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Identification of Candidate Genes Involved in Bud Growth in *Pinus pinaster* through Knowledge Transfer from *Arabidopsis thaliana* Models

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Abstract: *Pinus pinaster* is a plant species of great ecological and economic importance. Understanding the underlying molecular mechanisms that govern the growth and branching of *P. pinaster* is crucial for enhancing wood production and improving product quality. In this study, we describe a simple methodology that enables the discovery of candidate genes in *Pinus pinaster* by transferring existing knowledge from model species like *Arabidopsis thaliana* and focusing on factors involved in plant growth, including hormonal and non-hormonal pathways. Through comparative analysis, we investigated the main genes associated with these growth-related factors in *A. thaliana*. Subsequently, we identified putative homologous sequences in *P. pinaster* and assessed the conservation of their functional domains. In this manner, we can exclude sequences that, despite displaying high homology, lack functional domains. Finally, we took an initial approach to their validation by examining the expression levels of these genes in *P. pinaster* trees exhibiting contrasting growth patterns. This methodology allowed the identification of 26 candidate genes in *P. pinaster*. Our findings revealed differential expression patterns of key genes, such as *NCED3*, *NRT1.2*, *PIN1*, *PP2A*, *ARF7*, *MAX1*, *MAX2*, *GID1*, *AHK4*, *AHP1*, and *STP1*, in relation to the different growth patterns analyzed. This study provides a methodological foundation for further exploration of these genes involved in the growth and branching processes of *P. pinaster*. This will contribute to the understanding of this important tree species and open new avenues for enhancing its utilization in sustainable forestry practices.

Keywords: *Pinus pinaster*; growth regulators; growth; RT-qPCR; branching

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1. Introduction

Current climate change and the need to produce wood and other products determine the growing importance of green biology [1] and the urgency of finding roads that allow substantial forest improvement. This goal has to be multidisciplinary, covering applied aspects including plantation management but also other basic ones, i.e., the understanding of the bases of the growth and development of trees.

Plant growth and development are complex processes involving numerous mechanisms, from the formation of the embryo to the attainment of a fully mature individual. All of them combine systems of cell growth and differentiation that give rise to different tissues and organs. During post-embryonic development, growth starts from the meristems, cellular assemblies that remain in an embryogenic state indefinitely and whose division gives rise to the new cells that will constitute the adult plant. Focusing on the aerial part of the plant, it originates in the shoot apical meristem, located at the end of the stem. This, in

addition to length growth, plays an organogenic function on the phyllotaxis, forming the leaves and axillary buds from which future branches will arise. The phyllotaxis requires a fine-tuned regulation of the process that involves multiple factors, such as plant hormones, whose spatial control of their distribution leads to different arboreal architectures [2].

In addition to this, other non-hormonal mechanisms that promote growth and development in plants are also known, such as sugars and the red:far red light ratio, among others [3,4]. Sugars not only represent the plant's energy source but also regulate processes including flowering, anthocyanin synthesis, and meristematic proliferation, and light perception represents key information regarding changes in the surrounding environment.

Nowadays, relevant advances have been made to understand individual plant hormones, their mechanisms, and their processes in model species of angiosperms, like *Arabidopsis thaliana* [5]. An example of this is auxins. They were the first plant hormones discovered, synthesized in the stem apex and actively distributed through polar auxin transport, forming a gradient that is related to apical dominance and the inhibition of axillary bud development [3]. Because this phytohormone is involved in virtually all plant development processes, it is considered the most important signaling molecule [6,7]. However, understanding the molecular basis of the different responses offered by molecules related to auxins remains a pending challenge for the future [8].

It has recently been stated that, to be able to understand how plant hormones work together in the regulation of plant growth and development, connections between their pathways (the mechanism named crosstalk) need to be understood [9]. However, despite these advances, there is still much to learn about the molecular mechanisms underlying these processes [10].

Furthermore, it is not yet clear whether the knowledge acquired from studying angiosperm model species, e.g., *A. thaliana*, can be directly applied to gymnosperms such as *P. pinaster*. One of the main reasons is that the identification of candidate genes in *P. pinaster* through forward and reverse genetics poses an extremely challenging task. This is primarily due to the absence of defective mutants, difficulties in applying techniques like T-DNA insertional mutagenesis, and the fact that its genome has not been released yet.

P. pinaster is a species of great ecological and economic importance, with its ability to thrive in a wide range of environments [11] and its potential for use in forestry [12]. In addition, it can withstand cold or temperate climates and all kinds of substrates or environmental factors, including drought, and has high-quality wood [13,14]. Therefore, investigating the role of plant hormones in the growth and development of pine trees, such as *P. pinaster*, could have significant implications for our understanding of plant hormone function and for the development of sustainable forest management practices. Currently, important strides are being made in the field of pine plantations, utilizing a range of techniques from domestication and traditional plant breeding to genetic engineering [15].

Our starting working hypothesis is that stem development is a conserved process in the evolution of seed plants, and therefore the models proposed for *A. thaliana* can be extrapolated to conifers. To contrast this, we need to deepen our knowledge of the physiological and molecular factors involved in the shoot development of gymnosperms by using systems biology as a methodological framework that brings together different organizational levels, thus providing responses to the plasticity and performance of cells and tissues in different environments [16]. Transferring knowledge about growth and development from angiosperms to conifers can have great applicability for the timber industry, where the quality and quantity of wood are economically key. The first step in this knowledge transfer is to obtain the sequence of candidate genes. In this work, we present a simple methodology that enables the discovery of candidate genes in *P. pinaster* by transferring existing knowledge from model species such as *A. thaliana* and focusing on factors involved in plant growth, including hormonal and non-hormonal pathways.

In addition, we assessed the initial validation of the candidate genes obtained by examining the expression levels of these genes in *P. pinaster* trees exhibiting contrasting growth patterns. In Spain, *P. pinaster* has traditionally been divided into two types or

subspecies: (i) Atlantic or maritime, mainly located in the northwest; and (ii) Mediterranean or mesogeensis, representing the remaining *P. pinaster* stands. At the time of sampling, late summer (September), the Atlantic origin was in an active growth phase, while the Mediterranean origin was in a resting phase.

The identification of genes involved in plant growth and development in *P. pinaster* will facilitate molecular studies to characterize the function of key genes in gymnosperms.

2. Materials and Methods

2.1. Selection of Candidate Genes in Arabidopsis thaliana and Identification of Homologue Sequences in Pinus pinaster

2.1.1. Selection of Candidate Genes in *Arabidopsis thaliana*

A first selection of genes related to hormonal (abscisic acid, auxins, cytokinins, strigolactones, and gibberellins) and non-hormonal (sugars and red:far red light ratio) key factors was made according to the literature [17,18]. For each group of hormones, members of synthesis, transport, and signaling were represented. Both nucleotide and protein sequences, as the latter are more conserved between species, were compiled in FASTA format from the TAIR database (The Arabidopsis Information Resource, arabidopsis.org).

2.1.2. Identification of Homologue Sequences in *Pinus pinaster*, Sequence Search, and Comparison of Functional Domains

In order to identify homologue sequences in *P. pinaster*, we carried out a screening of the *P. pinaster* transcriptome and proteome data obtained in the frame of the European projects ProCoGen and SustainPine (http://www.scbi.uma.es/sustainpinedb/home_page, accessed on 31 March 2023), and in the PLAZA Gymnosperms (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/, accessed on 31 March 2023) database.

The TBLASTN and BLASTP algorithms [19], with default settings, were used for the screening using the *A. thaliana* protein sequences as queries. The sequences obtained were compared using the Multiple Sequence Alignment CLUSTALW tool [20], and the protein sequences encoded were analyzed using the InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/, accessed on 31 March 2023), Prosite (http://prosite.expasy.org/, accessed on 31 March 2023), and SMART (Simple modular architecture research tool, /smart.embl-heidelberg.de/) tools. After obtaining the domains, functions, and positions of each sequence, they were plotted with My domains (https://prosite.expasy.org/mydomains/, accessed on 31 March 2023) The similarity degree was represented by the E value. Sequence annotations were performed with the Geneious software v.11 (Biomatters Ltd., Auckland, New Zealand). Interactions between pairs of genes were analyzed using the STRING database (v.11.5).

2.2. Expression Analyses

2.2.1. Plant Material

To validate the candidate genes, samples from basal branches were harvested by the end of the summer (September). We collected the apical and whorled buds of the developing whorl (main apical buds and main whorl buds) and the apical buds from the last fully developed whorl (secondary apical buds) (Figure 1) in eleven year old *P. pinaster* clonal trees, representing the Mediterranean and Atlantic subspecies, characterized by a different model of development. Specifically, the Atlantic origin is characterized by extended and continuous growth, whereas the Mediterranean type presents a shorter growing period with several flushes within the same season. Three trees of each type were grown at the experimental plantation "La Mata" of the "Servicio Regional de Investigación y Desarrollo Agroalimentario de Asturias (SERIDA)" in Grado, Principado de Asturias (SPAIN). Samples were collected, frozen in liquid nitrogen for transport, and stored at −80 °C until use.

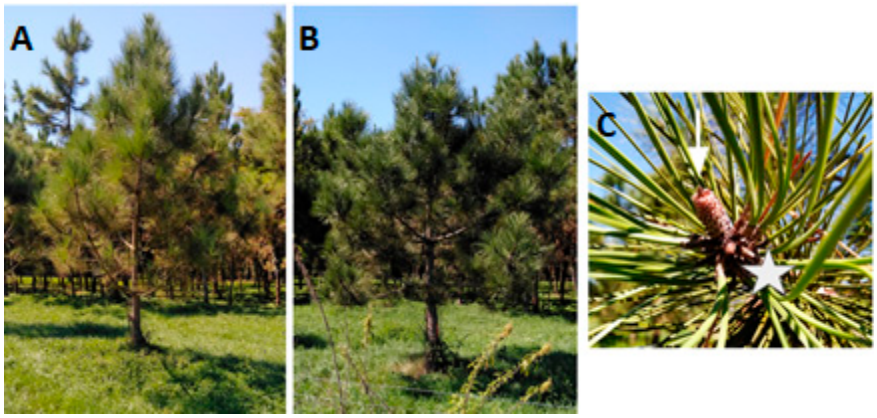

Figure 1. Prototypes of the studied individuals of *P. pinaster* of (**A**) Atlantic and (**B**) Mediterranean origin growing in the experimental plantation "La Mata" of the SERIDA in Grado, Principado de Asturias, Spain; and (**C**) detail of the main apical bud (arrow) and the main whorl buds (star).

2.2.2. RNA Isolation and cDNA Synthesis

The RNA was extracted from the samples using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland), and its quantity was measured spectrophotometrically at 260 nm. The integrity of the RNA was verified by performing agarose gel electrophoresis. For each sample, 1 µg of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) following the manufacturer's instructions.

2.2.3. Quantitative Real-Time PCR (RT-qPCR)

Gene expression analysis was performed by RT-qPCR in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), following the standards for this technique, like MIQE [21,22]. *P. pinaster* ubiquitin gene (Acc. AF461687) was used as an endogenous reference gene [23–25]. Specific primers for each gene (list available in Supplementary Table S1) were designed with Primer3 software v.4 [26], following the parameters recommended [21], to amplify an 80–100 bp fragment (amplicon). Three biological replicates and three technical replicates each were analyzed with 5 µL of Fast SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA, USA), oligonucleotide primers (0.20 µM), and 100 ng of cDNA in a final volume of 10 µL. The protocol used was: 95 °C for 20 s; 45 cycles of 95 °C for 3 s; and 60 °C for 30 s, with a final melting curve to assess for non-specific products. For this purpose, negative controls (no template) and RT-controls (non-retro-transcribed RNA) were also included.

2.2.4. Data Analysis

Analysis of the RT-qPCR data was performed with the qpcR package for R software v4.1.3 (www.dr-spiess.de/qpcR.html, accessed on 31 March 2023), which allows the fitting of the RT-qPCR fluorescence raw data to a five parameter sigmoidal model for obtaining essential PCR parameters such as efficiency, threshold cycle, and transcript abundance [27]. The relative abundance of each transcript was calculated as the mean of the technical duplicates and normalized to the expression value of the reference gene in each sample. Results were expressed as mean normalized expression values ± standard error of three biological replicates. Significant differences in mRNA levels were determined by *t*-test analysis or ANOVA using the Student–Newman–Keuls test for post hoc comparisons (SIGMA-PLOT v11 software, Chicago, IL, USA). In addition, a principal component analysis (PCA) of the gene expression data in both provenances was performed through R software v4.1.3 (https://www.r-project.org/, accessed on 31 March 2023).

3. Results

3.1. Selection of Candidate Genes in Arabidopsis thaliana and Identification of Homologue Sequences in Pinus pinaster

Given the complexity of the networks involved in plant growth processes, a first selection of the main factors involved in plant growth and development was divided into two large groups: hormonal and non-hormonal factors.

Regarding hormonal factors, the main hormonal groups studied were abscisic acid, auxins, cytokinins, gibberellins, and strigolactones. More specifically, within abscisic acid, selected genes were *NCED3* (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* 3), *ABCG40* (*ATP-BINDING CASSETTE G*), *NRT1.2* (*NITRATE TRANSPORTER 1.2*), *SNRK2.4* (*SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2*), and *ABI1* (*ABA INSENSITIVE 1*).

In the auxin case, the following genes were analyzed: *PIN1* (*PIN-FORMED 1*), *PP2A* (*SERINE/THREONINE PROTEIN PHOSPHATASE 2A*), *ABP1* (*AUXIN BINDING PROTEIN 1*), *TIR1* (*TRANSPORT INHIBITOR RESPONSE 1*), *ARF7* (*AUXIN RESPONSE FACTOR 7*), and *AXR1* (*AUXIN RESISTANT 1*).

Within the cytokinins group, the genes studied were: *CRE1/AHK4/WOL* (*ARABIDOPSIS HISTIDINE KINASE 4*), *AHP1* (*ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER 1*), *ARR* (*ARABIDOPSIS RESPONSE REGULATOR), CRF2 (CYTOKININ RESPONSE FACTOR 2*), *CYP735A* (*CYTOCHROME 735A*), *IPT* (*ISOPENTENYL TRANSFERASE*), *LOG* (*LONELY GUY*), and *PUPs* (*PURINE PERMEASE*).

In relation to strigolactones, the following genes were studied: *LBO1* (*LATERAL BRANCHING OXIDOREDUCTASE*), *BRC1* (*BRANCHED 1*), *MAX1* (*MORE AXILLARY BRANCHES 1*), *D14* (*DWARF 14*), and *MAX2* (*MORE AXILLARY BRANCHES 2*).

In relation to the gibberellins, the genes studied were: *GID1* (*GIBBERELLIN INSENSITIVE DWARF 1*), *GAI* (*GA-INSENSITIVE*), *RGA* (*REPRESSOR OF GA1-3*), and *SLY1* (*SLEEPY 1*).

On the other hand, other important factors involved in plant growth and development are sugar and light. The sugar genes selected were *STP1* (*SUGAR TRANSPORT PROTEIN 1*), *SWEET17*, and *WRKY20*, while concerning light, *PHYB* (*PHYTOCHROME B*) was studied.

3.2. Bioinformatic Analysis of Homologues in Pinus pinaster and Their Functional Domains

For this study, an E value of 10^{-30} was defined as the threshold. Some of the aforementioned proteins—IPT, CYP735A, LOG, PUPs, BRC1, and ARR—presented an E value higher than the one set as a threshold and, therefore, were discarded for subsequent experimental study at this analytical stage as they were considered poorly conserved. A set of 26 *P. pinaster* proteins were selected for further analysis (Table 1).

To confirm their possible homology, the functional domains of putative homologue proteins from *A. thaliana* and *P. pinaster* were analyzed using SMART, comparing them in pairs (example given at Supplementary Figure S1). The results showed a high degree of domain conservation in all *P. pinaster* sequences compared to *A. thaliana*, suggesting similar functional capabilities.

To show the relevance of plant hormone crosstalk in the regulation of plant growth and development, interactions between proteins used in this study were evaluated in silico by using the STRING database (Figure 2).

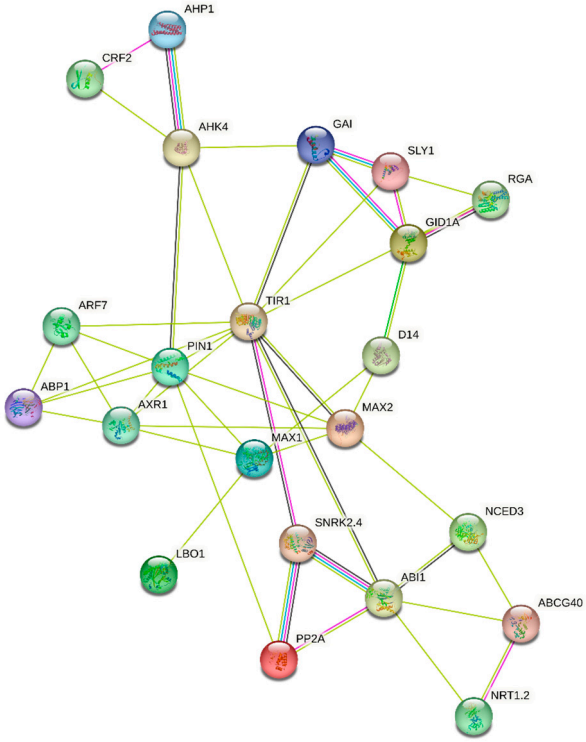

Figure 2. Interactions between proteins were analyzed with STRING. The represented proteins belong to abscisic acid (NCED3, ABCG40, NRT1.2, SNRK2.4, ABI1), auxins (PIN1, PP2A, ABP1, TIR1, ARF7, AXR1), cytokinins (AHK4, AHP1, CRF2), strigolactones (LBO1, MAX1, D14, MAX2), and gibberellins (GID1A, GAI, RGA, SLY1). The edges represent the predicted functional associations. A red line indicates the presence of fusion evidence; a green line—neighborhood evidence; a blue line—co-occurrence evidence; a purple line—experimental evidence; a yellow line—textmining evidence; a light blue line—database evidence; and a black line—co-expression evidence.

Table 1. Classification of the protein sequences object of this study in *Arabidopsis thaliana* and the results of homology in *P. pinaster* (the ID is indicated in TAIR and Gymnoplaza, respectively, and the length of the sequence in amino acids). The E value of the BLAST (from the sequence of *A. thaliana* versus *P. pinaster*) and the identity and positive percentage (BLOSUM62) (from the sequence of *P. pinaster* versus *A. thaliana*) are also indicated. (Only proteins that passed bioinformatics tests with a high degree of homology and/or conservation of their domains are shown.)

			Arabidopsis thaliana			*Pinus pinaster*				
			Protein	**ID**	**Length (aa)**	**ID**	**Length (aa)**	**E value**	**Identity %**	**Positive % (BLSM62)**
Hormonal	Abscisic acid	Synthesis	NCED3	AT3G14440	599	PPI00015334	412	0.0	68.3	81.1
		Transport	ABCG40	AT1G15210	1442	PPI00033391	1457	0.0	63.1	78.4
			NRT1.2	AT1G69850	585	PPI00013776	598	0.0	48.5	67.3
		Signaling	SNRK2.4	AT1G10940	371	PPI00050983	455	0.0	80.1	90.1
			ABI1	AT4G26080	434	PPI00056219	594	1.0×10^{-111}	49.9	65.9

Table 1. *Cont.*

			Arabidopsis thaliana			*Pinus pinaster*				
			Protein	**ID**	**Length (aa)**	**ID**	**Length (aa)**	**E value**	**Identity %**	**Positive % (BLSM62)**
Hormonal	Auxins	Transport	PIN1	AT1G73590	622	PPI00011546	695	0.0	57.1	67.8
			PP2A	AT1G69960	307	PPI00008039	306	0.0	90.8	97.4
		Signaling	ABP1	AT4G02980	198	PPI00009705	160	2.0×10^{-53}	49.9	65.9
			TIR1	AT3G62980	594	PPI00012072	574	0.0	65.4	79.3
			ARF7	AT5G20730	1165	PPI00041807	497	1.0×10^{-180}	71.5	83.3
			AXR1	AT1G05180	540	PPI00013097	560	0.0	67.0	82.4
	Cytokinins	Signaling	AHK4	AT2G01830	1080	PPI00064460	1036	0.0	56.7	70.3
			AHP1	AT3G21510	154	PPI00050577	156	4.0×10^{-62}	57.6	80.1
			CRF2	AT4G23750	343	PPI00010365	270	3.0×10^{-31}	43.1	56.9
	Strigolactones	Synthesis	LBO1	AT3G21420	364	PPI00010339	377	8.0×10^{-113}	47.1	67.3
			MAX1	AT2G26170	522	PPI00016810	421	3.0×10^{-134}	53.4	69.3
		Signaling	D14	AT3G03990	267	PPI00017643	267	4.0×10^{-133}	65.8	83.1
			MAX2	AT2G42620	693	PPI00014698	329	6.0×10^{-86}	54.5	67.1
	Gibberellins	Signaling	GID1A	AT3G05120	345	PPI00071627	357	1.0×10^{-162}	66.6	78.2
			GAI	AT1G14920	533	PPI00014310	458	3.0×10^{-174}	62.6	77.8
			RGA	AT2G01570	587	PPI00011857	594	5.0×10^{-174}	49.6	66.0
			SLY1	AT4G24210	151	PPI00016475	219	3.0×10^{-38}	48.9	63.7
Non-hormonal	Sugars	Transport	STP1	AT1G11260	522	PPI00012920	514	0.0	65.0	77.9
			SWEET17	AT4G15920	241	PPI00061954	280	8.0×10^{-75}	52.9	71.7
			WRKY20	AT4G26640	557	PPI00011874	679	4.0×10^{-90}	42.5	58.6
	Light	Signaling	PHYB	AT2G18790	1172	PPI00062444	1139	0.0	67.3	81.5

3.3. Expression Analyses

The expression levels of the genes coding for each of the 26 proteins were then studied in eleven year old individuals with different developmental patterns (Atlantic and Mediterranean shapes). All genes studied showed amplification, and the results indicated significant differences in the expression of 11 genes out of 26 (Table 2; gene expression values are shown in Supplementary Figure S2). All the groups studied, both hormonal and non-hormonal (except for the red:far red light ratio), showed at least one gene with a significant difference in expression (Supplementary Figure S2).

Table 2. Summary of gene expression differences in three types of buds; nd = no significant differences in expression were observed. A = higher expression was observed in that type of bud of Atlantic origin. M = higher expression was observed in people of Mediterranean origin. * = 90% confidence interval; ** = 95% confidence interval.

	Gene	**Main Apical Bud**	**Main Whorl Bud**	**Secondary Apical Bud**
Abscisic acid	*NCED3*	A **	nd	nd
	NRT1.2	M *	nd	M **
Auxins	*PIN1*	A *	nd	nd
	PP2A	nd	M *	nd
	ARF7	nd	nd	M *

Table 2. *Cont.*

	Gene	Main Apical Bud	Main Whorl Bud	Secondary Apical Bud
Cytokinins	*AHK4*	nd	M *	nd
	AHP1	nd	A *	nd
Strigolactones	*MAX1*	nd	nd	M *
	MAX2	A **	nd	nd
Gibberellins	*GID1*	M **	nd	nd
Sugars	*STP1*	nd	M *	M **

3.4. Multivariate Analyses of Gene Expression Data

Expression data were analyzed by PCA, which explained 60.1% of the variation observed. The Atlantic and Mediterranean types were separated by principal component two. The differences observed between groups were mainly explained by *AHP1*, *PIN1*, *MAX2*, *CRF2*, *NRT12*, *MAX1*, *ABCG40*, *GID1*, and *LBO* data (Figure 3).

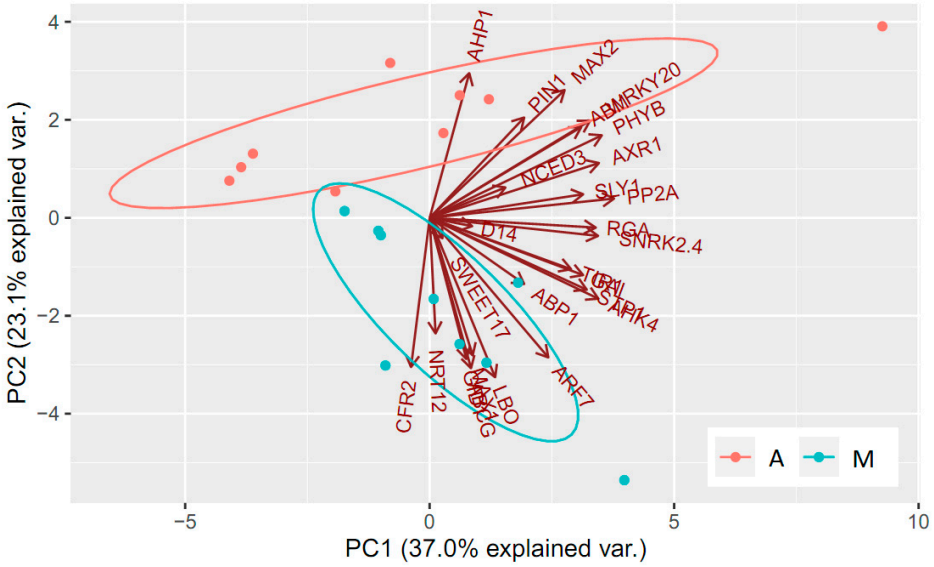

Figure 3. Analysis of gene expression data by principal component analysis (PCA). A: Atlantic type; M: Mediterranean type. Ellipses represent the distribution of projected data points in the space of principal components.

4. Discussion

Molecular studies in *P. pinaster* are still challenging as its genome has not been released yet, which, together with the lack of a collection of defective mutants, contributes to the complexity of conducting studies within this species. Transferring knowledge from angiosperm models can help overcome these difficulties, as has been shown in this study, where we used a straight-forward methodology to obtain candidate genes. To achieve this, we focused our study on factors involved in plant growth, including hormonal and non-hormonal pathways. We used the models described in *A. thaliana* to obtain the sequences of candidate genes. Subsequently, we searched for potential homologous sequences in available *P. pinaster* databases and compared functional domains, which led to the exclusion of sequences that, despite showing high homology, lacked functional domains.

The search for putative homologues revealed a high degree of conservation between angio- and gymnosperms. STRING analysis showed multiple interactions among the genes selected, highlighting the relevance of plant hormone crosstalk in the regulation of plant growth and development.

Once we achieved the main goal of this study, we assessed the initial validation of these candidate genes in *P. pinaster* trees from two subspecies (Atlantic and Mediterranean) exhibiting contrasting growth patterns. With this proof of concept, we could correlate the state of growth with certain hormonal gene dynamics, even determining if the expression of these genes could be critical. At the time of sampling, late summer (September), the Atlantic origin was in an active growth phase, while the Mediterranean origin was in a resting phase. The methodology chosen to carry out this initial validation was RT-qPCR, as it has the capacity to detect and measure minute amounts of nucleic acids in a wide range of samples. Its conceptual and practical simplicity, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification [22]. RT-qPCR results showed that within all groups of hormonal and non-hormonal factors, except light, there are significant differences.

In the case of the study of genes related to abscisic acid, significant differences in expression were observed in *NCED3* and *NRT1.2*. *NCED3* differences were detected in the main apical bud, being superior to their expression in *P. pinaster* of Atlantic origin. This dioxygenase catalyzes key stages in the local biosynthesis of ABA, causing the transformation of violaxanthine to xanthoxin, which is then translocated from the chloroplast to the cytosol [28], which will later lead to ABA. Therefore, a greater expression of *NCED3* would be associated with an increase in ABA.

On the other hand, the expression of *NRT1.2* is differential in both main and secondary apical buds, with greater expression in the Mediterranean origin (resting phase at the time of sample collection). This conveyor is key in transporting signals, and its greater presence is associated with buds in dormancy [28]. This NRT1.2 trend would be consistent with the resting phase associated with Mediterranean provenance at the sampling time.

Three genes related to auxins resulted in having different significant expressions: in the case of *PIN1*, its level at the main apical bud is superior in the origin with active growth (Atlantic origin). *PIN1* encodes a mediator of the active transport of auxins, which promotes the flow of polar transport. Polar auxin transport plays a crucial role in active growth in shoots by regulating various aspects of shoot development and architecture. In addition, it promotes apical dominance and inhibits axillary bud development [3]. Therefore, it can be expected that an increase in expression of this gene would lead to a more elongated phenotype, such as that of Atlantic origin.

A greater expression of *PP2A* has been observed in the main whorl bud of the Mediterranean type. This phosphatase promotes the direction of auxin flow and is key in the control of integrated cell functioning, in cell development in the face of stress, and in the membrane interactions of plant cells [29]. The results indicate that the main whorl bud has higher auxin transport and might therefore compete with the main bud for growth, suggesting that this strategy could lead to reduced growth in Mediterranean samples. Similarly, the positive regulator of auxin-mediated transcription, ARF7, presents higher expression in the secondary apical buds of Mediterranean origin.

Apart from that, two of the studied genes related to cytokinins showed significant differences in expression. *AHK4* encodes a signaling receptor protein [30,31], and the results showed significantly higher expression in the main whorl bud of the Mediterranean origin. Considering the antagonist effect that cytokinin exerts on auxin dynamics, the results here presented endorsed the higher expression of certain cytokinin-related genes in the non-active apical growth samples (Mediterranean). From a crosstalk perspective, AHK4 has been proposed as responsible for mediating greater stability of 1-aminocyclopropane-1-carboxylic acid, which is the ethylene precursor. As it is known, ethylene promotes inhibition of elongation [32], so the experimental results obtained are consistent, as individuals with higher expression of *AHK4* are in a resting phase.

On the contrary, the higher expression of *AHP1* in the Atlantic region could be associated with its involvement in cytokinin signaling and its influence on processes such as cell division, elongation, and differentiation.

Strigolactone-related genes also resulted in different significant expressions. A significantly higher expression of the biosynthesis-related gene *MAX1* has been observed in the secondary apical bud of *P. pinaster* of Mediterranean origin (with lower growth). This cytochrome transforms the precursor of strigolactones, carlactone, together with LBO [33], into a mobile, bioactive strigolactone.

A greater expression of *MAX2* was found in the main apical bud of the phenotype with greater growth (Atlantic origin). The protein encoded by this gene is involved in the polyubiquitination complex that, in the presence of strigolactones, activates the response to them [33,34]. In this study, a greater expression in the main apical bud was observed in individuals from the Atlantic model, fitting with the higher auxin content proposed and supporting the second messenger model, with a connection between auxin, strigolactone signaling, and promoted apical growth.

In the case of giberellins, a significantly higher expression of the gene coding its receptor, *GID1*, was observed in the main apical bud of the Mediterranean origin, pointing out the discrepancy between the expected active growth assumed with high GID1 levels and the lower growth phenotype assumed on this Mediterranean origin [2].

Significant differences in *STP1* (sugar-related gene) expression were observed in both secondary and main whorl buds, being significantly higher in individuals of Mediterranean origin. The function of this sugar carrier is key in the regulation of the absorption of monosaccharides from the environment, promoting growth [35,36]. In light of these data, it is not clear if the expression of *STP1* reflects where the resources are allocated. Indeed, even if its higher levels indicate a higher sugar content, STP1 cannot be considered an active growth marker, as the phenotype that presents less growth (the Mediterranean one) has significantly higher values.

Multivariant analysis by PCA showed that both origins were clearly separated by principal component two, suggesting that the different expression patterns of the genes studied could be in part responsible for the different growth phenotypes observed between Atlantic and Mediterranean provenances.

It can therefore be claimed that the methodology used in this study allowed the acquisition of 26 candidate genes in *P. pinaster* from *A. thaliana* models. By taking this approach, genes identified in this study will facilitate molecular studies to characterize the function and correlation of key genes in gymnosperms. In addition, the methodology described in this study may be applied to identify candidate genes involved in other processes in this species.

5. Conclusions

This study analyzed the hormonal and non-hormonal factors involved in plant growth and branching, using *A. thaliana* as a reference model. The methodology used in this study allowed the identification of 26 candidate genes in *P. pinaster.* In addition, we designed an experimental system for the initial validation of the candidate genes by studying their expression levels in three types of buds in individuals with contrasting growth. Our results revealed numerous significant differences in gene expression related to differential growth phenotypes. This methodology facilitates the transfer of knowledge from model plants to *P. pinaster*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14091765/s1, Figure S1: Representation of the functional domains of some of the studied proteins; Figure S2: Relative gene expression analyzed by RT-qPCR; Table S1: Specific primers for each gene.

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Data Availability Statement: Sequences used in this study are openly available in TAIR (https://www.arabidopsis.org/) and Gymnoplaza (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/) databases.

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