

Anatomy and Ultrastructure of Embryonic Leaves of the C₄ Species *Setaria viridis*

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Running title: Development in embryonic leaves of *Setaria viridis*

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23 ABSTRACT

24 • *Background and Aims*

25 *Setaria viridis* is being promoted as a model C₄ photosynthetic plant because it has a small
26 genome (~515 Mb), a short life cycle (~60 days) and it can be transformed. Unlike other C₄
27 grasses such as maize, however, there is very little information about how C₄ leaf anatomy
28 (Kranz) develops in *S. viridis*. As a foundation for future developmental genetic studies, we
29 aimed to provide an anatomical and ultrastructural framework of early shoot development in
30 *S. viridis*, focusing on the initiation of Kranz anatomy in seed leaves.

31 • *Methods*

32 *S. viridis* seeds were germinated and divided into five stages covering development from the
33 dry seed (stage S0) to 36h after germination (stage S4). Material at each of these stages was
34 examined using conventional light, scanning and transmission electron microscopy.

35 • *Key Results*

36 Dry seeds contained three embryonic leaf primordia at different developmental stages
37 (plastochron 1-3 primordia). The oldest (P3) leaf primordium possessed several procambial
38 centres whereas P2 displayed only ground meristem. At the tip of P3 primordia at stage S4, C₄
39 leaf anatomy typical of the NADP-ME subtype was evident in that vascular bundles lacked a
40 mestome layer and were surrounded by a single layer of bundle sheath cells that contained
41 large centrifugally-located chloroplasts. Two to three mesophyll cells separated adjacent
42 vascular bundles and one mesophyll cell layer on each of the abaxial and adaxial sides
43 delimited vascular bundles from the epidermis.

44 • *Conclusions*

45 The morphological trajectory reported here provides a foundation for studies of gene
46 regulation during early leaf development in *S. viridis* and a framework for comparative
47 analyses with other C₄ grasses.

48 KEY WORDS: C₄, *Setaria viridis*, Kranz anatomy, embryonic leaves, vascular development

INTRODUCTION

C₄ photosynthesis occurs in 19 angiosperm families that encompass both monocots and eudicots, with monocot representatives including ~4,500 grass species (Sage *et al.*, 2011; Williams *et al.*, 2012). The C₄ pathway differs from the C₃ pathway in that photosynthesis is split between two cell-types. CO₂ is initially fixed in the mesophyll (M) cells by phosphoenolpyruvate carboxylase and then after decarboxylation of a C₄ acid, it is re-fixed by ribulose biphosphate carboxylase/oxygenase in the bundle sheath (BS) cells (Von Caemmerer and Furbank, 2003; Langdale, 2011). In most C₄ plants, BS and M cell-types are arranged around leaf veins in a characteristic anatomy known as Kranz (Brown, 1975; Dengler *et al.*, 1985). Although Kranz anatomy is not a prerequisite for C₄ photosynthesis (Voznesenskaya *et al.*, 2001; Sage, 2002), it is present in all C₄ grasses.

C₄ plants have previously been classified into three subtypes, depending on the mechanism by which C₄ acids are decarboxylated in the BS cells. Although the biochemical distinction between these subtypes is now questioned (Furbank, 2011), there are distinct differences between BS cell anatomy in so-called NADP-ME, NAD-ME and PEP-CK subtypes (Brown, 1975; Edwards and Voznesenskaya, 2011). In NADP-ME species BS cell chloroplasts lack well-developed grana and are located centrifugally; in NAD-ME species BS chloroplasts are positioned centripetally and possess well-developed grana; and in PEP-CK species BS chloroplasts possess well-developed grana and are arranged in a centrifugal position. In addition to differences in chloroplast structure and arrangement, BS cells are distinguished from M cells by a range of other features including number, arrangement and size of mitochondria, degree of vacuolation and type of plasmodesmata. The thickness and composition of the cell wall can also differ (Dengler *et al.*, 1986; Edwards and Voznesenskaya, 2011). For example, the BS cells of several species in the NADP-ME and PEP-CK subgroups have a suberized lamella (Eastman, 1988; Edwards and Voznesenskaya,

2011). With at least 62 lineages of C₄ plants now identified (Sage *et al.*, 2011), further variation in structure and organisation of Kranz anatomy is sure to be discovered (Williams *et al.*, 2012), reinforcing the need for a clear and detailed understanding of both anatomy and metabolism in species used as C₄ models.

The C₄ grass *Setaria viridis* (L.) Beauvois is being proposed as a model for understanding C₄ metabolism and anatomy (Brutnell *et al.*, 2010; Diao *et al.*, 2014), with the genome sequence of closely related *S. italica* (Bennetzen *et al.*, 2012) and transformation protocols (Martins *et al.*, 2015; Van Eck and Swartwood, 2015; Saha and Blumwald, 2016) opening the way for comprehensive analyses of C₄ in a rapid cycling plant. *S. viridis* is a NADP-ME C₄ grass (Aliscioni *et al.*, 2015), but is phylogenetically related to switchgrass (NAD-ME subtype) and *Panicum virgatum* (PEP-CK subtype) (Brutnell *et al.*, 2010). Six subclasses of NADP-ME subtype anatomy were identified in Poaceae, including the most common ‘Classic’ subtype (Edwards and Voznesenskaya, 2011). The different anatomies mainly vary with respect to i) presence or absence of the mesophyll sheath and suberized lamella, ii) number of BS cell layers, and iii) ultrastructure of chloroplasts (Edwards and Voznesenskaya, 2011). Because photosynthetic BS cells are a defining feature of C₄ plants, understanding the ontogeny of this cell-type is central to any interpretation of the function of these different anatomical arrangements and of the relationship between species possessing them. We have therefore conducted a detailed analysis of the structure and the ultrastructure of BS cells in embryonic leaves of *S. viridis* during germination, from seed imbibition to coleoptile rupture by the first leaf. Data presented here on the pattern of Kranz differentiation in *S. viridis* seed leaves provides a platform for future morphological and comparative studies with this new C₄ model species.

101 MATERIALS AND METHODS

102 *Plant growth and sampling*

103 Fruit of *S. viridis* accession A10 were collected from greenhouse-grown plants at the
104 *Universidade Federal do Rio de Janeiro* - UFRJ (Brazil) in January 2015 and stored at room
105 temperature (25-28°C) for 10 months before use. Prior to sampling for microscopy, ~150
106 fruits were placed in 30 mM potassium nitrate solution for 24 h at room temperature and then
107 washed three times with gentle agitation in distilled water (modified from Sebastian *et al.*,
108 2014). Germination was then carried out on moistened Germitest paper® (Germilab, Brazil)
109 in Petri dishes in a BOD incubator (Eletrolab EL202, SP, Brazil) under 28°C under
110 continuous light.

111
112 For light and transmission electron microscopy (TEM), ten fruits with bracts were sampled at
113 each of the sampling times: 0 h (dry seed), 12 h, 15 h, 24 h and 36 h after imbibition.
114 Sampling times were established based on pilot studies that identified morphological
115 landmarks such as the protrusion of coleorhiza and the appearance of the primary root. The
116 developmental stage of the seed at each sampling time was classified as S0 (0 h), S1 (12 h),
117 S2 (15 h), S3 (24 h) and S4 (36 h). Seeds became swollen between stages S0 and S1, and the
118 coleorhiza emerged at stage S1. Stage S2 was characterized by the presence of hairs on the
119 coleorhiza and stage S3 by the appearance of the coleoptile and the primary root. By stage S4,
120 the first embryonic leaf had ruptured the coleoptile.

121
122 Five fruits without bracts were collected for scanning electron microscopy (SEM) at the same
123 sampling times. All bracts (two glumes, sterile lemma, fertile lemma and palea) were
124 removed from the fruits to facilitate SEM study of post germination events.

125
126 To examine the overall venation pattern in expanded leaves, the first leaf to emerge was

harvested around a week after seed imbibition. The leaf was cleared with ethanol at 90°C and stained in 1% safranin (modified from Kraus and Arduin, 1997).

Anatomical and ultrastructural studies

Plant material was fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 h and post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer for 1 h. For SEM, fruits without bracts were then dehydrated through an ethanol gradient, critical-point dried and sputter-coated with gold (Vega 3LMU Tescan, Brno, Czech Republic). Samples were viewed using a scanning electron microscope (Spirit Biotwin 12 FEI Company, Oregon, USA). For light microscopy, fruits with bracts were dehydrated in an ethanol-propylene oxide series, embedded in Spurr's resin (Premix Kit-Hard, TAAB Laboratory and Microscopy, Berks, England) and polymerized at 70°C overnight. Transverse thin sections (1µm) of the embedded material were cut from the tip to the base of the coleoptile using an ultramicrotome (RMC Products, Tucson, USA), stained with 0.05% toluidine blue and viewed in a Leica DM 2500 (Wetzlar, Germany) optical microscope. Sequential 1µm sections were used to create longitudinal diagrams of the leaf vascular pattern based on the structures present at each developmental stage. For TEM, ultrathin sections (60 nm) were cut using an ultramicrotome (ultra-RMC Products, Tucson, USA), mounted on copper grids, and stained with 1% uranyl acetate in water for 25 min followed by lead citrate for 3 min. Sections were examined in a transmission electron microscope (Spirit Biotwin 12 FEI Company, Oregon, USA). Measurements were carried out using the TEM Imaging Platform program.

RESULTS

General development during the first 36h of germination

SEM images of the diaspore (dispersion unit) of *S. viridis* revealed a caryopsis comprised of five bracts – two glumes, a sterile and a fertile lemma, plus a palea (Stage S0; Fig. 1A). The

coleorhiza ruptured the seed coat after 12 h imbibition (Stage S1; Fig. 1B) and after 15 h the absorbent hairs of the coleorhiza were visible (Stage S2; Fig. 1C). Disruption of the caryopsis by emergence of the coleoptile and the radicle occurred 24 h after imbibition (Stage S3; Fig. 1D), and 36 h after imbibition the first juvenile leaf ruptured the coleoptile (Stage S4; Fig. 1E, F). The diaspore will be referred to hereafter as the seed.

Anatomy of the mature embryo

The embryo of mature seeds averages ~800 µm in length and ~150 µm in width, excluding the coleoptile. The shoot apical meristem (SAM) and young leaf primordia are subtended by the mesocotyl, which is characterized by cells that are more densely cytoplasmic than in the SAM, and by the radicle in which longitudinal cell files are apparent. Three leaf primordia are present around the SAM (Fig. 2), referred to as plastochron (P) 1-3, where a plastochron is the time interval between initiation of sequential primordia. The youngest (P1) primordium has just initiated whereas the P2 and P3 primordia enclose the SAM, as is typical of grass leaf primordia. During the 0-36 h timeframe examined here, no new leaf primordia were initiated at the SAM.

Venation pattern in expanded leaves

To provide a framework on which to map the trajectory of vascular development in embryonic P1-P3 leaf primordia of *S. viridis*, venation patterns were first examined in the expanded leaf blade of the first leaf to emerge from the coleoptile (i.e. P3 in Fig. 2). Paradermal (Fig. 3A) and transverse (Fig. 3B) views revealed a venation pattern that is typical of monocots, with the longitudinal midvein (MV) and lateral veins (LV) separated by multiple intermediate veins (IV), that will ultimately be distinguishable as two classes (primary with sclerenchyma connecting to the epidermis, and secondary without sclerenchyma). At the widest point of the leaf the midvein is flanked on both sides by two LV

179 whereas at the leaf tip there is just one LV on each side. Transverse (T) veins connect the
180 longitudinal veins at intervals along the proximo-distal leaf axis (Fig. 3C).

182 *Vascular development during the first 36 hours of germination*

183 To analyse the developmental trajectory towards the expanded leaf venation pattern,
184 transverse sections of developing P3 primordia were examined at different points along the
185 proximo-distal leaf axis, at each of stages S0 to S4. Data from these sections were used to
186 reconstruct the venation pattern at the level of the whole primordium (Fig. 4). Over the first
187 15 hours, the primordium increased in size by only ~10% in the medio-lateral direction and
188 ~45% in the proximo-distal direction, and no new veins were initiated (Fig. 4A-C). A further
189 33% size increase in the medio-lateral direction between 15 and 24 hours led to the initiation
190 of a new intermediate vein at each leaf margin (Fig. 4D). Between 24 and 36 hours the
191 primordium increased in size by 50% in the medio-lateral axis and ~280% in the proximo-
192 distal axis, but no new veins were initiated (Fig. 4E).

194 *Differentiation of vascular bundles*

195 At the tip of the P3 primordium the midvein was flanked by a single LV on each side at all
196 stages of development. In the dry seed (S0), the P3 leaf tip comprised the ground meristem
197 sandwiched by the abaxial and adaxial protoderm. Three procambial centres were identified
198 as groups of small cells but there was no evidence of differentiated cell-types at this stage
199 (Fig. 5A). Cells in the procambial centre were more organized at S1 and the intervening
200 ground tissue comprised three layers (Fig. 5B). Protoxylem was evident in the MV at S2 (Fig.
201 5C), protophloem at S3 (Fig. 5D) and vacuolated BS cells at S4 (Fig. 5E). By S4, most leaf
202 tissues were differentiated: epidermal cells were expanded; stomata were developed on both
203 surfaces; two to three mesophyll cells were present between each pair of vascular bundles;
204 protoxylem, metaxylem and phloem were all present in the MV; chloroplasts were visible in

both bundle sheath and mesophyll cells; and sclerenchyma was present at the leaf margins (Fig. 5E).

At the widest part of the P3 primordium, 25 procambial centres were observed up to S2 (the MV, 4 LV and 20 IV) (Fig. 4AC; 6A-C), and 27 at S3 and S4 (two more IV having developed, one at each leaf margin) (Fig. 4D, E; 6D, E). A comparison of midvein anatomy at the middle and at the tip of the leaf, revealed the normal basipetal differentiation gradient seen in grass leaves. Midvein development was at an equivalent stage 24 h after imbibition at the tip and 36 h after imbibition in the middle of the leaf (compare Fig. 5D and 6G). The transition from undifferentiated procambium at the midvein (Fig. 6F) to visible protoxylem and protophloem (Fig. 6G) took place over 24 h.

Ultrastructure of developing leaf vascular centres

To determine the ultrastructure of developing vascular centres transmission electron microscopy (TEM) was carried out on the tip/middle portion of P3 primordia. TEM revealed organized clusters of essentially undifferentiated cells in the dry seed (S0) (Fig. 7A). BS cells could be distinguished by their concentric organization around the procambial cells and by their larger size. Neither protoxylem nor protophloem were differentiated at this point. All cells in these rudimentary vascular centres possessed numerous lipid bodies and the nuclei featured a prominent nucleolus (Fig. 7A). The presence of large amounts of lipid was confirmed with histochemical analysis (Sudan III and Sudan IV) (data not shown). By S1, the number of lipid bodies had decreased and cells in the procambial centre were more organized (Fig. 7B). Initiation of protoxylem development was marked by cell wall thickening at S2 (Fig. 7C), that was even more evident at S3 (Fig. 7D). At S4, protoxylem, protophloem and metaxylem with degenerated cytoplasm and thick cell walls were evident in the vascular tissue (Fig. 7E).

231

232 At S3, BS cells surrounding the developing vein exhibited dense cytoplasm, large nuclei, and
233 numerous plastids distributed around the cell periphery (Fig. 7D). By S4, BS cells were
234 vacuolated and chloroplasts contained multiple starch granules (Fig. 7E). BS chloroplasts
235 were qualitatively similar to M chloroplasts in terms of thylakoid stacking at S3 (Fig. 8A) but
236 at S4 M chloroplasts appeared to contain more grana (Fig. 8B, C). Plasmodesmatal
237 connections between BS and M cells were established by S4 (Fig. 8C).

238

239 DISCUSSION

240 The development of *Setaria viridis*, from germination to coleoptile rupture 36 h from
241 imbibition, has been investigated using optical, scanning and transmission electron
242 microscopy. Examination of dry seeds demonstrated that three leaves are initiated during
243 embryo development with P3 and P2 primordia encircling the SAM of the mature embryo and
244 P1 comprising just a few cells. 36 h after imbibition of dry seeds it was possible to identify i)
245 the NADP-ME ‘Classic’ subtype of Kranz anatomy with large agranal chloroplasts assuming
246 a centrifugal position in the BS cells and no mestome sheath; ii) vacuolated BS and M cells;
247 iii) two to three M cells between adjacent vascular bundles; iv) a single M cell layer between
248 minor veins and the epidermis; and v) protophloem, protoxylem and metaxylem in developing
249 veins.

250

251 During the first 12 h after seed imbibition, conspicuous and numerous lipid bodies were
252 present in cells of the embryonic *S. viridis* leaves (Fig. 7A, B) but by 24 h most of the lipid
253 reserve had been mobilized (Fig. 7D). This form of energy storage has previously been
254 reported in *S. lutescens*, which showed a decrease in lipid content before leaf emergence after
255 hydration of non-dormant embryos (Rost, 1972), and a similar mobilization of seed reserves
256 precedes cell-type differentiation in maize (Nikiforidis *et al.*, 2013). Distinct growth phases

are also seen in young leaves of the NADP-ME grass *Stenotaphrum secundatum* (Sud and Dengler, 2000) and in *Cleome angustifolia*, a NAD-ME eudicot species (Koteyeva *et al.*, 2014). During the first growth phase in *C. angustifolia* the meristematic zone of the leaf remains undifferentiated, with BS and M cells only becoming identifiable in the second period of growth. The third phase is characterized by BS cells with well-developed central vacuoles and, during the fourth phase, the characteristic NAD-ME type differentiation of the chloroplasts and mitochondria takes place (Koteyeva *et al.*, 2014). Events in these growth phases in *C. angustifolia* clearly parallel development during stages S0 to S4 in *S. viridis* (Fig. 5, 6, 7). It thus appears that cell-type differentiation in embryonic leaves is generally coordinated with regulation of the glyoxylate cycle such that reserves are appropriately mobilized prior to intracellular organization.

Whereas most previous reports have focused on vascular development in the seedlings of C₄ plants, this study has focused on development in embryonic leaves. In grasses the number of embryonic leaves in the seed and the number of juvenile leaves in the seedling are not necessarily the same (Sylvester *et al.*, 2001), and the developmental events determining this relationship are not understood (Bongard-Pierce *et al.* 1996; Sylvester *et al.*, 2001). For example, in maize the four to five embryonic leaves in the seed correspond to the juvenile leaves in the seedling (Bongard-Pierce *et al.*, 1996, Liu *et al.*, 2013,) whereas in bluegrass and rice there are two embryonic leaves but no juvenile seedling leaves (Sylvester *et al.*, 2001). *S. viridis* has four juvenile seedling leaves (Hodge and Doust, 2017) but only three leaves are initiated during embryogenesis, and only two of those exhibit any cell-type patterning in the mature embryo (Fig. 2, 6). As such, the signals that pattern vascular development in *S. viridis* operate in at least three different contexts: i) juvenile patterned during embryogenesis (first two leaves to emerge) ii); juvenile patterned post-germination (leaves 3 & 4); and iii) adult.

Vascular development in embryonic leaves of *S. viridis* closely resembles that of maize (Liu *et al.*, 2013) and of *Spartina alternifolia* (Walsh, 1990), with leaf primordia of the mature seed exhibiting several vascular centres at different stages of development (Fig. 5, 6). When the embryo of *S. viridis* initiated growth following imbibition, the MV and LVs differentiated more rapidly at the tip of the leaf primordium, suggesting that vascular differentiation occurred basipetally, despite the veins being initiated at the base. This same pattern is observed in maize and other grasses (Sharman, 1942; Bosabalidis *et al.* 1994; Nelson and Dengler, 1997). Studies in maize have suggested that the differentiation of BS and M cells in C₄ leaves is dependent on inductive signals from developing veins (Langdale *et al.*, 1988; Langdale *et al.*, 1989; Langdale and Nelson, 1991). BS cell development has also been proposed to act as a point of reference, guiding the differentiation of adjacent tissues (Nelson and Dengler, 1997). For example, the position of veins has been shown to play a critical role in the development of the epidermis in maize (Cerioli, *et al.*, 1994). In *S. viridis*, the consecutive initiation of vascular, BS and epidermal cell-types (Fig. 5, 7) supports the view that the formation of vascular bundles influences the organization of adjacent tissues.

The development of the C₄ photosynthetic apparatus requires coordinated gene expression to underpin both anatomical and metabolic traits. Transcriptomes of BS and M cells in maize and *S. viridis* show a high correlation of transcripts encoding C₄ metabolism proteins (John *et al.*, 2014) but similar datasets are not yet available for genes that are likely to regulate leaf anatomy. The anatomical and ultrastructural analyses reported here provide an empirical basis for such comparisons in the future.

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408 FIGURE CAPTIONS

409 Figure 1. Scanning electron microscopy of *S. viridis* diaspore during germination from dry
410 seed to 36 hours after imbibition (first embryonic leaf rupturing the coleoptile). (A) Stage S0 -
411 dry seed - with bracts: first glume (*), second glume (**), sterile lemma (arrow) and fertile
412 lemma (dashed arrow) enveloping the caryopsis (the palea remains covered by the sterile
413 lemma). (B) Stage S1 - 12 h after imbibition, the coleorhiza rupturing the caryopsis (arrow).
414 (C) Stage S2 - 15 h after imbibition, absorbent hairs of the coleorhiza (arrow) are visible. (D)
415 Stage S3 - 24 h after imbibition, coleoptile breaking through the caryopsis (arrow); the radicle
416 (*), the mesocotyl (dashed arrow) and coleorhiza (***) are visible. (E) Stage S4 – 36 h after
417 imbibition, left: intact coleoptile; right: the first juvenile leaf has ruptured the coleoptile
418 (arrow). (F) Ruptured coleoptile shown in detail. Scale bar = 500 μ m (A, B, D), 1mm (C), 2
419 mm (E), 200 μ m (F).

420

421 Figure 2. Three leaf primordia are evident at the shoot apical meristem (asterisk). The oldest
422 (P3) and P2 primordia encircle the meristem, whereas the youngest (P1) has just been
423 initiated. Scale bar = 20 μ m.

424

425 Figure 3. The vascular system of the first embryonic leaf of *S. viridis*. (A) Cleared leaf
426 showing the vascular pattern in longitudinal view with midvein (MV) and lateral veins (LV)
427 indicated. (B) Transverse section at the leaf tip showing MV flanked by a LV on each side,
428 with intermediate veins (IV) interspersed between the major veins. Three layers of ground
429 tissue (dashed red line) separate the two epidermal layers. (C) Vascular pattern in longitudinal
430 section showing MV, LV and transverse (T) veins. Scale bar = 2 mm (A), 50 μ m (B), 100 μ m
431 (C).

432

433 Figure 4. Vascular pattern in longitudinal view from midvein to margin on one side of *S.*
434 *viridis* leaf from stage S0 (dry seed) to S4 (36 h after imbibition). (A) S0: ~250 μm x ~330
435 μm ; (B) S1: ~330 μm x ~365 μm ; (C) S2: 360 μm x ~375 μm ; (D) S3: ~980 μm x ~500 μm ;
436 (E) S4: ~2740 μm x ~725 μm . Numbers in each case refer to length along proximo-distal axis
437 x width of half of medio-lateral axis. Arrows indicate the direction of vein formation -
438 acropetal: midvein (black arrow) and lateral veins (red arrows); basipetal: intermediate veins
439 (black dashed arrows). Scale bar = 125 μm .

440 Figure 5. Transverse sections at the tip of the P3 primordium. (A) Stage S0 (dry seed) at ~40
441 μm from the tip. MV – black dashed line and LV – red dashed line. (B-E) Sections at ~60 μm
442 from the tip: Stage S1 (12 h after imbibition) (B); Stage S2 (15 h after imbibition), black
443 arrow indicates protoxylem (C); Stage S3 (24 h after imbibition), yellow arrow indicates
444 protophloem (D); Stage S4 (36 h after imbibition) * = BS cell; ** = M cell; * = metaxylem;
445 red arrow points to terminating IV (E). All sections orientated with adaxial surface facing
446 upwards. Scale bar = 20 μm .

447 Figure 6. Transverse sections at the widest part of the P3 primordium. (A) Stage S0 (dry seed)
448 at ~180 μm from the tip. (B) Stage S1 (12h after imbibition) at ~230 μm from the tip. (C)
449 Stage S2 (15h after imbibition) at ~240 μm from the tip. (D) Stage S3 (24h after imbibition)
450 at ~690 μm from the tip. (E) Stage S4 (36h after imbibition) at ~1900 μm from the tip. (F)
451 High magnification of (B) showing the midvein. (G) High magnification of (E) showing the
452 midvein. (A-E): P3 primordium and MV – dashed black line, P2 primordium – dashed red
453 line, SAM – asterisk. (F, G): bundle sheath progenitor cells dashed black. Scale bar = 50 μm
454 (A-E), 20 μm (F, G).

455

Figure 7. Transmission electron micrographs of transverse sections of developing major veins at the tip of P3 primordia. (A, B) Sections at S0 (dry seed) (A) and S1 (B) showing procambial centres (red dashed line) surrounded by BS progenitor cells (dashed yellow line). Cells contain numerous lipid bodies (orange asterisk), dense cytoplasm, and prominent nucleoli (white asterisk). At S1, many lipid bodies have coalesced. (C) S2 section showing protoxylem cells in the procambial centre with thickened cell walls (blue asterisks). Lipid content in all cells is greatly reduced relative to S0 and S1. (D) S3 section showing BS cells with dense cytoplasm, large nuclei (N) and numerous chloroplasts with prominent thylakoid membranes (c). In the vascular centre, progressive thickening of xylem cell walls is evident (red asterisks) and protophloem is in the early stage of development (yellow asterisks). (E) S4 section showing differentiated procambial and BS cells. Protophloem (black asterisks), protoxylem and metaxylem (X) are evident in the vascular centre. BS cells are vacuolated (V) with numerous well-developed chloroplasts (C) that contain starch granules (yellow arrow). CC = Companion cells. Scale bar = 5 μm (A-E).

Figure 8. Transmission electron micrographs of transverse sections of chloroplasts at the tip of P3 primordia. (A) S3 section showing adjacent M, BS and procambium (Pc) cells. At this stage, some cells are vacuolated whilst others are densely cytoplasmic, and M cells can still contain numerous lipid bodies. Both BS and M cells contain small chloroplasts with minimal thylakoid stacking (black arrows). (B, C) Sections at S4 showing BS (B) and M (C) chloroplasts in vacuolated cells. Areas of stacked thylakoids are visible in chloroplasts of both cell-types (yellow arrows). Pc cells contain degenerating protoplasm. Plasmodesmatal connections have formed in the cell walls between BS and M cells (red asterisk). Scale bar = 2 μm (A), 1 μm (B, C).