition, the monitoring of light condition in both meadows along one year allowed us to asses a significant reduction in light availability in the disturbed vs preserved site for a period spanning from the winter to the first two months in the spring.

In the annual cycle of *P. oceanica*, spring is the season in which plants exhibit the highest growth rate (Alcoverro et al., 1995) and productivity, this latter leading to a massive storage of carbohydrate resources in the rhizome which allow the plants to survive during the following winter (Pirc, 1985; Pirc, 1989). On the contrary, photosynthetic activity decreases in summer when temperature and light availability are very high (Buia et al., 1992) and older leaves detach (Pirc, 1986). Taking into account this pattern of plant growth-production, we planned to analyze the distribution of cytokinins in plant at the end of winter and spring seasons. Shoots and rhizomes were selected as organs essential to the reiterated production of phytomers (i.e. plant growth unit) and reserve accumulation (Pirc, 1989) respectively. It is worth noting that among hormone classes, cytokinins play a key role in both biological processes (for a review Mok and Mok, 2001).

In line with the involvement of cytokinins in controlling resources translocation Mok and Mok, 2001). A clear seasonal increase in the levels of zeatin was detected in the rhizome of plants sampled from both meadows. However, no differences were evidenced when comparing plants collected in the different sites, whatever sampling period was taken into account. This result is consistent with the similar primary production of the two meadows (Rende et al., 2006), likely related to the relevant light availability recorded in both sites at the end of the spring. By contrast, clear differences in the level and pattern of zeatin distribution marked the shoot of plants growing in preserved vs disturbed sites in both seasonal periods. Indeed, zeatin was less abundant in the plants from disturbed site. In addition, its distribution in the apical dome appeared scattered and in some cases absent from the CZ, which represents the meristematic domain that replenishes the differentiating PZ and RM (Kerstetter and Hake, 1997). On the basis of key role played by cytokinins in cell proliferation and morphogenesis (Mok and Mok, 2001), the above features could

account for the surprising small size of shoot and consequently for the reduced rate of growth in these plants (Rende et al., 2006) in a period of expected high activity (Ott, 1980).

In this context we may recall that differences in cytokinin distribution, clearly related to environmental condition, have been previously described for leaves (Cozza et al., 2004). The extension of this behavior to the apical meristem, responsible of plant growth, strongly supports an effective role for these hormone class as cytophysiological marker of stress condition in *P. oceanica* plants. Furthermore, the absence of differences in cytokinin level and distribution exhibited by rhizomes growing in the different sites as compared to shoot and leaf behavior (Cozza et al., 2004) is in agreement with our hypothesis that response mechanism involving cytokinins could be related to light signal.

To further dissect the metabolic state of plants exposed to different light conditions we took into account the expression pattern of *PoCHL P* gene, which encodes an enzyme active in both chlorophyll and tocopherol biosynthetic pathways. RT-PCR analyses allowed us to detect that *PoCHL P* was prevalently expressed in leaves, according to the importance of gene activity in chloroplast differentiation (Keller et al., 1998; Giannino et al., 2004). Notably, *in situ* approach evidenced that *PoCHL P* transcripts mainly localized in the epidermis cells that in *Posidonia* leaf are very rich in chloroplasts, becoming the principal site of photosynthetic activity (Cozza et al., 2004). Moreover, *PoCHL P* signal in vascular bundles suggests a side role associated to the regulation of photoassimilates translocation related to tocopherols biosynthesis (Hofius et al., 2004).

Expression of *PoCHL P* resulted differ also in relation to developmental stage of leaf, being higher in young and intermediate leaves than in adult ones. However, the most interesting aspect of our results was the opposite trend detected in all the seasonal periods in the leaves of plants collected in stressed vs conserved site. The down regulation of *PoCHL P* expression detected in developing leaves exposed to a reduced light intensity well fits with the reduction of leaf growth rate in the disturbed site (Rende et al., 2005, 2006). To this matter, we may recall that in *Posidonia* plants the accumulation of carbohydrate reserves during spring is a crucial event for surviving when either temporary or long lasting reduction of light availability occurs and the C balance can be negative, like during the winter (Pirc et al., 1985; Alcoverro et al., 2001). Moreover, during winter a reduction of these reserves leads to a decrease in the production of leaves which are essential for the acquisition of N and C, probably implying a deterioration of plant status and a decreased survival (Alcoverro et al., 2001).

On the other hand, the high level of *PoCHL P* expression that we detected in adult leaves of disturbed plants support a strong requirement of gene product. This feature can be interpreted as a compensative response to stressful condition, likely requiring both chlorophylls and tocopherols. According to the multifunctionality of *PoCHL P*, chlorophyll content assay evidenced that pigment amounts were not proportionally correlated to *PoCHL P* expression levels. In this context it is worth noting that tocopherols shield plants from both photo-oxidative and ageing processes (Weaver and Amasino, 2001; Havaux et al., 2005), due to their antioxidant activity. Consequently, high activity of *PoCHL P* gene in adult leaves of stressed plants may be related to a protection mechanism against senescence which could precociously occur in absence of adequate levels of chlorophylls and cytokinins (Cozza et al. 2004) .

All together these results strongly suggest that *PoCHL P* expression pattern is modulated by environmental light-related conditions. This assumption is fully confirmed by the results of aquarium session, showing that in plants collected from both preserved and disturbed sites gene expression pattern changed in relation to light regime. Namely, at low light condition plants collected from the preserved site exhibited a pattern of gene expression tightly comparable to that recorded in stressed marine environment. Moreover, the exposure to light condition further compromised the performance of disturbed plants in respect to that observed in marine environment. A partial reversion of expression pattern was instead obtained in plants sampled in disturbed site following exposure to high light conditions, as evidenced by the delayed recovery of gene expression in young leaf. We suggest that plants sampled in the disturbed site didn’t completely reach the steady state level for *PoCHL P* expression following a week period at high light regime.

Interesting the expression of *RbcL*, used as marker of photosynthetic activity, differed in relation to leaf developmental stage and showed a pattern very similar to that observed for *PoCHL P*. To this concern it can be inferred that at high light intensity, the major photosynthetic sustain is charged to intermediate leaves. In a different manner, at low light conditions the highest expression level of *RbcL* as well as of *PoCHL P* was observed in adult leaves. Thus, it can be speculated that when light availability is reduced plant vitality is first guaranteed by adult leaves through the enhancement of photosynthesis. The upregulation in these leaves of *PoCHL P* expression could concur to this event by promoting resource translocation through the switch of tocopherols biosynthesis (Hofius et al., 2004), as previously discussed.

As for as *PoCHL P* expression is concerned, aquarium session allowed us to verify that during the darkness period our gene was expressed at very low level and strongly increased in response to the input light signal, according to data in literature (Giannino et al., 2004; Schmitt et al., 2004). In this context we may recall that for both *CHL P* ortholog gene of the photosynthetic cyanobacterium *Synechocystis* sp. (Schmitt et al., 2004) and *Prunus persica* (Giannino et al., 2004) a fast light-dependence expression has been demonstrated. Our results do not provide direct evidence for this feature, since the time course of light signal response was not monitored. On the contrary, the monitoring of *PoCHL P* expression along the whole day, clearly showed that at fluctuating light conditions the gene is expressed at same level.

In conclusion, in the present work we demonstrated that in *Posidonia oceanica* plants *PoCHL P* exhibited a organ-specific expression pattern which is modulated in relation to leaf developmental stage. We also demonstrated that in both environmental and controlled growth conditions, this pattern differed in relation to light regime. On the other hand, the levels of *PoCHL P* expression were not proportionally regulated by light intensity as proved by the monitoring of expression level along the day (aquarium session) and the year (marine environment). On the basis of all these evidences**,** we propose that the observed differences in the pattern of *PoCHL P* expression are related to light-induced changes on photosynthetic metabolism.. Thus, in our opinion, the 'inverted' pattern observed for *PoCHL P* at low light regime, both in environmental as well as under aquarium conditions, together to that of *RbcL*, represent a specific metabolic response occurring in relation to light conditions and leading to a lag in leaf development. Consequently, at metabolic level, the adult leaves play the role of intermediate ones, supporting plant life through photosynthesis and resources relocation. The involvement of *PoCHL P* in the biosynthesis of both chlorophyll and tocopherols, as well as the relationship between these latter and resources export (Hofius et al., 2004), strongly supports this assumption. By contrast, the metabolic similarity between intermediate and adult leaves observed in disturbed plants at exasperated low light conditions is still far from being well understood.

In this context, to add further insight into the complex network of metabolic changes related to light conditions, many concerns are still under investigations: first of all the effect of light quality influence on gene expression. At this aim we are monitoring the expression of *PoCHL P* in plants growing at different depths and sampled from other meadows. In addition, we started to investigate other genes related to plant light-response, such as phytochrome family (see annexes to the present thesis).

However, on the basis of discussed results, we propose that the expression pattern of *PoCHL P* represents a useful tool to assess long-lasting and low-light-related stressful conditions in *Posidonia oceanica* meadow.

Materials and methods

Fig. 32 Sampling sites

1. Sampling area

Plant material used in the present work was collected in two different meadows of *P. oceanica* , three kilometers away each other, spanning over a sandy substrate along north-western coasts of Calabria and located: i) at Praja a Mare (CS), a touristic area near the mouth of Noce river that provokes a high sedimentation rate and periodical raise of water turbidity; ii) at San Nicola Arcella (CS) a poorly anthropized trait of coast representing a useful control site (Fig. 32). Preliminar investigation allowed us to detect the occurrence of significant variation of light condition between the two selected meadows. Furthermore, on the basis of macrostructural features the two meadow had been classified, according to Giraud (1977), as 'sparse meadow of III type' and 'dense meadow of type II' respectively (Rende et al., 2005). From this moment on the two meadows will be referred to as disturbed site (Praja a Mare) and preserved site (San Nicola Arcella).

2. Sampling of plant material

Posidonia oceanica plants were sampled in the two meadows at 10 m depth and at different seasonal period: March, June and November 2005, April and June 2006. Shoots, leaves and rhizomes were excised from plants and epiphytes were rapidly removed from leaves using a razor sterile blade (Dauby and Poulicek, 1995). Leaves were classified according to Giraud (1977) as young leaf (<5cm, sheathless), intermediate leaf (>5 cm, sheath <2cm) and adult leaf (>5 cm sheath >2 cm) and for each category leaves were cut into three equivalent portions (basal, intermediate and apical) which were separately processed. Orthotropic and plagiotropic rhizomes were also separately sampled and after excision of apical shoot, the apical portion (about the first 5 cm) of excised rhizomes was cut into 1 mm thick slides All sampled material was immediately fixed or frozen for molecular and cytological analyses, respectively.

3. Growth condition in aquarium

P. oceanica plants sampled in April from both preserved and disturbed meadow were transferred into two aquarium (n= 20 plants for each) containing 100L of seawater, pH 7.8, assuring water flow-through and fluxing oxygen throughout. Settings of light condition (Fig.24) and samplings are illustrated in the result section.

4. Histological analysis

Sampled rhizome slices were fixed in paraformaldheyde 4% in phosphate buffer 1X (NaCl 1.3M, $Na_2HPO_4 \cdot 2H_2O$ 70 mM, $NaH_2PO_4 \cdot H_2O$ 30 mM) and kept at +4°C overnight. Then, material was dehydrated in ethanol crescent series, resin-embedded (Technovitt Istokulzer 800®) according to manufacturer instructions and sectioned at 2 μm by using a microtome LEICA RM2155. Sections were stained either according to PAS method or with Toluidine blue (0.1% in water w/v) dried at 37°C, mounted with Canada balm and observed at a transmission microscope Leica DMRB. Representative images were acquired through the software Leica IM50.

For qualitative fluorescence performed on shoot apexes and leaves, samples were fixed in paraformaldehyde (3%, w/v), glutaraldheyde (0.5%, v/v) and buffer phosphate solution (NaCl 135 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, K_2HPO_4 8 mM, pH 7.2). Subsequently, samples were dehydrated through a growing percentage series of ethanol solution, paraffin-embedded (paraplast, SIGMA) and sectioned at 10 μm thick using a LEICA RM2155 microtome. After remotion of paraffin, rehydrated sections were treated with a saline phosphate buffer (SPB: $CaCl_2$ 100 mg/L, KCl 200 mg/L, KH_2PO_4 200 mg/L, $MgSO_4$ 59 mg/L, NaCl 800 mg/L, $NaHPO_4$ 1150 mg/L) for 2' and then stained with 1:9 acridine orange/SPB solution (stock solution 1% , w/v) for 15'. After two washing with SPB sections were mounted with SPB. Slides were observed using a fluorescence microscope LEICA DMRB having an epicondenser and mercury lamp of 50W. Images were acquired with Leica IM50 software.

5. Zeatin immunolocalization

Immunolocalization of zeatin was performed, according to the procedure described by Dewitte et al. (1999). Samples were fixed in a 0.5% (v/v) gluteraldehyde and 3% (w/v) paraformaldehyde mixture in PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8 mM K2HPO4, pH7.2) at 4 8C for 3 h. Sections (20–25 mm) were cut using the vibratome (Leica VT1000E, Germany) and collected in PBS on ice in the wells of a tissue culture plate. Subsequently, floating vibratome sections were incubated in blocking buffer [0.5% (w/v) BSA, 0.1% (v/v) fish gelatin, 1% (v/v) normal goat serum, 20 mM glycine in PBS] three times for 10 min and in a 0.07% saponin solution in PBS for 20 min. Afterwards, the sections were incubated with a primary antibody against zeatin in a dilution of 1:200 in blocking buffer overnight at 4 8C, followed by 1 h at room temperature. After three washings (three times for 10 min) with PBS, sections were incubated for 3 h at room temperature with the secondary antibody goat anti-rabbit IgG (1:100, in blocking buffer) conjugated with alkaline phosphatase (Boehringer, Germany). After three washings (three times for 10 min) with PBS, sections treated with alkaline phosphatase conjugates were rinsed with Tris –HCl buffer (0.1 M Tris, 2 mM MgCl2, pH 9.5) and left to react in the presence of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Germany) for 3'. The enzymatic reaction

was stopped by 2 mM EDTA in PBS. Samples were mounted in a PBS/glycerin mixture (1:1 v/v) and immediately observed under a Leica DMRB photomicroscope.

6. Chlorophyll pigments extraction

For chlorophylls (0.5 g) extraction apical portions of frozen young, intermediate and adult leaves were used. Material was grinded in a mortar with liquid nitrogen, collected and weighted before treatment with 80% acetone 1:5 (w/v) for three hours, at dark condition. Subsequently a centrifugation step at 3'000 rpm and recover of the supernatant was performed. Pigment determination was carried out at dim light with a Jasco V-530 spectrophotometer, using the associated software: JASCO SPECTRA MANAGER – Ver. 1-10.00, V-500 series. Pigments concentration was determined by using Lichtenthaler's formula (1987): $C_a = 12.25A_{663.2} - 2.79A_{646.8}$ and $C_b = 21.50A_{646.8} - 5.10A_{663.2}$ for chlorophylls *a* and *b* respectively, $C_{a+b} = 7.15A_{663.2} - 18.71A_{646.8}$ for total chlorophylls, and $C_{x+c} = (1000A740 - 1.82Ca - 85.02Cb)/198$ for total carotenoids (xanthophylls and β-carotene). Formulas were multiplied for 0.01/weight to obtain a concentration expressed as mg g^{-1}. Three replicates were performed and for each replicate six measurements were carried out on each sample. ANOVA followed by Neuman-Keul's *post-hoc* test was used to evaluate differences between the considered groups of data.

7. Total RNA extraction and reverse transcription

Total RNA extraction was performed following Doyle and Doyle (1990) protocol, with some modification. All solutions were prepared with distilled water treated with 0.1% DEPC (Sigma®) to avoid RNases contamination. About 0.3 g of *Posidonia* tissue were grinded in liquid nitrogen adding 0.1 g PVP-40 (Sigma®) directly in the mortar. One milliliter of freshly prepared extraction buffer [200 mM Tris/HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA, CTAB 3% (w/v)] and thereafter β-mercaptoethanol (final concentration 1.3%) were added to samples. After 30' at 60°C, one volume of chlorophorm/isoamyl alcohol (49:1) was added and after centrifugation at 6'500 rpm (3'944 g) for 15'supernatant was recovered and precipitated with isopropanol at –20°C, overnight. After centrifugation at 13'000 rpm (15'776 g) for 15' and washing with sodium acetate 0.2M/ethanol 70% for 1 h , RNA was finally purified, resuspended and treated with 30 U of DNase I (Roche) for 15' at 37°C. cDNA was obtained by reverse transcription of about 1 µg of RNA with Superscript III according to the manufacturer's instructions (Invitrogen).

8. Isolation and sequence analysis of cDNA and genomic clones

To identify *PoCHL P* gene, RT-PCR reactions were performed using a Primus Thermal Cycler® according to the following program: 94°C for 3', 35 cycles of 94°C for 50'', 55°C for 40'', 72°C for 1', 72°C for 5'. The gene was isolated using leaf cDNA (2 µl) or gDNA (genomic DNA) (200 ng), 0.3 µM of primers, 0.5 mM dNTP, 2.5U *Taq* polymerase (GoTaq, Promega®), 1/5 *Taq*

Buffer 5X containing directly $MgCl_2$ (final concentration into the reaction mix: 1.5 mM), in a final volume of 50 µl.

All primers used in this work were designed with Primer3 software (http:// Frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). FW(5'-GTGCTGGCGGTNGAYGAYAG-3')and BW(5'-GCGGACGGGCTTNAGNAG-3') primers allowed us to identify a partial fragment of 911 bp. A second couple of primers (IFw: 5'-GGCCAGTAAGCCTTGTCAAC-3', IBw: 5'-TCTCCCAAGGACGATGTCTC-3'), designed on the identified sequence was used on gDNA to identify the presence of two introns, 91 bp and 735 bp respectively, harboring inside the 911 bp cDNA sequence. The obtained genomic sequence was aligned with coding cDNA using two programs available online: the AAT (dds-gap2) gene server (http://genome.cs.mtu.edu/aat/aat.html), ClustalW, and the Plant Gene Databank server (http://www.plantgdb.org/PlantGDB-cgi/GeneSeqer/PlantGDBgs.cgi) and in such a way it was possible to recognize the splice site sequence GT/AG.

Untranslated regions were identified by 3'RACE technique (Invitrogen®) using the FW1-(5'-CAAGGCCTAGAAGGTTGCTG-3'), while 5' UTR is not yet identified.

All RT-PCR products were analyzed on a 1.2% electrophoresis gel stained with ethidium bromide (Maniatis et al., 1982), using a GeneRuler 100 bp (Fermentas™) as ladder of molecular weight. All obtained sequences were extracted by using Qiaquick system (Qiagen), ligated in pGEM-T easy vector (Promega), cloned using *E. coli* JM109 strain (Promega®) and sent to sequencing service of GeneLab (ENEA, Rome).

9. DNA extraction and Southern blot analysis

DNA was extracted following Doyle and Doyle (1990) protocol. 5 µg of extracted total DNA was digested overnight at 37°C with 60U of Spe1 and Sph1 restriction enzymes (Promega). Subsequently, digestion was precipitated with isopropanol at –20°C overnight and checked on 1% agarose gel. All digested DNA was subjected to electrophoresis (20h at 45 V on 0.8% agarose gel) and blotted onto membranes Hybond-N+ (Amersham Pharmacia Biotech) by Vacuum Blotting System according to the manufacturer's instructions (Amersham Pharmacia Biotech). The membrane was oven-dried at 80°C for 2h. Prehybridization (3h at 52°C in 70 ml) and hybridization (overnight at 52°C in 30 ml) were carried out in 25% SSC20X (NaCl 3M, Sodium Citrate 300 mM), 0.01% sarkosyl (10%), 0.1% blocking reagent (Roche) (2.5% prepared in maleic acid buffer, pH 7.5) in a hybridiser HB-2D (Techne). Digoxygenin-labelled probe obtained by PCR on genomic DNA with SeQ primers using DIG-dNTPs (Roche), according to manufacturer instructions, was used for southern blot. The probe was denatured for 3' at 94°C and placed in ice before to be added to hybridization buffer. Concentration of the probe was calculated as indicated in Roche manual (www.roche-applied-science.com). Detection was performed with anti-DIG-AP (Roche) and nitro-

blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Roche) according to manufacturer instruction. Hybridization bands were visualized keeping the membrane at dark for 2 h .

10. Relative RT-PCR analysis

cDNAs was obtained from apical tips, young, intermediate and adult leaf were carried out as above described. PCRs were performed using gene-specific primers from cDNA of *PoCHL P* (SeQFw: 5'-ATCTCCGCCAAGTCCTTGTA-3' and SeQBw: 5'-ACGATGTCTCCCCTTACGTG-3') and *18S rRNA* of *Posidonia oceanica* (AY491942) with the primers 18SFw1: 5'-CATGGCCGTTCTTAGTTGGT-3' and 18SBw2: 5'-GTACAAAGGGCAGGGACGTA-3'. Primers were designed to yield 223 bp and 363 bp amplicons for *PoCHL P* and *18S* respectively. *PoCHL P*-primers were designed externally to an 91bp long intron .

The relative amounts of PCR products were readily quantified on TBE- agarose gel. To normalize for equal amounts of total RNA and efficiency of cDNA synthesis from various tissue samples, the intensities of each band were normalized with the intensity of the *18S* product. PCR conditions were the same used in paragraph 3.3a and 3.3b with the following variations: annealing at 58°C and extension at 40'', 18 cycles for *18S rRNA* gene and 30 cycles for *PoCHL P* gene. .

To mark photosynthetic activity, rubisco small subunit gene (*RbcL*) expression was monitored in parallel with *PoCHL P*. Primers used to this purpose gave an amplicon of 485 bp and were RFW 5'-ATCTTGGCAGCATTCCGAGTA-3', and RBW 5'-AGCCCACCACGCAGACATT-3', designed on the NCBI available sequence (U80719). Cycling was 30 cycles with annealing at 55°C for 40'' and extension at 72°C for 35''. Three independent replicates were performed for each thesis

11. RNA antisense probe and in situ hybridation (ISH)

RNA probe was synthesized using a cDNA fragment of *PoCHL P* 911 bp cloned in pGEM-T easy vector. The plasmid was linearized with NcoI and SpeI restriction enzymes (Promega). Digoxygenin-labelled (Roche) sense and antisense probes were generated by T7- and SP6 polymerase driven in vitro transcription, respectively. Probe integrity was checked on ethidium bromide stained gel.

Excised shoots and leaves were fixed, dehydrated, embedded in paraffin, cut into 8 lm sections and hybridized (55 °C) to a digoxygenin-labelled antisense RNA probe as described by Cañas, et al. (1994).

12. Statistical analysis

Statistical analysis was performed to validate data obtained from light conditions of the sites, chlorophyll quantification and from measurements of OD values for zeatin immunoreaction. All data set were analyzed by ANOVA followed by Neuman-Keul's *post-hoc* test.

References

Addlesee, H. A. and Hunter, C.N. (1999). Physical mapping and functional assignment of the geranylgeranyl-bacteriochlorophyll reductase gene, *bchP*, of *Rhodobacter sphaeroides*. *J. Bacteriol.* **181**,7248-7255.

Addlesee, H. A. and Hunter, C.N. (1999). Physical mapping and functional assignment of the geranylgeranyl-bacteriochlorophyll reductase gene, *bchP*, of *Rhodobacter sphaeroides*. *J. Bacteriol.* **181**,7248-7255.

Addlesee, H.A., Gibson, L.C.D., Jensen, P.E. and Hunter, C.N. (1996). Cloning, sequencing and functional assignment of the chlorophyll biosynthesis gene, *chlP*, of *Synechocystis* sp. PCC 6803. *FEBS Lett.* **389**, 126-130.

Aitzetmüller, K. (1997). Antioxidative effects of *Carum* seeds. *J. Am. Oil Chem. Soc.* **74**, 185.

Akiyoshi, D.E., Morris, R.O., Hinz, R., Mischke, B.S., Kosuge, T., Garfinkel, D.J., Gordon, M.P. and Nester, E.W. (1983). Cytokinin/auxin balance in crown gall tumours is regulated by specific loci in the T-DNA. *Proc. Natl. Acad. Sci.* USA **80**, 407-411.

Albergoni, F.G., Basso, B. and Tedesco, G. (1978). On the anatomy of *Posidonia oceanica* (*Zosteraceae*). *Plant Sys. Evol.* **130**, 191-201.

Alcoverro, T., Duarte, C.M. and Romero, J. (1995). Annual growth dynamics of *Posidonia oceanica*: contribution of large scale versus local factors to seasonality. *Mar. Ecol. Prog. Ser.* **120**, 203-210.

Alcoverro, T., Duarte, C.M. and Romero, J. (1997). Influence of herbivores on *Posidonia oceanica* epiphytes. *Aquat. Bot.* **56**, 93-104.

Alcoverro, T., Manzanera, M. and Romero, J. (2001). Annual metabolic carbon balance of the seagrass *Posidonia oceanica* (L.) Delile: the importance of carbohydrate reserves. *Mar. Ecol. Prog. Ser.* **211**, 105-116.

Ancora, S., Bianchi, N., Butini, A., Buia, M.C., Gambi, M.C. and Leonzio, C. (2004). *Posidonia oceanica* as a biomonitor of trace elements in the Gulf of Naples: temporal trends by lepidochronology. *Environ. Toxicol. Chem.* **23**, 1093-1099.

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takahashi, T., et al. (2005). Cytokinin oxidase regulates rice grain production. *Science* **309**, 741-745.

Atta-Asafo-Adjei, E., Lawon, M.P. and Philpot, R. (1993). Cloning, sequencing, distribution, and expression in *Escherichia coli* of flavin-containing monooxygenase 1C1. *J. Biol. Chem.* **268**, 9681-9689.

Balestri, E. (2004). Flowering of the seagrass *Posidonia oceanica* in a north-western Mediterranean coastal area: temporal and spatial variations. *Mar. Biol.* **145**, 61-68.

Balestri, E., and Cinelli, F. (2003). Sexual reproductive success in *Posidonia oceanica. Aquat. Bot.* **75**, 21-32.

Balestri, E., Piazzi, L., and Cinelli, F. (1998). Survival and growth of trasplanted and natural seedlings of *Posidonia oceanica* (L) Delile in a damaged coastal area. *J. Exp. Mar. Biol. Ecol.* **228**, 209-225.

Bay, D. (1984). A field study of the growth dynamics and productivity of *Posidonia oceanica* (L.) Delile in Calvi Bay, Corsica. *Aquat. Bot.* **20**, 43-64.

Benz, J., Wolf, C. and Rüdiger, W. (1980). Chlorophyll biosynthesis: hydrogenation of geranylgeraniol. *J. Plant Sci. Lett.* **19**, 225-230.

Blackwell, J.R. and Horgan, R. (1994). Cytokinin biosynthesis by extracts of *Zea mays*. *Phytochem.* **35**, 339-342.

Bollivar, D.W., Wang, S.J., Allen, J.P. and Bauer, C.E. (1994). Molecular genetic analysis of terminal steps in bacteriochlorophyll *a* biosynthesis: characterization of a *Rhodobacter capsulatus* strain that synthesizes geranylgeraniol-esterified bacteriochlorophyll *a*. *Biochem.* **33**, 12763-12768.

Boudouresque, C.F. and Meinesz, A. (1982). Découverte de l'herbier de *Posidonie*. *Parc. National de Port-Cros*, *Cahiers* **4**, 1-79.

Bramley, P.M., Elmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A. and Wagner, K-H. (2000). Vitamin E. *J. Sci. Food Agric.* **80**, 913-938.

Brzobohaty, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N. et al. (1993). Release of active cytokinin by a beta-glucosidase localized to the maize root meristem. *Science* **262**, 1051-1054.

Buia, M.C., and Mazzella, I. (1991). Reproductive phenology of the Mediterranean seagrasses *Posidonia oceanica* (L.) Delile, *Cymodocea nodosa* (Ucria) Aschers., and *Zostera noltii* (Hornem). *Aquat. Bot.* **40**, 243-262.

Buia, M.C., and Peirano, A. (1988). Record of a *Posidonia oceanica* (L) Delile seedling in the Egadi islands (Sicily, Italy). *Posidonia Newslett.* **2**, 19-22.

Buia, M.C., Zupo, V. and Mazzella, L. (1992). Primary production and growth dynamics in *Posidonia oceanica*. *P.S.Z.N.I. Mar. Ecol.* **13**, 2-16

Burkle, L., Cedzich, A., Dopke, C., Stransky, H., Okumoto, S. et al. (2003). Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis*. *Plant J.* **34**, 13-26.

Cahoon, E.B., Hall, S.E., Ripp, K.G., Ganzke, T.S., Hitz, W.D. and Coughlan, S.J. (2003). Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. *Nat. Biotechnol.* **21**, 1082-1087.

Campbell, M.L. (2003). Recruitment and colonisation of vegetative fragments of *Posidonia australis* and *Posidonia coriacea*. *Aquat. Bot.* **76**, 175-184.

Cañas, L.A., Busscher, M., Angenent, G.C., Beltrá, J.P. and van Tunen, A.J. (1994). Nuclear localization of the petunia *MADS* box protein FBP1. *Plant J.* **6**, 597-604.

Capiomont, A., Sandmeier, M, Caye, G. and Meinesz, A. (1996). Enzyme polymorphism in *Posidonia oceanica*, a seagrass endemic to the Mediterranean. *Aquat. Bot.* **54**, 265-277.

Caye, G. (1982). Etude sur la croissance de la posidonie, *Posidonia oceanica* (L.) Delile, formation des feuilles et croissance des tiges au cours de l'année. *Téthys*, France, **10**, 229-235.

Caye, G. and Meinesz, A. (1984). Observation sur la floraison et la fructification de *Posidonia oceanica* dans la baie de Villefranche et an Corse du Sud. In: *Boudouresque, C.F., Jeudy de Grissac, A. and Olivier, J. (Eds). Proceedings of International Workshop on Posidonia oceanica Beds, GIS Posidonie Publication, Marseilles, France*, pp. 193-201.

Collakova E, DellaPenna D (2003). Homogentisate phytyltransferase activity is limiting for tocopherol biosynthesis in *Arabidopsis*. *Plant Physiol* **131**, 632–642.

Contandiopoulos, J. and Verlaque, M. (1984). Contribution à l'étude caryologique de quelques populations de *Posidonia oceanica* [L.] DELILE du littoral Français. In: *Boudouresque, C.F., Jeudy de Grissac, A. and Olivier, J. (Eds). Proceedings of International Workshop on Posidonia oceanica Beds, GIS Posidonie Publication, Marseilles, France*, pp. 211-215.

Cozza, R., Chiappetta, A., Petrarulo, M., Salimonti, A., Rende, F., Bitonti, M.B. and Innocenti, A.M. (2004). Cytophysiological features of *Posidonia oceanica* as putative markers of environmental conditions. *Chem. Ecol.* **20**, 215-223.

Dahlgren, R.M.T., Clifford, H.T. and Yeo, P.F. (1985). The families of the monocotyledons. Springer-Verlag, Berlin.

Dalmazio, S., Cammarata, M., Carrillo, D., Calvo, S., Romancino, D.P., Cognetti, G. and Di Carlo, M. (2002). Molecular characterization of a variable tandem repeat sequence determined during RAPD analysis among *Posidonia oceanica* insular and coastline populations. *Rus. J. Genet.* **38**, 684-690.

Dauby, P. and Poulicek, M. (1995). Methods for removing epiphytes from seagrasses: SEM observations on treated leaves. *Aquat. Bot.* **52**, 217-228.

Dewitte, W., Chiappetta, A., Azmi, A., Witters, E., Strnad, M., Rembur, J., Noin, M., Chriqui, D. and Van Onchelen, H. (1999). Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. *Plant Physiol.* **119**, 111-122.

den Hartog, C. (1970). The seagrasses of the world. *Verh. K. ned. Akad. Wet.*, 59. The North-Holland Publ. Comp. Amsterdam-London, pp. 1-274.

den Hartog, C., Hennen, J., Noten, Th.M.P.A. and Van Wijk, R.J. (1987). Chromosome numbers of the European seagrasses. *Plant Syst. Evol.* **156**, 55-59.

Di Carlo, G., Badalamenti, F., Jensen, A.C., Koch, E.W. and Raggio, S. (2005). Colonisation process of vegetative fragments of *Posidonia oceanica* (L.) Delile on rubble mounds. *Mar. Biol.* **147**, 1261-1270.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus* **12**, 13-15.

Duarte, C.M. (1991). Seagrass depth limits. *Aquatic Bot.* **40**, 363-377.

Duarte, C.M. (1999). Seagrass ecology at the turn of the millennium: challenger for the new century. *Aquat. Bot.* **65**, 7-20

Feldman, L.J. (1975). Cytokinins and quiescent center activity in roots of *Zea mays*. In: *The development and function of roots, Torrey, J.G. and Clarkson, D.T. (Eds.)*. Academic Press, London, pp 55-72.

Fonseca, M.S., Kenworthy, W.J., and Thayer, G.W. (1998). Guidelines for the conservation and restoration of seagrasses in the United States and adjacent waters. *NOAA Coastal Ocean Program Decision Analysis Series No. 12. NOAA Coastal Ocean Office*, Silver Spring, Maryland.

Foote, C.S., Ching, T. and Geller, G.G. (1974). Chemistry of singlet oxygen. XVIII. Rates of reaction and quenching of α-tocopherol and singlet oxygen. *Photochem. Photobiol.* **20**, 511-513.

Fryer, M.J. (1992). The antioxidant effects of thylakoid vitamin E (*α*-tocopherol). *Plant Cell Environ.* **15**, 381-392.

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S. and Peč, P. (2001). Cytokinin oxidase or dehydrogenase? Mechanism of cytokinin degradation in cereals. *Eur. J. Biochem.* **268**, 450-461.

Gambi, M.C., Buia, M.C., and Mazzella, L. (1996). Record of a diffuse germination of *Posidonia oceanica* (L) Delile in the central Adriatic Sea (Croatia). *Biol. Mar. Medit.* **3**, 467-470.

Gan, S. and Amasino, R.M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270**, 1986-1988.

Ghirardelli, E. (1981). La vita nelle acque. *Utet, Torino*, 392-402.

Giannino, D., Condello, E., Bruno, L., Testone, G., Tartarini, A., Cozza, R., Innocenti, A.M., Bitonti, M.B. and Mariotti, D. (2004). The gene geranylgeranyl reductase of peach (*Prunus persica* [L.] Batsch) is regulated during leaf development and responds differentially to distinct stress factors. *J. Exp. Bot.* **55**, 2063-2073.

Giordani, T., Natali, L., Maserti, B.E., Taddei, S. and Cavallini, A. (2000). Characterization and expression of DNA sequences encoding putative type-II metallothioneins in the seagrass *Posidonia oceanica*. *Plant Physiol.* **123**, 1751-1782.

Giraud, G. (1977). Essai de classement des herbiers de *Posidonia oceanica* (Linnè) Delile. *Bot. Mar.* **20**, 487-491.

Grasses, T., Grimm, B., Koroleva, O. and Jahns, P. (2001). Loss of alpha-tocopherol in tobacco plants with decreased geranylgeranyl reductase activity does not modify photosynthesis in optimal growth conditions, but increases sensitivity to high light stress. *Planta* **213**, 620-628.

Gray, J.C. (1987). Control of isoprenoid biosynthesis in higher plants. *Adv. Bot. Res.* **14**, 25-91.

Grimm, B. (1998). Novel insights into the control of tetrapyrrole metabolism in higher plants. *Curr. Opin. Plant Biol.* **1**, 245-250.

Gyula, P., Schäfer, E. and Nagy, F. (2003). Light perception and signalling in higher plants. *Curr. Opin. Plant Biol.* **6**, 446-452.

Haas, B.J., Volkovsky, N., Town, C.D., Troukhan, M., Alexandrov, N., Feldmann, K.A., Flavell, R.B., White, O. and Salzberg, S.L. (2002). Full-length messenger RNA sequences greatly improve genome annotation. *Genom. Biol.* **3**, 0029.1-0029.12.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor end analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**, 95-98.

Halliwell, B. (1981). Toxic effects of oxygen in plant tissue. In *Chloroplast Metabolism: The structure and function of Chloroplast in Green Leaf Cells*, Chapter 8 pp 179-205, Clarendon Press Oxford.

Havaux, M., Bonfils, J., Lutz, C. and Niyogi, K. (2000). Photodamage of the photosynthetic apparatus and its dependence on the leaf development stage in the *npq1 Arabidopsis* mutant deficient in the xantophyll cycle enzyme violaxanthin de-epoxidase. *Plant Physiol.* **124**, 273-284.

Havaux, M., Eymery, F., Porfirova, S., Rey, P. and Dörmann (2005). Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. *Plant Cell* **17**, 3451-3469.

Havaux, M., Lutz, C. and Grimm, B. (2003). Chloroplast membrane photostability in *chlP* transgenic tobacco plants deficient in tocopherols. *Plant Physiol.* **132**, 300-310.

Heintze, A., Gorlach, J. Leuschner, C. and Hoppe, P. (1990). Plastidic isoprenoid synthesis during chloroplast development. Change from metabolic autonomy to division-of-labor stage. *Plant Physiol* **93**, 1121-1127.

Hemminga, M.A. and Duarte, C.M. (2000). Seagrass ecology. Cambridge University Press, Cambridge.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S. et al. (2004). In planta functions of the *Arabidopsis* cytokinin receptor family. *Proc. Natl. Acad. Sci.* USA **101**, 8821-8826.

Hofius, D., Hajirezaei, M-R., Geiger, M., Tschiersch, H., Melzer, M. and Sonnewald, U. (2004). RNAi-mediated tocopherol deficiency impairs photoassimilate export in transgenic potato plants. *Plant Physiol.* **135**, 1256-1268.

Hutchison, C.E. and Kieber, J.J. (2002). Cytokinin signaling in *Arabidopsis*. *Plant Cell* **14**, S47-S59.

Iijima, Y., Davidovich-Rikanati, R., Fridman, E., Gang, D.R., Bar, E., Lewinsohn, E. and Pichersky, E. (2004). The biochemical and molecular basis for the divergent patterns in the biosynthesis of terpenes and phenylpropenes in the peltate glands of three cultivars of basil. *Plant Physiol.* **136**, 3724-3736.

Inoue, T., Higuchi, M. Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K. and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* **409**, 1060-1063.

Invers, O., Kraemer, G.P., Pérez, M. and Romero, J. (2004). Effects of nitrogen addition on nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica*. *J. Exp. Mar. Biol. Ecol.* **303**, 97-114.

Jacqmard , A., Houssa, C. and Bernier, G. (1994). Regulation of the cell cycle by cytokinins. In: *Cytokinins: chemistry, activity and function. Mok, D.W.S. and Mok M.C. (Eds)*. Boca Raton, FL. USA, CRC Press, pp 197-215.

Jeudy de Grissac, A. and Boudouresque, C.F. (1985). Rôles des herbier de phanérogames marines dans les mouvements des sédiments côtiers: les herbiers à *Posidonia oceanica*. *Coll. Fr.-japon. Océanogr.* **1**, 143-151.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol.* **42**, 677-685.

Kamal-Eldin, A. and Appelqvist, L.A. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **31**, 671-701.

Keller, Y., Bouvier, F., D'Harlingue, A. and Camara, B. (1998). Metabolic compartmentation of plastid prenyllipid biosynthesis: evidence for the involvement of a multifunctional geranylgeranyl reductase. *Eur. J. Biochem.* **251**, 413-417.

Kerstetter, R.A. and Hake, S. (1997). Shoot meristem formation in vegetative development. *Plant Cell* **9**, 1001-1010.

Kevei, E. and Nagy, F. (2003). Phytochrome controlled signalling cascades in higher plants. *Physiol. Plant.* **117**, 305-313.

Klein, P., Kanehisa, M. and DeLisi, C. (1985). The detection and classification of membrane-spanning proteins *Biochem. Biophys. Acta* **815**, 468-476.

Koce, J.D., Vilhar, B., Bohanec, B. and Dermastia, B. (2003). Genome size of Adriatic seagrasses. *Aquat. Bot.* **77**, 17-25.

Koda, Y. and Okazawa, Y. (1980). Cytokinin production by asparagus shoot apex cultured *in vitro*. *Phys. Plant.* **49**, 193-197.

Kreuz, K. and Kleinig, H. (1984). Synthesis of prenyl lipids in cells of spinach leaf compartmentation of enzymes for formation of isopentenyl diphosphate. *Eur. J. Biochem.* **141**, 531-535.

Kubo, M. and Kakimoto, T. (2000). The *CYTOKININ-HYPERSENSITIVE* genes of *Arabidopsis* negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. *Plant J.* **23**, 385-394.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinfo.* **5**, 150-163.

Kuo, J. and McComb, A.J. (1989). Seagrass taxonomy, structure and development. In: *Biology of the seagrasses: a treatise on the biology of seagrasses with special reference to the Australian regions. Larkum, A.W.D., McComb, A.J. and Sheperd, S.A. (Eds.)*. Amsterdam, North Holland, Elsevier, pp. 6-73.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Lange, B.M., Rujan, T., Martin, W. and Croteau, R. (2000). Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA* **24**, 13172-13177.

Les, D.H. (1988). Breeding systems, population structure and evolution in high hydrophilous angiosperms. *Ann. Missouri Bot. Gardens* **75**, 819-835.

Les, D.H. and Haynes, R.R. (1995). Systematics of subclass Alismatidae: a synthesis of approaches. In *Monocotyledons: systematics and evolution. P.J. Rudall, P. Cribb, D.F. Cutler and C.J. Humphries (Eds.)*. Kew: Royal Botanic Gardens, pp. 353-377.

Les, D.H., Cleland, M.A. and Waycott, M. (1997). Phylogenetic studies in Alismatidae, II: evolution of marine Angiosperms (seagrasses) and hydrophily. *Sys. Bot.* **22**, 443-463.

Les, D.H., Garvin, D.K. and Wipee, C.F. (1993). Phylogenetic studies in the monocot subclass Alismatidae: evidence for a reappraisal of the aquatic order Najadales. *Mol. Phyl. Evol.***2**, 304-314.

Letham, D.S. (1994). Cytokinins as phytohormones – sites of biosynthesis, translocation and function of translocated cytokinin. In: *Cytokinins: chemistry, activity and function. Mok, D.W.S. and Mok M.C. (Eds)*. Boca Raton, FL. USA, CRC Press, pp. 57-80.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: *Douce, R. and Packer, L. (eds.)*: *Methods Enzymol.* **148**, 350-382, *Academic Press Inc.*, *New York*.

Lichtenthaler, H.K., Rohmer, M. and Schwender, J. (1997). Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol. Plant.* **101**, 643-652.

Logani, M.K. and Davies, R.E. (1980). Lipid oxidation: biological effects and antioxidants. *Lipids* **15**, 485-495.

Lopukhina, A., Dettenberg, M., Weiler, E.W. and Hollander-Czytko, H. (2001). Cloning and characterization of a coronatine-regulated tyrosine aminotransferase from *Arabidopsis*. *Plant Physiol.* **126**, 1678-1687.

Lorenti, M., Buia, M.C. and Mazzella, L. (1993). Growth patterns and energy allocation in the Mediterranean seagrass *Posidonia oceanica*. *XV International Botanical Congress*, Tokyo, 28-08/03-09-2003, pp. 326-327.

Lorenti, M., Mazzella, L. and Buia, M.C. (1995).Light limitation of *Posidonia oceanica* (L.) Delile leaves growth at different depths. *Rapports Commission International Mer Mediterranee* **34**, 34.

Maestrini, P., Giordani, T., Lunardi, A., Cavallini, A. and Natali, L. (2004). Isolation and expression of two aquaporin-encoding genes from the marine phanerogam *Posidonia oceanica*. *Plant Cell Physiol.* **45**, 1838-1847.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: A laboratory manual. *Cold Spring Harbour Laboratory Press, New York.*

Marbà, N., Duarte, C.M., Cebriàn, J., Gallegos, M.E., Olesen, B. and Sand-Jensen, K. (1996). Growth and population dynamics of *Posidonia oceanica* on the Spanish Mediterranean coast: elucidating seagrass decline. *Mar. Ecol. Prog. Ser.* **137**, 203-213.

Martin, R.C., Mok, M.C and Mok, D.W.S. (1999a). A gene encoding cytokinin enzyme zeatina O-xylosiltransferase of *Phaseolus vulgaris*. *Plant Physiol.* **120**, 553-557.

Martin, R.C., Mok, M.C and Mok, D.W.S. (1999b). Isolation of a cytokinin gene, *ZOG1*, encoding zeatin O-glucosyltranferase of *Phaseolus lunatus*. *Proc. Natl. Acad. Sci. USA* **96**, 284-289.

Martin, R.C., Mok, M.C, Habben, J.E. and Mok, D.W.S. (2001). A maize cytokinin gene encoding an O-glucosyltransferase specific to *cis*-zeatin. *Proc. Natl. Acad. Sci. USA* **98**, 5922-5926.

Matsushita, S., Terao, J. and Yamauchi, R. (1978). Photosensitised oxidation of unsaturated fatty acid ester and the quenching effects of tocopherol on singlet oxygen. In *Tocopherol, Oxygen and Biomembranes. de Duve C. and Hayashi O. (Eds).* Elsevier/North Holland Biomedical Press, Amsterdam, pp 23-29.

Mazzella, L. and Ott, J.A. (1984). Seasonal changes in some features of *Posidonia oceanica* (L.) Delile leaves and epiphytes at different depths. In: *C.F. Boudouresque, A. Jeudy de Grissac and J. Olivier (Eds), Int. Workshop on Posidonia oceanica beds*. GIS Posidonie publ., France, **1**, 119-127.

Meinesz, A. and Lefèvre, J.R. (1984). Régénération d'un herbier à *Posidonia oceanica* quarante années après sa destruction par une bombe dans la rade de Villefranche (Alpes-Maritimes). In: *Boudouresque C.F., Jeudy de Grissac A., Olivier J. (eds). Int. Workshop on Posidonia oceanica beds,* GIS Posidonie publ., France, **1**, 39-44.

Micheli, C., Paganin, P., Peirano, A., Caye, G., Meinesz, A. and Bianchi, C.N. (2005). Genetic variability of *Posidonia oceanica* (L.) Delile in relation to local factors and biogeographic patterns. *Aquat. Bot.* **82**, 210-221.

Migliaccio, M. and Procaccini, G. (2006). Spatial pattern of genetic diversity in the endemic Mediterranean seagrass *Posidonia oceanica*: from genetics to genomics. *International Conference Marine Genomics 28-10/01-11*, 25.

Miller, C.O., Skoog, F., von Saltza, M.H. and Strong, M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *J. Am. Chem. Soc.* **77**, 1329-1334.

Miyawaki, K., Matsumoto-Kitano, M. and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis* tissue specifity and regulation of auxin, cytokinin, and nitrate. *Plant J.* **37**, 128-138.

Mok, D.W. and Mok, M.C. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 89-118.

Mok, M.C. (1994). Cytokinins and plant development – An overwiev. In: *Cytokinins: chemistry, activity and function. Mok, D.W.S. and Mok, M.C. (Eds)*, pp 155-166, Boca Raton, FL. USA, CRC Press.

Molina-Torres, J. and Martinez, M.L. (1991). Tocopherols and leaf age in *Xanthium strumarium* L. *New Phytol.* **118**, 95-99.

Munné-Bosch, S. and Alegre, L. (2002a). The function of tocopherols and tocotrienols in plants. *Crit. Rev. Plant Sci.* **21**, 31-57.

Munné-Bosch, S. and Alegre, L. (2002b). Plant aging increases oxidative stress in chloroplasts. *Planta* **214**, 608-615.

Munné-Bosch, S. and Falk, J. (2004). New insights into the function of tocopherols in plants. *Planta* **218**, 323-326.

Nagatani, A. (2004). Light-regulated nuclear localization of phytochromes. *Curr. Opin. Plant Biol.* **7**, 708-711.

Neely, W.C., Martin, J.M. and Barker, S.A. (1988). Products and relative reactions rates of the oxidation of tocopherol with singlet molecular oxygen. *Phytochem. Photobiol.* **48**, 423-428.

Nesaretnam, K., Stephen, R., Dils, R. and Drabre, P. (1998). Tocotrienols inhibit the growth of human breast cancer cells irrespective of estrogen receptor status. *Lipids* **33**, 461-469.

Oster, U. and Rüdiger, W. (1997). The G4 gene of *Arabidopsis thaliana* encodes a chlorophyll synthase of etiolated plants. *Bot. Acta* **110**, 420-423.

Oster, U., Bauer, C.E. and Wolfhart, R. (1997). Characterization of chlorophyll *a* and bacteriochlorophyll *a* synthases by heterologous expression in *Escherichia coli*. *J. Biol. Chem.* **15**, 9671-9676.

Ott, J.A. (1979). Persistence of a seasonal growth rhythm in *Posidonia oceanica* (L) Delile under constant conditions of temperature and illumination. *Mar. Biol. Lett.* **1**, 99-104.

Ott, J.A. (1980). Growth and production in *Posidonia oceanica* [L.] Delile. *Mar. Ecol.* **1**, 4-64.

Packer, L., Weber, S.U. and Rimbach, G. (2001). Molecular aspects of α-tocotrienol antioxidant action and cell signalling. *J. Nutr.* **131**, 369S-373S.

Parker, R.A., Pearce, B.C., Clark, R.W., Gordon, D.A. and Wright, J.J. (1993). Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **268**, 11230-1238.

Pasqualini, V., Pergent-Martini, C., Clabaut, P. and Pergent, G. (1998). Mapping of *Posidonia oceanica* using aerial photographs and side scan sonar: application off the island of Corsica (France). *Estuar. Coast. Shelf Sci.* **47**, 359-367.

Peres, J.M. (1984). La régression des herbiers à *Posidonia oceanica*. In : *Boudouresque, C.F., Jeudy de Grissac, A., Olivier, J. (Eds) . Proceedings International Workshop Posidonia oceanica Beds. Marseille, France: GIS Posidonie*, pp. 445-454.

Pergent, G. (1990). Lepidochronological analysis of the seagrass *Posidonia oceanica* (L.) Delile: standardized approach. *Aquatic Bot.* **37**, 39-54.

Pergent, G., Pergent-Martini, C. and Boudouresque, C.F. (1995). Utilisation de l'herbier à *Posidonia oceanica* comme indicateur biologique de la qualité du milieu littoral en Méditerranée: état de connaissances. *Mésogée* **54**, 3-29.

Pergent-Martini, C. (1998). *Posidonia oceanica*: a biological indicator of past and present mercury contamination. *Mar. Env. Res.* **45**, 101-111.

Pergent-Martini, C., Leoni, V., Pasqualini, V., Ardizzone, G.D., Balestri, E., Bedini, R., Belluscio, A., Belsher, T., Borg, J., Boudouresque, C.F., Boumaza, S., Bouquegneau, J.M., Buia, M.C., Calvo, S., Cebrian, J., Charbonnel, E., Cinelli, F., Cossu, A., Di Maida, G., Dural, B., Francour, P., Gobert, S., Lepoint, G., Meinesz, A., Molenaar, H., Mansour, H.M., Panayotidis, P., Peirano, A., Pergent, G., Piazzi, L., Pirrotta, M., Relini, G., Romero, J., Sanchez-Lizaso, J.L., Semroud, R., Shembri, P., Shili, A., Tomasello, A. and Velimirov, B. (2005). Descriptors of *Posidonia oceanica* meadows: use and application. *Ecol. Ind.* **5**, 213-230.

Pergent-Martini, C., Rico-Raimondino, V. and Pergent, G. (1994). Primary production of *Posidonia oceanica* in the Mediterranean basin. *Mar. Biol.* **120**, 9-15.

Philbrick, C.T. (1988). Hydrophily: phylogenetic and evolutionary considerations. *Rhodora* **93**, 36-50.

Piazzi, L., Acunto, S. and Cinelli, F. (1999). In situ survival and development of *Posidonia oceanica* [L.] Delile seedlings. *Aquat. Bot.* **63**, 103-112.

Piazzi, L., Balata, D., Cinelli, F. and Benedetti-Cecchi, L. (2004). Patterns of spatial variability in epiphytes of *Posidonia oceanica*. Differences between a disturbed and two reference locations. *Aquat. Bot.* **79**, 345-356.

Pirc, H. (1984). Depth adaptation in *Posidonia oceanica* (L.) Delile. In: *C.F. Boudouresque, A. Jeudy de Grissac and J. Olivier (Eds), Int. Workshop on Posidonia oceanica Beds*. GIS Posidonie Publications, France, **1**: 227-234.

Pirc, H. (1985). Growth dynamics in *Posidonia oceanica* (L.) Delile. I. Seasonal changes of soluble carbohydrates, starch, free amino acids, nitrogen and organic anions in different parts of the plant. *P.S.Z.N.I. Mar. Ecol.* **6**, 141-165.

Pirc, H. (1986). Seasonal aspects of photosynthesis in *Posidonia oceanica*: influence of depth, temperature and light intensity. *Aquat. Bot.* **26**, 203–212.

Pirc, H. (1989). Seasonal changes in soluble carbohydrates, and energy content in Mediterranean seagrasses. *P.S.Z.N.I. Mar. Ecol.* **10**, 97-105.

Plumley, F.G. and Schmidt, G.W. (1995). Light-harvesting chlorophyll *a*/*b* complexes: interdependent pigment synthesis and protein assembly. *Plant Cell* **7**, 689-704.

Porfirova, S., Bergmuller, E., Tropf, S., Lemke, R. and Dormano, P. (2002). Isolation of an *Arabidopsis* mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis. *Proc. Natl. Acad. Sci. USA* **99**, 12495-12500.

Procaccini, G. and Mazzella, L. (1998). Population genetic structure and gene flow in *Posidonia oceanica* (L.) Delile populations form the West Mediterranean basin: a microsatellite analysis. *Mar. Ecol. Prog. Ser.* **169**, 133-141.

Procaccini, G., Orsini, L., Ruggiero, M.V. and Scardi, M. (2001). Spatial patterns of genetic diversity in *Posidonia oceanica*, an endemic Mediterranean seagrass. *Mol. Ecol.* **10**, 1413-1421.

Raedestorff, D., Elste, V., Aebischer, C. and Weber, P. (2002). Effect of either γ-tocotrienol or a tocotrienol mixture on the plasma lipid profile in hamsters. *Ann. Nutr. Metlab.* **46**, 17-23.

Rende, F. Cardilio, M., Cozza, R. and Innocenti, A.M. (2005). Monitoring of *Posidonia oceanica* (L) Delile beds in Dino island SIC site. *Biol. Mar. Med.* **12**, 436-441.

Rende, F., Cozza, R. and Innocenti, A.M. (2006). Growth dynamics of *Posidonia oceanica* (L) Delile meadows in different environmental conditions. *Biol. Mar. Med.***13**, 629-632.

Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H. (1993). Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem. J.* **295**, 517-524.

Romero, J. (1989). Seasonal pattern of *Posidonia oceanica* production: growth, age and renewal of leaves. In: *Boudouresque, C.F., Meinesz, A., Fresi, E., Gravez, V. (Eds.), International Workshop on Posidonia Beds*. GIS Posidonie, Marseille, pp. 63–68.

Ros, J.D., Romero, J., Ballesteros, E. and Gili, J.M. (1985). Diving in the blue water: the benthos. In: *Western Mediterranean. Margalef R.(Ed.), Pergamon, Oxford*, pp. 233-295.

Rüdiger, W. (1997). Chlorophyll metabolism: from outer space down to the molecular level. *Phytochem.* **46**, 1151-1167.

Sakakibara, H. (2005). Cytokinin biosynthesis and regulation. *Vit. Hor.* **72**, 271-287.

Sakakibara, H. (2006). Cytokinins: activity, biosynthesis and translocation. *Annu. Rev. Plant. Biol.* **57**, 431-449.

Samuelson, M.E. and Larsson, C-M. (1993). Nitrate regulation of zeatin riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. *Plant Sci.* **93**, 77-84.

Sanchez Lizsaso, J.L., Guillen Nieto, J.E. and Ramos Espla, A.A. (1990). The regression of *Posidonia oceanica* meadows in El Campello (Spain). *Rapp. Comm. Int. Mer Médit.* **32**, 7.

Sandorf, I. and Hollander-Czytko, H. (2002). Jasmonate is involved in the induction of tyrosine aminotransferase and tocopherol biosynthesis in *Arabidopsis thaliana*. *Planta* **216**, 173-179.

Savidge, B., Weiss, J.D., Wong, Y-H. H., Lassner, M.W., Mitsky, T.A., Shewmaker, C.K., Post-Beittenmiller, D. and Valentin, H.E. (2002). Isolation and characterization of homogentisate phytyltransferase genes form *Synechocystis* sp. PCC 6803 and *Arabidopsis*. *Plant Physiol.* **129**, 321-332.

Schmitt Jr., W.A., Raab, R. and Stephanopoulos, G. (2004). Elucidation of gene interaction networks through time-lagged correlation analysis of transcriptional data. *Gen. Res.* **14**, 1654-1663.

Schmülling, T., Werner, T., Riefler, M., Krupkova, E. and Bartrina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. *J. Plant Res.* **116**, 241-252.

Schoch, S., Lempert, U. and Rüdiger, W. (1977). On the last steps of chlorophyll biosynthesis intermediates between chlorophyllide and phytol-containing chlorophyll. *Z. Pflanzenphysiol.* **83**, 427-436.

Semroud, R. (1993). Données sur des floraisons anciennes de *Posidonia oceanica* (Linnaeus) Delile dans la région d'Alger (Algérie). *Posidonia Newslett.* **1**, 5-14.

Serbinova, E.A. and Packer, L. (1994). Antioxidant properties of α-tocopherol and α-tocotrienol. *Methods Enzymol.* **234**, 354-366.

Shani, E., Yanai, O. and Ori, N. (2006). The role of hormones in shoot apical meristem function. *Curr. Opin. Plant Biol.* **9**, 484-489.

Shepherd, S.A., McComb, A.J. , Bulthuis, D.A., Neverauskas, V., Steffensen, D.A., and West, R. (1989). Decline of seagrass. Pages 346–387 in *A.W.D. Larkum, A.J. McComb and S.A. Shepherd, editors. Biology of seagrasses*. Elsevier, Amsterdam, The Netherlands.

Shibata, M., Mikota, T., Yoshimura, A., Iwata, N., Tsuyama, M. and Kobayashi, Y. (2004). Chlorophyll formation and photosynthetic activity in rice mutants with alterations in hydrogenation of the chlorophyll alcohol side chain. *Plant Sci.* **166**, 593-600.

Shintani, D. and DellaPenna, D. (1998). Elevating the vitamin E content of plants through metabolic engineering. *Science* **282**, 2098-2100.

Shintani, D.K., Cheng, Z. and DellaPenna, D. (2002). The role of 2-methyl-6-phytylbenzoquinone methyltransferase in determining tocopherol composition in *Synechocystis* sp. PCC 6803. *FEBS Lett.* **511**, 1-5.

Short, F.T. and Wyllie-Echeverria, S. (1996). Natural and human-induced disturbance of seagrasses. *Env. Cons.* **23**, 17-27.

Shpilyov, A.V., Zinchenko, V., Shestakov, S.V., Grimm, B. and Lokstein, H. (2005). Inactivation of the geranylgeranyl reductase (ChlP) gene in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochem. Biophys. Acta* **1706**, 195-203.

Silverstein, K., Chiffings, A.W. and McComb, A.J. (1986). The loss of seagrass in Cockburn Sound, Western Australia. III. The effect of epiphytes on productivity of *Posidonia australis* Hook F. *Aquat. Bot.* **24**, 355–371.

Soll, J. (1987). *α*-Tocopherol and plastoquinone synthesis in chloroplast membranes. *Methods Enzymol.* **148**, 383-392.

Soll, J. and Schultz, G. (1979). Comparison of geranylgeranyl and phytyl substituted methylquinols in the tocopherol synthesis of spinach chloroplasts. *Biochem. Biophys. Res. Commun.* **91**, 715-720.

Soll, J. and Schulz, G. (1981). Phytol synthesis from geranylgeraniol in spinach chloroplasts. *Biochem. Biophys. Res. Commun.* **99**, 907-912.

Soll, J., Schulz, G., Rüdiger, W., Benz, J. (1983). Hydrogenation of geranylgeraniol: two pathways exist in spinach chloroplasts. *Plant Physiol.* **71**, 849-854.

Strnad, M. (1997). The aromatic cytokinins. *Physiol Plant.* **101**, 674-688.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H. and Mizuno, T. (2001). The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. *Plant Cell Phys.* **42**, 107-113.

Suzuki, Y.J., Tsuchiya, M., Wassall, S.R., Choo, Y.M., Govil, G., Kagan, V.E. and Packer, L. (1993). Structural and dynamic membrane properties of α-tocopherol and α-tocotrienol: implication to the molecular mechanism of their antioxidant potency. *Biochem.* **32**, 10692-10699.

Swaminathan, S. and Bock, R.M. (1977). Isolation and identification of cytokinins from *Euglena gracilis* transfer ribonucleic acid. *Biochem.* **16**, 1355-1360.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., Nagy, F., Schäfer, E. and Harter, K. (2001). Interaction of the response regulator ARR4 with the photoreceptor phytochrome B in modulating red light signaling. *Science* **294**, 1108-1111.

Takei, K, Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T. and Sakakibara, H. (2004b). *AtIPT3* a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* **45**, 1053-1062.

Takei, K., Sakakibara, H., Taniguchi, M. and Sugiyama, T. (2001). Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. *Plant Cell Physiol.* **42**, 85-93.

Takei, K., Yamaya, T. and Sakakibara, H. (2004a). *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *J. Biol. Chem.* **279**, 41866-41872.

Tanaka, R., Oster, U., Kruse, E., Rüdiger, W. and Grimm, B. (1999). Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylgeranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. *Plant Physiol.* **120**, 695-704.

The Arabidopsis genome initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796-815.

Thelin, I., and Boudouresque, C.F. (1985). *Posidonia oceanica* flowering and fruiting: recent data from an international inquiry. *Posidonia Newslett.* **1**, 5-14.

Theriault, A., Chao, J.T., Wang, Q., Gapor, A. and Adeli., K. (1999). Tocotrienol: a review of its therapeutic potential. *Clin. Biochem.* **32**, 309-319.

Thomas, H. (1997). Tansley review No.92 chlorophyll: a symptom and a regulator of plastid development. *New Phytol.* **136**, 163-181.

Tramontano, W.A., Ganci, D., Pennino, D. and Dierenfeld, E.S. (1992). Age dependent α-tocopherol concentrations in leaves of soybean and pinto beans. *Phytochem.* **31**, 3349-3351.

Tsegaye, Y., Shintani, D.K. and DellaPenna, D. (2002). Over-expression of the enzyme *p*-hydroxyphenylpyruvate dioxygenase in *Arabidopsis* and its relation to tocopherol biosynthesis. *Plant Physiol. Biochem.* **40**, 913-920.

Ueguchi, C., Koizumi, H., Suzuki, T. and Mizuno, T. (2001). Novel family of sensor histidine kinase genes in *Arabidopsis thaliana*. *Plant Cell Physiol.* **42**, 231-235.

Veach, Y.K., Martin, R.C., Mok, D.W., Malbeck, J., Vankova, R. and Mok, M.C. (2003). O-Glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. *Plant Physiol.* **131**, 1374-1380.

Walker, D.I. and Cambridge, M.L. (1995). An experimental assessment of the temperature responses of two sympatric seagrasses, *Amphibolis antarctica* and *Amphibolis griffithii*, in relation to their biogeography. *Hydrobiologia* **302**, 63-70.

Wang, L., Newmann, R.K., Newmann, C.W., Jackson, L.L. and Hofer, P.J. (1993). Tocotrienol and fatty acid composition of barley oil and their effects on lipid metabolism. *Plant Foods Hum. Nutr.* **43**, 9-17.

Wellburn, A.R. (1976). Evidence for chlorophyll esterified with geranylgeraniol in newly greened leaves. *Biochem. Physiol. Pflanzen* **169**, 265-271.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating

opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15**, 2532-2550.

Werner, T., Motyka, V., Strnad, M. and Schmülling, T. (2001). Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci. USA* **98**, 10487-10492.

Whippo, C.W. and Hangarter, R.P. (2003). Second positive phototropism results form coordinated co-action of the phototropins and cryptochromes. *Plant Physiol.* **132**, 1499-1507.

Wierenga, R.K., Terpstra, P. and Hol, W.G.J. (1986). Prediction of the occurrence of the ADP-binding βαβ fold in proteins, using an aminoacid sequence fingerprint. *J. Mol. Biol.* **187**, 101-107.

Wittmann, K.J. (1984). Temporal and morphological variations of growth in a natural stand of *Posidonia oceanica* (L.) Delile. *P.S.Z.N.I. Mar. Ecol.* **5**, 301-316.

Yamada, H., Suzuki, T, Terada, K., Takei, K., Ishikawa, K. Miwa, K. and Mizuno, T. (2001). The *Arabidopsis* AHK4 histidine kinase is a cytokinin binding receptor that transduces cytokinin signal across the membrane. *Plant Cell Physiol* **42**, 1017-1023.

Yamamoto, Y., Kobayashi, Y. and Matsumoto, H. (2001). Lipid peroxidation is an early symptom triggered by aluminium, but not the primary cause of elongation inhibition in pea roots. *Plant Physiol.* **125**, 199-208.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T. and Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize: differential ligand preferences and response to *cis*-zeatin. *Plant Physiol.* **134**, 1654-1661.

Zupo, V., Buia. M.C. and Mazzella, L. (1997). A production model for *Posidonia oceanica* based on temperature. *Est. Coast. Shelf Sci.* **44**, 483-492.

Annex: Additional work

A. - Characterization of *PHYTOCHROME B* gene in *Posidonia oceanica*: preliminary results

To gain further insight into light response of *P. oceanica* plants we began to study the *PHYTOCHROME B* gene.

Phytochromes are a class of red/far red light-absorbing photoreceptors that, together with blue light absorbing cryptochromes, the phototropins, and the yet uncharacterized UV light photoreceptors, mediate a variety of cellular and developmental responses to light (Fankhauser and Chory, 1997; Deng and Quail, 1999). Phytochromes have two major domains, an amino-terminal domain that carries a covalently attached tetrapyrrole chromophore and mediates light absorption and photoreversibility, and a carboxy-terminal domain that is required for signal transduction activity (Quail, 1997). Phytochromes are synthesized in the dark in a red light absorbing form called P_r. Upon exposure to red light, they switch to a far-red light absorbing form called P_{fr}, which can then be reconverted to the P_r form by exposure to far-red light. The P_{fr} is the active form of phytochrome, although there are evidences of responses to far-red light that complicate this simple picture. In particular Phytochrome B is involved in many photomorphogenetic responses like leaf and cotyledons expansion, stem growth, cell division and cell expansion regulation, shadow perceiving and flowering (Neff et al., 2000).

Phytochromes are present in all plants examined and also in some algae. In particular, in the model plant Arabidopsis thaliana, five phytochromes (PhyA, PhyB, PhyC, PhyD and PhyE) have been identified (Mathews and Sharrock, 1997) and it has been revealed that different phytochromes control distinct responses in some cases mediated by distinct light conditions (Fankahuser and Chory, 1997; Whitelam and Devlin, 1997; Deng and Quail, 1999). On the basis of recent data a model for phytochrome action has been proposed. According to this model phytochromes besides inducing a signaling cascade mediated by Ca^{2+} and cGMP in the cytoplasm, also function as a light-regulated kinase. Moreover its P_{fr} conformer can rapidly translocate into the nucleus, where it interacts with transcription factors directly regulating light-induced gene transcription (Nagy and Schäfer, 2002). Although this model is attractive, and it is well supported by experimental data, it must be noted that the molecular mechanism that mediates phytochrome import into the nucleus and retention in cytoplasm in the dark are still unknown (Gyula et al., 2003). On the

other hand, also molecular mechanisms regulating phytochrome stability and degradation in the nucleus and/or the cytoplasm are not yet known. Recently, is has been understood the stabilizing role of ARR4 protein (Arabidopsis Response Regulator) on PHYB which, in turn, became stabilized by the phenomenon of dark reversion (Sweere et al., 2004). Notably, the ARR4 protein is activated by cytokinin-triggered phosphorylation through a specific signal cascade referred as two-component system (Sweere et al., 2004). Thus, new light is shed within the complicated interactions occurring among light, hormones and cell responses.

In this context and because of the specific involvement of cytokinins which are one focus of our work, this latter evidence strongly influenced our choice in selecting *PHYTOCROME B* as candidate gene

Brief outline of preliminary results

A partial cds of *P. oceanica* was obtained from cDNA leaf with primers indicated in material and methods. The identified sequence (1281 bp) covers the phytochrome and GAF domain, which binds the phytochromobilin cofactor. Moreover, this partial sequence didn't show presence of introns at genomic level as usual for phytochrome gene family. The deduced partial protein (420 aminoacids) has an estimated molecular mass of 46.242 KDa

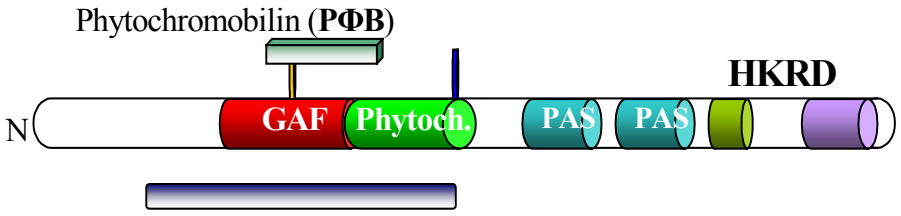


Fig.33 Schematic design of a PHYTOCHROME B protein. The azure bar indicates regions covered by *P. oceanica* PHYB.

calculated with BioEdit software (Hall, 1999). Furthermore, PFAM web site comparison on this partial protein yielded the following domains: GAF domain (118-294) and phytochrome domain (298-420). Phylogenetic analysis of this proteic fragment showed its clustering with B Phytochromes members (Fig.33 and 34B).

For the southern analysis, a digoxygenin labeled probe of 370 bp, spanning Phytochrome domain was used at 45°C to identify members of the *Phytochrome* superfamily in *P. oceanica* (Fig.35A). Five bands appeared on the blotted membrane.

A>Posidonia *PhyB*

AATGCTATCGATTTGTTGGACTTAACCCCACAATCTGTGCCTATCCTTGATAGAAAGCCTGAGGCCCTCACGGTGGGTAC
TGATGTGAGGACCCTTTTCACCTCTTCGAGCGCTCTCCCGCTCGAGAGGGCAGCTGGTGCCCGCGAACTTACCCTTCTGA
ATCCGATTTGGATTCATTCGAGGAATTCGGGGAAACCTTTCTATGCAATTTTACATAGGATTGATGTTGGCATTGTGATT
GATTTGGAGCCTGCTCGGTCAGAGGACCCTGCGCTTTCGATCGCTGGTGCGGTGCAGTCACAGAAACTTGCTGTTCATGC
TATCTCACGGTTGCAGGCTTTGCCTGGTGGAGATATCAAGCTCCTCTGTGACACAGTTGTGGACCATGTTAGGGAGCTCA
CAGGATATGATAGAGTCATGGTCTACAAGTTTCATGAGGATGAGCATGGGGAGGTTGTTGCAGAGAGTAAAAAGGATGAT
TTAGAGCCATATATAGGTCTCCATTACCCTGCCATAGACATACCTCAGGCCTCGAGGTTCTTATTTAAACAGAACAGAGT
TAGGATGATAGCTGATTGTCGTACAACTCCTGTCCGCATGATTCAAGATGAGAACCTGATGCAGCCGCTTTGCCTCGTGG
GATCCACGCTACGAGCCCCACATGGTTGCCATGCACAATACATGACTAGCATGGGTTCCATTGCATCTCTTGTTATGGCC
ATTATCGTTAATGGGGGTGAAGAGGAGGGAACAAGGAAGTCGATGAAACTATGGGGTCTTGTTGTTTGTCATCATACTTC
TCCACGATGCATTCCATTTCCACTGCGCTATGCATGTGAGTTCCTCATGCAGGCTTTAGGCCTTCAGCTTAATATGGAAC
TTAAGTTGGCCTCCCAATTATCTGAGAAACACATCTTAAGAACTCAGACCCCCCTGTGTGATATGCTTCTTCGGGATTCT
CCAATTGGAATTGTTACACAGAGTCCTAGCATAATGGATCTTGTGAAGTGTGATGGTGCAGCACTGTATTACCAGGGTAA
ATACTGGCCACTTGGTGTGACCCCCTCTGAGACACAGATTAAAGATATTGTGGGGTGGTTATTGGCAAGCCATGGAGACT
CCACAGGCTTGAGCACAGACAGTTTCGCTGATGCTGGGTACCCAGGTGCTGCTTCCCTTGGGGATGCAGTTTGTGGTATG
GCGGTGGCTCGCATCACCAGAAGAGATTTTTTGTTTTGGTTTAGATCCCACACAGCAAAAGAGATAAAGTGGGGTGGTGC
A

B>PhyB protein

NAIDLLDLTPQSVPILDRKPEALTVGTDVRTLFTSSSALPLERAAGARELTLLNPIWIHSRNSGKPFYAILH
RIDVGIVIDLEPARSEDPALSIAGAVQSQKLAVHAISRLQALPGGDIKLLCDTVVDHVRELTGYDRVMVYKF
HEDEHGEVVAESKKDDLEPYIGLHYPAIDIPQASRFLFKQNRVRMIADCRTTPVRMIQDENLMQPLCLVGST
LRAPHGCHAQYMTSMGSIASLVMAIIVNGGEEEGTRKSMKLWGLVVCHHTSPRCIPFPLRYACEFLMQALGL
QLNMESEKHILRTQTPLCDMLLRDSPIGIVTQSPSIMDLVKCDGAALYYQGKYWPLGVTPSETQIKDIVGWL
LASHGDSTGLSTDSFADAGYPGAASLGDAVCGMAVARITRRDFLFWFRSHTAKEIKWGGA

Fig.34 **A.** cds sequence for *PHYB* gene. **B.** deduced protein of *PHYB* gene. Red aminaocids indicate GAF domain, blue aminoacids indicates Phytochrome domain.

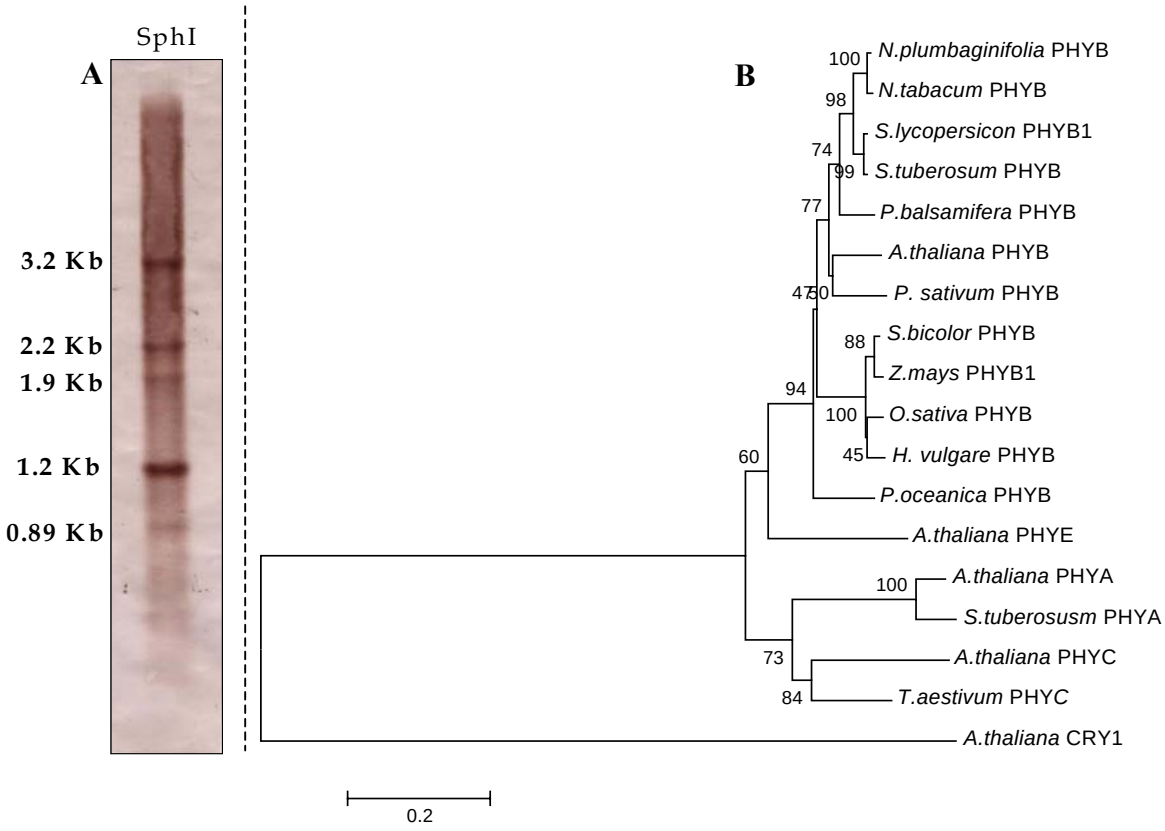


Fig.35 **A.** Southern analysis for *PHYB* genes in *P. oceanica*. **B.** Phylogram was constructed by MEGA 3.1 software (Kumar et al., 2004). Numbers on the tree indicate bootstrap (1000). Accession numbers of protein examined: *N. plumbaginifolia* PHYB (CAA74992), *N. tabacum* PHYB (P29130), *S. lycopersicon* PHYB1 (CAA05293), *S. tuberosum* PHYB (CAA74908), *P. balsamifera* (AAG25725), *A. thaliana* PHYB (AAW56590), *P. sativum* PHYB (AAF14344), *S. bicolor* PHYB (AAR30900), *Z. mays* PHYB1 (AAP06788), *O. sativa* PHYB (NP_001049910), *H. vulgare* PHYB (ABB13323), *A. thaliana* PHYE (At4g18130), *A. thaliana* PHYA (NM_100828), *S. tuberosusm* PHYA (DQ208423), *A. thaliana* PHYC (NM_122975), *T. aestivum* PHYC (AY673000), *A. thaliana* CRY1 (NM_116961).

B. - Characterization of two *HOMEOBOX* genes in *Posidonia oceanica:* preliminary results

Beyond the specific field of interest of my PhD research project, two homeobox genes of *P. oceanica* were also investigated in the context of their well documented relationship with cytokinins (Rupp *et al*., 1999; Frugis *et al*., 2001; Hamant *et al*., 2002),

Knotted1-like homeobox genes (*knox*) are important components that maintain cells in an indeterminate state (Smith et al., 1992; Sinha et al., 1993; Schneeberger et al., 1995; Weigel and Jurgens, 2002; Veit, 2003).

From a structural point of view, in a knotted-like protein can be identified four domains (Fig.36): a MEINOX domain, a GSE box, an ELK domain and an homeodomain. The MEINOX domain comprises two subdomains, KNOX1 and KNOX2, while the first plays a role in suppressing target gene expression, the second is essential for *knox* gene funtion and it is thought for homodimerization. The GSE box is a small domain involved in protein-to-protein interactions (Bürglin, 1997). The ELK domain (Vollbrecht et al., 1991) bears a highly conserved series of the aminoacids glutamate (E), leucine (L), lysine (K) and is also involved in DNA interaction. The homoeodomain and can be subdivided in a basic end of 9 aminoacids and three α-helixes with a total length of 60 aminoacids. The three α-helixes recognize specific sequences in the minor groove of DNA and conservation of some aminoacids suggests a functional implication related to a three-dimensional conformation of the knox protein to interact with other protein cofactors. All the homeodomain regions keep contact with sugar backbone of DNA with the exception of helix 1 (Fraenkel et al., 1998).

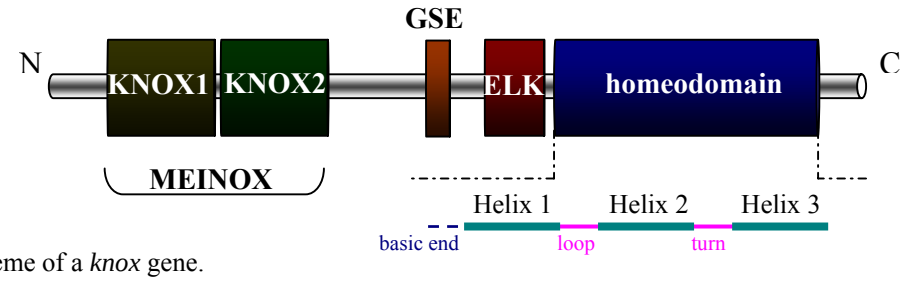


Fig.36 Scheme of a *knox* gene.

Based on comparative analysis of the conserved motifs of encoded proteins, the *knox* family has been subdivided in two classes, class I and class II (Kerstetter et al., 1994). The class I genes are mainly expressed in the shoot apical meristem (SAM) and they may play a role in the establishment and maintenance of meristematic activity. However, their early repression is necessary for leaf primordium initiation (Reiser et al., 2000). In dicots as well as in monocots, *knox* gain of function induces considerable alteration in leaf morphology and plant architecture (Hareven et al., 1996; Tamaoki et al., 1997; Frugis et al.,

2001). Moreover, ectopic expression of class I knox genes can restore morphogenetic potential in differentiated cells (Chuck et al., 1996; Sentoku et al., 2000; Gallois et al., 2002). Such a phenotype was also mimicked by the mutation of genes that negatively regulate the KN1-like genes (Schneeberger et al., 1998, Byrne et al., 2000; Daimon et al., 2003).

Many evidences exist of a tight relationship between *knox* genes and cytokinin signalling (Rupp *et al*., 1999; Frugis *et al*., 2001; Hamant *et al*., 2002), Moreover, several other studies have shown that *knox* expression is also linked to other hormonal activities, including gibberellic acid (Sakamoto *et al*., 2001; Hay *et al*., 2002), ethylene (Hamant *et al*., 2002) and polar auxin transport (PAT) (Tsiantis *et al*., 1999; Scanlon, 2003). In general, ectopic KNOX accumulation is correlated with a defective PAT, which in turn could influence cytokinin level and/or translocation (Mok and Mok, 2001).

Brief outline of preliminary results

The two homeobox genes were identified in apical tips. The genes were named *KNOPO1* (*knox Posidonia oceanica* 1) and *PoSTM* (*Posidonia oceanica shootmeristemless*) on the basis of sequence similarity with other gene sequences available in data bank. For both of them a partial sequence harboring the 3' end UTR and the knox-DNA-interacting domain (domain KNOX2) have been identified and the evaluated phylogram showed their clustering with class 1 *knox* genes. Moreover, southern analysis revealed presence of many members for these genes that are known to be members of a greater family (Kerstetter et al., 1994). In particular, *PoSTM* was close to *Arabidopsis thaliana STM*, while *KNOPO1* grouped with the *knox* gene *RS1* (*Rough Sheath 1*) of *Zea mays* (Schneeberger et al., 1995, 1998). *PoSTM* showed to be exclusively expressed in apical tips and rhizomes, whereas *KNOPO1* was also present at a very low levels in roots, apical portion of leaves and in the basal region of adult leaf (the sheath) (Fig.37).

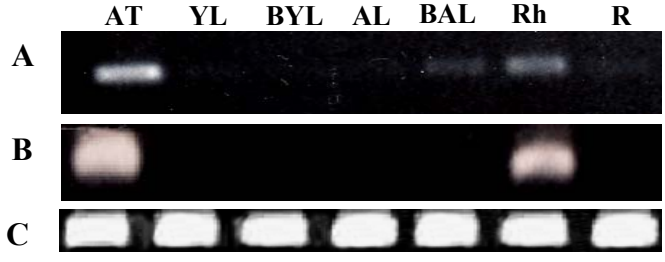


Fig.37 Expression organ specific for KNOPO1 (A) and PoSTM (B). AT: apical tips, YL: young leaf, BYL: basal young leaf, AL: adult leaf, BAL: basal adult leaf, Rh: rhizome, R: root. C. 18S rRNA gene.

The *KNOPO1* gene was isolated as indicated in 'material and methods'. The sequence available covers 766 bp and has an 3'UTR region of 139 bp. The 5' end has been not yet identified and it is expected to be about 500 bp. The deduced partial protein (208 aminoacids) has an estimated molecular mass of 12.970 KDa with the software BioEdit (Hall, 1999). Through PFAM web site, on this partial protein three domains, KNOX2 (3-45), ELK (87-108) and homeobox (110-169), were recognized (Fig. 39).

For the southern analysis, a digoxygenin labeled probe of 370 bp, spanning ELK domain and homeodomain, was used at 45°C to identify members of the *knox* superfamily in *Posidonia*. About thirteen bands appeared on the blotted membrane (Fig.38A).

PoSTM gene was isolated in apical tips. The sequence available covers 568 bp and comprises a 3'UTR of 213 bp. The deduced partial protein is 188 aminoacids long (estimated molecular mass of 21.668 KDa with the software Bioedit, Hall, 1999), and it bears an incomplete KNOX2 domain (1-22), an ELK domain (66-87) and the homeodomain (97-148) (values obtained through the PFAM web site) (Fig.40).

Phylogenetic analysis, performed by using MEGA 3.1 software (Kumar et al., 2004), showed that both *KNOPO1* and *PoSTM* clusterized with *knox* class1 genes (Fig. 38B).

Similarity matrix PAM 250	KNOPO1		PoSTM	
	% Similarity	% Identity	% Similarity	% Identity
KNAT1 BP	88.6	74.7	65.5	45.6
KN1 Triticum	82.0	70.5	65.0	42.4
KN2 Helianthus	87.6	73.8	64.3	47.4
KN1	85.0	72.0	69.9	45.3
Oskn2	64.8	44,5	61.6	40.0
Hordeum KN	69,3	59.7	55.9	38.1
RS1	85.9	73.8	70.0	46.6
OSH1	87.5	75.5	71.4	46.3
LeT6	71.2	54.1	78.7	64.4
STM_At	73.6	55.1	79.8	63.1
POTH1 solanum	64.6	45.8	57.6	42.4

Tab.3 Similarity and identity values between *KNOPO1* and *PoSTM* respect to other class 1 *knox* genes available in data bank. Accessions are indicated in Fig.38.

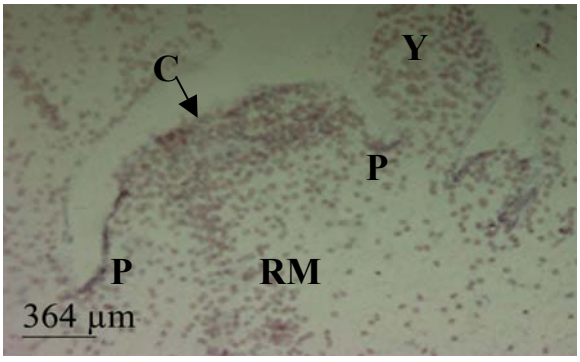


Photo 28. Localization of *KNOPO1* transcripts in longitudinal section *P. oceanica* SAM. The probe used spanned the 3'UTR. Signal is red-purple. Legend = CZ: central zone, PZ: peripheral zone, RM: rib meristem, YL: young leaf.

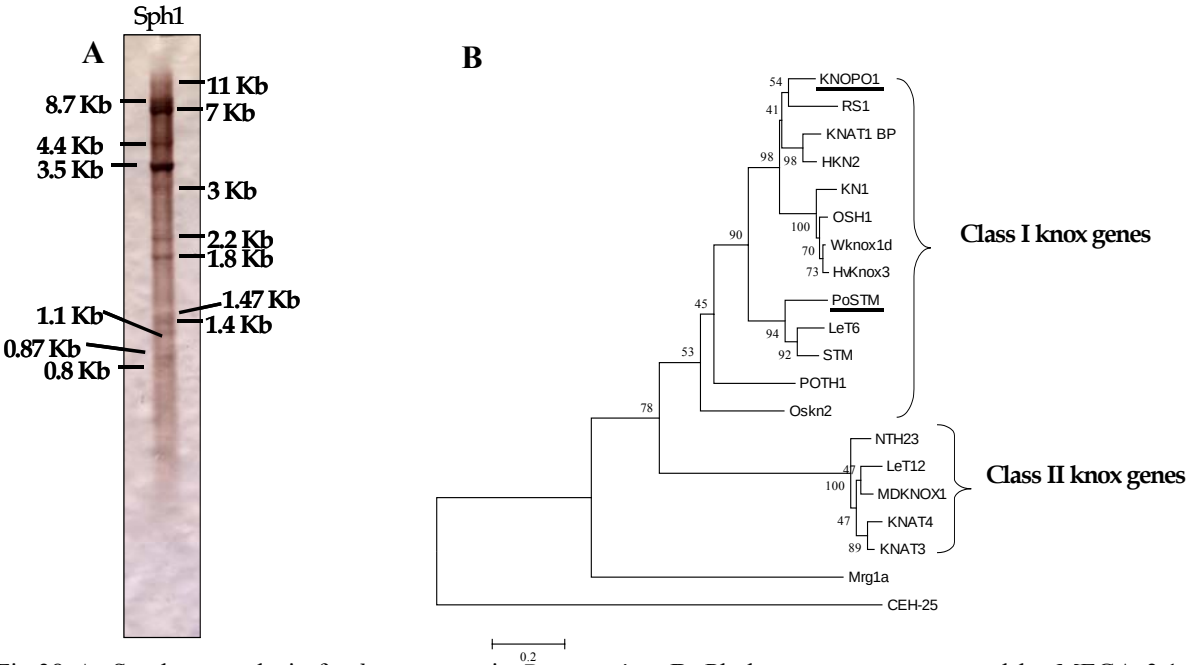


Fig.38 **A.** Southern analysis for *knox* genes in *P. oceanica*. **B.** Phylogram was constructed by MEGA 3.1 software (Kumar et al., 2004). Numbers on the tree indicate bootstrap (1000). Accession numbers of protein examined: *RS1* (*Zea mays*, L44133), *KNAT1 BP* (*Arabidopsis thaliana*, AT4g08150), *HKN2* (*Helianthus anuus*, AY096803), *KN1* (*Zea mays*, X61308), *OSH1* (*Oryza sativa*, D16507), *Wknox1d* (*Triticum aestivum*, AB182945), *HvKnox3* (*Hordeum vulgare*, AF022390), *LeT6* (*Solanum lycopersicum*, AF00141), *STM* (*Arabidopsis thaliana*, U32344), *POTH1* (*Solanum tuberosum*, U65648), *Oskn2* (*Oryza sativa*, AF050180), *NTH23* (*Nicotiana tabacum*, AB004797), *LeT12* (*Solanum lycopersicon*, AF000142), *MDKNOX1* (*Malus domestica*, Z71980), *KNAT4* (*Arabidopsis thaliana*, X92393), *KNAT3* (*Arabidopsis thaliana*, X92392), *Mrg1a* (*Mus musculus*, U68383), *CEH-25* (*Caenorhabditis elegans*, AJ000533).

>*KNOPO1*
TCCTGCTCTGGATCAGTTCATGGCGGTAAAAAATACGATATGCTGGTGAAGTACCGGGAAGAGCTAACACGGCCACTGCAAGAAGCCATGGATT
TCCTCCGGCGAGTAGAGTCGCAACTGAGCGTTCTCACCAATGGAGCTGCCCGTGTCTTCTCACCTGATGAGAAATGTGAAGGTGTGGGGTCTTC
TGAAGAGGATCAAGATGGTAGTGGGGGAGAAACAGAACTTCCTGAGATTGACCCGAGGGCTGAGGACAAGGAGCTTAAGCACCATCTTCTTAAG
AAGTACAGCGGATACCTGAGCAGCCTCAAACAAGAACTATCCAAGAAAAAAAAGAAAGGAAAACTACCTAAAGAAGCAAGGCAGAAACTGCTGA
ACTGGTGGGAAATGCACTACAAGTGGCCTTATCCTTCGGAGACAGAGAAGTTGGCCCTGGCAGAATCCACGGGTCTTGATCAGAAGCAAATAAA
CAACTGGTTTATTAATCAGAGGAAGCGCCACTGGAAGCCTTCCGAGGACATGCAGTTCGTTGTTATGGATGGCTACCACCCACAAAACGCTGCT
GCCTTTTACATGGATGGACATCACCACTTCGTTGGTGATGGCTCGTATCGACTAGGCCCTTGATTGCTAATCGGTCAGCAATGGCAGTGCCCAT
GAAATCTTTATATGTGTCATGTAAAGCATGATTTCTTTGTGAGCTTTATGTCTGTTTTTGGAAGTGTGTCGGCAGCGTTTTATCATTTGAATGT
ACCTTATCTTCATCAAAAAAAAAAAAAAAAAA

>KNOPO1
SCSGSVHGGKKYDMLVKYREELTRPLQEAMDFLRRVESQLSVLTNGAARVFSPDEKCEGVGSSEEDQDGSGGETELPEIDPRAEDKELKHHLLK
KYSGYLSSLKQELSKKKKKGKLPKEARQKLLNWWEMHYKWPYPSETEKLALAESTGLDQKQINNWFINQRKRHWKPSEDMQFVVMDGYHPQNAA
AFYMDGHHHFVGDGSYRLGP

Fig.39 cds sequence for KNOPO1 gene (*knox Posidonia oceanica*). The underlined region indicates the stop codon. Deduced protein of *KNOPO1* gene. The bold region indicates the GSE box. Green aminoacids indicate KNOX2 domain, red aminoacids indicate ELK domain, blue aminoacids indicate homeodomain.

>*PoSTM*
CAAACCCTTCAAGGAAGCCATGCTCTTCTTCTCAAGAATGGAGTCCCAGTTCAAGTCTCTCACACTTACTAGTGCATCCCCTTCCTCCCTTCTA
CCTCCTGGAGGATGTGGTGATACTGCAGACAAGAACGTCTCTTCCGAAGATGATGCTGTTGATGTGGATGATAACTACATCGATCCGCAAGCAG
AGGATCGAGAATTGAAGGGGCAGCTTCTTCGGAAATACAGTGGGTATCTTGGAAGTCTCAAAAAGGAGTTTCTTAAGAAGCGGAAAAAAGGGAA
GTTGCCTAAAGAAGCTAGACAGCAACTGCTGGACTGGTGGAGCAGACATTATAAGTGGCCTTACCCCTCGGAGTCTCAAAAGCTGGCACTCGCG
GAGACTACGGGTCTTGACCCGAAACAAATAAACAATTGGTTCATAAATCAAAGAAAGCGTCACTGGAAGCCTACAGATGACATGCAGTATGCGG
TGATGGATGCATCACATCCAACCCATTACTACATGGAAAACAACTTCGGTGGTGCAAATCCTTTCCCTCATCTTGATTGTGGCACTACTCTTTT
CTAAAGAAATTTATAAAAGATCGATCTGTTGGTGGAATATTTTATGCATGATGCACATTGTCTATGATATATGTGTAGTAGTGTAGCTTTTATT
ATATTTGCTGCCATTGTATTCCCAACTTTGTTGGTTCTGTAGTAACTCGAGACGAGGGTTTAATTGAGTTGTGGAAGTGTAATATGATCGTATG
TGTATGACCAAGTATTGCTGCTTCCGGTCAAAAAAAAAAAAAAAAAAAAAA

>PoSTM
KPFKEAMLFFSRMESQFKSLTLTSASPSSLLPPGGCGDTADKNVSSEDDAVDVDDNYIDPQAEDRELKGQLLRKYSGYLGSLKKEFLKKRKKGK
LPKEARQQLLDWWSRHYKWPYPSESQKLALAETTGLDPKQINNWFINQRKRHWKPTDDMQYAVMDASHPTHYYMENNFGGANPFPHLDCGTTLF

Fig.40 cds sequence for *PoSTM* gene (*Posidonia oceanica shootmeristemless*). The underlined region indicates the stop codon. Deduced protein of *KNOPO1* gene. Green aminoacids indicate KNOX2 domain, red aminaocids indicate ELK domain, blue aminoacids indicates homeodomain.

C. – Materials and Methods

1. RT-PCR

RT-PCR was performed as indicated in Paragraph 8 of materials and methods above. The same couple of degenerated primers (knox Fw CCDGMDYTRGAYCARTTCATGG, knox Bw ATRAACCARTTGTTKATYTGYTTC) was used to identify the first fragment of *KNOPO1* and *PoSTM* genes. Subsequently, 3' end was identified with 3'RACE-PCR with gene specific primers (Fw 5'-AATGGAGCTGCCCGTGTCTT-3' for *KNOPO1* and Fw 5'-TTCCGAAGATGATGCTGTTG-3' for *PoSTM*). A 591 bp fragment for the Phytochrome B gene was firstly identified with degenerate primers Fw1 (5'-AACATGGGKTCVATTGCRTC-3') and Bw1 (5'-TGCRCCACCCCAYTTKATCT-3'). Subsequently a second forward primer (Fw2: 5'-GCGGCGGGCAYATHCARC-3') coupled with Bw2 gave an amplicon of 1281 Kb.

2. Southern blot

Southern blot and probe labeling were performed as indicated in Paragraph 9 of materials and methods above. Probe size was of 370 bp for *knox* genes and 520 bp for *PHYB* gene.

References

Bürglin, T. R. (1997). The PBC domain contains a MEINOX domain: coevolution of Hox and TALE homeobox genes? *Dev. Genes Evol.* **208**, 113-116.

Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A. and Martienssen, R.A. (2000). *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* **408,** 967-971.

Chuck, G., Lincoln, C. and Hake, S. (1996). *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. *Plant Cell* **8,** 1277-1289.

Daimon, Y., Takabe, K. and Tasaka, M. (2003). The *CUP-SHAPED COTYLEDON* genes promote adventitious shoot formation on calli. *Plant Cell Physiol.* **44,** 113-121.

Deng, X-W. and Quail, P.H. (1999). Signalling in light-controlled development. *Sem. Cell Dev. Biol.* **10**, 121-129.

Fankhauser, C. and Chory, J. (1997). Light control of plant development. *Annu. Rev. Cell Dev. Biol.* **13**, 203-229.

Fraenkel, E., Rould, M. A., Chambers, K. A. and Pabo, C. O. (1998). Engrailed homeodomain-DNA complex at 2.2 Å resolution: a detailed view of the interface and comparison with other engrailed structures. *J. Mol. Biol.* **284**, 351-361.

Frugis, G., Giannino, D., Mele, G., Nicolodi, C., Chiappetta, A., Bitonti, M.B., Innocenti, A.M., Dewitte, W., Van Onckelen, H. and Mariotti, D. (2001). Overexpression of *KNAT1* in lettuce shifts leaf determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins. *Plant Physiol.***126,** 1370-1380.

Gallois, J.L., Woodward, C., Reddy, G.V. and Sablowski, R. (2002). Combined *SHOOT MERISTEMLESS* and *WUSCHEL* trigger ectopic organogenesis in *Arabidopsis*. *Development* **129,** 3207-3217.

Gyula, P., Schäfer, E. and Nagy, F. (2003). Light perception and signalling in higher plants. *Curr. Opin. Plant Biol.* **6**, 446-452.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor end analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**, 95-98.

Hamant, O., Nogué, F., Belles-Boix, E., Jublot, D., Grandjean, O., Traas, J. and Pautot, V. (2002). The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. *Plant Physiol.* **130**, 657-665.

Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y. and Lifschitz, E. (1996). The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell* **84,** 735-744.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S. and Tsiantis, M. (2002). The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Curr. Biol.* **12,** 1557-1565.

Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J. and Hake, S. (1994). Sequence analysis and expression patterns divide the maize *knotted1*-like homeobox genes in two classes. *Plant Cell* **6**, 1877-1887.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinfo.* **5**, 150-163.

Mathews, S. and Sharrock, R.A. (1997). Phytochrome gene diversity. *Plant Cell Environ.* **20**, 666-671.

Mok, D.W. and Mok, M.C. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 89-118.

Nagy, F. and Schäfer, E. (2002). Phytochromes control photomorphogenesis by differentially regulated, interacting signaling pathways in higher plants. *Annu. Rev. Plant Biol.* **53**, 329-355.

Neff, M.M., Fankhauser, C. and Chory, J. (2000). Light: and indicator of time and place. *Genes Dev.* **14**, 257-271.

Quail, P.H. (1997). An emerging molecular map of the phytochromes. *Plant Cell Environ.* **20**, 657-665.

Rupp, H.M., Frank, M., Werner, T., Strnad, M. and Schmülling, T. (1999). Increased steady state mRNA levels of the *STM* and *KNAT1* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. *Plant J.* **18**, 557-563.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S. and Matsuoka, M. (2001). KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. *Genes Dev.* **15,** 581-590.

Scanlon, MJ. (2003). The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. *Plant Physiology* **133,** 597-605

Schneeberger, R.G., Becraft, P.W., Hake, S. and Freeling, M. (1995). Ectopic expression of the *knox* homeobox gene *rough sheath1* alters cell fate in the maize leaf. *Genes Dev.* **9,** 2292-2304.

Schneeberger, R.G., Tsiantis, M., Freeling, M. and Langdale, J.A. (1998). The *rough sheath2* gene negatively regulates homeobox gene expression during maize leaf development. *Development* **125,** 2857-2865.

Sentoku, N., Sato, Y. and Matsuoka, M. (2000). Overexpression of rice *OSH* genes induces ectopic shoots on leaf sheaths of transgenic rice plants. *Dev. Biol.* **220,** 358-364.

Sinha, N.R., Wiliams, Jr E.E. and Hake S. (1993). Overexpression of the maize homeo box gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fate. *Genes Dev.* **7,** 787-795.

Smith, L.G., Greene, B. and Hake, S. (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in the leaf cells with altered fate. *Development* **116,** 21-30.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., Nagy, F., Schäfer, E. and Harter, K. (2001). Interacion of the response regulator ARR4 with the photoreceptor phytochrome B in modulating red light signaling. *Science* **294**, 1108-1111.

Tamaoki, M., Kusaba, S., Kano-Murakami, Y. and Matsuoka, M. (1997). Ectopic expression of a tobacco homeobox gene, *NTH15*, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant Cell Physiol.* **38,** 917-927.

Tsiantis, M., Schneeberger, R., Golz, J.F., Freeling, M. and Langdale, J.A. (1999). The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. *Science* **284**, 154-156.

Veit, B. (2003). Determination of cell fate in apical meristems. *Curr. Opin. Plant Biol.* **7,** 1-8.

Vollbrecht, E., Veit, N., Sinha, S., and Hake, S. (1991). The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* **350**, 241-243.

Weigel. D. and Jurgens, G. (2002). Stem cells that make stems. *Nature* **415,** 751–754.

Whitelam, G.C. and Devlin, P.F. (1997). Roles of different phytochromes in *Arabidopsis* photomorphogenesis. *Plant Cell Environ.* **20**, 752-758.

Publications

- **Bruno, L., Bruno, A., Giannino, D., Chiappetta, A. and Bitonti, M.B.** Expression pattern of *GERANYLGERANYL REDUCTASE* gene (*PoCHL P*) in plants of *Posidonia oceanica* (L) Delile. **(manuscript in preparation)**.

- **Bruno, A., Petrarulo, M., Chiappetta, A. and Bitonti, M.B.** Differences in cytokinins distribution in *Posidonia oceanica* mark stress condition in plants from preserved and disturbed sites. **(manuscript in preparation)**.

Meetings

- **Bruno, A., Bruno, L., Greco, M., Chiappetta, A., Bitonti, M.B. (2005)**. Expression pattern of a chlorophyll biosynthesis gene in plants of *Posidonia oceanica* from intact and stressed meadows. **VII National Congress Federazione Italiana Scienze della Vita, Riva del Garda, 22-25/09/2005, pg. D10.1**. *(oral symposium)*

- **Bruno, A., Bruno, L., Greco, M., Giannino, D., Chiappetta, A., Bitonti, M.B. (2006)**. Il pattern di espressione del gene della geranilgeranil reduttasi evidenzia una condizione di stress luminoso in praterie disturbate e di controllo della fanerogama *Posidonia oceanica* [L.] Delile. **XVII Convegno del Gruppo per l'Ecologia di Base "G. Gadio"**. *(oral symposium).*

- **Bruno, A., Bruno, L., Greco, M., Giannino, D., Chiappetta, A., Bitonti, M.B. (2006)**. Expression pattern of *CHL P* gene as a putative marker of light stress conditions in *Posidonia oceanica* [L.] Delile. **Mediterranean Seagrass Workshop, 29-05/03-06-2006** *(oral symposium)*.

- **Bruno, L., Bruno, A., Greco, M., Giannino, D., Chiappetta, A. and Bitonti, M.B. (2006)**. Pattern di espressione del gene che codifica per la geranilgeranil reduttasi (*PoCHL P*) in *Posidonia oceanica* (L) Delile. **CI Congresso Nazionale SBI, Caserta, settembre 2006**.

- **Bruno, L., Bruno, A., Greco, M., Giannino, D., Chiappetta, A. and Bitonti, M.B. (2006)**. Expression pattern of *GERANYLGERANYL REDUCTASE* gene (*CHL P*) in plants of *Posidonia oceanica* (L) Delile. **International Conference Marine Genomics, Sorrento, 28/10-01/11/2006, pg. 77**.