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Article

RNA Polymerases IV and V are Involved in Olive Fruit Development

Alicia Serrano [1,&], Martín Moret [1,&], Isabel Fernández-Parras [1], Aureliano Bombarely [2], Francisco Luque [1,*] and Francisco Navarro [1,*]

[1] Departamento de Biología Experimental, Instituto Universitario de Investigación en Olivar y Aceites de Oliva, Universidad de Jaén, 23071 Jaén, Spain; asg00144@ext.ujaen.es (AS); mmoret@ujaen.es (MM) ; ifparras@ujaen.es (IF); (FL) fjluque@ujaen.es; (FN) fngomez@ujaen.es.

[2] Instituto de Biología Molecular y Celular de Plantas (IBMCP), CSIC and Universitat Politécnica de Valencia, 46011 Valencia, Spain; abombarely@ibmcp.upv.es.

* Correspondence: fngomez@ujaen.es (F.N.); fjluque@ujaen.es (F.L.); Tel.: +34-953212771; +34-953212527

& Both authors contribute equally.

Abstract: Transcription is carried out in most eukaryotes by three multimeric complexes (RNA polymerases I, II and III). However, plants contain two additional RNA polymerases (IV and V), which have evolved from RNA polymerase II. RNA polymerases II, IV and V contain both common and specific subunits that may specialize some of their functions. In this study, we conducted a search for the genes that putatively code for the specific subunits of RNA polymerases IV and V, as well as the corresponding of RNA polymerase II in olive. Based on the homology with the genes of *Arabidopsis thaliana*, we identified 13 genes that putatively code for the specific subunits of polymerases IV and V, and 16 genes that code for the corresponding specific subunits of polymerase II in olive. The transcriptomic analysis by RNA-Seq revealed that the expression of the RNA polymerases IV and V genes was induced during the initial stages of fruit development. Given that RNA polymerases IV and V are involved in the transcription of long non-coding RNAs, we investigated their expression and observed relevant changes in the expression of this type of RNAs. Particularly, the expression of the intergenic and intronic long non-coding RNAs tended to increase in the early steps of fruit development, suggesting their potential role in this process. The positive correlation between the expression of RNA polymerases IV and V subunits and the expression of non-coding RNAs supports the hypothesis that RNA polymerases IV and V may play a role in fruit development throughout the synthesis of this type of RNAs.

Keywords: RNA polymerases, long non-coding RNAs, olive, stress conditions, fruit development.

1. Introduction

Transcription in bacteria and archaea is carried out by a single multimeric RNA polymerase, while most eukaryotes contains three multimeric complexes (RNA pol I, II, and III) [1–3]. Furthermore, plants contain two additional RNA pols (IV and V) that have evolved from RNA pol II [4–12]. RNA pol I consists of 14 subunits and synthesizes precursor rRNA 45S (35S in yeast) of the three largest rRNAs [3,13–15]. RNA pol III contains 17 subunits and transcribes tRNAs, 5S rRNA and other non-coding RNAs [13,16–18]. RNA pol II is composed of 12 subunits and synthesizes mRNAs and some non-coding RNAs [13,19–21]. Plant-specific RNA pol IV and V, which have evolved from RNA pol II through duplication and functional divergence, also contain 12 subunits. These two enzymes, that are involved in epigenetic regulation synthesize siRNAs, which play roles in transcriptional silencing via RNA-directed DNA methylation (RdDM), and non-coding RNAs participating in plant growth, development, response to environmental changes or plant immunity [4–12,22–24].

RNA pol II, IV and V contain specific conserved subunits that may specialise some of their functions [5,22,25]. This is the case of subunits NRPD1, NRPE1 and NRPB1 which correspond to RNA

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pols IV, V and II, respectively, in *Arabidopsis thaliana*. In addition, there are other subunits that are common to RNA pol IV and V, but are conserved in RNA pol II, such as the subunits NRPDE2 and NRPB2, RNPDE4 and NRPB4, and NRPDE7 and NRPB7, which are shared by RNA pol IV and V and conserved with RNA pol II [5,22,25–27]. Furthermore, several isoforms of the common subunit five, shared by all the RNA pols have been described, while a specific isoform, NRPE5, has been found for RNA pol V [28]. In addition, several paralogues have been described for these and other subunits in different plants [22,25,26,28–30]. Based on the existence of these paralogues, it has been proposed that these may perform new functions or be subject to different regulation. This is indeed the case of the distinct isoforms of the shared subunits from the RNA pols in cultivated olive trees 'Picual' (*Olea europaea L.* cv. Picual) [28].

RNA pol IV and V have been reported to be involved in the biogenesis and functionality of 24-nt siRNA, which participates in RdDM [9,12,25,30]. RNA pol IV and V have also been proposed to participate in the transcription of long non-coding RNAs (lncRNAs) [24,31–38]. Some of these lncRNAs are the intermediary of siRNA and are found within intergenic regions [34]. Although lncRNAs are also transcribed by RNA pol II, those synthesised by RNA pol IV and V show some structural differences as regards the RNA pol II ones, such as lack of poly-A at the 3' end region or lack of introns [4]. lncRNAs transcribed by RNA pol IV and V are poorly characterized, in part because of their low expression and instability [34,39]. However, well-studied examples of non-polyA lncRNAs have been reported [40–42]. Notably, the synthesis of non-polyA lncRNAs can be regulated by environmental conditions, as demonstrated in *A. thaliana* under abiotic stress [43–45].

In this work we searched for genes that putatively encode specific subunits of RNA pol IV and V, and for those corresponding to RNA pol II, in the olive 'Picual' cultivar given its economic, agronomic and agro-ecological importance as one of the most important fruit trees in the Mediterranean Basin [28,46–48]. The analysis allowed to identify paralogues for NRPD1, NRPE1 and NRPB1, NRPDE2 and NRPB2, NRPDE4 and NRPB4, NRPDE7 and NRPB7, and also for NRPB7-like, in addition to the putative pseudogenes, according to our transcriptomic analyses. The transcriptional studies from RNA-Seq data evidenced an increase for the corresponding RNA pol IV and V genes during fruit development. Furthermore, given the known role of RNA pol IV and V in the transcription of lncRNAs, we studied the lncRNA transcriptome during fruit development, which revealed important changes in their expression and differed based on the analysed lncRNA type. Accordingly, our data point to RNA pol IV and V involved in the regulation of lncRNAs during fruit development.

2. Materials and Methods

2.1. Plant Material

In order to analyse gene expression during fruit development in olive tree, flowers and fruit were collected from the 'Picual' olive cultivar growing in the experimental field of the University of Jaén (Jaén, Spain). Flower and fruit samples were collected from three different closely located trees and from south-facing branches to reduce environmental variability, as specified by [49]. Therefore, three independent biological samples were collected at eight different times from full bloom (flowering) to fruit ripening 6 months later (15 day AFB), and monthly from 1 to 6 months AFB. These samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

2.2. Transcriptomic Analysis

The total RNA from the triplicate samples of flowers and fruit at 15 days AFB was isolated using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. PoliA+ RNA was purified and sequenced from the samples collected during fruit development as indicated by [49]. Briefly, poliA+ RNA 150 bp x 2 paired-end Illumina sequences were obtained at Novogene (UK) and at least 50 M reads of Q30 sequences data were obtained from each biological replicate sample. The dataset is available at NCBI as BioProject: PRJNA870905.

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For this work, an additional RNA-Seq of the total RNA was done. In this case, 150 bp x 2 paired-end Illumina sequences were obtained at Ascires (Spain) from the flower and 15 days AFB samples, as well as a mix of RNAs from flower, fruit, root, leaf, meristem and stem. For total RNA sequencing, at least 100 M reads were obtained per sample. The dataset is available at NCBI as BioProject: PRJNA989401.

Other RNA-Seq data were used as described in [28]. Basically, a previous RNA-Seq from olive organ/tissues [50] and several stresses, such as cold, injury or *Verticillium dahliae* infection [51], were analysed. The datasets are available at BioProject PRJNA556567 and at NCBI accession numbers SRR1525051, SRR1525052, SRR1524949, SRR1524950, SRR1524951, SRR1524952, SRR1525086, SRR1525087, SRR1525113, SRR1525114) SRR1525231, SRR1525237, SRR1524947, SRR1524948, SRR1525213, SRR1525114, SRR1525224, SRR1525226, SRR1525284, SRR1525285, SRR1525286, SRR1525287, SRR1525415, SRR1525416, SRR1525436, and SRR1525437.

The RNA-Seq analysis was performed with DNAstar (ArrayStar 17, Rockville, MD, USA) for the RNA-Seq analyses (www.dnastar.com). Gene expression was carried out using a 95% false discovery rate (FDR).

2.3. Annotation of lncRNAs in Olive

Assessments of raw sequence quality were first performed using the FastQC software (version 0.11.5, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequences and reads shorter than 50 bp were trimmed with Fastq-mcf (EA-Utils version 1.04.759) (http://expressionanalysis.github.io/ea-utils/). Next clean reads were mapped to the olive genome of the 'Picual' cultivar [52] available in OliveTreeDB (https://genomaolivar.dipujaen.es/db/downloads.php) using the HISAT2 software (v2.2.1) [53]. Mapped reads were sorted and compressed by Samtools (v1.16.1) [54], and then assembled and merged using StringTie v2.2.1 [55]. The gffcompare tool (v0.12.6) [56] was used to identify the unannotated transcripts by comparing the assembled transcriptome to the reference 'Picual' transcriptome. Note that these unannotated transcripts corresponded to the non-polyA lncRNAs. Subsequently, the transcripts categorised as "u" (intergenic lncRNAs), "x" (antisense lncRNAs), "i" (intronic lncRNAs) and higher than 200 bp were selected as candidate lncRNAs.

However, as the selected transcripts could contain coding genes, they underwent another filtering process. The transfer RNAs were filtered using the tRNAscan-SE 2.0 tool [57]. Barrnap tool v0.7 (https://github.com/tseemann/barrnap) was applied to identify the ribosomal RNA genes and CPC2 software (http://cpc2.cbi.pku.edu.cn) [58] was applied to filter out those transcripts with coding ability. Finally, transcripts were analysed by the second script of GreeNC (https://github.com/sequentiabiotech/GreeNC) to discriminate any other non-coding transcripts from lncRNAs and to identify any possible miRNA precursors (Figure 1).

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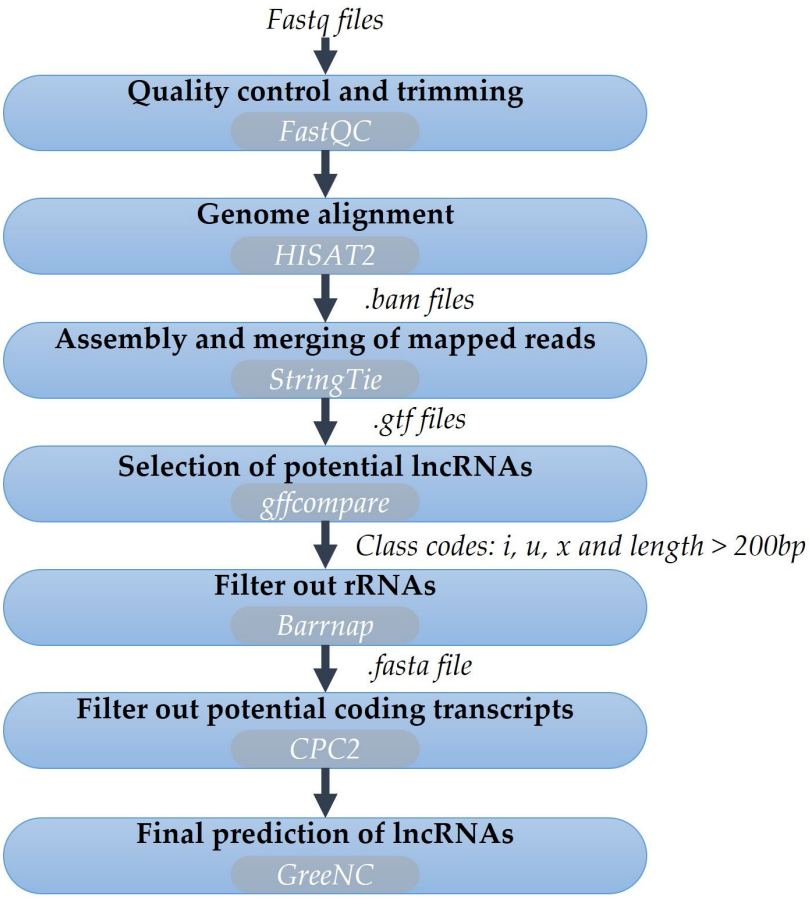

Figure 1. LncRNAs identification pipeline from RNAseq data sets.

2.4. Analysis of the Differentially Expressed lncRNAs

The expression analysis was performed with DNAstar (ArrayStar 17, Rockville, MD, USA, www.dnastar.com). Mapping was done with high-stringency parameters to differentiate between very similar paralogues, k-mer = 63 and 95% matches. Data were normalised based on reads per kilobase of transcript per million reads mapped (RPKM). A basal expression level of log2 RPKM = −2 was considered. Therefore, the genes with expression values above this threshold level were considered expressed, whereas those genes with expression values that equalled or were below the threshold level were considered not expressed. A comparison between samples was made using the parametric t-student test.

3. Results

We conducted a genome search for olive genes that are highly homologous to those that code for the specific subunits of RNA pol IV and V in *A. thaliana*. Then their expression profile was studied by RNA-Seq in different plant organs/tissues in response to environmental stresses, as well as during the fruit development process. As RNA pol IV and V have evolved from RNA pol II, the equivalent olive genes for the RNA pol II subunits were also identified. These genes coding for the RNA pol II subunits were used as references for the expression profiles and were compared to the RNA pol IV and V coding genes. An additional annotation and expression analysis of lncRNAs was also performed.

3.1. Olive Genes Coding for RNA pol IV and V Subunits

In order to search for the genes putatively coding for the specific subunits of RNA pol IV and V in olive, the *A. thaliana* sequences for NRP1, NRP2, NRP4 and NRP7 of RNA pol II, IV and V were

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used as a query to identify the corresponding homologues. This search yielded several paralogues: 16 for pol II and 12 for RNA pol IV and V (Table 1 and Figure 2). This was not surprising because olive ancestors have quite probably undergone two whole genome duplication (WGD) events in the last 65 M years [52,59]. Furthermore, putative pseudogenes were identified for RNA pol II, which were not expressed and contained inactivating mutations according to our RNA-Seq analyses under different conditions (see below).

Table 1. Coding genes for RNA pol II, IV and V subunits in olive (*Olea europaea L.* cv. Picual).

RNA pol II		RNA pol IV		RNA pol V	
NRPB1	Oleur061Scf2303g05021.1 Oleur061Scf0709g00017.1 Oleur061Scf1475g00008.1 Oleur061Scf0012g03006.1	**NRPD1**	Oleur061Scf8288g09024.1 Oleur061Scf3115g03008.1 Oleur061Scf1883g00024.1	**NRPE1**	Oleur061Scf1459g03013.1 Oleur061Scf0397g00013.1 Oleur061Scf0194g01004.1
NRPB2	Oleur061Scf0169g06007.1 Oleur061Scf0008g04041.1 Oleur061Scf3112g05037.1	**NRPDE2**	Oleur061Scf2342g06016.1		
NRPB4	Oleur061Scf7473g00034.1 Oleur061Scf0021g02004.1	**NRPDE4**	Oleur061Scf9139g02012.1 Oleur061Scf1057g07004.1 Oleur061Scf2091g00019.1		
NRPB7	Oleur061Scf0456g03006.1 Oleur061Scf1270g16022.1 Oleur061Scf3490g10013.1 Oleur061Scf0186g07027.1 Oleur061Scf0397g02002.1	**NRPDE7**	Oleur061Scf8086g00007.1 Oleur061Scf8230g00012.1		
NRPB7-like	Oleur061Scf4485g00001.1 Oleur061Scf7934g03011.1				

Probable pseudogenes with inactivating mutations are shown in red.

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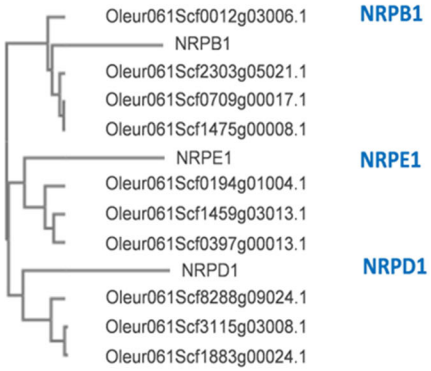
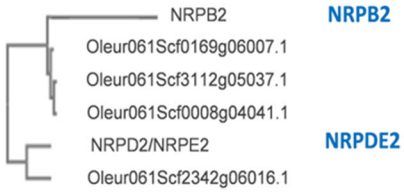
	NRPB1 (*O. eu.*)	NRPD1 (*O. eu.*)	NRPE1 (*O. eu.*)
NRPB1 (*A. th.*)	**79-85**	27-28	23-25
NRPD1 (*A. th.*)	26-28	**43-46**	26-27
NRPE1 (*A. th.*)	24-27	26-28	**44-52**



	NRPB2 (*O. eu.*)	NRPDE2 (*O. eu.*)
NRPB2 (*A. th.*)	**94-95**	33
NRPDE2 (*A. th.*)	35-36	**73**



	NRPB4 (*O. eu.*)	NRPDE4 (*O. eu.*)
NRPB4 (*A. th.*)	**59-67**	32-43
NRPDE4 (*A. th.*)	40-41	**40-43**

	NRPB7 (*O. eu.*)	NRPDE7 (*O. eu.*)	NRPB7-like (*O. eu.*)
NRPB7 (*A. th.*)	**81-89**	34-36	25
NRPDE7 (*A. th.*)	30-31	**52-55**	37
NRPB7-like (*A. th.*)	26-27	30-35	**41**

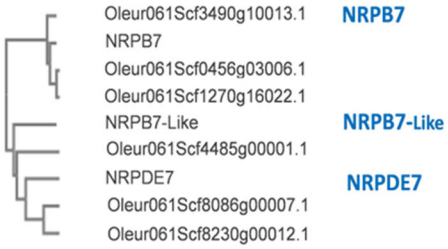


Figure 2. Left panel, amino acids identities calculated with the multialignment tool of Blast-P. Right panel, cladograms generated with Clustal Omega. The A. thaliana subunits are denoted by their NRP name.

3.2. Gene Expression Profile in Different Plant Organs/Tissues

In a previous study, we found that the genes coding for subunits shared by RNA pols in 'Picual' cultivar [28] were spatially and temporally regulated. To investigate whether the specific subunits of the RNA pol II, IV and V are spatially regulated in plant, a transcriptomic analysis was performed.

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The transcriptomic analysis of the genes coding for the specific subunits of RNA pol II, IV and V showed that some of them were regulated in the different analysed plant tissues (Figure 3). In line with this, the genes coding for RNA pol II subunits 1, 2, 4 and 7 generally exhibited relatively uniform expression across the different analysed tissues, with some exceptions. For instance, the four genes coding for subunit 1 of RNA pol II (NRPB1) showed a lower expression level in leaves than in the other tissues. Similarly, two genes coding for subunit 7 (NRPB7), specifically Oleur061Scf0186g07027.1 and Oleur061Scf3490g10013.1, seemed to be tissue-specific because they were expressed only in flowers. Furthermore, one of the genes coding for subunit 7 of RNA pols IV and V (Oleur061Scf8086g00007.1) showed the highest expression level for all tissues. The putative pseudogenes of the NRPB7 and NRPB7-like subunits were not expressed in any organ and tissue (not shown). Regarding the RNA pol IV and V subunits, the overall expression pattern was also quite homogeneous, except for the NRPD1 and NRPE1 subunits, which seemed to be expressed at variable levels depending on the specific plant tissue.

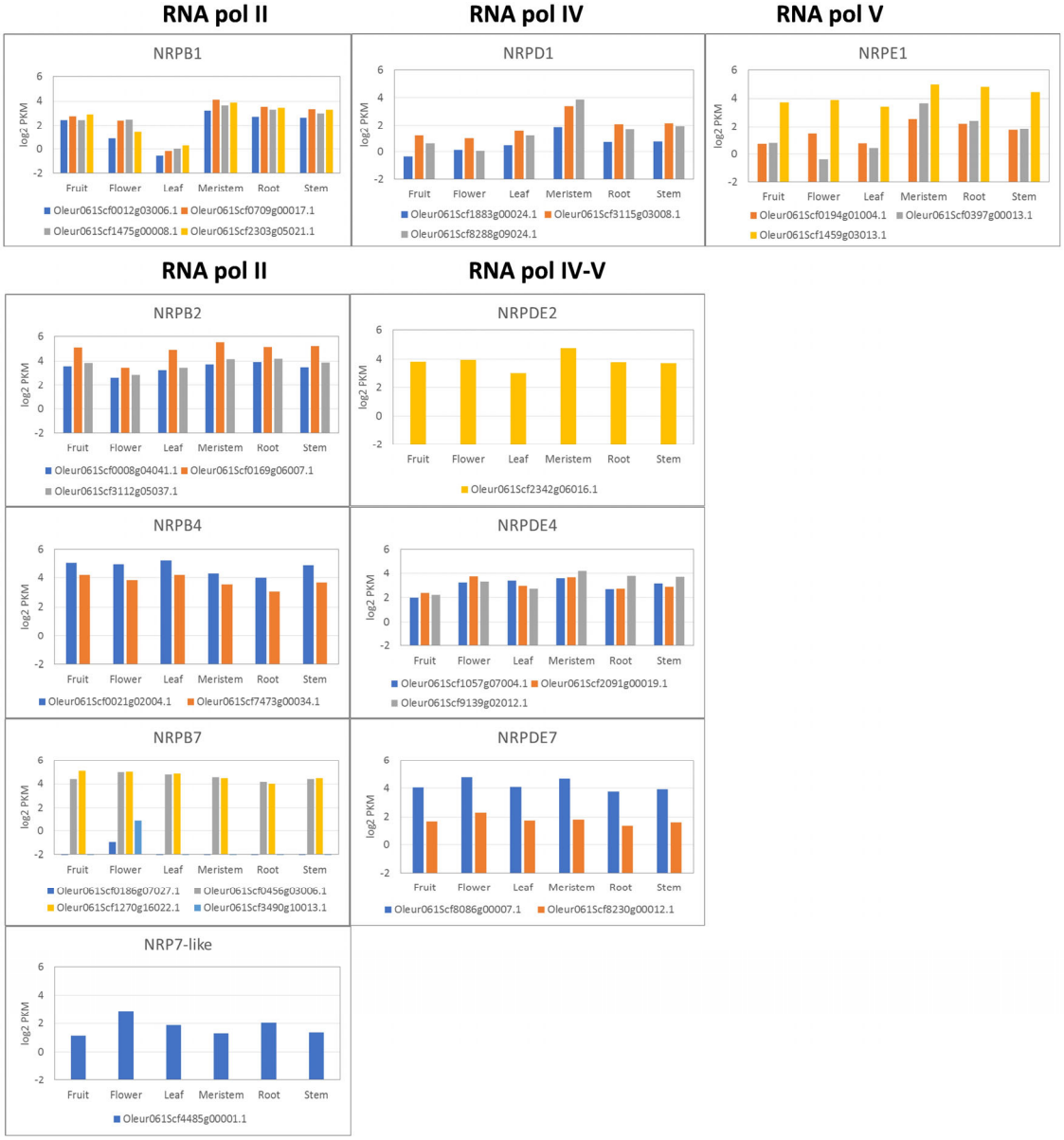

Figure 3. Expression profile of RNA pol II, IV and V subunits in different olive tree organs.

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3.3. Expression Profile in Response to Biotic and Abiotic Stresses

RNA pol IV and V might be involved in the response to stress stimuli to plants, according to reported data [12,60]. To examine this possibility, we studied the expression level of those genes coding for the different RNA pol II, IV and V subunits in response to root injury, *V. dahliae* infection and cold stress. As a result of the transcriptomic analysis of the genes encoding the specific RNA pol II, IV and V subunits (Figure S1), no consistent expression pattern in response to any of the studied stresses was observed. However, for root injury stress, some of the genes coding for the RNA pol IV and V subunits seemed to slightly reduce its expression following injury, and the original expression level were recovered after a 7-day follow-up. This behaviour was not consistent among all the subunits or among all the paralogue genes of the same subunit.

In addition, no specific response to biotic stress produced by *V. dahliae* infection was detected (Figure S2). In this case, minor changes were similar to the response observed in the case of root injury, which is performed to induce *V. dahliae* infection. Notably, some mayor changes were observed after 15 days post-inoculation. At the time of this follow-up, plants displayed clear severe disease symptoms. This fact could modify the general gene expression pattern, as previously described by [61].

Regarding response to cold stress, changes in the expression for some genes of the RNA pol II, IV and V specific subunits were observed, but, once again, no consistent pattern was found (Figure S3).

3.4. Expression Profile during Fruit Development

In line with the reported role of RNA pol IV and V in plant development, we investigated whether this could be the case in olive trees. To investigate this, we performed a transcriptomic analysis of the genes putatively code for the specific RNA polymerases II, IV and V subunits during fruit development. For this purpose, samples from three trees were analysed by RNA-Seq, which consisted of recently bloomed flowers and developing fruit at 15 days and every month from the flowering stage to fully ripe fruit (Figure S4) [49].

Notably, all the RNA pol IV and V specific subunits showed a significant induction at 15 days after full bloom (AFB) (Figure 4), with an average fold change of 3.2 (1.8 – 8.0). However, this induction at 15 days AFB was observed only for three of the genes of RNA pol II (Oleur061Scf0709g00017.1 of subunit 1; Oleur061Scf1270g16022.1 and Oleur061Scf0456g03006.1 for subunit 7), with fold change from 1.3 to 1.6. Therefore, no relevant changes in the gene expression were observed for the genes of the specific RNA pol II subunits, except for the NRPB7 genes Oleur061Scf0186g07027.1 and Oleur061Scf3490g10013.1 which, according to the organ/tissue specificity proposed above (Figure 2), were rapidly repressed once fruit development began. In addition, the two putative pseudogenes identified for NRPB7 and NRPB7-like were not expressed during fruit development.

Olive cultivar 'Picual', like other plants, contains a gene that codes for the specific RNA pol V subunit NRPE5, an isoform of subunit NRP5 shared by all eukaryotic RNA polymerases [28]. In line with this protein being specific for RNA pol V, the corresponding gene (Oleur061Scf4420g01012.1) showed a significant induction on the first 15 days of fruit development (Figure 4). Note that this was not the case of the other NRP5 paralogues identified in olive tree.

Taken together, these data suggest that RNA pol IV and V could play a major role in early fruit development steps. As RNA pol IV and V have been proposed to participate in the transcription of lncRNAs [24,31–38], we can speculate about a transcriptional response of these type of transcripts during this development process.

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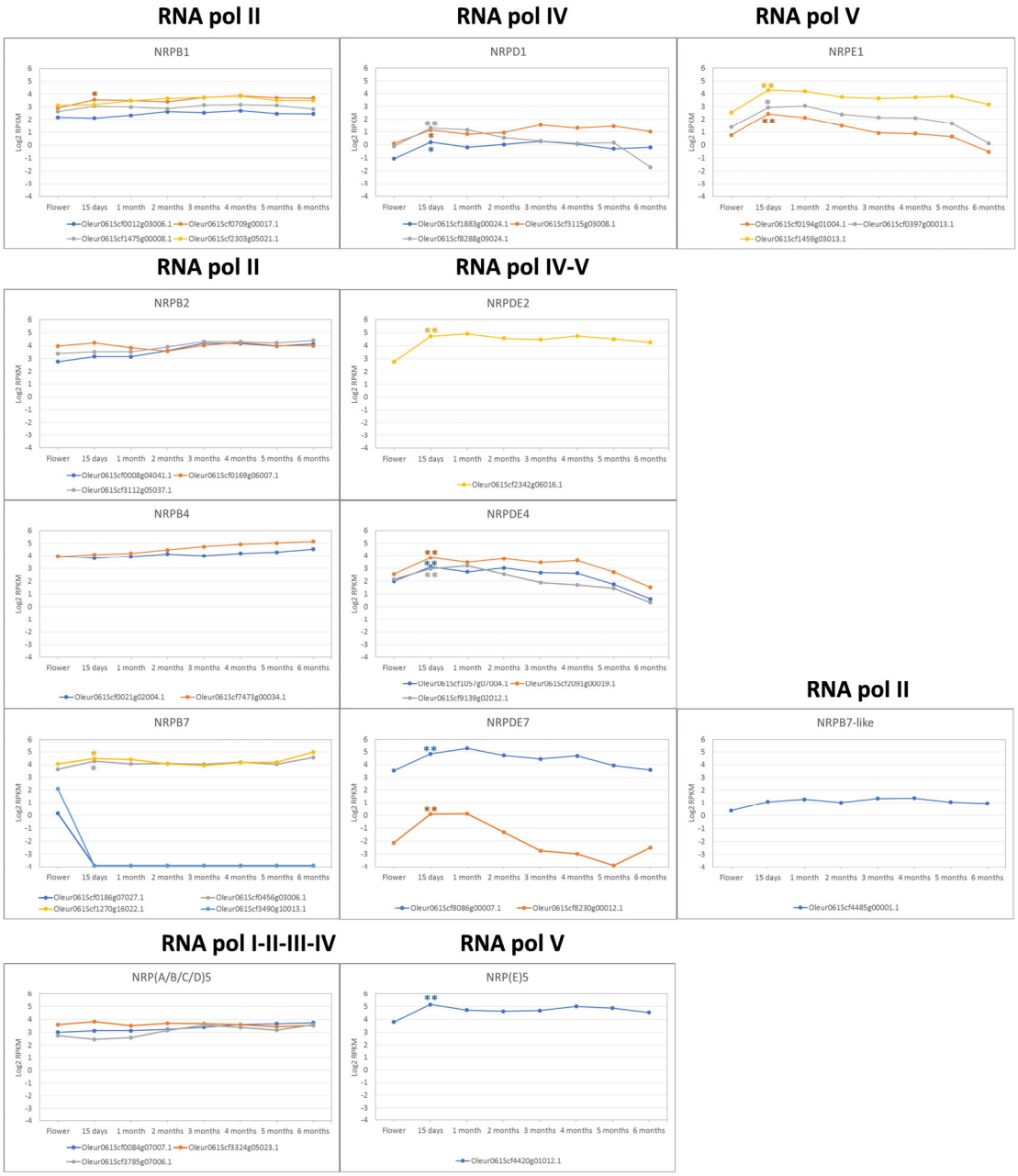

Figure 4. Expression profile of RNA pol II, IV and V subunits during fruit development. * p-value < 0.05 and ** p-value < 0.01 at comparing flower and after 15 days of fruit development.

3.5. Annotation and Expression of lncRNAs

In order to study the expression pattern of lncRNAs in olive, six strand-specific RNA-seq libraries were constructed (with three biological replicates each), using the total RNA of olive flower at full bloom and olive fruit at 15 days AFB,. An additional mix including different plant tissues was sequenced to obtain a broad representation of the lncRNA transcriptome in olive. From the total reads showing high quality (score > Q30), 744,825,368 clean reads were obtained from the seven libraries after trimming the adapters and reads that were shorter than 50bp (Table S1). These clean reads were aligned to the olive genome of the 'Picual' cultivar (https://genomaolivar.dipujaen.es/db/downloads.php). Alignment rates appeared to range from 60.24% to 86.07% (Table S1). Subsequently, 120,670 total transcripts were assembled using Stringtie, and 79,654 transcripts resulted after filtering transcripts by size ≥ 200 bp. The length of lncRNAs ranged from 200 bp to 21,212 bp, although most were shorter than 900 pb (Figure 5A). These transcript sequences were analysed to identify putative lncRNAs. As a result, 3,603 total transcripts

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from both experimental conditions were selected as intergenic, intronic or antisense after annotation with GffCompare. No tRNAs were identified when applying tRNAscan-SE. Furthermore, 146 rRNAs and 370 coding transcripts were discarded by applying Barrnap and CPC2, respectively. Finally, 2,303 candidate non-poly-A lncRNAs were identified as non-coding RNAs by GreenNC, including 1,814 intergenic, 261 antisense and 228 intronic transcripts (Figure 5B).

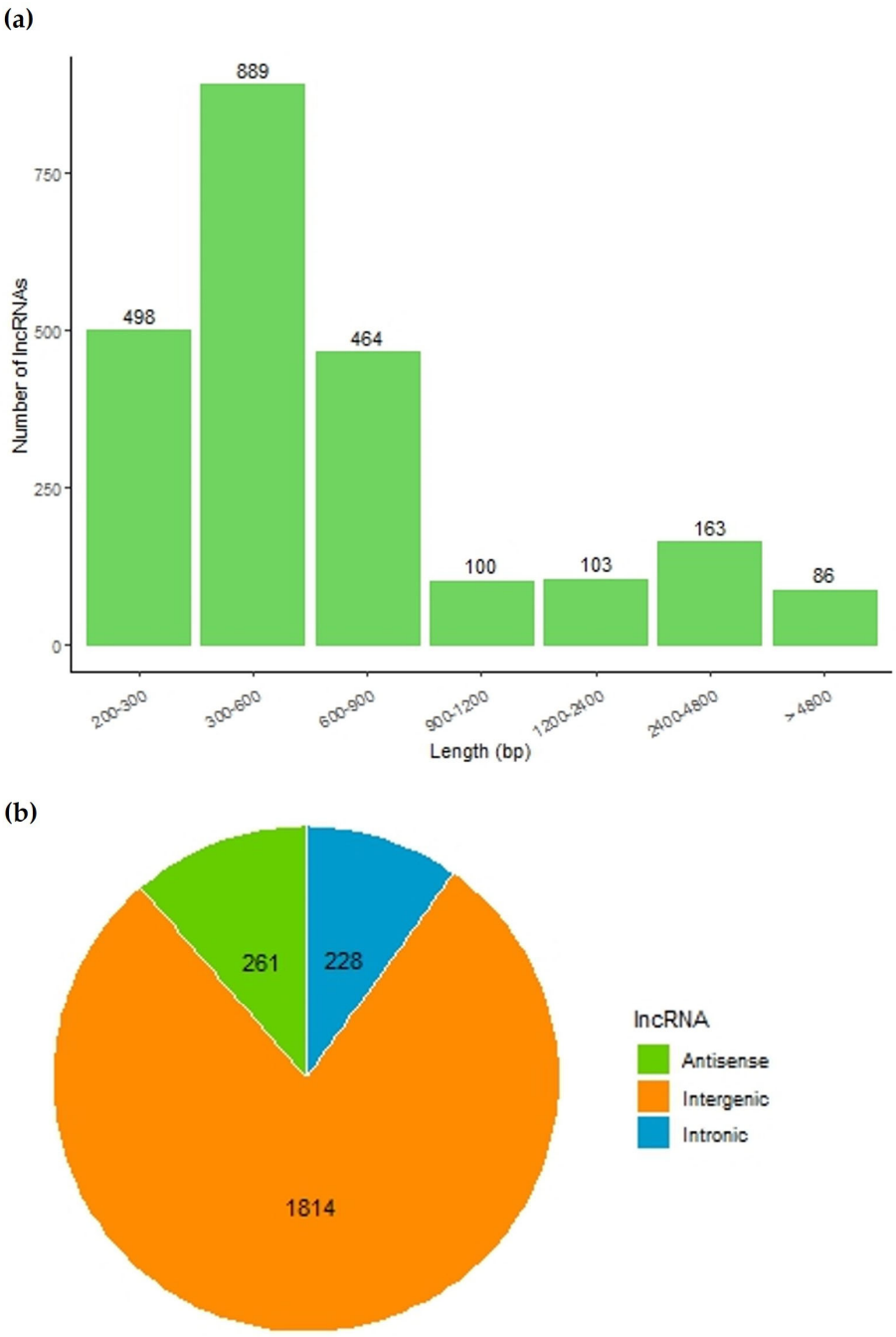

Figure 5. Classification of lncRNAs. **(a)** Length of lncRNAs; **(b)** Location of lncRNAs classified based on their genomic location regarding the neighbouring protein-coding genes: intergenic (transcript mapped to the unknown intergenic regions), intronic (transcripts mapped completely within the introns of the known protein-coding genes), and antisense (transcript mapped to the exon of the protein-coding gene but, on the opposite strand).

The analysis allowed us to identify 1,899 lncRNAs (non-polyA lncRNAs) in flowers and 1,915 from 15 days AFB, with 1,651 in common (Figure 6). In addition, changes in the expression pattern of lncRNAs were observed. During the transition from flowering to 15 days AFB, 143 lncRNAs were

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found to be up-regulated and 273 down-regulated, by using a false discovery rate (FDR) of 5% (Table 2). However, no major changes in the average expression of lncRNAs was found between the flower and 15 days AFB samples, with 718.68 and 733.22 RPKMs, respectively (p-value = 0.8365). Nevertheless, some differences between the flower and the 15 days AFB samples were observed when discriminating between types of lncRNAs(Table 2). Specifically, a tendency towards increased expression in lncRNAs at 15 days AFB was observed in the intronic lncRNAs (234.96 RPKM in flowers to 336.53 RPKM at 15 days AFB, p-value = 0.0023) and intergenic lncRNAs (525.43 RPKM in flowers and 594.10 RPKM at 15 days AFB, p-value = 0.0399). However, no significant differences were found for the antisense lncRNAs (2,484.33 RPKM in flowers and 2,046.71 RPKM at 15 days AFB, p-value = 0.4484) (Table 2).

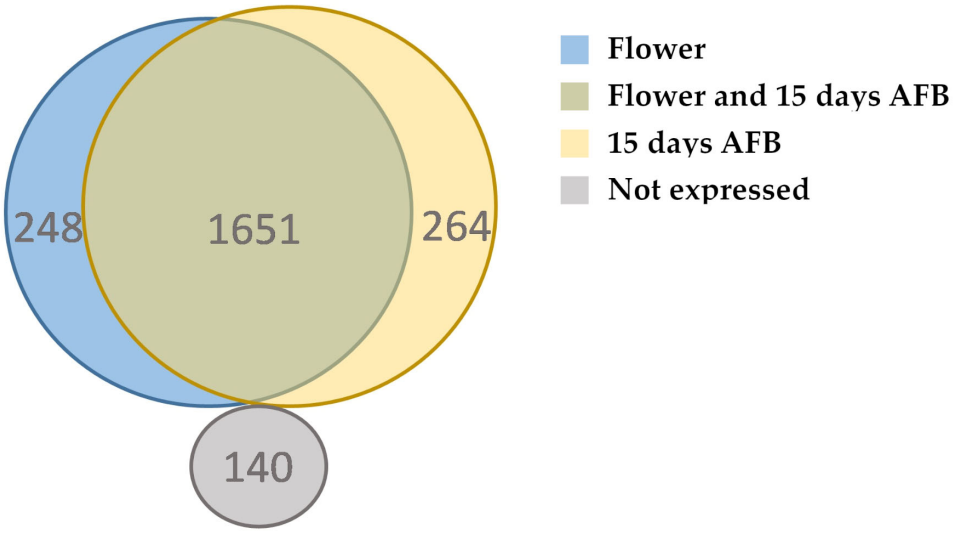

Figure 6. Venn diagram showing the expression pattern in flowers and developing fruits at 15 days AFB.

Table 2. lncRNA expression analysis in flowers and at 15 days AFB.

LncRNA type	lncRNAs		Average RPKMs		
	up regulated	down regulated	flower	15 days AFB	p-value
Intergenic	106	237	525.43	594.10	0.0399
Intronic	13	22	234.96	336.53	0.0023
Antisense	24	14	2484.33	2046.71	0.4484
Total	143	273	718.68	733.22	0.8365

Together, these data indicate that the changes in the gene expression level for the specific RNA pol IV and V subunits during fruit development were accompanied by important changes in the expression of lncRNAs, and these changes differed depending on the type of analysed lncRNA.

4. Discussion

The role of RNA pol IV and V in plants is still under study and is not fully understood. The olive tree is an important crop and is more complex than the model plant *A. thaliana*. In this work, we searched for the genes encoding the specific subunits of RNA pol IV and V, as well as the RNA pol II from which they have evolved. A comprehensive analysis of the expression profile of these genes was also performed, revealing an increase of mRNA expression for RNA pol IV and V subunits during fruit development. Furthermore, as RNA pol IV and V mediate lncRNA transcription, we

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analysed lncRNA transcriptome genome-wide and found a positive correlation. In concordance, our data suggest a role for RNA pol IV and V during fruit development through the expression of lncRNAs.

Several genes for the specific RNA pol IV and V subunits, and their RNA pol II counterparts, were identified by a blast-p search with the corresponding *A. thaliana* subunits [7,12,22]. Protein identity with *A. thaliana* homologues varied within the 40-55% range for RNA pol IV and V subunits, except for NRPDE2 with 73% identity (Figure 2). In contrast, the NRPB homologues exhibited notably higher identity levels (79-95%) indicating greater conservation compared to the NRPD and NRPE subunits that have allowed major variation during evolution. RNA pol IV and V have evolved from RNA pol II [4,5,22,62,63], and have apparently evolved more rapidly than the RNA pol II because their *A. thaliana* and *O. europaea* sequences have diverged more.

Several paralogues of the different specific RNA pol II, IV and V subunit genes were found for all the subunits, except for RNA pol IV/V subunit NRPDE2, which had only a single gene (Table 1). This was not the only case for the NRP1, NRP2, NRP4 and NRP7 subunits identified in this work, but also for an additional specific RNA pol V subunit (the previously described NRPE5) which has a paralogues for additional common subunits for all the RNA polymerases [28]. The presence of several paralogues is found for many genes in olive, as it is case for the RNA pol subunits shared by the five RNA pols [28]. These results are consistent with the olive cultivar genome that results from two independent whole-genome duplication (WGD) events, in addition to recent partial genome duplications [52,59]. In addition, several paralogues for RNA pols subunits have been identified in other organisms [1,5,22,25,64].

It has been demonstrated that RNA pol IV and V play a role in silencing, plant growth, development, response to environmental changes or plant immunity [4–12,22–24]. Therefore, we can speculate that the regulation of the gene expression of these RNA pols could be expected in response to some growth conditions. The transcriptomic analysis performed by several RNA-Seq experiments during stress or developing processes using the olive cultivar 'Picual' [50,51,61,65] has shown that all the genes coding for the specific subunits identified in this work are expressed under these conditions. Similarly, the genes corresponding to the RNA pol II subunits were also expressed. Although no common pattern for the changes in the expression of the RNA pol II, IV or V genes was observed, some cases of regulation by plant tissue were evident. Remarkably, two RNA pol II (NRPB7) genes presented strict organ specificity and were expressed only in flowers (Figures 3 and 4). Furthermore, the response to biotic *V. dahliae* infection [61] or abiotic stresses like cold [51] or root injury [61] showed null or weak changes in the expression profile of most genes.

However, and notably, clear gene regulation occurred for the RNA pol IV and V subunits during fruit development. A consistent and significant overexpression of the RNA pol IV and V genes was observed at the beginning of fruit development, contrasting with the behaviour of the RNA pol II subunits. This observation suggests a possible role of RNA pol IV and V during fruit development in agreement with the role of RNA pol IV and V during plant development and plant growth [12]. According to the role of RNA pol IV and V during the synthesis of ncRNAs, relevant changes in the lncRNAs expression pattern were observed. In line with this, the synthesis of the non-polyA lncRNAs has been demonstrated to be regulated by environmental conditions in *A. thaliana* under abiotic stress [43–45]. Indeed, we identified 2,303 lncRNAs (non-polyA) transcripts in flowers and fruit at 15 days AFB. The majority of these transcripts were intergenic, while intronic and antisense lncRNAs were less frequent (Figure 5). Relevant changes in expression were found, with 284 lncRNA transcripts expressed only in flowers and 264 only in fruit at 15 days AFB (Figure 6). Furthermore, a tendency toward an increasing expression was noted in the intronic and intergenic lncRNAs, although this was not observed in the antisense lncRNAs. Considering the role of RNA pol IV and V in the synthesis of the small ncRNAs involved in silencing, we cannot rule out that some of these lncRNAs could be processed to small ncRNAs, which has been reported for other plants [40,66]. Indeed this fact has been observed for intergenic lncRNAs in *A. thaliana* [40] and, in our case, in 39 intergenic transcripts in olive, which were also identified as putative siRNAs.

In summary, our study identified the genes that code for specific RNA IV and V subunits, and the corresponding ones in RNA pol II, in olive cultivar 'Picual'. The expression analysis performed of different organs/tissues, responses to biotic and abiotic stresses and of development process revealed that the expression of the RNA pol IV and V genes was induced during the early stages of fruit development. This induction was accompanied by relevant changes in the expression of lncRNAs, particularly an increase in the intergenic and intronic lncRNAs. These changes in the expression of lncRNAs may be important for controlling gene expression during fruit development. In addition, certain intergenic transcripts are susceptible to be processed and to become siRNAs, which are known to play a role in the gene expression control. This reinforces the hypothesis that RNA pol IV and V may contribute to the process of fruit development throughout the synthesis of lncRNAs.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Expression profile of genes coding for RNA pol II, IV and V subunits in response to root injury; Figure S2: Expression profile of genes coding for RNA pol II, IV and V subunits in response to the fungal infection by *V. dahliae*; Figure S3: Expression profile of genes coding for RNA pol II, IV and V subunits in response to cold stress; Figure S4: Sampling times for RNA-Seq analysis from recently bloomed flowers, developing fruit at 15 days after full bloom and every month from the flowering stage to fully ripe fruit;Table S1: Quality analysis of RNA-seq and reads alignment to the reference transcriptome of olive (*Olea europaea L.* cv. Picual).

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