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Research Note

Modulated gene expression during the cold acclimation process in tolerant and sensitive clones of cultivar Leccino (*Olea europaea* L.)

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Abstract

The plants are constantly exposed to environmental changes that need to be addressed with appropriate modifications of gene expression. The genetic approach to the study of the response to biotic and abiotic stresses is of great importance in plants, for the identification of the genes involved and their activation mechanisms. In our work, we have characterized the expression of some genes induced during the acclimation under low temperatures in plants of *Olea europaea* L., by isolating DNA sequences differentially expressed in a cold tolerant clone of Leccino cultivar by using the suppression subtractive hybridisation (SSH). The sequences obtained were analysed by sequencing. Some sequences of the libraries and from orthologous genes expressed in various abiotic/biotic stress treatments, were labelled and used as probes for slot blot hybridization with total RNAs extracted from cold sensitive and tolerant Leccino clones treated with decreasing temperatures down to -10 °C, thus mimicking a process of cold acclimation. Six genes were found to be of interest for the adaptation to cold stress, whose expression is differentially modulated in the two clones. The most important result was in a bimodal response during the acclimation phases, the causes of which have been discussed.

Key words: Olive, Leccino cultivar, Cold susceptibility, Suppression Subtractive Hybridization, cDNA differential clones.
Abbreviations: ABA_abscisic acid; BSP_bark storage protein; CA_carbonic anhydrase; CAB_chlorophyll a,b binding protein; CMS_cytoplasmic male sterility; hnRNP_heterogeneous nuclear ribonucleoprotein; JA_jasmonic acid; PAL_phenylalanine ammonia-lyase; PCMP-H_plant combinatorial and modular protein subfamily; PPR_pentatricopeptide repeat; ROS_reactive oxygen species; RRM_RNA recognition motif; SA_salicylic acid; snRNP_small nuclear ribonucleic particle; SSH_Suppression Subtractive Hybridization; TPR_tetratricopeptide repeat; WRKY_WRKY transcription factors; YCF2_chloroplast YCF2 protein.

Introduction

The study of abiotic stress in plants mainly consists in the identification of the genes involved in defence responses and of the mechanisms that regulate their activity. The temperature variations are one of the most important factors for the growth and productivity of the trees (Atkinson and Urvin, 2012). Many plants increase cold tolerance in response to low temperatures. Generally, the tolerance of plants to freezing is not constitutive, but induced by prolonged exposure to temperatures below 10 °C. This phenomenon is known as cold acclimation (hardening) (Thomashow, 1999; Lissarre et al., 2010; Miura and Furumoto, 2013). The metabolic and physical changes that occur after acclimatization include an increase of sugar, soluble protein, organic acids and altered lipid composition of plasma membranes and other modifications (Hughes and Dunn, 1996; Lissarre et al., 2010). Many of these biochemical and physiological changes are regulated by low temperatures through changes in gene expression: in recent years, several genes inducible by low temperatures have been cloned in monocots and dicots. Moreover, there is also an interaction between low temperatures and other environmental factors, in particular the photoperiod and water status (Thomashow, 1999; 2001). The interest in the current research is to determine the differences in cold tolerance in cultivated plant species and the molecular basis of cold acclimation, which is of primary scientific interest for genetic improvement to cold stress. The purpose of this work is to identify genes of potential importance for cold acclimation through analysis of differential gene expression induced by low temperatures in olive tree (*Olea europaea* L.). The olive tree does not tolerate prolonged thermal lowering; it is damaged by temperatures below -5 °C (Barranco et al., 2005). The choice of the biological material is clearly linked to the economic importance of this plant cultivated in the Mediterranean area. Since the best quality oil is produced in Italy from plants grown in areas with cold winters and subjected to frequent frosts, such as in Tuscany, it is necessary to detect a criterion to discriminate productive and hardy plants. For this purpose it is necessary to carry on methodologies for the selection of cultivars, through the use of markers isolated from tolerant plants and usable for massive screening of seedlings (Bartolozzi and Fontanazza,

1999). In this work we have used the technique of suppression subtractive hybridization (SSH) (Diatchenko et al., 1996; Ji et al., 2002; Casassola et al., 2013), which permitted the isolation of differentially expressed sequences in control and treated plants by using clones of Leccino cultivar that have been selected for cold sensitivity and tolerance. In addition, we carried out a study on the expression of some genes involved in cold sensitivity and tolerance, in order to understand how and to what extent these are regulated during the acclimation process.

Results

Library constitution

Two libraries were constructed using the differential SSH method of Diatchenko et al. (1996). cDNAs from control and cold treated plants permitted the separation and amplification of the products of genes activated by cold treatment (forward library) and of genes whose transcription is abolished or diminished (reverse library). The forward and reverse cDNAs were cloned, and about 500 independent positive clones were picked out from forward and reverse subtractive libraries. The results presented here refer to 350 randomly chosen clones from the two libraries. The sizes of the DNA fragments were comprised in a range of approximately 150-800 bp with a mean value of about 350 bp. We sequenced 40 differentially expressed cDNA clones. With the information collected from different databases, we identified and assigned a putative function (Table 1). The clones of the forward library are SAAC, SAAG3, FF301, FF304, FF401, and FF203. The clones of the reverse library are SABP, SABS, and SARF. Other clones were analysed, that contain shorter sequences coding for the same function and which are present in the sequences listed above: for this reason, they have not been annotated. The clones MDA12, MDE4, MDF1, and FF354 are Expressed Sequence Tags (ESTs), that represent portions of expressed genes for which there is no information on their function (no similarity match with sequences of DNA or protein in the databases).

Expression analysis of the cold gene transcripts

Quantitative slot-blot analysis for the selected clones confirmed that they are differentially modulated in *O. europaea*, after cold stress. Fig. 1 shows the results of five randomly selected clones: *chloroplast ycf2 protein* and *carbonic anhydrase* from forward library; *rubredoxin family protein, bark storage proteins* and *chlorophyll a,b binding proteins* from the reverse library. In order to assess the activation processes during treatments at various temperatures, analyses were carried out by means of slot blot analysis of the total RNA. The results obtained using, as probes, sequences coding for *chloroplast ycf2 protein, bark storage protein, carbonic anhydrase, chlorophyll a,b binding protein, PAL* and *WRKY* are reported in Fig. 2. In general, the expression values are very low at -10 °C with the exception of *ycf2* and *WRKY* in the tolerant strain that present a high increase at 0 °C and -5 °C. *PAL* shows an increase in the tolerant strain at temperatures above 0 °C, but with a decrease at 0 °C and -5 °C; at these temperatures, on the contrary there is an increment in the sensitive clone. The *carbonic anhydrase* expression strongly decreases in the two clones at low temperatures. The *chlorophyll a,b binding protein* expression presents an up regulation at 0 °C and -5 °C in the sensible clone. Interestingly, *chloroplast ycf2 protein* and *WRKY* show a very sharp increase from 0 °C to -10 °C in the tolerant strain and to a lesser degree in the sensitive strain: note a drastic drop of *WRKY* in the sensitive strain from 25 °C to 0 °C.

Discussion

Identification of the differentially expressed genes

The genes differentially expressed in response to the cold stress codify for proteins that are involved in several processes such as signal transduction, RNA processing, translation, protein processing, redox homeostasis, photosynthesis, photorespiration, and metabolisms.

Concerning the putative proteins of the sequences isolated from the forward library, the carbonic anhydrase (CA) is a Zn-dependent enzyme important for the assimilation of carbon (Tiwari et al., 2005), whose synthesis is inhibited by cold stress (Liu et al., 2013). The protein of the chloroplast gene *ycf2* is very important for various reasons: although not implicated in the processes of photosynthesis, it is essential for cell survival, as demonstrated by experiments of breaking or deletion of the corresponding gene (Drescher et al., 2000); moreover, it may be subjected to tissue specific regulation.

The serine/threonine protein kinases phosphorylate the -OH group of the serine and act as a "central processor unit", accepting input information from receptors that sense environmental conditions (Hardie, 1999). The phosphorylation plays an important role in regulating the expression of genes induced by cold and in the development of cold tolerance (Monroy et al., 1995). The ubiquitin activating enzyme E1 2 plays a very important role in the control of several processes, including those for signal transduction and regulation in transcription (Hershko and Ciechanover, 1998; Herrmann et al., 2007; Hurley and Stenmark, 2011). Recent reviews (Dreher and Callis, 2007; Lyzenga and Stone, 2011) evidenced a role of the ubiquitin/proteasome system (UPS) linked to hormonal cross-talk in defence against biotic threats. The heterogeneous nuclear ribonucleoprotein F-like contains a region for the RNA-binding proteins [RNA recognition motif (RRM) domain] superfamily. The RNA RRM motif found in RNA-binding proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and small nuclear ribonucleoproteins (snRNPs), are involved in different stages of the synthesis and maturation of mRNA such as the regulation of transcription, the splicing of pre-mRNAs, the mRNA transport and translation (Lambermon et al., 2002).

The putative filamin-like protein represents an intriguing gene, since the codified product till now was recognized as not present in plants. The sequence was found in the Picoplanktonic green alga *Micromonas pusilla* (Worden et al., 2009). In animals, filamin proteins cross-link cortical actins and interact with a large number of cellular proteins of great functional diversity (Feng and Walsh, 2004). At a lesser extent, similarity exists with the histone H3K4-specific methyltransferase SET7/9 N-terminal domain-containing protein of the green alga *Coccomyxa subellipsoidea* C-169 that contains filamin like domains (Blanc et al., 2012).

The pentatricopeptide repeat-containing protein At1g31920 belongs to the pentatricopeptide repeat (PPR) family. Aubourg et al. (2000) discovered in the *Arabidopsis thaliana* genome a new large family of the so-called *orphan genes* named *AtPCMP*, that are almost entirely plant specific. PPR proteins play constitutive, often essential roles in mitochondria and chloroplasts, probably by binding to organelle transcripts (Small and Peters, 2000; Lurin et al., 2004), thus suggesting that they are the nucleus encoded

Table 1. Identification of SSH cDNA sequences of differentially expressed genes from mature leaves of the tolerant olive clone.

Clone	Accession No.[(a)]	Accession No. of matching sequence[(b)]	Putative identification[(c)]	Biological process[(d)]
SAAC	FN814304	1707317A	Carbonic anhydrase	Response to abiotic or biotic stimulus
SABP	FN814305	AAD27878	Chlorophyll a/b binding protein	Cellular and metabolic processes
SAG3	FN814306	NP_054542	Chloroplast YCF2 protein	Probable ATPase of unknown function
SABS	HG530075	XP_002280745	Bark storage protein	Metabolic processes
SARF	HG798534	NP_001078598	Rubredoxin family protein	Response to abiotic or biotic stimulus
FF301	HG798535	ABC54583.1	Putative serine/threonine protein kinase	Cellular processes
FF304	HG798536	ABA95612.2	Ubiquitin-activating enzyme E1 2	Cellular and metabolic processes
FF203	HG798537	XP_003056733	Filamin-like protein	Cell organization and biogenesis
FF401	HG798538	XP_002285426	Putative heterogeneous nuclear Ribonucleoprotein F-like	Post-transcriptional gene expression Processes
MDA1	HG798539	XP_002275784	Pentatricopeptide repeat-containing protein At1g31920	Unknown
MDA12	HG798530		EST	
MDE4	HG798531		EST	
MDF1	HG798532		EST	
FF354	HG798533		EST	

The accession numbers (No.) of the SSH sequences[(a)] and of the matching sequences[(b)] are deposited in the data bank (Gene Bank / EMBL / DDBJ). Identification based on sequence similarity[(c)]. Biological processes according to the Arabidopsis Information Resource (TAIR) and MIPS database[(d)].

factor that controls the chloroplast gene expression. Their role in organellar RNA processing, fertility restoration in plant cytoplasmic male sterility (CMS), embryogenesis, and plant development is widely demonstrated (Fuji and Small, 2011). As for the putative proteins of the sequences isolated from the reverse library, the chlorophyll a,b binding proteins (Cab CP29) have an important role in the cold stress; in fact, plants with phosphorylated Cab CP29 are more tolerant to cold-induced photoinhibition (Bergantino et al., 1995).
The bark storage protein (BSP) plays a role in promoting the accumulation of nutrilites (mainly nitrogen) in autumn and their use for the development of new shoots in spring as demonstrated by Clausen and Apel (1991) in poplar tree.
The rubredoxin family proteins are iron-sulfur proteins that are involved in electron transfer in several biochemical pathways.

Expression analysis of transcripts during cold acclimation treatments

In the experiments of slot blots, we used homologous and orthologous genes. We are interested in comparing several genes involved in stresses that we have studied in other biological systems, to verify if there is a common basic response to biotic and abiotic stresses in plants. For this reason, we have analysed genes isolated in our department from different plant species, after treatment with abiotic stress such as cold in poplar (Maestrini et al., 2009), ozone in poplar (Rizzo et al., 2007), heavy metals in *Nicotiana* (Taddei et al., 2007) and with biotic stress by treatment with the fungus *Ceratocystis* in *Platanus acerifolia* (Fontana et al., 2008; Baccelli et al., 2013). Among the various genes tested, two, PAL and WRKY, evidenced very interesting results, that seem to confirm the hypothesis of a cross talk response to abiotic and abiotic stresses as previously described by other authors (Cheong et al., 2002; Fujita et al., 2006). *WRKY* belongs to a large transcription factor family that is expressed in many different biological processes involving signaling, transcription, chromatin remodeling, and others (Chen et al., 2012; Chi et al., 2013). Maré et al.

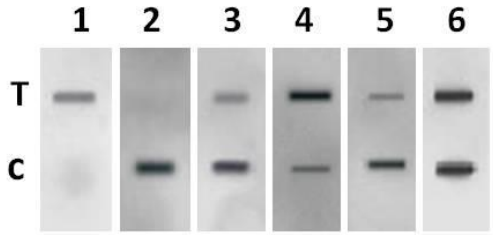

Fig 1. Slot blot analysis of total RNAs extracted from resistant plants, control (C) and cold treated (T), after hybridization with labelled probes: (1) chloroplast ycf2 protein; (2) rubredoxin family protein; (3) bark storage protein; (4) carbonic anhydrase; (5) chlorophyll a,b binding protein; (6) 5.8S ribosomal gene. The housekeeping gene 5.8S shows the same intensity in control and treated samples.

(2004) have isolated, by means of the technique of SSH, a sequence, activated both in the cold and drought stresses, with a clear homology with *WRKY*. In our material, the hybridization shows that in the resistant cultivar the sequence is expressed at room temperature but with low intensity, and subsequently increases at low temperatures (0, -5, -10 °C). In the sensitive cultivar, activation is very intense at high temperatures (25, +5, +10 °C) while, at low temperatures, the expression seems to decrease, until it disappears at the temperature of -10 °C. The trend of the expression seems to indicate that the sequence is involved in the resistance to cold stress. As far as the *PAL* family is concerned, *PAL* is involved in the first steps of the phenylpropanoid pathway. PAL plays an important role in disease resistance and levels of PAL activity vary with developmental stage and exposure to different stress stimuli (Bagal et al., 2012). Christie et al. (1994), observed the effects of low-temperature stress on general phenylpropanoid pathways in *Zea mays* seedlings. Time-course studies showed that *PAL* levels remained relatively constant for the first 12 hours of cold stress and dramatically increased over the next 12 hours. Our results in *O. europaea* showed a response, during the time-course

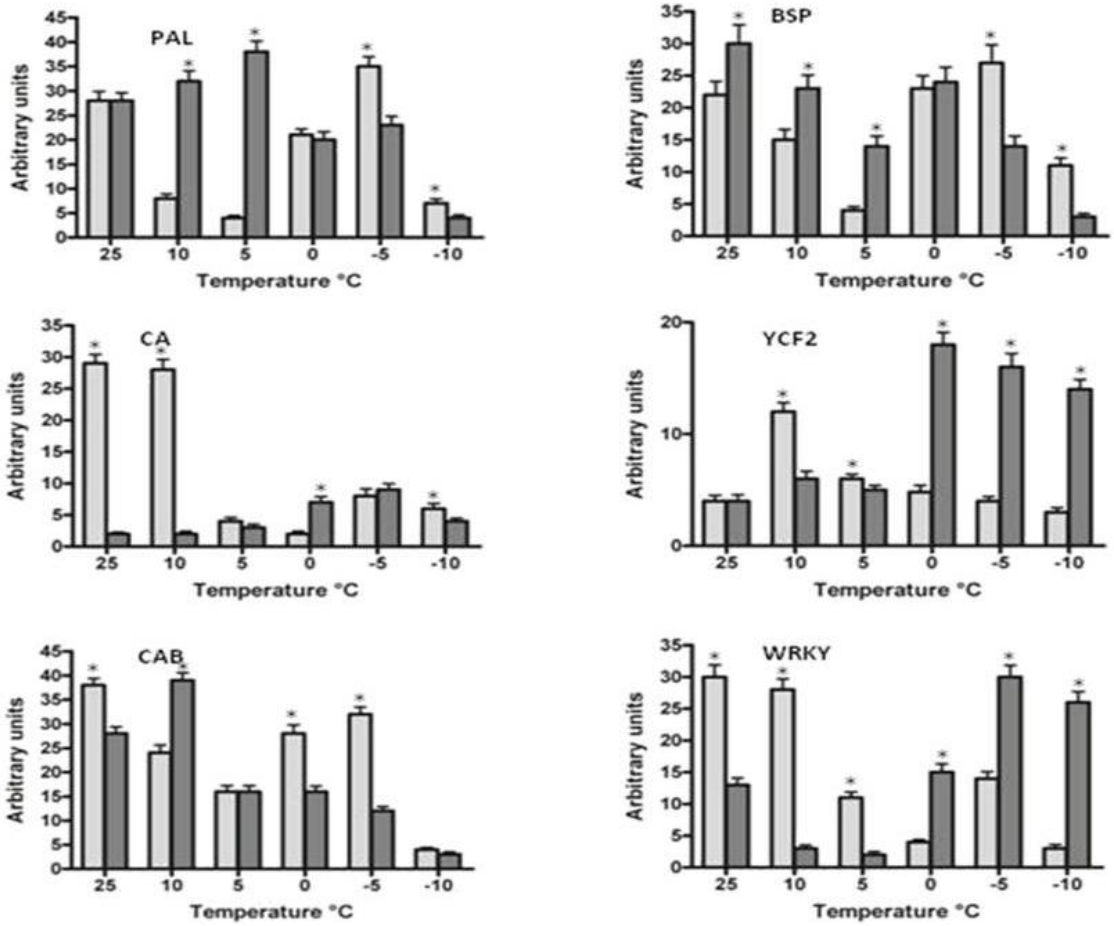

Fig 2. Slot blot analysis of total RNA extracted from plant leaves of sensitive (grey bars) and cold resistant (black bars) strains. Plants have been subjected to different temperatures (25 °C, 10 °C, 5 °C, 0 °C, -5 °C, and -10 °C, for the times indicated under methods). Hybridization was carried out with the six labelled probes: *phenylalanine ammonia-lyase* (PAL), *bark storage protein* (BSP), *carbonic anhydrase* (CA), *chloroplast ycf2 protein* (YCF2), *chlorophyll a,b binding protein* (CAB) and *WRKY transcription factor* (WRKY). The expression levels of transcripts were estimated by densitometric analysis using the UVPS GelBase TM Windows Software. Mean values (columns) ± SEM (bars) were calculated in three independent experiments with three biological replicates of leaf materials. Asterisks indicate significant difference at $P \leq 0.05$ level.

treatment, similar to that found during the analysis of the *WRKY* gene transcripts as described above. A similar result was found in the ozone-stressed resistant clone of poplar (Rizzo et al., 2007). An interesting feature of our acclimation experiments lies in the fact that the response of the expression of different genes at different acclimation temperatures occurs as biphasic: the intensity of expression generally tends to decrease at around 5 °C, then rise up to maximum values at -5 °C. Several examples of biphasic responses are present in different stress treatments. For instance, in biotic stresses, Wi et al. (2012) observed the biphasic production of ethylene and reactive oxygen species (ROS) in susceptible tobacco plants after shoot-inoculation with *Phytophthora parasitica*; van de Mortel et al., (2007), observed distinct biphasic mRNA changes in response to Asian soybean rust infection. Some examples of abiotic stresses are represented by metal ions interactions: Cuypers et al. (2000) showed a biphasic effect of copper on the leaves of *Phaseolus vulgaris* seedlings during the early stages of metal assimilation, and Sanità di Toppi et al. (2012) reported a biphasic response to Cadmium stress in carrot root. A working hypothesis could lie in the production of phytohormones such as abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA), which may control the expression of various genes. These newly synthesized hormones may cause a return or amplification of the initial signals to generate a second round of signalling pathways (Mahajan et al., 2005; Shao et al., 2007; Jeong et al., 2012).

Materials and Methods

Plant materials

Leccino is one of the most important cultivated varieties of *Olea europaea* L.. It is an ancient autochthonous cultivar of Tuscany, from which were selected cold resistant and sensitive accessions. A resistant accession (accession 18) and one sensitive (accession 4) to low temperatures of *Olea europaea* L. cultivar Leccino were used. This material was selected from plants, which had survived in Tuscany after exposure to the very low temperatures (-15 °C) in 1985 (La Porta et al., 1994; Bartolozzi and Fontanazza, 1999). The criterion for the choice of tolerant cultivars has been the apical re-vegetative capability, i.e., the only cultivars

considered suitable were those, which, after cold stress, sprouted in the apical part of the plant. Plants that have suffered severe damage from the cold and have produced only basal suckers with wide necrosis in the apical part were considered sensitive.

Stress treatments

Self-rooted plants (clones) derived from the two Leccino accessions (sensitive accession 4 and tolerant accession 18) were grown in a controlled greenhouse (minimum temperature of +13 °C to avoid hardening) for two years. The plants of the two clones (named clone 4 and clone 18) were prepared using 10-12 cm long cuttings with 4-6 leaves. With the plants thus obtained, two samples were prepared, one of which was maintained in a range with an average temperature of 25 °C (control), while the other was subjected to steps of decreasing temperatures in a climatic chamber, simulating a gradual decrease in temperatures as can occur during the transition from late autumn to intense winter cold. Stressed samples were sequentially treated as follows: 10 °C for 14 days, 5 °C for 7 days, 0 °C for 1 day, -5 °C for 3 hours and -10 °C for 23 hours. The leaves from control and treated samples were collected and frozen in liquid nitrogen. Test temperatures and times of treatments were selected according to previous work based on experimental trials in order to optimize the conditions (Bartolini et al., 1994).

RNA extraction from the leaves

Total RNA was isolated from control (25 °C) and cold stressed (-10 °C) mature leaves of the tolerant and sensitive cultivars according to the method of Logemann et al., (1987). In order to purify RNA from any contamination with DNA, RNA solutions were submitted to CsCl ultracentrifugation according to Fontana et al., (2008).

Construction of suppression subtractive cDNA libraries

Two libraries were constructed using the Suppression Subtractive Hybridization (SSH) according to Diatchenko et al., (1996). The main advantage of the SSH method consists in the enrichment of rare transcripts of the differentially expressed genes. The SSH technique is generally used to isolate genes induced by biotic and abiotic stresses (Ji et al., 2002; Casassola et al., 2013). Poly (A)+ RNAs were isolated from the pools of total RNAs extracted from control (25 °C) and cold stressed (-10 °C) mature leaves, using the PolyATract mRNA Isolation Systems (Promega, USA) according to the protocol described by the manufacturer. (SSH) was performed according to Diatchenko et al. (1996) using the PCR-Select cDNA Subtraction Kit (Takara Bio Europe/Clontech, France). The cDNAs from treated samples were respectively used as tester and driver for the forward and reverse subtraction according to the PCR-Select cDNA Subtraction Kit. Amplified cDNA sequences from forward and reverse subtractions were directly inserted into a T/A cloning vector using the TOPO TA Cloning Kit (Life Technologies, Italia) according to the manufacturer's instructions. The efficiency of subtraction was evaluated by PCR amplification using the 5.8S ribosomal gene (the subtraction was efficient if the 5.8S transcript was reduced); the primers were designed on the 5.8S rRNA gene and the internal transcribed spacers 1 and 2 of *Nicotiana tabacum* was registered to GenBank with the accession no. AJ012363.

Clones were primarily screened with the cDNA array approach using the Clontech PCR-Select Differential Screening (Takara Bio Europe/Clontech, France), according to the manufacturer's recommendations. Only clones revealing, by visual inspection, a subtracted/unsubtracted hybridization ratio of about 4 times were considered as representing differentially expressed transcripts.

Isolation of WRKY and PAL orthologous transcripts

In order to isolate a coding sequence for *WRKY transcription factor* (*WRKY*), degenerated primers were constructed on the homologous zones of a group of mRNA sequences selected by BLAST and subsequent CLUSTALW multi-alignment, to obtain the orthologous sequences in *O. europaea.* The degenerated primers for *WRKY* were: 14WLARPOPF: 5'-GCTCWYWTGCYCCAAGYTGCC-3', 14WRARPOPR: 5'-CCARTGCWGCTKTAAARYTYGGATC-3'.

RT-PCR products were sequenced, checked to ensure that they were a single sequencing product, and gene-specific primers were designed on the obtained *O. europaea* sequences.

For the isolation of the codifying sequence of *phenylalanine ammonia-lyase* (*PAL*) the primers were: PLAPALF: 5'-ACAACATCACTCCATGCTTGCCACT-3', PLAPALR: 5'-CAATTTGACCTGGATGGTGCTTCAA-3' of the *PAL* of *Platanus x acerifolia* registered to GenBank with the accession no.AJ012363.

RT-PCR products were sequenced, checked to ensure that they were a single sequencing product, and deposited in GenBank with the accession numbers HG798540 e HG798541 for *PAL* and *WRKY* respectively.

Sequence analysis

The plasmids containing the inserts of the clones obtained by differential screening of the cDNA library were sequenced in both directions with an automated sequencer. The obtained nucleotide sequences and the deduced amino acid sequences have been used for comparison tests with sequences deposited in the data bank (Gene Bank / EMBL / DDBJ) with the programs FASTA and BLAST or BLAST-N-X available in the network. A functional classification of the genes corresponding to the differentially regulated transcripts was made according to *The Arabidopsis Information Resource* (TAIR, http://arabidopsis.org) (Swarbreck et al., 2008) and MIPS database (http://www.helmholtz-muenchen.de/en/mips/projects/funcat) (Ruepp et al., 2004). The sequences obtained from the forward/reverse library were deposited in the data bank (Gene Bank / EMBL / DDBJ).

Slot blot and hybridization

For quantitative slot blot analyses, scalar dilutions from 5 µg to 0.625 µg of equivalent amounts of total RNA extracted from sensitive and cold-tolerant plants subjected to different temperatures (ambient (average 25 °C), +10 °C, +5 °C, 0 °C, -5 °C and -10 °C), were denatured by formaldehyde and bound to positively charged nylon membranes, Hybond-N+ (GE Healthcare Bio-Sciences AB,Uppsala Sweden), using a Minifold II blotting apparatus according to the manufacturer's protocol (Schleicher & Schuell SRC 072/0, Schleicher & Schuell, Inc., Keene, NH). The membranes were cross-linked by UV. All the experiments were repeated at least by three biological replica and triplicate samples were loaded for each dilution. The membranes were pre-hybridized

for 3 h. in the pre-hybridization buffer DIG easy Hyb (Roche, Germany), and then incubated overnight at 42 °C in the hybridization buffer DIG easy Hyb containing the labelled cDNA *inserts* or the PCR products *PAL* and *WRKY*, obtained by DIG-DNA Labelling Kit Nonradioactive (Roche, Germany). The filters were washed twice in 2x saline-sodium citrate (SSC) buffer, 0.1% SDS at room temperature for 5 min, and then washed twice in 0.1xSSC, 0.3% (w/v) SDS for 10 min, at 65 °C. The visualization of the hybridized probe was made by using the DIG Luminescent Detection Kit (Roche, Germany), after that the filters were exposed to the Hyperfilm β-max (GE Healthcare Bio-Sciences AB, Uppsala Sweden) with two intensifying screens at −70 °C for appropriate durations. Equal loading of RNA onto the same membrane after stripping was controlled using a randomly labelled nonradioactive 5.8S ribosomal gene, as described above. In order to quantify the mRNA expression levels, densitometric analysis was performed using the UVPS GelBase TM Windows Software (Ultraviolet Products Ltd., UK). The data obtained were used to construct the histograms. The Student's t-test was used to compare the two groups of data (sensible and tolerant for each temperature treatment). The analysis was performed using GraphPad InStat version 3.0 (GraphPad Software Inc., San Diego, CA, USA).

Conclusion

Leccino is a cultivar of known geographical origin (Tuscany, Italy) and is of ancient cultivation, so its genetic origin is unknown. Presumably, the cultivar was constituted, either by accessions derived from multiple founders, or by single seedling with somatic mutations. This justifies the differences in morphological and biochemical / molecular responses to biotic and abiotic stresses that have been observed. Our results are consistent with those observed by other authors, using different laboratory methods for discriminating between frost-tolerant and frost-sensitive genotypes (for more detailed information, see Barranco et al., 2005).
The major achievement of the present work is in the bimodal response of the gene expression during hardening, the causes of which we are currently investigating.

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