Nuclear and cytoplasmic changes associated with maturation in the vascular cambium of *Larix laricina*

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Received May 31, 1994

Summary We studied the effects of apical maturation on the vascular cambium of juvenile and mature scions of *Larix laricina* (Du Roi) K. Koch that had been grafted on seedling rootstocks. Comparisons between the juvenile and mature cambium in nuclear genome size, nuclear shape, DNA concentration, number and volume of nucleoli per nucleus, and concentration of extranuclear RNAs, proteins and insoluble carbohydrates were conducted on four occasions during the annual cycle of cambial activity and dormancy. All investigated variables exhibited strong annual oscillations, whereas differences between the two maturation stages were less prominent. Many of the differences between the two phases could be explained by delayed spring reactivation and accelerated onset of dormancy in the mature cambium compared with the juvenile cambium. At the time of reactivation and during activity, the mature cambium exhibited lower genome size, lower DNA concentration, fewer nucleoli per nucleus and a higher extranuclear concentration of insoluble carbohydrates than the juvenile cambium. The dormant mature cambium contained more extranuclear RNAs than the dormant juvenile cambium. The observed differences provide circumstantial evidence of changes in chromatin organization or functioning, or both, during maturation.

Keywords: conifers, cyclophysis, cytology, dormancy, eastern larch, genome size, phase change.

Introduction

Postembryonic ontogeny of both herbaceous and woody plants can be represented as a continuum of juvenile, mature vegetative and reproductive phases. Each phase is characterized by more or less distinctive morphological, anatomical, biochemical and physiological features (for recent reviews see Bonga 1982, Hackett 1985, Poething 1990, Greenwood and Hutchison 1993, Hackett and Murray 1993). Transition between the juvenile and mature phases occurs in the apical meristem although it may be affected by factors produced in other plant parts (Wareing and Frydman 1976, Poething 1990). Once a particular phase is attained by the apical meristem, it is conserved during cell division. At present, neither the induction nor maintenance of phase change is understood. Two mechanisms operating at the chromatin or DNA level have been postulated to account for the conservation of gene expression through successive cell cycles. The first is conservation of cytidine methylation, a well-known feature of the animal kingdom (Weissbach et al. 1989); however, no methylated, maturation-specific portions of the genome have been identified. A second mechanism, operating at the level of gene rearrangements, has been proposed for *Hedera helix* L. (Wareing and Frydman 1976, Kessler and Reches 1977, Schäffner and Nagl 1979, König et al. 1987); however, the evidence is contradictory and the hypothesis has not been tested in other species. Therefore, we have examined the cambial cells of juvenile and mature *Larix laricina* (Du Roi) K. Koch to determine whether nuclear DNA changes result in cytologically detectable shifts in nuclear genome size.

There is evidence that the secondary xylem, a direct product of the cambium, is affected by apical maturation (Rumball 1963, Sweet and Maddern Harris 1976, Olesen 1982, Dodd 1988, Dodd and Walker 1988, Takemoto and Greenwood 1993). Although effects of apical maturation on the cambium have not been studied in any species, it is known that the properties of cambial cells vary depending on position in the bole (Bailey 1920, Bannan 1964, Riding and Little 1986, Mellerowicz et al. 1990). For example, in *Abies balsamea* (L.) Mill., the size of the nuclear genome is 1.3 times larger in 20-year-old cambium from the trunk base than in 1-year-old cambium from the top of the crown (Mellerowicz et al. 1989). Because cambial cells at the top of the crown are derived from a more mature apex than cambial cells at the base of the tree, genome size might reflect the degree of apical maturation. Alternatively, differences in genome size might result from differences in internal conditions between the crown and the bole of the tree, or they might be associated with the course of cambial development with age at a specific location along the bole. To distinguish among these possibilities, we examined cambia produced by juvenile and mature apices of *L. laricina* following grafting of scions of the same age to seedling rootstocks. In this system, the cambial cells at the time of their formation were at a similar distance from the roots and foliage leaves. The samples collected were from internodes of the...
same age. Thus, the major difference between the scions was the stage of maturity of the original donor scion. The nuclear DNA content was assessed at four different stages of cambial activity, because the size of the nuclear genome exhibits annual oscillations in other temperate species (Mellerowicz et al. 1989, 1993, Lloyd et al. 1994). In addition to nuclear DNA content, we investigated nuclear DNA concentration, number and volume of nucleoli, and extranuclear concentrations of RNAs, protein and insoluble carbohydrates.

Materials and methods

One-year-old scions of eastern larch were obtained from the upper crown of 19-year-old trees (mature material) and from their 1-year-old open-pollinated progeny (juvenile material) as described by Takemoto and Greenwood (1993). They were grafted on 2-year-old potted eastern larch rootstocks and grown in an unheated greenhouse for 4 years.

Two families, each comprising a grafted scion from the mother and one from its offspring, were sampled four times during the annual growth cycle: December (fully dormant cambium), March (cambial reactivation), May (fully active cambium) and August (onset of cambial dormancy). The uppermost section of the 2-year-old internode was selected for sampling at the first sampling occasion. At each subsequent sampling, an additional 5-cm long main stem segment was removed from below the most distal long shoot in the selected internode. Decapitation of the plant probably affected cambial behavior, but both juvenile and mature plants received the same treatment and the cambium continued to function. The segments were divided into 1-cm pieces or 0.3-cm slices that were further cut into 2–4 wedges. All pieces were fixed in FAA (formaldehyde/glacial acetic acid/95% ethanol/water, 1/1/14/4, v/v) (Johansen 1940) for 48 h and stored in 70% ethanol until used. The 1-cm pieces were used to prepare cambial cell isolates to examine nuclei, and the slices were softened in pectinase, and cambial cells were isolated with a needle under a dissecting microscope, spread on a microscope slide and air-dried. For DNA determination, chicken erythrocytes (Sigma) were placed alongside to serve as an internal DNA standard (Dhillon et al. 1977). They were removed from ethanol, dehydrated through a tertiary-butyl-alcohol series, embedded in Paraplast, sectioned transversely on a rotary microtome at 10 µm and mounted on chemically clean slides (Johansen 1940) without adhesive. The RNAs were stained with azure B (Jensen 1962), total proteins were stained with aniline blue-black (Riding and Little 1986), and total insoluble carbohydrates were stained with the periodic acid–Schiff’s (PAS) reaction (Johansen 1940). Concentrations were measured in relative units of light absorption with a microspectrophotometer equipped with narrow-band light filters having transmission maxima of 600 nm for azure B, 450 nm for aniline blue-black, and 550 nm for PAS (Lillie 1969). Light absorption was measured in 10 plugs per tree per date on sections that did not include the nucleus. Plug size was selected to contain a cambial fusiform cell, including its cytoplasm and cell walls.

Cambial phenology

The PAS-stained cambial cross sections were used to determine cambial phenology. Presence of recent periclinal divisions and expanding cambial derivatives as well as the cambial cell shape and wall thickness were used to assess current cambial activity. An indication of past cambial activity was obtained by counting fully differentiated tracheids from the current growth increment along one radius per sample.

Statistical analysis

Data were subjected to analysis of variance with maturity stage, family and date as random factors, followed by Duncan’s multiple range test when appropriate.

Results

Cambial phenology

In December, the juvenile and mature cambia of both families were dormant as indicated by the thick radial walls of fusiform cells, their radially flattened shape and lack of periclinal divisions or radially expanding cambial derivatives. In March, the swelling of fusiform cells was visible in the juvenile cambium in both families and in the mature cambium in Family 11, but the mature cambium of Family 14 showed no signs of reactivation. By May 27, the juvenile and mature cambia of both
families were active; fusiform cells were thin-walled, radially expanded, and numerous periclinal divisions and expanding derivatives were seen. In August, the juvenile cambium was active in Family 11 and dormant in Family 14, whereas the mature cambium was dormant in both families. In the previous annual increment, the number of tracheids produced per ring by the mature cambium was 63% of that produced by the juvenile cambium (data not shown).

Nuclear shape

Nuclear length to width ratio (nuclear slenderness ratio) varied with maturation stage and date (Table 1), and there was a significant stage × date interaction. The nuclear slenderness ratio exhibited an annual oscillation of similar amplitude in both maturation stages, with a minimum in May and a maximum in December (Figure 1); however, in the mature cambium, the ratio appeared to decrease later and to increase earlier than in the juvenile cambium.

Nuclear DNA content

Distributions of nuclear interphase DNA contents were unimodal for the juvenile and mature cambia in December and August and for the mature cambium in March (Figure 2). Bimodal distributions were typical for the juvenile cambium in March and both stages of maturation in May. In the bimodal distributions, the larger population, which had the lower DNA values, represented nuclei with a 2C DNA content in the prereplicative phase (G1) of the cell cycle. This was confirmed by measurements of DNA contents in telophase nuclei (data not shown). The smaller population represented nuclei with a 4C DNA content in a postreplicative phase (G2) of the cell cycle. No nuclei had DNA contents higher than the 4C DNA value. In the juvenile and mature cambia, the modal values for both G1 and G2 populations showed distinct seasonal shifts, with maxima on March 15 and minima on August 19.

The average 2C DNA content of G1 nuclei varied with maturation stage, date and family (Table 1 and Figure 1). The seasonal patterns of change in 2C DNA content in the juvenile and mature cambia were similar. The 2C DNA content increased from December (2C = 25 pg for both stages) to a maximum in March (2C = 29 pg for mature and 33 pg for juvenile) and then declined to a minimum in August (2C = 15 pg for both stages).

The DNA concentration of G1 nuclei depended on maturation stage, date and family (Table 1). In both juvenile and mature cambia, the concentration was higher in March and May than in August and December (Figure 1). In May and August, the DNA concentration was lower in mature cambium than in juvenile cambium.

Nucleoli

The average number of nucleoli per nucleus was lower in mature cambium than in juvenile cambium as a result of the

Table 1. Probability values for type III analysis of variance.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage</td>
</tr>
<tr>
<td>Nuclear slenderness</td>
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</tr>
<tr>
<td>2C DNA content</td>
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</tr>
<tr>
<td>DNA concentration</td>
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</tr>
<tr>
<td>Nucleolar number</td>
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<tr>
<td>Nucleolar volume</td>
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<tr>
<td>Cytoplasmic proteins</td>
<td>0.2005</td>
</tr>
<tr>
<td>Cytoplasmic carbohydrates</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Figure 1. Seasonal changes in nuclear slenderness (length/width ratio), DNA content and DNA concentration in the G1 fusiform cells of the vascular cambium produced by juvenile and mature scions grafted on seedling rootstocks. Mean ± SE, n = 40. Means followed by the same letter are not significantly different (P = 0.05, Duncan’s multiple range test).
A low number of nucleoli observed in mature cambium in May (Table 1 and Figure 3). The maximum number of nucleoli per nucleus was five or six in both juvenile and mature cambia, depending on the date (data not shown). Total volume of nucleoli per nucleus was identical for both maturation stages and varied with date (Table 1 and Figure 3). It was threefold higher in May than in August, with intermediate values observed in December.

**Extranuclear RNAs**

Concentrations of cytoplasmic and cell wall RNAs varied with maturation stage and date, and the stage × date interaction was significant (Table 1). For both stages, the concentration declined from a maximum in December to a minimum in May, and it started to increase again in August (Figure 4). The mature cambium had a higher RNA concentration than the juvenile cambium at all dates except May, when it was similar for both maturation stages.

**Extranuclear proteins**

Concentrations of cytoplasmic and cell wall proteins varied with date, and there was a significant stage × date interaction (Table 1). In juvenile cambium, protein concentrations declined from a high in December to a low in May–August (Figure 4). The protein concentrations in the mature cambium were similar to those in the juvenile cambium for all dates except March.

**Extranuclear insoluble carbohydrates**

Concentrations of cytoplasmic and cell wall carbohydrates varied with maturation stage, date and family, and the stage × date interaction was significant (Table 1). The seasonal pattern was similar for both stages. The carbohydrate concentrations were highest in December and declined to a minimum in May (Figure 4). Mature cambium had higher carbohydrate concentrations than juvenile cambium in May and August.

**Discussion**

Cambial phenology differed between maturation stages. Mature cambium reactivated later in the year and entered dormancy earlier than juvenile cambium. As a result, seasonal changes in nuclear slenderness ratio and DNA concentration (Figure 1), and concentrations of cytoplasmic RNAs, proteins and carbohydrates (Figure 4) were delayed in the mature cambium during reactivation and accelerated during the onset of dormancy compared with seasonal changes in the juvenile cambium. These findings are in agreement with the patterns of seasonal variation in cytological features known to oscillate on an annual basis in the cambium (Bailey 1920, Riding and Little 1986, Mellerowicz et al. 1990). The observations are also consistent with a greater tendency for dormancy in mature apical meristems than in juvenile meristems (Borchert 1976, Franclet 1981, Greenwood 1984).

The nuclear DNA contents in the juvenile and mature cambia reflected cambial activity. Thus, active cambium had cycling cells in the G₁, S and G₂ phases with DNA amounts...
The seasonal pattern of changes in 2C DNA content of juvenile and mature cambia, with a maximum in March and a minimum in August (Figures 1 and 2), is similar to the patterns observed in *Abies balsamea* (Mellerowicz et al. 1989, 1992, Lloyd et al. 1994) and *Fraxinus americana* L. (Zhong et al. 1995), although the magnitude of the seasonal increase in larch (15 to 32 pg) is the highest observed. The nature of the fraction of the genome that increases is not known. In *Abies balsamea*, there is evidence that ribosomal DNA is transiently amplified in early spring concomitant with the resumption of cell cycling (Lloyd et al. 1994); however, a different fraction of the genome might be due to a delay in amplification rather than to a difference in the degree of DNA amplification. A more frequent sampling schedule might resolve this question. Kessler and Reches (1977) reported that mature shoots of *Hedera helix* had a smaller genome than juvenile shoots; in addition, mature shoots had a smaller proportion of middle repetitive and AT-rich sequences but a larger proportion of highly repetitive DNA than juvenile shoots. Other researchers did not confirm these observations (Wareing and Frydman 1976, Schäffner and Nagl 1979, König et al. 1987). It is possible that the genome size of *Hedera helix* oscillates with season as we have observed in *Larix laricina, Abies balsamea* (Mellerowicz et al. 1989, 1992, Lloyd et al. 1994) and *Fraxinus americana* (Zhong et al. 1995), thereby confounding the effects of maturation.

The lower springtime DNA amplification and lower number of nucleoli per nucleus observed in mature cambium compared with juvenile cambium (Figures 1 and 3) suggest that a gross change in chromatin organization is involved in maturation (cf. Kessler and Reches 1977, Schäffner and Nagl 1979, Polito and Aliata 1981, Polito and Chang 1984). Such a change could be mediated through DNA methylation or activation of mobile DNA elements (Poething 1990). No major differences in DNA methylation of fully expanded leaves were observed between juvenile and mature larch scions (Greenwood et al. 1989); however, analysis of meristematic tissue and specific DNA fractions might be more informative.

Whatever the nature of the genomic change associated with maturation, it results in modifications of cellular metabolism by affecting gene transcription and protein synthesis. Extranuclear RNAs, proteins and insoluble carbohydrates all varied with date (Table 1 and Figure 4), similar to trends described for *Abies balsamea* (Riding and Little 1986). Extranuclear RNAs and carbohydrates also differed between maturity stages (Table 1). Comparisons between juvenile and mature cambia made for December and May, when both cambia were at similar phases of their annual cycle, showed that mature cambium stored more extranuclear RNAs during dormancy and contained more insoluble carbohydrates during active growth than juvenile cambium (Figure 4). A similar difference in RNA content was found in leaves of a *Pyrus* species (Ali and Westwood 1966). The high carbohydrate content of the mature tissue is consistent with the proposed key role for carbohydrates in the induction of maturation (Allsopp 1953, Trippi 1990, Materi and Cumming 1991).

A comparison of our findings with the positional effects on cambial cytology reported by Mellerowicz et al. (1993) indicated that most differences in the properties of cambial cells between the top and bottom of the bole may be attributed to internal conditions within the bole and not to the maturation stage of the apex at the time the cambium was formed. Thus, staining intensity for RNA, total carbohydrates and proteins was higher at the top (more mature) than at the bottom (more juvenile) of a tree (Riding and Little 1986). Our results suggest that differences in concentrations of RNA and carbohydrate, but not protein, might be due to maturation (Figure 4). Although the nuclear genome size was larger in the lower portion of the bole throughout the year (Mellerowicz et al. 1989), the
difference in nuclear genome size between juvenile and mature states was confined to the period of reactivation (Figure 1). The number of nucleoli was higher at the top (more mature) than at the bottom (more juvenile) of a tree (Mellerowicz et al. 1993), whereas the volume of nucleoli exhibited an opposite trend. In contrast, we observed no differences in nucleolar volume between the juvenile and mature scions, although the juvenile material had more nucleoli per nucleus than the mature material (Figure 3). We conclude that cells of the vascular cambium incorporate changes in maturation state of the apical meristem at the time of cambium formation as well as in situ changes associated with the increase in distance from the apical meristem as the bole increases in length and diameter.

References


