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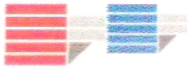

Dottorato di Ricerca in Ambiente Salute e Processi Ecosostenibili

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Structural and functional characterization of genes related to the quality of plant products in olive (*Olea europaea* L.)

Settore scientifico disciplinare - BIO/04

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Chapter 3

Structural and functional characterization of *Olea europaea ALCOHOL DEHYDROGENASE* gene (*OeADH*) involved in fruit flavour

Extended Abstract

Background. Olive (*Olea europaea* L.) is an emblematic species and one of the most widespread fruit tree in the Mediterranean basin. It is a member of the *Oleaceae* family, which includes 600 species within 25 genera distributed over all continents, from northern temperate to southern subtropical regions and from low to high altitudes. On the basis of several morphological synapomorphies, *Oleaceae* family is considered as monophyletic and is easily circumscribed. Within the family, phylogenetic relationships among genera have been assessed by a cladistic analysis of chloroplast non-coding DNA sequences. The different members of *Oleaceae* family are trees, shrubs or woody climbers and among them *Olea europaea* L. features as an evergreen schlerophyllous tree.

Native to the Mediterranean region, olive represents a unique species within the *Olea* genus that produces edible fruits. There are historical evidences that the utilization of wild olive as fruit tree can be dated to Neolithic period, as early as the 8th millennium B.C., and that its domestic cultivation was firstly undertaken on Crete island during the Minoan period, between 1500–3000 BC. From here, olive plants and their products were exported not only towards mainland Greece, but also to Northern Africa and Asia Minor. Today, olive is one of the most extensively cultivated crop species in the world and about 96% of olive cultivated area is within the Mediterranean basin (8,6 million of hectares, over 750 million of trees). Most of olive fruits is destined for oil production. About 73 % of the global oil production comes from European Countries and main producers among them are Spain, Italy and Greece. However, a considerable part of olive fruits is destined for direct human consumption as table olives, whose worldwide production amounts to about 17 million tons.

Primarily, the quality of olive products (e.g. table olive and oil) relies on agronomic and organoleptic features of fruits which according to botanical nomenclature are named “drupes”. On the other hand the distinctive features of drupe vary in relation to the genetic traits (cultivar) but also to ripening stage as well as to the environmental growth conditions (i.e. soil and climatic conditions). Note that, these latter potentially include different biotic and abiotic stressful factors that, besides influencing plant productivity, strongly impact on the quality of its product. Commonly, a “good” oil (i.e. obtained from healthy drupes collected at the optimal maturation stage) contains about 98% of acyl lipids while the remaining 2% includes several minor component such as polyphenols, esters, terpenes, chrorophyll, tocopherols, phylloquinones, vitamin K and unsaponifiable volatiles. The monounsaturated oleic acid represents the main component of lipidic fraction (up to 80%).

Other major fatty acids are the polyunsaturated linoleic acid (2.5–20%) and the saturated palmitic acid (10–20%).

Certainly, a strong impact on the perception of olive oil quality is played by sensory testing which in turn is tightly related, on one hand to a well-balanced composition of fatty acids and, on the other, to the profile of minor components. Chief among these components are different volatiles responsible for characteristic aroma which make olive products unique among other oils. Moreover, olive oil quality strongly relies on the presence of antioxidant compounds, such as polyphenols and tocopherols, which ensures the stability of fatty acids, acting as an effective scavenging system of reactive oxygen radicals and thus largely reducing lipid peroxidation.

Taking into account the above features it is clear that oil extraction process is rather relevant for preserving oil sensory properties and a high oxidative stability during storage . So far, olive oil is the only major dietary oil extracted by pressing rather than by solvent extraction. Therefore, no harsh treatment (high temperature, organic solvents and removal of these by evaporation) is generally applied before olive oil is ready for human consumption. This allows olive oil to maintain the integrity of its minor constituents, such as polyphenols, tocopherols and volatile compounds, which are partly water-soluble and, as above mentioned, play a key role in conferring oxidative stability and sensory properties.

It is worth noting that olive oil is a predominant and relevant component of so-called "Mediterranean diet". To day, a major attention is worldwide paid to this diet as a result of the even increasing evidences of its protective action against cardiovascular diseases and cancer, derived by both fundamental researches and epidemiological studies. Concerning olive oil, healthy property is related to the above mentioned high proportion of monounsaturated fats as well as to the high content of antioxidants (i.e. phenols, tocopherols, phylloquinones, vitamin K) (Green, 2002). Consistently with this role, worldwide consumption of olive oil multiplied 6-fold over the past 30 years.

Despite the relevant properties of olive plant products, breeding strategies for this crop species are still long term due to the long juvenile phase before flowering (20/25 years) and the inherent self-incompatibility. Currently, olive germoplasm is under an intensive survey but its characterisation is far from a clear definition, even though it has recently benefited by the availability of genomic polymorphic markers. Very recently, pyrosequencing technology provided large scale information on the structure and putative function of gene transcripts in relation to drupe development. Notwithstanding, the information on the genetic control underlying specific agronomic traits and/or physiological performance of olive plant is still limited. Hence, fundamental and applied research is required to improve olive breeding

programs. In particular, a wider characterization of genes related to both the quality of plant products and the adaptive mechanism to abiotic and biotic stresses could provide knowledge and tools for marker aided selection (MAS) and biotechnological approach, as well as for developing appropriate farming techniques leading to enhance productivity and quality of this unique oil tree species.

Aim and activities. In the context of the exposed item, in the present PhD project the attention has been focused on the genetic dissection of two relevant biochemical pathways, such as the biosynthesis of tocopherols and the lipoxygenase oxydation cascade along which different flavour metabolites are produced. The rational behind these focus relies on the link between the quality/ nutritional value of olive product and the high content of: i) tocopherols that, besides protecting plant under stress condition, confer exceptional stability to its products and exert beneficial effects on human health; ii) aroma profile which confer sensory properties and stability to plant products.

In particular, by using the approach of candidate genes the work was addressed to characterize in *Olea europaea* the following genes:

1) the *GERANYLGERANYL REDUTTASE* (*CHLP*) gene, encoding the NADPH-dependent geranylgeranyl reductase enzyme, that catalyses the formation of carbon double bonds in the phytolic side chain of chlorophyll (Chl) and tocopherols (TP);
2) the *ALCOHOL DEHYDROGENASE* (*ADH*) gene, encoding an enzyme that interconvertes aldehyde compounds, derived from the degradation of polyunsaturated fatty acids (linoleic and linolenic) into alcohols thus contributing to volatile chemical composition.

For both genes, named *OeCHLP* and *OeADH*, levels and pattern of gene expression were investigated by quantitative RT-PCR and *in situ* hybridisation. In order to functionally characterize *OeCHLP*, gene expression was monitored in fruits: i) at different developmental stages; ii) mechanically wounded; iii) infested by *Bactrocera olea* pest. *OeCHLP* expression was related to Chls and TP contents , estimated by Spectrophotomety and High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS) respectively. Likewise, *OeADH* gene expression was monitored in relation to quantity and quality of volatile compounds developed during fruit maturation, estimated by Solid-Phase Micro-Extraction method (SPME) in-line with a GC-MS ion trap Mass Spectrometer. To enlarge the information on the genetic control underlying aroma biogenesis in olive fruits the temporal expression pattern of

OeADH was monitored, jointly with that of *LIPOXYGENASE* (*LOX*) gene (ACG56281.1) acting in the same pathway.

Results and conclusion. Concerning *OeCHLP*, the obtained results showed that its encoded deduced protein is 51 kDa typically harbours a transit peptide for cytoplasm-to-chloroplast transport and a nicotinamide binding domain. Two identical copies of gene were estimated per haploid genome of the cv. Carolea used for gene identification. In line with the presence of ubiquitous TPs and/or Chls , *OeCHLP* transcripts were detected in various organs of plants. In drupes, *OeCHLP* was differentially expressed at the different developmental stages in relation to their specific morpho-physiological features. Interestingly, in drupes the expression pattern of *OeCHLP* fully matched whit the time-course of TP accumulations during ripening. Moreover, an early and transient enhancement of gene transcription was detected in drupe mechanically wounded. Moreover, *OeCHLP* strongly increased in the infested drupes and transcripts specifically localized in cell domains severely damaged by pathogen infestation. Globally, these data show that *OeCHLP* expression early responds to biotic and abiotic stressful factors. We suggest that in both cases gene activity could be related to the protective action of TPs against stress-related oxidative burst and therefore play a relevant role in avoiding the impairment of plant product quality.

[These results have been already published as "Role of geranylgeranyl reductase gene in organ development and stress response in olive (Olea europaea L) plants" by Bruno L., Chiappetta A.A.C., Muzzalupo I., Gagliardi C., Iaria D.L., Bruno A., Greco M., Giannino D., Perri E., Bitonti M.B.A. in *Functional Plant Biology* - 2009 - Vol. 36, pp. 370-381]

Concerning *OeADH* gene, our results showed that it encodes a deduced product of 41 kDa which exhibits a conserved multi-domain architecture. Genomic southern blot data were consistent with the presence of small gene family with at least two gene copies harboured per haploid genome of the cv. Carolea used for gene identification. Since the aroma imprint, expressed through well established and constant ratios of different flavourings, mainly relies on the genetic traits, gene expression levels and aroma-related profiles were investigated in drupes harvested at different developmental stages of two distinct cultivars: Carolea and Coratina. We found that both *OeADH* and *LOX* were transcriptionally modulated in relation to both fruit developmental stages and cultivar. In particular, early and sensible differences in the relative gene expression were detected between the two cultivars, while at the ripening stage expression pattern was quite comparable. As far as flavour profile is concerned, it resulted strongly differ between cultivars at the early developmental stages, while at the maturation phase a similar composition of selected volatiles was detected in the drupes of

both cultivars. Thus, the temporal variations in aroma profiles fully match with the differential regulation of gene expression detected over time during fruit maturation, suggesting that during the ripening of olive fruit, transcriptional modulation of selected genes play a role in aroma biogenesis.

[These results have been recently submitted as a paper "Interfacing between the expression pattern of two related genes in the lipoxygenase pathway and aroma biogenesis in olive (*Olea europaea* L.) fruits" by Iaria D.L., Bruno L., Chiappetta A.A.C., Macchione B., Tagarelli A., Sindona G., Bitonti M.B.A. to *Journal of Agricultural and Food Chemistry*].

In conclusion, on the basis of our results both selected genes features as putative good functional markers for distinctive fruit characteristics (i.e. stability/nutritional value and flavour) to be validate as a tool for olive cultivar selection and breeding program.

Aim of work

Olive (*Olea europaea* L.) tree is one of the oldest and most important crop species in the world and about 96% of olive cultivated area is within the Mediterranean basin. The nutritional value of olive products, oil and table olives, can be traced to some specific feautures such as a well-balanced composition of fatty acids and a high content of various antioxidant compounds. In particular olive oil contains small amounts of palmitate while being highly enriched in the monoenoic oleic acid. This makes olive oil fairly stable against auto-oxidation and suitable for human health. Nevertheless, it is the presence of minor components, such as phenols, tocopherols and various volatiles, contributing for oil's high oxidative stability, colour and flavour, that makes olive oil unique and with a high commercial value if compared with other plant oils. More specifically, phenols and tocopherols besides determining the sensory properties of products, have a direct beneficial effects on human health due to their antioxidant activities. Also unique to virgin olive oil is its characteristic aroma. This results mainly from the formation of volatile compounds, aldehydes and alcohols of five/six carbon atoms, which is triggered when olives are crushed during the process of oil extraction. Therefore, the quality of olive products is closely related to the amount of all these compounds which accumulate in the fruit during the development, at a rate and a level tightly depending on the variety and probably ecotype.

In this context, some distinctive biochemical traits of olive tree should be mentioned. *O. europaea* is one of the few species able to synthesize both polyols (mannitol) and oligosaccharides (raffinose and stachyose) as final products of photosynthetic CO_2 fixation. These carbohydrates, together with sucrose, can be exported from leaves to fruits to fulfil cellular metabolic requirements and act as precursors for oil synthesis. Additionally, developing olives contain active chloroplasts capable of fixing CO_2 and thus directly contributing to fruit metabolism during its development and ripening.

Conceivably, the overall quality of table olives and olive oil is influenced by the stage of fruit ripening which is a combination of physiological and biochemical changes and is influenced by environmental and cultural conditions, even if most events are under a strict genetic. On the other hand, also the productivity of olive plants is under the control of endogenous genetic factors and environmental conditions that strongly impact on flowering time and fruit set as well as on the susceptibility to pest/pathogen attack. The identification of olive cultivars that not only comply with the requirements of productivity and quality but also show resistance to biotic/abiotic stressful conditions, could, therefore, optimize production strategy by reducing treatments for pest control. Hence, the choice of both olive cultivars and

cultivation area are crucial parameters for developing a modern olive-culture, which aims to obtain excellent products, healthy for humans while simultaneously preserving environment quality.

However, genetic entities of Italian olive heritage have not yet been fully characterized so that their utilization does not fulfil the above mentioned requirements. Furthermore and more importantly, there is little information on the genetic pathways that control specific agronomic traits and/or physiological performance of olive plant. So far, this prevented the identification of functional markers to be used in both assisted breeding strategies and in the selection of appropriate farming techniques to increase productivity and quality. On this basis, a wide characterization of genes related to both the quality of plant products and adaptive mechanism to different stresses is strongly required.

Recently, in order to contribute to the exposed item, at the laboratory of Plant Physiology of University of Calabria, the research group of prof. M.B. Bitonti started a study dealing with the genetic dissection of tocopherol biosynthetic pathway and lipoxygenase oxidation cascade in *Olea europaea* L species. As integral part of this study, the present PhD project was focused on the structural and functional characterization of *GERANYLGERANYL REDUTTASE* (*OeGGH*) gene, involved in the biosynthesis of tocopherols, and *ALCOHOL DEHYDROGENASE* (*OeADH*) gene, involved in the biogenesis of aroma-related volatiles through the lipoxygenase oxidation cascade. The rational behind this focus relies on the link between quality/nutritional value of olive products and the high content: i) of tocopherols that, besides protecting plant under stress condition, confer exceptional stability to its products and exert beneficial effects on human health; ii) of different flavour metabolites which confer sensory properties and stability to plant products.

Chapter 1

Botanical and agronomic features of *Olea europaea* L.

1.1 Taxonomy, cytogenetics and germplasm data on olive

O. europaea is a member of the *Oleaceae* family. A biological classification is here reported:

Kingdom: Plantae

Division: Eudicots

Clade: Asterids

Order: Lamiales

Family: Oleaceae

Tribe: Oleeae

Genus: *Olea*

Species: *europaea*

Oleaceae family is a medium-sized family which includes 600 species within 25 genera distributed over all continents, with the exception of Antarctide, spanning from northern temperate to southern subtropical regions and from low to high altitudes [1]. On the basis of several morphological synapomorphies, *Oleaceae* family is considered as monophyletic and is easily circumscribed. The phylogenetic relationships among the recognized family genera have been assessed by a cladistic analysis of DNA sequences from two non-coding chloroplast loci, the *rps*16 intron and the *trn*L-F region [1]. The family's members are trees, shrubs or woody climbers with opposite, simple or compound leaves without stipules. The flowers are hypogynous and tetramerous, generally with two stamens while in some species flowers exhibit four stamen; the corolla is actinomorphic and usually sympetalous. From a botanical perspective, the olive tree is the only species, among over 600 that make up the *Oleaceae* family, which produces edible fruits.

Its has long been commonly accepted the distinction of *Olea europaea* species in two subspecies: *sativa* Loudon (communis) and *oleaster* Hoffmgg et Link (sylvestris) corresponding respectively to cultivated and wild forms [2-3]. The first covers all varieties of cultivated olive tree, the second subspecies collect shrubs or trees characterized by small rounded leaves, with small fruits exhibiting rather large pits and low oil content. However, according to the latest revisions of the *Olea europaea* L. taxonomy [4-5] based on morphology and geographical distribution this species is divided into six sub-species:

1) subsp. *europaea*, with the varieties *europaea* (common olive) and s*ylvestris* (wild olive), widely spread throughout the Mediterranean basin;
2) subsp. *cuspidata*, distributed from Southeast Asia to South-West of China, but also in the Arabian peninsula and in South Africa;

3) subsp. *laperrinei*, present only in the Sahara region;
4) subsp. *Maroccan*, present in Morocco;
5) subsp. *cerasiformis*, found on the island of Madeira;
6) subsp. *guanchica*, present in the Canary Islands.

Despite controversial opinions on the classification of the olive persist, varieties are almost all diploid with genome 2n = 46 and a genome size of about 1,800 MB [6-7]. Occasionally, examples of triploid and tetraploid plants and one case of polisomaty (2n = 55) have been reported [8]. The chromosome number of *Olea europaea L* was first determined by Breviglieri e Battaglia [9]. Based on chromosome morphology these authors suggested that this species probably originated by allopolyploidy from parents with haploid chromosome number n = 11 and n = 12, respectively. Recent cytological studies led to the conclusion that *Olea europaea L* evolved most likely by hybridization with subsequent chromosome doubling [8].

Currently, olive germoplasm is under an intensive survey and to assess genetic changes or stability between/within different olive genotypes/ ecotypes both phenotypic identification and DNA fingerprint are used. Phenotypic identification is based on the analysis of morphological and physiological characters and is the largely used even though it requires major observations of plants until the adult stage. In addition, genetic changes induced by *in vitro* culture can not be detected through phenotypic identification because they do not always affect plant phenotype. On the contrary DNA fingerprint allows one to detect whatever genetic change. Today different DNA markers are available, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) which enable plant characterization through a specific molecular profile [10].

Although many studies have been performed on plastid polymorphisms to evaluate taxonomy and phylogeny of *Olea* subspecies, only few polymorphic regions discriminating among the agronomically and economically important olive cultivars have been identified [11-12-13]. Thus, even though it has recently benefited by the availability of genomic polymorphic markers, olive characterisation is far from a clear definition. Within the several olive varieties numerous cases of synonymy (different name same genotype), and homonymy (same name different genotype) still occur. This makes rather difficult their identification and distinction. In many cases with "cultivar-population" is indicated the presence of multiple clones within the same variety, which differ in a more or less wide number of characters [14-15-16]. These clones can also correspond to ecotypes of the same variety which originated

occasionally from seed propagation followed by vegetative propagation. To date, approximately 2600 varieties have been estimated, including clones and subclones [17].

Recently, some specific chloroplast regions such as the two-locus combination of *rbc*L-*mat*K have been indicated as ideal for use in tests that discriminate between different land plants and recommended as a universal framework for plant barcoding [18-19]. The combination of *trn*H-*psb*A coupled with *rbc*L has been recommended for DNA barcoding to discriminate between lower taxonomic ranks such as genera or related species [20]. In highly valuable crop species, such as the olive, that have a variety of cultivars available in the market, however, typing at the species level is not sufficient. However, the availability of complete chloroplast genome sequences from a growing number of species offers the opportunity to evaluate many potentially polymorphic sites and identify new regions that could be used to define cultivar DNA barcodes. In addition to identifying mutations that may play a functional role in modifying the metabolism and adaptation of olive cultivars, the new chloroplast markers represent a valuable tool to assess the level of olive intercultivar plastome variation for use in population genetic analysis, phylogenesis, cultivar characterisation, DNA food tracking [21] and analysis of archaeological remains [22].

Thus, the development of reliable methods to rapidly and efficiently discriminate between cultivars has become a pressing need to fully turn to account olive collections present in all the main olive-growing countries such as Italy, Spain, France, Morocco. In Italy, many cultivated olive genotipes are catalogued on the Schedario Olivicolo (390 genotypes) [23] and others by FAO (538 genotypes) [24]. Italian olive germplasm is collected in different Italian regions and one of the largest collections is that of the CRA-OLI - Centro di ricerca per l'olivicoltura e l'industria olearia - in Rende (CS), currently constituted by over 500 Italian varieties. CRA-OLI collection also includes 50 varieties from other Mediterranean countries [25-26-27].

1.2 Morphology and biology of the olive

Olive is an evergreen tree with strong xerophyllous characters [28] which make it to be a thermopile and moderately drought- and salt- tolerant species. Olive plant is medium-sized (4-8 m high) but can reach and exceed 15 m in height and 1.5-2.0 m in diameter depending on the cultivar, environmental and cultural conditions. An example, widely known, is represented by olive trees growing in the area of Gioia Tauro (R.C., Italy) which exhibit a very big size and longevity as proved by the presence in the field of numerous ancient plants.

The natural shape of olive canopy is generally rounded or spherical, fairly dense, and over the years it turns into a hemispheric profile, with an expansion of the crown in a radial

direction rather than in height [29]. The roots, mostly adventitious, are very superficial, expanding generally not beyond 60-100 cm depth. The trunk is often twisted, cracked and empty, with a more expanded bottom (ciocco) from which suckers with shaped bulges (ovoli) easily arise. The leaves are opposite, oval / elliptical in shape, leathery, glossy green and whitish-gray on the adaxial and abaxial surface respectively. However, differences in leaf shape and size can be observed in relation to age, growth and environmental conditions. In general, in the juvenile phase, when plants undergo vegetative development but are not yet competent to flower, leaves are clearly different from those produced in the adult one. However, leaf polymorphism is evident only in trees that originate from seeds, whereas trees obtained by vegetative propagation do not produce the real juvenile form of leaves [30]. As far as the polymorphism related to environmental conditions is concerned, we may recall that the leaves of one branch can change shape and size in relation to the time of year when they are formed [29]. The flowers are hermaphrodite with a calyx formed by 4 sepals and a corolla formed by 4 greenish-white petals. Flowers are grouped in clusters called "mignole". Only a small percentage of flowers turns into fruits due to a high frequency of flower abortion.

The fruit is a drupe, variable in size (10-20g in weight), mostly oval in shape and its colour first green turns to purple-black during ripening. According to the different colour and size, which correspond also to a specific developmental stage, drupes are generally indicated as: immature green or herbaceous (about 70 DAF - days after flowering - depending on varieties and climatic conditions), turning black (about 180 DAF) ripened (about 200 DAF) and over ripened (over 250 DAF). Generally, mature drupe exhibits: a thin epicarp (1.5-3.5 % f.w), a deep mesocarp commonly named oily flesh or pulp (70-80% f.w.) and a lignified endocarp or pit (15-25% % f.w). Pulp adheres strongly to the pit surrounding the seed which accounts for the remaining 2.5-4 % of fruit fresh weight .

Olive plant exhibits a long juvenile phase and begins to flower and produce fruits when it is about 20-25 years old; plants obtained by in vitro propagation begin to produce fruits when are 3rd-4th year old, reaching a good production at the 9th-10th year and becoming full productive after 50 years. Flower bud differentiation requires high light intensity, so that in most varieties fruits are mainly localized on the surface of the crown [31]. Along the whole life cycle, olive plant produces fruits at one of every two years and requires specific horticultural practices (i.e. organic and mineral fertilization during the winter) to minimize or suppress this feature. This alternate fruit production of olive plant is related to endogenous metabolic factors and consequent annual growth rhythm consisting in that plant vegetative growth and fruiting are mutually exclusive. In other words, in the years in which

plant productivity is high, vegetative growth is very limited and thus, in the following year, its level need to be elevated before a new fruiting can be settled. Flowering and seed establishment assure sexual reproduction of olive plant. However, due to both its long juvenile phase and the necessity to keep constant olive varieties, standard grafting onto a rootstock as well as propagation by cuttings, suckers or ovules are frequently adopted.

Olive growth and consequent farming practices are strongly influenced by two peculiar features of this species such as the branch elongation pattern and a large bud complex. In particular, branches are characterized by relatively long internodes, small diameter and slow lignification process. Therefore, due to the weight of both leaves and fruits, branches bend themselves, letting other buds to be illuminated and become dominant, thus opening and producing new branches. Concerning bud complex, it consists of numerous latent buds and hyperplastic clusters of adventitious buds (sferoblasti), which exhibit a high morphogenetic potential. The ability of olive plant to reconstruct new stems and roots with juvenile traits is just due to these adventitious morphogenetic structures which also allow a drastic rejuvenation pruning [29].

1.3 Cycle and phenology of fruit development

Olive tree does not exhibit classical winter dormancy and undergoes continuous vegetative development even though growth rate is greatly slowed under cold conditions, which can impend on the plant for a period tightly dependent on climatic state. During this period any axillary bud of the previous year branches has the potential to generate a new shoot. Usually, starting from February and throughout March olive plant undergoes an intense vegetative activity with the growth of sprouts, followed by flower induction and the issue of mignole, a phase that according to geographic region lasts until April. Mignole formation has its climax in the middle of spring with the achievement of the final size. The inflorescences are still closed but flowers are fully formed. After flowering, which occurs in spring, plant start fruit set process (allegagione) approximately at mid-June. However, the percentage of flowers that undergoes this process is generally very low (less than 5%) since an abundant and early flower fall (colatura) usually occurs. Any drop in temperature as well water deficiency and hot winds may also affect fruit set process. At the beginning of this process corolla withers and dries, persisting until the ovary swelling causes its detachment. After fruit set is established, drupes undergo to a first growth phase which stops when endocarp becomes lignified. This occurs at early-August Usually and the entire developmental phase is referred to as drupe *immature-green stage*. Thereafter drupe growth relies only on the increase of mesocarp.

In non-irrigated areas, seasonal rains occurring from mid-August through the end of September strongly influence the size of drupe which is still green and photosyntetically very active. This phase is referred to as drupe *mature-green stage*. From October to December, depending on the cultivar, fruit ripening takes place and drupe colour progressively changes due to the synthesis of anthocyanins, spanning from a spotted green-yellow/black to total black feature, indicating that maturation is fully accomplished. Thus ripening includes two subsequent developmental stages referred to as drupe *turnig-black* and *black-ripened* stages. Note that the ripening is more or less gradual within the same plant and from plant to plant .

After ripening, dupes persist on the plant, also until the following spring but plant could suffer a more or less intense fruit drop. During this period referred to as drupe *over-ripened* stage, drupes undergo a progressive loss of water leading to a relative increase of oil yield but product quality rapidly declines due to the rise of oxidative processes [32]. Therefore, for good oil production it is advise to collect drupes as soon as they reached full ripening, preferably by hand picking, which prevents fruit damage as is the case of beating; picking up or shaking practices are also applied [33].

1.4 Chemical composition and organoleptic properties of olive products

Genetic traits, climatic factors such as temperature and water availability (rainfall or irrigation) and light and soil composition strongly influence not only fruit development and growth but also its chemical composition. Moreover chemical composition changes over time during fruit development and ripening .

1.4.1 General picture

As a very general picture, in mature drupe the flesh exhibits a very complex matrix which contains water, oil, carbohydrates, minerals, vitamins, proteins and pigments. In particular, unlike other drupes, olive fruits are rich in oil and low in soluble sugar. Moreover, distinctive features of olive drupe are a high content in phenolic compounds and the presence of unique bitter glicoside, oleuropein, whose level is markedly reduced by hydrolysis during fruit processing.

The water-soluble components are confined in cell vacuole, whereas early during drupe development oil is accumulated in oil-bodies (oleasomes) that coalesce against the vacuole over fruit maturation. The water content of olive flesh is around 60-70% w/w. Therefore the lower the water content of olive flesh is, the higher is its nutritive value. Water

content of drupe is responsible for its turgidity, but fruits can shrivel under extreme conditions or when undergo senescence.

About oil, its percentage depends on the variety, drupe maturation stage and growth conditions. Most of the oil fraction consists of triacylglycerols (98%), with the remaining fraction including diglycerides (1.1%) and free fatty acid (0.3%). Other oil soluble compounds in drupe flesh include sterols and tocopherols (vitamin E). Indicative ranges of main fatty acids in the flesh oil fraction at maturity stage are: oleic acid 70-80% , linolenic acid 1.5%, linoleic acid 5-10% , palmitic acid 10-15% and stearic acid. 2-3% .

Total carbohydrates in drupe flesh amount to 8-12% w/w and include soluble sugars and sugar polymers. The main soluble sugar are glucose, fructose, sucrose which are utilized in metabolic processes and mannitol which represents the only transported sugar. The amount of soluble sugars in drupe flesh ranges from 0.5% to over 5% w/w with glucose representing the predominant component. Soluble sugars level decreases as the olive fruit develops and oil synthesis begins. Sugar polymers include cellulose, hemicellulose or pectins and lignin. Hemicellulose and cellulose are structural component of cell wall and therefore contribute to the structural characteristics of drupe flesh; changes or reduction in these polysaccarides during ripening or processing can influence the organoleptic features of drupes destined to direct consume as table olives.

Phenolic compounds, also called polyphenols, are secondary metabolites which are present in plant tissues and play a key role in protective mechanisms against various stressors due to their antioxidant activitiy. In olive drupes polyphenols make up 2-3% w/w of drupe flesh and include oleuropein, hydroxytyrosol, caffeic acid and tyrosol. Among them oleuropein is the most abundant component.

Olive drupe flesh also contains low levels of soluble and insoluble proteins (1.5% w/w). Further components are water- or oil-soluble vitamins. In general, water-soluble vitamins, such as vitamin C, B1, B2 and B6, are mostly lost during drupe processing whereas the oil-soluble ones, including vitamin A and vitamin E, are retained.

1.4.2 Dynamic of chemical composition during fruit development and ripening

As previously mentioned chemical composition undergoes progressive changes over fruit development and ripening. The most important variations occurring in drupes at the main developmental stages are below described:

Immature and Mature Green Stage

The drupe, green and firm, is still photosynthetically active and soluble sugars reach their maximum concentration. The fats begins to accumulate in the form of triglycerides, following a dynamic which differs in relation to the cultivar and environmental conditions. Moreover, differences deal not only with the percentage ratio between different lipids but also with their fatty acid composition. At this stage, linoleic, palmitic, lauric and myristic acids are the most important component of lipid fraction. The amounts of polyphenols, tocopherols and squalene are also high [34-35].

Turning-Black Stage

The drupe begins to change colour because chlorophyll breaks down in pheophytin and phytin, and thus carotenes appearance turns fruits into a yellowish colour. With the fading of photosynthesis, the fruits accumulate abundant lipids in which oleic acid is the main fatty acid [35].

Black-Ripened Stage

At this stage, drupe has fully accomplished its ripening and oil, expressed as a percentage value of dry matter, reaches the highest level. However, during oil accumulation, the biosynthesis of fatty acids progressively decreases, with the highest effect on oleic acid. Other compounds such as oxalic acid and citric acid increase over time [36-37]. As far chlorophylls (a and b), carotenoids (β-carotene) and xanthophylls (lutein, violaxantin and neoxantin) are concerned, it is difficult to exactly follow the dynamic of these pigments during drupe maturation [38]. Although they are always present in the drupe, chlorophyll and carotenoid levels decrease during maturation while xanthophylls amount increases mainly as esterified compounds.

Over-Ripened Stage

This stage refers to the physiological processes that set in drupes that remain attached to the plant after ripening. During this phase several effects can be observed such as: water loss, reduction of respiratory activity, increase of pectolytic enzyme activity leading to cell wall, loss of natural antioxidants (polyphenols and tocopherols), triglyceride degradation, increased acidity [39-40]. All these effects drastically impair organoleptic features of drupes.

The described dynamic of chemical composition in the drupe is a relevant aspect for selecting the optimal harvesting time and assuring not only the quantity but also the quality of oil production. Therefore, it is important to choose the period corresponding to an optimal oil accumulation in the drupe together with a balanced ratio between antioxidants and aroma-related compounds, such as to obtain a product stable over time with a specific flavour and

taste. In absence of precise benchmarks, it is very difficult to determine this stage, even though several parameters have been taken into account to define a simple and reliable maturation index [40-41-42-43-44]. These parameters include: drupe growth, drupe colour, fruit respiration rate [45], changes in levels of fatty acids and their mutual ratios, total anthocyanins/tannic polyphenol and oxalic acid/citric acid ratios, dynamics of polyphenols, anthocyanins and sterols, free acidity and absorbance at 447 nm [44].

1.4.3 Effects of environmental conditions and agronomic practices

As previously mentioned environmental conditions, such as temperature, water availability, light and soil composition strongly impact on fruit growth and quality. First of all, the levels of most representative fatty acid are closely correlated with latitude and altitude: olive oils, from the southern areas have a higher percentage of saturated fatty acids than oil from the north; on the other hand the relative content of unsaturated fatty acids (oleic, linoleic, linolenic) is higher in oils deriving from areas at high than low altitude [46-47]. It is largely known that an adaptive response of plant to low temperatures consists in the modification of lipidic composition of cell membrane through an enhanced synthesis of unsatured fatty acids which reduce membrane fluidity [48]. In line with this adaptive mechanism, the drupes produced by plants growing in cold environments (i.e. high altitude and latitude), although less rich in oil, provide a product of higher value for the highest percentage of unsaturated fatty acids [49]. On the contrary, total polyphenol content of drupe is positively modulated at low altitudes [47].

With regard to water availability, the comparison between [50] oils produced by irrigated and un-irrigated farms showed that oils from non-irrigated farms were more rich in monounsaturated and polyunsaturated fatty acids than oils from irrigated farms which instead exhibited a higher relative content of saturated fatty acids. As far as the effects of water availability on total polyphenols content, literature data are somewhat discordant. Indeed, some authors state that polyphenol content decreases with increasing irrigation rate, other authors state that it is higher following irrigation with low intake [50-51-52] and some others found an increase (16%) of total polyphenols in drupes collected from plants grown in irrigated groves [53-54].

Light is an other important factor in that, besides the formation of chlorophyll and anthocyanins, its action through photosynthesis controls the biosynthesis of carbohydrates.

Moreover, light regulates stomatal aperture thus influencing transpiration rate and, indirectly, nutrient availability .

Wind and air humidity also impact on the productivity of olive plants: moderate winds during flowering favour pollination with repercussion on the phenomena of alternate production. Concerning air humidity, it affects transpiration rate and may favour the occurrence of pest and fungal diseases which cause severe damage to plant production at both quantitative and qualitative level. Finally, a good correlation has been found between phosphorus and nitrogen contents in the soil and sterols, squalene, oleic acid levels in the drupe [55].

In addition to environmental conditions which act directly on the stage of fruit ripening, also the farming practices such as pruning, fertilization, irrigation and pest management can exert an indirect impact by influencing the physiological response of the plant. For example it ha been observed that nitrogen-rich fertilizers delay drupe ripening and enhance the formation of oleic acid and stearic acid [56]. However it must be underlined that synergy or competition can settle between the different practices thus making still very difficult to quantify the real impact of each of them [57]. This event could account also for the discordant data above cited dealing with the effect of water irrigation.

1.4.4 Olive oil as component in Mediterranean diet

Olive oil is a predominant and relevant component of so-called "Mediterranean diet". Currently, an even increasing attention is worldwide paid to this diet as a result of the growing evidences of its protective action against cardiovascular diseases and cancer, deriving from both fundamental researches and epidemiological studies. Concerning olive oil, its healthy property is related to the above mentioned high proportion of monounsaturated fats as well as to a high content of antioxidants such as phenols, tocopherols, phylloquinones, vitamin K [5].

In particular, concerning the positive effects of olive oil on cardiovascular diseases, there is good evidence that the high relative content of oleic acid contributes to decrease low-density lipoprotein (LDL) and increase high-density lipoprotein (HDL) levels in the blood [58-59-60]. That is extremely important, since LDL deposits cholesterol in the arteries, thus increasing atherosclerosis process which represent a cardiovascular risk, whereas HDL carries cholesterol away from the arteries to the liver. Similar effects are induced by a-linolenic acid (which is an omega-3 fatty acid) and by certain phenolic compounds present in olive oil.

In this context, it is worth underlining that cancer incidence is significantly lower among the Mediterranean populations than in the rest of Europe and United States. Interestingly, this result has been related to the high dietary intake of plant foods and olive oil by these populations [61-62-63-64]. So far, which component of olive oil exerts this effect is not precisely known but oleic acid seems to be at least partially involved. Indeed, through studies on animal diets it has been verified that oleic acid prevents the over expression of HER2 (Her-2/neu, erB-2), a well-characterized oncogene that plays a key role in etiology, progression and response to chemotherapy and endocrine therapy, in approximately 20% of breast carcinomas [64-65].

There is, also, an increasing interest in the phenols present in olive fruit and oil, for their possible beneficial implications on human health due to their demonstrated action in the prevention of cancer and cardiovascular disease. Phenols exhibit intrinsic biological properties as antioxidant compounds and free radical scavengers, and contribute to the colour, flavour and high oxidative stability of plant final product (i.e.oil) during storage [66-67-68]. Recently, it has been demonstrated that polyphenol presents in virgin olive oil can modify the expression of atherosclerosis-related genes, leading to cardiovascular health benefits. Results also show that olive oil consumption in conjunction with a Mediterranean diet can positively impact lipid and DNA oxidation, insulin resistance, inflammation, carcinogenesis and tumour suppression [69].

Consistently whit this claimed protective role, worldwide consumption of olive oil multiplied 6-fold over the past 30 years [67-68-69].

1.5 Breeding strategy and functional genomic in *Olea europaea*

Despite the high impact of *Olea europaea* L. production on world-wide economy, breeding strategies for this crop species are still long term due to the long juvenile phase before flowering (20/25 years) and the inherent self-incompatibility. Currently, a certain number of genomic polymorphic markers is available to breeders but the characterisation of olive germoplasm is far from a clear definition [11-12-13]. Moreover, knowledge on the genetic control of molecular/cellular/organismal events and physiological process underlying olive growth and development is still very limited while representing a prerequisite for improving plant productivity and quality of its products. In particular, understanding gene regulatory network controlling key metabolic pathways during fruit development represents an essential goal for breeding strategies aiming to improve quality and healthy properties of olive oil.

To identify and characterize genes involved in these and many other processes, different genomic approaches (ESTs, large-scale microarrays, deep transcriptome profiling, 454 pyrosequencing technology etc.) have been used in several fruit species [70] and the body of information dealing with transcriptional networks and regulatory circuits involved in physiological and developmental processes increased massively during the last two decades. In tomato, knowledge of molecular mechanisms of fruit ripening and identification of transcription factors not previously associated with ripening was achieved through a large-scale EST sequencing projects [71-72]. Other extensive EST sequencing projects or extensive analysis of all the EST sequences available in public databases have been set up in grape berry [73-73] peach [75], melon [76], kiwifruit [77] and apple [78]. Recently this approach has been applied also in olive and a large sets of genes differentially expressed in developing fruits have been annotated [79-80]. Among them, genes involved in fatty acid biosynthesis as well as in the synthesis of different phenolic compounds and mainly in oleuropein synthesis were included [79-80].

Notwithstanding, so far in olive only few genes have been characterized in full details: one gene (*OeMST2*) [81], encoding a monosaccharide transporter whose expression increases during fruit maturation and other six genes, all related to fatty acid metabolism. More specifically, these latter include two *FATTY ACID DESATURASE* (*FAD2*) genes which encode microsomal oleate desatures responsible of fatty acid's desaturation. It has been demonstrated that these two genes exhibit a differential function in seed and fruit , respectively [82], while genes involved in acyl chains elongation still remain unknown [83-84-85-86-87]. Other data deal with one type-1 (LOX EC1.13-11.12) and two type-*2 LIPOXYGENASE* genes (*Oep1LOX2* and *Oep2LOX2*) which encodes enzymes responsible of regio- and stereo-specific dioxygenation of polynsatured fatty acids. It has been evidenced a differential role for these genes, with a major involvement of type-1 *LOX* in fruit senescence and *Oep2LOX2* in aroma biogenesis [88-89]. Very recently, a 13-*HYDROPEROXIDE LYASE* (*OepHPL*) encoding an enzyme which catalyzes the cleavage of polynsatured fatty acid hydroperoxides into aldehydes and oxacids and therefore involved in aroma biogenesis, has been also cloned [90].

Based on the recent availability of large set of ESTs provided by pyrosequencing results [79-80] it is hopeful that functional genomic characterization of olive could be rapidly enlarged providing new relevant tools for molecular aided breeding of this relevant crop species.

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Chapter 2

Structural and functional characterization of *Olea europaea GERANYLGERANYL REDUCTASE* gene (*OeCHLP*) involved in tocopherol biosynthetic pathway

2.1 Introduction

In this section, work was addressed to structurally characterize in *Olea europaea* L. the *GERANYLGERANYL REDUCTASE GENE* (*CHLP*), which encode the enzyme geranylgeranyl reductase, also named CHLP referring to the unit P of prokaryotic chlorophyll-synthase [1-2]. This enzyme is responsible for catalysing the formation of carbon double bonds in the phytolic side chain of chlorophylls (Chls) and tocopherols (TPs) and phylloquinones (PQs), thus, providing a potentially limiting substrate for the biosynthesis of these compounds [1-2-3]. In addition, the expression pattern of this gene and its relationship with Chls and TPS action has been investigated during drupe development and ripening as well as in fruits exposed to biotic/abiotic stress .

2.1.1 Chlorophyll: role in plant metabolism

As largely known Chls are the main pigments involved in photosynthetic process on which relies the whole plant metabolism. In the process, a specific range of energy (PAR, Photosynthetic Active Radiation) from sunlight is used for the conversion of carbon dioxide into organic compounds, especially sugars. During light-reactions process, Chls and other pigments present in the chloroplasts of photosynthetic tissue absorb PAR and conserve it as ATP and NADPH; simultaneously, O_2 is evolved. In the carbon-assimilation reactions, ATP and NADPH are used to reduce CO_2 to form triose phosphates, starch, and sucrose, and other products derived from them.

Chls are green pigments with polycyclic, planar structures resembling the protoporphyrin of hemoglobin, whit the exception of Mg^{2+}, not Fe^{2+}, occupying the central position. Chl molecule consists of two portions: a tetrapyrrole ring with four inward-oriented nitrogen atoms coordinated with the Mg^{2+} and a long phytol side chain, esterified to a carboxyl-group substituent in ring IV. The heterocyclic ring system that surrounds the Mg^{2+} has an extended polyene structure, with alternating single and double bonds. Such polyenes characteristically show strong absorption in the visible region of the spectrum and therefore Chls have unusually high molar extinction coefficients and are particularly well-suited for absorbing visible light during photosynthesis. The phytol side chain favours the insertion of chlorophyllmolecule into thylakoids membranes.

The biosynthesis of Chls involves two cellular compartments (cytoplasm and chloroplast) and occurs through two different pathways :

1) the tetrapyrrole pathway in which chlorophyllide *a*, a molecule bearing a tetrapyrrole ring with a magnesium atom in its centre, is synthesized starting from the precursor 5-

aminolevulinate [4-5-6-7];

2) the isoprenoid pathwayin in which isopentenyl pyrophosphate (IPP) represents the starting precursor for the synthesis of phytol side chain;

In the second pathway the final product is the 20C polyunsaturated geranylgeranyl pyrophosphate (GGPP) which can be either esterified immediately with chlorophyllide through chlorophyll synthase action to give geranylgeranylated chlorophyllide (ChlideGGPP), or converted into phytyl pyrophosphate (PhyPP) prior to be joined to mature chlorophyll [1]. The enzyme responsible of this reaction is geranylgeranyl reductase (CHL P) (EC 1.3.1), that in a NADPH dependent manner [2-3] reduces double bonds of GGPP at positions 6, 10 and 14 [8], converting it into mature phytol chain that confers hydrophobicity to chlorophylls [9]. In plants, in normal light exposure condition GGPP is reduced before condensation with chlorophyllide because PhyPP is the preferred substrate to the chlorophyll synthase [2-10]. In etiolated plants and during the first stages of leaf differentiation, ChlideGGPP is formed and then PhyPP synthesis occurs [11-12-13-14].

Terrestrial plants exhibit only two different types of chlorophyll: chlorophyll a and chlorophyll b, which differ respectively for a methyl group and a formyl group in the tetrapyrrole ring, respectively. But whithin algae, there are other variants: chlorophyll c1, c2, c3, and d in red algae (Rhodophyta). Chlorophyll, like all other photosynthetic pigments and all other components of the photosynthetic apparatus, are associated with special membrane systems, called photosynthetic membranes and orgaized as multimolecular complexes named photosystems.

In this context it must be underlined some specific features of carbon metabolism in olive and in particular in olive fruit. Developing drupe contains active chloroplasts capable of fixing CO2 and thus contributing to the carbon economy of the fruit Additionally, *O. europaea* is one of the few species able to synthesize both polyols (mannitol) and oligosaccharides (raffinose and stachyose) as final products of the photosynthetic CO2 fixation in the leaf. These carbohydrates, together with sucrose, can be exported from leaves to fruits to fulfil cellular metabolic requirements and act as precursors to oil synthesis.

2.1.2 Tocopherols: biological action in plants and impact on food value

TPs, commonly known as vitamin E, are compounds synthesized only by plants and cyanobacteria and, therefore, are essential components of animal diet [15]. So far, there are eight natural compounds with common chemical structure, having the biological activity of vitamin E. They are all derivatives of 6-chromanol with four methyl groups linked to aromatic

ring and a 16 carbon atoms isoprenoid side chain, saturated or unsaturated in position 2. Depending on the presence of a saturated or unsaturated chain, these compounds are divided into two groups: the tocopherols (α, β, γ, δ) and tocotrienols (α, β, γ, δ).

The biosynthesis of Tps involves only chloroplast compartment. The first step of TP pathway consists in the synthesis of isoprenic chain that will be joined to homogentisic acid, synthesized from p-hydroxyphenyl pyruvate by the enzyme p-hydroxyphenyl pyruvate dioxygenase (HPPD) [16]. Homogentisic acid is produced through shikimic acid pathway in which the precursor is the aminoacid tyrosine or the prephenate molecule [17]. After the joining of isoprenic chain, the homogentisate is condensed with PhyPP produced by the CHL P enzyme [18] and then converted into 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) [19]. The enzyme responsible of this reaction is named homogentisate phytyltransferase (HGPT) and it is rate limiting for tocopherols pathway [20]. Subsequently, MPBQ can follow two alternatives ways: a) conversion into δ-tocopherol [21] and then methylation to β-tocopherol, or b) methylation to α-tocopherol and then cyclization to produce γ-tocopherol [22-23].

TPs are produced by plants in a non-esterified form and therefore are unstable to light, being easily degraded by UV light, and to heat, although to a less extent; this instability is enhanced in presence of oxygen due to their anti-oxidant nature. The presence of TPs in plants is mainly related to the protection of the photosynthetic apparatus [24], playing an important role in the protection of PSII (Photosystem II) by photoinactivation [3-25]. This role of TPs in plant photoprotection is tightly related to their capacity, as antioxidant compounds, of quenching and scavenging reactive oxygen species (ROS) which are naturally and continually formed in plant body. ROS have strong tendency to donate oxygen to other molecules including all types of cell macromolecules and therefore, if not inactivated, their reactivity can cause cell damage [26]. The most famous chain reaction for free radicals is lipid peroxidation. Thus, the immediate damage, for cell metabolism, is the reaction of ROS with the acyl tails of PUFAs which reduces membrane fluidity [27]. Moreover, the photo-oxidation of lipids also contribute to the destruction of amino acids and proteins. The oxidized PUFAs, in fact, may induce cross-links in proteins [26]. Thus, as above mentioned, in photosynthetic tissues, TPs create an optimal environment for the photosynthetic machinery by protecting chloroplast membranes and photosystems from photo-oxidative damage [28-29]. However, as antioxidant compounds TPs are also involved in many other plant protection mechanisms, and indeed TPs accumulate in all organs of plants, especially seeds, which are often rich in lipid reserves, thus preventing the oxidation of polyunsaturated fatty acids [28]. Therefore, in seeds and fruits, TPs strongly contribute to product stability and post harvesting shelf life [28-30].

To the antioxidant action of TPs it is also related their healthy effect on humans. In this context it is noteworthy to recall that vitamin E was discovered in 1922 as a liposoluble factor able to prevent fetal and animal abortion and then turned out to be an essential nutrient for maintaining fertile laboratory rats. Later (1936), a factor exhibiting the same biological activity of vitamin E was isolated from wheat germ oil whose structure was determined in 1938 and called α-tocopherol. Since that time, other compounds with similar activities were isolated from many vegetable oils corresponding to tocotrienols (α, β, γ, δ) and β-, γ- and δ-tocopherol. However, since vitamin E deficiency is a rare condition, vitamin E was demonstrated to be essential for human only in 1968 [31].

The most active natural TP form is the α-tocopherol. Moreover, natural α-TP is represented solely by the D isomeric form which is more biologically active than L isomer present in synthetic vitamin E. Therefore, it is advised to use foods rich in natural TPs content rather than synthetic vitamin supplements as these are less effective [32]. As consequence, vegetable food rich in such compounds have a relevant nutritional value.

2.1.3 CHLP action in Chl and TP biosynthetic pathways

CHLP is a NADPH-dependent enzyme highly conserved in all photosynthetic organisms (bacteria, algae, plants) belonging to the family of hydrogenase [2]. GGH enzyme acts in reducing geranylgeranyl phosphate (GGPP), a plastidial isoprenoid, to phytil phosphate (PhyPP) by catalyzing the reduction of three of the four double C=C bonds present in GGPP, in position 6, 8 and 10, consuming three molecules of NADPH [8] (**Fig. 1**).

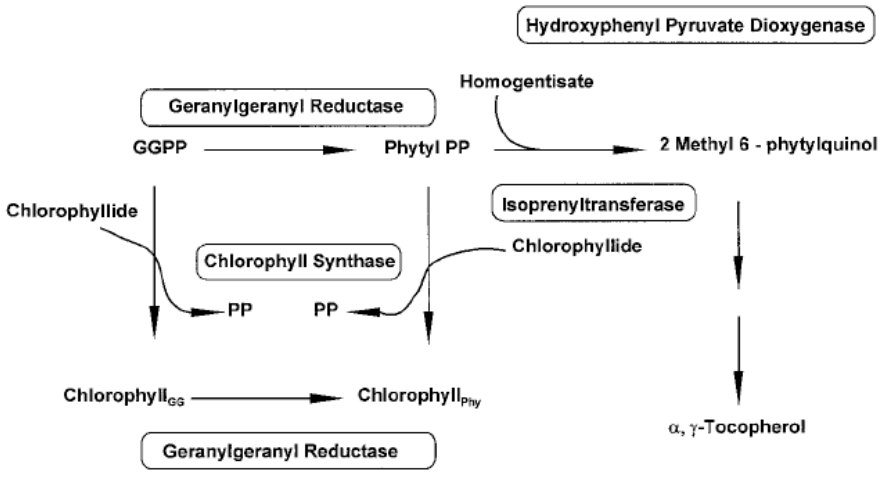

Fig. 1 Scheme of the branched pathway starting from GGPP to α-tocopherol, γ-tocopherol, or ChlPhy. CHL P, Chl synthase, and an isoprenyl transferase are indicated. CHL P uses GGPP and ChlGG as substrates and directs PhyPP to the tocopherol- and the Chlsynthesizing pathway.

GGPP and PhyPP are lipophilic molecules and promote the interaction with acyl chains of membrane fatty acids. PhyPP can be esterified with macromolecules including Chl, and TPs and represents the lipophilic tail needed for the integration of TP and PQ in the membrane and of Chl in protein complexes that constitute the photosystems [7-30-31-32]. [1-10-33-34].

In the pathway that leads to the synthesis of TPs, PhyPP is condensed to homogentisate, an intermediate of the shikimate pathway and then a series of methylation and cyclization of the quinol intermediate lead to the formation of TPs. In the metabolic pathway leading to phytil-Chl (ChlPhy) the GGPP can either be reduced by GGH to PhyPP and after esterified to Chl, or first prenylated to Chl to form geranylgeranyl-Chl (ChlGG) and then reduced to ChlPhy

GGPP presents a higher number of double bonds and is more rigid compared to PhyPP, thereby limiting the number of possible conformations of the isoprene tail in association with apoproteins to form the photosystems. Since double bonds are preferential target of free radicals, it is evident that Chl molecule esterified with GGPP will join in a more unstable way to the various proteins that form photosystems and will be potentially more vulnerable to light stress conditions which determine an increases of ROS presence [8].

Although the biosynthetic reactions leading to the formation of the ChlPhy and TPs take place respectively in the thylakoid membrane and outer membrane of plastids, it seems that in both cases the same enzyme presides over the catalysis. In vitro essay of CHLP catalytic activity in *Arabidopsis thaliana*, have highlighted its ability to reduce both the GGPP and ChlGG, demonstrating that the same enzyme can catalyze the synthesis of products in both metabolic pathways [2]. These results were corroborated by studies on transgenic tobacco transformed with antisense *CHLP* which showed a lack of TPs and a concomitant accumulation of ChlGG [3-24]. Probably due to its low hydrophobicity CHLP can easily be shared between the thylakoid membrane and the outer membrane of plastids and thus be recruited in both pathways [2].

2.1.4 Aim of the work

Taking into account the above discussed role of Chls and TPs, it is likely that transcriptional activity of GGH gene is tightly related to plant productivity as well as to product quality. Therefore it featured as a good candidate to enlarge the information on genetic pathways controlling physiological traits in olive plants . In this context we planned to characterize, at both structural and functional level, this gene in olive species, using Carolea cultivar, a widespread cultivated variety in Calabria region (Italy). The general aim was to

provide knowledge and putative tools for MAS and biotechnological approaches in this relevant agronomic species for which, as above discussed, breeding programs are still long-term even though it recently benefited by the availability of genomic polymorphic markers [35-36-37].

Here, we report important structural features of the *OeCHLP* (*Olea europaea CHLP*) gene and the *OeCHLP* expression pattern: i) in fruits at different developmental stages; ii) in fruits mechanically wounded; iii) in fruits infested by *Bactrocera olea* pest. The relationship of gene expression pattern with Chl and TP action has been also analyzed for understanding the general role of this gene in fruit development and stress response. The results of a conjoint study dealing with the role of *OeCHLP* gene in leaf development and its adaptive response to cold stressful condition are also discussed.

2.2 Materials and methods

2.2.1 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.

2.2.2 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 μmolm^{-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 and 30 min, 1, 2 and 4 h after wounding. Uninjured healthy drupes (n=25) were also collected at comparable times and used as controls.

2.2.3 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 RNasefree 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).

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

The cDNA from fruit RNA (Fig.2) 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'-CAAATCCACAGGTTCACCCTA-3', and kit anchor primer. The 5' *OeCHLP* end was amplified by using the degenerate primer FWdeg 5'-CCGGATGGCTTCCATTGCTC(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'-ATGGCTTCCATTGCTCTCAA-3' and BWend 5'-TAGCTTCTCCATCTCCCTCCT-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'-GACAAAACATATTGGCCTC CA-3'/BWend. PCR components were: gDNA (200 ng) and/or cDNA (100 ng), 1.5 μM each primer, 0.5mM dNTs, Taq DNA polymerase (Go Taq, Promega, Milan, Italy) 2.5 U, 1/10 of 10x Taq Buffer, 2.5mM $MgCl_2$, 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).

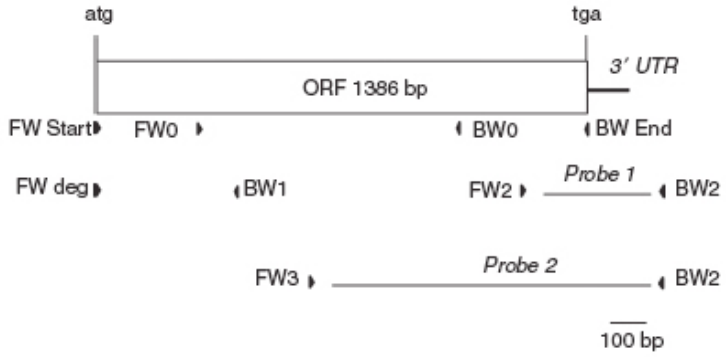

Fig. 2. Scheme of *OeCHLP* cDNA. Start and stop codons andpolyadenylation signals are typed. The probe fragments are represented byblack bars. Forward (FW) and backward (BW) directed primers are shown by arrows. UTR, untranslated regions. ORF, open reading frame; bp, base pairs.

2.2.5 In situ hybridisation

For in situ expression analysis, the specific probe 1 for *OeCHLP* spanned the 1134–1411stretch (**Fig. 2**) and was cloned after PCR amplification using the primers FW2/BW2 FW2 5'-GACAAAACATATTGGCCTC CA-3' / 5'-TCTTCTAAAACAATTAATTT-3'. The probe 1 was linearised by SpeI and NcoI 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-mm 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)

[38]. For immunological detection, the slides were incubated as described by Chiappetta et al. (2006) [39]. Transcript accumulation was visualised as a violet/brown staining. After stopping the reaction with TE (50mM Tris–HCl, pH 8.0, 5mM 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).

2.2.6 Quantitative real-time PCR (qRT-PCR)

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

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-Rad) containing the (iTaq DNA polymerase 50 units μL^{-1}, 6mm Mg^{2+}, 1mM ROX internal Reference DYE Stabilisers, 0.4mM of dATP-dCTP-dGTP and 0.8mM 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" [40] 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.

2.2.7 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) according to the strategies set up by Yokoyama and Nishitani [41]. 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 Ct values within the reactions of a triplicate.

The primers used for *OeCHLP* are FWrealtime 5'-CCAAGGGAGGCATTTGTAGA-3' and BWrealtime 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'.

2.2.8 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 (CT) values were obtained with the Genex software (Bio-Rad) and data were analysed with the $2^{-\Delta\Delta CT}$ method [42]. The means of *OeCHLP* expression levels were calculated from three biological repeats, obtained from three independent experiments.

2.2.9 Southern blot analysis

Total DNA was isolated from leaves frozen with liquid nitrogen, by the ctyltrimethylammonium bromide (CTAB) method [43]. A 10 mg sample was restricted overnight (o/n) at 37°C with 60 U of EcoRI, Pst1 and HindIII 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 45V 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. 2**). It was digoxygenin-labelled using the FW3 5'-TGCG CTTGTTACCAGAACAC-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 20x (NaCl 3M, sodium citrate 300mM), 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).

2.2.10 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.

2.2.11 Total chlorophyll content

Total Chl was extracted from 100 mg aliquot of the dry powder with 8mL of acetone: water (4:1, v/v) twice. The liquid phases were collected in 50mL tubes. The extraction was repeated three times until the pellet was colour-less. The combined acetone extracts were cleared by centrifugation step at 1500g for 15 min. Chl determination was conducted by using a spectrophotometer (model Cary 50Bio, Varian, Turin, Italy). A646.8 and A663.2 were determined and used for calculation of the total contents of Chl *a* and *b* according to the method by Lichtenthaler [44]. 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.

2.2.12 Tocopherol content

TPs quantification was performed according to Tanaka [3]. For each sample a precisely weighted 5mg 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) and 20 µL of this solution was analysed by HPLC (1100 Series, Agilent, Milan, Italy) using a column (4.6x250 mm) filled with C18 Nucleosil 50 (5 µm) at a flow rate of 1.5mL 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.

2.2.13 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 [45]. 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 [46-47]. Samples were ground in a mortar, and an aliquot (0.4 g) was suspended in extraction buffer (Tris–HCl 1M pH7.4, in which was added PVP-40 1.5% w/v). After centrifugation at 10 000g for 20 min, 1mL of supernatant was added to 4mL of trichloroacetic acid (TCA) 20% / thiobarbituricacid (TBA) 0.5%. Another 1mL of supernatant was added to 4mL 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 000g 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. [47].

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

2.3 Results and Discussion

2.3.1 Features of the OeCHLP transcript and genomic organisation

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 polyadenylation signal (stretch 1387–1511) in the 30UTR. The predicted protein (**Fig. 3**) 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/) 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 [48]. *In silico* analysis evidenced high homology of *OeCHLP* with other known homologous genes [2-49].

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 transpeptide. A phylogenetic tree, constructed using proteins devoid of the hyper variable tp region, confirmed monophyletic origin of plant CHLP proteins [49] with OeCHLP being closest to that of *Nicotiana tabacum*.

Southern blot analysis was performed using leaf gDNA digested with EcoRI, PstI and HindIII endonucleases, which did not cut in the probe 2 (**Fig. 2**). The band patterning (**Fig. 4**) 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. 2**). Differently from the homologous genes of *Arabidopsis thaliana*, *Glycine maxima* and *Prunus persica*, no introns were present in the genomic sequence of *OeCHLP*. The amplified products had identical size and sequence to those of cDNA. Moreover, several products retrieved from distinct primer couple amplification were sequenced and we did not score any nucleotide polymorphism. This suggested that the two *OeCHLP* estimated copies might be identical due to duplication events.

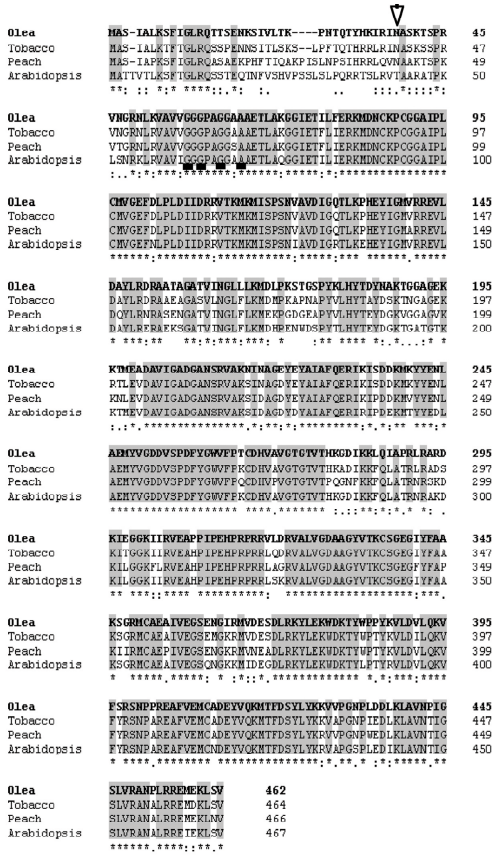

Fig. 3 Amino acidic alignment of deduced protein *OeCHLP* in bold (accession no. DQ424963) with homologues from other plant species. Strictly conserved amino acids are boxed in grey. The residues of the motif necessary to bind NADPH are underlined. The black arrow indicates the editing site for the transit peptide.

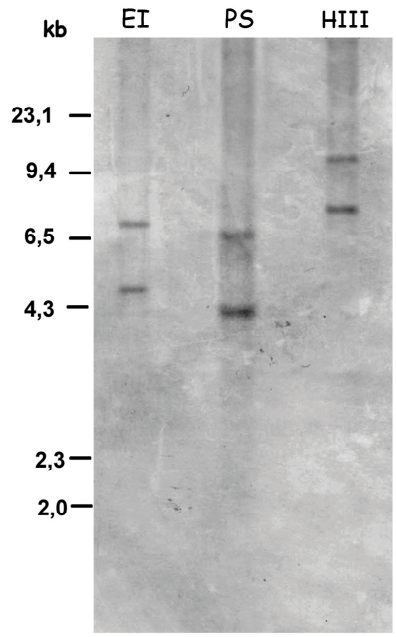

Fig. 4 Southern analysis: genomic DNA was digested with *Eco*RI(EI), *Pst1*(PS) and *Hind*III (HIII) endonucleases, electrophoresed on 0.8% agarose gel, blotted and hybridised with digoxygenin-labelled probe 2. The molecular weights of a co-migrating DNA marker are in kilo base pairs (Kb).

2.3.2 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 and its expression was detected in all the analyzed samples. 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, [1-2-3-28-50]. Further, in both leaves and fruits *OeCHLP* was expressed at different level.

Leaves and fruits were analysed in relation to the stage of organ growth and differentiation. *OeCHLP* resulted to be differentially expressed and the highest value was detected in mature DF fruits (**Fig. 5**). In particular, *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. 5**). By contrast, the relative level of gene expression resulted low in GF, then drastically increased at the last stage (DF) of fruit maturation (**Fig. 5**).

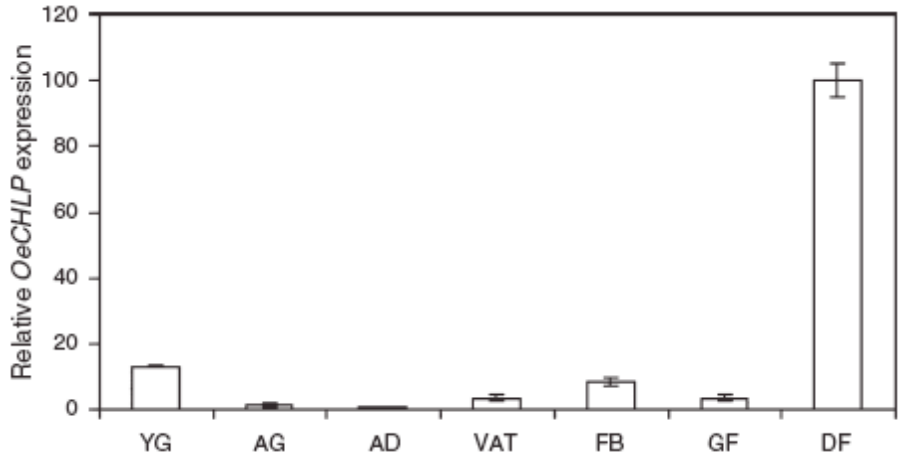

Fig. 5 Levels of *OeCHLP* expression in various organs of olive (Olea europaea) evaluated by qRT–PCR analysis. The results are presented as a proportion of the highest value after normalisation with respect to 18S rRNA expression level and represent the means ± s.e. of three independent biological replicates. 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.

It must be emphasized the higher expression level of *OeCHLP* in YG respect to AG and AD leaves, together with the lower Chl and similar TP level. In young leaves the high transcriptional activity seems to 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.

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.

An extensive analysis of transcripts localisation was also conducted by in situ hybridisation (**Fig. 6A–K**). We observed that in the apical tips *OeCHLP* transcripts were confined in developing leaflets (**Fig. 6A**), whereas in floral buds a strong message accumulation occurred in both leaflets and floral meristematic dome (**Fig. 6B**). *OeCHLP* transcripts were spread evenly in the leaf mesophyll (**Fig**. **6C, E**). In the fruits, *OeCHLP* signal was faintly diffuse in the epicarp and the whole mesocarp (**Fig. 6G**). Signal was not observed in leaf and fruit sections processed with sense probe (**Fig. 6D, H**).

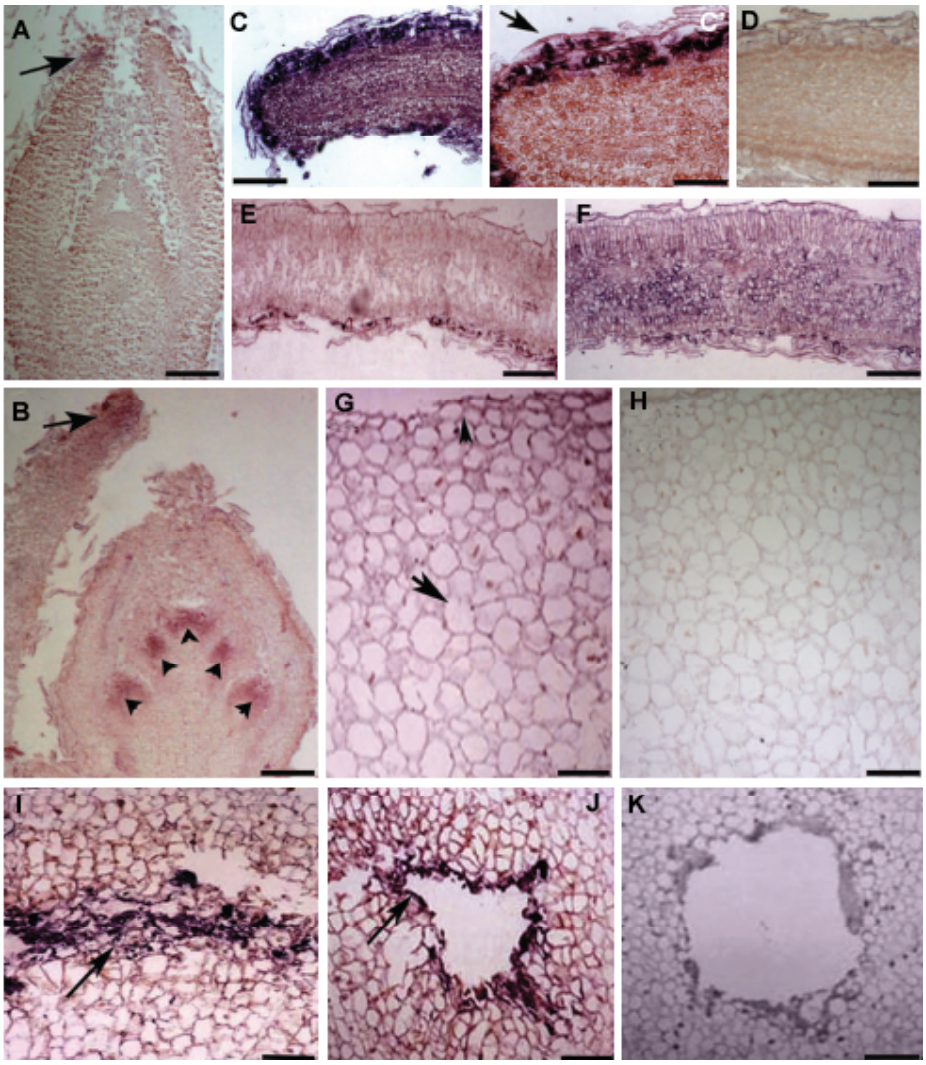

Fig. 6. Localisation of *OeCHLP* transcripts in various organs of olive by in situ hybridisation with dig-labelled *OeCHLP* antisense probe (A–K). Signal is evidenced by purple staining in: (A) longitudinal section of Vegetative Apical Tips (VAT); (B) longitudinal section of FB; (C, E, F,) cross-sections of YG, AG, and cold-stressed AD leaves, respectively; (C') magnification of (C); (G) longitudinal sections of green fruit (GF); longitudinal- (I) and cross- (J) section of infected green fruit (IGF). (D, H, K) control sections of YG,GF and IGF incubated with the sense probe. In (A, B) arrow indicates leaflet; in (B) arrow-head indicates floral dome; in (C') arrow indicates trichome cell; in (G) arrow-head indicates epicarp and arrow indicats mesocarp. Scale bars: (A) =80mm, (B) =60mm, (C) = 100 mm, (C', D) = 50 mm, (E, F) =70mm, (G, H) =50mm, (I–K) =20mm.

2.3.3 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 [51]. 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. 7**). During the gene response interval, the mRNA level was 5–10

fold higher than in untreated plants (**Fig. 7**). *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. 6F v. Fig. 6E**). Following cold exposure, the occurrence of oxidative stress in leaves was assessed estimating the amount of malondialdehyde (MDA) as an end product of lipid peroxidation [45]. A significant increase (~50%) in the level of MDA was observed in stressed leaves compared with control ones. Moreover, in both control and stressed leaves MDA level was slightly higher in YG than in AG and AD stages.

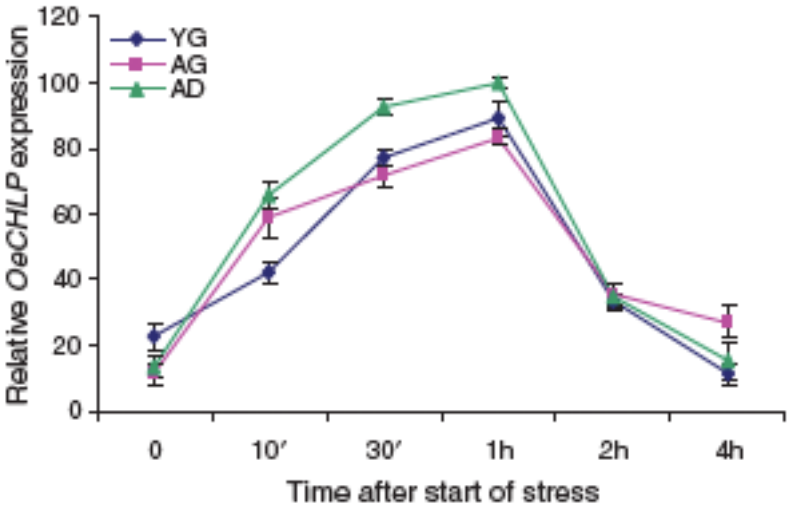

Fig. 7 *OeCHLP* expression analysed by qRT–PCR in leaves of olive (*Olea europaea*) exposed to cold (4°C) conditions for different times: 0 (unexposed leaves used as control), 10 min, 30 min, 1 h, 2 h or 4 h. Results are presented as a proportion of the highest value after normalisation with respect to 18S rRNA expression level and represent the means±s.e. of three independent biological replicates. YG, young green leaves 2–4 cm long; AG, adult green leaves up 6 cm long; AD, adult dark leaves up 6 cm long.

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. 8** and **Fig. 9**). A significant decrease of Chl content, mainly involving Chl b, was observed in cold-treted leaves starting from 2 h pt (**Fig. 8**). The consequent higher Chl a/b ratio is consistent with a reduced photosynthetic efficiency and a stress condition [52]. By contrast, TP content significantly increased in the leaves exposed to cold stress for 4 h (**Fig. 9**). 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 [53-54], and cellular signalling, as recently demonstrated for TPs [55]. In this context, it appears that the early responsiveness of *OeCHLP* which could be related to the interaction of TPs and ROS in signalling the cell redox state [54-56-57] following stress perception.

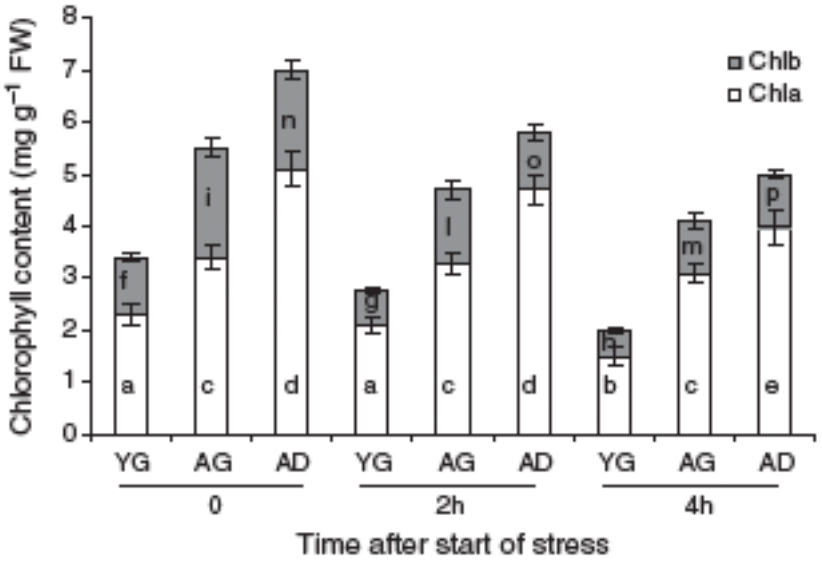

Fig. 8 Chl a and b content in leaves of olive exposed to cold (4°C) conditions for different times: 0 (unexposed leaves used as control), 2 and 4h. Values represent the means±s.e. of three independent biological replicates. Means signed with the same letters are not significantly different for P = 0.05 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.

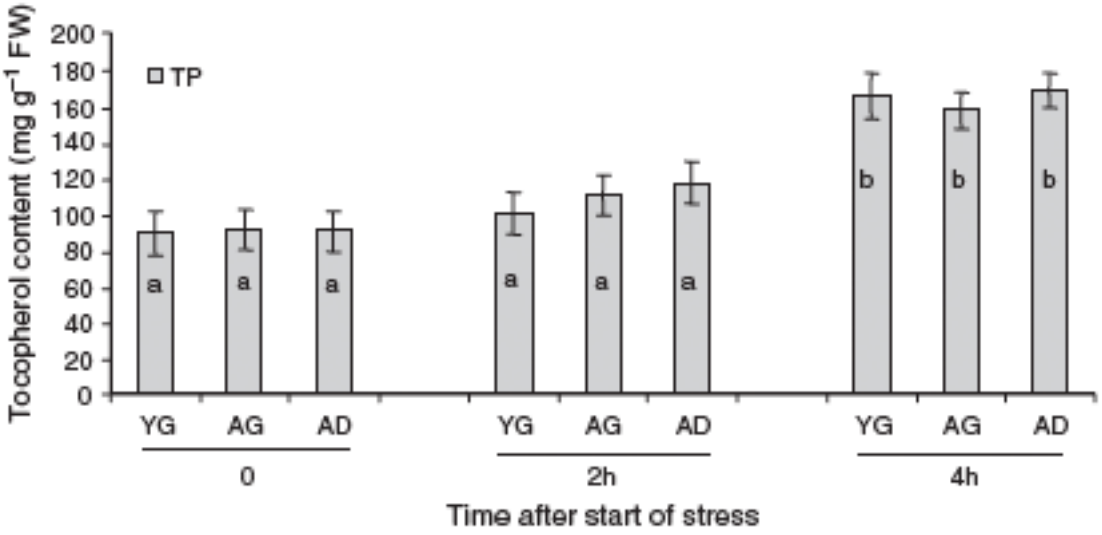

Fig. 9 Tocopherol (TP) total content in leaves of olive exposed to cold (4°C) conditions for different times: 0 (unexposed leaves used as control), 2 and 4h. Values represent the means±s.e. of three independent biological replicates. Means signed with the same letters are not significantly different 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.

An enhancement of *OeCHLP* transcriptional activity was also detected in fruits attacked by Bactrocera oleae pathogen, one of the main biotic stressor for olive plants, which lay one egg in young fruit epidermis and severely impairs drupe quality [58], as well as in fruits wounded by needle. *OeCHLP* expression was 2.5-fold higher in attacked (IGF) than healthy (GF) fruits. *In situ* experiments were performed on drupe sections harbouring damaged sectors, and the *OeCHLP* transcript highly accumulated in cell areas with lesions (**Fig. 6I**) and at the border of

mine traces (**Fig. 6J**). No signal was observed above background in fruit sections of infected sectors processed with sense probe (**Fig. 6K**). 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. 10**). Subsequently it declined at the level of uninjured fruits (**Fig. 10**).

In infected (IGF) and wounded fruits (WGF) both Chls and TPs levels were analysed (**Fig. 11 A** and **B**). Chl content drastically decreased (50%) in IGF, while it was unchanged in WGF fruits analysed at 2 and 4 h after stress started (**Fig. 11A**). For TP content, a significant increases was detected only in IGF infected by fly pathogen (**Fig. 11B**).

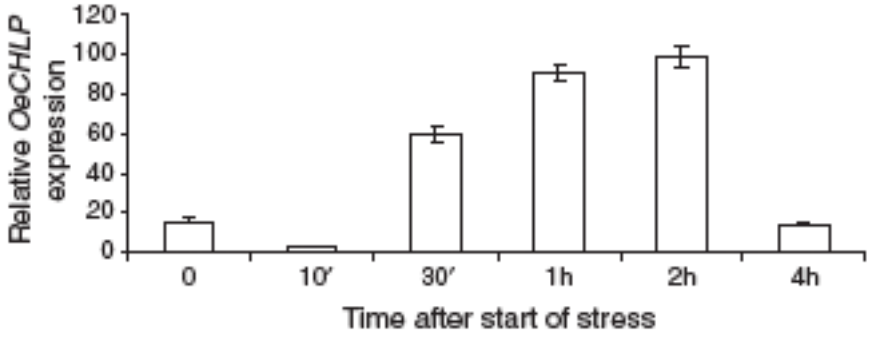

Fig. 10. Levels of *OeCHLP* transcripts analysed by qRT–PCR in green fruits of olive (*Olea europaea*) at different times from mechanical wounding: 0 (uninjured fruits used as control), 10, 30 min, 1, 2, or 4 h. Results are presented as a proportion of the highest value after normalisation with respect to 18S rRNA expression level and represent the means±s.e. of three independent biological replicates.

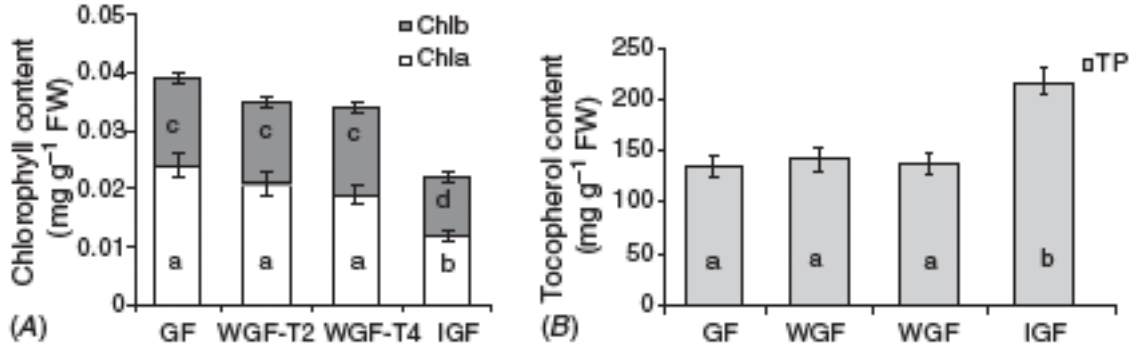

Fig. 11. (*A*) Chl *a* and *b*, and (*B*) total tocopherol (TP) content in uninjured, wounded and infected green fruits of olive. Values represent the means±s.e. of three independent biological replicates. Means signed with the same letters are not significantly different for $P = 0.05$ after ANOVA followed 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.

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 [59-60].

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.

[These results have been already published as "Role of geranylgeranyl reductase gene in organ development and stress response in olive (Olea europaea L) plants" by Bruno L., Chiappetta A.A.C., Muzzalupo I., Gagliardi C., **Iaria D.L.**, Bruno A., Greco M., Giannino D., Perri E., Bitonti M.B.A. in *Functional Plant Biology* - 2009 - Vol. 36, pp. 370-381]

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Chapter 3

Structural and functional characterization of *Olea europaea ALCOHOL DEHYDROGENASE* gene (*OeADH*) involved in fruit flavour

3.1 Introduction

Higher plants have the capacity to synthesize, accumulate and emit special compounds acting as aroma and flavour molecules due to interactions with human receptors. These substances which exhibit a low-molecular-weight derive from fatty acids, amino acid and carbohydrate pools. Therefore they constitute a heterogenous group of saturated and unsaturated molecules with straight- and branched-chains, cyclic structures bearing various functional groups (e.g. alcohols, aldehydes, ketones, esters and ethers) and also nitrogen and sulfur.

Many of these substances are commercially important for pharmaceutical, agricultural and chemical industries as "flavourant", drugs, pesticides and industrial feedstock. Moreover and more relevantly, due to the capacity to confer sensory properties their accumulation in plant organs and mainly in the fruits has a strong impact on the perception of food quality and consumer preference

Certainly, sensory testing has a strong impact on the perception of quality also for olive products (e.g. olive and oil) and consequently on its commercial value, thus the production of flavourful olive oil represents an important goal of olive breeding programs [1-2]. In this context, the second part of this PhD project aimed to obtain some insight into the genetic control of lipoxygenase pathway directly involved in the production of the major volatile compounds constituting the fruit aroma.

3.1.1 Formation of plant volatile compounds: the lipoxygenase pathway

Several metabolic pathways driven by appropriate enzymes are involved in the formation of plant volatiles. The majority of plant volatiles originate from saturated and unsaturated fatty acids. Fatty acid-derived alcohols, aldehydes, ketones, acids, esters and lactones are found ubiquitously in the plant kingdom at high concentrations, and are basically formed by three processes, α-oxidation, β-oxidation and the lipoxygenase pathway [3]. Among these, for olive fruit the most relevant is the lipoxygenase pathway on which relies the development of major volatile constituents of olive oil aroma.

As depicted in **Fig. 1**, C6 aldehydes and derivate alcohools, responsible for the characteristic green odour of olive products, are formed following the activation of a series of enzymatic reactions that are known as "lipoxygenase cascade" [4-5-6-7-8]. Along this pathway the enzyme lipoxygenase oxidises polyunsaturated fatty acids to yield hydroperoxide acid derivates which are cleaved into aldehydes by hydroperoxide lyase activity. Subsequently the

alcohol dehydrogenase catalyses the reversible reduction of aliphatic aldehydes to alcohols which are esterified by alcohol acyltransferase to produce esters.

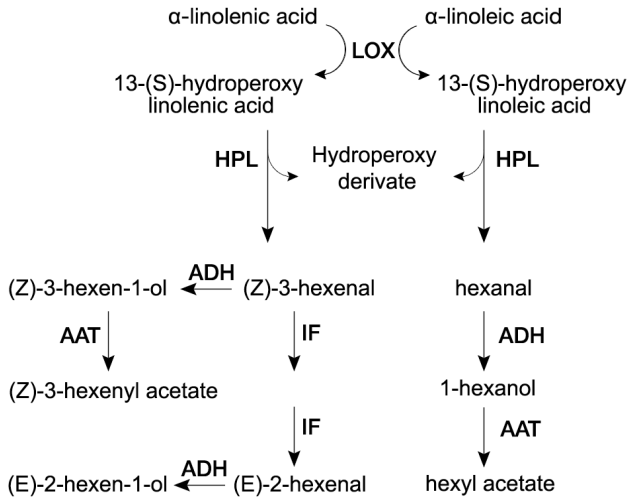

Fig. 1. Schematic representation of the lipoxygenase pathways involved in the production volatile compounds. LOX: lipoxygenase; HPL: hydroperoxide lyase; ADH: alcohol dehydrogenase; AAT: alcohol acyltransferase; IF: isomerization factor

3.1.2 Profile and role of plant volatiles compounds

In plants, volatiles compounds develop in different organs whereas either the quality and the quantity of different volatiles represent a genetic trait expressed through an established and constant relationship of several chemicals [4-9-10-11-12]. In planta they play a role on one hand in the attractive strategies exploited for sexual reproduction and on the other in the set-up of defence mechanisms against pest and predators [13-14]. Generally, volatiles act as direct repellents or toxicants for herbivores and pathogens, and some have the potential to eliminate reactive oxygen species. These latter also include root-emitted volatiles, which not only may act as antimicrobial or anti-herbivore substances but also exhibit allelopathic activities thus increasing the ecological competitiveness of plants [15-16]. In addition, there are evidences that some of these substances play an indirect role in defence mechanism acting through multi-trophic interactions: for example by attracting arthropods that prey upon or parasitize the herbivores, thus minimizing further damage to plant tissue. In the fruits, volatile accumulation and emission have probably evolved to facilitate seed dispersal by animals and insects. This putative function of fruit volatiles is supported by the fact that some of these compounds are specifically formed in ripened fruits while are absent in plant vegetative tissues as well as in immature fruits. However, it must be underlined that, unlike ripened fruits and flowers, vegetative tissues often produce and release many of these volatiles only after their cells are disrupted.

Concerning olive (*Olea europaea* L.) plants, volatiles compounds are mainly formed in the fruit throughout its development [6] and there is widespread scientific agreement that volatiles having 'green or grassy' and 'fresh' notes derive from lipoxygenase pathway [5-6-12-17-18-19-20-21]. Moreover, volatiles can also build-up following drupe attack by different pathogens and predators as well as following crushing and malaxation processes undertaken in the production of oil.

Clearly, the profiling of volatiles is correlated to the activity and expression of all the enzymes acting in the pathway (**Fig. 1**), which in turn is mainly affected by genetic factors (cultivar) and also by the stage of fruit ripeness. It should be also mentioned that fruit ripeness and the ability of fruits to reach the climacteric peak are characterized by a series of biochemical and physiological modifications which reflect changes in the activities of several enzymes [5-17-18]. Moreover, ripening stage is coupled with fruit tissue softening and senescence process [6] leading to the loss of cell integrity which cause the releasing of specific enzymes, physically separated from substrate in immature or unaffected fruit, which can interact with the formation of those metabolites due to the to lipoxygenase pathways [9]. Notably, oil extracted from olive fruits at the climacteric stage is particularly rich in volatiles compounds with a subsequent and progressive decrease beyond this stage [22-23]. However, many other factors, such i) pedoclimatic conditions characterizing plant growth area, ii) fruit storage state before processing as well as iii) the technology used for oil extraction, can affect the volatiles composition [5-24-25-26-27].

3.1.3 Selected gene : ALCOHOL DEHYDROGENASE

ALCOHOL DEHYDROGENASE gene (ADH, EC 1.1.1.1) encodes an enzymes which catalyzes the reversible conversion of aldehydes to the corresponding alcohols. ADHs have been found to be involved in the response of plants to a wide range of stresses, as well as to signaling triggered by either abscisic acid and various elicitors [28-29-30]. Among stress conditions, anaerobiosis is the most relevant for ADHs action being responsible for ethanol production. The involvement of ADHs in the formation of volatile alcohols was first suggested on the basis that macerated leaves and fruits of tomato were found to form these volatiles. Later on, the observation that the formation of alcohols by macerated tea leaves was inhibited by iodoacetic acid, and inhibitor of alcohol dehydrogenase, fully confirmed such an involvement [31]. As a general picture, ADH activities have been found to be responsible for the biosynthesis of six-carbon alcohols such as hexanol, E-2-hexenol and Z-3-hexenol, known as major responsible of so-called "green odour" [6-7].

It is worth noting that in several species, including kiwi, tomato, apple, grape and melon [18-20-32-33-34] the expression of *ADH* genes are expressed in the different tissues in a developmentally-regulated manner, particularly during fruit ripening [32-33-35-36]. For example, it has been demonstrated that in tomato fruit *Le-ADH2*, one of the two identified *ADH* genes, is highly active in producing alcohols, particularly Z-3-hexenol, during the ripening phase, thus participating in the formation of fruit flavour. Consistently, over-expression of *Le-ADH2* improved fruit flavour by increasing Z-3-hexenol level of [35]. Also in grapes, three different members of *ADH* genes are differentially expressed during fruit development. In particular in young developing berry *Vv-ADH1* and *Vv-ADH3* transcripts accumulate transiently, while *Vv-ADH2* transcripts strongly increase at the onset of ripening phase [34].

3.1.4 Aim of the work

Major progress in plant volatiles research has been made through the use of molecular and biochemical techniques and progresses have been achieved in defining the biochemical steps of aroma biosynthesis. Recently, a number of genes encoding enzymes involved in volatile compound biosynthesis have been reported for various plant species [18-19-20-21-33-34-35-36]. However, how transcriptional network leading to different chemicals compositions is modulated throughout the pathway is yet largely unexplored especially in olive species. So far, only two genes along the pathway have been characterized at the structural and functional level: the *LIPOXYGENASE* (*LOX*) and very recently the *HYDROPEROXIDE LYASE* (*HPL*) genes [7-37-38]. In order to reach further insight into the genetic control of lipoxygenase cascade in olive plants, the present work was addressed to characterize one member of *ALCOHOL DEHYDROGENASE* (*ADH*) gene family, whose encoded enzymes immediately follow LOX and HPL activity, thus catalyzing the formation of C6 volatile alcohols. The main objective was to define the interfacing between the temporal expression pattern of new identified (*ADH*) and existing *LOX* (ACG56281.1) [38] genes and aroma biogenesis during fruit development in two different cultivars of *Olea europaea* L. widely cultivated in southern Italy for olive and oil production (e.g “Carolea” and “Coratina” cultivars). Since climatic condition and soil composition also influence volatile profile, for both cultivars plants grown in the same area were used. Here, we report the structural features of an *OeADH* (*Olea europaea ADH*) gene identified in Carolea cultivar. The distinct, development-related expression patterns of *OeADH* and *LOX* genes simultaneously detected by qRT-PCR in the fruits of the two cultivars harvested at the different developmental stages, together with the

quantification of volatile products by a SPME-GC/ion trap method [27], are also reported.

3.2 Materials and methods

3.2.1 Plant material

Plants used were 10-year-old clonally propagated olive (*Olea europaea* L.) cv Carolea and Coratina. The plants belonged to the olive genome collection of the "Centro Sperimentale Dimostrativo Mirto" - CRA (Centro di Ricerca per l'Olivicoltura e l'Industria Olearia) in Mirto Crosia (Cosenza, Calabria, Italy) and grown in the same field conditions (latitude 39°37'04.57"N; longitude 16°45'42.00"E; altitude 8m).

Fruit (drupe) samples were harvested at different times after flowering corresponding to different developmental fruit stages: 70, 125, 150, 180 and 200 days after flowering (DAF), spanning from immature green stage to ripe black drupes. Fruits were picked from all around the external parts of the canopy of trees. Leaves were also collected from each cultivar and all samples were fixed in liquid nitrogen and stored at -80°C.

3.2.2 Biometrical analysis

At each sampling the following biometric parameters were estimated: pulp weight, pulp-pit ratio, polar and transverse diameter, polar and transverse thickness. For the analysis 50 drupes randomly collected were used for each stage and for each cultivar.

3.2.3 RNA isolation and sscDNA synthesis

Total RNA was isolated from drupes at different developmental stages, processed separately as previously described [39].

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 50 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(20), according to the manufacturer's instructions (Invitrogen, Milan, Italy) to synthesize first-strand cDNA.

3.2.4 Isolation and cloning of ADH gene from Olea europaea cv 'Carolea' (OeADH)

The sscDNA obtained from total RNA was PCR amplified by the degenerated primers ADHdFW 5'-GGACAyGAAGsAGTyGGyrTGA-3' and ADHdBW 5'-GCAACyGTmG AGCCrTTTTC-3', designed from the comparison between known plant *ADH* sequences of *Solanum tuberosum* (CAA63093) and *Arabidopsis thaliana* (AAM67260). The PCR reaction yielded a ~400 bp products, which was homologue to *ADH* of the above mentioned plants.

Both the 5' and 3' ends of the *OeADH* were amplified by the Rapid Amplification of cDNA Ends (RACE) methodology (Invitrogen). In 5' RACE reaction first strand cDNA synthesis is primed using a gene-specific antisense oligonucleotide, ADHsp1 5'-GATAG ACCCTCTTACGCCCC-3', allowing the specific conversion of mRNA. Following purification and tail steps, the specific cDNA is amplified by PCR using the provided Abridge Anchor Primer (AAP) and a nested gene-pecific primer ADHsp2 5'-ATGCCAAC TCCTTCATGTC-3'.

For the 3' RACE method a gene-specific primer, ADH3FW 5'-ACCACAGGTTTTGG ATCTGC-3', single stranded cDNAs synthesised by the oligo-(dT) Adapter Primer (AP), provided with the system. PCR was performed following the manufacturer's instructions with the gene-specific primer and Abridged Universal Amplification Primer (AUAP).

The full-length *ADH* transcript was re-checked by amplifying the cDNA of leaves and fruits using the primers FW 5'-CCATTTGCAACCAGGTATCC-3' and BW 5'-GGGAGACGCCTTGATAAACA -3'. All RACE fragments and the 1231bp full-length products were cloned into pGEM-T easy vector system, according to the manufacturer's instructions (Promega) and sequenced by the Genelab ENEA service (Rome, Italy).

3.2.5 Southern blot analysis

Southern blot as previously described [39], with the following major changes: 5 µg of genomic DNA sample was restricted overnight (o/n) at 37°C with 60 U of EcoRI, EcoRV, XhoI and DraI endonucleases (Promega), which do not cut in the probe.

The probe was digoxygenin-labelled following the manufacturer's instructions (Roche), using FW1 5'-GAAGCAATTGAGGGATCCAA-3' / BW1 5'-GGGAGGTCTGTTTGAGGTCTT-3' primers spanning the 834–989 stretch (probe 1) , and FWExo7 5'-TCAGCTGGATG AACTATTGA-3' / BWExo7 5'-TTTGATACAACTTTTAACACAGC-3' primers spanning the 1022–1122 stretch (probe 2). Hybond-N+ membranes were hybridized at 60°C and 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).

3.2.6 Alignments and phylogenetic analysis

The alignment of OeADH with other known plant ADH proteins was conducted by ClustalW (http://www.ebi.ac.uk.clustalw). Phylogenetic tree was constructed by MegaBlast3, with 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 ADH accession numbers were: *S. tuberosum* (CAA63093), *O. basilicum* (AAX83109), *R. communis* (EEF42402), *S. lycopersicum* (AAB33480), *Z. mays* (ACG35040), *A. thaliana* (AAM67260), *P. banksiana* (AAC49548), *V. vinifera* (AAG01383), *C. melo* (ABC02081), *D. longan* (ABF61806), *R. palustris* (RNC011004).

3.2.7 Quantitative real-time PCR (qRT-PCR)

Gene expression analysis was performed by quantitative real-time PCR on a BioRad Miniopticon (Bio-Rad, Milan, Italy) single colour thermocycler with Bio-Rad SYBR Green Supermix (Cat. No.170–8884). The oligonucleotide primer sets used for q-RT-PCR analysis were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/ primer3/primer3_www.cgi). Each primer pairs generates a single amplicon of 100-200 bp and are specific for the target sequence; primers also have compatible melting temperatures (within 5°C) and contain approximately 50% GC content.

The primers used for *ADH* are ADHqFW 5'- GAAGCAATTGAGGGATCCAA -3' and ADHqBW 5'-GGGAGGTCTGTTTGAGGTCTT-3'; while for *LOX* gene (ACG56281.1) are LOXqFW 5'-CCTGGAAAACCCGAGTATGA-3' and LOXqBW 5'-GAGCGTCTCTCTGT CCAAGG-3'. The length of all PCR products ranged from 150 to 200 bp. The average efficiency of all of the primer pairs ranged between 0.95 and 1.0.

The housekeeping *Olive Elongation factor 1* (*EF1*) gene (CAQ17046.1) was used as endogenous reference to normalize the expression levels; the primer sequences used were EF1FW 5'-CCTTGGTGTCAAGCAGATGA -3' and EF1BW 5'-TGTTGTCACCCTCA AAACCA -3'.

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-Rad), 0.4 mM 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 10s and annealing-extension at 58°C for 30s. After reaction, in order to confirm the existence of a unique PCR product the

"melting curve" [40] was evaluated by an increase of 0.5°C every 10s, from 60°C to 95°C, obtaining in our experiment a unique "melting peak" in every reaction.

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

The comparisons of cycle threshold (CT) values were obtained with the Genex excel macro (provided by Bio-Rad) analyzing data with the $2^{-\Delta\Delta CT}$ method [41]. The means of *OeADH* and *LOX* expression levels were calculated from three biological repeats, obtained from three independent experiments.

3.2.8 Headspace-SPME GC–MS

For volatile compounds analysis, drupes collected at 70, 150 and 200 DAF were used as the main stages of fruit maturation. Volatile compounds were identified by Solid Phase Micro-Extraction technique in headspace mode followed by Gas Chromatography/Mass Spectrometry analysis (HS-SPMEGC/MS). The olive drupes were destoned and crushed to obtain a homogeneous paste. The preparation of samples and the most suitable solid-phase microextraction (SPME) conditions for quantitative assay of the four selected compounds, hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol and 1-hexanol were described in a previous study [27]. Briefly, 2 g of olive paste (*OP1* and *OP2*) samples were placed in a septum-closed vial and the extraction was performed in the headspace volume (~8 mL) at 40 °C for 20 min by a SPME divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 65 μm fiber (Supelco Co., Bellefonte, PA). The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 250 °C for 3 min. The quantitative analysis was performed according to Benincasa et al. [27] using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS ion trap system in positive chemical ionization mode, with isobutane as reagent gas, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 210 °C with an ionization time of 2 ms, a reaction time at 50 ms, and a scan rate at 1000 ms. The transfer line temperature was set at 230°C. The column was a 30 m Varian Factor Four 5-ms (0.25 mm i.d., 0.25 μm film thickness). The gas chromatography (GC) oven temperature was initially held at 40 °C for 3 min, then increased at 1 C/min to 70°C, increased again at 20 °C/min to 250 °C, and held for 8 min. The carrier gas was helium at 1 mL min^{-1}. Analyses were performed in splitless mode. For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used.

3.3 Results and discussion

3.3.1 OeADH exhibited a conserved multi-domain architecture

The full-length cDNA (Genbank accession not yet assigned) of *OeADH* consisted of 1248 nucleotides, including an open reading frame of 1128 bp (stretch 41–1168) (**Fig. 2**). The predicted protein was made of 375 amino acids (aa) with a calculated mass of 41 kDa (http://www.expasy.ch/tools/pi_tool.html). The entire OeADH protein was blasted in NCBI database and shared the highest identity with that of *Solanum tuberosum* (68%) followed by *Ocimum basilicum* (65%), *Ricinus communis* (56%).

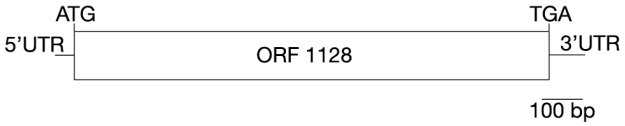

Fig. 2. Schematic representation of *OeADH* full-length cDNA. Start and stop codons are typed. UTR, untranslated regions. ORF, open reading frame; bp, base pairs. Scale bar 100 bp.

Based on the visualization at NCBI's Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [42-43] our *ADH* sequence exhibited a conserved multi-domain architecture, with the catalytic domain, the zinc binding site and the NAD binding domain. This latter consisted in a series of beta-alpha-beta folds tipycal of the NAD(P)-binding Rossmann-fold, supported by PSIpred and PFP-FunDSeqE prediction (**Fig. 3**) (www.expasy.ch/tools/#proteome; http://www.csbio.sjtu.edu.cn/bioinf/PFP-FunDSeqE) [44-45-46-47].

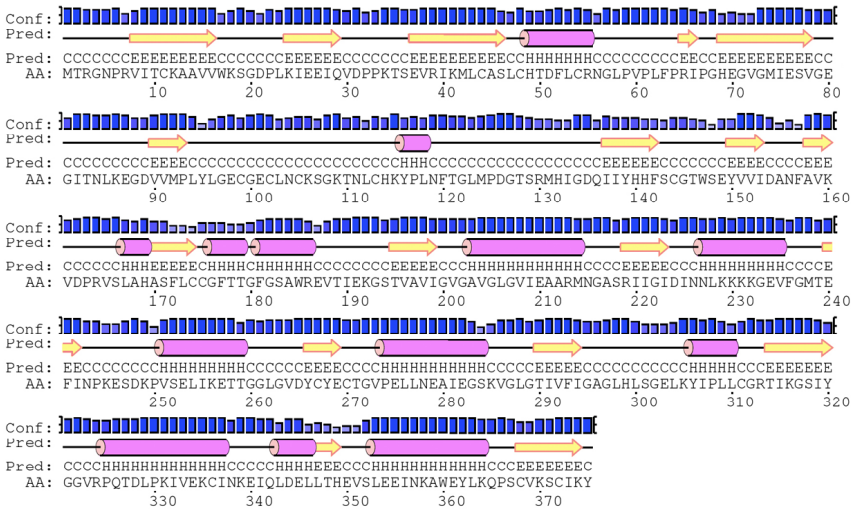

Fig . 3 The predicted secondary structure of *OeADH* from PSIPRED. The H stands for helix, C for coil, and E for strand. The blue bars for each amino acid represents the confidence of each prediction. The taller the bar, the higher the confidence.

A phylogenetic tree was constructed using other known plant ADH proteins, and OeADH resulted closest to that of *Solanum tuberosum* (**Fig. 4**).

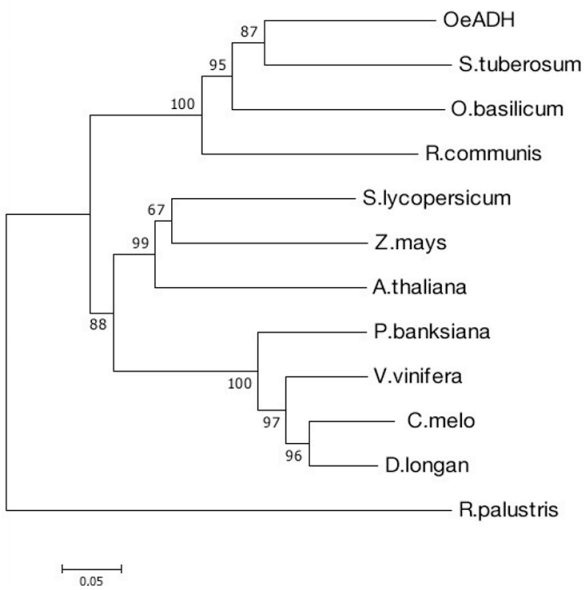

Fig. 4. Phylogenetic tree of OeADH proteins. OeADH amino acid sequence was compared to other ADHs proteins of dicotyledonous and monocotyledonous species available in database; trees were rooted with the Rhodopseudomonas palustris has used to create an out-group. Bootstraps values (at the branching points) are given for major nodes only and were based on 1000 repetitions. The list of protein accession numbers are: *S. tuberosum* (CAA63093), *O. basilicum* (AAX83109), *R. communis* (EEF42402), *S. lycopersicum* (AAB33480), *Z. mays* (ACG35040), *A. thaliana* (AAM67260), *P. banksiana* (AAC49548), *V. vinifera* (AAG01383), *C. melo* (ABC02081), *D. longan* (ABF61806), *R. palustris* (RNC011004).

3.3.2 OeADH belongs to a multigene family and at least two copies are harboured in olive genome.

Genomic organization of *OeADH* was investigated through southern blot analysis on leaf genomic DNA digested with EcoRI, EcoRV, XhoI and DraI endonucleases by using probe 1 and 2. The alignment of *OeADH* cDNA sequence with the genomic ADH sequence of *Arabidopsis thaliana* (NM_106362) using ClustalW program, make possible to predict that the probe1 spanned the exon 5 to exon 6, corresponding to NAD binding domain, witch is highly conserved within gene family, while the probe 2 spanned the exon 7 corresponding to 3' ending (**Fig. 5A**). It was also verified through digestion of PCR genomic fragments that probe1 contained one DraI site in the intergenic region whereas no restriction sites for the used enzymes were present in the probe 2.

The multiple band patterning obtained whit probe 1, spanning the conserved domain, pointed to the presence of *ADH* gene family (**Fig. 5B**). On the contrary, using probe2 which targets a specific region of *OeADH* gene, whatever digestion was applied only two hybridization bands were revealed, suggesting that in the cultivar Carolea at least two copies of this gene were harboured per haploid genome (**Fig. 5C**).

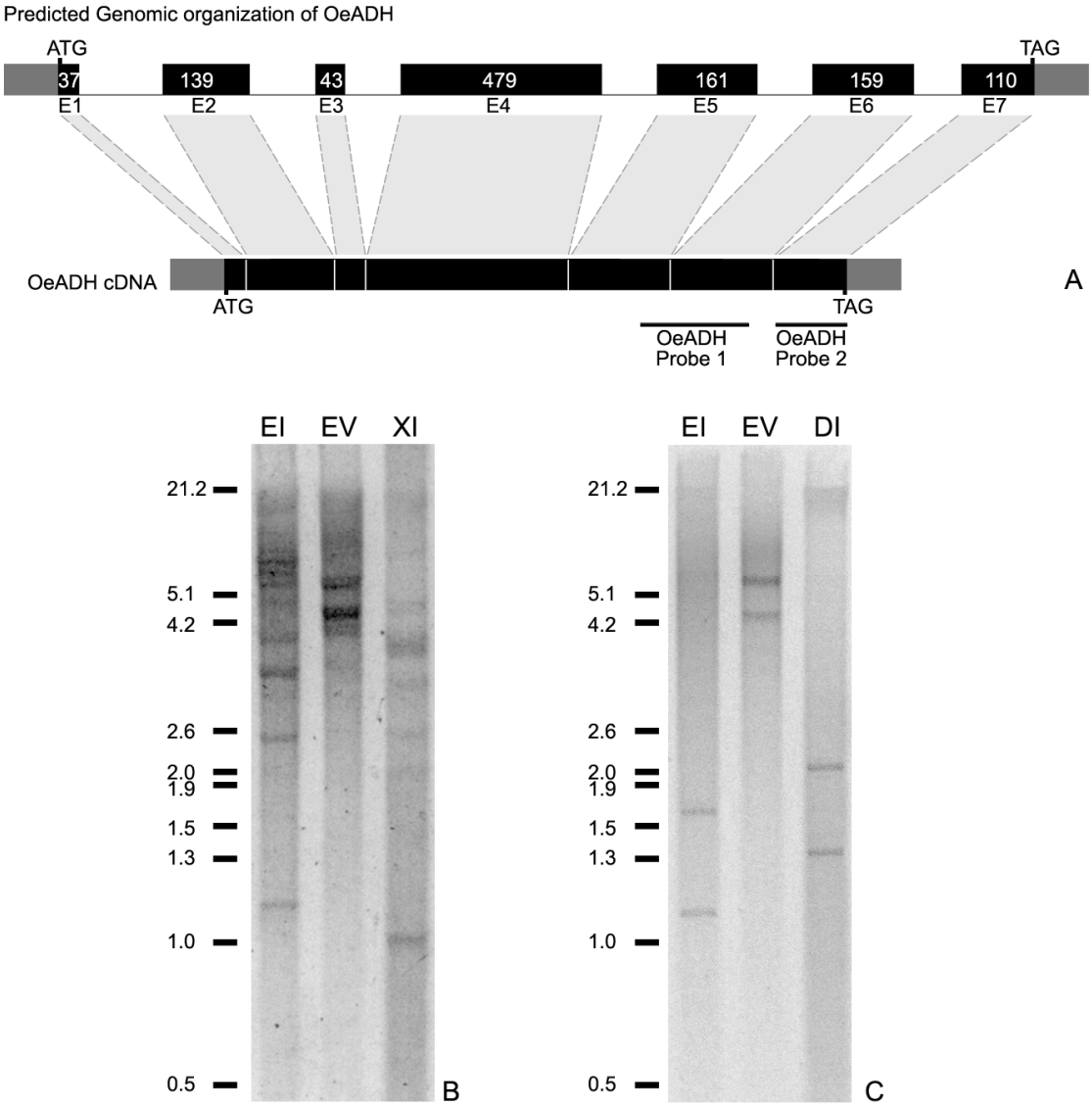

Fig. 5. Southern blot hybridization of leaves gDNA from cultivar Carolea with *OeADH* probe1 and probe2. (A) Representation of the predicted genomic organization of the *OeADH* gene deducted from the nucleic sequence alignment of the *OeADH* cDNA with the *Arabidopsis thaliana ADH* gene (NM_106362). Boxes represent the exons and the line between boxes represents the introns. The translation start and stop codons are typed. (B) Genomic DNA digested with *Eco*RI (EI), *Eco*RV (EV) and *Xho*I (XI) hybridized with probes 1; (C) Genomic DNA digested with *Eco*RI (EI), *Eco*RV (EV) and *Dra*I (DI) hybridized with probes 2. DNA size standards in kb are shown on the left.

3.3.3 OeADH and LOX expressions are differentially modulated in relation to both fruit stage and cultivar.

The expression levels of new identified *OeADH* and known *LOX* were simultaneously monitored by q-PCR in drupes of Carolea and Coratina cultivars harvested at different days after flowering (DAF), corresponding at the morphological level to: immature green (70 and 125 DAF), mature green (150 DAF) , turning black (180 DAF) and black ripe (200 DAF) stages (**Fig. 6**).

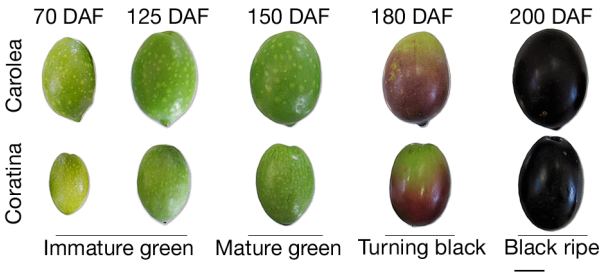

Fig. 6. Fruits sampled from Carolea and Coratina cultivar. Fruits were harvested at different times after flowering corresponding to different developmental stages: 70, 125, 150, 180 and 200 days after flowering (DAF), spanning from immature green to ripe black drupe. Scale bar 1 cm.

To carefully follow the time-course of drupe development, at each stage the following biometric parameters were monitored: drupe polar and transverse diameter, pulp weight, pulp-pit ratio, pulp polar and transverse thickness (**Fig. 7**). As a general rule, drupe size was constantly higher in Carolea than in Coratina cultivar. Moreover, an identical developmental time-course was observed in both cultivars with an increase of all the analyzed parameters mainly during the transition from turning black (180DAF) to black ripe (200 DAF) stage (**Fig. 7**).

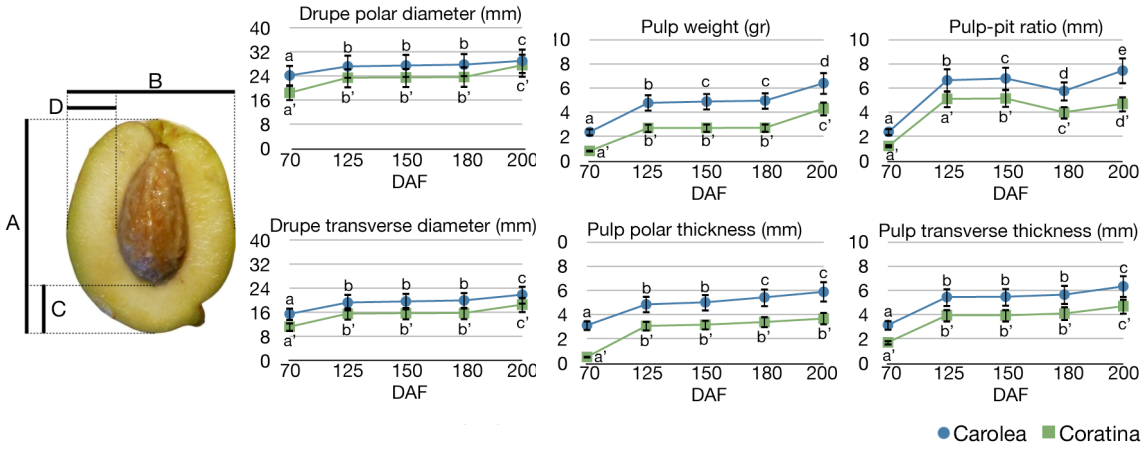

Fig. 7. Schematic representation of parameters sampled: drupe polar and transverse diameter (A, B), pulp polar and transverse thickness (C, D); developmental course of monitored parameters: drupe polar and transverse diameter, pulp weight, pulp-pit ratio, pulp polar and transverse thickness. Values represent the means ± s.e. Means signed with the same letters are not significantly different for P = 0.05 after ANOVA followed by Bonferroni post hoc test.

Concerning gene expression analysis, in both cultivars the relative levels of *OeADH* and *LOX* transcripts significantly differed in relation to drupe stage, thus pointing out a development-related modulation of gene transcriptional activity (**Fig. 8**). In particular, in Carolea cultivar the expression level of both genes progressively increased over maturation, reaching the highest value at the last stage of maturation (200DAF). Whereas, in Coratina cv a drop in the levels of gene expression was observed in the transition from 70 to 125 DAF, followed by a progressive recovery leading to final levels quite comparable to those observed in Carolea cultivar. These results are in line with previous studies [10, 23, 24] performed on

different olive cultivars, showing that the expression of *LOXs* and *HPL* genes increased over fruit development, reaching the highest levels at 28 weeks after flowering (approximately 200 DAF). However, it must be underlined that Authors [10, 23] looked at the expression pattern of these genes in the same pathway by distinct experiments. Whereas, by monitoring concurrently the expressions of *OeADH* and *LOX* genes, our study provides for the first time in olive plants a simultaneous picture of their temporal transcriptional pattern in lipoxygenase pathway.

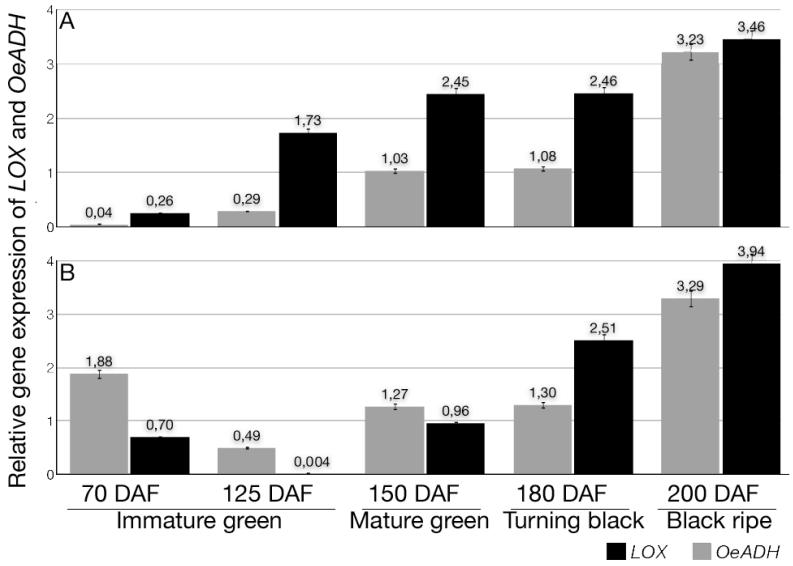

Fig. 8. Levels of OeADH (grey bar) and LOX (black bar) expression in fruits from cultivar Carolea and Coratina harvested at different developmental stages, evaluated by qRT–PCR analysis. The results (log scale) are presented as a proportion of the highest value after normalisation with respect to the expression level of house-keeping gene (EF1)and represent the means ± s.e. of three independent biological replicates.

Furthermore, beside the different temporal modulation of gene expression, also the relative levels of *OeADH* and *LOX* transcripts, detected at each stage, was found to differ in Coratina vs Carolea cultivar (**Fig. 8**), despite the similar time-course of fruit maturation observed at morphological level (**Fig. 7**). Indeed, at the early immature green stage (70 DAF) the levels of both *OeADH* and *LOX* transcripts were higher in Coratina then in Carolea drupes, while at both 125 and 150 DAF a very high level of *LOX* expression was detected in the drupes of Carolea compared to Coratina cultivar (**Fig. 8**). Moreover, by comparing within each cultivar the expression levels of the two genes it was evident that in Carolea cultivar *LOX* transcripts were constantly more abundant than those of *OeADH* gene. Whereas in Coratina cultivar *OeADH* transcriptional activity was largely preminent compared to that of *LOX* from 70 to 125 DAF, followed by an opposite trend only at the last two stages of maturation (180 and 200DAF) (**Fig. 8**). That is consistent with data on ADH and LOX enzymatic activity, assayed in olive drupes harvested at different developmental stage,

showing the achievement of a peak at about twenty five weeks (180 DAF) after flowering [34, 35].

All together these results allow us to conclude that the expression of these two related genes in lipoxygenase pathway is modulated in relation to both fruit developmental stage and cultivar.

3.3.4 Cultivar specific composition of aroma is tightly linked to differential gene expression.

In order to define whether and how transcriptional modulation of investigated genes was correlated with aroma biogenesis, the composition of four selected volatile compounds developed in immature green (70 DAF), green mature (150 DAF) and black ripe (180 DAF) drupes was analyzed by headspace-SPME GC-MS. According to literature [3-4-7-12-23-26], the following four secondary metabolites were selected as markers of the lipoxygenase oxidation pathway: hexanal, 1-hexanol, (E)-2-hexenal, (E)-2-hexen-ol.

It was evident that in both cultivars the relative amounts of selected compounds changed during drupe maturation (**Fig. 9**). Moreover and more interestingly, deep differences were observed by comparing the two cultivars, mainly at the early developmental stages of drupe development. In particular, at 70 DAF the drupes of cv Carolea were particularly rich in aldehyde compounds (**Fig. 9A**) and among these hexanal was the most abundant (**Fig. 9C**). By contrast, an opposite pattern was observed in the Coratina cv, since alcohols resulted the dominant component (**Fig. 9B**), largely represented by 1-hexanol (**Fig. 9D**). This observation matchs with the different pattern of gene expression recorded at 70 DAF in the two analyzed cultivars, in that the higher expression of *ADH* than *LOX* detected in Coratina cultivar provides an explantion for its high 1-hexanol content compared to Carolea cultivar.

The transition from 70 to 150 DAF was characterized, in both cultivars, by a clear reduction of fruit volatile components. Although we did not estimated presence and content of esters, we suggest that such decrease may be related to a ready conversion of aldehydes to alcohols and then to esters, due to a high activity of ADH and Alcohol Acyl Transferase (AAT), respectively. This suggestion is partially, even though indirectly, supported by the rise of *ADH* expression detected at150 DAF. It is likely that these two enzymatic activities work in concert at a rate that prevents the accumulation of derived volatiles. Further investigations on the expression of *AAT* gene and the activity of encoded enzyme could be greatly helpful for defining this step of aroma biogenesis in olive fruit.

From 150 to 200 DAF, in both cultivar, a consistent increase in the amount of aldehydes and alcohols was observed, due to an accumulation of hexanal and 1-esanolo.

However, this step is also characterized by a drop in (E)-2-hexenal and (E)-2-hexen-ol (**Fig. 9E and F**).

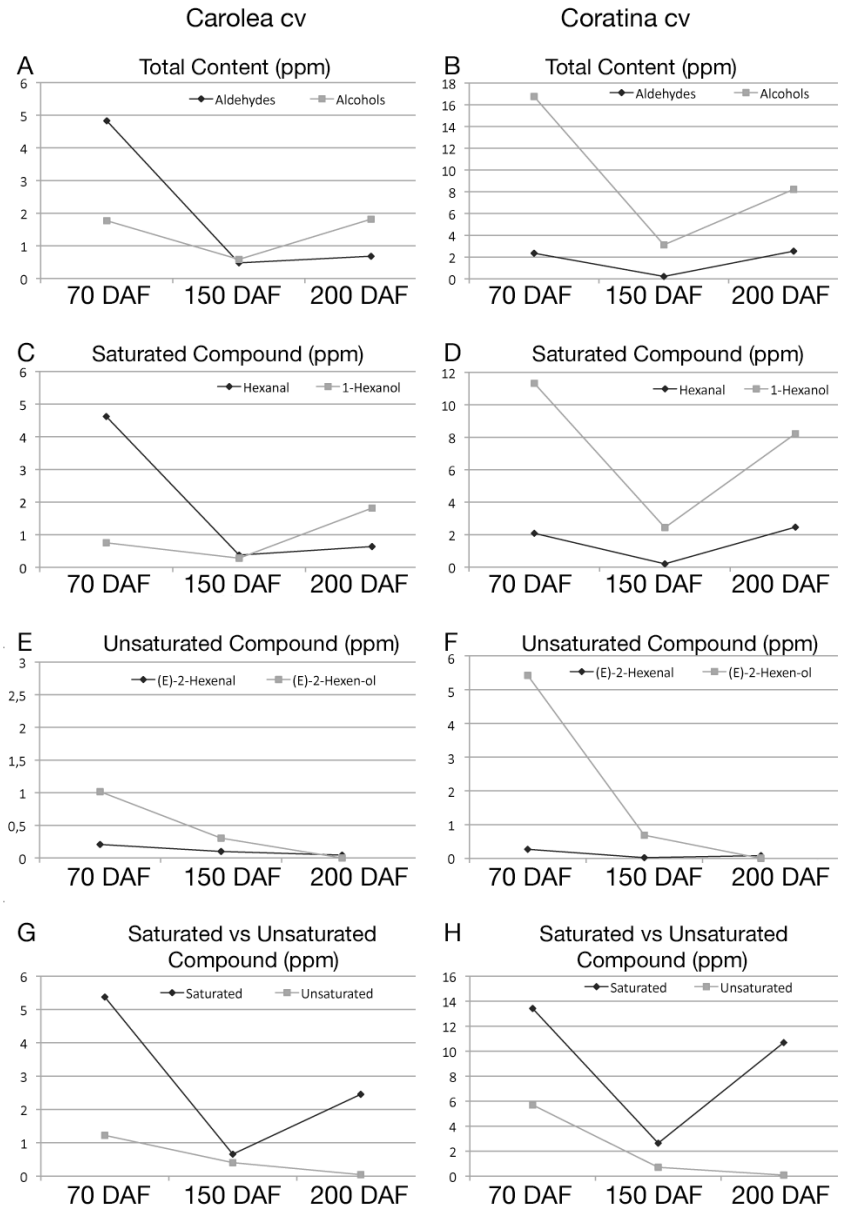

Fig. 9. Change of selected volatile chemical during Carolea (left panel) and Coratina's drupes (right panel) ripening. Distribution of: (A)(B) aldehydes (hexanal + (*E*)-2-hexenal) and alcohols (1-hexanol +(*E*)-2-hexen-1-ol), (C)(D) Hexanal and 1-Hexanol, (E)(F) (*E*)-2-hexenal and (*E*)-2-hexen-1-ol variation, (G)(H) Saturated vs. unsaturated compound at each developmental stages. Data on aldehydes and alcohols contents are expressed in ppm.

Several progress in plant volatile compound research has been made through the use of molecular and biochemical techniques in different species [18-20-32-33-34-35-36]. As far as olive plant is concerned, data in literature deal exclusively with oil [5-7-17-22-25]. On the basis of our experimental data on fruit, two main conclusions can be drawn. The first conclusion is that from mature to black ripe stage deep changes in aroma composition of olive

fruit occurred and starting from a very different initial conditions, at the ripening stage drupes of both cultivars showed a quite comparable pattern with respect to selected volatile compounds. The second deals with the observation that for all analyzed stages and for both cultivar, saturated compounds (i.e. hexanal and 1-hexanol) strongly impact on the amount of volatiles (**Fig. 9G and H)**. This result can be related to either a high ratio between linoleic and linolenic acids content and/or substrate specificity, and/or specific activities of enzyme working upstream and within the LOX pathway.

In conclusion, the results here discussed appear to match well with the differential regulation of gene expression detected over time in the fruits of the two evaluated cultivars. Namely they exhibited early differences in the relative expression of related *OeADH* and *LOX* genes which could account for the sensible differences in the flavour profiles at the green mature stages. As drupes undergo ripening process both gene expression and volatile composition became comparable in line with a more general role of lipoxygenase pathway in fruit senescence process [38]. On the basis of this tight relationship, we propose that investigated genes feature as good putative molecular markers for particular flavour characteristic occurring in very short temporal window and thus provide a molecular tool for olive cultivar selection and breeding program.

[These results have been recently submitted as a paper "Interfacing between the expression pattern of two related genes in the lipoxygenase pathway and aroma biogenesis in olive (*Olea europaea* L.) fruits" by **Iaria D.L.**, Bruno L., Chiappetta A.A.C., Macchione B., Tagarelli A., Sindona G., Bitonti M.B.A. to *Journal of Agricultural and Food Chemistry*].

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