

Xylogenesis: Coniferous Trees of Temperate Forests Are Listening to the Climate Tale during the Growing Season But Only Remember the Last Words!¹

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The complex inner mechanisms that create typical conifer tree-ring structure (i.e. the transition from large, thin-walled earlywood cells to narrow, thick-walled latewood cells) were recently unraveled. However, what physiological or environmental factors drive xylogenesis key processes remain unclear. Here, we aim to quantify the influence of seasonal variations in climatic factors on the spectacular changes in the kinetics of wood cell differentiation and in the resulting tree-ring structure. Wood formation was monitored in three sites over 3 years for three coniferous species (Norway spruce [*Picea abies*], Scots pine [*Pinus sylvestris*], and silver fir [*Abies alba*]). Cell differentiation rates and durations were calculated and related to tracheid final dimensions and corresponding climatic conditions. On the one hand, we found that the kinetics of cell enlargement and the final size of the tracheids were not explained by the seasonal changes in climatic factors. On the other hand, decreasing temperatures strongly constrained cell wall deposition rates during latewood formation. However, the influence of temperature was permanently written into tree-ring structure only for the very last latewood cells, when the collapse of the rate of wall deposition was no longer counterbalanced by the increase of its duration. Our results show that the formation of the typical conifer tree-ring structure, in normal climatic conditions, is only marginally driven by climate, suggesting strong developmental control of xylogenesis. The late breakage of the compensatory mechanism at work in the wall deposition process appears as a clue to understand the capacity of the maximum latewood density to record past temperature conditions.

A remarkable aspect of the wood formation process (i.e. xylogenesis) is its capacity to generate various wood forms in response to developmental or environmental constraints (Carlquist, 1975; Rowe and Speck, 2005). Contrasting wood anatomy can thus be observed between different phylogenetic groups (Carlquist, 1975) but also between different individuals of the same group, between different organs inside the same individual (Lachenbruch et al., 2011), or even during the ontogenic trajectory of the plant for the same organ (Bao

et al., 2001). On top of this, wood anatomy also is known to adapt to changes in environmental conditions either in space or time (Jansen et al., 2004; Fonti et al., 2010). However, some of the most spectacular variations in wood anatomy occur within a single tree ring. For example, in conifers, the diameter and wall thickness of the most abundant cells (called tracheids) are divided and multiplied by a factor 5, respectively, when going from the beginning to the end of a tree ring (Vaganov et al., 2006; Cuny et al., 2014). These anatomical changes are generally well ordered along tree rings presenting a typical structure characterized by a transition, more or less progressive depending on the species and the environmental conditions, from large-diameter, thin-walled earlywood tracheids to narrow-diameter, thick-walled latewood tracheids (Schoch et al., 2004). Yet, to what extent these anatomical changes are related to climatic constraints or developmental control is still unclear. Also, which and how climatic factors influence xylem cell differentiation and the resulting tree-ring structure have not been unraveled yet (Vaganov et al., 2011).

During xylogenesis, cell enlargement and secondary cell wall deposition and lignification (hereafter named wall thickening) are the two fundamental subprocesses

¹ This work was supported by the French National Research Agency as part of the Investissements d'Avenir Program (grant no. ANR-11-LABX-0002-01, Laboratory of Excellence ARBRE), the Swiss National Science Foundation (grant no. 160077, CLIMWOOD), and the FPS COST Action STReESS (grant no. FP1106).

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C.B.K.R. conceived the experimental device; H.E.C. created the data, performed the research, and analyzed the data with the help of C.B.K.R.; H.E.C. wrote the article and prepared the figures with the assistance of C.B.K.R.

www.plantphysiol.org/cgi/doi/10.1104/pp.16.00037

that shape xylem cell dimensions and the resulting tree-ring structure (Skene, 1969; Wodzicki, 1971; Cuny et al., 2014). The duration and rate of enlargement determine the final size of a xylem cell, while the duration and rate of wall thickening determine its final weight; cell wall thickness is the result of the total amount of wall material deposited for one cell, relative to its final size. Finally, the complex interplays between the durations and rates of xylogenesis subprocesses determine the changes in cell features (e.g. cell and lumen diameter, lumen area, and wall thickness) that, in turn, create the anatomical structure driving the wood density profile (Cuny et al., 2014).

Two schools of thought have developed to explain the intriguing changes in cell dimensions along conifer tree rings. The developmental theory supposes that tree-ring formation is primarily and directly regulated by internal signals (Uggla et al., 1996, 1998; Sundberg et al., 2000). According to this view, a morphogenetic gradient of phytohormone (e.g. auxin) concentration shapes the zonation of the developing xylem by giving positional information to the forming cells (Uggla et al., 1996). Plant development results in changes in signal concentration or in tissue sensitivity through the growing season, creating the typical tree-ring structure (Uggla et al., 2001; Bhalerao and Fischer, 2014). Such internal control may be tightly coupled with daylength, which has long been regarded as a stable indicator of the time of year that helps to coordinate plant development by influencing the balance of growth regulators (Larson, 1962; Wodzicki, 1964; Jackson, 2009).

In contrast, the environmental theory proposes that the within-ring anatomical changes are driven mainly by climatic constraints (Larson, 1963; Deleuze and Houllier, 1998; Fritts et al., 1999; Vaganov et al., 2011). However, a precise assessment of the influence of climatic factors on tree-ring formation is complex, because the processes involved are tightly coupled and depend on the whole-tree physiology (carbon and hormonal balances), which itself is under the influence of the environment (Denne and Dodd, 1981). Consequently, the influence of climatic factors can be direct, through physical inhibition or stimulation of xylogenesis processes, or indirect (i.e. mediated by the carbon balance or growth regulators; Denne and Dodd, 1981). For example, unusual climatic events may occasionally indirectly influence tissue development by altering the phytohormonal gradient (Schrader et al., 2003), showing that the developmental and environmental theories are not mutually exclusive. Moreover, the direct or indirect nature of the climatic influence depends on the factor considered. For example, in addition to possible indirect effects, temperature and water availability can directly influence wood formation processes by modulating cellular metabolic activity and/or cell water turgor pressure. In contrast, the effects of light conditions are undoubtedly indirect, being mediated by

photosynthates (in the case of the amount of light radiation) and/or phytohormones (in the case of daylength).

Furthermore, cell enlargement and wall thickening are expected to be influenced by different factors, as they are very different processes in nature. Thus, cell enlargement requires water, exerting pressure on the cell wall and enhancing its extensibility (Nonami and Boyer, 1990b; Cosgrove, 1997). When a drought occurs, cell enlargement is physically and physiologically inhibited (Nonami and Boyer, 1990a, 1990b), resulting in a rapid reduction of the diameter of the cells produced (Abe et al., 2003; Rossi et al., 2009b). Cell enlargement is thus often depicted as the plant process most sensitive to water stress (Hsiao, 1973). This has fueled the long-lasting belief that decreasing water availability triggers the transition from the wide earlywood cells to the narrow latewood cells (Kramer, 1964; Deleuze and Houllier, 1998). In contrast, wall thickening involves high energetic costs and represents the bulk of biomass allocation in trees (Demura and Ye, 2010; Cuny et al., 2015). Thus, this process depends mainly on the carbohydrates produced by photosynthesis, for which light is the indispensable energy source (Kozłowski and Pallardy, 1997). However, wall thickening involves numerous and complex metabolic processes, which are considered the most sensitive to temperature among all the physiological processes related to tree carbon balance (Donaldson, 2001; Körner, 2003; Simard et al., 2013; Cuny et al., 2015). Finally, we expect that the environmental factors influence the rate of the processes, which directly reflects the physiological state of the plant as well as the influence of the environment, rather than their duration, which is tightly associated with genetic control and developmental constraints (Uggla et al., 1996, 2001; Tuominen et al., 1997).

In this study, we aim to assess how the seasonal variations in environmental factors influence the dramatic changes in cell dimensions observed along conifer tree rings. For that, we quantified the cell development kinetics and the resulting cell dimensions along the tree rings of three coniferous species: silver fir (*Abies alba*), Norway spruce (*Picea abies*), and Scots pine (*Pinus sylvestris*). We then capitalized upon recently published works linking cell differentiation kinetics with tree ring structure (Cuny et al., 2014; Balducci et al., 2016) in order to relate the kinetics (duration and rate) and final result (cell radial diameter and wall cross area) of each differentiation process (cell enlargement and cell wall thickening) to the environmental factors occurring during the corresponding time window. Based on the knowledge reported previously, we hypothesize that the soil water balance directly drives the seasonal changes in cell enlargement kinetics and the resulting cell size, while seasonal changes in wall thickening kinetics and the resulting cell weights are directly constrained by temperature.

RESULTS

Seasonal Cycles of Environmental Factors

The climatic conditions of the studied area followed seasonal patterns representative of a cool, temperate area (Fig. 1). We observed different levels but very similar seasonal patterns between the different sites and years (Supplemental Figs. S1–S3). Daylength followed a symmetric bell curve (Fig. 1A), reaching a maximum of about 16 h at the summer solstice (June 21) and dropping to a minimum of 8 h at the winter solstice (December 21). Light radiation followed the course of daylength, but with large day-to-day variations (Fig. 1A). By contrast, the daily air temperatures followed a bell curve slightly skewed to the right (Fig. 1B), peaking at $17^{\circ}\text{C} \pm 3.2^{\circ}\text{C}$ during summer (July–August) and dropping to $1.7^{\circ}\text{C} \pm 4.5^{\circ}\text{C}$ during winter (December–January). At the Abreschviller site, the cambium temperature measured in one silver fir and one Scots pine tree during 2008 and 2009 perfectly followed air temperature, demonstrating that the air temperature measured for the three sites and used in further analyses can be considered a good indicator of cambium temperature (Supplemental Fig. S4).

Precipitation was quite regularly distributed over the year (112 ± 61 mm per month on average), with two slightly drier periods in April and May (77 ± 63 mm per month) and in September and October (77 ± 55 mm per month; Fig. 1C). Because of these abundant and regular

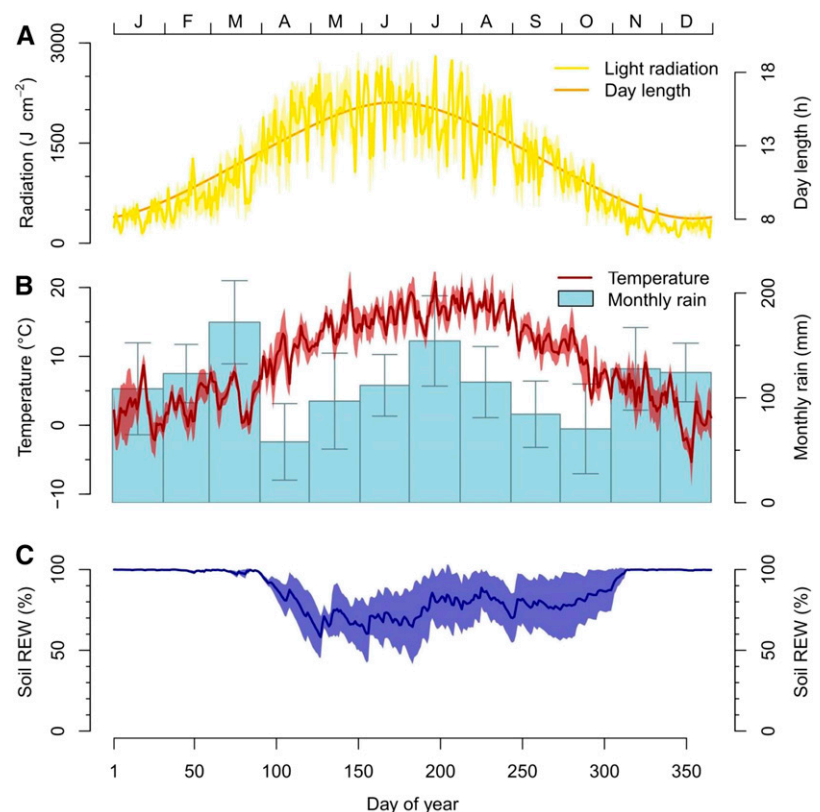
rains, the relative extractable water (REW) in the soil was usually maintained at a high level ($86\% \pm 21\%$ on average; Fig. 1C). It was generally lower in May and June ($68\% \pm 21\%$), due to the rapid increase of temperature and the slight decrease in precipitation. During the 3 years of monitoring and for the three sites, dry spells (REW below 40%) occurred mainly at the lowest site (Walscheid) and were of weak intensity (Supplemental Fig. S1; Supplemental Table S1).

Climatic Conditions Experienced during Xylem Cell Differentiation

Overall, the complete formation of the tree rings lasted from mid-April to mid-November, with similar timings of cellular development observed between the three species, sites, and years (Supplemental Figs. S5–S7). Earlywood cells developed from mid-April to mid-July; transition wood, from mid-June to mid-August; and latewood, from the beginning of July to mid-November. However, each cell in a tree ring experienced a unique cocktail of environmental signals (Fig. 2; Supplemental Fig. S8), potentially shaping its final dimensions in a unique way, since each differentiation phase occurred within a specific time window.

The daylength and daily light radiation were maximal during earlywood formation, before decreasing regularly during transition wood and latewood formation (Fig. 2, B and C). In contrast, the temperatures

Figure 1. Annual evolution of the monitored environmental factors. A, Light radiation and daylength. B, Temperature and monthly sums of rain. C, Soil REW. Lines represent the means for the 3 years (2007–2009) and the three sites, and colored areas around lines represent 95% confident intervals around means.



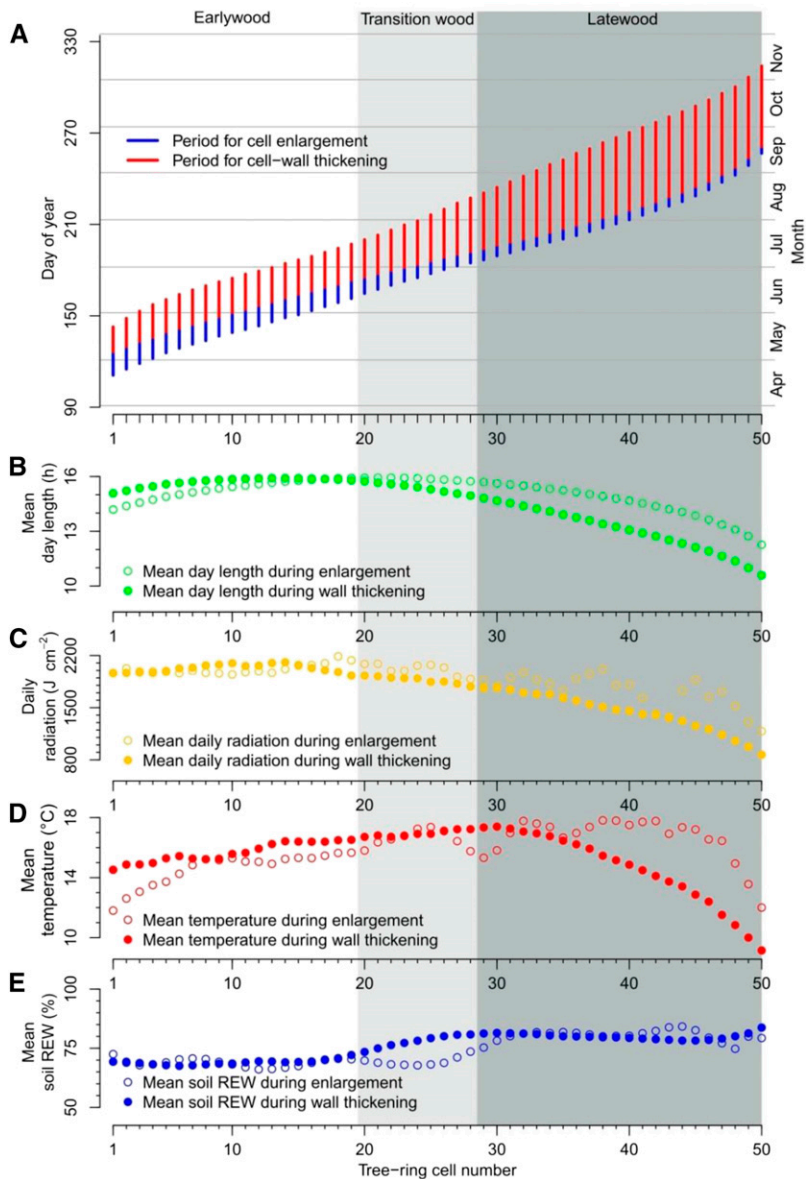


Figure 2. Timing of cell differentiation and environmental conditions experienced during cell differentiation. For each successive cell along the tree ring, shown are the timing of cell enlargement and cell wall thickening (A) and the mean environmental conditions experienced by the studied trees during the periods of cell enlargement and cell wall deposition, with daylength (B), cumulative daily light radiation (C), temperature (D), and soil REW (E). Average values for the three species, sites, and years (2007–2009) are shown.

experienced during differentiation increased slightly for the successive cells of the rings until latewood, in which it decreased abruptly (Fig. 2D). In particular, the temperature for wall thickening increased slightly during earlywood formation (+2°C on average from the first to the last earlywood cells), whereas it decreased abruptly during latewood formation, passing from $17.3^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for the first latewood cells to $8.6^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$ for the last ones.

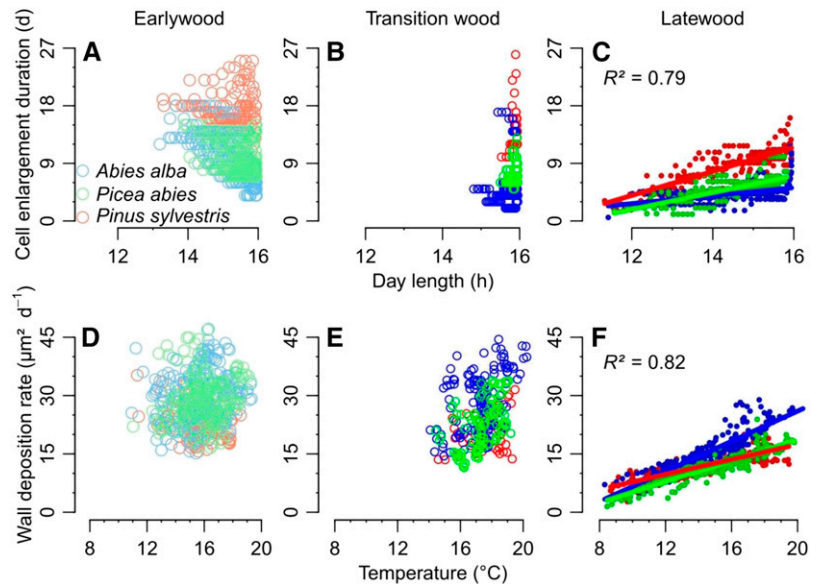
Because of the abundant and regular precipitation at the studied sites, all successive cells of the ring experienced a high soil REW during their development (Fig. 2E). However, surprisingly, the water availability was higher during the enlargement of the latewood cells (soil REW = $80 \pm 3\%$) than during the enlargement of the early- and transition-wood cells (soil REW = $69 \pm 3\%$).

Influence of Climatic Factors on the Kinetics of Xylem Cell Differentiation

Whatever the considered type of wood cells, we did not find any significant effects of the considered environmental factors prevailing during cell enlargement on the seasonal changes in the kinetics of cell enlargement (Supplemental Figs. S9 and S10). However, we observed a significant, positive effect of daylength on the duration of enlargement in latewood ($P < 0.001$; adjusted $r^2 = 0.79$; $n = 697$; Fig. 3C; Supplemental Table S2).

In contrast, we found highly significant effects of light radiation and temperature on the wall deposition rate during latewood formation (Supplemental Figs. S11 and S12). Yet, the temperature and radiation prevailing during cell wall thickening were highly

Figure 3. Influences of daylength on cell enlargement duration and of temperature on cell wall deposition rate. A to C, Relationship between the daylength experienced during cell enlargement and the cell enlargement duration in earlywood (A), transition wood (B), and latewood (C). D to F, Relationship between the temperatures experienced during wall thickening and the wall deposition rate in earlywood (D), transition wood (E), and latewood (F). Each point represents a cell along a tree ring of one of the three species at one of the three studied sites and during 1 year in the period 2007 to 2009. In C and F, the lines represent species-specific linear relationships, and the r^2 value is for the whole model.



correlated in our data set ($P < 0.0001$; adjusted $r^2 = 0.84$; $n = 697$), but the construction of a statistical model expressing the daily rates of wall deposition as a function of temperature, radiation, and soil REW reveals a highly significant and positive effect of temperature, with radiation and soil REW containing no additional information (Table I).

Indeed, highly significant, species-specific, linear, and positive relationships were found between the mean daily temperatures experienced during wall thickening and the mean daily rate of wall deposition during latewood formation ($P < 0.001$; adjusted $r^2 = 0.82$; $n = 697$; Fig. 3F; Table II) but not during earlywood and transition wood formation (Fig. 3, D–E). The highest temperature sensitivity of the wall deposition rate was observed in silver fir (slope = 1.94), then in Norway spruce (slope = 1.34), while the lowest sensitivity was found in Scots pine (slope = 0.96).

Whatever the process considered, the relationships were always better when using the environmental factors prevailing during the corresponding phase rather than other phases. For example, the species-specific relationship found between the temperature and the

wall deposition rate remained significant but was largely weakened ($P < 0.001$; adjusted $r^2 = 0.14$; $n = 697$) when using the temperature experienced by the cells when they were in the cambial zone.

Mechanisms of Signal Registration in Tree-Ring Structure

For the majority of the cells along the ring, the variations in the rate and duration of wall deposition were inversely proportional: variations in the daily rate of wall deposition were accompanied by opposite variations of the same magnitude in process duration (Fig. 4A). Because of this compensation, the cell wall cross area exhibited little change along a large part of the ring. Only in latewood did the compensation effect by duration decrease; it was minimal for the last latewood cells (Fig. 4A), for which variations in the wall deposition rate engendered equivalent variations in the wall cross area.

Because of the compensation effect played by duration, the influences of the environmental factors on the rate of wall deposition could not be translated into deposited amounts of wall material and so be recorded permanently in the tree-ring structure (Fig. 4B). Thus, for cells identified with a compensation effect, influences of temperature on wall deposition rate were not traced in the wall cross area (Fig. 4B), whereas for cells without compensation, highly significant, species-specific, linear, and positive relationships were found between the wall cross area and the temperature during the period of wall formation ($P < 0.001$; adjusted $r^2 = 0.87$; $n = 398$; Fig. 4C; Table III). Consistent with the species-specific temperature sensitivities found for the wall deposition rate, the highest temperature sensitivity of the wall cross area was observed in silver fir (slope = 81.7), then in Norway spruce (slope = 74.6), while the lowest sensitivity was found in Scots pine (slope = 48.1).

Table I. Estimated coefficients and associated SE values, Student's t test (t) values, and two-sided P values of the linear model linking the cell wall deposition rate in latewood with the radiation (R_w), soil REW (REW_w), and temperature (T_w) prevailing during cell wall thickening

Source	Estimated Coefficient	SE	t	P
Intercept	-8.976	1.635	-5.489	1.11e-07***
R_w	0.002	0.002	0.762	0.45
REW_w	0.008	0.014	0.549	0.58
T_w	1.205	0.227	5.303	2.76e-07***

Asterisks highlight significant effects (* for $P \leq 0.05$, ** for $P \leq 0.01$ and *** for $P \leq 0.001$).

Table II. Covariance analysis of the relationship between the temperature during wall thickening (T_w) and the daily rate of cell wall deposition in latewood among the three species (Sp)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F	P
T_w	1	8,636	8,636	2,125	<0.001***
Sp	2	2,122	1,061	261	<0.001***
$T_w:Sp$	2	723	362	89	<0.001***
Residuals	691	2,691	4		

Asterisks highlight significant effects (* for $P \leq 0.05$, ** for $P \leq 0.01$ and *** for $P \leq 0.001$).

DISCUSSION

Sensor of Environmental Influence: Is There a Transition Zone in Xylem Patterning?

A long-lasting source of debate about the influences of environmental factors on tree-ring formation concerns the place and the moment at which external signals could be sensed. By analogy with the root apex (Baluška et al., 2010), some authors suspect the existence of a transition zone located between the cambial and enlargement zones (Vaganov et al., 2011). Such a transition zone would act as a sensor of environmental conditions and would translate them as adaptive responses during further stages of xylem cell differentiation. However, we found that the influences of climatic factors on xylogenesis processes were highest when using the exact climate conditions prevailing during each process occurrence. Therefore, as defended by other authors (Wodzicki, 1971; Antonova and

Stasova, 1993, 1997), we argue that each xylogenesis subprocess is particularly sensitive to the environmental conditions prevailing at the time of its occurrence.

Environmental Control of Cell Enlargement and Tracheid Final Diameter

In contrast with our hypothesis that soil water availability (REW) is the driver of the seasonal changes in cell enlargement kinetics and the associated impressive decrease in cell dimension along tree rings, we did not find any relationship, either between REW and the rate and duration of cell enlargement or between REW and the final diameter of tracheids. In fact in the studied area, the narrow latewood cells even benefited from higher water availability to enlarge than the large earlywood cells (Fig. 2E). Such results refute the long-lasting belief that water conditions systematically trigger latewood formation (Kramer, 1964; Deleuze and

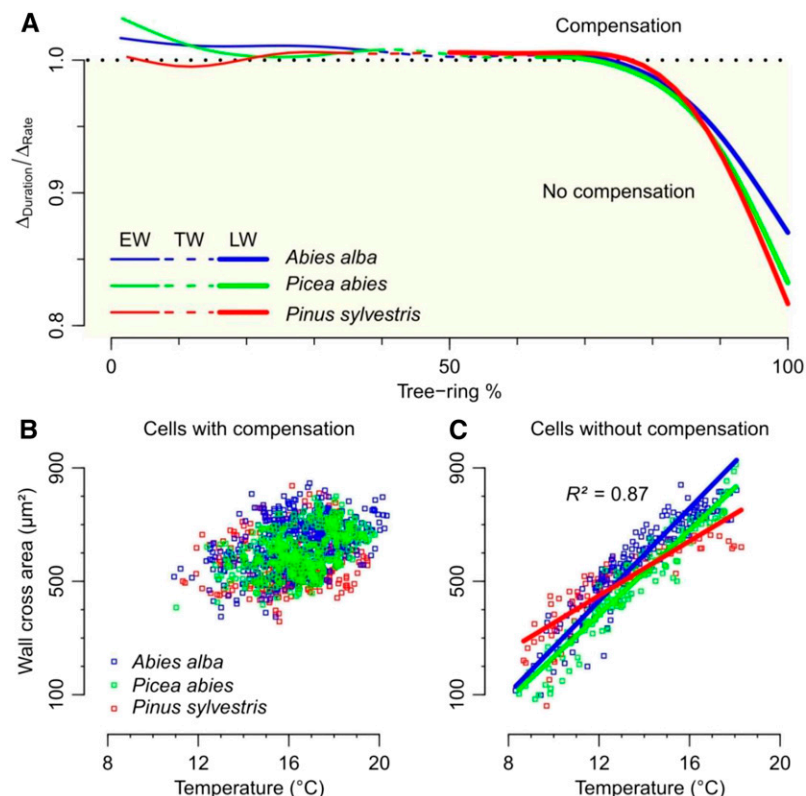


Figure 4. Mechanisms of temperature signal registration in tree-ring structure. A, Compensation effect in the kinetics of wall deposition. B, Influence of temperature on final wall cross area for cells with a compensation effect. C, Influence of temperature on final wall cross area for cells without a compensation effect. In A, for each species, the line represents the ratio between the changes in the duration of wall thickening and the changes in the rate of wall deposition along the tree ring (means for the three sites and years). Different line types distinguish earlywood (EW), transition wood (TW), and latewood (LW). The horizontal dotted line represents the value of a perfect compensation effect. The area below this line illustrates values without a compensation effect. In B and C, each point represents a cell along a tree ring of one of the three species at one of the three studied sites and during 1 year in the period 2007 to 2009. In C, the lines represent species-specific linear relationships, and the r^2 value is for the whole model.

Table III. Covariance analysis of the relationship between the temperature during wall thickening (T_w) and the final cell wall cross area in latewood cells having no compensation effect among the three species (Sp)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F	P
T_w	1	9,549,656	9,549,656	2,558	<0.001***
Sp	2	284,628	142,314	38	<0.001***
T_w :Sp	2	239,449	119,724	32	<0.001***
Residuals	392	1,463,454	3,733		

Asterisks highlight significant effects (* for $P \leq 0.05$, ** for $P \leq 0.01$ and *** for $P \leq 0.001$).

Houllier, 1998). By contrast, they are in agreement with observations that narrow latewood cells also are formed in irrigated trees (Zahner et al., 1964; Rossi et al., 2009b).

Climatic Control of Wall Thickening and Wall Amount in Tracheids

We also hypothesized that the seasonal changes in cell wall thickening and cell wall content are related to temperature. We actually found that the daily rate of wall deposition was strongly and positively related to the temperature, but only during latewood formation (Fig. 3, D–F). Such contrasting sensitivities of earlywood and latewood cells to temperature are in agreement with dendroclimatologists' observation that latewood conveys a stronger climatic signal than earlywood (Lebourgeois, 2000; Wimmer and Grabner, 2000; Wang et al., 2002). They could be linked to the large changes in developmental signaling associated with latewood formation (Uggla et al., 2001) and reveal the strong interactions between environment and development during tree-ring growth. Moreover, the relationships were species specific because of slightly different levels in the wall deposition rate and different climatic sensitivities between species. Notably, the higher slope was observed in silver fir, which is known to have the highest climatic sensitivity of the three species, whereas the lowest slope was observed in Scots pine, which is the least sensitive species (Lebourgeois et al., 2010).

The nature of the influence of temperature is complex, as it can affect all the processes influencing photosynthate availability (photosynthesis, respiration, storage, transport) as well as the processes directly responsible for wall thickening (cellulose and lignin biosynthesis, cell wall material deposition). However, most results in the literature suggest a direct effect of temperatures on wall-thickening processes, which, for example, are inhibited at temperatures still favorable for photosynthesis (Körner, 1998, 2003; Donaldson, 2001; Rossi et al., 2008; Simard et al., 2013). Lignification notably appears to be highly sensitive to temperature (Donaldson, 2001), in particular during latewood formation (Gindl et al., 2000).

We found a tight coupling between the duration and the rate of wall deposition (Wodzicki, 1971; Doley, 1979; Antonova and Stasova, 1993, 1997), and we

highlighted its crucial involvement in terms of the response of trees to their environment (Balducci et al., 2016) as well as its consequences in terms of tree-ring structure. Throughout the growing season, increasing durations of wall thickening counterbalanced decreasing wall deposition rates, except at the end of the season, during the formation of the last latewood cells (Fig. 4A). The direct consequence of this compensation is to erase the trace the climate may have left in tree-ring structure via its influence on the rate of wall deposition. Thus, despite the strong influence of the temperatures on the wall deposition rate, the actual amount of deposited wall material was related to the temperature only in the very last latewood cells, when the compensatory effect of the duration ceased (Fig. 4, B and C).

Climatic Versus Developmental Control of Tree-Ring Structure

The results of this study allow us to draw a picture of which and how environmental factors influence the seasonal changes in xylogenesis processes creating the tree-ring structure for conifers grown in the temperate zone when no particular environmental stresses are involved (Fig. 5). We did not find any clear influence of climatic factors on enlargement kinetics and associated cell size, suggesting that the regular decrease in cell size along a tree ring is primarily under developmental control and not linked to changes in climatic conditions. Indeed, we showed in a previous study (Cuny et al., 2014) that the final tracheid diameter depends primarily on the duration of enlargement, whereas the rate of enlargement exhibits few changes during the season. We associated the high importance of the enlargement duration with a developmental control, in which auxin would be of primary importance. This hormone is distributed across the cambial and enlargement zones according to a steep gradient, which serves as a positional signal for the developing cells (Uggla et al., 1996, 1998; Tuominen et al., 1997; Sundberg et al., 2000). According to its position along the gradient, a cell has the ability to divide or to enlarge without dividing. However, the width of the gradient decreases during the season, implying a reduction of the enlargement zone and decreases in cell enlargement duration and cell diameter (Uggla et al., 1998, 2001). This strong developmental control may be coupled with daylength. We actually found a significant positive effect of

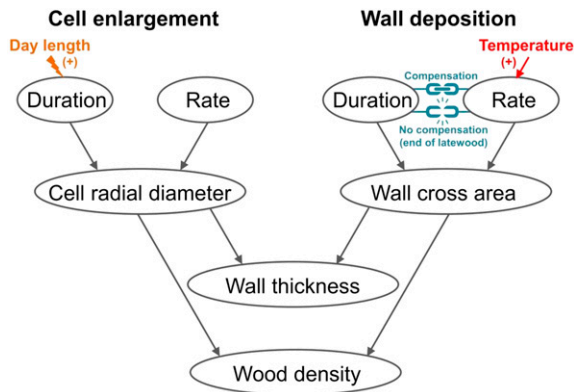


Figure 5. Graphical model of which and how environmental factors influence xylogenesis processes and the resulting cell dimensions during latewood formation of conifer tree rings. For daylength, the lightning bolt symbolizes an indirect effect on the duration of cell enlargement, whereas for temperature, the arrow symbolizes a direct effect on the rate of wall deposition. Signs of the relationships are given below in parentheses. For wall deposition, the chain links symbolize the coupling between the duration and the rate of the process; this coupling is broken during the formation of the last latewood cells.

daylength on cell enlargement duration (Fig. 5), but only during latewood formation. However, such a positive influence of daylength on cell enlargement and the resulting cell diameter has been experimentally documented for a long time, with long days inducing large diameters by promoting the synthesis of molecular substances (notably auxin) that stimulate enlargement (Larson, 1962; Richardson, 1964; Wodzicki, 1964; Balatinecz and Farrar, 1968).

Such a strong developmental control does not fully prevent climatic influences, as well as complex interplays between developmental and environmental factors, particularly in more stressful environments. Indeed, the auxin gradient can undergo rapid modifications in response to extreme climatic events such as freezing temperatures (Schrader et al., 2003). Moreover, many observations argue for strong influences of water availability on cell enlargement and the resulting diameter under harsh conditions. For example, the formation of intraannual density fluctuations, characterized by the presence of narrow latewood-like tracheids within the earlywood or large earlywood-like tracheids within the latewood, has been attributed to unusual hydric conditions, notably drought for earlywood intraannual density fluctuations (Wimmer et al., 2000; Bouriaud et al., 2005; Hoffer and Tardif, 2009). In fact, an accelerating decline in cell sizes happens below some critical value of water potential (Von Wilpert, 1991). Cell enlargement is physically inhibited and its rate collapses (Nonami and Boyer, 1990, 1990); hence, the rapid reduction in diameter when a drought occurs (Abe et al., 2003; Rossi et al., 2009b). This is why differences in the average cell diameter are generally observed when comparing sites or years with contrasting water conditions (Gruber et al., 2010). But the drastic

reduction in cell size along tree rings does not necessarily result from changing water conditions.

The regular decrease in cell size is a major determinant of the whole conifer tree-ring structure, as it is the primary driver of the increase in wall thickness, and so of the transition from earlywood to latewood and of the tree-ring density profile (Cuny et al., 2014). In opposition to ring width, which is very sensitive to environmental variations (Briffa et al., 2002; Esper et al., 2002), tree-ring structure appears much more conservative. This echoes the observations from genetic studies that cell diameter, cell wall thickness, and tree-ring density are highly heritable while tree-ring width is not (Zhang and Morgenstern, 1995; Lenz et al., 2010). So, while tree-ring width is largely related to environmental conditions, we believe that tree-ring structure is mainly related to developmental control and genetic variability. An important exception concerns the tag affixed by the temperature on the latest latewood cells, for which the variation of the rate of wall deposition was no more counterbalanced by its duration (Fig. 5).

This late breakage of the compensatory mechanism at work in the wall material deposition process appears as a clue to explain why maximum latewood density, a parameter of the tree-ring structure localized at the end of latewood, is one of the most valuable tree-ring proxies for temperature reconstruction (Hughes et al., 1984; Briffa et al., 1998; Barber et al., 2000). As the wall thickening takes almost 2 months for the last cells, the final part of the tree-ring structure possibly integrates several months of environmental influence, explaining why the maximal latewood density is usually correlated with the temperature on monthly to seasonal time scales (Hughes et al., 1984; Briffa et al., 1998). Finally, we speculate that recent warming may have decreased the temperature constraints on the cell wall deposition rate in latewood and the proportion of cells able to register the temperature signals, contributing to the explanation of the reduced sensitivity of maximal wood density to temperature over the last century (Briffa et al., 1998).

CONCLUSION

In this study, we assessed how the seasonal variations in climatic factors influence the spectacular changes in tracheid dimensions characterizing the typical conifer tree-ring structure. We found consistent results for three conifer species grown under natural conditions in a temperate forest. Our results bring new insights on the developmental regulation and climatic sensitivity of tree-ring formation. We showed strong and positive effects of temperature on the wall deposition rate in latewood cells, but not in earlywood and transition wood cells, revealing the complex interactions between development and environment during tree-ring formation. On the other hand, no climatic influence was observed on cell enlargement. The narrow latewood cells even benefited from higher water

availability to enlarge than the large earlywood cells. This absence of a clear climatic determinism of the decreasing tracheid diameter along the tree ring, a crucial determinant of the whole ring structure, argues for a tight developmental control of conifer tree-ring structure under temperate climate and in the absence of major stress. A monitoring of wood formation under greenhouse gases, with known provenances, controlled environmental conditions, and quantification of internal growth regulators, could help to even further disentangle the complex regulation of tree-ring formation.

Our work also unravels the fine mechanisms by which climatic factors are recorded into the tree-ring structure. We notably revealed the existence of a compensatory mechanism that prevents the preservation of temperature signals in tracheid cell walls. Thus, we found that the climatic influences on the rate of wall deposition were offset by the compensation in the duration of wall deposition. Only for the last latewood cells, because the duration leveled off, did the temperature influences on the wall deposition rate directly translate into the resulting amount of wall material deposited into the cells. This lack of compensatory effect for the last latewood cells appears as a clue to explain the supremacy of maximum latewood density as a proxy for past climatic conditions, and temperature in particular.

MATERIALS AND METHODS

Study Area

Three sites were selected in mixed stands composed of silver fir (*Abies alba*), Norway spruce (*Picea abies*), and Scots pine (*Pinus sylvestris*) in northeast France, in the temperate forest of the Vosges Mountains. The three sites were spread on a north-south axis of about 15 km, on the slope of the Donon massif, and were named according to the closest village: Walscheid (370 m above sea level, 48° 38'N, 7°09'E), Abreschviller (430 m above sea level, 48°36'N, 7°08'E), and Grandfontaine (650 m above sea level, 48°28'N, 7°08'E). Based on complete inventories of the stands, five dominant and healthy silver fir, Norway spruce, and Scots pine trees were selected on each site, for a total of 45 studied trees (5 trees × 3 species × 3 sites; Supplemental Table S3). On each site, two soil pits were dug in order to assess soil characteristics (soil type, number of layers and their depth, and proportion of fine roots in each layer).

Sampling, Preparation, and Microscopic Observations of the Developing Xylem

To monitor tree-ring formation, microcores were collected weekly at breast height on the stem of the selected trees from April to November during three years (2007–2009). The orientation on the stem of the first sampling was randomly selected among the four cardinal points, and successive microcores were then taken about 1 cm apart from each other and following a slightly ascending spiral pattern to avoid wound reaction. Microcores were then prepared in the laboratory, and 5- to 10- μ m-thick transverse sections were cut with a rotary microtome (HM 355S; MM France). Sections were stained with Cresyl Violet acetate and permanently mounted on glass slides using Histolaque LMR. Overall, about 4,300 anatomical sections were observed using an optical microscope (AxioImager.M2; Carl Zeiss) under visible and polarized light at 100× to 400× magnification to distinguish the different phases of development among the cells. Thin-walled enlarging cells were discriminated from cambial cells by their larger size. In contrast to enlarging cells, cells in the thickening zone developed secondary walls that shined under polarized light because of the orientation of cellulose microfibrils (Abe et al., 1997). Cresyl Violet acetate, which stains cellulose in purple and lignin in blue (Kutscha et al., 1975), was

used to follow the advancement of lignification. Thickening cells exhibited violet and blue walls, indicating that lignification was in progress, whereas mature tracheids presented entirely lignified and thus completely blue walls.

According to Rossi et al. (2003), count data were standardized by the total number of cells of the previous ring using the R package CAVIAR (Rathgeber, 2012). This standardization process reduced the noise in the data, increasing the signal-to-noise ratio of about 50% (Cuny et al., 2014).

Tracheid Dimension Measurements

For each tree, at the end of each year, a well-preserved section of the entirely formed tree ring was selected to characterize the dimensions of the cells produced during the year. Digital images of the selected tree rings were analyzed using WinCell (Regent Instruments) in order to measure the lumen radial and tangential diameters, the lumen area, and the wall radial thickness of tracheids along at least three radial files (Supplemental Fig. S13). From these measurements, the cell radial diameter and the wall cross area were calculated (for details on the measurement and calculation of tracheid dimensions, see Cuny et al., 2014) and considered as the final results of the enlargement and wall deposition phases, respectively.

To show variation in tracheid dimensions along a ring, cell morphological measurements were grouped by radial file in profiles called tracheidograms (Vaganov, 1990). Because the number of cells varied between radial files within and between trees, tracheidograms were standardized according to the method of Vaganov (1990) using a dedicated function of the R package CAVIAR (Rathgeber, 2012). This standardization allows adjusting the length of the profiles (cell numbers) without changing their shape (cell dimensions; Vaganov, 1990). We checked visually that this standardization did not alter the shape of the anatomical profiles. The standardized tracheidograms were then averaged to obtain species-specific annual tree-ring structures for each year and each site.

Mature tracheids were classified into three different types of wood: earlywood, transition wood, and latewood, according to Mork's criterion (MC; Denne, 1988), which is computed as the ratio between 4 times the wall radial thickness divided by the lumen radial diameter (for details, see Supplemental Table S4). According to Park and Spiecker (2005), tracheids were classified as follows: $MC \leq 0.5$, earlywood; $0.5 < MC < 1$, transition wood; and $MC \geq 1$, latewood.

Assessing the Kinetics of Tracheid Development

In order to accurately characterize wood formation dynamics, generalized additive models were fitted on the standardized numbers of cells for each phase of xylem development, each year, and each individual tree (Cuny et al., 2013), using the R package mgcv (Wood, 2006; R Core Team, 2014). The values of the fitted models were then averaged in order to calculate the mean wood formation dynamics of each species during each year and at each site.

For each species, we used the average cell numbers predicted by generalized additive models to calculate the date of entrance of each cell into each zone (cambial, enlargement, wall thickening, and mature) of xylem cell production and differentiation along the developing tree ring, based on the method described by Cuny et al. (2013). From these dates, the residence durations of each cell i in the cambial ($d_{C,i}$), enlargement ($d_{E,i}$), and wall thickening ($d_{W,i}$) zones and the total duration of its formation ($d_{F,i}$) were computed.

In order to have a complete characterization of the kinetics of differentiation of each cell i , we also estimated the rate of enlargement ($r_{E,i}$) and wall deposition ($r_{W,i}$) by dividing its final dimensions (CRD_{*i*} for cell radial diameter and WCA_{*i*} for wall cross area) by the duration ($d_{E,i}$ and $d_{W,i}$) it spent in the corresponding phase (enlargement and wall deposition; Supplemental Table S4).

Meteorological Variable Recording and Soil Water Balance Modeling

In order to characterize the annual cycle of environmental factors, daily meteorological data (temperature, precipitation, cumulative global radiation, wind speed, and relative humidity) of the period 2007 to 2009 were gathered from three meteorological stations in the studied area, following the disposition of the selected sites. In addition, at the Abreschviller site, six temperature sensors (Campbell Scientific) were inserted in April 2008 under the bark of two trees (one silver fir and one Scots pine) according to different orientations in order to measure cambium temperature.

Moreover, the model Biljou was used to assess the daily water balance of the three studied stands (<https://appgeodb.nancy.inra.fr/biljou/>; Granier et al., 1999). In addition to the daily meteorological data mentioned above, the model takes as input some soil (e.g. number and depth of layers as well as proportion of fine roots per layer) and stand (forest type and maximum leaf area index) parameters and gives as output the REW on a daily scale. The REW is a relative expression of the filling state of the soil: REW is 100% at field capacity and 0% at the permanent wilting point. Water stress is assumed to occur when the soil REW drops below a threshold of 40%, under which transpiration is gradually reduced due to stomata closure (Granier et al., 1999).

Assessing the Climatic Influence on Tracheid Development and Final Dimensions

In order to study accurately the mechanisms by which the climate influences tree-ring structure, xylogenesis kinetics and the resulting xylem cell features were associated with the exact climate conditions prevailing during their development. For example, the rate of enlargement and the final radial diameter of a cell were related to the climatic factors that were occurring during the enlargement of this very same cell. Similarly, the rate of wall deposition and the final wall cross area of a cell were related to the climatic factors that were occurring during the wall thickening phase of this cell.

Furthermore, in order to test the existence of a transition zone sensing environmental stimuli and transmitting them to further stages of cell differentiation (Vaganov et al., 2011), we performed the same analyses but using the climatic factors that were occurring when the cell was in the cambial zone (i.e. during the time between its appearance in the cambial zone and its entrance into the enlargement zone).

Relationships between climatic, kinetics, and anatomical variables were assessed using linear models. In particular, covariance analyses were performed to evaluate the effect of species on the relationships. Moreover, analyses were conducted according to earlywood, transition wood, and latewood modalities in order to test the possibility of different climatic sensitivities between these different developmental stages of tree-ring formation.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Annual evolution of the monitored environmental factors during the three studied years at the Walscheid site.

Supplemental Figure S2. Annual evolution of the monitored environmental factors during the three studied years at the Abreschviller site.

Supplemental Figure S3. Annual evolution of the monitored environmental factors during the three studied years at the Grandfontaine site.

Supplemental Figure S4. Comparison of air and cambium temperatures measured at the Abreschviller site during the 2008 to 2009 period.

Supplemental Figure S5. Timing of tracheid development for the three studied coniferous species during the three studied years at the Walscheid site.

Supplemental Figure S6. Timing of tracheid development for the three studied coniferous species during the three studied years at the Abreschviller site.

Supplemental Figure S7. Timing of tracheid development for the three studied coniferous species during the three studied years at the Grandfontaine site.

Supplemental Figure S8. Mean environmental conditions experienced by the successive cells of the tree ring during their differentiation for the three studied coniferous species.

Supplemental Figure S9. Relationships between the environmental factors prevailing during cell enlargement and the rate of cell enlargement.

Supplemental Figure S10. Relationships between the environmental factors prevailing during cell enlargement and the duration of cell enlargement.

Supplemental Figure S11. Relationships between the environmental factors prevailing during wall thickening and the rate of wall thickening.

Supplemental Figure S12. Relationships between the environmental factors prevailing during wall thickening and the duration of wall thickening.

Supplemental Figure S13. Illustration of the wood anatomical quantifications made using WinCell.

Supplemental Table S1. Hydric conditions for the three monitored sites and years.

Supplemental Table S2. Covariance analysis of the relationship between the daylength during cell enlargement and the duration of cell enlargement in latewood among the three species.

Supplemental Table S3. Main characteristics (mean \pm SE) of the monitored trees.

Supplemental Table S4. Variables used in this work.

ACKNOWLEDGMENTS

We thank E. Cornu, E. Farré, C. Freyburger, P. Gelhay, and A. Mercanti for field work and monitoring; M. Harroué for sample preparation in the laboratory; B. Longdoz of the Forest Ecology and Ecophysiology Team and the Association for the Study and Monitoring of Air Pollution in Alsace for the meteorological data; M. Nicholas of the French Permanent Plot Network for the Monitoring of Forest Ecosystems for the meteorological data and the description of the soil profiles; and P. Fonti, D. Frank, and M. Fournier for discussions on the article.

Received January 9, 2016; accepted March 28, 2016; published March 30, 2016.

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