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THE CONTROL OF VASCULAR DEVELOPMENT[1]

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INTRODUCTION

Vascular development is a central theme in aspects of biology as diverse as evolution and cell biology. Our current conception of the control of development of this tissue is extremely rudimentary in comparison to the strides which have been made in other aspects of biology in recent years. Perhaps this is not surprising considering the complexity and subtleness of the

[1]Abbreviations used: AT, adenine thymidine; BAP, benzylamino purine; BUdR, bromuridine deoxyriboside; EM, electron microscope; ER, endoplasmic reticulum; FUdR, fluorouridine deoxyriboside; GA, gibberellin; NAA, naphthalene acetic acid; PAL, phenylalanine ammonia-lyase; QC, quiescent center; TdR, thymidine deoxyriboside.

problem. The study of the control of vascular development, however, offers an open field of investigation for the imaginative investigator interested in either basic aspects of eukaryotic developmental biology in general or in vascular plant physiology and development specifically. There have been a number of reviews of the subject from a variety of points of view: primary vascular development (29, 30); shoot apical meristem biology (140); root apical meristem biology (131); phloem regeneration (60); eukaryotic development (135); and xylem development in general (101, 102). The present review is an attempt to integrate more recent work on the developmental biology of vascularization with the earlier physiological and anatomical literature. The goal is to identify what we presently know about vascular development and to point out directions future work might take in order to expand our conceptualization of vascular development.

Vascular development involves first the formation of cells (procambium) which are committed at some point to become xylem and phloem as opposed to pith, cortex, mesophyll, and epidermis. Secondly, it involves the overt cytodifferentiation of these procambial cells into xylem, phloem, and cambium. What is the nature of these potential vascular cells which are currently called procambium during primary development or cambium during secondary development? Have they, at the time at which they become cytologically identifiable, actually progressed toward differentiation into xylem or phloem, or are they simply ordinary meristematic cells? Answers to these questions can only be obtained when the following questions are also answered: (*a*) What are the early biochemical markers of vascular development? (*b*) What regulates the spatial distribution of vascular cell formation within the stem, leaf, or root? (*c*) What mechanisms operate to prevent the formation of vascular elements in mature tissues or to stimulate their formation in mature tissues?

PROCAMBIUM: DEVELOPMENT IN THE SHOOT

The anatomical approach to vascular development in shoot apical meristems has generated some controversy concerning what should properly be called procambium. Until overt cytodifferentiation occurs, however, it is only possible to define these cells as being larger or smaller, denser or less dense, etc relative to surrounding cells. One cannot define these cells in terms of either their developmental *commitment* or their real developmental *state*. Thus, it seems presumptive at this point to state more than that one can observe some of the cytological changes in the progression of cells toward maturation as part of the vascular system. As yet, experiments have not been done which would allow one to define the stage at which the cells are committed to xylem or phloem differentiation unless they have overtly differentiated.

Esau (30) has taken the simplest view that in angiosperms the residual meristem, a residium of the shoot apical meristem, extends from the region of the apical initials into the differentiating region of the meristem where differential parenchymatization of the pith and cortex reveal its existence. Portions of the residual meristem are considered to differentiate into procambium, a meristem for the vascular system. Wardlaw (140) takes a different point of view based on his work with ferns. He observed a zone of cells which extends into the apical dome above the insertion of the youngest leaf primordium and which he considers to be procambium. In his view, vascular development is initiated prior to the development of leaf primordia. The development of the leaf primordia, in fact, causes disruption of this initially continuous procambium, resulting in the formation of parenchymatous leaf gaps in the stem. Surgical removal of the primordia resulted in elimination of gap formation (139). Thus Wardlaw believes that the cells above the leaf primordia represent procambial cells committed to vascular development unless specifically altered by influences from developing leaves. The interpretation of the histological preparations is clearly a subjective matter since one does not know from these kinds of experimental approaches whether the cells are committed to vascular development, to another fate, or not committed at all. Wardlaw's view can only be accepted when rigid criteria of procambium are developed and when those criteria are successfully applied to the cells he observed in the apical dome or when those cells are successfully isolated and induced to develop into vascular cells in vitro with relatively simple input.

Experimental approaches to the nature of procambium and the role of leaves in vascular development were taken by Helm (50) and Young (146). Both observed that in angiosperms the removal of the young leaf primordia caused the already recognizable procambium to develop into *parenchyma* and not to develop into vascular tissue nor even remain identifiable as procambium. Furthermore, Young (146) found that supplying auxin in the place of an excised leaf primordium prevented the change in the appearance of the procambium but did not stimulate its development into vascular tissue. More recently, McArthur & Steeves (75) attempted to clarify questions about the nature of procambium and the effects of leaves in *Geum chloense*. In this angiosperm the removal of the leaf primordia did not eliminate leaf gap parenchyma formation within the presumptive vascular cylinder as observed by Wardlaw (139) in ferns, although the cells did not differentiate into parenchyma as observed by Helm (50) and Young (146). McArthur & Steeves (75) concluded that the early phase of vascular development is not under specific control of the leaf since "provascular" tissue increased in longitudinal extent in the absence of the leaves. In *Geum* they found that vascular development progressed normally when auxin plus sucrose was applied in the absence of the leaf primordium but not with

auxin alone. In the absence of knowledge of the effect of the auxin plus sucrose in Young's (146) work with *Lupinus,* it is not possible to make any generalizations concerning the nature of the stability of provascular or procambial tissues in the absence of developing leaves. However, from the fact that presumptive vascular tissue did not undergo parenchymatization in *Geum* while it did in *Lupinus* in the absence of leaves, it may be that there are distinct differences in the regulation of the formation and in the stability of these cells.

Procambium is considered to "differentiate" continuously acropetally (e.g. it appears to develop from differentiated regions toward undifferentiated regions in the meristem) in the shoot (29, 76, 77, 136). Ball (3) found procambium formation to be initiated near a leaf base and to then differentiate both basipetally and acropetally. Esau (30) questioned this interpretation on the grounds that it is extremely difficult to discriminate procambium without intimate knowledge of the pattern of vascular development in the given shoot. Again, identification of these cells is a relatively subjective matter. In axillary shoots procambium may develop acropetally from the main shoot to the axillary bud (29, 42), or basipetally from the bud to the shoot (44). The direction is reported to be basipetal in adventitious buds (140). To state that the procambium is propagated continuously acropetally within the meristem would be conceptually clearer, because "differentiate" has a specific meaning which has not yet been demonstrated for procambium.

If one accepts that procambium is a meristem producing the vascular tissues, then it is clear that the continued functioning of this meristem in the shoot depends upon leaf formation. The effect of the leaf is in some cases replaceable by auxin or auxin plus sucrose. It remains questionable whether or not early changes in cellular appearance represent obligate changes in the course of vascular development. Without specific characteristics attributable to procambium, e.g. a specific enzyme activity, protein complement, etc, it is virtually impossible to adequately study the development of procambium per se, but rather, one must deal with the later stages of vascular element maturation.

THE CONTROL OF PRIMARY VASCULAR DIFFERENTIATION IN THE SHOOT

Primary vascular tissue development has been pursued both descriptively and experimentally in *Coleus* by Jacobs and collaborators. They report that in *Coleus* the differentiation of the phloem precedes the differentiation of the xylem with the first formed phloem elements appearing at the base of a leaf, and then differentiation proceeds acropetally into the leaf and basipe-

tally to connect with the mature vascular cells (reviewed in 60). A day or so later the xylem elements appear in the procambium at the same level at which the first phloem elements had earlier differentiated and the xylem too subsequently differentiates bidirectionally. Jacobs & Morrow (61) pursued the question of the role of the leaf in the induction of vascular development and found that the relative rates of auxin production by the leaf correlated well with the rates of xylem differentiation in the leaf trace. They concluded that auxin limits primary xylem differentiation in the leaf trace of *Coleus.* Wangermann (138) subsequently demonstrated that exogenous auxin could replace the leaf effect in the differentiation of the primary xylem of *Coleus.* It is of interest that the primary xylem in *Coleus* consists only of vessels, and thus this work agrees well with the results in *Phaseolus* of Jost (65), who found that xylem development stopped when leaves were excised. However, Jost (65) found that the application of auxin only restored vessel element differentiation. Leaf removal caused cessation of xylem fiber differentiation from the cambium in *Xanthium,* but vessels developed in normal numbers (106). NAA restored normal vascular development in that case (107).

Kinetin was first implicated in vascular development in intact plants as a result of the studies of Sorokin, Mathur & Thimann (122). Sorokin & Thimann (123) concluded that kinetin was responsible for the differentiation response, although the effects of the kinetin stimulation of growth were not experimentally separated from the xylogenesis response.

While it is generally believed that the leaf plays a dominant regulatory role in the overt cytodifferentiation of the procambium into vascular tissues, the various contributions of a leaf vs the rest of the shoot have not been fully revealed. Auxin is one of the principal regulatory agents supplied by the leaf. Although sucrose has been shown to improve the auxin effect, it is not likely that this is ordinarily derived from the leaf, because net exportation of sucrose from developing leaves is unlikely until after leaves are well beyond the stages under consideration in early vascular development. It seems that elucidation of the other regulatory components in primary vascular differentiation could be revealed in a rather straightforward way via meristem culture.

PROCAMBIUM: DEVELOPMENT IN THE ROOT

Vascular tissue development in the root was reviewed extensively by Torrey (131) and by Esau (30). As they have indicated, the root meristem is a less complex experimental tissue because there are no complications due to terminal appendages. Here the stages of vascular development occurred in a true linear sequence. The major questions concerning development in

roots have revolved around: (*a*) the nature of the pattern-forming influences, e.g. is it from within the meristem or imposed by mature tissues; (*b*) the nuclear cytology of procambium development into xylem; and (*c*) the ultrastructural changes in the cytoplasm associated with phloem development. This section deals primarily with the pattern-forming influences. As in the shoot, procambium is distinguished early from other tissues in the root by enlargement of cells which will constitute the epidermis and cortex. This results in a central procambial cylinder rather than a ring (131). This central cylinder, whose outermost boundary is the pericycle, eventually differentiates with a variety of flexible arrangements (within and between organisms) into vascular and, in some cases, pith tissue. At a young stage the cells within the central cyclinder do not have unique cytological features at the electron microscope level which might suggest their early commitment to different fates (89).

In Torrey's view (131) the ability of the central cylinder to differentiate into vascular tissue with a variety of patterns suggests that the term procambium is appropriate. This interpretation of procambium cannot carry any connotation of a commitment to vascular development and suggests that procambium in the root, as in the shoot, ought to be considered a meristem within which vascular development may be induced, as was suggested earlier in this review.

The formation of procambium from the apical initials follows a continuous acropetal sequence (reviewed in 131). Procambium development into vascular tissue is marked by the radial enlargement of the future metaxylem vessel elements very close to the apical initials (10, 11). This produces (in cross-sectional view) a linear array of xylem (or a triarch or hexarch, etc array of xylem) with phloem developing laterally to the xylem or between the points of the triarch, hexarch, etc arrays of xylem. Development at any point in time is further advanced in the basipetally located cells producing an axial developmental sequence. Details of overt differentiation in root and shoot systems at the cytological, cytochemical, and biochemical levels are treated together in the next section.

The sequence of vascular development allows for the possibility of pattern induction either by the mature tissues or patterning inherent in the design of the apical meristem. Thoday (126) suggested that the meristem could be autonomous in pattern formation. Bünning (11) performed experiments which were perhaps overinterpreted to support that concept. Clowes (13) took a more direct experimental approach of surgical manipulation of either the apex or the mature tissues and found an effect on the pattern of vascular development (triarch/hexarch, etc) only when the apex per se was treated but not when the mature tissues were manipulated. Torrey (129, 130) and Reinhard (98) unequivocally demonstrated that the apex deter-

mines the pattern of vascular development. They cultured 0.5 mm root tips (129, 130) or 0.7 mm root tips (98) aseptically on defined medium and found that these very small root tips (though not necessarily other small pieces of the root) produced roots with vascular cells with normal organization. Torrey (130) observed that the number of xylem strands (triarch, hexarch, etc) differentiating in the procambium correlated well with the diameter of the procambial cylinder. Auxin treatment of these cultured root tips both increased the size of the procambial cylinder and converted the normal triarch xylem pattern to hexarch (130). Odhnoff (87) found that GA caused xylem to differentiate closer to the root tip and concluded that GA has a direct effect on xylogenesis.

More recently Feldman & Torrey (37) found that in *Zea mays* (cv. Kelvedon) the complexity of the vascular pattern and the size of the QC could be manipulated coordinately. When the QC is small the vascular pattern is simple, and when it is large the pattern is more complex. As a result of changing the size of the QC, there is a shift in the position of the proximal meristem from which all of the cells of the axis except the root cap are derived. Feldman (35) found that in corn the vascular pattern is evident within 63 to 110 μm of the root cap junction. He proposed that changes in the size of the QC shift the point of pattern initiation to areas of the procambium which differ in diameter, and thus more or less area is actually available for the patterning influence to operate.

CYTOLOGICAL AND BIOCHEMICAL ASPECTS OF VASCULAR DIFFERENTIATION

Cytological Aspects

Basic cytological aspects of overt vascular cell differentiation were summarized by Esau (30). The status of phloem cytology specifically at the light and EM levels was summarized by Esau (31) and Cronshaw (17). Torrey, Fosket & Hepler (135), O'Brien (86), and Roberts (102) reviewed aspects of xylem cell differentiation. The following discussion begins with an account of the cytology of overt vascular cell differentiation and then proceeds to a more detailed presentation of aspects which have received considerable attention regarding mechanisms or possible markers of differentiation.

Within the phloem the sieve elements undergo considerable elongation during differentiation from procambium (33). They develop a thickened cell wall which does not lignify. Sieve areas form at rather specific positions in the wall. Neuberger & Evert (84) consider the initiation of wall thickening to be an early diagnostic feature. Significant changes in the nucleus or nucleolus have not been reported except that nuclear degeneration may be nearly completed when wall thickening and modification are completed.

The development of callose (β-1,3-glucan) is another diagnostic feature of sieve cells. Callose develops as a pad covering the developing sieve area. At maturity the pores of the sieve area are lined with callose, but they are not plugged in functioning cells. There are also early structural changes in the plastids of sieve elements, the main change being the formation of a protein crystalloid within the plastid (83). In many species P-protein formation is diagnostic of sieve element development (28, 31). P-protein can be observed in extremely young cells and may be the first indication of the fate of the cell (32). P-protein has been identified in phloem since Hartig's (49) description of phloem "slime," but it is not present in all species and may exist in phloem cells other than the sieve elements (31, 34). Northcote & Wooding (85) observed P-protein to be bound in membrane which they interpreted as ER in origin. Sieve elements are characterized by extensive development of the ER. The cell biology and biochemistry of P-protein development could be a fertile area of research. The interested reader should consult the following accounts of phloem development: (*a*) root protophloem (33); (*b*) gymnosperm phloem (82–84); (*c*) the general review by Cronshaw (17). The basic features are similar in each plant system as well as in callus (144) and hormone-induced phloem development in excised tobacco pith (17). P-protein structure per se has been explored also (88).

Cytological aspects of xylem cell differentiation were reviewed by O'Brien (86) and by Torrey, Fosket & Hepler (135). Regardless of the origin of the cell (procambium or mature parenchyma), there are several overlapping stages of development. The problem is to ascertain which of these are critical and then to determine how they are regulated. Cell expansion is normal in development from procambium or cambium but not pronounced (if it occurs at all) in development from mature parenchyma. Thus, nuclear or nucleolar changes relevant to expansion growth in procambium may confuse studies of xylogenesis. This problem should not occur in xylogenesis in cultured parenchyma. This problem then becomes one of identifying the time and place of xylem development in order to study nuclear changes.

Secondary wall deposition and lignification are diagnostic features of xylogenesis. The control of specific patterns of wall deposition has received considerable attention (reviewed in 102, 135). Pickett-Heaps (94) observed changes in the distribution of microtubules during xylem development. He found that initially they were evenly spaced along the cell wall, but later they grouped into bands and the cell wall thickenings were deposited adjacent to these bundles of microtubules. Pickett-Heaps (94) showed that the antimicrotubular drug colchicine disrupted the microtubules and the wall pattern. Cellulose microfibril orientation was also altered by colchicine in

developing xylem cells (51). How microtubules affect cell wall deposition remains an unsolved problem.

Lignin deposition is considered to be an early aspect of xylogenesis and it accompanies secondary wall deposition (145). However, as O'Brien (86) pointed out, we really do not have a stain for lignin at the EM level. Thus it is not possible to study very reliably with EM techniques how lignin deposition and wall formation are coordinated.

When secondary wall deposition and lignification near completion, portions of the primary wall may become hydrolyzed. This is followed or accompanied by protoplast autolysis. Little if anything is known about the control of these processes. At maturity the water-conducting cells (vessels, tracheids) consist of elaborately constructed cells without protoplasts but with variously modified wall regions which allow rather free liquid movement.

As noted previously, in the root the metaxylem is first detected by the radial expansion of these cells very near the apical initials (131). Large increases in nuclear DNA in these cells were reported (125). These observations generated the concept that increased ploidy was basic to xylem development (73). As will be seen, this correlation is not universal within a tissue or between organisms.

Corsi & Avanzi (15) reported an eightfold increase in the DNA of metaxylem cells of *Allium cepa* relative to pericycle cells which remained at the 2C level. They also reported changes in the DNA/histone ratios, but it is not clear that these were consistent or significant. Innocenti & Avanzi (56) interpret their observations of this system as follows: The developing metaxylem cells first undergo chromosome endo-reduplication very close to the apical initials. This is followed by amplification of the nucleolar organizer region of the DNA (rDNA coding for rRNA) which in turn is followed by extrusion of the nucleoli. Metaxylem cells showed a sixfold increase in rDNA (2). This cycle is repeated in the 2 to 5mm region from the tip (cap included in measurements) and the cells then stop DNA synthesis. During this time the metaxylem cells undergo expansion from 20 μm in length to 750 μm. Innocenti (55) reported a decrease in the histone/DNA ratio in developing metaxylem. The work cannot be evaluated adequately since the data were not presented except as ratios. Similar results were reported from work in tracheary element differentiation in the corn leaf (70, 124). As these investigators indicate, these observations need closer examination in systems more suitable for biochemical work. However, these studies suggest some experimental approaches that are not readily apparent from biochemical methods. The primary confusing aspect of these studies is the inability to separate events relevant to expansion from these relevant to overt xylo-

genesis. As will be seen, biochemical approaches have failed (unnecessarily) to separate replication-relevant events from tracheary element differentiation.

Biochemical Aspects

There are a few studies of vascular development at the biochemical level. Thornber & Northcote (128) studied changes in carbohydrate and lignin contents of cells developing from the cambium. The results were confusing because of the inadequate methods for such studies at that time. Jeffs & Northcote (63) found an increase in the xylose/arabinose ratio in bean callus after 1 to 2 months of hormone-stimulated xylogenesis. New methods are now available, and such analyses have been elegantly performed on primary cell walls (67) but not related to secondary wall development or vascularization.

Lignification of secondary cell walls begins very soon after secondary thickening is initiated (52, 145). The enzyme PAL catalyzes the conversion of phenylalanine to cinnamic acid and is a key reaction in the diversion of carbon from protein synthesis to lignin synthesis. PAL activity in various tissues is correlated positively with vascular development in the plant (105) and in hormone-treated callus (104). Most recently Haddon & Northcote (46) followed the time course of PAL activity during vascular nodule formation in bean callus. In this case the increased PAL activity correlated fairly well with the initiation of xylogenesis in the early stages. PAL activity declined later when it would seem that it ought to remain at least constant.

Haddon & Northcote (46, 47) also examined β-1,3-glucan synthetase activity as a marker of callose formation during phloem development. β-1,3-glucan synthetase activity followed a time course similar to PAL activity and to sieve cell differentiation. It is difficult to analyze this work since hormone-treated replicating (but nondifferentiating) controls were not examined.

Dalessandro & Northcote (21–23) initiated studies of the activities of various enzymes involved in nucleoside diphosphate sugar reactions which are expected to be involved in carbohydrate metabolism associated with vascular development. Enzyme activities were measured: (*a*) in the cambium, differentiating xylem or differentiated xylem of two angiosperms and two gymnosperms, or (*b*) in cultured Jerusalem artichoke tuber tissue in which hormone-induced vascular development occurred. Enzyme activity (cpm in product/mg protein) did not reveal consistent or large differences in the various tissues examined in *a*. The Jerusalem artichoke experiments suffered from the lack of adequate controls.

THE CONTROL OF NONPRIMARY VASCULAR DIFFERENTIATION

Differentiation from the Cambium

As the primary vascular tissues differentiate from procambium and the tissue ceases elongation, some of the procambial cells initiate replication in which the cell plate is formed in the longitudinal plane. These cells are now considered to be cambium and they will form all of the secondary vascular cells, e.g. the wood (xylem, cells differentiated interior to the cambium) and phloem (cells differentiated exterior to the cambium). In the stem the cells between the vascular (fascicular) bundles may initiate replication to form the interfascicular cambium. When this happens the cambium becomes a sheath of meristematic cells. The *initiation* of replication in the fascicular and interfascicular cambium and seasonal reactivation of the cambia are attributed to factors derived from the shoot (48, 118). Coster (16) suggested that a hormone was responsible and Snow (118) demonstrated the hormonal nature of the cambial stimulus. Snow (119) effectively replaced shoot stimulation with exogenous auxin. However, auxin is not always sufficient (96).

Siebers (112–114) studied the activation of the interfascicular cambium of *Ricinus communis.* He found that the *activation* of this cambium and the differentiation of xylem occurred in interfascicular tissue isolated and cultured on simple media without exogenous auxin. However, younger tissue required kinetin (113). He concluded that the development of this meristem was determined at a very young stage during procambium development and was not dependent upon homogenetic induction from the fascicular cambium.

Generally, studies of vascular development from the cambium have centered on explorations of the effects of combinations of hormones or photoperiod on both cambial division and differentiation in qualitative terms. Auxin stimulation of cambial division in decapitated plants may result in division and vascular differentiation (119) or cambial division without normal differentiation (65, 95, 118–121). In some cases normal vascular development may be elicited by GA (68) or may require auxin plus GA (25, 141). GA tends to be sufficient in rooted tissue but not in isolated stems.

Removal of young leaves and buds from *Xanthium* allows cambial activity to continue, once initiated, but the majority of the cells (fibers) do not differentiate. However, vessel elements, though smaller than normal in diameter, develop in normal numbers (106). In this case fiber differentiation is restored briefly by allowing one leaf to develop acropetal to this region

of the stem or by adding an auxin (NAA) instead (107). The derivatives of the cambium in *Xanthium* are not programmed as a result of their cambial origin; rather, there must be hormonal input to the new cells either during cell formation or subsequent to cell formation or both. If the auxin application is delayed, the cells derived in the meantime fail to respond in terms of fiber differentiation and remain as thin-walled unlignified parenchyma (107). Similar results were observed in other systems (6, 53).

Experimentally Induced Vascular Development

WOUND-INDUCED VASCULARIZATION Vascular tissue development may occur in portions of the plant other than from procambium in root and shoot meristems. The bulk of the vascular tissues are secondary and produced by the vascular cambium. However, the literature on secondary vascular tissue formation, although extensive, reflects a great interest in the alteration of subtle characteristics of the differentiated cells, e.g. wall thickness, cell length, etc. Very little of this literature deals specifically with the problem of the control of vascular cell differentiation. The literature on the cambium was reviewed by Brown (8) and by Phillipson, Ward & Butterfield (93). While some experimental work on vascular development from the cambium will be considered in this section, the emphasis of this section will be on experimentally induced vascular development in explanted parenchymatous tissue, callus, or wounded stems.

Vascular regeneration Vascular regeneration in parenchyma of wounded stems was first reported by Crüger (18). It was observed to progress basipetally around a wound (115), and Janse (62) suggested that a hormonal stimulus was responsible. von Kaan Albest (66) demonstrated that leaves acropetal to the wound produced factors necessary for vascular regeneration. She also showed that the vascular system per se must be wounded to obtain regeneration. Xylem regeneration may involve the transformation of some cells directly into xylem without cell division (117), although one does not know what unobserved biosynthetic events may be prerequisite. Parenchyma is not converted directly to sieve elements without cell division (66).

The major break in understanding vascular regeneration came from Jacobs' work (57–59), which showed that the role of the leaf in xylem regeneration was replaceable by auxin. Subsequently, La Motte & Jacobs (71, 72) showed that phloem regeneration was similarly controlled. Surprisingly, in view of studies in other systems, sucrose did not limit in the *Coleus* system (59, 72). In *Coleus* the leaves need to be present for 48 hours after wounding to obtain a xylogenic response (100). An inductive effect was evident since the vascular strands did not complete differentiation until 72

hours after wounding (1). The dependence of phloem regeneration on roots is variable. Houck & La Motte (54) report that roots are essential but can be replaced by a cytokinin (zeatin). However, previous work had shown no role for the roots in the same clone of *Coleus* (59).

The studies on hormone involvement are, with the exception of the auxin studies, in conflict. In addition to the differences reported concerning the role of roots and cytokinin noted above, there is a clear indication that kinetin inhibits wound vessel regeneration in a different clone of *Coleus* (40). In that clone GA enhanced the auxin effect on regeneration (103). But Thompson (127) found GA to have no effect on regeneration in the Princeton clone of *Coleus*. The manner of hormone presentation may be critical because Thompson (127) found that concentrations of auxin which are effective when applied apically are not effective in regeneration when applied basally.

Ultimately, regeneration is a wound-induced redevelopment of some mature parenchyma, but it is important to establish how these newly formed cells are "plumbed" into the vascular system. Is the new vascular tissue in the wound region "plumbed" directly into the old vascular system, e.g. old cells → new cells → old cells, or is there a more extensive replacement resulting in the insertion of the regenerated cells into a new vascular strand, e.g. new cells → new (regenerated) cells → new cells? These questions were posed by Benayoun et al (5). They interpret their work to support the replacement view, e.g. new cells → new (regenerated) cells → new cells. In their work the act of wounding actually causes a reduction in the *rate* of formation of vascular cells. It is probable that a variety of patterns of regeneration may be produced (D. E. Fosket, personal communication). Jacobs (60) has reviewed the earlier work on regeneration.

VASCULARIZATION IN CULTURED TISSUE Significant progress toward understanding the control of vascular development came when Camus (12) showed that strands of xylem elements could be induced to differentiate in the parenchymatous cells of endive callus as the result of the insertion of a growing bud. Ball (4) observed a similar effect when buds were regenerated in *Sequoia sempervirons* callus. One of the roles of the inserted bud is to supply auxin (12). Wetmore & Sorokin (143) performed similar experiments with lilac callus and buds and found that auxin imitated the bud effect in forming xylem strands only when supplied from a point source. The need for sucrose was demonstrated by Wetmore & Rier (142), and it was shown in *Parthenocissus tricuspidata* callus that tracheary element production varied quantitatively with changes in sucrose concentration when auxin was held constant (99). Furthermore, the ratio of xylem to phloem varied with sucrose concentration if auxin levels were constant: low sucrose (ca 1%)

elicited relatively more xylem while high sucrose (ca 4%) elicited relatively more phloem.

Those reports of carbohydrate effects led to the investigation of effects of alternative carbohydrate supplies which are still not satisfactorily resolved. In *Phaseolus* neither glucose plus fructose nor glucose alone replaced sucrose in producing organized nodules of xylem and phloem (64). However, glucose alone caused the production of scattered xylem elements. Fructose, mannose, xylose, rhamnose, arabinose, galactose, mannitol, or α-methyl glucoside at 2% did not elicit any differentiation, while cellobiose, lactose, and raffinose (2%) all elicited some xylem differentiation but not organized nodules of xylem and phloem. The sucrose effect on nodule differentiation was partially replaced quantitatively by 2% maltose or αα-trehalose (64). Sequential treatments of sucrose then IAA, or IAA then sucrose, indicated that nodule formation occurred only when IAA preceded or was accompanied by sucrose (64).

Helianthus tuberosa tissue forms tracheids in response to NAA and BAP in the presence of sucrose or glucose or trehalose, or to a lesser extent maltose, while other carbohydrates are very much less effective (78). Soluble starch (4%) stimulated xylogenesis in *H. tuberosa* quantitatively above that produced by optimal (4–8%) sucrose (78). Phillips & Dodds (90) were unable to confirm either that observation or the trehalose stimulation. Minocha & Halperin (78) observed an inhibition of differentiation without concomitant effects on cell division when increasing amounts of glucose were presented with optimal levels of sucrose. Increased amounts of glucose or sucrose alone did not have the same effect, but data on the glucose response was not included, They postulate a competitive uptake effect which could be tested. Replotting their data (percentage of differentiation vs total cell number) indicates that the glucose effect may be a simple shift of the percentage of differentiated cells curve up on the cell number scale. Whether this means a simple delay in the initiation of xylem differentiation or a decrease in rate of formation of xylem cannot be deduced. We are not at a point where the specific effects of carbohydrate sources on differentiation can be explained satisfactorily. The situation is made even worse by the inability of investigators to see the same qualitative responses in the same tissue. However, it appears that only those carbohydrates which support significant cell division also support tracheary element formation. The disproportionate effect of carbohydrates on differentiation relative to cell division should be investigated in a time course experiment to determine how carbohydrates affect the initiation and rate of xylogenesis.

The requirement for auxin observed in vascular differentiation relative to primary, secondary, or regenerated vascular cells is also observed consistently in all forms of tissue culture, e.g. callus (12), pith (14), pea root parenchyma (134), Jerusalem artichoke tuber parenchyma (20, 78), or let-

tuce pith (19, 24). The specific roles of auxin have not been deciphered but may be related to aspects of cell division or gene expression or carbohydrate metabolism or all these. Fosket (39) found that IAA did not alter the time lag preceding wound vessel member formation in *Coleus* where exogenous auxin increases the numbers of vascular cells formed but is not essential for their formation. Auxin has only a small affect on DNA synthesis in *Coleus* (39) and no affect by itself in pea (116), and so it cannot be assumed to increase the numbers of differentiated cells via increasing the numbers available for differentiation. It is not clear how auxin affects RNA or protein synthesis in those systems where auxin suffices to induce vascular differentiation since "state-of-the-art" biochemical approaches have not been utilized.

Cytokinins were first shown to stimulate xylogenesis in cultured tissue in conjunction with exogenous auxin by Bergmann (7). That observation was confirmed in a qualitative manner in several systems by Torrey (132). The first quantitative analysis was achieved by Fosket & Torrey (41), who used a soybean callus which required these hormones for replication as well. Increasing concentrations of kinetin stimulated xylogenesis relatively more than replication. The soybean system used no longer exists. It is an unfortunate fact that callus cultures are notoriously capricious and develop altered hormone responses as they age. It is essential to develop and employ adequate means of tissue preservation if these studies are to achieve their true potential in developmental studies.

Torrey & Fosket (134) observed similar responses to auxin and cytokinin in pea root parenchyma. This system is known to contain a heterogeneous cell population. However, xylogenesis occurs in the hormone-stimulated replicating cortical parenchyma (134). Phillips & Torrey (91) refined the analysis by punching out the central vascular cylinder. They established that xylogenesis and replication of these cells absolutely required auxin plus cytokinin and that replication preceded xylogenesis by several days. Shininger & Torrey (111) then established that, like the soybean system, increasing concentrations of cytokinin stimulated the rate of xylem cell formation relatively more than cell replication. Furthermore, these cells showed a continuous requirement for the hormones for xylogenesis while cell divisions were induced by shorter periods of cytokinin treatment and continued after cytokinin "removal." This may not be true in the Jerusalem artichoke tissue since preliminary work suggested that xylogenesis could continue after cytokinin withdrawal (78). Cytokinin is required in lettuce pith (19), carrot tissue (80), and *Zinnia* mesophyll (69) as well as pea (91, 111, 134) and artichoke (78). The specific role of cytokinin remains to be determined in all systems.

Cell replication is observed to precede and accompany xylogenesis in nearly all culture systems. In those cases where replication is not observed

it has *not* been shown that DNA synthesis did not occur. Within the plant, cell division normally accompanies primary and secondary vascular development as well as wound-induced or hormone-induced vascular development. On rare occasions tracheary element formation can also be observed in single cells in suspension cultures, but in general, replication is coincident (133). Do these observed cell divisions have any bearing on the problem of the regulation of vascular differentiation? I think they do.

In animal systems a considerable body of evidence has developed to support the concept that some forms of differentiation require cell replication (reviewed in 97). Rigorous evidence in plant systems is minimal and, like animal systems, depends upon the use of inhibitors (reviewed in 102 and 109).

In plants the first direct approach to the question was focused on the need for cell division in toto. Fosket (38) found that both FUdR and mitomycin C blocked replication and xylogenesis in *Coleus* explants. The FUdR effect was prevented by simultaneous TdR treatment. This indicates a need for some aspect of the division process. Although colchicine had similar effects in this system, it is less clear where the effect was manifest (D. E. Fosket, personal communication). Turgeon (137) concluded that DNA synthesis is not necessary for vascular differentiation, since FUdR did not prevent xylogenesis in lettuce pith. This interpretation may be premature because it was not shown rigorously that DNA synthesis was prevented. It is not adequate to demonstrate a lack of increase in cell numbers or to measure DNA microspectrophotometrically in 0.5% of the cells (when 6% will differentiate) and conclude that DNA synthesis was totally blocked. In fact, FUdR had a pronounced effect on vascular differentiation since it reduced vascular differentiation to 3% of the control level.

The evidence from gamma plantlets (45) is frequently used to support the view that vascular development does not require recent cell divisions. However, recent cell divisions have only been considered essential as a result of studies where it is known that they are occurring coincidently in time with the agents which actually induce differentiation. It is not known when induction takes place relative to overt cytodifferentiation in the gamma plantlets. We do not yet know why replication is essential although the pea system is yielding some new insight.

Endopolyploidy is frequently observed to be correlated with in vivo xylogenesis (73). Phillips & Torrey (92) and Dodds & Phillips (26) carefully eliminated this possibility as being a causal relationship in pea and Jerusalem artichoke respectively. However, the need for normal replication in the pea system remains.

Shininger (108) demonstrated a reversible FUdR inhibition of xylogenesis in the pea system, and with the use of BUdR (a thymidine analog)

demonstrated a specific role of DNA synthesis per se in the differentiation process. BUdR did not block cell replication. Both FUdR and BUdR effects are prevented and reversed by simultaneous or subsequent TdR treatment. Both replication and xylogenesis were blocked within 48 hours of FUdR treatment and both recovered within 72 hours of TdR addition (108). How BUdR elicits its effect is unknown. BUdR incorporation into DNA is considered essential (74). BUdR is incorporated into the DNA of pea cells (108). In *Tetrahymena* BUdR blocks RNA synthesis in synchronized cells but only if it is available to cells during DNA synthesis and most specifically during replication of the ribosomal genes (74). Lykkesfeldt & Andersen (74) postulated that this BUdR effect results from its incorporation into AT-rich regions. Thus, the transcription of all AT-rich regions may be selectively inhibited, and this could conceivably block RNA synthesis related to xylogenesis but not that which is necessary for cell replication.

Factors other than hormones have not been extensively investigated relative to their use in studies of vascular development. Increased pressure stimulates xylem differentiation in stems (9). The mechanism has not been investigated, but Roberts (102) suggested it occurred through induced ethylene production. Doley & Leyton (27) found lowered water potential to stimulate xylogenesis and suggested that this might be the basis of the observed sucrose stimulation. Other investigators have not found osmotic agents in general to stimulate vascular development as efficiently as sucrose.

In Jerusalem artichoke tuber tissue it was observed that (*a*) reducing the nitrogen concentration of the medium, and (*b*) reducing the volume of medium are both stimulatory to xylogenesis (90). The volume effect does not seem due to a "conditioning" of the medium (90).

Light generally inhibits xylogenesis. In the Jerusalem artichoke, a brief exposure to dim white light inhibited cell replication and xylogenesis relative to a dim green light control (90). Continuous white light (relative to a brief exposure to white light) had little effect on replication but inhibited xylogenesis (90). Similar results have been observed in the pea system (T. L. Shininger, unpublished observations). In carrot culture, light can be a requirement for xylogenesis (79–81), but if so, it is replaceable by cytokinin (79). Studies of the light effect in other systems need to be conducted more conscientiously.

Temperature as a probe of vascular development has not been utilized generally. Gautheret (43) reported that in the Jerusalem artichoke callus temperatures less than 17°C were inhibitory. This was confirmed in freshly excised tubers as well (90). Naik (82) reported that a high temperature (35°C) stimulates xylogenesis in this system, but this was not confirmed by subsequent work (90). In any case, these effects have not been distinguished from effects on replication.

In a recent series of experiments with the pea system, I have found some specific effects of temperature on xylogenesis (110). Briefly, low (10°C) or high (30°C) temperatures inhibit xylogenesis relatively more than replication. Low temperature was studied extensively, and it was found to delay the initiation of xylem cell appearance by several days. However, once initiated, these cells appeared at essentially normal rates. Cell replication was not affected in this case. This is not caused by an affect on overt cytodifferentiation because transfer of cells to low temperature after the initiation of xylogenesis did not cause a drop in the rate of xylem cell appearance for at least 7 days. When cultures were initiated at low temperature and then shifted up, there was no transient increase in the rate of vascular cell appearance to suggest they had accumulated at a specific temperature-sensitive point in development. Brief low temperature treatments (24, 48 hours, etc) indicated that xylogenesis was temperature sensitive before replication of the cortical parenchyma was initiated. The results suggest that since xylogenesis can be uncoupled from the early rounds of replication, these early replications have no unique role in development. Since it is unlikely that cells committed to xylogenesis exist in the cortical parenchyma, it follows that xylogenesis in this system can be induced by defined exogenous stimuli whenever the cells are replicating at an appropriate temperature.

The pea system offers some interesting points for further analysis. At 25° both xylogenesis and nonxylem cell formation show nearly exponential kinetics. How this relates to the fate of individual replicating cells is not yet known. The possibilities are that one cell provides two daughters which differentiate or that one cell provides one cell for continued replication and one for vascular development or some complication of this scheme. Observations of sections have not yet revealed isolated single tracheary elements, but it is premature to state that they are not to be found in this system.

It is also interesting to note that in this system one can observe linearity between the log of the vascular cell number and log of the nonvascular cell number. This relationship is not disturbed by temperature changes. Nor is it altered by changes in cytokinin concentration which are known to affect the rates of xylem and nonxylem cell formation differentially. This may mean that the *rates* of these processes are coupled. If so the result of this is that changes in replication rates generate tissues with different percentages of vascular elements rather than a proportionate increase in the number of xylem and nonxylem cells. The log/log relationship is disturbed by BUdR. My reanalysis of other systems indicates that where adequate data exist the same relationship occurs (41, 90). Exactly how these responses are coupled remains to be investigated. It seems clear that this relationship indicates a basic developmental control mechanism.

CONCLUSIONS: Control of Vascularization in the Shoot and Root

The process of vascularization involves first the production of a population of cells in which vascular differentiation can be induced. The process ought to be viewed as a continuum of developmental events involving as a first identifiable step the distinguishing of the potential vascular cells from the nonvascular by differential parenchymatization. The nonparenchyma cells may now be considered to be in the pathway to vascular development but are not obligated to that fate because removal of exogenous stimuli (auxin, sucrose) allows them to divert to the parenchyma state. Procambium is a term that should be employed to describe potential vascular cells which are at this state of development. It should be possible to further break down the stages of differentiation within this concept of procambium. When these cells irreversibly change cytologically toward either the xylem or the phloem, they can be considered overtly differentiated and no longer procambial. In shoots, stimuli are derived in part from the developing leaves and in part from the mature shoot. In the root these factors may be derived in part from the terminal portions of the meristem (root cap, QC, and proximal meristem) and in part from more mature sections of the root.

In the shoot it is clear that the leaves play an important regulatory role in the formation and development of procambium as it is currently identified. However, we do not have a meaningful concept of procambium at a biochemical or ultrastructural level, and the development of concepts at these levels is important. Both auxin and sucrose are critical to procambium formation and to the formation of vascular cells in cultured tissue. We do not know the roles of either of these agents in the differentiation process.

Overt differentiation probably can be monitored at the biochemical level and certainly can be monitored at the ultrastructural level. It is conceivable that phloem differentiation could be followed immunologically in those organisms which form P-protein or biochemically with careful analyses of β-1,3-glucan synthetase activity in conjunction with cytological studies. It is not clear if significant nuclear or nucleolar changes occur in sieve element differentiation. The changes in the nuclei of differentiating tracheary elements may have been misleading, however.

Biochemical analyses of tracheary element differentiation may be more difficult until secondary cell wall composition is characterized in greater detail. Lignification probably can be used as a valid marker of xylem differentiation, especially since one really wants a marker characteristic of terminal aspects of cytodifferentiation. There exists the possibility that there is considerably more microtubular protein in differentiating xylem cells if microtubules are really associated with secondary wall deposition. Such a quantitative change might be detectable.

DNA synthesis seems critical to xylem differentiation. With biochemical markers of xylogenesis, it may be possible to determine the reason for the need for DNA synthesis.

The various roles of auxins, cytokinins, and occasionally gibberellins need meaningful exploration. We do not have an adequate understanding of these hormones even in simpler response systems. These hormones can only be understood when plant scientists generally become willing to explore hormone responses with truly rigorous and contemporary biochemical or genetic approaches. The exploitation of temperature and light may be useful and, because of the "neatness" of such experiments, the results might be extremely important.

The development of suspension cultures that will differentiate reasonably synchronously would be extremely useful. The recent work of Torrey (132) makes one optimistic comment about that approach.

The recent manipulations of the QC effect on development and attempts at physiological replacement of the QC with hormones bound to resin coated beads (36) provide impetus to explore these systems more imaginatively. Finally, the autonomous nature of the root tip makes it ideal for exploration of many of the sorts of questions which previously have been considered only in tissue culture. The chief advantage of the root tip culture is the production of vascular cells in *predictable* time and space. Thus it ought to be possible to define procambium in the root in terms of its responses to specific metabolic analogs or physical treatments in ways analogous to the early work of Torrey (132).

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