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An overview of ROS and RNS metabolism in the olive reproductive process

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An overview of ROS and RNS metabolism in the olive reproductive process

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A mis hijos Adrián y Elsa

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Abstract

The olive tree (*Olea europaea* L.) has a high economical and social importance in the Mediterranean area. Spain as the main world producer, and specially Andalusia, has very significant positions regarding these aspects. The peculiarities of the olive tree in relation with the reproductive process make its study an important tool to achieve satisfactory yield production. We are beginning to understand that Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), far from their traditional roles related to toxicity and stress, have much to say in Plant Reproductive Biology as in the many processes that have been described to by regulated by these two groups of molecules.

As a first approach, and aimed to study the overall implications of ROS and RNS in the Reproductive Biology of Plants, we used flowers from a variety of Higher Plant families with different types of stigmas and self-incompatibility systems (as well as an ancient representative of Angiosperms) at different developmental stages. We analysed the presence of ROS (H_2O_2)/NO in these stigmas by fluorescence stereomicroscopy. Next, we performed a detailed and individualised detection of H_2O_2, O_2^- and NO in the olive stigmas, anthers and pollen grains over several developmental stages under stereomicroscopy and high-resolution confocal laser scanning microscopy by using highly selective probes.

In order to gain knowledge about the molecular basis of the observed changes taking place in ROS and NO metabolism during the reproductive development, a molecular approach was designed by preparing subtractive (SSH) libraries able to dissect differential expression of transcripts amongst the reproductive (pollen, pistil) and vegetative (leaf) tissues of the olive. Moreover, the molecular polymorphism, expression levels, ranks of biochemical activity, allergenic characteristics and cell localization of a key component of the antioxidant enzyme pool of olive pollen (the Cu,Zn superoxide dismutase enzyme) (Cu,Zn-SOD), were characterized. Localization was accomplished by means of a broad panel of cytological and immunocytochemical approaches. The high polymorphism of olive pollen SOD transcripts, obtained from SSH libraries, transcriptomic approaches and experimental cloning, was further assessed by using bioinformatics methods.

A large number of interesting and novel conclusions were reached after using such multidisciplinary study: ROS were detected in the stigmas of the different plant species inspected, but not significantly in the style or ovary. ROS accumulation was present in multiple and varied patterns, considering their presence at the unopened flower, those phases considered "receptive" to pollen interaction, or post-fertilization stages. At this

regard, no clear phylogenetic relationships, or associations with the type of stigma (dry or wet) or with the self-incompatibility system were observed. ROS accumulation in primitive

Angiosperms of the *Magnolia* genus might indicate a preferential function for these chemicals in defence functions, which could later evolve to more focused signalling functions as those of signalling. In the reproductive tissues of the olive tree, ROS and NO are produced by following stage- and tissue- specific profiles. We assigned different putative functions to these products depending on early flowering stages (defence functions) and stages where there is an intense interaction between pollen and pistil which may determine the presence of a receptive phase in the stigma Finally ROS have a well known function in senescence, which could also be applied to the stigma. The study confirms the decrease in the presence of ROS in the olive stigma when NO is actively produced by pollen grains and pollen tubes during the receptive phase, which has been described already in other plant species.

The construction of six SSH subtractive libraries between each two of pollen, pistil and leaf libraries allowed identifying transcripts with important roles in these respective tissues. The functions of many of the transcripts obtained are intimately related, and most of them are of crucial importance in defence, pollen-stigma interaction and signalling. Several of these transcripts are directly involved in ROS metabolism, and include those of superoxide dismutases. Many of these SOD sequences were retrieved from the libraries and from additional transcriptome approaches made by next generation sequencing procedures. After experimental cloning and confirmation, the sequences were used for further scrutiny by bioinformatics methods, in order to identify potential relationships, conservation, and the detection of several unique forms, including a deleted variant of the Cu,Zn-SOD. Moreover, 3-D models of the main alternative forms were generated, and bioinformatic predictions on the antigenicity/allergenicity of these molecules were obtained.

Biochemical assays of SOD activity, carried out on pollen extracts, did not show correlation to pollen viability. New isoforms of Cu,Zn-SODs (including chloroplastidic forms not previously described in this material) were detected in the extracts, as well as differentially expressed Mn, Fe and Cu,Zn-SODs isoforms present in the pollen extracts obtained from different olive cultivars. Proteins cross-reacting to an Cu,Zn-SOD antibody were also identified for the first time in the pollen of a variety of allergenic species, thus indicating a potential relevance of this molecule as panallergen. Finally, both relevant forms of Cu,Zn-SOD (complete and deleted) were expressed as recombinant proteins, and subjected to SOD activity analyses. The deleted recombinant enzyme showed a slightly differential SOD activity when compared to the complete form. Such difference might

contribute to finely regulate ROS balance in a differential form. Several antibodies to the Cu,Zn-SOD enzyme were obtained, and used for immunolocalization purposes. Such studies, particularly those performed at ultrastructural level, confirmed the previously reported localization of the enzyme in the cytosol and the olive pollen exine and apertures. In addition, we also detected the enzyme in amyloplasts present in young pollen grains, and in low-differentiated plastids existing in the mature pollen grain. The Cu,Zn-SOD enzyme was also detected in peroxisomes present in the cytoplasm of the vegetative cell, which were also positive to catalase as a peroxisomal marker.

The physiological relevance of all these findings is broadly discussed and contrasted In comparison with the available literature about the matter.

Resumen

El olivo (*Olea europaea* L.) posee una gran importancia económica y social en el área mediterránea. España como el mayor productor mundial, y especialmente Andalucía, tiene una posición muy significativa en relación con estos aspectos. Las peculiaridades del olivo en relación a los procesos reproductivos hacen de su estudio una importante herramienta para alcanzar una producción satisfactoria. Estamos empezando a conocer que las especies de oxígeno reactivo (ROS) y las especies de nitrógeno reactivo (RNS), lejos de sus tradicionales funciones implicadas en toxicidad y estrés, tienen mucho que decir en la Biología Reproductiva de las Plantas, tanto como en los numerosos procesos regulados por estos dos grupos de moléculas.

Como una primera aproximación, y con objeto de estudiar las implicaciones generales de las ROS y RNS in la Biología Reproductiva de las Plantas, usamos flores de varias familias de Plantas Superiores con diferentes tipos de estigmas y de sistemas de autoincompatibilidad (así como un representante primigenio de las Angiospermas) en diferentes estadios de desarrollo. Analizamos la presencia de ROS (H_2O_2)/NO en dichos estigmas mediante estereomicroscopía de fluorescencia. A continuación, realizamos una detección detallada e individualizada de H_2O_2, O_2^- y NO en los estigmas, anteras y granos de polen de olivo en diferentes estadios de desarrollo mediante técnicas de estereomicroscopía y microscopía láser confocal de alta definición, usando sondas altamente selectivas.

Con objeto de mejorar nuestro conocimiento sobre las bases moleculares de los cambios observados en el metabolismo ROS y NO durante el desarrollo reproductivo, se diseñó una aproximación molecular consistente en la preparación de bibliotecas sustractivas (SSH), capaces de diseccionar la expresión diferencial de transcritos entre los tejidos reproductivos (polen, pistilo) y vegetativos (hoja) del olivo. Además, se caracterizaron el polimorfismo molecular, los niveles de expresión, las tasas de actividad bioquímica, las características alergénicas y la localización celular de un componente clave del conjunto de enzimas antioxidantes del polen del olivo (el enzima Cu,Zn superóxido dismutasa) (Cu,Zn-SOD). La localización fue realizada mediante un amplio panel de aproximaciones citológicas e inmunocitoquímicas. El elevado polimorfismo de los transcritos de SOD obtenidos de las bibliotecas SSH, de aproximaciones transcriptómicas, y de su clonación experimental fue evaluado posteriormente mediante métodos bioinformáticos.

Un gran número de interesantes y nuevas conclusiones fueron obtenidas tras realizar el presente estudio multidisciplinario: las ROS fueron detectadas en los estigmas de las diferentes especies de plantas inspeccionadas, pero no aparecieron de forma significativa en el estilo o en el ovario. La acumulación de ROS se presentó en forma de patrones múltiples y variados, teniendo en cuenta su presencia en la flor sin abrir, en aquellas fases consideradas "receptivas" a la interacción con el polen, o en los estadios de post-fertilización. A este respecto, no se encontraron relaciones filogenéticas claras, o asociaciones con el tipo de estigma (seco o húmedo), o con el sistema de autoincompatibilidad. La acumulación de ROS en Angiospermas primitivas del género *Magnolia* podría indicar una función preferencial de estos químicos en defensa, que podría haber evolucionado posteriormente hacia funciones de señalización más especializadas como las de señalización. En los tejidos reproductivos del olivo, las ROS y el NO son producidos según perfiles específicos de estadio y de tejido. Hemos asignado funciones potenciales a esos productos, dependiendo de los estadios tempranos de floración (funciones de defensa) y de los estadios donde existe una intensa interacción entre polen y pistilo, que pueden determinar la presencia de una fase receptiva en el estigma. Finalmente, las ROS tienen una función bien conocida en senescencia, que serían igualmente aplicables a este fenómeno en el estigma. El estudio confirma la disminución de la presencia de ROS en el estigma del olivo cuando se produce activamente NO en los granos de polen y tubos polínicos, durante la fase receptiva, que ha sido anteriormente descrita en otras especies vegetales.

La construcción de seis bibliotecas sustractivas SSH entre cada dos (each two of) de las bibliotecas de polen, pistilo y hoja, permitió la identificación de transcritos con papeles fundamentales en los respectivos tejidos reproductivos. Las funciones de muchos de los transcritos obtenidos están íntimamente relacionadas, y muchas de ellas son de importancia capital en defensa, interacción polen-estigma y señalización. Algunos de estos transcritos están directamente implicados en el metabolismo ROS, e incluyen aquellos de superóxido dismutasas. Muchas de estas secuencias de SODs fueron extraídas de las bibliotecas SSH y de aproximaciones transcriptómicas adicionales, realizadas mediante procedimientos de secuenciación de última generación. Tras la clonación por medios experimentales y su confirmación, las secuencias fueron usadas para posterior escrutinio mediante métodos bioinformáticos, con objeto de identificar relaciones potenciales, grado de conservación, y la detección de varias formas únicas, que incluyen una variante delecionada de la Cu,Zn-SOD. Además, se generaron modelos 3-D de las formas alternativas fundamentales, así como predicciones bioinformáticas sobre la antigenicidad/alergenicidad de estas moléculas.

Los ensayos bioquímicos de actividad SOD, llevados a cabo sobre extractos de polen, no mostraron ningún tipo de correlación con la viabilidad polínica. Se detectaron nuevas formas de Cu,Zn-SODs (incluyendo formas cloroplastídicas no descritas previamente en este material), así como isoformas de Mn, Fe y Cu,Zn-SODs expresadas diferencialmente en extractos de polen obtenido de diferentes cultivares. También fueron identificadas proteínas con reacción cruzada frente a un anticuerpo anti Cu,Zn-SOD de polen de olivo en los extractos de polen de diversas especies alergénicas, lo que indica una relevancia potencial de esta molécula como panalérgeno. Finalmente, ambas formas relevantes de Cu,Zn-SOD (completa y delecionada) fueron expresadas como proteínas recombinantes , y fueron sometidas a análisis de actividad SOD. El enzima delecionado recombinante mostró una actividad SOD ligeramente diferencial a la de la forma completa del enzima. Esta diferencia podría contribuir a regular de forma muy fina el equilibrio ROS. Varios anticuerpos frente al enzima Cu,Zn-SOD fueron obtenidos, y usados para inmunolocalización. Estos estudios, particularmente los realizados a nivel ultrastructural, confirmaron la localización del enzima, previamente indicada en el citosol y en la exina y las aperturas del grano de polen del olivo. Además, el enzima fue detectado en amiloplastos presentes en los granos de polen jóvenes, y en plástidos poco diferenciados existentes en el grano de polen maduro. El enzima Cu,Zn-SOD fue detectado también en peroxisomas presentes en el citoplasma de la célula vegetativa, que fueron también positivos a catalasa, utilizada como marcador peroxisomal.

La relevancia fisiológica de estas observaciones es ampliamente discutida y contrastada con la literatura disponible sobre el tema.

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OVERALL INTRODUCTION

ROS & RNS. Concept and diversity

Between 1% to 3% of all oxygen consumed by respiration is inevitably reduced to superoxide radicals and hydrogen peroxide (H_2O_2). These and other oxygen-derived molecules with moderate to very high reactivity are known as Reactive Oxygen Species (ROS). The term includes free radicals (molecules with one or more unpaired electrons, such as the superoxide and hydroxyl radicals) and non-free radicals (molecules with no unpaired electrons, such as H_2O_2 and singlet oxygen) (Matamoros et al., 2003) (Table 1). All organisms produce a range of Reactive Oxygen Species, including superoxide, hydroxyl radical and hydrogen peroxide during the course of normal metabolic processes (Maxwell et al., 1999). ROS have been viewed as toxic by- products of cellular metabolism; however, a growing body of evidence suggests that they function as signalling molecules in eukaryotes, leading to specific downstream responses (Rentel and Knight, 2004). The evolution of aerobic metabolic processes such as respiration and photosynthesis unavoidably lead to the production of Reactive Oxygen Species in mitochondria, chloroplasts, and peroxisomes (Apel and Hirt, 2004). Free-radicals in living systems are a source of study which has increased exponentially in the last decades.

Similarly, the term Reactive Nitrogen Species (RNS) refers to nitrogen-derived molecules with variable reactivity and includes free radicals and non-free radicals (Table 1). Nitric oxide (NO) is involved in many key physiological processes in animals and, as shown in recent years, also in plants. It reacts with a broad panel of chemicals, including the superoxide radicals to form peroxynitrite, with thiol compounds to form nitrosothiols, as well as with lipids, proteins, vitamin E, and nucleic acids (Corpas et al., 2009; Fazzari et al., 2014). Nitric oxide is a free radical that had been known for many years simply as a toxic air pollutant. The discovery of enzymatic NO production in many living organisms has established a new paradigm: NO being an essential molecule endogenously produced in the cells. In Plant Science it has been suggested that NO acts as a plant hormone equivalent to ethylene; that is, as a gaseous signal transmitter. A major difference between plants and animals is that the growth and development of plants is closely linked to the surrounding environment where NO levels vary according to biotic and abiotic activities. This

fundamental difference may make the NO-signalling network system of plants larger and more complicated than that of vertebrates (Yamasaki, 2005).

ROS and RNS have been found to be ubiquitous intermediates in natural biochemical reactions. However, their unbalance is responsible for biochemical dysfunctions by causing damage to lipids, DNA and proteins. When ROS production exceeds the anti-oxidizing capacity, this can lead to cell damage and ultimately cell death by ROS toxicity and/or specific ROS-activated cell-death-inducing signalling events (Tripathy and Oelmüller, 2012). Although the study of these molecules is increasing, there are currently many processes that are not well understood yet.

Reactive Oxygen Species (ROS)			
Superoxide anion	$\cdot O^{2-}$	Ozone	O_3
Hydroxyl radical	$\cdot OH$	Peroxide	$\cdot O_2 \cdot$
Hydroxyl ion	OH^-	Alcoxyl radical	$RO \cdot$
Singlet oxygen	$^1O_2$	Hypochloric acid	HOCL
Hydrogen Peroxide	H_2O_2	Peroxynitrite	ONOO-
Peroxyl radical	$ROO \cdot$	Lipid peroxyl	$LOO \cdot$
Hydroperoxyl radical	$\cdot OH_2$	Organic hydroperoxide	ROOH
Reactive Nitrogen Species (RNS)			
Nitrous oxide	$NO \cdot$	Nitroxyl anion	NO-
Peroxynitrate	$OONO^-$	Nitrogen dioxide	$NO_2 \cdot$
Peroxynitrous acid	ONOOH	Dinitrogen trioxide	N_2O_3
Nitryl chloride	NO_2Cl	Nitrous acid	HNO_2

Table 1. Major ROS/RNS present in biological systems

The beginnings and the nowadays of the study of ROS & RNS in animals

The story of the study of ROS in animals can be considered recent as it started in 1954 with the discovery of the toxicity of oxygen due to partially reduced forms of oxygen (Gerschman et al., 1954). In the same year the presence of free radicals in a variety of lyophilised biological materials was reported (Commoner et al., 1954). Only two years later, the role of free radicals in ageing process (Harman, 1956) was proposed, and this

represented the turning point in the study and interest towards these molecules. A few years later happened a new highlighted event: the discovery of the SOD enzyme (McCord and Fridovich, 1969). It was also relevant the discovery of the evidence that the hydroxyl radical stimulates the activation of guanylate cyclase, and the formation of the "second messenger": cyclic guanosine monophosphate (cGMP) (Mittal and Murad, 1977).

Reactive oxygen species, oxidative stress, and oxidative damage are increasingly assigned important roles in biomedical science as deleterious factors in pathologies and aging. There is the growing recognition that many ROS are important mediators in a range of biological processes such as signalling (Murphy et al., 2011) (Table 2). Reactive oxygen species, oxidative stress, and oxidative damage are also increasingly assigned important roles in biomedical science as deleterious factors in pathologies and aging (Table 3).

Process	References
Tumour suppression	(Vurusaner et al. 2012)
Regulation of glucose metabolism	(Styskal et al., 2012)
Regulation of intracellular signalling cascades in phagocytic cells	(Thannickal and Fanburg, 1995); (Jones et al., 1996); (Robinson, 2008).
Regulation of vascular tone	(Ignarro and Kadowitz, 1985); (Wolin et al., 1999); (Dorner et al., 2003).
Oxygen concentration sensor	(Acker and Xue, 1995)
Cell adhesion	(Albelda et al., 1994)
Regulation of immune responses	(Guzik et al., 2003); (Wink et al., 2011); (Yang et al., 2013).

Table 2. ROS & RNS-controlled physiological processes in animals.

On the other hand, altered redox regulation has also pathophysiological implications, including:

Physiopathology	References
Atherosclerosis	(Dalle-Donne et al., 2006); (Leonarduzzi et al., 2012).
Retinal disease	(Cervellati et al., 2014)
Neurodegenerative diseases	(Sayre et al., 2001); (Emerit et al., 2004)
Ischemia heart	(Gey et al., 1987)
Cancer	(Gey et al., 1987)
Cardiovascular disease	(Dhalla et al., 2000); (Afanas'ev, 2011)
High blood pressure	(Paravicini and Touyz, 2006)
Inflammation	(Guzik et al., 2003)
Allergy	(Bowler and Crapo, 2002); (Ckless et al., 2011); (Manzo et al., 2012).
Parkinson	(Jenner, 2003)
Asthma	(Ghosh and Erzurum, 2011); (Prado et al., 2011); (Ritz and Trueba, 2014);
Aging related diseases	(Harman, 1956); (Ames et al., 1993); (Berlett and Stadtman, 1997); (Manton et al., 2004)

Table 3. ROS and RNS-controlled pathophysiological processes in animals.

Aging is the progressive accumulation of diverse, deleterious changes with time that increases the chance of disease and death. The involvement of free radicals in aging is related to their key role in the origin and evolution of life. Aging changes are commonly attributed to development, genetic defects, the environment, and disease. The latter produces aging changes at an exponentially increasing rate with age, becoming the major risk factor for disease and death for humans after the age of 28 years in the developed countries (Harman, 2006).

When the antioxidant defence in the human body becomes overwhelmed, oxidative stress to the cellular components often occur, inducing inflammatory, adaptive, injurious, and reparative processes. Besides, lifestyle and nutrition might play an important role against environmental oxidant exposure and damage (Poljšak and Fink, 2014).

The story of NO in mammals started when Hauschildt et al. (1990) realized that there was a production of NO in macrophages cultured with bacterial lipopolysaccharides. Later, the role of NO in the regulation of smooth muscle contraction was researched (Ignarro et al., 1990). In animals, NO production is mainly generated by deamination of arginine by nitric oxide synthase (NOS), to form citrulline and NO. Nitric oxide synthase (homologous to P450 cytochrome c reductases) belongs to an enzymatic group, which is sub-classified as $Ca^{2+/}$calmodulin activated brain NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The reduction of NO_2 in the mitochondrion in mammalian tissues also generates NO (*via* cytochrome c oxidase, reduction at complex II, or nitrate reductase) (Mur et al., 2013).

The beginnings and the nowadays of the study of ROS & RNS in plants

Although basic oxygen metabolism is similar in plants and animals, there are several aspects unique to plants. Plants have photosynthetic electron transport in the chloroplast that produces oxygen and is accompanied by the production of reactive oxygen species (ROS). Other processes unique to plants that involve ROS are lignification of plant cell walls, which is important in normal growth, as well as pathogenesis and senescence, which is essential to fruit ripening, seed production and overwintering (Baker and Orlandi, 2002).

The story of the study of ROS started a little bit earlier than in animals. Several highlighted events marked the key point of the story. In 1951 happened the discovery of the Mehler reaction in the photosystems (superoxide and hydrogen peroxide production) (Mehler, 1951). By the 1990´s, the ascorbate-glutathione cycle was characterized (Foyer and Halliwell, 1976; Asada, 1999). Simultaneous studies on plant ROS started to be focused over tissues infected by pathogens and the production of ROS (Doke, 1983). The production of ROS in response to environmental stress was also a starting research topic. The evidences indicated that ROS also functioned as signalling molecules in plants involved in regulating development and pathogen defence responses (Apel and Hirt, 2004). The new role of ROS, not only involved in harmful processes, but also as signalling molecules began to be a strong branch of study for numerous scientists around the world.

In 2005, the term "oxidative signalling" was coined by Foyer and Noctor (Foyer and Noctor, 2005).

The main sources of ROS in plants under physiological conditions are respiration, photosynthesis, and N_2 fixation. In addition, ROS are produced at high rates when plants are exposed to abiotic, biotic, or xenobiotic stress (Matamoros et al., 2003).

ROS are involved in a variety of plant physiological functions, as it is displayed in Table 4.

Physiological response of ROS/RNS	References
Programmed cell death	(Bolwell, 1999)
Stomatal closure in response to abscisic acid and elicitors	(Pei et al., 2000)
Modulation cytoskeletal functions	(Yemets et al., 2011)
Root hair growth	(Foreman et al., 2003)
Auxin signalling and gravitropism	(Joo et al., 2001)
Expression of genes encoding proteins required for peroxisome biogenesis	(Lopez-Huertas et al., 2000)
Salinity and drought	(Miller et al., 2010)
Excess of light	(Li et al., 2009)
Cold	(O´Kane et al., 1996)
Ozone	(Langebartels et al., 2002)
UV-B	(Green and Fluhr, 1995)
Defence	(Torres et al., 2006); (Leitner et al., 2009); (Gaupels et al., 2011); (Mur et al., 2013).

Table 4. ROS & RNS-controlled physiological processes in plants.

During the process of plant pathogen defence, ROS are produced by plant cells via plasma membrane-bound NADPH-oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast (Grant and Loake, 2000).

On the other hand, the study of RNS (mainly NO) started to be a hot topic a few years later, since NO was recognized as an important molecule in plant defence response in concert with ROS (Dangl, 1998; Delledonne et al., 1998; Durner et al., 1998). Until that

moment, NO had long been considered only as a harmful air pollutant. The discoveries of the enzymes that produce NO with NADPH and L-arginine have entirely changed the recognition of NO from just an air pollutant to an essential signalling molecule. Such a historical big wave in science, of course, has led plant biologists to the idea that plants may possess a NO signalling system similar to that found in animals. The search for mammalian-type NO synthase (NOS) in plants was the primary interest at that time. Indeed, much biochemical research did suggest that plant cells seem to include a mammalian-type NOS activity (Delledonne et al., 1998). However, there has been no evidence for the presence of a plant NOS structurally similar to the mammalian one (del Río et al., 2004). It is now a consensus for plant biologists that NO is an important gas molecule that is comparable with the plant hormone ethylene (Yamasaki, 2005). The molecular mechanisms for plant NO synthesis remain elusive up to date.

Interaction of ROS & RNS

The study of ROS and RNS nowadays goes hand in hand (Figure 1), and they co-ordinately regulate plant defence responses to biotic stress. In addition, evidence has accumulated demonstrating that there is a strong cross-talk between oxidative and nitrosative signalling upon abiotic stress conditions (Molassiotis and Fotopoulos, 2011). The increasing interest in the studying of these molecules is due to the fact they are involved in a huge number of plant processes, being considered as nearly ubiquitous plant signalling molecules.

The interaction of ROS and NO in plants exposed to unfavourable conditions inevitably produce ROS, but not necessarily NO accumulation. ROS can arise as a toxic by-product of disturbed energy metabolism, and/or can be produced for signalling purposes. In contrast, NO is rather a highly specialized second messenger, which modifies ROS signalling or acts independently of ROS. Significantly, ROS and NO bursts are often triggered simultaneously -sometimes even in the same cellular compartment-. Particularly chloroplasts and peroxisomes are hot spots of NO-ROS interactions. NO, ROS, and antioxidants chemically react resulting in the formation of RNS such as $ONOO^-$, NO_2, N_2O_3, and GSNO. More indirect interactions include induction of NO synthesis by H_2O_2 and accumulation of ROS due to inhibition of antioxidant enzymes by NO-dependent

protein modifications. Uncontrolled self-amplification of ROS/RNS signalling might provoke nitrosative stress and ultimately programed cellular dead. Therefore, plants have developed efficient measures for controlling NO levels by GSNOR, haemoglobins and other RNS scavenging enzymes (Corpas et al., 2013a; Groß et al., 2013).

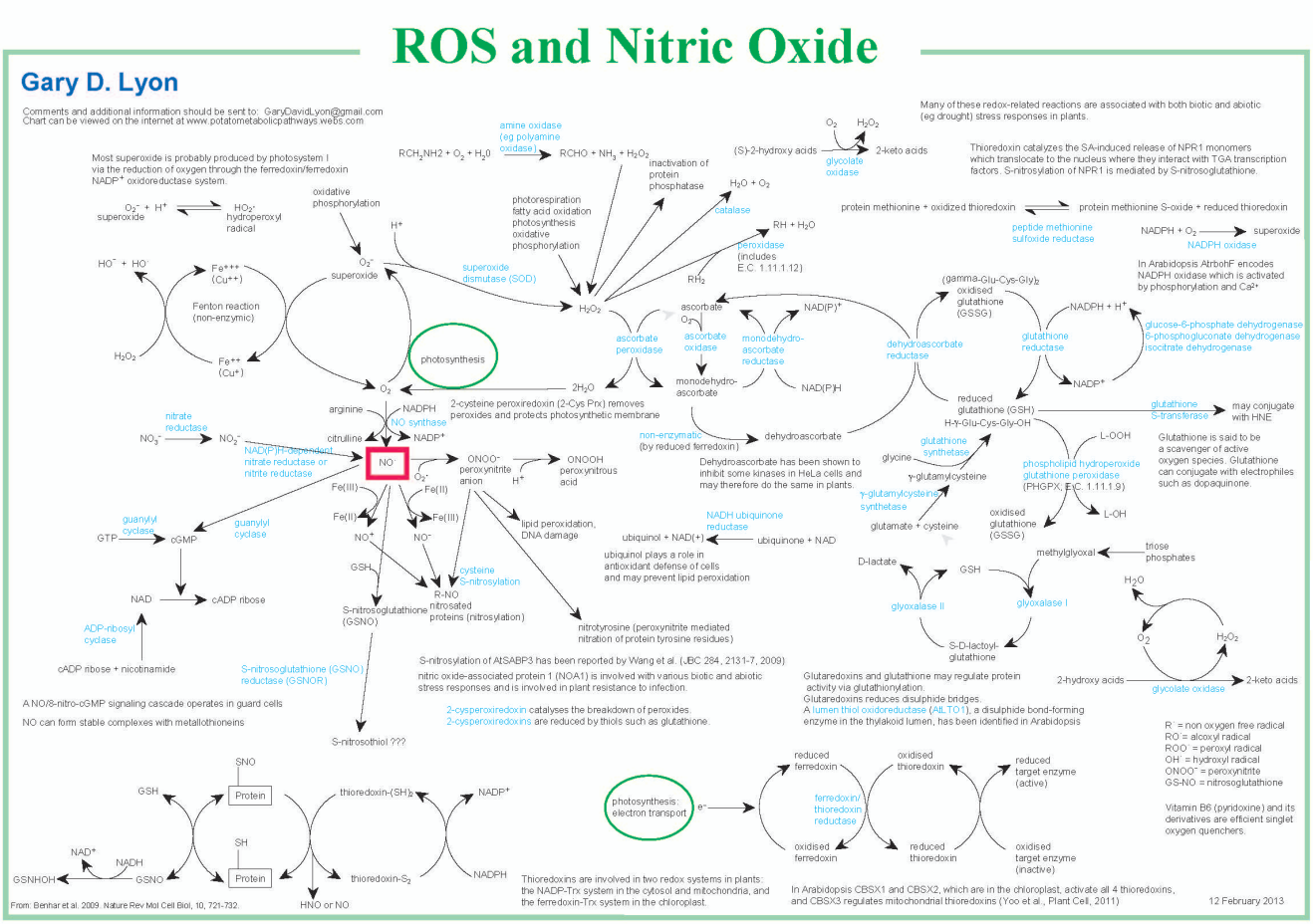

Figure 1. Metabolic pathways involving ROS and nitric oxide (NO). Diagram last up-dated 12 February 2013 and available at http://potatometabolicpathways.webs.com/ROS_and_Nitric_oxide.pdf

ROS and RNS are also involved in signalling processes through modifications of biomolecules. Protein tyrosine nitration is a post-translational modification mediated by nitric oxide-derived molecules. Tyrosine nitration can change the function of the protein, and may influence in many signal transduction pathways, because this modification prevents phosphorylation of tyrosine and consequently affects regulatory mechanisms. Peroxynitrite -a reactive nitrogen species formed usually under stress conditions by the chemical reaction between two radicals, nitric oxide and superoxide anion-, is a frequent

nitrating agent. Another nitrating reagent is the nitrogen dioxide radical formed from hydrogen peroxide and nitrite (Corpas et al., 2013b). Other biomolecules also potentially modified by RNS are lipids and nucleid acids. Preliminary data have demonstrated that the exogenous application of nitrolinolenate (a nitro-fatty acid, $LnNO_2$) to roots can induce an increase of NO content in both roots and leaves, suggesting the presence of a signalling process between both organs (Sánchez-Calvo et al., 2013).

ROS & RNS in Reproductive Biology

Reactive oxygen species are important regulators of cell and organ growth and are thought to operate by controlling the extensibility of the cell wall and modulating intracellular signalling processes. By increasing elasticity of the wall they promote growth, and by cross-linking polymers they increase rigidity and repress growth. ROS produced by NADPH oxidase proteins are also important regulators of tip growth in root hairs and pollen tubes, where they not only control wall rigidity, but also cell signalling events involving calcium and MAP kinases cascades (Bell et al., 2009). Xie et al., (2014) consider that ROS produced by NADPH oxidases, together with other signalling pathways, are key players in the control of the tapetal transcriptional network ensuring proper timing of tapetal PCD. Several authors have described the importance of the OH• in anther and pollen development. However, the role of extracellular OH• in pollen tube elongation remains to be determined (Richards et al., 2014).

The interaction or ROS with other molecules are also fine tune regulators of the reproductive process. It is possible that the interaction of ROS and cytosolic-free Ca^{2+}, both generated at the tip of the pollen tube, act as signal to pollen tube growth (Wudick and Feijó, 2014).

The NO is also relevant in reproductive biology as it has been described to participate in complex regulatory mechanisms controlling polarized and oscillating pollen growth, as well as pollen´s responses to external stimuli (Šírová et al., 2011).

In Angiosperms, the interaction between pollen and stigma is one of the most important stages in the life cycle of the plant, because its outcome determines whether fertilization will occur, and thus whether seed will set (McInnis et al., 2006a). During Angiosperm sexual reproduction, pollen grains land over receptive stigma, producing a pollen tube, which enlarges in its apical region with growing rates exceptionally high. This pollen tube penetrates through the style to deliver the male gametes to the ovules and the polar nuclei, thus participating in the double fertilization, which is a characteristic of Angiosperms. The fertilization process requires a continuous interchange of both physical and chemical signals between partners (pollen, stigma, ovules…) which has to take place in a narrow time frame, otherwise limitation or absence of seeds is the result of the failed fertilization (Herrero, 2003). In order to achieve this goal, pollen tubes have evolved to develop an extremely high metabolism, with high-energy requirements resulting in the generation of large quantities of ROS, as an inevitable consequence of the aerobic metabolism. ROS are also generated in a controlled manner.

Recent studies have shown that reactive oxygen species and nitric oxide are involved in many signalling processes taking place during the interactions pollen-pistil in several plants. Thus, different signalling networks have been described to promote pollen germination and/or pollen tube growth on the stigma (McInnis et al., 2006a; Prado et al., 2004; Cárdenas et al., 2006; Potocký et al., 2007; He et al., 2007; Wu et al., 2010; Smirnova et al., 2013; Potocký et al., 2012), as well as drive pollen tube guidance and embryo sac fertilization (Domingos et al., 2015). Kaya et al., (2015) suggest that apoplastic ROS derived from RbohH and RbohJ are involved in pollen tube elongation into the stigmatic papillae by affecting the cell wall metabolism.

The putative presence of ROS in the stigma exudate has been also described as a defence mechanism against microbe attack, similar to the secretion of nectar (McInnis et al., 2006b; Carter and Thornburg, 2004). These chemicals (hydroxyl free radicals) have been also involved in the mediation of pollen tube rupture to release sperm cells for fertilization, in a Ca^{+2} dependent process involving Ca^{+2} channel activation (Duan et al., 2014).

As regard to pollen-stigma interactions, low levels of NO were detected by McInnis et al. (2006a) in stigmas, whereas NO was observed at high levels in pollen. An interesting suggestion to explain the biological function of ROS/H_2O_2 in stigmas and NO in pollen was proposed by Hiscock and Allen (2008), who observed a reduction of these molecules in the stigmatic surface when either pollen grains or NO were artificially added. They propose that the main function of stigmatic ROS/H_2O_2 can be defence against pathogens, whereas pollen NO may cause a localized reduction of these molecules, thus breaching this defence system.

Self-Incompatibility related to ROS & RNS

ROS and RNS have also been shown to be involved in SI mechanisms. In the *Papaver rhoeas* model, allele-specific interaction was shown to induce a rapid and transitory increase of ROS and NO in pollen tubes. In this model, SI is triggered by an increase of intracellular Ca^{2+} in the pollen tube, which ultimately originates actin reorganization and programmed cell death resulting in the destruction of the self-pollen. ROS/NO seem to act mediating the signal between calcium and PCD. In the S-RNase-based model of SI response, the S-RNase specifically disrupts tip localized ROS of incompatible pollen tubes of *Pyrus pyrifolia* via arresting ROS formation in mitochondria and cell walls (Wang et al., 2010a; Wang et al., 2010b; reviewed by Traverso et al., (2013). Moreover, ROS and NO have been reported to control male-female cross-talk during fertilization in olive to regulate PCD in self-incompatible pollen, providing a highly effective way to prevent self-fertilization (Serrano et al., 2015).

ROS/NO in pollen grains and the allergy issue

Pollen exposure induces allergic airway inflammation in sensitized subjects. The role of several antigenic pollen proteins in the induction of allergy is well characterized.

An important molecule regarding both ROS metabolism and allergy is the Ole e 5 allergen, initially purified, characterized and partially sequenced by Boluda et al., (1998) (GenBankTM/ EMBL P80740). It consist of a 16 kDa acidic protein (Ip 5.1-6.5) displaying relevant homology with Cu/Zn SODs from different species like spinach (Sakamoto et al., 1990), pineapple (Lin et al., 2000) and maize (Cannon and Scandalios, 1989). Its incidence

in type I hypersensitivity reactions is of 35% (Boluda et al., 1998). Several Mn-SODs have also been identified as allergens in different species, like *Hevea brasilensis* (Rihs et al., 2001; Wagner et al., 2001) and *Aspergillus fumigatus* (Mayer et al., 1997). In addition, a Cu,Zn-SOD, highly homologous to Ole e 5, has been described as allergen in tomato (Kondo et al., 2001). Cloning and full sequencing of Ole e 5 was achieved by Butteroni et al., (2005) (GenBank AJ428575). The sequence obtained, together with previous sequences reported by Boluda et al., (1998), Tejera et al., (1999) (GenBank P80740), Alché et al. (GenBank AF191342) and Corpas et al., (2006) (GenBank AF426829) shows a high degree of conservation with Cu,Zn SODs from other plant sources (80-90%).

Considering that pollens produce molecules such as ROS and RNS, it is possible that pollen-derived soluble signals such as NAD(P)H oxidase-generated $\cdot O^{2-}$, NO-, or its metabolite nitrite, may directly affect human airway cells, contributing to the initiation of an allergenic response (Boldogh et al., 2005; Bright et al., 2009). Pollen grain ROS represent a first, crucial signal which primes and magnifies a cascade of events in the allergic response (Speranza et al., 2012). These hypotheses are supported by different experimental evidences: i) the production of superoxide by NOX activity has been described in various allergenic pollens (Wang et al., 2009); ii) peroxynitrite has been demonstrated to play a major role in the late phase nasal blockage induced by antigen challenge in guinea pigs sensitized to Japanese cedar pollen (Mizutani et al., 2008); iii) iron-mediated dismutation of superoxide anion augments antigen-induced allergic inflammation induced by ragweed pollen extracts in a sensitized mouse experimental model (Chodaczek et al., 2007).

Agronomical importance of the olive tree in the Mediterranean area

The olive tree (*Olea europaea* L.) has a high economic and social importance in the Mediterranean area. In spite of this significance, few studies are available on the metabolism of ROS and NO in the olive tree.

The Mediterranean area produces 97% the world's olive oil. Most groves conserve traditional production methods. Specifically, Spain is the major producer country with 44% of world production and an area of 2.58 Mha. More than 74% of olive groves are traditional olive orchards (Sola-Guirado et al., 2014). The 94.4% of this area corresponds

olive trees producing olive oil, a 3% is destined to table olives, and a 2,6% of the groves has a dual purpose. Within Spain, the region of Andalucía concentrates most of the olive tree groves (60,2%) of the national surface, this representing 43,9% of the cultivated areas of the region. The province of Jaén is the main producer of olive oil, with 43,41% of the cultivated areas corresponding to olive groves. The provinces of Córdoba, Málaga, Granada and Sevilla are also of high importance in this sector (MAGRAMA, 2013).

The olive tree as a model for the analysis of Reproductive Biology

The olive tree is a dicotyledonous species, with some peculiarities in its reproductive organs. The presence of self-incompatible genotypes in this species has been described. Moreover, most olive varieties display some degree of Self-Incompatibility (SI). They produce little or no fruit in pure orchards, and even for those partially Self-Fertile (SF) ones, cross-pollination favours higher and more regular yields (Breton and Bervillé, 2012). Olive tree fertilization can be named as mainly allogamous. This characteristic refers to the fact that flowers are preferentially fertilised by pollen grains from a different cultivar. Such pollen grains have been demonstrated to be able of flying over large distances (Pinillos and Cuevas, 2009). The Self- Incompatibility system in this plant has been proposed to respond to the gametophytic type, although it has not been well determined yet (Breton et al., 2014).

A combination of photoperiod and temperature determines the olive flowering period. Floral induction takes place during the previous summer, and a chilling period is required by floral buds, in order to break winter dormancy. Subsequently, a warm period is required to allow flower development. Logically, topography plays an important role in floral development, which varies depending on different photoperiods and weather conditions. Annual vegetative and reproductive cycles and, therefore, annual fruit yield are strongly influenced by environmental conditions. The complex phenological response to milder winter temperatures provides a reliable bioindicator of the impact of climate change in a number of plant species, including the olive tree (Oteros et al., 2013).

OBJECTIVES

The general purpose of the present work was to deep into the knowledge about the involvement of oxidative metabolism in the Reproductive Physiology of higher plants, with a particular reference to what we consider a relevant crop (the olive tree) as regard to its economical importance, and its ability to perfectly adapt to the Mediterranean climate and their ecological conditions. Within the oxidative metabolism, ROS and RNS are molecules involved in key physiological processes including pollen viability, germination ability, self-incompatibility, signalling, as well as in allergenicity. The specific objectives were as follows:

1. Investigate the different patterns of ROS and NO accumulation in the stigmas of a broad panel of Angiosperm species, with different types of stigmas and self-incompatibility systems.

2. Determine whether relevant ROS and NO are present in the stigmatic surface and other reproductive tissues of the olive over different key developmental stages.

3. Identify key gene products involved in the function of olive pollen and pistil, in order to elucidate the events and signalling processes happening during their development, courtship, pollen grain germination and fertilization in the olive.

4. Characterize the presence of distinctive variants of Superoxide Dismutase enzymes in the olive pollen analyzing their relationships, variability and involvement in allergy.

5. Determine cellular and subcellular localization of Cu,Zn Superoxide Dismutases in the olive pollen.

CHAPTER 1

Patterns of ROS accumulation in the stigmas of Angiosperms and visions into their multi-functionality in plant reproduction

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Accumulation of reactive oxygen species (ROS) and NO in the stigma of several plant species has been investigated. Four developmental stages (unopened flower buds, recently opened flowers, dehiscent anthers and flowers after fertilization) were analyzed by confocal laser scanning microscopy using different fluorophores. In all plants scrutinized, the presence of ROS in the stigmas was detected at higher levels during those developmental phases considered "receptive" to pollen interaction. In addition, these molecules were also present at early (unopened flower) or later (post-fertilization) stages, by following differential patterns depending on the different species. The biological significance of the presence ROS/NO may differ between these stages, including defence functions, signaling and senescence. Pollen-stigma signaling is likely involved in the different mechanisms of self-incompatibility in these plants. The study confirms the enhanced production of NO by pollen grains and tubes during the receptive phase, and the decrease in the presence of ROS when NO is actively produced.

Finally, the distribution of ROS/NO in primitive Angiosperms of the genus Magnolia was determined. The production of such chemical species in these plants was several orders of magnitude higher than in the remaining species evoking a massive displacement towards the defence function. This might indicate that signaling functions of ROS/NO in the stigma evolved later, as fine tune likely involved in specialized interactions like self-incompatibility.

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INTRODUCTION

The term Reactive Oxygen Species (ROS) defines molecules derived from the metabolism of oxygen such as hydrogen peroxide or superoxide radical. In a similar way, RNS includes reactive molecules derived from nitrogen metabolism, mainly the nitric oxide (NO). The presence of ROS and RNS must be balanced to maintain the correct cellular functions. When they are present in high concentrations, they may cause damage to the cell or even cell death. So the role of the antioxidants is very important, in order to keep the correct balance of these species.

The study of both ROS and RNS in the Reproductive Biology of plants is an emerging discipline. These molecules are able to modulate and control the complex signaling cascades regulating the pollen-pistil interactions in Angiosperms. Several studies have been carried out in plants considered as model like *Lilium longiflorum, Arabidopsis, Petunia* and a invasive plant in the United Kingdom such as *Senecio squalidus* (see review of Traverso et al., 2013). McInnis et al., (2006a,b) explored the amounts of reactive oxygen species (ROS), particularly hydrogen peroxide, in stigmas and pollen from various different angiosperms by using the ROS probes $DCFH_2$-DA and TMB. They demonstrated that constitutive accumulation of ROS/H_2O_2 appears to be a feature of angiosperm stigmas, and discussed these results in terms of a possible role for stigmatic ROS/H_2O_2 and pollen-derived NO in pollen–stigma interactions and defence.

A former work by Zafra et al., (2010) was aimed to determine whether relevant ROS and RNS were present in the stigmatic surface and other reproductive tissues in the olive over different key developmental stages of the reproductive process. The olive tree is an important crop in

Mediterranean countries. It is a dicotyledonous species, with some peculiarities in its reproductive organs. The presence of self-incompatible genotypes in this species has been described, as well as fertilization mainly allogamous (this means that a flower will be preferentially pollinated by pollen from a different cultivar) (Mookerjee et al., 2005). The self-incompatibility system in this plant is of gametophytic type, although not well determined yet.

The main conclusions of this work were that both ROS and NO are produced in the olive reproductive organs in a stage- and tissue- specific manner, and that these chemicals may play different functions depending on these parameters. Thus, ROS and NO may foster defence functions against microbial or fungal attacks at the early flowering stages, whereas they also may determine the presence of a receptive phase in the stigma later on, or regulate pollen-pistil interaction. This work developed on olive also confirmed the emission of NO through the apertural regions of the pollen grains and the pollen tubes, the absence of these chemicals in the style or the ovary, and the decrease in the presence of ROS present in the stigma when NO was actively produced by pollen grains reaching this floral structure.

Some emerging literature has also described ROS and NO in the reproductive biology of other species like *Glycine max* (Li et al., 2012), *Corylus avellana* (Beltramo et al., 2012), *Helianthus* (Sharma and Bhatla, 2013), *Elaeocarpus hainanensis* and *Michelia alba* (Liu and Lin, 2013).

We have recently conducted similar analyses in species with different types of stigmas and systems of self-compatibility in order to reach general conclusions regarding the physiological roles of these products in plant reproduction.

MATERIALS AND METHODS

The conspicuous changes in the distribution and proportion of different ROS/NO occurring in the reproductive tissues of the olive throughout flower development have been used as a model to compare this topic in other plants. Several stages (unopened flower buds, recently opened flowers, dehiscent anthers and flowers after fertilization) have been studied by using $DCFH_2$-DA fluorophore and confocal laser scanning microscopy. The study was carried out in species with different types of stigmas and systems of compatibility.

Dissected floral buds or complete flowers were immersed in 50 μM $DCFH_2$-DA (Calbiochem) in MES (2-[N- morpholino]ethanesulfonic acid)-KCl buffer (5 μM KCl, 50 μM $CaCl_2$, 10 mM MES, pH 6.15) for 10 minutes followed by a wash step in fresh buffer for 15 minutes, and then observed in a Nikon C1 confocal microscope using an Ar-488 laser source. Negative controls were treated with MES-KCl buffer only.

The intensity of the green fluorescence was quantified by using the Nikon EZ-C1 viewer (3.30) software. Both average and standard deviation were calculated after measurement of a minimum of nine images corresponding to three independent experiments.

RESULTS

Differences in the flower developmental patters of the selected species were in many cases obvious (**Figure 1**, panels **A**), although a selection of similar stages was made based in the criteria described next. Flowers at stage 1 corresponded to unopened, generally green flower buds of the smaller size. Such flowers were usually dissected in order to make gynoecium available to the fluorochrome solution. At stage 2, flowers used were larger in size although still unopened; therefore removal of petals and sepals was frequently needed to make gynoecium prone to fluochrome incubation. Flowers at stage 3 were just opened and showed in most cases a significant change in the color of the petals. Gynoecia in these flowers were more easily accessible. Anthers in stage 3 contained pollen grains in most cases although anthers were not dehiscent yet. Stage 4 was characterized for anther dehiscence, with numerous pollen grains present on the stigma surface, whereas stage 5 corresponded to flowers already pollinized, displaying fallen corollas or degeneration of petals. For *Arabidopsis thaliana*, four stages were selected only, as this was the only species analyzed in which anther dehiscence took place before flower bud opening.

After treatment with $DCFH_2$-DA fluorophore, low-magnification observations detected green fluorescence in all samples analyzed. This fluorescence was in all cases restricted to the stigma, and absent from the remaining parts of the gynoecium (this is the style and the ovary), which only displayed red fluorescence assigned to autofluorescence. Such autofluorescence was comparable to that one present in the negative controls where the $DCFH_2$-DA fluorophore was omitted (**Figure 1**, panels **B**). Anthers only showed fluorescence at dehiscence stages and during senescence. When higher magnification was used, the green fluorescence of the stigmas, resulting from $DCFH_2$-DA, was observed both in the stigmatic papillae and in the stigma exudate of those species where this later was present (results not shown).

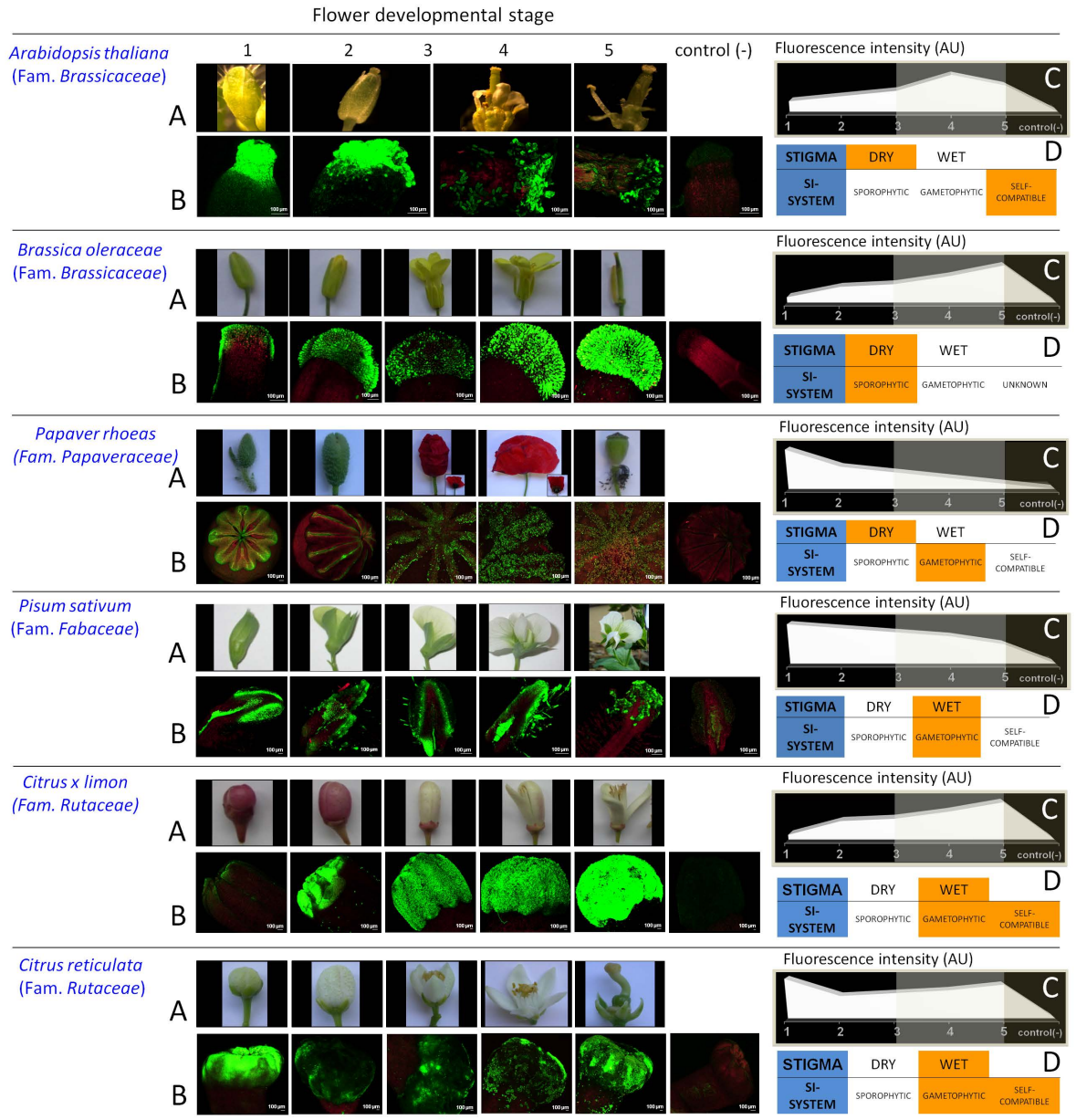

FIGURE 1: **Patterns of ROS/NO in different Angiosperm species. Panels A:** Pictures showing flower developmental stages from different Angiosperm families. Five different stages were differentiated (4 stages in *A. thaliana*, displaying dehiscent anthers before flower bud opening). **Panels B:** 3-D reconstructions of the whole stigma surface from multiple optical sections. The presence of ROS is shown as a fluorescent signal (green) exclusively obtained in the range of 515-560 nm emission wavelengths, whereas tissues autofluorescence was recorded in red. Green fluorescence was extensively localized in the exudate (wet stigmas), as well as in stigmatic papillae. Identical settings were used for image capture in both control/test experiments in order to ensure reproducibility. **Panels C:** Developmental patterns resulting from fluorescence quantification (AU: arbitrary units). **Panels D:** Additional details as regards to type of stigma (dry, wet) and self-incompatibility system for each plant analysed.

Green fluorescence was analysed and quantified in replica of the different experiments for each one of the species analysed and the five different developmental stages. Results of quantification are represented in **Figure 1,** panel **C** by using arbitrary units of fluorescence. Different patterns of ROS/NO accumulation were observed. In *A. thaliana, P. rhoeas, P. sativum and C. reticulata*, high levels of fluorescence were already

observed at the very early flower buds (stage 1), whereas *B. oleraceae* and *C. x lemon* displayed low levels of fluorescence at this same stage.

Stage 5, characterized by senescence of several flower organs after fecundation, also represented a differential step concerning fluorescence accumulation. In this case, high levels of fluorescence were observed in *B. oleraceae, C. reticulate* and *C. x lemon*, whereas low level of stigmatic fluorescence appeared in the remaining species.

Relatively low fluorescence levels in comparison with the surrounding stages were observed in stages 3 and 4 for all species analysed, coincidentally with the flower bud aperture and the putative presence of pollen grains over the stigma surface.

ROS/NO detection was also performed in flowers of *Magnolia grandiflora* (Fam. *Magnoliaceae*), a representative species considered one of the most ancient lineages of present flowering plants (**Figure 2**). Flowers of this species are bisexual and display protogynous dichogamy in order to prevent self-incompatibility. First, female flowers open, and then a delayed second opening occurs after some time, with the flower functionally acting as a male (Losada et al., 2014 and references therein). Evidence indicates that stigma receptivity in plants of this family is brief, and has been reported to be finely coordinated with the secretion of AGPs (arabinogalactan proteins) in the stigma (Losada et al., 2014).

For this species, five developmental stages were defined as regard to female development (**Figure 2**, panel **A**), including flower buds before and immediately after opening of green and white tepals (stages 1 and 2, respectively), recently opened tepals with green stigmas revealing curled tips (stage 3), coloured stigmas with curled tips (stage 4), and senescent stigmas (brown) together with dehiscent anthers (stage 5).

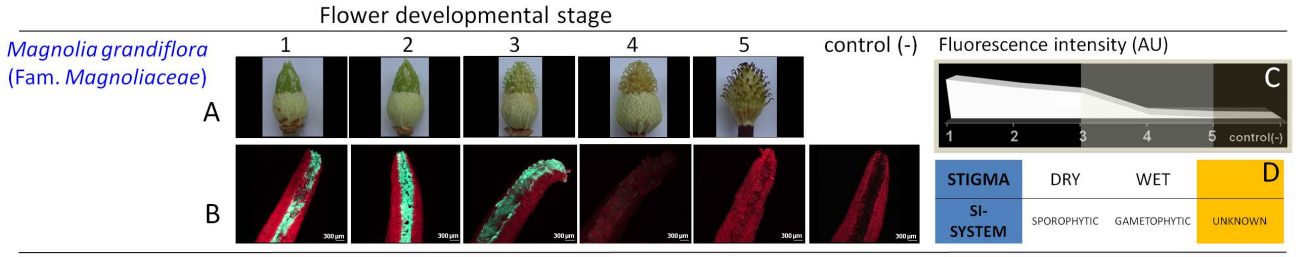

FIGURE 2: **Patterns of ROS/NO in *Magnolia grandiflora*. Panels A:** Pictures showing flower developmental stages (tepals removed). **Panels B:** 3-D reconstructions of the whole stigma surface from multiple optical sections. The presence of ROS is shown as a fluorescent signal (green) exclusively obtained in the range of 515-560 nm emission wavelengths, whereas tissues autofluorescence was recorded in red. Green fluorescence was extensively localized at the stigmatic surface. Identical settings were used for image capture in both control/test experiments in order to ensure reproducibility. **Panels C:** Developmental patterns resulting from fluorescence quantification (AU: arbitrary units). **Panels D:** Additional details as regards to type of stigma (dry, wet) and self-incompatibility system for each plant analysed.

After treatment with $DCFH_2$-DA fluorophore, medium-magnification observations detected green fluorescence over the stigmas surface only, mainly at stages 1-3. Green fluorescence was absent from the anthers and other areas of the flower at the stages analyzed, which showed red autofluorescence only, comparable to that one present in the negative controls where the $DCFH_2$-DA fluorophore was omitted (**Figure 2**, panels **B**). Green fluorescence was analysed and quantified. For this plant, the intensity of green fluorescence at the different replica was much higher than in the remaining species analysed here, even after using the same experimental procedure and identical settings for image capture of the fluorescence under the same microscope equipment. Therefore, images were acquired using modified settings in order to prevent saturation of the microscope detectors. The resulting profile is displayed in **Figure 2**, panels **C**, consisting in high levels of signal at the early stages (1 to 3), that quickly diminishes through stages 4 and 5 to nearly negligible levels.

DISCUSSION

Although a succinct number of plant species have been assayed to date, also through a limited number of developmental stages, several guidelines may arise from the present study. First, and coincidentally with the studies of (McInnis et al., 2006a; Hiscock et al., 2007; Zafra et al., 2010, Sharma and Bhatla, 2013), our findings confirm that production of ROS is a prevalent feature of Angiosperm

stigmas, detected in all species analysed on this aspect so far.

A second feature demonstrated through the present and former studies consist in the limitation of the presence of detectable amounts of ROS to the tissues of the stigma surface, with the remaining floral organs lasting unlabelled (exception made to the anthers and pollen at dehiscent stages). One of the unique features of numerous stigmas in comparison with other floral organs is the presence of stigmatic exudate, extremely rich in nutrients, including sugars, lipids and proteins, which has been detected to accumulate ROS in many species (i.e. *P. sativum, O. europaea* both *Citrus* species studied here, etc.) (Serrano et al., 2008; Shakya and Bhatla, 2010; Suárez et al., 2012; Rejón et al., 2013). Moreover, we have also detected a massive presence of ROS in *Magnolia grandiflora*, which also produces abundant secretions (Losada et al., 2014). Accumulation of ROS in the stigmatic exudate has been proposed by Hiscock et al., (2007) as a mechanism to protect against pathogen attack, on the same basis than flower nectars (Carter and Thornburg, 2000, 2004). However, and although this might represent a plausible explanation, we have detected ROS accumulation in species displaying stigmas of the dry-type *like A. thaliana, B. oleraceae* and *P. rhoeas*, therefore lacking of a significant stigma exudate. Plant species with dry and semi-dry stigmas have been described to harbour a thin pellicle which overlays the cuticle, often containing associated peroxidases (McInnis et al., 2006b). High-resolution microscopy studies would be necessary in order to assign the production of each one of the major ROS components to the tissue constituents of such stigmas.

As disclosed here, accumulation patterns for these chemicals through stigma development -a topic much less studied- offer a high level of variability among plant species. In spite of the still scarce number of stages and limited number of plants analysed, different basic outlines have been observed. Apparently, patterns do not follow clear phylogenetic criteria, as different species from the same family do not share identical o similar models of ROS accumulation (e.g. *A. thaliana* and *B. oleraceae*, both *Citrus* species studied here, and some other examples not shown -*Olea europaea* and *Jasminum excelsior*-).

Sharp differences among species are visible just at the very early stages of flower development (stages 1-2), corresponding to unopened flowers. Zafra et al., (2010) discuss many of the physiological scenarios which may concur at such stages, including the presence of high metabolic rates at the papillae and the surrounding tissues, and the defence issue mentioned above. What seems doubtful at these stages is the involvement of ROS in stigmatic receptivity and/or pollen-pistil signalling, as such stages do not physically involve pollen-pistil interaction. Then, why some plant species do not show high levels of ROS at early flowering stages? Discrepancies among species might therefore occur as the result of different rates of ROS production, for example because differences in the timing and intensity of the generated exudate, the growth rate of the floral tissues or metabolic rates (Liu and Lin, 2013).

ROS (mainly H_2O_2) scavenging has been widely correlated with launching of stigmatic receptivity, by means of the increased activity of enzymes like superoxide dismutases and peroxidases, even through the expression of new isoforms (McInnis et al., 2006b and cites therein; Sharma and Bhatla, 2013). Thus, tests for peroxidase activity have become the election method to measure pistil receptivity (Dafni and Motte Maués, 1998). Although not performed in the present study, such enzyme activities and ROS levels have been described to exhibit reverse trends during pollen–stigma interaction, the same tendency that occurs with regard to the production of NO by pollen grains reaching the stigmatic surface (McInnis et al., 2006a,b; Zafra et al., 2010; Sharma and Bhatla, 2013). In the majority of species tested here, ROS accumulation at stages 3 and 4, was overall lower than in the remaining stages. Although not particularly tested here, these observations are in good agreement with both situations: reaching of maximum stigmatic receptivity, and pistil interaction with pollen grains likely emitting NO.

Although the fine involvement of ROS, and particularly PCD in self-incompatibility mechanisms is beginning to be undercover (Thomas and Franklin-Tong, 2004; Goldraij et al., 2006; Gao et al., 2010; Serrano et al., 2010, 2012; Allen et al., 2011; Wang and Zhang, 2011), no clear relationships between the differential patterns of ROS accumulation in the stigmas and the self-incompatibility mechanism applying for each species have been detected either. As an example, *B. oleraceae* and *Citrus x lemon* display quite similar patterns of ROS accumulation, in spite of concealing different self-incompatibility systems. On the contrary, species with similar self-incompatibility systems may differ broadly in their ROS-accumulating profiles (i.e. both *Citrus* species analysed here, *P. rhoeas* and additional species not shown). Moreover, *A. thaliana* (self-compatible) and *P. rhoeas* (gametophytic SI) share rather similar profiles. Finally, *Magnolia grandiflora* shows a nearly unique ROS-accumulating pattern. The prevalence of this type of profiles among other ancient species (either with protogynous dichogamy in order to prevent self-incompatibility, or other considered more evolved systems) is yet to be determined. The great contrast between the huge presence of ROS at the early stages and the near absence of these chemicals in *M. grandiflora* stigmas at those stages with pollen-stigma interactions might suggest that ROS function in these stigmas could be strongly

unbalanced towards the defence function. Finely tuned signaling interactions among pollen and stigma, might be then reduced or absent in this primigenous and singular plant, and appear later in evolution. However, these premises have to be further assessed.

An additional topic to be comprehensively examined is flower senescence, particularly stigma senescence as per stage 5 in the present study. Rise in ROS production at this stage, as the consequence of programmed cell death (PCD), although frequent and widely described for petals (Rogers, 2012), shouldn't be considered a fully general trend in stigmas. Alternative patterns, with low production of ROS at stage 5 have been detected here in *Papaver* and *Magnolia*, again depending on the species analyzed.

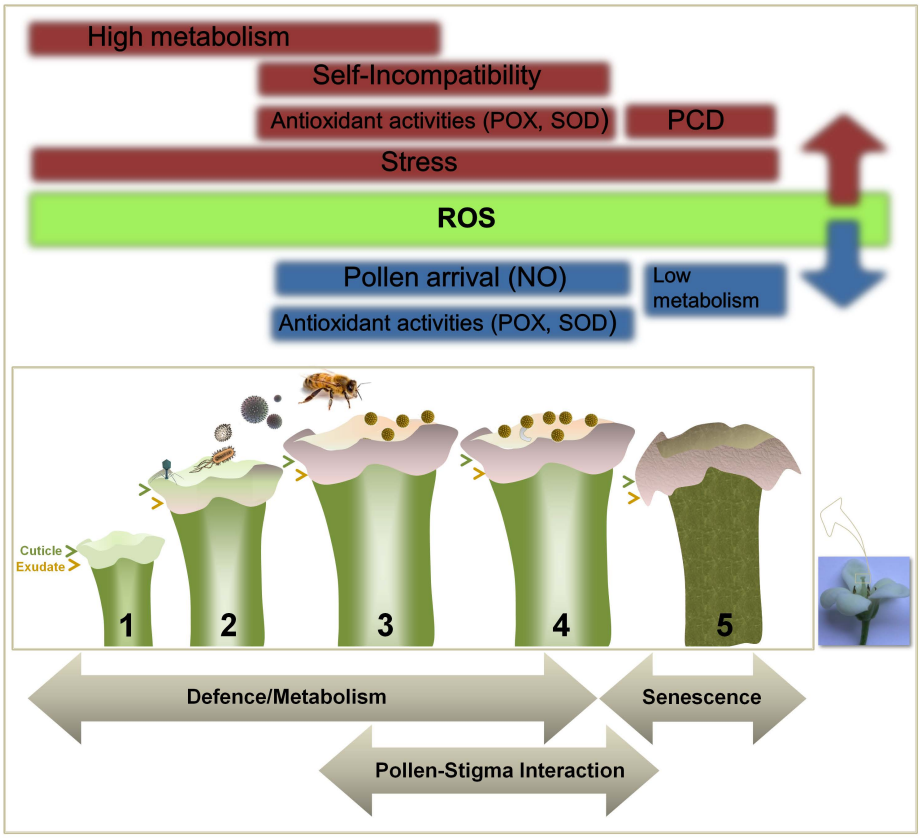

FIGURE 3: Factors affecting ROS accumulation in Angiosperm stigma. Numerous intrinsic and extrinsic factors may increase (red) or reduce/scavenge (blue) basal levels of ROS produced through metabolism and present mainly on the stigma surface (green), modulating their accumulation and generating different profiles highly depending on the plant species. These factors can be grouped into three major categories: defence, pollen-pistil interaction and senescence.

In conclusion, the multifunctional nature of ROS, generated as a consequence of metabolism, involved in numerous stress, defence and signaling functions, and modulated through numerous enzymatic and non-enzymatic systems makes their presence a valuable marker of plant (flower) physiology. The presence of ROS in pollen and stigma (Figure 3) is likely influenced by a number of intrinsic (histochemical composition of the stigma, presence of exudate, cuticle, differential timing of floral development for each species, self-incompatibility mechanisms) and probably also extrinsic (model of pollen dispersion, pollen viability, stress) factors. The developmental changes observed involve many biochemical systems and molecular mechanisms, which both promote and counteract the increase of ROS (Cavaiuolo et al., 2013). This should be further analyzed in the different models for reproductive biology by means of the numerous tools available.

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CHAPTER 2

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BMC Plant Biology

RESEARCH ARTICLE Open Access

Cellular localization of ROS and NO in olive reproductive tissues during flower development

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Abstract

Background: Recent studies have shown that reactive oxygen species (ROS) and nitric oxide (NO) are involved in the signalling processes taking place during the interactions pollen-pistil in several plants. The olive tree (*Olea europaea* L.) is an important crop in Mediterranean countries. It is a dicotyledonous species, with a certain level of self-incompatibility, fertilisation preferentially allogamous, and with an incompatibility system of the gametophytic type not well determined yet. The purpose of the present study was to determine whether relevant ROS and NO are present in the stigmatic surface and other reproductive tissues in the olive over different key developmental stages of the reproductive process. This is a first approach to find out the putative function of these signalling molecules in the regulation of the interaction pollen-stigma.

Results: The presence of ROS and NO was analyzed in the olive floral organs throughout five developmental stages by using histochemical analysis at light microscopy, as well as different fluorochromes, ROS and NO scavengers and a NO donor by confocal laser scanning microscopy. The "green bud" stage and the period including the end of the "recently opened flower" and the "dehiscent anther" stages displayed higher concentrations of the mentioned chemical species. The stigmatic surface (particularly the papillae and the stigma exudate), the anther tissues and the pollen grains and pollen tubes were the tissues accumulating most ROS and NO. The mature pollen grains emitted NO through the apertural regions and the pollen tubes. In contrast, none of these species were detected in the style or the ovary.

Conclusion: The results obtained clearly demonstrate that both ROS and NO are produced in the olive reproductive organs in a stage- and tissue- specific manner. The biological significance of the presence of these products may differ between early flowering stages (defence functions) and stages where there is an intense interaction between pollen and pistil which may determine the presence of a receptive phase in the stigma. The study confirms the enhanced production of NO by pollen grains and tubes during the receptive phase, and the decrease in the presence of ROS when NO is actively produced.

Background

Both reactive oxygen species (ROS) and nitric oxide (NO) are involved in numerous cell signalling processes in plants, where they regulate aspects of plant cell growth, the hypersensitive response, the closure of stomata, and also have defence functions [1-5]. In *A. thaliana* stigmas, ROS/H_2O_2 accumulation is confined to stigmatic papillae and could be involved in signalling networks that promote pollen germination and/or pollen tube growth on the stigma [6]. In addition, the putative presence of ROS in the stigma exudate could be a defence mechanism against microbe attack, similar to the secretion of nectar [6,7]. Several studies have implicated ROS and NO as signalling molecules involved in plant reproductive processes such as pollen tube growth and pollen germination [8-11] and pollen-stigma interactions [6,12]. Low levels of NO was detected by these authors in stigmas, whereas NO was observed at high levels in pollen. An interesting suggestion to explain the biological function of ROS/H_2O_2 in stigmas and NO in pollen was proposed by Hiscock and Allen [13], who observed a reduction of these molecules in the stigmatic surface when either pollen grains of NO were artificially added. They propose that the main function of stigmatic

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ROS/H_2O_2 can be defence against pathogens, whereas pollen NO may cause a localized reduction of these molecules, then breaching this defence system. Evidence for the connections between Ca^{2+} and NO signalling pathways is also beginning to emerge [14-18]. Although there are diverse modes of NO production in plants [4,19], not all of them are regulated by calcium ions.

The presence of numerous specific ROS-related activities (catalases, superoxide dismutases, ascorbate peroxidase, monodehydroascorbate reductase and GSH-dependent dehydroascorbate reductase, peroxidases, glutathione S-transferases) has been characterized in pollen grains [20,21]. Recently, NADPH oxidase activity has been shown to be present at the tip of the pollen tube [10]. However, less is known about these enzymes in the stigma, where only a specific stigma peroxidase has been detected up to date [22]. Most of these studies have been carried out in model species like *Lilium*, *Arabidopsis* and *Petunia*, and in the UK-invading species *Senecio squalidus*. More effort is needed to determine whether the presence of these molecules throughout the reproductive tissues is a general feature of all Angiosperms.

The olive tree (*Olea europaea* L.) has a high economical and social importance in the Mediterranean area. Although several studies are beginning to uncover the details of the reproductive biology in this plant [23,24], much is still unknown. Olive pollination is mainly anemophilous. Paternity tests have revealed a certain degree of self-incompatibility (SI) in several olive cultivars [25,26]. The pistil of the olive tree (*O. europaea* L. c.v. *Picual*) is composed of a two-lobed wet stigma, a solid style and a two-loculus ovary with four ovules. The exudate of the olive stigmatic receptive surface is heterogeneous, including carbohydrates, lipids and proteins in its composition [23,24]. All these structural and cytochemical features of the pistil in olive are in good agreement with the presence of a SI mechanism of the gametophytic type in this plant, in accordance with general consensus and previous observations carried out in olive and other *Oleaceae* species [23,24,27-29].

The purpose of this study was to first approach the possible implications of ROS and NO during flower development and the pollen-pistil interactions in the olive. For this purpose, several of these molecules have been precisely localized in the stigma and the pollen during the main developmental stages of flowering.

Results

Developmental stages of olive flowering

Five major developmental stages were established to better scrutinize flower development in the olive (Figure 1). Very early stages were omitted, as olive flower buds were completely covered by solid trichomes which made dissection very difficult without compromising the integrity of anthers and gynoecium, and therefore altering the presence of ROS/NO. Flower buds at the "green bud" stage (stage 1) had an average size of 2.5 ± 0.2 mm length × 1.7 ± 0.1 mm width. All flower organs were green coloured. This stage lasted for 8 days on the average. At the "white bud" stage (stage 2), the floral buds were 3.3 ± 0.1 mm length × 2.7 ± 0.7 mm width on the average. Petals have changed from green to whitish colour although they were still wrapping the remaining organs into the unopened flower. This stage lasted an average of 4 days. At the "recently opened flower" stage (stage 3), of two days of duration, the four white petals turned out to be separated, leaving the remaining floral structures visible: the anthers coloured in yellow, and the stigma, style and ovary which remained in green colour. At the "dehiscent anther stage" (stage 4), two days long, one or the two anthers became dehiscent, releasing the pollen grains, which also covered the stigma. In the last developmental step (stage 5), anthers and petals were abscised. The apex of the stigma appeared clearly brown-coloured. Only the two first days of this stage were considered.

Light Microscopy detection of H_2O_2

Ligh microscopy (LM) detection of H_2O_2 with TMB (3,5,3',5'-tetramethylbenzidine-HCl) solution was assayed in olive flowers during different stages of its development (Figure 2). Once the chemical was added, a progressive change of colour was observed in both the stigmas and the anthers, as the result of the presence of a dark purple

Figure 1 Developmental stages of the olive flower. Stage 1: "green bud". Stage 2: "white bud". Stage 3: "recently opened flower". Stage 4: "dehiscent anther". Stage 5: "abscised anthers and petals".

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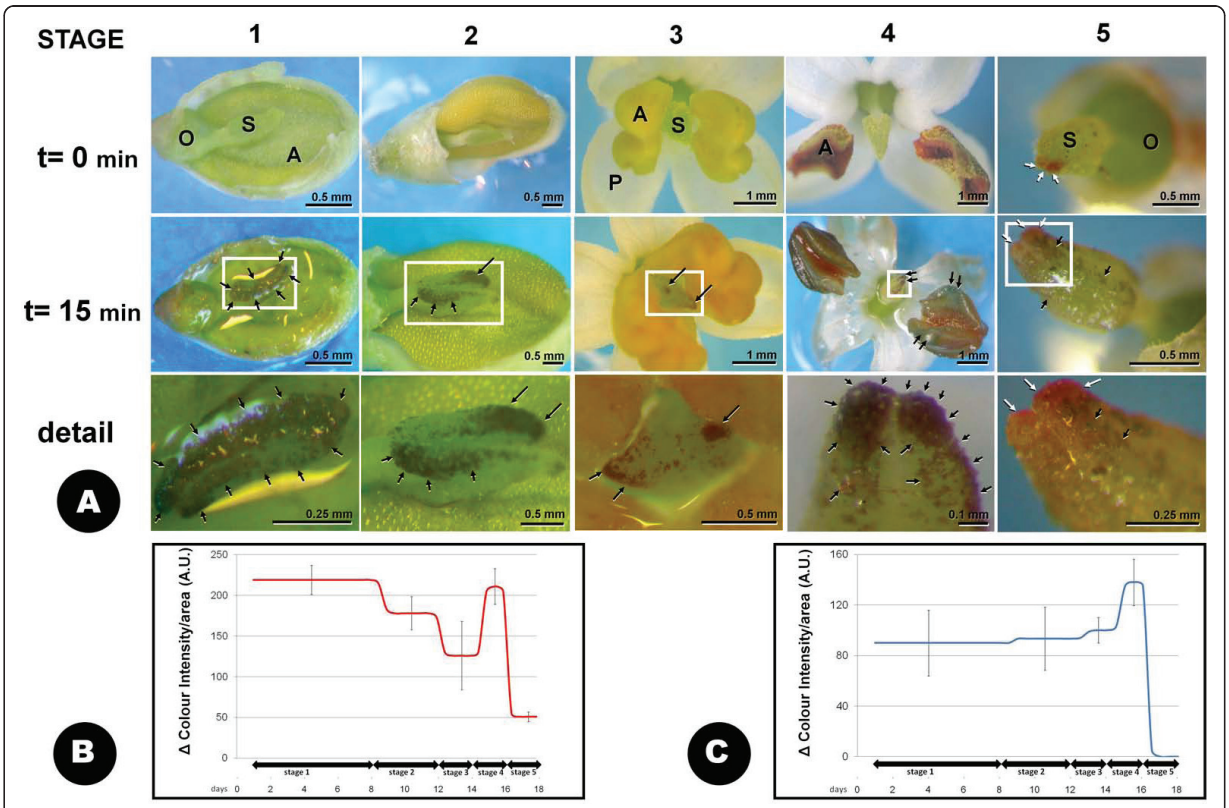

Figure 2 LM detection of H_2O_2 with TMB at different developmental stages of the olive flower. **A:** the presence of H_2O_2 is shown by a dark purple precipitate appearing shortly (c 15 minutes) after the incubation with the appropriate medium (black arrows). This precipitate is clearly distinguishable from the dark brown colour appearing at the latest stages of flower development (white arrows). The last row of pictures shows some details of the labelling at larger magnification. **B:** quantification of the labelling intensity detected over the stigma surface. **C:** quantification of the labelling intensity over the anther surface. Both the average and the standard deviation displayed in the graphs correspond to the measurement of a minimum of nine images, on three independent experiments. A: anther; AU: arbitrary units; O: ovary; P: petal; S: stigma.

precipitate. Neither the style nor the ovary tissues were coloured. The appearance and localization of H_2O_2 was not homogenous in all the developmental stages studied: during stage 1, the precipitate started to accumulate at the very distal part of the stigma shortly after de beginning of the treatment, spreading throughout the borders of the stigma until covering almost all its surface. Anthers showed no change of colour at the green bud stage. White buds stigmas (stage 2) also started to be coloured in the distal part of the stigma. However, the progressive appearance of the precipitate was relatively slower and finally covered less area of the stigma and showed lower intensity than in stage 1, becoming limited to the peripheral regions of the stigma. As in stage 1, no H_2O_2 was detected in the anthers in this stage. The stigmas of the newly opened flowers (stage 3) started to be coloured soon after the initiation of the histochemical staining. In this case, the presence of the purple precipitate was restricted to the distal part of the stigma and to some small spots on the remaining stigma surface.

At stage 4, the distribution of the coloured precipitated over the stigma was even more limited, focusing into the stigma two-lobed apex only. At this stage we detected an intense purple coloration corresponding to the massive presence of H_2O_2 in the dehiscent anthers even after 5 minutes of treatment. Finally, over the last stage (stage 5), very little purple colour appeared in the stigma, even after long periods of incubation with the reagent. As described above, anthers are absent at this stage.

Confocal Laser Scanning Microscopy detection of ROS

The $DCFH_2$-DA (2',7'-dichlorodihydrofluorescein diacetate) fluorochrome was used to detect ROS by Confocal Laser Scanning Microscopy (CLSM). Low magnification CLSM allowed the observation of both stigmas and anthers at stages 1, 2 and 3 whereas they were observed separately at stage 4 (Figure 3A). The presence of these chemicals produced a green fluorescence in the stigma and the anthers, which showed different degrees of

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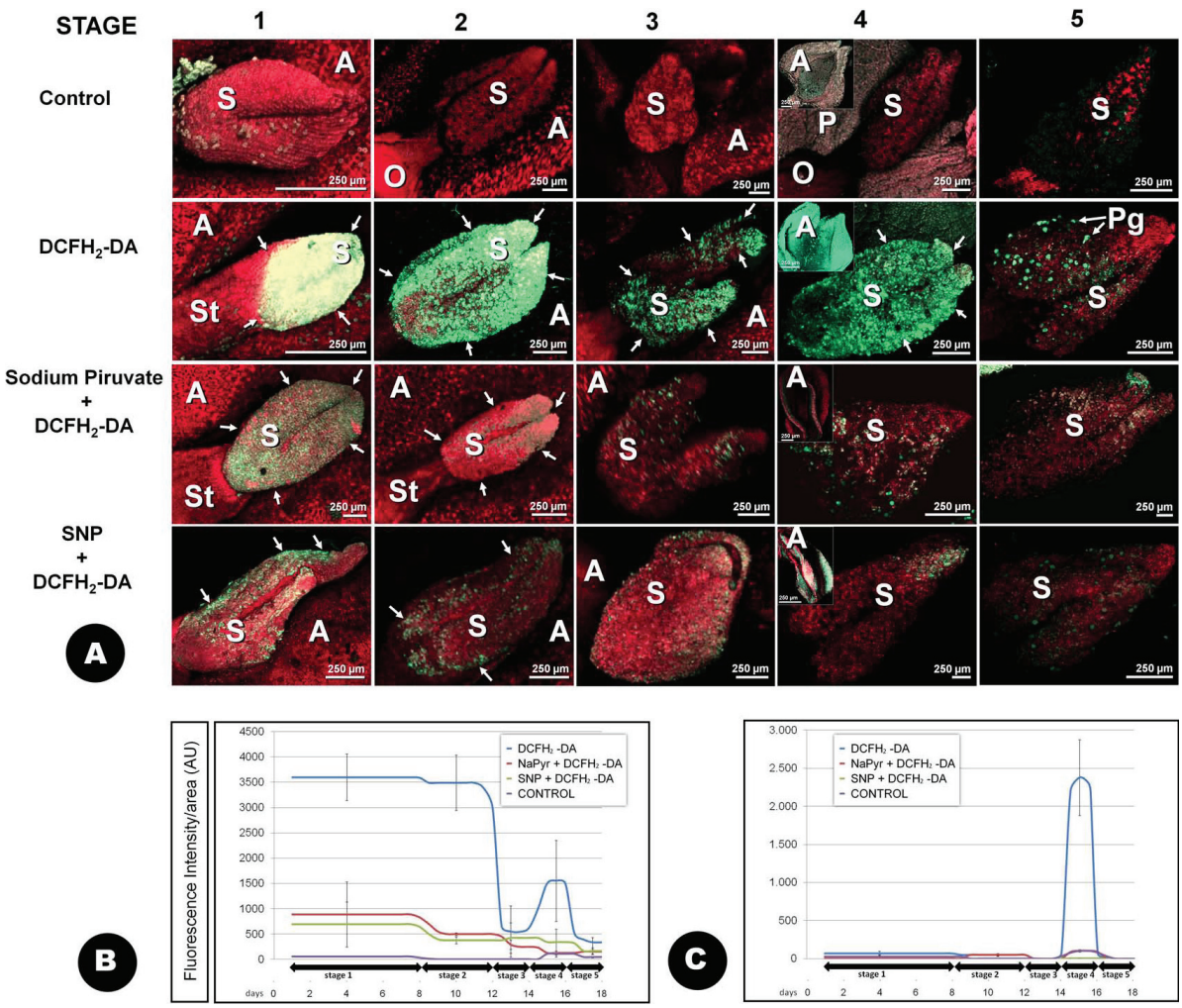

Figure 3 Low-magnification CLSM detection of H_2O_2 with $DCFH_2$-DA at different developmental stages of the olive flower. Projections of section stacks **A:** the presence of H_2O_2 is shown by green fluorescence (arrows), which is clearly distinguishable from the tissues autofluorescence, showed here in red colour. Co-localization of both fluorescence sources results in yellow colour. Three different treatments are displayed ($DCFH_2$-DA alone or in combination with sodium pyruvate or SNP), as well as untreated samples (control). **B:** quantification of the fluorescence intensity owing to $DCFH_2$-DA under the different treatments over the stigma surface. **C:** the same over the anther surface. Both the average and the standard deviation displayed in the graphs correspond to the measurement of a minimum of nine images, on three independent experiments. A: anther; AU: arbitrary units; O: ovary; P: petal; Pg: pollen grain; S: stigma; St: style.

intensity depending on the stages analyzed (Figure 3B, C). Although the fluorescence was present all through the stigma surface, it was slightly more intense at the distal side of the stigma (the apex of both stigma lobules) than in the basal region of the stigma. The tissue situated between both stigma lobules frequently appeared unlabelled. No fluorescence over the background or the control experiments was detected in the tissues of the ovary or the style at any of the stages analyzed. Autofluorescence of the floral tissues was recorded in red. Stigmas at the stage 1 exhibited the greatest relative intensity of fluorescence per area analysed, in comparison with other developmental stages (Figure 3A,B; additional file 1). High magnification CLSM images of the stigma at the same stage showed the fluorescence to localize in association with the stigmatic papillae present throughout the stigma surface. (Figure 4A).

At stages 2 and 3, stigma size was considerably larger than at the previous stage. Although the distribution of fluorescence was similar to the previous stage, a dramatic decrease in the fluorescence intensity detected on the stigmatic surface was measured (Figure 3B; additional files 2 and 3). Similarly to stage 1, fluorescence

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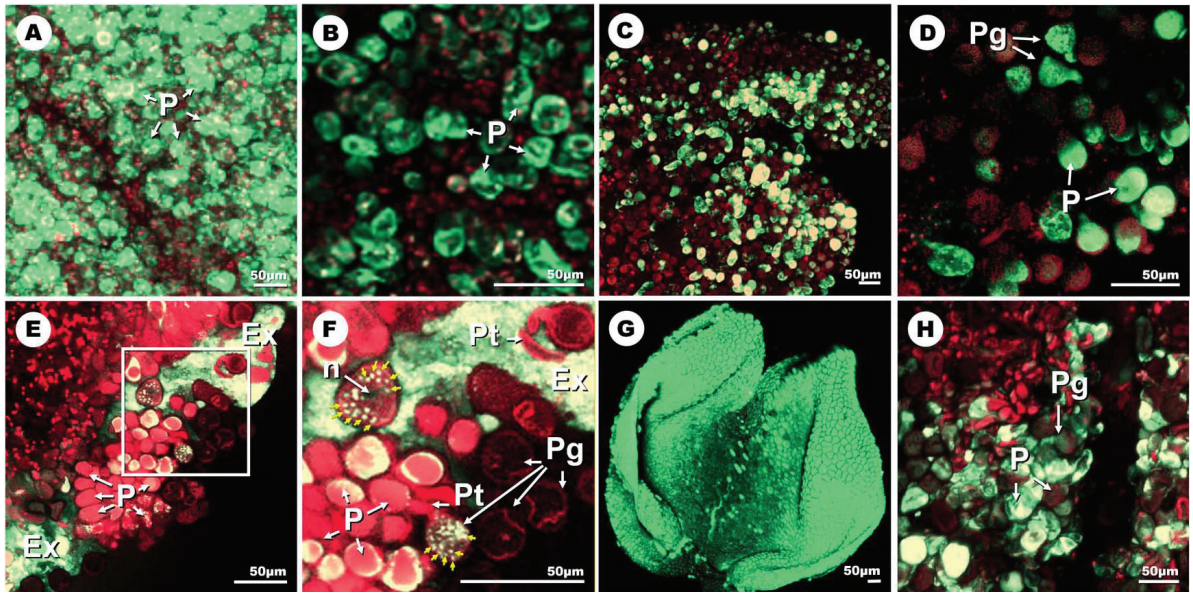

Figure 4 High-magnification CLSM detection of H_2O_2 with $DCFH_2$-DA at different developmental stages of the olive flower. **A and B:** projections of section stacks of the stigma surface at stages 1 and 3, respectively. The fluorescence localizes in association with the stigmatic papillae. **C and D:** optical section -and an enlarged view- of the stigmatic surface in an area lacking exudates at stage 4. **E and F:** optical section -and an enlarged view- of the stigmatic surface at stage 4. Green fluorescence extensively localizes in the exudate, as well as in stigmatic papillae and in small organelles inside some pollen grains (yellow arrows). **G:** projection of section stacks of the anther surface at stage 4. **H:** projection of section stacks of the stigma surface at stage 5. Fluorescence remains associated to the papillae and the pollen grains. Ex: exudate; n: nuclei; P: papillae; Pg: pollen grain; Pt: pollen tube.

concentrated in the stigmatic papillae at these stages (Figure 4B). The stage 4 was characterized by the presence of the stigmatic exudate, which was particularly visible when high magnification observations were carried out. This stigmatic exudate resulted to be intensely fluorescent (Figures 4C and 4D). Pollen grains over the surface of the stigma were observed from stage 3 onwards, and were easily identified even at low magnification (Figure 3A), due to their high levels of fluorescence. At high magnification, fluorescence was in some cases located in small individualised organelles clearly visible inside the pollen grains when observed in single optical sections by CLSM (additional file 4). At this stage, the dehiscent anthers which until now had remained practically free of fluorescence became intensely stained (Figures 3A,B, 4E; additional file 5). Finally, the fluorescence became restricted to the pollen grains over the surface of the stigma at stage 5 (Figure 4F).

The incubation of the samples with the H_2O_2 scavenger Na-pyruvate, prior to the treatment with the fluorochrome [6], resulted in a substantially lower intensity of the fluorescence in all the stages and the floral organs assayed (Figure 3A). A similar reduction in the overall levels of fluorescence intensity was observed when the samples were treated with SNP (sodium nitroprusside), a NO donor (Figure 3A). In both cases, the intensities of the residual fluorescence were practically identical to those of the untreated -control- samples (Figures 3A and 3B).

CLSM detection of $O_2^{\cdot-}$

The incubation of the samples with the DHE (dihydroethidium) fluorophore produced green fluorescence in the presence of $O_2^{\cdot-}$ when compared to the control samples (Figures 5A, B). Autofluorescence of both the anthers and the gynoecium was recorded in red. The fluorescence was located in the stigma, mainly at stages 2 to 5, with a maximum of intensity at stage 3 (Figure 5A, B; additional files 6, 7). In this case, the fluorescence was centred at the basal and central region of the stigma, with the apex of both stigma lobules practically unlabelled. The equivalent samples previously incubated with the $O_2^{\cdot-}$ scavenger TMP (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy) [30] displayed much reduced fluorescence intensity all over the stigma (Figure 5A). No relevant fluorescence was detected in either the ovary or the style. The anthers presented high levels of fluorescence, particularly at stage 4 (Figure 5C). Images at higher magnification allowed us to determine that fluorescence was particularly evident in particular areas of the anther corresponding to the stomium (Figure 6F; additional file 8). The observation of the samples at high magnification also allowed us to allocate the signal in

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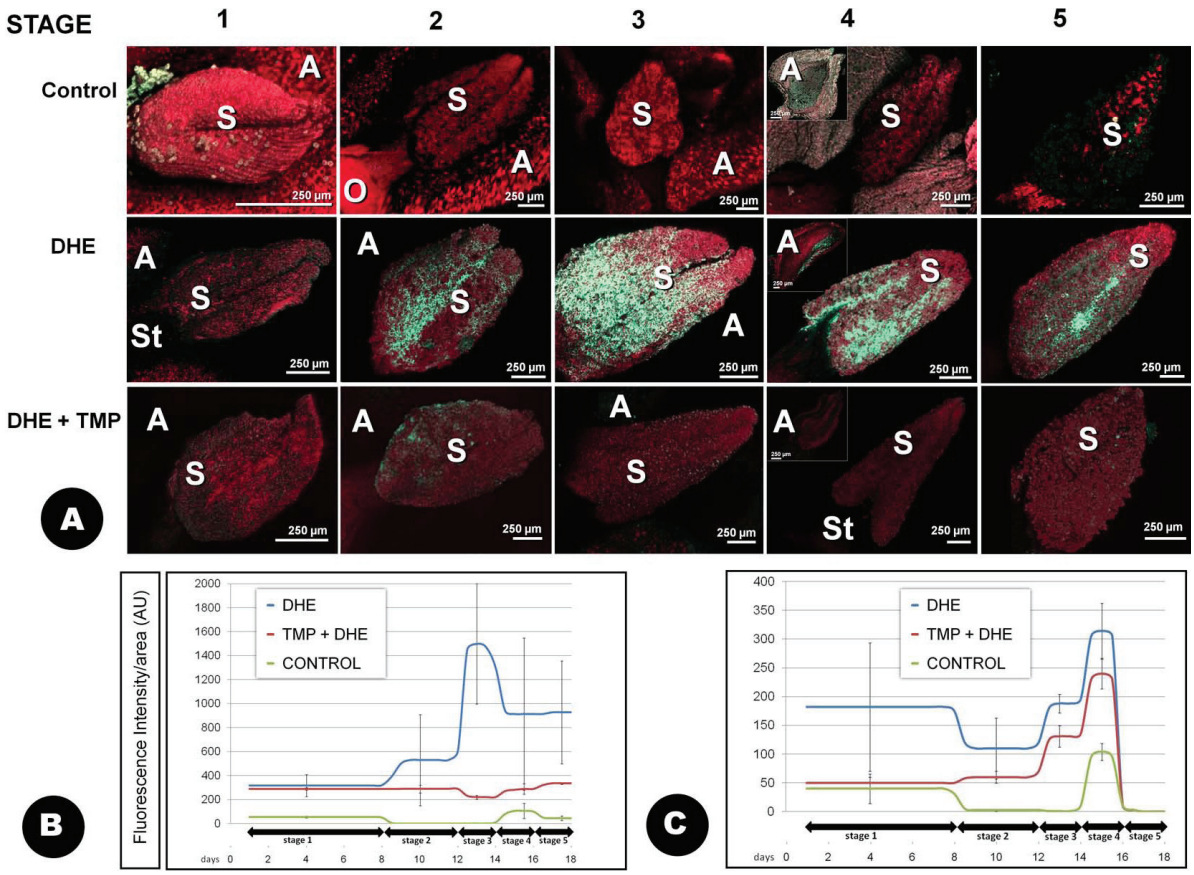

Figure 5 Low-magnification CLSM detection of superoxide anion ($O_2^{\cdot-}$) with DHE at different developmental stages of the olive flower. Projections of section stacks **A:** the presence of $O_2^{\cdot-}$ is shown by green fluorescence, which is clearly distinguishable from the tissues autofluorescence (red colour). Two different treatments are displayed (DHE alone or in combination with TMP), as well as untreated samples (control). **B:** quantification of the fluorescence intensity owing to DHE under the different treatments over the stigma surface. **C:** the same over the anther surface. Both the average and the standard deviation displayed in the graphs correspond to the measurement of a minimum of nine images, on three independent experiments. A: anther; AU: arbitrary units; O: ovary; S: stigma; St: style.

the stigma mainly to the stigmatic papillae (Figures 6A, E), the exudate and the pollen grains and pollen tubes (additional file 9). Conspicuous differences in the exudate texture and fluorescence intensity were detected between the distal area of the stigma (Figure 6B), and the basal/central area (figure 6C). The pollen grains attached to the stigma exhibited intensely labelled particles or organelles frequently grouped in clusters in the pollen cytoplasm (Figure 6D). Pollen tubes on the surface of the stigma also showed a weak labelling in their cytoplasm, which increased in intensity in the area of the pollen tube in close contact with the stigmatic papillae and the exudates (Figure 6E).

CLSM detection of NO

The presence of NO in the olive floral organs was examined by using the DAF-2 DA (2',7'-dichlorodihydrofluorescein diacetate) fluorochrome by CLSM. As it also happened with the $DCFH_2$-DA and DHE fluorophores, fluorescence was not observed to occur over the background or the control experiments in the tissues of the ovary or the style at any of the stages analyzed (Figure 7A). Autofluorescence in these tissues was documented in red. Fluorescence was practically negligible over the developmental stages 1, 2 and most of the stage 3, to rise at stage 4, coincidentally with the presence of numerous pollen grains over the stigma surface (Figure 7A, B). At this "dehiscent anther" stage, fluorescence accumulated for the most part at both tips of the two-lobed stigma. The samples treated with cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) prior to the incubation with NO showed comparatively reduced levels of fluorescence in all stages studied (Figure 7A). Detailed localization at higher magnification showed that NO started in fact to accumulate at the very

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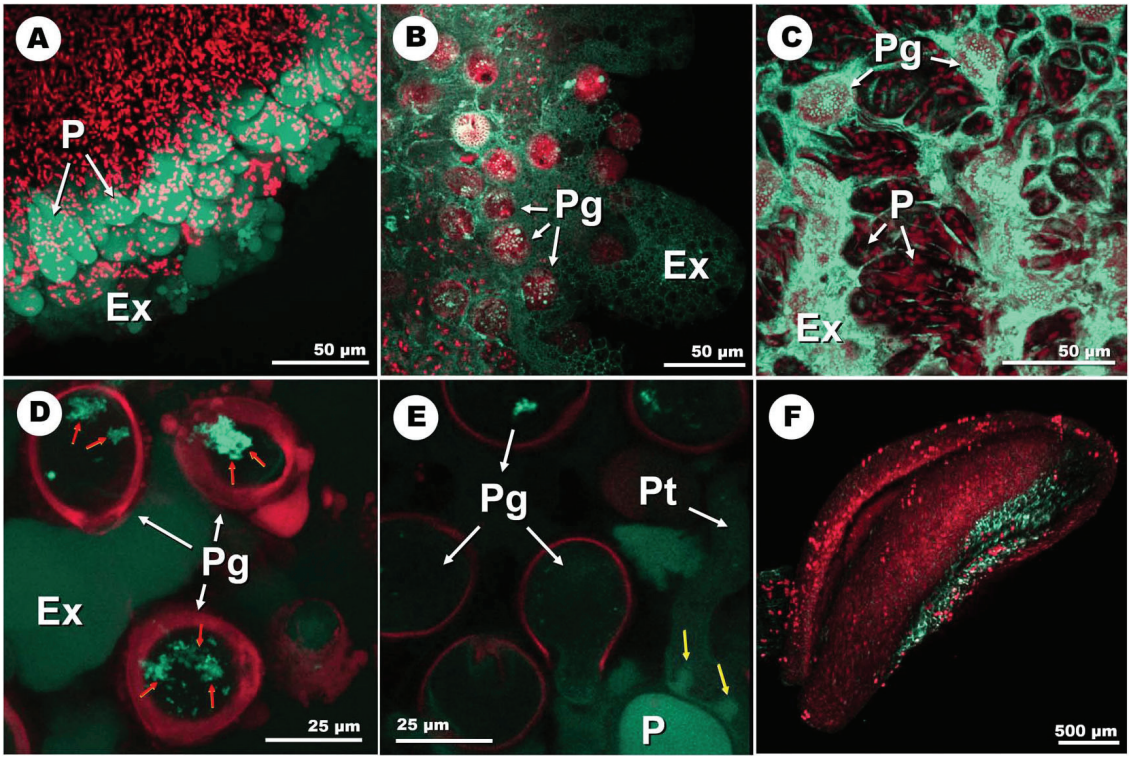

Figure 6 High-magnification CLSM detection of superoxide anion ($O_2^{\cdot-}$) with DHE at different developmental stages of the olive flower, **A:** projection of section stacks of the stigma surface at stage 3. The fluorescence localizes in association with the stigmatic papillae. **B:** stacks projection of the surface of the distal area of the stigma at stage 4. **C:** stacks projection of the surface of the central area of the stigma at stage 4. Note the differences in both the texture of the exudate, and the intensity of the labelling. **D:** optical section of several pollen grains on the stigmatic surface at stage 4. Several clusters of pollen organelles are intensely labelled (red arrows). **E:** optical section of several pollen grains germinating on the stigmatic surface at stage 4. The cytoplasm of the pollen tube appears weakly labelled. However the fluorescence becomes more intense in the contact areas between the pollen tube and the papillae (yellow arrows). **E:** projection of section stacks of the anther at stage 4. Fluorescence localizes in the stomium. The pollen grains show red autofluorescence. Ex: exudate; p: papillae; Pg: pollen grain; Pt: pollen tube.

end of stage 3, partially in the stigmatic papillae, and mainly in both the apertural regions and the pollen tubes of the scarce pollen grains landed on the stigma surface at this stage (Figure 8A-C; additional files 10, 11). It is at stage 4 when NO was extensively localized in the stigmatic papillae, the pollen tubes and apertures of the numerous pollen grains settled on the stigma. The stigmatic exudate, when present, was also intensely fluorescent. (Figure 8D; additional files 12, 13). The anthers only displayed relevant labelling at stage 4 (Figure 7C), in the form of high levels of autofluorescence and signal co-localization at the stomium. The pollen grains inside the sacs were also fluorescent (Figure 8E; additional file 14). Finally, at stage 5, only residual fluorescence was detected in association with the remaining pollen grains (Figure 8F).

Discussion

The present study confirms that the olive tree shares several features with other Angiosperms, as regard to the presence of ROS and NO in reproductive tissues. The first of these features is that H_2O_2 is the most prominent ROS in the olive stigma, at least in early stages (1-3). This conclusion is the result of the application of the same criteria already described by [6], mainly the reduction in $DCFH_2$-DA fluorescence after the application of the scavenger sodium pyruvate, the strong reaction of the stigmas to TMB (with a practically identical distribution of the labelling by TMB and $DCFH_2$-DA), and the relative low presence of other ROS and NO in these stages (as showed by the DHE and DAF-2 DA fluorophores) (Figure 9). The average level of $DCFH_2$-DA fluorescence in olive stigmas slightly decreases at stages 3-4, where pollen grains adhere and emit pollen tubes over the stigma. $DCFH_2$-DA fluorescence is also notoriously reduced after the addition of SNP, a NO donor. This observation is similar to those described for *Senecio squalidus* [6]. Although olive pollen and pollen tubes are clearly demonstrated in this paper to be major

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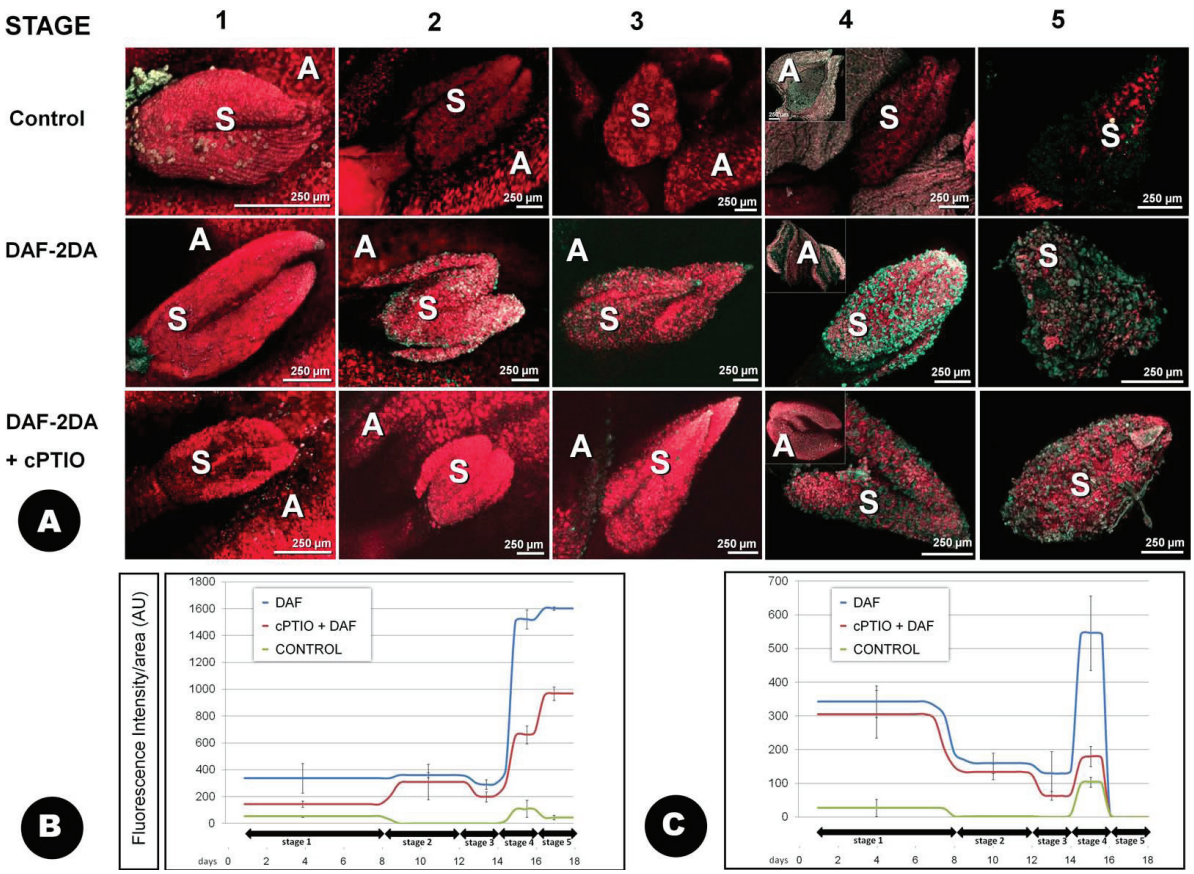

Figure 7 Low-magnification CLSM detection of NO with DAF-2 DA at different developmental stages of the olive flower. Projections of section stacks **A:** the presence of NO is shown by green fluorescence (arrows), which is clearly distinguishable from the tissues autofluorescence, showed here in red colour. Co-localization of both fluorescence sources results in yellow colour. Two different treatments are displayed (DAF-2 DA alone or in combination with cPTIO), as well as untreated samples (control). **B:** quantification of the fluorescence intensity owing to DAF-2 DA over the stigma surface. **C:** the same over the anther surface. Both the average and the standard deviation displayed in the graphs correspond to the measurement of a minimum of nine images, on three independent experiments. A: anther; AU: arbitrary units; O: ovary; P: petal; Pg: pollen grain; S: stigma; St: style.

sources of NO, our results do not provide a causal link between NO generated by pollen and this decrease in H_2O_2 levels. This and some other possibilities of signalling cross-talk between pollen and stigma have yet to be investigated. This NO production by pollen has now being reported in a number of plant species [8-11,31], and has been connected with the regulation of the rate and orientation of pollen tube growth at the pollen tube tip. Moreover, a possible link between production of NO and nitrite to pollen-induced allergic responses has been proposed [31]. In the case of olive pollen, (a highly allergenic source in Mediterranean countries), further investigation regarding the putative interaction between pollen-produced NO and the immune system is also needed.

The present study is the first to report the presence and distribution of ROS and NO in plant reproductive tissues in a developmental manner. The differential presence of ROS/NO throughout stages 1-5 is likely to correspond to different physiological scenarios. The massive presence of ROS/H_2O_2 in the stigma at early stages of flower development (stages 1 and 2) will doubtfully reflect the presence of a receptive phase in the stigma, as flowers at these stages are still unopened, and temporally far from pollen interaction. In this context, some other hypotheses should be taken into account: high levels of ROS/H_2O_2 may be generated as the result of the high metabolic activity of the stigmatic papillae and the surrounding tissues, which start to accumulate starch and lipid materials as well as pectins, arabino-galactan proteins and many other components integrating not only the stigma tissues, but also the stigma exudate and a clearly distinguishable cuticle [23,24]. Major differences in starch content have been

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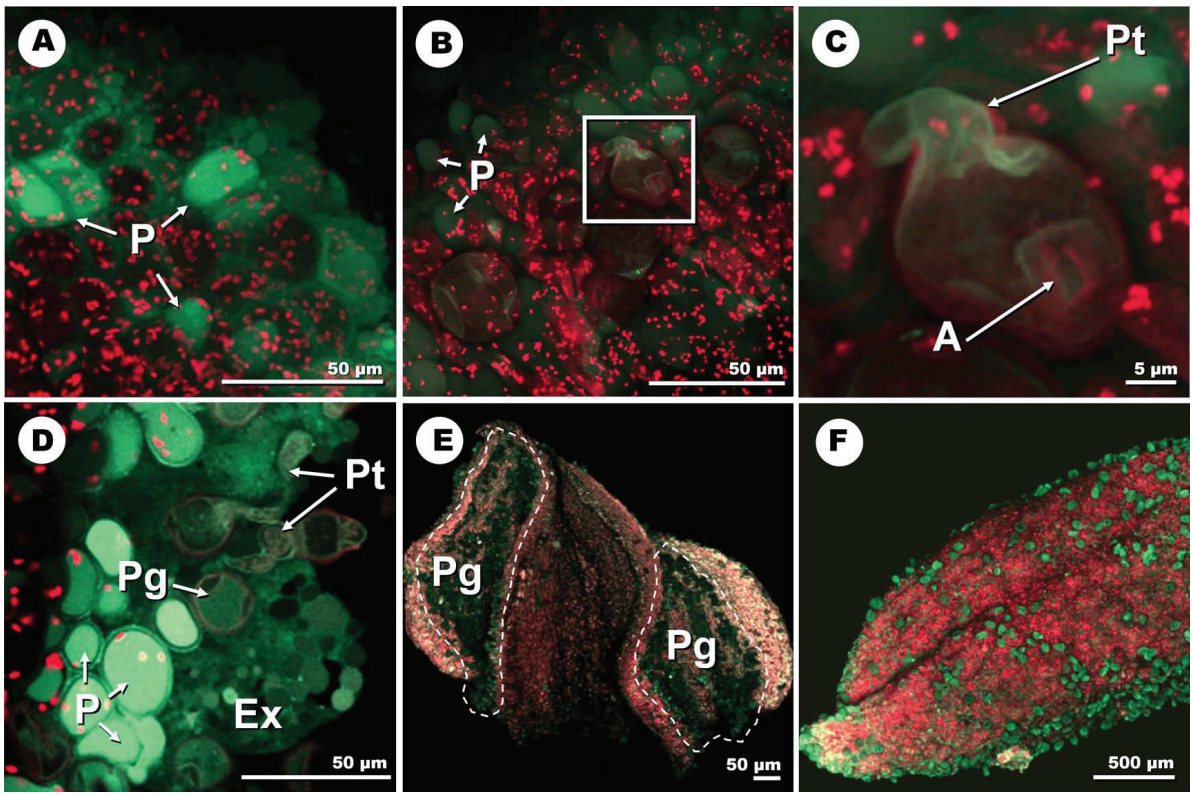

Figure 8 High-magnification CLSM detection of NO with DAF-2 DA at different developmental stages of the olive flower. **A:** projection of section stacks of the stigma surface at stage 3. The fluorescence localizes in association with the stigmatic papillae. **B and C:** projection of section stacks -and an enlarged view- of the stigmatic surface at the end of stage 3. Green fluorescence labels the stigmatic papillae and the pollen surface, mainly the apertural region and the emerging pollen tube. **D and E:** optical section -and an enlarged view- of the stigmatic surface at stage 4. NO extensively accumulates in the stigmatic papillae, and in the pollen grains, the pollen tubes and the exudate. **F:** projection of section stacks of the dehiscent anther surface at stage 4. NO labelling occurs in the dehiscent loculi, associated to the numerous pollen grains. Ap: aperture; Ex: exudate; p: papillae; Pg: pollen grain; Pt: pollen tube.

recently described between staminate and hermaphrodite flowers in the olive tree. Differences in pistil development between these two types of flowers have been related to differences in their sink strength [32]. ROS are likely required for cell expansion during the morphogenesis of the stigma, as has been widely reported for other organs such as roots and leaves [33]. H_2O_2 is likely to participate in the peroxidation reactions driven to the formation of the cells walls and many other metabolic reactions, and its levels are tightly regulated by peroxidases, some of them stigma-specific [12,22]. On the other hand, ROS/H_2O_2 may also have a putative role in flower defence functions at these early stages. Olive flowers are tightly closed at the very early stages of flower development and until stages 1-2. Many of flower organs are protected by numerous trichomes (Rejón et al., unpublished results), which physically protect them from both desiccation and biotic stresses. High levels of ROS may represent an additional barrier to several pathogens which may include bacteria, fungi and even insects, in a similar manner than in nectar (as widely reviewed by [6,12]).

Once we progress into flower development, different types of interactions start to occur: when the receptive phase of the stigma is reached, high levels of ROS/H_2O_2 may harm the pollen grains/pollen tubes growing at the stigma surface. Numerous studies have reported to date the presence of enhanced levels of peroxidase activity in Angiosperm stigmas at maturity [34-37]. Providing that olive stigmas behave similarly, a putative increase in peroxidase activity is therefore likely to take place in olive stigmas at stages 3-4. Peroxidases reduce H_2O_2 to water while oxidizing a variety of substrates including glutathione, ascorbate and others. Therefore, they are important enzymatic components of the ROS-scavenging pathways of plants [33]. These high levels of peroxidase activity would be responsible for the observed decrease in the levels of ROS/H_2O_2occurred at the later

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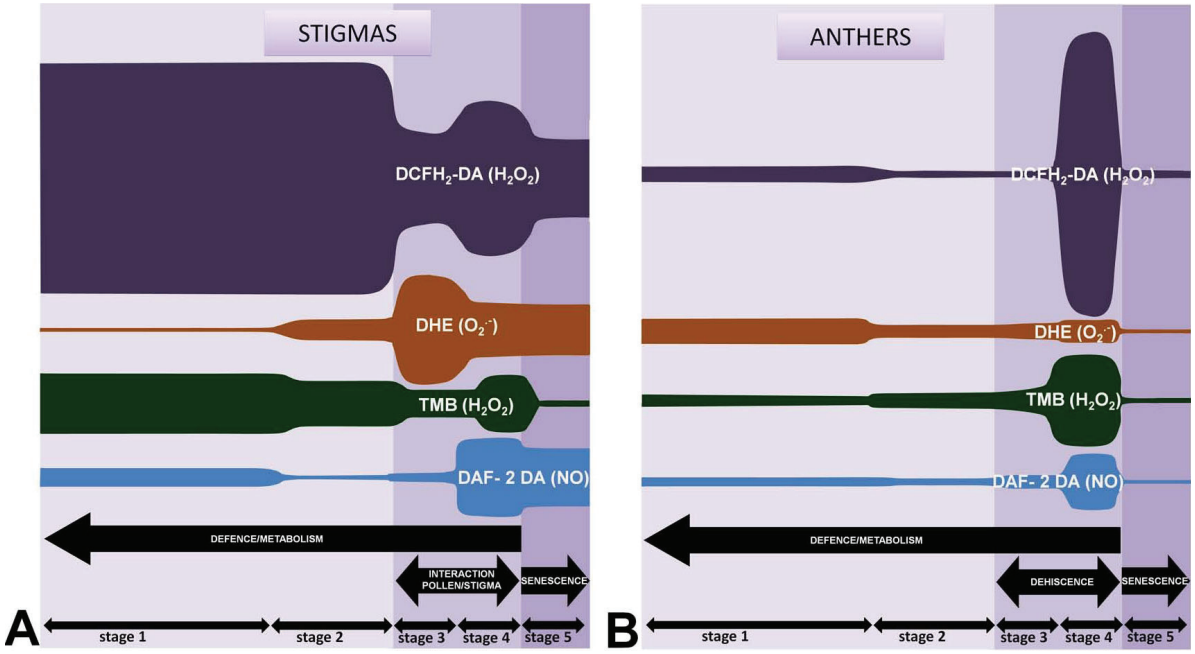

Figure 9 Summary diagram of the overall presence of ROS and NO in the olive stigma and anther. **A:** diagram showing the relative abundance of ROS and NO in the stigma at the different developmental stages, as the result of the different histochemical determinations, and proposed functions of these species in the stigma physiology. **B:** the same in the anther.

stage, coincidentally with the enhanced receptivity of the stigma to pollen. A forthcoming step in this research is therefore to determine whether this described reduction in the levels of ROS/H_2O_2 at the receptive phase is a general feature of Angiosperm stigmas.

Much is still to learn about the source of the described ROS/H_2O_2 and NO in the plant reproductive tissues, as showed in this paper. In pollen, plasma membrane-localized NADPH oxidase (NOX) has been described as an active source of superoxide, needed to sustain the normal rate of pollen tube growth in *Nicotiana* [10]. This $O_2^{\cdot-}$ readily forms other ROS including H_2O_2 and $HO^{\cdot}$ either spontaneously or by the intermediation of other enzymes involved in oxygen metabolism. In the olive pollen, different isoforms of superoxide dismutase (SOD), with extracellular and cytosolic localization have been described [38], and there is clear evidence of the presence of NOX activity (Jiménez-Quesada et al., unpublished observations). However data regarding the stigma tissues are still lacking. In the olive leaves, the presence of different SOD forms has been described [39]. In these tissues, recycling of NADPH by different enzymes, including glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme and ferredoxin-NADP reductase seems to have an important role in controlling oxidative stress caused by high-salt conditions in olive somatic tissues [40]. As regards to NO production, both NO synthase (NOS) and nitrate reductase activities are considered putative enzymatic sources for NO in pollen, although the presence of other enzymatic sources cannot be excluded [41]. Even though the presence of L-arginine- dependant NOS activity in plant tissues is widely accepted, the identification of the enzyme responsible for this nitric oxide generation is still a matter of controversy [42]. Therefore, much effort is still necessary to characterize these systems in the reproductive tissues of the olive and other Angiosperms. In addition, many of the ROS and NO can be generated in multiple cellular localizations. Peroxisomes have been described as subcellular organelles particularly active in the generation of these signal molecules [43,44]. Further research in order to characterize these organelles in the olive reproductive tissues should be carried out. The extreme ability of these molecules to diffuse may lead to the localization of ROS and NO in some areas as described here, for example, the stigmatic exudate.

The superoxide anion ($O_2^{\cdot-}$) is the only detected ROS having a slight increase over the stages 3/4 in the stigma (Figure 9). The rise in the levels of this species can be attributed to the massive presence of pollen grains and growing pollen tubes over the surface of the stigma at these stages, with putatively high rates of NOX activity

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[10]. In addition, a reduction in the activity of SOD forms can also occur.

The occurrence of ROS/NO at stage 5 of the stigma is coincident with the presence of morphological features indicating senescence of this structure. Decay in plant antioxidant capacity has been described at the terminal phase of senescence for different plant organs, which is frequently coincident with increased release of ROS [45,46]. In *Arabidopsis* flowers, senescence has been connected with low levels of ascorbic acid and therefore alterations of the endogenous levels of both giberelic and abscisic acid [47]. In addition to hormonal imbalance, numerous modifications in the expression of senescence associated genes (SAGs) have been described [48]. Many of these gene products include antioxidant barriers, and thus an increase of the ROS present in the senescent floral organs is likely to occur. Whether this can be considered a mechanism for apoptosis or programmed cell death (PCD) is still a matter of controversy [47-49].

ROS/NO maintain steady low levels in the anther tissues until stage 4, in which a rapid increase takes place (Figure 9B). At this stage, release of mature pollen is produced by breakdown of the anther cells at the stomium, a specialized structure situated at the side of the anthers. Dehiscence of the anther involves a number of PCD mechanisms involving degeneration of the endothecium and the surrounding connective tissues, and selective cytotoxin ablation of the stomium [50]. These changes lead to massive ROS release at this stage, whereas NO is mainly produced by the mature pollen grains.

Conclusion

Conspicuous changes in the distribution and the proportion of different ROS/NO occur in the reproductive tissues of the olive throughout flower development. These changes correspond to different physiological circumstances (defence, metabolism, signalling...) and reveal the complex interrelationships taking place between the plethora of enzymatic activities involved in their production, the high number of potential substrates and products involved in their metabolism, and the presence of complex signalling pathways. Most changes in ROS occur at stages 3-4, coincidentally with the presence of high levels of NO. Therefore, special attention has to be addressed in the future to the different ROS/NO-signalling pathways present in plant reproductive tissues [51].

Methods

Plant material

Olea europaea flowers (cv. Picual) at different stages were obtained from adult olive trees growing at the Estación Experimental del Zaidín (Granada, Spain) over the blooming period (fifteen-twenty days throughout the months of May-June). Five different stages were differentiated attending to macroscopic differences. Flowers at the developmental stages 3 to 5 were directly used for ROS and NO determinations. However, flower buds (stages 1 and 2) were dissected by gently removing one of the anthers and the associated petals in order to gain visual access and to allow the contact of chemicals with the gynoecium and the remaining anther.

Light microscopy

H_2O_2 was detected by using the H_2O_2 indicator dye TMB (Sigma). Dissected buds or complete flowers at the different stages were soaked in a solution containing 0.42 mM TMB in Tris-acetate, pH 5.0 buffer [52]. The appearance of blue colour was monitored at different times after the initiation of the incubation in a multipurpose zoom microscope Multizoom AZ-100 (Nikon Instruments Company). Images were gathered with a Nikon Coolpix 4500 digital camera with a resolution of 2272 × 1704 dpi after 15 minutes of incubation (no substantial changes were further observed after that time).

Confocal Laser Scanning Microscopy

ROS were detected using the fluorescent indicator dye $DCFH_2$-DA (Calbiochem). Dissected floral buds or complete flowers were immersed in 50 μM $DCFH_2$-DA in MES (2- [N-morpholino]ethanesulfonic acid)-KCl buffer (5 μM KCl, 50 μM $CaCl_2$, 10 mM MES, pH 6.15) for 10 minutes followed by a wash step in fresh buffer for 15 minutes and then observed at the confocal microscope. Parallel sets of floral buds/complete flowers at equivalent stages were treated with a) 1 M sodium pyruvate (Sigma-Aldrich) in MES-KCl buffer for 30 min, or b) 500 μM SNP (Sigma-Aldrich) in MES-KCl buffer prior to the treatment whit $DCFH_2$-DA as above. Negative controls were treated with MES-KCl buffer only [6].

The presence of the superoxide anion ($O_2^{\cdot-}$) was analysed as above by incubating the samples 30 minutes in a 20 μM solution of the fluorophore DHE (Sigma) in Tris-HCl buffer (10 mM, pH 7.4). Equivalent samples were treated with the $O_2^{\cdot-}$ scavenger TMP (Calbiochem) in Tris-HCl buffer (10 mM, pH 7.4) for 60 minutes, prior to the treatment with DHE (modified from [30]).

The NO indicator dye DAF-2 DA (Calbiochem) was used to detect NO in flowers. Dissected buds or complete flowers were immersed in MES/KCl pH 6.15 for 10 min, transferred to 10 μM DAF-2 DA for 10 min, followed by a wash step (with MES/KCl buffer) for 15 min and then observed in the microscope [6]. Parallel sets of samples were treated the same, although they were previously incubated for 1 hour with the NO-scavenger cPTIO

(Sigma) in a concentration of 400 μM in Tris-HCl 10 mM, pH 7.4 [30]. Negative controls were treated with MES-KCl buffer only instead of DAF-2 DA.

Observations were carried out in a Nikon C1 confocal microscope using an Ar-488 laser source and different levels of magnification (20× to 60×). Small pinhole sizes (30 μm) were used even in combination with low-magnification, dry-objectives. Multiple optical sections were captured and processed to generate 3-D reconstructions of the whole stigma surface. 3-D reconstructions of small areas of the stigma surface were also generated from high-magnification immersion-objectives. The fluorescent signal was obtained exclusively in the range of the 515-560 nm emission wavelengths with both fluorochromes, and was recorded in green colour. Autofluorescence (mainly due to the presence of chlorophyll and other pigments and secondary metabolites) was isolated and displayed in red. For each fluorochrome, identical settings were used for image capture in both control/test experiments in order to ensure reproducibility and accurate quantification.

Colour and fluorescence quantification

The intensity of both the dark purple-coloured precipitate and the green fluorescence was quantified for each organ at the different stages studied by using the Nikon EZ-C1 viewer (3.30) software. Both average and standard deviation were calculated after measurement of a minimum of nine images corresponding to three independent experiments.

For quantification of the dark purple-coloured precipitate, an independent subtraction of the background was performed for each sample. For this purpose, images of the samples were also captured prior to the addition of the chemicals.

Additional file 1: Animated 3-D reconstruction of CLSM detection of ROS in a flower at stage 1 with$DCFH_2$-DA at low magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S1.AVI]

Additional file 2: Animated 3-D reconstruction of CLSM detection of ROS in a flower at stage 2 with$DCFH_2$-DA at low magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S2.AVI]

Additional file 3: Animated 3-D reconstruction of CLSM detection of ROS in a flower at stage 3 with$DCFH_2$-DA at low magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S3.AVI]

Additional file 4: z-Animated 3-D reconstruction of CLSM detection of ROS in pollen on olive stigmaat stage 4 with $DCFH_2$-DA at high magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S4.WMV]

Additional file 5: 3-D reconstruction of CLSM detection of ROS in olive anther at stage 4 with$DCFH_2$-DA at low magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S5.AVI]

Additional file 6: 3-D reconstruction of CLSM detection of superoxide anion in olive flower at stage 3with DHE at low magnification.
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[http://www.biomedcentral.com/content/supplementary/1471-2229-10-36-S6.AVI]

Additional file 7: 3-D reconstruction of CLSM detection of superoxide anion in olive stigma at stage 4with DHE at low magnification.
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Additional file 8: 3-D reconstruction of CLSM detection of superoxide in olive anther at stage 4 withDHE at low magnification.
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Additional file 9: z-Animated 3-D reconstruction of CLSM detection of superoxide in pollen on olivestigma at stage 4 with DHE at high magnification.
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Additional file 10: 3-D reconstruction of CLSM detection of NO in pollen on stigma surface at stage 3with DAF-2 DA at medium magnification.
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Additional file 11: 3-D reconstruction of CLSM detection of NO in pollen on stigma surface at stage 3with DAF- 2 DA at high magnification.
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Additional file 12: z-Animated 3-D reconstruction of CLSM detection of NO in pollen on stigmasurface at stage 4 with DAF-2 DA at high magnification.
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Additional file 13: 3-D reconstruction of CLSM detection of NO in pollen on stigma surface at stage 4with DAF-2 DA at high magnification.
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Additional file 14: 3-D reconstruction of CLSM detection of NO in olive anther at stage 4 with DAF-2DA at low magnification.
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Abbreviations

AU: arbitrary units; CLSM: confocal laser scanning microscopy; cPTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-2 DA: diaminofluorescein diacetate; $DCFH_2$-DA: 2',7'-dichlorodihydrofluorescein diacetate; DHE: dihydroethidium; LM: light microscopy; MES: 2-(N-morpholino)ethanesulfonic acid; NOX: nicotinamide adenine dinucleotide phosphate-oxidase; PCD: programmed cell death; ROS: reactive oxygen species; SAG: senescence associated gene; SI: self-incompatibility; SNP: sodium nitroprusside; SOD: superoxide dismutase; TMB: 3,5,3',5'-tetramethylbenzidine-HCl; TMP: 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy.

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Authors' contributions
JDA and MIR conceived the study. JDA and AZ designed and carried out the experiments. AZ performed quantification. The three authors discussed the results and prepared the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3

Identification and Functional Annotation of Genes Differentially Expressed in the Reproductive Tissues of the Olive Tree (*Olea europaea* L.) through the Generation of SSH Libraries

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Abstract:

Background: The olive tree gives a crop of high socio-economical importance in the Mediterranean area. Sexual reproduction in this plant is an essential process which determines the yield. Successful fertilization is more favourable, with sometimes a requirement, in the presence of pollen grains from a different cultivar, as the olive uses a self-incompatibility system, probably of the gametophytic type. In spite of its commercial importance, no reproductive transcriptomes, nor a genome, are available for this plant. The purpose of the present study was to identify key gene products involved in the function of olive pollen and pistil, in order to help elucidate the events and signalling processes which happen during the courtship, pollen grain germination, and fertilization in olive. The use of subtractive SSH libraries constructed at specific stages may help to reveal the specific transcripts involved in these events. Such libraries have also been created by subtracting vegetative mRNAs (from leaves), in order to identify reproductive sequences only.

Results: A variety of transcripts have been identified in the mature pollen grains and in the pistil at the receptive stage. Among them, those related to defence, transport and oxidative metabolism are highlighted mainly in the pistil libraries where transcripts related to stress, and response to biotic and abiotic stimulus have a prominant position. Extensive lists containing information as regard to the specific transcripts determined for each stage and tissue are provided, as well as functional classifications of these gene products. Moreover, the unique presence of these transcripts has been validated by means of PCR approaches.

Conclusions: The construction of SSH libraries using pistil and pollen, considering the high interaction between male-female counterparts, allowed the identification of transcripts with important roles in stigma physiology. The functions of many of the transcripts obtained are intimately related, and most of them are of pivotal importance in defence, pollen-stigma interaction and signalling.

Keywords:
Olive, pollen, gynoecium, reproductive tissues, SSH, leaf, transcripts, self-incompatibility, germination

Background:

The olive (*Olea europaea* L.) is an important crop in Mediterranean countries. The fruit is used for the production of olive oil. Olive oil yield, organoleptic properties, quality, fatty acid content and many other parameters are highly depending on the procedures used for olive oil production, including which olive cultivars are used. Asexual propagation of this tree, achieved by different methods (Böhm, 2013), is the usual practice since its domestication. This practice results in very high heteroplasmy, as assessed by the accumulation of mutations in a non-coding sequence of the mitochondrial genome when vegetative propagation is maintained for a long period of time (García-Díaz et al., 2003). However, olive production relies on the successful achievement of sexual reproduction. This plant has been suggested to harbour a self-incompatibility (SI) system of the gametophytic type (Wu et al., 2002; Cuevas and Polito, 1997; Ateyyeh et al., 2000), as described for the Oleaceae family (Igic and Kohn, 2001). However, the mechanisms governing this system are not well known, especially when considering the divergence of incompatibility mechanisms which can occur between members of the same family, as seen in Arabidopsis (Kusaba et al., 2001). The SI mechanism described in the olive involves the preferential presence of pollen grains from a different cultivar for successful fertilization (allogamy). The main worry of the growers is the yield, which is affected by the pollinisers–pollinator relationship (Breton and Bervillé, 2012). In the case of the olive, wind is the main factor affecting the yield as the dispersion of the pollen in olive is mainly anemophylous.

A number of recent studies have described the generation of several olive transcripts. Recent publications identified the expressed sequence tags (ESTs) from different olive tissues and developmental stages (Muñoz-Mérida et al., 2013; Ozgenturk et al., 2010; Galla et al., 2009), as well as small RNAs (sRNAs) (Donaire et al., 2011). However, little attention has been devoted to the generation of reproductive transcriptomes in olive, in spite of their significance for the study of reproductive process in this plant. In this work we make the first approach to the events happening in the olive reproductive process by the analysis of the differentially expressed transcripts.

The present study was based on the construction of several cDNA libraries that were subtracted using the SSH method. The aim was to study the reproductive biology of the olive. For that purpose we used reproductive tissues (pollen and pistil) as well as vegetative tissues (leaf as the subtractive item). For each pair combination, the forward and reverse libraries were constructed.

Results and discussion:

Six libraries were generated by using the combination of tester/driver tissues indicated in Table 1. Special attention was paid to the P(Po), P(L), and Po(L) libraries. Table 1 includes information as regards to the number of clones identified and sequenced from each one of the six SSH libraries generated.

The P(Po) library provided information about those transcripts that are expressed during the pollen tube germination in comparison with the mature pollen grains, within the context of the whole pistil as in this stage, the pistil is full of germinating pollen grains. The P(L) library reveals the presence of transcripts in a tissue which is a distinct form from the leaf, being in addition a reproductive tissue. Lastly, the Po(L) shows the transcripts of a reproductive dormant tissue (pollen) from which transcripts from vegetative tissue have been subtracted.

Name of the library	Tester tissue	Driver tissue	No. Sequenced clones	Total ESTs	Total contigs	Mean contig(pb)	Mean ESTs (pb)	% Redundancy	BLAST Hits(%) (NCBIdb)	BLAST Hits(%) (OLEA ESTdb)	e-value median (NCBIdb)	e-value median (OLEA ESTdb)
Po(P)	Pollen	Pistil	288	127	46	588	440	3,17	76	90	4,27E-29	6,00E-12
P(Po)	Pistil	Pollen	288	200	30	589	431	3,10	57	97	4,71E-30	1,50E-29
P(L)	Pistil	Leaf	192	116	17	615	523	2,51	64	97	1,18E-43	1,00E-44
L(P)	Leaf	Pistil	192	60	34	576	447	2,15	74	98	4,71E-44	7,00E-53
Po(L)	Pollen	Leaf	192	129	28	543	500	2,40	70	92	1,78E-37	2,00E-08
L(Po)	Leaf	Pollen	192	158	16	565	525	2,52	77	95	1,97E-51	4,00E-47
Total			**1344**	**790**	**171**	**579**	**478**	**2,64**	**70**	**95**	**7,90E-30**	**3,33E-09**

Table 1: SSH libraries constructed and descriptive parameters about the clones sequenced. Key: P = Pistil; Po = pollen; L= leaf.

A total of 1344 clones were sequenced. From those 790 resulted in ESTs and 171 in contigs. The mean length of the ESTs compared to the contigs showed an increase of 25% for Po(P), 27% for P(Po), 15% for P(L), 22% for L(P), 8% for Po(L), and 7% for L(Po). The redundancy levels were relatively low, ranging between 3.17 to 2.15%. BLAST analysis was carried out by using two alternative databases. The

percentage of BLAST hits averaged 70% when the alignment was carried out with the NCBI database (www.ncbi.nlm.nih.gov) (Altschul et al., 1990) (e-value e-4), and averaged 95% when the alignment was made against the OLEA EST database (Alagna et al., 2009). Also, lower median threshold e-values (e-1) were obtained after using the Olea EST database, constructed with the transcriptome data obtained from olive (Olea europaea L.) mesocarp than after using the NCBIdb (Table1).

The averages of the median e-values were 7.90e-30 and 3.30e-9, respectively, and were significantly lower for the OLEA ESTdb than for the NCBIdb, as the result of the low values of both pollen libraries Po(P) and Po(L). This could be explained by the origin of OLEA ESTdb, which was generated using the olive mesocarp. The results seem to indicate that pollen transcripts differ more from mesocarp transcripts than those of pistil or leaf.

In order to assess the subtractive efficiency of the libraries, PCR-amplified samples of the DNA inserts of each clone were transferred to membranes and subjected to multiple hybridizations with: a) unsubtracted tester, b) unsubtracted driver, c) forward-subtracted, and d) reverse-subtracted probes. An example of the procedure is displayed in Fig.1.

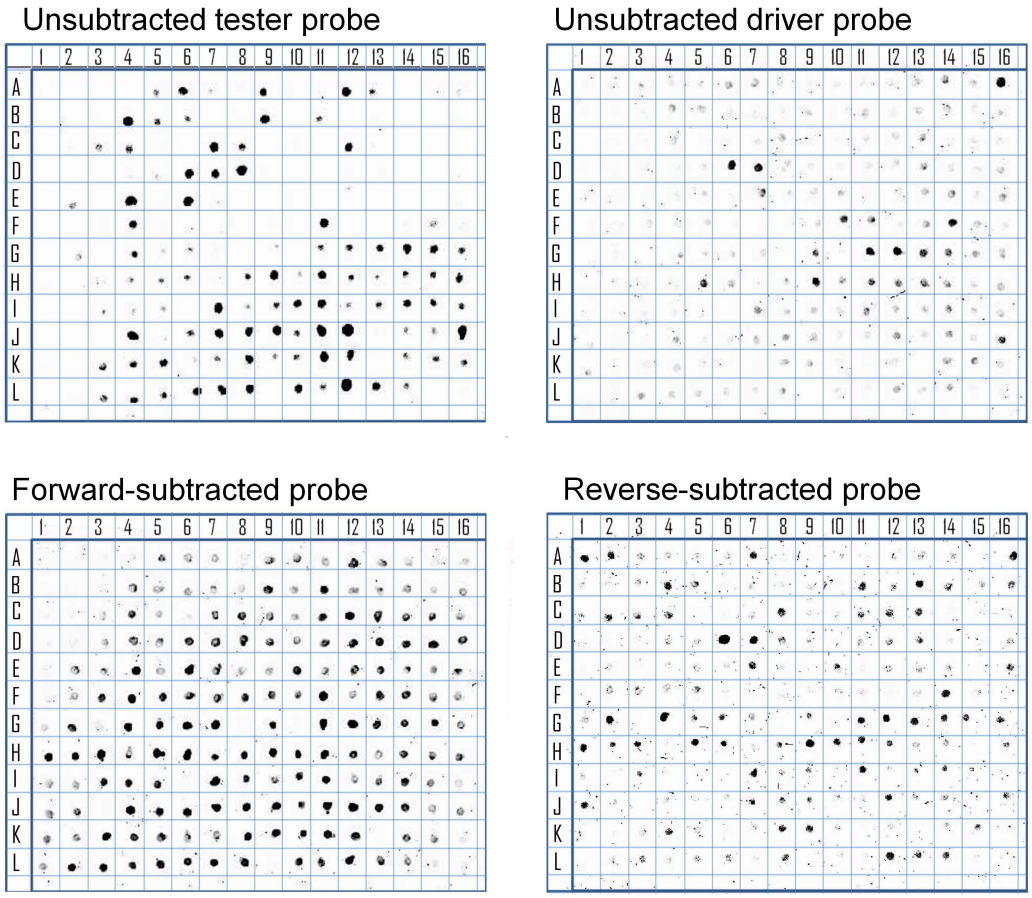

Figure 1: Example of screening carried out in order to check the efficiency of subtraction procedure. Four membrane replicates harbouring the amplified clones to be assessed were hybridized with the unsubtracted tester, the unsubtracted driver, the forward-subtracted, and the reverse-subtracted probes, respectively.

The criteria followed in order to define clones as regard to their tissue-specificity were as follows: clones hybridizing exclusively with the forward-subtracted probe were considered to be differentially expressed and low abundance transcripts. The clones that hybridize to the forward-subtracted probe and the unsubtracted tester probe also correspond to differentially expressed genes with a 95% of probability. Clones that hybridize to all the four probes correspond to non-differentially expressed clones. The results from the screening (additional Tables 2A, 2B, 2C, 2D, 2E, 2F) revealed that 33.3% of the clones were differentially expressed in the Po(P) library, 47.9% in the P(Po) library, 29.1% in the P(L) library, 41.6% in the L(P) library, 73% in the Po(L), and 68.2% in the L(Po).

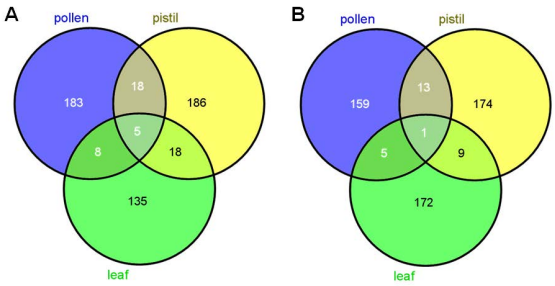

Figure 2: Venn diagrams showing the number of specific and common transcripts to the tree tissues tested. (A) Output data after using OLEA ESTdb, and NCBIdb (B), respectively (http://bioinfogp.cnb.csic.es/tools/venny/).

The use of subtractive libraries allowed us to obtain a relatively large number of tissue-specific transcripts. The number of such putative tissue-specific transcripts was highly dependant on the database searched by means of BLAST. Thus, the number of specific reproductive sequences (from pollen and pistil) was larger after using the OLEA EST db than after using NCBI db. The opposite tendency occurred with vegetative (leaf) transcripts (Figure 2).

In order to validate the results obtained after the construction and screening of the SSH libraries, 6 genes were selected for further corroboration of their expression profiles by means of ReverseTrasncriptase-PCR on the basis of representing a panel of different expression situations (Table 2). Three transcripts (L-ascorbate oxidase, Pectin methyl esterase 2.1 and Pathogenesis related protein-1) were selected according to their unique presence in pollen. After PCR amplification (primers are listed in additional Table 1) the transcripts were confirmed to be present in pollen exclusively, with PCR product sizes of 118, 130 and 99 bp, respectively (Figure 3), which was in good agreement with the expected amplification sizes.

Name of the transcript	Po(P)	P(Po)	P(L)	L(P)	Po(L)	L(Po)
L-Ascorbate Oxidase	+	-	-	-	+	-
Pectin Methyl Esterase 2.1	+	-	-	-	-	-
Pathogenesis Related Protein-1	+	-	-	-	+	-
14-3-3 protein 4	-	+	-	-	-	-
Disease Resistance Response Protein-206	-	+	-	-	-	-
Pathogenesis Related Proein-5	-	+	+	-	-	-

Table 2: Selected gene products for validation of their presence in the generated SSH libraries.

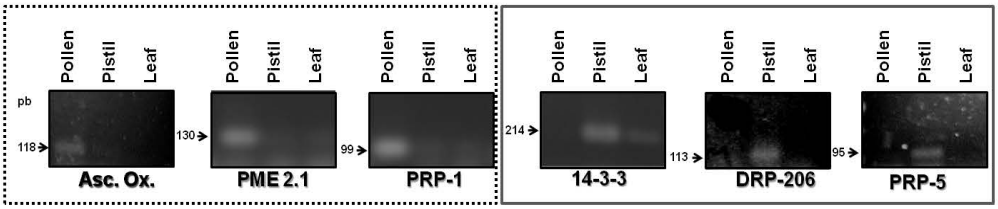

Figure 3: Validation of the generation of SSH libraries by PCR amplification of selected transcripts. Several pairs of primers were designed for pollen (left panel) and pistil (right panel) preferentially expressed transcripts, according to Table 2. Asc. Oxidase: L-ascorbate oxidase; PME2.1: Pectin methyl rsterase 2.1; PRP-1: Pathogenesis related protein-1; 14-3-3: 14-3-3 protein 4; DRP-206: Disease resistance response protein-206; PRP-5: Pathogenesis related protein-5.

Using transcripts putatively differentially expressed in the olive pistil (Table 2), three transcripts were selected. These were 14-3-3 protein 4, Disease resistance response protein-206, and Pathogenesis related protein-5. The results of PCR amplification showed respectively products of 214, 113 and 95 bp, also compatible with the expected sizes. Both DRP-206 and PRP-5 were amplified exclusively in the pistil. However, 14-3-3 sequences appeared as a PCR reaction product in the pistil but also weakly in the leaf. This may reflect a situation in which the 14-3-3 protein 4 sequences from leaf were not subtracted when the P(L) library was created, perhaps because they correspond to slightly different sequences, although able to be amplified by the primer combination selected in this case.

The normalization of the libraries allowed us to identify low abundance transcripts but with the drawback of missing details about their real abundance. However, analysis of the distribution of Gene Ontology terms provided a first approach to define the implication of these transcripts (Figure 4A, B and C). The two pollen subtractive libraries (Po(L) and Po(P)) (Figure 4A) included exclusive transcripts involved in biological processes related to the categories of pollination, responses to extracellular stimulus and post-embryonic development, and a high abundance of transcripts connected with cell differentiation, cell growth, and cellular component organization, in comparison to the rest of the libraries. The large presence of transcripts involved in cellular component organization may suggest that

although the mature pollen grains have not yet started to germinate, many transcripts needed for pollen tube formation are already accumulated inside the pollen grains. In the same way, transcripts connected to transport activity also occupy a relevant role in both pollen libraries (Po(P) and Po(L)). Therefore, the presence of such "preparative" transcripts in the mature pollen grain may be a determinant for the correct development of the pollen tube once the pollen grain arrives on the stigma and starts germinating through the style.

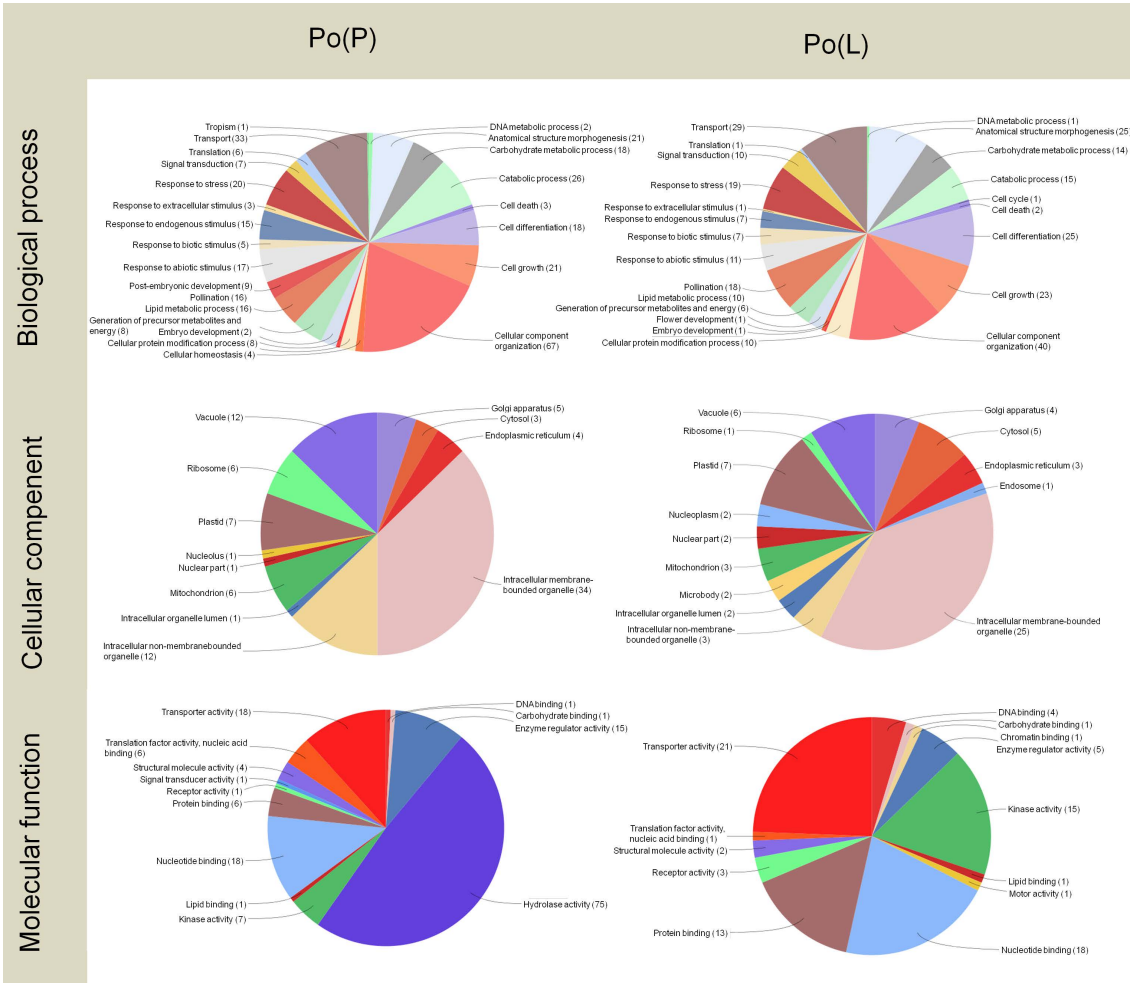

Figure 4A: Gene Ontology terms distribution of the Po(P) and Po(L) libraries. The distribution of biological processes and molecular function was assessed to a sequence cut off=1. The distribution of cellular components corresponds to a level=6. The graphs were performed using the Blast2Go software.

As regard to the distribution of transcripts among the different cellular components, the presence of transcripts apparently assigned to plastids is surprising, as olive pollen grains contain only poorly-differentiated plastids, probably lacking a highly structured biochemical machinery (Rodriguez García and Garcia, 1978; Rodríguez-García et al., 1995). In both the Po(P) and Po(L) libraries, transcripts likely assigned to intracellular membranes or non-membrane bounded organelle represent approximately half of the total transcripts.

Regarding the pie charts showing molecular function, considerable differences between the two pollen subtractive libraries were observed. These differences mainly result from the massive presence of Pectin Methyl Esterases (PMEs) in pollen. Approximately half of the transcripts in the Po(P) library were involved in hydrolase activity. On the other hand, the Po(L) library did not show a high abundance of these transcripts. The subtraction carried out to create the Po(P) library possibly removed most of the PMEs of the pollen, and allowing the identification a wide variety of PME isoforms, as well as one pollen-specific PME. Therefore, the information provided by the pie charts for molecular functions delivers particular evidence on the processes happening within the pistil at stage 4.

In the pistil subtractive libraries P(Po) and P(L) (Figure 4B), the presence of an exclusive transcript related to symbiosis (encompassing mutualism through parasitism) was detected. Further to this, both the pistil and the leaf tissues contain wide pools of transcripts related to stress and defence. A highlight here is the presence of transcripts linked to responses to biotic stimulus in the P(L) library as it has been proposed that certain SI processes may have evolved from pathogen-defence mechanisms (de Nettancourt, 1997; Hodgkin et al., 1988; Elleman and Dickinson, 1999; Hiscock and Allen, 2008). Within these transcripts, we found Pathogenesis related proteins-1, 5, and 10, Beta-glucosidases and PME Inhibitors.

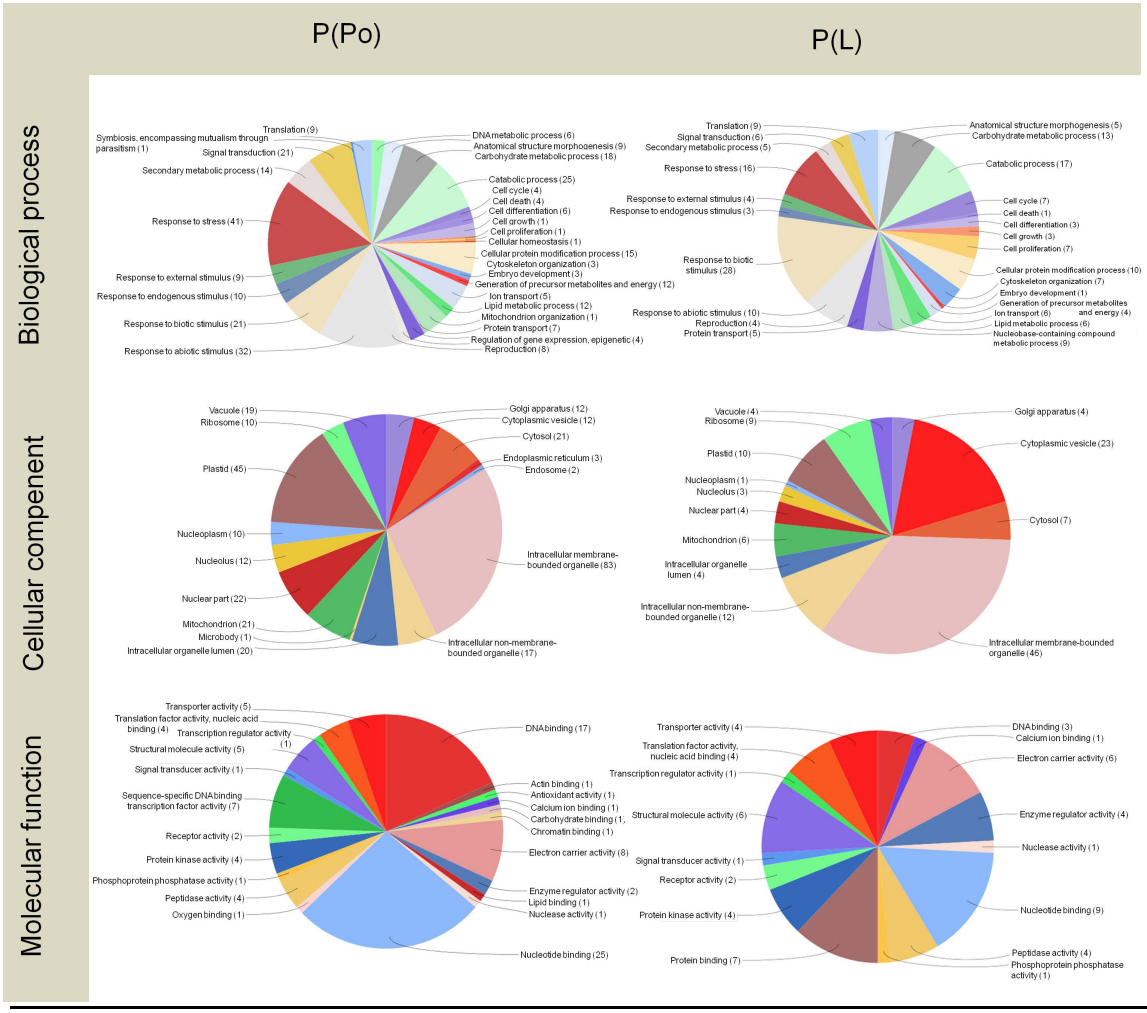

Figure 4B: Gene Ontology terms distribution of the P(Po) and P(L) libraries. The distribution of biological processes and molecular function was assessed to a sequence cut off=1. The distribution of cellular components corresponds to a level=6. The graphs were performed using the Blast2Go software.

On the other hand, the pistil harboured numerous PME inhibitor transcripts (revealed in the P(L) library), but they did not appear in the P(Po) library as the consequence of the subtraction carried out. This is likely due to the homology between PME and the PME inhibitors present in the stigmatic exudate of the olive (Rejón et al., 2013). These results are consistent with the localization of the PME inhibitors described at the pollen tube apex (Röckel et al., 2008), which were detected in the pistil library, probably as a result of the presence of pollen tubes growing through the stigma-style.

In a similar way to both pollen libraries, there was a large presence of transcripts for proteins associated to intracellular membranes or non-membrane bounded organelles in the P(Po) and P(L) libraries. Among these, transcripts associated to plastids scored as an important proportion, and were more numerous in the P(Po) library than in the P(L) one.

The analysis of the molecular functions of the transcripts present in pistils revealed the noticeable presence of transcripts for proteins with electron carrier activity in both cases, which did not appeared

in the pollen libraries. The number of transcripts for proteins with DNA-binding was almost six fold higher in the P(Po) library compared to the P(L) library. Even though transcripts within the nucleotide-binding category were abundant in both libraries, they were almost three times more abundant in the P(Po) library than in the P(L) one.

DNA-binding proteins are key players in the process of expression and regulation of new proteins as such interactions are considered to be central for many basic biological processes, including transcription regulation, DNA replication and DNA repair (Bonocora and Wade, 2015). The high levels of transcripts encoding DNA-binding proteins in the pistil could be indicative of the ability to have quick responses, where finely tuned regulation is needed. On the other hand, the presence of transcripts for nucleotide binding proteins could also be related to the changes happening outside the gynoecium cells, as the plant disease resistance genes have been described to frequently encode nucleotide binding proteins (Meyers et al., 1999).

Regarding the leaf subtractive libraries L(Po) and L(P) (Figure 4C), most of the transcripts corresponded, not surprisingly, to proteins involved in photosynthetic metabolism, with the detection of a large proportion of transcripts for proteins located at plastids as well as a majority of transcripts implicated in biological processes such as carbohydrate metabolism and the generation of precursor metabolites and energy. This is also consistent with the large presence of transcripts for proteins with electron carrier function. As expected, these transcripts are also present in the pistil, although less abundantly, and absent in pollen.

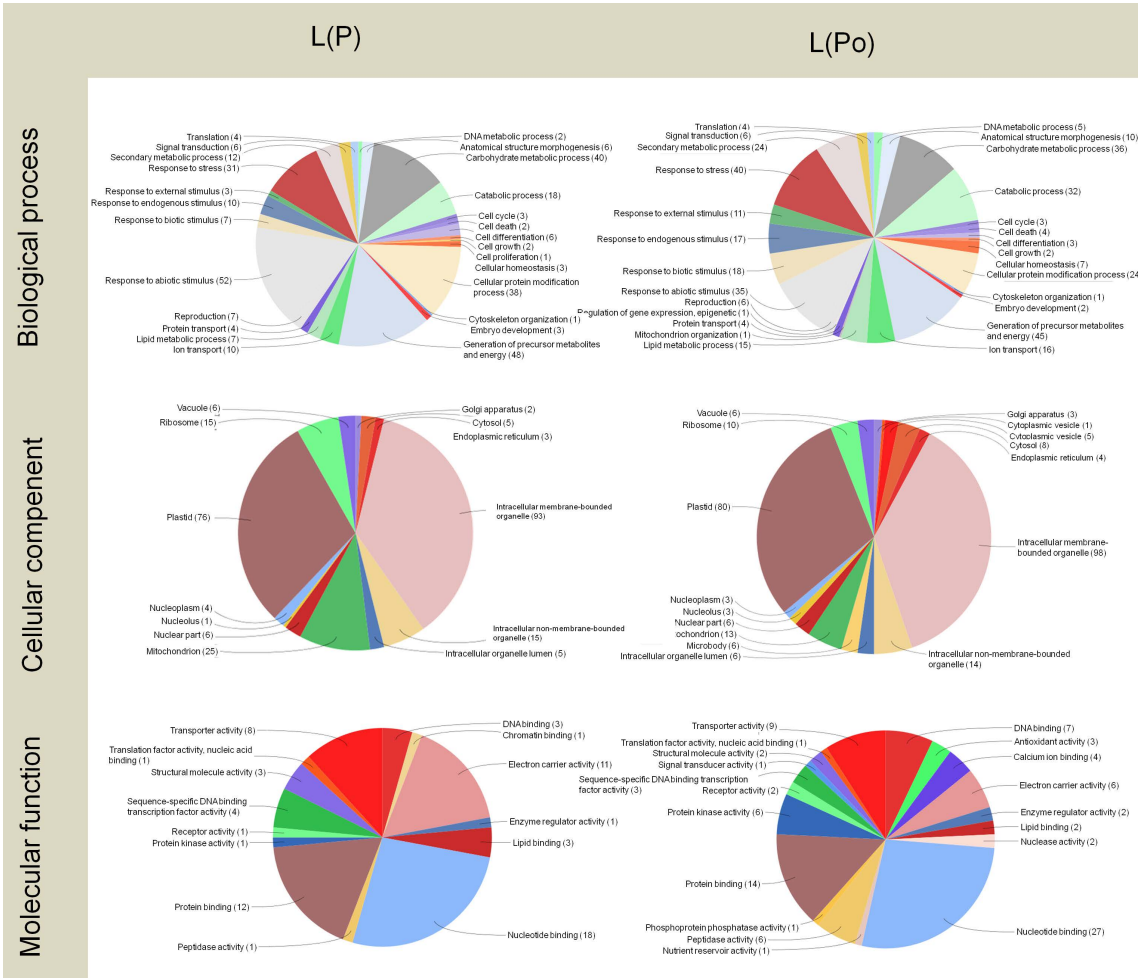

Figure 4C: Gene Ontology terms distribution of the L(P) and L(Po) libraries. The distribution of biological processes and molecular function was assessed to a sequence cut off=1. The distribution of cellular components corresponds to a level=6. The graphs were performed using the Blast2Go software.

To sum up and as discussed above, the putative origins, biological processes, cellular localizations and molecular functions of the transcripts identified in the different subtractive libraries analyzed are in good agreement with the predicted nature of such transcripts derived from the methods and tissues used for their construction, as displayed in Table 3. It is necessary to take into account that pistil tissues are expected to include dehiscent (mature) olive pollen, as well as hydrated pollen grains and even germinating pollen grains and pollen tubes, as described in the materials and methods section.

Name of the library	Tester tissue	Driver tissue	Expected Biological significance
Po(P)	Pollen	Pistil	Transcripts expressed exclusively in mature pollen grains, not expressed in pistil nor in hydrated, or geminated pollen grains.
P(Po)	Pistil	Pollen	Transcripts expressed exclusively in pistil and hydreted and/or germinated pollen grains but not expressed in mature pollen grains.
P(L)	Pistil	Leaf	Transcripts expressed exclusively in pistils, but not in vegetetive tissues (leaf) despite their common evolutive origin.
L(P)	Leaf	Pistil	Transcripts expressed exclusively in vegetative tissues (leaf) and hydrated or germinated pollen grains, but not in pistil,
Po(L)	Pollen	Leaf	Transcripts expressed exclusively in male reproductive tissues (pollen grains), but not in vegetative tissues (leaf).
L(Po)	Leaf	Pollen	Transcripts expressed exclusively in vegetative tissues (leaf), but not in mature pollen grains.

Table 3: Putative composition and nature of the transcripts present in the generated SSH libraries

Therefore, taking into account the origins for the described transcripts, the SSH libraries which best describe the pollen-pistil interactions in olive and the pollen hydration and pollen tube growth are Po(P) and P(Po). For both of these SSH libraries, the corresponding GO terms have been identified, and been represented together for comparison purposes (Figure 5).

Within the pool of transcripts present in the pistil from which pollen has been subtracted P(Po)], those involved in regulation, response to stress/stimulus, and signalling/cell communication are highly represented in terms of number of copies. On the other hand, the library of pollen from which pistil has been subtracted [Po(P)], is mainly rich in transcripts involved in cellular organization, localization, developmental processes, pollination and growth. Detailed lists of the transcripts detected for each pollen SSH library [Po(P) and Po(L)], together with BLAST relevant scores for each one are listed in additional Tables 2A and 2E. Amongst these pollen transcripts LAT52 plays a role in pollen hydration and germination. The presence of SF21 confirms the importance of these transcripts in reproduction, with a putative function in pollen-pistil interaction. However, no transcripts of SF21 were found in the pistil [see additional Tables 2B and 2C, corresponding to P(Po) and P(L)], despite a putative function in pollen tube guidance (Allen et al., 2010a). The SNARE proteins within the mature pollen grains are also present in the spore, with a role in the pollen tube movements (Bushart and Roux, 2007).

In the pistil (additional Tables 2B and 2C), the response to stress is mainly represented by the Pathogenesis related proteins (PRPs); the signalling processes occurring in the pistil is emphasised by the presence of auxin responsive factors. Regulation is carried out by the interaction with the pollen specific auxin induced/repressed proteins (present in the mature pollen; they were found in both pollen libraries). The output results from the pistil libraries showed a similarity to that seen with auxin-induced root cultures (Neuteboom et al., 1999), but with an unknown function in the mature pollen grains. The auxin responsive proteins present in both pistil libraries are important for pollen tube formation (Yang et al. 2013), which indicates the presence of growing pollen tubes. However, they are not present in the mature pollen grains (additional Tables 2A and 2E). The 14-3-3 protein is also important in pollen germination as it is involved in the regulation of turgor pressure of the pollen tube (Pertl et al., 2010).

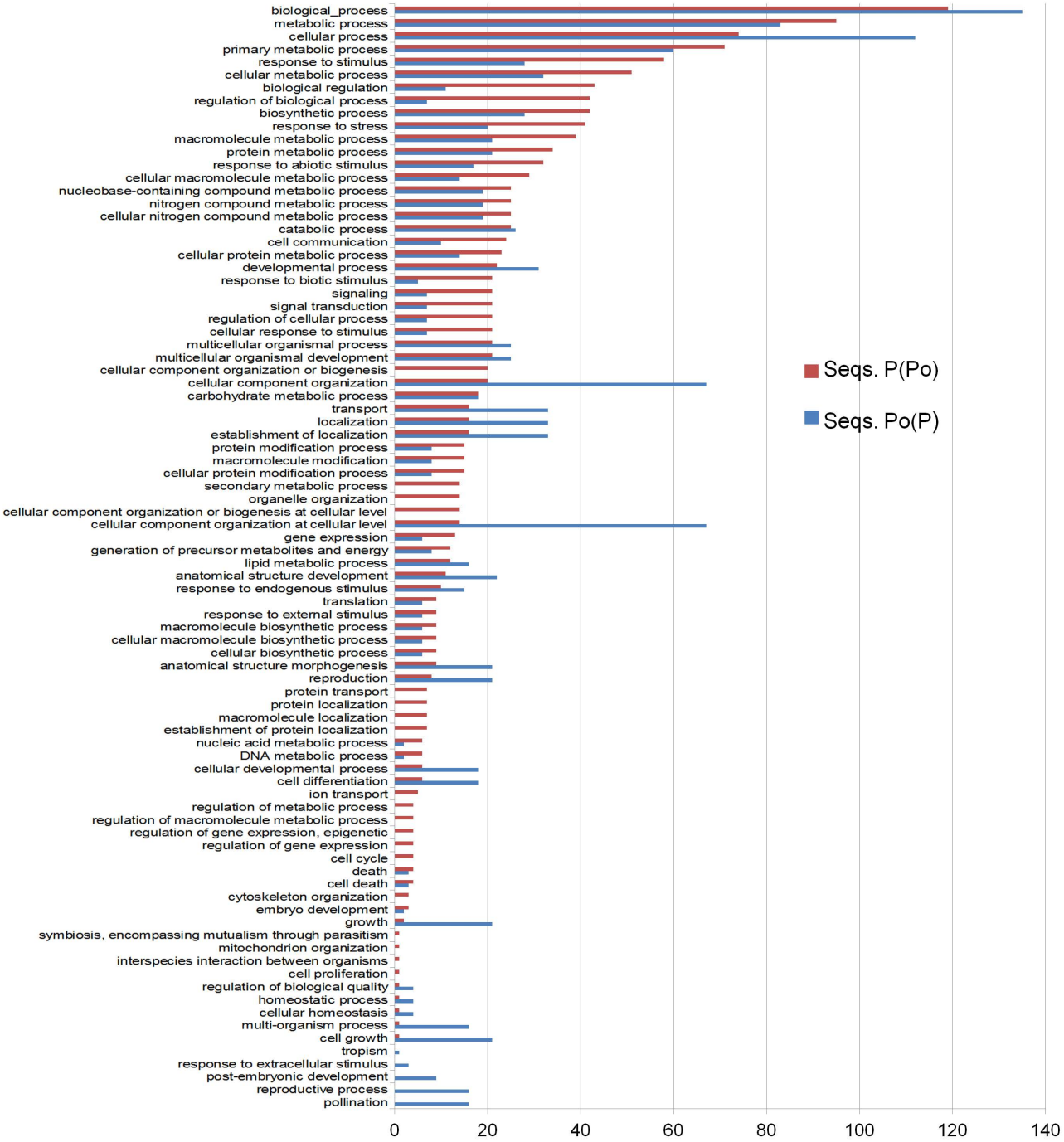

Figure 5: Differential Gene Ontology terms between the Po(P) and P(Po) libraries.

In order to obtain clues as regard to the phylogenetic relationships between the olive tree and other plants, the taxonomical origin of the top-hit BLAST results from the olive transcripts present in the SSH libraries using the NCBIdb are shown in Figure 6. However, as the number of sequences available in NCBIdb for each plant species is unequal, a normalization procedure was applied.

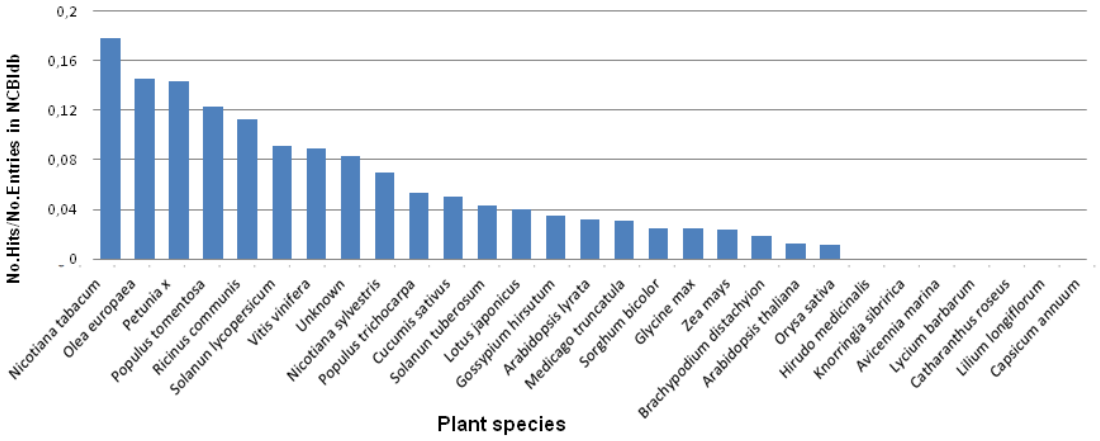

Figure 6: Plant species distribution of the hits resulting from analysis using BLAST for all six SSH libraries against the NCBIdb, after normalization of the distribution of the species in relation to the total number of entries in the NCBIdb. Most abundant hits corresponded to *Nicotiana tabacum*, whereas *Olea europaea* occupies the second position.

The higher relative number of BLAST hits after analysis using BLAST for the SSH olive libraries to NCBIdb corresponded to *Nicotiana tabacum*, with a higher number of matches than Olea europaea. Third place (*Petunia* x, also a member of the Solanaceae family, closely related to the Oleaceae family of the olive tree), *Populus tomentosa*, *Ricinus communis*, *Solanun lycopersicum* and *Vitis vinifera* occupying the subsequent positions. It was surprising to find *Nicotiana tabacum* in the first position instead of *Olea europaea*, which would have been expected. However, owing to the high specificity of the transcripts in the reproductive tissues and that *Nicotiana tabacum* is the second most studied species in the reproductive field, behind *Arabidopsis thaliana*, perhaps this is not so unexpected. Furthermore, most reproductive transcripts from *Olea europaea* are unknown and therefore are not present in NCBIdb to date. On the other hand, *Arabidopsis*, a highly characterised plant species, seems to be not so closely related to the olive as *Nicotiana tabacum*, with respect to this study of reproductive tissues.

The use of BLAST analysis of the SSH-retrieved sequences against the OLEA ESTdb, specifically containing sequences from olive mesocarp only, provided in many cases further information through the annotation of our sequences. For example, the described pistil-specific thaumatin/PRP-5 (Sassa et al., 2002; Kuboyama, 1998) is identified in the OLEA ESTdb as "Thaumatin-like protein, Pathogenesis–related protein 5", whereas the NCBIdb identify them with the general term "Thaumatin-like protein". We were able to discriminate between two different thaumatin-like proteins: the Pathogenesis–related protein 5, which was only found in the pistil, and the Pathogenesis–related protein 1, which was pollen specific. Moreover, three isolated transcript from different thaumatins were found in the pistil, the first one identified as "STS14 protein/ Pathogenesis-related protein 1C", and the others as "Osmotin-like protein" (OSML13 and OSM34, respectively). The STS14 protein is proposed to be involved in the protection the outer tissues of the pistil from pathogen attack or guidance of the pollen tubes through the pistil. It is highly expressed in the stigma and stylar cortex around 120 hours before anthesis and increases towards the end of flower development, with a maximum at anthesis (Van Eldik et al., 1996).

As an example, three groups of transcripts were selected (defence, oxidative metabolism and transport: Figure 7) with the aim to further analyse and discuss the expression of transcripts with high abundance as well as their key putative roles in the pollen-pistil interaction, pollen tube germination and growth. The genes considered putative homologues to Arabidopsis from each group were analysed throughout the anatomy tool of Genvestigator. The specificity of the transcripts and the biological implications of their differential expression are described and discussed below.

Different transcripts putatively involved in defence are present in the pistil

One of the stigma-specific transcripts detected in olive was that corresponding to the Pathogenesis-related protein 5 (PRP-5). Members of this protein group have been associated with resistance to fungal infection and to responses to biotic/abiotic stresses, disease resistance or hormonal responses by inducing transcripts such as DOR, MYB, AP2 and WRKY

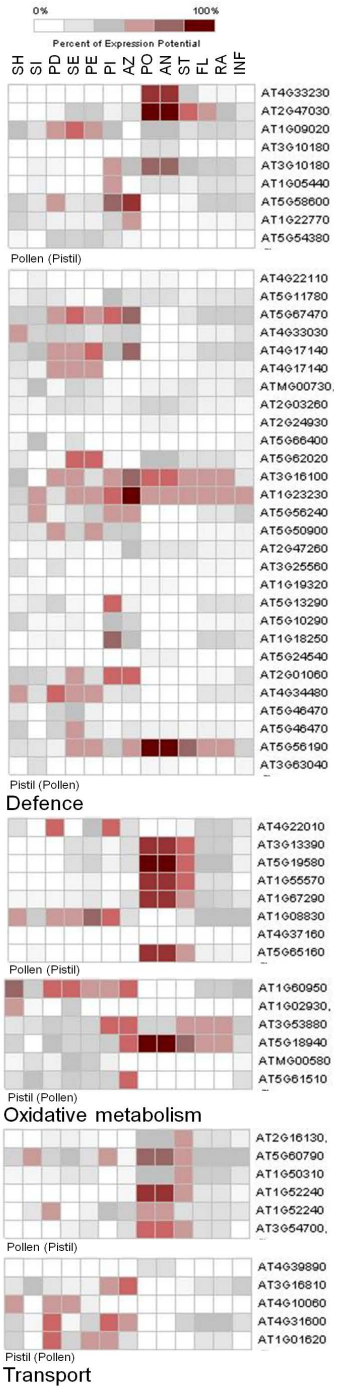

Figure 7: Level of expression across several reproductive/vegetative tissues of genes considered putative homologues to Arabidopsis using the anatomy tool Genvestigator. Three categories were considered for analysis of the levels of expression: oxidative metabolism (upper part), defence (middle part), and transport (lower part). The transcripts of two selected libraries were considered only: Po(P) and P(Po). SH:shoot; SI:silique; PD:pedicel; SE:sepal; PE:petal; PI:pistil; AZ:abcision zone; PO:pollen; AN:anther; ST:stamen; FL:flower; RA:raceme; INF:inflorescence

(El-kereamy et al., 2011). A pistil-specific thaumatin/PRP-5 has been described in Japanese pear (Sassa et al., 2002) and in tobacco (Kuboyama, 1998), where the maximum levels of the transcript were reached at anthesis. This gene product has been proposed to play a role in pollen recognition and pollen tube pathways. Among the stigma-specific PRP-5 sequences obtained in olive, we observed high homology with the SE39B specific-stigma thaumatin from tobacco (Kuboyama, 1998) and also with a specific thaumatin from the fruit of Olea europaea (Corrado et al., 2012). Another olive stigma-specific transcript of interest, involved in defence, and also considered an allergen, is Mal d 1 from apple (Vanekkrebitz et al., 1995). This belongs to the PRP-10 group. These gene products have been described throughout several developmental stages and plant tissues, with a dual role associated with defence functions and regulation/signalling (Choi et al. 2012; Zubini et al. 2009). Pectin Methyl Esterases (PMEs) and their inhibitors (PMEIs) have been considered to be involved in defence function in vegetative tissues (Boudart et al., 1998; McMillan et al., 1993; Wydra and Beri, 2007; Lionetti et al., 2007; Ann et al., 2008; Körner et al., 2009; Volpi et al., 2011; Lionetti et al., 2012; Volpi et al., 2013). Thus, the PMEs have been reported to enhance RNAi action, acting in gene-regulatory mechanisms (Dorokhov et al., 2006), which include virus-induced gene silencing (VIGS) and the fight against others pathogens (Collmer and Keen, 1986). Previous proteomic work has also identified PMEs in the olive stigmatic exudate. These proteins contain N-terminal pro-regions with homology to PMEIs (Rejón et al., 2013). Interestingly, it has been suggested that PMEIs might be internalized by endocytosis at the flanks of the pollen tube tip, regulating pollen-tube wall stability by locally inhibiting pollen PME activity (Röckel et al., 2008). It has also been suggested that PMEIs are able to reduce the activity of cell wall PMEs, leading to a drop in pollen tube stability (Paynel et al., 2014). Interestingly, a pollen specific PMEI was described in broccoli triggering partial male sterility and decreased seed set by inhibition of pollen tube growth (Zhang et al., 2010).

The large presence of cysteine proteinase in the pistil may be attributed to defence mechanisms, similar to that already described by Grudkowska and Zagdańska (2004). Another defence mechanism which seems to work actively in the olive pistil is the "disease resistance response protein 206", that has been described to be induced in pea in response to the infection by F. solani f. sp. phaseoli (Culley et al., 1995). Within the LEA (Late Embriogenesis Abundant) proteins, the pistil possesses transcripts for the dehidrin Rab 18. The LEA-18 transcript decreases during the germination process in pea, though it is present again in the emerging hypocotyls. Therefore, this transcript might be related to the

elongation process under optimal growing conditions (Colmenero-Flores et al., 1999). In the case of the pistil, the presence of these transcripts could be related to the elongation process occurring in the growing pollen tubes within the stigma/style. Beta-glucosidases, late blight resistance proteins, WRKY genes, mitogen-activated kinase proteins, and the MYB genes expressed in the olive pistil are also involved in defence (Pandey and Somssich, 2009; Engelhardt et al., 2012). The MYB transcription factor itself has been described to be involved in pollen development (Katiyar et al., 2012; Niwa et al., 1993). A pistil specific nodulin has been also described in the pistil of several species (Allen et al., 2010b), being involved in a successful fertilization (Shi et al., 2012).

Different transcripts putatively involved in defence are present in the pollen grain

Defence genes highly expressed in the olive pollen comprise PME, the PME inhibitor U1, and a panel of Pathogenesis related proteins.

The olive pollen PME is considered a highly prevalent allergen present in the olive pollen (Salamanca et al., 2010). PMEs are enzymes present in higher plants, fungi and bacteria. They catalyze the demethylesterification of homogalacturonan residues of pectin, releasing methanol as the reaction product. Such modification is responsible for changes in the pectin molecule, which can then be cross-linked by calcium, and this further results in changes in the mechanical properties of the plant cell wall, altering its plasticity. This particularly affects the ability for growth and guidance of pollen tubes (Castro et al., 2013). Pollen specific PMEs have been described in other species (Tian et al., 2006; Gómez et al., 2013), with key roles during pollen germination (Leroux et al., 2015), during pollen tube elongation along the transmitting tract and when the pollen tube reaches the embryo sac in the ovule (Gómez et al., 2013).

PRP-1 was detected exclusively in olive pollen subtracted Po(P) and Po(L) libraries. To date, PRP-1 has only been described to be involved in food allergy (Asensio et al., 2004), as the precise function of these proteins is not yet known. The specific expression of the heat stress transcription factor (HsfA2) was also detected. HsfA2, together with chaperones, are important protectors of the pollen maturation, viability and pollen tube germination from heat damage (Zinn et al., 2010; Frank et al., 2009; Giorno et al., 2010).

Oxidative metabolism in the pistil:

Closely related to defence mechanisms, oxidative metabolism interplays a dual role, keeping the balance between defence and signalling. In the case of the pistil, these two functions are even more finely tuned as the signalling processes are very important for a successful reproduction. Therefore, it is important to highlight the presence of transcripts corresponding to Glutathione S-transferases (GSTs), Ferredoxin-1, NAD(P)H-dependent oxidoreductase, Peroxidase 72 and Quinone oxidoreductases. Most of these transcripts have not been described as pistil-specific in Arabidopsis (Figure 7). Among these, stigma-specific peroxidases have been previously studied in several species (McInnis et al., 2005; Beltramo et al., 2012; Swanson et al., 2005), with the implication in the pollen-pistil interaction, pollination process, and signalling. The glutathione S-transferase has been classified as an allergenic protein as well in animal species (Yu and Huang, 2000; Huang et al., 2006). Later it was identified in birch pollen (Deifl et al., 2014). However, when compared to other birch pollen allergens such as Bet v 1, the release kinetics of GST from pollen grains upon contact with water and different physiologic solutions was much slower. It was suggested that the amount of glutathione S-transferases released during this time period was too low to induce allergic sensitization (Deifl et al., 2014).

Oxidative metabolism in the pollen grain

The presence of transcripts from Tpr repeat-containing thioredoxin ttl1-like was observed. Such gene products have been described to accumulate in response to osmotic stress and abscisic acid (ABA), and also may be involved in pollen compatibility (Haffani et al., 2004). Using analysis to look for members of the oxidoreductase family of proteins we could find transcripts for galactose oxidase, glyoxal oxidase and a specific L-ascorbate oxidase homolog (Pollen-specific protein NTP303). To our knowledge, the presence of galactose oxidase has not been connected to any particular characteristic of the plant reproductive tissues. On the contrary, the enzyme glyoxal oxidase has been described to be involved in male sterility, jointly to other enzymes implicated in cell wall expansion (Ming et al., 2012; Suzuki et al., 2013). The presence of L-ascorbate oxidase transcripts has been described in in vitro germinating pollen grains (Weterings et al., 1992), although we failed to find these mRNAs in the olive pistil, which also contains in vivo growing pollen tubes. It is interesting to highlight the presence of the olive pollen allergenic protein Cu,Zn SOD which is involved in the protection against oxidative stress during pollen development. Its dual role, ie as an allergen and as part of the antioxidant/signalling metabolism, makes its study particularly interesting (Butteroni et al., 2005; Alché et al., 1998). It also serves as a positive control for the present study. Moreover, it has been described to be implicated in the development of the male reproductive tissues of the olive tree (Zafra et al., 2012).

Transcripts connected with transport of molecules in the pistil

Pollen-stigma interactions and the growth of the pollen tube throughout the pistil tissues encompass a large exchange of molecules among these tissues, either positively or negatively regulating and/or permitting such growth, throughout providing energy, ions or structural molecules. Among the pistil exclusive transcripts detected in this work, several have been attributed with functions facilitating transport of such molecules. This is the case for the Ras-related transport protein, which facilitates proteins movement through membranes, and the mitochondrial import inner membrane translocase subunit Tim13. Transcripts from a member of the solute carrier family 35 (B1) are also present in the olive gynoecium. Other transporters that have been described also in primary roots (with a growing processes comparable to that of pollen tubes within the style of receptive flowers) are the specific lipid-transfer protein (LTP) AKCS9 (present in membranes) and aquaporins, both specifically present within the olive pistil transcripts and with described vegetative/reproductive differential meanings: LTPs were correlated with root hair deformation and pistil abortion (Krause et al., 1994; Shi et al., 2012) whereas specific aquaporins were found in the region adjacent to the root tip and have been demonstrated to be required for the self-incompatibility process displayed for members of the family Cruciferae (Sakurai et al. 2008; Ikeda et al. 1997).

Transcripts connected with transport of molecules in the pollen grain:

Transcripts for several transporters were found in the mature olive pollen grain. The sugar transport protein must represent a key transcript in pollen and pollen germination as it has been described in tobacco (Lemoine et al., 1999). Also, the polyol transporter present in the olive pollen could share similar functions to the polyol/monosaccharide transporter 2 expressed in mature pollen grains, growing pollen tubes, hydathodes, and young xylem cells (Klepek et al., 2010). Moreover, boron transporters expression reveals the regulatory role of boron in pollen germination and pollen tube growth (Qinli et al., 2003). Nitrate transporters also act as a nitrate sensor that triggers a specific signalling pathway stimulating lateral root growth (Guo et al., 2001), which may have a similar significance in pollen tube growth. The presence of the cation proton exchanger is critical for maintaining polarity, directing pollen growth toward the ovule, and to allow cell expansion and flower development (Lu et al. 2011; Bassil et al. 2011). The transcripts encoding ABC transporters, also found in the olive pollen, could be related to the transport of sporopollenin precursors for exine formation in developing pollen (Choi et al., 2011). Rho guanine nucleotide exchange factors are crucial in polar growth of pollen tubes (Zhang and McCormick, 2007). Finally, phosphate transporters have also been described as central for gametophyte development (Niewiadomski et al., 2005).

The present analysis also has reported some unexpected results. As an example, anther-specific proline-rich protein APG transcripts have been found in the pistil, when they have been considered to be confined to the anther during the period of microspore development, with a dramatic decline during pollen maturation (Roberts et al., 1993). This result could be explained by the implication of the proline-rich protein APG in the pollen tube during the germination process, through a process yet to be determined.

Even though our data still do not reveal substantial information as regards some key aspects of the olive reproductive biology which are still open, such as the demonstration of the presence of a SI system of the gametophytic type (largely suspected), the data here are highly valuable due to the current absence, to our knowledge, of an openly available genome or reproductive transcriptome for this plant, in spite of its huge economical and clinical interest. Many of the transcripts detected here (either tissue-specific or not) are of great interest for the further characterization of the species, and in some cases for important issues like olive pollen and stigma physiology, as discussed above. Current knowledge of olive pollen allergenicity can also be improved, as several of the identified transcripts correspond to potential allergenic molecules already described in other species, but as such not yet described in olive. This is the case, for example, with glutathione S-transferase, considered a minor allergen in birch pollen (Zwicker, 2013; Deifl et al., 2014). Gene products corresponding to transcripts detected in the resulting SSH libraries P(Po) and P(L) described here are also consistent with proteins characterized in the olive stigma exudate by means of proteomic approaches (Rejón et al., 2014) which may act as additional positive controls for the present methodology, because the presence of olive pollen originated peptides among those detected by was almost completely avoided.

Conclusions:

The generation and analysis of different SSH subtractive libraries has provided a dataset of sequences, consisting in about a thousand entries of great value for the understanding of the physiological processes taking place in olive pollen and pistil during their development and interaction. They are particularly important as many of these inputs have been demonstrated to be exclusively or preferentially expressed in the reproductive tissues, and not in the

leaf tissues, as this material was used to build the subtractive strategy.

The subtractive transcripts have been annotated according to their homology as regard to two main databases: an olive-specific database constructed from mesocarp, and a general plant database provided by the NCBI. Moreover, they have been extensively classified and their presence discussed as regard to their putative biological function, cellular localization, and the molecular functions expected to exert.

Such information will be used in the near future as the basis to examine further aspects of the olive reproductive biology through the specific analysis of the expression of these products. These aspects may include compatibility, cell-to-cell communication, pollen tube growth and guidance, and pollen allergenicity among others.

Methods:

Plant material:

The different tissues were obtained from adult olive trees (Olea europaea, cv. Picual) growing at the Estación Experimental del Zaidín (Granada, Spain). Pistils were excised from the complete flower at the stage of development 4 (dehiscent anthers, as defined by Zafra et al., 2010). These pistils normally include a relatively high number of mature (dehiscent) pollen, hydrated pollen grains, and even germinating pollen grains and pollen tubes, either over the stigma surface or through the transmitting tissues of the style or the ovary. The mature pollen grains were collected during the anthesis period using large paper bags by vigorously shaking the inflorescences. Pollen was sequentially sieved through a mesh in order to separate the grains from the debris. Young leaves were also selected. In all the three cases the different tissues were quickly frozen in liquid nitrogen and stored at –80◦C.

Construction of the suppression subtractive hybridization (SSH) libraries:

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), and the contaminating genomic DNA was removed by DNAase I (Quiagen) treatment followed by a clean-up with the RNeasy MinElute Cleanup kit (Qiagen). cDNA was then synthesized from pistil, leaf, and mature pollen total RNA using the SMART PCR cDNA Synthesis kit (Clontech). The subtracted libraries were constructed with the PCR-Select cDNA Subtraction Kit (Clontech). A total of 6 libraries were constructed: 1. Pistil subtracted with pollen [P(Po)]; 2. Pollen subtracted with pistil [Po(P)]; 3. Pistil subtracted with leaf [P(L)]; 4. Leaf subtracted with pistil [L(P)]; 5. Pollen subtracted with leaf [Po(L)]; 6. Leaf subtracted with pollen [L(Po)], according to the manufacturer's instructions. Two rounds of PCR amplifications were also performed according to the manufacturer´s protocol in order to enrich differentially regulated genes, by using the PCR Primer 1 and the Nested PCR primer 1 and 2R as indicated in the manufacturer´s instructions and provided by the kit.

Cloning and differential screening:

The secondary PCR products were cloned into the T/A cloning vector pGEM-T Easy (Promega) according to the manufacturer´s instructions and transformed into DH5α *E. coli* cells. The colonies containing inserts were picked and used as template for PCR. The primers used in this case were SP6 and T7. Sanger sequencing of PCR products was carried out at the Estación Experimental del Zaidín DNA Sequencing Service (CSIC, Granada, Spain), the Laboratório de Biologia Molecular de Plantas (Universidade de São Paulo, Brazil), and other commercially available facilities. With the aim to perform the differential screenings, a number of membrane replicates were prepared, each one containing 1 µl dot of the PCR products, which were transferred onto nylonmembranes and fixed with a brief wash in 2x SSC followed by baking at 120◦C during 30 minutes. The membrane replicates were probed with the forward-subtracted probe, the reverse-subtracted probe, the unsubtracted tester probe, and the unsubtracted driver probe in each case. The labelled probes were generated from the secondary PCRs products described in the (SSH) library construction section, which were purified using the MinElute PCR Purification Kit (Qiagen). DIG-DNA labelling, determination of labelling efficiency, hybridization, and immunological detection were carried out as described in the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) instruction manual. The membranes were revealed with the CSPD ready-to-use chemiluminiscent substrate (Roche), exposed to ChemiDoc XRS system (Bio-Rad). Images were gathered with a supersensitive 12-bit CCD after 30 minutes of exposition.

Sequencing and data analysis:

Singletons were compared against non-redundant protein databases at the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990) (e-value e-4) and also against the non-redundant proteins unique transcripts Olea EST database (Alagna et al., 2009). The Blast2Go (https://www.blast2go.com/) software was used (Conesa et al., 2005) to carry out the statistical analysis of GO terms. For the analysis of the contigs obtained from the singletons, the Codon Code Aligner software was used (http://www.codoncode.com/aligner/).

The Venn diagrams were constructed using the transcripts of the 6 SSH libraries analysed. Tree groups were considered, corresponding to pollen, pistil and leaf transcripts. The VENNY software (http://bioinfogp.cnb.csic.es/tools/venny/) was used for this purpose. With the aim to compare the output results after the comparison against the NCBI and Olea EST databases, two diagrams were performed separately.

To retrieve the putative Arabidopsis homologues of the olive clones obtained, the sequences from the transcripts of two selected libraries (Po(P) and P(Po)) were compared against the Arabidopsis Information Resource (TAIR) webpage (http://www.arabidopsis.org/Blast/). A BLASTn against the TAIR10 Transcripts (-introns, +UTRs)(DNA) was carried out. The matrix weight was Blosum45, the nucleic mismatch -3, gapped alignments ON. The output results were used as input data in the plant biology resource from Genvestigator (https://genevestigator.com/). The anatomy tool from this webpage was used to construct the heatmap representing the level of expression of the transcripts corresponding to defence, oxidative metabolism and transport.

PCR validation:

Primers were designed for transcripts specific of each tissue. The length of the primers were 20-21 bp, with a 48-60% GC content, and a Tm= 57-59◦C. The expected amplicons sizes ranged from 95 to 214 bp (see additional Table 1).

List of abbreviations:

DRP: Disease Response Protein
EST: Expressed Sequence Tag
GO: Gene Ontology
Hsf: Heat stress factor
LEA: Late Embriogenesis Abundant
LTP: Lipid Transfer Protein
PME: Pectin Methyl Esterase
PMEI: Pectinmetielesterase Inhibitor
PRP: Pathogenesis Related Protein
ROS: Reactive Oxygen Species
SI: Self-Incompatibility
SNARE: Soluble NSF Attachment Protein Receptor
SOD: Superoxide Dismutase
sRNA: small RNA
SSH: Supression Substractive Hybridization
VIGS: Virus-Induced Gene Silencing

Competing interests:

The authors declare no conflict of interests.

Authors' contributions:

A. Zafra, J.D. Alché designed the experiments and redacted the manuscript. A. Zafra performed the experiments and analysed the results. J.A. Traverso was particularly involved in the work with the databases and tools on the web servers. M.H. Goldman participated in the sequencing and interpretation of results.

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CHAPTER 4

Identification of Distinctive Variants of the Olive Pollen Allergen Ole e 5 (Cu,Zn Superoxide Dismutase) throughout the Analysis of the Olive Pollen Transcriptome

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Abstract. Ole e 5 is an olive pollen allergen displaying high identity with Cu,Zn superoxide dismutases. Previous studies characterized biochemical variability in this allergen, which may be of relevance for allergy diagnosis and therapy. The generation of an olive pollen transcriptome allowed us to identify eight Ole e 5 sequences, one of them including a 24 nt deletion. Further *in silico* analysis permitted designing primers for PCR amplification and cloning from both cDNA and gDNA. A large number of sequences were retrieved, which experimentally validated the predictive NGS sequences, including the deleted enzyme. The PCR-obtained sequences were used for further scrutiny, including sequence aligment and phylogenetic analysis. Two model sequences (a complete sequence and a deleted one) were used to perform 3-D modeling and a prediction of the T- and B-cell epitopes. These predictions interestingly foreseed relevant differences in the antigenicity/allergenicity of both molecules. Clinical relevance of differences is discussed.

Keywords: allergen, deletion, Ole e 5, pollen, Superoxide Dismutase.

1 Introduction

Olive pollen is one of the most important causes of respiratory allergy in the Mediterranean area [1]. To date, twelve allergens have been identified in olive pollen, and one in olive mesocarp, named as Ole e 1 to Ole e 13 [2,3]. Several studies have shown that olive pollen allergens posses a relatively high level of microheterogeneity in their sequence, which has been extensively characterized for allergens Ole e 1, Ole e 2 and

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Ole e 7 [3,4,5]. Moreover, olive pollen contains a complex mix of allergen forms, which is highly dependent of the genetic background (olive cultivar) used for the analysis [4].

Allergen polymorphism is a relevant issue concerning allergy diagnosis and treatment. Heterogeneity of vaccines used for specific immunotherapy (SIT) is considered a challenging factor their efficacy or even worse, it may represent a cause for secondary sensitization of patients or the development of undesirable reactions. Therefore, accurate identification and quantification of the different allergenic forms present in the extracts and its reactivity is a major concern nowadays.

Ole e 5 is considered a minor allergen in olive pollen, with prevalence around 35%, which has been identified as a Cu,Zn superoxide dismutase (SOD). Due to its ubiquity, it is considered as a cross-reactive allergen in the pollen-latex-fruit syndrome [6]. Studies carried out over Ole e 5 show that it is a 16 kDa protein with a high identity to Cu,Zn SODs from other species. In olive pollen, the presence of up to five isoforms has been detected [4, 7-9]. Concerning the biological function of this protein in pollen, SODs catalyse the dismutation of the superoxide anions into molecular oxygen and hydrogen peroxide, therefore acting as an antioxidant able to remove reactive oxygen species (ROS) [10]. ROS are produced in both unstressed and stressed cells. Under unstressed conditions, the formation and removal of O_2 are in or close to balance. Within a cell, the SODs constitute the first line of defence against ROS [11] and they have been localized in cytosol, chloroplasts, peroxisomes and the apoplast [12,13]. ROS may also act as signalling molecules. Thus, the interaction pollen-pistil is mediated by the accumulation or ROS in the stigma, where Ole e 5, is considered one of the main actors [14,15]. Under stress conditions, a clear rise of Cu,Zn-SOD has also been detected [16].

Actual olive tree transcriptomes mainly rely on libraries from vegetative tissues using different NGS strategies. The peculiarity of the reproductive tissues, and the widely reported presence of numerous tissue-specific transcripts in pollen grains, made us to attempt a similar approach in the later. Hence, reproductive cDNA libraries were prepared to be sequenced using 454/Roche Titanium+ platform. This information is highly valuable to elucidate the mechanisms governing the allergy process, as well as its implication in the olive pollen metabolism through germination, stigma receptivity, and the interaction pollen-stigma.

2 Materials and Methods

2.1 Construction of the Olive Pollen Transcriptome

For the construction of the pollen transcriptome, mature pollen obtained from dehiscent anthers of the olive cultivar 'Picual' was used. The samples were thoroughly grinded with a pistil and liquid N_2 followed by the extraction of the total RNA as RNeasy Plant Mini Kit (Qiagen) manual instructions recommends. RNA integrity was checked by formaldehyde gel analysis [17]. The mRNAs were purified using the Oligotex mRNA mini kit (Qiagen). The concentration and quality of the mRNAs were determined by the Ribogreen method (Quant-it RiboGreen RNA Reagent and kit) and

the Agilent RNA 6000 Pico assay chip (Bioanalizer 2100). The isolated mRNAs were subjected to 454/Roche Titanium+ sequencing.

2.2 Pre-processing

All reads obtained were pre-processed using the SeqTrimNext pipeline, in order to remove unsuitable reads, such as low quality, low complexity, linkers, adapters, vector fragments, polyA/polyT tails and contaminated sequences [18].

2.3 Assembling

The assembling strategy relies on the combination of two completely different algorithms to compensate assembling biases. After several assembling attempts, the best assembler combination was MIRA3 (based on overlap-layout-consensus algorithm) with Euler-SR (based on de Bruijn graphs) and a final reconciliation with CAP3.

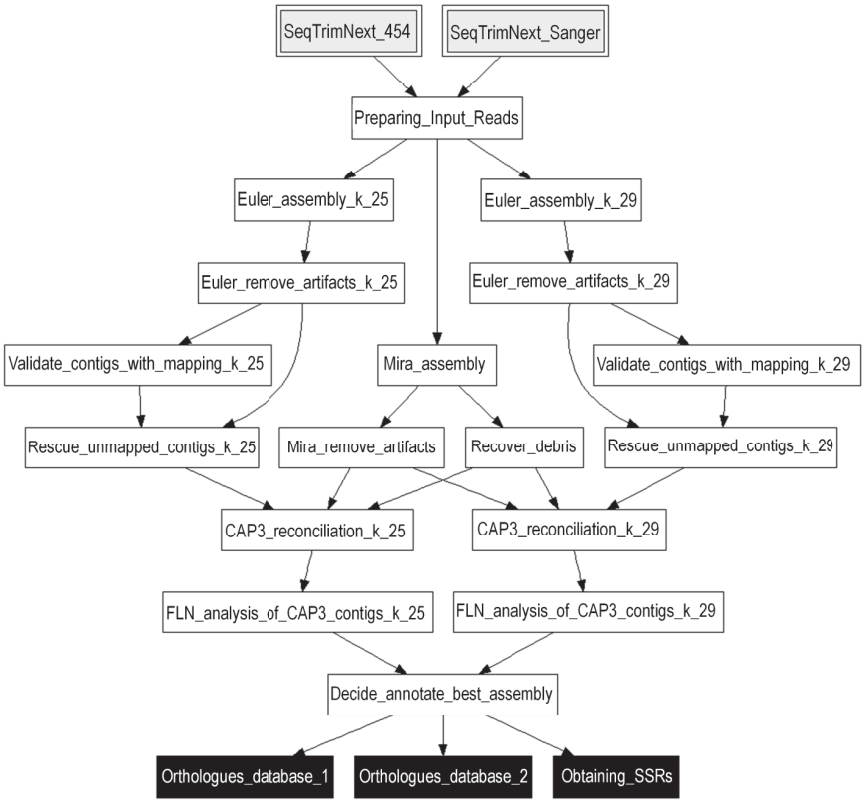

Fig. 1. Pipeline for the assembling of transcripts

2.4 Functional Annotation

A preliminary unigene analysis was performed using Full-LengtherNext. It provided a gene description, full-length unigenes, putative start and stop codons, the putative protein sequence, putative ncRNAs, an unknown unigenes. This gives a quick preview of the olive pollen transcriptome content. Full annotation was carried out using Sma3 to provide another gene description.

2.5 Selection of Cu,Zn-SOD Transcripts

Different strategies were defined to select such transcripts. First, EC codes of *A. thaliana* orthologous to olive sequences were extracted from annotations and subjected to analysis into the *Arabidopsis* reactome database of plant biological pathways. Finally, annotations were manually screened for specific enzymes selected from well-established bibliography resources.

2.6 Cloning of full Cu,Zn-SODs Sequences on the Basis of the Retrieved Sequences

Genomic DNA was obtained from the olive leaves of 7 cultivars and the extraction was carried out with the REDExtract-N-Amp Plant PCR (Sigma). Total RNAs corresponding to olive pollen from 17 olive cultivars were obtained using the RNeasy Plant Mini kit (Qiagen) and subjected to reverse transcription using the SuperScript II reverse transcriptase kit (Invitrogen). The gDNAs and cDNAs obtained were amplified with the following primers:

5´ ATG GTG AAG GCC GTA ACA GTC 3´ (forward) and
5´ TCA ACC CTG AAG GCC AAT G 3´ (reverse).

The PCR products were analyzed electrophoretically, and the amplified bands were excised, and purified with the MBL-Agarose Quikclean kit (Dominion). The purified PCR products were cloned in pGEM-Teasy vector system (Promega). *E. coli* DH5α were transformed with the vector followed by a selection of the colonies: the plasmidic DNAs were extracted as indicated in the Real mini-prep turbo kit (Real) manual instruction. After double-checking the correct insertion of the fragment, it was sequenced in the Sequencing Service of the Institute of Parasitology and Biomedicine "López-Neyra" (IPBLN-CSIC, Granada, Spain). At least 3 clones of each cultivar were sequenced.

2.7 Alignment and *in silico* Analysis of the Sequences

The alignment of all the nucleotidic sequences obtained was performed by using the Clustal W software (http://www.ebi.ac.uk/Tools/clustalw/). The WinGene 1.0 software (Henning 1999) was used to generate the translation to aminoacidic sequences, which were aligned using the Clustal W software as well. The ScanProsite software

(http://www.expasy.org/tools/scanprosite) was used to identify potential post-translational modifications and SOD consensus sequences.

2.8 Phylogenetic Tree

Phylogenetic trees were constructed with the aid of the software Seaview [19] using the maximum likelihood (PhyML) method implemented with the JTT model of the most probable amino acid substitution calculated by the ProtTest 2.4 server [20]. The branch support was estimated by bootstrap resampling with 100 replications.

2.9 3D-Modeling

The two most distinctive forms of Cu,Zn SODs were subjected to 3D reconstruction (http://swissmodel.expasy.org/workspace/)[21,22,23,24] by using the 2q21 template (annotated as a dimer) available as PDB by means of the DeepView v3.7 software.

2.10 Identification of B- and T-cell Epitopes

To determine linear B- and T-cell, as well as conformational B-cell epitopes we made use of a set of tools as described for other olive pollen allergens like Ole e 12 [25].

3 Results

3.1 Screening of the Olive Pollen Transcriptome for Cu,Zn SOD Sequences

A total of eight inputs corresponding to Cu,Zn SOD were obtained from the database generated (Table1). The sequences were named OePOlee5-1 to OePOlee5-8 (*Olea europaea* Pollen allergen Ole e 5-1 to 8).

Table 1. Selected sequences from the olive pollen transcriptome identified as Cu,Zn SOD. Lenghts from the sequences obtained after the assembly and from the codificant protein are indicated, as well as the missing aminoacids. Putative cellular localization is also shown.

Identifier	Nucleotides (NGS)	Aa (no UTRs)	Seq. description	Subcellular location
OePOlee5-1	677	128	Lacking N-terminal(24A)	Cytosol
OePOlee5-2	532	150	Lacking N-terminal (2aa)	Cytosol
OePOlee5-3	450	144	Deleted sequence (8 aa)	Cytosol
OePOlee5-4	621	152	Complete sequence	Cytosol
OePOlee5-5	587	192	Lacking C-terminal (36aa)	Chloroplast
OePOlee5-6	615	155	Complete sequence	Cytosol
OePOlee5-7	407	39	Lacking N-terminal (189aa)	Chloroplast
OePOlee5-8	758	152	Complete sequence	Cytosol

3.2 Alignment of the NGS-Retrieved Sequences of Cu,Zn SODs to the GenBank Database and Phylogenetic Analysis

BLAST query of the individual sequences against the GenBank database confirmed all of them as Cu,Zn SODs, either highly homologous to Cu,Zn SODs previously described in olive (Sequences OePOlee5-1,3,4,6, and 8), or in other tree species (sequences OePOlee5-2,5, and 7). Sequences alignment allowed the detection of microheterogeneities among the sequences, affecting several positions, however a high level of identity was the main characteristic observed (data not shown). The most distinctive feature observed was the presence of one relevant deletion (24 nucleotides) in one of the sequences (OePOlee5-3). The phylogenetic tree generated by including most relevant BLAST scores showed the presence of two differentiated clusters, putatively corresponding to those previously described as chloroplastidic and cytosolic proteins, respectively (Figure 2). However, not all the sequences used for the phylogeny were annotated as chloroplastidic or cytosolic by the describing authors.

3.3 Alignment of the Cloned Sequences from Different Olive Cultivars

The nucleotide sequences experimentally obtained from cDNA (51 sequences) and gDNA (21 sequences) were aligned separately (not shown). In both cases, most of the sequences were 456 nt long. Three exceptions were found where the length of the sequences were 432 nt in all cases, with a deletion of 24 nt positioned between the nt 252-276. These three exceptions corresponded to clones obtained from different olive cultivars and origins ('Arbequina' and 'Empeltre' from cDNA; 'Loaime' from gDNA).

All 72 aminoacidic sequences were aligned in order to identify postraslational modifications (glucosylations and phosphorylations), cysteines putatively involved in 3D structure, Cu-binding histidines, and consensus sequences for SODs (figure not shown). The deletion observed in three of the sequences was positioned between the aa 85-92. All experimentally obtained sequences of Cu,Zn SOD clustered coincidentally with sequences OePOlee5-1,3,4 and 8 (Figure 2).

3.4 3D-Modeling of the Complete and Deleted Forms of Cu,Zn SODs and Putative Involvement of Modifications in Allergenicity

Sequences OePOlee5-4 and OePOlee5-3 were used as representative forms of the complete and deleted forms of Cu,Zn SOD, respectively. The modeling approaches performed confirmed that the missing part of the protein in the deleted form (8 aa) matched to an external loop which does not form part of the active centre neither binds to the Cu,Zn atoms (Figure 3).

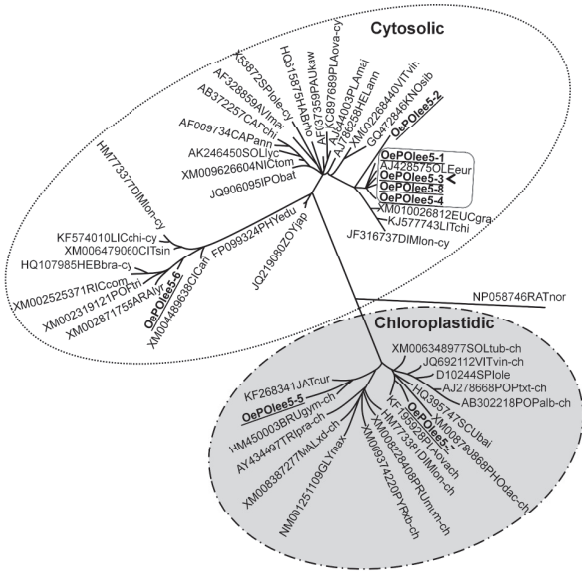

Fig. 2. Phylogenetic tree including olive Cu,Zn SODs sequences obtained either by NGS (underlined) or cloning strategies (which are situated inside the box -not fully listed-), and most relevant heterologous forms from other species (identified by their accession number, shortened name, and -ch or -cy if identified as a chloroplastidic or cytosolic form respectively). Two clusters, corresponding to chloroplastidic and cytosolic sequences are clearly defined. Deleted sequence OePOlee5-3 is pointed out with an arrowhead. The names of the experimentally obtained sequences are not fully displayed. These 72 sequences cluster with OePOlee5-1, 3, 4 and 8 within the rectangle inside the cytosolic cluster. A sequence from rat was included for rooting purposes.

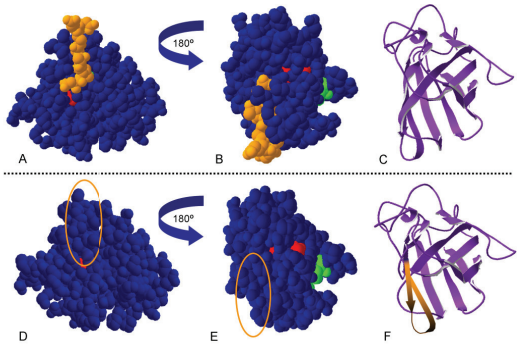

Fig. 3. 3D-modelling of the complete (A-C) and deleted forms (D-F) of the enzyme. The missing sequence is coloured in yellow.

The use of predictive software allowed us to identify the presence of T- and B- epitopes along the two representative forms of SOD mentioned above (OePOlee5-4 and -3). The short sequence (aa 85-92), which is absent from the deleted form, takes part of a defined T-epitope (aa 88-95), a predicted B-linear epitope (aa 87-102), and a conformational T-epitope in the complete sequence. Moreover, the 3-D restructuration occurring in the deleted form when compared to the complete form slightly alters the distribution of other T- and B- epitopes not directly involving the missing loop (results not shown).

4 Discussion

The transcriptomic approach used here greatly helped to define further strategies to identify the variability of the Ole e 5 (Cu,Zn SOD) pollen allergen both within and among olive cultivars, by allowing the identification of the most common variants, which included a deleted form of the enzyme. Such strategies comprised the design of accurate primers for PCR amplification. The presence among pollen transcripts of an even larger number of variants than those expected on the basis of transcriptome analysis was further confirmed by standard cloning procedures, which allowed an experimental validation of the predicted sequences generated by NGS assembly.

Nucleotide polymorphism in the olive pollen allergen Ole e 5 (Cu,Zn SOD) is however relatively lower than that of other olive pollen allergens like Ole e 1 [26] and the considered “highly conserved” allergen Ole e 2 [27] (profilin). Microheterogeneities observed in the sequence cannot be considered artefacts as the result of the miss-incorporation of nt by the polymerase as they have been detected in Cu,Zn SODs at much higher rate than the described for the enzyme. The observed substitutions do not affect key amino acids for the structure and/or function of the enzyme, like Cys involved in disulphide bridges, His residues involved in Cu-binding, of those motifs described as consensus for Cu,Zn SODs. Therefore, it is likely that the resulting gene products may represent active enzymes. The deleted forms of Cu,Zn SOD observed in both the sequences assembled after NGS as well as after experimental cloning developed in this work are likely not to be greatly affected in their functionality, as neither the reading frame nor the presence of key amino acids were disturbed. However, it is conceivable the presence of slight modifications either in the molecular weight, the isoelectric point of other properties of the protein. Moreover, subtle changes in the activity of the protein, including its kinetics could occur, and should be experimentally tested further.

As regard to the antigenicity/allergenicity of the deleted form of Cu,Zn SOD, predictive tools clearly report a modification of the overall ability of the protein to be recognized as a potential antigen/allergen. T-cell epitopes play a crucial role in immune responses for the induction of cytotoxic T-cell responses and in providing help to B cells for the development of antibody responses, whereas the identification of B-cell epitopes contributes to improve our understanding of structural aspects of

allergens and of the pivotal role they play in the induction of hypersensitivity reactions against small molecules and allergens [28]. The observed differences in the distribution of T- and B-epitopes among the complete and deleted sequences of Cu,Zn SODs may help to develop biotechnological approaches aimed to improve allergy vaccines. These approaches include the generation of hypoallergenic variants including isoforms and folding variants and the engineering of vaccines combining B- or T-cell epitopes. Most recent studies at this regard, resulting in an increased safety profile and reduced side-effects compared with allergen extracts have been recently reviewed [28].

5 Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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CHAPTER 5

Mature pollen from olive (*Olea europaea* L.) comprehends a wide panel of Superoxide Dismutase isozymes, including cytosolic, plastidial and a functional deleted form of Cu,Zn-SOD

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ROS (Reactive Oxygen Species) production in plants occurs during biotic and abiotic stresses, being also associated to molecular signalling. In plant reproductive biology, the presence of ROS has been described to be a key player involved in pollen tube growth, guidance through the pistil and has became a maker widely used to assess stigma receptivity. Due to the dual role of ROS in toxicity and signalling, their levels in cells are tightly controlled by a wide antioxidant system. Among them, the SOD family is an important actor catalyzing the disproportion of superoxide radicals. SODs are metallozymes containing Fe, Mn, or Cu,Zn as prosthetic group in plants. Besides their role as antioxidants, Cu,Zn-SODs in olive pollen are also allergenic proteins so-called Ole e 5. Aimed to deep into the knowledge of the different forms of SODs present in the olive pollen, and the analysis of the molecular variability of pollen Cu,Zn-SOD family, we used mature pollen grains from different olive cultivars and from other allergenic species to carry out biochemical, transcriptomic and localization studies. We observed that total SOD activity in olive pollen does not show correlation to pollen viability, however this intrinsic property could be related to the germinability of pollen. 1-D and 2-D SDS-PAGE and immunoblotting by using an specific antibody to Cu,Zn-SOD allowed us to identify new isoforms of Cu,Zn-SODs (among them chloroplastidic forms), as well as differentially expressed Mn, Fe and Cu,Zn-SODs isoforms among cultivars through the olive pollen. The later have also been identified in the pollen of a variety of allergenic species. Ultrastructural localization of Cu,Zn-SOD using the raised antibody confirmed the previously reported localization of the enzyme in the cytosol and the pollen exine and apertures. However, it is now reported its presence in amyloplasts and low-differentiated plastids present in the pollen grain.

In addition to the newly reported presences of a pollen Fe-SOD, and a chloroplastidic Cu,Zn-SOD, we also account the occurrence of a deleted form of the Cu,Zn-SOD, which has been expressed as a recombinant protein in *E. coli*, together with the complete form of the enzyme. As previously foreseen, the deleted enzyme shows a slightly differential SOD activity, when compared to the complete form of the enzyme.

Keywords: Amyloplast, Cu,Zn-SOD, deleted, Fe-SOD, isoforms, Mn-SOD, olive, pollen, superoxide dismutase

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Abbreviations: ROS, Reactive Oxygen Species; RNS, Reactive Nitrogen Species; H_2O_2, Hydrogen peroxide; $HO^{\cdot}$, Hydroxyl radical; $^1O_2$, Singlet oxygen; $O_2^{\cdot -}$, Superoxide radical; NO, Nitric Oxide; $ONOO^{-}$, Peroxinitrite; SOD, Superoxide Dismutase; Cu,Zn-SOD, Cupper Zinc Superoxide Dismutase; Mn-SOD, Manganese Superoxide Dismutase; Fe-SOD, Iron Superoxide Dismutase; KCN, Potassium Cyanide; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, Dithiothreitol; PVPP,Polyvinylpolypyrrolidone.

Introduction

Approximately 1% of O2 consumed by plants is diverted to produce ROS (Reactive Oxygen Species) at various subcellular loci (Bhattacharjee, 2005). ROS is a term including hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide radical ($O2^{-}$), and singlet oxygen ($_1O^2$). In plant kingdom, ROS production and ROS-induced damage occur during biotic and abiotic stresses, ROS however are associated to molecular signalling as well (Apel and Hirt, 2004; Mittler et al., 2004; Foyer and Noctor, 2005; Gechev et al., 2006). In higher plants, enhanced oxidation is also a signal for the appropriate adjustments of gene expression and cell structure in response to environmental and developmental cues (Shao et al., 2008). The different ROS molecules have very different properties: whereas H_2O_2 is relatively stable and its concentration in plant tissues is in the micromolar to low millimolar range, the other ROS have very short half-lives and are probably present at very low concentrations (Rinalducci et al., 2008). H_2O_2 plays different roles in plants: whereas at low concentrations it acts as a signal molecule involved in acclimatory signalling triggering tolerance to various abiotic and biotic stresses, at high concentrations it orchestrates programmed cell death. Besides, it acts also as a signal molecule during normal growth and development (Quan et al., 2008).

There is a tight relationship between ROS (H_2O_2 in particular), and plant reproductive biology. When pollen grain reaches the stigma, the pollen tube is generated and grows through the stigma and pistil tissues towards the ovary, where it delivers the sperm cells to the ovule. Pollen tubes growing have high energetic requirements and ROS appear as consequence of aerobic metabolism. Besides, pollen tube growth is guided through the pistil thanks to the interchange of signals between pollen and the stigma and pistil tissues and ROS seem to participate as signals in this process. ROS, mainly H_2O_2, are constitutively accumulated in Angiosperm stigmas and high levels of peroxidase activity have been detected when stigmas reach maturation and are receptive to pollen grains (McInnis et al., 2006a). ROS accumulation in stigma is related to NO production in pollen grains (McInnis et al., 2006b), which seems to negatively modulate H_2O_2 when pollen grains stick to stigmatic papillae (McInnis et al., 2006a; McInnis et al., 2006a; Zafra et al., 2010). Besides, a clear association has been established between ROS and the oscillatory cycles of the pollen tube growth in *Lillium* pollen tubes (Cárdenas et al., 2006).

Because of ROS toxicity as well as their important signalling role, the levels of ROS in cells are tightly controlled by a vast gene network termed the "ROS gene network" (Miller et al., 2008). ROS are produced during metabolic processes or stress conditions and are reduced by a wide antioxidant system. Antioxidants can be enzymatic or non-enzymatic. The non-enzymatic system includes ascorbic acid, tocopherol, glutathione, flavonoids, alkaloids, and carotenes (Pitzschke et al., 2006). The enzymatic antioxidant system is composed by superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Blokhina et al., 2003) among others. The SOD family catalyzes the disproportion of superoxide ($O_2^{\cdot-}$) radicals in biological systems to form H_2O_2 (McCord and Fridovich, 1969), playing an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments. SODs are metallozymes containing Fe, Mn, or Cu,Zn as prosthetic group (in plants).

The number and type of SOD isozymes can change depending on the plant species, age of development and environmental conditions (Bridges and Salin, 1981; Bowler et al., 1994; Kliebenstein et al., 1998; Alscher et al., 2002). SOD isoform distribution also depends on cell type (Corpas et al., 2006). Each SOD subtype has different molecular characteristics.

Fe-SODs probably constitute the most ancient SOD group (Alscher et al., 2002), they are distributed in all plant kingdom (Salin and Bridges, 1981).They are inactivated by H_2O_2 and resistant to KCN inhibition. Two different groups of Fe-SOD have been described. The first one is a homodimer formed by two identical 20 kDa subunits, with 1-2 Fe atoms in the active centre. The second Fe-SOD group, found in most higher plants, is a tetramer with a molecular weight of 80-90 kDa with 2-4 Fe atoms in the active centre (Alscher et al., 2002). Fe-SODs have been found mainly in chloroplasts (Salin, 1988; Asada, 1994) but also in peroxisomes (Droillard and Paulin, 1990), and the cytosol (Kanematsu et al., 2013).

Mn-SODs are functional homodimers and homotetramers formed by 23 kDa subunits (Bannister et al., 1987). Mn-SODs are inhibited neither by cyanide nor by H_2O_2 and are present in mitochondria and peroxisomes (Baum and Scandalios, 1981; del Rio et al., 1983; Palma et al., 1986; Sandalio and del Rio, 1987; Bowler et al., 1994; Corpas et al., 1998; del Rio et al., 2003). Mn-SOD gene is expressed in all cell types, being the unique SOD present in vascular tissues (Corpas et al., 2006).

Cu,Zn-SODs, also called SOD A, are very stable and seem to be present in practically all the eukaryotic cells (plants or animals) (Halliwell and Guteridge, 1985). Cu,Zn-SODs display a dimeric form formed by two identical sub-unities, including a Cu atom and a Zn atom in each one. Copper takes part in the dismutation reaction, whereas Zn is a key player to stabilize the enzyme, to keep the correct catalytic activity, and to catalyze folding of SOD in physiological buffer (Haliawell and Gutreridge, 1985; Nedd et al., 2014; Szpryngiel et al., 2015). The molecular weight of the dimeric form is approximately 32

kDa. It is reversibly inhibited by cyanide and H_2O_2 concentrations higher than 10 μM (Fridovich, 1978). Cu,Zn-SODs resist to temperature variations and denaturation by substances like guanidine chloride, dodecyl sodium sulphate, or urea (Halliwell and Guteridge, 1985). Cu,Zn-SODs have been located in cytosol, chloroplasts, peroxisomes and the apoplast (Sandalio and del Rio, 1987; Sandalio and del Rio, 1988; Salin, 1988; Kanematsu and Asada, 1991; Ogawa et al., 1996; Ogawa et al., 1997; Sandalio et al., 1997; Corpas et al., 1998; del Rio et al., 2002; Zafra et al., 2012). There are some differences between cytosolic and chloroplastic Cu,Zn-SODs, these two subgroups diverged early in plant evolution. Whereas chloroplastic Cu,Zn-SODs are 153 amino acids long and the terminal amino acid can be leucine, isoleucine or valine, the cytosolic SODs are 152 amino acids long, being the last amino acid always a glycine. The rest of the sequence has a high degree of homology (Fink and Scandalios, 2002).

The presence of different SOD isoforms in olive is dependent on the cell type. Fe-SOD, followed by Mn-SOD is the most abundant isoform in palisade mesophyll cells. However, in the phloem cells Fe-SOD was the predominant isoform, and Mn is the only isoform present in the xylem cells (Corpas et al., 2006). Alché et al., (1998) described the presence of four isoforms of Cu,Zn-SOD in olive pollen. Olive pollen extracts cross-reacted with a polyclonal antibody to cytosolic Cu,Zn-SOD, which was also used to report the ultrastructural localization of this enzyme in the cytoplasm of both the vegetative and the generative cells. More recently, Cu,Zn-SOD has also been associated to peroxisomes from mature olive pollen grains (Zafra et al., 2012).

Besides their role as antioxidants, Cu,Zn-SODs in olive pollen are also allergenic proteins so-called Ole e 5. Olive tree (*Olea europaea* L.) is a widespread crop in the Mediterranean area. Its pollination is mainly anemophylous and olive trees produce high quantities of pollen. The olive pollen is one of the first causes of respiratory allergy in Mediterranean areas (Liccardi et al., 1996). The incidence of Ole e 5 in type I allergenic reactions is 35% (Boluda et al., 1998). Ole e 5 was first purified, characterized and partially sequenced by Boluda et al. (1998). It is a 16 kDa protein (pI 5.1-6.5) with a high homology to Cu,Zn-SOD from spinach (Sakamoto et al., 1990) and maize (Cannon and Scandalios, 1989). Several studies have shown that allergenic proteins have a high degree of polymorphism, as a consequence of modifications like glycosylation, generation of dimers and formation of glycolic components (Villalba et al., 1993; Villalba et al., 1994; Lombardero et al., 1994; Rodriguez et al., 2002). Moreover, olive allergens have also a high degree of polymorphism in their chain sequence: Ole e 1 and Ole e 2 have been demonstrated to display numerous heterogeneities (Asturias et al., 1997; Martinez et al., 2002; (Hamman-Khalifa et al., 2008; Castro et al., 2010; Jimenez-Lopez et al., 2012; Jimenez-Lopez et al., 2013) as well as Ole e 7 (Tejera et al., 1999). Ole e 5 sequences obtained from several authors are quite conserved and share a high degree of identity with other plant species (Butteroni et al., 2005; Boluda et al., 1998; Tejera et al., 1999; Alché et al., 1998; Corpas et al., 2006). However, the generation of an olive pollen transcriptome allowed Zafra et al., (2015) to identify eight Ole e 5 sequences (Cu,Zn-SODs), one of them including a 24 nt deletion. Further *in silico* analysis permitted discriminate differential features in these molecules on a predictive basis.

Here we deep into the knowledge of the different forms of SODs present in the olive pollen, revealing the presence of new SOD forms. We also analyse the molecular variability of pollen Cu,Zn-SOD family in several olive tree cultivars and discuss the possible implications of this variability in relation to reproductive biology and as allergenic protein.

Materials and Methods

Plant material:

Mature pollen was individually collected from 16 different cultivars of *Olea europaea* L. belonging to different Spanish olive germplasm banks: Olive World Germplasm Bank, CIFA Alameda del Obispo (Córdoba), CIFA Venta del Llano (Jaén), Olive Oil Museum (Baeza, Jaén), Estación Experimental La Mayora (CSIC) (Málaga) and Estación Experimental del Zaidín (CSIC) (Granada). Pollen samples were collected in paper bags by vigorously shaking the olive inflorescences, and then sieved through 150 and 50 μm mesh nylon filters to remove debris and stored at -80°C.

Mature pollen from different allergenic species, including *Parietaria judaica, Salsola kali, Phleum pratense, Artemisia vulgaris, Platanus hybrida, Chenopodium album, Plantago lanceolata, Festuca pratensis,* and *Dactylis glomerata* was generously provided by Inmunal SAU (Madrid, Spain).

Protein extraction:

Proteins extracts from olive pollen of the 16 varieties were obtained by stirring 0.1 g of material in 1.5 ml of extracting buffer [50 mM phosphate buffer (pH 7.8) and 1 mM PMSF] for 4 h at 4°C. Samples were then centrifuged at 12000 x g for 20 min at 4°C, and supernatants were filtered through a 0.22 μm filter (MillexGP, Millipore) and stored at − 20°C until use.

Proteins from pollen of the 9 allergenic plant species were obtained by stirring 0.1 g of material in 2.5 ml of extracting buffer (40 mM Tris-HCl (pH 7.0), 2% Triton X-100, 60 mM DTT and 10 µl of protease inhibitor cocktail for 2 h at 4°C. The extracts were treated with a PD-10 Desalting Column (Ge Healtcare Biosciences AB) and finally precipitated by using 20% (w/v) TCA in chilled acetone at -20ºC overnight. The samples were centrifuged at 10000 x g during 30 min at 4°C. The supernatants were discarded and the pellet was dried at RT under a fume hood for 5-10 min until total removal of the acetone. Finally the samples were resuspended in 40mM Tris-HCl (pH 8.8).

Proteins for 2-D were extracted as indicated by Zienkiewicz et al. (2014). Briefly, olive pollen grains were stirred in extraction buffer [0.05 M Tris – HCl, pH 7.4, 1 % (v/v) Triton X-100, 4 % (w/v) CHAPS, 40 mM DTT, 5 % (w/v) PVPP, and 10 μL/mL plant protease inhibitor cocktail] for 4h at 4°C. After that samples were centrifuged at 20000 x g for 30 min at 4°C. The aqueous supernatant was recovered and filtrated trough PD-10 Desalting Columns. The elution obtained was precipitated with 20% TCA and 0.2% DTT in chilled acetone overnight at -20°C followed by a centrifugation at 20000 x g for 30 min at 4°C. The pellet was washed with 0.2% DTT in chilled acetone for 3 times and finally was let to partially dry. Semidry pellet was resuspended in an appropriate volume of 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 40 mM DTT, 0.5 % (v/v) carrier ampholytes pH 3 – 10, and 0.002 % (w/v) bromophenol blue.

Protein quantification

Extracts from olive pollen were quantified by Bradford method (Bradford, 1976), and extracts from allergenic species and 2D extracts using the 2D Quant Kit (Amersham Biosciences) according to the manufacturer's instructions.

SOD activity

Total SOD activity of the 16 pollen extracts was assayed according to the ferricytochrome c spectrophotometric method of McCord and Fridovich (1969).

In addition, SOD isozymes from the different extracts were separated by native PAGE on 15% acrylamide gels performed in a Hoefer Electrophoresis Unit SE 600 series (Amersham Pharmacia Biotech) and their activity was visualized by the photochemical method of Beauchamp and Fridovich (1971). Aproximatelly 375 µg of total protein was loaded per lane, aimed to detect very low abundant isoforms. Parallel native PAGE gels of the different extracts were also incubated in the reaction buffer containing 5mM H_2O_2 or 15mM KCN during 20 minutes at dark and room temperature, and afterwards SOD activity was detected as described by Beauchamp and Fridovich (1971).

MALDI-TOF

Once identified the bands with SOD activity as described above, bands were excised and subjected to MALDI-TOF analysis in the Proteomic Facilities of the Institute of Parasitology and Biomedicine "López-Neyra" (Granada, Spain).

Pollen viability:

Pollen grains were stained for cell viability using fluorescein diacetate (FDA, Sigma); following the viability test designed by Heslop-Harrison and Heslop-Harrison (1970), which labels viable pollen with green fluorescence. Observations were carried out in a Zeiss Axioplan fluorescence microscope equipped with a CCD camera.

Recombinant proteins and antibody generation

Two olive pollen sequences (GenBank EU20770.1 and EU250769.1), representative for the complete and deleted Cu,Zn-SODs, respectively were used to generate the recombinant proteins. The sequences were obtained as described in Zafra et al., (2015). They were prepared as recombinant proteins with a His-tag fusion in its N-terminus, expressed in *E. coli* and purified by Rekom Biotech S.L. Protein fingerprints by mass spectrometry was performed with MALDI TOF/TOF model UltrafleXtreme (Bruker), and was carried out in the Institute of Parasitology and Biomedicine "López-Neyra" (CSIC), Granada, Spain. The protein were spectrophotometrically quantified at 280 nm, and the protein concentration was performed with the theoretical extinction coefficient of the recombinant protein obtained from Gill and Hippel (1989).

The antibody against the olive Cu,Zn-SOD was generated in rabbits after immunisation with the whole recombinant protein (deleted isoform) by Davids Biotechnologie GmbH (Germany).

SDS-PAGE and Immunoblotting

Proteins were separated by sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE). Gels were dyed with Coomassie Blue/Flamingo staining or transferred onto polyvinylidenedifluoride (PVDF) membranes. Membranes were blocked with 10% defatted milk during 1h at RT and incubated with the primary antibody (diluted 1/5000) during 12 h at 4°C and inmunodetection was performed using a secondary antibody (Anti-Rabbit IgG Alexa Fluor 532, Molecular Probes) diluted 1/10000 during 1h at RT. Bands were visualized in a Pharos FX system (Bio-Rad) and analysed with the Quantity One Software v.4.6.2 (Bio-Rad).

2-D gel electrophoresis

Rehydration of 11 cm IPG strips (pH 3-10 NL, BioRad) was performed overnight at RT including the sample (200µg of total protein). The IEF was carried out at 20°C in a Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) as follows: 300 V for 1 min, 1000 V for 10 min, 8000 V for 90 min, and finally a total of 30 kVh. Reduction and alkylation steps were performed as described by (Castro 2010). The second dimension was performed in a CriterionTM Cell (BioRad). Immunoblotting was performed as described above.

Preparation of samples for TEM immunocytochemistry

Mature pollen grains from cv. 'Picual' were fixed for 24 h at 4°C with a mix of 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Samples were then dehydrated throughout an ethanol series and embedded in Unicryl resin (BBInternational, UK). After ultraviolet light polymerization of samples at -20°C for 48h, thin sections (1 μm), and ultra-thin sections (70 nm) were obtained using a Reichert-Jung Ultracut E microtome (Leica Microsystems, Germany). Thin sections were stained with methylene blue/azur B. Ultra-thin sections were mounted on formwar coated 200 mesh nickel grids.

For immunolocalization, the samples were blocked with BSA 1% prepared in PBS for 1h at RT, followed by incubation with the anti Cu,Zn-SOD antibody generated (diluted 1/200 in PBS plus 1% BSA). The incubation was carried out overnight at 4°C in a wet chamber. The samples were washed with PBS (3 x 5min) and incubated with the secondary antibody labelled with 30 nm gold particles (BBInternational) (diluted 1/50 in PBS plus 2% BSA). The samples were washed with miliQ water (3 x 5 min) and contrasted with 5% uranyl acetate for 20 min. Finally, grids were washed with miliQ water (3 x 5 min) and let to dry over Whatman paper.

Results

In vitro SOD activity in olive pollen from different cultivars

The different pollen extracts display very different total SOD activities. Due to the fact that the pollen viability may change depending on the cultivar and environmental conditions, we tested the viability for each cultivar. No relationship was found when comparing total SOD activity and the viability of the same pollen samples [Pearson r = 0,1942 (Cohen, 1988; Evans, 1996)] (Figure 1). The SOD total activity ranged from 3.9 ('Blanqueta') to 23.7 U/mg ('Bella de España') of total protein.

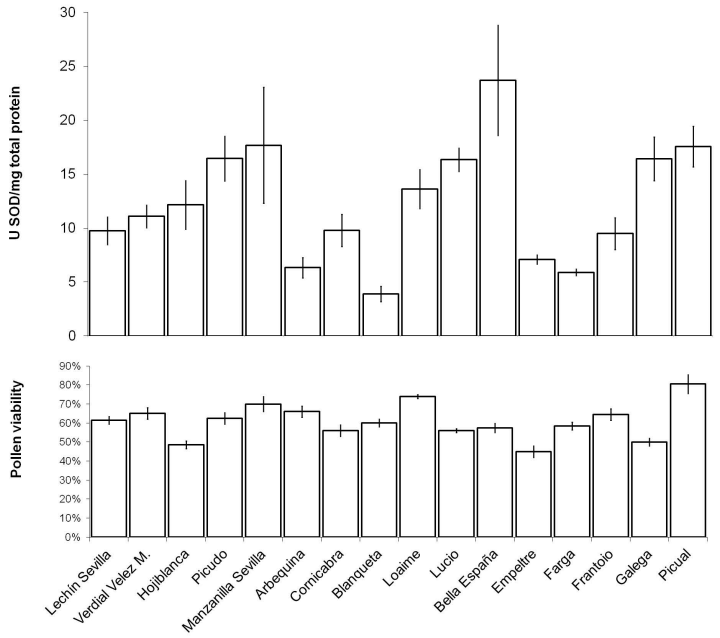

Figure 1: Upper panel: Total SOD activity (U/mg of total protein) of olive pollen extracts from 16 different cultivars. **Lower panel**: Percentage of pollen viability of the same samples. Cultivars used are labelled at the lower panel.

In order to discriminate between different SOD isoforms on native polyacrilamide gels, 15 cm large gels were used. In the same way, a relatively large amount of total protein (375 µg) was loaded per lane, and only freshly prepared extracts were used. Distinctive SOD band profiles were revealed for each cultivar (Figure 2). The use of specific inhibitors (CN^- and H_2O_2), allowed us to identify a variable number of Fe, Mn, and Cu,Zn-SODs in each cultivar. We were able to corroborate de presence of the three forms of SOD in olive pollen extracts for most of the cultivars. However, the cultivar 'Empeltre' was the only exception, with no Fe-SOD activity detected. A common pattern including seven bands was present in most of the cultivars. The upper band (1) corresponded to a Mn-SOD, which was present in 15 of the 16 cultivars analysed, and was only absent in the cultivar 'Bella de España'. The second and third bands corresponded to a Cu,Zn and Mn-SOD, respectively and they were present in all cultivars. The band number 4 appeared in the 'Picudo' and 'Picual' cultivars only. The inhibition assays showed that this band corresponded to a Cu,Zn-SOD. The band number 5 was identified as a Fe-SOD for all cultivars but 'Empeltre' (where this fifth band was replaced by a Cu,Zn-SOD instead). The bands positioned in the sixth and seventh place corresponded also to Cu,Zn-SODs, which were differentially expressed over the cultivars. Thus, band number 6 was displayed in 4 of the cultivars: 'Verdial de Vélez Málaga', 'Hojiblanca', 'Farga', and 'Frantoio'. The last band (seventh) was present in 14 of the 16 cultivars assayed, with only 'Empeltre' and 'Galega' lacking of this band.

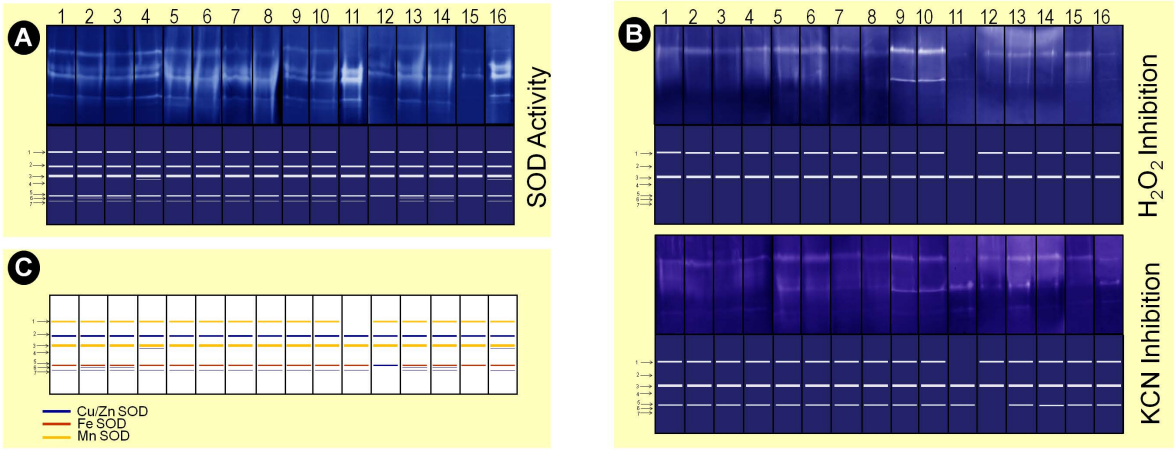

Figure 2: **Total SOD activity and SOD inhibitions assays tested over native extracts from olive pollen from different cultivars. Panel A:** Total SOD activity and the corresponding zymogram. **Panel B:** Activity gels (upper) and zymogram after H_2O_2 inhibition. Activity gels (lower) and zymogram after KCN inhibition. **Panel C:** Zymogram corresponding to the different bands after their assignment to Cu,Zn, Mn, and Fe-SODs on the basis of their inhibition profiles. 1: 'Lechín de Sevilla', 2: 'Verdial de Vélez Málaga', 3: 'Hojiblanca', 4: 'Picudo', 5: 'Manzanilla de Sevilla', 6: 'Arbequina', 7: 'Cornicabra', 8: 'Blanqueta', 9: 'Loaime', 10: 'Lucio', 11: 'Bella de España', 12: 'Empeltre', 13: 'Farga', 14: 'Frantoio', 15: 'Galega', 16: 'Picual'.

The presence of Mn-SOD and Cu,Zn-SOD was corroborated when sequencing a protein from excised bands on a SOD activity assayed gel. The results form MALDI-TOF revealed that band 3 disclosed 3 positives matches to Mn-SOD, and band 7 corresponded to Cu,Zn-SOD (4 positive matches). Band 1 and 2 did not showed positive matches with SOD sequences.

Cu,Zn-SOD variability in olive pollen cultivars

Coomassie-stained SDS-PAGE gels (Figure 3) show protein profiles of mature pollen from 16 olive cultivars representing a selection of the germplasm of this species. Sharp differences in the protein profiles are visible, mainly concerning the 18-20 kDa bands, which correspond to the major olive pollen allergen. The immunoblot probed with the anti Cu,Zn-SOD revealed up to 5 different bands in most of the extracts, with Mws of approximately 75, 50, 37, 25 and 15 kDa. The intensity of the bands clearly varied between the different cultivars.

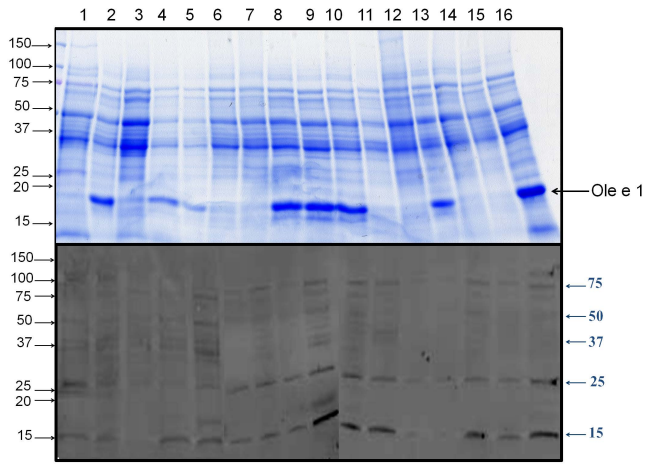

Figure 3: Olive pollen extracts from different cultivars. Upper panel. Coomassie blue stained gel. **Lower panel**: Immunobloting tested with the antibody to Cu,Zn-SOD. 30 µg of total protein was loaded per lane. 1: 'Lechín de Sevilla'; 2: 'Verdial de Vélez Málaga'; 3: 'Hojiblanca'; 4: 'Picudo'; 5: 'Manzanilla de Sevilla'; 6: 'Arbequina'; 7: 'Cornicabra'; 8: 'Blanqueta'; 9: 'Loaime'; 10: 'Lucio'; 11: 'Bella de España'; 12: 'Empeltre'; 13: 'Farga'; 14: 'Frantoio'; 15: 'Galega; 16: 'Picual'.

Similarly to 1D, the 2-D gels of whole protein extracts from two olive cultivars ('Picual' and 'Arbequina') displayed similar distribution of spots, with one significant difference: the substantial presence of Ole e 1 spots at around 18-20 kDa (Figure 4). The pollen extracts from 'Picual' cultivar exhibited at least 7 well-represented spots of Ole e 1 that are lacking in 'Arbequina'. After probing the corresponding immunoblot with the Cu,Zn-SOD antibody, different spots with molecular weights of approximately 15, 22, 32, 40 and 60 KDa showed reactivity to the antibody. Overlapping of the gel-immunoblotting pictures allowed identifying the localization of the spots assigned to Cu,Zn-SODs in the 2-D gels of olive pollen extracts after Flamingo staining.

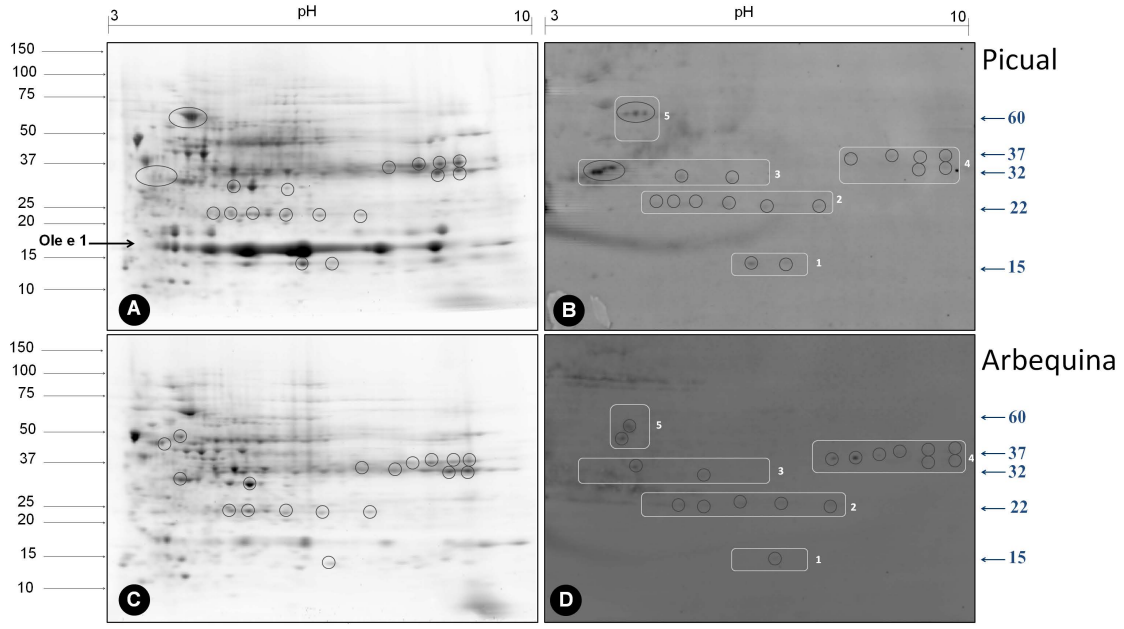

Figure 4: Two-dimensional separation of the proteins of olive pollen extracts from two cultivars. 200 µg of total protein was loaded on the IEF strips. **A & B**: Flamingo stained gel and immunoblotting membrane tested with the generated Cu,Zn-SOD antibody, respectively, corresponding to mature pollen extracts from the cultivar 'Picual'. **C & D:** Flamingo stained gel and immunoblotting membrane tested with the generated Cu,Zn-SOD antibody, respectively, corresponding to mature pollen extracts from the cultivar 'Arbequina'. Grey circles in A & C show the spots matching to the immunoreactive spots from B & D. Five groups were determined taking into account similar molecular weights.

The distribution of the immunoreactive spots showed the presence of several isoforms with Ips focused along the whole membrane. Five different groups of spots were distinguished according to the similarity of their molecular weights. The distribution of spots in the groups is quite similar in both the 'Picual' and 'Arbequina' extracts. However, some different spots were noticed. Group 1 corresponded to peptides of approximately 15 kDa, and

showed 1 spot in cultivar 'Arbequina', and two spots in 'Picual'. The group 2 corresponded to approximately 22 kDa, and displayed 5 spots in 'Arbequina' and 6 in 'Picual'. The group 3 corresponded to approximately 32 kDa and 'Arbequina' had 2 spots whereas 'Picual' had 3 spots. The group 4 displayed several spots with slight molecular weight differences, ranging between 37-40 kDa approximately. The number of spots in 'Arbequina' was 8, and 6 in 'Picual'. The group 5 presented the higher molecular weights (approximately 60 kDa) in their spots. 'Arbequina' showed 2 spots for this group, whereas 'Picual' pictured 3 of these spots.

Cu,Zn-SOD variability in pollen from different allergenic species

The allergenic nature of Cu,Zn-SOD (Ole e 5 allergen) in the olive pollen drove us to assess the presence of Cu,Zn-SODs reactive to the generated antibody in the pollen grains of different allergenic species. For this purpose, a panel of 9 species were selected, which were tested using the same experimental procedure described for olive pollen.

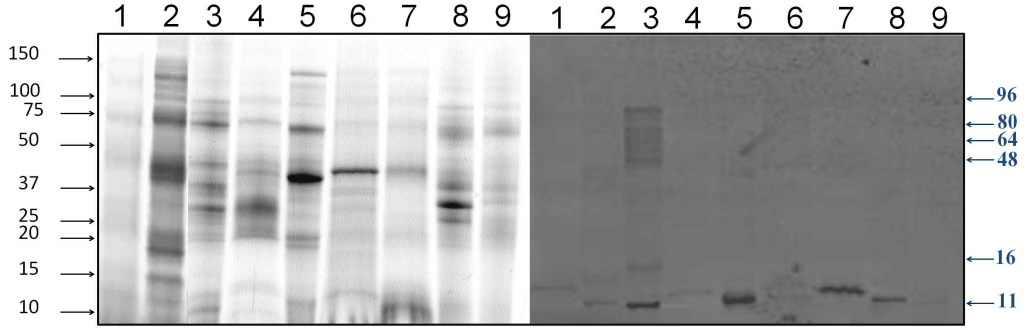

Figure 5: SDS-PAGE protein profiles of mature pollen from 9 allergenic species. **Left panel**: Commassie stained gel. **Right panel**: Immunoblot tested with the anti Cu,Zn-SOD antibody from olive pollen. 30 µg of total protein was loaded per lane. 1: *Parietaria judaica*; 2: *Salsola kali*; 3: *Phleum pratense*; 4: *Artemisia vulgaris*; 5: *Platanus hybrida*; 6: *Chenopodium album*; 7: *Plantago lanceolata*; 8: *Festuca pratensis*; 9: *Dactylis glomerata.*

SDS-PAGE protein profiles of allergenic species displayed very differential patterns of protein distribution. In several cases, major bands were identified, corresponding to well-described allergens through the specialised literature (Figure 5). After using the Cu,Zn-SOD antibody generated here, a number of cross-reactive bands were detected in the different species analysed.

The immunoreactive bands corresponded to different molecular weights for the different species. The range of sizes oscillated between 12-15 kDa in most of the cases with one single band per specie. Only *Phleum pratense* displayed reactivity to a related band of 16 KDa (low intensity signal). Moreover, the presence of 6 extra immunoreactive bands in the same species was detected, with molecular weights including 48 kDa, 64 KDa, 80 KDa, and 96 KDa. Such sizes may indicate the presence of multimeric forms ranging from trimers, tetramers, pentamers and hexamers, repectively. One additional band of lower molecular size (c.a. 11kDa) was observed in this species. The low molecular weight immunoreactive band had significativelly more intensity and was positioned in a similar range of molecular weight than in the other species. The *Chenopodium album* pollen extracts did not show reactivity to the antibody.

TEM Immunocytochemical localization of SOD in olive pollen and microspores

Immunocytochemistry was carried out on olive pollen samples at the mature (dehiscent) and early pollen grain stages, respectively (Figure 6). The mature pollen stage was characterized among other subcellular characteristics by the existence of a fully differentiated pollen exine and the presence of the vegetative cell, completely engulfing the generative cell. At this mature stage, gold labelling was detected mainly in the cytoplasm of the vegetative cell, the pollen exine, and associated to poorly differentiated organelles, which may correspond to plastids and peroxisomes.

Ultrastructural localization was also performed at the early pollen stage, identified in this case for the lateral position of the generative cell, which was not fully surrounded by the vegetative cell. At this younger stage labelling was also present in the cytoplasm of the vegetative cell. However, some of the labelled organelles were identified as amyloplast in spite of their low degree of differentiation, due to the presence of starch granules (Figure 6).

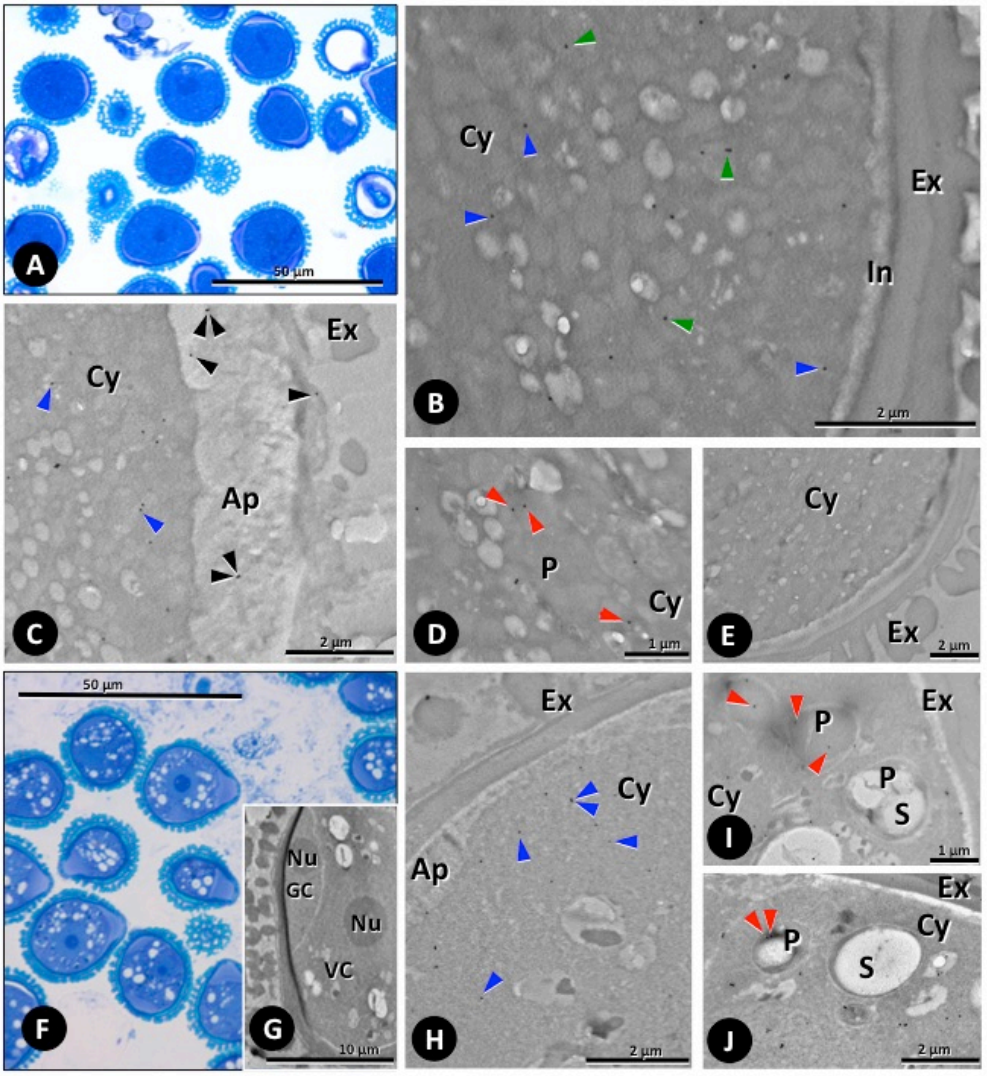

Figure 6: **A and F:** Stained thin sections of mature and young pollen grains, respectively observed at light microscopy. **B to E:** Transmission electron microscopy immunolocalization of Cu,Zn-SOD using the generated antibody. mature (dehiscent) olive pollen grains. **G to J:** TEM immunolocalization in young pollen grains within the anther, prior to dehiscence. Blue, black, red and green arrowheads point out the cytosolic, exine/aperture, plastidic and undetermined-organelle immunolocalization, respectively. **E:** negative control treated by omitting the primary antibody. Ap, aperture; Cy, cytoplasm; Ex, exine; GC, generative cell; P, plastid; S, starch; Nu, nucleolus; VC, vegetative cell.

In vitro expression of the complete and deleted forms of olive pollen Cu,Zn-SOD

Both proteins were expressed as His-tag fusions, displaying molecular weights determined by SDS-PAGE of approximately 22 and 20 kDa, respectively (Figure 7). Relative molecular mass and isoelectric points calculated from amino acid sequence were 21742,9 Da/6.36 for the complete protein (EU20770.1) and 20957,1 Da/6.65 for the deleted protein (EU250769). The purified fusion proteins ran at molecular weights slightly different to the predicted molecular weight, likely due to the presence of the His-tag. A lower-Mw band (c.a. 16 kDa) was also detected in the expressed deleted form of the enzyme. The immunoblots probed with the generated anti-Cu,Zn-SOD antibody showed that the antibody recognizes all three bands corresponding to the recombinant proteins. Thus, the 16 kDa band likely corresponds to a degradation or truncated product of the deleted form of the enzyme.

Both recombinant enzymes were active, as revealed in native acrylamide gels; nevertheless the specific activity was apparently lower in the deleted enzyme.

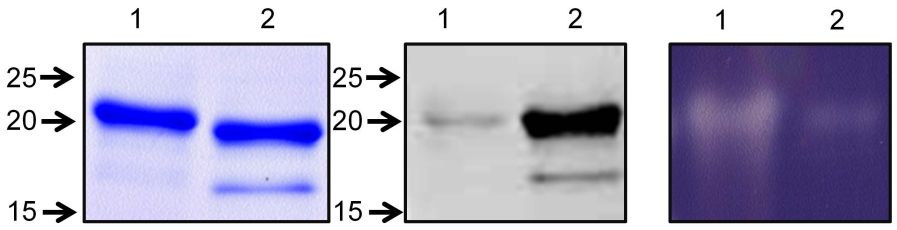

Figure 7: Left panel: SDS-PAGE of the complete and deleted protein stained with Coomassie blue. **Middle pannel:** Immunobloting of the complete and deleted protein tested with the generated antibody to Cu,Zn-SOD. **Right panel:** SOD in-gel activity. 10 µg of protein was loaded per lane. 1: Complete sequence (EU20770.1). 2: Deleted sequence (EU250769).

The protein fingerprints of both recombinant proteins obtained by mass spectrometry are displayed in Table 1 and 2. Matches correspond in both cases to Cu,Zn-SODs from different sources.

Table 1: Fingerprint report of the complete Cu,Zn-SOD protein (EU20770.1)

Uni Prot ID: **Q8L5E0.2**	Score: **83.40**	N.peptides: **9**	Name of the recombinant protein: **completeSOD**		
m/z meas.	**Δ m/z [ppm]**	**z**	**P**	**Range**	**Sequence**
2054,9637	-6,181	1	0	2-21	GSTSGSGHHHHHHSAGLVPR
1870,8137	-11,28	1	0	39-57	QHMDSPDLGTGGGSGIEGR
1886,8149	-7,869	1	0	39-57	QHMDSPDLGTGGGSGIEGR
1081,4452	-18,97	1	0	133-142	EHGAPGDENR
2280,1236	-10,08	1	0	143-165	HAGDLGNITVGEDGTAAINIVDK
1388,7845	-11,80	1	0	166-178	QIPLTGPHSIIGR
1379,6722	-13,87	1	0	179-191	AVVVHSDPDDLGR
2088,033	-6,656	1	1	179-198	AVVVHSDPDDLGRGGHELSK
719,3377	-7,561	1	0	199-206	STGNAGGR

Table 2: Fingerprint report of the **deleted** Cu,Zn-SOD protein (EU250769)

Uni Prot ID: **Q8L5E0.2**	Score: **69.00**	N.peptides: **8**	Name of the recombinant protein: **deletedSOD**			
m/z meas.	**Δ m/z [ppm]**	**z**	**P**	**Range**	**Sequence**	**Modification**
20549,637	-6,181	1	0	2-21	GSTSGSGHHHHHHSAGLVPR	
1870,08137	-11,28	1	0	39-57	QHMDSPDLGTGGGSGIEGR	
11886,8149	-7,869	1	0	39-57	QHMDSPDLGTGGGSGIEGR	Oxidation: 3
1081,4452	-18,97	1	0	133-142	EHGAPGDENR	
1388,7845	-11,80	1	0	158-170	QIPLTGPHSIIGR	
1379,6722	-13,87	1	0	171-183	AVVVHSDPDDLGR	
2088,033	-6,656	1	1	171-190	AVVVHSDPDDLGRGGHELSK	
719,3377	-7,561	1	0	191-198	STGNAGGR	

Discussion

In vitro SOD activity, pollen viability and *in vitro* germination

Although highly variable and depending on the cultivar assayed, olive pollen harbours a relatively high rate of SOD activity (c.a. 5-25 U/mg protein), when compared to other olive tissues as leaves, with rates of 1.7 U/mg protein (Corpas et al., 2006). This may be due to the high accumulation of this antioxidant enzyme in the mature pollen, which is ready to balance the extremely high metabolic rates of the pollen grain upon hydration and germination. No other additional data concerning the quantitation of SOD activity in pollens are available in the literature according to our knowledge, with the exception of the work of Moon (2006) who also reported apparently high levels of SOD activity in the pollen of *Petunia*. Reported activity ranged in this case between 2.1 to 4.8 U/flower. These results are not easily comparable with those reported here because of the use of very different units.

The absence of a correlation between the SOD activity and the viability of pollens from different cultivars may reflect different causes. First, the method used here to calculate pollen viability. Although broadly recognized as a useful way to measure both the integrity of membranes and the presence of a metabolically active environment, the fluorochromatic reaction of the fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970) is based in the presence of esterases, rather than SODs. Second, although SODs may accumulate prior to the mature stage, their function is likely needed in a higher degree a little bit later, over pollen hydration and through the germination and growth of the pollen tube (Morales, 2012). Previous studies carried out in tomato and maize, have shown a direct correlation between the increase in Cu,Zn-SOD and tolerance to intense dryness (Arora et al., 2002). It has been also reported that overexpression of SOD genes in transgenic plants increases tolerance to dryness and freezing in several species (Gupta et al., 1993; Mckersie et al., 1993; Mckersie et al., 1996). These findings suggest that the expression of SOD enzymes may confer tolerance to dehydration, thus revealing the importance of maintaining high levels of SODs in the mature pollen, which represents the stage with the higher rates of dehydration.

TEM immunolocalization experiments, which trace the enzyme in the olive pollen exine and apertures (Alché et al. 1998, this work), explain how this protein, in addition to its immunological effects may be released from the pollen grain, thus favouring its allergenic character (Morales, 2012).

Cu,Zn, Mn and Fe-SODs are differentially expressed through the olive pollen, depending on the olive cultivar

The presence of several Cu,Zn-SOD isoforms and a Mn-SOD in the olive pollen of the 'Picual' cultivar was already described by Alché et al. (1998) by using in gel assays of rod-slab gels. The increased sensitivity of thin layer gels allowed in the present paper to identify through inhibition assays an additional form of SOD corresponding to a Fe-SOD. The unexpected presence of this isoform has also been corroborated by means of transcriptomic approaches, after detailed annotation of an olive pollen transcriptome (Zafra et al., 2015), generated by 454/Roche Titanium+ sequencing (results not shown). This is the first time that a Fe-SOD has been clearly evidenced to be present in pollen grains according to our knowledge. Previous evidence of the presence of this isoform, consisting in in gel inhibition assays of activity, was reported in *Petunia* pollen grains (Moon, 2006). Fe-SODs have been typically related to chloroplasts, however they have been recently localized also in the cytoplasm (Kanematsu et al., 2013). Moreover, the presence of several isoforms of Cu,Zn-SODs was confirmed by means of high-resolution 2-D electrophoresis and further immunoblotting experiments as we will discuss later.

The differences observed through the olive germplasm include both presence/absence of bands, but also quantitative differences in the relative intensity of the bands. Such broad polymorphism seems to be involved in the physiology of the olive reproductive system (Alché et al., 2007), and may participate in key events for pollen functionality. As an example, ROS and in particular H_2O_2 participate as signals in pollen-pistil interactions (McInnis et al., 2006a,b). The presence of distinctive SOD isoforms in pollen from different olive cultivars may be related with self-incompatibility processes which are mediated through ROS signalling (Wilkins et al., 2011; Eaves et al., 2014; Serrano et al., 2015), with a preference for allogamous fertilization, already described for olive pollen (Mookerjee et al., 2005) and with the events driving to the polarization of pollen tube growth (Potocký et al., 2007; Hiscock and Allen, 2008). Some work has described a positive correlation between the presence of Cu,Zn-SOD in pollen and climate factors like minimal temperature, rainfall or relative humidity for several cultivars like 'Blanqueta' and 'Verdial de Vélez' (Morales, 2012).

Cu,Zn-SOD (Ole e 5 allergen in the olive pollen) is present in numerous species emitting allergenic pollen

Superoxide dismutases (Cu,Zn-SODs, Mn-SODs and Fe-SODs) have been reported to be allergenic proteins in many different sources including insects (cockroaches, *Drosophila*) fungi (*Alternaria, Aspergillus, Penicillium, Cochliobolus, Malassezia*, grasses (*Phleum pratense*), *Lycopersicon esculentum* and trees like *Hevea*, *Olea europaea* and *Pistacia* (www.allergome.org/index.php and literature therein). Here we have analysed the presence of Cu,Zn-SODs in a relatively high number of species documented by their ability to produce allergy-eliciting pollens. The conclusion reached is that most of them carried forms of this enzyme recognized by the antibody to the protein in olive.

This allergen is considered a minor allergen of the olive pollen, which in spite of its low prevalence is still responsible for symptoms, IgE and/or skin prick tests (SPT) reactivity in about 35% of the analysed allergenic population allergic to this pollen (Boluda et al., 1998). Cross-reactivity of the allergen among species has not been extensively analysed yet (Villalba et al., 2014). However, on the basis of the results exposed here, it is likely that Cu,Zn-SODs, and perhaps other SOD forms may take part of the complex allergograms of the species analysed here as well. On support of this hypothesis we could account the similar low-molecular weight of the cross-reacting bands, the high level of conservation of the enzyme in many different sources, the ability to be quickly and easily released from pollen grains, etc. This has to be further analysed by using specific approaches like SPTs, ELISA, immunoCAP or others.

High-resolution 2-D electrophoresis allows assignment of multiple forms of SOD

As discussed by Momcilovic et al., (2014), implemented methods to the traditional one of in-gel activity assay are needed to reveal the presence of the SODs isoform. In this study, the in-gel activity assay was complemented with 1-D and 2-D immunobloting tested with the generated antibody. The specific molecular weights of the bands/spots could correspond to monomeric/polymeric forms of both cytosolic and chloroplastidic Cu,Zn-SOD isoforms. Such enzymes are one of the most stable globular protein families studied. They maintain the native dimeric stucture under high reducing conditions, and high temperature. This stability is due to hydrophobic interfaces, the packing of the stranded β-barrel scaffold, the presence of an intra-chain disulphide bridge, and the stabilizing effect of metal cofactors (Tuteja et al., 2015). The calculated Isoelectric points of the cytosolic and chloroplastidic monomers vary between 5.1 to 6.5. However, our results indicate that the putative dimeric isoforms had a lower or higher Ip than the monomeric isoforms. We propose 5 differentiated groups of Cu,Zn-SOD isoforms, based on their molecular weights and Ip. The group 1 could correspond to monomeric cytosolic isoforms. The group 2 could localize the monomeric chloroplastidic isoforms. The group 3 could correspond to dimeric cytosolic isoforms. The group 4 could correspond to the dimeric chloroplastidic isoform. Finally, the group 5 could correspond to tetrameric isoforms of the cytosolic Cu,Zn-SOD. We postulate a change of the Ip if compared the monomeric/dimeric isoforms, being present in the acidic part the multimeric forms of the cytosolic Cu,Zn-SOD, and in the basic part, the dimeric form of the chloroplastidic Cu,Zn-SOD. It is frequent that antibodies directed to cytosolic Cu,Zn-SODs may also recognize the chloropastidic counterparts (Tanaka et al., 1996) due to the high homology between both sequences.

Some commercially available antibodies to cytosolic Cu,Zn-SODs have been also reported to cross-react in some degree with the chloroplastidic equivalent.

Immunocytochemical localization of Cu,Zn-SOD and transcriptomic approaches provide further evidence of the presence of chloroplastidic isoforms of Cu,Zn-SOD in olive pollen

Pioneer study on SODs carried out in mature olive pollen (Alché et al. 1998), although managed to demonstrate the presence of cytosolic Cu,Zn-SOD, failed to report the putative presence of chloroplastidic isoforms. In the present work, we have accumulated evidence as regard to the existence of such enzymes in the mature pollen, mainly based in their differential molecular weights after SDS-PAGE and immunoblotting.

Mature olive pollen organelles are often undifferentiated and in many cases, plastids, mitochondria, peroxisomes are undistinguishable unless specific preparation methods or the use of specific markers is applied. This is in most cases due to the absence of easily distinguishable structures like mitochondrial cristae or plastidial thylakoids or grana. Sometimes, the high abundance of dilated cisternae of endoplasmic reticulum may increase this plethora of organelles in this pollen (Rodríguez-García and Fernandez, 1990). Chloroplastidic Cu,Zn-SODs are described to be present in the thylakoid membranes (at the stromal face, close to the PSI) of the vegetative tissues (Ogawa et al., 1995). The presence of amyloplasts in mature pollen grains was described by Rodriguez García and Garcia (1978) who proposed the callose special wall of the tetrad as the possible source of the starch. More recent studies revealed that overproducing starch mutants of rice display accumulation of starch in pollen amyloplasts (Matsushima et al., 2014). The high demand of energy needed during pollen maturation and

pollen growth could be generated by pollen plastids (Selinski and Scheibe, 2014). These authors propose the role of the plastidial glycolisis in pollen, together with mitochondrial respiration, fermentation or cytosolic glycolysis among the ways to obtain energy for the metabolism during pollen maturation and tube growth. A weak detection of the core component of the chloroplast division machinery (the FtsZ) in pollen was performed by Fujiwara et al., (2012). The presence of this proteins which marks the constriction site of dividing chloroplasts was detected in pollen, via FtsZ1 – GFP, as diffuse signals, and occasionally as ring-like deposits, within plastids.

In the present work, additional localizations of Cu,Zn-SODs have been reported. In this case, it is notorious the presence of gold signals in structures of the mature pollen, which are compatible with plastids. Moreover, when the localization was performed in young pollen grains, signal was undoubtedly attributed to amyloplasts, easily distinguished by their starch content.

Moreover, the presence of specific chloroplastidic forms of Cu,Zn-SODs derives from the identification of sequences corresponding to such forms, obtained through the detailed annotation of an olive pollen transcriptome (Zafra et al., 2015). Two partial sequences of chloroplastidic Cu,Zn-SOD were obtained after NGS. The phylogenetic tree grouped these two sequences far from other cytosolic Cu,Zn-SOD, and within the same group of chloroplastidic Cu,Zn-SOD from several species. The contig obtained with the two partial sequences generated a complete sequence displaying 75% of identity to chloroplastidic Cu,Zn-SOD from *Sesamum indicum*. Surprisingly, the olive pollen transcriptome generated also displayed a total of 1015 output unigenes related to chloroplast, with enzymes that are clearly involved in the chloroplastidial enzymatic machinery as the RuBisCO (results not shown) in spite of the absence of well-structured chloroplasts in the mature pollen grain.

Transcriptome analysis allowed identifying a unique deleted Cu,Zn-SOD form, maintaining functionality

Among the Cu,Zn-SOD sequences identified in the olive pollen transcriptome, one of them included a relevant 24 nt deletion (Zafra et al., 2015). Further *in silico* structural analysis permitted the authors to predict that the resulting gene products may represent active enzymes. Thus, the deleted forms of Cu,Zn-SOD observed in both the sequences assembled after NGS as well as after experimental cloning developed by them were likely not to be greatly affected in their functionality, as neither the reading frame nor the presence of key amino acids were disturbed. The authors also indicated that the presence of slight modifications either in the molecular weight, the isoelectric point of other properties of the protein like subtle changes in the activity of the protein, including its kinetics could occur. Several of these properties have been tested in the present manuscript, and most of these predictions have been assessed. The heterologous expression of both the complete and deleted SODs resulted in both cases in a proper folding at least in a certain although low proportion of both proteins, as demonstrated by the detection of superoxide dismutase activity for both expressed forms. The antibody generated to the deleted form was able to react to both recombinants proteins and also to the natural forms of the enzyme, as stated all through the present work. In spite of this evidence, we detected that the stability of the enzyme was very low (not shown). No reproducible results were obtained when spectrophotometric assays of SOD activity were carried out. This can be due to the fact that both of the proteins appeared in the monomeric form and/or to the lack of adequate concentrations of the ions needed for proper folding. The Zn atom has been described to influence the Cu site directly by adjusting its reduction potential and geometry (Nedd et al., 2014). Characteristics like instability of the monomeric form and loss of activity have been widely described (Lin et al., 200; Rodriguez et al., 2002a; Hadji et al., 2007; Mishra et al., 2014). On the other hand, negative charge at physiological pH has also been linked to Cu,Zn-SOD stability (Calamai et al., 2003). Even Cu,Zn-SOD with high isoelectric point has been demonstrated to be stable when dimeric forms are formed. Therefore, the absence of disulphide bonds and probably the absence of metal atoms through folding provided highly un-stable Cu,Zn-SOD forms. Further improvements in the production of these proteins will help to test the differential properties of these enzyme forms.

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CHAPTER 6

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Peroxisomal localization of CuZn superoxide dismutase in the male reproductive tissues of the olive tree

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Superoxide dismutases (SODs) are a class of antioxidant enzymes which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, therefore controlling cellular levels of Reactive Oxygen Species (ROS). In the mature pollen grains of the olive tree, the presence of several forms of CuZn-SOD and the cytosolic localization of the enzyme have been described [1]. The present study was aimed to elucidate the adaptation of the oxidative metabolism to the changing conditions occurring during the course of olive pollen formation, hydration and pollen tube emergence and growth. We used a polyclonal antibody (raised against a KLH-linked synthetic peptide including a consensus sequence for CuZn-SODs in olive pollen) in immunocytochemical experiments carried out by Fluorescence (FM) and Transmission Electron Microcopy (TEM).

CuZn-SOD immunolocalization by FM revealed the presence of differences in the expression of the enzyme depending on the developmental stage and the analyzed reproductive tissue (Figure 1). In the anther tissues, the fluorescent signal became highly visible at the stage of tetrad, where most of the cells of the tetrad (particularly at their periphery) and the cells of the anther wall and the tapetum revealed green fluorescence. The fluorescent signal displayed an increase at the stages of "early microspore" and "mature pollen grain inside the anther", with an intense signal observed in the different anther wall layers, including the senescent tapetum. Both the microspores and the mature pollen grains showed fluorescence labeling localized in the cytosol and the microspore/pollen wall. A majority of the pollen grains presented a "spotty" labeling pattern, probably corresponding to peroxisomes. Although CuZn-SOD is considered as a characteristic matrix enzyme of peroxisomes in different tissues like oilseed cotyledons [2], the presence of the enzyme has not been previously associated to these organelles in olive pollen.

In order to dip into this possibility, we carried out TEM immunolocalization experiments by using the same antibody to olive CuZn-SOD (Figure 2). In addition to the labeling associated to the cytosol, the apertural region and the pollen wall (already described for olive pollen), intense labeling was detected in roundly-shaped structures with a slight electron-dense matrix, present in the cytoplasm of the vegetative cell. Confirmation of their nature as peroxisomes was accomplished by immunolocalization using an antibody reactive to catalase, a peroxisomal marker enzyme, prepared against a synthetic peptide designed using a consensus sequence of different plant catalases. Co-localization of both enzymes in these structures was also detected.

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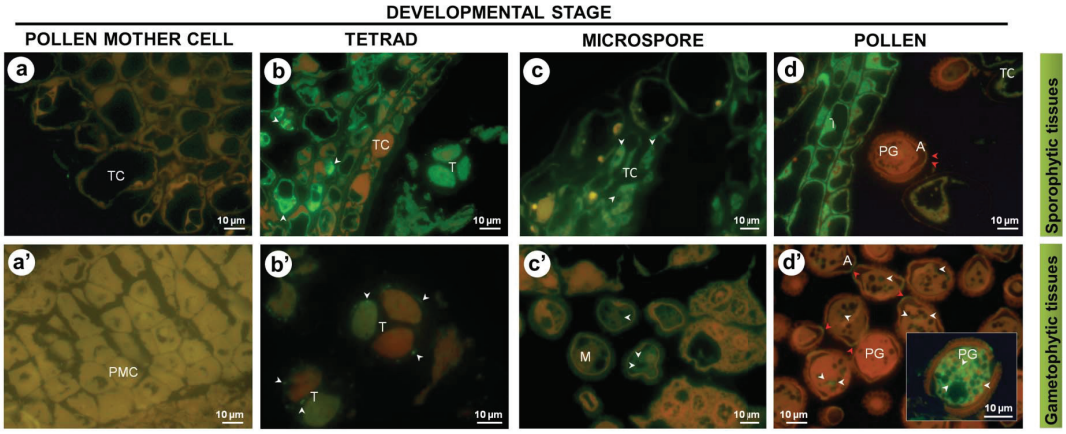

Figure 1. FM immunofluorescence localization of CuZn-SOD in the anther tissues throughout different developmental stages. Green fluorescence reveals the presence of the enzyme (Alexa fluor 488 secondary antibody). The red color is due to the autofluorescence. White arrowheads point to CuZn-SOD localization. Red arrowheads indicate signal in the apertural region of the mature pollen grains. Images were gathered with a digital camera attached to a Zeiss Axioplan Fluorescence Microscope using a standard FITC filter combination. A: aperture; M: microspore; PG: pollen grain; PMC: pollen mother cells; T: tetrad; TC: tapetal cells.

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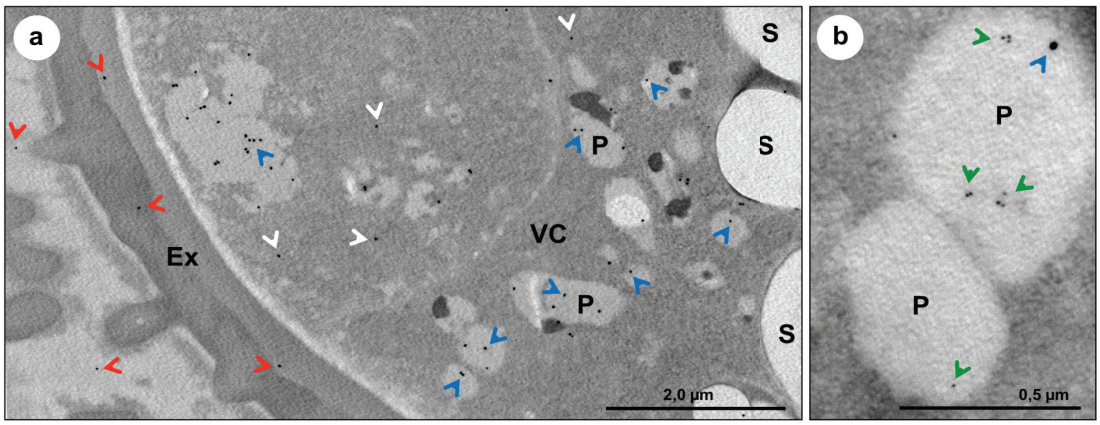

Figure 2. a: TEM immunolocalization of CuZn-SOD revealed its presence in the exine and the material adhered to this structure (red arrowheads). The enzyme is also localized in the cytosol (white arrowheads), and organelles of different shapes and sizes, frequently more or less rounded (blue arrowheads). b: co-localization of catalase (10 nm particles, yellow arrowheads) and CuZn-SOD (25 nm particles, blue arrowheads) in putative peroxisomes. Ex: exine; P: peroxisomes; S: starch; VC: vegetative cell cytoplasm.

OVERALL DISCUSSION

The importance of the study of Reactive oxygen species (ROS), Reactive Nitrogen Species (RNS), as well as other emerging reactive species such as Reactive Sulphur Species (RSS) in plant signalling processes is increasing in the last years. These molecules that were considered poisonous in the past, are now well known to be involved in a fine tune regulation of many processes. Plant reproductive processes are not aside from this tendency, and the present doctoral thesis has been focused on the study of derivates from the oxygen and nitrogen metabolism and their involvement in the sexual reproductive process, taking the olive tree as the model.

ROS/RNS metabolism in plant reproductive tissues includes numerous chemical forms, multiple interplaying pathways, and unique enzyme forms

The studies carried out in this work, and those described up to date in the literature, confirm both the presence of multiple chemical forms of ROS/RNS (including at least H_2O_2, superoxide, NO and GSNO), and numerous enzyme systems regulating their interplay in plant reproductive tissues. This has as the result the appearance of numerous and distinctive developmental patterns of ROS accumulation as described here. ROS/RNS metabolism in reproductive tissues, as it happens in other plant tissues, possesses many other actors:

The superoxide radical is mainly produced by NADPH oxidase (NOX) that transfers electrons from NADPH to an electron acceptor leading to the formation of superoxide radical. NADPH oxidases in plants are named Respiratory burst oxidase homolog (Rboh). They have been shown to play key roles in numerous physiological processes, such as root hair and pollen tube tip growth, stomatal closure, and various stress responses (Kärkönen and Kuchitsu, 2015). In *Arabidopsis thaliana*, the tissue-specific distribution falls into three basic classes; expression throughout the plant (AtrbohD and F), in the roots (Atrboh A–G, I), and in a pollen-specific manner (Atrboh H and J). The phylogenetic distribution of tissue-specific expression shows that H and J form a small subclade (Sagi and Fluhr, 2006). RbohH and RbohJ, play a critical role in pollen tube tip growth (Kärkönen and Kuchitsu, 2015). Recently, NADPH oxidase-dependent signalling pathway has been described to induce sperm release and enable fertilization in flowering plants (Duan et al., 2014). NADPH oxidase activity in pollen tubes is affected by calcium ions, signalling phospholipids and Rac/Rop GTPases (Potocký et al., 2012).

The generation process of the NO in plants has been controversial for many years. The discovery of the presence of a Nitric Oxide Synthase (NOS) in animals developed an increasing interest to find the homologous in plants. The majority of NO synthesized in plants is thought to be derived from two unrelated enzyme-based pathways, one involving two functionally redundant nitrate reductase (NIAs) and the other requiring the undefined action of NO-associated protein 1 (AtNOA-1) (Gibbs et al., 2014). Recently, a NOS gene from the unicellular marine alga *Ostreococcus tauri* (OtNOS) has been discovered and characterized (Foresi et al., 2015).

Glutathione, which is one of the major antioxidants in plant cells, representing a critical multifunctional metabolite in redox homeostasis, signalling and defence reactions. Moreover, this metabolite can react with nitric oxide (NO) to generate S-nitrosoglutathione (GSNO), which is considered a mobile reservoir of NO in plants. Glutathione, in the reduced and oxidized form together with the GSNO, have been pointed out as important metabolites in the reproduction process (Zafra et al. unpublished results). It was observed that GSH and GSSG decreased along *in vitro* pollen germination, meanwhile GSNO experimented a light increase in the initial steps of pollen germination to finally decrease after 14 hours of germination. Besides, two glutathione *S*-transferases have been associated to microsporogenesis in wheat (Sánchez-Díaz et al., 2013). Ascorbate metabolism, tightly associated to that of gluthatione is also enhanced in pollen grain of particular species (de Gara et al., 1993).

A recent study reveals that ROS play a crucial role during female gametogenesis and fertilization (Martin et al., 2013). Megagametogenesis is dependent on a mitochondrial Mn-superoxide dismutase (MSD1) in the following way: the mature embryo sacs show ROS in the central cell, which appears to be the main source of ROS before pollination. MSD1 expression is elevated in the egg apparatus at maturity but is down-regulated in the central cell. ROS burst depends on stigma pollination but precedes fertilization, suggesting that embryo sacs sense the imminent arrival of pollen tubes and respond by generating an oxidative environment. ROS and NO are produced by the ovule, and have been identified to play important roles during pollen tube elongation, repulsion, and death (Dresselhaus and Franklin-Tong, 2013), as well as in pollen tube burst during fertilization (Duan et al., 2014). NO and ROS were also proposed to participate in gamete fusion blockage (Reviwed by Domingos et al., 2015).

Other antioxidant enzymes described in plant reproductive tissues include catalases (Acevedo and Scandalios, 1990; Wang et al., 2010b) and peroxidases, in some cases specific

to these tissues and restricted to particular plants (McInnis et al., 2006; Sharma and Bhatla, 2013), and frequently associated to stigma receptivity (Dafni and Motte Maués, 1998).

Despite the overview disclosed above, our knowledge of these enzyme systems in the olive tree is still limited, and very contemporary. However, advances in all areas described here are currently underway in the Plant Reproductive Biology Group at the EEZ-CSIC, where the present work has taken place. This is a particular consequence of the recent advances carried out under transcriptomic approaches.

Transcriptomic approaches to the study of reproductive biology represent global, highly informative improvements

The search for new clues in the ROS metabolism in pollen/pistil very much relays on transcriptomic approaches over the last years. Finding the molecules involved in the ROS metabolism as well as allergenic proteins in the precise physiological stage during the pollen development, pollen-pistil interaction, and/or pollen hydratation and germination is a goal to understand the intimate details of Reproductive Biology. Increasing number or manuscripts are been published related to reproductive processes in many species, indicating that massive sequencing is a trendy as it can reveal many aspects of the sexual reproduction.

Transcriptomes have been prepared in some cases from whole flowers, including many reproductive variants and involving a wide panel of research topics. A recent study of naturally occurring asexual reproduction was performed in *Hypericum perforatum* L. by *de novo* sequencing and generation of the flower transcriptome (Galla et al., 2015). The obtained results allowed annotating a large number of transcripts related to meiosis, gametophyte/gamete formation, and embryogenesis, as well as genes that are exclusively or preferentially expressed in sexual or apomictic libraries. Illumina sequencing and *de novo* assembly allowed the analysis of differentially abundant unigenes in two pepper lines (a cytoplasmic male sterile line and its near-isogenic restorer), prompting to identify new fertility-associated genes and elucidate the mechanisms of cytoplasmic male sterility (CMS) and fertility recovery (Liu et al., 2013). Studies of the flower transcriptome have identified candidates that could be involved in the genetic regulation of inflorescence evolution and disease resistance (Zhang et al., 2013), as well as stress-responsive pathways and a GA-signaling pathway, proposed to have a key role in flowering (Gao et al., 2014). Ilumina sequencing has also been applied to study cytoplasmic male sterility in cotton, and revealed

that transcripts related to ROS homeostasis are key players in the CMS (Yang et al., 2014). In the same way, a study on CMS carried out in *Brassica napus* proposed a model showing how the expression of some genes located in the nucleus and related to pollen development were inhibited (An et al., 2014). Transcriptomic analysis has also been used to determine the influence of the herbicide Monosulfuron Ester Sodium (MES) in the CMS of *Brassica napus* (Li et al., 2015). Moreover, transcriptomic data on pollen–pistil interactions indicated a time course-specific modulation of AtNOA1 and NR1 and NR2 transcripts, which putatively may trigger an NO signaling pathway (Boadavida et al., 2011).

A detailed review has been recently published by Rutley and Twell (2015) appraising historical evidence for male haploid gene expression and the wealth of pollen transcriptome data available through the last decade. They state that studies have progressed from genetic studies to the direct analysis of RNA and from gene-by-gene studies to analyses on a genomic scale, registering that microarray and/or RNA-seq data can now be accessed for all phases and cell types of developing pollen from at least 10 different angiosperms.

The present study represents a clear example on how high throughput analyses carried out by using next generation sequencing methods may help to improve our knowledge on a particular topic of plant biology as Plant Reproduction. Although we have focused exclusively in SOD sequences to date, most transcripts encoding enzymes involved in ROS and NO metabolism are available, and have been already characterized as regard to their sequences and even to their polymorphism in olive pollen and pistil, through the generation of an olive reproductive transcriptome called ReprOlive (http://reprolive.eez.csic.es/olivodb/) (Carmona et al., 2015 under review). These data will certainly speed ROS and NO metabolism research in these tissues.

ROS/RNS are highly involved in Self-Incompatibility in several model species; however SI in the olive tree remains uncharacterized

Self-incompatibility (SI) is one of the plant physiological mechanisms where ROS and RNS have been widely described. ROS and Nitric Oxide (NO) signalling and the second messenger Ca^{2+}, as well as different proteins, are key players in the intercellular communication between the pollen grain/pollen tube with the maternal tissues and the cells of the female gametophyte (Dresselhaus and Franklin-Tong, 2013). The involvement of ROS in SI is often mediated through programmed cell death (PCD) phenomena. In

Pyrus pyrifolia, S-RNase specifically disrupts tip-localized ROS of incompatible pollen tubes via arrest of ROS formation in mitochondria and cell walls. The tip-localized ROS disruption decreases Ca^{2+}, depolymerizes the actin cytoskeleton and induces nuclear DNA degradation (Wang et al., 2010a). Self-Incompatibility has also been described to be controlled by apoplastic calmodulin, which enhances calcium influx and ROS concentration (Jiang et al., 2014). In the case of *Papaver rhoeas*, the self-incompatibility mechanism is determined by the multi-allelic S-locus, that allows discrimination between self (incompatible) and non-self (compatible) pollen by the pistil. SI signalling is initiated by Ca^{2+}, that triggers several downstream events, including increment in ROS and NO. As result, there is an inhibition of pollen tube growth and PCD in incompatible pollen (Eaves et al., 2014). PCD has also been associated to the acidification of the cytosol of the pollen tube (Wilkins et al., 2015).

In conclusion, ROS and NO signalling form an integral part of SI-PCD. As stated above, such activity is frequently mediated through chemical modification of other key actors in metabolism. These modifications include nitration and S-nitrosylation. Evidence of the involvement of these modifications in olive reproductive organs is beginning to be described (Serrano et al., 2012a,b; 2015). However, in the olive tree, many of these potential mechanisms have to be further explored yet. For example, the identification of factors like S-RNases in the olive remains elusive. Transcriptomic approaches have failed to detect such products so far (Dr. A.J. Castro, EEZ-CSIC Granada, personal communication).

ROS/RNS are important players in allergy through different mechanisms

Key antioxidant enzymes may represent themselves allergenic proteins present in pollen grains. This is the case of several peroxidases, catalases, Mn-SODs (allergome database: http://www.allergome.org) and a glutathione-S-transferase considered a minor allergen in birch pollen (Zwicker, 2013; Deifl et al., 2014). Several of these allergens have been reported to be present in pollen exudate as well (Zaidi et al., 2012). This may help these proteins establishing contact with the human immune system. Olive pollen Cu,Zn-SOD, (namely Ole e 5 allergen) has been detected here to have heterologous counterparts in other allergenic pollen sources. Although yet to be determined whether these enzymes may represent relevant allergens in such sources, the broad presence of this enzyme in many plant and even animal materials could led to consider its potential consideration as a panallergen (Hauser et al., 2010) even though its prevalence in olive pollen is low.

Controversy exists about whether the production of ROS by "respiratory burst oxidase homologues" (RBOHs) in pollen, and further modulation of ROS levels may trigger the inflammatory response in allergy episodes. An increasing number of studies are demonstrating that, in addition to the allergens generated and released by pollen grains, other soluble signals like $O_2^{\cdot-}$, NO, or nitrite contribute to the development of allergy symtoms (Boldogh et al., 2005; Dharajiya et al., 2008; Bright et al., 2009; Wang et al., 2009). Among these, $O_2^{\cdot-}$ production by NADPH oxidase activities seem to occur in an important number of allergenic pollens, representing a clear cause for exacerbation of symptoms, and acting as a early signal, prior to antigen presentation to the human immune system (Wang et al., 2009; Speranza et al., 2012). However, such involvement of ROS has been brought to a doubt by (Shalaby et al., 2013), who demonstrated that hyper-reactivity to an inhaled extract of birch pollen was independent from NADPH oxidase activity.

It is also suggested the implications of NO in hay fever, NO derived from hydrating pollen could directly impact upon respiratory tract cells to initiate an immune response or augment antigenicity. This plant cell–human cell interaction could prompt the initiation of an allergic response via interaction with dendritic cells (Bright et al., 2009). The high NO levels in the oxidative environment of the asthmatic airway lead to greater formation of reactive nitrogen species (RNS) and subsequent oxidation and nitration of proteins, which adversely affect protein functions that are biologically relevant to chronic inflammation. In contrast to the high levels of NO and nitrated products, there are lower levels of beneficial S-nitrosothiols (RSNO), which mediate bronchia dilation, due to greater enzymatic catabolism of RSNO in the asthmatic airways (Ghosh and Erzurum, 2011).

Many of these aspects are yet controversial, have been described in a limited number of species, and have to be confirmed.

CONCLUSIONS

1. ROS were largely detected in the stigmas of all different plant species scrutinized, while absent from the style and ovary. Interestingly, multiple patterns of ROS accumulation were observed, considering their presence at the unopened flower, those phases considered "receptive" to pollen interaction, or post-fertilization stages. Surprisingly, no clear phylogenetic relationships were observed, or with the type of stigma (dry or wet) or the self-incompatibility system applied. Analysis of primitive Angiosperms might indicate that signalling functions of ROS/NO in the stigma might have evolved from defence functions.

2. ROS and NO are produced in the olive reproductive organs in a stage- and tissue-specific manner. The biological significance of the presence of these products may differ between early flowering stages (defence functions) and stages where there is an intense interaction between pollen and pistil which may determine the presence of a receptive phase in the stigma. The study confirms the enhanced production of NO by pollen grains and tubes during the receptive phase, and the decrease in the presence of ROS when NO is actively produced.

3. The construction of SSH libraries using pistil and pollen within a context of high interaction between male-female counterparts allowed identifying transcripts with important roles in stigma physiology. The functions of many of the transcripts obtained are intimately related, and most of them are of pivotal importance in defence, pollen-stigma interaction and signalling.

4. The use of SSH libraries together with transcriptomic approaches and further experimental cloning permitted exploring the paramount importance of superoxide dismutase enzymes in maintaining ROS balance in olive pollen, as numerous sequences of the enzyme were retrieved. Such variability was further scrutinized by means of bioinformatics tools, which allowed identifying unique forms of Cu,Zn-SOD, including a deleted form, and generate 3-D models of these main forms. Bioinformatic predictions also interestingly envisaged relevant differences in the antigenicity/allergenicity of these molecules.

5. Biochemical analyses of SOD activity in olive pollen rendered new and relevant information. As examples, SOD activity did not show correlation to pollen viability. New isoforms of Cu,Zn-SODs (among them chloroplastidic forms) were detected, as well as differentially expressed Mn, Fe and Cu,Zn-SODs isoforms among cultivars through the olive pollen. The later was also identified in the pollen of a variety of allergenic species, thus reporting a potential relevance of this molecule as a putative panallergen. Finally, and as previously foreseen, the deleted recombinant enzyme showed a slightly differential SOD activity when compared to the complete form, likely contributing to a fine regulation of ROS, compatible with signalling events.

6. Light Microscopy studies, and particularly TEM ultrastructural localization of Cu,Zn-SOD, contributed to confirm the previously reported localization of the enzyme in the cytosol and the olive pollen exine and apertures. Moreover, we also detected the enzyme in amyloplasts and low-differentiated plastids present in the pollen grain, and in roundly-shaped structures, present in the cytoplasm of the vegetative cell, which were confirmed as peroxisomes through co-localization with a peroxisomal marker.

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ANNEXES

AU: Arbitrary Units

BSA: Bovine Serum Albumin

cGMP: cyclic Guanosine monophosphate

CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CLSM: Confocal Laser Scanning Microscopy

CMS: Cytoplasmic Male Sterility

cPTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

Cu,Zn-SOD: Cupper Zinc Superoxide Dismutase

DAF-2 DA: Diaminofluorescein diacetate

$DCFH_2$-DA: 2',7'dichlorodihydrofluorescein diacetate

DHE: Dihydroethidium

DNA: Deoxyribonucleic Acid

DRP: Disease Response Protein

DTT: Dithiothreitol

EST: Expressed Sequence Tag

FDA: Fluorescein diacetate

Fe-SOD: Iron Superoxide Dismutase

FM: Fluorescence Microcopy

GO: Gene Ontology

GSNO: S-Nitrosoglutathione

GSNOR: S-Nitrosoglutathione Reductase

H_2O_2: Hydrogen peroxide

Hsf: Heat stress factor

IEF: IsoElectroFocusing

Ip: Isoelectric point

KCN: Potassium Cyanide

kDa: KiloDalton

LEA: Late Embriogenesis Abundant

LM: Light microscopy

LTP: Lipid Transfer Protein

MES: Monosulfuron Ester Sodium

Mn-SOD: Manganese Superoxide Dismutase

Mw: Molecular weight

NGS: Next Generation Sequencing

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NOX: Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase)

$O_2^{\cdot-}$: Superoxide radical

ONOO-: Peroxinitrite

OSML: Osmotin-like protein

PAGE: Polyacrylamide Gel Eelectrophoresis

PBS: Phosphate-Buffered Saline

PCD: Programmed Cell Death

PCR: Polymerase Chain Reaction

PME: Pectin Methyl Esterase

PMEI: Pectin Methyl Esterase Inhibitor

PRP: Pathogenesis Related Protein

PVDF: Polyvinylidenedifluoride

PVPP: Polyvinylpolypyrrolidone.

Rboh: Respiratory burst oxidase homolog

RNA: Ribonucleic acid

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RSNO: S-Nitrosothiols

RT: Room Temperature

SAG: Senescence Associated Gene

SDS: Sodium Dodecyl Sulfate

SF: Self-Fertile

SI: Self-Incompatibility

SIT: Specific Immunotherapy

SNARE: Soluble NSF Attachment Protein Receptor

SNP: Sodium Nitroprusside

SOD: Superoxide Dismutase

SOD: Superoxide Dismutase

sRNA: small RNA

SSH: Suppression Subtractive Hybridization

TCA: Trichloro Acetic

TEM: Transmission Electron Microcopy

TMB: 3,5,3',5'-tetramethylbenzidine

TMP: 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy

VIGS: Virus-Induced Gene Silencing

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Selected talk: Molecular characterization and polymorphism of superoxide dismutase (SOD) in olive (Olea europaea L.) pollen. Putative roles in the interaction pollen-stigma
XX International Congress on Sexual Plant Reproduction. Brasilia (Brazil). August 2008
URL: http://hdl.handle.net/10261/32891

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Talk: Localization of ROS (Reactive oxygen species) and nitric oxide in olive reproductive tissues by stereomicroscopy and CLSM
XXIV Congress of the Spanish Microscopy Society. XLIV Annual meeting of the Portuguese society for microscopy. Segovia (Spain). June 2009
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Poster: Preliminary characterization of NADPH oxidase (NOX) activity in olive reproductive tissues
XVIII Reunión de la Sociedad Española de Fisiologia Vegetal (SEFV). XI Congreso Hispano-Luso de Fisiología Vegetal. Zaragoza (Spain). September 2009
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Poster: Localization of ROS and NO in olive reproductive tissues during flower development
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Poster: Developmental changes in the presence of ROS (reactive oxygen species) in the stigma and their relationships with stigma functionality
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Talk: Nitric oxide production during olive flowering and in vitro pollen germination
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Talk: Patterns of ROS distribution during flower development. The olive tree as a model
XXI International Congress on Sexual Plant Reproduction. Bristol (UK). August 2010
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Poster: Detection of peroxidase activity in olive (Olea europaea L.) floral organs
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Zafra A, Jiménez-Quesada MJ, Rejón-García JD, Rodríguez-García MI; Alché JD.
Talk: Detection of Reactive Oxygen Species and Nitric Oxyde in Floral Tissues
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Talk: Characterization of NADPH oxidase (NOX) activity in olive reproductive tissues
10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest (Hungary). July 2011
URL: http://hdl.handle.net/10261/40115

Zafra A, Rodríguez-García MI, Alché JD.
Talk: Cellular localization of ROS and NO in pollen grains and stigma tissues during olive flower development
10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest (Hungary). July 2011
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Zafra A, Jiménez-Quesada MJ, Traverso JA, Rodríguez-García MI, Alché JD.
Talk: Localization of CuZn- and Mn-Superoxide Dismutases in olive pollen throughout *in vitro* germination
2nd Joint Congress of the Portuguese and Spanish Microscopy Societies. Aveiro (Portugal). October 2011

Jiménez-Quesada MJ, Traverso JA, **Zafra A**, Castro AJ, Rodríguez-García MI, Alché JD.
Poster: Nitric oxide production in *Olea europaeae* L. pollen during *in vitro* germination
Redox Signaling and oxidative stress in health and disease. Valencia (Spain). June 2012

Jiménez-Quesada MJ, **Zafra A**, Potocký M, Traverso JA, Castro AJ, Rodríguez-García MI, Zàrský V, Alché JD, Pleskot R.
Poster: Enzymes involved in oxygen metabolism in the olive (*Olea europaea* L.) pollen, and their contribution to allergy.
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Poster: Detection and quantification of NO and S-Nitrosoglutathione (GSNO) in olive (Olea europaea) pollen during *in vitro* germination.
NO Club. Edinburgh (UK). July 2012

Zafra A, Jiménez-Quesada MJ, Traverso JA, Corpas FJ, Rodríguez-García MI, Alché JD.
Poster: LC-ES/MS detection and quantification of chemical species related to Reactive Oxygen Species and Nitric Oxide (NO) metabolism in olive pollen (*Olea europaea* L.) throughout *in vitro* pollen germination.
Plant Biology Congress jointly organized by FESPB and EPSO. Freiburg (Germany). August 2012

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Poster: Imaging Reactive Oxygen Species and NO in plant reproductive tissues
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Zafra A, Carmona R, Jiménez-Quesada MJ, Traverso JA, Castro AJ, Rodríguez-García MI, Bautista R, Claros MG, Alché JD .
Poster: *de novo* assembly of the olive tree (*Olea europaea* L.) reproductive transcriptome
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Morales S, Castro AJ, Jiménez-López JC**, Zafra A**, Jiménez-Quesada MJ, Florido F, Rodríguez-García MI, Alché JD.
Talk: A multiplex system to analyse the expression of allergens and other gene products of interest in the olive pollen. Pollen Biotechnology, Diversity and Function in a Changing
2nd International APLE-APLF Congress. Warsaw (Poland). September 2013

Ortiz MA, **Zafra A,** Rodrigues LA, Perez-Espona S, Hiscock SJ.
Poster: Genetic diversity in alien species: The case of Senecio Squalidus (Asteraceae)
Adapting to Global Change. In the Mediterranean Hot Spot. Sevilla (Spain). September 2013

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Poster: Localizatión of NADPH Oxidase and Superoxide Dismutase Transcripts in Olive Reproductive Tissues by Using Double-Labelled Oligonuceotide Probes"
Microscopy at the frontiers of science 2013. 3rd Joint Congress of the Portuguese and Spanish Microscopy Societies and Israel Society for Microscopy as invited guest. Tarragona (Spain). September 2013

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Invited talk: Imaging of living pollen tubes across in vitro germination as a tool to analyze pollen physiology and performance.
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Poster*: De novo* assembly, functional annotation and applications of the olive tree (Olea europaea L.) reproductive transcriptome
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Sociedad Española de Ciencias Hortícolas (SECH)

National Conferences:

Alché JD, Castro AJ, Jiménez-López JC, Morales S, **Zafra A**, Rodríguez-García MI. Talk: Carácter alergénico diferencial del polen de olivo de distintas variedades
I Jornadas Grupo de Olivo de la Sociedad Española de Ciencias Hortícolas (SECH). Córdoba (Spain). October 2006
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Poster: Biología reproductive del olivo
10th III Jornadas Nacionales del grupo de Olivicultura de la SECH. Sevilla (Spain). October 2011

Carmona R, **Zafra A**, Seoane P, Castro AJ, Alché JD, Claros MG.
Talk*:* From annotated transcriptome to a browseable database in the seek of olive tree allergens
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