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RAPID COMMUNICATION

Gibberellin-induced formation of tension wood in angiosperm trees

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Abstract After gibberellin had been applied to the vertical stems of four species of angiosperm trees for approximately 2 months, we observed eccentric radial growth that was due to the enhanced growth rings on the sides of stems to which gibberellin had been applied. Moreover, the application of gibberellin resulted in the formation of wood fibers in which the thickness of inner layers of cell walls was enhanced. These thickened inner layers of cell walls were unlignified or only slightly lignified. In addition, cellulose microfibrils on the innermost surface of these thickened inner layers of cell walls were oriented parallel or nearly parallel to the longitudinal axis of the fibers. Such thickened inner layers of cell walls had features similar to those of gelatinous layers in the wood fibers of tension wood, which are referred to as gelatinous fibers. Our anatomical and histochemical investigations indicate that the application of gibberellin can induce the formation of tension wood on vertical stems of angiosperm trees in the absence of gravitational stimulus.

Keywords *Fraxinus* · Gibberellin · *Kalopanax* · *Populus* · *Quercus* · Tension wood formation

Abbreviations

FE-SEM	Field emission-scanning electron microscopy
GA_3	Gibberellic acid
UV	Ultraviolet

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Introduction

When herbaceous plants are displaced from their normal position in space by an environmental force, such as wind, flooding or a landslide, differential longitudinal growth of elongating stems generally occurs to correct for the change in orientation. However, woody plants respond differently to such environmental changes because the extension growth of most parts of their stems, which are the sites of secondary growth, has ceased. Thus, woody plants respond to positional displacement by an environmental force by developing of a special type of secondary tissue in stems that is referred to as reaction wood (Wardrop 1964; Timell 1986). Angiosperm trees generally form reaction wood, known also as tension wood, on the upper sides of leaning stems. As tension wood develops by gravitational stimulus, it generates a strong tensile force, which can pull a large inclined stem into a vertical or near vertical position, along the grain of the upper side of the living stem (Okuyama et al. 1994; Yamamoto et al. 2002). Thus, the formation of tension wood is an important morphogenetic response to gravitational stimulus in angiosperm trees and allows them to maintain the appropriate form.

The characteristic anatomical and chemical features of tension wood have been the focus of extensive study because they are closely related to the quality of wood and its products (Onaka 1943; Wardrop 1964; Côté and Day 1965; Norberg and Meier 1966; Côté et al. 1969; Araki et al. 1982; Chaffey et al. 2002; Clair et al. 2005, 2006; Ruelle et al. 2006, 2007). However, the physiological and molecular mechanisms responsible for the formation of tension wood remain poorly understood (Timell 1986; Little and Pharis 1995; Mellerowicz et al. 2001; Pilate et al. 2004). Although plant hormones, in particular auxin and ethylene, have been suggested as key factors, their roles in

the formation of tension wood are not conclusively established (Cronshaw and Morey 1965; Kennedy and Farrar 1965; Morey and Cronshaw 1968a, c; Moyle et al. 2002; Andersson-Gunnerås et al. 2003, 2006; Du and Yamamoto 2003; Hellgren et al. 2004).

This study was designed to examine the role of another plant hormone gibberellin in the formation of tension wood. The application of exogenous gibberellin affected differentiation and lignification of fibers in stems of *Coleus blumei* (Aloni 1979; Aloni et al. 1990). In addition, the application of exogenous gibberellin accelerated formation of well-developed tension wood in branches of *Prunus spachiana* (Baba et al. 1995; Yoshida et al. 1999). Although experiments by the application of gibberellin have not proven the final conclusion concerning the role of gibberellin in the control of differentiation of fibers, these observations suggest a possible role for gibberellin in the formation of tension wood. Therefore, we applied gibberellin to the vertical stems of four species of angiosperm trees during the middle part of the season of active cambial growth. Then, we investigated the anatomical and histochemical features of the wood fibers in the treated trees 2 months later. We provide here the first evidence, to our knowledge, that gibberellin can induce the formation of tension wood on vertical stems of angiosperm trees.

Materials and methods

Plant materials

Straight-growing trees of four species of angiosperms, namely, six approximately 5-year-old specimens of *Fraxinus mandshurica* var. *japonica*, six 10-year-old specimens of Quercus *mongolica* var. *grosseserrata*, two 4-year-old specimens of *Kalopanax pictus* and five 3-year-old specimens of *Populus sieboldii*, growing in the Experimental Forest of Hokkaido University in Sapporo or Tomakomai, were used in this study.

We applied a 1% (w/w) mixture of gibberellic acid (GA_3) in lanolin once to one side of each vertical stem, at breast height, in June or July, during the middle part of the season of active cambial growth. We also applied lanolin alone once to the opposite side of each stem as a control. For each application, we used 1 g of lanolin. Prior to the application, a portion of the outer bark (approximately 15 mm × 20 mm) was carefully removed with a scalpel to facilitate penetration. The application point was covered with aluminum foil after GA_3 in lanolin or lanolin alone had been applied. After approximately 2 months, all of the trees were felled. Small blocks containing phloem, cambial zone cells and xylem were taken from the stems at the sides of application of GA_3 in lanolin or lanolin alone. Each block was trimmed to 8–10 mm cubes and slivers that were 2–3 mm thick.

Light microscopy

The 8–10 mm cubes were fixed in FAA (a mixture of 50% ethanol, acetic acid and formaldehyde, 18:1:1, by vol.). Transverse sections of 15 μm in thickness were cut from the FAA fixed cubes on a sliding microtome (LS-113; Yamatokohki, Saitama, Japan). Sections were stained with a 1% solution of safranin-fast green. They were observed with a light microscope (BHS-2; Olympus, Tokyo, Japan).

Ultraviolet microscopy

The 2–3 mm thick slivers were fixed in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at room temperature. They were then washed with the buffer, dehydrated through a graded ethanol series and were embedded in epoxy resin. Transverse sections of 1 μm in thickness were cut from epoxy-embedded samples with a glass knife, placed on quartz slides and mounted in glycerine with quartz coverslips. Photographs were taken under an ultraviolet (UV) microscope (MPM-800; Carl Zeiss, Jena, Germany) at a wavelength of 280 nm for determination of the extent of lignification (Nakaba et al. 2006; Watanabe et al. 2006).

Field emission-scanning electron microscopy (FE-SEM)

The 2–3 mm thick slivers were fixed in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at room temperature and then washed with the buffer and with distilled water. The longitudinal radial surfaces that were to be examined were exposed with a sliding microtome. The samples were then treated with a dilute solution of sodium hypochlorite for 1 min to remove cytoplasm. After further washing with water, the samples were post-fixed in 1% OsO_4 in distilled water for 2 h at room temperature. After washing with distilled water, the samples were dehydrated in a graded ethanol series, dried by a *t*-butyl alcohol freeze–drying method and lightly coated with platinum in a vacuum evaporator (Abe et al. 1991, 1995). Specimens were observed with a field emission-scanning electron microscope (JSM-6301F; JEOL Co., Tokyo, Japan) at an accelerating voltage of 2.5 kV (Sano and Jansen 2006).

Results and discussion

In angiosperm trees the formation of tension wood is often accompanied by eccentric radial growth towards the upper

side of a leaning stem (Wardrop 1964). In addition, tension wood is usually characterized by the presence of gelatinous fibers, which contain a thickened the inner layer of cell walls, known as gelatinous layer. The main characteristic features of the gelatinous layer are that it is composed entirely or almost entirely of cellulose and unlignified or only partially lignified (Wardrop 1964; Côté and Day 1965; Côté et al. 1969; Timell 1986). Cellulose microfibrils in the gelatinous layer are oriented parallel or nearly parallel to the longitudinal axis of the fibers (Côté and Day 1965; Prodhan et al. 1995a, b).

Approximately 2 months after the application of GA_3, we observed, in all cases, eccentric radial growth that was due to the wider growth ring on the side to which GA_3 had been applied than on the control side (Fig. 1a). In addition, the application of GA_3 resulted in similar changes in the anatomical features of the wood fibers in all species examined. All treated trees had formed wood fibers in which the

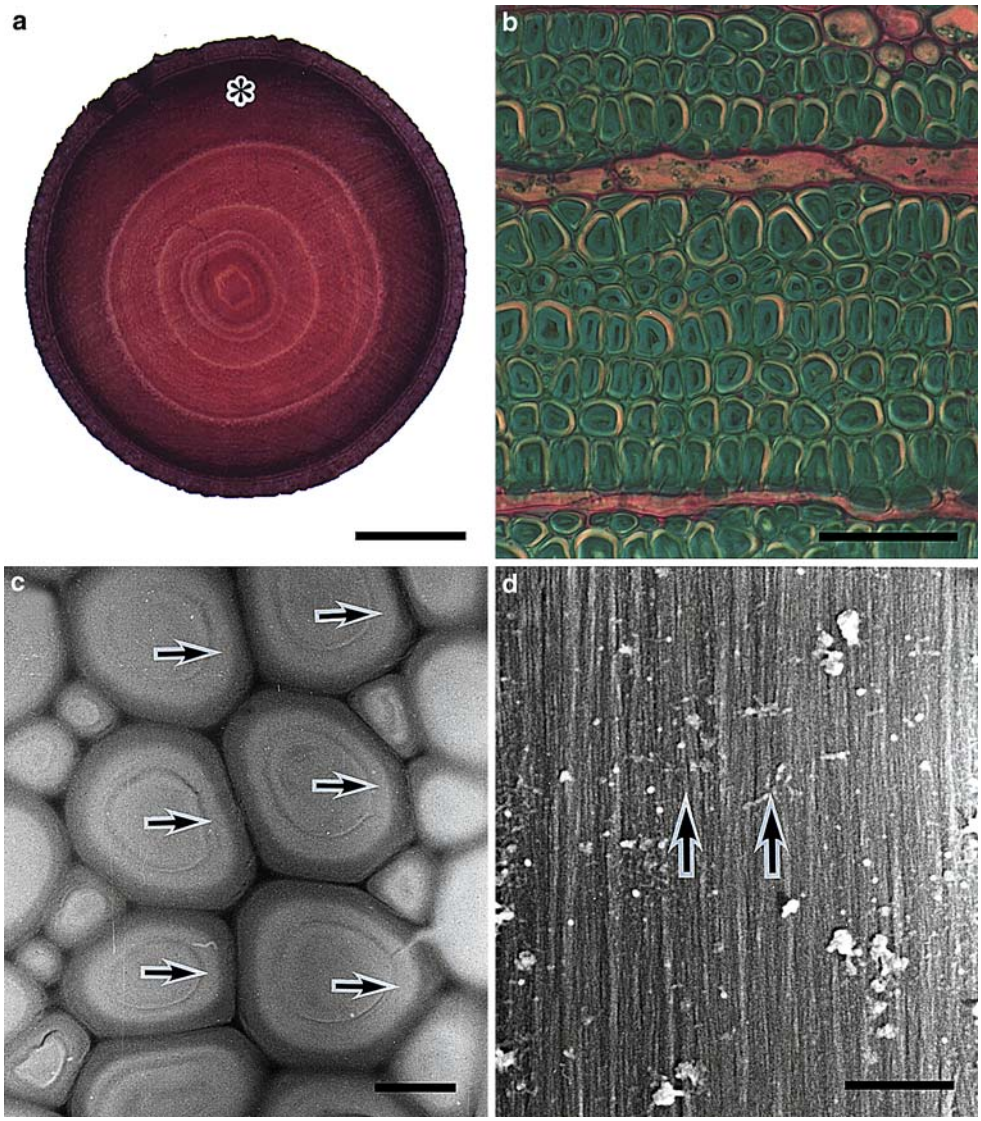

Fig. 1 Anatomical and histochemical features of vertical stems of angiosperm trees after the application of gibberellin. **a** *Fraxinus mandshurica* var. *japonica.* A cross section of a stem at the application point of gibberellin in lanolin or lanolin (control). A wider growth ring on the gibberellin-applied side (*asterisk*) than on the control opposite side is shown. *Scale bar* 1 cm. **b** *Quercus mongolica* var. *grosseserrata*. Light photomicrograph of a transverse section of a stem on the gibberellin-applied side showing wood fibers with thickened inner layers of cell walls. The inner layers of cell walls are stained *green* by the solution of safranin-fast green and are pulled slightly away from the remainder of the cell wall. *Scale bar* 100 μm. **c** *Kalopanax pictus*. Ultraviolet photomicrograph at a wavelength of 280 nm of a stem on the gibberellin-applied side showing the absence or only a very low level of absorption in the inner layers of cell walls of wood fibers (*arrows*). *Scale bar* 10 μm. **d** *Populus sieboldii*. Field emission-scanning electron micrograph on the innermost surface of the inner layer of the cell wall of wood fiber of a stem on the gibberellin-applied side. The parallel orientation of cellulose microfibrils (*arrows*) relative to the fiber axis is shown. *Scale bar* 0.5 μm

thickness of inner layers of cell walls was enhanced at the site of application of GA_3 (Fig. 1b). These inner layers of cell walls were stained green by a solution of safranin-fast green, an indication that they contained large amounts of cellulose but very small amounts of lignin. No similar thickened inner layers of cell walls were found in wood fibers on the control sides of trees. The secondary cell walls of the wood fibers strongly absorbed UV light at 280 nm, an indication of lignification (Fig. 1c). By contrast, the inner layers of cell walls absorbed little or no UV light. Therefore, the thickened inner layers of cell walls were unlignified or only slightly lignified. The presence of such thickened inner layers of cell walls is a characteristic feature of the wood fibers in tension wood, which are referred to as gelatinous fibers.

Examination of cellulose microfibrils on the innermost surface of the inner layers of cell walls by FE-SEM revealed that the microfibrils were close to each other and oriented parallel or nearly parallel to the longitudinal axis of the fibers (Fig. 1d). These characteristics of cellulose microfibrils are also typical of gelatinous layers. Therefore, the wood fibers on the gibberellin-applied sides of vertical stems of all the angiosperm trees examined had anatomical and histochemical features similar to those of the gelatinous fibers in the tension wood that is formed on the upper sides of leaning stems.

Considerable evidence indicates that gibberellin plays an important role in the radial and longitudinal growth and differentiation of secondary xylem cells in trees (Little and Savidge 1987; Little and Pharis 1995; Aloni et al. 2000; Eriksson et al. 2000; Israelsson et al. 2005; Dünisch et al. 2006; Björklund et al. 2007). However, it has been unclear whether gibberellin plays a key role in the formation of tension wood because inconsistent results have been reported. In *Acer rubrum*, the application of gibberellin stimulated the rate of xylem production but did not induce the formation of tension wood in vertical shoots (Cronshaw and Morey 1968; Morey and Cronshaw 1968b). These results suggest that gibberellin might play only a minor role in the formation of tension wood. Furthermore, the application of uniconazol-P, an inhibitor of the biosynthesis of gibberellin, to horizontally positioned stems of *F. mandshurica* var. *japonica* inhibited upward bending (negative gravitropism) and xylem production but did not prevent the formation of gelatinous fibers on the upper sides of stems (Jiang et al. 1998a, b). By contrast, the application of exogenous gibberellin to the apical buds of elongating branches of a weeping-type cherry, *Prunus spachiana*, caused branches to bend upward (Nakamura et al. 1994). This phenomenon was apparently due to strong tensile growth stress on the upper sides of branches, which was generated by the gibberellin-accelerated formation of well-developed tension wood (Baba et al. 1995; Yoshida et al. 1999). These observations suggested a possible role for gibberellin in the signaling required for the formation of developed tension wood in branches.

Our present results show clearly that the application of gibberellin can induce the formation of tension wood on vertical stems of several species of angiosperm trees in the absence of gravitational stimulus. Therefore, it is likely that the application of gibberellin and the gravitational stimulus elicit similar responses with respect to secondary xylem development in the stems of angiosperm trees. In addition, the application of gibberellin resulted in the widening of growth rings, as compared to controls, and such widening is indicative of active cambial growth. We propose that the application of gibberellin induces the expression of specific genes that are involved in the division of cambial cells and the xylem differentiation of cambial derivatives in a manner similar to the induction that occurs in response to the gravitational stimulus. Thus, gibberellins might be involved in the signal-transduction pathways whereby angiosperm trees respond to gravitational stimulus and they might play an important role in the maintenance of tree form.

Numerous experiments by the application of exogenous auxin or auxin-transport inhibitors have suggested that tension wood is induced by a difference in levels of auxin around the stem and deficiency of auxin (Timell 1986; Little and Savidge 1987). However, Hellgren et al. (2004) found that tension wood was formed without any obvious alternations in balance of endogenous auxin in the cambial region. Therefore, direct information on the endogenous status of plant hormones is required for the correct interpretation of experiments by the application of exogenous plant hormones. A recent reliable study in vertical stems of *Populus tremula* demonstrated that endogenous bioactive gibberellins showed peak levels in expanding xylem cells but very low levels in cambial cells and differentiating xylem cells that were in the process of secondary wall formation (Israelsson et al. 2005). Thus, if gibberellins are involved in the active division of cambial cells and the differentiation of gelatinous fibers, endogenous bioactive gibberellins across the cambial region might be redistributed by gravitational stimulus. To establish the role of gibberellins in the formation of tension wood, we need to investigate levels of endogenous gibberellins in cambial cells and differentiating xylem cells in leaning stems of angiosperm trees.

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