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# Chapter 4

## The Molecular Mechanisms of Reaction Wood Induction

**Kurt Fagerstedt, Kévin Tocquard, David Lopez, Mélanie Decourteix,  
Bernard Thibaut, Jean-Louis Julien, Philippe Label, Nathalie Leblanc-  
Fournier, and Patricia Roeckel-Drevet**

### 4.1 Introduction

Reaction wood (RW) develops in stems and branches in response to the perception 9  
of endogeneous and environmental stimuli caused by a change in the natural 10  
position. As a result the stem or branch bends back towards its original position. 11  
It is generally stated that in most cases, in gymnosperms RW is formed on the lower 12  
side of branches and bent stems and is called compression wood (CW). In angio- 13  
sperms it is formed on the upper side of branches and bent stems and is called 14  
tension wood (TW). Wood formed on the other side of branches and bent stems is 15  
called opposite wood (OW). 16

It was commonly believed until the end of the 1980s that RW was induced by the 17  
stress state of the new wood layer at cambium vicinity. The bottom of a branch 18  
should be in compression thus promoting CW in gymnosperms, the top being in 19  
tension should promote TW in angiosperms. For experimentation the challenge was 20  
to submit the external layer of a living stem to high tensile or compressive axial 21  
stress without any other signal such as gravity or light, and without too big a 22

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23 physiological stress due to the mechanical loading. Fournier et al. (1994) showed  
24 that the cross section grows while it is loaded. For each material point, the  
25 superposition of stress and strain begins from the time the material is differentiated.  
26 That comes from the obvious assumption that “a tissue cannot be loaded before it  
27 exists”. The main consequence is that the new wood layer does not contribute at all  
28 to support the load of the existing trunk or branch and the resulting support stress  
29 should be zero at the stem periphery. A change of paradigm therefore had to be  
30 made: it is not the stress that induces RW formation but the RW formation that  
31 produces different stress levels (in tension or compression) in the new wood layer.

32 Very often, successive growth events are used by trees “to solve” some mechan-  
33 ical problem, in addition to building of the prescribed structure, in order to restore  
34 the posture of an inclined tree (Thibaut et al. 2001; Moulia et al. 2006), to search for  
35 the light, to change the tree architecture after death of a major axis, and so on. RW is  
36 a solution for a drastic and sudden change in the existing wooden structure of the  
37 tree. It is commonly used by all trees, particularly in the juvenile stage. RW is  
38 created very locally in answer to a global mechanical problem for the tree by  
39 creating a step change in the pre-stressing state of the new layer. According to  
40 modelling simulations, the curving efficiency of asymmetrical stressing of the axis  
41 using RW is nearly five times higher than the best solution using normal wood  
42 asymmetry alone (Almeras and Fournier 2009).

43 Solving the mechanical problems of a tree through growth is possible because of  
44 the flexibility in growth of the meristematic tissues in the length or ramification  
45 (primary growth) and the diameter (secondary growth) of each axis (trunks and  
46 branches). This structure needs to be mechanically sound and able to respond to  
47 most hazards faced by the tree. In the tree, each elementary growth event has to be  
48 precisely managed: action or dormancy, rapid or slow cell division, cell differen-  
49 tiation and cell wall formation. And each of these events has mechanical conse-  
50 quences. In addition, the new growth events that involve primary and/or secondary  
51 growth seem to be coordinated at the whole tree level. RW originates from cambial  
52 activity (secondary meristem), which adjusts the number of cell division to modify  
53 the axis diameter and in particular its second moment of area, as well as the  
54 proportion of fibres and the cell wall thickness to modify the density and the  
55 mechanical properties of the new layer. Through cambial activity the microfibril  
56 angle (MFA) in the S2 layer of the cell wall is also adjusted to modify the axis  
57 flexibility both by changing the modulus of elasticity for a given tissue density and  
58 the maximum allowed strain before damage (more flexibility appears to be an  
59 adaptation to wind); this may also modify the pre-stressing state of the new wooden  
60 layer. Last but not least, cambial activity adjusts the chemistry of cell wall com-  
61 ponents to modify the pre-stressing state of the new wooden layer; this may be done  
62 in conjunction with the adjustment of MFA (these changes are discussed further in  
63 Chaps. 2, 3, 5 and 6).

64 Hence, the following questions are raised concerning the induction of RW  
65 through modulation of cambial activity. What are the different external or internal  
66 signals related to secondary growth in order to solve different mechanical require-  
67 ments? Where are the perception sites for the new mechanical requirements for the

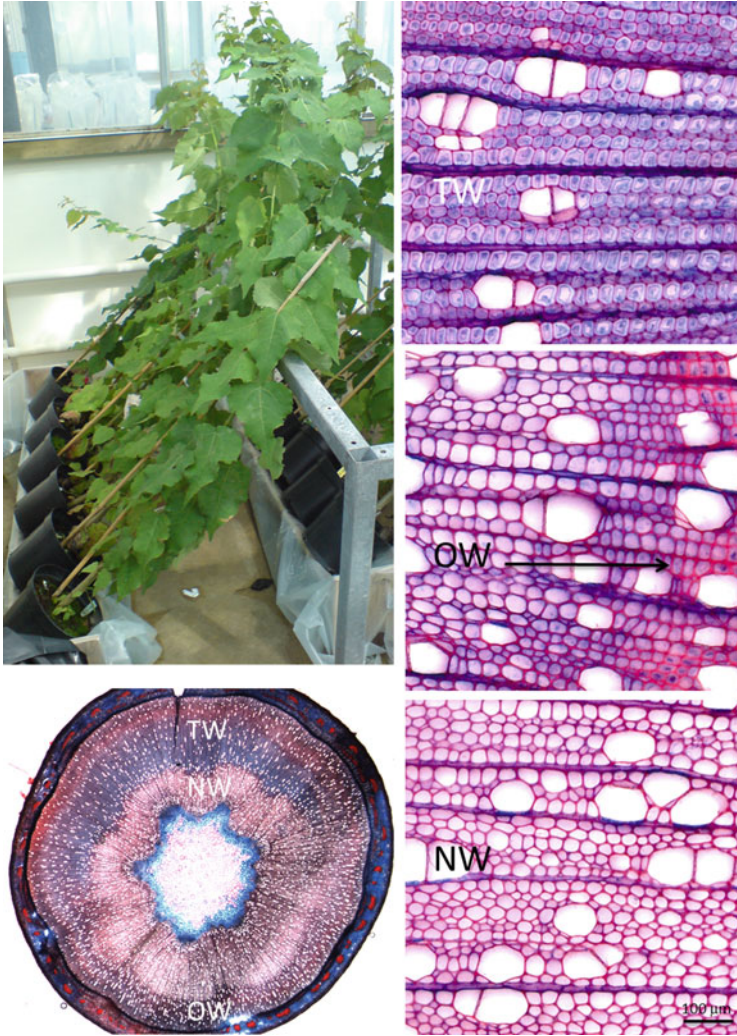
tree? Could a signal get to particular cells in the cambium in order to manage new growth? If such a signal exists from the perceptive cells to the cambium, what about the conversion of the perception into messages transmitted to the secondary meristem? What is the process of “regulation”? How are these messages transcribed in the making of RW? Also most of the questions raised for RW formation could also be addressed to the regulation of primary growth since both primary and secondary growth must be coordinated at the whole tree level.

In this chapter, after reviewing different kinds of signals (gravity, light, mechanical strain) that can induce a mechanical reaction causing RW formation, we will focus on the molecular mechanisms that might be involved in the perception and response to gravity and other mechanical stimuli. Since it is quite clear that the signal perception gives way to synthesis of proteins guiding the production or translocation of various plant hormones, we will review their implication in the gravitropic or phototropic mechanical response inducing the making of RW. We will also discuss the insights provided by global approaches such as transcriptomic, proteomic and metabolomics, made possible by the sequencing and annotation of the genome of trees such as poplar and eucalyptus. In particular, these global approaches gave new information on genes involved in RW formation.

## **4.2 Perception and Signal Transduction**

### ***4.2.1 Physical Parameters Inducing RW***

To maintain a branching architecture that is optimal for growth and reproduction, plant stems continuously control their posture to counterbalance environmental physical parameters such as gravity, wind and light, that shift their orientation from the vertical (Mouliia et al. 2006). In trees, this postural control has been mainly studied in response to gravity (Du and Yamamoto 2007). In the primary growing zone of stems, reorientation of woody plant organs involves local differential elongation growth between opposite sides of the stem. In stem parts undergoing radial growth, sectors of RW are produced that can be associated with eccentric cambial growth. In angiosperm woody species, TW is often characterized by fewer vessels and the formation of fibres with smaller diameter containing a gelatinous layer inside the S2 layer of secondary cell walls where cellulose microfibrils are aligned into a vertical orientation (Fig. 4.1, Mellerowicz et al. 2008; Chap. 3) with a lower lignin content (Pilate et al. 2004). In conifers, CW is characterized by tracheids with a thicker secondary cell wall than in normal wood, with higher lignin content, intracellular spaces at cell corners, and a realignment of cellulose microfibrils in the S2 layer orientation with respect to the axis of the stem (Timell 1986). These differences in secondary cell wall biochemical composition and architecture of RW generate internal growth stresses in the stem (Chap. 5), and because of its



**Fig. 4.1** Induction of TW by tilting of hybrid aspen trees (*Populus tremula* × *tremuloides*). After 17 days of tilting, a band of TW (TW) is seen on the upper side of the tilted stems. The mature normal wood (NW) produced before tilting, and the opposite wood (OW) produced at the lower side of the stems are shown at higher magnification on the right side of the figure. The sections were stained with safranin and alcian blue. Picture courtesy of Ewa Mellerowicz

106 unilateral formation in the stem, it induces a directional movement, bending the  
 107 stem towards a favourable position.

108 As discussed recently by Felten and Sundberg (2013), many experiments were  
 109 performed where branches or stems are tilted, bent into complicated shapes, grown  
 110 on clinostats or centrifuges, to identify if a single stimulus is responsible for the  
 111 induction of RW. For loop experiments on shoots or branches, the localization of

RW suggested that its induction depends rather more on positional sensing (sensing 112 of the local angle of the growing organ relative to the gravitational field) than 113 sensing of mechanical stresses such as tensile or compressive stresses (Spurr and 114 Hyvärinen 1954). 115

However, RW formation is not only induced by gravitational stimulus. TW has 116 been reported to form in the vertical stems of rapidly growing poplar (*Populus*) 117 trees (Telewski 2006). RW was also observed in branches and stems contributing to 118 crowns reshaping after loss of apical dominance (Wilson and Archer 1977) and is 119 part of the mechanism allowing up-righting of apricot tree stems in response to 120 increased shoot and fruit load (Almeras et al. 2004). Plant exposure to wind spray or 121 to repeated stem bending to mimic the wind triggers (1) transitory inclination of the 122 stem, but with a duration of stem inclination much shorter than the presentation 123 time necessary to observe the induction of RW (Jourez and Avella-Shaw 2003) and 124 (2) mechanical signals such as stresses and strains. Exposition of poplar stems to 125 repeated transitory bendings produced a flexure wood with anatomical similarities 126 to TW (Pruyn 1997; Pruynt et al. 2000). In *Abies fraseri*, the morphology and 127 function of wood developed after daily flexures (<20 s) were more closely related 128 to CW than normal wood (Telewski 1989). CW was also detected in wind-treated 129 *Pinus* (Berthier and Stokes 2005). One of the best examples demonstrating that RW 130 is not exclusively induced in response to positional sensing came from analysis of 131 the kinematics of stem straightening (for review, see Moulia et al. 2006; Moulia and 132 Fournier 2009). The characterization of the spatio-temporal curvature field during 133 stem straightening allowed the recognition of a biphasic pattern: an initial phase of 134 spatially homogeneous up-curving due to gravitropic response and a second phase 135 of stem decurving that propagates basipetally to finally reach a steady state where 136 the curvature is concentrated to the base of the growth zone. In some plants, this 137 decurving phase occurred before the shoot apex had overshoot the vertical, demon- 138 strating that this phenomenon was not due to the perception of the inclination angle, 139 and was called autotropism. This autotropic decurving has been observed in many 140 plants (Moulia et al. 2006). Studying this process in poplars (*Populus nigra* × 141 *Populus deltoides*), Coutand et al. (2007) observed that no part of the trunk overshoot 142 the vertical during stem straightening. Indeed, during the initial phase of 143 up-righting, arcs of RW were detected homogeneously all along the upper side of 144 the trunk, whereas a second sector of RW was produced on the initially lower side 145 in the most distal part of the trunk, contributing to this autotropic decurving. 146 Recently, Bastien et al. (2013) studied the gravitropism kinematics of different 147 organs from 11 angiosperms by time lapse photography, including both primary 148 elongation zones and zones of secondary growth in which active bending is 149 achieved through the production of TW. The biphasic pattern of tropic reactions 150 described above was found to be generic whatever the type of the organ, and it was 151 shown to lead to a steady state shape in which the apical part is straightened 152 whereas the curvature is more concentrated at the base of the stem. However, 153 inter- and intra-specific variability occurred in the steady states and in the trans- 154 sients: whereas some plant organs reached a steady state without overshooting the 155 vertical, others exhibited oscillations around the vertical axis. Bastien et al. (2013) 156

157 also demonstrated that the minimal dynamic model cannot involve only  
158 gravisensing but the simultaneous sensing of the local curvature, referred by the  
159 authors as proprioception. When the organ is tilted and straight, gravisensitive  
160 control is dominant and the organ bends up actively. However as curvature  
161 increases, the inclination angle decreases and proprioceptive control takes over  
162 and autotropic counter-bending is produced starting in the apical parts of the  
163 organs, allowing it to straighten and align with the gravity vector. These data also  
164 suggest that the different shapes observed along the straightening response reflected  
165 a different ratio between graviceptive and proprioceptive sensitivities.

166 Another physical parameter that can influence reorientation of the stem by RW  
167 formation is light interception. Because of their co-occurrence under natural condi-  
168 tions gravi- and phototropism are intrinsically linked (Correll and Kiss 2002).  
169 Remarkably, phototropism sensing converges to common molecular actors with  
170 gravitropism and notably auxin transport and perception (Hohm et al. 2013). Typ-  
171 ically, stems and stem-like organs have positive phototropism and negative  
172 gravitropism. Additive or synergetic effects were reported (Kern and Sack 1999)  
173 making the identification of their relative contribution and their possible interac-  
174 tions problematic. To address this issue, gravity effects were experimentally  
175 reduced or abolished (e.g. microgravity, space flight) while applying directional  
176 light source to induce phototropism (Millar and Kiss 2013). To date, it remains  
177 technically difficult to alleviate or reduce gravity effects on trees. The few possi-  
178 bilities offered to researchers consist in the manipulation of gravity orientation by  
179 tilting potted trees in combination with directional light modifications. Matsuzaki  
180 et al. (2006) report phototropism in trees submitted to different gravitational  
181 stimulations as observed on mountain slopes. Basal parts of the trees did not  
182 show bending in response to tropisms, which was limited to upper parts of stems.  
183 The authors suggested that reorientation could be achieved by asymmetric radial  
184 growth due to the formation of RW as is the case for gravitropism and further  
185 proposed trees inclination on slopes depends on phototropism. The same team later  
186 proved that the mechanism involved in phototropism required differential xylem  
187 production (Matsuzaki et al. 2007). In a recent study, Collet et al. (2011) studied  
188 long-term (4 years) phototropic response of *Fagus sylvatica* and *Acer*  
189 *pseudoplatanus* after canopy opening in natural conditions. Plants reacted by  
190 righting themselves towards the light source and this involved reorientation of the  
191 lignified parts of the stems through asymmetrical formation of RW. Herrera  
192 et al. (2010) noticed changes in the orientation of apical part of pine seedlings but  
193 not in the basal parts even after 22 days of light and gravi-stimulation. Although  
194 limited to primary growth, this work provided molecular data on the interaction of  
195 these two tropisms, scarce for tree models. Interestingly, photo- and gravitropic  
196 responses of potato plants were different depending on the time of the day  
197 suggesting they were also regulated by an endogenous circadian clock to some  
198 extent (Vinterhalter et al. 2012). Such complexity, far from being completely  
199 understood in herbaceous plants, still needs to be established in trees where  
200 secondary growth in reaction to phototropism and gravitropism is still a matter of  
201 exploration.

All these data converge on the induction of RW during plant postural control. 202  
Clearly the triggering of RW formation during tropic reaction not only is related to 203  
the sensing of the inclination of the stem versus gravity but also involves curvature 204  
sensing. TW has been shown to systematically be formed on the lower side of the 205  
branch when autotropism dominates gravitropism, allowing for curvature compen- 206  
sation (Coutand et al. 2007). Similar shifts in the location of RW along the tropic 207  
motion have also been described for CW in conifers (Sierra de Grado et al. 2008). 208  
But, how these diverse physical parameters (gravity, local curvature, light) are 209  
perceived by plant cells in order to induce RW remains unclear. Are all these 210  
physical parameters perceived by a common sensing mechanism or is there any 211  
crosstalk at a later stage during the signaling pathway? 212

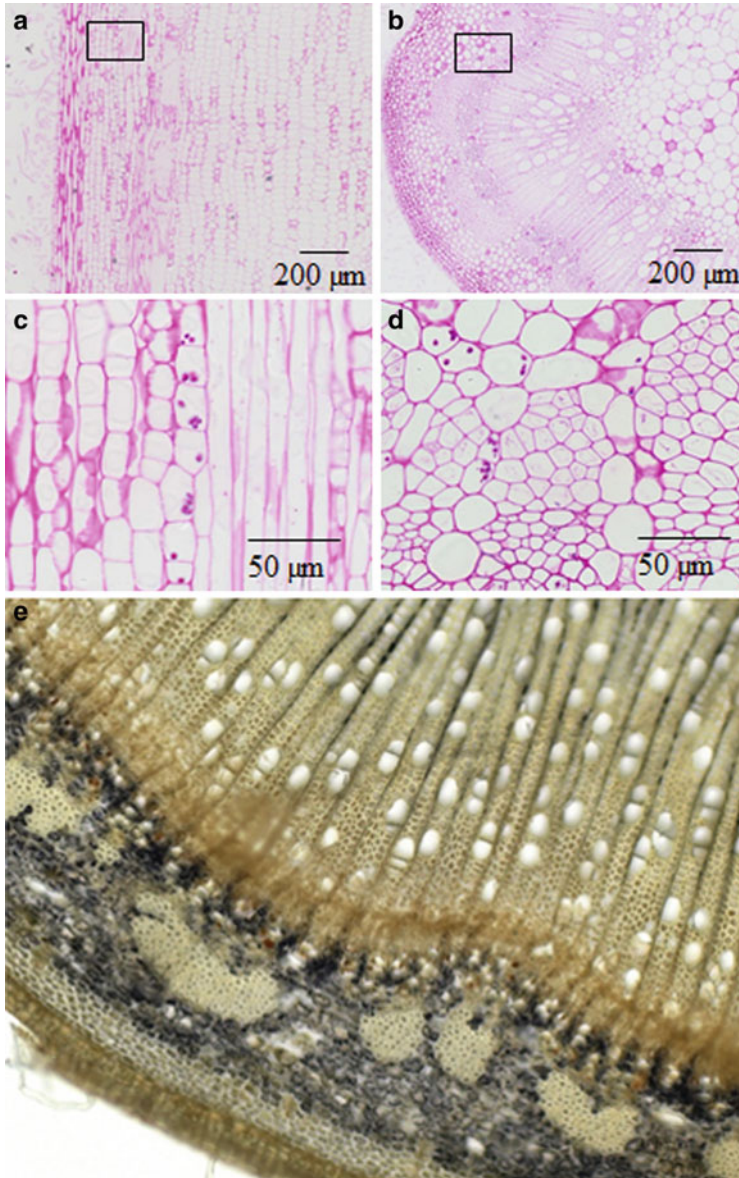
#### **4.2.2 Molecular Mechanisms Involved in the Perception of Mechanical Stimulation Leading to RW Formation** 213 214

In case of a gravitational stimulus, the resulting physical forces can deform or move 215  
objects of specific mass inside the cell. Two hypotheses are currently favoured: 216  
(1) the amyloplast-sedimentation in specialized cells named statocytes and the 217  
perception of the direction of this sedimentation and (2) the weight of the proto- 218  
plasm itself triggering mechanical deformation of subcellular structures such as 219  
membrane, cytoskeleton elements and cell wall (Baluška and Volkmann 2011). 220

The role of starch-filled amyloplast sedimentation during graviperception is well 221  
documented in *Arabidopsis* (Morita 2010). In young shoots, statocytes are localized 222  
in the endodermis layer surrounding vascular tissues. These cells are highly vacu- 223  
olated and equipped with prominent F-actin bundlets (Morita et al. 2002). The 224  
studies of different mutants affected either in starch formation (*pgm*) or in intra- 225  
cellular components such as the vacuolar membrane (VM) or actin microfilaments 226  
(AFs) that both modify cytoplasm viscosity and activity showed that amyloplast 227  
dynamics are important during shoot gravisensing (for review, Hashiguchi 228  
et al. 2013). Recently, by using a centrifuge microscope to analyse gravitropic 229  
mutants in *Arabidopsis*, Toyota et al. (2013) confirmed the importance of amylo- 230  
plast movement perception during shoot gravisensing. In woody species, amylo- 231  
plast localization in endoderm cells has been observed (Nakamura 2003) in the 232  
young shoots of Japanese flowering cherry tree (*Prunus spachiana*) and in young 233  
poplars (*Populus tremula* × *alba*) (Fig. 4.2a–d). However, a link between amylo- 234  
plasts sedimentation and RW formation has not yet been demonstrated. Moreover, 235  
the endoderm is disrupted by secondary growth. In cross sections of older poplar 236  
stem, lugol-stained starch grains are observed in whole bark tissue as well as in the 237  
wood rays (Fig. 4.2e). 238

The cellular mechanism underlying curvature proprioception is unknown 239  
(Bastien et al. 2013). The sensing of cell deformation (strain sensing) or more 240  
precisely of the deformation of some cellular component is a good candidate 241





**Fig. 4.2** Sections of poplar stems (*Populus tremula* × *alba*). Photographs (a–d) represent sections from the fourth bottom internode of a young plant with 20 developed internodes. Longitudinal sections (a, c) or transversal sections (b, d) were stained using Periodic acid/Schiff (PAS) reaction to detect starch and polysaccharides. Photographs (c) and (d) are, respectively, enlarged views of the photographs (a) and (b) (*black rectangle area*). Photographs courtesy of Wassim Azri. (e) This picture represents a cross section of a poplar stem, 40 cm under the apex. The tree height was 2.35 m and the diameter of the section is 5.3 mm. The freehand cut was stained by lugol. Photographs courtesy of Kevin Tocquard

(Wilson and Archer 1977; Bastien et al. 2013). Moreover the perception of the deformation of cellular components is suggested also through amyloplast sedimentation, in the gravitational pressure model (Baluška and Volkmann 2011), and through wind sensing (Moullia et al. 2011). A few molecular candidates have been identified as mechanoperceptors of this deformation. In trees, these are only putative. Results obtained from the herbaceous model plant *Arabidopsis thaliana* suggest molecular candidates at the CPMCW (cytoskeleton—plasma membrane—cell wall) continuum. They would be able to sense the mechanical signal from the plant cell wall and convert it into a molecular signal in the cell (Baluška et al. 2003; Telewski 2006).

The first molecular candidates are mechanosensitive (MS) ion channels. MS ion channels are membrane proteins that allow the transit of ions through cellular membranes. They open either directly by the force applied on the membrane or indirectly through links between the channels and/or both the cytoskeleton and cell wall components (Haswell et al. 2011). To date the involvement of MS ion channels in the perception of mechanical signals has yet to be clearly established. Nevertheless, several pieces of evidence show that ionic changes occurred after mechanical signals (Haley et al. 1995). In plants, MS ion channels ( $\text{Cl}^-$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ ) have been identified at plasma membranes using patch-clamp electrophysiology (Cosgrove and Hedrich 1991; Ding and Pickard 1993; Haswell et al. 2008). Currently three main groups of MS ion channels have been described in plants. First, the mechanosensitive channel of the small conductance (MscS) family from *Escherichia coli* which releases osmolytes from the cell (Booth and Blount 2012) has ten homologues (MSL1–10) in the *A. thaliana* genome (Pivetti et al. 2003). MscS homologues may release osmolytes in response to membrane tension and may be modulated by additional signals (Monshausen and Haswell 2013). The second Mid1-complementing activity (MCA) family is structurally unique to the plant kingdom. The MCA proteins are located in the plasma membrane and promote calcium influx upon mechanical stimulation (Nakagawa et al. 2007). Finally, the Piezo proteins are a class of MS cation channels which respond to mechanical stimuli (Coste et al. 2012; Kim et al. 2012). In *Arabidopsis*, there is a single Piezo protein (Coste et al. 2010; Kurusu et al. 2013; Monshausen and Haswell 2013) but it has yet to be characterized.

Other molecular candidates are receptor-like kinases proteins (RLKs) that are a family of proteins with an extracellular domain, a single transmembrane region and an intracellular cytoplasmic kinase (Shiu and Bleecker 2001). Among RLKs, wall-associated kinases (WAKs) are the most well-studied potential cell wall status receptors (He et al. 1999; Verica and He 2002). They have a particular interest because WAKs extracellular domains are able to bind to the cell wall (He et al. 1996). Notably, Wagner and Kohorn (2001) showed that *At*WAKs covalently bind the cell wall pectins, in a calcium-induced conformation (Decreux and Messiaen 2005). Moreover, a reduction of WAK expression inhibited cell elongation and altered morphology (Lally et al. 2001; Wagner and Kohorn 2001), indicating an activity in growth control. Therefore, WAKs are interesting candidates as sensors of cell wall integrity. Another candidate among RLKs for sensing

287 the cell wall integrity is the *Catharanthus roseus* RLK1-like subfamily  
288 (CrRLK1L). The 17 members present an extracellular malectin-like domain  
289 (Lindner et al. 2012). Malectin proteins bind to glycoproteins in animal endoplas-  
290 mic reticulum (Qin et al. 2012). The hypothesis that the malectin-like domain of  
291 CrRLK1L proteins binds cell wall polysaccharides or glycoproteins in plants has  
292 been proposed (Monshausen and Haswell 2013). In this subfamily THESEUS1  
293 (THE1), a particular member with a plasma membrane location, could be a candi-  
294 date for cell wall integrity sensing in *Arabidopsis*. THE1 was identified at a  
295 suppressor screen of cellulose-deficient mutant *cesA6* (Hématy et al. 2007). The  
296 *the1* mutant partially restored the hypocotyl elongation of the *cesA6*. However,  
297 mutations or over-expression of the *THE1* gene did not exhibit any effects in  
298 *Arabidopsis*. Consequently, THE1 was therefore proposed as a sensor of the cell  
299 wall status and modulator of cell elongation during perturbed cellulose synthesis.

300 Members of ArabinoGalactan proteins (AGPs) bind pectins and are also hypoth-  
301 esized to be cell wall integrity sensors. Indeed, AGPs are highly glycosylated  
302 proteins located in the cell wall (for more detail, see Chap. 3). Some AGPs bind  
303 cell wall pectin (Serpe and Nothnagel 1995) and could be attached to the plasma  
304 membrane via GPI anchors which would mediate intracellular signaling (Hum-  
305 phrey et al. 2007).

### 306 **4.2.3 Mechanical Signal Transduction: Secondary** 307 **Messengers**

308 Regarding secondary messengers, few data are available in trees and almost lacking  
309 in the context of gravitropic stimulation and/or stem bending. However, herbaceous  
310 plant model data can be a starting point for future studies on tree models. According  
311 to Toyota and Gilroy (2013), calcium is an important and ubiquitous cell secondary  
312 messenger. Its specific role as a secondary effector in MS signaling has been  
313 extensively investigated in *Arabidopsis* and *Nicotiana* (Knight et al. 1991, 1992;  
314 Haley et al. 1995; Plieth and Trewavas 2002; Toyota et al. 2008). Gravity stimu-  
315 lation of *Arabidopsis* seedlings indicated a cytosolic  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_{\text{cyt}}$   
316 increase with a first sharp increase followed by another less intense but longer  
317 signal (Plieth and Trewavas 2002). Wind also induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  increase in *Nico-*  
318 *tiana* and *Arabidopsis* seedlings (Knight et al. 1992; Haley et al. 1995; Plieth and  
319 Trewavas 2002). Experiments conducted on trees support the involvement of  $\text{Ca}^{2+}$   
320 in RW formation. By using a  $\text{Ca}^{2+}$  chelator (EGTA) or a calcium channel inhibitor  
321 ( $\text{LaCl}_3$ ) which allowed modification of  $\text{Ca}^{2+}$  availability, CW formation was  
322 inhibited in *Taxodium distichum* gravistimulated stems (Du and Yamamoto  
323 2003). The involvement of calcium in TW induction has also been suggested by  
324 indirect data obtained from several global approaches (see Sect. 4.4) notably  
325 through regulation of protein accumulation such as calreticulin, a  $\text{Ca}^{2+}$  storage

protein (Azri et al. 2009). The overall results suggested  $\text{Ca}^{2+}$  ion as a second messenger in the early stages of mechanical signal transduction.

Other secondary messengers have been identified as important after mechanical/gravitational stimulation. Azri et al. (2009) suggested the involvement of reactive oxygen species (ROS) in poplar TW formation induced through the accumulation of glutathione-dehydroascorbate reductase (GSH-DHAR), glutathione S-transferase (GST) and thioredoxin *h* (Thr *h*) proteins. Azri et al. (2013) further showed an induction of the *Thr h* gene in response to gravitropic stimulus. With an immuno-chemistry approach, they co-located Thr *h* proteins with amyloplasts in stem endoderm cells, thus providing a coherent framework for graviperception. More evidence from herbaceous models demonstrates that an interplay of ROS and  $\text{Ca}^{2+}$  could mediate mechanosensing: ROS can stimulate  $[\text{Ca}^{2+}]_{\text{cyt}}$ , and an increase of  $[\text{Ca}^{2+}]_{\text{cyt}}$  can also lead to a ROS production via NADPH oxidase (Mori and Schroeder 2004).

Molecular candidates to convert the transient ionic  $\text{Ca}^{2+}$  signal to long-term biochemical signal are mainly calmodulins (CaM) and  $\text{Ca}^{2+}$ -dependent protein kinases (CDPK). Calmodulins are  $\text{Ca}^{2+}$ -dependent regulation proteins linking calcium to MS. CDPK are cytosolic proteins with a kinase domain, an autoinhibitory domain, and a calmodulin-like domain that bind calcium ions (Hrabak et al. 2003). In poplar, the expression of calmodulin encoding genes is up-regulated as soon as 10 min after a transient stem bending (Martin et al. 2009). Although very likely, a role for these proteins in the  $\text{Ca}^{2+}$  signaling after mechanical stimulation still needs to be demonstrated.

Phosphatidylinositol signaling is another part of the gravitropism sensing network (Strohm et al. 2012). Membrane phospholipid Phosphatidylinositol 4,5-bisphosphate (PIP2) releases inositol 1,4,5 triphosphate (IP3 or InsP3) after phospholipase C (PLC) hydrolysis. InsP3 is accumulated during gravitropic response which later is repressed through PLC inhibition (Perera et al. 2001). InsP3, like  $\text{Ca}^{2+}$ , is a second messenger suggested as triggering intracellular calcium flow from vacuole (Allen et al. 1995).

Taken together, these results indicate that complex and interactive signaling pathways are involved after mechanical/gravitational stimulation. To date, no study has yet addressed these mechanisms in tree stems. Numerous questions remain to be resolved to understand the induction of RW. Why RW is produced in only one side of the stems, whereas the physical parameters triggering RW production are potentially perceived on both sides of the stem. Are the putative receptors uniformly localized in cells? Furthermore, if the endodermis cells are considered as sensitive cells, how is the inductive signal transmitted to cambium cells or developing xylem cells to modify the secondary wall composition and architecture? What cells act as gravisensor in older stems? How and where does crosstalk between gravisensing and proprioception occur?

## 367 **4.3 Signal Transmission to Reaction Wood-Forming Cells**

### 368 **4.3.1 Hormone Action and Reaction Wood**

369 The anatomical and biochemical features of RW have been the subject of extensive  
370 studies, which are described and reviewed in Chaps. 2 and 3. However the phys-  
371 iological and molecular signals inducing the formation of RW remain poorly  
372 described. Several plant hormones have been implicated in the regulation of  
373 cambial cell proliferation activity and in wood cell differentiation (for review, see  
374 Elo et al. 2009; Nieminen et al. 2012; Ursache et al. 2013). Until recently, their role  
375 in RW formation was studied through the application of exogenous compounds, the  
376 hormones themselves, their antagonists or transport/perception inhibitors. These  
377 experiments proved to be very informative as a first step towards deciphering  
378 whether hormones have the potential to induce RW formation. However, the  
379 possibility to genetically transform poplar and the use of high throughput molecular  
380 technics are, and will be continue to be, of great help in confirming and completing  
381 these observations.

#### 382 **4.3.1.1 Auxin**

383 The application of auxin antagonists or transport inhibitors on both gymnosperm  
384 and angiosperm tree stems led to the assumption that auxin plays a role in RW  
385 formation. Increased amounts of auxin could induce CW formation in gymno-  
386 sperms while a deficiency in auxin was required to form TW in angiosperm (for  
387 review, see Du and Yamamoto 2007; Felten and Sundberg 2013). However, such  
388 experiments do not prove the function of auxin under natural conditions because of  
389 the risk of uncontrolled modification of auxin homeostasis. To be validated, they  
390 need a better documentation of *in planta* auxin concentrations/amounts. Reports on  
391 the relationship between endogenous auxin levels and the formation of CW or TW  
392 are scarce and sometimes contradictory. For example, while Wilson et al. (1989)  
393 did not find a clear and conclusive correlation between the occurrence of CW and  
394 auxin concentrations, Funada et al. (1990) working on *Cryptomeria japonica* and  
395 Du et al. (2004) working on *Metasequoia glyptostroboides* found a higher amount  
396 of endogenous IAA (indole-3-acetic acid) in the cambial region producing  
397 CW. More recently, Hellgren et al. (2004) conducted a kinetic survey of endoge-  
398 nous IAA distribution across the cambial region of Aspen (*P. tremula*) and Scots  
399 pine (*Pinus sylvestris*) trees after bent-stem gravistimulation. These experiments  
400 showed that RW could be formed even when the pattern of IAA distribution in the  
401 cambial region is unmodified. The authors concluded that modulation of the auxin  
402 concentration gradient across the cambial zone might not be the signal that main-  
403 tains the cells in an RW developmental state.

404 Data found in the literature are contradictory and were obtained on different  
405 species with different techniques and at different time points after stimulation. It is

therefore difficult to give a clear model of auxin role in RW formation. However, an involvement of auxin in the early steps of the induction process has not yet been ruled out. Actually, several reports showed that auxin signaling is responsive to gravistimulation. After 6 h of stem bending, the expression of two *AUX/IAA* genes in poplar (*P. tremula* × *tremuloides*) (Moyle et al. 2002) and one in *Eucalyptus* (Paux et al. 2005) was altered in TW compared to normal wood in unbent trees. In yellow poplar (*Liriodendron tulipifera*), a species that does not produce a typical G-layer, the expression of ARFs and *AUX/IAA* genes as well as other auxin-related genes is modified in TW compared to OW (Jin et al 2011). Similar results were also obtained in poplar (*P. tremula* × *tremuloides*) after 3 weeks of TW induction by leaning the stem (Andersson-Gunnerås et al. 2006).

High throughput approaches have already helped in improving our knowledge of auxin function in TW formation. Coupling these with functional genomic approaches could help to gain a better understanding of the role of auxin in TW formation.

#### 4.3.1.2 Ethylene

The gaseous hormone ethylene has long been known to be produced in response to diverse stresses including mechanical solicitations (for review, see Braam 2005; Telewski 2006; Du and Yamamoto 2007) such as bending and tilting. These stresses usually stimulated wood production by increasing cambial activity and sometimes led to the production of RW. Like auxin, ethylene involvement in RW formation was first investigated by measuring ethylene or its precursor (ACC: 1-aminocyclopropane-1-carboxylic acid), and by using application experiments. For example, in the vascular cambium of *Pinus contorta* Dougl. ssp. *latifolia* branches, endogenous ACC was detected in association with CW differentiation, but not with OW (Savidge et al. 1983). Applications of ethrel, an ethylene releasing compound, stimulated wood production and were able to modify anatomical features of xylem in gymnosperm and angiosperm trees (Du and Yamamoto 2007). In angiosperm, although some of these features, like fewer and smaller vessels, can be reminiscent of TW characteristics (Little and Savidge 1987; Du and Yamamoto 2007; Love et al. 2009), it has to be noted that there is no report of G-layer induction by ethylene treatments.

Molecular approaches have helped to gain new insights about regulation of TW formation by ethylene. In bent poplars (*P. tremula* × *tremuloides*), a clear induction of *PttACO1* (ACC oxidase the last enzyme in the ethylene biosynthesis pathway) expression and relative activity were observed in the TW-forming tissues (Andersson-Gunnerås et al. 2003). Therefore, *PttACO1* may represent a major control of ethylene asymmetric production during TW formation. In poplars leant for 3 weeks, the expression of genes related to ethylene signaling was also modified in TW compared to OW (Andersson-Gunnerås et al. 2006). In *L. tulipifera*, such modifications were seen as soon as 6 h after bending of the stem (Jin et al. 2011). Love et al. (2009) combined the use of ethylene-insensitive trees, ethylene-

448 overproducing trees, and the application of the ethylene-perception inhibitor MCP  
449 (1-methylcyclopropene) to explore ethylene physiological function in  
450 gravistimulated poplars. They showed that ethylene could be responsible for the  
451 stimulation of cambial cell divisions on the upper TW-forming side of leaning  
452 stems. In 2013, Vahala et al. identified 170 gene models encoding ERFs (ethylene  
453 response factors) in the *Populus trichocarpa* genome. Among these, 17 had a  
454 minimum of a twofold induction of expression in TW compared to normal wood.  
455 Over-expression of some of them in poplar resulted in anatomical or wall chemistry  
456 modifications that are reminiscent of TW features.

457 Ethylene and its signaling pathway seem therefore to control part of the molec-  
458 ular and physiological modifications underlying RW formation, especially the  
459 asymmetric increase in radial growth. However, it seems that the establishment  
460 of the full characteristics of RW involves ethylene in combination with yet  
461 unidentified other signaling factors (Love et al. 2009; Vahala et al. 2013; Felten  
462 and Sundberg 2013).

#### 463 4.3.1.3 Gibberellins

464 Gibberellins (GAs) constitute another group of plant hormones known to promote  
465 secondary growth and xylem fibre length (Eriksson et al. 2000; Mauriat and Moritz  
466 2009; Gou et al. 2011). Applications of exogenous GAs or GA inhibitors to tree  
467 stems can only provide indirect evidence for a role of GA in RW formation. In  
468 gymnosperms, the possibility of a role of GAs in CW formation has not yet been  
469 clearly demonstrated. However, experiments conducted on upright or tilted angio-  
470 sperm trees helped to establish a correlation between GA and TW formation. For  
471 example, it has been shown that the application of GA to vertical stems of *Fraxinus*  
472 *mandshurica*, *Quercus mongolica*, *Kalopanax pictus* and *Populus sieboldii* induced  
473 the development of TW with typical G-fibres in the absence of gravistimulus  
474 (Funada et al. 2008). When tilted *Acacia mangium* seedlings were treated with  
475 GA their negative gravitropism was stimulated. On the contrary, when they were  
476 treated with paclobutrazole or uniconazole-P, inhibitors of gibberellin biosynthesis,  
477 the gravistimulated upright movement of the acacia stems was inhibited and the  
478 formation of TW was suppressed (Nugroho et al. 2012, 2013).

479 No functional genomic experiment has yet proved that gibberellins could control  
480 RW formation. However, the use of a natural weeping mutant of *P. spachiana*  
481 brought some evidence for a role of GAs in TW formation. Exogenous application  
482 of GAs on branches of these Japanese cherry trees (*P. spachiana*) stimulated  
483 cambial growth and promoted TW formation on the upper side of branches  
484 resulting in an upright movement (Nakamura et al. 1994; Baba et al. 1995; Yoshida  
485 et al. 1999).

486 Together these results indicate that GAs seem to be involved in RW formation.  
487 However, more direct supporting evidence and a better understanding of the  
488 involved signaling factors is still needed to make a clearer conclusion.

As mentioned above, although sometimes quite indirect, an important amount of data indicates a role for auxin, ethylene and gibberellins in RW formation. This holds especially true for angiosperms since knowledge obtained on CW is less advanced. On the contrary, no such relation has been identified for cytokinins, abscissic acid or brassinosteroids. Most of the experiments designed to gain a better understanding of the role of plant hormones in TW formation focused on a single hormone, studied independently. It is, however, important to keep in mind that many hormones have been shown to interact with each other in a synergistic or inhibitory manner. For example, GA is known to stimulate polar auxin transport (Björklund et al. 2007) and IAA to promote ethylene biosynthesis (Abeles et al. 1992). Although the studies made so far have greatly improved our knowledge of RW biology, the use of high throughput molecular technics combined with functional genomics has started to, and should in the future, help to gain a deeper understanding of the processes underlying RW development. Moreover, hormones are currently mostly regarded as upstream primary responses to TW sensing (Felten and Sundberg 2013), but studies on hormone distribution and transport are still too scarce and contradictory to rule out the possibility of their involvement in the transmission of the signal from the perceptive to the RW-forming cells.

### **4.3.2 Other Candidates for Signal Transmission to Reaction Wood-Forming Cells**

miRNAs are small non-coding RNA molecules (about 21 nucleotides) which cleave or degrade messenger RNA targets. In plants, they are involved in the regulation of a large number of physiological processes (Jones-Rhodes et al. 2006) through the targeting of cell metabolism, signal transduction and stress response mRNAs. Different authors (Griffiths-Jones et al. 2008; Lu et al. 2005, 2008; Zhang et al. 2010) have characterized mechanical stress-responsive miRNAs in *P. trichocarpa*, especially miRNA that were differentially regulated by bending. The predicted target genes encode transcription factors and proteins involved in various cellular processes. For example, the function of the target of miR1446 is a gibberellin response modulator-like protein and the target of miR160 is an auxin responsive factor. Although a direct link between miRNAs and RW has never been proven, the above-mentioned data indicate that these small molecules could be good candidates to explore the molecular network controlling RW formation. To do so, further genome-wide identification of miRNAs using a different experimental design (inclination) is needed, as well as functional characterization of the identified miRNA and corresponding targets.

Recently, several authors reported intercellular signaling by miRNAs and showed that some can move from one cell to another or over long distances (for review, see Marín-González and Suárez-López 2012). Since signaling from the cells that perceive the RW-generating stimuli to the RW-forming cells may require



529 long distance regulation of gene expression, it is tempting to consider miRNAs as  
530 good candidates for the signal transmission from perceptive cells to RW-forming  
531 cells.

## 532 **4.4 Insights from Global Approaches**

533 Despite the economic impact of RW occurrence in industrial process and its  
534 importance from a tree developmental point of view, the molecular mechanisms  
535 involved in the perception and response to the gravitational stimulus have not been  
536 extensively studied. Furthermore, very few studies have addressed this question by  
537 global approaches, which require the genome of the studied tree species to be  
538 sequenced and annotated.

539 Investigating the induction of RW is also a very complex question RW is formed  
540 very locally in answer to a global mechanical problem for the tree. In addition most  
541 of the regulations used in RW formation (division rate, cell elongation, cell wall  
542 thickening, MFA setting) are also used for normal wood formation. Experimental  
543 setups have to take into account this point to specifically address the question of  
544 RW induction. Most of the studies on genes or proteins acting as regulators of RW  
545 making were done on inclining experiments. By inclining the whole tree system by  
546 an angle of around 30° and letting it grow afterwards (see Chap. 5) a pure, long-  
547 lasting RW formation is induced at the base of the stem (see Fig. 4.1). At inclination  
548 angles of this magnitude there is a strong perception of disequilibrium, secondary  
549 growth processes are very active and no new primary axillary growth is observed.  
550 This is in contrast to very inclined (nearly horizontal) trees, which use growth  
551 through axillary buds (i.e. primary growth) to create new vertical axes.

### 552 **4.4.1 Transcriptome Analysis**

553 Transcriptomics of RW is still in its infancy. Quite limited reports are available  
554 although studies have been conducted for about two decades. Regarding the vast  
555 majority of transcriptomics works on normal wood, the reader should refer to the  
556 most recent review of Zhong and Ye (2013). Early work addressed gene expression  
557 during RW formation through target genes approaches, leading progressively to  
558 recent transcriptome-wide overviews. Tools for deciphering gene involvement in  
559 the control of RW formation are becoming increasingly available and although  
560 most recent gene expression measurement tools, such as RNAseq, are still under-  
561 used in this research field, hopefully this will change in the near future.

562 Most studies of angiosperm RW formation using transcriptomics have been  
563 conducted with poplar species and less frequently with *Eucalyptus grandis*, *Euca-*  
564 *lyptus globulus* and *Eucalyptus nitens*. Other angiosperms species have been rarely  
565 studied with the exceptions of *A. thaliana* and the tulip tree (*Liriodendron* sp.). In

gymnosperms, most studies have been conducted with *Picea taeda* although *Pinus* 566  
*pinaster*, *Pinus radiata*, *Picea abies*, *Picea glauca* and *Chamaecyparis obtusa* have 567  
also been examined. Target gene studies began in this research area with the 568  
reporting of the involvement of 4-coumarate:coenzyme A ligase (4CL) during 569  
CW formation (Zhang and Chiang 1997). Along with up-regulation of 4CL trans- 570  
cripts, the corresponding enzyme activity was also increased and its impact on 571  
lignin composition was observed. Regulators of lignin biosynthesis have been 572  
targeted as well, namely MYB factors in *P. glauca* (Bedon et al. 2007). 573

Meanwhile, transcriptome profiling started with the pioneering work showing 574  
*Pinus taeda* transcripts down-regulated for genes involved in lignin biosynthesis 575  
(Allona et al. 1998) and later in *C. obtusa* (Yamashita et al. 2008). At the same time, 576  
a large diversity of responses was reported for carbohydrates enzymes, 577  
i.e. xyloglucan endo-transglycosylase (XET), in CW using 1,097 ESTs in a 578  
co-expression clustering study (Allona et al. 1998). AGPs were reported as 579  
key-players through cDNAs encoding six novel so-called cell wall-associated pro- 580  
teins in CW formation by the same approach (Zhang et al. 2000). These results were 581  
further developed with a set of 2,400 ESTs from a cDNA microarray where 33 out of 582  
69 transcripts were differential in CW and related to monolignols biosynthesis 583  
(Whetten et al. 2001). Following this, studies on angiosperms provided additional 584  
information by deciphering the gelatinous layer (G-layer) deposition in the 585  
so-called G-fibres during TW formation. Fasciclin-like arabinogalactan (FLA) 586  
proteins and their corresponding transcripts have been intensively studied empha- 587  
sizing their involvement as a hypothetical adhesion factor facilitating cellulose 588  
deposition in the G-layer during TW formation in *P. tremula* × *tremuloides* 589  
(Andersson-Gunnerås et al. 2003, 2006), *P. tremula* × *alba* (Lafarguette 590  
et al. 2004), *E. grandis* (Qiu et al. 2008), *E. nitens* and *A. thaliana* (MacMillan 591  
et al. 2010). Along with FLA studies, transcriptional mechanisms of saccharide 592  
metabolism and deposition were also functionally dissected, even down to the cell 593  
scale using microgenomic tools (Goué et al. 2008). Major works outlined the role of 594  
sucrose synthase (SuSy) in *P. tremula* × *alba* (Déjardin et al. 2004), cellulose 595  
synthase (CesA) in *E. globulus* (Paux et al. 2005), XET and xylo-glucan endo- 596  
transglycosylase/hydrolase (XTH) in *P. tremula* × *tremuloides*, *Populus alba* and 597  
*P. tremula* (Nishikubo et al. 2007) and XET in *L. tulipifera* (Jin et al. 2011). 598

With the improvement of sequencing facilities and transcriptome-wide studies, 599  
development of collections of ESTs related to wood formation transcriptomics were 600  
set-up, some of them including data related to RW formation as for 601  
*P. tremula* × *tremuloides* (Sterky et al. 2004), but also including expression data 602  
in RW for several other poplar species (Sjodin et al. 2009), for *P. abies* (Koutaniemi 603  
et al. 2007), for *P. radiata* (Ramos et al. 2012) and for *P. pinaster* (Villalobos 604  
et al. 2012). 605

A striking point with timescale studies of RW formation is that very few reports 606  
deal with early molecular events of the process. In other words, most of the 607  
published works dealing with transcriptomics of RW formation, either gene- 608  
targeted or genome-wide, focus at developmental stages when RW is already 609  
histologically observable in the xylem. Precursor work in the field of the induction 610

611 of RW does exist but is only gene-targeted at the moment. ZFP2 transcription factor  
612 was firstly reported in *Juglans regia* (Leblanc-Fournier et al. 2008) and  
613 *P. tremula* × *alba* (Martin et al. 2009). This ZFP2 is coined “mechano-sensitive”  
614 and addresses the xylem cell response to mechanical stress at a very early stage in a  
615 timely and structured manner in the transduction pathway to TW formation in trees,  
616 along with TCH2 and TCH4 as reported in *P. tremula* × *alba* from quantitative  
617 PCR studies (Martin et al. 2010). TCH4, reported as encoding for an XET in  
618 *A. thaliana* (Xu et al. 1995), draws attention to enzymatic-oriented cellulose  
619 modifications in the cell wall. The field of early RW induction, at the cell level  
620 and before any macroscopic tissue organization can be observed in the stem, is  
621 hopefully a must in any forthcoming experiments.

#### 622 4.4.2 Techniques for Proteome Measurement

623 Proteomics is a powerful molecular tool for describing proteomes at the organelle,  
624 cell, organ or tissue levels and for showing the modifications of protein expression  
625 in response to environmental changes (Abbasi and Komatsu 2004). Proteomics  
626 completes the large-scale analysis of the transcriptome. On many occasions, the  
627 level of mRNA is not always correlated with protein expression level. One tran-  
628 script may be translated into more than one protein because of alternative splicing  
629 or alternative post-translational modifications. In addition, post-translational mod-  
630 ifications such as phosphorylation and glycosylation may modify protein activities  
631 and subcellular localization (Yan et al. 2005).

632 Although attempts have been made at identifying proteins whose abundance,  
633 localization, and/or post-transcriptional modifications are altered by gravisti-  
634 mulation, most studies were conducted on *A. thaliana* seedlings and tended to  
635 unravel the mechanisms that control root gravitropism (for review, see Harrison  
636 et al. 2008). As for the understanding of the response of tree shoots to gravity, both  
637 gymnosperm and angiosperm species should be considered separately since the  
638 structure and properties of CW are different from those of TW. Although studies  
639 have been conducted to elucidate wood formation in trees, few of them have  
640 addressed the problem at the proteomic level, and even less focussed on RW  
641 genesis.

642 Among the first global attempts to unravel xylogenesis in trees, two-dimensional  
643 (2D) electrophoresis has been used to characterize xylem maritime pine proteins  
644 (Costa et al. 1999) or seasonal changes in protein expression in wood-forming  
645 tissues of poplar (Minsbrugge et al. 2000). The first description of the proteome of  
646 maritime pine wood-forming tissue (identification of 175 proteins) was provided by  
647 Gion et al. (2005). The variations in the proteome of differentiating xylem collected  
648 from the top to the bottom of adult maritime pine (*P. pinaster*) trees have provided a  
649 list of candidate genes for wood properties (Paiva et al. 2007). Using a large-scale  
650 approach, regeneration of the secondary vascular system in poplar was studied after  
651 peeling of the bark and sampling by scraping regenerating tissues (Juan et al. 2006).

A dynamic view of the changes occurring during the juvenile wood formation in the proteome of *E. grandis* has been provided using xylem tissues from 3- and 6-year-old trees (Celedon et al. 2007). More recently, a focussed analysis of plasma membrane proteomes from different tissues isolated from 3 to 4 m high poplar trees identified 108 proteins that were specifically expressed in the xylem (Nilsson et al. 2010). The authors proposed a schematic model for wood formation, integrating proteins expressed in the xylem such as cellulose-synthesizing complex, receptors, glucan synthase, AGPs, and enzymes of lignin biosynthesis. In particular the thorough investigation of cellulose synthase complexes in differentiating *Populus* xylem has been realized using complementary approaches including laser microdissection, immunolocalization along with proteomic analysis (Song et al. 2010).

With the aim of understanding TW induction or formation, proteomic analyses have been conducted on *Poplar* and *Eucalyptus*. These species are used as models in forest genetics and woody plant studies because they grow rapidly, they can be genetically transformed and the size of their genome is relatively small (5 to  $6 \times 10^8$  bp) (Plomion et al. 2001). As for CW, different pine species, Sitka spruce (*Picea sitchensis*) and Japanese cypress (*C. obtusa*) have been used because of their economic and ecological interest. To investigate differentially expressed proteins in response to gravity, most studies report 2D polyacrylamide gel electrophoresis (PAGE) patterns and include identification of proteins by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), mass spectrometry (MS) or by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). One study using Multidimensional Protein Identification Technology (MudPIT) reported on the proteome of *Populus* developing xylem (Kalluri et al. 2009). The proteins were extracted from subcellular fractions of xylem stems, enzymatically digested and the resulting peptides were analysed using LC-MS/MS. However, this study was not specifically addressing RW induction/formation. In the next paragraphs, proteomic studies on TW induction/formation are first presented, followed by data on CW. Depending on the studies, the gravistimulation design, the organs and tissues used for protein extraction have been very different. In addition, studies on TW formation after bending using constraining strings are also presented. Because of the diversity of experimental designs, the synthesis of the results remains problematic.

#### 4.4.3 Measured Changes in Proteome

Azri et al. (2009) studied young poplars (14–20 internodes) inclined at 35° from the vertical axis. Whole internodes from the basal and apical regions of vertical and gravistimulated stems were collected. The purpose of this experimental procedure was to allow analysis of the differential expression caused by gravistimulation between regions showing different motors for stem reorientation. The apical region responds to inclination by differential growth due to elongation of primary tissues

693 while reorientation is due to asymmetrical formation of RW at the base. After  
694 45 min of gravistimulation, the stem showed no reorientation. After 1 week, RW  
695 was observed at the base of the stem. Differential protein expression was reported  
696 between inclined or non-inclined conditions and also between the regions of the  
697 stem. Among 300 protein spots, 40 % showed significant changes after inclination.  
698 Sixty protein spots whose staining intensity was altered by gravistimulation were  
699 identified by mass spectrometry. These 60 proteins fell into a large range of  
700 functional categories. Interestingly, the patterns of expression of these selected  
701 proteins differed strongly between the conditions tested (apical and basal regions,  
702 45 min and 1 week of inclination). At 45 min and 1 week, respectively, three and  
703 four proteins were similarly regulated by gravistimulation between the top and the  
704 basal regions. These observations suggested that different metabolisms and signal-  
705 ing pathways were involved in each region of the stem following a short (45 min) or  
706 a long (1 week) exposure to gravistimulation. At 45 min, before any visible  
707 reorientation of the stem, some of the proteins regulated by gravistimulation may  
708 be involved in graviperception. At the top of the stem (where reorientation will later  
709 occur through differential elongation of primary tissues), the results suggested the  
710 implication of ROS (regulation of oxidative stress-responsive enzymes). The reg-  
711 ulation of actin and tubulin subunits, or microtubule-binding proteins showed the  
712 importance of cell wall—plasma membrane—cytoskeleton structural continuum  
713 for graviperception. Several proteins suggested some signaling via the  
714 endomembrane system and that calcium and phosphoinositides might act as cellular  
715 messengers (calreticulin, phosphatidyl inositol transfer protein SEC14). At the base  
716 of the stem (where orientation will later occur through the formation of RW), the  
717 most noticeable enzymes that were differentially expressed by gravistimulation  
718 were involved in lignin biosynthesis (phenylcoumaran benzylic ether reductase,  
719 S-adenosylmethionine synthase). However, members of the S-adenosyl-L-methio-  
720 nine-synthase gene family, which serve as universal methyl-group donors, are  
721 potentially involved in lignin as well as in ethylene biosynthesis pathways.

722 In *Eucalyptus gunnii*, proteins were extracted from xylem tissue harvested from  
723 a crooked tree. Two-dimensional gel electrophoresis images from normal and TW  
724 were compared showing that 12 proteins out of 140 proteins analysed were differ-  
725 entially expressed (Plomion et al. 2003). However, none of these proteins were  
726 identified.

727 A different approach was carried out by Kaku et al. (2009) who focused on the  
728 proteome of the G-layer in poplar TW. Leaning stems and branches from field-  
729 grown poplars were used as sources for isolation of G-layers from TW. Among the  
730 proteins separated by 2D gel electrophoresis, 108 were identified. Most abundant  
731 were lignin synthesis-related proteins although the G layer did not contain lignin  
732 itself. Cytoskeleton proteins, methionine synthesis-related proteins and cell wall-  
733 related proteins were also identified. Lignification in TW is still a matter of debate.  
734 Andersson-Gunnerås et al. (2006) using a global analysis reported a decrease in  
735 monolignol biosynthesis in TW compared with normal wood. However, on-going  
736 lignification was observed during G layer deposition in the compound middle  
737 lamella, S1 and S2 layers in poplar TW (Yoshinaga et al. 2012). An assay based

on protein cleavage isotope dilution mass spectroscopy (PC-IDMS) has been developed for quantification of proteins regulating monolignol biosynthesis in *P. trichocarpa* (Shuford et al. 2012) and could potentially bring valuable data to decipher lignification in RW.

In conifers, CW is formed in response to gravitropic stimulus or environmental disturbances such as prevailing winds, and “pushes” the stems toward a vertical orientation. In the same way as for the TW studies, proteomic analyses of CW formation concerned either developing CW or inclined stems where no CW had been formed yet.

A comparative protein-based approach to identify proteins specifically expressed in CW was conducted with branches of Sitka spruce (*P. sitchensis*) (McDougall 2000). The developing xylem was sampled from the compression and non-compression sides of the branches. The comparison of polypeptides patterns by SDS-PAGE led to the identification of a laccase-type polyphenol oxidase that was over-expressed in compression tissues. This enzyme is thought to be involved in lignin biosynthesis.

On a larger scale, the identification of CW responsive proteins has been conducted with a 22-year-old crooked maritime pine (*P. pinaster* Ait.) (Plomion et al. 2000). Wood samples were mechanically and chemically characterized by measuring growth strains and lignin and cellulose contents, respectively. Of the 137 spots studied, 19 % were associated with growth strain effect. The results indicated the importance of ethylene in CW formation. The implication of 1-aminocyclopropane-1-carboxylate (ACC) oxidase which catalyses the final reaction of the ethylene biosynthetic pathway in CW formation has also been suggested by Yuan et al. (2010). These authors examined PtACO1 and *PtACO1*-like (encoding putative ACC oxidases) transcript levels by quantitative PCR in loblolly pine seedling stems that were bent to a 90° angle using constraining strings. They observed an increase in these transcripts levels starting at 30 min and peaking at 3 h after bending. *PtACO1*-like transcripts were higher in CW than in opposite wood (OW). Besides, Plomion et al. (2000) have found that lignin biosynthesis was also affected during CW formation and that enzymes involved in Krebs cycle, sucrose and starch metabolism were up-regulated.

In another study, sampling of compression and OW was done with 16-year-old maritime pines bent to a 15° angle by tying their trunks to neighbouring trees for 2 years (Gion et al. 2005). Other types of wood were also analysed (juvenile and mature woods, early and late woods). The clustering of 215 proteins identified over the six types of wood was presented. It appeared that 20 % of the identified proteins exhibited distinctive expression patterns between CW and OW. Profilin, actin and nucleoside diphosphate kinase, 40S ribosomal S12 proteins were under-expressed in CW.

LC-MS analysis of Golgi-enriched membrane fraction from developing *P. radiata* CW has been done following in-solution digestion with trypsin (Mast et al. 2010). CW was sampled from 6-year-old trees in late summer to maximize the identification of proteins involved in secondary cell wall formation. As expected most proteins detected were involved not only in cell wall synthesis (i.e. cellulose

783 synthase, laccase, phenyl alanine ammonia-lyase) but also in hormone biosynthesis  
784 and signaling (i.e. auxin-induced proteins, ACC synthase) and stress and defence  
785 response. Within this last putative functional category, numerous receptors were  
786 found (CC-NBS-LRR protein, NBS/LRR, TIR/NBS, TIR/NBS/LRR disease resistance  
787 protein).

788 Gravitropism is not the only process that determines stem orientation; photot-  
789 ropism is also an important factor that can lead to RW production. The interaction  
790 between these two processes has not been extensively studied in trees. Herrera  
791 et al. (2010) have presented a proteomic analysis of inclined pine seedlings sub-  
792 mitted to an orthogonal light source. However, the apices were collected instead of  
793 the basal part of the stem where undergoing secondary growth takes place. Thus this  
794 study mainly identified differentially expressed proteins in the primary response to  
795 stem tilting.

796 Proteomic studies have been realized with different organs and tissues (stem,  
797 branches, whole internodes, xylem, G layer) from seedlings to trees aged from  
798 2 month-old to 22 year-old. In some cases the plants were inclined or mechanically  
799 bent and the proteome was analysed after varying times depending on the study  
800 (from 45 min to days or weeks). In other cases, aged plants showing RW were used.  
801 The problem is to discriminate between overlapping events such as induction,  
802 signal transduction (first events following stimulation) in the stimulated cells  
803 which are not clearly identified yet, reorientation of cambial cell programming,  
804 and differentiation of newly formed cells in the developing xylem. In addition,  
805 perception of the gravitropic stimulus and response probably occur in different  
806 cells. The proteomic approach has been executed either on whole internodes or on  
807 xylem tissue which may not contain the perceptive cells. Among the proteins  
808 listings published, large functional categories appear such as primary metabolism,  
809 cytoskeleton organization and biogenesis, cell wall synthesis, hormone biosynthe-  
810 sis and signaling. However, the role of most proteins is still hypothetical. Focused  
811 studies are needed to evaluate the role of the proteins brought forward by global  
812 proteomic analyses.

#### 813 **4.4.4 Metabolomics and RW Formation**

814 Metabolomics is a global approach used in biology for systematic metabolite  
815 quantification, a metabolite being any intermediate or product of the metabolism,  
816 e.g. amino acids, carbohydrates, hormones, and many more. Although several  
817 studies targeted some metabolites related to carbon and secondary metabolism  
818 that potentially play a role in RW formation (Yeh et al. 2005, 2006; Shi and Li  
819 2012), “without *a priori*” approaches have been marginally exploited in this field.  
820 To date, only Andersson-Gunnerås et al. (2006) have published work using this  
821 technique. A combination of metabolomics and transcriptomics recently gave  
822 precious insights on the *Arabidopsis* gravitropism and phototropism interplay  
823 (Millar and Kiss 2013). Andersson-Gunnerås et al. (2006) used a similar approach

to gain access to the G-layer formation in poplar TW induced after 11 days bending. 824  
Conclusions from biochemical measurements follow predictions coherent with 825  
gene expression showing in TW: an increased activity of cellulose synthesis and 826  
pectin degradation-related genes while those involved in lignin biosynthesis are 827  
decreased. An advantage of their transcriptomic approach consists in fine identifi- 828  
cation of differentially expressed genes from multigenic families, thus refining the 829  
implication of gene candidates individually. Based on their expression and meta- 830  
bolic profiles, they propose extensive relational models for carbon metabolism and 831  
lignin biosynthesis in TW. Despite the importance of this work that provides a 832  
coherent framework based on quantitative and qualitative data on TW chemistry 833  
and gene expression, the earlier steps remain a matter of discovery. 834

## 4.5 Concluding Remarks

835

The formation of RW allows woody structures to adapt their position in response to 836  
gravitational and mechanical stimulation and/or a change in the light environment. 837  
The deciphering of the molecular mechanisms underlying this particular growth 838  
response is complex. It requires at the very least, tree models with sequenced 839  
genomes, which allow global approaches such as transcriptomics and proteomics. 840  
Functional genomics which aims to elucidate the function of proteins encoded by 841  
candidate genes is limited by a scarcity of mutants and the long generation times of 842  
forest trees. In the face of these complex challenges, Wyatt et al. (2010) presented 843  
*A. thaliana* as a model for a molecular and genetic analysis of the mechanisms of 844  
RW formation. In addition, RW formation having many external or internal causal 845  
agents (gravity, light, interactions of both stimuli, inherent patterning mechanisms), 846  
it is difficult to set up an experimental design that addresses the impact of one 847  
particular stimulus in trees. Most studies utilized inclined trees in greenhouse 848  
conditions, although both phototropic and gravitropic reactions occurred in such 849  
conditions. Signalisation pathways leading to tree stature adjustments are different 850  
whether starting from a light or a gravi-mechanical stimulus. For a short period of 851  
time, dark or isotropic light conditions could be used to gain insight into gravitropic 852  
signalisation pathway leading to RW formation. Moreover the issues around stak- 853  
ing also need to be considered since different molecular pathways may be induced 854  
if stem deformation is allowed or not. 855

Hypotheses about perception of gravistimulation were previously defined 856  
through studies using *A. thaliana* mutants. However, in trees the question about 857  
the role of amyloplasts remains since starch is present in high level and everywhere 858  
in old woody structures. In trees, the tissue/cell that perceives gravistimulation is 859  
not clearly identified. As for the early events of signal transduction, one has to 860  
emphasize that very few studies were done at the very beginning of induction of 861  
RW, before any macroscopic observation of RW formation. Mechano-receptors 862  
involved in RW induction have still to be characterized. Global approaches suggest 863  
the role of calcium and ROS as second messengers and some signaling via the 864



865 endomembrane system and phosphoinositides. Although components of the signal-  
866 ing network have been identified through global analyses, the way they relate to one  
867 another in space and time is still unknown. Wyatt and Kiss (2013) speak about a  
868 “more or less amorphous gray cloud” when relating to the understanding of the  
869 signaling network. More precision could come from microdissection of chosen  
870 tissues or cells prior to molecular investigations. Typically, the early events of  
871 signal transduction are supposed to lead to hormonal response (i.e. synthesis,  
872 degradation, redistribution, reallocation, compartmentalization, and so on) that  
873 will finally provoke the growth and cell differentiation response. The involvement  
874 of ethylene gibberellin and auxin has been discussed but more studies are needed in  
875 order to decipher hormone signaling crosstalk in RW induction at the cell level, and  
876 also at the organ and whole plant level. For example, Azri et al. (2009) suggested  
877 that different signaling pathways occurred at the top and the base of a tilted poplar  
878 stems.

879 In conclusion, global approaches reveal the complexity of the RW induction  
880 both on a temporal scale and as a function of the location in the tree. Therefore,  
881 although the transcriptional network, the organization of protein synthesis and the  
882 subsequent hormonal response at the whole tree level is still unknown, the begin-  
883 ning of an understanding of how trees manipulate RW formation to solve their  
884 mechanical requirements is emerging.

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