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Role of geranylgeranyl reductase gene in organ development and stress response in olive (*Olea europaea*) plants

Leonardo Bruno[A,E], *Adriana Chiappetta*[A,E], *Innocenzo Muzzalupo*[B,C], *Cinzia Gagliardi*[A], *Domenico Iaria*[A], *Alessandro Bruno*[A], *Maria Greco*[A], *Donato Giannino*[D], *Enzo Perri*[B] and *Maria Beatrice Bitonti*[A,F]

[A]Department of Ecology, University of Calabria, Ponte Bucci, 87036 Arcavacata di Rende, Cosenza, Italy.
[B]CRA – Centro di Ricerca per l'Olivicoltura e l'Industria Olearia (CRA-OLI), C.da Li Rocchi, 87036 Rende (CS), Italy.
[C]CRA – Centro di Ricerca per le produzioni Foraggere e Lattiero-Casearie (CRA-FLC), Via A. Lombardo, 11, 26900, Lodi, Italy.
[D]Institute of Biology and Agricultural Biotechnology, National Research Council of Italy (CNR), via Salaria km 29300, 00015 Monterotondo Scalo, Rome, Italy.
[E]These authors contributed equally to this work.
[F]Corresponding author. Email: b.bitonti@unical.it

Abstract. The NADPH-dependent geranylgeranyl reductase gene (*OeCHLP*) was characterised in olive (*Olea europaea* L.). OeCHLP catalyses the formation of carbon double bonds in the phytolic side chain of chlorophyll, tocopherols and plastoquinones and, therefore, is involved in metabolic pathways related to plant productivity and stress response, besides to nutritional value of its products. The nuclear *OeCHLP* encodes a deduced product of 51 kDa, which harbours a transit peptide for cytoplasm-to-chloroplast transport and a nicotinamide binding domain. Two estimated identical copies of gene are harboured per haploid genome of the cv. 'Carolea' used in the present study. Levels and cytological pattern of *OeCHLP* transcription were investigated by quantitative RT–PCR and *in situ* hybridisation. In line with the presence of ubiquitous tocopherols and/or chlorophyll, *OeCHLP* transcripts were present in various organs of plants. In leaves and fruits at different developmental stages, *OeCHLP* was differentially expressed in relation to their morpho-physiological features. An early and transient enhancement of gene transcription was detected in leaves of different age exposed to cold treatment (4°C), as well as in fruits mechanically wounded. Moreover, *OeCHLP* transcripts locally increased in specific cell domains of fruits severely damaged by the pathogen *Bactrocera olea*. Combined, these data show that *OeCHLP* expression early responds to biotic and abiotic stressful factors. Levels of tocopherols also increased in leaves exposed to cold conditions and fruits severely damaged by pathogen. We suggest that gene activity under stress condition could be related to tocopherol action.

Additional keywords: developmental regulation, drupe, *geranylgeranyl reductase*, leaf, stress factor.

Introduction

Olive (*Olea europaea* L.) is a crop species that is spread throughout the Mediterranean basin. It represents a unique species within the *Olea* genus that is able to produce edible fruits with a high content of healthy antioxidants such as tocopherols (TP), phylloquinones (PQ), vitamin K and phenols (Green 2002). The quality of both fruits and oil impacts on the Mediterranean diet, and is of agro-economic relevance.

The olive plant is an evergreen tree which does not undergo wintertime dormancy. The formation of leaves occurs continuously, and these persist on the plant for 1–2 years (Barone and Di Marco 2003). Fruits (drupes) reach maturation at winter time, and the level of antioxidant compounds accumulating in the drupe is related to both plant genotype, soil and climatic condititions, and especially temperature and water availability (Larcher 1970; D'Angeli and Altamura 2007). Notably, the quality of plant products is enhanced in plants growing at the high limit of cultivation area, notwithstanding the low cold tolerance of olive tree (Larcher 1970). Moreover, in most Mediterranean countries drupe is severely impaired by the fly *Bactrocera olea*, which lay one egg in the epidermis of young fruit. Damage may result from ovipositor injury, but mostly (30% loss) from the tunnelling of carpophagus larvae (see Fig. S1 in the accessory publication available from the online version of *Functional Plant Biology*) (Burrack and Zalom 2008).

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Olive plants also exhibit a long juvenile phase before flowering (20–25 years), as well as an inherent self-incompatibility. Hence, breeding programs are long-term even though have recently benefited by the availability of genomic polymorphic markers (Baldoni *et al.* 2006; Consolandi *et al.* 2007; Muzzalupo *et al.* 2008). Currently, olive germoplasm is under an intensive survey, but its characterisation is still far from complete and only a few genetic pathways that control physiological and crucial agronomic traits have been investigated (Hernández *et al.* 2005; Baldoni *et al.* 2006; D'Angeli and Altamura 2007; Giannoulia *et al.* 2007). Hence, a wide characterisation of genes related to both plant productivity and stress response could provide knowledge and tools for MAS and biotechnological approaches.

One of our aims has been to characterise in olive plants a NADPH-dependent geranylgeranyl reductase gene (CHLP), which is important in catalysing the formation of carbon double bonds in the phytolic side chain of chl, TPs and PQs, thus, providing a potentially limiting substrate for the biosynthesis of these compounds (Bollivar *et al.* 1994; Keller *et al.* 1998; Tanaka *et al.* 1999). In line with this role, in transgenic tobacco plants expressing antisense *CHLP* there was a significant decrease in TPs and chl, mostly present as chl_{GG} forms, together with a decrease in resistance to photo-oxidative stress (Tanaka *et al.* 1999; Graβes *et al.* 2001; Havaux *et al.* 2003). In plants, a major role of TPs is to prevent lipid peroxidation by quenching and scavenging reactive oxygen species (ROS) and lipid peroxyl radicals (Fukuzawa and Gebicki 1983; Munnè-Bosch and Alegre 2002). Thus, in photosynthetic tissues, TPs create an optimal environment for the photosynthetic machinery by protecting chloroplast membranes from photo-oxidative damage (Fryer 1993; Munnè-Bosch and Alegre 2002). In seeds and fruits, TPs confer not only nutritional value (Valk and Hornstra 2000), but also contribute to product stability and post-harvesting shelf life (Goffman and Bohme 2001) by protecting storage oil from oxidative damage (Munnè-Bosch and Alegre 2002; Sattler *et al.* 2004).

Here, we report important structural features of the *OeCHLP* (*Olea europaea CHLP*) gene and its pattern of expression and mRNA localisation in different organs of plant. In a previous work, we demonstrated that in peach deciduous leaves *CHLP* gene was differentially expressed in relation to developmental stage and stressful conditions (Giannino *et al.* 2004). Therefore, work was also addressed to verify a general role of this gene in organ development and stress response by analysing *OeCHLP* expression and its putative relationship with chl and TP action: (i) in olive pluriannual leaves as well in fruits at different developmental stages; (ii) in leaves responding to cold treatment; and (iii) in fruits responding to mechanical wounds and to damages caused by the pest *Bactrocera oleae*. We found that *OeCHLP* expression was modulated during leaf and fruit development and rapidly enhanced under stress conditions.

Materials and methods

Plant material

Plants used were 10-year-old clonally propagated olive (*Olea europaea* L.), cultivar 'Carolea'. Cultivar 'Carolea' was selected because of its diffuse cultivation in Calabria Region (Italy) for both oil and olive production. The plants belonged to the olive genome collection of the CRA (Centro di Ricerca per l'Olivicoltura e l'Industria Olearia) in Rende (Calabria, Italy) and grown under field conditions. Leaves and vegetative apical tips (shoot apical meristem and surrounding leaflets) were sampled from plants ($n = 10$) during spring; the leaves were grouped in three developmental classes on the basis of both length and colour: 2–4 cm long young green leaves (YG), up 6 cm long adult green leaves (AG), borne on the branches produced in the current year, up 6 cm long adult green-dark leaves (AD), borne on the branches formed during previous year. For all the samples branches exhibiting similar length and the same number of internodes were selected. Floral buds were sampled in late spring–early summer. Fruit samples were collected at 90 days (G*F* = green fruit) and 150 days (D*F* = dark fruits) after anthesis (full blooming).

Samples were picked from all around the external parts of the canopy of trees. Green fruits manifesting recognisable signs of damage caused by infection with *Bactrocera oleae* were also collected.

Stress assay conditions

Three-year-old 'Carolea' plants, belonging to the above described clonal line, were moved from outdoors and acclimated for 1 week in a growth chamber at 22°C, at 100 μmol m^{-2} s^{-1} PAR, under 16/18 h of light/dark regime. Subsequently, temperature was rapidly lowered to 4°C and leaves were collected from primary and secondary shoots after 10 and 30 min, 1, 2 and 4 h of cold exposition. Leaves of acclimated plants not exposed to cold treatment were used as control samples.

In fruit wounding assays, the above described 'Carolea' plants growing in open field were used. Healthy GF drupes were carefully selected and punctured with a needle in the morning of a sunny day on the plan (December). Injured drupes ($n = 10$ for each thesis) were picked from the plant at 10 or 30 min, 1, 2 or, 4 h after wounding. Uninjured healthy drupes ($n = 25$) were also collected at comparable times and used as controls.

RNA isolation and reverse transcription

Total RNA was isolated from tissues of leaves, inflorescences and fruits at different developmental stages, processed separately. Tissues frozen with liquid nitrogen (100 mg) were processed with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In the elution step, RNA was resuspended in a volume of 50 μL of RNase-free water and incubated at 37°C for 30 min with DNase I in a final volume of 100 μL. DNAse I was inactivated at 70°C for 15 min. RNA was precipitated and finally resuspended in 40 μL of RNase-free water. Quality and quantity of total isolated RNA were controlled with a NanoDrop Spectrophotomer ND-1000. The total RNA (3–5 μg) from each sample was used, with the SuperScript III Reverse Trascriptase with the oligo dT(22), according to the manufacturer's instructions (Invitrogen, Milan, Italy).

Isolation and cloning of CHLP gene from Olea europaea *cv 'Carolea' (OeCHLP)*

The cDNA from fruit RNA was PCR amplified by the primers: FW0 5′-CAAATCCACAGGTTCACCCTA-3′ and BW0 5′-TGGAGGCCAATATGTTTTGTC-3′, which were selected within the region of highest conservation between tobacco and *Arabidopsis*. The PCR reaction yielded a ~900 bp products, which was homologue to *CHLP* of the above mentioned plants. The 3′ end of the *OeCHLP* was amplified by the rapid amplification of cDNA ends (RACE) methodology, using single stranded cDNAs synthesised by the oligo-(dT)-AP primer and following the manufacturer's instructions (Invitrogen). PCR was performed with the FW0 5′-CAAATCCACAGGTTCAC CCTA-3′, and kit anchor primer. The 5′ *OeCHLP* end was amplified by using the degenerate primer FWdeg 5′-CCGGAT GGCTTCCATTGCTC(C/T)CAA-3′, based on the conserved aminoacids of *Arabidopsis* and tobacco, and the BW1 primer 5′-TGGCGTTGTAATCCGTGTAA-3′. The full-length *OeCHLP* transcript was re-checked by amplifying the cDNA of leaves and fruits using the primers FWStart 5′-ATGGCTTC CATTGCTCTCAA-3′ and BWend 5′-TAGCTTCTCCATCT CCCTCCT-3′.

The 1389 bp products were cloned and several of them were sequenced, and nucleotide differences were not encountered, indicating that the same gene was expressed in these tissues. To screen for introns, genomic DNA (gDNA) was used as PCR template using the following primer combinations: FWstart/ BW1, FW0/BW0 and FW2 5′-GACAAAACATATTGGCCT CCA-3′/BWend.

PCR components were: gDNA (200 ng) and/or cDNA (100 ng), 1.5 μM each primer, 0.5 mM dNTs, Taq DNA polymerase (Go Taq, Promega, Milan, Italy) 2.5 U, 1/10 of 10× Taq Buffer, 2.5 mM $MgCl_2$, in a final volume of 50 μL.

PCR conditions: starting cycle at 95°C for 4 min; 35 cycle at 95°C for 60 s, 55°C for 50s and 72°C for 60 s, final extension at 72°C for 7 min. All PCR fragments were cloned into pGEM-T easy vector system according to the manufacturer's instructions (Promega) and sequenced by the Genelab ENEA service (Rome, Italy).

Alignments and phylogenetic analysis

The alignment of OeCHLP with other CHLP proteins was conducted by ClustalW (http://www.ebi.ac.uk.clustalw, accessed 9 July 2006) and optimised by visual inspection (PILEUP program). Phylogenetic trees were constructed by MegaBlast3 (based on the minimum evolution criterion), using bootstrap values performed on 1000 replicates and the 50% value was accepted as an indicative of a well supported branch. The sequences of *Rhodopseudomonas palustris* (Molisch) Kluyver and van Niel 1937 was used to create an out-group. The CHLP accession numbers were: *Arabidopsis thaliana* L. (AY059860.1); *Brassica rapa* L. (DQ886527.1); *Nicotiana tabacum* L. (AJ007789.1); *Prunus persica* L. Basch (AY230212); *Lotus corniculatus* L. (DQ013361.1); *Medicago truncatula* Gaertn. (AY960125.1); *Glycine max* Merr. L. (AF068686.3); *Triticum aestivum* L. (AAZ67145); *Oryza sativa* L. (AP001080.1). The *R. palustris* accession numbers of the out–group was (NC_007958.1).

In situ *hybridisation*

For *in situ* expression analysis, the specific probe 1 for *OeCHLP* spanned the 1134–1411stretch (Fig. 1*A*) and was cloned after PCR amplification using the primers FW2/BW2 5′-TCT TCTAAAACAATTAATTT-3′. The probe 1 was linearised by *Spe*I and *Nco*I endonucleases and the resulting products were used as a template to synthesise digoxigenin-labelled RNA sense and anti-sense probes, by T7 and SP6 polymerase driven *in vitro* transcription, respectively. DIG-RNA labelling Kit protocol, (Roche Diagnostic GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Excised leaf and fruit tissues were fixed, dehydrated and embedded in paraplast (Sigma-Aldrich, Milan, Italy). Samples were cut with RM 2125 RT microtome (Leica, Milan, Italy) into 8-μm sections that were transferred to charged slides and hybridised at 50°C to a digoxigenin-labelled antisense RNA probe 1 as previously described by Cañas *et al*. (1994). For immunological detection, the slides were incubated as described by Chiappetta

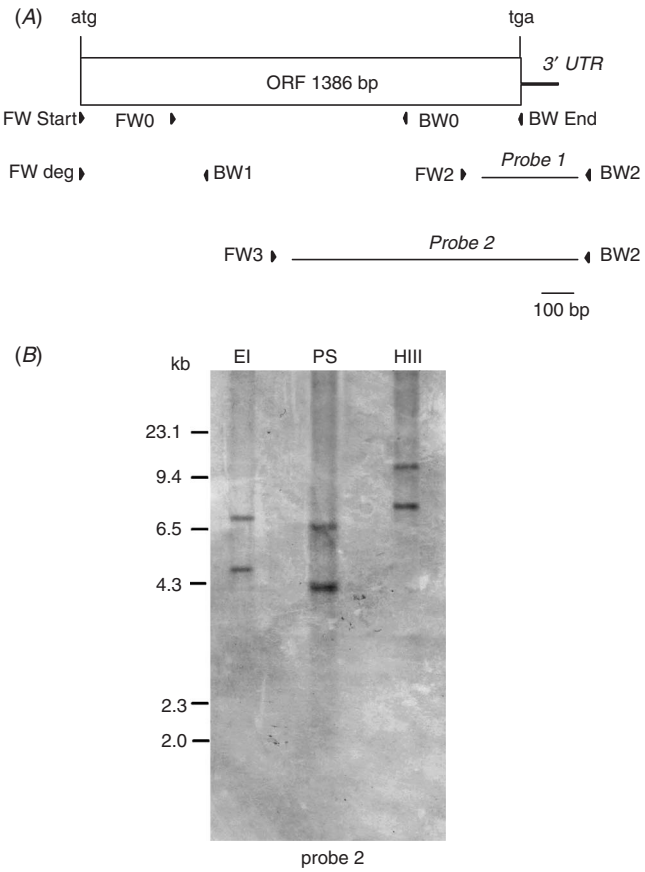

Fig. 1. *Olea europaea OeCHLP* genomic features. (*A*) Scheme of the genomic organisation of *OeCHLP*. Start and stop codons and polyadenylation signals are typed. The probe fragments are represented by black bars. Forward (FW) and backward (BW) directed primers are shown by arrows. UTR, untranslated regions. ORF, open reading frame; bp, base pairs. (*B*) Southern analysis: genomic DNA was digested with *Eco*RI (EI), *Pst1* (PS) and *Hind*III (HIII) endonucleases, electrophoresed on 0.8% agarose gel, blotted and hybridised with digoxygenin-labelled probe 2. The molecular weights of a co-migrating DNA marker are in kilo base pairs (Kb).

et al. (2006). Transcript accumulation was visualised as a violet/brown staining. After stopping the reaction with TE (50 mM Tris–HCl, pH 8.0, 5 mM EDTA), sections were mounted with 50% glycerol in TE and analysed with an optical microscopy (Leica DMRB). Images were taken with a digital camera (Leica DFC 320).

Light microscopy

Leaves and drupes at different stages were fixed in formaldehydeacetic acid (3.7% [w/v] formaldehyde, 50% [w/v] ethanol, and 5% [w/v] acetic acid in water), dehydrated in an ethanol series, and resin-embedded. Sections were cut at 2 μm with a tungsten knife, mounted on slides, and stained with 0.1% (w/v) toluidine blue and periodic acid-Schiff's reagent.

Qualitative fluorescence

Total RNA was stained by acridine orange as follows: excised leaves ($n = 5$ for each sample) were fixed in 4% paraformaldehyde in 1 × PBS pH 7.0 and incubated for 24 h at 4°C. After dehydration through an alcohol series, the material was embedded and longitudinally sectioned (8 μm) using a RM 2125 RT microtome (Leica). The slides were dewaxed, rehydrated and stained with acridine orange and examined as described by Bitonti *et al*. (2002).

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed on a Bio-Rad Miniopticon (Bio-Rad, Milan, Italy) Single colour thermocycler with Bio-Rad SYBR Green Supermix (Cat. No.170–8884).

Primer design

The oligonucleotide primer sets used for qRT-PCR analysis were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 11 January 2006) according to the strategies set up by Yokoyama and Nishitani (2001). Experimentally optimal primers were identified based upon their ability to meet several standards: (a) robustness—successful amplification over a range of annealing temperatures, (b) specificity—generation of a single significant peak in the melting curve, and (c) consistency highly reproducible of *C*t values within the reactions of a triplicate. The primers used for *OeCHLP* are FWrealtime 5′-CCAAGGGAGGCATTTGTAGA-3′ and BW realtime 5′-TGGATTCACAGCCAATTTCA-3′. The length of all PCR products ranged from 150 to 200 bp. The average amplification efficiency of each primer pair was determined, and primers performing poorly were replaced. The average efficiency of all of the primer pairs discussed in this study ranged between 0.95 and 1.0. After checking independent trials of several housekeeping genes, *18S rRNA* produced the most reproducible results across various cDNAs, and was used as a normalisation control. The primer sequence of *18S rRNA* was FW18S 5′-AAACGGCTACCACATCCAAG-3′ and BW18S 5′-CCTCCAATGGATCCTCGTTA-3′.

Amplification conditions

Amplification reactions were prepared in a final volume of 25 μL by adding 12.5 μL of the iTaq SYBR-Green Super Mix with ROX (Bio-Rrad) containing the (iTaq DNA polymerase 50 units mL^{-1}, 6 mm Mg^{2+}, 1 μM ROX internal Reference DYE Stabilisers, 0.4 mM of dATP-dCTP-dGTP and 0.8 mM dUTP), 0.4 μM of primers, and 2 μL (25 ng) of cDNA. All reactions were run in triplicate in 48-well reaction plates, and negative controls were set. The cycling parameters were as follows: one cycle at 95°C for 3 min to activate the *Taq* enzyme, followed by 40 cycle of denaturation at 95°C for 10 s and annealing-extension at 58°C for 30 s.

After reaction, in order to confirm the existence of a unique PCR product the 'melting curve' (Lekanne Deprez *et al.* 2002) was evaluated by an increase of 0.5°C every 10 s, from 60°C to 95°C. We obtained a unique 'melting peak' in every reaction and the PCR products were verified by 1% agarose gel-electrophoresis.

Data analysis

The results of real-time PCR were analysed using Opticon Monitor: quantification real-time PCR Detection System (Bio-Rad), a program that facilitates the analysis of the kinetics of each performed reaction.

Cycle threshold (C_T) values were obtained with the Genex software (Bio-Rad) and data were analysed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The means of *OeCHLP* expression levels were calculated from three biological repeats, obtained from three independent experiments.

Southern blot analysis

Total DNA was isolated from leaves frozen with liquid nitrogen, by the ctyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). A 10 μg sample was restricted overnight (o/n) at 37°C with 60 U of *Eco*RI, Pst1 and *Hind*III endonucleases (Promega), which do not cut in the probe, in a 200 μL final volume. The digested DNA was precipitated at −20°C o/n, resuspended in 50 μL volume and an 5 μL aliquot was rapidly checked by electrophoresis. DNA fragments were separated by electrophoresis (20 h at 45 V on 0.8% agarose gel) and blotted onto Hybond-N+ membranes (Amersham Pharmacia Biotech, Milan, Italy) by vacuum blotting according to the manufacturer's instructions (Amersham Pharmacia Biotech). The membrane was oven-dried at 80°C for 2 h. The probe 2 spanning the 542–1411 stretch was used (Fig. 1*A*). It was digoxygenin-labelled using the FW3 5′-TGCGCTTGTTA CCAGAACAC-3′/BW2 primers, following the manufacturer's instructions (Roche). Membrane prehybridisation (3 h at 52°C) and hybridisation (overnight at 58°C) were carried out in 25% SSC 20× (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, Milan, Italy). Detection was performed with anti-DIG-AP antibodies and nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate following the manufacturer's instruction (Roche). Signal bands were visualised by keeping the membrane at dark for 2 h and then blocking the reaction with TE at pH 8. Signals were computer scanned (UMAX SPEED II).

Biochemical analyses

Chl and TP quantification was performed using samples separately pulverised in liquid nitrogen with a mortar and pestle and thereafter lyophilised.

Lipid peroxidation assay was performed on samples separately frozen with liquid nitrogen and stored at −80°C.

Total chlorophyll content

Total chl was extracted from 100 mg aliquot of the dry powder with 8 mL of acetone: water (4 : 1, v/v) twice. The liquid phases were collected in 50 mL tubes. The extraction was repeated three times until the pellet was colour-less. The combined acetone extracts were cleared by centrifugation step at 1500*g* for 15 min.

Chl determination was conducted by using a spectrophotometer (model Cary 50Bio, Varian, Turin, Italy). *A*646.8 and *A*663.2 were determined and used for calculation of the total contents of chl *a* and *b* according to the method by Lichtenthaler (1987). Three replicates were performed and for each replicate six measurements were carried out on each sample. ANOVA followed by Bonferroni post-hoc test was used to evaluate differences between the considered groups of data.

Tocopherol content

TPs quantification was performed according to Tanaka *et al.* (1999). For each sample a precisely weighted 5 mg aliquot of the dry powder was extracted four times in a pre-cooled mortar with 350 µL each of dioxane: *n*-hexane (1 : 1, v/v), and the combined supernatants were cleared by centrifugation and evaporated. The residue was dissolved in 100 µL of dioxane: *n*-hexane (3 : 97, v/v). The residue was dissolved in 100 µL of dioxane: *n*-hexane (3 : 97, v/v) and 20 µL of this solution was analysed by HPLC (1100 Series, Agilent, Milan, Italy) using a column (4.6 × 250 mm) filled with C18 Nucleosil 50 (5 µm) at a flow rate of 1.5 mL min^{-1} with dioxane: *n*-hexane (3 : 97, v/v).

Peaks of TPs were identified by comparing their retention times with commercially available authentic standards. Total TP content was estimated at 295 nm_{ex} and 325 nm_{em} using a fluorescence detector. TP concentration was calculated by using a calibration curve obtained with a commercial standard (Sigma-Aldrich).

Three replicates were performed and for each replicate six measurements were carried out on each sample. ANOVA followed by Bonferroni post-hoc test or Student's *t*-test were used to evaluate differences between the considered groups of data.

Lipid peroxidation assay

Lipid peroxidation in the leaves of cold-stressed plants was analysed through the thiobarbituric acid (TBA) test which allows to quantify the malondialdehyde (MDA) as an end product of polyunsaturated fatty acids oxidation (Dhindsa *et al.* 1981) The MDA was checked in leaves at different times post treatment (pt): 0, 2, or 4 h. Because olive tissues are rich in substances such as polyphenols, a protocol reliable in reducing any interference was used (Blokhina *et al.* 1999; Hodges *et al.* 1999). Samples were ground in a mortar, and an aliquot (0.4 g) was suspended in extraction buffer (Tris–HCl 1 M pH 7.4, in which was added PVP-40 1.5% w/v). After centrifugation at 10 000*g* for 20 min, 1 mL of supernatant was added to 4 mL of trichloroacetic acid (TCA) 20%/thiobarbituric acid (TBA) 0.5% (solution + TBA). Another 1 mL of supernatant was added to 4 mL of TCA 20% (solution – TBA). Both solutions were mixed vigorously, heated at 95°C for 30 min and then cooled on ice. After centrifugation at 10 000*g* for 20 min, the supernatant was recovered and diluted before absorbance measurements by using a Jasco (Lecco, Italy) V-530 spectrometer. Measurements were performed at 532 nm for MDA, 600 nm for unspecific signals and at 440 for sucrose. MDA equivalents were calculated following Hodges *et al.* (1999).

ANOVA followed by Bonferroni post-hoc test was used to evaluate differences between the considered groups of data.

Results

Features of the OeCHLP transcript and the deduced protein

The full-length *OeCHLP* cDNA (Genbank accession no. DQ424963) consisted of 1511 nucleotides, including an open reading frame of 1386 bp (stretch 1–1386) and a canonical poly-adenylation signal (stretch 1387–1511) in the 3′UTR. The predicted protein (Fig. S2*A*) was made of 462 amino acids (aa) with a calculated mass of 51 kDa (BioEdit Alignment Sequence Editor, Carlsbad, CA, USA). The transit peptide (tp) for cytoplasm-to-chloroplast transport (aa: 1–39) was identified by computational analysis (Chlorop 1.1 Server, http//www.cbs.d.tu.dk/service/, accessed 27 November 2006) and the 423 aa mature portion weighed 47 kDa. Within the tp stretch, the GRLQ (aa: 11–14) and a few other residues (indicated by *) were tightly conserved. The GXGXXG motif (aa: 48–81), which is associated to the binding of nicotinamide nucleotides, was assigned (Atta-Asafo-Adjei *et al.* 1993).

The entire OeCHLP protein was blasted in NCBI database and shared the highest identity with that of *Nicotiana tabacum* (86%), followed by *Glycine max* (82%), *Lotus corniculatus* (81%), *Medicago truncatula* and *Prunus persica* (79%). The identity grade of OeCHLP increased with respect to the above mentioned proteins when the analysis was conducted by excluding the trans-peptide. A phylogenetic tree, constructed using proteins devoid of the hyper variable tp region, confirmed monophyletic origing of plant CHLP proteins (Giannino *et al*. 2004) with OeCHLP being closest to that of *Nicotiana tabacum* (Fig. S2*B*).

Genomic organisation

Southern blot analysis was performed using leaf gDNA digested with *Eco*RI, *Pst*I and *Hind*III endonucleases, which did not cut in the probe 2 (Fig. 1*A*). The band patterning (Fig. 1*B*) consisted of two band signals per each digestion type, suggesting that at least two copies are harboured per haploid genome of the 'Carolea' cultivar.

In order to search for introns, PCR experiments were performed using gDNA and primers pairs designed along the gene cDNA (Fig. 1*A*). The amplified products had identical size and sequence to those of cDNA, indicating that the gene did not contain any introns. Moreover, several products retrieved from distinct primer couple amplification were sequenced, but we did not score any nucleotide polymorphism. This suggested that the two *OeCHLP* estimated copies might be identical due to duplication events.

Cytological and metabolic features of leaf and fruit at different developmental stages

A preliminary characterisation of leaf and fruit developmental stages was carried out taking into account both cytological and metabolic features. YG leaves (2–4 cm long) showed a compact mesophyll where only one layer of palisade tissue began being evident (Fig. 2*A*, *B*). Otherwise, AG and AD leaves (up 6 cm long) showed a typical dorsoventral organisation with a two-three layered palisade tissue and a well distinguishable spongy tissues (Fig. 2*C*, *D*). Moreover, YG leaves exhibited an intricate network of thrycomes along both abaxial and above all adaxial epidermis (Fig. 2*A*, *B*, *E*, *E*′, *F*), and AG and AD leaves showed a very thick layer of cuticola (Fig. 2*C*, *D*, *G*, *G*′, *H*, *H*′).

Distinct metabolic features also marked the leaf developmental stages. A higher orange acridine staining accounting for a higher transcriptional activity was observed in YG *v.* AG and AD leaves (Fig. 2*E–H*, *E*′, *G*′, *H*′). Moreover, quantitative and qualitative differences in chl and TP level were also detected: total chl amount was significantly lower in YG than in AG and AD leaves, while the relative percentages of chl *a* and *b* were quite comparable in all the stages (Table 1). Total TP amount was instead roughly equal in YG, AG, AD leaves but a progressive increase in the relative percentage of the form α− and a simultaneous decrease in the percentage of form β− were observed from young to adult stages (Table 2).

GF and DF fruits showed very similar cytological features, with mesocarp cells appearing largely occupied by oil bodies and rich in cellular inclusions (Fig. 2*I–N*). An evident decrease in total chl content together with an inversion in the relative percentages of chl a and b was observed in DF *v.* GF fruits (Table 1). By contrast, in DF fruits characterised by strong oil accumulation (Sanchez and Harwood 2002; Conde *et al.* 2008) total TP content was higher than in GF fruits (Table 2). This feature is consistent with the protective action

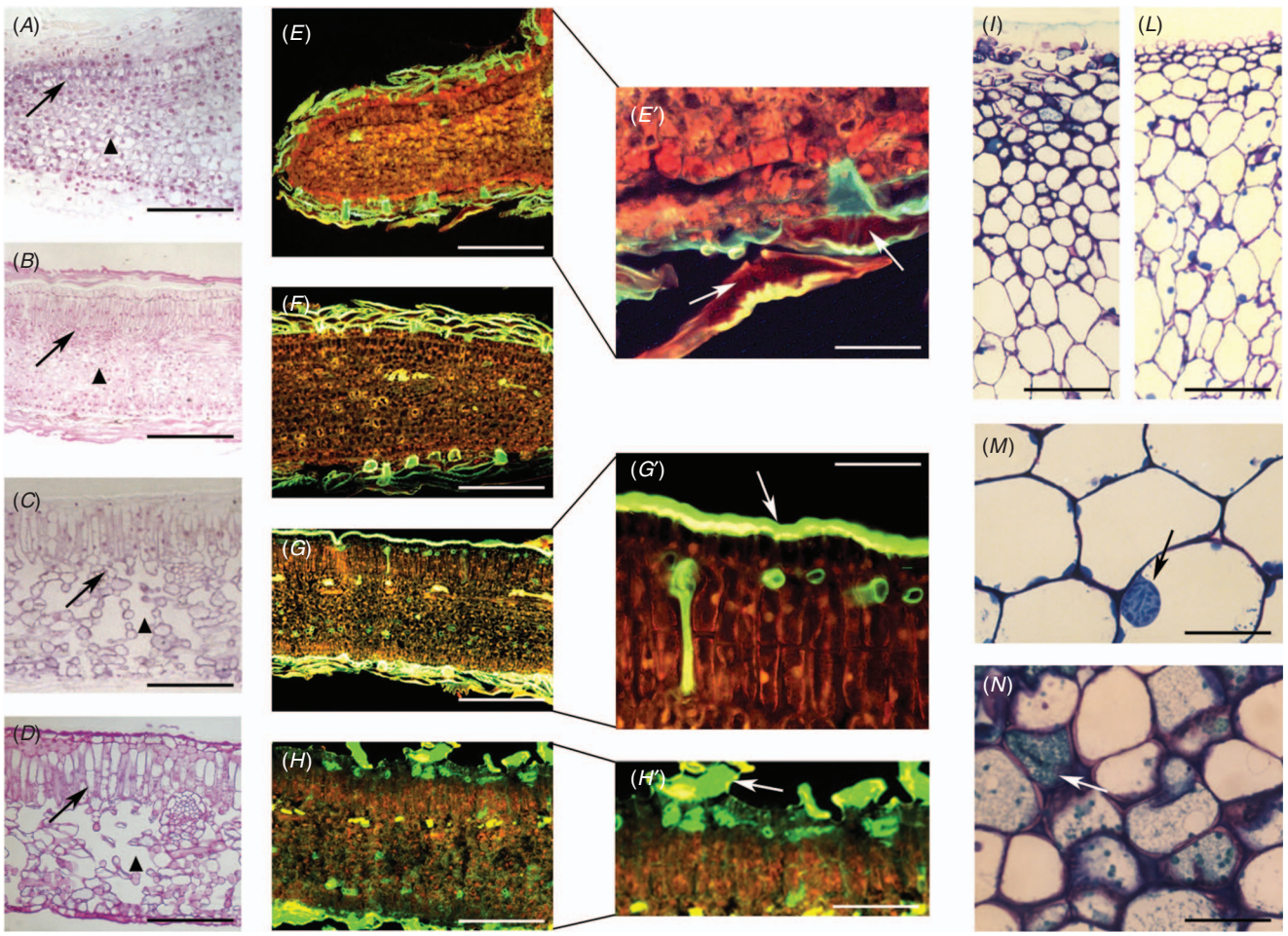

Fig. 2. Cytological features of leaves and fruits of olive (*Olea europaea*) at different developmental stages: (*A–D*) leaf cross-sections stained with periodic acid-Schiff's; (*E–H*) leaf cross-sections stained with orange acridine; (*I–N*) fruit longitudinal sections stained with toluidine blue; (*A*, *E*) 2 cm long YG, (*B*, *F*) 4 cm long YG, (*C*, *G*) AG, (*D*, *H*) AD leaves; (*E*′, *G*′, *H*′) magnification of (*E*, *G*, *H*). In (*A–D*), arrow indicates palisade tissue and arrow-head indicates spongy tissue; in *E*′ arrow indicates trichome cell; in *G*′, *H*′ arrow indicates cuticola layer and wax deposition, respectively; in (*M*) arrow indicates nucleus; in (*N*) arrow indicates cellular inclusions. Scale bars: (*A–H*) = 50 µm; (*E*′, *G*′, *H*′) = 25 µm; (*I*, *L*), 100 µm; (*M*) = 20 µm; (*N*) = 30 µm. YG, young green leaves 2–4 cm long; AG, adult green leaves up 6 cm long; AD, adult dark leaves up 6 cm long.

Table 1. Amount of total chlorophyll and single forms (chl a, chl b) in olive (*Olea europaea*) organs

Percentage value indicates the relative fraction respect to the total content. Values represent the means ± s.e. of three independent biological replicates. Leaf and fruit data were statistically evaluated by using ANOVA followed by Bonferroni post hoc and Student's *t*-test, respectively: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. YG, young green leaf; AG, adult green leaf; AD, adult dark-green leaf; GF, green fruit; DF, dark fruit

Samples	Chlorophyll *a* (mg g^{-1} DW)		Chlorophyll *b* (mg g^{-1} DW)		Chlorophylls *a*+*b* (mg g^{-1} DW)
	Mean	%	Mean	%	Mean
YG	2.574 ± 0.24	69.5	1.129 ± 0.17	30.5	3.703 ± 0.21
AG	3.796 ± 0.20	66.6	1.903 ± 0.11	33.4	5.699 ± 0.15*
AD	5.243 ± 0.48	69.9	2.257 ± 0.41	30.1	7.500 ± 0.45**
GF	0.029 ± 0.02	60.1	0.019 ± 0.02	39.9	0.048 ± 0.03
DF	0.007 ± 0.07	33.9	0.014 ± 0.02	66.1	0.021 ± 0.02*

of such compounds on storage oil from oxidative damage (Fukuzawa and Gebicki 1983; Munnè-Bosch and Alegre 2002; Sattler *et al.* 2004).

In both leaves and fruits OeCHLP *expression is modulated in relation to developmental stages*

OeCHLP expression was estimated through qRT–PCR analysis in various organs of plants such as leaves, vegetative apical tips, floral buds and fruits. Leaves and fruits were analysed at the developmental stages previously characterised. *OeCHLP* resulted to be differentially expressed and the highest value was detected in mature DF fruits (Fig. 3*A*). Furthermore, *OeCHLP* transcripts were 9–10-fold more abundant in YG than AG and AD leaves, and values did not significantly differ between AG and AD leaf types (Fig. 3*A*). By contrast, the relative level of gene expression resulted low in GF, then drastically increased at the last stage (DF) of fruit maturation (Fig. 3*A*).

An extensive analysis of transcripts localisation was also conducted by *in situ* hybridisation (Fig. 3*B–L*). We observed that in the apical tips *OeCHLP* transcripts were confined in developing leaflets (Fig. 3*B*), whereas in floral buds a strong message accumulation occurred in both leaflets and floral meristematic dome (Fig. 3*C*). *OeCHLP* transcripts were spread evenly in the leaf mesophyll (Fig. 3*D*, *F*). A high accumulation of transcripts was detected in trichome cells (Fig. 3*D*, *D*′, *F*), which resulted also intensely stained by orange acridine (Fig. 2*E*′). In the fruits, *OeCHLP* signal was faintly diffuse in the epicarp and the whole mesocarp (Fig. 3*H*). Signal was not observed in leaf and fruit sections processed with sense probe (Fig. 3*E*, *I*).

OeCHLP *transcription and TPs amount are increased in response to biotic and abiotic factors affecting leaves and drupes*

Olive tree production may be strongly affected by low temperatures during the vegetative phase (Larcher 1970). Consequently we tested the *OeCHLP* behaviour in young and adult leaves of plants exposed to cold treatment (4°C).

In all leaf types, the gene expression exhibited the same response kinetics: the raise of transcript level occurred 10 min post treatment (pt), peaked at 1 h pt, and restored the starting levels at four hours pt (Fig. 4*A*). During the gene response interval, the mRNA level was 5–10 fold higher than in untreated plants (Fig. 4*A*). *In situ* hybridisation performed in AG leaf at 1 hour pt evidenced intense *OeCHLP* transcript accumulation in both the palisade layer and spongy tissues, around vascular heath, as compared with unstressed leaves (compare Fig. 3*G v.* Fig. 3*F*).

The occurrence of oxidative stress condition in leaves following cold exposure was assessed by estimating the amount of malondialdehyde (MDA) as an end product of lipid peroxidation (Dhindsa *et al.* 1981). A significant increase (~50%) in the level of MDA was detected in stressed leaves compared with control ones (Fig. S3). Moreover, in both control and stressed leaves MDA level was slightly higher in YG than in AG and AD stages (Fig. S3).

To add further insights on the metabolic conditions induced in the leaves by cold treatment, chl and TP levels were evaluated at different times after stress started (Fig. 4*B*, *C*). A significant decrease of chl content, mainly involving chl *b*, was observed in cold-treted leaves starting from 2 h pt (Fig. 4*B*). The consequent higher chl *a*/*b* ratio is consistent with a reduced photosynthetic efficiency and a stress condition (Nilsen and Orcutt 1996). By contrast, TP content significantly increased in the leaves exposed to cold stress for 4 h (Fig. 4*C*) No significant differences were observed in the relative percentages of different TP forms (data not shown).

One of the main biotic stressor for olive plants is the fly *Bactrocera olea*, which lay one egg in young fruit epidermis and severely impairs drupe quality (Burrack and Zalom 2008). Therefore, *OeCHLP e*xpression was evaluated through

Table 2. Amount of total tocopherols and single forms (α–, β–, γ–, δ–) in olive (*Olea europaea*) organs

Percentage value indicates the relative fraction respect to the total content. Values represent the means ± s.e. of three independent biological replicates. Leaf and fruit data were statistically evaluated by using ANOVA followed by Bonferroni post hoc and Student's *t*-test, respectively: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. YG, young green leaf; AG, adult green leaf; AD, adult dark-green leaf; GF, green fruit; DF, dark fruit

Samples	α-tocopherol (ug g^{-1} DW)		β-tocopherol (ug g^{-1} DW)		γ-tocopherol (ug g^{-1} DW)		δ-tocopherol (ug g^{-1} DW)		Tocopherols total content (ug g^{-1} DW)
	Mean	%	Mean	%	Mean	%	Mean	%	
YG	38.39 ± 5.45	44.5	36.33 ± 3.00	42.1	9.35 ± 1.03	10.8	2.19 ± 0.24	2.5	86.26 ± 17.1
AG	40.28 ± 7.04	48.0	26.37 ± 2.46	31.4	11.59 ± 1.76	13.8	5.67 ± 0.55	6.7	83.91 ± 16.4
AD	83.29 ± 4.68	76.9	16.25 ± 2.77	15.0	6.49 ± 1.37	6.0	2.27 ± 0.24	2.1	108.32 ± 14.9
GF	109.98 ± 12.97	72.0	20.09 ± 3.47	13.1	19.75 ± 5.54	12.9	2.92 ± 0.61	1.8	152.75 ± 18.1
DF	151.44 ± 12.80	75.6	22.69 ± 2.77	11.3	14.62 ± 0.80	7.3	11.44 ± 3.93	5.7	200.19 ± 13.9*

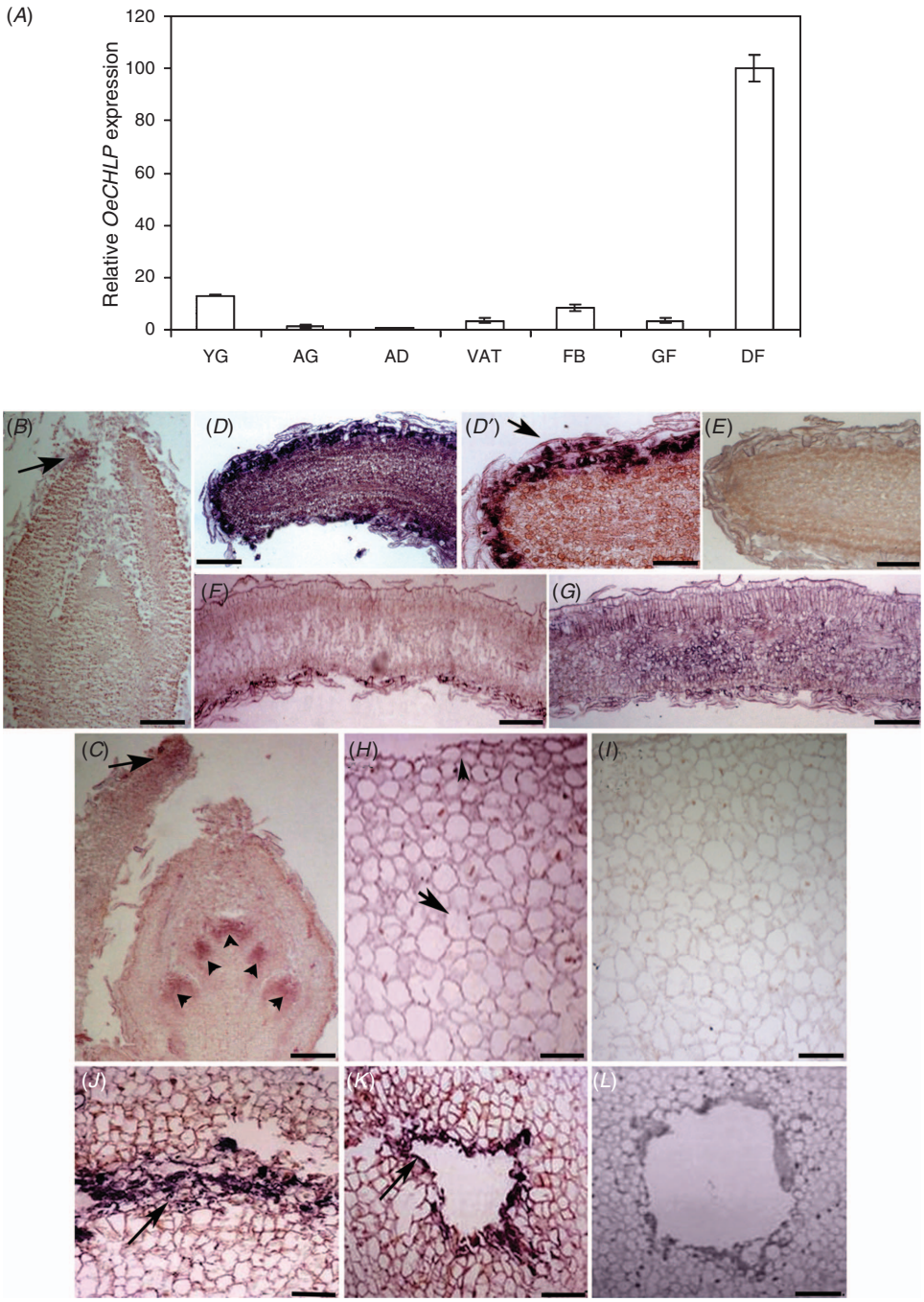

Fig. 3. (*A*) Levels of *OeCHLP* expression in various organs of olive (*Olea europaea*) evaluated by qRT–PCR analysis. The results are presented as a proportion of the highest value after normalisation with respect to *18S* rRNA expression level and represent the means ± s.e. of three independent biological replicates. (*B–L*) Localisation of *OeCHLP* transcripts in various organs of olive by *in situ* hybridisation with dig-labelled *OeCHLP* antisense probe. Signal is evidenced by purple staining in: (*B*) longitudinal section of Vegetative Apical Tips (VAT); (*C*) longitudinal section of FB; (*D*, *F*, *G*,) cross-sections of YG, AG, and cold-stressed AD leaves, respectively; (*D′*) magnification of (*D*); (*H*) longitudinal sections of green fruit (GF); longitudinal- (*J*) and cross- (*K*) section of infected green fruit (IGF). (*E*, *I*, *L*) control sections of YG, GF and IGF incubated with the sense probe. In (*B*, *C*) arrow indicates leaflet; in (*C*) arrow-head indicates floral dome; in (*D′*) arrow indicates trichome cell; in (*H*) arrow-head indicates epicarp and arrow indicats mesocarp. Scale bars: (*B*) = 80 µm, (*C*) = 60 µm, (*D*) = 100 µm, (*D′*, *E*) = 50 µm, (*F*, *G*) = 70 µm, (*H*, *I*) = 50 µm, (*J–L*) = 20 µm. YG, young green leaves 2–4 cm long; AG, adult green leaves up 6 cm long; AD, adult dark leaves up 6 cm long; FB, floral buds; DF, dark fruits.

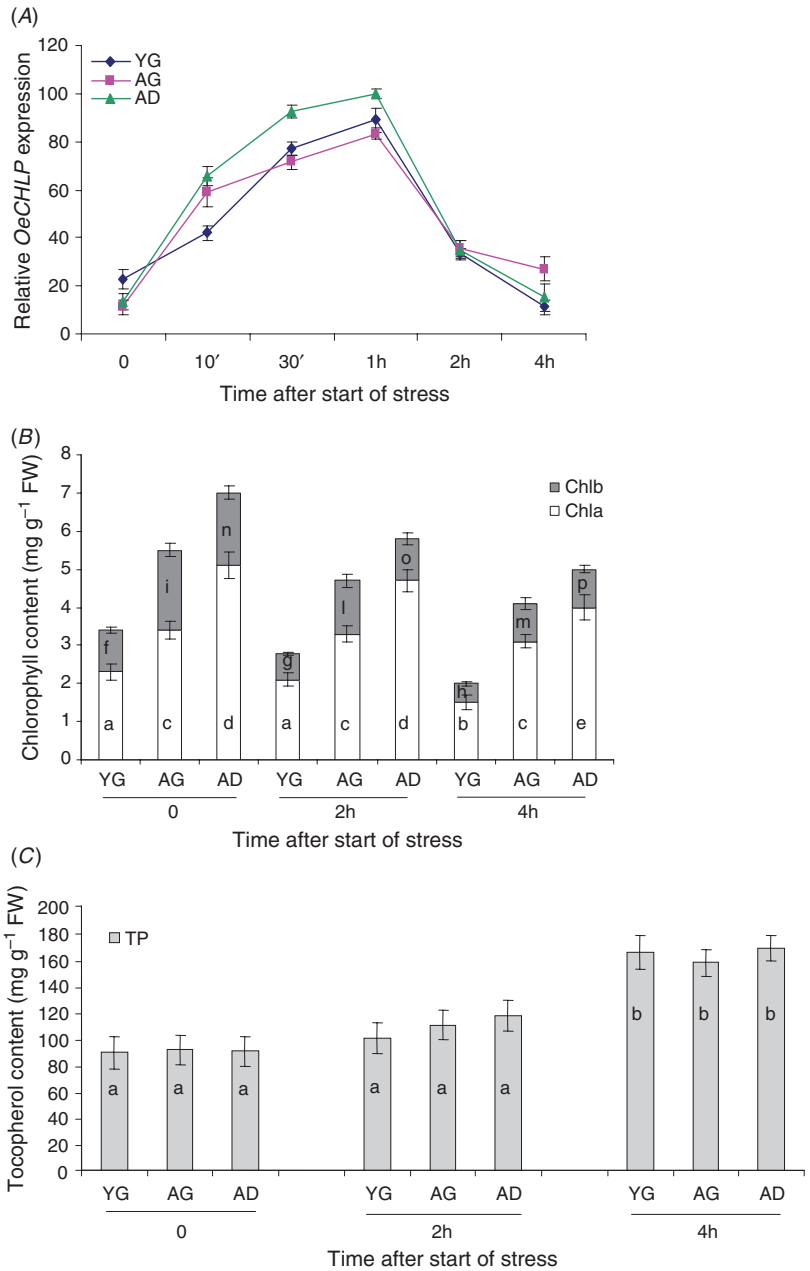

Fig. 4. (*A*) *OeCHLP* expression analysed by qRT–PCR in leaves of olive (*Olea europaea*) exposed to cold (4°C) conditions for different times: 0 (unexposed leaves used as control), 10 min, 30 min, 1 h, 2 h or 4 h. Results are presented as a proportion of the highest value after normalisation with respect to *18S* rRNA expression level and represent the means ± s.e. of three independent biological replicates. (*B*) Chl *a* and *b*, and (*C*) total tocopherol (TP) content in leaves of olive exposed to cold (4°C) conditions for different times: 0 (unexposed leaves used as control), 2 and 4h. Values represent the means ± s.e. of three independent biological replicates. Means signed with the same letters are not significantly different for $P = 0.05$ after ANOVA followed by Bonferroni post hoc test. YG, young green leaves 2–4 cm long; AG, adult green leaves up 6 cm long; AD, adult dark leaves up 6 cm long.

qRT–PCR also in relation to fly damage and resulted to be 2.5-fold higher in attacked (IGF) than healthy (GF) fruits (data not shown). *In situ* experiments were performed on drupe sections harbouring damaged sectors, and the *OeCHLP* transcript highly accumulated in cell areas with lesions (Fig. 3*J*) and at the border of mine traces (Fig. 3*K*). No signal was observed above background in fruit sections of infected sectors processed with sense probe (Fig. 3*L*).

Finally, *OeCHLP* expression was monitored in green drupes mechanically injured by needle punctures. Drupes were sampled 10, 30 min, 1, 2 and 4 h after needle puncture. Uninjured fruits were used as starting point of stress. Early after the injury, the level of *OeCHLP* expression decreased within 10 min, thereafter suddenly increased within 30 min and peaked at 2 h after wounding, becoming 5-fold higher than T0 (Fig. 5*A*). Subsequently it declined at the level of uninjured fruits (Fig. 5*A*).

In infected (IGF) and wounded fruits (WGF) both chls and TPs levels were analysed (Fig. 5*B*, *C*). Chl content drastically decreased (50%) in IGF, while it was unchanged in WGF fruits analysed at 2 and 4 h after stress started (Fig. 5*B*). For TP content, a significant increases was detected only in IGF infected by fly pathogen (Fig. 5*C*).

Discussion

In this work we characterised in *Olea europaea* the nuclear *OeCHLP* gene which encodes a chloroplastic enzyme involved in the formation of phytolic side chain of chl, TPs and PQs. *In silico* analysis evidenced high homology of *OeCHLP* with other known homologous genes (Keller *et al.* 1998; Giannino *et al.* 2004). Differently from the homologous genes of *Arabidopsis thaliana*, *Glycine maxima* and *Prunus persica*, no introns were present in the genomic sequence of *OeCHLP*. Moreover, two copies of *OeCHLP* gene per haploid genome were detected in the 'Carolea' cultivar used in the present study. Such feature is common to other Drupoideae species, such *Prunus amygdale*, *Prunus avium* and *Prunus dulcis*, whereas one single copy gene was estimated in both *Prunus persica* and *Arabidopsis thaliana*. (Keller *et al.* 1998; Giannino *et al.* 2004). The absence of any nucleotide polymorphism strongly suggests that the estimated copies of *OeCHLP* might be due to duplication events.

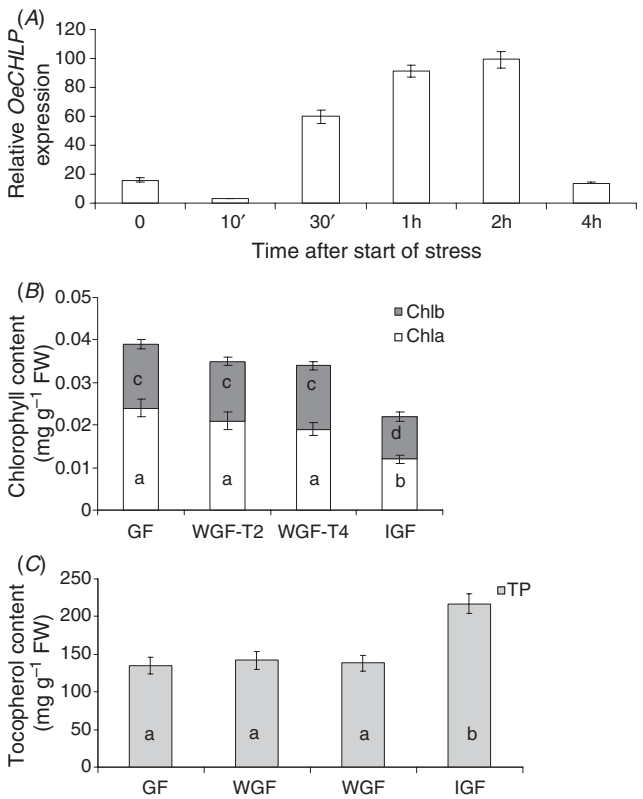

Fig. 5. (*A*) Levels of *OeCHLP* transcripts analysed by qRT–PCR in green fruits of olive (*Olea europaea*) at different times from mechanical wounding: 0 (uninjured fruits used as control), 10, 30 min, 1, 2, or 4 h. Results are presented as a proportion of the highest value after normalisation with respect to *18S* rRNA expression level and represent the means ± s.e. of three independent biological replicates. (*B*) Chl *a* and *b*, and (*C*) total tocopherol (TP) content in uninjured, wounded and infected green fruits of olive. Values represent the means ± s.e. of three independent biological replicates. Means signed with the same letters are not significantly different for $P = 0.05$ after ANOVA followed by Bonferroni post hoc test. GF, uninjured green fruits; WGF-2 h, green fruit 2 h after wounding; WGF-4 h, green fruit 4 h after wounding; IGF, green fruits infected by *Bactrocera oleae* pathogen.

OeCHLP transcripts were detected in various organs of olive plants. This result is in line with the involvement of this gene in the biosynthesis of several compounds, of which chls, present in plant aereal organs and fruits, and ubiquitous TPs, (Threlfall and Whistance 1970; Bollivar *et al.* 1994; Keller *et al.* 1998; Tanaka *et al.* 1999; Munnè-Bosch and Alegre 2002). Notably, a very strong accumulation of *OeCHLP* transcripts were detected in leaf trichomes where many protective products, including terpenes, accumulated (McCaskill and Croteau 1995; Aziz *et al.* 2005). This result is rather relevant, adding further information on the set of genes expressed in trichomes, an aspect not yet globally defined.

Further, in both leaves and fruits *OeCHLP* was expressed at different level in relation to the stage of organ growth and differentiation. For lateral organs, a higher level of *OeCHLP* transcripts was detected in developing YL than AG and AD leaves. Notably, in decidous leaves of peach, gene expression was demonstrated to be higher in adult than young leaves (Giannino *et al.* 2004). We suggest that the pattern of gene expression in leaves is related not only to the specific time course of mesophyll differentiation but also to the physiological features underlying leaf life-cycle (deciduous *v.* evergreen plants). In fruits gene expression was enhanced in DF *v.* GF very likely in relation to the increase in mature fruits of the level of total TPs. All together these results are consistent with a regulatory modulation of *OeCHLP* expression in relation to developmental factors.

Due to the contemporary involvement of *CHLP* gene in different biosynthetic pathways and because we did not experimentally investigate the fluxes through pathways, it was not within the aim of the present work to tightly relate gene expression to chl and TP levels. In addition data in literature suggest that CHLP is likely a minor component in the regulation of both chl and TP biosynthesis (Collakova and DellaPenna 2003). Notwithstanding, the higher level of *OeCHLP* expression, together with the lower chl and similar TP level, in YG respect to AG and AD leaves is noteworthy. We postulate that in young leaves the high transcriptional activity of gene might be related to a high turnover of either chls or TPs compared with a steady-state reached in adult leaves. Notably, MADS level was higher in YG than in AG and AD leaves, indicating that YG leaves are more subjected to photo-oxidative damage. Their cyto-physiological features, such as the absence of a thick cuticula and wax deposition, strongly support this assumption.

Thus, the high level of *OeCHLP* gene in young leaves could represent an adaptative response to stressful environmental conditions.

The above assumption is supported by the enhancement of *OeCHLP* expression detected in cold-stressed leaves. Notably, besides a higher chl *a*/*b* ratio, an increase of both lipid peroxidation and TP level was observed in stressed leaves compared with control leaves. These results are consistent with other evidences showing that cold and wounding induce in plants the production of ROS and antioxidant compounds, involved in both defence mechanism (Hasegawa *et al.* 2000; Foyer and Noctor 2005), and cellular signalling, as recently demonstrated for TPs (Munnè-Bosch 2005). In this context, it appears that the early responsiveness of *OeCHLP* which could be related to the interation of TPs and ROS in signalling the cell redox state (De Pinto and De Gara 2004; Foyer and Noctor 2005; Tokunaga *et al.* 2005) following stress perception. However, it must be recalled that olive is characterised by a very slow capacity to cold acclimation (Larcher 1970; D'Angeli and Altamura 2007). Both these features together with the different time course in the evaluation of gene expression could account for olive differential behaviour respect to both *Arabidopsis* and peach, which exhibited a downward trend in *CHLP* expression after prolonged cold treatment (Collakova and DellaPenna 2003; Giannino *et al*. 2004).

An enhancement of *OeCHLP* transcriptional activity was also detected in fruits attacked by *Bactrocera oleae* pathogen as well as in fruits wounded by needle. A very high level of TPs, likely related to cell damage and oxidative burst induced by pathogen, was measured in infected *v.* healthy fruits (Baron and Zambryski 1995; Ebel 1998; Klessig *et al.* 2000). By contrast, despite the increase in the level of gene expression, TP amount was almost unchanged in wounded fruits, at least in the short time for our analysis. In this context, we note that fly infection represents a long-lasting stressful condition compared with experimental wounding. Hence, the time course of metabolic changes induced by stress application were not strictly comparable. In addition, *in situ* analyses on fruits infected by carpophagus larvae evidenced that transcripts accumulation was confined in the damaged regions. This cytological pattern strongly suggests an involvement of gene into a very localised synthesis of antioxidant compounds in damaged area (Baron and Zambryski 1995; Klessig *et al.* 2000).

In summary, we clearly demonstrate that in olive plants *OeCHLP* gene is differentially expressed in relation to tissue differentiation and organ development as well to stress factors. Moreover, under stressful conditions an increase of both *OeCHLP* transcripts and TP level was detected which links *OeCHLP* gene transcriptional activity to TPs action. All together these results clearly indicate that the modulation of *OeCHLP* gene expression is part of the complex genetic network underlying plant development and stress response.

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References

Atta-Asafo-Adjei E, Lawton MP, Philpott RM (1993) Cloning, sequencing, distribution, and expression in *Escherichia coli* of flavin-containing monooxygenase. Evidence for a third gene subfamily in rabbits. *Journal of Biology and Chemistry* **268**, 9681–9689.

Aziz N, Paiva NL, May GD, Dixon RA (2005) Transcriptome analysis of alfalfa glandular trichomes. *Planta* **221**, 28–38. doi: 10.1007/s00425-004-1424-1

Baldoni L, Tosti N, Ricciolini C, Belaj A, Arcioni S, Pannelli G, Germanà MA, Mulas M, Porceddu A (2006) Genetic structure of wild and cultivated olives in the central Mediterranean basin. *Annals of Botany* **98**, 935–942. doi: 10.1093/aob/mcl178

Baron C, Zambryski PC (1995) The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? *Annual Review of Genetics* **29**, 107–129. doi: 10.1146/annurev.ge.29.120195.000543

Barone E, Di Marco L (2003) 'Morfologia e ciclo di sviluppo.' In *Olea* Trattato di olivicoltura Edagricole. pp. 13–31. (Fiorino P. Edagricole: Bologna)

Bitonti MB, Cozza R, Chiappetta A, Giannino D, Castiglione MR, Dewitte W, Mariotti D, Van Onckelen H, Innocenti AM (2002) Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach [*Prunus persica* (L.) Batsch]. *Journal of Experimental Botany* **53**, 1047–1054. doi: 10.1093/jexbot/53.371.1047

Blokhina OB, Fagerstedt K, Chirkova TV (1999) Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiologia Plantarum* **105**, 625–632. doi: 10.1034/j.1399-3054.1999.105405.x

Bollivar DW, Wang SJ, Allen JP, Bauer CE (1994) Molecular genetic analysis of terminal steps in bacteriochlorophyll a biosynthesis: characterization of a *Rhodobacter capsulatus* strain that synthesizes geranylgeraniol-esterified bacteriochlorophyll *a*. *Biochemistry* **33**, 12763–12768. doi: 10.1021/bi00209a006

Burrack HJ, Zalom FG (2008) Olive fruit fly (*Diptera*: *Tephritidae*) ovipositional preference and larval performance in several commercially important olive varieties in California. *Journal of Economic Entomology* **101**, 750–758. doi: 10.1603/0022-0493(2008)101[750:OFFDTO]2.0.CO;2

Cañas LA, Busscher M, Angenent GC, Beltran JP, van Tunen AJ (1994) Nuclear localization of the petunia MADS box protein FBP1. *The Plant Journal* **6**, 597–604. doi: 10.1046/j.1365-313X.1994.6040597.x

Chiappetta A, Michelotti V, Fambrini M, Bruno L, Salvini M, Petrarulo M, Azmi A, Van Onckelen H, Pugliesi C, Bitonti MB (2006) Zeatin accumulation and misexpression of a classI *knox* gene are intimately linked in the epiphyllous response of the interspecific hybrid EMB-2 (*Helianthus annuus* × *H. tuberosus*). *Planta* **223**, 917–931. doi: 10.1007/s00425-005-0150-7

Collakova E, DellaPenna D (2003) The role of homogentisate phytyltransferase and other tocopherol pathway enzymes in the regulation of tocopherol synthesis during abiotic stress. *Plant Physiology* **133**, 930–940. doi: 10.1104/pp.103.026138

Conde C, Delrot S, Geros H (2008) Physiological, biochemical and molecular changes occurring during olive development and ripening. *Journal of Plant Physiology* **165**, 1545–1562. doi: 10.1016/j.jplph.2008.04.018

Consolandi C, Palmieri L, Doveri S, Maestri E, Marmiroli N, *et al*. (2007) Olive variety identification by ligation detection reaction in a universal array format. *Journal of Biotechnology* **129**, 565–574. doi: 10.1016/j.jbiotec.2007.01.025

D'Angeli S, Altamura MM (2007) Osmotin induces cold protection in olive trees by affecting programmed cell death and cytoskeleton organization. *Planta* **225**, 1147–1163. doi: 10.1007/s00425-006-0426-6

De Pinto MC, De Gara L (2004) Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. *Journal of Experimental Botany* **55**, 2559–2569. doi: 10.1093/jxb/erh253

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany* **32**, 93–101. doi: 10.1093/jxb/32.1.93

Ebel J (1998) Oligoglucoside elicitor-mediated activation of plant defense. *BioEssays* **20**, 569–576. doi: 10.1002/(SICI)1521-1878(199807)20:7<569::AID-BIES8>3.0.CO;2-F

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* **17**, 1866–1875. doi: 10.1105/tpc.105.033589

Fryer MJ (1993) Evidence for the photoprotective effects of vitamin E. *Photochemistry and Photobiology* **58**, 304–312. doi: 10.1111/j.1751-1097.1993.tb09566.x

Fukuzawa K, Gebicki JM (1983) Oxidation of α-tocopherol in micelles and liposomes by the hydroxyl, perhydroxyl, and superoxide free radicals. *Archives of Biochemistry and Biophysics* **226**, 242–251. doi: 10.1016/0003-9861(83)90290-4

Giannino D, Condello E, Bruno L, Testone G, Tartarini A, Cozza R, Innocenti AM, Bitonti MB, 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. *Journal of Experimental Botany* **55**, 2063–2073. doi: 10.1093/jxb/erh217

Giannoulia K, Banilas G, Hatzopoulos P (2007) Oleosin gene expression in olive. *Journal of Plant Physiology* **164**, 104–107. doi: 10.1016/j.jplph.2006.03.016

Goffman FD, Bohme T (2001) Relationship between fatty acid profile and vitamin E content in maize hybrids (*Zea mays* L.). *Journal of Agricultural and Food Chemistry* **49**, 4990–4994. doi: 10.1021/jf010156y

Graβes T, Grimm B, Koroleva O, Jahns P (2001) Loss of alphatocopherol 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. doi: 10.1007/s004250100532

Green PS (2002) A revision of *Olea* (L.). *Kew Bulletin* **57**, 91–140. doi: 10.2307/4110824

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 463–499. doi: 10.1146/annurev.arplant.51.1.463

Havaux M, Lutz C, Grimm B (2003) Chloroplast membrane photostability in chlP transgenic tobacco plants deficient in tocopherols. *Plant Physiology* **132**, 300–310. doi: 10.1104/pp.102.017178

Hernández ML, Mancha M, Martínez-Rivas JM (2005) Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (FAD2) from olive. *Phytochemistry* **66**, 1417–1426. doi: 10.1016/j.phytochem.2005.04.004

Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* **207**, 604–611. doi: 10.1007/s004250050524

Keller Y, Bouvier FD, Harlingue A (1998) Metabolic compartmentation of plastid prenyllipid biosynthesis: evidence for the involvement of a multifunctional geranylgeranyl reductase. *European Journal of Biochemistry* **251**, 413–417. doi: 10.1046/j.1432-1327.1998.2510413.x

Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, *et al*. (2000) Nitric oxide and salicylic acid signaling in plant defense. *Proceeding of the National Academy of Sciences of the United States of America* **97**, 8849–8855. doi: 10.1073/pnas.97.16.8849

Larcher W (1970) Kälteresistenz und Uberwinterungsvermögen mediterraner Holzpflanzen. *Oecologia Plantarum* **5**, 267–286.

Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF (2002) Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Analytical Biochemistry* **307**, 63–69. doi: 10.1016/S0003-2697(02)00021-0

Lichtenthaler HK (1987) Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. *Methods in Enzymology* **148**, 350–382.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods* **25**, 402–408. doi: 10.1006/meth.2001.1262

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha* × *piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. *Planta* **197**, 49–56.

Munnè-Bosch S (2005) The role of alpha-tocopherol in plant stress tolerance. *Journal of Plant Physiology* **162**, 743–748. doi: 10.1016/j.jplph.2005.04.022

Munnè-Bosch S, Alegre L (2002) The function of tocopherols and tocotrienols in plants. *Critical Reviews in Plant Sciences* **21**, 31–57. doi: 10.1016/S0735-2689(02)80037-5

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321–4326. doi: 10.1093/nar/8.19.4321

Muzzalupo I, Lombardo N, Salimonti A, Perri E (2008) Molecular characterization of Italian olive cultivars by microsatellite markers. *Advances in Horticultural Science* **22**, 142–148.

Nilsen ET, Orcutt DM (1996) 'Physiology of plants under stress: abiotic factors.' (John Wiley & Sons: New York)

Sanchez J, Harwood JL (2002) Biosynthesis of triacylglycerols and volatiles in olives. *European Journal of Lipid Science and Technology* **104**, 564–573. doi: 10.1002/1438-9312(200210)104:9/10<564::AID-EJLT564>3.0.CO;2-5

Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, DellaPenna D (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *The Plant Cell* **16**, 1419–1432. doi: 10.1105/tpc.021360

Tanaka R, Oster U, Kruse E, Rudiger W, 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 Physiology* **120**, 695–704. doi: 10.1104/pp.120.3.695

Threlfall DR, Whistance GR (1970) Biosynthesis of phytoquinones. Homogentisic acid: a precursor of plastoquinones, tocopherols and alpha-tocopherolquinone in higher plants, green algae and blue-green algae. *The Biochemical Journal* **117**, 593–600.

Tokunaga T, Miyahara K, Tabata K, Esaka M (2005) Generation and properties of ascorbic acid-overproducing transgenic tobacco cells expressing sense RNA for l-galactono-1,4-lactone dehydrogenase. *Planta* **220**, 854–863. doi: 10.1007/s00425-004-1406-3

Valk EE, Hornstra G (2000) Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man. *International Journal for Vitamin and Nutrition Research* **70**, 31–42. doi: 10.1024/0300-9831.70.2.31

Yokoyama R, Nishitani K (2001) A comprehensive expression analysis of all members of a gene family encoding cell-wall enzymes allowed us to predict cis-regulatory regions involved in cell-wall construction in specific organs of Arabidopsis. *Plant & Cell Physiology* **42**, 1025–1033. doi: 10.1093/pcp/pce154

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