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Differentially Expressed Transcription Factors during Male and Female Cone Development in *Pinus halepensis*

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Abstract: The molecular regulation of induction and development of reproductive organs is well established in angiosperms, while it is slowly accumulating in gymnosperms. Here, we conducted comparative transcriptomic profiles at different stages of male and female cone development in *Pinus halepensis*. Our aim was to reveal transcription factor encoding genes involved in reproductive induction and development. For accurate developmental stage identification, histological analysis preceded the molecular analysis. The flowering induction genes *FT/TFL* were expressed mainly at the late developmental stages of the male cone, suggesting involvement in vegetative bud dormancy instead of flowering induction. The male cone development was associated with the expression of the C-class *PhMADS2*, and the B-class genes *PhDAL11* and *PhDAL13*, while the female cone development was associated with the expression of the C-class *MADS1* and *DAL14* genes. This study adds valuable knowledge to the profile of transcription factors and MADS-box genes regulating cone development in gymnosperms.

Keywords: flowering induction; conifers; cone development; MADS-box; *Pinus halepensis*

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1. Introduction

Reproduction in plants includes induction and organ differentiation stages. The molecular regulation of these processes is well characterized in angiosperms compared to gymnosperms [1–5]. Yet, the current genomic tools permit a slow bridging of this gap and provide valuable knowledge to understand cone development in conifers.

Flowering induction in angiosperms is regulated by transcription factors, including LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL), and FRUITFUL (FUL) [1]. Other key regulators are genes belonging to the *FLOWERING LOCUS T* (*FT*)/*TERMINAL FLOWER 1* (*TFL1*) family [6,7]. The FT protein (also known as florigen) is produced in the leaves, then transported to the meristem, where it forms a complex with the FLOWERING LOCUS D (FD) transcription factor, to induce flowering. In contrast, TFL1 is produced in the meristem and forms a complex with FD that inhibits the expression of the flowering-promoting genes *LFY* and the MADS-box gene, *APETALA1* (*AP1*), preventing flowering induction [8,9]. *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), encoding a MADS-box transcription factor, is another key gene in flowering induction [2,10,11]. The photoperiodic regulation of *SOC1* is mediated by *CONSTANS* (*CO*), mainly through *FT*, which acts as an activator. SOC1 directly activates *LFY* and *AGL24*, a MADS-box transcription factor [2]. Genes encoding basic helix-loop-helix-type (bHLH) transcription factors are also related to flowering induction. These include *FLOWERING BHLH* (*FBH1-4*), which

together with *TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR* (*TCP*) positively regulate *CO* [3].

Studies on induction of reproductive organs in gymnosperms, specifically in the *Pinaceae*, indicate similar and dissimilar mechanisms to angiosperms [12]. Contrary to the one copy of *LFY* in angiosperms, gymnosperms possess two copies; *LFY* and *Needly* (*NLY*) [13–15]. Both are expressed in vegetative buds, male, and female developing cones [14,16–18]. However, the *Picea abies LFY* (*PaLFY*) mRNA levels are slightly increased prior to the female cone setting in the early-cone-setting mutant *acrocona* [18]. Similarly, the *Pinus caribaea LFY* homolog, *PcLFY*, is found to be expressed dominantly in the female cone in early developmental stages [19]. Furthermore, both *LFY* and *NLY* from *Pinus radiata* as well as *LFY* from *P. caribaea* largely rescued the Arabidopsis *lfy* mutant phenotype, suggesting a role in transition to the reproductive stage [15,17,19].

The gymnosperm homologous of *FT/TFL1* was identified in *Pinaceae* [20,21]. The expression levels of the *P. abies* homologs *PaFTL1* and *PaFTL2* reached their maximum level before growth cessation, suggesting a role in dormancy regulation. Moreover, expression of *FTL1* and *FTL2* from *P. abies* and *Pinus tabuliformis* in Arabidopsis inhibited floral transition, demonstrating the functional homology of these genes to the *FT/TFL1* in angiosperms [21,22]. A *SOC1* homolog of *P. abies*, *DEFICIENCE-AGAMOUS*-LIKE 19 (*DAL19*) was highly expressed in the *acrocona* mutant prior to cone set, suggesting a role in flower induction similarly to *SOC1* [18].

Following flower induction, the development of flower organs is regulated mainly by transcription factors from the MADS-box family [23]. The ABC model, later expanded to the ABCDE model, describes the interactions between gene groups that regulate the different floral organs development [5,24]. In Arabidopsis, overlapping expression of E-class genes, which include *SEPALLATA*1,2,3,4 (*SEP*1,2,3,4) and *AGL6*-like, and A-class genes, which include *AP1* and *APETALA2* (*AP2*), induce sepal formation; E-class together with B-class, which include *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and A-class induce petal formation; E-class together with B- and C-class, which include *AGAMOUS* (*AG*) genes, form stamens; E- and C-class induce carpels, while E- together with C- and D-classes are responsible for ovule formation [5,25].

Many genes homologous to angiosperm floral organ determining genes were found in gymnosperms [5,21]. It appears that B-class homologous genes, including the *P. radiata PrDGL* and *DAL11-13* from *P. abies* and *P. tabuliformis*, are expressed specifically in developing male cones [21,26,27]. Gymnosperm genes homologous to angiosperm C-class genes include *PrMADS1-3* in *P. radiata*, as well as *DAL2* in *P. abies* and *P. tabuliformis* [21,28,29]. The *DAL2* homologs in other conifers such as *Podocarpus reichei* and *Taxus globose* exhibit female cone expression [30]. Interestingly, the *DAL10* MADS-box gene from *P. abies*, which do not show homology to any known angiosperm gene, exhibits a male specific function similar to that of B-class genes [31]. The overexpression of the *P. tabuliformis DAL10* homolog in Arabidopsis resulted in very early flowering induction, suggesting also a role in the transition to the reproductive phase in gymnosperms [21].

According to the gymnosperms model, overlapping C- and B-class gene expression results in male cone development, while the expression of only C-class genes causes female cone development. In both cases, the tetramers contain A class proteins [5]. A recent study revealed several physical interactions among MADS-box proteins in *P. tabuliformis*, supporting the hypothesis of the requirement for tetramerization of MADS-box proteins [21]. Furthermore, the large number of MADS-box homologous genes in conifers suggest complex regulatory interaction.

P. halepensis is a monoecious species (bearing male and female cones on the same tree), which begins its reproductive phase with the production of female cones approximately at the age of five years. Once the tree grows sufficiently in size, it begins to form male cones as well, usually one to two years later [32,33]. An early female cone development and a spatial distribution of the cones on a mature tree (female cones at the upper part and male cones at the middle and lower part, Supplementary Figure S1) facilitate outcrossing, ensuring

high genetic diversity [32]. Female cone buds occur in a lateral position on a vegetative shoot bud. The female cone contains ovule-bearing scales (megasporophylls) attached to bracts and arranged spirally on a central axis, while the male cones are organized in clusters around a central axis (Figure 1). Vegetative to reproductive transition occurs in October–November depending on the provenance and environmental conditions, and cone development continues until March when pollination takes place [34].

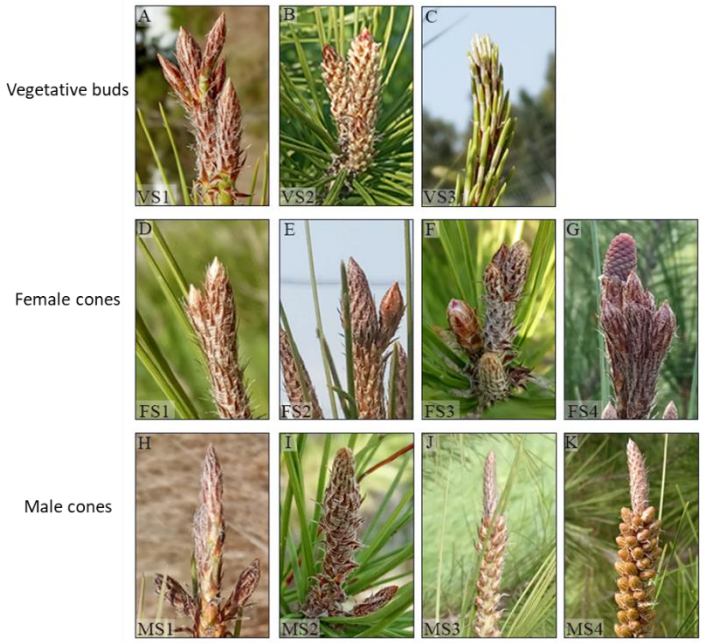

Figure 1. Developmental stages of vegetative buds, and female and male cones in *P. halepensis*. (**A**–**C**) Vegetative buds at stages 1–3. (**A**) VS1, Early vegetative buds. (**B**) VS2, Dwarf shoots are visible. (**C**) VS3, Needles are visible. (**D**–**G**) Female cones. (**D**) FS1, Tightly enclosed bud. (**E**) FS2, Elongated and swelled bud. (**F**) FS3, Cone emergence through the top of the scales. (**G**) FS4, A visible cone. (**H**–**K**) Male cones. (**H**) MS1, Tightly enclosed bud. (**I**) MS2, Apparent buds. (**J**) MS3, Cone emergence from the scales. (**K**) MS4, Fully developed cones.

To gain knowledge on the molecular regulation of the reproductive phase of *P. halepensis*, we analyzed differentially expressed genes encoding for transcription factors at different stages of male and female cones development. The transcriptomic analysis was accompanied by histological analysis to achieve an accurate developmental stage identification. We identified several TF families that were dominantly expressed in either female or male cones, and also showed the cone specific expression of several MADS-box genes.

2. Materials and Methods

2.1. Plant Material

Plant material was collected from *Pinus halepensis* trees that grow at the Volcani Center (31.9872, 34.8212, Rishon LeZion, Israel). For transcriptome analysis, samples (13–18 shoot apices from 3–5 trees) were collected every week from December 2019 to March 2020 between 10:00 and 11:00 a.m. to minimize the effect of the circadian rhythm. Each shoot apex was immediately cut longitudinally into two parts. One part was frozen in liquid nitrogen and stored at −80 °C for mRNA extraction and sequencing, while the other half was fixated in formaldehyde-acetic acid–alcohol (FAA, 10:5:50 in double-distilled water) solution for histological analysis. The aim of the histological analysis was to determine the developmental stage and meristem identity (male/female/vegetative) of each collected sample.

For candidate gene analysis, samples were collected in a similar way from October 2020 to February 2021.

2.2. Histological Analysis

Samples in FAA were put in a vacuum chamber for 15 min and were stored overnight at 4 °C. Following dehydration in an ethanol series (70, 80, 90, and 100%, 30 min each), the samples went through a gradual Histoclear solution change up to 100% Histoclear. Overnight incubation at room temperature with Paraplast chips (Leica, Wetzlar, Germany, Paraplast Plus) was followed by several hours of incubation at 42 °C. Dissolved pure paraffin was changed twice a day for four days at 62 °C oven. The samples were embedded in blocks, sectioned into 10 µm width longitudinal sections (using Leica, RM2245 Microtome), and mounted on slides, which were incubated overnight on a 40 °C plate. The sections were then stained with Fast Green and Safranin [35], observed in a light microscope (Nikon Eclipse Ni-E), and images were taken using DS-Ri2 camera (Nikon, Tokyo, Japan).

2.3. Sample Selection for RNA Sequencing

Following histological analysis, terminal buds were chosen for mRNA sequencing according to their developmental stage [36] (Table 1). All developmental stages were identified, except for stage 1 for vegetative, male, and female, which was absent (probably due to late sampling). For each stage, we included three biological replicates.

Table 1. Developmental stages of vegetative shoot, and male and female buds.

Meristem	Stage	Description
Vegetative	1 *	Undifferentiated lateral meristems.
	2	Differentiated non-visible needles.
	3	Visible elongated needles.
Male	1 *	Male flowering bud clearly identified.
	2	Differentiated microsporophyll.
	3	Developed pollen sacs with a tapetal layer on the edges, containing pollen mother cells.
Female	1 *	Female flowering bud (ovulate cone) clearly identified.
	2	Bract differentiation, ovuliferous scales are not differentiated.
	3	Ovuliferous scales are at early differentiation.
	4	Ovuliferous scales are differentiated, ovules are visible.

* Stages that were missing from the collected samples for RNA-seq.

2.4. RNA Isolation, Library Preparation and Sequencing

The selected buds were ground with liquid nitrogen, using a mortar and pestle. Total RNA was extracted with SpectrumTM Plant Total RNA Kit (Sigma, St Louis, MO, USA) following the manufacturer's instructions, and the RNA quantity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA samples were sent to Macrogen Inc. (Amsterdam, the Netherlands) for the following sequencing procedure: RNA quality analysis using the 2200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA), mRNA enrichment, library preparation using Illumina TruSeq RNA Library v2 kit, and 100 bp paired end sequencing by Illumina NovaSeq (Illumina, San Diego, CA, USA).

2.5. Transcriptome Analysis: Assembly and Differential Expression Analysis

A transcriptome database was de novo assembled using Trinity software (version: v2.1.1) [37] based on the sequences derived from the RNA sequencing. The transcript quantification from the RNA-Seq data was performed using the Bowtie2 aligner [38] and the expectation maximization method (RSEM) by estimating maximum likelihood expression levels [39] via the perl script align_and_estimate_abundance.pl with –est_method RSEM from Trinity protocol [40]. According to the sample correlation matrix (Supplementary Figure S2), three samples showed expression patterns which were not sufficiently correlated with their equivalent biological replicates. These samples (Male stage 3 replicate 1, Female

stage 2 replicate 2, and Vegetative stage 1 replicate 1) were excluded from further analyses. Differential expression analyses were done using the edgeR R package [41].

2.6. Sequence Similarity and Functional Annotation

The merged transcriptome was used as a query term for a search of the NCBI non-redundant (nr) protein database that was carried out with the DIAMOND program [42]. Homologous sequences were also identified by searching the Swiss-Prot database with the BLASTx tool [43] and an E-value threshold of 10^{-5}. The search results were imported into Blast2GO version 4.0 [44] for gene ontology (GO) assignments. Enzyme codes and KEGG pathway annotations were based on the mapping of GO terms to their enzyme codes. The KAAS tool (Kegg Automatic Annotation Server; http://www.genome.jp/tools/kaas/, accessed on 1 July 2021) was used for KEGG orthology and KEGG pathway assignments. Gene ontology enrichment analysis was carried out using the Blast2GO program based on Fisher's exact test [45] with multiple testing correction of false discovery rate (FDR) [46]. The threshold was set as FDR with a corrected *p*-value of less than 0.05. Gene ontology analysis was done by comparing the GO terms in the test sample to the GO terms in a background reference. Every comparison had hundreds to thousands of DE genes. In order to focus on transcription factors, the differentially expressed (DE) genes were blasted against the transcription factors database PlantFTDB (http://planttfdb.gao-lab.org/, accessed on 1 September 2021). In addition, the homologs of the MADS-box genes were isolated from the identified DE transcription factors.

2.7. Phylogenetic Analysis

A phylogenetic tree of the MADS-box transcription factors was constructed with MEGA X [47] using the maximum likelihood method. In silico translated amino acid sequences were used for this analysis. One isoform was selected from each gene, according to the highest protein annotation score. The *P. halepensis* proteins were aligned using the MUCSLE algorithm [48] with MADS-box protein sequences from Arabidopsis (retrieved from The Arabidopsis Information Resource, TAIR) and other conifer MADS-box proteins (retrieved from UniProt, https://www.uniprot.org/, accessed on 1 September 2021). The conserved MADS and K domains together with the linker between them were used for the alignment. A thousand replicates were used for bootstrapping. The evolutionary history was inferred by using the maximum likelihood method and JTT matrix-based model [49]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with a superior log likelihood value.

2.8. Quantitative Polymerase Chain Reaction (qPCR) Analysis

Candidate genes were analyzed by qPCR in samples that were collected in the second year (see above). Primers were designed to amplify 70–200 bp segment of each candidate gene (Supplementary Table S1). Each type of bud at each developmental stage was represented by three to four biological replicates, with two technical replicates for each. Since sample collection for the qPCR analysis started earlier in the second year than in the first year, it included earlier developmental stages; 1, 3 and 4 for female, and 2 and 3 for male. RNA was extracted as described above from all the samples and diluted equally to reach a concentration of 1000 ng/µL. DNA was degraded using RQ1 RNase-Free DNase (Promega, Madison, WI, USA). cDNA was synthesized using the qScript cDNA Synthesis Kit (QIAGEN, Hilden, Germany). Finally, qPCR was done using ABsolute Blue QPCR Mix, SYBR Green, ROX (Thermo Scientific™, Waltham, MA, USA). The expression level of the chosen genes was normalized according to the expression levels of the *P. halepensis* homologs of *UPL6* (E3 ubiquitin-protein ligase) TRINITY_DN211436_c2_g2_i2. This gene showed little variation in expression levels between the different bud types, suggesting reliability for qPCR relative expression levels normalization. The relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method [50], using the housekeeping gene CT

values, and the average CT values of vegetative stage 1 tissues, defined as the control group. The derived values were converted to its logarithmic base 2 for stabilization of variances.

3. Results

3.1. Phenotypic Characterization of the Developmental Stages

To gain insights into the genes involved in reproduction in *P. halepensis*, we collected the three different bud types, vegetative, female and male at different developmental stages (Figure 1), for RNA-seq, and a year later for qPCR analysis as described in Material and Methods. The buds presented in Figure 1 and the histology analysis in Figure 2 represent all stages identified in this study.

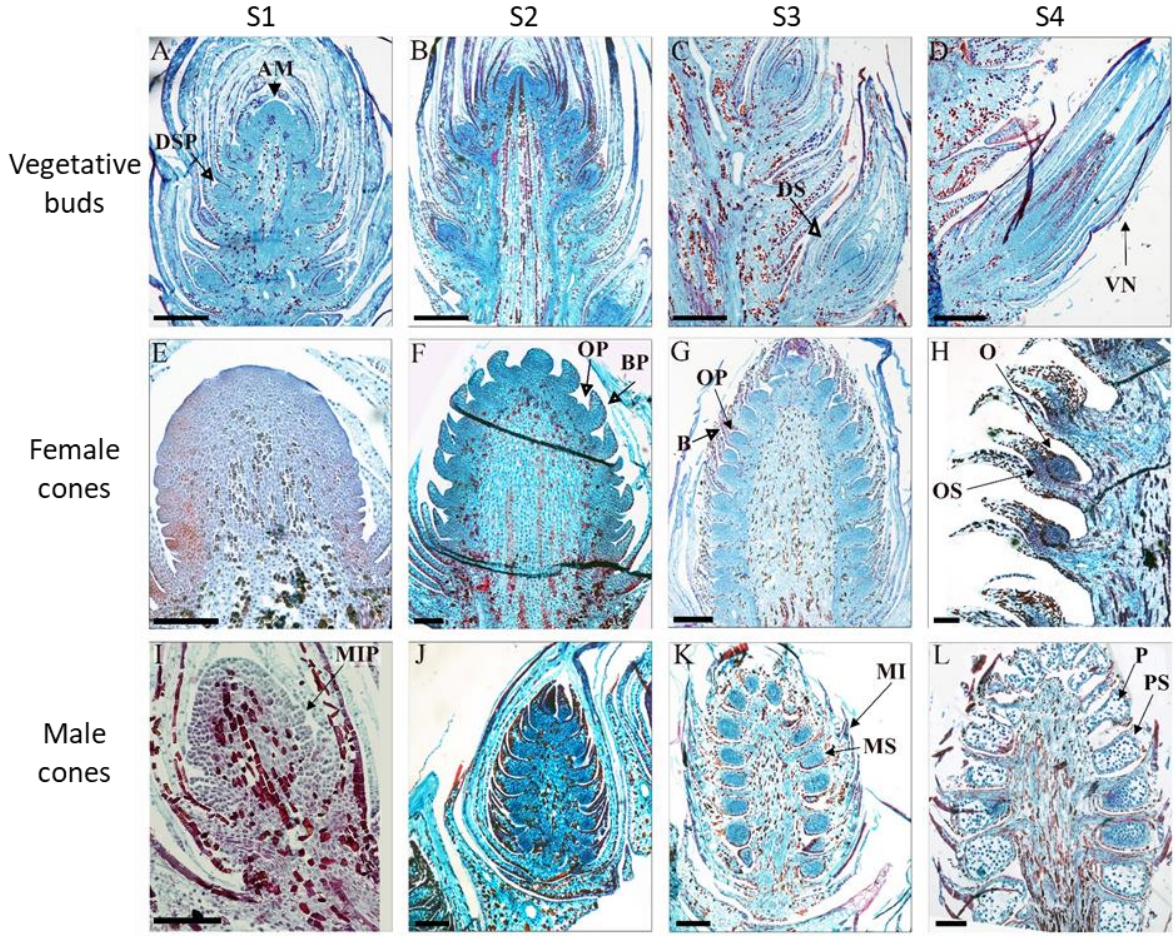

Figure 2. Longitudinal sections of vegetative, female and male *P. halepensis* buds at different developmental stages. (**A**–**D**) Vegetative buds developmental stages. (**A**) S1, Dwarf shoot (Brachyblasts) primordia (DSP) with apical meristem (AM). (**B**) (S2) and (**C**) (S3), Dwarf shoots (DS) emerge through the scales. (**D**) S4, Visible needles (VN). (**E**–**H**) Female cones developmental stages. (**E**) S1, Bulge-like structure enclosed within the scales with few lateral bract anlagen. (**F**) S2, bract primordia (BP) and ovuliferous primordia (OP). (**G**) S3, Bracts (B) are fully developed covering the developing ovuliferous scales. (**H**) S4, bract and ovuliferous scale (OS) complex with developing ovule (O). (**I**–**L**) Male cones developmental stages. (**I**) S1, Lateral microsporophyll primordia (MIP). (**J**) S2, Young cones hidden within the scales, contains developing microsporophyll. (**K**) S3, Cone emergence from bud scales and the microsporophyll (MI) contains microsporangium (MS). (**L**) S4, fully developed cones with pollen sacs (PS) and pollen mother cells (P). Scale Bar, (**A**–**E**,**I**) 500 μM, (**F**–**H**,**J**–**L**) 250 μM.

At their early developmental stage, the vegetative, female and male closed buds look similar (Figure 1A,D,H). Soon after the buds elongate and swell, their different morphology

becomes clearly visible (Figure 1B,E,I). The vegetative shoot develops needles, which further elongate (Figure 1C), and the female cone emerges through the top of the scales (Figure 1F,G), while the many small male cones emerge from the bud scales to be ready for pollen release (Figure 1J,K).

3.2. Histological Analysis

Histological analysis that was performed on half of each sample (see Materials and Method) revealed the developmental stages of the three different bud types (Figure 2). In early developmental stage, the lateral meristems of the vegetative bud are still not fully developed and enclosed within the bud scales, this stage was called "vegetative stage 1" (VS1). At stage 2 (VS2), the lateral meristems are apparent, and the developing brachyblasts (dwarf shoots) are growing outside the bud scales. At stage 3 (VS3), the bud is fully developed and the elongated needles are visible.

At stage 1 of the female cone developmental (FS1), a huge swollen structure with a few lateral primordial bracts appear, enclosed within the bud scales. At stage 2 (FS2), the bud is enlarged in size, the bract primordia are elongated, and the ovuliferous scale anlagen becomes visible. During stage 3 (FS3), developed bracts cover the developing ovuliferous scales. At stage 4 (FS4), the cone is fully developed with ovules, ready to be pollinated. The developmental stages presented in Figure 2 for the female cone are consistent with the previously defined stages [51].

The first stage of the male cone development is characterized by lateral microsporophyll primordia. In the second stage (MS2), a swollen structure, hidden within the bud scales, contains developing microsporophyll. At MS3, the cones are emerged from the bud scales, and the microsporophyll contains a microsporangium (pollen sac), with pollen mother cells surrounded by a tapetal layer. In the final stage of male cone development (MS4), the cone contains fully developed pollen grains ready to be carried by wind for pollination.

3.3. Transcriptome Analysis of the Three Bud Types at Early and Late Differentiation Stages

Based on the developmental stages determined by the histology analysis in the first year, we realized that we started our sampling a little late. Thus, the selected buds for RNA-seq represented stages 2 and 3 for vegetative bud (VS2 and VS3) and male cone (MS2 and MS3), and stages two to four for the female cone (FS2-FS4). Twenty-one cDNA libraries yielded an average of 44 million 100 bp paired-end reads, which were assembled using Trinity as described in Material and Methods. The transcriptome catalog contained 546,815 transcripts corresponding to 414,456 genes. The average contig length was 735.42 bp, with an N50 size of 1487 bp corresponding to a total length of 402 Mbp. Annotating the transcriptome catalog resulted in 81,521 contigs. Differential gene expression analyses between the three buds, both at early and late developmental stages, revealed that the number of DE genes between male and vegetative types is the highest, whereas between the female and the vegetative types is the lowest (Table 2, full list of genes in Supplementary Table S2). Moreover, more genes were DE at late developmental stages than in early developmental stages (Table 2), reflecting the advanced differentiation of each organ. Comparison between stage 2 and stage 3 of each bud type revealed that the vegetative bud exhibits the smallest number of DE between the two stages, whereas the male cones exhibited the largest number of DE genes. A similar number of genes were up- and down-regulated between stage 2 and stage 3 in all three bud types (Table 2). Shared expressed genes between the three developmental stages of the female cone are presented in Figure 3. It appears that 649 genes were uniquely up-regulated in developmental FS3 compared to FS4, suggesting that at the latter stage, some processes were completed.

Table 2. Number of differentially expressed genes between the different bud types, and between the developmental stages in each bud type.

	Comparison	Up-Regulated	Down-Regulated	Total
Early developmental Stage	FS2 vs. MS2	417	405	822
	FS2 vs. VS2	92	88	180
	MS2 vs. VS2	519	689	1208
Late developmental Stage	FS3 vs. MS3	2382	2115	4497
	FS3 vs. VS3	1020	1300	2320
	MS3 vs. VS3	2042	2521	5563
Vegetative bud development	VS2 vs. VS3	139	224	363
Male cone development	MS2 vs. MS3	2200	2155	4355
Female cone development	FS2 vs. FS3	558	738	1296
	FS2 vs. FS4	1219	1023	2242
	FS3 vs. FS4	1283	832	2115

S, developmental stage; F, female; M, male; V, vegetative; DE, differentially expressed.

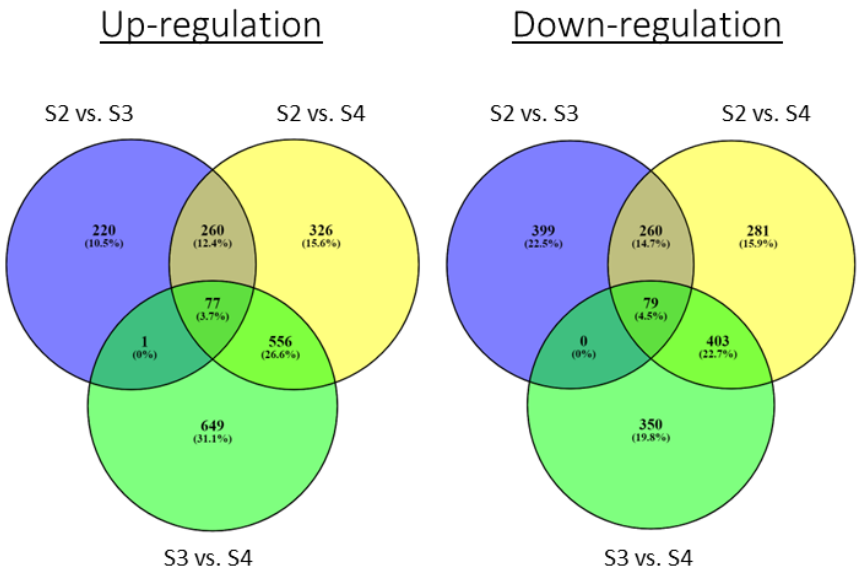

Figure 3. Venn diagram of up-regulated and down-regulated genes showing shared genes between the female cone three developmental stages. S2–S4, developmental stages.

3.4. Transcription Factors Families Involved in Early and Late Stages of Cone Development

To identify transcription factors related to cone development, we blasted the DE genes against the transcription factors database PlantFTDB (http://planttfdb.gao-lab.org/, accessed on 1 September 2021) and grouped the genes into TF families (Figure 4). Included in Figure 4 are TF families with at least three members differentially expressed in at least one comparison. The number of TFs that were DE during the male cone development was dramatically higher than DE TFs during the female cone development (Figure 4). For female cone development, more TFs were up-regulated in the late than in the early developmental stages. The most represented TF families (>10 members in both stages) in the male cone were bHLH, ERF, MYB-related, NAC, and MIKC-MADS (Figure 4). The MYB-related family was highly represented in the late female stages, while the ERF, bHLH, and MYB families were highly represented in the most advanced developmental stage, FS4. Interestingly, the HD-ZIP family was dominantly expressed at MS2 with 15 family members. In addition, male cone development exhibited TFs (GRF, HB-other, bZIP, ZF-HD, NF-YC, FAR1, and Nin-like) that were not DE during the female or vegetative bud development. MICK_MADS and M-type_MADS were enriched in the early and late stages of male and female cone development (Figure 4).

a Up -Regulated TFs at Stage 2

TF Family	MS2/MS3	FS2/FS3	FS2/FS4	VS2/VS3
bHLH	21	2	13	0
ERF	16	6	17	1
MYB_related	15	3	9	0
HD-ZIP	15	1	2	0
NAC	13	5	9	0
MIKC_MADS	10	4	9	1
C2H2	9	4	6	1
LBD	9	2	8	1
NF-YC	8	0	2	0
MYB	8	2	12	1
C3H	8	1	3	0
WRKY	7	1	9	0
Trihelix	7	0	1	1
G2-like	7	2	3	0
FAR1	6	1	2	0
ARF	5	1	1	0
GeBP	5	1	1	1
* GRF	5	0	0	0
HSF	4	2	1	0
M-type_MADS	4	1	7	0
AP2	4	2	5	0
* HB-other	4	0	0	0
* bZIP	3	0	0	0
WOX	3	0	1	0
* ZF-HD	3	0	0	0
B3	3	0	1	0
YABBY	3	0	4	0
NF-YB	3	0	2	0
Total :	208	41	128	7

b Up -Regulated TFs at Stage 3

TF Family	MS3/MS2	FS3/FS2	FS4/FS2	VS3/VS2
ERF	24	6	27	1
bHLH	24	7	10	1
MYB_related	22	12	14	2
NAC	18	5	8	0
MYB	16	6	12	0
C2H2	16	3	4	1
LBD	15	4	5	0
MIKC_MADS	14	4	4	0
Trihelix	12	4	2	0
M-type_MADS	8	3	6	0
C3H	8	0	2	0
WRKY	7	4	7	1
bZIP	7	2	6	0
GATA	7	0	2	1
* NF-YC	6	0	0	0
B3	5	2	1	0
TCP	4	1	1	0
* FAR1	4	0	0	0
ZF-HD	4	4	2	1
ARF	3	0	2	1
* Nin-like	3	0	0	0
HD-ZIP	3	6	3	0
YABBY	3	0	1	0
AP2	3	2	1	0
CO-like	3	1	1	0
GRAS	3	0	2	0
NF-YB	1	0	5	6
Total :	243	76	128	15

Figure 4. Heat-map of up-regulated transcription factors (TF) grouped into families involved in cone development. Included are families which showed at least three up-regulated members in at least one meristem type in the early (**a**) and late (**b**) stages of cone development. Color intensity of the heat-map reflects the number of genes. Common families between the early and late stages share the same color, while uncommon families are not colored. Asterisks indicate TF families found only in the male cone. MS2, Male Stage 2; MS3, Male Stage 3; FS2, Female Stage 2; FS3, Female Stage 3; FS4, Female Stage 4; VS2, Vegetative Stage 2; VS3, Vegetative Stage 3.

3.5. Differentially Expressed TFs Involved in Flowering Induction

The *LFY* homolog gene (TRINITY_DN212345_c0_g2) exhibiting 97.5% similarity to the *P. radiata NLY* (Accession U76757) had higher expression level in MS2 compared to MS3 (log2FC = 2.48, Supplementary Table S2). Except for that, *LFY* homologs did not show differential expression between meristem types or developmental stages.

Two *FT/TFL1* homologous contigs (TRINITY_DN197941_c0_g2 and TRINITY_DN19 7941_c0_g3) were up-regulated in the male cones at S3, compared to vegetative buds at S3 (log2FC = 10.33 and 5.75, respectively), and also up-regulated in MS3 compared to MS2 (log2FC = 4.8 and 10.5, respectively). Both contigs exhibit high similarity (93% and 97%, respectably) to the *Pinus armandii* FT/TFL protein. TRINITY_DN197941_c0_g3 (*FT/TFL1*) was found to decline in female cones as development proceeded. In addition, a MOTHER OF FT and TFL1-like homolog (MFT) (TRINITY_DN203996_c0_g3) was highly expressed at S3 of the female cone (Supplementary Table S2, Figure S3).

From the bHLH family, 31 TFs were identified as DE among the six comparisons between different bud types at early and late developmental stages (Figure 5). DE *bHLH* TFs were more pronounced in the male than the female cones. Early differentiated female cones had one up-regulated gene, a MYC2–like homolog. Early differentiated male cones had seven up-regulated genes, including homologous to *HEC2*, *EAT1* and *bHLH63*. Three down-regulated genes in early differentiated male cones included homologs of *ABORTED MICROSPORES*, *bHLH112* and *bHLH16*. Late differentiated female cones had two up-regulated genes (*bHLH094*, *bHLH49* homologs) and one down-regulated gene (*BHLH140*). Like MADS-box genes, the most DE bHLH TFs were up-regulated in late differentiated male cones. Substantial down-regulation of the *bHLH041* homolog was observed in late differentiated male cones (Figure 5).

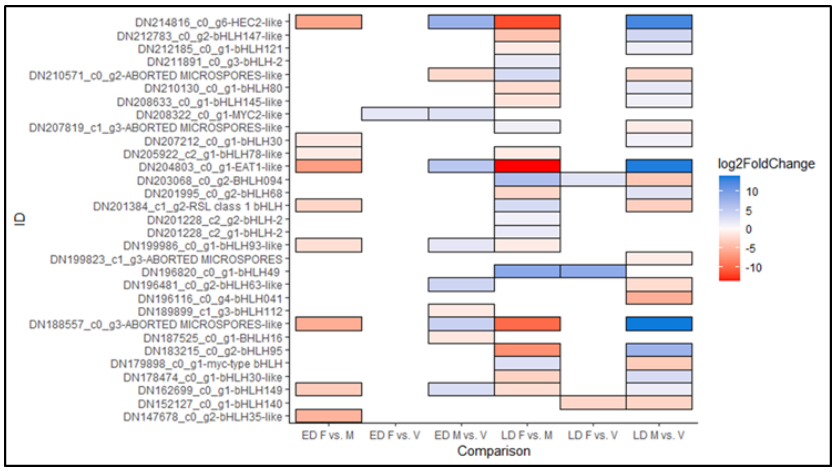

Figure 5. *bHLH* TFs differentially expressed in six comparisons. Gene ID is composed of the TRINITY accession ID, followed by NCBI BLASTP derived homolog. ED, Early development (Stage 2); LD, Late development (Stage 3); F, female; V, vegetative; M, male.

3.6. Differentially Expressed TF Involved in Female Cone Differentiation

Altogether, 25 MADS-box TFs were identified as DE in six comparisons (Figure 6). Three of them, homologs of *DAL3* were up-regulated in early differentiating female cones. Late differentiating female tissues had six up-regulated genes, including *DAL14*, *MADS1*, *MADS2* and *MADS5* homologous genes. Those tissues had five down-regulated genes as well, among them are *DAL13*, *AGL1* and *SOC1* homologous genes, together with two putative MADS-box genes.

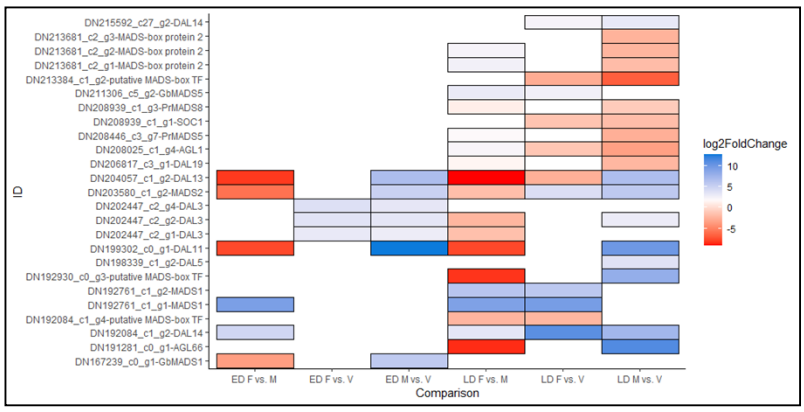

Figure 6. MADS-box TFs differentially expressed in six comparisons. Gene ID is composed of the TRINITY accession ID, followed by NCBI BLASTP derived homolog gene. ED, Early development (Stage 2); LD, Late development (Stage 3); F, female; V, vegetative; M, male.

3.7. Differentially Expressed TF Involved in Male Cone Differentiation

Seven genes, including homologs of *MADS1*, *MADS2*, *DAL3*, *DAL11* and *DAL13*, were up-regulated in early differentiating male cones. Late differentiated male cones were the most prominent bud type in terms of the magnitude of DE MADS-box genes, with nine up-regulated genes (*DAL3*, *DAL5*, *DAL11*, *DAL13*, *DAL14*, *MADS2*, *AGL66*, and a putative MADS-box homologous gene), and seven down-regulated genes (*MADS-box 2*, *MADS8*, *SOC1*, *MADS5*, *AGL1*, *DAL19*, and a putative MADS-box homologous gene).

3.8. Phylogenetic Analysis

The importance of MADS-box genes in flowering regulation as well as other developmental processes, led us to focus on those genes for further analysis. We found that most of MADS-box homologs had several isoforms. Thus, we generated a phylogenetic

tree of the 25 identified MADS-box TFs together with homologs from Arabidopsis and representative gymnosperms, including *P. radiata*, *Ginkgo biloba*, and *P. abies*. The 25 TFs were divided into 14 different clades, while the clade containing the higher number of *P. halepensis* MADS-box proteins was the sister clade of the Arabidopsis *SOC1* TF, which included the *P. abies* proteins *DAL3* and *DAL19* as well (Supplementary Figure S4).

3.9. Differentially Expressed Gene Validation

Eight MADS-box genes were chosen for further characterization by qPCR. We chose them due to their potential role in cone development manifested by their up-regulation in at least one developmental stage (Figure 6; Supplementary Table S3). Each gene was represented by its most expressed isoform. The selected genes were named according to their closest coniferous homologous inferred from their closest NCBI BLASTP results, as well as the derived phylogenetic tree.

Homogeny of variances between the different bud types was determined by the Bartlett test. We found the variances to be unequal in *PhDAL11* and *PhMADS1*. Therefore, significant differences in *PhDAL11* and *PhMADS1* were determined by six different t-tests, under the assumption of unequal variances. Bonferroni correction was applied in *PhMADS1* and *PhDAL11* resulting in α values of 0.0083. The Tukey–Kramer HSD test supported significant differences found by the independent t-tests. Therefore, the differences in relative expression levels of all genes were determined using the Tukey–Kramer HSD test.

The qPCR showed that *PhDAL11* and *PhDAL13* were highly expressed across the male cone developmental stages (Figure 7). *PhDAL14*, *PhMADS1*, and *PhMADS2* were highly expressed across the female cone developmental stages; however, they were also highly expressed at late developmental stages of the male cone. The expression of *PhMADS1* was increasing gradually throughout the female cones development, while *PhMADS2* showed a weak correlation with cone development. The expression patterns of all genes described above, corresponded to the RNA-seq results (Figure 6, Supplementary Table S3). However, the expression patterns of *PhDAL3.1*, *PhDAL3.2*, and *PhMADS5* were not consistent with the RNA-seq results (Figure 7).

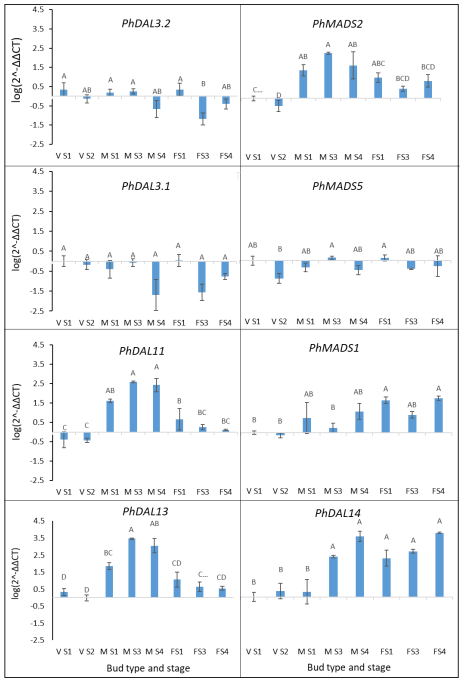

Figure 7. Relative expression levels of eight MADS-box genes in vegetative buds, male, and female cones during several developmental stages. Uppercase letters indicate significant differences ($p < 0.05$). Bars represent standard errors. VS1, Vegetative Stage 1; VS2, Vegetative Stage 2; MS1, Male Stage 1; MS3, Male Stage 3; FS1, Female Stage 1; FS3, Female Stage 3; FS4, Female Stage 4.

4. Discussion

This study describes the involvement of transcription factors in the induction and development of the male and female cones in *P. halepensis*. Identification of the different developmental stages through histology prior to the molecular analysis allowed us to identify differentially expressed TFs at early and late developmental stages. This study enriches our knowledge on the regulation of reproductive development in gymnosperms, and particularly in pines.

By analyzing the transcriptome of vegetative, male, and female tissues at different developmental stages, we found that the higher number of DE genes correlated with the male tissues (Table 2). That may be attributed to a more complexed regulation of male compared to female cone development [5]. Male cone development follows the BC model, according to which expression of both B- and C-class TFs are required, whereas female cone development requires solely C-class TFs [5]. DE genes were also prominent in all tissues in late compared to early developmental stages, suggesting that cone structure and tissue specification at late developmental stages are complex.

4.1. Reproductive Development Is Accompanied by Enriched TF Expression

TFs that were DE in early and late cone developmental stages were enriched in the five families bHLH, ERF, MYB-related, NAC, and MIKC-MADS (Figure 4). These TF families are essential in many processes throughout the plant's life cycle. In cone development, the bHLH family was prominent with more than 20 genes in early and late male cone development stages. This suggests that *bHLH* genes may have a significant regulatory role in reproductive organ development in conifers, similar to what was previously described in Arabidopsis [3]. A study on Arabidopsis and the liverwort *Marchantia polymorpha* showed that *bHLH* genes are crucial for the formation of generative cells that give rise to the male gametes, suggesting that *bHLH* may be key regulators of reproductive development across plant species [52]. Recently, it was shown that the *bHLH* expression in *P. tabuliformis* is age dependent, suggesting a role in the vegetative growth transition [53]. In light of these reports, the current study results, which suggest massive involvement of bHLH genes in the reproductive stage, encourage additional studies in conifers. Another noticeable TF family is the ERF, a subfamily within the AP2/ERF super-family [54]. We found that most *ERF* genes were up-regulated in late female cone development (Figure 4). *AP2* homologs, *AP2L1* and *AP2L2*, were identified in *P. abies* and *Pinus thunbergii*. *AP2L1* was dominantly expressed in the female cone, whereas *AP2L2* was expressed in both cone types in both species [55,56]. In addition, *PaAP2L2* was able to rescue the *ap2-1* Arabidopsis mutant phenotype [57]. These functional studies imply that ERF TFs in *P. halepensis* likely possess *AP2/ERF* homologs regulating late stages of female cone development.

4.2. Floral Induction Homologous Genes

Unexpectedly, we found only one *LFY* homolog, which was DE in the male cone tissue only. Its expression level was higher at stage 2 compared to stage 3. This result is inconsistent with previous studies that showed high expression of *LFY* and *NLY* in early stages of male and female cone development [58]. It has been shown that *NLY* is expressed in reproductive as well as in vegetative tissue in pines. However, its capacity to rescue the Arabidopsis *lfy* mutant implies that it also plays a role in flowering induction [17]. In the current study, the absence of *LFY* homologs in the DE genes may be due to late tissue collection for the RNA-seq analysis, after the expression peak of *LFY* and *NLY*.

The expression patterns of the two *FT/TFL1* genes imply a role in late male cone development (Figure S3A). One of those genes (TRINITY_DN197941_c0_g3) had the highest expression level at FS2 and then declined as development progressed (Figure S3B). The *FT/TFL* homologs exhibited a similar expression level in vegetative and female buds. Yet, an additional *FT/TFL* homolog (a MOTHER OF FT and *TFL1*-like, Table S2) was DE between the three developmental stages of the female cone, with highest expression level seen at stage 3, suggesting that *FT/TFL* may have a role in female cone development. The opposite

expression pattern of the *FT/TFL1* homologs in female and male cones indicates a different function, which may be antagonistic. It has been shown that *FT/TFL1* homologs from conifers act similarly to *TFL1* in repressing instead of promoting flowering, and do not seem to have a major role in the reproductive development of pines [20,22]. A recent publication reporting on age-related gene expression in *Pinus tabuliformis* suggests that interactions between the MADS-box genes *MADS11* and *DAL1* mediate flowering induction in pines [53]. The same study showed that the over-expression of *PtMADS11* in Arabidopsis rescued the late flowering *ft* phenotype, implying a role in flowering induction [53]. Since in our study we did not include pre-cone differentiation stages in the RNA-seq analysis, we could not confirm that *MADS11* and *DAL1* are involved in the flowering induction in *P. halepensis*.

The *PhDAL3.1* and *PhDAL3.2* genes exhibited high expression level during early differentiation stages of both cone types. The closest homologous gene of *PhDAL3.1* and *PhDAL3.2* is *DAL3*, which belongs to an orthologous sister clade and thus to the entire angiosperm SOC1 clade [58]. Our phylogenetic analysis also classified the *P. halepensis* DAL3 homologs in a sister clade of the SOC1 clade, together with the *P. abies* DAL3 (Figure S4). The central role of SOC1 in the transition to flowering in Arabidopsis through multiple pathways [2] also implies a central role for *PhDAL3.1*, *PhDAL3.2* in pine, assuming functional similarity between angiosperms and gymnosperms. The qPCR results showed no difference in *PhDAL3.1*, *PhDAL3.2* expression between both cone types and the vegetative buds, in all developmental stages. The only significant difference observed was a lower expression of *PhDAL3.2* in FS3 cones. Therefore, our data suggest a role for *PhDAL3.1* and *PhDAL3.2* in the development of all bud types, including vegetative growth.

Although the RNA-seq data showed that *PhMADS5* is up-regulated in late developing female cones, the qPCR results found no difference in the expression levels of this gene in any specific tissue (Figure 7). The closest Arabidopsis homolog of *PhMADS5* is *SHORT VEGETATIVE PHASE* (*SVP*), involved in flowering time regulation and transition to flowering [59,60]. Like *SOC1* orthologs, *SVP* is a multiple copies family in gymnosperms [61], which indicates that like *SOC1*, different *SVP* paralogs may have a different role in developmental processes in conifers.

4.3. Genes Involved in Male and Female Cone Development

We found that the MADS-box genes *PhDAL11* and *PhDAL13* expressed dominantly in the Male cones. These genes were previously found to be phylogenetically related to B- class MADS-box genes, expressed specifically in male cones of *P. abies* and *P. tabuliformis* [58,62], and had similar phenotypic effects to endogenous Arabidopsis B-class proteins when expressed ectopically in Arabidopsis [27]. A new study published recently further demonstrated the male cone specific expression of *DAL11* and *DAL13* in *P. tabuliformis*, and showed physical interactions between these two proteins [21]. Together with these studies, our study supports the model proposing that B- class genes are essential for male identity determination [5]. An additional B-class gene examined in the current study, *PhMADS2*, was up-regulated in both cone types, yet most prominently in the male cone. Niu et al. [21] showed that PtMADS2 interacts with PtDAL11 and PtDAL13, supporting the involvement of PhMADS2 in the male cone development.

PhMADS1 was up-regulated in female cones (Figure 7). The most compatible alignment of PhMADS1 in Arabidopsis was the AP1 protein, which is an A-class TF, important for both floral meristem identity transition and the development of petal and sepals [1,5]. *MADS1* in *P. radiata* was shown to be highly expressed in early cone development and was localized to the ovuliferousscale primordia [13]. MADS1 homologs from *P. tabuliformis*, *PtMADS1* and *PtMADS10* were also specifically expressed in the developing female cone [21]. They were shown to physically interact with other female-related MADS-box proteins, such as PtDAL14 and PtDAL1, to regulate the female cone development. It is likely that PhMADS1 plays a similar role in *P. halepensis*.

In our study, *PhDAL14* was highly expressed throughout the female cone development; however, it was also up-regulated in the male cone during the middle to late developmental stages (Figure 7). This expression pattern is in agreement with *PaDAL14* expression in *P. abies* and *P. tabuliformis* female and male cone development [21,58]. AGL6 is the most closely related Arabidopsis protein to PhDAL14. AGL6 has an E function of floral organ development, with additional functions including floral meristem regulation, ovule and seed development [63]. The function of AGL6 and other E class proteins in angiosperms seem similar to that of *PhDAL14* in conifers. This suggests that *PhDAL14* is an essential component for both female and male cone development [28,64]. Physical interactions between DAL14 and the male-specific proteins DAL11 and DAL13 in *P. tabuliformis* support its involvement in regulating both cone types [21].

Function of the genes presented in the current study is confirmed by heterologous complementation done in Arabidopsis mutants in similar studies, as mentioned above. However, gene silencing via the CRISPR/Cas9 approach offers a better understanding of gene function, assuming that transformation and regeneration platforms are available for coniferous species. Silencing key genes in the development of the female pine cone can result in sterile trees, an outcome desirable for both commercial and ecological purposes. Sterile trees might direct the energy to vegetative growth, offering a great economic potential for timber production. In particular, sterility in *P. halepensis* might prevent the negative environmental impacts caused by uncontrolled regeneration in forests, thereby increasing the efficiency of forest management.

5. Conclusions

Our study contributes to the accumulating evidence of the molecular regulation of the reproduction phase in conifers. The expression pattern of the *P. halepensis* MADS-box genes was very similar to that found in other conifers, as well as to the newly studied homologs from *P. tabuliformis* [21], suggesting highly conserved mechanisms in Pinaceae. Yet, the large number of transcription factors revealed in our study suggests that the reproductive phase in Pinaceae is much more complex. We believe that this study will urge further investigation into the regulation of reproductive development in conifers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071588/s1, Figure S1: A *P. halepensis* tree with male and female cones; Figure S2: Heatmap of Pearson correlation analysis evaluating the similarity between samples; Figure S3: Differential expression of *FT/TFL1* homologs between different developmental stages of the male (A) and female (B) cones. Figure S4: Maximum likelihood phylogenetic analysis of 25 MADS-box proteins from *P. halepensis* related to MADS-box proteins from other species. Table S1: Primers used in qPCR; Table S2: Transcriptome analysis of *Pinus halepensis* cones development; Table S3: Selected *P. halepensis* MADS-box genes and their expression patterns.

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