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Review

Wood Formation under Changing Environment: Omics Approaches to Elucidate the Mechanisms Driving the Early-to-Latewood Transition in Conifers

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Abstract: The global change scenarios highlight the urgency of clarifying the mechanisms driving the determination of wood traits in forest trees. Coniferous xylem is characterized by the alternation between earlywood (EW) and latewood (LW), on which proportions the wood density depend, one of the most important mechanical xylem qualities. However, the molecular mechanisms triggering the transition between the production of cells with the typical features of EW to the LW are still far from being completely elucidated. The increasing availability of omics resources for conifers, e.g., genomes and transcriptomes, would lay the basis for the comprehension of wood formation dynamics, boosting both breeding and gene-editing approaches. This review is intended to introduce the importance of wood formation dynamics and xylem traits of conifers in a changing environment. Then, an up-to-date overview of the omics resources available for conifers was reported, focusing on both genomes and transcriptomes. Later, an analysis of wood formation studies using omics approaches was conducted, with the aim of elucidating the main metabolic pathways involved in EW and LW determination. Finally, the future perspectives and the urgent needs on this research topic were highlighted.

Keywords: genomic resources; gymnosperms; transcriptome; wood density; xylogenesis

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1. Introduction

1.1. Xylogenesis, Ring Width, Xylem Traits, and Global Warming: What Is the Matter?

The predicted increase in air temperature at the global scale will rise from 1.7 to 4.8 °C by 2100 [1], with the warming more pronounced at the high latitude of the northern hemisphere [2,3]. The increase in temperature will affect the water cycle at the local scale and an increase in precipitation is expected in cold temperate and boreal regions because of higher water vapor concentration in the troposphere. On the contrary, a decrease in precipitation is assumed in arid and semi-arid regions, with an increased likelihood of extreme events [4]. In this scenario, the woody species located at high latitude should benefit from the lengthening of the growing season caused by climate changes, while tree radial growth in the southern regions might be negatively affected by high temperatures and drought [5]. A rapid warming of cold environments can generate a shift in tree phenology that is thought to determine, in the short term, an acclimation/adaptive response to cope with the changing environment, while in the long term, an increase in species competition, altering the composition of forest communities [6].

The dependence of wood formation (i.e., xylogenesis) to the temperature is widely demonstrated, even if the long-term effect of warming on woody traits remains to be

clarified. Recent findings have highlighted that in conifers, the lengthening of the growing season would induce a decrease in wood-specific gravity [7], because of higher production of earlywood (EW)-like cells, in response to an earlier cambium resumption in spring and a longer duration of cambium activity [8]. The specific gravity (dry mass to volume ratio) is considered the main trait to evaluate the wood technological properties [9], and in conifers, it is determined by the EW–latewood (LW) proportion within each ring [10,11]. In conifers, the EW portion is negatively correlated to the specific gravity, whilst the LW portion has a positive correlation [12], but a general trend between the proportion between EW and LW within each ring has never been found. In addition, the size and proportion of tracheids in EW and LW within a woody ring is affected by cambial age [13] and changes significantly along tree axial height [14,15]. Results obtained through manipulative experiments in conifers have shown that temperature increase during dormancy could drive the increase in EW/LW ratio [16–18], whilst an enrichment of LW-like cells can be induced by a decrease in stem temperature during EW formation [19]. On the contrary, an increase of 5 °C in stem temperature from the rest to the maximum cambium activity induced LW-like cell formation and reduced the stem growth [20], showing a crucial role in the interaction between chilling and forcing temperature, rather than a temperature threshold in the duration and timing of EW formation [21,22]. However, LW formation appeared more sensitive to climate variables than that of EW, as shown in Corsican pine [23]. The EW–LW proportion within a ring has a direct effect on the xylem hydraulic conductance, defined as the flux for a specific driving force [24]. As the volumetric flow through a conduit is proportional to the fourth power of conduit radius, as given by the Hagen–Poiseuille equation [25], the higher EW tracheid lumen area could support most of the total sap flow in the stem. Indeed, EW has about 11-times the specific conductivity (k_s) of LW, supporting over 90% of the total stem flux in Douglas fir, and besides, LW features caused a higher vulnerability to cavitation at high trunk water potentials than those of EW [26]. This apparent contradiction can be explained through a higher control of the xylem conductivity by the end wall, tracheid length, and pit membrane size, rather than lumen diameter alone [27].

These contrasting results point out the need to increase the research on wood formation and the mechanisms regulating the phenological shift of cambium physiology and the effect on EW–LW proportion, to predict the forest productivity and related ecosystem services, in view of global warming. Indeed, while the EW portion is fairly constant in the xylogenesis process, LW production is more influenced by environmental factors. The evaluation of clonal differences in the timing of LW formation in Japanese larch highlighted small variations in EW to LW transition timing, but higher fluctuations in the duration of LW formation and, thus, in wood density [28]. Therefore, between the others, one of the most recurrent questions in tree physiology and forestry is: how will the expected shift in cambium phenology affect woody traits, specific gravity, and hydraulic architecture because of a longer growing season?

1.2. Earlywood vs. Latewood Traits in Conifers: The Control of the Environmental Cues over Phenophases?

The woody traits are a sum of two biological processes: the mitotic activity of cambium, determining the number of cells/tracheids within each ring, and the differentiation in the derivative cambial cells undergoing to expansion, secondary wall deposition, programmed death, and lignification, by which woody cells assume their final geometry and function within the xylem [29,30]. Schematically, after the resumption of cambium activity in spring, the high mitotic activity is associated with a higher turgor pressure, a rapid cell expansion, and a low lignification rate, which determine the formation of wide tracheids with thin cell walls, large lumen area, and low density, named EW. Summer solstice is often reported as the maximum of cambium growth rate, and the reduction in cambium activity in summer is associated with a lower cell turgor, a reduction in cell expansion, and an increase in lignification rate, resulting in small tracheids with thick cell walls, restricted lumen area,

and high density, named LW [31]. Examples of the anatomical features of EW and LW are reported in the anatomical observations of a Monterey pine xylem cross section in Figure 1.

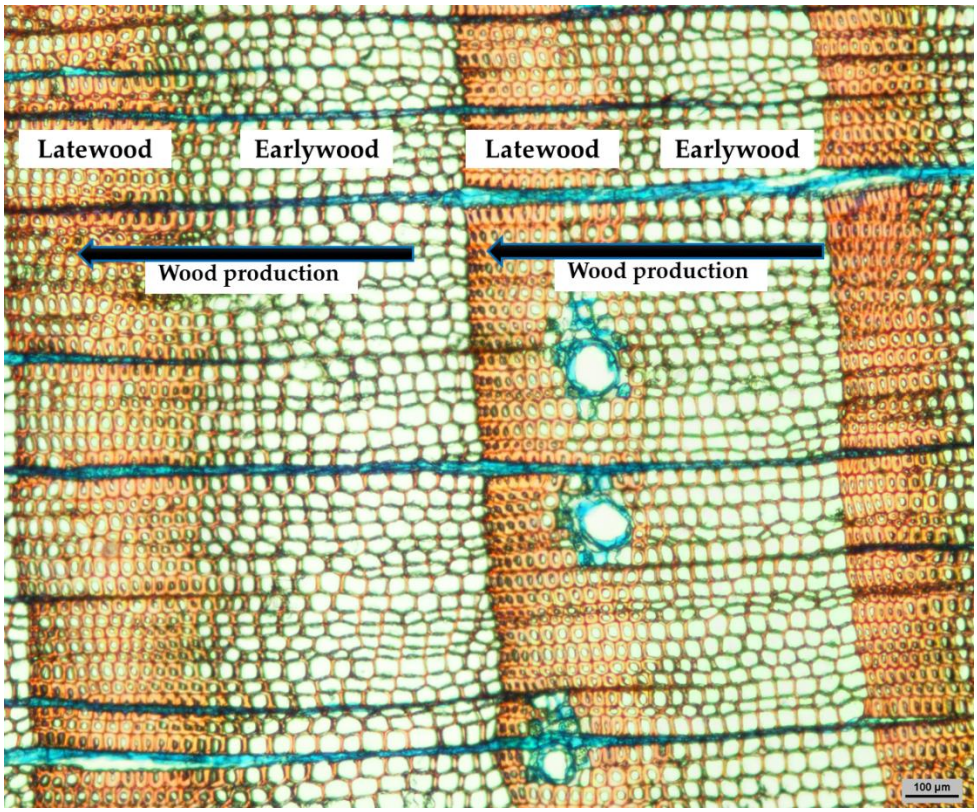

Figure 1. Earlywood and latewood portions along two annual woody rings are shown in stem tangential sections of *Pinus sylvestris*. Stem cross section was prepared by a rotary microtome (Leica RM 2245, Wetzlar, Germany), stained with Lugol solution (Merck KGaA, Darmstadt, Germany), and the image was acquired using a light microscope (Nikon Eclipse 800E, Tokyo, Japan).

The cell wall components significantly differ between EW and LW in conifers. Altogether, EW contains significantly more lignin than LW, whilst hemicellulose and pectins show the opposite trend [32]. The differences in lignin composition between LW and EW were mainly determined by the modulation of monolignols biosynthesis and dehydrogenative polymerization, which determine the final enrichment in G or S-H units [33]. Although the cellulose content is similar, the intrinsic crystallinity increases from EW to LW, being negatively correlated to the growth rate [34].

Under normal climatic conditions, the transition between EW and LW production is regulated by a developmental control, rather than determined by the environmental parameters [35]. EW and LW formation results from the interaction between genetic features and plant hormones, as well as external stressors [36]. Indeed, a recent model [37] proposed that auxin regulates the cell enlargement rate, while a different signal controls the cell division and auxin polar transport, e.g., cytokinin, tracheary element differentiation inhibitory factors, and/or the mechanical pressure exerted by the bark. The differences in the contrasting cell morphology between EW and LW could, therefore, be driven by the amplitude of a morphogenetic hormonal gradient in the cambial region, mainly induced by the auxin indole-3-acetic acid (IAA) concentration. According to the auxin gradient theory, the cambial zone width is determined by a high concentration of IAA while the acquisition of xylem cell identity, i.e., the xylogenesis, occurs along an IAA decreasing concentration gradient [38,39]. Thus, the transition between EW and LW cell morphology would be explained through the rate and duration of the differentiating process occurring along the IAA gradient within the cambial region, as shown in Japanese [40] and Scots [38] pines.

The tissue hormone concentrations are supposed to be strongly affected by environmental conditions. High temperature induced an increase in auxin concentration and hypocotyl elongation in *Arabidopsis thaliana* [41] and the IAA level in the cambium was very sensitive to frost hardiness, as shown in balsam fir [42]. Besides the IAA fluctuations, carbon metabolism and the related sugar-metabolizing enzymes are strongly involved in the EW to LW transition in conifers as well [38,43]. Indeed, carbohydrate content in the maturing xylem of Norway spruce has been shown to be modulated by cellulose metabolism during the EW formation and by starch metabolism during the cambium dormancy [44], and similar results have been reported in Scots pine [45]. Multiple environmental factors are known to control cellulose biosynthesis, considered a temperature-sensitive process [46], as well as the lignin metabolism [47]. In this frame, the investigation on the role of hormones and carbon metabolism in the EW to LW transition would have a crucial importance to assess future changes in woody traits under global warming. So far, to the best of our knowledges, we are not yet able to fully understand the main mechanisms driving the cambium physiology and the cambial cell derivatives' fate, and many efforts should be made to fill this gap by linking the competences of several disciplines, including wood anatomy, physiology, and genomics.

1.3. How Could Genomics Help to Disentangle the Physiological Processes Related to the Early-to-Latewood Transition?

Along with anatomical, phenological, and physiological observations and models, the genomic data are fundamental to describe the general wood formation process and more specific issues, such as EW to LW transition. Recent technological advances, e.g., the Next Generation Sequencing (NGS) techniques, along with the implementation of new bioinformatic approaches and tools, have deeply impacted omics studies, as well as in the plant sector, enabling a fast accumulation of genomes, transcriptomes, Single Nucleotide Polymorphisms (SNPs)-based population genomic surveys, Genome Wide Association Studies (GWAS), etc. These new methods may represent significant breakthroughs in research on conifers, considering the difficulties in using forward and reverse genetics for functional genomics, due, among other factors, to long life cycles, large genomes, high heterozygosity, difficulties in propagation, genetic transformation, and generation of mutant collections [48,49]. Indeed, the identification of the molecular mechanisms controlling the xylem density could highlight targets to be engineered or selected in breeding programs to face the ongoing climate change conditions [50]. Moreover, the growing availability of complete or draft genomes and transcriptomes opens the possibility to perform comparative whole-genome phylogenomic studies, comparing evolutionary distant species to investigate the origin and evolution of genes and metabolic pathways, leading to specific traits. SNP data can be more easily obtained at the genomic scale to implement GWAS [51], along with high-throughput phenotyping scanning [52] and references therein [53].

To facilitate and increase the accuracy and potency of functional studies, it is desirable or necessary to have a strong integration of different omics data, with a special emphasis on gene expression, the complex process leading from DNA gene sequence to protein synthesis, even if, erroneously, the term is now often used to indicate RNA accumulation studies (e.g., RNAseq). Differential (m)RNA accumulation studies, under a plethora of stressors/developmental stages/organs/cells, including the wood formation process, have been extensively conducted for plant species and, to a lesser extent, in conifers. Thus, an overview of the currently available genomic resources for conifers is reported within the Section 2. Later, an up-to-date discussion about the molecular players involved in the EW to LW transition in conifers, identified through omics studied, will be presented in Section 3.

2. Genomic Resources for Conifer Wood Formation Studies

2.1. Available Conifer Genomes

To date, 759 reference genomes for Magnoliopsida have been reported, among which, broadleaf forest tree species are well represented, with 17 *Populus* spp., 28 representative *Fagales*, and 34 *Eucalyptus* spp. genomes deposited at NCBI, just as an example. Moreover, large genome-sequencing projects, such as the 10KP (10,000 Plants) [54] and Earth Biogenome Project [55], have recently been launched. However, despite being keystone species in many temperate forest ecosystems, and regardless of being often economically relevant, conifers have a smaller available dataset of omics resources because of the huge genome dimension (17–35 Gb) and redundancy, mainly related to the accumulation of transposable elements that can account for up to 80% of the total genome [56,57]. Indeed, the first conifer-released genome belonging to Norway spruce is one-hundred-times larger than that of *Arabidopsis*, even if it contains a similar number of predicted genes [58]. The technical challenges posed by the size and complexity of coniferous genomes have only been partially answered by the development of long-read single molecule sequencing technology, such as Pacbio (https://www.pacb.com) and Oxford Nonopore (https://nanoporetech.com) that can be used in combination with Hi-C libraries [59], or BioNano optical maps [60], for scaffolding short reads. Table 1 reports the representative coniferous nuclear genomes deposited in the NCBI genome assembly database. It is evident how genomics has changed pace in conifers only in recent years, considering that the first *Arabidopsis* genome was released back in 2000 (the Arabidopsis Genome Initiative [61]) and that of black cottonwood, the first available forest tree genome, was published in 2006 [62]. Genome sequences can allow for the implementation of genome-editing tools, such as CRISPR/Cas9 [63–65], as their application requires precise knowledge of the target sequence. Thus, the availability of new omics resources would increase the research in this sector, partially avoiding some limitations that have hampered research, breeding, and propagation of conifers, especially regarding the genetic manipulation (mutant libraries, production of transgenic plants, EST banks, etc.).

For the present excursus, only members of the division Pinophyta, commonly referred to as "conifers", are considered; this taxonomic group includes 13 out of 14 representative Acrogymnospermae nuclear genomes deposited in the NCBI (Table 1). Genomes are also available for two species belonging to the *Gnetophyta* class, the last Acrogymnospermae reference genome, i.e., *Gnetum montanum*, a relevant species for evolutionary studies that shows peculiar wood anatomy features [77], and *Welwitschia mirabilis* [78], whose genome is not, however, scored as reference.

Unfortunately, despite having reached a "chromosome scale" status, many gymnosperm genomes are still poorly assembled and annotated, partially hampering comparative genomics and functional studies. A high-quality chromosome-level genome has recently been released for the Chinese pine [79], deposited in the NCBI under the BioProject (PRJNA784915), and in the CNSA of the China National GeneBank Database (CNP0001649). Moreover, the genome of *Taxus yunnanensis* has been presented [80] and deposited in the CNGBdb (https://db.cngb.org/search/assembly/CNA0020892/). It is important to remember that in some cases, resequencing efforts and population genomic projects are ongoing, so more than one genome for the same species might be available.

Extending the overview for the non-representative or uncomplete genomes, a draft sequence is available for silver fir [81] (sequences available at https://treegenesdb.org/FTP/Genomes/Abal/). A draft genome for Western red cedar is deposited in the Joint Genome Initiative database JGI (phytozome-next.jgi.doe.gov/info/Tplicata_v3_1) [82]. Low-coverage draft sequences are also available for European and Japanese larches [83].

Complete chloroplast and mitochondrial genome sequences are also represented, with 185 plastomes [84–86], but only a few mitogenomes [87–90], including those coming from whole genome sequencing projects.

Table 1. List of 13 complete and representative coniferous nuclear genomes as reported to date by NCBI.

Species	Assembly Status	Year of First Publication/Release	Reference(s)	Genbank Assembly Database	Genbank Accession Number
Larix kaempferi (Japanese larch)	Contig	2020	-	ASM1317126v2	GCA_013171265.2
Larix sibirica (Siberian larch)	Scaffold	2019	[66]	LarixSibirica0.1	GCA_004151065.1
Picea abies (Norway spruce)	Scaffold	2013	[58]	Pabies01	GCA_900067695.1
Picea engelmannii (Engelmann's spruce)	Scaffold	2020	-	Se404-851_v1	GCA_009831015.1
Picea glauca (white spruce)	Contig	2013	[67,68]	PG29_v5	GCA_000411955.6
Picea sitchensis (Sitka spruce)	Contig	2020	-	SNQJ01	GCA_010110895.1
Pinus lambertiana (sugar pine)	Scaffold	2016	[69]	Sugar pine JHU assembly	GCA_001447015.2
Pinus taeda (loblolly pine)	Scaffold	2014	[70,71]	Ptaeda2.0	GCA_000404065.3
Pseudotsuga menziesii (Douglas-fir)	Scaffold	2017	[72]	DougFir1.0	GCA_001517045.1
Sequoia sempervirens (Coast redwood)	Scaffold	2022	[73]	SESE.2.2	GCA_007258455.2
Sequoiadendron giganteum (Giant sequoia)	Chromosome	2020	[74]	SEGI.2.0	GCA_007115665.2
Taxus chinensis (Chinese yew)	Chromosome	2021	[75]	Ta-2021	GCA_019776745.2
Taxus wallichiana var. *yunnanensis* (Himalayan yew)	Chromosome	2021	[76]	ASM1834077v1	GCA_018340775.1

Genome sequences must be deposited in publicly accessible databases, in addition to well-known molecular biology portals, including GenBank, EMBL-EBI, CNGBdb, Phytozome. Genome sequencing initiatives often build and maintain a dedicated database, usually containing gene annotations, sequence homology search, and comparative genomic tools (Table 2).

2.2. Transcriptomic Resources and Functional Genomic Studies

For fully sequenced genomes, reference transcriptomes are, in general, also available, as this step is usually needed to perform the gene prediction. This task is also particularly difficult for conifers because of the typical long introns of their genes [68] and, therefore, strongly benefits from a reference transcriptome. Given that mRNA accumulation is intrinsically transient, a reference transcriptome must contain data from different datasets (stressors/developmental stages/organs/cell types) to maximize the possibility of capturing all potential mRNAs, including splicing variants. The generation of genome-level transcriptomes started before the advent of NGS technologies, with the development of expression sequence tag (EST). EST techniques provided valuable data for coniferous genomics and, since timber is the main product of conifers, many studies focused on wood formation processes [92–95]. Nevertheless, ESTs had several technical disadvantages: they implied the construction of costly cloned cDNA libraries that were labor demanding to build and maintain and had to be screened and individually sequenced using the Sanger technique. A main pitfall was, nevertheless, in their incapacity to adequately represent the transcriptome, as the number was limited and, in general, did not allow for the study of rare transcripts and splicing variants.

Table 2. List of databases containing genomic raw data, assemblies, and annotations in addition to the NCBI. Many of the databases reported in the table also store other types of data, such as SNPs and gene expression results. The same genome sequences and other omics data can be accessible form different databases as a result of platform integration and multiple submissions.

Database Name	Content	Link
TreeGenes database	Genomic assemblies and raw sequences and annotation for: • *Pseudotsuga menziesii* • *Sequoia sempervirens* • *Abies alba* • *Picea abies* • *Picea glauca* • *Pinus lambertiana* • *Pinus taeda* Blast searches Comparative genomic tools	https://treegenesdb.org [91]
Norway spruce genome project—Congenie	Genomic assemblies and raw sequences and annotation for *Picea abies* Blast searches Comparative genomic tools	https://congenie.org/
Spruce-Up Project and SMarTForests	Genomic assemblies and raw sequences for *Picea glauca*	https://spruce-up.ca/ https://www.smartforests.ca
Phytozome	Genomic assemblies and raw data for *Thuja plicata* Plant comparative genomics portal	https://phytozome-next.jgi.doe.gov
China National GeneBank Database	Genomic assemblies and raw sequences and annotation for: • *Taxus chinensis* (Chinese yew) • *Pinus tabuliformis* (Chinese pine) Blast searches	https://db.cngb.org
PLAZA	Comparative genomic data for several conifers	https://bioinformatics.psb.ugent.be/plaza/
European nucleotide archive	Genomic assemblies and raw sequences and annotation for *Larix decidua* and *L. kaempferi*	https://www.ebi.ac.uk/ena
Earth Biogenome projects	About 300 coniferous ongoing genomes with links to raw data and sequencing projects	https://www.earthbiogenome.org
10KP: 10,000 Plant Genomes Project	About 80 coniferous ongoing genomes with links to raw data and sequencing projects	https://db.cngb.org/10kp/

EST libraries were also used as probes to spot first-generation commercial or custom microarrays, before the advent of synthetic oligonucleotide chips; a main limitation of such early custom-made cDNA microarrays was that the probes were cloned and physically preserved and then spotted on the slide surface, which was time consuming and costly, as well as that the analysis of transcriptomes was limited by the representativity of EST collection used to print the array. Nevertheless, cDNA microarray technology represented a major benchmark in transcriptomics studies before the rise of NGS approaches, and enabled the measurement of differential RNA accumulation simultaneously, for thousands of genes. Microarrays, even if their use is decreasing, were and still are widely used in coniferous transcriptome analyses, even if suffering from a lack of sensitivity compared to RNAseq [96]. On the contrary, the sensitivity and accuracy of RNAseq is related to the quantity of generated sequences (depth) and by the initial representativity, as a reference of the samples analyzed. The advent of commercially manufactured oligonucleotide arrays, usually guaranteeing greater accuracy, enabled a more representative coverage of the

transcriptome, using data from genome sequencing and larger databases, and allowed for analyses on different species; for example, the wound xylem formation in Canary Island pine has been studied using a loblolly-pine-developed oligonucleotide array, containing more than 180,000 probes (GEO platform GPL21977) [97].

Even if cDNA microarrays' use is declining in favor of NGS transcriptome sequencing for RNA accumulation studies, spotted arrays are still widely used for genotyping in GWAS studies, both in hardwood and conifers. SNP arrays have some limitations, such as the presence of SNPs close to or within coding regions that may potentially produce biased data, but they are still the technology of choice to obtain reproducible and reliable data that are straightforward to process and analyze [98]. Moreover, SNPs' arrays are important for population genomics studies, especially for conifers, as the re-sequencing costs and bioinformatic processing is still prohibitive. For example, Bernhardsson and coworkers [99] used a whole genome resequencing (WGS) approach in Norway spruce to develop a 50K SNPs array to implement GWAS and genomic selection (GS) studies. SNP microarrays can be also developed with mixed approaches, involving candidate gene sequencing, RNAseq data, and whole-genome-level data. A Douglas fir array based on transcriptome data, containing more than 55K potential SNPs, is available [100], and a mixed transcriptome and candidate gene sequencing technique has been used to produce a nearly 50K SNP array for different pine species [101]. Similar mixed approaches have been used to design SNP arrays for maritime pine [102], black spruce [103], white spruce [104], and employed for wood formation studies as well [105]. Recently, a nearly 50K array, specific for Scots pine, has been designed [106].

Regardless of the technology used, all transcriptomics data are deposited in databases: two main repositories are the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and EMBL-EBI ArrayExpress (https://www.ebi.ac.uk/arrayexpress). GEO contains, to date, 145 datasets (that may contain many samples) of RNA accumulation studies for Acrogymnospermae, using different technologies and starting from a plethora of biological questions, including wood-formation-related studies. EMBL-EBI ArrayExpress currently reports 38 and 36 transcriptomic experiments (microarrays and RNAseq) for *Picea* spp. and *Pinus* spp., respectively. ArrayExpress also embeds an Expression Atlas tool, designed to study the mRNA accumulation patterns of genes across different species, biological conditions, or even for single cell experiments, but no conifer model species has yet been included (https://www.ebi.ac.uk/gxa/home) [107]. More specific databases and analysis tools have also been developed, often in concomitance with genome sequencing initiatives; for example, Norwood [108] is now implemented in the Congenie database (https://congenie.org/exnet), enabling the analysis of wood formation gene-related RNA accumulation patterns in Norway spruce. Notably, the 1K plant transcriptomes initiative has produced transcriptomes for more than 1300 Viridiplantae species, including 84 Acrogymnospermae [109,110] (raw data available in the bioproject PRJEB4922 of NCBI Sequence Read Archive and in the China Nation al GenBank database at https://db.cngb.org/datamart/plant/DATApla4/); this initiative has provided a very useful platform for comparative studies, even if the data were obtained from a limited number of tissues and/or conditions and only in a few cases are immediately usable for wood-formation studies.

2.3. Proteomic Resources

Along with utility, as a guide for genome annotations, or as a reference to map-sequenced reads in RNA-Seq experiments, transcriptomes may be useful to generate virtual proteomes, but to date, just one reference conifer proteome is available in the Universal Protein Resource (UniProt) database (http://www.uniprot.org/), i.e., the white spruce proteome (UP000242691).

Unfortunately, mainly due to technical and interpretation difficulties, experimentally generated proteomic data have not increased at the same pace compared to other omics resources in the last two decades. This lack represents a key limitation in building strong

transcriptome–phenotype associations, as transcript abundance is not always a perfect predictor of the active protein pool. To date, only proteomic studies based on 2-dimensional polyacrylamide gel electrophoresis (2-DE) and mass spectrometer techniques are available in the literature and only a few analyze wood formation in conifers, with most works related to embryogenesis or abiotic and biotic stress response; early studies focused mainly on maritime pine [111–113]. Proteome has also been studied in white spruce [114] and Monterey pine [115,116]. With few exceptions, e.g., for Douglas fir [117] and loblolly pine [118], the preponderance of metabolomic studies in conifer is not centered on wood formation or wood phenotyping, but rather related to organogenesis and development and, more recently, to plant–pathogen interaction, with a strong focus on VOCs metabolic profiling, which may be implemented as a high-throughput technique in phenotyping [119].

In general, it is, therefore, important to underline, highlighting also a very important target for future research, that a strong association between genomics and transcriptomics resources and proteomic and phenotypic data (including metabolic profiling) is still largely lacking; this implies that a reliable functional annotation specific to conifers is still missing. A major pitfall is that annotations remain mainly based on sequence homology and are usually just "electronically inferred", without, or with limited, human supervision. Thus, a reliable and experimentally supported reference annotation is really needed. Indeed, no KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/) -specific metabolic pathways have been reported for conifer trees, while more than 110 non-conifer plant species' specific pathway maps are available, including those for 8 forest tree species. Likewise, no PlantCyc-specific database (https://plantcyc.org) has been implemented for any Acrogymnospermae species [120].

3. Disentangling the Molecular Mechanisms Underlying the Early-to-Latewood Transition in the Omics Era

The increasing availability of omics resources for conifers is allowing researchers to shed light on some molecular pathways involved in wood-formation steps, such as in the EW to LW transition. However, most of the more targeted works are still based on microarray analyses, while omics approaches are still used principally for more general works. Some metabolic pathways that seem to be stronger determinants in EW and LW traits have been highlighted in different conifer species. As an example, functional studies, using microarray analyses on white spruce [121], highlighted that the genes included in metabolic pathways related to basic cellular activities, such as the metabolism of lipid/carbohydrate reserves and secondary products, were more transcribed in EW than in LW, supporting the hypothesis that EW production is a developmental-determined process, while genes related to the stress responses were representative of the LW transcriptome, supporting that variations in wood density will occur in a changing environment.

Following a recent model based on ecophysiology and wood phenology, EW formation is related to a low amount of available soluble sugars in cambium, allowing a longer cell expansion phase, while in late summer, a high level of soluble sugars determines the production of thicker xylem cell walls, resulting in LW formation [122]. Thus, a possible signal triggering the EW to LW transition could be the concentration of available free sugars. Indeed, in loblolly pine, in two different studies using microarrays, several genes were found to be more transcribed in LW than in EW, many of which were for enzymes involved in cell wall biosynthesis, such as a cellulose synthase (*CesA*) and a sucrose synthase (*Sus*), as well in monolignol biosynthesis and lignin polymerization [123,124]. Similar results were found by comparing transcripts in the juvenile wood of Monterey pine, with high and low density [125], confirming a dependence of transcriptome on the cell wall thickness of newly formed cells. In Monterey pine, the study of transcriptome differences between juvenile and mature wood confirmed that primary-cell-formation-related genes are more transcribed in juvenile xylem [126], as also found in EW in a precedent study [127], while genes more transcribed in mature xylem were related to the secondary wall production [126], as also found in LW [127]. Analyses through real-time PCR in Norway spruce

also highlighted a high transcription of *CesA* and *Sus* genes [38], as well as of genes related to lignin biosynthesis [128] during LW production. The two gene families, *CesA* and *Sus*, were also found up-regulated in the xylem of a Japanese larch clone, with higher wood density, when compared with a clone with low-wood density [129]. In the same species, the genes related to sugar metabolism, carbohydrate transport, and intracellular trafficking were also up-regulated in EW, supporting the control of wood formation through carbohydrate concentration at the beginning of the growing season, while β-tubulin and several transcription factors were up-regulated in LW [130]. Indeed, different transcription factors likely have a key role in the shift between EW-to-LW transcriptomes and should be a focus for future research, as omics resources are accumulating. As an example, in white spruce, the transcription factor *NAC7* has been shown to have a positive correlation with the EW genes, while a negative correlation with the LW genes [113], whereas the transcription factor *NAC8* has a positive association with the EW traits [114]. In Norway spruce, the RNA-dependent RNA polymerase (*RDR*), a post-transcriptional gene silencer [130], has been found to be involved in large-scale transcriptome reprogramming during cambial growth shift to LW formation. Moreover, arabinogalactan protein transcripts, *AGPs*, are usually present in transcriptome variations between EW and LW in pine species [127]. These differences in expression during the transition between EW and LW were, in general, less marked in Japanese cedar, in which most genes involved in carbohydrate metabolism or lignin biosynthesis were induced from April until the end of the growing season, maybe because of belonging to a different lineage than *Pinaceae* [131]. Variations at genome level can also have a fundamental role in shaping the differences observed in the EW to LW transition process, both between different coniferous species and different populations; comparative genomics and association studies can help in identifying genes involved in this step of wood production. In Norway spruce, GWAS highlighted that the EW/LW ratio had a significant association with ten SNPs, identifying three putative candidate genes that might be crucial in this step of wood formation, i.e., DNA-3-methyladenine glycosylase II enzyme, phytochrome kinase substrate 1, and glycosyltransferase [132]. Further GWAS data identified regulatory regions of transcription factors involved in EW (i.e., *NF-YA7*) or transition wood (i.e., *ICE2*) proprieties, as well as highlighted a general involvement of auxin pathways [51]. Studies on genetic association in loblolly pine using SNPs and wood traits highlighted the possibility to reveal associations between, as an example, the *cad* SNP M28 with EW-specific gravity, or between the *4cl* SNP M7 with LW percentage [133].

The increasing availability of genomic resources will further clarify the metabolic pathways involved in EW to LW transition, increasing the possibility of guided breeding or genome-editing approaches aimed at engineering the coniferous wood density traits.

4. Conclusions and Future Perspectives

The growing interest in the study of the mechanisms controlling the EW to LW transition is driven by the necessity of understanding the processes involved in the definition of xylem morphology, which would be extremely important in predicting future wood quality, as well as the resilience of forests, from the perspective of global changes. As a main determinant of wood density, the EW/LW ratio represents a powerful proxy of the acclimation/adaptation of trees to environmental constrains and one of the main traits to consider for future breeding strategies. Although the metabolic pathways related to the cell wall synthesis have been deeply investigated in the last decades, future efforts must focus on the characterization of genes involved in the cambium phenology and response to temperature and photoperiod. In the higher latitudes of the northern hemisphere, the increasing temperature and the relatively long photoperiod in the early fall would induce delayed dormancy, but in the lower latitudes, the short photoperiod could limit the length of the growing season, even if the temperatures are favorable for growth. Thus, the characterization of genes related to photoperiod and involved in cambium activation and cell wall synthesis could be useful to disentangle the role of the length of the day over the xylem traits.

Even if information is accumulating on coniferous genomes and transcriptomes, deeper investigations, also with classical forward genetic approaches, must be conducted to obtain the functional annotation of many genes and to clarify the relations existing between genomes and phenomes. This last step can benefit from GWAS experiments, conducted with high-throughput phenotyping strategies [134,135], including metabolomics [136]. A deep integration of omics data is, indeed, still lacking, as well as, probably more importantly, a fine-scale experimental verification of predicted gene/metabolic pathways' actual function.

Our excursus on representative genes of EW and LW highlights the involvement of cellulose and lignin metabolic pathways in LW features, but a specific target for LW determination must still to be identified. However, the greater ease of using omics techniques on conifers and the availability of new genetic engineer methods, such as CRISPR/Cas9, can help in the identification of key genes in EW and LW features, through pointed mutagenesis. Indeed, CRISPR/Cas9-mediated targeted mutagenesis have been recently successfully used in Japanese cedar [137] and Monterey pine [65]. These new techniques offer promising opportunities for both the creation of edited lines and to drive breeding programs for reforestation.

Thus, the climate change scenario seems to remark the urgency to implement target experiments to elucidate gene function and regulation. The huge amount of data obtained with high-throughput omics techniques can help to guide forward genetic approaches and/or gene editing, when in association with powerful, and so far only partially available, in silico analysis tools, able to mine robust genotype–phenotype associations and/or robust regulatory networks to target.

Considering the quality of available genomics and transcriptomics resources, the xylem features' representativity, and their importance as timber/wood-related products sources, Norway spruce and loblolly pine are potentially the most suitable species to be used as model species for the investigation of the molecular mechanisms underlying the EW to LW transition. Among the emerging model species, Chinese pine also deserves to be mentioned for the availability of high-quality genomic resources.

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