Biology, Chemistry and Structure of Tension Wood

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Abstract Trees maintain and adjust their stature by developing reaction wood in stems and branches. The physical properties of reaction wood result in a higher strain than in normal wood. Because reaction wood is only formed at one side of the stem, this unilateral strain creates a force and hence a movement of the stem or branches towards a more favorable position. The spectacular modification of cambial growth, cell shape, cell-wall chemistry, and ultrastructure observed in reaction wood has attracted generations of scientists to study its features and molecular regulation. In the early literature, the physiology of reaction wood induction was much studied, especially the relative importance of positional and mechanical sensing for its induction. Even today this is still a matter of debate and confusion, as discussed in the first part of this chapter. In angiosperm trees, reaction wood is denoted tension wood (TW), and in many tree species TW fibers develop an inner cellulose-rich gelatinous layer (G-fibers). Much research has been devoted to understand the chemistry and ultrastructure of the gelatinous layer and its function in creating tension stress in the wood. Less attention has been paid to TW without G-fibers, although it has similar physical properties and function as TW with G-fibers. The chemistry and structural variation of TW, and their importance for TW function, are discussed in the second part of this chapter. Not much is known about the molecular control of TW formation. However, some information has been gained about the role of plant hormones as signaling components in TW induction. The last part of the chapter summarizes this knowledge.

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J. Fromm (ed.), *Cellular Aspects of Wood Formation*, Plant Cell Monographs 20,
DOI 10.1007/978-3-642-36491-4_8,

1 Physiology of Sensing and Signaling Mechanisms for Reaction Wood Induction

Primary shoots of trees and other plants grow in response to inherent patterning mechanisms (e.g., apical dominance) and environmental factors (e.g., light and gravity) to establish a branching architecture that is optimal for growth and reproduction. However, large and long-lived organisms like trees often have a requirement to adjust the position of their stems and branches, for example, when crown architecture or the entire tree has been perturbed by abiotic or biotic factors (e.g., wind, snow, grazing, self-weight). Such growth correction of stems is accomplished by the unilateral formation of reaction wood, which exerts a strain that induces a directional movement of the stem towards a more favorable position (Scurfield 1973). With few exceptions (Kojima et al. 2012), reaction wood in angiosperm trees is under tension and denoted tension wood (TW), and, in gymnosperms, it is under compression and denoted compression wood. Development of reaction wood is commonly associated with increased cambial growth, a modified wood anatomy and xylem cell morphology, and a change in the ultrastructure and chemistry of the tracheid/fiber secondary cell wall (Timell 1986; Jourez et al. 2001; Mellerowicz and Sundberg 2008; Donaldson and Singh 2013). In this chapter, we discuss some aspects pertaining to the biology of reaction wood induction and signaling. Although TW and compression wood are different both from a structural and mechanical sense, the basic mechanisms for sensing the stimuli and forming the reaction wood seem to be similar in angiosperms and gymnosperms and are therefore discussed in a broader sense. We then focus on the anatomy, chemistry, and ultrastructure specifically for TW and finally summarize knowledge on the role of plant hormones for its formation.

It has long been discussed in the literature whether reaction wood is induced in response to positional or mechanical sensing. A main reason for this is that experimental studies on reaction wood induction often involve displacement of stems that normally cause both a positional change and mechanical stress in the organ. Jaccard (1938) performed pioneering studies to distinguish between positional or mechanical sensing in the reaction wood response. By bending stems into loops (Jaccard's loop), he demonstrated that reaction wood formed unilaterally in the upper and lower part of the loop where cambial tissues were under tension and compression, respectively, and not along the whole loop although all tissues in the loop were under mechanical stress. He concluded, in accordance with studies from Hartmann (1932), that reaction wood forms at places where the resulting strain will make the stem move towards its original position, irrespectively of tension or compression forces in cambial tissues. These findings by Jaccard, later supported in other studies (reviewed in Timell 1986), strongly suggest that positional sensing is a primary mechanism for the induction of reaction wood. Thus, the fact that TW is formed at the upper side of leaning stems (and vice versa for compression wood) is not because this side is under tension stress, but because this will induce the desired movement of the stem towards the vertical.

A similar positional memory was also demonstrated for gravitropic responses during primary shoot growth of branches, where displaced shoots restore their angle towards the "gravitropic set-point angle" (Digby and Firn 1995). The gravitropic set-point angle was defined as "the angle with respect to gravity at which an organ shows no gravity induced differential growth in order to correct its orientation." This observation suggests a common sensing and signaling mechanism in gravitational responses in both primary shoots and secondary stems. In primary organs, this is translated into asymmetric elongation growth, and in secondary stems, it will induce unilateral reaction wood formation in a way that will result in the movement towards a favorable position.

The relative importance of gravitational and mechanical stimuli for reaction wood induction has been evaluated in many experiments. Experiments in which mechanical stimuli were applied to stems in the absence of any gravity factor, by rotating plants on a clinostat, showed that the gravity factor is important for reaction wood induction. Nevertheless other experiments have pointed towards an importance for mechanical sensing (Timell 1986; Kwon et al. 2001). It is also observed that trees exposed to wind sway often respond by reaction wood formation, which supports the idea that the mechanical stimulus is an important factor (Timell 1986; Telewski 2006). Even in vertically growing trees, self-weight of the crown during growth will induce both mechanical forces and displacement of the stem from vertical, and the tree needs to develop reaction wood in order to maintain itself upright (Almeras and Fournier 2009). This phenomenon is particularly important in slender stems of, for example, young trees. Arcs of TW frequently form in greenhouse grown *Populus* sp., which are often used as a model species in molecular research on trees. Such wood heterogeneity may cause problems when stems are used for experimental studies on wood biology and wood chemistry (Bjurhager et al. 2010).

In addition to gravitational and mechanical stimuli, reaction wood is also formed in response to inherent growth patterning. For example, removal of the leader shoot of trees induces reaction wood in the underlying whorl of branches and an upward movement towards vertical in order to replace the leader shoot (Hartig 1901; Hartmann 1932; Wilson and Archer 1981, 1983). This response evidently takes place without any change in position or any mechanical stimulus. Taken together, reaction wood develops in response to inherent patterning mechanisms, to a positional change, and probably also to mechanical stimuli. The relative importance of the latter two is not resolved, and they may very well act in concert.

The positional change or mechanical stimulus that results in the induction of reaction wood formation in a displaced stem will be sensed along the whole stem. But the development of reaction wood is directed to one side, and even to a specific position of the stem as observed in Jaccard's loops, in order to induce a movement towards the favorable position. This suggests that there is a mechanism for spatial separation of sensing the inductive stimuli and forming reaction wood. Moreover, the inductive signal is active across cells at all stages of xylem development and not only in cells at early developmental stages in cambial tissues. This was deduced from experiments where conifer stems were displaced and reverted back to the original position. It was observed that developing tracheids that were in the stage of expansion and secondary wall formation at the time of displacement were

responding by partly exhibiting compression wood characteristics (Kennedy and Farrar 1965; Yoshizawa et al. 1984). It does not seem unlikely that this observation holds true also for TW formation in angiosperm trees, although similar experiments to show this are missing. Little, if anything, is known about the molecular mechanisms underlying sensing and signaling of the reaction wood response. However, all reaction wood characteristics are not always observed together (i.e., altered wood anatomy/chemistry can be observed in the absence of increased growth). Consequently, inductive signal(s) can independently act on different stages of xylem cell development, probably by interacting with the complex genetic and molecular machinery regulating cell division, cell expansion, ultrastructure, and biosynthesis of secondary cell-wall formation. This will lead to the remarkable wood plasticity presented in reaction wood.

2 The Chemical and Structural Variation of Tension Wood Fibers

The TW response often (but not always) results in an increased eccentric cambial growth and a decreased frequency of vessel development (see Fig. 1). This will increase the xylem area of TW fibers, which is the load-bearing cell type in the wood and responsible for exerting the tensile strain in the tissue. However, variation in TW anatomy, chemistry, and fiber wall ultrastructure is diverse between species (Onaka 1949; Côté et al. 1969; Clair et al. 2006b; Ruelle et al. 2006, 2007). In particular, the secondary cell wall of TW fibers has attracted large interest because it is highly modified in order to exert a longitudinal shrinkage that leads to the tensile stress in the wood.

2.1 Tension Wood with a Gelatinous Layer

The most spectacular TW fibers observed in many species are those that develop a cellulose-rich gelatinous layer (G-layer) on the lumen side of the fiber (so-called G-fibers) (Norberg and Meier 1966; Côté et al. 1969; Clair et al. 2006b; Mellerowicz and Gorshkova 2012) (Fig. 1). Depending on the tree species, G-fibers can arrange in very different patterns, e.g., be restricted to earlywood as seen in *Fagus grandifolia*, be absent from fibers in the vicinity of vessels like in *Ulmus americana*, or be present either in clusters or as sparse isolated G-fibers like in certain tropical species (Côté et al. 1969; Clair et al. 2006b). Furthermore the G-layer can vary in its thickness and can replace either wholly or partially the S2 or the S3 cell-wall layer, or be added in addition to those layers (Wardrop and Dadswell 1955). The gelatinous-looking structure of the G-layer inspired researchers early on to investigate its ultrastructure and composition. Based on microscopy and optical birefringence

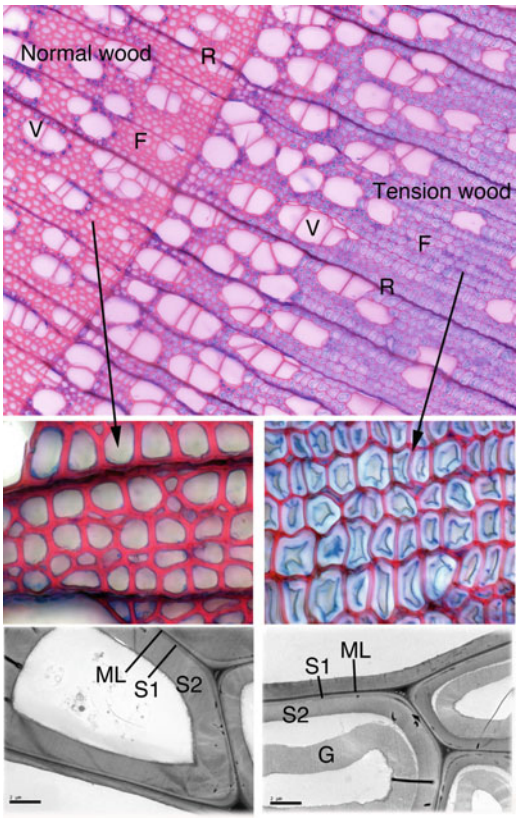

Fig. 1 Normal wood and tension wood across two annual rings of *Populus tremula*. Sections are stained with safranin/alcian blue for light microscopy. A decrease in vessel frequency and size is visible in TW. Magnification of NW and TW areas shows the presence of G-layers (*light blue*) in TW, whereas in NW, only secondary cell-wall layers (*pink*) are visible. TEM analyses highlight the cell-wall structure in NW and TW. The thick, detached G-layer in TW is visible. Scale bars are 2 µm. Vessel (V), fiber (F), ray (R), middle lamella (ML).

analyses, Norberg and Meier (1966) suggested the existence of numerous water-filled pores in G-layers. A high water content of G-layers was later observed by Raman spectroscopy (Gierlinger and Schwanninger 2006). The mesoporosity (mesopores are pores smaller than 50 nm) of TW was analyzed using nitrogen adsorption by Clair et al. (2008) in *Castanea sativa* and by Chang et al. (2009) in six different tropical tree species. Chang et al. found a large variation in sizes, numbers, networks, and shapes of the pores between G-layers of different species. A common conclusion from both authors was that porosity in TW is normally higher than in NW within the same species. They associated this high porosity mostly with the G-layer itself. Thus, it seems evident that the high porosity of G-layers gives room for water incorporation, which causes the gelatinous appearance. The high water content provides a capacity of the G-layer to swell or shrink transversally. How important these hygroscopic properties are for the longitudinal shrinkage and tension stress in TW is, however, not known (Clair et al. 2005a; Mellerowicz and Gorshkova 2012).

2.2 The Gelatinous Layer

2.2.1 Cellulose and Its Ultrastructure

The attachment of G-layers to the outer layers of the secondary wall is weak (Clair et al. 2005b) (Fig. 1). G-layers can therefore be isolated from transverse wood sections for chemical analysis. Norberg and Meier (1966) analyzed G-layers from *Populus tremula* isolated by ultrasonication in ethanol. Based on paper chromatography of hydrolyzed G-layers, they detected almost only glucan and concluded that the G-layer was composed of pure cellulose. Although this view is modified by recent studies that reported the presence of about 25 % matrix polymers in the G-layer (Nishikubo et al. 2007; Kaku et al. 2009), cellulose is by far the major component. The cellulose microfibrils in the G-layer have a parallel or almost parallel orientation (low microfibril angle, MFA) to the axis of the fiber in all species investigated (Norberg and Meier 1966; Côté et al. 1969; Clair et al. 2011; Lautner et al. 2012). The cellulose crystals in microfibrils are larger in the G-layer than in the S2-layer, with diameter values of 6.5 nm in G-layers and 3.1 nm in S2-layers of the G-fiber (Muller et al. 2006). Translated into cellulose chain number of microfibrils, this corresponds to a fourfold increase in G-layers compared to S2-layers. Muller et al. (2006) speculated that this could be due to a higher number of cellulose biosynthesis complexes in each rosette or because the low hemicellulose content of the G-layer facilitates microfibril aggregation and increased crystallization.

Clair et al. (2006a) have assessed lattice spacing of cellulose microfibrils in TW using synchrotron X-ray diffraction. Lattice spacing describes the distance between successive cellulose monomers along a cellulose microfibril. They found that lattice spacing increased with the mechanical stress measured in TW and was related to deformation of the microfibrils. In a later study, lattice spacing was recorded in developing wood at successive stages of cell-wall formation in TW and NW fibers using synchrotron radiation microdiffraction (Clair et al. 2011). In this study, the authors concluded that lattice spacing was increased in the G-layer already during its biosynthesis. This suggests that the tensile stress measured on a macroscopic level in TW can be traced down to the ultrastructure of the cellulose in the G-layer. It also provides evidence for the idea that the tensile stress is caused by longitudinal shrinkage of the cellulose crystals, established when TW is formed (Okuyama et al. 1994; Clair et al. 2011).

2.2.2 Noncellulosic Polymers

Lignin

Even though cellulose is a major compound of the G-layer and its longitudinal shrinkage is thought to be a major factor for stress generation in TW, the G-layer has a more complex composition that involves other polymers. Different studies

have reported the presence of lignin, xyloglucans, pectins, and arabinogalactan proteins (AGPs) in G-layers. The presence of lignin in the G-layer has been much debated despite its very low abundance (Pilate et al. 2004). Several studies have used UV microscopy and stainings to indicate the presence of lignin in the G-layer in different woody species such as *Tristania conferta, Grevillea robusta, Hakea laurina, Fraxinus mandshurica, Robinia pseudoacacia,* and *Populus euramericana* (Scurfield and Wardrop 1963; Araki et al. 1982; Prodhan et al. 1995; Yoshida et al. 2002a). Immunolocalization with antibodies against synthesized lignin epitopes also indicated the presence of particularly S-type lignin at the lumen side of the G-layer in *Populus deltoides* × *P. trichocarpa* (Joseleau et al. 2004). The observation of lignin towards the lumen side of the G-layer was also supported by Raman spectroscopy imaging in *Populus nigra* × *P. deltoides* (Gierlinger and Schwanninger 2006). The Raman spectra contained several bands characteristic for lignin, and the authors claimed that this demonstrates the presence of a lignin polymer rather than mono- or oligolignols. Lignin traces in G-layers of other species, such as *Acer* ssp., *Fagus sylvatica,* or *Quercus robur,* were detected by Raman spectroscopy in concentric rings or spots within the G-layer (Lehringer et al. 2008). Prodhan et al. (1995) reported stronger lignin signals towards the S-layer side of G-layers in *Fraxinus mandshurica* using $KMnO_4$ staining and TEM. In addition to these chemical indications of lignin in the G-layer, a protein analysis of the G-layer revealed that some of the most abundant proteins were related to metabolism of lignin and/or phenolic compounds (Kaku et al. 2009). Although lignin, or lignin-like compounds, can be detected in the G-layer, it has always been reported in low abundances and sometimes not detected at all. It is possible that studies that fail to detect lignin used methods with insufficient sensitivity. But it is also possible that the lignin polymer is not a general feature of G-layers. Conclusive analytical evidence for the presence of a lignin polymer in the G-layer is still missing, and it cannot be excluded that stainings and UV spectroscopy detect phenolic compounds rather than lignin. Also, the trace amount of lignin-like compounds that may be present in the G-layer suggests little significance of lignin for TW function.

Hemicelluloses

A role for hemicellulose in stress generation in TW fibers has been proposed from recent studies (Nishikubo et al. 2007; Mellerowicz and Gorshkova 2012). Norberg and Meier (1966) found in addition to cellulose a low amount (1.5 %) of xylose in their chemical analysis of isolated G-layers but did not investigate this any further. The presence of significant amounts (5–18 %) of xylose in G-layers of *Populus* ssp. was demonstrated later by different authors (Furuya et al. 1970; Nishikubo et al. 2007; Kaku et al. 2009). Xylose is a building block of the hemicelluloses xylan and xyloglucan. Results of immunolocalization (Nishikubo et al. 2007; Sandquist et al. 2010) and linkage analysis of extracted polymers have proposed that xyloglucan is present in G-layers (Nishikubo et al. 2007; Kaku et al. 2009). Further support of

xyloglucan in *Populus* G-layers is the presence of xyloglucan:xyloglucosyl transferase (XET) enzyme and activity in the G-layer shown by proteomics (Kaku et al. 2009), immunolocalization, and incorporation of a labeled xyloglucan oligosaccharide into fresh wood sections, respectively (Nishikubo et al. 2007). The linkage analysis of polymers extracted from the G-layer did not detect xylan, and likewise immunolocalization with xylan antibodies (LM10, AX1 (arabinoxylan)) indicated that xylan was absent from G-layers, in contrast to S2-layers where it is highly abundant (Bowling and Vaughn 2008; Decou et al. 2009; Kim et al. 2012). Opposed to these findings, Kim et al. (2012) claimed the presence of xylan in the G-layer based on positive signals from the LM11 antibody. The presence of small amounts of mannan, another hemicellulose, was detected in isolated G-layers (Nishikubo et al. 2007), and glucomannan was also indicated using the LM21, LM22, and BGM C6 antibodies (Kim et al. 2012).

Tension stress is thought to be induced by the longitudinal shrinkage of the G-layer. The tension needs to be transferred from the G- to the S2-layer and to the entire fiber, which is a potential problem as these layers are weakly linked. It was hypothesized that xyloglucan is located at the border between the G- and S2-layer to facilitate this stress transfer. Broken bonds due to tension stresses could continuously be repaired by XET activity (Nishikubo et al. 2007; Mellerowicz and Gorshkova 2012). This hypothesis was strengthened by Sandquist et al. (2010) who used TEM-/SEM-combined immunolocalization with CCRC-M1 antibodies (binding to xyloglucan and rhamnogalacturonan) and reported labeling at the boundary between S2- and G-layer in *Populus* where the transfer of tension stress would occur. However, using another antibody against (fucosylated) xyloglucan (CBM FXG-14b), they observed signals only at the lumen side of the G-layer. In studies of *Liquidambar styraciflua* and *Celtis occidentalis,* Bowling and Vaughn (2008) detected labeling with CCRC-M1 in a thin layer at the lumen side of the G-layer but not in the G-layer or at the G-S interface. Taken together, most data indicate that xyloglucan is the major hemicellulose in the G-layer. A role for xyloglucan in transferring tension stress from G- to S-layer has been suggested in *Populus*, but whether this is a general function in other species remains to be experimentally investigated.

Pectins and Arabinogalactan Proteins

Early studies showed the presence of higher amounts of galactose in TW compared to NW in different species (Gustafsson et al. 1952; Meier 1962). Further studies identified galactans with complex structures that were not characterized in detail but nevertheless suggested an increased amount of pectins in TW (Meier 1962; Kuo and Timell 1969). Pectins are matrix polymers usually found in primary walls. The cell-wall localization of the detected pectinous structures in these early studies could not be determined since whole wood rather than isolated G-layers was analyzed. Pectins can be grouped into three classes based on the subunits of the polymer: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II.

Specific antibodies against these pectin types were used to examine the G-layer for the presence of pectins. No homogalacturonan was detected by immunolocalization (JIM5 antibodies) in G-layers (Arend 2008; Bowling and Vaughn 2008). However, Bowling and Vaughn detected strong labeling of G-layers in *L. styraciflua* or *C. occidentalis* with CCRC-M10 and CCRC-M22 antibodies that bind rhamnogalacturonan I (RG I). RG I can have different side chains that can be identified by specific antibodies. Arend (2008) detected labeling of *Populu*s G-layers with LM5 antibodies that bind to epitopes on 1,4-(β)-galactan sidechains of RG I. Interestingly the signal was weak within the G-layer but strongest at the interface of G-and S2-layer, like for xyloglucan as discussed in the previous section. Again there appears to be variation between species since Bowling and Vaughn (2008) did not observe any signal with LM5 antibodies in G-layers in *L. styraciflua*.

Several *fasciclin-like AGPs* (*FLAs*) are among the most upregulated genes during TW formation in *Populus* based on transcript analysis (Lafarguette et al. 2004; Andersson-Gunneråsetal. 2006). Moreover, both protein analysis and sugar linkage analysis of polymers extracted from isolated G-layers have identified the presence of FLAs (Nishikubo et al. 2007; Kaku et al. 2009). Immunolocalization with JIM14 antibodies suggested the localization of FLAs to a thin layer that lines the G-layer on its lumen side in *P. tremula* × *P. alba* (Lafarguette et al. 2004). Also Bowling and Vaughn (2008) detected a signal with this antibody in G-layers of *L. styraciflua* and *C. occidentalis*. They observed the signal in a patchy pattern across the G-layer. In *Populus*, *FLA*s belong to an expanded gene family with members that have high and specific expression in developing secondary xylem (Lafarguette et al. 2004; Andersson-Gunneråsetal. 2006). The large set of wood specific *Populus FLA*s has only two close homologs in Arabidopsis (*AtFLA11* and *12*) that are also specifically expressed during secondary wall formation. A double At*fla11/12* mutant was demonstrated to have decreased tensile stiffness and strength of the stem, together with modified cell-wall composition (particularly decreased cellulose content) (MacMillan et al. 2010). From this observation, it was suggested that AtFLA11/12 were important for both cellulose biosynthesis and as a structural component of the cell-wall matrix. The role of FLAs in TW is not known, but their high expression suggests that they may be an important matrix component and have an influence on cellulose structure and high MFA in the G-layer.

In summary, it can be concluded that the early view by Norberg and Meier (1966) that the G-layer was almost entirely composed of cellulose has been revised by the use of different analytical tools. Xyloglucans, pectins, AGPs, and lignin-like compounds have all been detected by chemical analysis in isolated G-layers or by microanalytical tools such as immunolocalization or Raman spectroscopy. Their detection and localization may be dependent on the analytical tools used but may also vary between species. Monoclonal antibodies against specific sugar epitopes are a highly powerful method, but results must be carefully interpreted due to possible cross-reactions with nontarget epitopes and different epitope accessibility in the distinct layers. It should also be noted that it is difficult to estimate the abundance of minor cell-wall polymers in the G-layer because isolation of G-layers by sonication in ethanol will unavoidably dissolve some of the extractives (such as

phenolic compounds, pectins, and AGPs) and immunolocalization and spectroscopic studies give at best only a crude estimate of quantities.

2.3 The S2-Layer of Gelatinous Fibers

Many studies have investigated the chemistry and ultrastructure of the G-layer, but less is known about the S2-layer in G-fibers and whether this is modified in comparison to the S2-layer of fibers in NW. The overall chemical composition of TW shows an increase in the concentration of cellulose and a corresponding decrease in both the major and minor matrix polymers (Timell 1969; Hedenström et al. 2009). This is primarily a result from the additional cellulose-rich G-layer, and the extent of cellulose enrichment in the TW will depend on the proportion of G- to S-layer in the G-fibers. In the early literature, it was correctly stated that the concentration of matrix polymers is not necessarily decreased in the S2-layer per se of the G-fiber (Timell 1969). This author further claimed that the concentration of lignin and xylan is higher in the S2-layer of G-fibers compared to the NW fibers, but this statement was based on unsupported assumptions of similar cellulose content in the two types of wood. Bentum et al. (1969) further visualized lignin in the S2-layer of G-fibers and NW fibers after removal of polysaccharides with hydrofluoric acid. This showed abundant lignin in the S2-layer of both fiber types, but quantitative comparisons are not possible from the published information. It is however not trivial to compare the S2-layer of G-fibers with that of normal fibers because the S2-layer per se is not homogenous. Thus, a fully formed S2-layer in NW may be different from a partly formed S2-layer in G-fibers, even if the outer (and comparable) layer of the S2 wall is similar in the two types of wood. Therefore similar sub-layers of the S2 should be compared between the two fiber types to come to a valid conclusion. Using immunogold labelling combined with electron microscopy, Kim et al. (2012) found no difference between S2 of TW fibers and outer S2 of NW fibers using the anti-xylan antibodies LM10 and LM11, and also no clear difference could be observed for the anti-glucomannan antibodies LM21, LM22, and BGM C6 when comparing the corresponding S2-sublayers. Another approach to compare corresponding S2-sublayers in G-fibers and NW fibers is to record successive stages of fiber cell-wall formation during S2 formation in developing wood. This was done in a study by Clair et al. (2011) who recorded MFA in *Populus deltoides* × *P. trichocarpa*, using synchrotron radiation microdiffraction. They found a MFA of about 25° to the longitudinal fiber axis in both NW and TW when comparing S2 walls at the same (early) developmental stage. In TW, the MFA decreased dramatically towards 0° when the G-layer started to form. But they also concluded a decrease in the MFA to about 10° of the inner layer of S2 of NW fibers. In contrary, Goswami et al. (2008) measured a MFA of about 36° in the S2-layer of matured TW fibers from *Populus nigra* × *P. deltoides* after enzymatic removal of the G-layer. However, as pointed out by Clair et al. (2011), it cannot be excluded that this measure of average MFA was also influenced by the high MFA in the S1

layer (estimated to 45° in Clair et al. 2011). Taken together, there are no consistent and conclusive data of quantitative or qualitative differences between comparable S2-layers in TW G-fibers and NW, although such differences cannot be excluded.

2.4 TW Without Gelatinous Fibers

Even though the presence of G-fibers often is used as an indicator of TW, a significant number of tree species form TW with high tensile strain but without typical G-fibers (Onaka 1949; Okuyama et al. 1994; Bailleres et al. 1995; Yoshida et al. 2002b; Clair et al. 2006b; Ruelle et al. 2006; Sultana et al. 2010). As a further example of the variation of TW fibers between species, several species within the *Flacourtiaceae* family form a multilayered secondary cell-wall layer in the TW fibers (Clair et al. 2006b; Ruelle et al. 2006). This TW also exerts high tensile growth strain. Yet, despite the lack of a G-layer, the TW fibers in these species are in many cases different from the corresponding opposite wood fibers. The emerging picture is that they have a lower MFA, an increased cellulose to lignin ratio, and, for certain species, an increased S to G ratio (Sugiyama et al. 1993; Okuyama et al. 1994; Bailleres et al. 1995; Yoshizawa et al. 2000; Yoshida et al. 2002b; Ruelle et al. 2007). In TW from *Eucalyptus globulus*, Aguayo et al. (2010) found that the decrease in lignin was followed by an increase in xylose, whereas the cellulose concentration was not affected. The high mesoporosity of the G-layer is not shared by the TW fibers lacking a G-layer (Chang et al. 2009). This suggests that the swelling properties of the G-layer are not critical for creating tension stress. Despite the high diversity in TW anatomy and ultrastructure of TW fibers among tree species, all types of TW are able to exert the required tensile stress to induce a movement in the displaced stem. No correlation between TW structure (with and without G-fibers) and the occurrence of the tensile stress that develops in TW was found when comparing several species (Clair et al. 2006b). In species with G-layers, it is clear that this layer is critical for the tensile stress in TW resulting from the longitudinal shrinkage of the cellulose crystals and always combined with a low MFA (Clair and Thibaut 2001; Fang et al. 2008; Goswami et al. 2008; Clair et al. 2011). The low MFA and increased cellulose in the S2-layer of fibers in TW without G-layer strongly suggest that these are the general attributes of TW, and that tensile stresses are generated by similar mechanisms as in G-fibers. Thus, there is no obvious advantage of a G-layer as such (McLean et al. 2012). The G-layer could rather be a complication since the tensile stress in the G-layer caused by cellulose shrinkage must be transmitted to the S2-layer. The exact mechanism behind tensile stress generation in the cellulose microfibrils of TW fibers, mostly investigated in species with G-fiber, is still a matter of debate (Goswami et al. 2008; Clair et al. 2011; Mellerowicz and Gorshkova 2012).

3 Hormone Signaling and TW Development

Most experimental approaches on inductive signaling molecules involved in TW formation have been dealing with plant hormones, typically auxin, gibberellins (GAs), and ethylene. It seems plausible that these in turn interact with the transcriptional machinery regulating wood development and further downstream with the regulatory machinery controlling cell division, expansion, and cell-wall biosynthesis (Nieminen et al. 2012; Zhong and Ye 2013). The large number of gene transcripts involved in all these processes are reflected in several studies comparing transcript abundance in TW- and NW-forming tissues (Dejardin et al. 2004; Paux et al. 2005; Andersson-Gunnerås et al. 2006; Hobson et al. 2010; Jin et al. 2011). Here we will discuss experiments evaluating the role of plant hormones in TW formation in trees.

The classical approach to study plant hormone function is by applying the hormone or inhibitors of its biosynthesis, response, or transport and observing their effects on molecular events and various aspects of growth and development. But application studies may also cause aberrant compartmentalization and/or unnormal tissue concentrations of the hormone, and such experiments must therefore be interpreted with caution. However, more conclusive interpretations can be made from application experiments when the resulting hormone balance is measured in the tissue of interest. Correlations between the endogenous hormone balance and the physiological event(s) studied can further provide information about hormone function. More solid evidence about hormone function, however, requires mutants either blocked in the biosynthetic pathway or in the perception of the hormone. This approach is problematic for hormones such as auxin and GAs due to their requirement in many aspects of plant development. Unless inducible systems and tissue-/cell-type-specific promoters are used, such mutants will unavoidably result in plants with more or less aberrant growth, where primary and secondary effects of hormone action are difficult to distinguish.

3.1 Auxin

Auxin affects most aspects of wood development when applied to cambial tissues (reviewed in Sundberg et al. (2000)). Indole-3-acetic acid (IAA) that is apically supplied to decapitated stems enters the polar auxin transport pathway (Sundberg and Uggla 1998) and stimulates cambial cell division and xylogenesis basipetally from the application site along the internode. In such experiments a dose response correlation between the resulting internal IAA levels and the cambial growth was established both in conifers (Scots pine) and angiosperm trees (hybrid aspen) (Sundberg and Little 1990; Björklund et al. 2007). The importance of auxin for most aspects of wood formation is also reflected in the large part of the transcriptome that is affected when auxin is applied to auxin-depleted internodes

of hybrid aspen (Björklund et al. 2007; Nilsson et al. 2008). Endogenous IAA is exhibiting a steep concentration gradient across wood-forming tissues with a peak level in the cambial zone both in conifers and angiosperm trees. The auxin concentration gradient was suggested to provide positional signaling required for coordinated wood development (Uggla et al. 1996; Tuominen et al. 1997). In Scots pine a correlation was found between cambial growth and the amount of IAA across the cambial tissues, rather than with its concentration in the cambial zone. In line with the idea that auxin provides positional information, it was suggested that it regulates the radial number of dividing cambial cells, which is important for the rate of wood cell production (Uggla et al. 1998). The multiple roles of auxin in wood development are also suggested from the expression of putative auxin-signaling genes across the different wood-forming tissues (Moyle et al. 2002). The expression pattern of auxin-signaling genes varied for the different genes across the developing wood, hence they did not correlate with the auxin concentration gradient. A similar non-overlap between endogenous auxin and the auxin-responsive *GH3* promoter was shown in *Populus* trees expressing a *GH3::GUS* construct (Teichmann et al. 2008). Transgenic hybrid aspen trees expressing a mutated version of the IAA signaling gene *PttIAA3* exhibited a decrease in auxin responsiveness and also in cambial growth and xylem cell size (Nilsson et al. 2008). It is however unclear how the decreased auxin responsiveness in these trees affected overall plant growth and development and whether the effect on cambial growth was a result of dwarfing.

The potential of applied auxin to affect all aspects of wood development suggests that it could be involved in signaling in the TW response. In angiosperm trees, application of auxin and auxin transport inhibitors has been observed to induce TW fibers with characteristic G-layers, suggesting a role for auxin in the TW response (Cronshaw and Morey 1968; Morey and Cronshaw 1968). However, the results from these and similar experiments are inconsistent and contradictory and therefore difficult to interpret [summarized in Little and Savidge (1987) and Hellgren et al. (2004)].

When the endogenous IAA distribution across wood-forming tissues was compared in TW and normal wood of *Populus tremula*, no clear difference was observed, despite the higher cambial growth rate in the TW-forming trees (Hellgren et al. 2004). However, at the opposite, lower side of the leaning trees IAA levels were decreased, as was cambial growth. Despite any obvious change in IAA balance across wood-forming tissues during TW formation, the transcript abundance of several putative auxin transporters and signaling genes is affected in association to the TW response in *Populus* (Moyle et al. 2002; Paux et al. 2005; Andersson-Gunneråas et al. 2006). This suggests that auxin-signaling pathways somehow are involved in TW formation. Possibly the responsiveness to auxin is modified in leaning stems as suggested from experiments where IAA was applied to horizontal shoots and stimulated cambial growth at the upper but not at the lower side (Wareing et al. 1964). However, experimental indications supporting a role for auxin per se in TW signaling are meager.

3.2 Gibberellins

GAs stimulate both cambial cell division and fiber elongation when applied to woody tissues or when GA levels or perception is increased in genetically modified *Populus* trees (Little and Savidge 1987; Ridoutt et al. 1996; Eriksson et al. 2000; Dünisch et al. 2006; Mauriat and Moritz 2009; Gou et al. 2011). GA stimulation of cambial growth is strongly synergistic with auxin, and at a certain auxin-to-GA balance fewer and smaller vessels are formed similar to what is observed in TW (Digby and Wareing 1966; Little and Savidge 1987; Björklund et al. 2007). The synergy with auxin can at least partly be explained by the stimulating effect of GAs on polar auxin transport (Björklund et al. 2007). When GAs are applied on their own to auxin-depleted decapitated stems, they still stimulate cell division. But the produced cells form dedifferentiated structures of parenchyma-like cells, and consequently differentiation into typical fibers or vessels was not observed (Little and Savidge 1987; Björklund et al. 2007). Transgenic *Populus* trees deficient in GAs due to increased activity of GA2 oxidase (which catabolizes active GAs) are dwarfs and, as a consequent of dwarfism, also have decreased cambial growth (Busov et al. 2003; Mauriat and Moritz 2009; Gou et al. 2011).

Endogenous bioactive GAs measured across developing wood tissues in *Populus* exhibit a peak concentration localized to the zone of cell expansion, with low levels in the cambial meristem (Israelsson et al. 2005). This observation questions whether the main function of endogenous GAs is to stimulate cambial cell division. It can not be excluded that application experiments and ectopic transgene expression of GA metabolism/perception genes could result in abnormal GA distribution/perception, and the resulting phenotype may therefore not reflect endogenous GA function. Information about the balance of endogenous GAs or IAA-to-GAs ratios in wood-forming tissues of angiosperm trees with different growth rates is not yet available.

Several application experiments have been reported that point towards a role for GAs in TW formation. Weeping Japanese cherry (*Prunus spachiana*) is a variety with slender weeping branches due to poor cambial growth, and no TW is observed in these branches. When GAs were applied to weeping branches, they stimulated cambial growth and induced upright movement of the branch as a consequence of TW formation at the upper side (Nakamura et al. 1994; Baba et al. 1995; Yoshida et al. 1999). The mutation causing the weeping phenotype is not known. But these experiments suggest that the weeping phenotype is due to a lack of GA biosynthesis and that GAs are required for TW formation. When bioactive GAs were applied to tilted stems of *Fraxinus mandshuricas* and *Acacia mangium*, they stimulated the upward bending of the stem compared to control trees by increasing cambial growth and the amount of TW formed (Jiang et al. 1998; Nugroho et al. 2012). When GA biosynthesis inhibitors (paclobutrazole and/or uniconazole-P) were applied to the same experimental systems, the upward bending was negated and the formation of TW much reduced. However, the limited amount of TW that did form exhibited

typical G-fibers. Together these studies indicate that the induced cambial growth associated to TW formation is dependent on GAs, but they do not provide evidence that GAs are involved in G-fiber differentiation. However, when GAs were applied to wood-forming tissues of upright trees from *Quercus, Kalopanax, Fraxinus,* and *Populus* ssp., they stimulated cambial growth and induced fibers with an inner cellulose-rich layer similar to G-fibers, despite the absence of any gravitational stimulus (Funada et al. 2008). Thus, applied GAs also have the potential to induce the developmental program of G-fibers. Altogether, these application studies strongly indicate a role for GAs in TW formation. Applied GAs stimulate cambial growth, enhance fiber to vessel ratio, and can trigger the induction of G-fibers. They also stimulate the TW response in tilted stems, and applied GA biosynthesis inhibitors inhibit the TW wood response. Supporting evidence from measurements of endogenous GA-to-IAA balance during TW formation or from GA insensitive mutants is still missing.

3.3 Ethylene

Applied ethylene (or its precursor ACC) stimulates cambial growth, modifies xylem cell shape, and, in *Populus*, induces a xylem anatomy with fewer and smaller vessels as observed in TW (Little and Savidge 1987; Du and Yamamoto 2007; Love et al. 2009). However, applied ethylene has not yet been reported to induce G-fibers in angiosperm trees. Ethylene is the only plant hormone that has been shown to increase in association to reaction wood formation, both in conifers and angiosperm trees (Du and Yamamoto 2007). In *Populus* ACC oxidase, ACO (the last enzyme in ethylene biosynthesis) is heavily induced in TW-forming tissues and probably responsible for the unilateral induction of ethylene biosynthesis in leaning stems (Andersson-Gunnerås et al. 2003).

Ethylene is sensed by ER-membrane bound receptors (Alonso and Stepanova 2004). A dominant negative mutation in the Arabidopsis ETR1 receptor (*etr1-1*) gives rise to ethylene insensitivity (Bleecker et al. 1998). Studies of ethylene insensitive Arabidopsis mutants showed that ethylene is not required for normal vegetative growth or xylogenesis (Tholen et al. 2004). The physiological function of environmentally induced ethylene is therefore ideally studied in ethylene insensitive transgenic plants. Ethylene insensitive hybrid aspen trees were produced by heterologous expression of *etr1-1* and used in combination with experiments based on application of the gas 1-Methylcyclopropene (1-MCP) that blocks ethylene perception on wild-type trees to dissect the physiological role of ethylene in the TW response (Love et al. 2009). Ethylene insensitivity did not inhibit the formation of fibers with typical G-layers in response to leaning. But it was demonstrated that the stimulation of cambial growth and stem eccentricity was at least partly a response to endogenous ethylene. This is the first conclusive demonstration of a

role for a plant hormone in the TW response. The function of endogenous ethylene in other aspects of the TW response needs further investigation.

Downstream ethylene signaling pathways have been unraveled in Arabidopsis, and the transcription factors ethylene response factors (ERFs) have been shown to be involved in this signaling (Solano et al. 1998). Applied ethylene (or its precursor ACC) was found to modify the transcript abundance (fold change >5 times) of 72 out of 169 *ERF*s in *Populus* stems (Vahala J, Felten J, Love J, Gorzsás A, Gerber L, Lamminmäki A, Kangasjärvi J and Sundberg B, unpublished). Some of these *ERF*s were also induced in TW-forming tissues in leaning stems and had the potential to modify different aspects of wood formation when overexpressed in transgenic hybrid aspen, suggesting that they may be part of the transcriptional network regulating TW formation.

3.4 Reflections on the Role of Plant Hormones in the TW Response

Both IAA and GAs are required for normal cambial growth and xylem development. The question is whether a drastic change in the level of one or both of them is part of the TW signaling. There is little evidence from application studies or measurements of endogenous levels that a change in IAA concentration is part of the TW induction per se. But the decrease in IAA level on the lower side of tilted stems, and the different growth response of upper and lower side when IAA is applied to horizontal stems, suggest that auxin transport and/or signaling is directly or indirectly part of the TW signaling (Hellgren et al. 2004; Wareing et al. 1964). On the same theme, it can be pointed out that redistribution of auxin transport is of great importance for gravitropic responses of primary shoots (Friml et al. 2002). The GA application experiments with weeping Japanese cherry and tilted seedlings of *Acacia* and *Fraxinus* support the idea that an increase in GAs is part of TW signaling (Nakamura et al. 1994; Baba et al. 1995; Yoshida et al. 1999; Jiang et al. 1998; Nugroho et al. 2012). Moreover, applied GAs mimic the induction of G-fibers in several tree species (Funada et al. 2008). The resulting concentration of GAs and IAA in the developing wood tissues is unknown in these experiments, which complicates their interpretation. However, in the presence of auxin, applied GAs caused a large stimulation on cambial growth in *Populus* (resembling the TW response), and resulting internal concentrations were found to be physiological relevant (Björklund et al. 2007). Information on endogenous GA-to-IAA balance during TW formation would be helpful to evaluate their roles in TW signaling. Ethylene levels do increase during TW formation and are important for the increased cambial growth associated to TW formation (Love et al. 2009). Applied ethylene also induce typical TW anatomy with fewer and smaller vessels, but conclusive evidence for other roles of ethylene in TW formation needs further studies. Despite the indications that both GAs and ethylene are part of TW

signaling, neither ethylene insensitivity nor GA inhibitors could inhibit the formation of G-layers. It cannot be excluded that an increase in both hormones takes place during TW formation and that their function is partly redundant. Moreover, most likely TW signaling involves cross-talk between plant hormones as well as additional, yet not identified, components. The plant hormones are, however, most likely rather upstream primary responses to TW sensing.

Acknowledgments We kindly thank Kjell Olofsson and Dr. Melissa Roach for providing graphical material for Fig. 1 and Drs Urs Fischer and Totte Niittylä for reviewing this chapter. We also thank FORMAS, Swedish Research Council, VINNOVA, and Bio4Energy (the Swedish Programme for renewable energy) for funding.

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