OXFORD UNIVERSITY PRESS | Tree Physiology

Proteometabolomic characterization of apical bud maturation in *Pinus pinaster*

Journal:	*Tree Physiology*
Manuscript ID	TP-2020-237.R2
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Valledor, Luis; University of Oviedo, Organisms and Systems Biology Guerrero, Sara; University of Oviedo, Organisms and Systems Biology García-Campa, Lara; University of Oviedo, Organisms and Systems Biology Meijon, Monica; Universidad de Oviedo, Organisms and Systems Biology
Keywords:	Bud aging, Metabolomics, Proteomics, Integrative approach, Conifer

SCHOLARONE™
Manuscripts

Title: Proteometabolomic characterization of apical bud maturation in *Pinus pinaster*

Running Title: Proteometabolomic characterization of apical bud

Luis Valledor*, Sara Guerrero, Lara García-Campa, Mónica Meijón*

Plant Physiology, Department of Organisms and Systems Biology, University of Oviedo, Oviedo 33071, Asturias, Spain

**Corresponding authors: valledorluis@uniovi.es; meijonmonica@uniovi.es*

KEYWORDS: Conifer, Bud aging, Metabolomics, Proteomics, Integrative approach

Number of words: 10647

Number of words: 6555 (without References, Funding, Acknowledgments)

Number of figures included in manuscript: 5

Number of Supplementary figures: 1 (S1)

Number of Supplementary tables: 5 (S1 to S5)

For Peer Review

ABSTRACT (300 words)

Bud maturation is a physiological process which implies a set of morphophysiological changes which lead to the transition of growth patterns from young to mature. This transition defines tree growth and architecture, and in consequence traits such as biomass production and wood quality. In *Pinus pinaster*, a conifer of great timber value, bud maturation is closely related to polycyclism (multiple growth periods per year). This process causes a lack of apical dominance, and consequently increased branching that reduces its timber quality and value. However, despite its importance, little is known about bud maturation. In this work, proteomics and metabolomics were employed to study apical and basal sections of young and mature buds in *P. pinaster*. Proteins and metabolites in samples were described and quantified using (n)UPLC-LTQ-Orbitrap. The datasets were analyzed employing an integrative statistical approach, which allowed the determination of the interactions between proteins and metabolites and the different bud sections and ages. Specific dynamics of proteins and metabolites such as HISTONE H3 and H4, RIBOSOMAL PROTEINS L15 and L12, CHAPERONIN TCP1, 14-3-3 protein gamma, gibberellins A1, A3, A8, strigolactones and ABA, involved in epigenetic regulation, proteome remodeling, hormonal signaling and abiotic stress pathways showed their potential role during bud maturation. Candidates and pathways were validated employing interaction databases and targeted transcriptomics. These results increase our understanding of the molecular processes behind bud maturation a key step towards improving timber production and natural pine forests management in a future scenario of climate change. However, further studies are necessary by using different *P. pinaster* populations that show contrasting wood quality and stress tolerance in order to generalize the results.

For Peer Review

INTRODUCTION

Pines are key players of forest ecosystems since most species have the ability to colonize a great variety of niches, act as good CO_2 sinks due to its fast growth (Allona et al., 1998), and are demanded by forest industry due to their quality timber, paper pulp and resins (Canales et al., 2014). Current production is not enough to cover current and predicted timber demand (FAO, 2009) and new strategies should be designed for implementing a more sustainable forest management also considering the climate change scenario (FAO, 2015). The use of fast-growth species such as *Pinus pinaster* Aiton, commonly known as maritime pine, may have an important role to this aim. This species has a major ecological and industrial role in southern Europe both at Mediterranean and Atlantic basins (González et al., 2016; Meijón et al., 2016; Cano et al., 2018). Its provenances adapted to different geoclimatic conditions across its distribution exhibit specific adaptions, allowing its optimal growth under a wide range of environments, from mild humid Atlantic regions to warm dry Mediterranean. These adaptions are genetically encoded, having this species a great genetic variability (Meijón et al., 2016; Vizcaíno-Palomar et al., 2016; Vázquez-González et al., 2019).

However, the architecture of this species can be greatly affected by harsh environmental conditions like drought periods, which can ultimately affect tree productivity. *Pinus pinaster* is a species with a characteristic polycyclic growth (multiple shoot flushes in a single season) (Zas et al., 2004). This growth pattern, which reduces tree value due to a loss of apical dominance and increased branching, is inheritable and genetically conditioned, with provenances more polycyclic than others (Sabatier et al., 2003; Zas et al., 2004; Meijón et al., 2016). Polycyclism also presents an important environmental component, being conditioned by water availability, soil composition or radiation (Sabatier et al., 2003; Girard et al., 2011). It has been shown that periods of drought reduce its appearance and also tree growth, and that fertile soils and good irradiation lead to increase growth cycles and branching (Sabatier et al., 2003; Verdú and Climent, 2007). Polycyclism is also conditioned by the maturation state of the apical bud (Sabatier et al., 2003; Girard et al., 2011), being buds with young phenology more polycyclic than mature, although the molecular mechanisms that explain these differences have not yet been described.

Bud maturation implies a number of phenological changes, altering growth pattern, morphogenetic competence and increasing abiotic stress resistance (Jordy, 2004; Brunner et al., 2017). Maturation is regulated by internal and environmental factors. Among internal factors, plant hormones (Meijón et al., 2009) and epigenetic mechanisms regulating differential gene expression are required for bud maturation (Valledor et al., 2010b; Valledor et al., 2015; Conde et al., 2017). This differential gene expression should lead to changes in proteome and, in consequence, in metabolome, altogether resulting in the physiological and morphological characteristics of each ontogenetic age (Haffner et al., 1991; Meijón et al., 2016; Großkinsky et al., 2017). Surprisingly, and despite its importance for adaptive responses, tree growth or polycyclism, little is known about the molecular processes that are behind bud maturation process and different developmental stages and how they interact with environment. The great complexity

For Peer Review

that these processes seem to have along with the large number of variables potentially involved and not previously described suggest an unmanaged and massive strategy as the most appropiate to address this problem.

The availability of high throughput alternatives for molecular phenotyping such as gel-free proteomics and metabolomics give us an unprecedented capability to address this gap (Valledor et al., 2018). These techniques usually associated to model species can currently be applied with high confidence in almost any organism, since they do not require extended genome information. Despite unsequenced, *Pinus pinaster* has extensive transcriptomic data available that ease protein identification (Romero-Rodríguez et al., 2014). In this species, there are several contributions in the proteomics field but focused on the study of wood forming tissues (Paiva et al., 2008; Garcés et al., 2014) and somatic embryogenesis (Morel et al., 2014; Trontin et al., 2016). As the final reflection of genomes and its variation, metabolome analyses allowed to define population structures and evolution in this species (Meijón et al., 2016; López-Goldar et al., 2019), and also adaptive responses to abiotic/biotic stress (Cañas et al., 2015; de Simón et al., 2017; López-Goldar et al., 2020). The combination of different omics greatly increases the power of analysis since the datasets of the different levels complement each other in a synergistic way (Mochida and Shinozaki, 2011; Kim et al., 2012). This type of integrative studies has already been carried out successfully in conifers to study needle development combining proteomics and transcriptomics (Valledor et al., 2010a) or to comprehensively analyze response to heat (Escandón et al., 2017) or ultraviolet stress comparing proteomics and metabolomics (Pascual et al., 2017). However, there is no information related to bud maturation in pines.

Therefore, the main aim of this work was to study the bud maturation process in *Pinus pinaster* using integrative omics approach combining proteomics and metabolomics. The integration of both levels allowed the extensive characterization of bud maturation processes. Specific dynamics of proteins and metabolites related to epigenetic regulation, proteome remodeling, hormonal signaling, and abiotic stress response (such as histones, ribosomal proteins, strigolactones, gibberellins, ABA, and chaperones) showed an essential role during bud maturation. In addition, the interconnection of these elements and its relation to different polycyclic capacity and stress tolerance of each maturation state of the bud was revealed through an integrative approach.

MATERIAL AND METHODS

Plant material and growth conditions

Apical buds in young and mature stages were sampled from two-years-old *Pinus pinaster* seedlings (plant size around 20 ± 3 cm) just after they exhibited young/mature apical phase change. These plants were grown in a greenhouse with seasonal fertirrigation.

Buds in the young stage show leaf primordia differentiate into photosynthetically active primary needles around of the shoot apical meristem (SAM). However, when the mature stage is reached,

For Peer Review

leaf primordia differentiate into scale leaves, and photosynthetic activity is shifted to long needles differentiated on brachyblasts in more distal positions on the stem (Jordy, 2004). Apical buds of 18 seedlings of the same age, half of them having mature morphology and half of them young, were sampled and dissected into their apical and basal sections, corresponding to apical and axillary/foliar meristems, respectively (Figure 1). Three biological replicates for each treatment (apical and basal parts of the bud, young and mature buds) were constituted pooling basal or apical parts of the buds of three different seedlings with the same bud developmental stage. Samples were immediately frozen in liquid nitrogen and kept at -80 ° C until biomolecule extraction. Metabolites, proteins, and RNA were isolated from the same sample following the protocol of Valledor et al. (2014a) using 75 mg of fresh weight per sample.

Metabolome analysis

High-performance liquid chromatography (Dionex Ultimate 3000, ThermoFisher Scientific, USA) was coupled to a LTQ-Orbitrap XL high resolution mass spectrometer equipped with a HESI II (heated electrospray ionization) source and controlled by Xcalibur version 2.2 (Thermo Fisher Corporation). Polar fraction of each sample was analyzed twice, first using the positive and then the negative ion modes. Samples were run according to the procedure described by Meijón et al. (2016). Instrument was operated in full-scan mode with a resolution of 60 000, and spectra were acquired in mass range m/z 50–1000 in the positive mode, and 65–1000 in the negative mode. The resolution and sensitivity were controlled by the injection of a standard mix (caffeine, proline, and sucrose) after the analysis of each batch and resolution was also checked with the aid of lock masses (phthalates). Blanks were also analyzed during the sequence. With the aim of improving metabolite assignation, one sample of each treatment was additionally reanalyzed including an ion fragmentation step. Chromatrographic and analytical conditions were the same, but top-three ions of each scan were fragmented (30 s dynamic exclusion window). Parent ions (minimum intensity of 500) were fragmented by CID (normalized collision energy of 35, activation Q 0.25, and activation time 90 ms). These spectra were employed for MS/MS metabolite identification as described below.

RAW files were directly processed employing MZMINE v2.14 (Pluskal et al., 2010). Spectra were filtered establishing noise threshold at 2×10^4 and minimum peak height at 2.5×10^5. Peaks were smoothed and deconvoluted using a local minimum search algorithm (95 % chromatographic threshold, minimum retention range 0.2 min, minimum relative height of 5 %, and minimum ratio top/edge of 0.5). Chromatograms were aligned using the RANSAC algorithm with a tolerance of 5 ppm of m/z and 0.2 min of retention time. Peak areas were used for quantification.

Peaks were identified following a sequential approach. The first stage was performed against our in-house library (>100 compounds) and manual annotation considering its m/z and retention times. In a second stage, MS/MS data was used for identification employing Compound Discoverer software (Thermo Scientific, USA) and custom scripts for comparing experimental data to MS/MS databases Metlin, HMDB and FooDB. A positive identification was defined when

For Peer Review

parent mass was below 5-ppm threshold compared to analyte in DB and at least, two main ions of fragmentation spectra were identified. The last stage assigned potential identity to masses by direct comparsion of ion masses using a 5-ppm threshold and KEGG, HMDB, FooDB, Plantcyc, and MassBank databases. Those metabolites that were defined after the comparison with our standard compound library or by a matching of MS/ MS were considered as unquestionably 'identified', while were considered 'tentatively assigned' those molecular ions with exact masses corresponding to identified metabolites in databases. Metabolite identification against our library was confirmed by RT, mass, and isotopic patterns.

Protein identification and quantitation using nLC-Orbitrap-MS analysis

Sixty µg of total protein were cleaned, digested, and desalted following the protocol described by Valledor and Weckwerth (2014). Peptide chromatography and mass spectrometric analysis were performed according to Pascual et al. (2017) with only a slight modification in the effective gradient, which was set to 90 min from 5 % to 45 % acetonitrile/0.1 % formic acid (v:v) with a later column regeneration step of 27 min. The employed column was a Chromoltih RP-18R 15 cm length 0.1 cm inner diameter (Merck, Germany).

Spectra were processed in Proteome Discoverer 2.0 (Thermo Scientific, USA). Protein identification threshold was established at 5% and 1% false discovery rates (FDR) at peptide and protein levels, respectively. Only proteins with at least two identified peptides and one of them unique were considered as identified. Four databases were used: *Pinus sylvestris* and *Pinus taeda* (34063 accessions) (Proost et al., 2014), and against in-house databases, *Pinus pinaster* (117080 accessions) and *Pinus radiata* (67647 accessions) that were built following the procedure described by Romero-Rodríguez et al. (2014). Proteins were also functionally classified according to Mapman (Thimm et al., 2004) functional bins. Identified proteins were quantified by a label-free approach based on the estimation of the areas of the three most abundant peaks assigned to each protein by Proteome Discoverer.

Targeted transcriptomic analysis of candidate genes

RNA abundance was determined in a microdrop spectrophotometer NB1 (Nabi, South Korea) and its integrity was checked by agarose gel electrophoresis. One µg of RNA was reversed transcribed using the RevertAid kit (Thermo Scientific, USA) and random hexamers as primers following the manufacturer's instructions. qPCR reactions were performed in a CFX96™ Real-Time System (Biorad, USA) with RealQ Plus Master Mix Green, no ROX (2X) (Ampliqon, Denmark); four biological and two analytical replicates per treatment were made for each gene. Expression levels of *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH*) and *UBIQUITINE (UBI)* were used as endogenous control and the results were analyzed by Bio-Rad CFX Manager 3.1 (Biorad, USA) software using the cycle threshold comparative method (ΔC_T). Detailed information about the primers used for qPCR experiments is available in Supplementary Table S1.

For Peer Review

Statistical and bioinformatics analysis

All statistical procedures were conducted with the R programming language running under the open source computer software R v3.5.0 (R Development Core Team, 2015) and RStudio v1.1.423 (RStudio Team, 2016) using the packages pRocesomics[1] and mixOmics (Rohart et al., 2017).

Three biological replicates per treatment were used for metabolome and proteome analysis. Proteome and metabolome datasets were pre-processed following the recommendations of Valledor and Jorrín (2011) and Valledor et al. (2014b). In brief, missing values were imputed using a Random Forest approach, and variables were filtered out if they were not present in at least all of replicates corresponding to one treatment or in at least 45% of the analyzed samples. Data were normalized and transformed following a samplecentric approach followed by log transformation. Centered and scaled values (z-scores) were subjected to univariate (one-way ANOVA followed by a Tukey HSD post-hoc test, $P<0.05$.) and Venn diagrams, and heat map clustering. To avoid variable noise only those variables with interquartile range 50% greater than average were selected for multivariate analyses. Integrative analysis was based on the use of DIABLO algorithm and network representation. Cytoscape v. 3.7 (Shannon et al., 2003) was employed in network representation and analysis. Proteins were annotated according to Mapman classification employing protein sequences and Mercator online tool v3.6 (Lohse et al., 2014) while metabolites were manually classified according to this classification.

RESULTS

Proteomic and metabolomic characterization of buds in *Pinus pinaster*

Proteomic analyses of young and mature buds and their sections allowed the identification of 1609 proteins. Proteins were annotated according to Mapman, classifying 1540 proteins in 34 functional bins. 951 proteins showed abundances and consistencies above threshold for their use in quantitative analyses. Out of these, 142 were differentially accumulated between treatments (ANOVA p-value <0.05; Supplementary Table S2). At metabolome level, 3670 peaks were detected, being 2267 suitable for quantification (Supplementary Table S3). From these, 133 peaks were unequivocally identified using the in-house database or MS/MS spectra, corresponding to 105 unique compounds, and 974 were tentatively assigned by comparing their very accurate masses to those available in public databases (Supplementary Table S4). 75 metabolites were classified according to Mapman. Despite the high number of identified/assigned metabolites, their complete classification according to Mapman was not possible, mainly due to the difficulty of classifying secondary metabolites. However, this potential bias does not affect the most studied and preserved functional groups such as those related to primary metabolism. From all detected compounds, 914 were differentially expressed between analyzed samples (ANOVA 5% FDR; Supplementary Table S3).

[1] https://github.com/Valledor/pRocessomics

For Peer Review

Venn analyses revealed qualitative differences between ontogenic stages and bud sections. At protein level (Figure 2A), young buds (apical and basal sections) showed the highest number of characteristic proteins (126) but, interestingly, the apical section of the mature bud was the most differentiated organ with 59 characteristic proteins. At metabolome level (Figure 2B), mature tissues showed the highest rate of unique compounds (569), while the apical section of the young bud exhibited the highest number of characteristic metabolites (194).

Quantitative analyses of Mapman classified proteins (Figure 2C) and metabolites (Figure 2D) pointed the differential pathways among bud sections and developmental stages. At proteome level, increased pathway clusters related to stress, hormone, lipidic, energetic, and major carbohydrate metabolism pathways can be correlated to the different tissues that were analyzed. Heatmap classified samples according to bud section, which may be suggesting a greater variation between apical and basal meristem proteomes than between mature and young bud proteomes. Contrary to proteome, metabolome allowed the classification of samples in relation to their ontogenetic age. This metabolomic differentiation was mainly caused by differences in photosynthesis, aminoacid synthesis and metabolism, redox regulation, and nucleotide metabolism.

These differences between mature and young buds and their basal and apical sections may be related to the location of the shoot apical meristem (SAM) activity in the apical section of the bud and also to the differential growth and developmental patterns of young and mature buds, as it was demonstrated by the differential accumulation of sample-specific pathways. Furthermore, the accumulation of a higher number of specific metabolites in comparison to proteins revealed the potential effect over the metabolome of the changes related to a smaller number of proteins. Alterations of key enzymes of metabolic pathways may lead to changes in abundance of a large number of metabolites. On the other hand, samples with a great ontogenic and functional differentiation (young vs mature) shared numerous proteins, which difficulted their classification according to their developmental stage. However, metabolites allowed their classification, reflecting the importance of metabolomic specificity in functional differentiation. The metabolome is the final downstream product of gene transcription and, therefore, changes in it are amplified relative to the changes in transcriptome and proteome (Das et al., 2015; Escandón et al., 2017). As young buds present more active development than differentiated mature buds, it is expected an overaccumulation of metabolites related to active processes of development, such as redox activity or aminoacid synthesis.

The integrative analysis of proteome and metabolome unmasked potential interaction networks involved in bud maturation and differentiation in *Pinus pinaster*

The combination of different omic levels in an integrative analysis supposes a major analytical advantage since the different levels can be used for cross-validation and, at the same time, to get a global overview of the physiological processes (Singh et al., 2018). For this purpose, we

For Peer Review

employed DIABLO algorithm (Rohart et al., 2017) to analyze proteome and metabolome datasets (Figure 3). This approach provided a better clustering of the samples at proteome and metabolome levels (Figure 3A). The joint analysis of both datasets correctly classified studied tissues (Figure 3B). The basis of this classification relied on the biological source of variation gathered by two main components, which collectively accounted 59% of the total variance (Supplementary Table S5). The analysis of the variables exhibiting highest loadings to these components (Figure 3C-D; Supplementary Table S5) allowed a biological interpretation of these results.

Component 1 gathered the variation related to ontogenic differentiation, distinguishing between young and mature buds. Enzymes related to energy, protein biosynthesis, lipid metabolism, and signaling/differentiation showed the highest correlations to this component (Figure 3C). Young tissues were characterized by an increased accumulation of energy-related ATPases or PSII reaction center proteins as well as several ribosomal- and RNA-related proteins. Lipids are supposed to be relevant players in this differentiation, and despite no differential lipids were identified after metabolome analysis, enzymes related to their metabolism were key nodes of our models. ACETYLCOA CARBOXYLASE CARBOXYL TRANSFERASE SUBUNIT ALPHA, which is a key lipidic enzyme as seen above (Harwood, 1996), a START-like domain, whose function in lipid regulation in plants was previously suggested (Ponting and Aravind, 1999), and GDSL ESTERASE LIPASE, being described its function in development, defense, synthesis of secondary metabolites, and morphogenesis in some plant species (Chepyshko et al., 2012), were some of the most highlighted lipidic-related enzymes.

On the other hand, RAS proteins, 14-3-3 transcription factors and DNA regulatory proteins by epigenetic mechanisms were re-emphasized as key processes in bud differentiation (Figure 3B). HISTONE 3 and HISTONE 4 (Tariq and Paszkowski, 2004; Valledor et al., 2010b; Bräutigam et al., 2013), GLYCOSYL HYDROLASES FAMILY 100 (Penterman et al., 2007), and RAS related proteins (Kamada et al., 1992; Alonso et al., 2007) regulate developmental processes, and were important to explain the ontogenic differences in the analyzed tissues. Many of these proteins were correlated to primary and secondary metabolites, some of which are characteristic of specific physiological stages, and therefore, having a potential involvement in development. Even though none of the most significant metabolites were identified in public databases, it would be interesting to highlight some of them due to their possible importance in differentiation interaction networks. Specifically, in ontogenic differentiation, significant metabolites (P0340, N1518, P0320, and N1292) seemed to differentiate young from mature buds (Figure 3D; Supplementary Table S5).

Second component distinguished between the apical and basal parts of the bud. Enzymes related to protein biosynthesis and folding-related, as well as peroxidases, were characteristic of this component (Figure 3C). Apical sections of the bud showed a positive correlation, and were characterized by increased abundance of stress-related proteins such as CHAPERONIN-LIKE TCP1 and others (Wang et al., 2004), a MANGANESE BINDING SITE from a GERMIN, which is related to abiotic and biotic stresses response in plants (Woo et al., 2000), and a PEROXIDASE.

On the other hand, key variables defining the basal parts of the buds showed negative correlation to this component. Basal sections were characterized by higher growth and proliferation rates than apical, fact that explains the importance of proteins related to cell division and reorganization such as PROFILIN 1 or PROHIBITIN 1 in this model. This last protein was only found in basal sections of mature bud, having multiple functions related to plant development and stress tolerance (Chen et al., 2005). In the same way, several transferases such as PYRIDOXAL PHOSPHATE DEPENDENT TRANSFERASE or PHOSPHORIBOSYLGLYCINAMIDE FORMYLTRANSFERASE were characteristic of the basal section of the bud. Most of the metabolites associated to this component were not identified. Among identified metabolites, loganin and deoxyloganin were only detected in apical sections of the bud, while basal had only highlighted unidentified metabolites (P1560 + P1340, N2243 and P1379) (Figure 3D; Supplementary Table S5).

The clustering and heatmap visualization of this integrative analysis (Figure 3B; Supplementary Figure S1) complemented results described above, revealing four different sets of variables: those proteins and metabolites over-accumulated in apical section of young bud and down-accumulated in the rest of the samples; those over-acumulated in basal section of young bud and down-accumulated in the rest of the samples; those over-acumulated in young buds and not in matures; and those over-acumulated in mature buds but not in young ones. First set of variables was mainly composed by metabolites and proteins already referenced such as GERMIN MANGANESE BINDING PROTEIN, PHOSPHOGLUCONATE DEHYDROGENASE or ribosomal proteins; conversely, second set of proteins was essentialy constituted by proteins from different metabolic pathways such as photosynthesis, redox mechanism or signaling. Above all the differential variables found in the third set of proteins, this group was characterized by numerous proteins belonging on the one hand to stress pathways such as the previously described CPN10 or H-TYPE THIOREDOXIN (Zhang et al., 2011) and EPOXIDE HYDROLASE (Morisseau, 2013) like proteins, and on the other hand, to energetic routes with a large number of proteins identified (ATP-DEPENDENT 6-PHOSPHOFRUCTOKINASE 2, MALATE DEHYDROGENASE, CYTOCHROME C, V-TYPE PROTON ATPase SUBUNIT D, SUCCINATE DEHYDROGENASE UBIQUINONE FLAVOPROTEIN SUBUNIT). Likewise, previously highlighted proteins related to lipid metabolism (ACETYL-COA ACETYLTRANSFERASE) and cellular reorganization (PROFILIN 1 and PROHIBITIN 1) were found in this pattern. Last set, including those proteins characteristic of mature buds, differentiation was established by a large number of non-identified metabolites and a small number of proteins, including ribosomal proteins, 14-3-3, HISTONE H3 or GLYCOSYL HYDROLASE FAMILY 100 previously described.

The analysis of proteome-metabolome interaction combined with targeted transcriptomics allowed a deeper characterization of the bud differentitation process

The integration of metabolome and proteome datasets also allowed the definition of a protein-metabolite interaction network based on the different correlations between variables of different types. The resulting network (Figure 4; correlation >0.75) showed two interaction clusters. First cluster (Figure 4A, left) gathered proteins and metabolites more abundant in the apical sections of young buds (Figures 4B-D). Proteins in this cluster are related to protein biosynthesis and transport (ribosomal-related, CHAPERONIN TCP1, TMP21), transcriptional response to stress (GERMIN, NUCLEIC ACID BINDING PROTEIN), and a PHOSPHOESTERASE similar to Arabidopsis PURPLE ACID PHOSPHATASE, PAP14 (At2g46880), required for petal differentiation and expansion (Zhu et al., 2005). These proteins positively correlated to different terpenoids (mascaroside, loganin, menthane) and the flavonoid luteolin. The abundance of these variables greatly diminished during the transition to basal section and also in mature buds. 4,4'-ditolylthiourea, the only metabolite in this cluster whose abundance is maximal in the basal section of mature buds, showed negative correlations to all of its linked nodes.

Second cluster (Figure 4A, right) was not as selective in its variable categories as first cluster, since groups variables peaking at each developmental stage and bud section. However, there was a major presence of variables more accumulated in mature tissues and/or basal sections of the buds (Figures 4C, D). Young buds are characterized by an increased photosynthesis (active center of PSII, GAPDH) and glycolytic (PYRUVATE KINASE, PHOSPHOFRUCTOKINASE) pathways. HISTONE H4, GDSL ESTERASE LIPASE, and elements related to protein biosynthesis (eRF1, protease inhibitor SERPIN, ribosomal proteins L15 and L50) and redox (THIOREDOXIN, START-like domain protein) were also more abundant in young buds. Most of these enzymes were positively correlated to N1751, an unknown metabolite, and were characteristic of basal sections of the bud. On the other hand, mature buds had increased energetic (fructose-6-P and ATPase), signaling and gene regulation (14-3-3 protein gamma, HISTONE H3, regulator of ribonuclease activity), antioxidant/detoxification activities (dihydrolipoate), and proteome remodeling (ribosomal proteins and peptidases). 2-alpha-(S)-Strictosidine, a key compound in monoterpene indol alkaloyds biosynthetic pathway (Rüffer et al., 1978), was mostly accumulated in the basal sections.

Interestingly, HISTONE H3, and other proteins related to epigenetic regulation of gene expression formed a cluster that had a greater accumulation in mature buds. Despite having a positive correlation to most of the metabolites of this cluster, it negatively correlated to those metabolites accumulated in young buds. HISTONE H4, characteristic of young tissues, negatively correlated to metabolite P0265 and through it to glycolytic and carbon-related enzymes, suggesting its role in regulation of energetic pathways. Finally, 14-3-3 Protein gamma, involved in the signaling pathways of the main plant hormones (Camoni et al., 2018), was also accumulated in mature buds in the apical section. However, in this case, it was negatively correlated to most of the metabolites in this cluster, some of them as relevant as diphyllin, lignin (Hemmati et al., 2007), or fructose-6-P.

For Peer Review

In order to support hypotheses raised after proteometabolomic dataset, six genes related to the different activities of the clusters depicted above were analyzed by qPCR (Figure 5). According to transcriptomics results, the different treatment had different expression patterns of the analyzed genes. Apical section of young buds had a differential overexpression of *ADENOSYLHOMOCYSTEINASE (SAHH)*, related to epigenetic regulation (Tanaka et al., 1997; Rocha et al., 2005), and *GLUTATHIONE S-TRANSFERASE (GST)* and *PHENYLALANINE AMMONIA LYASE (PAL),* both genes involved in secondary metabolism and hormonal signaling (Hahlbrock and Scheel, 1989; Rivero et al., 2001; Dixon et al., 2010; Czerniawski and Bednarek, 2018). The expression level of these genes was similar in mature buds and in the basal part of the young bud.

S-ADENOSYLMETHIONINE SYNTHASE (SAM SYNTHASE), a central element in DNA methylation (Gómez-Gómez and Carrasco, 1998) was more expressed in young buds, as *NEDD8-ACTIVATING ENZYME E1 CATALYTIC SUBUNIT (NEDD8-E1)*, involved in proteome remodelling and DNA repair (Brown and Jackson, 2015; Brown et al., 2015). Finally, it is important to highlight the expression pattern of the *MORE AXILLARY GROWTH 1 (MAX1)* gene, which is required for hormonal biosynthesis of a shoot-branching inhibiting signal (Booker et al., 2005). *MAX1* increased its expression in young basal section, while its expression decreased drastically in mature basal section.

DISCUSSION

The study of the transition between young and mature buds, and how it is reflected at metabolome and proteome levels, is not only crucial in order to understand shoot growth and tree architecture, but also to improve relevant traits for forestry such as total growth or polycyclism (Cabezas et al., 2015). Growth-related traits are influenced not only by their inherent genetic factors (most of them polygenic) but also by the environment (Zas and Fernández-López, 2005). Environmental conditions (light, temperature, rainfall) in combination with the genotype define not only the yearly tree growth period, ontogenic stage and flowering time, but also tree architecture, modulating for instance, polycyclism (Meijón et al., 2016; de Simón et al., 2018). The combination of genetic and environmental factors, together with the different growth patterns of mature and young buds sometimes mixed in trees of the same age, makes the apical growth in *Pinus pinaster* very complex at physiological and molecular levels (Nguyen et al., 1995). Consequently, the characterization of the proteome and metabolome of buds in different developmental stages is a necessary first step towards the fully understanding of all of these processes.

The employment of an integrative approach allowed the characterization of bud maturation from a holistic perspective, identifying the key molecular pathways of this process. Two main sources of variation were clearly distinguished after clustering and multivariate analyses. The first was the bud maturation status (young vs mature) and the second the presence of apical or lateral meristems (apical vs basal section of the buds), each of them with a different set of characteristic biomolecules.

For Peer Review

Mature buds exhibit specific phenology and growth patterns distinct from young buds. In the young stage, leaf primordia differentiate into photosynthetically active primary needles. Axillary buds develop either into auxiblasts in basal positions or into randomly distributed brachyblasts in more distal positions on the stem. When trees reach the mature stage, leaf primordia differentiate into scale leaves, and photosynthetic activity is shifted to long needles differentiated on brachyblasts (Nguyen et al., 1995; Jordy, 2004). The physiological competence of mature buds was probably imposed by the increased abundance of ABA and specific gibberelins (bioactive GA1 and intermediary GA19; Supplementary Table S3). ABA has not only a role in dormancy, but also in dormancy release and bud set in combination with specific gibberellins (Zheng et al., 2015; Maurya et al., 2018; Vimont et al., 2019). Interestingly, young buds had slightly lower concentrations of ABA but an increased abundance of bioactive GA3, and GA8, a degradation product of GA1. Both gibberellins, GA1 and GA3, are related to shoot elongation (Little and MacDonald, 2003); however, is complicated to venture which specific role are playing each one in the different development stage of the bud. Additionally, in relation to hormonal signaling, a key element was identified in the network, 14-3-3 protein. A possible role for 14-3-3 proteins in the coordination of GA and ABA signaling has emerged in the last years (Camoni et al., 2018). In fact, the overexpression of ABA responsive, 14-3-3-interacting transcription factors ABF1-3 impairs GA action, indicating that they act as negative regulators of GA signaling and that 14-3-3 proteins may function by sequestering ABF1-3 in the cytoplasm. However, the mechanism of 14-3-3 action and all the elements involved in the ABA and GA coordination are still unknown.

As pointed by datasets and correlation networks, having the enzymatic machinery to increase metabolic rate allowing burst and growth seems to be essential in young buds, since enzymes of photosynthesis and glycolytic pathways were increased compared to mature (Figures 2 and 4). Interestingly, the accumulation of fructose-6-P in mature buds indicate not only a differential allocation of sugars between ontogenic stages, but also specific hormonal, growth (Eveland and Jackson, 2012), and maturation patterns (Uggla et al., 2001). Lipids such as pimelate and dihydroxylipoate with a dual redox and transcriptional regulation role (Sen and Packer, 1996) were also accumulated in mature buds. The mature secondary metabolism was also reflected by the accumulation of flavonoids. Flavonoids and tannins may be involved in cellular detoxification (Meijón et al., 2016) and also in plant resistance against herbivores or other stresses (Treutter, 2008). At the end of the growing season, accumulation of lipid and starch is positively correlated with the onset of dormancy in mature buds (Jordy, 2004). Bud development is also associated to different abiotic stress tolerance and proteins related to detoxification. In all analyzed tissues, there were a great abundance of heat shock or chaperonin-like proteins (TCP1, CPN10) and ROS detoxifying enzymes (peroxidases, thioredoxines); however, mature buds were characterized by higher abundances of these protein families, suggesting that the greater tolerance to stress exhibited by these buds (Miller et al., 2008) relies on the overaccumulation of these molecules, compared to young tissues.

The different cell competence is defined by specific protein sets consequence of differential transcriptional and post-transcriptional regulation (del Mar Castellano et al., 2004; Dembinsky et

al., 2007). Mature and young buds have a differential set of DNA/RNA interacting proteins and ribosomal proteins. Among them, START-like domain proteins were up-accumulated in young tissues, despite one characteristic of mature buds (PPI00057470). The specific dynamic of this family, which is required for regulating transcription factors needed for cell differentiation after binding a lipid ligand (Schrick et al., 2014; Grabon et al., 2019), illustrates the complexity of bud maturation. Young buds were characterized by the serin protease inhibitors SERPIN (+5-fold change compared to mature) (Supplementary Table S2), which has been proposed to have also a non-peptidase inhibitory functions negatively regulating stress-induced cell death or reducing gene expression by compacting chromatin (Cohen et al., 2019). Epigenetic regulation elements were also differential between mature and young buds (discussed below).

The complexity of a pine bud was also reflected in the direct comparison of its apical and basal sections. The former containing the shoot apical meristem, and the later the lateral meristems and needle primordia (Fernando, 2014). This organization implies physiological and metabolic differences, which has been validated through this work. Starch, lipid reserves, and tannins are known to accumulate in the shoot tip as pines become older (Jordy et al., 2000; Jordy, 2004). The accumulation of flavonoids and its biosynthetic machinery in the apical part of the buds reinforces its role in the protection of the meristem (Meijón et al., 2016) and also in the vegetative bud outgrowth. Differential organogenetic activity in apical and basal sections, changeable across the bud maturation, is also related to environment conditions and apical dominance regulation (Jordy, 2004; Hover et al., 2017). Thus, the high activity of *MAX1* gene in both sections of young bud suggests the essential role of strigolactone hormone regulating inhibition of axillary bud outgrowth in this phase; however, in mature bud, *MAX1* expression is higher in apical section (Figure 5). The current models in relation to control of apical dominance suggest complex interaction networks where sugars and ABA could be responsible for initial release of an apical bud, while auxins, strigolactones and cytokinins seem to determine sustained outgrowth of axillary buds (Nguyen and Emery, 2017). Gibberellins, despite being their role well known in shoot elongation, need more investigation to determine their function inside this network. However, some reports on their interaction with strigolactone suggest that increased gibberellin levels could repress axillary bud outgrowth (Luisi et al., 2011).

The basal part of the bud is prepared to burst, contrary to apical whose function is keeping and protecting apical meristem, and many cell division and development-related proteins like FTSZ needed for plastid division (Schmitz et al., 2009), dormancy/associated or DNA repair machinery (Os08g0519400 like protein) were up-accumulated. LEA proteins and TMP21, associated to less differentiated organs (Zimmerman, 1993), were characteristic of the apical part of the buds probably helping to maintain meristem identity together with specific abundances of growth regulators and nucleic acid binding proteins and histone modifications regulating gene expression.

The great amount of differential proteins and metabolites involved in epigenetic regulation suggests the key role of these mechanisms in bud development. DNA methylation is a well-known

epigenetic mark of transcriptional gene silencing, but also in the establishment of heterochromatin, transposon control and genomic imprinting (Galindo-González et al., 2018). Two of the key enzymes regulating the methylation cycle, SAM SYNTHASE and SAHH, showed an overaccumulation in young buds and more specifically in its apical part (Figure 5), suggesting that bud maturation is concomitant to increased DNA methylation levels as previously reported in *Pinus radiata* (Fraga et al., 2002). ARGONAUTE, a key enzyme involved in RNA-mediated DNA methylation, was overaccumulated in mature apical buds, which will be probably related to the hypermethylation associated to development. Despite the employed analytical procedures were not intended to describe post-translational modifications defining histone code, specific forms of HISTONE H3 and H4 were characteristic of each developmental stage, reinforcing the hypothesis that bud maturation is the consequence of a complex interaction between epigenetic mechanisms, transcription factors and hormonal regulators.

Overall, our study provided novel insights over bud maturation and the machinery involved in its development and growth and stress resilience at different molecular levels and pathways. The comprehensive overview of this process allowed the validation of proteins and metabolites involved in bud development that were previously described, but also the involvement of novel proteins, metabolites, and pathways. However, further studies will be necessary to validate these new set of candidate molecules by using buds coming from different populations that show contrasting wood quality and stress tolerance.

DATA AND MATERIALS AVAILABILITY

All relevant data can be found within the manuscript and supplementary materials.

SUPLEMENTARY MATERIAL

Table S1. Genes and primers employed in quantitative PCR analyses.

Table S2. Proteins identification according to SEQUEST (scores, % of coverage, number of common, unique, and razor peptides), quantification (mean ± SD of three biological replicates), univariate analysis (p and q values; TukeyHSD p values for all paired comparisons).

Table S3. Peaks obtained after UPLC-MS analysis of polar metabolites. Peaks were aligned with mzMine 2.10 avoiding redundancies between positive (P) and negative (N) modes. This table shows peak ID, adduct, m/z, retention time, normalized peak areas for each analyzed sample, and univariate analysis (p and q values; TukeyHSD p values for all paired comparisons). Peak compound description is provided according to Supplementary Table S4.

Table S4. Identification of metabolites in the 3670 peaks that were analyzed. a) 133 peaks were unequivocally identified (those metabolites that were defined after the comparison to our compound library or by comparison of the MS/MS to *online* databases). b) 987 peaks were tentatively assigned after comparing its very accurate mass to reference compound databases. Delta ppm and compound exact mass are provided. Annotation source, molecular form, and

For Peer Review

accessions of KEGG, FooDB and other databases are provided for all identified/assigned compounds.

Table S5. DIABLO integrative analysis of proteome and metabolome datasets. a) Sample scores for components 1 and 2 in both datasets, b) variance explained for each component, and c) variable loadings for both datasets.

Figure S1. Integrative clustering of proteome and metabolome analysis (High resolution Figure 3B).

DISCLOSURES

The authors have no conflicts of interest to declare.

FUNDING

This publication is an output of the National Project Vampiro (AGL2017-83988-R, Spanish Ministry of Economy and Competitiveness). M.M. and L.V. were supported by Ramón y Cajal program (RYC-2014-14981 and RYC-2015-17871, respectively; Spanish Ministry of Economy and Competitiveness). L.G.C and S.G. were supported by Severo Ochoa Predoctoral Program (BP19-146 and BP19-145, respectively; Principality of Asturias, Spain).

ACKNOWLEDGMENTS

The authors wish to thank Eloy A. Ron (SERPA S.A, Vivero Forestal de La Mata) for providing the *Pinus pinaster* seedlings employed in this work.

AUTHORS' CONTRIBUTIONS

MM and LV designed the experiments, performed mass-spectrometry analyses, and metabolome-related computational analysis. LV and SG performed proteome-related computational analysis. LGC performed targeted-transcriptomics analyses. LV and SG performed statistical analyses. All authors wrote the manuscript, read, and approved the final version of the manuscript.

For Peer Review

REFERENCES

Allenbach, L., and Poirier, Y. (2000). Analysis of the alternative pathways for the β-oxidation of unsaturated fatty acids using transgenic plants synthesizing polyhydroxyalkanoates in peroxisomes. *Plant Physiol.* 124**,** 1159-1168. doi: 10.1104/pp.124.3.1159

Allona, I., Quinn, M., Shoop, E., Swope, K., Cyr, S.S., Carlis, J., et al. (1998). Analysis of xylem formation in pine by cDNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 95**,** 9693-9698. doi: 10.1073/pnas.95.16.9693

Alonso, P., Cortizo, M., Cantón, F.R., Fernández, B., Rodríguez, A., Centeno, M.L., et al. (2007). Identification of genes differentially expressed during adventitious shoot induction in *Pinus pinea* cotyledons by subtractive hybridization and quantitative PCR. *Tree Physiol.* 27**,** 1721-1730. doi: 10.1093/treephys/27.12.1721

Baldermann, S., Homann, T., Neugart, S., Chmielewski, F.-M., Götz, K.-P., Gödeke, K., et al. (2018). Selected plant metabolites involved in oxidation-reduction processes during bud dormancy and ontogenetic development in sweet cherry buds (*Prunus avium* L.). *Molecules* 23**,** 1197. doi: 10.3390/molecules23051197

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., et al. (2005). *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a carotenoid-derived branch-inhibiting hormone. *Dev. Cell.* 8**,** 443-449. doi: 10.1016/j.devcel.2005.01.009

Bräutigam, K., Vining, K.J., Lafon-Placette, C., Fossdal, C.G., Mirouze, M., Marcos, J.G., et al. (2013). Epigenetic regulation of adaptive responses of forest tree species to the environment. *Ecol. Evol.* 3**,** 399-415. doi: 10.1002/ece3.461

Brown, J.S., and Jackson, S.P. (2015). Ubiquitylation, neddylation and the DNA damage response. *Open Biol.* 5**,** 150018. doi: 10.1098/rsob.150018

Brown, J.S., Lukashchuk, N., Sczaniecka-Clift, M., Britton, S., le Sage, C., Calsou, P., et al. (2015). Neddylation promotes ubiquitylation and release of Ku from DNA-damage sites. *Cell Rep.* 11**,** 704-714. doi: 10.1016/j.celrep.2015.03.058

Brunner, A.M., Varkonyi-Gasic, E., and Jones, R.C. (2017). "Phase change and phenology in trees," in *Comparative and evolutionary genomics of angiosperm trees*, eds A. T. Groover and Q. C. B. Cronk (Cham: Springer International Publishing AG), 227-274.

Byrne, M.E. (2009). A role for the ribosome in development. *Trends Plant Sci.* 14**,** 512-519. doi: 10.1016/j.tplants.2009.06.009

Cabezas, J.A., González-Martínez, S.C., Collada, C., Guevara, M.A., Boury, C., de María, N., et al. (2015). Nucleotide polymorphisms in a pine ortholog of the Arabidopsis degrading enzyme cellulase KORRIGAN are associated with early growth performance in *Pinus pinaster*. *Tree Physiol.* 35**,** 1000-1006. doi: 10.1093/treephys/tpv050

Camoni, L., Visconti, S., Aducci, P., and Marra, M. (2018). 14-3-3 proteins in plant hormone signaling: doing several things at once. *Front. Plant Sci.* 9**,** 297. doi: 10.3389/fpls.2018.00297

Canales, J., Bautista, R., Label, P., Gómez-Maldonado, J., Lesur, I., Fernández-Pozo, N., et al. (2014). De novo assembly of maritime pine transcriptome: implications for forest breeding and biotechnology. *Plant Biotechnol. J.* 12**,** 286-299. doi: 10.1111/pbi.12136

Cano, M., Morcillo, A., Humánez, A., Mendoza-Poudereux, I., Alborch, A., Segura, J., et al. (2018). "Maritime Pine (*Pinus Pinaster* Aiton)," in *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume I Forestry Sciences 84,* eds S. M. Jain and P. Gupta (Cham: Springer International Publishing AG), 167-179.

Cañas, R.A., Canales, J., Muñoz-Hernández, C., Granados, J.M., Ávila, C., García-Martín, M.L., et al. (2015). Understanding developmental and adaptive cues in pine through metabolite profiling and co-expression network analysis. *J. Exp. Bot.* 66**,** 3113-3127. doi: 10.1093/jxb/erv118

Chen, J.C., Jiang, C.Z., and Reid, M.S. (2005). Silencing a prohibitin alters plant development and senescence. *Plant J.* 44**,** 16-24. doi: 10.1111/j.1365-313X.2005.02505.x

Chepyshko, H., Lai, C.-P., Huang, L.-M., Liu, J.-H., and Shaw, J.-F. (2012). Multifunctionality and diversity of GDSL esterase/lipase gene family in rice (*Oryza sativa L. japonica*) genome: new insights from bioinformatics analysis. *BMC Genomics.* 13**,** 309. doi: 10.1186/1471-2164-13-309

Cohen, M., Davydov, O., and Fluhr, R. (2019). Plant serpin protease inhibitors: specificity and duality of function. *J. Exp. Bot.* 70**,** 2077-2085. doi: 10.1093/jxb/ery460

Conde, D., Moreno-Cortés, A., Dervinis, C., Ramos-Sánchez, J.M., Kirst, M., Perales, M., et al. (2017). Overexpression of DEMETER, a DNA demethylase, promotes early apical bud maturation in poplar. *Plant Cell Environ.* 40**,** 2806-2819. doi: 10.1111/pce.13056

Czerniawski, P., and Bednarek, P. (2018). Glutathione S-transferases in the biosynthesis of sulfur-containing secondary metabolites in Brassicaceae plants. *Front. Plant Sci.* 9**,** 1639. doi: 10.3389/fpls.2018.01639

Das, A., Paudel, B., and Rohila, J.S. (2015). "Potentials of proteomics in crop breeding," in *Advances in plant breeding strategies: breeding, biotechnology and molecular tools*, eds J. M. Al-Khayri, S. M. Jain and D. V. Johnson (Cham: Springer International Publishing AG), 513-537.

De Simón, B.F., Cadahía, E., and Aranda, I. (2018). Metabolic response to elevated CO_2 levels in *Pinus pinaster* Aiton needles in an ontogenetic and genotypic-dependent way. *Plant Physiol. Biochem.* 132**,** 202-212. doi: 10.1016/j.plaphy.2018.09.006

De Simón, B.F., Sanz, M., Cervera, M.T., Pinto, E., Aranda, I., and Cadahía, E. (2017). Leaf metabolic response to water deficit in *Pinus pinaster* Ait. relies upon ontogeny and genotype. *Environ. Exp. Bot.* 140**,** 41-55. doi: 10.1016/j.envexpbot.2017.05.017

Del Mar Castellano, M., Boniotti, M.B., Caro, E., Schnittger, A., and Gutierrez, C. (2004). DNA replication licensing affects cell proliferation or endoreplication in a cell type–specific manner. *Plant Cell* 16**,** 2380-2393. doi: 10.1105/tpc.104.022400

Dembinsky, D., Woll, K., Saleem, M., Liu, Y., Fu, Y., Borsuk, L.A., et al. (2007). Transcriptomic and proteomic analyses of pericycle cells of the maize primary root. *Plant Physiol.* 145**,** 575-588. doi: 10.1104/pp.107.106203

Dixon, D.P., Skipsey, M., and Edwards, R. (2010). Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry* 71**,** 338-350. doi: 10.1016/j.phytochem.2009.12.012

Escandón, M., Valledor, L., Pascual, J., Pinto, G., Cañal, M.J., and Meijón, M. (2017). System-wide analysis of short-term response to high temperature in *Pinus radiata*. *J. Exp. Bot.* 68**,** 3629-3641. doi: 10.1093/jxb/erx198

Eveland, A.L., and Jackson, D.P. (2012). Sugars, signalling, and plant development. *J. Exp. Bot.* 63**,** 3367-3377. doi: 10.1093/jxb/err379

FAO (2009). *Global demand for wood products. Rome: Italy: The Food and Agricultural Organization of the United Nations*. Available at: http://www.fao.org/3/i0350e/i0350e00.htm

FAO (2015). *Global forest resources assessment 2015. Rome, Italy*. FAO Forestry Paper No. 1. Available at: http://www.fao.org/forest-resources-assessment/past-assessments/fra-2015/en/

Fernández, H., Fraga, M., Bernard, P., and Revilla, M. (2003). Quantification of GA1, GA3, GA4, GA7, GA9, and GA20 in vegetative and male cone buds from juvenile and mature trees of *Pinus radiata*. *Plant Growth Regul.* 40**,** 185-188. doi: 10.1023/A:1025070707899

Fernando, D.D. (2014). The pine reproductive process in temperate and tropical regions. *New For.* 45**,** 333-352. doi: 10.1007/s11056-013-9403-7

Fraga, M.F., Rodríguez, R., and Cañal, M.J. (2002). Genomic DNA methylation–demethylation during aging and reinvigoration of *Pinus radiata*. *Tree Physiol.* 22**,** 813-816. doi: 10.1093/treephys/22.11.813

Galindo-González, L., Sarmiento, F., and Quimbaya, M.A. (2018). Shaping plant adaptability, genome structure and gene expression through transposable element epigenetic control: Focus on methylation. *Agronomy* 8**,** 180. doi: 10.3390/agronomy8090180

Garcés, M., Le Provost, G., Lalanne, C., Claverol, S., Barré, A., Plomion, C., et al. (2014). Proteomic analysis during ontogenesis of secondary xylem in maritime pine. *Tree Physiol.* 34**,** 1263-1277. doi: 10.1093/treephys/tpt117

Girard, F., Vennetier, M., Ouarmim, S., Caraglio, Y., and Misson, L. (2011). Polycyclism, a fundamental tree growth process, decline with recent climate change: the example of *Pinus halepensis* Mill. in Mediterranean France. *Trees Struct. Funct.* 25**,** 311-322. doi: 10.1007/s00468-010-0507-9

Gómez-Gómez, L., and Carrasco, P. (1998). Differential expression of the S-Adenosyl-L-Methionine Synthase genes during pea development. *Plant Physiol.* 117**,** 397-405. doi: 10.1104/pp.117.2.397

For Peer Review

González, Á.C., Díaz, I.F., de la Torre, C., Vázquez, J.P., Colina, F.J., Valledor, L., et al. (2016). Nuevos marcadores de calidad de madera en *Pinus pinaster*: Estrigolactonas y ramificación. *Tecnología agroalimentaria: Boletín informativo del SERIDA* 17**,** 21-27.

Grabon, A., Bankaitis, V.A., and McDermott, M.I. (2019). The interface between phosphatidylinositol transfer protein function and phosphoinositide signaling in higher eukaryotes. *J. Lipid Res.* 60**,** 242-268. doi: 10.1194/jlr.R089730

Großkinsky, D.K., Syaifullah, S.J., and Roitsch, T. (2017). Integration of multi-omics techniques and physiological phenotyping within a holistic phenomics approach to study senescence in model and crop plants. *J. Exp. Bot.* 69**,** 825-844. doi: 10.1093/jxb/erx333

Haffner, V., Enjalric, F., Lardet, L., and Carron, M. (1991). Maturation of woody plants: a review of metabolic and genomic aspects. *Ann. For. Sci.* 48, 615-630. doi: 10.1051/forest:19910601

Hahlbrock, K., and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Biol. Plant. Mol. Biol.* 40**,** 347-369. doi: 10.1146/annurev.pp.40.060189.002023

Harwood, J.L. (1996). Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta* 1301**,** 7-56. doi: 10.1016/0005-2760(95)00242-1

Hemmati, S., Schneider, B., Schmidt, T.J., Federolf, K., Alfermann, A.W., and Fuss, E. (2007). Justicidin B 7-hydroxylase, a cytochrome P450 monooxygenase from cell cultures of *Linum perenne* Himmelszelt involved in the biosynthesis of diphyllin. *Phytochemistry* 68**,** 2736-2743. doi: 10.1016/j.phytochem.2007.10.025

Hover, A., Buissart, F., Caraglio, Y., Heinz, C., Pailler, F., Ramel, M., et al. (2017). Growth phenology in *Pinus halepensis* Mill.: apical shoot bud content and shoot elongation. *Ann. For. Sci.* 74**,** 39. doi: 10.1007/s13595-017-0637-y

Considine, M.J., and Foyer, C.H. (2014). Redox Regulation of Plant Development. *Antioxid. Redox Signal.* 21**,** 1305-1326. doi: 10.1089/ars.2013.5665

Jordy, M.-N., Danti, S., Favre, J.-M., and Raccchi, M.L. (2000). Histological and biochemical changes in *Pinus* spp. seeds during germination and post-germinative growth: triacylglycerol distribution and catalase activity. *Aust. J. Plant Physiol.* 27**,** 1109-1117. doi: 10.1071/PP00069

Jordy, M.N. (2004). Seasonal variation of organogenetic activity and reserves allocation in the shoot apex of *Pinus pinaster* Ait. *Ann. Bot.* 93**,** 25-37. doi: 10.1093/aob/mch005

Kamada, I., Yamauchi, S., Youssefian, S., and Sano, H. (1992). Transgenic tobacco plants expressing *rgp1*, a gene encoding a RAS-related GTP-binding protein from rice, show distinct morphological characteristics. *Plant J.* 2**,** 799-807. doi: 10.1111/j.1365-313X.1992.tb00149.x

Kim, D., Shin, H., Song, Y.S., and Kim, J.H. (2012). Synergistic effect of different levels of genomic data for cancer clinical outcome prediction. *J. Biomed. Inform.* 45**,** 1191-1198. doi: 10.1016/j.jbi.2012.07.008

Little, C., and MacDonald, J.E. (2003). Effects of exogenous gibberellin and auxin on shoot elongation and vegetative bud development in seedlings of *Pinus sylvestris* and *Picea glauca*. *Tree Physiol.* 23**,** 73-83. doi: 10.1093/treephys/23.2.73

Lohse, M., Nagel, A., Herter, T., May, P., Schroda, M., Zrenner, R., et al. (2014). Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. *Plant Cell Environ.* 37**,** 1250-1258. doi: 10.1111/pce.12231

López-Goldar, X., Villari, C., Bonello, P., Borg-Karlson, A.K., Grivet, D., Sampedro, L., et al. (2019). Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. *J. Ecol.* 107**,** 2464-2477. doi: 10.1111/1365-2745.13159

López-Goldar, X., Zas, R., Sampedro, L. (2020). Resource availability drives microevolutionary patterns of plant defences. *Funct. Ecol.*, 1–13. doi: 10.1111/1365-2435.13610

Luisi, A., Lorenzi, R., and Sorce, C. (2011). Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*). *Plant Growth Regul.* 65**,** 415-419. doi: 10.1007/s10725-011-9603-0

Maurya, J.P., Triozzi, P.M., Bhalerao, R.P., and Perales, M. (2018). Environmentally sensitive molecular switches drive poplar phenology. *Front. Plant Sci.* 9**,** 1873. doi: 10.3389/fpls.2018.01873

Meijón, M., Feito, I., Oravec, M., Delatorre, C., Weckwerth, W., Majada, J., et al. (2016). Exploring natural variation of *Pinus pinaster* Aiton using metabolomics: Is it possible to identify the

region of origin of a pine from its metabolites? *Mol. Ecol.* 25, 959-976. doi: 10.1111/mec.13525

Meijón, M., Rodríguez, R., Cañal, M.J., and Feito, I. (2009). Improvement of compactness and floral quality in azalea by means of application of plant growth regulators. *Sci. Hortic-Amsterdam* 119, 169-176. doi: 10.1016/j.scienta.2008.07.023

Miller, G., Shulaev, V., and Mittler, R. (2008). Reactive oxygen signaling and abiotic stress. *Physiol. Plantarum* 133, 481-489. doi: 10.1111/j.1399-3054.2008.01090.x

Mochida, K., and Shinozaki, K. (2011). Advances in omics and bioinformatics tools for systems analyses of plant functions. *Plant Cell Physiol.* 52, 2017-2038. doi: 10.1093/pcp/pcr153

Moon, J., Parry, G., and Estelle, M. (2004). The ubiquitin-proteasome pathway and plant development. *Plant Cell* 16, 3181-3195. doi: 10.1105/tpc.104.161220

Morel, A., Trontin, J.-F., Corbineau, F., Lomenech, A.-M., Beaufour, M., Reymond, I., et al. (2014). Cotyledonary somatic embryos of *Pinus pinaster* Ait. most closely resemble fresh, maturing cotyledonary zygotic embryos: biological, carbohydrate and proteomic analyses. *Planta* 240, 1075-1095. doi: 10.1007/s00425-014-2125-z

Morisseau, C. (2013). Role of epoxide hydrolases in lipid metabolism. *Biochimie* 95, 91-95. doi: 10.1016/j.biochi.2012.06.011

Nguyen, A., Dormling, I., and Kremer, A. (1995). Characterization of *Pinus pinaster* seedling growth in different photo-and thermoperiods in a phytotron as a basis for early selection. *Scand. J. Forest Res.* 10, 129-139. doi: 10.1080/02827589509382876

Nguyen, T.Q., and Emery, R.N. (2017). Is ABA the earliest upstream inhibitor of apical dominance? *J. Exp. Bot.* 68, 881-884. doi: 10.1093/jxb/erx028

Paiva, J.A., Garcés, M., Alves, A., Garnier-Géré, P., Rodrigues, J.C., Lalanne, C., et al. (2008). Molecular and phenotypic profiling from the base to the crown in maritime pine wood-forming tissue. *New Phytol.* 178, 283-301. doi: 10.1111/j.1469-8137.2008.02379.x

Pascual, J., Cañal, M.J., Escandón, M., Meijón, M., Weckwerth, W., and Valledor, L. (2017). Integrated physiological, proteomic, and metabolomic analysis of ultra violet (UV) stress responses and adaptation mechanisms in *Pinus radiata*. *Mol. Cell. Proteomics* 16, 485-501. doi: 10.1074/mcp.M116.059436

Penterman, J., Zilberman, D., Huh, J.H., Ballinger, T., Henikoff, S., and Fischer, R.L. (2007). DNA demethylation in the Arabidopsis genome. *Proc. Natl. Acad. Sci. U.S.A* 104, 6752-6757. doi: 10.1073/pnas.0701861104

Pluskal, T., Castillo, S., Villar-Briones, A., and Orešič, M. (2010). MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* 11, 395. doi: 10.1186/1471-2105-11-395

Ponting, C.P., and Aravind, L. (1999). START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends Biochem. Sci.* 24, 130-132. doi: 10.1016/s0968-0004(99)01362-6

Proost, S., Van Bel, M., Vaneechoutte, D., Van de Peer, Y., Inzé, D., Mueller-Roeber, B., et al. (2014). PLAZA 3.0: an access point for plant comparative genomics. *Nucleic Acids Res.* 43, 974-981. doi: 10.1093/nar/gku986

R Development Core Team. (2015). *A Language and Environment for Statistical Computing: Vienna: R Foundation for Statistical Computing*.

Richards, D.E., King, K.E., Ait-Ali, T., and Harberd, N.P. (2001). How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signaling. *Ann. Rev. Plant Biol.* 52, 67-88. doi: 10.1146/annurev.arplant.52.1.67

Rivero, R.M., Ruiz, J.M., Garcıa, P.C., Lopez-Lefebre, L.R., Sánchez, E., and Romero, L. (2001). Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci.* 160, 315-321. doi: 10.1016/S0168-9452(00)00395-2

Rocha, P.S., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., et al. (2005). The Arabidopsis *HOMOLOGY-DEPENDENT GENE SILENCING1* gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. *Plant Cell* 17, 404-417. doi: 10.1105/tpc.104.028332

Rohart, F., Gautier, B., Singh, A., and Lê Cao, K.-A. (2017). mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* 13, e1005752. doi: 10.1371/journal.pcbi.1005752

Romero-Rodríguez, M.C., Pascual, J., Valledor, L., and Jorrín-Novo, J. (2014). Improving the quality of protein identification in non-model species. Characterization of *Quercus ilex* seed and *Pinus radiata* needle proteomes by using SEQUEST and custom databases. *J. Proteomics* 105, 85-91. doi: 10.1016/j.jprot.2014.01.027

RStudio Team. (2016). *RStudio: Integrated development environment for R*. Boston, MA.

Rüffer, M., Nagakura, N., and Zenk, M.H. (1978). Strictosidine, the common precursor for monoterpenoid indole alkaloids with 3 α and 3 β configuration. *Tetrahedron Lett.* 18**,** 1593-1596. doi: 10.1016/S0040-4039(01)94613-1

Sabatier, S., Baradat, P., and Barthelemy, D. (2003). Intra-and interspecific variations of polycyclism in young trees of *Cedrus atlantica* (Endl.) Manetti ex. Carrière and *Cedrus libani* A. Rich (Pinaceae). *Ann. For. Sci.* 60**,** 19-29. doi: 10.1051/forest:2002070

Sadka, A., Dahan, E., Cohen, L., and Marsh, K.B. (2000). Aconitase activity and expression during the development of lemon fruit. *Physiol. Plantarum* 108**,** 255-262. doi: 10.1034/j.1399-3054.2000.108003255.x

Sangster, T.A., and Queitsch, C. (2005). The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. *Curr. Opin. Plant Biol.* 8**,** 86-92. doi: 10.1016/j.pbi.2004.11.012

Schmitz, A.J., Glynn, J.M., Olson, B.J., Stokes, K.D., and Osteryoung, K.W. (2009). *Arabidopsis* FtsZ2-1 and FtsZ2-2 are functionally redundant, but FtsZ-based plastid division is not essential for chloroplast partitioning or plant growth and development. *Mol. Plant* 2**,** 1211-1222. doi: 10.1093/mp/ssp077

Schrick, K., Bruno, M., Khosla, A., Cox, P.N., Marlatt, S.A., Roque, R.A., et al. (2014). Shared functions of plant and mammalian StAR-related lipid transfer (START) domains in modulating transcription factor activity. *BMC Biology* 12**,** 70. doi: 10.1186/s12915-014-0070-8

Sen, C.K., and Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *Faseb J.* 10**,** 709-720. doi: 10.1096/fasebj.10.7.8635688

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13**,** 2498-2504. doi: 10.1101/gr.1239303

Singh, A., Gautier, B., Shannon, C.P., Rohart, F., Vacher, M., Tebutt, S.J., et al. (2018). DIABLO: from multi-omics assays to biomarker discovery, an integrative approach. *bioRxiv*. doi: 10.1101/067611

Tanaka, H., Masuta, C., Uehara, K., Kataoka, J., Koiwai, A., and Noma, M. (1997). Morphological changes and hypomethylation of DNA in transgenic tobacco expressing antisense RNA of the S-adenosyl-L-homocysteine hydrolase gene. *Plant Mol. Biol.* 35**,** 981-986. doi: 10.1023/A:1005896711321

Tariq, M., and Paszkowski, J. (2004). DNA and histone methylation in plants. *Trends Genet.* 20**,** 244-251. doi: 10.1016/j.tig.2004.04.005

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., et al. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* 37**,** 914-939. doi: 10.1111/j.1365-313x.2004.02016.x

Treutter, D. (2005). Significance of Flavonoids in Plant Resistance and Enhancement of Their Biosynthesis. *Plant Biol*. 7, 581-591. doi:10.1055/s-2005-873009

Trontin, J.-F., Klimaszewska, K., Morel, A., Hargreaves, C., and Lelu-Walter, M.-A. (2016). "Molecular aspects of conifer zygotic and somatic embryo development: a review of genome-wide approaches and recent insights," in *In vitro embryogenesis in higher plants*. (Cham: Springer International Publishing AG), 167-207.

Uggla, C., Magel, E., Moritz, T., and Sundberg, B. (2001). Function and dynamics of auxin and carbohydrates during earlywood/latewood transition in Scots pine. *Plant Physiol.* 125**,** 2029-2039. doi: 10.1104/pp.125.4.2029

Valdés, A.E., Fernández, B., and Centeno, M.L. (2004). Hormonal changes throughout maturation and ageing in *Pinus pinea*. *Plant Physiol. Bioch.* 42**,** 335-340. doi: 10.1016/j.plaphy.2004.02.004

Valledor, L., Carbó, M., Lamelas, L., Escandón, M., Colina, F.J., Cañal, M.J., et al. (2018). "When the tree let us see the forest: systems biology and natural variation studies in forest species" in *Progress in Botany*, eds Cánovas F., Lüttge U., Leuschner C., and Risueño MC (Cham: Springer International Publishing AG), 353-375.

Valledor, L., Escandón, M., Meijón, M., Nukarinen, E., Cañal, M.J., and Weckwerth, W. (2014a). A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *Plant J.* 79**,** 173-180. doi: 10.1111/tpj.12546

For Peer Review

Valledor, L., and Jorrín, J. (2011). Back to the basics: maximizing the information obtained by quantitative two dimensional gel electrophoresis analyses by an appropriate experimental design and statistical analyses. *J. Proteomics* 74**,** 1-18. doi: 10.1016/j.jprot.2010.07.007

Valledor, L., Jorrín, J.s.V., Rodríguez, J.L., Lenz, C., Meijón, M., Rodríguez, R., et al. (2010a). Combined proteomic and transcriptomic analysis identifies differentially expressed pathways associated to *Pinus radiata* needle maturation. *J. Proteome Res.* 9**,** 3954-3979. doi: 10.1021/pr1001669

Valledor, L., Meijón, M., Hasbún, R., Cañal, M.J., and Rodríguez, R. (2010b). Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of in vitro organogenic capability. *J. Plant Physiol.* 167**,** 351-357. doi: 10.1016/j.jplph.2009.09.018

Valledor, L., Pascual, J., Meijón, M., Escandón, M., and Cañal, M.J. (2015). Conserved epigenetic mechanisms could play a key role in regulation of photosynthesis and development-related genes during needle development of *Pinus radiata*. *PLoS One* 10**,** e0126405. doi: 10.1371/journal.pone.0126405

Valledor, L., Romero-Rodríguez, M.C., and Jorrin-Novo, J.V. (2014b). "Standardization of data processing and statistical analysis in comparative plant proteomics experiment," in *Plant Proteomics*. (Cham: International Publishing Springer AG), 51-60.

Valledor, L., and Weckwerth, W. (2014). "An improved detergent-compatible gel-fractionation LC-LTQ-Orbitrap-MS workflow for plant and microbial proteomics," in *Plant Proteomics: Methods and Protocols*. (Cham: Springer International Publishing AG), 347-358.

Vázquez-González, C., López-Goldar, X., Zas, R., and Sampedro, L. (2019). Neutral and climate-driven adaptive processes contribute to explain population variation in resin duct traits in a mediterranean pine species. *Front. Plant Sci.* 10, 1-12. doi: 10.3389/fpls.2019.01613

Verdú, M., and Climent, J. (2007). Evolutionary correlations of polycyclic shoot growth in *Acer* (Sapindaceae). *Am. J. Bot.* 94**,** 1316-1320. doi: 10.3732/ajb.94.8.1316

Vimont, N., Schwarzenberg, A., Domijan, M., Beauvieux, R., Arkoun, M., Jamois, F., et al. (2019). Hormonal balance finely tunes dormancy status in sweet cherry flower buds. *bioRxiv***,** 423871. doi: 10.1101/423871

Vizcaíno-Palomar, N., Ibáñez, I., González-Martínez, S.C., Zavala, M.A., and Alía, R. (2016). Adaptation and plasticity in aboveground allometry variation of four pine species along environmental gradients. *Ecol. Evol.* 6**,** 7561-7573. doi: 10.1002/ece3.2153

Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci*. 9**,** 244-252. doi: 10.1016/j.tplants.2004.03.006

Woo, E.-J., Dunwell, J.M., Goodenough, P.W., Marvier, A.C., and Pickersgill, R.W. (2000). Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat. Struct. Mol. Biol*. 7**,** 1036. doi: 10.1038/80954

Zas, R., and Fernández-López, J. (2005). Juvenile genetic parameters and genotypic stability of *Pinus pinaster* Ait. open-pollinated families under different water and nutrient regimes. *For. Sci.* 51**,** 165-174. doi: 10.1093/forestscience/51.2.165

Zas, R., Merlo, E., and Fernández-López, J. (2004). Genetic parameter estimates for Maritime pine in the Atlantic coast of North-west Spain. *For. Genet.* 11, 45-53. doi: 10261/101373

Zhang, C.-J., Zhao, B.-C., Ge, W.-N., Zhang, Y.-F., Song, Y., Sun, D.-Y., et al. (2011). An apoplastic h-type thioredoxin is involved in the stress response through regulation of the apoplastic reactive oxygen species in rice. *Plant Physiol.* 157**,** 1884-1899. doi: 10.1104/pp.111.182808

Zheng, C., Halaly, T., Acheampong, A.K., Takebayashi, Y., Jikumaru, Y., Kamiya, Y., et al. (2015). Abscisic acid (ABA) regulates grape bud dormancy, and dormancy release stimuli may act through modification of ABA metabolism. *J. Exp. Bot.* 66**,** 1527-1542. doi: 10.1093/jxb/eru519

Zhu, H., Qian, W., Lu, X., Li, D., Liu, X., Liu, K., et al. (2005). Expression patterns of purple acid phosphatase genes in *Arabidopsis* organs and functional analysis of *AtPAP23* predominantly transcribed in flower. *Plant Mol. Biol.* 59**,** 581-594. doi: 10.1007/s11103-005-0183-0

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5**,** 1411. doi: 10.1105/tpc.5.10.1411

FIGURE LEGENDS

Figure 1. Plant material employed in this analysis. Two-year old seedlings exhibiting an apical bud with young **(A)** or mature **(B)** morphology. Comparison of young (left) and mature (right) buds **(C)**. Dissection of young (left) and mature (right) buds into their apical and basal parts **(D)**. Vertical bars represent 0.5 cm length.

Figure 2. Venn diagrams showing the qualitative differences between bud sections and ontogenic stages at proteome **(A)** and metabolome **(B)** levels. Heatmap clustering of proteins **(C)** and metabolites **(D)** classified according to Mapman categories. Distances were established employing Manhattan distance and aggregated according to Ward's method.

Figure 3. Integrative analysis of proteome and metabolome. **(A)** Plot of samples scores at proteome and metabolome levels, showing components 1 and 2 in horizontal and vertical axes, respectively, **(B)** integrative heatmap clustering, **(C)** loadings plot showing the proteins with greatest correlation to components 1 and 2, **(D)** and loadings plot showing the metabolites with greatest correlation to components 1 and 2. Color code of the horizontal bar of the heatmap represents proteins (cyan) or metabolites (purple), while vertical shows treatments. Euclidean distances and complete-linkage algorithms were employed for classifying samples. Color of the protein loading bars represent the treatment with higher protein abundance.

Figure 4. Integrative analysis of proteome and metabolome during bud maturation. **(A)** sPLS-based network built after DIABLO analysis. Correlation cut-off was 0.75 and edge color reflect positive (red) or negative (green) interactions. Node color indicate Mapman functional bin and shape indicate proteins (circle) or metabolites (square). Same representations in which node color indicates that protein/metabolite is more abundant in **(B)** one of the treatments, **(C)** in the apical/basal part of the bud, or **(D)** in mature/young buds.

Figure 5. Whisker box representation of the qPCR analysis of target genes in the different bud sections. Dots indicate expression values of the different biological replicates normalized vs the expression of control genes (ΔCq Target/ΔCq Controls). Significant differences between bud sections and developmental status (ANOVA/Tukey HSD, $p < 0.001$) were highlighted (***).

For Peer Review

Title: Proteometabolomic characterization of apical bud maturation in *Pinus pinaster*

Running Title: Proteometabolomic characterization of apical bud

Luis Valledor*, Sara Guerrero, Lara García-Campa, Mónica Meijón*

Plant Physiology, Department of Organisms and Systems Biology, University of Oviedo, Oviedo 33071, Asturias, Spain

**Corresponding authors: valledorluis@uniovi.es; meijonmonica@uniovi.es*

KEYWORDS: Conifer, Bud aging, Metabolomics, Proteomics, Integrative approach

Number of words: 106475~~3~~

Number of words: 655560 (without References, Funding, Acknowledgments)

Number of figures included in manuscript: 5

Number of Supplementary figures: 1 (S1)

Number of Supplementary tables: 5 (S1 to S5)

For Peer Review

ABSTRACT (300 words)

Bud maturation is a physiological process which implies a set of morphophysiological changes which lead to the transition of growth patterns from ~~juvenile~~young to ~~adult~~mature. This transition defines tree growth and architecture, and in consequence traits such as biomass production and wood quality. In *Pinus pinaster*, a conifer of great timber value, bud maturation is closely related to polycyclism (multiple growth periods per year). This process causes a lack of apical dominance, and consequently increased branching that reduces its timber quality and value. However, despite its importance, little is known about bud maturation. In this work, proteomics and metabolomics were employed to study apical and basal sections of ~~juvenile~~young and ~~adult~~mature buds in *P. pinaster*. Proteins and metabolites in samples were described and quantified using (n)UPLC-LTQ-Orbitrap. The datasets were analyzed employing an integrative statistical approach, which allowed the determination of the interactions between proteins and metabolites and the different bud sections and ages. Specific dynamics of proteins and metabolites such as HISTONE H3 and H4, RIBOSOMAL PROTEINS L15 and L12, CHAPERONIN TCP1, 14-3-3 protein gamma, gibberellins A1, A3, A8, strigolactones and ABA, involved in epigenetic regulation, proteome remodeling, hormonal signaling and abiotic stress pathways showed their potential role during bud maturation. Candidates and pathways were validated employing interaction databases and targeted transcriptomics. These results increase our understanding of the molecular processes behind bud maturation a key step towards improving timber production and natural pine forests management in a future scenario of climate change. However, further studies are necessary by using different *P. pinaster* populations that show contrasting~~ant~~ wood quality and stress tolerance in order to generalize the results.

For Peer Review

INTRODUCTION

Pines are key players of forest ecosystems since most ~~of the~~ species have the ability to colonize a great variety of niches, act as good CO_2 sinks due to its fast growth (Allona et al., 1998), and are demanded by forest industry due to their quality timber, paper pulp and resins (Canales et al., 2014). Current production is not enough to cover current and predicted timber demand (FAO, 2009) and new strategies should be designed for implementing a more sustainable forest management also considering the climate change scenario (FAO, 2015). The use of fast-growth species such as *Pinus pinaster* Aiton, commonly known as maritime pine, may have an important role to this aim. This species has a major ecological and industrial role in southern Europe both at Mediterranean and Atlantic basins (González et al., 2016; Meijón et al., 2016; Cano et al., 2018). Its provenances adapted to different geoclimatic conditions across its distribution exhibit specific adaptions, allowing its optimal growth under a wide range of environments, from mild humid Atlantic regions to warm dry Mediterranean. These adaptions are genetically encoded, having this species a great genetic variability (Meijón et al., 2016; Vizcaíno-Palomar et al., 2016; Vázquez-González et al., 2019).

However, the architecture of this species can be greatly affected by harsh environmental conditions like drought periods, which can ultimately affect tree productivity~~despite its great adaptive capacity, the productivity of this species is greatly affected by environmental conditions and specifically drought periods since they can greatly alter tree architecture~~. *Pinus pinaster* is a species with a characteristic polycyclic growth (multiple shoot flushes in a single season) (Zas et al., 2004). This growth pattern, which reduces tree value due to a loss of apical dominance and increased branching, is inheritable and genetically conditioned, with provenances more polycyclic than others (Sabatier et al., 2003; Zas et al., 2004; Meijón et al., 2016). Polycyclism also presents an important environmental component, being conditioned by water availability, soil composition or radiation (Sabatier et al., 2003; Girard et al., 2011). It has been shown that periods of drought reduce its appearance and also tree growth, and that fertile soils and good irradiation lead to increase growth cycles and branching (Sabatier et al., 2003; Verdú and Climent, 2007). Polycyclism is also conditioned by the maturation state of the apical bud (Sabatier et al., 2003; Girard et al., 2011), being buds with ~~juvenile~~young phenology more polycyclic than ~~adult~~mature, although the molecular mechanisms that explain these differences have not yet been described.

Bud maturation implies a number of phenological changes, altering growth pattern, morphogenetic competence and increasing abiotic stress resistance (Jordy, 2004; Brunner et al., 2017). Maturation is regulated by internal and environmental factors. Among internal factors, plant hormones (Meijón et al., 2009) and epigenetic mechanisms regulating differential gene expression are required for bud maturation (Valledor et al., 2010b; Valledor et al., 2015; Conde et al., 2017). This differential gene expression should lead to changes in proteome and, in consequence, in metabolome, altogether resulting in the physiological and morphological characteristics of each ontogenetic age (Haffner et al., 1991; Meijón et al., 2016; Großkinsky et al., 2017). Surprisingly, and despite its importance for adaptive responses, tree growth or

For Peer Review

polycyclism, little is known about the molecular processes that are behind bud maturation process and different developmental stages and how they interact with environment. The great complexity that these processes seem to have along with the large number of variables potentially involved and not previously described suggest an unmanaged and massive strategy as the most appropiate to address this problem.

The availability of high throughput alternatives for molecular phenotyping such as gel-free proteomics and metabolomics give us an unprecedented capability to address this gap (Valledor et al., 2018). These techniques usually associated to model species can currently be applied with high confidence in almost any organism, since they do not require extended genome information. Despite unsequenced, *Pinus pinaster* has extensive transcriptomic data available that ease protein identification (Romero-Rodríguez et al., 2014). In this species, there are several contributions in the proteomics field but focused on the study of wood forming tissues (Paiva et al., 2008; Garcés et al., 2014) and somatic embryogenesis (Morel et al., 2014; Trontin et al., 2016). As the final reflection of genomes and its variation, metabolome analyses allowed to define population structures and evolution in this species (Meijón et al., 2016; López-Goldar et al., 2019), and also adaptive responses to abiotic/biotic stress ~~responses to abiotic/biotic stress and adaption~~ (Cañas et al., 2015; de Simón et al., 2017; López-Goldar et al., 2020). The combination of different omics greatly increases the power of analysis since the datasets of the different levels complement each other in a synergistic way (Mochida and Shinozaki, 2011; Kim et al., 2012). This type of integrative studies has already been carried out successfully in conifers to study needle development combining proteomics and transcriptomics (Valledor et al., 2010a) or to comprehensively analyze response to heat (Escandón et al., 2017) or ultraviolet stress comparing proteomics and metabolomics (Pascual et al., 2017). However, there is no information related to bud maturation in pines.

Therefore, the main aim of this work was to study the bud maturation process in *Pinus pinaster* using integrative omics approach combining proteomics and metabolomics. The integration of both levels allowed the extensive characterization of bud maturation processes. Specific dynamics of proteins and metabolites related to epigenetic regulation, proteome remodeling, hormonal signaling, and abiotic stress response (such as histones, ribosomal proteins, strigolactones, gibberellins, ABA, and chaperones) showed an essential role during bud maturation. In addition, the interconnection of these elements and its relation to different polycyclic capacity and stress tolerance of each maturation state of the bud was revealed through an integrative approach.

MATERIAL AND METHODS

Plant material and growth conditions

Apical buds in young and mature stages were sampled from two-years-old *Pinus pinaster* seedlings (plant size ~~about~~ around 20 ± 3 cm) just after they exhibited young/mature apical phase change. These plants were grown in a greenhouse with seasonal fertirrigation.

For Peer Review

Buds in the young stage show leaf primordia differentiate into photosynthetically active primary needles around of the shoot apical meristem (SAM). However, when it is reached the mature stage is reached, leaf primordia differentiate into scale leaves, and photosynthetic activity is shifted to long needles differentiated on brachyblasts in more distal positions on the stem (Jordy, 2004). Apical buds of 18 seedlings of the same age, half of them having mature morphology and half of them young, were sampled and dissected into their apical and basal sections, corresponding to apical and axillary/foliar meristems, respectively (Figure 1). Three biological replicates for each treatment (apical and basal parts of the bud, young and mature buds) were constituted pooling basal or apical parts of the buds of three different seedlings with the same bud developmental stage. Samples were immediately frozen in liquid nitrogen and kept at -80 ° C until biomolecule extraction. Metabolites, proteins, and RNA were isolated from the same sample following the protocol of Valledor et al. (2014a) using 75 mg of fresh weight per sample.

Metabolome analysis

High-performance liquid chromatography (Dionex Ultimate 3000, ThermoFisher Scientific, USA) was coupled to a LTQ-Orbitrap XL high resolution mass spectrometer equipped with a HESI II (heated electrospray ionization) source and controlled by Xcalibur version 2.2 (Thermo Fisher Corporation). Polar fraction of each sample was analyzed twice, first using the positive and then the negative ion modes. Samples were run according to the procedure described by Meijón et al. (2016). Instrument was operated in full-scan mode with a resolution of 60 000, and spectra were acquired in mass range m/z 50–1000 in the positive mode, and 65–1000 in the negative mode. The resolution and sensitivity were controlled by the injection of a standard mix (caffeine, proline, and sucrose) after the analysis of each batch and resolution was also checked with the aid of lock masses (phthalates). Blanks were also analyzed during the sequence. With the aim of improving metabolite assignation, one sample of each treatment was additionally reanalyzed including an ion fragmentation step. Chromatrographic and analytical conditions were the same, but top-three ions of each scan were fragmented (30 s dynamic exclusion window). Parent ions (minimum intensity of 500) were fragmented by CID (normalized collision energy of 35, activation Q 0.25, and activation time 90 ms). These spectra were employed for MS/MS metabolite identification as described below.

RAW files were directly processed employing MZMINE v2.14 (Pluskal et al., 2010). Spectra were filtered establishing noise threshold at 2×10^4 and minimum peak height at 2.5×10^5. Peaks were smoothed and deconvoluted using a local minimum search algorithm (95 % chromatographic threshold, minimum retention range 0.2 min, minimum relative height of 5 %, and minimum ratio top/edge of 0.5). Chromatograms were aligned using the RANSAC algorithm with a tolerance of 5 ppm of m/z and 0.2 min of retention time. Peak areas were used for quantification.

Peaks were identified following a sequential approach. The first stage was performed against our in-house library (>100 compounds) and manual annotation considering its m/z and retention times. In a second stage, MS/MS data was used for identification employing Compound

For Peer Review

Discoverer software (Thermo Scientific, USA) and custom scripts for comparing experimental data to MS/MS databases Metlin, HMDB and FooDB. A positive identification was defined when parent mass was below 5-ppm threshold compared to analyte in DB and at least, two main ions of fragmentation spectra were identified. The last stage assigned potential identity to masses by direct comparsion of ion masses using a 5-ppm threshold and KEGG, HMDB, FooDB, Plantcyc, and MassBank databases. Those metabolites that were defined after the comparison with our standard compound library or by a matching of MS/ MS were considered as unquestionably 'identified', while were considered 'tentatively assigned' those molecular ions with exact masses corresponding to identified metabolites in databases. Metabolite identification against our library was confirmed by RT, mass, and isotopic patterns.

Protein identification and quantitation using nLC-Orbitrap-MS analysis

Sixty µg of total protein were cleaned, digested, and desalted following the protocol described by Valledor and Weckwerth (2014). Peptide chromatography and mass spectrometric analysis were performed according to Pascual et al. (2017) with only a slight modification in the effective gradient, which was set to 90 min from 5 % to 45 % acetonitrile/0.1 % formic acid (v:v) with a later column regeneration step of 27 min. The employed column was a Chromoltih RP-18R 15 cm length 0.1 cm inner diameter (Merck, Germany).

Spectra were processed in Proteome Discoverer 2.0 (Thermo Scientific, USA). Protein identification threshold was established at 5% and 1% false discovery rates (FDR) at peptide and protein levels, respectively. Only proteins with at least two identified peptides and one of them unique were considered as identified. Four databases were used: *Pinus sylvestris* and *Pinus taeda* (34063 accessions) (Proost et al., 2014), and against in-house databases, *Pinus pinaster* (117080 accessions) and *Pinus radiata* (67647 accessions) that were built following the procedure described by Romero-Rodríguez et al. (2014). Proteins were also functionally classified according to Mapman (Thimm et al., 2004) functional bins. Identified proteins were quantified by a label-free approach based on the estimation of the areas of the three most abundant peaks assigned to each protein by Proteome Discoverer.

Targeted transcriptomic analysis of candidate genes

RNA abundance was determined in a microdrop spectrophotometer NB1 (Nabi, South Korea) and its integrity was checked by agarose gel electrophoresis. One µg of RNA was reversed transcribed using the RevertAid kit (Thermo Scientific, USA) and random hexamers as primers following the manufacturer's instructions. qPCR reactions were performed in a CFX96™ Real-Time System (Biorad, USA) with RealQ Plus Master Mix Green, no ROX (2X) (Ampliqon, Denmark); four biological and two analytical replicates per treatment were made for each gene. Expression levels of *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH*) and *UBIQUITINE (UBI)* were used as endogenous control and the results were analyzed by Bio-Rad CFX Manager 3.1 (Biorad, USA) software using the cycle threshold comparative method (ΔC_T).

For Peer Review

Detailed information about the primers used for qPCR experiments is available in Supplementary Table S1.

Statistical and bioinformatics analysis

All statistical procedures were conducted with the R programming language running under the open source computer software R v3.5.0 (R Development Core Team, 2015) and RStudio v1.1.423 (RStudio Team, 2016) using the packages pRocesomics[1] and mixOmics (Rohart et al., 2017).

Three biological replicates per treatment were used for metabolome and proteome analysis. Proteome and metabolome datasets were pre-processed following the recommendations of Valledor and Jorrín (2011) and Valledor et al. (2014b). In brief, missing values were imputed using a Random Forest approach, and variables were filtered out if they were not present in at least all of replicates corresponding to one treatment or in at least 45% of the analyzed samples. Data were normalized and transformed following a samplecentric approach followed by log transformation. Centered and scaled values (z-scores) were subjected to univariate (one-way ANOVA followed by a Tukey HSD post-hoc test, $P<0.05$.) and Venn diagrams, and heat map clustering. To avoid variable noise only those variables with interquartile range 50% greater than average were selected for multivariate analyses. Integrative analysis was based on the use of DIABLO algorithm and network representation. Cytoscape v. 3.7 (Shannon et al., 2003) was employed in network representation and analysis. Proteins were annotated according to Mapman classification employing protein sequences and Mercator online tool v3.6 (Lohse et al., 2014) while metabolites were manually classified according to this classification.

RESULTS

Proteomic and metabolomic characterization of buds in *Pinus pinaster*

Proteomic analyses of ~~juvenile~~young and ~~adult~~mature buds and their sections allowed the identification of 1609 proteins. Proteins were annotated according to Mapman, classifying 1540 proteins in 34 functional bins. 951 proteins showed abundances and consistencies above threshold for their use in quantitative analyses. Out of these, 142 were differentially accumulated between treatments (ANOVA p-value <0.05; Supplementary Table S2). At metabolome level, 3670 peaks were detected, being 2267 suitable for quantification (Supplementary Table S3). From these, 133 peaks were unequivocally identified using the in-house database or MS/MS spectra, corresponding to 105 unique compounds, and 974 were tentatively assigned by comparing their very accurate masses to those available in public databases (Supplementary Table S4). 75 metabolites were classified according to Mapman. Despite the high number of identified/assigned metabolites, their complete classification according to Mapman was not possible, mainly due to the difficulty of classifying secondary metabolites. However, this potential bias does not affect the most studied and preserved functional groups such as those related to

[1] https://github.com/Valledor/pRocessomics

For Peer Review

primary metabolism. From all detected compounds, 914 were differentially expressed between analyzed samples (ANOVA 5% FDR; Supplementary Table S3).

Venn analyses revealed qualitative differences between ontogenic stages and bud sections. At protein level (Figure 2A), ~~juvenile~~young buds (apical and basal sections) showed the highest number of characteristic proteins (126) but, interestingly, the apical section of the ~~adult~~mature bud was the most differentiated organ with 59 characteristic proteins. At metabolome level (Figure 2B), ~~adult~~mature tissues showed the highest rate of unique compounds (569), while the apical section of the ~~juvenile~~young bud exhibited the highest number of characteristic metabolites (194).

Quantitative analyses of Mapman classified proteins (Figure 2C) and metabolites (Figure 2D) pointed the differential pathways among bud sections and developmental stages. At proteome level, increased pathway clusters related to stress, hormone, lipidic, energetic, and major carbohydrate metabolism pathways can be correlated to the different tissues that were analyzed. Heatmap classified samples according to bud section, which may be suggesting a greater variation between apical and basal meristem proteomes than between ~~adult~~mature and ~~juvenile~~young bud proteomes. Contrary to proteome, metabolome allowed the classification of samples in relation to their ontogenetic age. This metabolomic differentiation was mainly caused by differences in photosynthesis, aminoacid synthesis and metabolism, redox regulation, and nucleotide metabolism.

These differences between ~~adult~~mature and ~~juvenile~~young buds and their basal and apical sections may be related to the location of the shoot apical meristem (SAM) activity in the apical section of the bud and also to the differential growth and developmental patterns of ~~juvenile~~young and ~~adult~~mature buds, as it was demonstrated by the differential accumulation of sample-specific pathways. Furthermore, the accumulation of a higher number of specific metabolites in comparison to proteins revealed the potential effect over the metabolome of the changes related to a smaller number of proteins. Alterations of key enzymes of metabolic pathways may lead to changes in abundance of a large number of metabolites. On the other hand, samples with a great ontogenic and functional differentiation (~~juvenile~~young vs ~~adult~~mature) shared numerous proteins, which difficulted their classification according to their developmental stage. However, metabolites allowed their classification, reflecting the importance of metabolomic specificity in functional differentiation. The metabolome is the final downstream product of gene transcription and, therefore, changes in it are amplified relative to the changes in transcriptome and proteome (Das et al., 2015; Escandón et al., 2017). As ~~juvenile~~young buds present more active development than differentiated ~~adult~~mature buds, it is expected an overaccumulation of metabolites related to active processes of development, such as redox activity or aminoacid synthesis.

The integrative analysis of proteome and metabolome unmasked potential interaction networks involved in bud maturation and differentiation in *Pinus pinaster*

For Peer Review

The combination of different omic levels in an integrative analysis supposes a major analytical advantage since the different levels can be used for cross-validation and, at the same time, to get a global overview of the physiological processes (Singh et al., 2018). For this purpose, we employed DIABLO algorithm (Rohart et al., 2017) to analyze proteome and metabolome datasets (Figure 3). This approach provided a better clustering of the samples at proteome and metabolome levels (Figure 3A). The joint analysis of both datasets correctly classified studied tissues (Figure 3B). The basis of this classification relied on the biological source of variation gathered by two main components, which collectively accounted 59% of the total variance (Supplementary Table S5). The analysis of the variables exhibiting highest loadings to these components (Figure 3C-D; Supplementary Table S5) allowed a biological interpretation of these results.

Component 1 gathered the variation related to ontogenic differentiation, distinguishing between ~~juvenile~~young and mature buds. Enzymes related to energy, protein biosynthesis, lipid metabolism, and signaling/differentiation showed the highest correlations to this component (Figure 3C). ~~Juvenile~~Young tissues were characterized by an increased accumulation of energy-related ATPases or PSII reaction center proteins as well as several ribosomal- and RNA-related proteins. Lipids are supposed to be relevant players in this differentiation, and despite no differential lipids were identified after metabolome analysis, enzymes related to their metabolism were key nodes of our models. ACETYLCOA CARBOXYLASE CARBOXYL TRANSFERASE SUBUNIT ALPHA, which is a key lipidic enzyme as seen above (Harwood, 1996), a START-like domain, whose function in lipid regulation in plants was previously suggested (Ponting and Aravind, 1999), and GDSL ESTERASE LIPASE, being described its function in development, defense, synthesis of secondary metabolites, and morphogenesis in some plant species (Chepyshko et al., 2012), were some of the most highlighted lipidic-related enzymes.

On the other hand, RAS proteins, 14-3-3 transcription factors and DNA regulatory proteins by epigenetic mechanisms were re-emphasized as key processes in bud differentiation (Figure 3B). HISTONE 3 and HISTONE 4 (Tariq and Paszkowski, 2004; Valledor et al., 2010b; Bräutigam et al., 2013), GLYCOSYL HYDROLASES FAMILY 100 (Penterman et al., 2007), and RAS related proteins (Kamada et al., 1992; Alonso et al., 2007) regulate developmental processes, and were important to explain the ontogenic differences in the analyzed tissues. Many of these proteins were correlated to primary and secondary metabolites, some of which are characteristic of specific physiological stages, and therefore, having a potential involvement in development. Even though none of the most significant metabolites were identified in public databases, it would be interesting to highlight some of them due to their possible importance in differentiation interaction networks. Specifically, in ontogenic differentiation, significant metabolites (P0340, N1518, P0320, and N1292) seemed to differentiate ~~juvenile~~young from ~~adult~~mature buds (Figure 3D; Supplementary Table S5).

Second component distinguished between the apical and basal parts of the bud. Enzymes related to protein biosynthesis and folding-related, as well as peroxidases, were characteristic of this

component (Figure 3C). Apical sections of the bud showed a positive correlation, and were characterized by increased abundance of stress-related proteins such as CHAPERONIN-LIKE TCP1 and others (Wang et al., 2004), a MANGANESE BINDING SITE from a GERMIN, which is related to abiotic and biotic stresses response in plants (Woo et al., 2000), and a PEROXIDASE. On the other hand, key variables defining the basal parts of the buds showed negative correlation to this component. Basal sections were characterized by higher growth and proliferation rates than apical, fact that explains the importance of proteins related to cell division and reorganization such as PROFILIN 1 or PROHIBITIN 1 in this model. This last protein was only found in basal sections of ~~adult~~mature bud, having multiple functions related to plant development and stress tolerance (Chen et al., 2005). In the same way, several transferases such as PYRIDOXAL PHOSPHATE DEPENDENT TRANSFERASE or PHOSPHORIBOSYLGLYCINAMIDE FORMYLTRANSFERASE were characteristic of the basal section of the bud. Most of the metabolites associated to this component were not identified. Among identified metabolites, loganin and deoxyloganin were only detected in apical sections of the bud, while basal had only highlighted unidentified metabolites (P1560 + P1340, N2243 and P1379) (Figure 3D; Supplementary Table S5).

The clustering and heatmap visualization of this integrative analysis (Figure 3B; Supplementary Figure S1) complemented results described above, revealing four different sets of variables: those proteins and metabolites over-accumulated in apical section of ~~juvenile~~young bud and down-accumulated in the rest of the samples; those over-acumulated in basal section of ~~juvenile~~young bud and down-accumulated in the rest of the samples; those over-acumulated in ~~juvenile~~young buds and not in ~~adult~~matures; and those over-acumulated in ~~adult~~mature buds but not in ~~juvenile~~young ones. First set of variables was mainly composed by metabolites and proteins already referenced such as GERMIN MANGANESE BINDING PROTEIN, PHOSPHOGLUCONATE DEHYDROGENASE or ribosomal proteins; conversely, second set of proteins was essentialy constituted by proteins from different metabolic pathways such as photosynthesis, redox mechanism or signaling. Above all the differential variables found in the third set of proteins, this group was characterized by numerous proteins belonging on the one hand to stress pathways such as the previously described CPN10 or H-TYPE THIOREDOXIN (Zhang et al., 2011) and EPOXIDE HYDROLASE (Morisseau, 2013) like proteins, and on the other hand, to energetic routes with a large number of proteins identified (ATP-DEPENDENT 6-PHOSPHOFRUCTOKINASE 2, MALATE DEHYDROGENASE, CYTOCHROME C, V-TYPE PROTON ATPase SUBUNIT D, SUCCINATE DEHYDROGENASE UBIQUINONE FLAVOPROTEIN SUBUNIT). Likewise, previously highlighted proteins related to lipid metabolism (ACETYL-COA ACETYLTRANSFERASE) and cellular reorganization (PROFILIN 1 and PROHIBITIN 1) were found in this pattern. Last set, including those proteins characteristic of ~~adult~~mature buds, differentiation was established by a large number of non-identified metabolites and a small number of proteins, including ribosomal proteins, 14-3-3, HISTONE H3 or GLYCOSYL HYDROLASE FAMILY 100 previously described.

The analysis of proteome-metabolome interaction combined with targeted transcriptomics allowed a deeper characterization of the bud differentitation process

The integration of metabolome and proteome datasets also allowed the definition of a protein-metabolite interaction network based on the different correlations between variables of different types. The resulting network (Figure 4; correlation >0.75) showed two interaction clusters. First cluster (Figure 4A, left) gathered proteins and metabolites more abundant in the apical sections of ~~juvenile~~young buds (Figures 4B-D). Proteins in this cluster are related to protein biosynthesis and transport (ribosomal-related, CHAPERONIN TCP1, TMP21), transcriptional response to stress (GERMIN, NUCLEIC ACID BINDING PROTEIN), and a PHOSPHOESTERASE similar to Arabidopsis PURPLE ACID PHOSPHATASE, PAP14 (At2g46880), required for petal differentiation and expansion (Zhu et al., 2005). These proteins positively correlated to different terpenoids (mascaroside, loganin, menthane) and the flavonoid luteolin. The abundance of these variables greatly diminished during the transition to basal section and also in ~~adult~~mature buds. 4,4'-ditolylthiourea, the only metabolite in this cluster whose abundance is maximal in the basal section of ~~adult~~mature buds, showed negative correlations to all of its linked nodes.

Second cluster (Figure 4A, right) was not as selective in its variable categories as first cluster, since groups variables peaking at each developmental stage and bud section. However, there was a major presence of variables more accumulated in ~~adult~~mature tissues and/or basal sections of the buds (Figures 4C, D). ~~Juvenile~~Young buds are characterized by an increased photosynthesis (active center of PSII, GAPDH) and glycolytic (PYRUVATE KINASE, PHOSPHOFRUCTOKINASE) pathways. HISTONE H4, GDSL ESTERASE LIPASE, and elements related to protein biosynthesis (eRF1, protease inhibitor SERPIN, ribosomal proteins L15 and L50) and redox (THIOREDOXIN, START-like domain protein) were also more abundant in ~~juvenile~~young buds. Most of these enzymes were positively correlated to N1751, an unknown metabolite, and were characteristic of basal sections of the bud. On the other hand, ~~adult~~mature buds had increased energetic (fructose-6-P and ATPase), signaling and gene regulation (14-3-3 protein gamma, HISTONE H3, regulator of ribonuclease activity), antioxidant/detoxification activities (dihydrolipoate), and proteome remodeling (ribosomal proteins and peptidases). 2-alpha-(S)-Strictosidine, a key compound in monoterpene indol alkaloyds biosynthetic pathway (Rüffer et al., 1978), was mostly accumulated in the basal sections.

Interestingly, HISTONE H3, and other proteins related to epigenetic regulation of gene expression formed a cluster that had a greater accumulation in ~~adult~~mature buds. Despite having a positive correlation to most of the metabolites of this cluster, it negatively correlated to those metabolites accumulated in ~~juvenile~~young buds. HISTONE H4, characteristic of ~~juvenile~~young tissues, negatively correlated to metabolite P0265 and through it to glycolytic and carbon-related enzymes, suggesting its role in regulation of energetic pathways. Finally, 14-3-3 Protein gamma, involved in the signaling pathways of the main plant hormones (Camoni et al., 2018), was also accumulated in ~~adult~~mature buds in the apical section. However, in this case, it was negatively

For Peer Review

correlated to most of the metabolites in this cluster, some of them as relevant as diphyllin, lignin (Hemmati et al., 2007), or fructose-6-P.

In order to support hypotheses raised after proteometabolomic dataset, six genes related to the different activities of the clusters depicted above were analyzed by qPCR (Figure 5). According to transcriptomics results, the different treatment had different expression patterns of the analyzed genes. Apical section of ~~juvenile~~young buds had a differential overexpression of *ADENOSYLHOMOCYSTEINASE (SAHH)*, related to epigenetic regulation (Tanaka et al., 1997; Rocha et al., 2005), and *GLUTATHIONE S-TRANSFERASE (GST)* and *PHENYLALANINE AMMONIA LYASE (PAL),* both genes involved in secondary metabolism and hormonal signaling (Hahlbrock and Scheel, 1989; Rivero et al., 2001; Dixon et al., 2010; Czerniawski and Bednarek, 2018). The expression level of these genes was similar in ~~adult~~mature buds and in the basal part of the ~~juvenile~~young bud.

S-ADENOSYLMETHIONINE SYNTHASE (SAM SYNTHASE), a central element in DNA methylation (Gómez-Gómez and Carrasco, 1998) was more expressed in ~~juvenile~~young buds, as *NEDD8-ACTIVATING ENZYME E1 CATALYTIC SUBUNIT (NEDD8-E1)*, involved in proteome remodelling and DNA repair (Brown and Jackson, 2015; Brown et al., 2015). Finally, it is important to highlight the expression pattern of the *MORE AXILLARY GROWTH 1 (MAX1)* gene, which is required for hormonal biosynthesis of a shoot-branching inhibiting signal (Booker et al., 2005). *MAX1* increased its expression in ~~juvenile~~young basal section, while its expression decreased drastically in ~~adult~~mature basal section.

DISCUSSION

The study of the transition between ~~juvenile~~young and ~~adult~~mature buds, and how it is reflected at metabolome and proteome levels, is not only crucial in order to understand shoot growth and tree architecture, but also to improve relevant traits for forestry such as total growth or polycyclism (Cabezas et al., 2015). Growth-related traits are influenced not only by their inherent genetic factors (most of them polygenic) but also by the environment (Zas and Fernández-López, 2005). Environmental conditions (light, temperature, rainfall) in combination with the genotype define not only the yearly tree growth period, ontogenic stage and flowering time, but also tree architecture, modulating for instance, polycyclism (Meijón et al., 2016; de Simón et al., 2018). The combination of genetic and environmental factors, together with the different growth patterns of mature and ~~juvenile~~young buds sometimes mixed in trees of the same age, makes the apical growth in *Pinus pinaster* very complex at physiological and molecular levels (Nguyen et al., 1995). Consequently, the characterization of the proteome and metabolome of buds in different developmental stages is a necessary first step towards the fully understanding of all of these processes.

The employment of an integrative approach allowed the characterization of bud maturation from a holistic perspective, identifying the key molecular pathways of this process. Two main sources of variation were clearly distinguished after clustering and multivariate analyses. The first was the bud maturation status (~~juvenile~~young vs ~~adult~~mature) and the second the presence of apical or

For Peer Review

lateral meristems (apical vs basal section of the buds), each of them with a different set of characteristic biomolecules.

~~Adult~~Mature buds exhibit specific phenology and growth patterns distinct from ~~juvenile~~young buds. In the ~~juvenile~~young stage, leaf primordia differentiate into photosynthetically active primary needles. Axillary buds develop either into auxiblasts in basal positions or into randomly distributed brachyblasts in more distal positions on the stem. When trees reach the ~~adult~~mature stage, leaf primordia differentiate into scale leaves, and photosynthetic activity is shifted to long needles differentiated on brachyblasts (Nguyen et al., 1995; Jordy, 2004). The physiological competence of ~~adult~~mature buds was probably imposed by the increased abundance of ABA and specific gibberelins (bioactive GA1 and intermediary GA19; Supplementary Table S3). ABA has not only a role in dormancy, but also in dormancy release and bud set in combination with specific gibberellins (Zheng et al., 2015; Maurya et al., 2018; Vimont et al., 2019). Interestingly, ~~juvenile~~young buds had slightly lower concentrations of ABA but an increased abundance of bioactive GA3, and GA8, a degradation product of GA1. Both gibberellins, GA1 and GA3, are related to shoot elongation (Little and MacDonald, 2003); however, is complicated to venture which specific role are playing each one in the different development stage of the bud. Additionally, in relation to hormonal signaling, a key element was identified in the network, 14-3-3 protein. A possible role for 14-3-3 proteins in the coordination of GA and ABA signaling has emerged in the last years (Camoni et al., 2018). In fact, the overexpression of ABA responsive, 14-3-3-interacting transcription factors ABF1-3 impairs GA action, indicating that they act as negative regulators of GA signaling and that 14-3-3 proteins may function by sequestering ABF1-3 in the cytoplasm. However, the mechanism of 14-3-3 action and all the elements involved in the ABA and GA coordination are still unknown.

As pointed by datasets and correlation networks, having the enzymatic machinery to increase metabolic rate allowing burst and growth seems to be essential in ~~juvenile~~young buds, since enzymes of photosynthesis and glycolytic pathways were increased compared to ~~adult~~mature (Figures 2 and 4). Interestingly, the accumulation of fructose-6-P in ~~adult~~mature buds indicate not only a differential allocation of sugars between ontogenic stages, but also specific hormonal, growth (Eveland and Jackson, 2012), and maturation patterns (Uggla et al., 2001). Lipids such as pimelate and dihydroxylipoate with a dual redox and transcriptional regulation role (Sen and Packer, 1996) were also accumulated in ~~adult~~mature buds. The mature secondary metabolism was also reflected by the accumulation of flavonoids. Flavonoids and tannins may be involved in cellular detoxification (Meijón et al., 2016) and also in plant resistance against herbivores or other stresses (Treutter, 2008). At the end of the growing season, accumulation of lipid and starch is positively correlated with the onset of dormancy in ~~adult~~mature buds (Jordy, 2004). Bud development is also associated to different abiotic stress tolerance and proteins related to detoxification. In all analyzed tissues, there were a great abundance of heat shock or chaperonin-like proteins (TCP1, CPN10) and ROS detoxifying enzymes (peroxidases, thioredoxines); however, mature buds were characterized by higher abundances of these protein families,

For Peer Review

suggesting that the greater tolerance to stress exhibited by these buds (Miller et al., 2008) relies on the overaccumulation of these ~~mechanisms~~molecules, compared to ~~juvenile~~young tissues.

The different cell competence is defined by specific protein sets consequence of differential transcriptional and post-transcriptional regulation (del Mar Castellano et al., 2004; Dembinsky et al., 2007). ~~Adult~~Mature and ~~juvenile~~young buds have a differential set of DNA/RNA interacting proteins and ribosomal proteins. Among them, START-like domain proteins were up-accumulated in ~~juvenile~~young tissues, despite one characteristic of ~~adult~~mature buds (PPI00057470). The specific dynamic of this family, which is required for regulating transcription factors needed for cell differentiation after binding a lipid ligand (Schrick et al., 2014; Grabon et al., 2019), illustrates the complexity of bud maturation. ~~Juvenile~~Young buds were characterized by the serin protease inhibitors SERPIN (+5-fold change compared to ~~adult~~mature) (Supplementary Table S2), which has been proposed to have also a non-peptidase inhibitory functions negatively regulating stress-induced cell death or reducing gene expression by compacting chromatin (Cohen et al., 2019). Epigenetic regulation elements were also differential between ~~adult~~mature and ~~juvenile~~young buds (discussed below).

The complexity of a pine bud was also reflected in the direct comparison of its apical and basal sections. The former containing the shoot apical meristem, and the later the lateral meristems and needle primordia (Fernando, 2014). This organization implies physiological and metabolic differences, which has been validated through this work. Starch, lipid reserves, and tannins are known to accumulate in the shoot tip as pines become older (Jordy et al., 2000; Jordy, 2004). The accumulation of flavonoids and its biosynthetic machinery in the apical part of the buds reinforces its role in the protection of the meristem (Meijón et al., 2016) and also in the vegetative bud outgrowth. Differential organogenetic activity in apical and basal sections, changeable across the bud maturation, is also related to environment conditions and apical dominance regulation (Jordy, 2004; Hover et al., 2017). Thus, the high activity of *MAX1* gene in both sections of ~~juvenile~~young bud suggests the essential role of strigolactone hormone regulating inhibition of axillary bud outgrowth in this phase; however, in mature bud, *MAX1* expression is higher in apical section (Figure 5). The current models in relation to control of apical dominance suggest complex interaction networks where sugars and ABA could be responsible for initial release of an apical bud, while auxins, strigolactones and cytokinins seem to determine sustained outgrowth of axillary buds (Nguyen and Emery, 2017). Gibberellins, despite being their role well known in shoot elongation, need more investigation to determine their function inside this network. However, some reports on their interaction with strigolactone suggest that increased gibberellin levels could repress axillary bud outgrowth (Luisi et al., 2011).

The basal part of the bud is prepared to burst, contrary to apical whose function is keeping and protecting apical meristem, and many cell division and development-related proteins like FTSZ needed for plastid division (Schmitz et al., 2009), dormancy/associated or DNA repair machinery (Os08g0519400 like protein) were up-accumulated. LEA proteins and TMP21, associated to less differentiated organs (Zimmerman, 1993), were characteristic of the apical part of the buds

probably helping to maintain meristem identity together with specific abundances of growth regulators and nucleic acid binding proteins and histone modifications regulating gene expression.

The great amount of differential proteins and metabolites involved in epigenetic regulation suggests the key role of these mechanisms in bud development. DNA methylation is a well-known epigenetic mark of transcriptional gene silencing, but also in the establishment of heterochromatin, transposon control and genomic imprinting (Galindo-González et al., 2018). Two of the key enzymes regulating the methylation cycle, SAM SYNTHASE and SAHH, showed an overaccumulation in ~~juvenile~~young buds and more specifically in its apical part (Figure 5), suggesting that bud maturation is concomitant to increased DNA methylation levels as previously reported in *Pinus radiata* (Fraga et al., 2002). ARGONAUTE, a key enzyme involved in RNA-mediated DNA methylation, was overaccumulated in ~~adult~~mature apical buds, which will be probably related to the hypermethylation associated to development. Despite the employed analytical procedures were not intended to describe post-translational modifications defining histone code, specific forms of HISTONE H3 and H4 were characteristic of each developmental stage, reinforcing the hypothesis that bud maturation is the consequence of a complex interaction between epigenetic mechanisms, transcription factors and hormonal regulators.

Overall, our study provided novel insights over bud maturation and the machinery involved in its development and growth and stress resilience at different molecular levels and pathways. The comprehensive overview of this process allowed the validation of proteins and metabolites involved in bud development that were previously described, but also the involvement of novel proteins, metabolites, and pathways. However, further studies will be necessary to validate these new set of candidate molecules by using buds coming from different populations that show ~~contrastant~~ contrasting wood quality and stress tolerance.

DATA AND MATERIALS AVAILABILITY

All relevant data can be found within the manuscript and supplementary materials.

SUPLEMENTARY MATERIAL

Table S1. Genes and primers employed in quantitative PCR analyses.

Table S2. Proteins identification according to SEQUEST (scores, % of coverage, number of common, unique, and razor peptides), quantification (mean ± SD of three biological replicates), univariate analysis (p and q values; TukeyHSD p values for all paired comparisons).

Table S3. Peaks obtained after UPLC-MS analysis of polar metabolites. Peaks were aligned with mzMine 2.10 avoiding redundancies between positive (P) and negative (N) modes. This table shows peak ID, adduct, m/z, retention time, normalized peak areas for each analyzed sample, and univariate analysis (p and q values; TukeyHSD p values for all paired comparisons). Peak compound description is provided according to Supplementary Table S4.

For Peer Review

Table S4. Identification of metabolites in the 3670 peaks that were analyzed. a) 133 peaks were unequivocally identified (those metabolites that were defined after the comparison to our compound library or by comparison of the MS/MS to *online* databases). b) 987 peaks were tentatively assigned after comparing its very accurate mass to reference compound databases. Delta ppm and compound exact mass are provided. Annotation source, molecular form, and accessions of KEGG, FooDB and other databases are provided for all identified/assigned compounds.

Table S5. DIABLO integrative analysis of proteome and metabolome datasets. a) Sample scores for components 1 and 2 in both datasets, b) variance explained for each component, and c) variable loadings for both datasets.

Figure S1. Integrative clustering of proteome and metabolome analysis (High resolution Figure 3B).

DISCLOSURES

The authors have no conflicts of interest to declare.

FUNDING

This publication is an output of the National Project Vampiro (AGL2017-83988-R, Spanish Ministry of Economy and Competitiveness). M.M. and L.V. were supported by Ramón y Cajal program (RYC-2014-14981 and RYC-2015-17871, respectively; Spanish Ministry of Economy and Competitiveness). L.G.C and S.G. were supported by Severo Ochoa Predoctoral Program (BP19-146 and BP19-145, respectively; Principality of Asturias, Spain).

ACKNOWLEDGMENTS

The authors wish to thank Eloy A. Ron (SERPA S.A, Vivero Forestal de La Mata) for providing the *Pinus pinaster* seedlings employed in this work.

AUTHORS' CONTRIBUTIONS

MM and LV designed the experiments, performed mass-spectrometry analyses, and metabolome-related computational analysis. LV and SG performed proteome-related computational analysis. LGC performed targeted-transcriptomics analyses. LV and SG performed statistical analyses. All authors wrote the manuscript, read, and approved the final version of the manuscript.

For Peer Review

REFERENCES

Allenbach, L., and Poirier, Y. (2000). Analysis of the alternative pathways for the β-oxidation of unsaturated fatty acids using transgenic plants synthesizing polyhydroxyalkanoates in peroxisomes. *Plant Physiol.* 124**,** 1159-1168. doi: 10.1104/pp.124.3.1159

Allona, I., Quinn, M., Shoop, E., Swope, K., Cyr, S.S., Carlis, J., et al. (1998). Analysis of xylem formation in pine by cDNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 95**,** 9693-9698. doi: 10.1073/pnas.95.16.9693

Alonso, P., Cortizo, M., Cantón, F.R., Fernández, B., Rodríguez, A., Centeno, M.L., et al. (2007). Identification of genes differentially expressed during adventitious shoot induction in *Pinus pinea* cotyledons by subtractive hybridization and quantitative PCR. *Tree Physiol.* 27**,** 1721-1730. doi: 10.1093/treephys/27.12.1721

Baldermann, S., Homann, T., Neugart, S., Chmielewski, F.-M., Götz, K.-P., Gödeke, K., et al. (2018). Selected plant metabolites involved in oxidation-reduction processes during bud dormancy and ontogenetic development in sweet cherry buds (*Prunus avium* L.). *Molecules* 23**,** 1197. doi: 10.3390/molecules23051197

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., et al. (2005). *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a carotenoid-derived branch-inhibiting hormone. *Dev. Cell.* 8**,** 443-449. doi: 10.1016/j.devcel.2005.01.009

Bräutigam, K., Vining, K.J., Lafon-Placette, C., Fossdal, C.G., Mirouze, M., Marcos, J.G., et al. (2013). Epigenetic regulation of adaptive responses of forest tree species to the environment. *Ecol. Evol.* 3**,** 399-415. doi: 10.1002/ece3.461

Brown, J.S., and Jackson, S.P. (2015). Ubiquitylation, neddylation and the DNA damage response. *Open Biol.* 5**,** 150018. doi: 10.1098/rsob.150018

Brown, J.S., Lukashchuk, N., Sczaniecka-Clift, M., Britton, S., le Sage, C., Calsou, P., et al. (2015). Neddylation promotes ubiquitylation and release of Ku from DNA-damage sites. *Cell Rep.* 11**,** 704-714. doi: 10.1016/j.celrep.2015.03.058

Brunner, A.M., Varkonyi-Gasic, E., and Jones, R.C. (2017). "Phase change and phenology in trees," in *Comparative and evolutionary genomics of angiosperm trees*, eds A. T. Groover and Q. C. B. Cronk (Cham: Springer International Publishing AG), 227-274.

Byrne, M.E. (2009). A role for the ribosome in development. *Trends Plant Sci.* 14**,** 512-519. doi: 10.1016/j.tplants.2009.06.009

Cabezas, J.A., González-Martínez, S.C., Collada, C., Guevara, M.A., Boury, C., de María, N., et al. (2015). Nucleotide polymorphisms in a pine ortholog of the Arabidopsis degrading enzyme cellulase KORRIGAN are associated with early growth performance in *Pinus pinaster*. *Tree Physiol.* 35**,** 1000-1006. doi: 10.1093/treephys/tpv050

Camoni, L., Visconti, S., Aducci, P., and Marra, M. (2018). 14-3-3 proteins in plant hormone signaling: doing several things at once. *Front. Plant Sci.* 9**,** 297. doi: 10.3389/fpls.2018.00297

Canales, J., Bautista, R., Label, P., Gómez-Maldonado, J., Lesur, I., Fernández-Pozo, N., et al. (2014). De novo assembly of maritime pine transcriptome: implications for forest breeding and biotechnology. *Plant Biotechnol. J.* 12**,** 286-299. doi: 10.1111/pbi.12136

Cano, M., Morcillo, A., Humánez, A., Mendoza-Poudereux, I., Alborch, A., Segura, J., et al. (2018). "Maritime Pine (*Pinus Pinaster* Aiton)," in *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume I Forestry Sciences 84,* eds S. M. Jain and P. Gupta (Cham: Springer International Publishing AG), 167-179.

Cañas, R.A., Canales, J., Muñoz-Hernández, C., Granados, J.M., Ávila, C., García-Martín, M.L., et al. (2015). Understanding developmental and adaptive cues in pine through metabolite profiling and co-expression network analysis. *J. Exp. Bot.* 66**,** 3113-3127. doi: 10.1093/jxb/erv118

Chen, J.C., Jiang, C.Z., and Reid, M.S. (2005). Silencing a prohibitin alters plant development and senescence. *Plant J.* 44**,** 16-24. doi: 10.1111/j.1365-313X.2005.02505.x

Chepyshko, H., Lai, C.-P., Huang, L.-M., Liu, J.-H., and Shaw, J.-F. (2012). Multifunctionality and diversity of GDSL esterase/lipase gene family in rice (*Oryza sativa L. japonica*) genome: new insights from bioinformatics analysis. *BMC Genomics.* 13**,** 309. doi: 10.1186/1471-2164-13-309

Cohen, M., Davydov, O., and Fluhr, R. (2019). Plant serpin protease inhibitors: specificity and duality of function. *J. Exp. Bot.* 70**,** 2077-2085. doi: 10.1093/jxb/ery460

Conde, D., Moreno-Cortés, A., Dervinis, C., Ramos-Sánchez, J.M., Kirst, M., Perales, M., et al. (2017). Overexpression of DEMETER, a DNA demethylase, promotes early apical bud maturation in poplar. *Plant Cell Environ.* 40**,** 2806-2819. doi: 10.1111/pce.13056

Czerniawski, P., and Bednarek, P. (2018). Glutathione S-transferases in the biosynthesis of sulfur-containing secondary metabolites in Brassicaceae plants. *Front. Plant Sci.* 9**,** 1639. doi: 10.3389/fpls.2018.01639

Das, A., Paudel, B., and Rohila, J.S. (2015). "Potentials of proteomics in crop breeding," in *Advances in plant breeding strategies: breeding, biotechnology and molecular tools*, eds J. M. Al-Khayri, S. M. Jain and D. V. Johnson (Cham: Springer International Publishing AG), 513-537.

De Simón, B.F., Cadahía, E., and Aranda, I. (2018). Metabolic response to elevated CO_2 levels in *Pinus pinaster* Aiton needles in an ontogenetic and genotypic-dependent way. *Plant Physiol. Biochem.* 132**,** 202-212. doi: 10.1016/j.plaphy.2018.09.006

De Simón, B.F., Sanz, M., Cervera, M.T., Pinto, E., Aranda, I., and Cadahía, E. (2017). Leaf metabolic response to water deficit in *Pinus pinaster* Ait. relies upon ontogeny and genotype. *Environ. Exp. Bot.* 140**,** 41-55. doi: 10.1016/j.envexpbot.2017.05.017

Del Mar Castellano, M., Boniotti, M.B., Caro, E., Schnittger, A., and Gutierrez, C. (2004). DNA replication licensing affects cell proliferation or endoreplication in a cell type–specific manner. *Plant Cell* 16**,** 2380-2393. doi: 10.1105/tpc.104.022400

Dembinsky, D., Woll, K., Saleem, M., Liu, Y., Fu, Y., Borsuk, L.A., et al. (2007). Transcriptomic and proteomic analyses of pericycle cells of the maize primary root. *Plant Physiol.* 145**,** 575-588. doi: 10.1104/pp.107.106203

Dixon, D.P., Skipsey, M., and Edwards, R. (2010). Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry* 71**,** 338-350. doi: 10.1016/j.phytochem.2009.12.012

Escandón, M., Valledor, L., Pascual, J., Pinto, G., Cañal, M.J., and Meijón, M. (2017). System-wide analysis of short-term response to high temperature in *Pinus radiata*. *J. Exp. Bot.* 68**,** 3629-3641. doi: 10.1093/jxb/erx198

Eveland, A.L., and Jackson, D.P. (2012). Sugars, signalling, and plant development. *J. Exp. Bot.* 63**,** 3367-3377. doi: 10.1093/jxb/err379

FAO (2009). *Global demand for wood products. Rome: Italy: The Food and Agricultural Organization of the United Nations*. Available at: http://www.fao.org/3/i0350e/i0350e00.htm

FAO (2015). *Global forest resources assessment 2015. Rome, Italy*. FAO Forestry Paper No. 1. Available at: http://www.fao.org/forest-resources-assessment/past-assessments/fra-2015/en/

Fernández, H., Fraga, M., Bernard, P., and Revilla, M. (2003). Quantification of GA1, GA3, GA4, GA7, GA9, and GA20 in vegetative and male cone buds from juvenile and mature trees of *Pinus radiata*. *Plant Growth Regul.* 40**,** 185-188. doi: 10.1023/A:1025070707899

Fernando, D.D. (2014). The pine reproductive process in temperate and tropical regions. *New For.* 45**,** 333-352. doi: 10.1007/s11056-013-9403-7

Fraga, M.F., Rodríguez, R., and Cañal, M.J. (2002). Genomic DNA methylation–demethylation during aging and reinvigoration of *Pinus radiata*. *Tree Physiol.* 22**,** 813-816. doi: 10.1093/treephys/22.11.813

Galindo-González, L., Sarmiento, F., and Quimbaya, M.A. (2018). Shaping plant adaptability, genome structure and gene expression through transposable element epigenetic control: Focus on methylation. *Agronomy* 8**,** 180. doi: 10.3390/agronomy8090180

Garcés, M., Le Provost, G., Lalanne, C., Claverol, S., Barré, A., Plomion, C., et al. (2014). Proteomic analysis during ontogenesis of secondary xylem in maritime pine. *Tree Physiol.* 34**,** 1263-1277. doi: 10.1093/treephys/tpt117

Girard, F., Vennetier, M., Ouarmim, S., Caraglio, Y., and Misson, L. (2011). Polycyclism, a fundamental tree growth process, decline with recent climate change: the example of *Pinus halepensis* Mill. in Mediterranean France. *Trees Struct. Funct.* 25**,** 311-322. doi: 10.1007/s00468-010-0507-9

Gómez-Gómez, L., and Carrasco, P. (1998). Differential expression of the S-Adenosyl-L-Methionine Synthase genes during pea development. *Plant Physiol.* 117**,** 397-405. doi: 10.1104/pp.117.2.397

For Peer Review

González, Á.C., Díaz, I.F., de la Torre, C., Vázquez, J.P., Colina, F.J., Valledor, L., et al. (2016). Nuevos marcadores de calidad de madera en *Pinus pinaster*: Estrigolactonas y ramificación. *Tecnología agroalimentaria: Boletín informativo del SERIDA* 17**,** 21-27.

Grabon, A., Bankaitis, V.A., and McDermott, M.I. (2019). The interface between phosphatidylinositol transfer protein function and phosphoinositide signaling in higher eukaryotes. *J. Lipid Res.* 60**,** 242-268. doi: 10.1194/jlr.R089730

Großkinsky, D.K., Syaifullah, S.J., and Roitsch, T. (2017). Integration of multi-omics techniques and physiological phenotyping within a holistic phenomics approach to study senescence in model and crop plants. *J. Exp. Bot.* 69**,** 825-844. doi: 10.1093/jxb/erx333

Haffner, V., Enjalric, F., Lardet, L., and Carron, M. (1991). Maturation of woody plants: a review of metabolic and genomic aspects. *Ann. For. Sci.* 48, 615-630. doi: 10.1051/forest:19910601

Hahlbrock, K., and Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Biol. Plant. Mol. Biol.* 40**,** 347-369. doi: 10.1146/annurev.pp.40.060189.002023

Harwood, J.L. (1996). Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta* 1301**,** 7-56. doi: 10.1016/0005-2760(95)00242-1

Hemmati, S., Schneider, B., Schmidt, T.J., Federolf, K., Alfermann, A.W., and Fuss, E. (2007). Justicidin B 7-hydroxylase, a cytochrome P450 monooxygenase from cell cultures of *Linum perenne* Himmelszelt involved in the biosynthesis of diphyllin. *Phytochemistry* 68**,** 2736-2743. doi: 10.1016/j.phytochem.2007.10.025

Hover, A., Buissart, F., Caraglio, Y., Heinz, C., Pailler, F., Ramel, M., et al. (2017). Growth phenology in *Pinus halepensis* Mill.: apical shoot bud content and shoot elongation. *Ann. For. Sci.* 74**,** 39. doi: 10.1007/s13595-017-0637-y

Considine, M.J., and Foyer, C.H. (2014). Redox Regulation of Plant Development. *Antioxid. Redox Signal.* 21**,** 1305-1326. doi: 10.1089/ars.2013.5665

Jordy, M.-N., Danti, S., Favre, J.-M., and Raccchi, M.L. (2000). Histological and biochemical changes in *Pinus* spp. seeds during germination and post-germinative growth: triacylglycerol distribution and catalase activity. *Aust. J. Plant Physiol.* 27**,** 1109-1117. doi: 10.1071/PP00069

Jordy, M.N. (2004). Seasonal variation of organogenetic activity and reserves allocation in the shoot apex of *Pinus pinaster* Ait. *Ann. Bot.* 93**,** 25-37. doi: 10.1093/aob/mch005

Kamada, I., Yamauchi, S., Youssefian, S., and Sano, H. (1992). Transgenic tobacco plants expressing *rgp1*, a gene encoding a RAS-related GTP-binding protein from rice, show distinct morphological characteristics. *Plant J.* 2**,** 799-807. doi: 10.1111/j.1365-313X.1992.tb00149.x

Kim, D., Shin, H., Song, Y.S., and Kim, J.H. (2012). Synergistic effect of different levels of genomic data for cancer clinical outcome prediction. *J. Biomed. Inform.* 45**,** 1191-1198. doi: 10.1016/j.jbi.2012.07.008

Little, C., and MacDonald, J.E. (2003). Effects of exogenous gibberellin and auxin on shoot elongation and vegetative bud development in seedlings of *Pinus sylvestris* and *Picea glauca*. *Tree Physiol.* 23**,** 73-83. doi: 10.1093/treephys/23.2.73

Lohse, M., Nagel, A., Herter, T., May, P., Schroda, M., Zrenner, R., et al. (2014). Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. *Plant Cell Environ.* 37**,** 1250-1258. doi: 10.1111/pce.12231

López-Goldar, X., Villari, C., Bonello, P., Borg-Karlson, A.K., Grivet, D., Sampedro, L., et al. (2019). Genetic variation in the constitutive defensive metabolome and its inducibility are geographically structured and largely determined by demographic processes in maritime pine. *J. Ecol.* 107**,** 2464-2477. doi: 10.1111/1365-2745.13159

López-Goldar, X., Zas, R., Sampedro, L. (2020). Resource availability drives microevolutionary patterns of plant defences. *Funct. Ecol.*, 1–13. doi: 10.1111/1365-2435.13610

Luisi, A., Lorenzi, R., and Sorce, C. (2011). Strigolactone may interact with gibberellin to control apical dominance in pea (*Pisum sativum*). *Plant Growth Regul.* 65**,** 415-419. doi: 10.1007/s10725-011-9603-0

Maurya, J.P., Triozzi, P.M., Bhalerao, R.P., and Perales, M. (2018). Environmentally sensitive molecular switches drive poplar phenology. *Front. Plant Sci.* 9**,** 1873. doi: 10.3389/fpls.2018.01873

Meijón, M., Feito, I., Oravec, M., Delatorre, C., Weckwerth, W., Majada, J., et al. (2016). Exploring natural variation of *Pinus pinaster* Aiton using metabolomics: Is it possible to identify the

region of origin of a pine from its metabolites? *Mol. Ecol.* 25**,** 959-976. doi: 10.1111/mec.13525

Meijón, M., Rodríguez, R., Cañal, M.J., and Feito, I. (2009). Improvement of compactness and floral quality in azalea by means of application of plant growth regulators. *Sci. Hortic-Amsterdam* 119**,** 169-176. doi: 10.1016/j.scienta.2008.07.023

Miller, G., Shulaev, V., and Mittler, R. (2008). Reactive oxygen signaling and abiotic stress. *Physiol. Plantarum* 133**,** 481-489. doi: 10.1111/j.1399-3054.2008.01090.x

Mochida, K., and Shinozaki, K. (2011). Advances in omics and bioinformatics tools for systems analyses of plant functions. *Plant Cell Physiol.* 52**,** 2017-2038. doi: 10.1093/pcp/pcr153

Moon, J., Parry, G., and Estelle, M. (2004). The ubiquitin-proteasome pathway and plant development. *Plant Cell* 16**,** 3181-3195. doi: 10.1105/tpc.104.161220

Morel, A., Trontin, J.-F., Corbineau, F., Lomenech, A.-M., Beaufour, M., Reymond, I., et al. (2014). Cotyledonary somatic embryos of *Pinus pinaster* Ait. most closely resemble fresh, maturing cotyledonary zygotic embryos: biological, carbohydrate and proteomic analyses. *Planta* 240**,** 1075-1095. doi: 10.1007/s00425-014-2125-z

Morisseau, C. (2013). Role of epoxide hydrolases in lipid metabolism. *Biochimie* 95**,** 91-95. doi: 10.1016/j.biochi.2012.06.011

Nguyen, A., Dormling, I., and Kremer, A. (1995). Characterization of *Pinus pinaster* seedling growth in different photo-and thermoperiods in a phytotron as a basis for early selection. *Scand. J. Forest Res.* 10**,** 129-139. doi: 10.1080/02827589509382876

Nguyen, T.Q., and Emery, R.N. (2017). Is ABA the earliest upstream inhibitor of apical dominance? *J. Exp. Bot.* 68**,** 881-884. doi: 10.1093/jxb/erx028

Paiva, J.A., Garcés, M., Alves, A., Garnier-Géré, P., Rodrigues, J.C., Lalanne, C., et al. (2008). Molecular and phenotypic profiling from the base to the crown in maritime pine wood-forming tissue. *New Phytol.* 178**,** 283-301. doi: 10.1111/j.1469-8137.2008.02379.x

Pascual, J., Cañal, M.J., Escandón, M., Meijón, M., Weckwerth, W., and Valledor, L. (2017). Integrated physiological, proteomic, and metabolomic analysis of ultra violet (UV) stress responses and adaptation mechanisms in *Pinus radiata*. *Mol. Cell. Proteomics* 16**,** 485-501. doi: 10.1074/mcp.M116.059436

Penterman, J., Zilberman, D., Huh, J.H., Ballinger, T., Henikoff, S., and Fischer, R.L. (2007). DNA demethylation in the Arabidopsis genome. *Proc. Natl. Acad. Sci. U.S.A* 104**,** 6752-6757. doi: 10.1073/pnas.0701861104

Pluskal, T., Castillo, S., Villar-Briones, A., and Orešič, M. (2010). MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* 11**,** 395. doi: 10.1186/1471-2105-11-395

Ponting, C.P., and Aravind, L. (1999). START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends Biochem. Sci.* 24**,** 130-132. doi: 10.1016/s0968-0004(99)01362-6

Proost, S., Van Bel, M., Vaneechoutte, D., Van de Peer, Y., Inzé, D., Mueller-Roeber, B., et al. (2014). PLAZA 3.0: an access point for plant comparative genomics. *Nucleic Acids Res.* 43**,** 974-981. doi: 10.1093/nar/gku986

R Development Core Team. (2015). *A Language and Environment for Statistical Computing: Vienna: R Foundation for Statistical Computing*.

Richards, D.E., King, K.E., Ait-Ali, T., and Harberd, N.P. (2001). How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signaling. *Ann. Rev. Plant Biol.* 52**,** 67-88. doi: 10.1146/annurev.arplant.52.1.67

Rivero, R.M., Ruiz, J.M., Garcıa, P.C., Lopez-Lefebre, L.R., Sánchez, E., and Romero, L. (2001). Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci.* 160**,** 315-321. doi: 10.1016/S0168-9452(00)00395-2

Rocha, P.S., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., et al. (2005). The Arabidopsis *HOMOLOGY-DEPENDENT GENE SILENCING1* gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. *Plant Cell* 17**,** 404-417. doi: 10.1105/tpc.104.028332

Rohart, F., Gautier, B., Singh, A., and Lê Cao, K.-A. (2017). mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* 13**,** e1005752. doi: 10.1371/journal.pcbi.1005752

Romero-Rodríguez, M.C., Pascual, J., Valledor, L., and Jorrín-Novo, J. (2014). Improving the quality of protein identification in non-model species. Characterization of *Quercus ilex* seed and *Pinus radiata* needle proteomes by using SEQUEST and custom databases. *J. Proteomics* 105**,** 85-91. doi: 10.1016/j.jprot.2014.01.027

RStudio Team. (2016). *RStudio: Integrated development environment for R*. Boston, MA.

Rüffer, M., Nagakura, N., and Zenk, M.H. (1978). Strictosidine, the common precursor for monoterpenoid indole alkaloids with 3 α and 3 β configuration. *Tetrahedron Lett.* 18**,** 1593-1596. doi: 10.1016/S0040-4039(01)94613-1

Sabatier, S., Baradat, P., and Barthelemy, D. (2003). Intra-and interspecific variations of polycyclism in young trees of *Cedrus atlantica* (Endl.) Manetti ex. Carrière and *Cedrus libani* A. Rich (Pinaceae). *Ann. For. Sci.* 60**,** 19-29. doi: 10.1051/forest:2002070

Sadka, A., Dahan, E., Cohen, L., and Marsh, K.B. (2000). Aconitase activity and expression during the development of lemon fruit. *Physiol. Plantarum* 108**,** 255-262. doi: 10.1034/j.1399-3054.2000.108003255.x

Sangster, T.A., and Queitsch, C. (2005). The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. *Curr. Opin. Plant Biol.* 8**,** 86-92. doi: 10.1016/j.pbi.2004.11.012

Schmitz, A.J., Glynn, J.M., Olson, B.J., Stokes, K.D., and Osteryoung, K.W. (2009). *Arabidopsis* FtsZ2-1 and FtsZ2-2 are functionally redundant, but FtsZ-based plastid division is not essential for chloroplast partitioning or plant growth and development. *Mol. Plant* 2**,** 1211-1222. doi: 10.1093/mp/ssp077

Schrick, K., Bruno, M., Khosla, A., Cox, P.N., Marlatt, S.A., Roque, R.A., et al. (2014). Shared functions of plant and mammalian StAR-related lipid transfer (START) domains in modulating transcription factor activity. *BMC Biology* 12**,** 70. doi: 10.1186/s12915-014-0070-8

Sen, C.K., and Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *Faseb J.* 10**,** 709-720. doi: 10.1096/fasebj.10.7.8635688

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13**,** 2498-2504. doi: 10.1101/gr.1239303

Singh, A., Gautier, B., Shannon, C.P., Rohart, F., Vacher, M., Tebutt, S.J., et al. (2018). DIABLO: from multi-omics assays to biomarker discovery, an integrative approach. *bioRxiv*. doi: 10.1101/067611

Tanaka, H., Masuta, C., Uehara, K., Kataoka, J., Koiwai, A., and Noma, M. (1997). Morphological changes and hypomethylation of DNA in transgenic tobacco expressing antisense RNA of the S-adenosyl-L-homocysteine hydrolase gene. *Plant Mol. Biol.* 35**,** 981-986. doi: 10.1023/A:1005896711321

Tariq, M., and Paszkowski, J. (2004). DNA and histone methylation in plants. *Trends Genet.* 20**,** 244-251. doi: 10.1016/j.tig.2004.04.005

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., et al. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* 37**,** 914-939. doi: 10.1111/j.1365-313x.2004.02016.x

Treutter, D. (2005). Significance of Flavonoids in Plant Resistance and Enhancement of Their Biosynthesis. *Plant Biol*. 7, 581-591. doi:10.1055/s-2005-873009

Trontin, J.-F., Klimaszewska, K., Morel, A., Hargreaves, C., and Lelu-Walter, M.-A. (2016). "Molecular aspects of conifer zygotic and somatic embryo development: a review of genome-wide approaches and recent insights," in *In vitro embryogenesis in higher plants*. (Cham: Springer International Publishing AG), 167-207.

Uggla, C., Magel, E., Moritz, T., and Sundberg, B. (2001). Function and dynamics of auxin and carbohydrates during earlywood/latewood transition in Scots pine. *Plant Physiol.* 125**,** 2029-2039. doi: 10.1104/pp.125.4.2029

Valdés, A.E., Fernández, B., and Centeno, M.L. (2004). Hormonal changes throughout maturation and ageing in *Pinus pinea*. *Plant Physiol. Bioch.* 42**,** 335-340. doi: 10.1016/j.plaphy.2004.02.004

Valledor, L., Carbó, M., Lamelas, L., Escandón, M., Colina, F.J., Cañal, M.J., et al. (2018). "When the tree let us see the forest: systems biology and natural variation studies in forest species" in *Progress in Botany*, eds Cánovas F., Lüttge U., Leuschner C., and Risueño MC (Cham: Springer International Publishing AG), 353-375.

Valledor, L., Escandón, M., Meijón, M., Nukarinen, E., Cañal, M.J., and Weckwerth, W. (2014a). A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *Plant J.* 79**,** 173-180. doi: 10.1111/tpj.12546

For Peer Review

Valledor, L., and Jorrín, J. (2011). Back to the basics: maximizing the information obtained by quantitative two dimensional gel electrophoresis analyses by an appropriate experimental design and statistical analyses. *J. Proteomics* 74**,** 1-18. doi: 10.1016/j.jprot.2010.07.007

Valledor, L., Jorrín, J.s.V., Rodríguez, J.L., Lenz, C., Meijón, M., Rodríguez, R., et al. (2010a). Combined proteomic and transcriptomic analysis identifies differentially expressed pathways associated to *Pinus radiata* needle maturation. *J. Proteome Res.* 9**,** 3954-3979. doi: 10.1021/pr1001669

Valledor, L., Meijón, M., Hasbún, R., Cañal, M.J., and Rodríguez, R. (2010b). Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of in vitro organogenic capability. *J. Plant Physiol.* 167**,** 351-357. doi: 10.1016/j.jplph.2009.09.018

Valledor, L., Pascual, J., Meijón, M., Escandón, M., and Cañal, M.J. (2015). Conserved epigenetic mechanisms could play a key role in regulation of photosynthesis and development-related genes during needle development of *Pinus radiata*. *PLoS One* 10**,** e0126405. doi: 10.1371/journal.pone.0126405

Valledor, L., Romero-Rodríguez, M.C., and Jorrin-Novo, J.V. (2014b). "Standardization of data processing and statistical analysis in comparative plant proteomics experiment," in *Plant Proteomics*. (Cham: International Publishing Springer AG), 51-60.

Valledor, L., and Weckwerth, W. (2014). "An improved detergent-compatible gel-fractionation LC-LTQ-Orbitrap-MS workflow for plant and microbial proteomics," in *Plant Proteomics: Methods and Protocols*. (Cham: Springer International Publishing AG), 347-358.

Vázquez-González, C., López-Goldar, X., Zas, R., and Sampedro, L. (2019). Neutral and climate-driven adaptive processes contribute to explain population variation in resin duct traits in a mediterranean pine species. *Front. Plant Sci.* 10, 1-12. doi: 10.3389/fpls.2019.01613

Verdú, M., and Climent, J. (2007). Evolutionary correlations of polycyclic shoot growth in *Acer* (Sapindaceae). *Am. J. Bot.* 94**,** 1316-1320. doi: 10.3732/ajb.94.8.1316

Vimont, N., Schwarzenberg, A., Domijan, M., Beauvieux, R., Arkoun, M., Jamois, F., et al. (2019). Hormonal balance finely tunes dormancy status in sweet cherry flower buds. *bioRxiv***,** 423871. doi: 10.1101/423871

Vizcaíno-Palomar, N., Ibáñez, I., González-Martínez, S.C., Zavala, M.A., and Alía, R. (2016). Adaptation and plasticity in aboveground allometry variation of four pine species along environmental gradients. *Ecol. Evol.* 6**,** 7561-7573. doi: 10.1002/ece3.2153

Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci*. 9**,** 244-252. doi: 10.1016/j.tplants.2004.03.006

Woo, E.-J., Dunwell, J.M., Goodenough, P.W., Marvier, A.C., and Pickersgill, R.W. (2000). Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nat. Struct. Mol. Biol*. 7**,** 1036. doi: 10.1038/80954

Zas, R., and Fernández-López, J. (2005). Juvenile genetic parameters and genotypic stability of *Pinus pinaster* Ait. open-pollinated families under different water and nutrient regimes. *For. Sci.* 51**,** 165-174. doi: 10.1093/forestscience/51.2.165

Zas, R., Merlo, E., and Fernández-López, J. (2004). Genetic parameter estimates for Maritime pine in the Atlantic coast of North-west Spain. *For. Genet.* 11, 45-53. doi: 10261/101373

Zhang, C.-J., Zhao, B.-C., Ge, W.-N., Zhang, Y.-F., Song, Y., Sun, D.-Y., et al. (2011). An apoplastic h-type thioredoxin is involved in the stress response through regulation of the apoplastic reactive oxygen species in rice. *Plant Physiol.* 157**,** 1884-1899. doi: 10.1104/pp.111.182808

Zheng, C., Halaly, T., Acheampong, A.K., Takebayashi, Y., Jikumaru, Y., Kamiya, Y., et al. (2015). Abscisic acid (ABA) regulates grape bud dormancy, and dormancy release stimuli may act through modification of ABA metabolism. *J. Exp. Bot.* 66**,** 1527-1542. doi: 10.1093/jxb/eru519

Zhu, H., Qian, W., Lu, X., Li, D., Liu, X., Liu, K., et al. (2005). Expression patterns of purple acid phosphatase genes in *Arabidopsis* organs and functional analysis of *AtPAP23* predominantly transcribed in flower. *Plant Mol. Biol.* 59**,** 581-594. doi: 10.1007/s11103-005-0183-0

Zimmerman, J.L. (1993). Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5**,** 1411. doi: 10.1105/tpc.5.10.1411

FIGURE LEGENDS

Figure 1. Plant material employed in this analysis. Two-year old seedlings exhibiting an apical bud with young **(A)** or mature **(B)** morphology. Comparison of ~~juvenile~~young (left) and ~~adult~~mature (right) buds **(C)**. Dissection of ~~juvenile~~young (left) and ~~adult~~mature (right) buds into their apical and basal parts **(D)**. Vertical bars represent 0.5 cm length.

Figure 2. Venn diagrams showing the qualitative differences between bud sections and ontogenic stages at proteome **(A)** and metabolome **(B)** levels. Heatmap clustering of proteins **(C)** and metabolites **(D)** classified according to Mapman categories. Distances were established employing Manhattan distance and aggregated according to Ward's method.

Figure 3. Integrative analysis of proteome and metabolome. **(A)** Plot of samples scores at proteome and metabolome levels, showing components 1 and 2 in horizontal and vertical axes, respectively, **(B)** integrative heatmap clustering, **(C)** loadings plot showing the proteins with greatest correlation to components 1 and 2, **(D)** and loadings plot showing the metabolites with greatest correlation to components 1 and 2. Color code of the horizontal bar of the heatmap represents proteins (cyan) or metabolites (purple), while vertical shows treatments. Euclidean distances and complete-linkage algorithms were employed for classifying samples. Color of the protein loading bars represent the treatment with higher protein abundance.

Figure 4. Integrative analysis of proteome and metabolome during bud maturation. **(A)** sPLS-based network built after DIABLO analysis. Correlation cut-off was 0.75 and edge color reflect positive (red) or negative (green) interactions. Node color indicate Mapman functional bin and shape indicate proteins (circle) or metabolites (square). Same representations in which node color indicates that protein/metabolite is more abundant in **(B)** one of the treatments, **(C)** in the apical/basal part of the bud, or **(D)** in ~~adult~~mature/~~juvenile~~young buds.

Figure 5. Whisker box representation of the qPCR analysis of target genes in the different bud sections. Dots indicate expression values of the different biological replicates normalized vs the expression of control genes (ΔCq Target/ΔCq Controls). Significant differences between bud sections and developmental status (ANOVA/Tukey HSD, $p < 0.001$) were highlighted (***).

For Peer Review

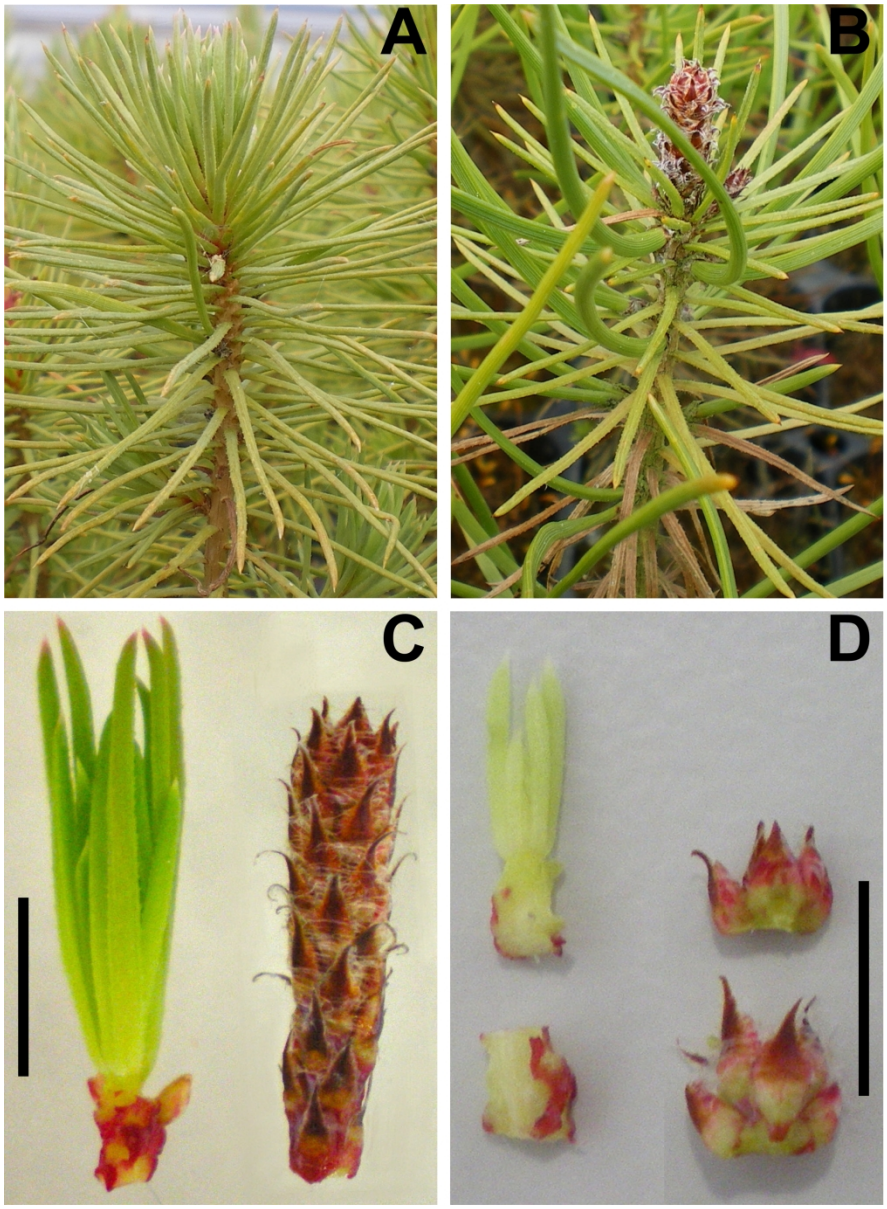

Figure 1. Plant material employed in this analysis. Two-year old seedlings exhibiting an apical bud with young (A) or mature (B) morphology. Comparison of juvenile (left) and adult (right) buds (C). Dissection of juvenile (left) and adult (right) buds into their apical and basal parts (D). Vertical bars represent 0.5 cm length

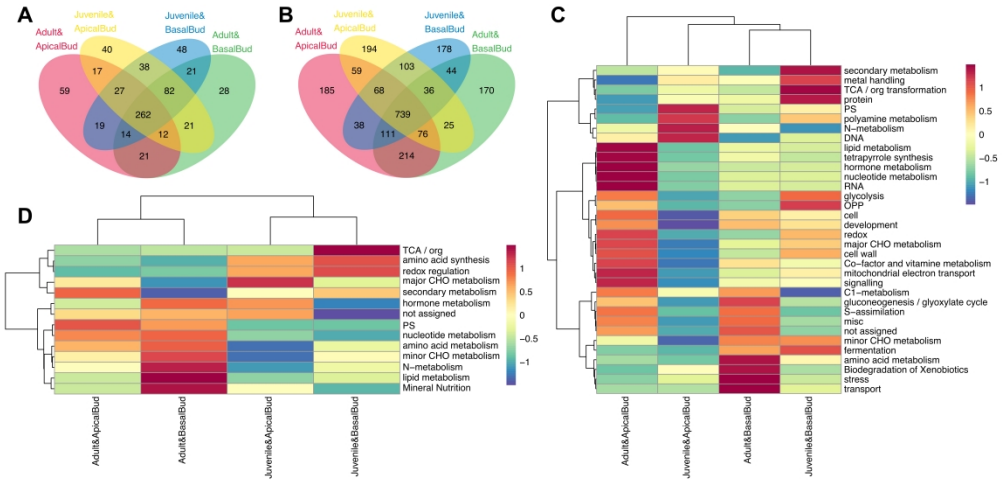

Figure 2. Venn diagrams showing the qualitative differences between bud sections and ontogenic stages at proteome (A) and metabolome (B) levels. Heatmap clustering of proteins (C) and metabolites (D) classified according to Mapman categories. Distances were established employing Manhattan distance and aggregated according to Ward's method.

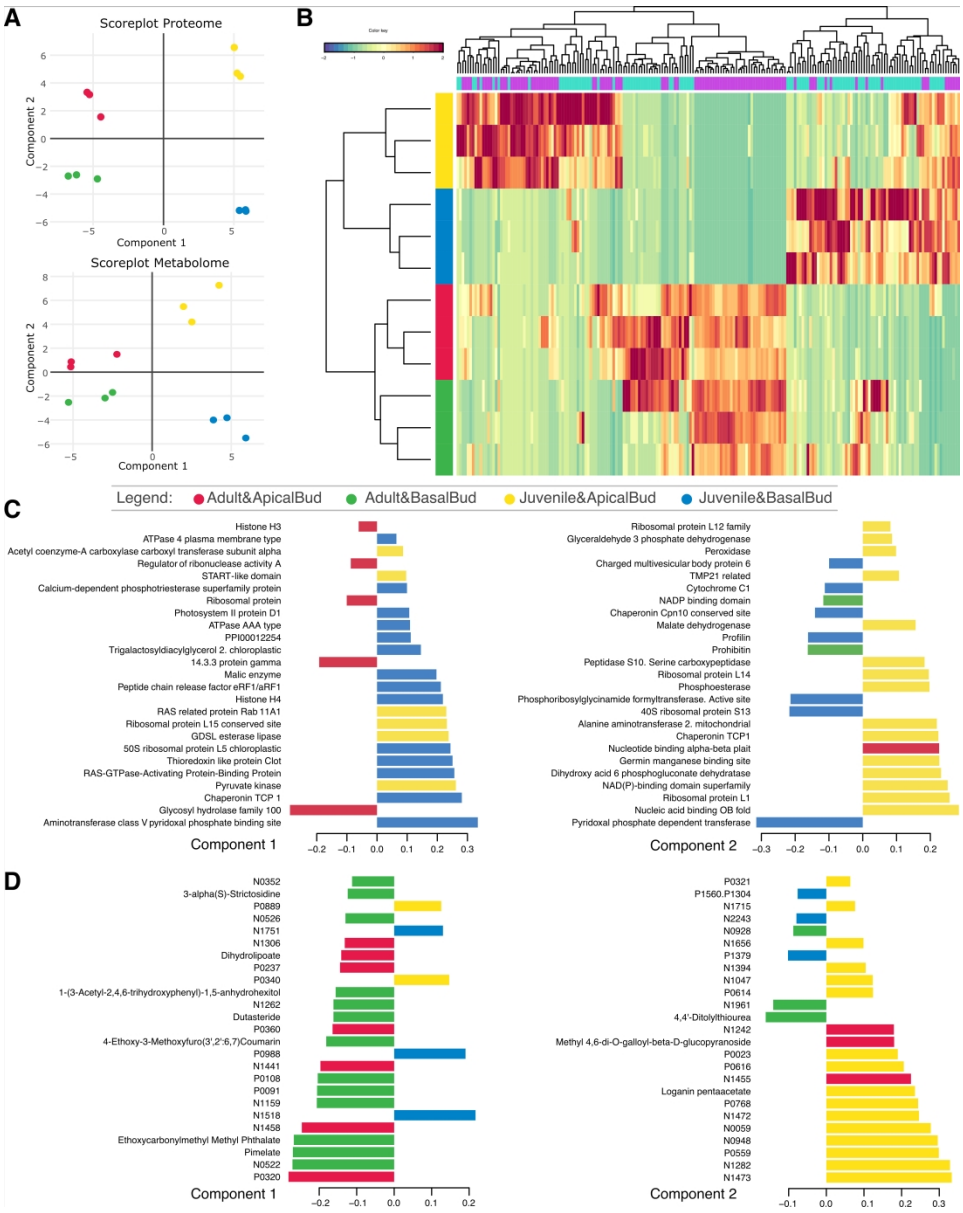

Figure 3. Integrative analysis of proteome and metabolome. (A) Plot of samples scores at proteome and metabolome levels, showing components 1 and 2 in horizontal and vertical axes, respectively, (B) integrative heatmap clustering, (C) loadings plot showing the proteins with greatest correlation to components 1 and 2, (D) and loadings plot showing the metabolites with greatest correlation to components 1 and 2. Color code of the horizontal bar of the heatmap represents proteins (cyan) or metabolites (purple), while vertical shows treatments. Euclidean distances and complete-linkage algorithms were employed for classifying samples. Color of the protein loading bars represent the treatment with higher protein abundance.

421x527mm (300 x 300 DPI)

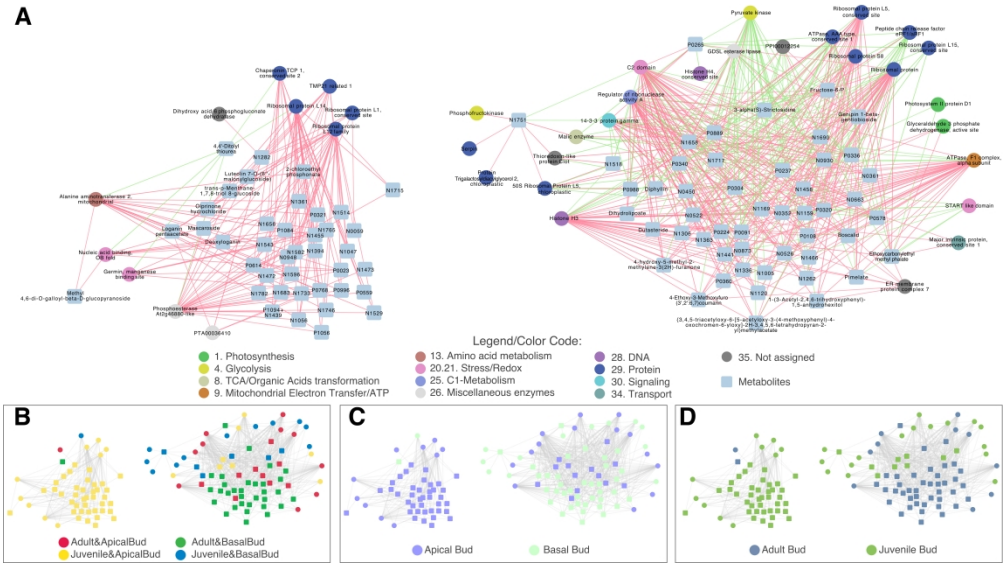

Figure 4. Integrative analysis of proteome and metabolome during bud maturation. (A) sPLS-based network built after DIABLO analysis. Correlation cut-off was 0.75 and edge color reflect positive (red) or negative (green) interactions. Node color indicate Mapman functional bin and shape indicate proteins (circle) or metabolites (square). Same representations in which node color indicates that protein/metabolite is more abundant in (B) one of the treatments, (C) in the apical/basal part of the bud, or (D) in adult/juvenile buds.

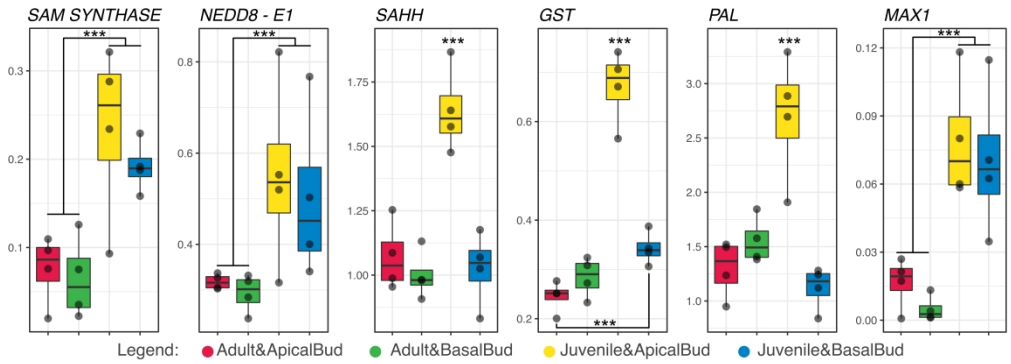

Figure 5. Whisker box representation of the qPCR analysis of target genes in the different bud sections. Dots indicate expression values of the different biological replicates normalized vs the expression of control genes (□Cq Target/□Cq Controls). Significant differences between bud sections and developmental status (ANOVA/Tukey HSD, $p < 0.001$) were highlighted (***).