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Intraspecific variability in *Alloteropsis semialata* (R. Br.) Hitchc.

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ABSTRACT

Intraspecific variability in *Alloteropsis semialata* (R. Br.) Hitchc. is shown ultrastructurally with particular regard to the structure of the bundle sheath and the development of the dimorphic chloroplasts in both the C_3 and C_4 forms of this species.

A. semialata is known to possess two distinct anatomical forms — Kranz and non-Kranz — within one species and occurring within a single ecological niche. Kranz and non-Kranz anatomy is known to be correlated with C_4 and C_3 physiology respectively.

Transverse sections of leaf portions taken at midsheath, at the ligule and at midlamina show plastids with different morphologies at different ontogenetic stages. Plastid form is related to the stage of development, the influence of light on the emerging leaf and the C_3 or C_4 form of *A. semialata*. Stages from amyloplast to chloroplast are investigated with regard to fine structure. Leaf transverse sections are examined microscopically and formation of new bundles, chiefly in the lamina is traced. Differences in anatomy and distribution of vascular bundles are more evident in the lamina than in the colourless leaf sheath.

Both C_3 and C_4 forms of *A. semialata* are found to show chloroplast dimorphism in vascular bundle sheath and mesophyll cells. This is shown to differ in the two forms. The specialized chloroplasts of the Kranz sheath are shown to develop in the inner or mestome sheath, and not in the parenchyma sheath as in some other members of the Kranz Panicoideae.

Features of *A. semialata*, such as the double bundle sheath, granal chloroplasts and large numbers of mitochondria in bundle sheath cells, in the C_4 form, question the current classification of this grass as a malate former.

RÉSUMÉ

VARIABILITÉ INFRASPÉCIFIQUE CHEZ ALLOTEROPSIS SEMIALATA *(R. BR.) HITCHC.*

Une variabilité infraspécifique chez Alloteropsis semialata *(R. Br.) Hitchc. est apparente dans son ultrastructure, particulièrement lorsqu'on examine la structure de la gaine du faisceau et le développement des chloroplastes dimorphiques dans les deux formes, C_3 et C_4, de cette espèce.*

*On sait qu'*A. semialata *possède deux formes anatomiques distinctes, 'Kranz' et 'non Kranz' — au sein de la même espèce et dans une même niche écologique. On sait aussi que l'anatomie 'Kranz' et 'non Kranz' est en corrélation avec la physiologie respectivement en C_4 et C_3.*

*Des sections transversales de morceaux de feuilles prélevées au milieu de la gaine, sur la ligule et au milieu du limbe montrent des plastes ayant une morphologie différente aux divers stades ontogénétiques. La forme du plaste est en relation avec le stade de développement, l'éclairement de la feuille qui émerge et la forme C_3 ou C_4 d'*A. semialata. *Les stades de l'amyloplaste au chloroplaste sont examinés quant à leur structure fine. Les sections transversales des feuilles sont soumises à un examen microscopique et la formation de nouveaux faisceaux, surtout dans le limbe, est observée. Les différences dans l'anatomie et la répartition des faisceaux vasculaires sont plus évidentes dans le limbe que dans la gaine foliaire incolore.*

Les deux formes C_3 et C_4 de A. semialata *révèlent un dimorphisme des chloroplastes dans la gaine des faisceaux vasculaires et dans les cellules du mésophylle. Ce dimorphisme est différent dans les deux formes. Les chloroplastes spécialisés de la gaine 'Kranz' se développent dans la gaine interne ou méstome et non dans la gaine parenchymateuse comme chez certains autres membres des Panicoïdées Kranz.*

*Les caractères d'*A. semialata, *tels que la double gaine des faisceaux les chloroplastes à granums et la grand nombre de mitochondries dans les cellules de la gaine des faisceaux, dans la forme C_4, remet en question la classification actuelle de cette graminée dans les producteurs de malate.*

INTRODUCTION

A close correlation has been reported between mature leaf anatomy and the biochemical type of CO_2 fixation in higher plants (Björkman & Berry, 1973; Hattersley, Watson & Osmond, 1977), particularly with regard to distribution and structure of chlorenchymatous tissue. High frequency of chloroplasts in the vascular bundle sheath cells, together with other specialized cytological features, constitutes Kranz anatomy (Carolin, Jacobs & Vesk, 1973; Johnson & Brown, 1973; Downton, 1971), and is one of the characteristics of C_4 photosynthetic plants (Black & Mollenhauer, 1971; Laetsch, 1974). In C_4 plants photosynthetic CO_2 fixation involves a pathway in which the primary products are C_4 dicarboxcyclic acids produced in the mesophyll cells (Hatch & Slack, 1966).

Mesophyll and bundle sheath chloroplasts may show both structural (Hodge, McLean & Mercer, 1955; Johnson & Brown, 1973) and ontogenetic dimorphism (Laetsch & Price, 1969). Chloroplast structure and arrangement within the bundle sheath (such as centripetal or centrifugal disposition of the chloroplasts and the presence or absence of grana) provide important distinguishing features which have been correlated with particular enzymes for decarboxylation of the 4-carbon products, malate

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and aspartate. (Gutierrez, Gracen & Edwards, 1974; Hatch, Kagawa & Craig, 1975).

Many C_3 plants which produce a three carbon compound as the primary product of CO_2 fixation and show non-Kranz anatomy, also possess bundle sheaths in which chloroplasts may be present (Miyake & Maeda, 1978), although in fewer numbers and without Kranz specialization. Whether the C_3 bundle sheath chloroplasts function as mesophyll cells or have a specialized function of their own, remains at present undetermined. From all these facts the structure of bundle sheath cells and the chloroplasts they contain, is seen to be important in both C_3 and C_4 plants.

Vascular bundle sheath structure in the Poaceae is well documented. Bundle sheaths may be double or single. The outer, the inner, or both bundle sheaths may be Kranz (Brown, 1975), or they may show non-Kranz anatomy. These and other specific anatomical features have been correlated with the C_3 (non-Kranz) and C_4 (Kranz) syndrome (Downton & Tregunna, 1968; Smith & Brown, 1973). The C_4 category has been further subdivided according to the carboxylation product, malate or aspartate (Downton, 1970; Kortschak, Hartt & Burr, 1965). C_4 grasses have been classified, according to the predicted dominant decarboxylating enzyme in the bundle sheaths, on the basis of anatomy and cytology alone (Hattersley & Watson, 1976; Ellis, 1977). Structural and biochemical criteria are also considered to reflect phylogenetic and evolutionary tendencies (Brown, 1975) as well as being correlated with ecological distribution patterns of C_3 and C_4 grasses which show different environmental preference (Teeri & Stowe, 1976; Vogel, Fuls & Ellis, 1978).

In much of the anatomical and physiological work done on the C_3/C_4 syndrome the midlamina region of mature leaves has been used. Ontogeny of chloroplasts and vascularization of grass leaves has not been dealt with extensively in the literature. Sharman (1945) deals with the leaf primordium only. Leech, Rumsby & Thomson (1973) and Leech, Thomson & Platt-Aloia (1981), describe plastid division in meristematic cells of young *Zea mays* leaves, but do not follow further plastid development in mature cells. Laetsch & Price (1969) show development of dimorphic chloroplasts in sugar cane, and Hinchman (1972) shows ultrastructural morphology and ontogeny of oat coleoptile plastids. Miyake & Maeda (1978) compare starch in chloroplasts of C_3 and C_4 plants of the Poaceae.

Bundle sheath structure and chloroplast ontogeny is of particular interest in *Alloteropsis semialata*, since this species shows two anatomical forms, Kranz and non-Kranz (Ellis, 1974) occurring within a single environment (Frean, Barrett & Cresswell, 1980). The latter authors have shown that non-Kranz and Kranz forms are associated respectively with C_3 and C_4 photosynthetic characteristics.

This unique situation where two anatomical and photosynthetic types occur in one species may indicate a genome able to express itself in different forms. This species also shows great morphological variability and wide geographical distribution (Chippindall & Crook, 1976). Within the sample used in this study Frean & Cresswell (1979) have shown that the C_3 form is more pubescent than the C_4 form. (The terms Kranz and C_4, non-Kranz and C_3 are used synonymously throughout this study.)

The aim of this investigation was to compare the development of chloroplasts in young leaves of comparative age in Kranz and non-Kranz forms of *A. semialata* in order to establish the developmental stage at which they show structural divergence.

METHODS

Glabrous (C_4) and pubescent (C_3) plants of *A. semialata*, removed from the same site in Sandton, Transvaal, were established in containers and kept in the open. Tillers considered to be of comparable age were removed from parent tussocks. Voucher specimens were lodged in the Moss Herbarium (J), University of the Witwatersrand, Johannesburg.

The most recently emerged leaves where the ligule is defined only as a ring of hairs, were taken from five C_3 and five C_4 tillers. Sections were taken at three levels; midsheath (colourless), the leaf immediately above the ligule (pale green) and at midlamina (green).

For electron microscopy leaf material was cut into small pieces (2 mm^2) from tissue between midrib and margin. Material was fixed in 2% glutaraldehyde in 0,1 M cacodylate buffer at pH 7,2 for 2 hours, rinsed in buffer and post-fixed in 1% osmium tetroxide for 2 hours. Subsequent treatment consisted of dehydration in a graded alcohol series and embedment in Spurr's resin (Spurr, 1969). Blocks were sectioned at 60–90 nm, using glass knives on a Reichert OM U_2ultra-microtome, stained with Reynolds's (1963) lead citrate enhanced with uranyl acetate and viewed in a JEM 100 S transmission electron microscope.

For light microscopy monitor sections were cut from the same blocks at 1–2 μm, and stained with 1% toluidine blue in 1% borax. Leaf transverse sections were cut on a freezing microtome at 20–25 μm. Presence of starch was confirmed by staining with iodine potassium-iodide.

The C_4 photosynthetic pathway enzymes were extracted as described by Raghavendra & Das (1978) with 1% (w/u) BSA added to the extraction medium. RUBP-carboxylase activity was determined according to the method of Björkman (1968), PEP-carboxylase according to Maruyama, Easterday, Chang & Lane (1966), aspartate aminotransferase and alanine aminotransferase, Edwards & Gutierrez (1972), NADP malic dehydrogenase and NAD/NADP malic enzyme, Raghavendra & Das (1978), and PEP carboxykinase according to Edwards, Kanai & Black (1971). Chlorophyll was determined by the method of Arnon (1949).

RESULTS

The size and position of the most recently emerged leaf on a C_3 and a C_4 tiller is shown in Fig. 1a, b and levels at which sections were taken are

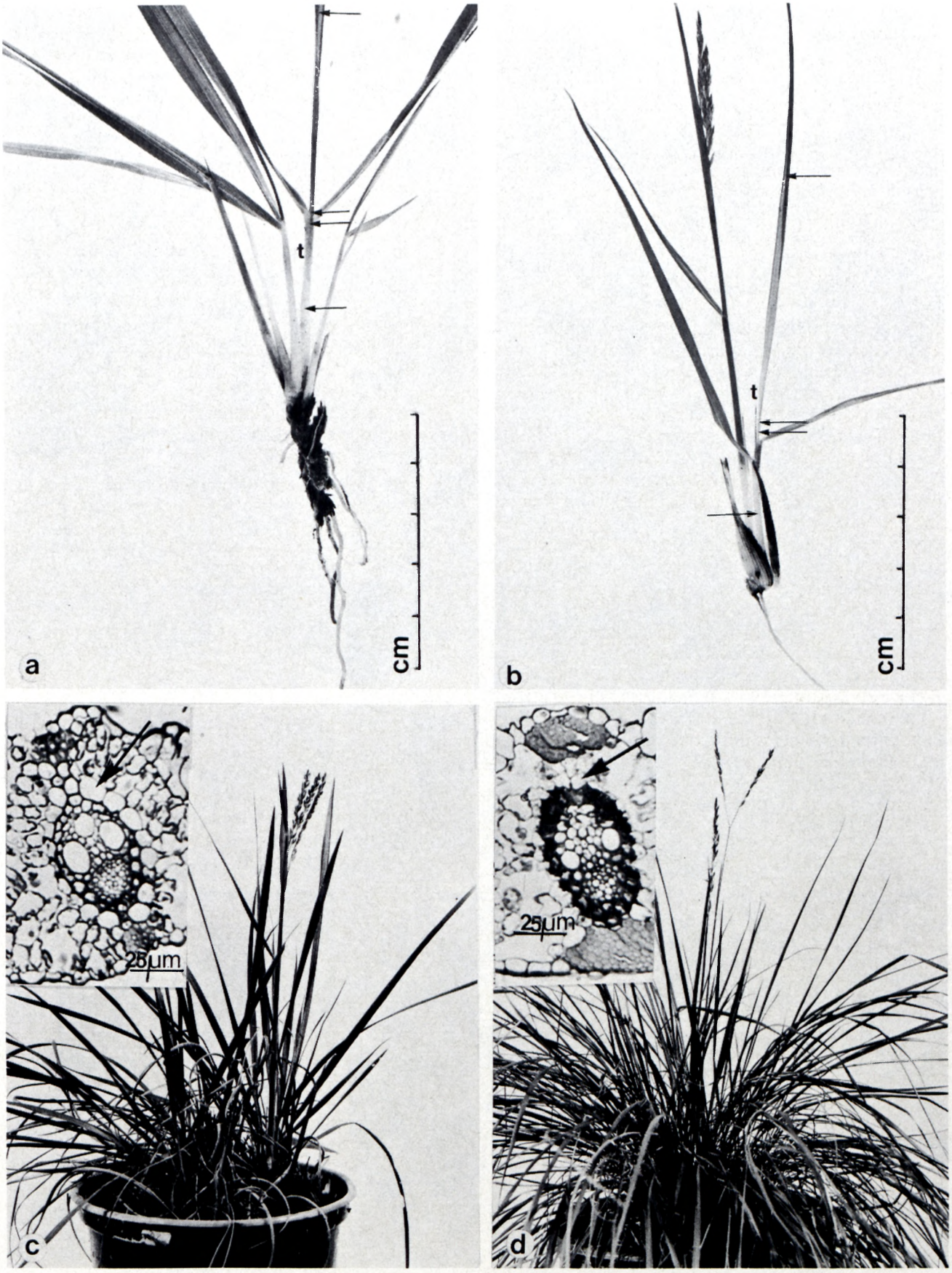

FIG. 1.—C_3 and C_4 tillers (t) of *Alloteropsis semialata*. Arrows indicate points at which sections were taken. The ligule is situated between the two arrows which are close together. **a,** C_3 tillers, natural size. **b,** C_4 tillers, natural size. **c,** C_3 tussock, one third natural size. Inset, transverse section of large (or first order) bundle, outer bundle sheath arrowed. Note radiate mesophyll cells. **d,** C_4 tussock, one third natural size. Inset, transverse section of large bundle, outer bundle sheath arrowed. Note specialized inner Kranz sheath and loosely arranged mesophyll cells.

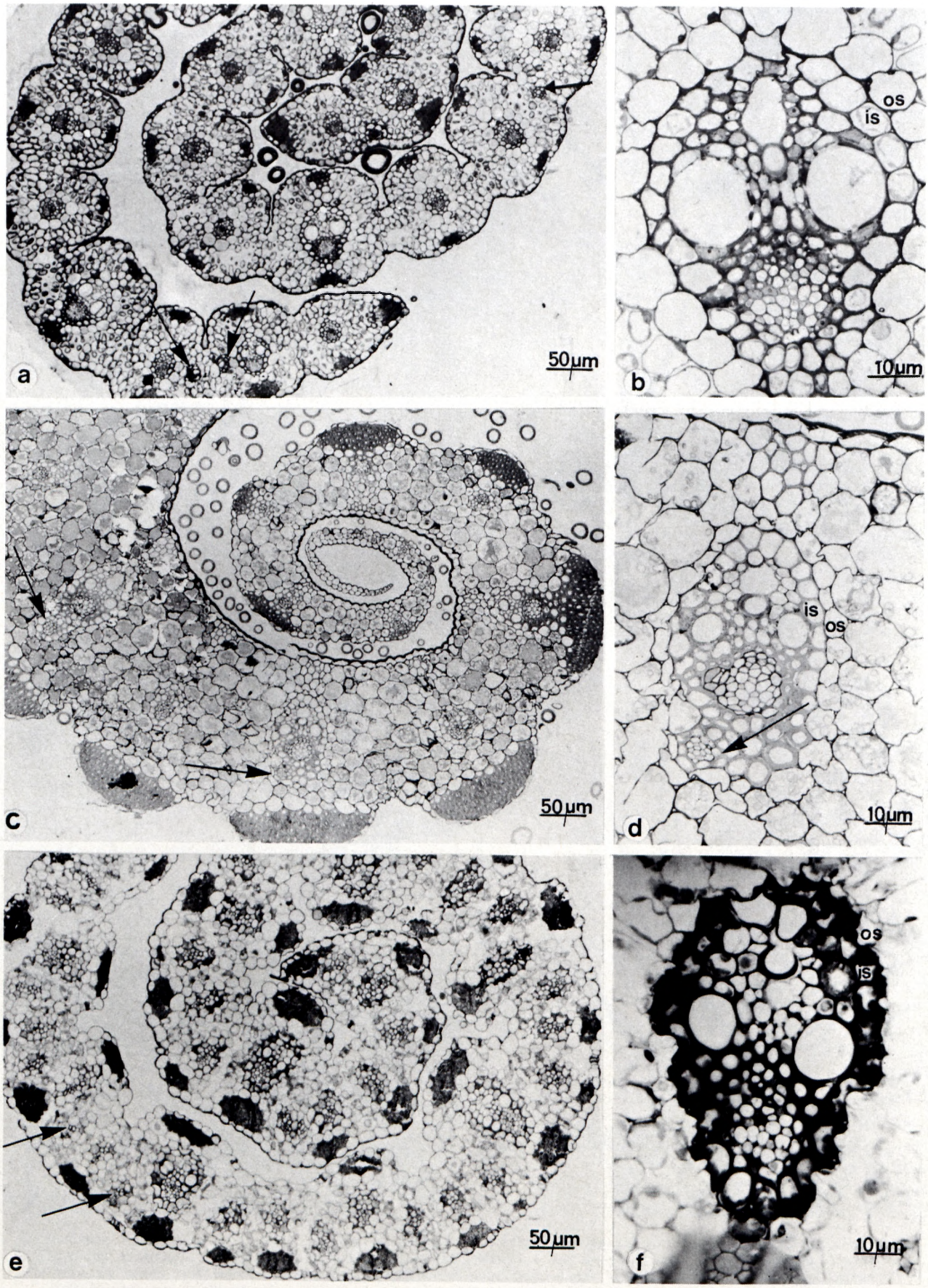

FIG. 2.—Light micrographs of transverse sections of C_3 and C_4 leaves. C_3: **a,** ligule region (pale green). Newly formed bundles arrowed. **b,** large (first order) bundle in ligule region. is = inner (mestome) sheath; os = outer sheath. C_4: **c,** mid-sheath. Newly formed bundles arrowed. **d,** large (first order) bundle in mid-sheath. Note new bundle (arrowed); is = inner sheath; os = outer sheath. **e,** ligule region. Newly formed bundles arrowed. **f,** large (first order) bundle in ligule region; is = inner (mestome) sheath; os = outer sheath.

indicated. Fig. 1c, d show respectively the differing growth habit of C_3 pubescent and C_4 glabrous forms of *A. semialata*, with the related non-Kranz and Kranz anatomy

Light microscopy

Fig. 2a, b show typical vascular bundle sheath structure of the C_3 leaf, regardless of where sections are taken. There is a double bundle sheath, with an inner mestome sheath of small thickwalled cells and an outer sheath of parenchymatous cells.

At the light microscope level there is not much visible cell-content in either layer. Bundle sheaths of the C_3 leaf are similar in appearance regardless of whether they occur in the colourless leaf sheath, the pale green ligule region, or at midlamina. They are also remarkably similar in appearance to the colourless mid-sheath bundles of the C_4 leaf (Fig. 2c, d) except that in the C_4 bundle the parenchyma sheath is less well defined. This similarity is noteworthy, since parts of the C_3 leaf, viz. lamina and ligule have been exposed to light, whereas the C_4 leaf sheath has not. Investigation of the C_4 ligule area, which has been light exposed (Fig. 2e, f) shows that the vascular bundle of the C_4 ligule now shows the thickwalled Kranz inner sheath packed with chloroplasts; the outer parenchyma sheath of small thin-walled cells is poorly defined (at light microscope level) and poorly populated with chloroplasts. (cf. Fig. 2b with Fig. 2d, f).

New bundles appear to be forming in all three leaves shown in Fig. 2a, c, e. These apparently form close to an established bundle (Fig. 2d) and then are separated from it by the formation of a new bundle sheath and the intervening mesophyll cells (Fig. 2a, e). Serial sections would confirm this method of formation. In the C_3 leaf new bundles form equidistant from ab- and adaxial surface. In the C_4 leaf the older bundles occupy a more central position in the leaf, while the newer bundles form close to the abaxial surface. Only the largest (first order) bundles show metaxylem vessels in both C_3 and C_4 leaves.

Electron microscopy

Leaf sheath (colourless)

In both C_3 and C_4 leaves, in the colourless leaf sheath tissue amyloplasts are present in mesophyll cells and in both inner and outer bundle sheath cells (Fig. 3a, b).

Amyloplasts are more numerous in the C_4 mesophyll cells than in the C_3. Amyloplasts of the mesophyll cells show starch grains which may be intensely osmiophilic. The reason for this intense staining is not known (Fig. 3d, e). The C_3 mesophyll cells contain fewer amyloplasts with smaller grains. Both C_3 and C_4 mesophyll cells show chloramyloplasts i.e. plastids which have starch grains but also contain stroma lamellae (Fig. 3d) and plastids with prolamellar bodies (Fig. 3c) These two plastid types

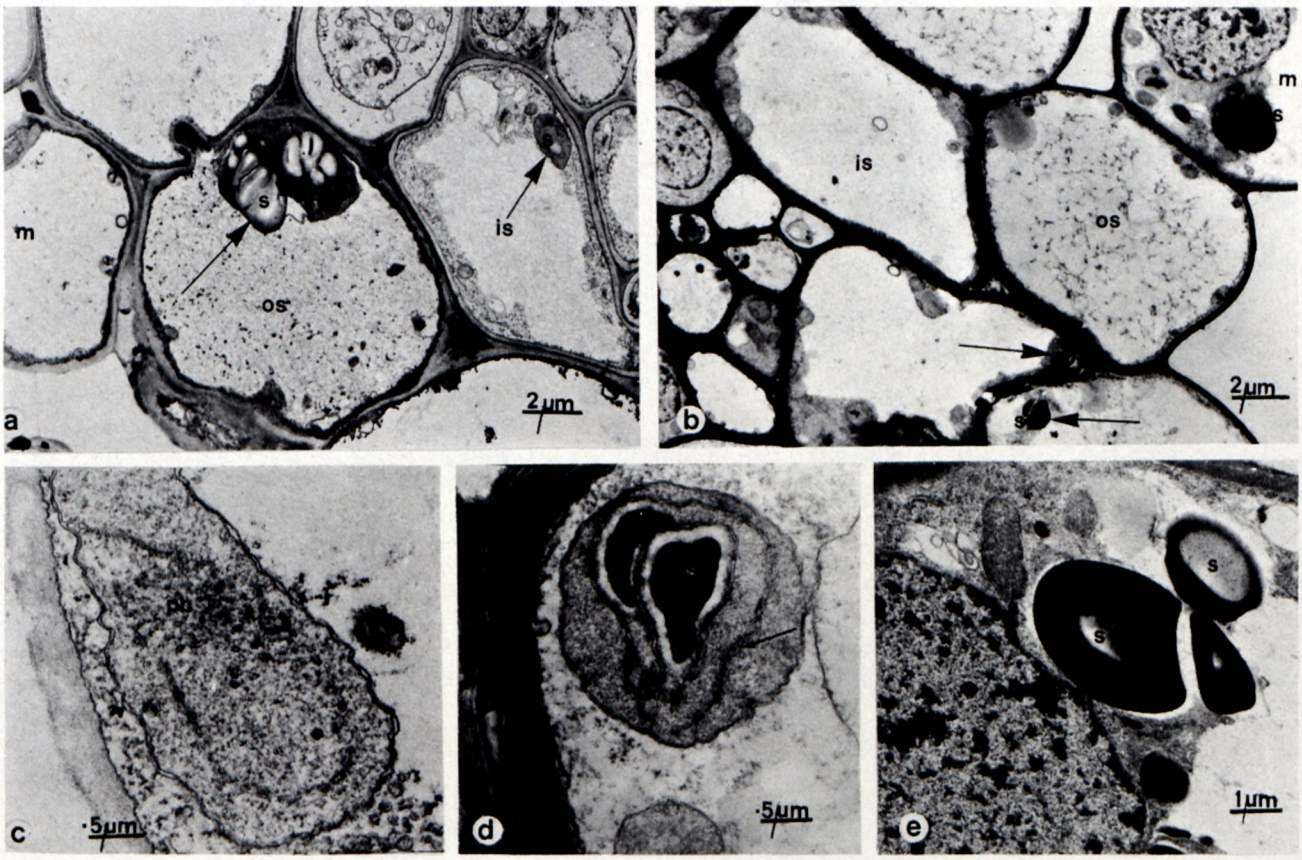

FIG. 3.—Tissue of the colourless leaf sheath. **a,** C_3 leaf sheath; inner (is) and outer (os) bundle sheath cells; amyloplasts (arrowed); m = mesophyll; s = starch. **b,** C_4 leaf sheath; inner (is) and outer (os) bundle sheath cells; amyloplasts (arrowed); m = mesophyll; s = starch. Note: amyloplasts are present in both inner and outer sheath (refer Fig. 2d). **c,** etioplast; pb = prolamella body. **d,** chloramyloplast containing starch grains (s) and lamellae (arrowed). **e,** amyloplast. Note osmiophilic starch grains (s).

are more frequently seen in the C_3 mesophyll, while the C_4 mesophyll shows more amyloplasts. Average size of amyloplast profiles is 5 μm across.

Ligule region

All bundles regardless of size are surrounded by two sheaths in both C_3 and C_4 leaves (Fig. 4a, b). In the C_4 form the inner cells show enlargement and plastid proliferation in the form of numerous chloramyloplasts (Fig. 4e) and many etioplasts, a recognized stage in chloroplast formation (Fig. 4f).

Lamellae and lipid droplets are present in the stroma. Division figures are frequently seen (Fig. 4e). Due to their pleiomorphic shapes plastid profiles are difficult to measure, but are small averaging approximately 2 μm in length. In the C_4 leaf fewer plastids are seen in the parenchyma outer sheath than in the inner sheath. By comparison with the C_4 inner bundle sheath, there are also fewer plastids in the small cells of the C_3 inner bundle sheath (Fig. 4c, d). In the latter, plastids have more or less the same conformation as those in the C_4 inner sheath and are of similar small size. They show fewer division figures.

Midlamina (green)

At midlamina C_3 and C_4 leaves are clearly very different in appearance. Compare Fig. 5a & b.

C_4 leaf

The Kranz of specialized inner bundle sheath cells is well defined (Fig. 5a). Bundle sheath cells are filled with large chloroplasts, profiles averaging from 5–7 μm in length and 5–6 μm in width. Bundle sheath chloroplasts have stacked grana, numerous starch grains and large lipid droplets (Fig. 6a). Chloroplasts show a tendency towards centrifugal arrangement within the cells but this is not obvious. The outer bundle sheath chloroplasts often show a disrupted appearance and/or reduction in size (Fig. 5a). The thickened cell wall shows numerous plasmodesmata (Fig. 5c) both between inner and outer sheath and between inner sheath and mesophyll cells.

Mesophyll cell chloroplasts are peripherally arranged. They compare favourably with bundle sheath cells with regard to starch and lipid content. Starch grains appear similar in size in bundle sheath and mesophyll cells and appear in comparable numbers in the mesophyll (Fig. 6b). There is a size difference in chloroplast profiles. The chief size difference is seen in profile width. They are of comparable length. Mesophyll chloroplast profiles measure approximately 5 μm in length and 3 μm in width.

C_3 leaf

Chloroplasts in the inner mestome sheath are reduced in size and in number. (Fig. 5b, d). Profiles

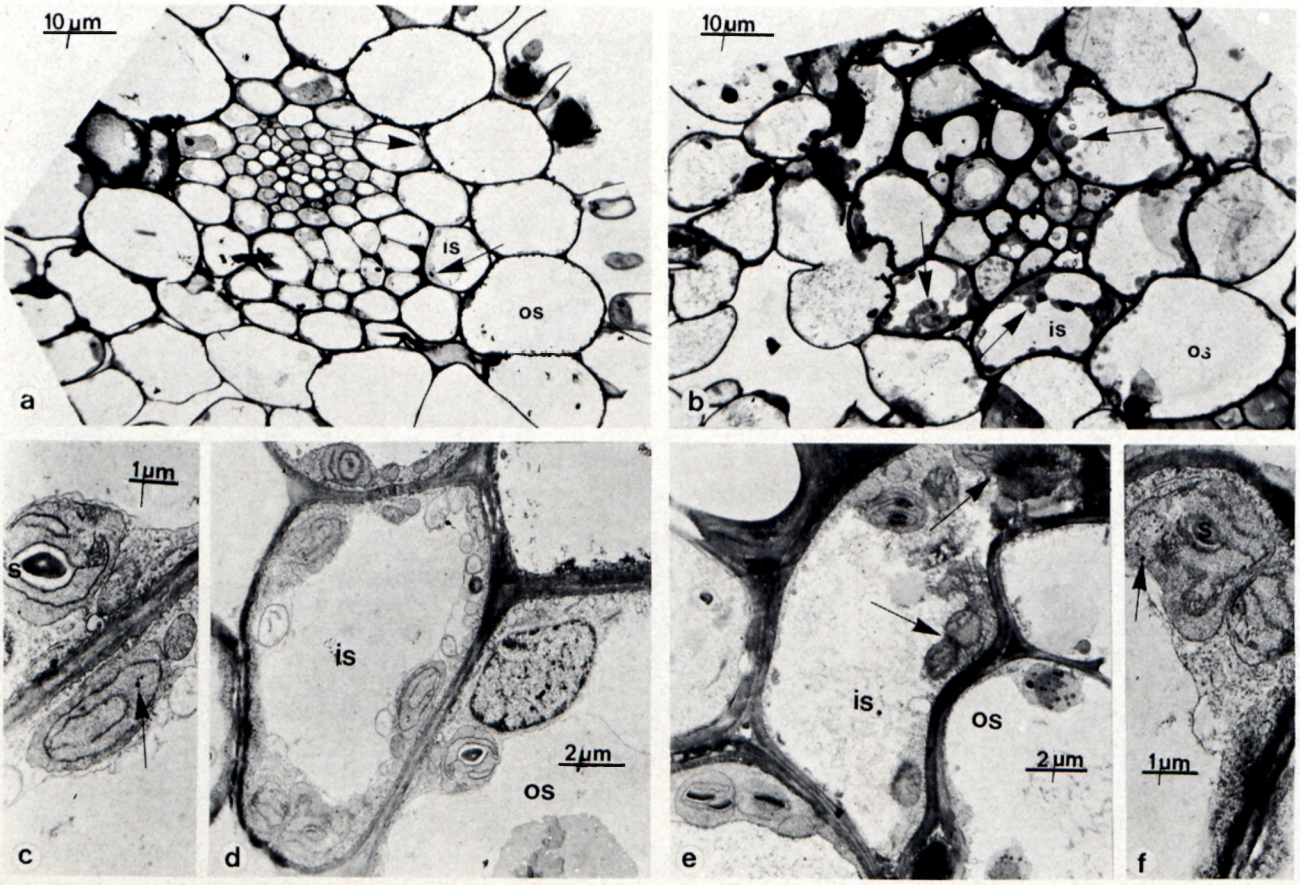

FIG. 4.—Tissue of the ligule region. **a,** C_3 leaf. Entire bundle; note that the inner sheath (is) contains few plastids (arrowed); os = outer sheath. **b,** C_4 leaf. Entire bundle; note plastid proliferation in inner or mestome sheath (is); os = outer sheath; plastids arrowed. **c.** C_3 leaf; chloramyloplasts in mesophyll cells. Lipid arrowed; s= starch. **d.** C_3 leaf inner (is) and outer (os) bundle sheath cells showing plastid population. **e,** C_4 leaf; inner (is) and outer (os) bundle sheath cells showing proliferating plastid population. Division profiles arrowed. **f,** C_4 leaf. Chloramyloplasts in mesophyll cells, lipid arrowed; s = starch.

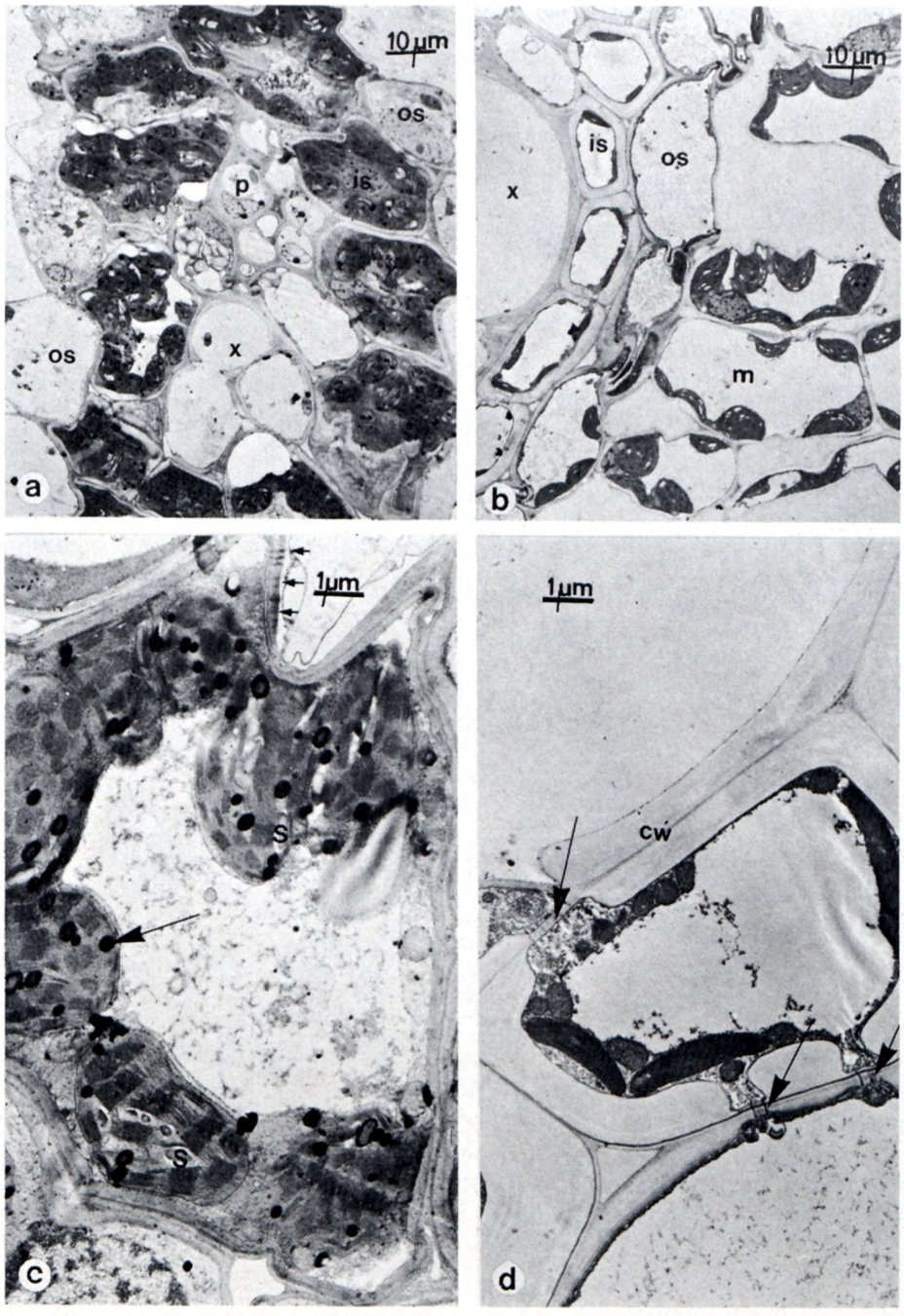

FIG. 5.—Tissue of the mid-lamina. **a,** C_4 leaf; entire bundle. Note the well-defined chloroplasts and the outer parenchymatous sheath (os) almost devoid of chloroplasts; p = phloem; x = xylem. **b,** C_3 leaf; portion of bundle. Note the small thick-walled mestome (inner) sheath (is) both with reduced peripheral chloroplasts; m = mesophyll; x = xylem. **c,** C_4 inner bundle sheath cell with large chloroplasts; plasmodesmata, small arrows; lipid, larger arrows; s = starch. **d,** C_3 inner bundle sheath cell with the thick-wall (cw) and reduced chloroplasts. Note plasmodesmata in embayments in wall (arrowed).

show marked reduction in width and length measuring approximately 1,5 µm across and 3–4 µm in length. They occupy a peripheral position in the cells which show very thick walls (Fig. 5d). Plasmodesmata are seen between inner and outer sheath cells and between inner sheath and vascular bundle. Where they occur the inner sheath wall is reduced in thickness forming embayments in the wall. Chloroplasts are virtually absent from the outer sheath.

Mesophyll cells show peripheral chloroplasts; they are characteristically lens-shaped (Fig. 6d). Profiles measure approximately 8 µm in length and from 2–3 µm across. They show stacked thylakoids, and numerous relatively small starch grains and lipid droplets (Fig. 6c, d).

Results of enzyme assays are shown in Table 1.

DISCUSSION

It is apparent from this study of chloroplast formation in *A. semialata* that ontogenetic studies require many different approaches. For this reason, points for discussion are dealt with under separate sections, viz ontogeny, taxonomy physiology and morphology.

Chloroplast and vascular bundle ontogeny

Four chloroplast types are present in *A. semialata:* mesophyll and specialized bundle sheath chloroplasts in the C_4 form and mesophyll and reduced bundle sheath chloroplasts in the C_3 form. All the chloroplasts present in the mature C_3 and C_4 leaves appear to be formed via an ontogenetic pathway similar to the one described by Hinchman (1972) in oat coleoptiles, as follows (Fig. 8): Amyloplasts are

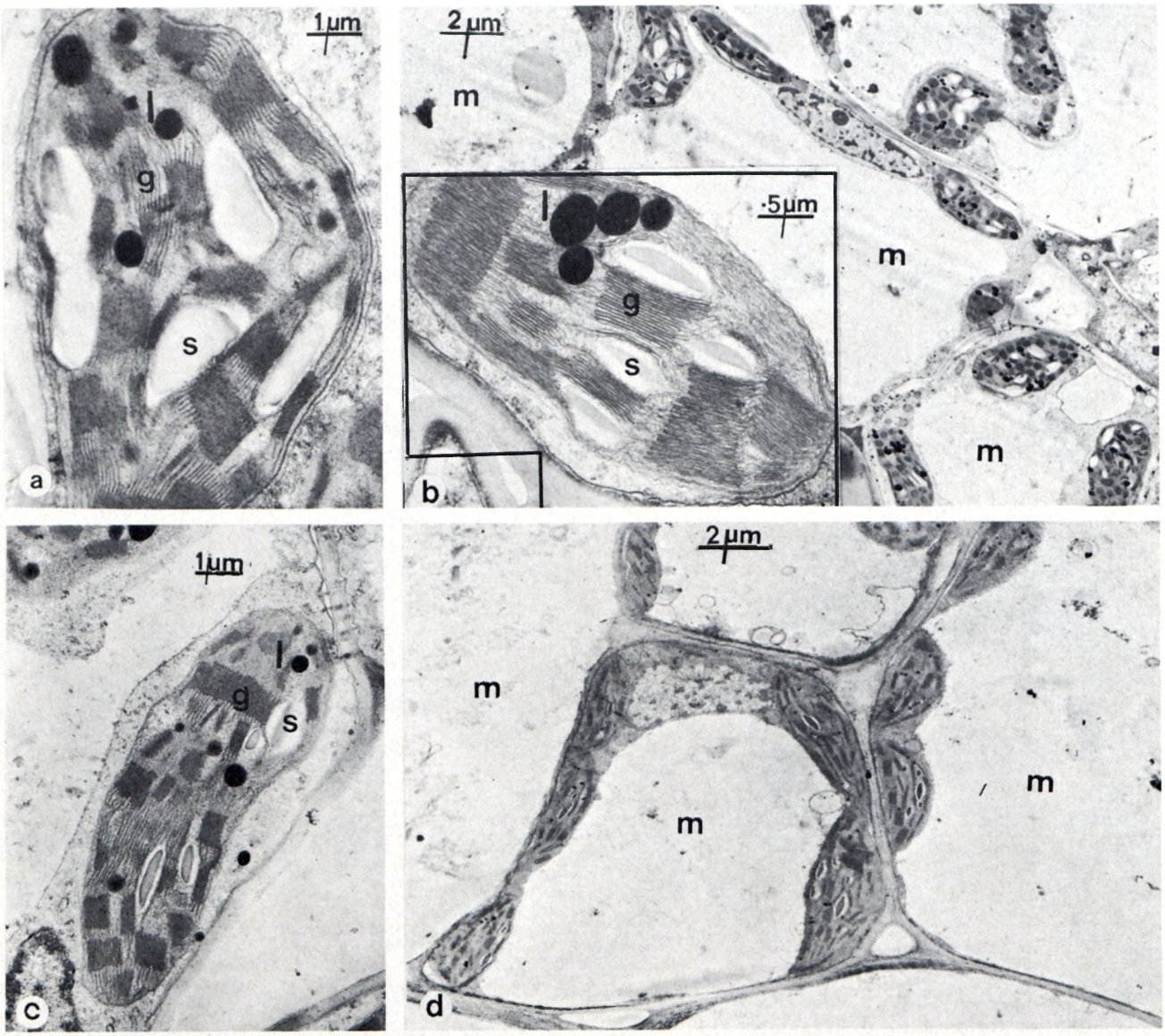

FIG. 6.—Mature chloroplasts in C_3 and C_4 leaves at midlamina. **a,** C_4 bundle sheath cell chloroplast. Well-stacked grana (g), large lipid droplets (l) and starch grains (s) are seen. Note absence of peripheral reticulum. **b,** C_4 mesophyll cells (m). Chloroplasts are peripherally arranged. Insert mesophyll chloroplast; g = grana; l = lipid; s = starch. **c,** C_3 mesophyll cell chloroplast with stacked grana (g) lens-shaped starch grains (s) and lipid droplets (l). **d,** C_3 mesophyll cells (m) with peripheral chloroplasts.

formed from proplastids in the coleoptile parenchyma. As the storage starch in the amyloplast is hydrolyzed, the amyloplast either reverts back to a 'permanent' proplastid with lamellae and ribosomes and no starch, (Fig. 8) or develops into a 'chloramyloplast', i.e. a plastid containing both lamellae and starch grains. This takes place in either the dark or the light. On exposure to light the chloramyloplast then either develops directly into a structurally mature chloroplast (Fig. 8a) or develops via an etioplast (Fig. 8b) which contains only unstacked lamellae and prolamellar bodies, into a chloroplast. Stages from amyloplast to chloramyloplast (Fig. 3c–e) are shown in both photosynthetic forms of *A. semialata*. In relating Hinchman's hypothesis to the current observations, it is proposed in this study that some proplastids are present in all the leaf cells whatever their age and position.

Hinchman (1972) established with buoyant density DNA studies that the plastid DNA component was identical irrespective of the plastid form, supporting his concept of a common origin for all plastids from the primary proplastid. The flow diagram of light related plastid ontogeny in the oat coleoptile (Hinchman, 1972) is interpreted in terms of plastid development in *A. semialata* (Fig. 8), which is light related in a normal growth situation.

The developmental sequence from proplastid to chloroplast on exposure to light is well known (Cran & Possingham, 1974; Engelbrecht & Weier, 1967). Stages shown in this investigation from amyloplast to chloroplast are less well known. These stages are important not only in chloroplast formation but also from the viewpoint of starch storage and translocation in grass leaves.

Vascular bundle ontogeny in the C_3 and C_4 forms of *A. semialata* has been traced by Frean & Cresswell (1981; see Fig. 7), who show provascular tissue in the lower leaf base and consider that *A. semialata* follows the general pattern of development

TABLE 1.—Activity of photosynthetic pathway enzymes extracted from *Alloteropsis semialata* plants grown inside and outside the greenhouse

Enzyme	μ moles NAD (P)/H or CO_2 mg Chl^{-1} min^{-1}	
	Inside greenhouse temp. max = 28°C min = 10°C	Outside greenhouse temp. max = 16°C min = 4°C
NADP-malic enzyme	47,1	5,3
NAD-malic enzyme	1,4	0
NADP-malic dehydrogenase	4,8	2,1
NAD-malic dehydrogenase	143,9	192,9
PEP-carboxylase	37,5	50,4
Aspartate aminotransferase	22,5	283,9
Alanine aminotransferase	1,4	77,6
PEP-carboxykinase	0,1	7,8
RUBP-carboxylase	43,4	44,9

NADP = nicotinamide adenine dinucleotide phosphate.
NAD = nicotinamide adenine dinucleotide.
PEP = phosphoenolpyruvate.
RUBP = ribulose-1,5-bisphosphate.

as described in *Zea mays* (Kirk, 1971). In both these plants the youngest cells are near the base of the leaf and oldest near the tip. Leech, Rumsby & Thomson (1973) find that there is a progressive sequence of plastid differentiation from the base of the primordial leaf sheath to the lamina. It is difficult to relate this early plastid differentiation in the leaf base to formation of Kranz cells in the smallest (third order) bundles of the lamina. These small bundles arise chiefly in the lamina (but are also seen in leaf sheath and ligule extensions), whereas first and second order bundles form from the provascular tissue in the leaf base early in the development of the leaf in both forms of *A. semialata*. The increase in leaf breadth, is accompanied by an increase in number of third order bundles.

The formation of the third order bundles may be compared with the formation of the smallest bundles in the leaf of *Zea mays* (Sharman, 1942), where there is considered to be a 'double wave' of procambial (= provascular) differentiation, i.e. the first wave of differentiation proceeds from the base towards the tip in the early ontogenetic stages when the larger strands are formed. Then the next wave proceeds from the tip towards the base when the smallest bundles are formed together with the lateral anastomoses which are the last strands to appear. This appears to be the pattern of vascularization in *A. semialata* where the first and second order bundles correspond to Sharman's larger strands, and the third order bundles to his smallest bundles.

The formation of the small (third order) bundles can be explained on the basis of some proplastids being present in all the leaf cells. Cells and proplastids could become activated in delayed meristems (Esau, 1953) in the ground tissue to form the small bundles, in a second wave of vascular differentiation as described by Sharman (1942). More evidence is needed to prove this point. Autoradiographic labelling of a plastid component such as a nucleotide might provide an answer to this problem. Labelling in the presence or absence of specific inhibitors of chloroplast protein biosynthesis may assist in the elucidation of the rôle of the cytoplasmic genome and of the organelle in these aspects.

Chloroplast development in *A. semialata* indicates both cell determined and light influenced morphological changes in these semi-autonomous organelles; this is deduced from the fact that exposure to light results in the formation of Kranz sheath chloroplasts in the C_4 form only.

Laetsch & Price (1969) in following formation of dimorphic chloroplasts in sugar cane, leave unsolved the question of whether the structure of the chloroplast is programmed by the cell in which they occur, or whether they control their own development. If C_3 and C_4 forms of *A. semialata* are indeed controlled by one genome, an interesting new aspect has been added to the problem of chloroplast dimorphism which differs in C_3 and C_4 forms of *A. semialata*.

Brown (1975) points out that Kranz cells in most taxa are derived from the ground parenchyma, but that they can also be derived from procambium which develops into the vascular bundle, including the mestome sheath. Fig. 8 shows possible derivation of Kranz chloroplasts from the proplastids of vascular tissue. This derivation may apply to third order bundle formation as well as to formation of first and second order bundles.

Taxonomy

Brown (1975) proposes that all single-sheathed Panicoideae may have the Kranz sheath homologous with the mestome sheath. In those members of the Paniceae with both a mestome (inner) and a parenchymatous (outer) sheath, it is the parenchymatous sheath which is homologous with the Kranz sheath. Brown suggests that the single Kranz mestome sheath represents typical panicoid bundle sheath structure and that evolution of a Kranz sheath homologous with the mestome sheath indicates that in the Panicoideae there have been

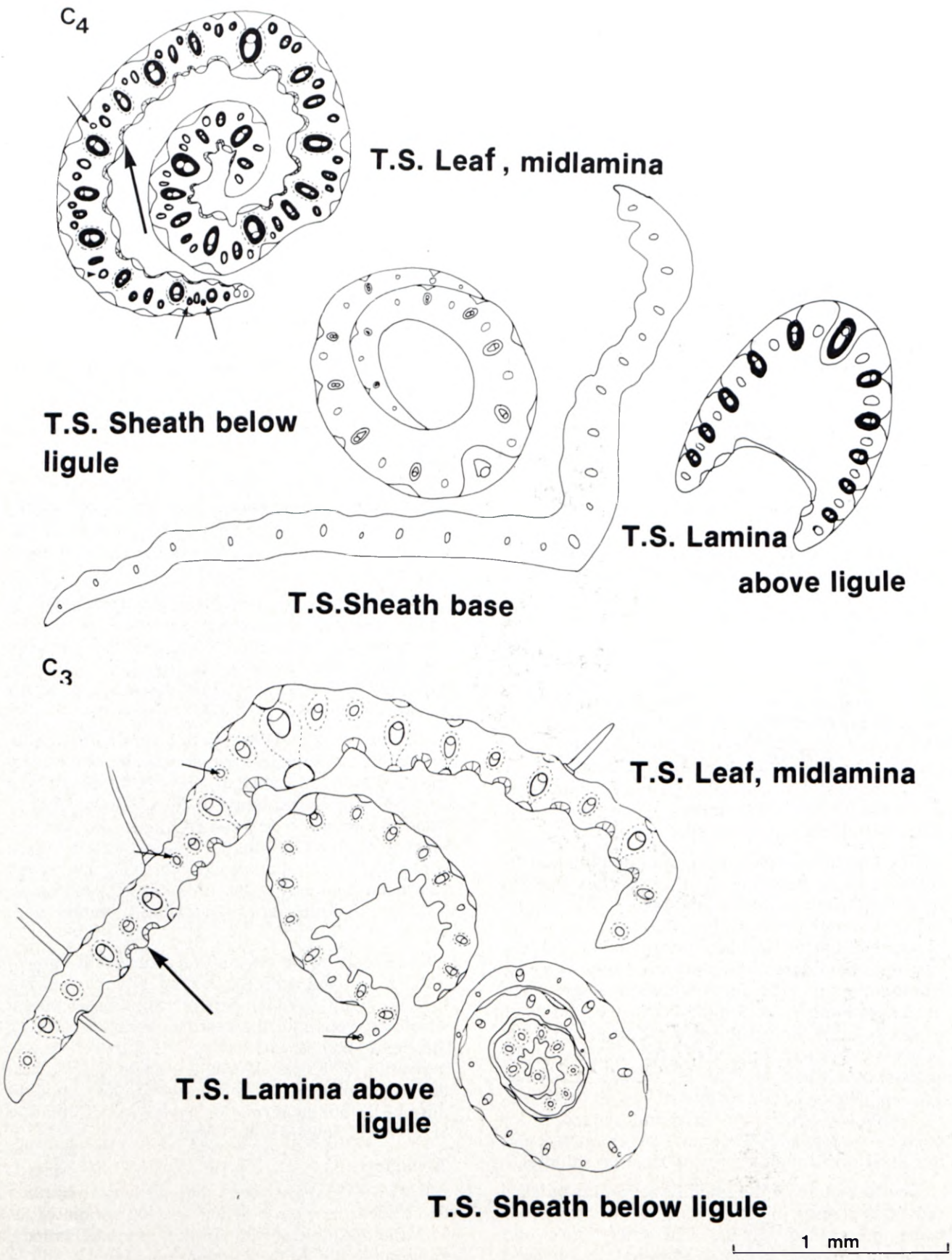

FIG. 7.—Plan of transverse sections of C_3 and C_4 leaves showing distribution of vascular bundles in leaf tissue. Note new bundles in midlamina section in both C_3 and C_4 leaves (small arrows). Xylem faces adaxial surface (large arrow). Modified from Frean & Cresswell (1981).

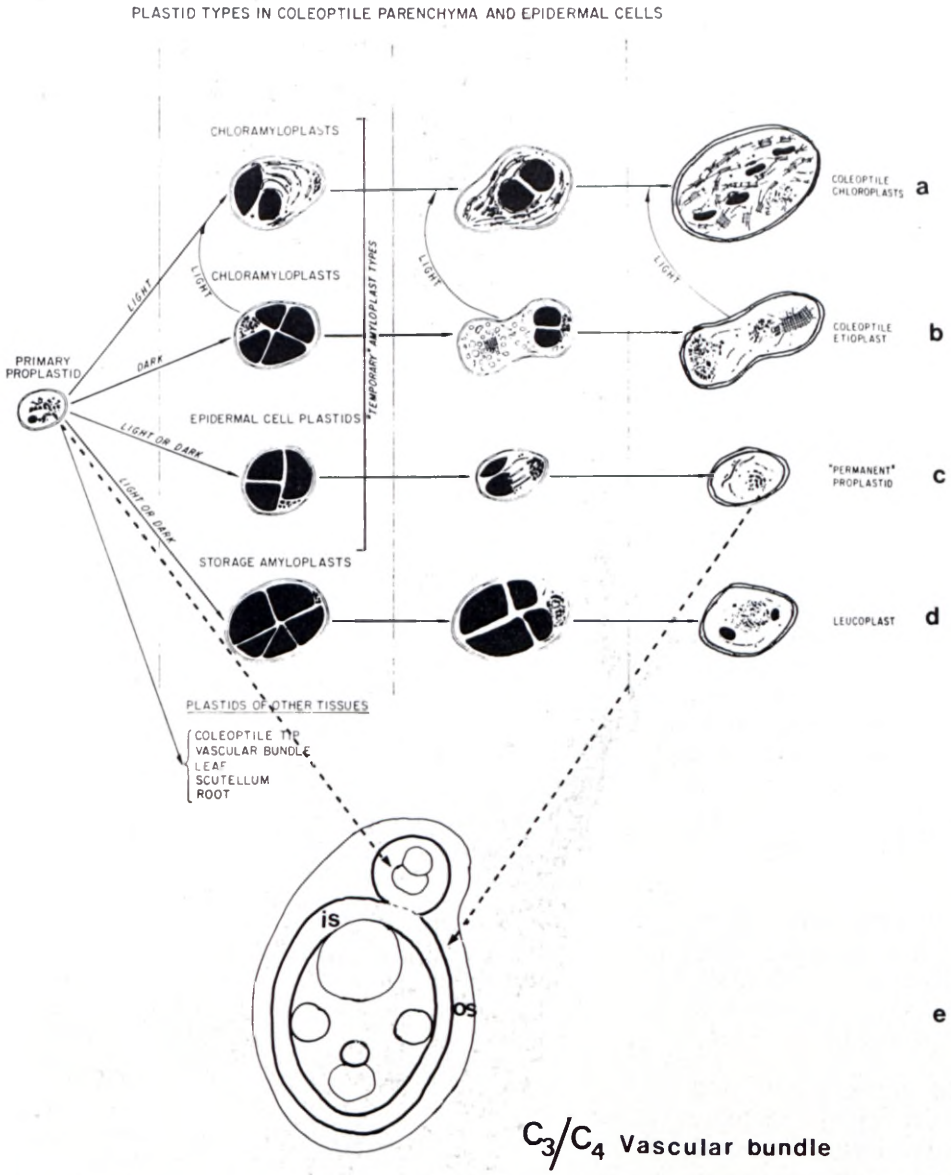

FIG. 8.—Flow diagram (modified from Hinchman, 1972) showing plastid ontogeny. **a,** from primary proplastid via amyloplast and chloramyloplast to chloroplast; **b,** from primary proplastid via amyloplast and etioplast to chloroplast; **c,** from primary proplastid via amyloplast to permanent proplastid; **d,** not followed in *A. semialata;* **e,** vascular bundle of C_3 or C_4 leaf showing possible derivation of plastids in inner (mestome) sheath from primary proplastids of the pro cambium and in the outer parenchyma sheath from permanent proplastids in the ground parenchyma.

two origins of Kranz tissue since some members have a Kranz parenchyma sheath and others a Kranz mestome sheath.

In the C_4 form of *A. semialata* in this study, not only are Kranz and mestome sheath homologous but there is a persistent parenchyma sheath almost devoid of chloroplasts. This is an unusual situation in the Poaceae generally and in the Panicoideae in particular. Brown (1975) notes three anatomical types in *A. semialata:*

1, non-Kranz in a South African specimen of the variety *ecklonii* (Stapf) Stapf similar to the C_3 form discussed in this study;

2, Kranz, with only one bundle sheath, homologous with the mestome sheath in an Australian specimen and

3, an intermediate anatomy as described in the C_4 form in this study, i.e. a Kranz mestome (inner) sheath, with parenchyma (outer) sheath cells smaller but still 'essentially empty' (and mesophyll cells not radially arranged).

Ellis (1974) describes this anatomy as representing an intermediate stage in the evolution of NADP-type anatomy. This matter is discussed in the following paragraphs.

Physiological anatomy

C_4 grasses, in which the primary product of CO_2 assimilation (oxaloacetate) is reduced to a high proportion of malate, are characterized by a single Kranz bundle sheath (Brown 1975; Hattersley & Watson, 1976; Ellis, 1977). Ultra-structurally the sheath cells surrounding the larger bundles are seen to have agranal chloroplasts with centrifugal arrangement and low mitochondrial frequency in contrast to grasses where a high proportion of oxaloacetate is aminated to aspartate, where there is a double bundle sheath with an inner or mestome sheath between the vascular tissue and the Kranz sheath.

This investigation using routine light and transmission electron microscope techniques, shows that the C_4 form of *A. semialata* does not conform

anatomically to the single Kranz bundle sheath diagnostic for malate formers. The presence of grana in the Kranz sheath chloroplasts and the presence of large numbers of mitochondria in the bundle sheath cells, as well as the lack of definite centrifugal arrangement of chloroplasts, is not in agreement with the classification of this grass anatomically as an NADP-malic enzyme species, as suggested by Ellis (1977).

The presence of grana in the bundle sheath chloroplasts would place it into one of two non malate forming groups namely NAD-malic enzyme type or PEP-carboxykinase type; this is further supported by the presence of large numbers of Kranz sheath mitochondria and the presence of an outer parenchymatous bundle sheath.

Enzyme studies

Preliminary studies on the decarboxylating enzyme activities show that although the predominant activity is found to be NADP-malic enzyme, enzyme activity varies with temperature. It is lower in plants grown in the open (under winter conditions) than in plants kept in the greenhouse. There is a marked increase in PEP-carboxykinase with a decline in NADP-malic enzyme in the outdoor plants. Negligible activity of NAD-malic enzyme was found under either set of growth conditions.

It is noted that these results differ from those obtained by Frean, Ariovich & Cresswell (1980) where low levels of both NAD-malic and NADP-malic enzyme were found in C_4 plants grown at a temperature range of 16/30°C–20/30°C min./max.

It is concluded from this investigation that classification of C_4 grasses on the basis of anatomy and cytology is valid; but classification may become confusing if the different anatomical forms are given a biochemical nomenclature, particularly in view of the labile nature of any one particular decarboxylating enzyme with environmental changes.

Morphology

In the sample used in this investigation glabrous and pubescent morphologies were found to show, respectively, C_4 and C_3 anatomical and photosynthetic characteristics. However, the degree of pubescence is known to vary considerably in this widely distributed species (personal observations); no consistent morphological differences of sufficient significance have been found to warrant separation of the C_3 and C_4 forms taxonomically (Clayton, 1980: pers. comm.).

This investigation succeeds in its immediate aim in that it shows that plastid dimorphism exists in both C_3 and C_4 forms of *A. semialata* and that plastids become structurally distinguishable in young leaves on exposure to light only in those portions which are chlorenchymatous. However, no explanation of the resulting dimorphism can be given. Chloroplast and vascular bundle structure is related also to other aspects of the C_3/C_4 syndrome with particular reference to the Poaceae.

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