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RESEARCH

Identification of Low Temperature Stress Regulated Transcript Sequences and Gene Families in Italian Cypress

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Abstract Cold acclimation is a complex transcriptionally controlled process regulated by many different genes and genic-interactions in plants. The northward spreading of woody species is mainly limited by winter harshness. To increase our knowledge about the biological processes underlying cold acclimation, plants evolved in warmer climates can serve as models. In this work, a Suppression Subtractive Hybridization approach using PCR-select was used to isolate Italian cypress (*Cupressus sempervirens* L.) transcript sequences putatively expressed under low temperature stress. After assessing the reliability of the subtractive step, a total of 388 clones were selected and sequenced. Following sequence assembly and removal of the redundant cDNAs, 156 unique transcripts were identified and annotated in order to assign them a putative functional class. Most of the identified transcripts were functionally classified pertaining to stress in cellular and chloroplast membranes, which are previously known to be severely damaged by cold treatment. Among the identified functional gene families, the extensively represented ones were dehydrins, early light-inducible proteins, senescence-associated genes and oleosins. The last three gene families were further selected for phylogenetic analysis, with the corresponding protein sequences across the complete genomes of the model plants *Populus trichocarpa*, *Vitis vinifera*, *Physcomitrella patens,* and *Arabidopsis thaliana*. The relationship with the ortholog sequences coming from these species and their further implications are discussed.

Keywords SSH · Gene expression · ELIPs · SAGs · Oleosins · Dehydrins

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Introduction

Cold is demonstrated to be one of the most limiting factors influencing plant growth. Winter sub-zero temperatures capable of severely damaging plant tissues are common in all temperate climates. Nonetheless, many plant species have the ability to increase their freezing tolerance when exposed to low but non-freezing temperatures (0–15 °C). This process, biologically known as cold acclimation, involves a number of physiological and molecular changes [1]. As a consequence, many genes and gene families are up- or down-regulated during cold acclimation in order to enable the plant to better resist to sub-zero temperatures.

Cryoprotective proteins [2, 3], osmolites such as sugars and certain amino acids [4, 5], dehydrins [6] and protein families such as late embryogenesis abundant (LEA) [7] and early light-inducible proteins (ELIP) [8], are known to accumulate in plants in response to low temperature exposure. Most of the studies related to cold tolerance have been performed on herbaceous model species such as *Arabidopsis* [9] or using cereal species such as rice [10] or barley [11]. Even if the mechanism of cold acclimation appears to be quite conserved among plants, differences still exist, especially for species that represent different phylogenetic lineages. During the last few years a number of molecular studies have been performed also on perennial woody species, in order to highlight similarities and peculiarities in low temperature response [12–14].

Italian cypress (*Cupressus sempervirens* L.) is a woody species originating in the Mediterranean area and is capable to adapt to a wide range of climates, including the continental one. During winter, cypress can cope with temperatures below −10 °C or even −15 °C [15]. Since the Roman time this conifer has been introduced in areas very far from its native range and nowadays it is a common landscape tree, not only in all Mediterranean regions [16] but also in colder European regions and at lower elevation in mountains, where cypress is often used as ornamental tree or as a windbreak [17]. As cypress clones show considerable variation in cold tolerance [18], the tree can serve as a model plant to better understand the processes that underlie cold acclimation, with the ultimate aim to generate more cold resistant cypress genotypes.

From a physiological and molecular point of view, cypress is still a poorly studied species. However, the few studies available on this species indicate that low temperature can heavily affect photosynthesis in Italian cypress [19] as well as in other Cupressaceae [20] and consequently significantly impair the tree growth. Photosynthesis in cypress is significantly influenced also by aging [21], and by canker infection [22], with different molecular mechanisms [23]. Nevertheless, the current climate change would be expected to shift the distribution of cypress, as plants expand in newly favorable areas and decline in increasingly hostile locations [17, 24].

In order to provide insights into the mechanisms of low temperature response of cypress, a genome- or transcriptome-wide analysis would be the ideal solution. Nowadays, methods such as RNA-seq provide a powerful tool to analyze hundreds or even thousands of transcripts linked to putative genes within a single experiment. Nevertheless, such techniques are also rather challenging due to the necessity to assembling millions of short reads and require the availability of genome sequence to link the transcript abundance to certain genes (for review see [25]). Suppression subtractive hybridization (SSH) provides a possible alternative to RNA-seq. SSH eliminates most of the redundant sequences between two given conditions, one major advantage of the technique being the production of cDNA libraries enriched in rare transcripts [26–29]. In the present paper, we describe the use of SSH technique for the cloning of cypress sequences putatively regulated by low temperature. Some of the highly represented gene families were then phylogenetically analyzed in comparison with the most similar sequences coded by the complete genomes of *Populus thrichocarpa*, *Vitis vinifera*, *Physcomitrella patens,* and *Arabidopsis thaliana*.

Materials and Methods

Plant Material and Treatment Conditions

A single cypress genotype (Clone 8), derived from a breeding program carried out by IPP-CNR of Florence [30], was used in cold treatment experiments. Five ramets of the same genotype were grown in pots in 50 % sand and 50 % soil to the height of about 60 cm. Before cold treatment the plants were acclimated at 22 °C in a growing chamber for 21 days (12 h light 160 µmol photons $m^{-2}s^{-1}$ and 12 h dark). The plants were cold treated in a daily regime of 12 h light (160 µmol photons $m^{-2}s^{-1}$) and 12 h dark at 3 °C for 7 days. Leaf samples were collected from each plant before cold treatment (control) and after 7 days at 3 °C. All samples were immediately frozen in liquid nitrogen and kept at −80 °C for mRNA isolation.

mRNA Extraction and Subtraction Technique

All the collected samples were ground in liquid nitrogen, then total RNA was extracted using a modified hot borate method as described in Moser et al. [31]. Three grams of cypress tissue was used for each extraction. Messenger RNA was obtained from total RNA using the Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, starting from 250 µg of total RNA. In order to identify cypress sequences putatively regulated by low temperature, a subtractive approach was performed using PCR-select cDNA subtraction kit (Clontech, Mountain view, CA, USA) according to the manufacturer's instructions. Subsequently, two different subtraction reactions (forward and reverse subtraction) were performed.

Gene Cloning and Sequencing

The sequences obtained from PCR-select were cloned into pCR®2.1-TOPO® cloning vector. An aliquot of 2 µg of subtracted cDNA was loaded onto a 1,5 % agarose gel and all the bands between 300 and 1,000 bp were eluted and

cloned into the vector with TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. A total of 500 single colonies were examined by digestion of the inserted product with *Alu*I enzyme. All the clones showing different restriction patterns were chosen and sequenced using an ABI PRISM 3100 Genetic Analyzer, Applied Biosystems. Sequencing reactions were prepared using the Perkin Elmer Big Dye DNA sequencing kit (Perkin Elmer, Foster City, CA, USA). Sequencing reactions were cleaned using the Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA). In this way 156 unique sequences were found and used for similarity search with known genes in GenBank using the BLASTx algorithm.

Dot Blot Hybridization

Plasmid DNA was purified using QuickLyse Miniprep Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The inserts were amplified by PCR using M13 forward and reverse primers. PCR amplification was carried out in a final volume of 15 µl with 1.5 µl of 10× buffer, 2 mM MgCl2, 0.2 mM dNTPs, 1.5 µM of each primer and 0.2 U of Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA). The cycling regime consisted of a denaturing step at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, with a final extension of 5 min at 72 °C. Dot blot hybridization was performed as described in Simon et al. [32]. PCR-amplified fragments were denatured by heating at 95 °C before blotting on positively charged nylon membranes (Boehringer Mannheim, Germany). Membranes were pre-hybridized for at least 2 h without probe and then incubated overnight with digoxygenin (DIG)-labeled probes (DIG oligonucleotide 3′-end Labeling Kit, Boehringer Mannheim) at 55 °C. Pre-hybridization and hybridization solutions contained 0.1 mg ml^{-1} of Poly-A to prevent non-specific binding of the tailed probes. Chemiluminescence detection was performed using Chemiluminescent Substrate For Alkaline Phosphatase (CSPD) as a substrate (DIG detection kit, Boehringer Mannheim). All steps were performed according to the manufacturer's instructions. Membranes were exposed to X-ray film for 20, 30, or 60 min.

Functional Annotation and Phylogenetic Analysis

The unigene dataset obtained was annotated by doing BLASTx searches (cut off E-value 1e-10^{-5} against NCBI (http://www.ncbi.nlm.nih.gov/) and TAIR (http://www.arabidopsis.org/) database. Following BLASTx searches, we performed Gene Ontology classification. The obtained ontologies were further slimmed according to Plant GO Slim category available from www.geneontology.org. In order to characterize the ESTs obtained as significantly expressed during cold treatment, a subset of low temperature responsive genes were further selected for phylogenetic analysis and namely: senescence-associated proteins—SAG (*EU430719, EU430725, EU430726, FJ237440, FJ237451, FJ237454, FJ237465, FJ237479, FJ237482, FJ379995*), early light-induced proteins—ELIP (*EU430722, FJ237435, FJ237483, FJ237486, FJ379970, FJ380014*), oleosins (*EU430721, FJ237443, FJ237481, FJ237494, FJ380021)* and dehydrins *(FJ237450, FJ237457, FJ237480, FJ379968, FJ379975, FJ379996, FJ379998, FJ380015*, and *FJ380020*). The selected sequences were compared with the complete genomes of the model plants *Populus trichocarpa*, *V. vinifera*, *P. patens,* and *A. thaliana* for the identification of the putative homologs. Conceptually translated amino acid sequences of the ESTs cloned from cypress were aligned with MUSCLE [33] and the alignments were trimmed to eliminate low homology regions. Curated final alignments were then imported in MEGA5 [34] to build Neighbor-Joining trees using the JTT substitution model with 1,000 bootstrap support.

Results

Gene Cloning and Functional Classification

In the present work, we constructed a subtracted cDNA library enriched in sequences putatively regulated by low temperature. mRNA extracted from a single cypress genotype (Clone 8) before and after 7 days of low temperature treatment was used as driver and tester RNA and two subtracted libraries, enriched in induced and repressed sequences, respectively, were obtained. All the cDNA fragments shorter than 300 bp were discarded prior to analysis. Electrophoresed fragments longer than 300 bp were gel eluted and subsequently cloned in *E. coli*. To assess the robustness and reliability of the subtraction step, 48 colonies from the induced library and 48 colonies from the repressed ones were randomly selected for dot blot hybridization. After purification of plasmid DNA, the inserts were amplified by PCR and blotted in triplicate onto nylon membrane. cDNA obtained from mRNA extracted from cold-treated cypress (Clone 8) was fluorescently labeled as described in Materials and Methods and used as a probe for hybridization with the spotted sequences (Fig. 1). The great majority of the fragments extracted from the repressed library showed a very weak or no signal after hybridization (Fig. 1a) while all the fragments extracted from the induced library showed a clear signal (Fig. 1b), suggesting that most of the fragments of each library were actually specific to the corresponding treatment condition. A total of 500 colonies were randomly

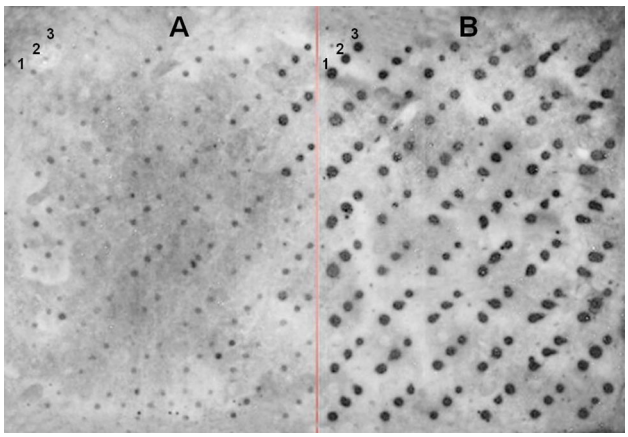

Fig. 1 Dot blot analysis of subtracted libraries. 48 random colonies from *Cypress* repressed library (**a**) and induced library (**b**) were spotted in triplicate [1–3] onto the nylon membrane and hybridized with fluorescently labeled cDNA from cold-treated cypress (Clone 8)

selected and tested for insert presence by direct colony PCR. Out of these, 388 showed an insert, which was sequenced using Sanger chemistry.

Following the sequencing, chromatograms were observed, vectors were trimmed off and the sequencing assembly was performed using the CAP3 program [35], resulting in 156 unigenes (contigs + singeltons). The average sequence length of the UniGene set was 394 bp, the longest sequence being 550 bp and the shortest 224 bp (Supplementary material, table S1). The most represented protein families are shown in Table 1 (see also supplementary material, Table S1 for the complete list of sequences). Based on their putative functions, the cDNA clones were characterized by grouping them into functional classes according to gene ontology (GO) classification followed by Plant GO Slim classification [36]. GO slim classification revealed three main "biological processes": "glycolysis", "gluconeogenesis", and "response to cold", followed by some more general categories such as "oxidation–reduction process", "fatty acid biosynthetic process" and "phosphorylation" (Fig. 2a). Additionally, some other stress-related groups of sequences which were found represented were "response to water stimulus", "abscisic acid-mediated signaling pathway" and "response to salt stress" (Fig. 2a). GO slim classification of the "cellular component" revealed an abundance of GO categories associated with chloroplasts and membranes, such as"thylakoid membrane", "integral to membrane", and "chloroplast membrane" categories (Fig. 2b). However, GO slim "molecular functions" revealed that "ATP binding" is by far the highest class of the GO term involved in molecular function (Fig. 2c).

Phylogenetic Analysis

When the 156 cypress sequences were categorized according to their putative function, it was noticed that a number of genes actually belonged to few specific gene families (Table 1; Supplementary materials, Table S1). In order to deepen the understanding of the highly represented gene families, and specifically the ELIP family, the SAG family, the oleosins family and dehydrin family, a phylogenetic analysis was performed. This approach was applied to map the newly cloned and annotated ESTs from cypress in a genomic and functional context (regardless of the best blast hit) providing a deeper annotation that might highlight targets for further investigation such as multi-gene family members differential expression (sub-functionalization

Table 1 Most represented protein families from the *Cypress* cold-regulated UniGene set after BLAST search

Putative function	No. of sequences	GenBank ID	Best homology (%)
Dehydrin	11	EU430717; FJ237450; FJ237457; FJ237480; FJ379975; FJ379968; FJ379998; FJ379996; FJ380020; FJ380015; FJ379973	87
Senescence-associated protein	10	EU430726; FJ237454; EU430725; FJ237482; FJ237465; FJ237440; EU430719; FJ379995; FJ237479; FJ237451	94
Cold-induced protein	9	FJ379984; FJ379992; FJ380007; FJ380016; FJ379980; EU430718; FJ379969; FJ237485; EU430723	85
Chlorophyll *a/b*-binding protein	7	EU430724; FJ380036; FJ237489; EU430707; FJ380017; FJ237471; FJ237473	100
Early light-induced protein	6	FJ237486; FJ237483; FJ237435; EU430722; FJ380014; FJ379970	82
Oleosin	5	FJ237481; FJ380021; EU430721; FJ237443; FJ237494	89
Ribulose bisphosphate carboxylase	5	FJ237488; FJ379983; FJ237462; FJ380006; EU430711	87
Aquaporin	3	EU430708; FJ380011; FJ379986	91

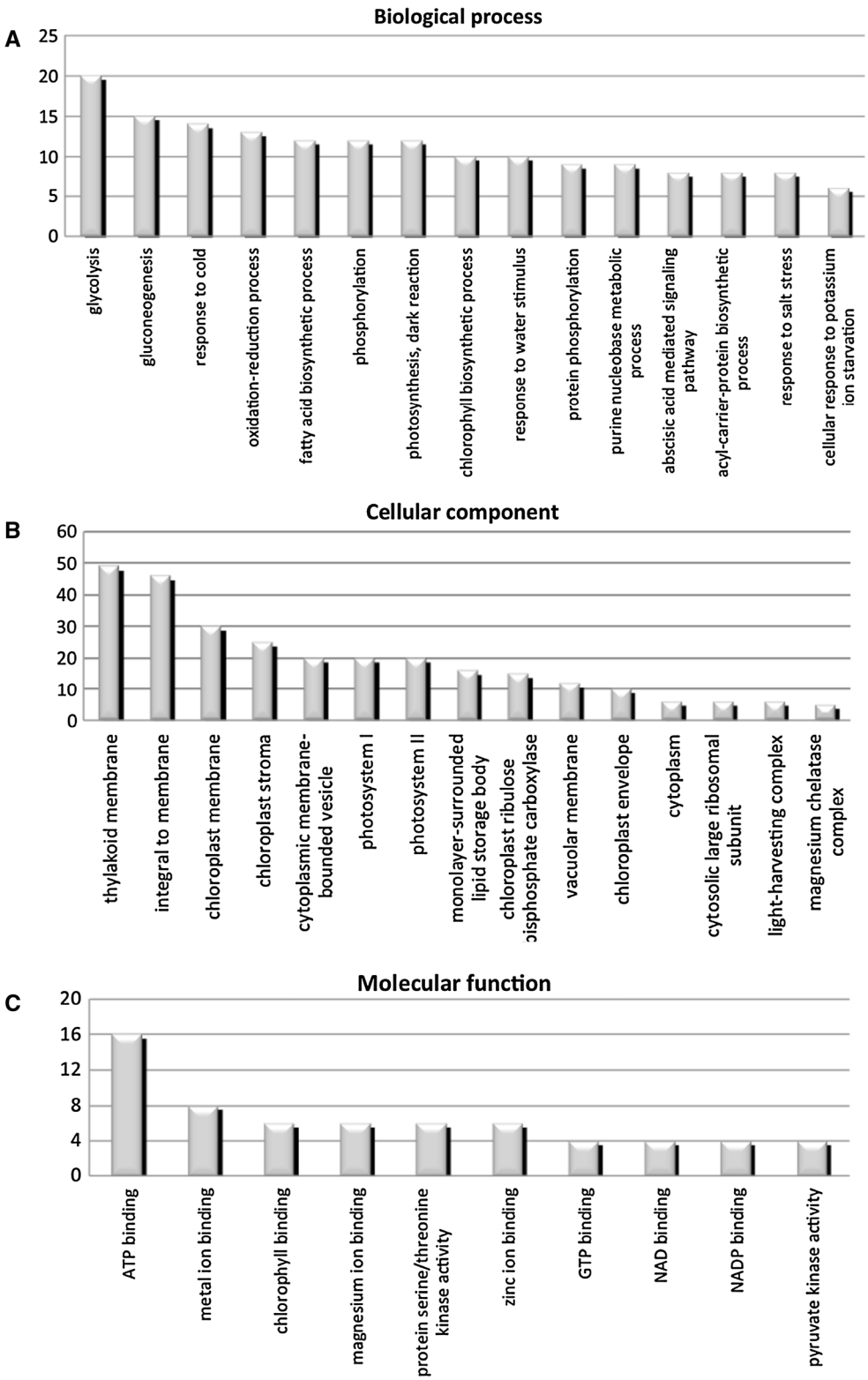

Fig. 2 Distribution of Cypress cold-regulated UniGene set within the gene ontology (GO) functional assignments. The *number of sequences* belonging to each category is indicated on the *y* axis

evolutionary processes). *C. sempervirens* ORF from EST sequences were identified using the frame correction as implemented in FrameD [37] and then BLASTed against the genome predicted coding regions of *P. trichocarpa*, *V. vinifera*, *P. patens*, and *A. thaliana* to identify the putative homologs. In addition to these complete genomes, additional sequences were added in case of well characterized gene families mined from the literature, as in the case of the

Coffea canephora oleosin gene family [38], the *Rhododendron catawbiense* ELIP family [39], and the *Pinus sylvestris* dehydrin family [40]. Despite the low bootstrap support of few nodes, due to the divergence of sequences belonging to species spanning a wide evolutionary range and the limited number of sites used to build the dendrograms, it is possible to depict patterns of phylogenetic classification of the cypress ESTs cloned. The dendrogram for the six cypress sequences along with the homologs in the other plant species in ELIP family is shown in Fig. 3. Three of the sequences (*EU430722, FJ237435,* and *FJ237483*) were clustered together but quite far from all the other ELIPs. Two sequences (*FJ237486* and *FJ379970*) showed higher similarity to ELIPs from other species but still were clustered separately, while only *FJ380014* was included in the *R. catawbiense* group (Fig. 3). This might reflect that the *ELIP* family is represented with in-paralogs in cypress. It is noteworthy to mention that previously seven highly homologous *ELIP*s (*RcELIP1-7*) have been characterized in *R. catawbiense* [39].

For the SAG family, six sequences (*FJ237454, FJ237482, EU430726, FJ237440, FJ237465*, and *EU430719*) were found clustered together with homologous SAGs from *P. trichocarpa*, *V. vinifera* and *A. thaliana* (Fig. 4). Three sequences (*EU430725, FJ237479* and *FJ379995*) clustered together but showed very divergent patterns of evolution from all the other senescence-associated genes from cypress and the other species considered. The last cypress SAG (*FJ237451*) was found to belong to a distinct group of sequences (Fig. 4).

Oleosin a*nalysis resulted in qu*ite a complex dendrogram (Fig. 5) in which at least two main groups of sequences were recognizable, each group containing genes from most of the species in analy*sis. The cypress oleosins were represented in both groups: FJ237485, FJ380021, and FJ237494* in the first and *EU430719* and *FJ237443* in the second group. Finally, the dendrogram built with dehydrin sequences was not considered enough accurate due to a lack of bootstrap support and therefore was not further analyzed (see supplementary material Fig. S1).

Discussion

Understanding the transcriptional control of genes, their up- and down-regulation under cold stress can suggest potential grounds for developing strategies to cope with the genetic loss due to cold conditions [41]. In plant functional genomic research, ESTs sequences are a valuable tool to study gene expression, especially for those species where the complete genomic sequence is not yet available [42, 43]. SSH represents a fast and economic way to identify and develop characterization approaches for sequences regulated during different developmental stages or biotic and abiotic stresses [26–29]. In order to identify cypress ESTs potentially regulated during low temperature exposure, SSH analysis was applied and 156 unique sequences were isolated. During cloning a cut-off value of 300 bp was applied in order to avoid all the shortest sequences that could be preferentially inserted in vectors during the cloning step. At the end, after applying such length threshold, we obtained 21 out of 156 sequences (13.4 %), which were shorter than 300 bp, and only 9 out of 156 (5.7 %), which were shorter than 250 bp (Table S1).

The efficiency of the enrichment step during SSH can greatly vary according to several factors, such as plant species, treatment conditions and RNA extraction efficiency [29, 44, 45]. In the present study, random colonies were selected and analyzed by dot blot hybridization to prove the efficiency of the protocol. Most of the sequences extracted from the induced library displayed a strong signal when labelled cDNA from cold-treated cypress plants was used as a probe (Fig. 1). On the contrary, only very few colonies of the repressed library contained sequences capable to hybridize with the probe, suggesting that most of the sequences in each group were differentially expressed during cold treatment. Even though this first test cannot give an accurate indication about the nature of the inserted sequence, it can be effectively used to assess the quality of the subtractive step, before proceeding with further analysis. The annotation of the cloned sequences also provided a good indication of the success of the enrichment step during SSH. When sequences were annotated according to the GO main term "biological process", the two more represented groups were "glycolysis" and "gluconeogenesis", which is well in accordance with previous findings in other species where cryoprotective metabolites, such as sugars, are known to be accumulated as a consequence of low temperature [41, 46, 47]. Changes in starch metabolism and sugar content during cold acclimation seem to be part of a conserved mechanism common to both herbaceous and woody plants [48, 49]. It is known that soluble sugars act as osmolytes [50, 51] in over-wintering plant tissues, so the breakdown of starch, may represent (as well as a source of carbohydrates to provide energy for acclimation) an active process to produce osmolytes for hardening.

As further indication of a successful enrichment step during SSH lies in the fact that the third observed biological category was specifically "response to cold", while sequences belonging to biological processes related to ABA signaling [52], response to water changes [53] and salt stress [54] were also found among the more represented groups.

Moreover, some genes identified in the present work by SSH have been previously used for expression studies by qRT-PCR in different cypress accessions during cold

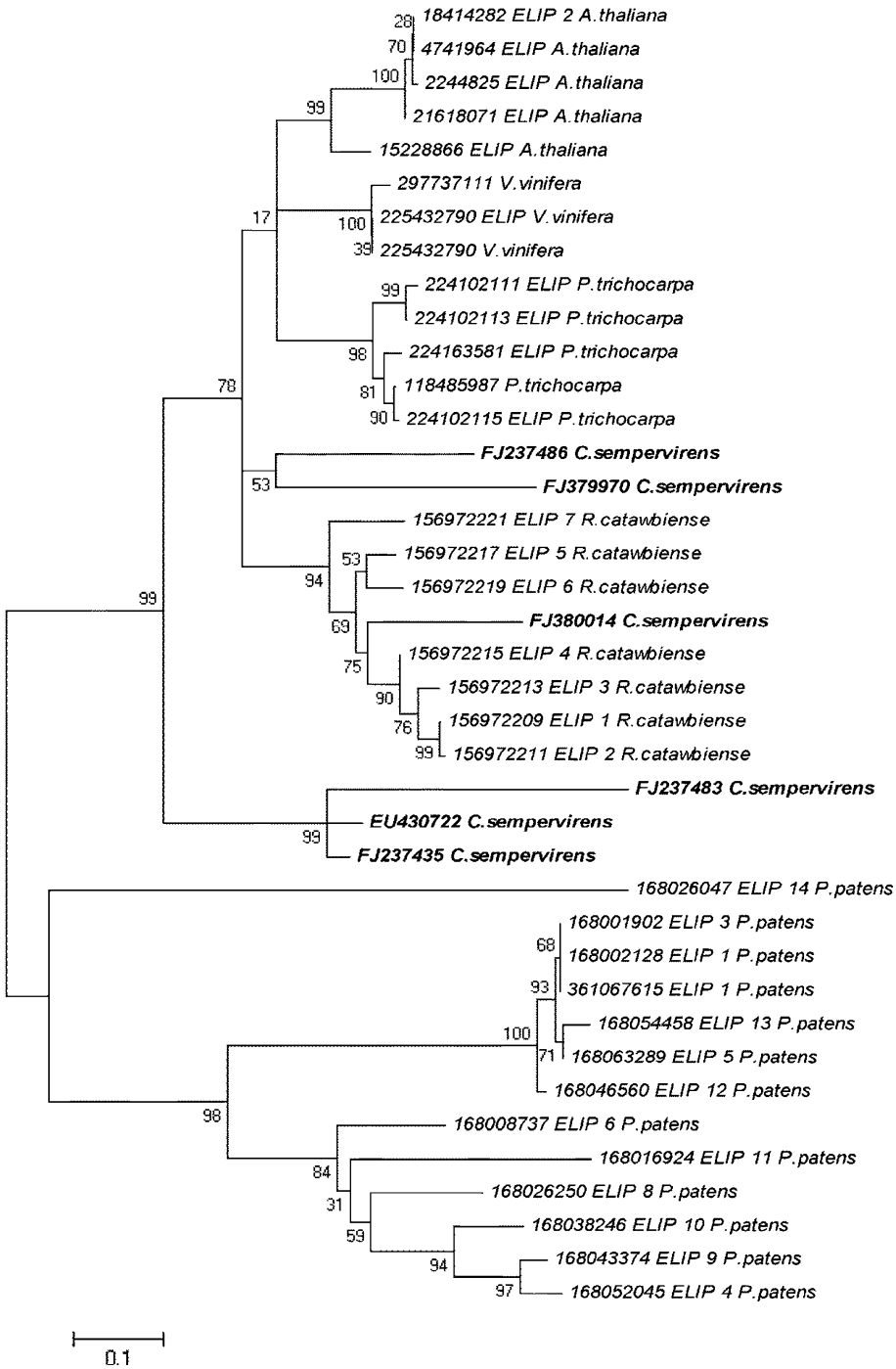

Fig. 3 Dendrogram based on the alignment of amino acid sequences from cypress putative early light-inducible proteins (ELIP) clones and sequences coded by *Populus thrichocarpa*, *Vitis vinifera*, *Physcomitrella patens, Arabidopsis thaliana*, and *Rhododendron catawbiense*. Bootstrap values based on 1,000 replications are indicated on the branches. In *bold* are the sequences characterized in this study

acclimation [28, 55]. Such genes resulted regulated in all the accessions tested, this suggesting a possible role for these sequences during cypress response to low temperature.

The involvement of chloroplasts and biological membranes during cypress response to low temperature is evident when the sequences annotated according to the GO main term "cellular component" are considered (Fig. 2b). Our results are in line with previous findings in several species, where damage to cellular membranes and to the photosystem was demonstrated during cold stress [56]. In particular, changes in plasma membrane proteins during acclimation have been identified as a crucial step for plant adaptation to low temperature [57]. Induction of several dehydrin families, disassembly of microtubules and increased ATPase activity represent some of the most relevant changes [58, 59]. During the induction of cold stress, plants have to cope also with photo-inhibition, due to excess of light absorption [60] and in cypress, even in absence of cold stress, excess of light

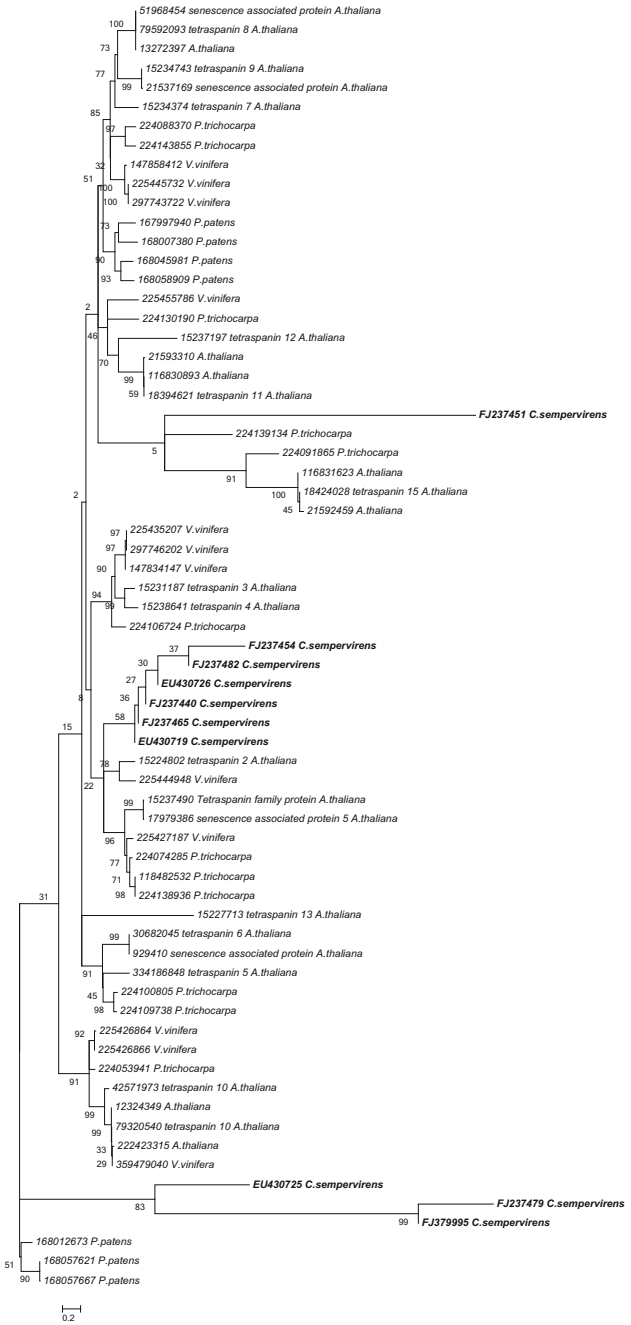

Fig. 4 Dendrogram based on the alignment of amino acid sequences from cypress putative senescens-associated genes (SAG) clones and sequences coded by *Populus thrichocarpa*, *Vitis vinifera*, *Physcomitrella patens, Arabidopsis thaliana*. Bootstrap values based on 1,000 replications are indicated on the branches. In *bold* are the sequences characterized in this study

absorption induces significant photo-inhibition by changes of photosystem II [61]. The response is a complex modulation of gene expression aimed at limiting photosystem damage [62].

Among the functionally identified classes representing the major transcripts identified in this study, dehydrins were found among the most abundant families (Table S1 in supplementary material). Dehydrins, a wide group of hydrophilic proteins that contain a large number of charged amino acids, belong to the group II of late embryogenesis abundant (LEA) protein family. This multi-family proteins present low sequence similarity characterized only by some small motifs that are conserved [63]. They display a broad repertoire of functional motifs and wide variations in the expression among the orthologs and in-paralogs [64]. Functionally, dehydrins play a protective role in the plant cell during drought, salt and low temperature stresses as well as during biotic stresses [6, 65]. Dehydrins show a random coil structure which can change the function, according to the environment where they act: this characteristic is called "moonlighting" [66]. To avoid bias in the phylogenetic analysis with inclusion of the extremely divergent functional sites, which can lower the reliability of the phylogenetic analysis, we prefer to exclude this family from the phylogenetic analysis. However, the dehydrin dendrogram is shown in the supplementary material (Fig. S1).

Numerous ELIP sequences were also identified among the cypress cold-induced genes. ELIPs are stress-induced thylakoid proteins localized in the stroma lamellae [67] and are thought to play a protective role in oxidative stress by binding free chlorophylls released during photoinhibition [68]. ELIPs from this study were divided into different groups when phylogenetic analysis was performed (Fig. 3). According to our results, only *FJ380014* was clearly clustered with ELIPs from other species, namely from *R. catawbiense*. From this species, seven highly homologous ELIPs (*RcELIP1-7*) were recently characterized [39]. All RcELIPs were up-regulated in leaves during winter, when photosynthesis was down-regulated [39, 69], thus suggesting that RcELIPs might protect winter evergreen leaves from sun [39]. Nonetheless, some recent studies showed that in *A. thaliana* suppression of two ELIPs (*ELIP*1 and *ELIP*2) does not change plant resistance to oxidative stress [70]. Taken together, these results may indicate the existence of several ELIP classes with functional in-paralogs as a result of genome duplication in plants, as also suggested by our analysis.

The second protein family chosen for phylogenetic analysis was SAG. During leaf senescence a number of physiological changes have been identified in chlorophyll content, photochemical efficiency, enzyme activities, protein levels, membrane ion leakage and so on [71]. Such changes are associated to a complex regulation of gene expression. Many of the SAGs can be induced by other environmental factors, including temperature stress, pathogen infection and plant hormones [72]. In a recent work on wheat, two members of this family, *TaSAG*3 and *TaSAG*5, responded differently to low temperature. After 7 days of exposure to 4 °C, only *TaSAG*3 was found up-regulated, while the *TaSAG*5 transcript level remained unchanged

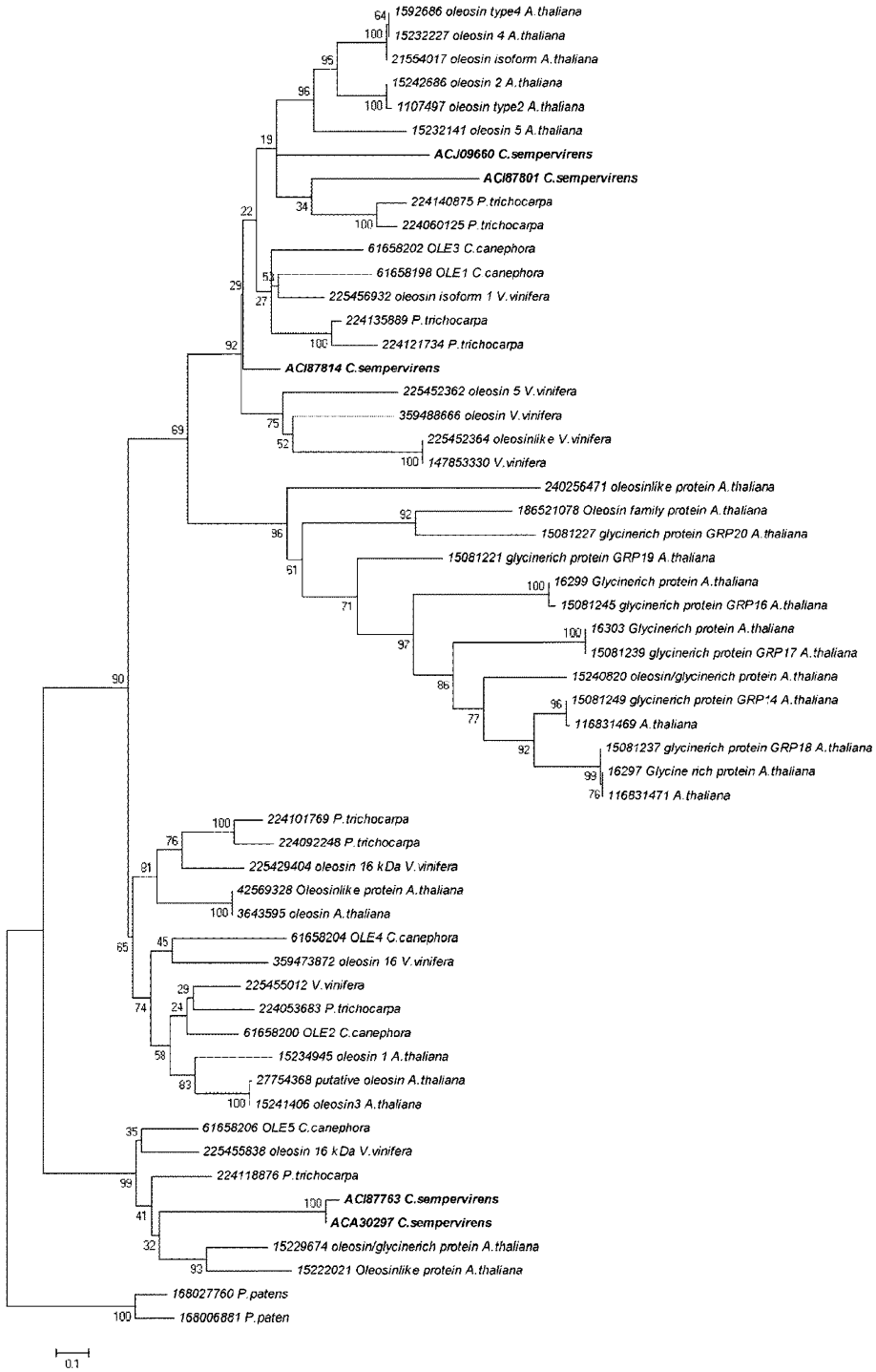

Fig. 5 Dendrogram based on the alignment of amino acid sequences from cypress putative Oleosin clones and sequences coded by *Populus thrichocarpa*, *Vitis vinifera*, *Physcomitrella patens, Arabidopsis thaliana*, and *Coffea canephora*. Bootstrap values based on 1,000 replications are indicated on the branches. In *bold* are the sequences characterized in this study

during all the treatment [73]. In a previous study, an expression analysis of some cypress SAG sequences (*EU430726, EU430725, FJ237440*) was published by our group [55], showing that three members of this family are overexpressed during cold acclimation. Such results can suggest that some members of SAG family may have a functional role also when cypress has to cope with low temperatures. Phylogenetic clustering of the cypress SAGs clearly distinguished them into two completely separated clades (Fig. 4), suggesting the existence of two functional classes for SAGs in cypress, which is in accordance with the previous reports from several plant species [72, 74].

The last protein family selected for phylogenetic analysis was the oleosin family (Fig. 5), whose members are not a classical cold-regulated genes but recently have been shown to be involved in seed freezing tolerance by

Shimada et al. [75]. Moreover oleosins have been found to be inducible by ABA [76] a plant hormone which is a known regulator of several cold-responsive genes [77, 78]. From the phylogenetic inferences presented, it is clear that at least two distinct oleosin groups exist in cypress, as in the case of most of the plant species studied (Fig. 5). Oleosin classification is usually performed according to their size as high- or low-molecular weight oleosins [79]. Recently, oleosins have been classified in different groups in *Arabidopsis* according to tissue-specific expression [80]. Therefore, it would be not surprising that different oleosin groups were regulated by different environmental factors, even if a specific study would be needed to prove it.

Conclusion

Italian cypress response to low temperature is a key factor to obtain new improved genotypes adapted to northern environments where this tree is being introduced. In this work, we described the isolation of 156 cypress sequences putatively regulated by low temperature. The subtractive approach used was not as extensive as, for example, RNAseq studies; nevertheless, it proved to be effective to isolate and identify novel sequences in a relatively poorly studied tree species as Italian cypress. By phylogenetic analysis, it was possible to highlight peculiarities and similarities between some cypress gene families putatively regulated by cold and the corresponding families of several other species. Even though a more detailed expression and functional study of the isolated genes is still needed to confirm which sequences are actually involved in low temperature response, the present work can be considered a successful attempt to provide a set of cold-regulated sequences in an economically important woody species such as Italian cypress.

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