Protoplasma 130, 12–26 (1986)

PROTOPLASMA

Wound Phloem in Transition to Bundle Phloem in Primary Roots of *Pisum sativum* L.

I. Development of Bundle-Leaving Wound-Sieve Tubes

A. SCHULZ *

Zellenlehre, Universität Heidelberg

Received March 15, 1985
Accepted July 13, 1985

Summary

48 hours after interrupting the root stele of *Pisum*, wound phloem initiated (proximally or distally to the wound) to reconnect the vascular stumps was found to contain some nucleate wound-sieve elements. At the elongating end of an incomplete wound-sieve tube these elements exhibit a sequence of ultrastructural changes as known from protophloem-sieve tubes. Elongation occurs by the addition of newly divided (wound-) sieve-element/companion-cell complexes. In order to dedifferentiate and assume a new specialization formerly quiescent stelar or cortical cells require at least one (mostly more) preliminary division. Companion cells are consequently obligatory sister cells to wound-sieve elements.

By reconstruction using serial sections it could be shown that wound-sieve tubes elongate bidirectionally, starting in an early activated procambial cell of the stele. The elongation is directed by the existence of plasmodesmata, preferably when lying in primary pit fields, and by the plane of preceding divisions. Thus, the developing wound-sieve tube can deviate from the damaged bundle and radiate into the cortex as soon as the plane of the preceding divisions is favourable. In the opposite direction, elongating wound-sieve tubes run parallel to pre-existing phloem traces, thus broading their base at the bundle for the deviating part of the wound-sieve tube. Frequently an individual wound-sieve tube is supplemented at the bundle by a further wound-sieve tube which is partly running parallel to it. Both sieve tubes are interlinked with sieve plates by three-poled sieve elements.

Ultrastructurally, the developmental changes of nucleate wound-sieve elements follow the known pattern. In spite of its contrasting origin and odd shape a mature wound-sieve element eventually has the same contents as regular sieve elements: sieve-element plastids, mitochondria, stacked ER and small amounts of P-protein within an electronlucent cytoplasm.

* Correspondence and Reprints: Zellenlehre, Universität Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, Federal Republic of Germany.

Keywords: Phloem initiation; Phloem regeneration; *Pisum*; Sieve element, sequential differentiation; Wound-sieve elements.

1. Introduction

Following interruption of all or part of their vascular bundles some dicotyledons can restitute the severed bundle by developing new vascular traces, *i.e.* wound phloem and wound xylem, in the cortex parenchyma. Both traces deviate from the interrupted bundle and, after by-passing the wounded tissue, are directed either back towards the same or towards another vascular strand. Light and electron microscopic studies of the transition area between wound and regular phloem revealed that wound-sieve tubes do not connect directly to pre-existing sieve tubes, but open a new tier of sieve elements by cambial activity (ESCHRICH 1953, BENAYOUN *et al.* 1975, BEHNKE and SCHULZ 1980). The development of direct plasmatic connections , *i.e.* sieve pores, between differentiating wound-sieve elements and enucleate bundle-sieve elements is doubtful because of the considerable difference in metabolic activity between the cells involved, dependent on their different ontogenetic states, observed ultrastructurally by a difference in cytoplasmic density. However, some plasmatic contact, between wound-induced differentiating sieve elements and pre-existing conductive sieve tubes, which are somewhat isolated from the symplasmatic space of non-specialized cells, seems to be a precondition of phloem restitution which is functionally realized as soon as assimilates are conducted

through wound-sieve tubes. The development of plasmatic contact is a prerequisite, not only for phloem regeneration after experimental incision into vascular bundles, but also for vascular connection between stock and scion following horticultural or experimental (KOLLMANN *et al.* 1983) grafting. In both cases it is unlikely that all pre-existing sieve tubes in the entire internodium could be compensated by new tiers of wound initiated and cambially developed sieve tubes.
Experimental interruption of the sieve-element continuum initiates the development of laterally deviating wound-sieve tubes at a given time and in a defined area. As a result, early physiological changes within this area which initiate the development of vascular elements can be pursued. For instance, by histochemical detection of esterase activity within an arc-forming strand of the root cortex RANA and GAHAN (1983) demonstrated that 18 hours after the root stele of *Pisum* was severed some parenchyma cells are already programmed for vascular element formation. Similarly, early structural changes preceding the vascular element development within formerly quiescent tissue can be examined and are accessible to, *e.g.*, inhibition experiments.

The timing of wound-phloem development depends on the intensity of bundle severance. 5–6 days after *single* bundles in the stem of *Impatient* or *Coleus* were cut, the first complete wound-sieve tubes link the vascular bundles (ESCHRICH 1953, BEHNKE and SCHULZ 1980). However as early as 2–3 days after severing *all* bundles of the root stele of *Pisum* ROBBERTSE and MCCULLY (1979) observed the first wound-sieve tubes by-passing the wound and reconnecting the bundles. Wound-sieve elements in both *Coleus* and *Pisum* show comparable ultrastructure to regular sieve elements (BEHNKE and SCHULZ 1980, HARDHAM and MCCULLY 1982 a). As described earlier, the development of species-specific sieve-element plastids in wound phloem of *Coleus* and *Pisum* includes the remarkable conversion of amylo- or chloroplasts to sieve-element plastids (BEHNKE and SCHULZ 1983). By influencing the regenerating root with inhibitors HARDHAM and MCCULLY (1982 b) investigated, whether a preceding cell division is a precondition for the reprogramming of cells to become (wound-) vascular elements.

The investigation reported in this and in the accompanying paper deals with the early development of wound-sieve tubes near pre-existing bundle-sieve tubes in order to elucidate cell differentiation into wound-sieve elements and to obtain information on the kind of plasmatic contact between wound- and bundle-sieve tubes. The ultrastructure of differentiating members of deviating wound-sieve tubes and the state of bundle sieve elements 48 hours after cutting the root stele were followed in several examples, including serial sections through a regular phloem trace and its adjacent cortex parenchyma. The course of the depicted wound-sieve tubes and differentiation of their elements are described. The accompanying paper reports on the ultrastructure of severed bundle-sieve tubes and in particular the plasmatic contact between wound and regular phloem.

2. Material and Methods

Seeds of *Pisum sativum* L. c.v. "Kleine Rheinländerin" were surface-sterilized, rinsed, soaked and grown on Agar for three days as described earlier (BEHNKE and SCHULZ 1983). The vascular cylinder of the primary root was completely severed by a horizontal razor blade incision leaving only a small cortex bridge. In order to prevent adventitious root development the root was injured about 3–5 mm from the root-stem transition. The wounded plantlets were usually allowed 48 hours regeneration, to obtain early development stages of wound phloem.
Longitudinal hand sections containing wound and regenerating areas were immediately immersed in a buffered formaldehyde-glutaraldehyde-fixation mixture, in which they were kept for 3 hours at room temperature. The sections were washed in cacodylate buffer, post-fixed in 1% buffered OsO_4 and dehydrated through acetone. After polymerization the Epon/Araldite-embedded material was cut into semithin sections (about 1 µm thick) and surveyed with the light microscope for wound sieve tubes near regular phloem [using J_2KJ or aniline blue as dyes after dissolving the embedding material with NaOH-saturated ethanol (*cf.*, LANE and EUROPA 1965)] before ultrathin sections were cut using glass knives.
Since wound-sieve tubes run very irregularly, a representative case of the transition area between wound and regular phloem was cut as serial ultrathin sections, including only few semithin sections. The course of the wound-sieve tubes was analyzed by 3-D-reconstruction of the serial sections, using plasticine for the single members (see Fig. 13). In order to show correlation between sieve-tube members and their plasmatical condition a "symplanar projection" of electron micrographs supplements the analysis (see Figs. 6 and 10, 7 and 11, respectively). After tracing a wound-sieve tube through the series of sections, micrographs of different sections were mounted together. Thus, the differentiation sequence within the chosen, partly nucleate, wound-sieve tubes is projected onto one image plane (since it is concerned with sieve elements the "symplanar projection" has breaks in the image plane of neighbouring parenchyma cells).
To ease orientation, all sieve tubes within the serially sectioned area are denoted with capital letters, and their sieve-tube members numbered, beginning with the youngest, or the first sieve element sectioned near the wound.

3. Results

As described earlier (*cf.*, BEHNKE and SCHULZ 1983), complete interruption of the vascular cylinder initiates enhanced mitotic activity, radiating from the vascular

bundles into the cortex parenchyma, causing cell division of formerly quiescent cells. New cells walls are usually oriented in parallel arcs around the wound, resulting in similarly orientated smaller cells. At the earliest 54 hours after the experimental disruption of the vascular continuum first complete wound-sieve tubes reconnect the vascular stumps using the outer arcs of these small cells. At the same time wound-xylem elements develop within the innermost arc of cells, frequently abutting wound-bordering cells.

Hand sections through regenerating roots of *Pisum* show delicate new cell walls traversing the original cortex-parenchyma cells which have thicker walls (Fig. 1, 60 hours after wounding). Callose-positive parts of the original walls mark sieve plates (Fig. 2: arrows) thus characterizing adjoining cells as wound-sieve elements (Fig. 2: *se*). Smaller amounts of callose are found over the walls between sieve elements and companion cells (Fig. 2: small arrows). It is obvious, that the original cortex-parenchyma cell is not directly converted into a wound-sieve element, but divides at least twice to produce a sieve element and companion cell. The first division (*e.g.*, Fig. 1: *1*) results in a transitional cell (Fig. 1: *tc*) and a phloem mother cell. A second (or further) division initiates the specific differentiation of the sieve element and the companion cell. More than one division usually precedes (wound-) sieve-element/companion-cell complex formation, thus dividing the original cell into several small daughter cells.

Fig. 4 shows a wound-sieve tube above the wound which with three elements has already entered the cortex 48 hours after wounding. The youngest element of this sieve tube was found at the greatest distance from the vascular bundle. Obviously, the elongation of a wound-sieve tube into the cortex is caused by the continued addition of newly divided sieve-element/companion-cell complexes to the incomplete sieve tube.

The course, ultrastructure and sequential differentiation of wound-sieve tubes are demonstrated below by two examples of wound phloem 48 hours after wounding.

3.1. Course of Wound-Sieve Tubes

In order to leave the interrupted stele a developing wound-sieve tube must cross the pericycle and endodermis. Figs. 3–5 show a wound-sieve tube (*first example*) with sieve elements near pre-existing bundle-sieve tubes (above the wound) and parallel to immature (wound) tracheary elements (Fig. 4: *tr*). Deviating slightly from the vertically arranged cells of the stele it first crosses the procambial cells of the pericycle (Figs. 3 and 4: wound-sieve elements labelled with *8, 7, 6,* and *5*) and reaches the cortex via an endodermal cell, which has also divided and given rise to a sieve element (Figs. 3 and 4: *4*). Following the direction of preceding divisions, three sieve elements developed within the cortex parenchyma (Fig. 4: *3, 2,* and *1*) which is marked by the occurrence of intercellular spaces (Fig. 4: *in*). Very young wound-sieve elements (not in tiers as within the bundles) can only be identified by the callose deposits in their future sieve plates. Thus, the youngest members of this sieve tube could only be recognized in ultrathin section after a subsequent semithin section was stained with aniline blue (*cf.*, Fig. 4 with Figs. 3 and 5: *1, 2,* and *3*). The sieve plate between the youngest sieve elements (Fig. 5: *1* and *2*) shows the typical arrangement of primary pit fields: three groups of plasmodesmata,

Figs. 1 and 2. Longitudinal section through wound phloem 60 hours after wounding, stained with aniline blue

Fig. 1. Cortex cells repeatedly subdivided and clearly bordered by pre-existing cell walls. Consecutive divisions (*1* and *2*) within one cortex cell, resulting in a transitional cell (*tc*), a sieve element (*se*) and a companion cell (*cc*). Nomarski-Interference contrast. × 380

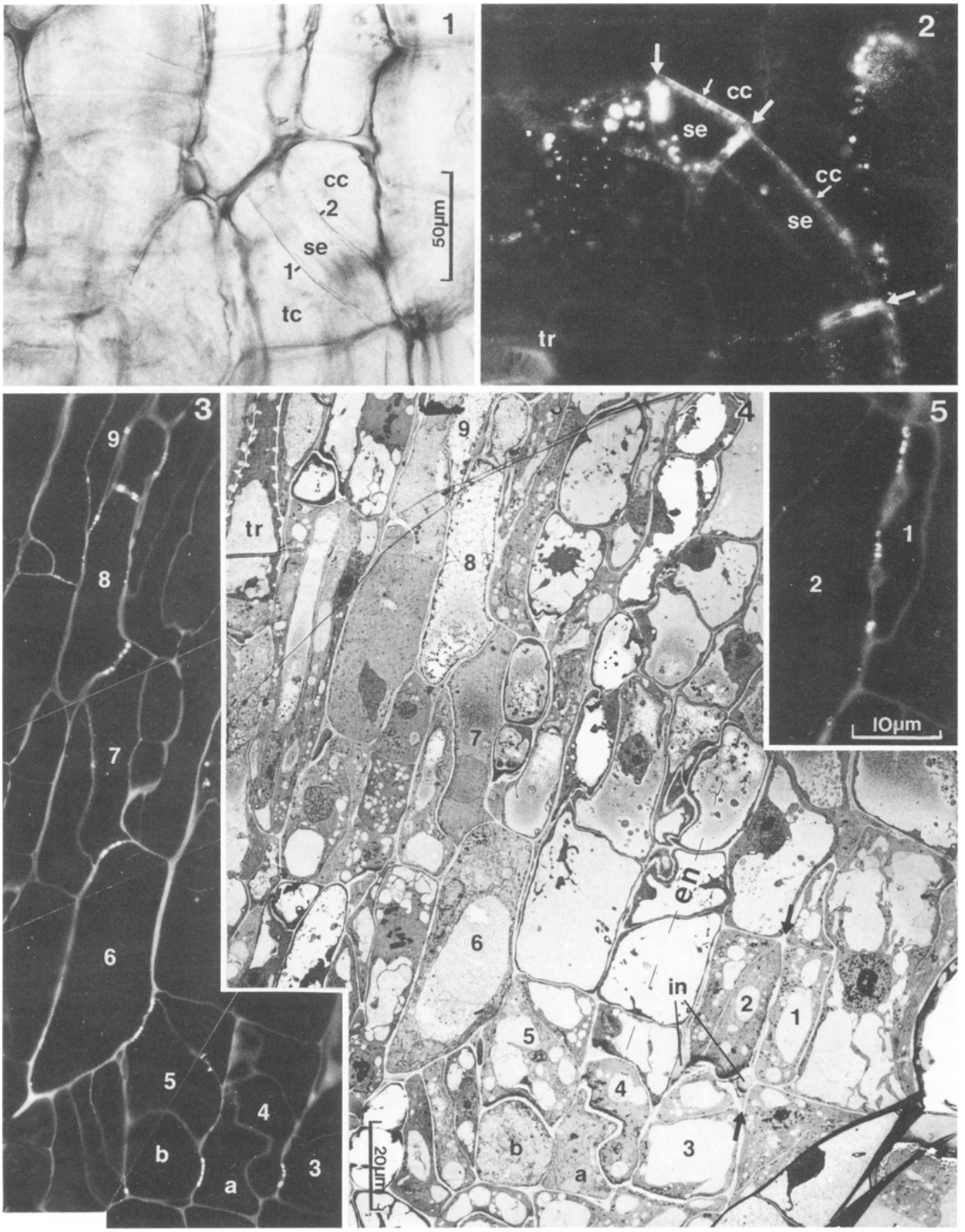
Fig. 2. (Fluorescence micrograph of Fig. 1) Sieve plates (arrows) are located in the pre-existing walls. Common walls between sieve elements (*se*) and companion cells (*cc*) are marked by callose spots (small arrows). Wound tracheary element (*tr*) with secondary wall thickening. × 380

Figs. 3–5. (Semithin- and ultrathin sections from a series, 48 hours after wounding.) Wound-sieve tube near a vascular bundle interrupted below the area shown. The sieve elements are numbered consecutively (*1—9*); beginning with the youngest

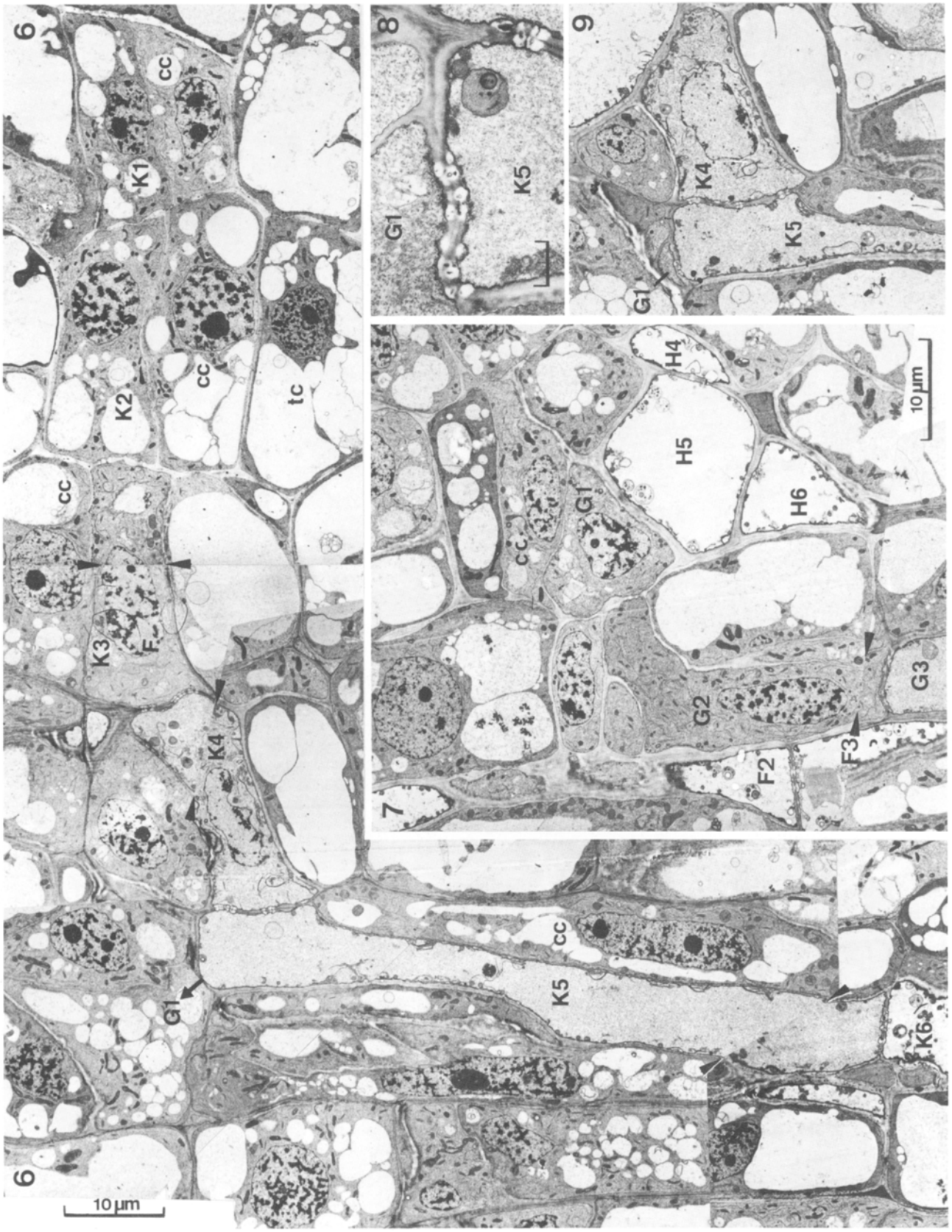
Fig. 3. Future sieve plates are indicated by callose fluorescence and link the sieve elements (*3—9*). Aniline blue, fluorescence microscopy. × 800. Marker in Fig. 4

Fig. 4. Ultrathin section showing the sequence of differntiating sieve elements *1—9* within the wound-sive tube. A branch of this sieve tube with two elements (*a* and *b*) leads out of the plane of sectioning. Sieve elements (*a* and *4*) traverse the original endodermis (*en*). The cortex is marked by intercellular spaces (*in*). Arrows indicate location of Fig. 5. × 800

Fig. 5. Next (semithin) section of the series. Future sieve pores linking sieve elements *1* and *2* and marked by callose fluorescence are arranged in groups. These sieve areas resemble pit fields separated by thicker wall parts. × 1,500


1
cc
2
se
1
tc
50µm
2
cc
se
cc
se
tr
3
9
8
7
6
5
4
b
a
3
4
tr
9
8
7
6
5
4
b
a
3
2
1
en
in
20µm
5
1
2
10µm

Figs. 1–5



Figs. 6–13. (Double-plate.) Contact area between wound phloem and a regular phloem bundle, which was interrupted about 150 µm above the area shown (48 hours after wounding). Fig. 6 continues in Fig. 10, Fig. 7 in Fig. 11 Figs. 6 and 10. Symplanar projection (see Material and Methods) of the developing wound-sieve tube *K*, with some nucleate sieve elements. Initially orientated parallel to earlier developed sieve tubes (*F* and *H*, see *F 5* and *H 9*) (with its members *K 8*—*K 5*) the wound-sieve tube *K* with four members (*K 4*—*K 1*) deviates from the bundle. *cc* companion cell, *tc* transitional cell. × 1,800

Figs. 7 and 11. (Symplanar projection.) Wound-sieve tube *G* lies parallel to the earlier initiated wound-sieve tube *K* in its bundle-associated part (see Fig. 26). Four sieve elements are nucleate (*G 1*, *G 2*, *G 3*, and *G 7*—*G 7* not shown). A third plasmatic contact (besides of *G 1*/*K 5*—Figs. 8 and 9, and *G 8*/*K 12*) is established via sieve element *GK* between the wound-sieve tubes *G* and *K*. × 1.800

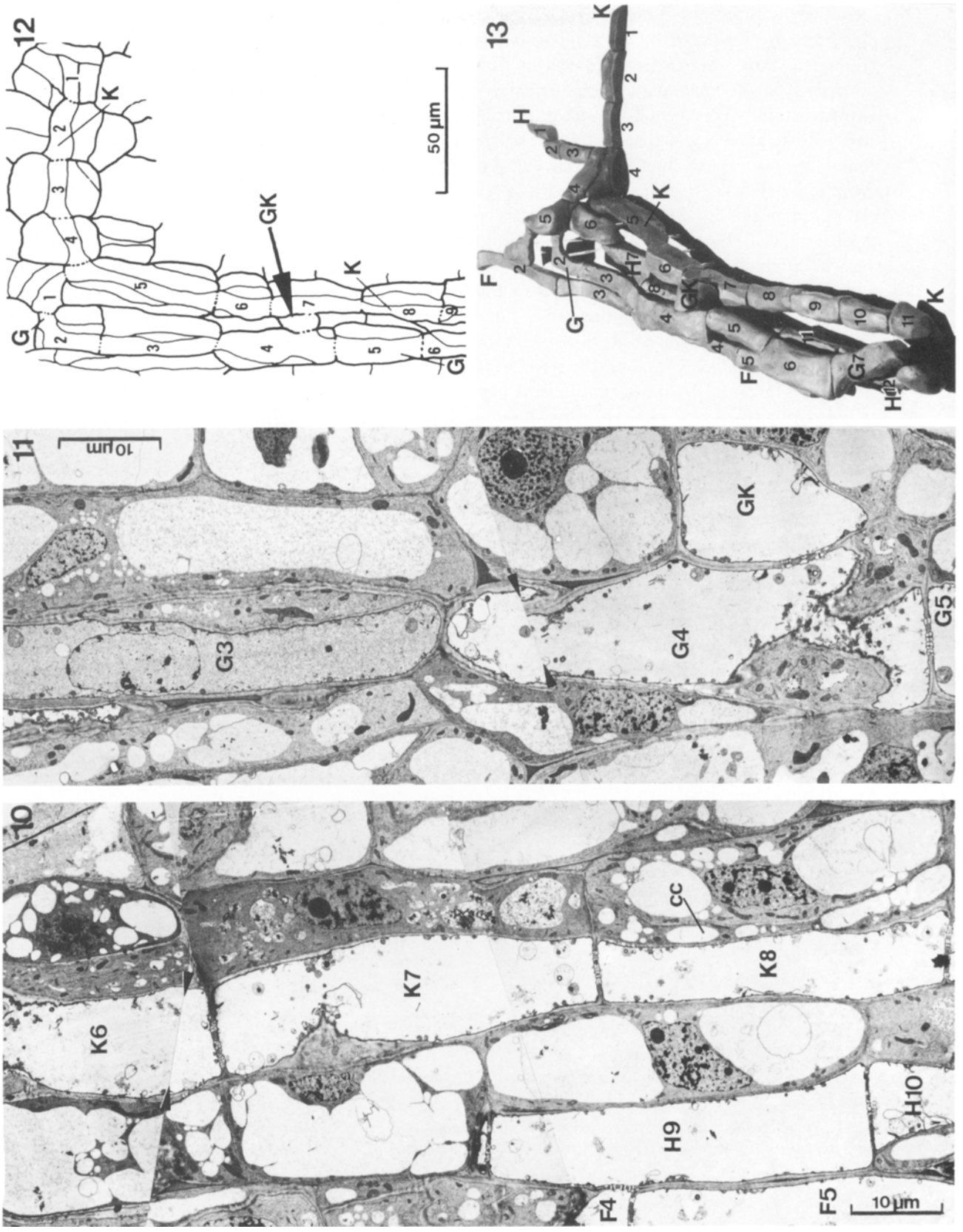


Fig. 8. Future sieve pores with callose deposition. × 8,400. Marker = 1 µm

Fig. 9. Section about 1 µm away from Fig. 8. Glancing section of sieve element *G 1*. × 1,800

Fig. 12. Drawing showing the relationship between wound-sieve tube *G* and *K* (projected in one plane). Sieve plates (dotted lines) are located in the pre-existing cell walls (thick lines) of the original cells. × 450

Figs. 13. 3-D-plasticine model formed of sieve elements developed after wounding. Ultrastructure of wound-sieve tubes *K* and *G* shown in Figs. 6–11. Nearly mature wound-sieve tube *H* bases in two bundle associated branches shown here but partly hidden *F 2—F 5* and *H 8—H 12*. The deviating sieve elements *H 7*, *H 6* and the three-poled sieve element *H 5* radiate into the cortex (*H 4—H 1*)

identified as future sieve pores by their callose fluorescence, are separated by thicker portions (Fig. 5). Ultimately, three sieve areas would develop from these three pit fields to constitute a compound sieve plate.
Ultrastructurally, the youngest sieve elements are similar to neighboring, meristematic parenchyma cells containing nuclei (not shown) and vacuoles in dense cytoplasm (Fig. 4: *1–3*). Changes in organelles, cytoplasm and future sieve pores are here visible from the fourth cell (Fig. 4: *4–7*) and are comparable to known differentiation patterns of bundle sieve elements. The oldest sieve elements in Fig. 4 (*8* and *9*) are enucleate and seem already mature.
The wound-sieve tube described above is connected via a three-poled member (Figs. 3 and 4: *5*) to further sieve elements ("three-poled" means that a sieve element is connected by three sieve plates to three different sieve elements—*cf.*, ESCHRICH 1953). This branch of the sieve tube, represented here by two elements (Figs. 3 and 4: *a* and *b*), also originates near bundle-sieve tubes, but in another plane.
Wound-sieve tubes are initiated above and below the wound. In an analysis of the entire regeneration area of a wounded plant, a developing wound-sieve tube, existing of only a few members, was found near bundle-sieve tubes above the wound (not shown), while below the wound two wound-sieve tubes were withdrawing further from the regular phloem (Figs. 6–13—*second example*). A 3-D-model of this area, consisting only of sieve elements developed after wounding (viz. during the 48 hours regeneration time) is composed of a wound-sieve tube with five (Fig. 13: *K5–K1*)[1], and another with at least seven sieve elements (Fig. 13: *H7–H1*) deviating from the regular course of the dissected vascular bundle. The latter wound-sieve tube (*H*) consists of mature sieve elements, *i.e.*, enucleate cells (*e.g.*, Fig. 10: *H9* and *H10*) linked by sieve plates, a few of which however contain pores which have not fully extended yet (between the outermost sieve elements *H1–H6*). Continuation of this sieve tube beyond its member *H1* was not followed. Close to the pre-existing bundle-sieve-tubes the wound-sieve tube *H* with its three-poled member *H5* branches into two paths, denoted by *H6–H12* and *F1–F5* (Fig. 13, partly hidden!). Eventually, both branches of the wound-sieve tube leave the investigated area, still as tiers of cells parallel to the regular phloem trace.
In addition to these nearly mature sieve tubes (*H, F*) another wound-sieve tube developed, which contains nucleate sieve elements. The irregular course of this wound-sieve tube (see Fig. 13: *K1–K10*), precluding observation within a single section, can be surveyed ultrastructurally only by a projection of planes ("symplanar projection", see Material and Methods). The sequence of differentiating sieve elements, thus visible in a single plane (Figs. 6 and 10: *K1–K9*), begins with three nucleate and vacuolate elements which are located furthest away from the bundle (Fig. 6: *K1–K3*). Compared with these, the next elements (Fig. 6: *K4* and *K5*) which have lost their vacuoles, are characterized by less dense cytoplasm. The sequence continues with elements which run parallel to pre-existing sieve tubes and are comparable to mature bundle-sieve elements in having electron-translucent cytoplasm (Fig. 10: *K6–K8*).
A further, partly nucleate wound-sieve tube (Figs. 7 and 11: *G*); consisting of eight elements, developed within the studied area running parallel to a part of wound-sieve tube *K* (*cf.*, Fig. 26: *G1–G8* and *K5–K10*). Both sieve tubes are linked by three-poled members of sieve tube *K* (Figs. 8 and 9: *K5*, Fig. 26: *K10* respectively), and by a mediating sieve element (in the middle of the mutual course—Figs. 11 and 12: *GK*). Consequently the supplementary wound-sieve tube *G*, which is laterally linked by sieve plates at three points with wound-sieve tube *K*, doubles the number of

[1] For nomenclature, see Material and Methods!

Figs. 14–18. Differentiating sieve elements of wound sieve tube *K* (*cf.*, with the symplanar projection Figs. 6 and 10)

Fig. 14. Wound-sieve element *K2* with a spherical nucleus (*n*), elongated proplastids (*p*), mitochondria and several vacuoles (*v*). Future sieve plates (*sp*) link with sieve elements *K1* and *K3*. Traversing the pre-existing cell wall, plasmodesmata (arrows) also join a parenchyma cell

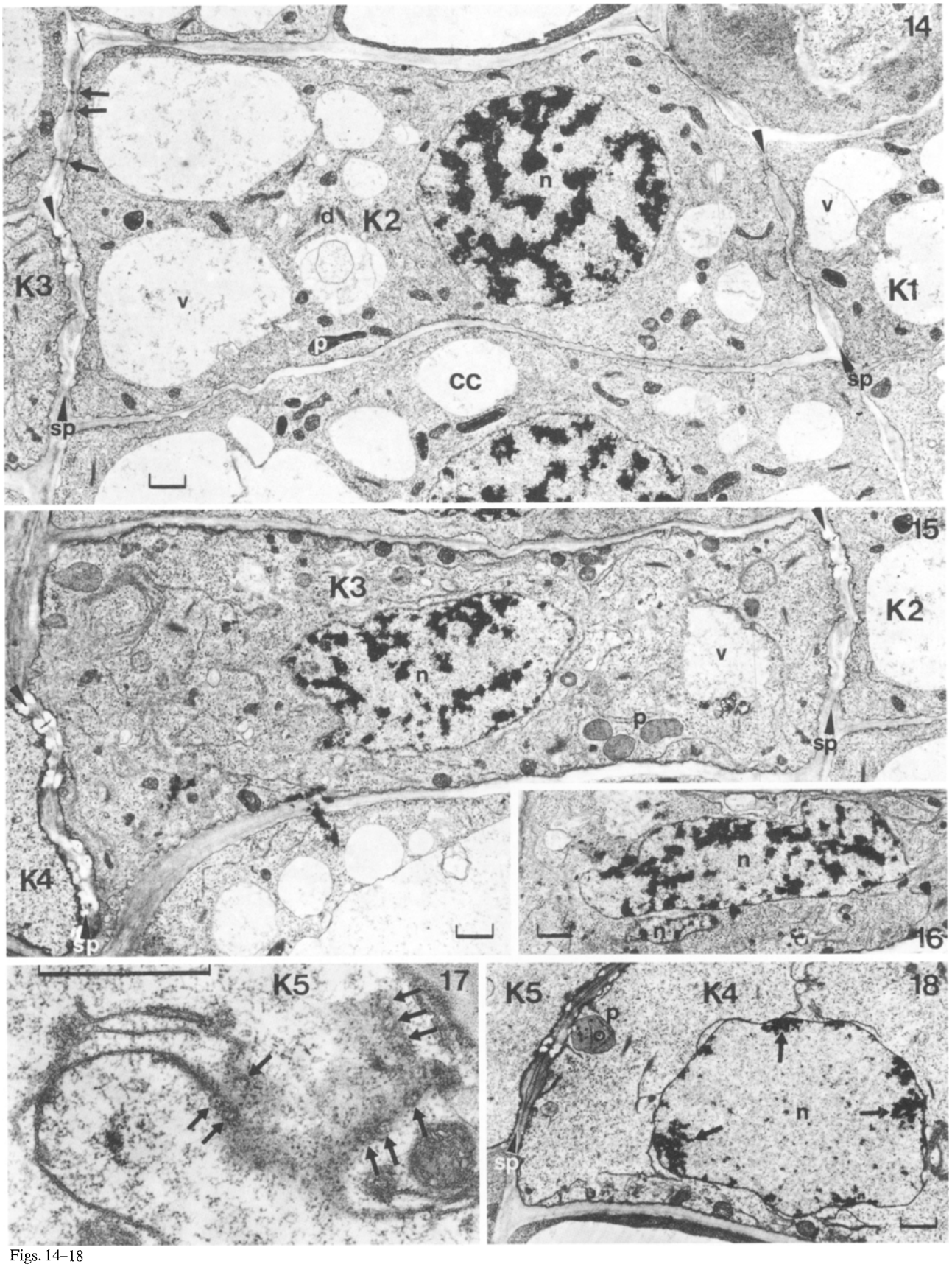
Fig. 15. Wound-sieve element *K3* with a lobed nucleus (*n*), oval plastids (*p*) and few vacuolar spaces (*v*), embedded in dense cytoplasm. The sieve plate (*sp*) connecting with sieve element *K4* shows typical callose platelets

Fig. 16. Section of the series, few µm away from Fig. 15. Due to the plane of sectioning, the nucleus (*n*) of *K3* is depicted in two portions

Fig. 17. Enucleate sieve element (*K5*) with remnants of the nuclear envelope lying among ER-cisternae. Arrows = nuclear pores.

Fig. 18. Last nucleate sieve elements (*K4*) within the depicted sequence, containing a disintegrating nucleus (*n*), a plastid (*p*) and some membranes within granular cytoplasm. Chromatin (arrows) is condensed just inside the nuclear envelope

Figs. 14–16 and 18. × 6,500. Fig. 17. × 31,000. Marker = 1 µm


14
K3
K2
K1
d
n
v
v
p
cc
sp
sp
15
K3
K2
n
v
p
sp
K4
sp
16
n
n
17
K5
18
K5
K4
p
n
sp

Figs. 14–18

bundle-associated sieve elements, and thus provides wound-sieve tube *K* with a broader base at the bundle before it radiates into the cortex.

3.2. Ultrastructure of Differentiating Wound-Sieve Elements

The wound-sieve tube *K* exhibits different stages of sieve element development, from youngest (*K1*) to nearly mature sieve elements (*e.g., K6*). The first and the second elements largely correspond to their companion cells and to other neighbouring cells (Fig. 6, *cf., K1, K2, cc,* and *tc*). They contain spherical nuclei (Fig. 14: *n*), elongated proplastids with a dense matrix, mitochondria, granular-cisternal endoplasmic reticulum, dictyosomes and several small vacuoles (Fig. 14). The cell wall is thin and plasmodesmata connect to adjacent sieve elements within future sieve plates, to companion cells and, also to parenchyma cells (Fig. 14: arrows).

The nucleus of the third sieve element (*K3*) is lobed (Figs. 15 and 16), the plastids are rounded off and include spherical starch grains (Fig. 20). ER cisternae approach one another without forming obvious stacks or networks (Figs. 19, 21, and 23: small arrows). Few vacuoles are sectioned. Dictyosomes, distributed all over the cell, are surrounded by many vesicles. Small aggregations of dense granular or filamentous material appear which possibly represent precursors of P-protein (Fig. 21: *pp*). The plasmodesmata between the sieve elements *K2* and *K3* already are surrounded by callose (Fig. 23:*), but more typically the prospective sieve pores linking sieve element *K3* with *K4* are marked by paired callose platelets (Fig. 21:*) and, additionally, by ER-cisternae covering the future pore site (Fig. 21: arrows). Plasmatic connections to the companion cell are characteristic: on the sieve-element side they have differentiating sieve pores (viz., callose platelets are located around the plasmatic channel, Fig. 19:*), but within the common wall this channel splits reaching the companion cell as two or more branches (Fig. 19: arrows).

If one compares the cytoplasmic contents of sieve elements *K3* and *K4* a drastic change is evident. The cytoplasm of sieve element *K4* is less dense (*cf.,* Figs. 15 and 18) and the degenerating nucleus contains only small amounts of chromatin close to the nuclear envelope (Fig. 18: arrows). Simultaneously the vacuole disappears (Fig. 18, see also Fig. 6). The next sieve element (*K5*) has a similar substructure, but is already enucleate. However, in one of the serial sections, remnants of the nuclear envelope marked by persistent, typical nuclear pores were detected among cisternae of the ER (Fig. 17: arrows).

The last step of sieve-element differentiation involves the degeneration of cytoplasmic contents apart from plastids, mitochondria, small stacks of ER (Fig. 22: arrow) and small amounts of filamentous material, the latter now dispersed over the cell lumen (*e.g.,* Fig. 25). Within the sieve elements *K4* and *K5* ribosomes still exist and are evenly distributed in the cell, but are lost in the following sieve elements. The cytoplasmic contents of the enucleate and electron-translucent sieve elements (beginning with *K6*) resemble the mature state of regular sieve elements.

Figs. 19–25. Wound-sieve tube *K*, parts of the cell wall and plastids (mounted to correspond with the overview of Figs. 6 and 10)

Fig. 19. Common wall between sieve element *K3* and its companion cell (*cc*). The branching (arrows) of the plasmodesmal channel on the companion-cell side is accompanied by callose deposition (*) on the sieve-element side. Small arrows = ER-cisternae close together, *n* nucleus

Fig. 20. Differentiating sieve-element plastid within wound-sieve element *K3* including starch grains (*st*) and some thylakoids (arrow) in a dense matrix

Fig. 21. Future sieve plate linking wound-sieve element *K3* with *K4*. Callose (*) surrounds the plasmodesmal tube without penetrating the middle lamella where the plasmodesmal tube is widening (arrowhead). ER-cisternae (arrows) overlie the future pore site. *d* dictyosome, *m* mitochondrion, *p* plastid, *pp* filamentous P-protein, small arrows = ER-cisternae close together

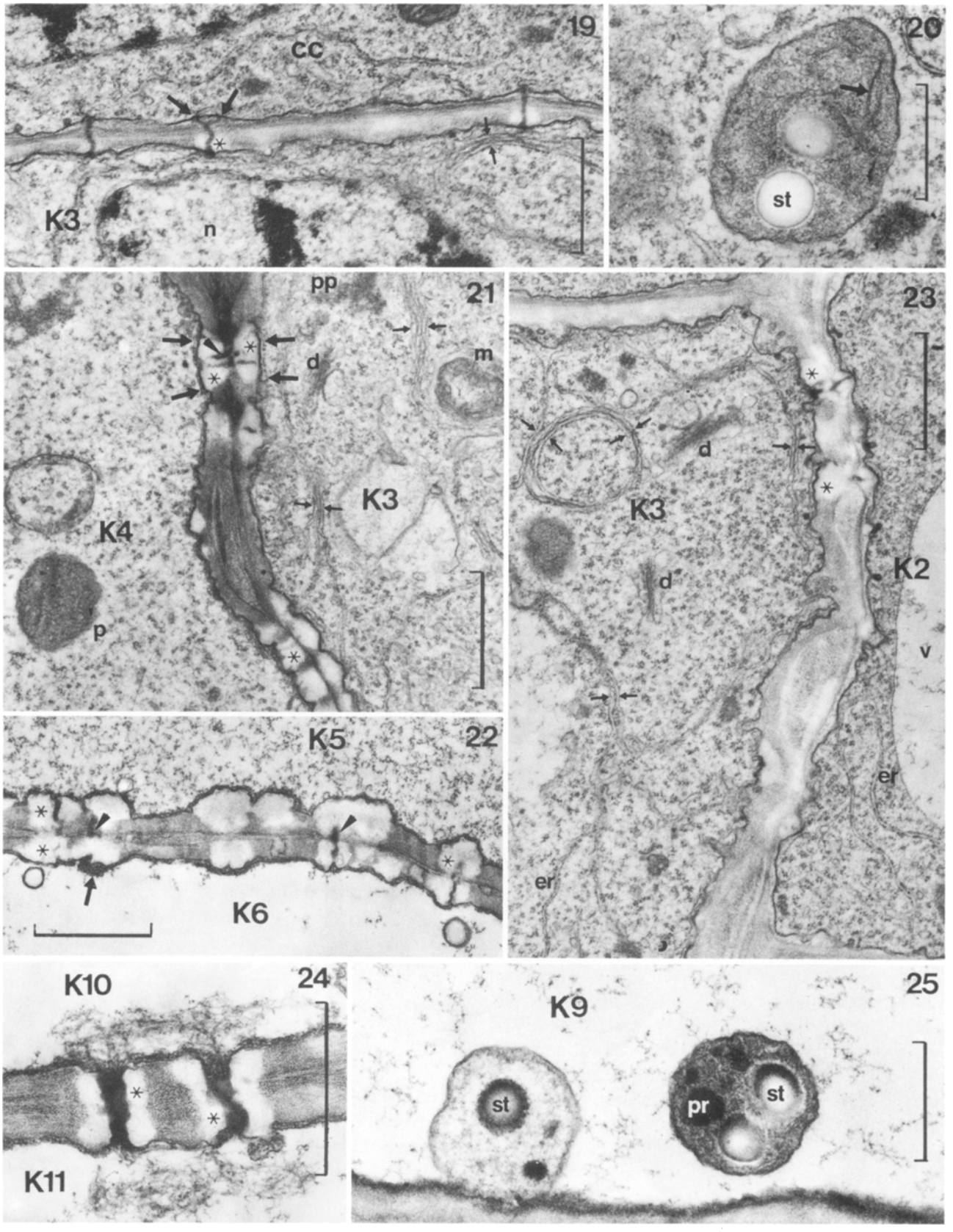
Fig. 22. Sieve plate between sieve elements *K5* and *K6*. Callose platelets (*) are still separated by the middle lamella. arrowhead = widened plasmodesmal tube, arrow = small stack of ER

Fig. 23. Future sieve plate between sieve elements *K2* and *K3*. Callose deposition(*) preponderates on the side of the older sieve element *K3*. ER-cisternae seem to aggregate (small arrows) within the cytoplasm. *d* dictyosomes, *er* ER-cisternae, *v* vacuole

Fig. 24. Sieve pores connecting sieve elements *K10* and *K11*. Callose collars (*) completely surround the open pores. Filamentous material, presumably P-protein, overlies both sides of the sieve plate and fills the sieve-pore lumina

Fig. 25. Sieve element plastids in sieve element *K9*. The thinning of the plastidal matrix is synchronized neither with the cytoplasm nor with other plastids. *pr* protein crystal, *st* starch grain

Figs. 19–23 and 25. × 21,000. Fig. 24. × 31,000. Marker = 1 μm


19
cc
K3
n
20
st
21
pp
d
m
K3
K4
p
22
K5
K6
23
d
K3
K2
d
v
er
er
24
K10
K11
25
K9
st
pr
st

Figs. 19–25

The opening of sieve pores starts with a widening of the plasmodesmal channel (Fig. 22: arrowheads). A small median cavity is already present within the future sieve pores between the sieve elements *K4* and *K5* (Fig. 21: arrowhead). The maturation of sieve pores is finished between the sieve elements *K10* and *K11* (Fig. 24). The pore lumina, now completely surrounded by callose, are filled with filamentous material.

Plastids of mature wound-sieve elements include starch grains and protein crystals. Within a single cell they may contain a very dense or nearly electron-transparent matrix (Fig. 25: *K9*).

3.3. The Sequence of Differentiation Within Wound-Sieve Tubes

The described sequence of differentiation within the wound-sieve tube *K* shows the age of sieve elements (at the moment of fixation) in a spatial order. It is possible to obtain information about the age, resp. the sequence of initiation, of sieve elements only as long as they are nucleate (*e.g.*, members of the wound-sieve tubes *K* and *G*) and not fully mature. The wound-sieve tube *K* was initiated by sieve elements which had developed from procambial cells about 500 µm below the wound, *i.e.*, in the oldest elements according to open pores (*e.g.*, *K11*). In uninjured roots procambial cells eventually differentiate into secondary phloem. After wounding some are activated earlier.

Beginning with the first established elements the wound-sieve tube *K* was extended by the addition of adjacent, new-divided sieve-element/companion-cell complexes, which also originated from early activated cambial cells. This tier of sieve elements runs parallel to pre-existing bundle-sieve tubes. The course of the young wound-sieve tube is directed both by the plane of preceding divisions and the location of pre-existing plasmodesmata. So the deviating sieve elements *K5* and in particular *K4* allow deflection of the wound-sieve tube away from the regular phloem. As described earlier, sieve tube *K* contains several three-poled elements, one of which (*K5*) provides a second connection to an early cambium-derived sieve tube, wound-sieve tube *G* (*cf.*, Fig. 12). As shown schematically in Fig. 26 the elements of this sieve tube do not continue the developmental sequence established in the elements *K1–K5*. Beginning with sieve element *G1* a new sequence is developed. Sieve elements *G1* to *G3* are nucleate (Figs. 7 and 11: *G1*, *G2*, and *G3*). The nucleus of sieve element *G3* however, is in an early phase of degeneration (Fig. 11). A total overview of this wound-sieve tube, which provides sieve elements *K5* and *K12*

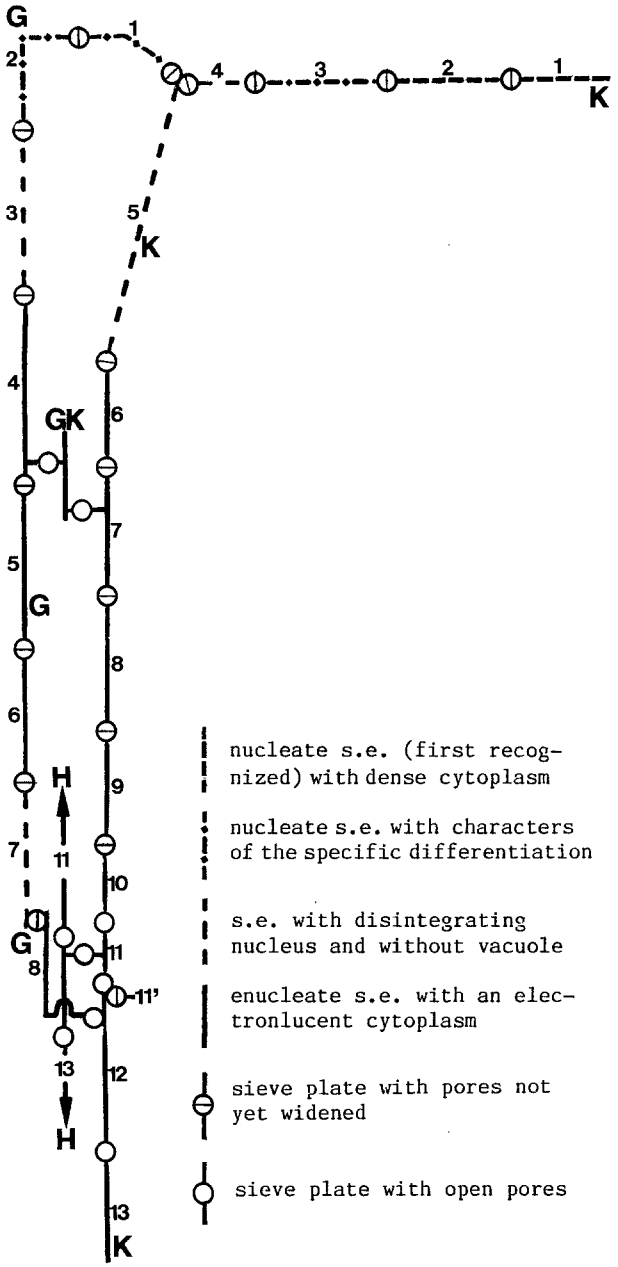


Fig. 26. Schematic overview of the development sequences within the wound-sieve tubes *K* and *G*. Wound-sieve tube *G* exhibits bidirectional development, with nucleate sieve elements *G1—G3*, nearly mature sieve elements *G4—G6*, and another nucleate sieve element *G7*

with a supplementary connection, shows that the most mature (and presumably oldest) sieve element is located in the middle of this bridge (Fig. 26: *G4*). This sieve element *G4* is three-poled and also linked to wound-sieve tube *K* via the mediating sieve element *GK* (Figs. 11, 12, and 26: *GK*). Thus, the following sequence of events is probable. The starting point of wound-sieve tube *G* was the mediating sieve element *GK* and its

laterally adjacent sieve element *G 4*. Elongating in both directions (parallel to pre-existing sieve tubes), wound-sieve tube *G* followed existing plasmodesmata and caused inequal divisions in the respective sieve-element/companion-cell complexes. Its bidirectional development is indicated by the fact that sieve element *G 7* (see Fig. 26) is still nucleate. This elongation at least led to further plasmatic contact (via pre-existing plasmodesmata) with wound-sieve tube *K*: at one end via sieve element *G 1* (*G 1/K 5*) and at the other via the nucleate element *G 7* and a mediating sieve element (*G 8*). These contacts consist of developing sieve plates linking cells which are developmentally rather far apart.

The two described wound-sieve-tube systems (*H, F, K,* and *G*), are relatively independent, being respectively nearly mature (*H* and *F*) and still partly nucleate (*K* and *G*). Only a few plasmatic connections each consisting of single, unwidened pores link the two systems (between members *K3/H4, H6/G 1,* and *G 3/F 3*). The only (lateral) sieve plate between the systems developed between the mature sieve elements *K 11* and *H 12* (*cf.,* Fig. 26), possibly indicating the starting point for the development of wound-sieve tube *K*. This sieve plate is derived from a lateral pit field in a member (*H 12*) of the earlier initiated wound-sieve tube *H* (see accompanying paper Fig. 24!).

4. Discussion

By reconstruction of serial sections the developmental changes within incomplete wound-sieve tubes were shown which are an example of sieve element differentiation under extreme conditions. While the origin of cells later differentiating into sieve-elements may be quite different, the sequence of developmental changes within an elongating wound-sieve tube accords remarkably with that demonstrated for protophloem-sieve elements (*e.g.,* ESAU and GILL 1972, MELARAGNO and WALSH 1976, THORSCH and ESAU 1981, ELEFTHERIOU and TSEKOS 1982) and for secondary sieve elements (BEHNKE and KIRITSIS 1983). *I.e.,* within the protoplast the gradual disintegration of the nucleus, the conversion of granular ER-cisternae to agranular ER-stacks and the disappearance of the vacuole seem to be obligatory for the differentiation of dicotyledonous assimilates-conducting cells, even when not derived from procambial tissue which is predisposed for such differentiation. In addition, specific sieve-element plastids develop also within wound-sieve elements, in *Pisum* containing protein crystals and starch grains (BEHNKE and SCHULZ 1983).

By tracing the sequence of differentiation it could be demonstrated that wound-sieve tubes are initiated above and below the wound. Starting from the interrupted vascular bundles they develop towards the cortex directed by plasmodesmata and the plane of preceding divisions. This development seems to be independent of source and sink positions in the root prior to wounding. Sieve tubes regularly elongate from source, *e.g.,* the seed, towards a sink, *e.g.,* the root apex. However, if wound-sieve tubes which start at the phloem trace below the wound and radiate into the cortex are considered this developmental direction seems reversed. At the same time, by precise analysis of wound phloem near the pre-existing phloem trace, it could be shown, that wound-sieve tubes elongate bidirectionally. The following initiation scheme could be derived: specific differentiation starts in one early activated (pro-)cambial cell, probably initiated by local efflux of assimilates and/or phytohormones from the interrupted phloem trace. Radiating from this initial cell vertically abutting cells, connected by plasmodesmata are induced, by an unknown stimulus, to divide. The final division is always inequal. Thus inside the root stele a tier of cambial cells is early-activated and a new sieve tube is formed which extends continuously in two directions, towards the wound and towards the shoot apex or the root apex above and below respectively. Deviation of the elongating wound-sieve tube towards the cortex (in order to by-pass the wound) can only be effected when the angle of preceding divisions and the existence of plasmodesmata permit it. This chiefly occurs near the wound where the previously vertical flux of the unknown stimulus might be diverted (*cf.,* the induction of circular vessels near a horizontal wound, presumably effected by circular flux of inductive signals, SACHS and COHEN 1982). By elongating in the opposite direction, the wound-sieve tube runs parallels to existing sieve tubes thus increasing the potential number of lateral connections. Finally, a network of newly developed sieve elements, connected by sieve plates at their lateral and end walls, provides a broad area for reconnection of the vascular stumps by wound-sieve tubes.

Wound-sieve elements are not only provided with companion cells when they originate from procambial cells, as shown for *Impatiens* (ESCHRICH 1953), *Coleus* (BEHNKE and SCHULZ 1980) and the root stele, but also when they originate from previously quiescent parenchyma cells of the root cortex of *Pisum*. Wound-companion cells are regularly characterized by specific plasmatic connections to their sieve elements allowing

early identification of these cells, which immediately after the final inequal division are otherwise ultrastructurally quite similar (see Fig. 14: *K2, cc*). Later, the dense cytoplasm of the companion cell with many mitochondria contrasts strongly with that of the maturing sieve element, which is gradually becoming more transparent.

Within the central cylinder in *Pisum* the pattern of divisions which eventually give rise to wound-sieve elements and companion cells is in accordance with that of secondary root phloem (see ZEE and CHAMBERS 1969). After the first division of a (pro-)cambial cell, which can immediately result in a transitional (parenchyma) cell and the sieve-element/companion cell complex, an inequal division, usually parallel to the plane of the preceding one(s), initiates differentiation into sieve element and companion cell. At least two divisions are required for differentiation of a sieve element and its companion cell, which develop regularly (ZEE and CHAMBERS 1969) or as a result of the disruption of existing sieve tubes within the central cylinder. Wound phloem development in stems of herbaceous plants is limited to the procambial cylinder between the pith and cortex parenchyma. The number of divisions in this tissue which give rise to wound-sieve elements is higher (ESCHRICH 1953, BEHNKE and SCHULZ 1980) than in the otherwise comparable procambial tissue of the *Pisum* root stele. As shown for *Coleus* stems at least four divisions are necessary for wound-sieve-element differentiation (BEHNKE and SCHULZ 1980). Possibly, the minimum number of divisions required for this differentiation is genetically fixed. If so, early activation of a few procambial cells to produce wound phloem follows a similar pattern to the regular cambial activation for secondary phloem development. Only the plane of divisions must alter, in order to direct the wound-sieve tubes towards the cortex.

Outside the central cylinder of *Pisum* the pattern and number of divisions also seem to be genetically determined. Direct conversion of a parenchyma cell into a wound-sieve element, as suggested by HARDHAM and MCCULLY (1982 a), was never found, even though many wounded plantlets were studied and several sectioned serially. Two or more divisions are usually necessary for the reprogramming of a cortical cell towards sieve-element differentiation, including dedifferentiation and new specialization. The wound-induced cell cycle begins with a meristematic division and ends with an inequal one (of one of the daughter cells) which initiates the differentiation of sieve element and companion cell. The combination of these two in a single division (*i.e.*, that a cortical cell is already a sieve-element/companion-cell complex) seems extremely rare. Suggestion of such a coincidence was found only once, when a wound-sieve element and its companion cell were probably the only daughter cells of a cortical cell. It is also most likely that functioning of a wound-sieve tube demands companion cells associated with its members. During complete inhibition of cell division by 0.2% colchicine, wound phloem consisting of wound-sieve elements built by direct conversion of parenchyma cells (HARDHAM and MCCULLY 1982 b, Figs. 26–31), developed but is presumably unable to conduct assimilates. This concentration of colchicine inhibits root growth without which it would slowly recover, attaining its normal rate 3–4 days after wounding (HARDHAM and MCCULLY 1982 a). This delay conforms with the time required for the restitution of translocation via wound-sieve tubes, *i.e.*, 72 hours (SCHULZ 1986). The observation by HARDHAM and MCCULLY (1982 b), therefore, that root growth cannot be resumed under the influence of 0.2% colchicine possibly reflects not only inhibition of the apical meristem, but also reveals persistent non-functioning of wound-sieve tubes which (unassociated with companion cells) are unable to transport assimilate around the wound. Consequently, it is suggested that the sieve element and its companion cell are not only the smallest structural but also functional unit for conducting assimilates in angiosperms.

Wound-sieve elements are interconnected via sieve plates (ESCHRICH 1953, BEHNKE and SCHULZ 1980, HARDHAM and MCCULLY 1982 a). As is known for regular phloem their sieve pores develop from plasmodesmata which must however provide contact not only through the thin procambial cell walls but also through the original cell walls of dedifferentiated and subdivided cortical cells. Comparable to the plasmatic communications between cells of the stock with those of the scion after grafting (KOLLMANN and GLOCKMANN 1985) these plasmodesmata might be formed *de novo* in the walls of original cortex parenchyma cells after a series of cells is destined to differentiate into sieve elements. However, there are no indications confirming such a secondary development of plasmodesmata. On the contrary, observations suggest that sieve pores originate from plasmodesmata already present between the cortical cells. Moreover, elongation of wound-sieve tubes (effected by the addition of newly divided sieve-element/companion-cell complexes) seems to be directed by the presence and position of plasmodesmata in the original walls. Therefore, the events preceding sieve

elements differentiation, including multiple mitoses of parenchyma cells, require the pre-existence of plasmodesmata (mostly arranged in primary pit fields), which direct the orientation and then give rise to wound-sieve pores. Without formulating the consequences of this, viz. that the location of pre-existing pit fields directs elongating wound-sieve tubes, ESCHRICH (1953) already described how pit fields in order to become sieve plates were successively encircled by new cell walls, which (standing perpendicular to the pit-field containing wall) prepared the development of sieve elements.

The differentiating of wound-sieve pores, beginning from plasmodesmata traversing parenchyma cell walls singly or in groups, shows a similar development to that of regular sieve pores described in detail, as early as 1966 by NORTHCOTE and WOODING and more recently by ESAU and THORSCH (1984). Within wound phloem this development is also characterized by local deposition of callose in the cell wall around plasmodesmata linking nucleate sieve elements. The deposition results in paired callose platelets which represent the future pore width. In addition single ER-cisternae mark the future pore sites, covering the plasmalemma where it lines the callose platelets.

The timing of sieve-element differentiation and wound-sieve pore development particularly is in accordance with that in regular phloem: The lobing of the nucleus accompanies the surrounding of a plasmodesma with callose. Only plasmodesmata conducting to other members of the wound-sieve tube or their companion cells are involved. On the companion-cell side of the common wall the typical branching of the plasmodesmal channel, beginning in the middle of the wall, is seen. Disintegration of nuclear material, especially of chromatin, begins while cellulose and callose deposition in the cell wall is continuing (*cf.*, ESAU and THORSCH 1984). Sieve elements at the start of sieve-pore opening usually have protoplasts without vacuoles or remnants of nuclear material, except for some nuclear-pore studded membranes sometimes found between the aggregated or stacked endoplasmic reticulum. Dictyosomes and ribosomes disappear simultaneously, so that mature wound-sieve elements as well as mature regular sieve elements (mature as defined by their open pores) contain only typical sieve-element plastids, mitochondria, stacked or otherwise aggregated endoplasmic reticulum and perhaps P-protein. Wound-sieve elements, which developed during the first 48 hours of regeneration, have only small amounts of filamentous material. Ultrastructurally, this is comparable with "fine-fibrous precursors" of P-protein as described by ESAU (1978, p. 5) for *Phaseolus*, and with filamentous P-protein originating near polysomes as demonstrated by BEHNKE (1974) in several angiosperms. Crystalline P-protein bodies typical for all *Fabaceae* (BEHNKE and POP 1981) are only found within wound-sieve elements which develop considerably more than 48 hours after wounding (SCHULZ, in prep.).

The restitution of an experimentally wounded plant is dependent on rapid recovery of the interrupted transport paths. Thus severance of all pre-existing phloem traces induces the regenerating tissue to form sieve elements as quickly as possible. Consequently all cell functions, as, *e.g.*, dividing and synthesizing capabilities, are reduced to a minimum, until the first "emergency"-reconnections are able to conduct. Examination of these wound-sieve tubes shows that in spite of their different origin, the equipment typical of mature bundle-sieve elements is also realized in wound-sieve elements. Differences in the origin of sieve-element plastids, as amylo- or chloroplasts (BEHNKE and SCHULZ 1983), and in the origin of sieve pores in pit-field plasmodesmata of cortex parenchyma are minimized as the wound-sieve elements differentiate. Within mature wound phloem only the very irregular course of sieve tubes, and the unusual and variable shape of its elements attest to the fact that this differentiation was experimentally induced in previously quiescent tissue. Small amounts of P-protein in early developed wound-sieve elements are not unusual. Developing protophloem-sieve tubes near the root apex of *Pisum* also contain only small amounts of filamentous P-protein (unpubl. data). This feature is common in root protophloem (*e.g.*, *Nicotiana*, see ESAU and GILL 1972), and possibly reflects conditions within rapidly-growing sieve elements.

Acknowledgements

This investigation was part of a Ph.D. thesis guided by Prof. Dr. H.-D. BEHNKE, whom I thank for criticism and valuable discussion.

References

BEHNKE, H.-D., 1974: Comparative ultrastructural investigations of angiosperm sieve elements: Aspects of the origin and early development of P-protein. Z. Pflanzenphysiol. **74**, 22–34.

— KIRITSIS, U., 1983: Ultrastructure and differentiation of sieve elements in primitive angiosperms. I. *Winteraceae*. Protoplasma **118**, 148–156.

BEHNKE, H.-D., POP, L., 1981: Sieve-element plastids and crystalline P(hloem)protein in *Leguminosae*: Micromorphological characters as an aid to the circumscription of the family and subfamilies. In: Advances in Legume Systematics (POLHILL, R. M., RAVEN, P. H., eds.), pp. 707–715. London: HMSO.

— SCHULZ, A., 1980: Fine structure, pattern of division, and course of wound phloem in *Coleus blumei*. Planta **150**, 257–365.

— — 1983: The development of specific sieve-element plastids in wound phloem of *Coleus blumei* (S-type) and *Pisum sativum* (P-type), regenerated from amyloplast-containing parenchyma cells. Protoplasma **114**, 125–132.

BENAYOUN, J., ALONI, R., SACHS, T., 1975: Regeneration around wounds and the control of vascular differentiation. Ann. Bot. **39**, 447–454.

ELEFTHERIOU, E. P., TSEKOS, I., 1982: Development of protophloem in roots of *Aegilops comosa* var. *Thessalica*. II. Sieve-element differentiation. Protoplasma **113**, 221–233.

ESAU, K., GILL, R. H., 1972: Nucleus and endoplasmic reticulum in differentiating root protophloem of *Nicotiana tabacum*. J. Ultrastruct. Res. **41**, 160–175.

— THORSCH, J., 1984: The sieve plate of *Echium* (*Boraginaceae*): Developmental aspects and response of P-protein to protein digestion. J. Ultrastruct. Res. **86**, 31–45.

ESCHRICH, W., 1953: Beiträge zur Kenntnis der Wundsiebröhren-Entwicklung bei *Impatiens holsti*. Planta **43**, 37–74.

HARDHAM, A. R., MCCULLY, M. E., 1982 a: Reprogramming of cells following wounding in pea (*Pisum sativum* L.) roots. I. Cell division and differentiation of new vascular elements. Protoplasma **112**, 143–151.

— — 1982 b: Reprogramming of cells following wounding in pea (*Pisum sativum* L.) roots. II. The effects of caffeine and cholchicine on the development of new vascular elements. Protoplasma **112**, 152–166.

KOLLMANN, R., DÖRR, I., SCHULZ, A., BEHNKE, H.-D., 1983: Funktionelle Differenzierung der Assimilatleitbahnen. Ber. dtsch. bot. Ges. **96**, 117–132.

— GLOCKMANN, C., 1985: Studies on graft unions. I. Plasmodesmata between cells of plants belonging to different unrelated taxa. Protoplasma **124**, 224–235.

LANE, B. P., EUROPA, D. L., 1965: Differential staining of ultrathin sections of Epon-embedded tissue for light microscopy. J. Histochem. Cytochem. **13**, 579–582.

MELARAGNO, J. C., WALSH, M. A., 1976: Ultrastructural features of developing sieve elements in *Lemna minor* L.—The protoplast. Amer. J. Bot. **63**, 1145–1157.

NORTHCOTE, D. H., WOODING, F. B. P., 1966: Development of sieve tubes in *Acer pseudoplatanus*. Proc. Roy. Soc. B **163**, 524–537.

RANA, M. A., GAHAN, P. B., 1983: A quantitative cytochemical study of determination for xylem-element formation in response to wounding in roots of *Pisum sativum* L. Planta **157**, 307–316.

ROBBERTSE, P. J., MCCULLY, M. E., 1979: Regeneration of vascular tissue in wounded pea roots. Planta **145**, 167–173.

SACHS, T., COHEN, D., 1982: Circular vessels and the control of vascular differentiation in plants. Differentiation **21**, 22–26.

SCHULZ, A., 1986: The beginning of translocation in wound phloem. In: International Conference on Phloem Transport (CRONSHAW, J., ed.). New York: Alan R. Liss, Inc. (in press).

THORSCH, J., ESAU, K., 1981: Ultrastructural studies of protophloem sieve elements in *Gossypium hirsutum*. J. Ultrastruct. Res. **75**, 339–351.

ZEE, S. Y., CHAMBERS, T. C., 1969: Development of secondary phloem of the primary root of *Pisum sativum*. Aust. J. Bot. **17**, 199–214.