Concomitant analysis of cambial abscisic acid and cambial growth activity in poplar

Matthias Arend · Jörg Fromm

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Abstract Endogenous levels of cambial region abscisic acid (ABA) were quantified by immunoassay and assessed together with cambial growth activity in poplar (*Populus nigra* L. × *P. maximowiczii* Henry, clone *Kamabuchi*) over the course of a growing season. The level of cambial region ABA increased from spring to late-summer but decreased sharply in autumn. Cambial growth activity, measured as the radial number of undifferentiated cambial cells and enlarging xylem cells, also increased from spring to summer and decreased sharply in autumn, indicating the onset of cambial dormancy. Exogenous ABA, applied laterally to poplar stems at two times within the growing season, enhanced cambial growth activity, as the radial number of undifferentiated cambial cells increased in ABA-treated trees subsequent to the two application times. Xylem cell development was also affected by exogenous ABA as fibre length increased significantly in ABA-treated trees at both application times. The positive correlation of cambial region ABA and cambial growth activity as well as the positive effects of exogenous ABA application thereon sheds new light on the role of this hormonal growth regulator.

Keywords ABA · Cambium · Fibre cell · Seasonality · Vessel cell · Wood formation

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M. Arend (✉)
Federal Institute for Forest, Snow and Landscape Research
WSL, Zürcher Strasse 111, 8903 Birmensdorf, ZH, Switzerland
e-mail: matthias.arend@wsl.ch

J. Fromm
Institute für Holzbiologie, Universität Hamburg,
Leuschner Strasse 91, 21031 Hamburg, Germany

Introduction

Wood formation in trees underlies stringent regulation by a complex of endogenous and exogenous factors. Plant hormones are central players in this regulatory network, affecting different aspects of cambial growth activity and wood cell development (Little and Pharis 1995; Savidge 1996). While much of the tree physiology research on cambial growth activity and xylem development has focused on auxin, several other hormones, such as gibberellins and cytokinins, are also implicated as important growth regulators (Little and Savidge 1987; Little and Pharis 1995).

In contrast, the plant hormone abscisic acid (ABA) is commonly considered to be a growth inhibitor modulating plant metabolism in response to environmental stress or unfavourable growth conditions (Trewavas and Jones 1991; Rock and Quatrano 1995; Munns and Cramer 1996). Accordingly, many attempts have been made by tree biologists to correlate endogenous levels of ABA with different aspects of cambial activity, in particular with late wood formation in late-summer and the onset of cambial dormancy at the end of the growing season (Webber et al. 1979; Wodzicki and Wodzicki 1980; Little and Wareing 1981; Savidge and Wareing 1984; Funada et al. 1988, 2001; Mwange et al. 2005). Assessed together, however, these studies gave no consistent relationship between the endogenous level of ABA and the seasonal cycle of cambial activity. More specifically, high levels of endogenous ABA did not appear to be associated with late wood formation or the onset of cambial dormancy (Little and Savidge 1987). Nevertheless, it has been shown that high levels of exogenous ABA can inhibit radial stem growth when applied to shoots from spruce (Little and Eidt 1968), balsam fir (Little 1975) or willow (Fromm 1997).

Altogether, these conflicting results have led to some uncertainty about the actual role of ABA in regulating cambial growth activity (Lachaud 1989).

Most recently, views on the role of ABA in the regulation of plant growth have shifted from ABA being simply a growth inhibitor to rather an opposite function, that of maintaining vegetative growth. In fact, studies on ABA-deficient or ABA-signalling mutants of *Arabidopsis*, maize and tomato, suggest that endogenous ABA is required to maintain both shoot and root development under stress and non-stress conditions (Sharp et al. 2000; LeNoble et al. 2004; Barrero et al. 2005). Furthermore, decreased leaf and root growth has been recently shown in transgenic poplar insensitive to ABA (Arend et al. 2009). These former findings shed new light on the role of ABA in plant growth and development and could explain inconsistent results given by previous studies on the function of ABA in regulation of cambial growth activity.

In this context, the present study was undertaken to re-examine the relationship between ABA and cambial growth activity in a deciduous hardwood species. Poplar was chosen for this study because of the importance of the genus *Populus* as a model system for tree biology (Chaffey 1999; Wullschlegel et al. 2002; Tuskan et al. 2006). Two different approaches were followed to gain information on the relationship between ABA and cambial growth activity: (1) seasonal changes of endogenous levels of ABA were measured in micro-samples of cambial tissue by enzyme-linked immunoassay and related to anatomical analysis of cambial growth activity and (2) exogenous ABA was applied to poplar stems to evaluate its effects on cambial region growth activity and xylem cell development.

Materials and methods

Plant material and growth conditions

Poplar cuttings (*Populus nigra* × *P. maximowiczii* Henry, clone *Kamabuchi*) with a height of 1 m and a basal stem diameter of approximately 1 cm were grown under field conditions (48°09'N, 11°33'E) in 80-l pots with a mix of sand, humus and loamy soil (40:40:20). Trees were watered every third day and supplied with a complete fertilizer once a month during the growing season. Trees for ABA application experiments were randomly assigned to two groups, grown for 4 weeks with or without a supply of exogenous ABA and then completely harvested for anatomical analysis. Two ABA application experiments were performed, the first one in early-summer (20 Jun–21 Jul) and the second one in late-summer (23 Aug–23 Sep).

ABA application

The natural isomer of abscisic acid (100 µM (+)-cis, trans-ABA dissolved in H₂O, Olchemim Ltd. Czech Republic) was applied laterally to the surface of the basal stem part. Before applying ABA, small parts of the outer epidermis were carefully scraped with a razorblade to enhance the permeability of the stem surface. The basal part of the stem was then covered with a thin layer of gauze soaked with an aqueous ABA solution and subsequently wrapped with thin layers of plastic and aluminium foil to prevent evaporation and light degradation. The ABA solution was replaced every second day by a new solution. Control trees were treated identically but without ABA.

ABA determination

Basal stem segments were separated from the bark and immediately frozen in liquid N₂. After freeze-drying, a very thin layer of tissue from the cambial region (1–3 mg) was carefully scraped from the stem surface. Microscopic investigations verified that scraped tissue samples consisted only of cells from the cambial region (fusiform and ray initials and young enlarging xylem cells lacking secondary cell walls, see also Fig. 1). The freeze-dried tissue was extracted for 12 h at 4 °C with 80 % aqueous methanol (v/v) and BHT (butylated hydroxytoluene, 0.1 mg/ml). The

Fig. 1 Transverse stem section in September with active cambium (*cam*), differentiating xylem cells undergoing cell enlargement (*xce*) and secondary cell wall formation (*scw*). Numbered arrows give examples for newly formed fibres that define the xylem region considered for anatomical analysis (fibres and vessels). Scraped tissue samples for ABA analysis consisted only of cells from the cambial region (cambial cells and enlarging xylem cells)

methanol extracts were purified by TLC [silica gel₆₀, ethyl acetate:methanol (3:1)], ABA containing fractions re-extracted with absolute methanol and evaporated to dryness under vacuum. The dry residues were taken up in tris buffered sodium chloride (TBS) and the ABA content quantified by a competitive enzyme-linked immuno assay (Phytodetek ABA, Agdia Inc., USA) according to the manufacturer's instruction. The antibody used in this immunoassay was a monoclonal antibody with a very high specificity for physiologically active 2-cis-(S)-ABA (Mertens et al. 1983). The presence of interfering contaminants was checked by analysing dilutions of extracts, together with a range of dilutions of ABA standard solutions (Caruso et al. 1995). Based on parallel dilution assays, we concluded that interfering contaminants were not present.

Anatomical analysis

Stem tissue from the point of ABA application was fixed with 3 % (w/v) formaldehyde in phosphate-buffered solution (PBS) for 2 h, washed in buffer and dehydrated in a graded series of ethanol. After embedding in LR White acrylic resin, semi-thin sections of 1 µm were cut with a diamond knife on a LKB Ultramicrotome (LKB, Uppsala, Sweden) and stained with 0.05 % (w/v) Toluidine Blue O in 0.2 M Na₂HPO₄ for light microscopy. Four images from different areas of each microscopic section were taken with a Zeiss Axiophot light microscope (Zeiss, Germany) and an Axiocam digital camera (Zeiss, Germany). Digital images were analysed for anatomical parameters using a digital image analysis system (Zeiss Axio Vision, Germany). To ensure that all analysed wood tissues were formed under the influence of ABA application, only the most recently formed xylem, defined by the first four layers of fully expanded fibre cells, was considered for anatomical analysis (Fig. 1). Xylem increment was measured on microscopic sections as an absolute amount of radial xylem increment from the beginning of wood growth in April to the sampling dates in July and September. For determination of fibre length, thin layers of wood tissue (<0.1 mm) were carefully separated from the surface of the stems and macerated with 15 % (v/v) nitric acid at 70 °C. Images from separated fibres were taken with a Zeiss Axiophot light microscope (Zeiss, Germany) and an Axiocam digital camera (Zeiss, Germany). Digital images were analysed using a digital image analysis system (Zeiss Axio Vision, Germany).

Statistical analysis

Relationships between ABA levels, radial number of cambial and enlarging xylem cells and fibre length were analysed using *Spearman's* rank correlation. Comparisons

of parameter means were analysed using *Student's t* test. Differences between parameter means were considered significant when $P < 0.05$. All means represent samples from four replicate trees.

Results

Seasonal changes of cambial region ABA and cambial growth activity

The level of endogenous ABA measured in micro-samples from the cambial region showed a clear and significant change during the course of the growing season (Fig. 2a). The values increased from the first sampling date in spring (24 May) to a maximum level in late-summer (13 September), remained at this level in early-autumn (7 October) and dropped sharply to a lower level at the last sampling date in autumn (18 October). The cambial growth activity, measured as a radial number of undifferentiated cambial cells and differentiating xylem cells undergoing cell enlargement, showed a similar significant change in comparison with cambial region ABA (compare Fig. 2a with 2b, c). The mean number of cambial cells and differentiating xylem cells increased from spring (24 May) to a maximum level in mid-summer (9 August), remained at this level till early-autumn (7 October) but decreased sharply to a lower number in autumn (18 October). Only a few small xylem cells were actively differentiating at the time of the last sampling date, indicating the onset of cambial dormancy. The sharp decrease in cambial region ABA and cambial growth activity in autumn coincided with the beginning of leaf colour change in the poplar trees and subsequent leaf fall.

Effect of exogenous ABA on cambial growth activity and xylem development

After lateral application of exogenous ABA to the growing poplar stems in early- and late-summer, levels of the cambial region ABA increased at both application times (Table 1). Further anatomical analysis of stem sections revealed that application of exogenous ABA also enhanced cambial growth activity, i.e. significantly increased the radial number of undifferentiated cambial cells (Table 2). Furthermore, there was a (non-significant) tendency for a higher number of enlarging xylem cells and increased xylem increment in ABA-treated trees (Table 2). Interestingly, when comparing individual trees treated with ABA in late-summer, the highest number of undifferentiated cambial cells was found in trees with the highest level of ABA, while the lowest number of undifferentiated cambial

Fig. 2 Seasonal change of cambial region ABA and cambial growth activity. **a** Level of endogenous ABA in micro-samples from the cambial region, **b** radial number of undifferentiated cambial cells and **c** radial number of differentiating xylem cells undergoing cell enlargement. All values are mean $\pm$ SE; $n = 4$. Minimal values in spring and autumn were significantly different from maximal values in summer at 5 % level using Student's *t* test. Correlation analysis yielded $R = 0.77$, $P < 0.1$ for ABA levels versus cambial cells and $R = 0.83$, $P < 0.05$ for ABA levels versus enlarging xylem cells (Spearman's rank correlation)

Table 1 Effect of lateral ABA application on detectable levels of ABA in the cambial region

Treatment	ABA content [pmol mg ⁻¹ dw]	
	H ₂ O	H ₂ O + ABA
Early-summer	2.7 $\pm$ 0.4	3.3 $\pm$ 0.8
Late-summer	3.2 $\pm$ 0.6	16.9 $\pm$ 5.8

Values represent the content of ABA at the end of each application period (mean $\pm$ SE, $n = 4$)

Differences between treatments were not significant at 5 % level using Student's *t* test

cells was found in trees with the lowest level of ABA (data not shown).

The higher cambial growth activity after application of ABA was associated with significant alterations in xylem

Table 2 Effect of lateral ABA application on cambial region characteristic and xylem cell development in basal stem segments in early (es) and late (ls) summer (mean $\pm$ SE, $n = 4$)

		Treatment of basal stem segments with	
		H ₂ O	H ₂ O + ABA
Cambial cells (number per radial file)	es:	4.8 $\pm$ 0.2*	5.8 $\pm$ 0.2*
	ls:	5.0 $\pm$ 0.4*	6.4 $\pm$ 0.3*
Expanding xylem cells (number per radial file)	es:	1.6 $\pm$ 0.1	1.9 $\pm$ 0.2
	ls:	1.5 $\pm$ 0.5	2.8 $\pm$ 0.3
Fibre length (μ m)	es:	514 $\pm$ 7*	554 $\pm$ 9*
	ls:	591 $\pm$ 7*	635 $\pm$ 10*
Vessel cross sect. area (μ m ²)	es:	1241 $\pm$ 78	1243 $\pm$ 129
	ls:	299 $\pm$ 17	520 $\pm$ 124
Vessel density (number per mm ²)	es:	232 $\pm$ 17	246 $\pm$ 26
	ls:	746 $\pm$ 56*	415 $\pm$ 50*
abs. xylem increment (μ m)	es:	421 $\pm$ 36	506 $\pm$ 40
	ls:	803 $\pm$ 119	882 $\pm$ 120

* Significant differences between treatments at 5 % level using Student's *t* test

cell development. ABA-treated trees formed longer fibre cells at both application times and less, but larger, vessel cells in late-summer (Table 2). Other anatomical parameters such as cross-sectional area of fibre cells and vessel length were not affected by exogenous ABA (data not shown).

Discussion

The present study provides two lines of evidence that suggest a positive role of ABA in the regulation of cambial growth activity. A positive relationship was found between endogenous levels of cambial region ABA and cambial growth activity. Additionally, exogenous ABA applied laterally to the stem base, enhanced cambial growth activity and fibre growth. These observations contrast with previous reports suggesting a role of ABA in inhibiting cambial growth (Little and Eidt 1968; Webber et al. 1979; Wodzicki and Wodzicki 1980; Fromm 1997; Mwange et al. 2005) and other studies which have not found consistent relationships between endogenous ABA and cambial activity (Little and Wareing 1981; Savidge and Wareing 1984; Funada et al. 1988, 2001).

While in our study the promotion of cambial growth activity and fibre cell development by ABA is an unexpected result, such a response is not unknown. Indeed, exogenous ABA has been reported to enhance radial growth of xylem cells when injected laterally into growing stems of *Pinus radiata* (Pharis et al. 1981) and recent studies on herbaceous species strongly suggest a role for endogenous

ABA as a promoter of vegetative growth (Sharp et al. 2000; LeNoble et al. 2004; Barrero et al. 2005). Nevertheless, the strong discrepancy between results of the present study and previous research on cambial ABA remains an open question that might be explained by differences in experimental setups. For instance, in many previous studies, the analysis of ABA in stems has been often done with samples containing considerable amounts of phloem and xylem. The amount of ABA in these mixed tissues, however, differs strongly from cambial region ABA (Mwange et al. 2005) and because of that these earlier measurements likely do not reflect the situation in the cambial region where wood formation takes place. Furthermore, all experiments that report an inhibition of cambial growth activity by exogenous ABA were done on detached shoots submersed with the basal cutting surface into a hormone containing solution. This approach, however, has shown to decrease transpiration and photosynthesis (Little 1975) which in turn affect cambial activity due to the reduced supply of assimilates and growth promoting substances (Denne and Dodd 1981). In contrast, the present study minimized such artificial conditions by applying ABA laterally to a restricted area of the basal stem surface of intact trees. Interestingly, Pharis et al. (1981) who used a similar approach also reported a growth promotion of xylem cells in ABA-treated seedlings of *Radiata* pine.

Further, circumstantial evidence supporting a positive correlation between cambial region ABA and cambial growth activity comes from observations showing that ABA in non-stressed plants is mainly synthesized in green shoots and leaves and then transported via phloem and xylem towards different plant organs (Eliasson 1975; Everat-Bourbouloux and Charnay 1982; Lachaud 1989; Wolf et al. 1990). Assuming shoots and leaves are also the main source for cambial region ABA, a change of hormone synthesis in these plant organs would have a strong influence on the level of ABA in the cambial zone. This assumption is in line with our finding that the level of cambial region ABA decreases sharply with the beginning of autumnal leaf senescence and subsequent leaf fall. Thus, a reduction of ABA synthesis in senescent leaves in autumn might explain the lower level of ABA found in the cambial region from poplar at this time of the season. A similar conclusion has also been drawn by Alvim et al. (1976) who reported a strong decline in xylem sap ABA in stems from willow after leaves started to yellow and abscise in autumn.

The positive relationship between cambial region ABA and cambial growth activity as well as the promotion of cambial growth by exogenous ABA raises the question of the physiological significance of this plant hormone for cambial growth. Although a plethora of fundamental research has been conducted on ABA and its particular role in stress physiology, little is known about possible

functions in plant growth regulation under non-stress conditions. However, there is some evidence that ABA not only regulates plant responses to environmental stress but also plays a key role in assimilating translocation and metabolism. Indeed, studies on herbaceous species showed that ABA promotes phloem unloading of assimilates in sink organs and their subsequent accumulation and metabolism in cells surrounding the phloem (Saftner and Wyse 1984; Schussler et al. 1984; Ackerson 1985; Clifford et al. 1986; Ross et al. 1987; Brenner and Cheikh 1995). In trees, the active vascular cambium is considered to be a strong sink for assimilates as cell division and differentiation strongly depends on sufficient nutrient supply for synthesis of cellular structures (Krabel 2000; Sauter 2000). It might, therefore, be hypothesized that ABA creates a strong sink for photoassimilates in the cambial region tissues. The significance of this physiological link, however, has to be proven by future work.

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