

Function and Dynamics of Auxin and Carbohydrates during Earlywood/Latewood Transition in Scots Pine¹

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In temperate regions the annual pattern of wood development is characterized by the formation of radially narrow and thick walled latewood cells. This takes place at the later part of the growing season when cambial cell division declines. To gain new insight into the regulation of this process, micro-analytical techniques were used to visualize the distribution of indole-3-acetic acid (IAA), soluble carbohydrates, and activities of sucrose (Suc)-metabolizing enzymes across the cambial region tissues in Scots pine (*Pinus sylvestris*). The total amount of IAA in the cambial region did not change with latewood initiation. But its radial distribution pattern was altered, resulting in an increased concentration in the cambial meristem and its recent derivatives. Thus, initiation of latewood formation and cessation of cambial cell division is not a consequence of decreased IAA concentrations in dividing and expanding cells. Rather, IAA most likely has a role in defining the altered developmental pattern associated with latewood formation. Carbohydrates and enzyme activities showed distinctive radial distribution patterns. Suc peaked in the phloem and decreased sharply to low levels across the cambial zone, whereas fructose and glucose reached their highest levels in the maturing tracheids. Suc synthase was the dominating Suc cleaving enzyme with a peak in the secondary wall-forming tracheids and in the phloem. Soluble acid invertase peaked in dividing and expanding cells. Suc-phosphate synthase had its highest activities in the phloem. Activities of cell wall bound invertase were low. The absence of major seasonal variations indicates that carbohydrate availability is not a trigger for latewood initiation. However, steep concentration gradients of the sugars suggest a role for sugar signaling in vascular development.

The annual transition from earlywood to latewood formation is a conspicuous developmental switch in temperate region trees. Latewood is induced during the later part of the growing season, when cell division activity in the cambial meristem declines. It involves a reduction in radial expansion and an increase in wall thickening of the cambial derivatives. Thus, earlywood is characterized by large-diameter and thin-walled tracheids, whereas latewood is composed of narrow diameter tracheids with thick cell walls. The induction of latewood cells and cambial dormancy offers a natural system by which we can gain new insight into the regulation of the basic growth processes of cell division and cell morphology.

Early investigators concluded that the initiation of latewood formation was induced by shortening of the photoperiod, and was associated with cessation of apical and needle growth at a time when current

year needles had become net exporters of photosynthetic assimilate (Richardson and Dinwoodie, 1960; Larson, 1964; Gordon and Larson, 1968). It was also observed that exogenous IAA could cause cambia that were forming narrow-diameter latewood tracheids to revert to forming large-diameter earlywood tracheids (Larson, 1960). These observations have led to a long-lasting dogma that propounds that the induction of both narrow-diameter latewood tracheids and growth cessation are induced by a reduction in IAA concentration in cambial tissues, whereas the induction of thick cell walls associated with the latewood tracheids is a result of an increased carbohydrate availability (Larson, 1969b).

This idea has inspired several investigations on the seasonal variation of auxin and carbohydrates in wood-forming tissues. Investigations into the concentration of IAA in samples containing the combined tissues from the cambial region (i.e. mature phloem plus dividing, expanding, and maturing phloem and xylem cells) collected during the earlywood/latewood transition have yielded conflicting results (Little and Wareing, 1981; Savidge et al., 1982; Savidge and Wareing, 1984; Sandberg and Ericsson, 1987; Sundberg, et al., 1987, 1990, 1993; Little and Pharis, 1995; Eklund et al., 1998) This may partly be explained by the fact that endogenous indole-3-acetic

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acid (IAA) is present as a steep concentration gradient across developing secondary vascular tissues, as recently demonstrated in Scots pine and hybrid aspen (Uggla et al., 1996, 1998; Tuominen et al., 1997; Sundberg et al., 2000). From this observation it is evident that the IAA concentration in the cells of specific developmental stages, such as division and expansion, cannot be judged from estimates based on samples comprising the combined cambial region tissues. Moreover, seasonal variation of the IAA concentration as calculated in earlier studies will be perturbed by the unavoidable variation in the amount and proportion of different cambial region tissues in the samples and will not necessarily reflect the amount of IAA supplied to cambial tissues by polar transport (Uggla et al., 1998; Sundberg et al., 2000). Therefore, these data are difficult to interpret. However, the observation of a steep concentration gradient across developing cambial tissues inspired the idea that IAA influences developmental patterns by providing positional information (Uggla et al., 1996) with support being obtained from studies with both Scots pine and hybrid aspen (Tuominen et al., 1997; Uggla et al., 1998; Sundberg et al., 2000).

There have only been a few studies on carbohydrates in relation to seasonal variation in cambial growth (Gordon and Larson, 1968; Parkerson and Whitmore, 1972; Savidge, 1991; Sundberg et al., 1993). The distribution patterns of carbohydrates across the different tissues of the cambial region have not been determined and, in analogy to the case of IAA, available information is therefore difficult to evaluate. Moreover, it is not just the presence of carbohydrates that is important, the capacity of tissues to use them in metabolic processes is just as significant or perhaps even more so. Thus, information concerning the activities of sugar-metabolizing enzymes would be very helpful. Such information has not been available for the separate tissues involved in wood formation. An understanding of the sugar dynamics in cambial tissues is also of interest in the light of recent evidence for sugar signaling in developmental control. Soluble carbohydrates such as Suc and Glc induce gene expression and seem to interact in, for example, light and hormonal responses (Sheen et al., 1999).

To gain new insight about the regulation of the earlywood/latewood transition, a detailed analysis of IAA and carbohydrates was performed in Scots pine (*Pinus sylvestris*) to evaluate their role in the transition process. The use of tangential cryosectioning of the cambial region, in combination with microscale analytical techniques, enabled us to report herein on the radial distribution patterns of IAA, Suc, Glc, Fru, and activities of Suc synthase (SuSy), acid invertase (AI), and Suc-phosphate synthase (SPS), across the tissues of the cambial region during latewood initiation.

RESULTS

Developmental Patterns and Anatomy

The sampling dates were selected to represent typical phases during the earlywood to latewood transition (Fig. 1). On July 4, an average of 21 ± 1.2 sd tracheary elements had been formed, still without any signs of latewood characteristics. The cambial zone, which consisted of 7.6 ± 0.4 cells per radial file, was still actively dividing. Samples from July 29 represent the initial stage of latewood formation as judged from the narrow radial diameter of the 4 to 7 most recent secondary wall-forming tracheids, in comparison with previously formed cells. At this date, an average of 29 ± 6.7 tracheids had been formed, and the cambial zone consisted of 5.9 ± 0.2 cells per radial file, still actively dividing. On August 19, an average of 37 ± 5.1 tracheids had been produced. The expansion zone was almost absent, and the number of cells in the cambial zone had decreased to 4.9 ± 1.0 and cell production was essentially complete. Typical latewood tracheids had been produced and cell wall thickening was still ongoing in the most recent tracheids. During the sampling period, the number of cells per radial cell file in the zone of tracheid expansion decreased from 8.1 ± 0.2 cells on July 4, to 3.1 ± 0.6 cells on July 29, and finally to 1.3 ± 0.6 cells on August 19. The radial diameter of the tracheids produced decreased as well. Fully ex-

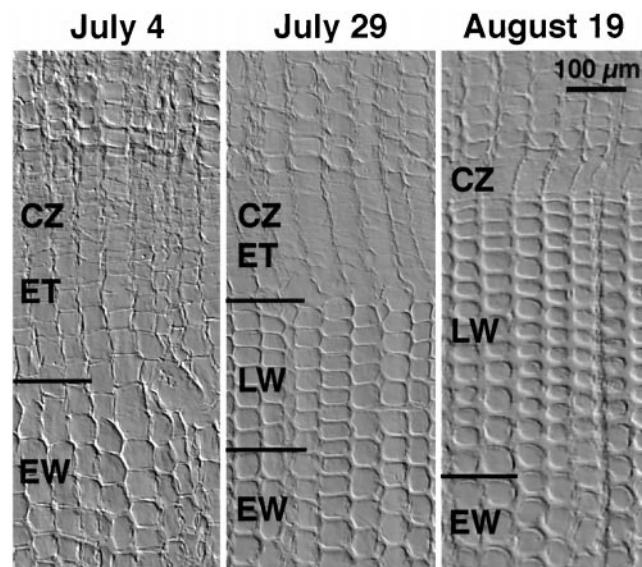


Figure 1. Representative transverse sections from the cambial region of Scots pine trees during the earlywood to latewood transition. On July 4, typical earlywood tracheids had been formed. On July 29, the radial width of the expansion zone had decreased, and tracheids undergoing secondary wall formation were radially narrow. On August 19, the expansion zone was almost absent, the most recent tracheids were very narrow and the thick wall of the latewood tracheids was obvious. CZ, Cambial zone; ET, expanding tracheids; EW, tracheids with earlywood characteristics; LW, tracheids with latewood characteristics.

panded earlywood tracheids had radial diameters of approximately 52 μm , compared with approximately 32 μm for typical latewood tracheids. The two most recently formed fully expanded latewood tracheids in the August 19 sample had radial diameters of approximately 13 μm . The number of cells per cell file in the zone of secondary wall formation increased slightly during the sampling period, e.g. 13 ± 1.3 cells on July 4, 14 ± 2.8 on July 29, and 16 ± 5.5 on August 19.

Distribution Patterns of IAA, Carbohydrates, Enzyme Activities, and Total Protein across the Cambial Region

IAA

From a peak level associated with the cambial zone, the IAA concentration steeply decreased toward phloem and xylem, reaching low and relatively stable levels close to the transition between expanding and secondary wall-forming cells (Fig. 2). This pattern of a steep concentration gradient across the developing cambial derivatives has been described and discussed earlier for Scots pine (Uggla et al., 1996, 1998) and hybrid aspen (Tuominen et al., 1997). An exceptional distribution pattern was evident in tree 2 sampled on July 4, where the peak IAA level was associated with expanding xylem derivatives. This anomaly is ascribed to a longitudinal waviness of the cambial zone. During the sampling period a consistent increase in peak concentration of IAA was evident, but the amount of IAA (IAA_{tot}) in all cambial region tissues was fairly constant. It is of consequence that the radial width of the IAA distribution decreased in close association with the decrease in the combined radial width of the cambial and expansion zones.

Carbohydrates and Enzyme Activities

Suc is supplied to the tissues of the cambial region via phloem sieve cells and indeed the phloem contained the highest levels of Suc (Fig. 2). Peak values from 100 $\mu\text{g cm}^{-2}$ section up to 400 $\mu\text{g cm}^{-2}$ section were recorded. Based on estimates of the total protein content, this would correspond to approximately 8.3 and 33 $\mu\text{g } \mu\text{g}^{-1}$ protein, respectively. From dry weight measurements of samples and previous determinations of a water content of 70% in the phloem (Uggla et al., 1996), this would result in a maximum concentration of Suc in the phloem ranging from 6% to 23% of the water content (97 to 390 mM), or 130 to 530 mg per g dry weight. From the phloem, the level of Suc steeply decreased toward the cambial zone, reaching lowest levels in the developing xylem cells.

The radial distributions of Fru and Glc across cambial region tissues were different from that of Suc. Both compounds were present in low levels in the phloem and increased toward the xylem, reaching their highest levels at 20 to 60 $\mu\text{g cm}^{-2}$ section (1.2–

3.8 $\mu\text{g } \mu\text{g}^{-1}$ protein) in the zone of secondary wall formation. The water content in this zone was estimated to be 60%. A Glc or Fru content of 50- $\mu\text{g cm}^{-2}$ section would therefore correspond to a concentration of 92 mM or 3.3% of the water content. The centripetal increase in concentration was initiated earlier for Fru than for Glc, resulting in a Fru to Glc ratio of approximately 2 in the cambial zone. The ratio decreased toward 1 in the zone of secondary wall formation. From this position and inwards, the level of Fru consistently decreased, whereas the level of Glc remained stable across the entire zone of maturing xylem. Both sugars were present in low levels in the mature xylem. Due to failure in MS analysis, data from August 19 is missing for one of the trees.

The extractable activity of the Suc metabolizing enzymes, SuSy, AI, and SPS, was also analyzed (Fig. 3). SuSy was found to be the dominating Suc cleaving enzyme with a consistent peak activity in the zone of secondary wall formation, reaching values of 200 to 400 pkat cm^{-2} section (12–25 pkat μg^{-1} protein). A tendency of increasing values during the earlywood to latewood transition was evident. Another peak of approximately 100 pkat cm^{-2} section (8.3 pkat μg^{-1} protein) was found in the phloem. The other Suc cleaving enzyme, AI, was present in two forms, denoted AI_{sol} and AI_{cw}. The AI_{sol} activity was highest in the zones of cambial cell division and cell expansion, and, to a lesser degree, toward the point of transition between functional and non-functional phloem with activities approximately 10 to 20 pkat cm^{-2} section (0.7–1.3 pkat μg^{-1} protein). The AI_{cw} activity was low (approximately 0.2 pkat cm^{-2} section) in all cambial region tissues. The activity increased to rates between 3 and 7 pkat cm^{-2} section only in mature xylem (lack of expression on a protein basis is due to non-detectable levels of total protein in this tissue).

The strong dominance of SuSy in Suc cleaving activity in the phloem and the secondary wall forming xylem is apparent from the SuSy to AI_{sol} activity ratios, which was between 20 and 400, and between 50 and 3,700, respectively. In the cambial and expansion zones, however, the SuSy to AI_{sol} ratio was lower, indicating a more pronounced role for AI_{sol} in these tissues. SPS, which is the key enzyme for Suc synthesis, showed considerable activity in all sampled tissues, although the highest values, between 20 and 50 pkat cm^{-2} section (1.7–4.2 pkat μg^{-1} protein), were restricted to the phloem.

The general appearance of the radial distribution pattern of carbohydrates and Suc-metabolizing enzymes across the cambial region, as described above, did not change in any typical trend during the sampling period except for an increase in the SuSy activity associated with latewood tracheids forming secondary walls. However, the amounts and peak values varied considerably between different trees and dates.

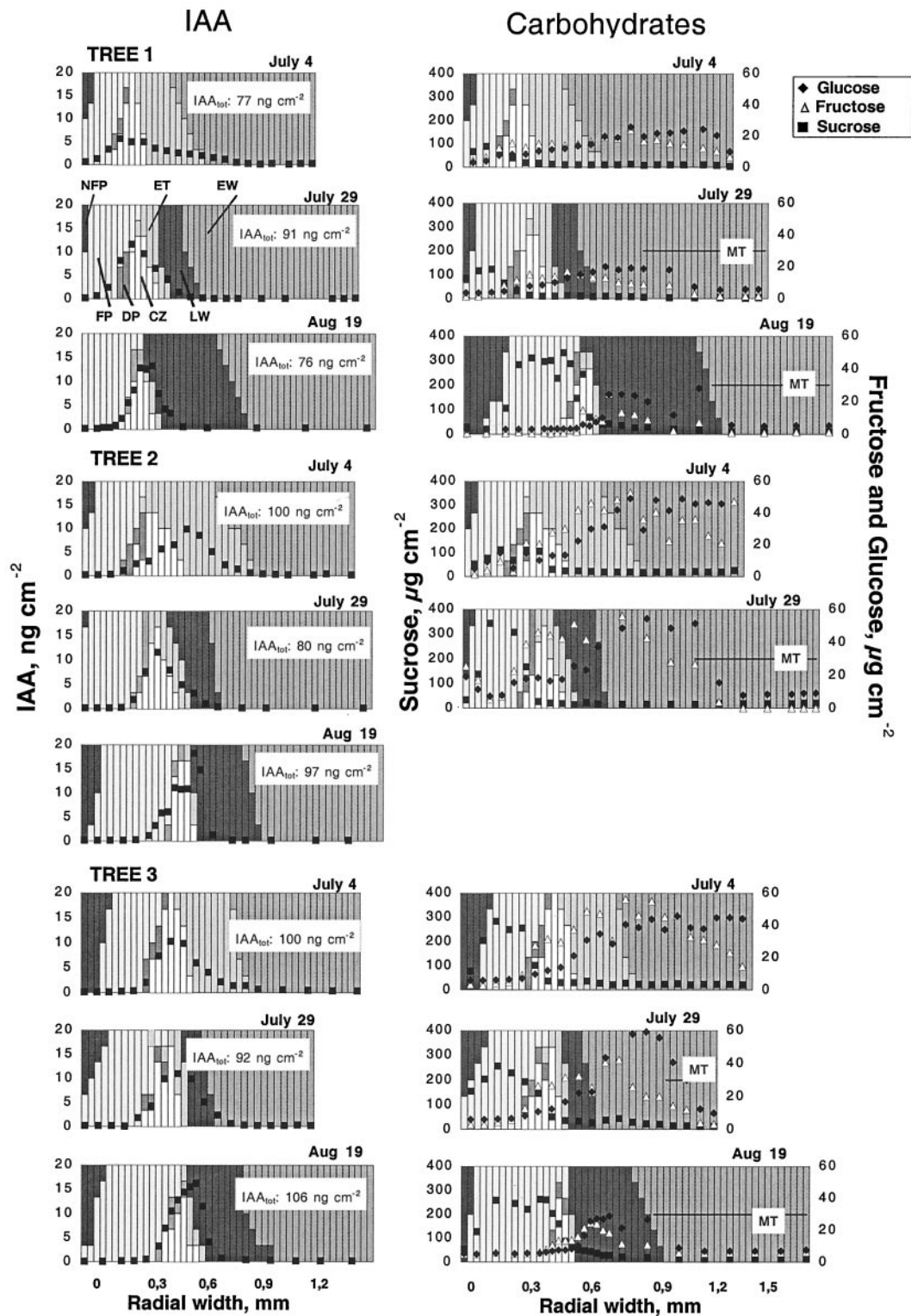


Figure 2. Radial distribution patterns of IAA and carbohydrates across the cambial meristem and its differentiating and mature derivatives at different dates in three different trees. Each column represents a 30- μ m-thick tangential section and its relative composition of different tissues. The different shadings of the columns are explained in the figure. Endogenous content of the substance per cm^2 section is indicated by the symbol in the column. For IAA, the total amount per cm^2 of all cambial region tissues (IAA_{tot} ; i.e. the integrated area below the gradient) is indicated at the upper right corner for each position. NFP, Nonfunctional phloem; FP, functional phloem; DP, developing phloem; CZ, cambial zone; ET, expanding tracheids; EW, maturing earlywood tracheids forming secondary wall; LW, maturing latewood tracheids forming secondary wall; MT, mature (dead) tracheids (only indicated in the right column).

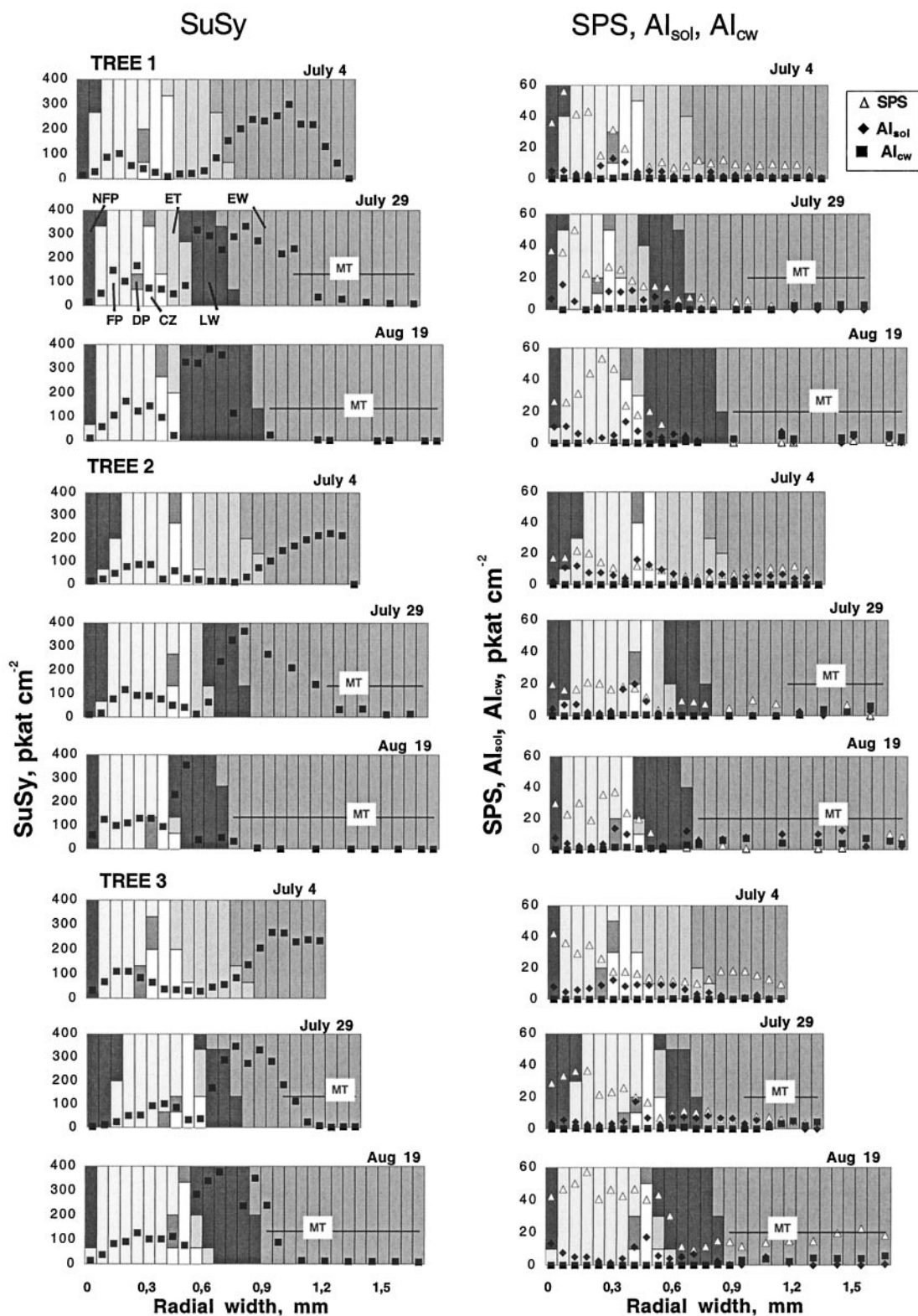


Figure 3. Radial distribution patterns of Suc-metabolizing enzyme activities across the cambial meristem and its differentiating and mature derivatives at different dates in three different trees. Each column represents two 30- μ m-thick tangential sections and their relative composition of different tissues. For explanations, see Figure 2.

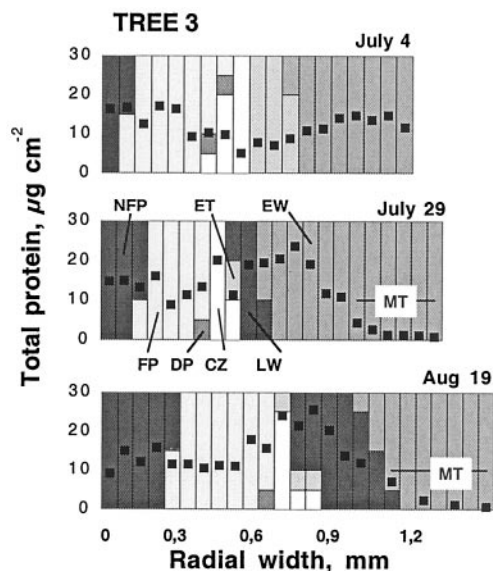


Figure 4. Radial distribution patterns of total protein content across the cambial meristem and its differentiating and mature derivatives at different dates. For explanations, see Figure 2.

Protein

The radial distribution pattern of total protein across the cambial region was determined for one tree (Fig. 4). Protein content varied from 5 to 25 $\mu\text{g cm}^{-2}$ section, except within the zone of mature tracheids, where it decreased to non-detectable levels. The protein data are used as an alternative basis for the expression of carbohydrate and enzyme activity. However, as the variation in protein content was relatively small between different tissues, this did not change the radial profiles of the enzyme activities very much. The one exception is for the estimates in samples from the late phase of secondary wall formation and mature tracheids where expression on a protein basis would increase the estimates.

DISCUSSION

The morphology of tracheary elements is determined by the rate and duration at which the developing cambial derivatives expand and form their secondary walls (Whitmore and Zahner, 1966; Wilson and Howard, 1968; Skene, 1969, 1972; Wodzicki, 1971; Denne, 1974; Dodd and Fox, 1990). The developmental phases of cell expansion and secondary wall formation are separated in space, giving rise to discrete developmental zones in a radial direction. The duration each derivative will remain in a particular developmental zone is governed by the radial width of the zone and the rate of cell production from the cambial meristem. Thus, the morphology of tracheary elements is a result of the combined effects of factors that: (a) determine developmental patterns, and (b) affect rates of cell division, cell expansion, and secondary wall formation. The formation of late-

wood, in particular, is a result of slower rates of cell division, decreased rates and duration of cell expansion, and a longer duration of secondary wall formation (Whitmore and Zahner, 1966; Wilson, 1966; Gregory, 1971; Wodzicki, 1971; Dodd and Fox, 1990).

The earlywood to latewood transition was not associated with a decreased supply of IAA to the cambial tissues (as reflected in total amount of IAA in the cambial region) (Figs. 2 and 5). This agrees with earlier studies of IAA contents in slow-growing cambial tissue at the base of Scots pine trees during the same transitional phase (Sundberg et al., 1990, 1993). Although a decrease in the amount of IAA has been observed in the more rapidly-growing cambial tissue in balsam fir during the earlywood to latewood transition (Sundberg et al., 1987), it must be concluded that latewood formation is not induced by a reduction in IAA supply. The detailed analysis in this study revealed that the earlywood to latewood transition was correlated with a reduction in the width of the radial distribution of IAA, and consequently, an increased peak concentration of IAA in the cambial meristem and its most recent derivatives. This demonstrates unequivocally that cessation of cambial cell division activity at the end of the growing season is not induced by a decreased IAA concentration in meristematic tissue. This is in accordance with the finding that IAA concentrations measured in dormant cambial zone cells at mid-winter were similar to concentrations in the dividing meristem at mid-summer

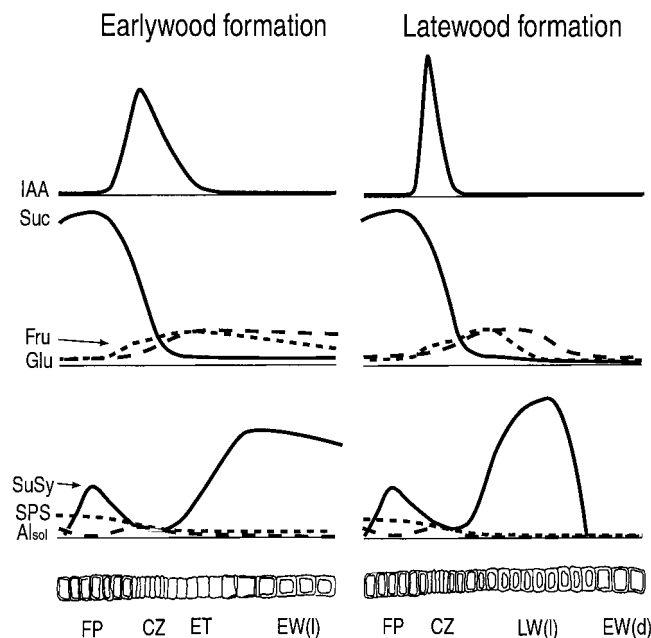


Figure 5. Schematic drawing describing the generalized distribution patterns of IAA, carbohydrates, and Suc-metabolizing enzyme activities across the cambial meristem and its differentiating and mature derivatives at earlywood and latewood formation. FP, Functional phloem; CZ, cambial zone; ET, expanding tracheids; EW(l), maturing (living) earlywood tracheids; EW(d), mature (dead) earlywood tracheids; LW(l), maturing (living) latewood tracheids.

(Uggla et al., 1996). It also agrees with the repeated finding of high IAA levels in cambial region tissues during dormancy (Little and Wareing, 1981; Savidge and Wareing, 1984; Sandberg and Ericsson, 1987; Sundberg et al., 1990; Savidge, 1991; Eklund et al., 1998). Furthermore, it is consistent with the observation that cessation of cambial activity cannot be inhibited by exogenous supply of IAA (Little and Bonga, 1974; Sundberg et al., 1987). The factor(s) inducing cessation of cell division activity in the cambial meristem as a consequence of shorter photoperiods therefore remains to be identified.

It can also be concluded that the decreased radial diameter of latewood tracheids is not due to a decreased IAA concentration in the expanding tracheids, ruling out a role for IAA in regulating the reduced rate of cell expansion observed to be involved in latewood formation (Dodd and Fox, 1990). However, our results show that the point along the IAA radial gradient where the concentration dropped steeply to low levels was closely related to the transition from the zone of expansion to the zone of secondary wall formation. This is consistent with previous findings in old stems of Scots pine (Uggla et al., 1996, 1998). Thus, we suggest that the developmental regulation of decreased width of the expansion zone is mediated by the decreased width of the IAA gradient. It is clear, however, that the altered appearance of IAA distribution is not a consequence of a decreased IAA supply but is caused by the yet unidentified mechanism(s) that determines the distance between the IAA peak in the cambial meristem and the margin of the concentration gradient.

The radial patterns of carbohydrates and of Suc metabolizing enzymes found in this study were related to tissue-specific processes (Figs. 2, 3, and 5). The Suc synthetic capacity (SPS activity) is predominantly confined to the functioning and non-functioning phloem. With Suc contents of up to 53% of dry matter, these tissues constitute an important source of carbon. Similar Suc contents (up to 66%) have been reported for source needles of gymnosperms (Blechsmidt-Schneider et al., 1997). As the compartmentation of Suc between axial translocation paths (sieve cells) and storage tissue (parenchyma) is not known, one can only speculate about the association between SPS-activities and Suc-pools and thus the possible occurrence of starch-Suc interconversions. The starch content in the living bark of Norway spruce and Scots pine (Sundberg et al., 1993; Egger et al., 1996) and outer sapwood of Scots pine (Höll, 1997) steadily decreases from the onset of cambial activity until late summer. This indicates that Suc in the radial translocation path (the rays) is partly derived from stored starch. However, unloading from the phloem is also likely and the dominant activities of SuSy may supply energy for unloading and loading in the phloem (Fu and Park, 1995).

Across the dividing and expanding tissues of the cambial region, the levels of Suc steeply decreased toward the xylem side, whereas the monomeric sugars gradually increased. This is associated with the peak activity of AI_{sol} in accordance with observations of high activities of AI_{sol} in a wide range of expanding tissues, such as young sink leaves (Morris and Arthur, 1984; Pate et al., 1985), elongating pods (Sung et al., 1994), expanding internodes, gravistimulated pulvini, fibrous roots, flower petals, and early stages of fruit expansion (Quick and Schaffer, 1996). Soluble AI has been demonstrated to be induced by exogenous IAA in a variety of tissues (Morris, 1996). Here, we demonstrate a close correlation between the presence of endogenous IAA and AI_{sol} activity in the primary walled tissues, confirming recent observations reported from eggplant and melon (Lee et al., 1997a, 1997b, 1997c). However, the increased AI_{sol} activity toward the non-functional phloem was not correlated with increased IAA content. Although our data support the view that IAA induces soluble AI activity, this induction seems to be differently regulated in different tissues.

SuSy activity was at its lowest level in the primary walled tissues and peaked in the zone of maturing tracheids where the secondary wall is formed. Here SuSy became the predominant enzyme and may be a measure of the sink strength of this tissue (Sung et al., 1993). The high SuSy activity in tracheids that are forming secondary walls may reflect its association with the synthesis of cell wall polysaccharides; SuSy has been suggested to be a part of the cellulose synthase complex (Amor et al., 1995). Moreover, it is very likely that SuSy is needed to sustain lignin biosynthesis (Hauch and Magel, 1998). This idea is supported by the high activities of SuSy, as expressed on a total protein basis, during the lignification process (data not shown) in the late phase of cell maturation when cellulose deposition is decreasing (Larson, 1969a). In the mature xylem, levels of soluble carbohydrates and enzyme activities were low. SuSy and AI_{sol} activities were partly replaced by AI_{cw} . This could be taken as evidence for apoplastic transport of Suc in rays of the mature xylem.

The only consistent variation in carbohydrates and activities of Suc metabolizing enzymes found during the earlywood/latewood transition was an increase in SuSy activity in tracheids undergoing secondary wall formation. But the impact of this increase on Suc turnover is probably minor. The lack of obvious seasonal changes in carbohydrate availability in our data is consistent with previous observations that latewood formation is under developmental, rather than metabolic, control. That is, it is the duration and not the rate of wall material deposition that causes the thicker cell walls of the latewood tracheids (Whitmore and Zahner, 1966; Skene, 1969, 1972; Wodzicki, 1971; Denne, 1974).

Although no variation in soluble sugars during earlywood/latewood transition was observed it is of interest to note that, similar to IAA, Suc, Glc, and Fru all exhibited steep concentration gradient across the developing vascular tissues. Much molecular and genetic evidence have recently emerged showing that soluble sugars are not only resources for energy and components for structures and storage but are also developmental regulators in plants, and cross talk between sugar and plant hormone signaling have been revealed (Sheen et al., 1999). A concentration gradient of Glc across developing cotyledons of *Vicia faba* was recently documented, and Glc was suggested to act as a morphogen in embryo development (Borisjuk et al., 1998). In callus and pith tissue a role for Suc as a developmental signal in the induction of xylem and phloem elements is well established (Wetmore and Rier, 1963; Jeffs and Northcote, 1966, 1967; Warren Wilson et al., 1994). When investigating the specificity of different sugars, Jeffs and Northcote (1967) found that Suc together with maltose and trehalose were the only sugars that induced both xylem and phloem elements. Glc and Fru induced xylem elements only, whereas other monosaccharides investigated had no effect. Moreover, ratios of Suc and auxin was observed to affect the direction of differentiation into phloem and xylem (Wetmore and Rier, 1963; Jeffs and Northcote, 1966), and opposing concentration gradients of Suc and auxin has been hypothesized to provide positional information for xylem and phloem development (Warren Wilson and Warren Wilson, 1984). Our data provide the first evidence for concentration gradients of soluble sugars across developing vascular tissues in plants, and with the accumulating evidence for sugars as developmental signals it seems likely that these gradients provides positional information for pattern formation in cambial growth.

MATERIALS AND METHODS

Plant Material

The plant material consisted of 44-year-old Scots pine (*Pinus sylvestris*) trees growing in northern Sweden (64° 21' N, 19° 46' E). Three trees were sampled at eight dates from early July to early September. Blocks (2 by 10 cm), consisting of extraxylary tissues and a few annual rings, were collected at breast height around the stem. The blocks were immediately frozen in liquid nitrogen, transported to the laboratory on dry ice, and stored at -80°C. Small samples (2 by 2 cm) were collected in parallel, and, after transverse sectioning with a razor-blade, were subjected to microscopic inspection to monitor the progression of latewood formation. On the basis of this assessment, samples from three dates, representative of different phases during the earlywood to latewood transition, were selected for further analysis. The dates were: July 4, during formation of earlywood tracheids; July 29, during the initial stage of late-

wood formation; and August 19, during the late stage of latewood formation (Fig. 1).

Sample Preparation and Anatomical Characterization

Analysis was performed on 30- μ m-thick, longitudinal tangential sections from tissues of the cambial region. The sections were obtained by centripetal tangential cryosectioning of an approximate 3- by 15-mm specimen obtained from a 2- by 10-cm block. The sampling and anatomical characterization of the tissues in each section was performed in accordance to Uggla et al. (1996, 1998).

IAA and carbohydrates were measured in alternate sections from the same series of sections for the two first collection dates and in separate section series for the last date. Suc metabolic enzymes and total protein were measured in separate series of sections, and each sample consisted of two pooled sections. For total protein, only one tree per sampling date was analyzed.

The number of cells per radial cell file in the cambial zone, the zones of expansion and secondary wall formation, and the mature earlywood and latewood, were determined for each series of sections. The outer radial diameters of earlywood, mid-latewood, and the most recently formed latewood tracheids were measured for three tracheids in each of nine radial files on a transverse section from the latest sampling date. As the border between earlywood to latewood was determined before secondary wall thickening had been completed, the often-used Mork definition of latewood (Denne, 1988) could not be used. Therefore, latewood tracheids were distinguished by their relatively narrow radial diameters (Fig. 1).

Quantification of IAA

Endogenous IAA was quantified using an isotope dilution mass spectrometry technique, according to the method of Edlund et al. (1995). One to 6 ng [¹³C₆]IAA (Cambridge Isotope Laboratories, Woburn, MA) was added to each sample as an internal standard. Analysis was performed by gas chromatography (GC)-selected reaction monitoring-mass spectrometry (MS), using a JMS-SX/SX102A instrument (JEOL, Tokyo).

Quantification of Carbohydrates

In the sections destined for carbohydrate analysis, Suc, Fru, and Glc were measured. Before extraction, each section was freeze-dried, weighed, and then placed in boiling water for 10 min in a sealed tube (Eppendorf Scientific, Westbury, NY) to inactivate endogenous hydrolytic enzyme activities. The sample was then homogenized in liquid nitrogen with a conical metal pestle connected to an electrical drill. Five-hundred microliters of distilled water, containing 20 μ g phenyl- β -D-glucoside (Sigma, St. Louis) as an internal standard, was added to the Eppendorf tube, and the sample was extracted with continuous shaking for 1 h. After centrifugation, the supernatant was transferred to a test tube and evaporated to dryness. Prior to GC-MS

analysis the sample was derivatized by incubation for 60 min at 70°C in 100 μ L 3% (w/v) hydroxylamine (Sigma) in freshly distilled pyridine. It was then silylated with 25 μ L *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% (w/v) trimethylchlorosilane (Pierce Chemical, Rockford, IL) for 15 min at 70°C, before dilution with heptane to approximately 10 mL. The derivatization procedure resulted in oxime-trimethylsilyl derivatives of Fru and Glc and the trimethylsilyl derivative of Suc.

Quantitative analysis was performed by GC-high resolution-selected ion monitoring-MS. The samples were injected split-less by an autosampler (model 7,673, Hewlett-Packard, Palo Alto, CA) into a gas chromatograph (model 5,890, Hewlett-Packard) equipped with a 25 m \times 0.25 mm i.d.-fused silica capillary column with a chemically bound 0.25 μ m CP-SIL-5 CB/MS stationary phase (Chrompack, Amsterdam). The injector temperature was 260°C, and the column temperature was held at 70°C for 2 min, then increased by 30°C min⁻¹ to 160°C, and finally by 5°C min⁻¹ to 230°C. The column effluent was introduced into the ion source of a JEOL, JMS-SX/SX102A tandem mass spectrometer (JEOL). The mass spectrometer was used in the single MS-mode. The interface and the ion source temperatures were 270°C. Ions were generated with 70 eV at an ionization current of 150 μ A. High resolution-selected ion monitoring measurements at a resolution of 5,000 were performed using accelerating voltage switching from 10 kV. The dwell time was 50 ms, and *m/z* 205.115, *m/z* 217.116, *m/z* 307.1581, *m/z* 319.1581, and *m/z* 361.1687 values were recorded. Perfluorokerosene was used as a reference compound.

Calibration curves were obtained using 1.25 ng to 40 ng Fru, Glc, and Suc with 7.5 ng phenyl-*b*- β -glucoside as the internal standard. As syn- and anti-conformation resulted in double peaks for the analysis of Fru and Glc, both peaks were integrated for standard curves and samples. The identity of the quantified compounds were verified by full scan mass spectrometry. The absence of interfering compounds in the quantitative analysis was verified by comparing ratios between different fragments. All data were processed by the MS-MP7010D data system (JEOL).

Measurement of Enzyme Activities

In the freeze-dried sections destined for analysis of Suc-metabolizing enzymes, the activities of SPS, SuSy, and the two forms of AI, denoted soluble acid invertase (AI_{sol}), and cell wall bound acid invertase (AI_{cw}) were measured. After weighing on an ultramicro balance (Sartorius Ultramicro, Göttingen, Germany), each pair of sections, ranging between 0.2 and 1.5 mg dry weight, were transferred to Pyrex tubes (2.3 mm i.d.), containing 7 mg of Polyclar AT. The material was homogenized under liquid nitrogen cooling, using a high-speed dental drill (Meisinger 21/023H; Guttenberger et al., 1998). Crude extracts were prepared by the subsequent addition of 255 μ L of extraction medium (0.1 M/0.3 M Tris/borate, pH 7.5, containing 1% [w/v] bovine serum albumin [BSA] and 14 mM β -mercaptoethanol), gentle mixing on ice for 20 min and centrifugation (8 min,

8,000g). AI_{cw} was extracted by re-extracting the pellet with extraction medium supplemented with NaCl (100 μ L, 1 M NaCl final concentration). Ten microliters of each of the crude extracts was assayed for enzyme activities using a microplate reader system (340 ATTC, SLT, Salzburg, Austria) according to Egger and Hampp (1993), modified for the specific characteristics of the pine enzymes. SPS activity was measured under non-limiting conditions (21.5 mM Fru-6-P, 7.7 mM UDP-Glc, 55 mM Glc-6-P, all final concentrations; pH 7.5; 65 μ L total volume) via the formation of UDP. UDP was assayed as the consumption of NADH in the presence of pyruvate kinase and lactate dehydrogenase (0.36 mM NADH, 1 mM phosphoenolpyruvate, 1.2 mM MgCl₂, 15.5 units of lactate dehydrogenase/mL, 8.6 units of pyruvate kinase/mL). The determination of SuSy activity was based on a two-step assay. First, UDP-Glc was formed by the enzyme in the specific step (total volume of 40 μ L, pH 7.1, 250 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 250 mM Suc, 6.25 mM UDP). Second, the product, UDP-Glc, was quantified via UDP-Glc dehydrogenase in the indicator step (300 mM Gly buffer, 14 mM NAD, 0.043 unit of UDP-Glc dehydrogenase/mL; pH 8.3; total volume of 70 μ L). Activities of AI_{sol} and AI_{cw} were assayed by measuring the amount of Glc and Fru (150 mM HEPES, 1.5 mM MgSO₄, 1.55 mM NADP, 4.07 mM ATP, 4.3 units of phospho-Glc-isomerase/mL, 2.2 units of Glc-6-P dehydrogenase/mL, 9.2 units of hexokinase/mL; pH 7.0; total volume of 70 μ L) formed from Suc in the specific step (68 mM citrate/86 mM phosphate buffer, 220 mM Suc; pH 4.0; total volume of 45 μ L). For all enzyme assay, blanks were run for each individual sample and thus for each tissue. In the blanks the specific substrate was omitted to assay unspecific background, such as endogenous metabolite pools of e.g. UDP-glucose, pyridine nucleotides, or tissue endogenous enzyme activities.

Measurement of Protein

Protein was determined by a dot-blot assay combining high sensitivity with minimal interference by extract constituents (Guttenberger et al., 1991). The assay is based on the binding of protein (2 μ L of aliquots of crude extracts prepared using extraction medium without BSA) to a cellulose acetate membrane. After staining (with the fluorescent dye, benzoxanthene yellow) and washing procedures the spots were eluted and the protein was fluorometrically quantified against BSA standards.

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