REVIEW ARTICLE

Cotyledon organogenesis

John W. Chandler*

Department of Developmental Biology, University of Cologne, Gyrhofstrasse 17, D-50923 Cologne, Germany

Received 15 April 2008; Revised 15 May 2008; Accepted 19 May 2008

Abstract

The cotyledon represents one of the bases of classification within the plant kingdom, providing the name-giving difference between dicotyledonous and monocotyledonous plants. It is also a fundamental organ and there have been many reports of cotyledon mutants in many species. The use of these mutants where they have arisen in Arabidopsis has allowed us to unravel some of the complexities of embryonic patterning and cotyledon development with a high degree of resolution. The cloning of genes involved in cotyledon development from other species, together with physiological work, has supported the hypothesis that there exists a small number of orthologous gene hierarchies, particularly those involving auxin. The time is therefore appropriate for a summary of the regulation of cotyledon development gleaned from cotyledon mutants and regulatory pathways in the model species Arabidopsis and what can be inferred from cotyledon mutants in other species. There is an enormous variation in cotyledon form and development throughout the plant kingdom and this review focuses on debates about the phylogenetic relationship between mono- and dicotyledon, discusses gymnosperm cotyledon development and pleiocotyly in natural populations, and explores the limits of homology between cotyledons and leaves.

Key words: Arabidopsis, auxin, cotyledon, dicots, evolution, monocots, natural variation, phylogeny.

Introduction

The patterning mechanisms responsible for generating any plant organ are complex and involve many gene hierarchies, and co-ordinated spatially and temporally regulated programmes of cell divisions, gene expression, and hormone function. The developmental regulation of leaf and root growth has been extensively described and reviewed (Fleming, 2005; Hochholdinger and Zimmermann, 2008), as have the patterning processes during embryogenesis responsible for establishing the meristems, mainly in Arabidopsis (Laux et al., 2004; Jenik and Barton, 2005; Jenik et al., 2007; Nawy et al., 2008), and maize (Vernoud et al., 2005). However, the development of the cotyledon as an organ has been neglected, although cotyledons are often the first aerial organ to differentiate and their initiation is inherent in embryonic patterning processes. With the explosion in the use of cotyledon mutants as tools for gene cloning (see Table 1 for a summary of cotyledon mutants in Arabidopsis and other species), many genetic pathways have been elucidated, which are usually included in reviews on embryonic patterning. However, one alternative consideration which provides a framework for thinking about cotyledon development, is the similarity between leaf and cotyledon development. Although dicot cotyledons and leaves have different functions in terms of storage andphotosynthesis and morphological differences such as trichomes and stipules on leaves, on the basis of comparative morphology, they can be considered homologous organs according to the following criteria: the homologous apical positioning of both sets of organs on the shoot, the presence of meristems at their bases during primordium growth, similar organ expansion programmes and mature morphology, and that intermediate structures exist between cotyledons and leaves (quoted in Kaplan and Cooke, 1997). In addition to morphological homology, cotyledon and leaf initiation share significant signalling determinants, most notably resulting from the asymmetric distribution of auxin; in a similar way to the pre-patterning of cotyledon primordia outgrowth, leaf primordia initiate at positions of maximum auxin concentration/perception resulting from polar PIN1 localization (Reinhardt et al., 2003; Reinhardt, 2005; Heisler et al., 2005; Jöransson et al., 2006). One important difference

* To whom correspondence should be addressed. E-mail: john.chandler@uni-koeln.de

© The Author [2008]. Published by Oxford University Press [on behalf of the Society for Experimental Biology]. All rights reserved.
For Permissions, please e-mail: journals.permissions@oupjournals.org
between cotyledon and leaf initiation resides in phyllotaxis: dicot cotyledons arise opposite each other, but leaves are subsequently initiated in many different phyllotactic patterns, however, the similarities provide an analogous framework for comparing the developmental genetic mechanisms in each case. With this analogy as a background, this review consolidates what is known about the initiation and outgrowth of cotyledons in the model eudicot Arabidopsis thaliana and surveys mutants known to affect cotyledon development in this and other species.

The formal division of angiosperm species based on morphological characteristics into Dicotyledonae and Monocotyledonae classes was first created by John Ray in his Methodus Platarum Nova in 1682. Cotyledon number is one of the characteristics involved in this division, with the Monocotyledonae (monocots) having a single cotyledon and containing approximately 50,000 lineages and theDicotyledonae comprising about 200,000 species. Similarly for leaves and flowers, the cotyledon as an organ displays huge variation in morphology and developmental programmes throughout the plant kingdom and the evolutionary origin of the cotyledon will also be reviewed here, together with considerations of natural variation in cotyledon number and development.

**Cotyledon diversity**

In dicots, the cotyledons are lateral organs and the shoot apical meristem (SAM) is at a central position, whereas in monocots the cotyledon is terminal and the SAM at a lateral position. Cotyledons are formed during embryogenesis and are defined as either epigeal, if they emerge above ground, expand following germination and become photosynthetic, or hypogeal, if they remain below ground, do not expand, and remain non-photosynthetic. Cotyledons can be ephemeral structures, only persisting for a few days following emergence, or can be retained by the plant throughout its vegetative life. They may have petioles, or be sessile.

For most dicot species, the number of cotyledons is invariable at two (Fig. 1A), however, exceptions include some species of Acer, Juglans, and Coffea which have three cotyledons (Eames, 1961; Duke, 1969) as well as Raphanus (Dube et al., 1981) and Sesamum (Pillai and Goyal, 1983), some members of the Ranales which have

---

**Table 1. Loci, mutations and redundant gene families affecting cotyledon organogenesis or pleiocotyly in various species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Locus or mutant</th>
<th>Cotyledon phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>ALTERED MERISTEM PROGRAM1</td>
<td>Supernumerary cotyledons</td>
<td>Chaudhury et al., 1993</td>
</tr>
<tr>
<td></td>
<td>ASYMMETRIC LEAVES1</td>
<td>Distorted cotyledons</td>
<td>Byrne et al., 2000</td>
</tr>
<tr>
<td></td>
<td>DORNRÖSCHEN/DORNRÖSCHEN-LIKE</td>
<td>Fused cotyledons, variable cotyledon number</td>
<td>Chandler et al., 2007a</td>
</tr>
<tr>
<td></td>
<td>EXTRA COTYLEDON1</td>
<td>Malformed cotyledons</td>
<td>Conway and Poethig, 1997</td>
</tr>
<tr>
<td></td>
<td>FACKEL</td>
<td>Cotyledons reduced or absent</td>
<td>Schrick et al., 2000</td>
</tr>
<tr>
<td></td>
<td>GURKE</td>
<td>Pleiocotyly</td>
<td>Torres-Ruiz et al., 1996</td>
</tr>
<tr>
<td></td>
<td>HYDRA1</td>
<td>Cotyledons lacking</td>
<td>Topping et al., 1997</td>
</tr>
<tr>
<td></td>
<td>laterne (pinoid, enhancer of pinoid)</td>
<td>Homeotic transformation of cotyledons into leaves</td>
<td>Treml et al., 2005</td>
</tr>
<tr>
<td></td>
<td>LEC/FUSCA</td>
<td>Cotyledon fusion variable cotyledon number</td>
<td>Meinke et al., 1994; Keith et al., 1994</td>
</tr>
<tr>
<td></td>
<td>MACCHI-BOU4 ENHANCER OF PINOID</td>
<td>Single cotyledons</td>
<td>Furutani et al., 2007</td>
</tr>
<tr>
<td></td>
<td>MONOPTEROS</td>
<td>Fused cotyledons, Pleiocotyly</td>
<td>Bennett et al., 1995</td>
</tr>
<tr>
<td></td>
<td>PINOID</td>
<td>Partially fused cotyledons</td>
<td>Aida et al., 1999</td>
</tr>
<tr>
<td></td>
<td>SHOOTMERISTEMLESS</td>
<td>Temperature-dependent cotyledon defects</td>
<td>Long et al., 2002</td>
</tr>
<tr>
<td></td>
<td>TOPLESS</td>
<td>Variable cotyledon number</td>
<td>Vernon et al., 2001; Vernon and Meinke, 2004</td>
</tr>
<tr>
<td></td>
<td>TWIN1</td>
<td>Single cotyledons</td>
<td>Dharmasiri et al., 2005b</td>
</tr>
<tr>
<td></td>
<td>TIR family</td>
<td>Fused cotyledons</td>
<td>Aida et al., 1997, 1999; Vroeman et al., 2003</td>
</tr>
<tr>
<td></td>
<td>CUP-SHAPED COTYLEDON (CUC) family</td>
<td>Single cotyledons or absence</td>
<td>Prigge et al., 2005</td>
</tr>
<tr>
<td></td>
<td>HD-ZIP Class III family</td>
<td>Altered cotyledon differentiation</td>
<td>Izhaki and Bowman, 2007; Eshed et al., 2001; Siegfried et al., 1999</td>
</tr>
<tr>
<td></td>
<td>KANADI/YABBY families</td>
<td>Altered cotyledon differentiation</td>
<td>Friml et al., 2003</td>
</tr>
<tr>
<td></td>
<td>PINFORMED1 family</td>
<td>Single and fused cotyledons</td>
<td>Cheng et al., 2007a</td>
</tr>
<tr>
<td></td>
<td>YUCCA family</td>
<td>Cotyledon fusion</td>
<td>Stubbe, 1966</td>
</tr>
<tr>
<td></td>
<td>Antirrhinum</td>
<td>CONNATA</td>
<td>Weir et al., 2004</td>
</tr>
<tr>
<td></td>
<td>CUPULIFORMIS</td>
<td>Cotyledon fusion</td>
<td>Stubbe, 1966</td>
</tr>
<tr>
<td></td>
<td>BLEI Cotyledon NEA</td>
<td>Pleiocotyly</td>
<td>Souer et al., 1996</td>
</tr>
<tr>
<td>Petunia</td>
<td>NO APICAL MERISTEM</td>
<td>Partial cotyledon fusion</td>
<td>Keddie et al., 1998</td>
</tr>
<tr>
<td>Tomato</td>
<td>DESTRUCTIVE EMBRYO AND MERISTEM</td>
<td>Pleiocotyly</td>
<td>Avasarala et al., 1996</td>
</tr>
<tr>
<td></td>
<td>LANCEOLATE</td>
<td>Cotyledons sometimes lacking</td>
<td>Al Hammadi et al., 2003; Madishetty et al., 2006</td>
</tr>
<tr>
<td></td>
<td>POLYCOTYLEDON</td>
<td>Pleiocotyly</td>
<td>Liu et al., 1999</td>
</tr>
<tr>
<td>Pea</td>
<td>SINGLE COTYLEDON</td>
<td>Cotyledon fusion</td>
<td></td>
</tr>
</tbody>
</table>
three or four cotyledons (Datta, 1988) and some species of *Persoonia* (Protaceae) (Fletcher, 1909). Conversely, the genus *Pinguicula* is anomalous amongst eudicots for sometimes having a single cotyledon (Degtjareva et al., 2006) and some parasitic plants such as those from the Orobanchaceae, Santalales, and Rafflesiales have no cotyledons (Burgher, 1998). Monocots have a scutellum (Fig. 1B), considered by many to be a single monocot cotyledon, which does not emerge above ground and can therefore only be shown in a seedling cross-section (Fig. 1B). As the scutellum remains underground it does not photosynthesize and therefore is not green, in contrast to most epigeal dicot cotyledons. Diversity exists in monocot cotyledon number, with some species such as *Commelina, Dioscorea, Tamus,* and *Tinantia* having a second vestigial cotyledon (Datta, 1988). Gymnosperms, distinct from angiosperm cotyledon classification, have variable cotyledon numbers between species, ranging from two, to 24 for *Pinus maximartinezii*; the largest number of cotyledons known for a plant (Farjon and Styles, 1997). Cotyledon number is also flexible within many gymnosperm species, for example, 5–7 for *Pinus radiata* and 7–13 for *Pinus jeffreyi* (Mirov, 1967), or 5 for *Pinus sylvestris* (Fig. 1C).

In addition to cotyledon number, cotyledons can be classified according to size; dicots usually demonstrate isocotyly: the development of two equally sized cotyledons. Anisocotyly, however, is the prolonged growth of one cotyledon over the other, to give a large macrocotyledon and a small microcotyledon. This is demonstrated by *Claytonia virginica* (Cook), *Capparis* species (Franceschini and Tressens, 2004) and members of the Gesneriaceae including *Streptocarpus rexii* (Mantegazza...
et al., 2007) and Monophyllaea horsfieldii (Tsukaya, 1997; Fig. 1D–F), where the macrocotyledon develops from a basal meristem to resemble a large foliage leaf up to 30 cm long, with no additional leaves being initiated before floral induction. Syncotyly relates to partial or complete fusion of cotyledons, as exists in species of Calophyllum, Swietenia, Guarea, and Carapa, where cotyledons are distally fused (Datta, 1988). The corollary of syncotyly is schizocotyly, applied to cotyledon bifurcation or lobing which can result in supernumerary cotyledons. Schizocotyly occurs in seedlings of the Chenopodiaceae, where one of two cotyledons may be cleft, to give hemitrocotyly, or one cotyledon of a tricotyledonous plant bifurcated to give a hemitetracotyl (Karschon, 1973). Various types of cotyleden syncotyly or pleiocotyly are demonstrated by some developmental mutants in Arabidopsis such as dornröschen and dornröschen-like, different alleles of which show pleiocotyly and syncotyly at low penetrance (Fig. 1G–L). The large diversity in cotyledon form and morphology throughout the plant kingdom argues against a single conserved ontogenetic programme.

The evolution of cotyledony

Cotyledons exist across the plant kingdom in gymnosperms, monocots, and eudicots. A distinction should be made here between dicots as a group, containing all plants with two cotyledons, where the term dicot is used as a form of morphology only, and the eudicots, a monophyletic phylogenetic group containing most dicot species. The Angiosperm Phylogeny Group website has consolidated current views of land plant phylogeny (http://www.mobot.org/MOBOT/research/APweb/) and it is believed that the gymnosperms diverged about 385 MYA (Zimmer et al., 2007) from eudicot progenitors and the monophyletic monocot group about 200 MYA (Zimmer et al., 2007) (Fig. 2). There has been a long-standing debate as to the origin of monocotyledony, but since the dicot state exists across all eudicot groups, including the Gnetopsids, it is accepted to be ancestral to monocotyly (Dean, 2002), suggesting that the monocot state is derived (is an apomorphy). The exact position of the Gnetopsids is also a subject of debate, with the anthophyte hypothesis placing them as a sister group to the angiosperms, but most molecular evidence places them as a sister group to the conifers (Chaw et al., 2000; Fig. 2). Dicotyly in diverse groups could also be the result of independent coevolution and the debate as to whether mono- or dicotyly represents the more primitive developmental programme is ongoing, with several alternative theories: a single monocot cotyledon could have derived from the fusion of two cotyledons, or dicotyly might represent an evolutionary organ duplication or cotyledon splitting event from the monocot state. Alternatively, the monocot cotyledon state could have resulted from the loss or suppression of both cotyledons and subsequent novel apomorphic innovation, or by functional sub-specification, whereby one cotyledon became retarded to form the first plumular leaf and the other retained cotyledon position and function, i.e. heterocotyly.

The taxonomic allocation into monocots or dicots has never been entirely clear for some species of paleoherbs such as Nymphaea (Tillich, 1990), which has a single cotyledon with two lobes, which could also be interpreted as two fused cotyledons. It has been suggested that Nymphaea is a monocot (Titova and Batygina, 1986), although molecular studies confirm a dicot association (Nickrent and Soltis, 1995). Alternatively, the cotyledon syncotyly of Nymphaea may represent a transitional cotyledon form. Some members of the Hydatellaceae have a sheathing structure which is similar to both a single monocot cotyledon and to the two fused cotyledons of Nymphaeales. The recent reclassification of the Hydatellaceae from the monocot order Poales to the Nymphaeales (Sokoloff et al., 2007) illustrates how unclear morphological characteristics defining the monocot–dicot categories can be and Hydatellaceae may demonstrate how a monocot–dicotyledonous condition could have arisen from the dicotyly present in water lilies. The phenomenon of dicotyledonous embryo formation in the monocot Agapanthus and the observation of stable transitional forms between mono- and dicotyledonous embryos supports the hypothesis of homology between monocot and dicot cotyledons, and Titova (2003) has advanced the hypothesis that, at least in this species, monocotyly derived from the dicotyledous situation via congenital fusion of two cotyledons and subsequent suppression of the growth of one of them.

The question whether dicotyly is plesiomorphic to monocotyly depends on the fundamental question of

![Fig. 2. Schematic phylogeny of the plant kingdom, showing the branching of the monocots and gymnosperms from eudicot ancestors about 200 and 385 million years’ ago (MYA), respectively. The dicotyledonous synapomorphy and monocot and gymnosperm cotyledon apomorphies are represented by circles on the cladogram. The debated position of the Gnetales is also shown, according Chaw et al. (2000).](https://academic.oup.com/jxb/article-abstract/59/11/2917/608661)
homology between both, i.e. a continuous evolutionary origin should give rise to homologous structures (Burgher, 1998). Burgher claims that monocot and dicot cotyledons are not homologous for the following reasons: firstly, both sets of organs do not share equivalent positions, with the terminal cotyledon of monocots not equivalent to the lateral dicot organs. The second criterion is homology of structure; dicot cotyledons are morphologically distinct from succeeding leaves, whereas monocot cotyledons share homology with succeeding leaves. Assuming homology, intermediate or transitional structures should be found between both sets of cotyledons. Despite occasional instances of unsterotypic cotyledons amongst both monocots and dicots, these are considered by Burgher to be exceptions and the structures in question do not completely resemble the respective mono- or dicot ontogeny. Finally, an assumption of homology is that mono- and dicot cotyledons cannot co-occur in one plant at the same developmental stage. It has been interpreted that the third leaf in Nymphaea corresponds to the monocot cotyledon (references quoted in Burgher, 1998), and therefore, that dicot and monocot characteristics co-occur in Nymphaea. These lines of evidence against a shared homology between mono- and dicot cotyledons still leaves the question of the origin of both sets of organs unresolved. The situation is confused since dicot plants are not a monophyletic group (Soltis and Soltis, 2004) and not all monocots possess a single cotyledon (Tillich, 1995).

With respect to homology between monocot and dicot cotyledons, there is debate as to whether the scutellum or coleoptile represents the monocot cotyledon. The grass scutellum and dicot cotyledon are functionally equivalent in being reserve lipid and protein storage organs, but to what extent the grass scutellum represents the whole cotyledon, part of it or an alternative structure is open (Vernoud et al., 2005). The coleoptile protects the plummule during germination and has also been considered by some to be equivalent to the cotyledon or part of it (Satoh et al., 1999). This debate is also related to whether the coleoptile is part of the scutellum, the first leaf, or a novel organ (reviewed in Vernoud et al., 2005) and is influenced by the observation that the development of the scutellum and coleoptile are genetically distinct (Satoh et al., 1999). The evolution of gymnosperm cotyledons is not clear, but they probably arose as a derived or apomorphic character via cotyledon duplication or schizocotyly within an ancestral angiosperm.

Homology between cotyledons and leaves

Despite developmental similarities between cotyledons and leaves, mutants exist which completely fail to develop cotyledons, but correctly initiate leaf primordia. These include several mutant combinations in Arabidopsis in genes involved in auxin biosynthesis or PIN1 expression, such as the laterne phenotype, caused by mutations in both PINOID and MACCHI-BOU 4/ENHANCER OF PINOID (Treml et al., 2005; Furutani et al., 2007) or DORNÖSCHEN (DRN) and DORNÖSCHEN-LIKE (DRNL) (Chandler et al., 2007), NON-PHOTOTROPIC HYPOCOTYL3 and PINOID or a loss of YUCCAI, YUCCA4, and PINOID (Cheng et al., 2007a, b). In addition, some mutants fail to establish a SAM, such as sti in Arabidopsis, no apical meristem in Petunia (Sauer et al., 1996), and leaf pattern mutants in pea (Marx, 1987), but produce normal cotyledons. This genetic uncoupling of cotyledon and leaf development demonstrates that they must each possess at least partly independent developmental programmes.

Transformation of cotyledons into leaves and leaves into cotyledons

Several mutants in Arabidopsis inform a discussion as to whether leaves and cotyledons are homologous structures or sui generis organs. These mutant phenotypes either demonstrate partial transformation of leaves into cotyledons or cotyledons into leaves, respectively. The LEAFY COTYLEDON (LEC) class of genes, LEC1 and LEC2, and FUSCA3 (FUS3) provide functions required for normal seed development. When mutated, cotyledons display features usually associated with vegetative leaves, such as trichomes, storage products, desiccation tolerance, and more complex vasculature (West et al., 1994; Meinke et al., 1994; Keith et al., 1994; Stone et al., 2001), and over-expression results in seedlings with embryonic characteristics (Stone et al., 2001). The lec cotyledon phenotype has been interpreted as representing partial homeosis (Meinke et al., 1994), or a disturbance in heterochrony (Keith et al., 1994), leading to an altered timing of developmental programmes. Similar to lec mutants, mutants in the HYDRA1 or HYDRA2/FACKEL genes in Arabidopsis have cotyledons with trichomes, thereby having a mixed phase phenotype (Topping et al., 1997; Jang et al., 2000; Schrick et al., 2000). Supplementation is defined as when the first true leaf is cotyledonary in character and three such mutations have been characterized in Arabidopsis (Conway and Poethig, 1997); extra cotyledon1 (xct1), xct2, and altered meristem program1 (amp1). These mutants produce leaves which phyllotactically develop perpendicular to the cotyledons, but possess partial or complete cotyledonary characteristics such as few or no trichomes, a simple venation pattern, and protein, lipid, and starch storage bodies. Some amp mutants show true polycotyly, with a true extra cotyledon being produced (Chaudhury et al., 1993), but the basis for the supplementation phenotypes is the precocious initiation of leaves during embryogenesis, and the mutations
are therefore heterochronic, rather than homeotic. However, the simultaneous presence of cotyledon- and leaf-specific characteristics in xtc1, xtc2, amp, and the lec mutants demonstrates that both cotyledons and leaves must at least share some common regulatory programmes.

Phase change

Phase-change from embryonic to vegetative development programmes has been little studied, but the question has been addressed in *Brassica napus*, which has non-dormant embryos (Fernandez, 1997). Precociously germinating embryos either produce extra cotyledons in a spiral phyllotaxy, possessing stipules, both leaf developmental characteristics, or chimeric organs with sectors of leaf tissue, characterized by trichomes and cotyledon tissue. Expression of molecular storage protein markers, normally restricted to the embryo, showed that the fate of a particular primordium was determined by the identity of the cells immediately surrounding it, implicating short range signalling pathways in primordium fate determination. As organ identity is related to the age of the embryo in *Brassica napus*, homeosis of cotyledons and leaves is a consequence of heterochrony, i.e. differentiation is dependent on temporal events in cellular environments. In anisocotylous plants such as *Streptocarpus wendlandii*, growth of the macrocotyledon is accompanied by trichome initiation in the basal region, which is characteristic for leaves (Nishii et al., 2004). Cytokinin application can convert the microcotyledon into a macrocotyledon in this species (Nishii et al., 2004), suggesting that endogenous cytokinin may play a role in cotyledon to leaf phase change.

Do cotyledons arise from the SAM?

If cotyledons and leaves share homology, the question arises whether they develop from the SAM or independently from it (Kaplan and Cooke, 1997; Barton and Poethig, 1993; Bowman and Eshed, 2000). If cotyledons originate from stem cell precursors in the SAM, what causes the phase-change switch to the initiation of leaf primordia in a different phyllotaxy to cotyledons? and if cotyledon development is independent of the SAM, what are the instructive signals determining cotyledon precursor cell fate? The SAM is usually defined in relation to its position between the cotyledons and, although in *Arabidopsis*, fully mature SAM histology becomes apparent only after cotyledon outgrowth, in some species, such as *Pinus strobus*, the SAM is visible before cotyledon initiation (Spurr, 1949). In addition, changes in gene expression domains which pre-pattern cotyledon outgrowth and SAM initiation in *Arabidopsis* are already established in the globular embryo, and clonal analyses cannot alone define the presence and activity of the SAM (Kaplan and Cooke, 1997). Long and Barton (1998) argue against the origin of both leaves and cotyledons from the SAM, on the basis that most cotyledon cells have never expressed *STM*, in contrast to those of leaf primordia, and that full SAM structure and activity is established following cotyledon outgrowth. In anisocotylous species such as *Streptocarpus wendlandii* (Nishii et al., 2004), the macrocotyledon enlarges by basal meristem activity, but as no leaves are subsequently produced, only an inflorescence, the meristem cannot be considered as a classical SAM. The question of the origin of the cotyledons can not be solved by histology alone, partly because of the huge developmental variation between species, and the danger of using *Arabidopsis* as a single paradigm, but only at the resolution of transcriptional cell profiling in single cells to identify the cell fate determinants of the cotyledon precursor cell(s).

**Cotyledon morphogenesis in Arabidopsis**

**Cotyledon initiation and outgrowth**

The initiation and outgrowth of cotyledons in eudicots as exemplified by *Arabidopsis* is morphologically visible at the late globular stage and represents a symmetry-breaking event, taking the embryo from a radially symmetrical embryo to one having two planes of bilateral symmetry (Chandler et al., 2008b). This re-definition of symmetry creates two domains within the apical embryo region which are each characterized by diagnostic gene expression patterns: an apical central domain, not to be confused with the embryo central domain situated between apical and basal domains, and an apical peripheral domain, with a central-peripheral axis linking them (Fig. 3A). The differential expression of several genes in these domains leads to the establishment of the SAM in the central apical domain. A feature of cotyledon outgrowth is the necessity to maintain separate boundaries between the central embryo domain and the enlarging cotyledon primordium and between the two primordia and the role of the cup-shaped cotyledon (*CUC*) genes is fundamental in this process: *Arabidopsis* possesses three redundant NAC domain transcription factors, *CUC1*, *CUC2*, and *CUC3*. Double *cuc1 cuc2* and triple *cuc1 cuc2 cuc3* mutants show complete cotyledon fusion to create cup-shaped cotyledons and seedlings lack a SAM, demonstrating that *CUC* gene functions are upstream of *STM* (Aida et al., 1999; Takada et al., 2001; Vroeman et al., 2003). The expression of *CUC* genes in the medial domain, between apical central and peripheral regions, in the heart stage embryo represses cell proliferation and differentiation in this domain, thereby creating a boundary which separates the developing cotyledons. In addition, *CUC* genes positively regulate *STM* expression in the central apical domain (Aida et al., 1999), where *STM* also represses
CUC expression (Fig. 3B), leading to mutually exclusive expression domains (Aida et al., 1999). KNAT6 is also expressed at the boundary between the SAM and cotyledons and acts redundantly with STM in organ separation via CUC genes (Belles-Boix et al., 2006). The boundary of CUC gene expression is also regulated by miR164 (Laufs et al., 2004) and TCP transcription factors (Koyama et al., 2007) and the STM expression domain is also positioned by DRN/DRNL (Chandler et al., 2008a). A fundamental aspect of the transition phase of embryogenesis, when apical and basal organ fate is established, is the repression of root-promoting gene cascades in the apical embryo domain, which is provided by the activity of the TOPOLESS (TPL) and TOPOLESS-RELATED (TPR) proteins (Long et al., 2006), co-repressors which function together with AUX/IAA proteins in auxin-mediated transcriptional repression (Szemenyi et al., 2008).

ASYMMETRIC LEAVES1 (AS1), a MYB domain transcription factor is required for normal cotyledon development and is involved in the control of cell differentiation. AS1 expression marks the sub-epidermal domain of the cotyledon initials and is later negatively regulated by STM in the developing SAM, leading to AS1 expression in a sub-epidermal domain of the developing cotyledons but is absent in the central domain (Byrne et al., 2000). Two redundant homeodomain proteins ATML1 and PDF2 which maintain the identity of L1 cells are necessary for cotyledon initiation (Abe et al., 2003), and the serine protease ALE1 and receptor like kinases ALE2 and ACR4 have overlapping roles in regulating protoderm-specific gene expression and cotyledon development (Tanaka et al., 2007). This provides some circumstantial evidence that appropriate protoderm differentiation in the embryo is a prerequisite for cotyledon formation. This is further supported by rplk1 toad2 double mutants, deficient in two receptor-like kinases, redundantly required for cotyledon development, which have defects in the central domain protoderm of globular embryos subjacent to cotyledon defects (Nodine et al., 2007; Nodine and Tax, 2008). How embryo central domain protoderm function is linked to cotyledon initiation is an open question, but it implicates an additional signalling mechanism involved in cotyledon initiation to those involving the apical central embryo domain.

Establishing central and peripheral embryo domains and cotyledon differentiation

Continuing the theme of the analogous development of cotyledons and leaves, polarity or sidedness is established in both organs with respect to the SAM, with the adaxial or upper side being that closest to the plant axis or SAM and deriving from the embryo apical central domain, and the abaxial side the opposite or underside generated by the peripheral embryo domain (Fig. 3C). The adaxial and abaxial surfaces of cotyledons are morphologically distinct with respect to cell size and stomatal density: the adaxial epidermis possesses cells of uniform size and has a flat surface with low stomatal density, whilst the abaxial surface has interspersed large and small cells, a rough surface and high stomatal density (Siegfried et al., 1999). This polarization during cotyledon differentiation, similarly to that of leaves, is established by three gene families: the class III HD-ZIP genes promote adaxial cell fate and KANADI and YABBY families promote abaxial cell fate (Fig. 3B). These roles are supported in each case by gene expression domains, loss-of-function and gain-of-function phenotypes.

Of the five class III HD-ZIP gene family members, PHAVOLUTA (PHV) and PHABULOSA (PHB) contribute to a functional apical meristem and REVOLUTA (REV) towards vasculature development, but all are also responsible for specifying the adaxial domain of the leaves and cotyledons. PHV, PHB, and REV are expressed from the early globular stages, but as the cotyledon develops, its adaxial side derives from cells from the central domain, whilst the abaxial side results from cells from the peripheral domain and PHV and PHB transcripts localize to the adaxial side of the emerging and developing cotyledons (Emery et al., 2003; Prigge et al., 2005). The
YABBY (YAB) genes FILAMENTOUS FLOWER and YAB3 are expressed from transition and heart stage embryos onwards, respectively, and subsequently at the abaxial side of the developing cotyledons, in cells derived from the peripheral domain (Sawa et al., 1999; Siegfried et al., 1999). Similarly, KAN1, KAN2, and KAN3, encoding GARP family plant-specific transcription factors (Eshed et al., 2001) are expressed in abaxial regions of developing embryos and cotyledons (Kerstetter et al., 2001; Eshed et al., 2004) and KAN4 expression is mainly ovule-specific (McAbée et al., 2006). Although the exact interaction between class III HD ZIP genes and KANADI/YABBY genes is not clear, the mutually exclusive expression domains of class III HD-ZIP genes and YABBY and KANADI genes has led to models of mutual antagonism (Emery et al., 2003; Eshed et al., 2004, Fig. 3D). In addition, the boundaries of class III HD-ZIP gene expression are partly delineated by miRNA control (Williams et al., 2005) and via interaction with the class of LITTLE ZIPPER (ZPR) genes (Wenkel et al., 2007; Fig. 3D).

Dominant gain-of-function alleles of PHV, PHB, and REV alleles cause the adaxialization of lateral organs (McConnell et al., 2001; Emery et al., 2003). Conversely, a triple phb phv rev mutant develops a single abaxialized cotyledon lacking bilateral symmetry (Emery et al., 2003), showing that the genes are necessary for adaxial cell fate and involved in hierarchies leading to the establishment of bilateral symmetry. Constitutive over-expression of KAN1, KAN2 or KAN3, or the YABBY genes FILAMENTOUS FLOWER1 (FIL) or YAB3 leads to constitutive wild-type abaxial characteristics in cotyledons (Eshed et al., 2001; Siegfried et al., 1999), suggesting that these genes promote abaxial cell fate. Constitutive expression of FIL or YAB3 (Siegfried et al., 1999) or the KAN genes (Eshed et al., 2001) also results in SAM arrest and together with the fact that phb-l/d mutants have an enlarged meristem, shows that SAM formation correlates with alterations in expression boundaries of genes determining adaxial or abaxial cotyledon fate, and thereby, that SAM maintenance and cotyledon differentiation involves two-way signalling between them (Siegfried et al., 1999; Golz, 2006). Not all single mutant YABBY and KANADI genes display a mutant phenotype, but double fil yab3 mutants result in a loss of polarity in leaves, with a uniform mesophyll and the epidermis is comprised of mosaics of abaxial and adaxial tissue (Siegfried et al., 1999). Increasingly, severe polarity phenotypes are observed in higher order kan mutants (Eshed et al., 2004), such that kan1 kan 2 kan4 triple mutants exhibit radialized leaf-like outgrowths on the abaxial side of the developing cotyledons and hextuple kan1 kan2 kan4 fil yab3 yab5 mutants initiate three narrow cotyledons (Izhaki and Bowman, 2007). Polarity defects in higher order class III HD-ZIP or KANADI mutants (phb phv rev and kan1 kan 2 kan4) can be explained by an altered PIN1 expression pattern, leading to altered auxin maxima and, consequently, abnormal organ initiation (Izhaki and Bowman, 2007).

The hormonal control of cotyledon development

Auxin synthesis, transport and perception

Auxin is a key regulator of cotyledon development as shown by mutant phenotypes of many of the loci in Table 1 which are involved with either auxin biosynthesis, polar transport or response, via the auxin receptor TIR1, or genes known to be up- or downstream of auxin response or sensitivity. In addition, many of these loci are members of redundant gene families, such as PINFORMED (PIN; Friml et al., 2003), YUCCA (Cheng et al., 2007a) and TIR (Dharmasiri et al., 2005b). Two lines of evidence demonstrate the importance of auxin synthesis or concentration in cotyledon organogenesis: the first is the use of the synthetic auxin-responsive DR5 promoter which has routinely been used to provide a read-out of auxin concentration (Ulmasov et al., 1997; Benková et al., 2003; Friml et al., 2003), although an absence of DR5 expression does not prove an absence of auxin maxima. DR5 expression concentrates in the tips of the incipient cotyledons in transition stage embryos and the tips of developing cotyledons (Cheng et al., 2007a; Fig. 4A) and correlates with an accumulation of IAA (Benková et al., 2003). Secondly, expression of the YUCCA1 and YUCCA4 genes, encoding flavin monoxygenase, a key enzyme in Trp-dependent auxin biosynthesis is strong in the central apical domain of the globular embryo and subsequently peaks in the tips of cotyledons (Cheng et al., 2007a). Auxin synthesized in the central apical domain is redirected by PIN1-mediated polar auxin transport (Friml et al., 2003).

It has been known for many years that treatment of embryos with various polar auxin inhibitors inhibits cotyledon and SAM development in wheat (Fischer-Iglesias et al., 2001), Brassica napus (Ramesar-Fortner and Yeung, 2001), Brassica juncea (Liu et al., 1993), Arabidopsis (Benková et al., 2003) and, more recently, in Norway Spruce (Larsson et al., 2008) and moss (Fujita et al., 2008). Auxin is transported in Arabidopsis embryos via the family of PIN auxin efflux transporters (Friml et al., 2003). PIN1 is polarly localized in the L1 layer of the developing embryo, such that the polarity correlates with auxin accumulation in cotyledon tips (Benková et al., 2003; Fig. 4B, C). Within the developing cotyledon, PIN1 becomes localized on the basal side of developing vasculature cells to direct auxin flow into the centre of the primordium and basipetally within the embryo (Benková et al., 2003). Thus polar auxin transport is responsible for creating auxin maxima necessary for...
correct cotyledon specification. The activity of the PIN proteins is regulated by the phosphorylation state controlled by the PINOID serine/threonine protein kinase (Benjamin et al., 2001) and PP2A phosphatase (Michniewicz et al., 2007) and PIN1 polar localization is also regulated by ENHANCER OF PINOID/MACCHI BOU4 (Trembl et al., 2005; Furutani et al., 2007). pin1 pid double mutants completely lack cotyledons and the embryos are characterized by an expansion in the expression domains of CUC1, CUC2, and STM, demonstrating that the negative regulation of the expression domains of these genes by PIN1 and PID is necessary for normal cotyledon initiation (Furutani et al., 2004).

The auxin receptor has been identified as TIR1 (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a), a member of a family of related AFB F-box proteins (Dharmasiri et al., 2005b). Auxin binds to a hydrophobic pocket within the TIR1 protein (Tan et al., 2007), thereby stabilizing the interaction between TIR1 and Aux/IAA proteins, targeting them for degradation and thereby relieving auxin response factor (ARF) transcription factors from transcriptional repression (Quint and Gray, 2006). Higher order mutants between TIR1 and AFB proteins show cotyledon development defects (Dharmasiri et al., 2005b), demonstrating that TIR and AFB proteins regulate auxin responses during cotyledon development (Dharmasiri et al., 2005b). Arabidopsis possesses 23 ARFs (Guilfoyle and Hagen, 2007) which bind to auxin responsive elements (AREs) in target genes and lead to their activation or repression. The DR5 promoter contains canonical AREs (Chandler et al., 2008a), providing further corroboration that auxin-controlled gene expression is relevant for cotyledon development.

Cytokinins

Some evidence implicates cytokinins in cotyledon development: in hybrid larch, exogeneous application of benzyladenine caused a decrease in cotyledon number (von Aderkas, 2002) and a monocotyledonous mutant of Catharanthus roseus could be restored to normal dicotyledon by the application of kinetin, suggesting that the mutant phenotype resulted from developmental-stage-specific cytokinin deficiency (Rai and Kumar, 2000). The Arabidopsis amp mutant has a pleiotropic phenotype, including polyctotyly and considerably higher cytokinin levels than the wild type (Chaudury et al., 1993), showing that appropriate cytokinin levels play a role in normal cotyledon initiation. The DORNÖSCHEN (DRN)/ENHANCER OF SHOOT REGENERATION1 (ESR1) and DRN-LIKE/SUPPRESSOR OF PHYTOCHROME/ BOLITA/ESR2 genes have a role in cotyledon development, with mutants showing syncotyly and pleiocotyly (Ikeda et al., 2006; Chandler et al., 2007) and they have an additional role in cytokinin-independent shoot regeneration and are themselves regulated by cytokinins (Banno et al., 2001; Ikeda et al., 2006).

Cotyledon development in other eudicots and monocots

Although Arabidopsis has become the developmental paradigm for eudicot patterning processes, it must be reiterated that it is atypical amongst eudicot embryo development in having an inflexible hallmark cell division pattern. Embryo and cotyledon development in other species have not been extensively studied at the molecular level, only on a morphological descriptive basis (Wardlaw, 1955). Embryos of monocots and eudicots are basically morphologically indistinguishable at the early embryo stages and differences only become apparent at the globular stage when, in eudicots, the outgrowth of the cotyledons results in a heart-stage embryo and, in monocots, the embryo is more elongated as the cotyledon defines the primary axis (Burgher, 1998). Homologues and orthologues of several Arabidopsis genes involved in cotyledon organogenesis have been isolated from other species and show a conservation of function: CUPULIFORMIS (CUP) from Antirrhinum is a CUC orthologue.
(Weir et al., 2004), as is NO APICAL MERISTEM (NAM) in Petunia (Souer et al., 1996) and mutations in these genes lead to cotyledon phenotypes. In maize, ZmCUC3 is a CUC3 orthologue and ZmNAM1 and ZmNAM2 are CUC1/CUC2 orthologues (Zimmermann and Werr, 2005) and their expression pattern supports a function for ZmCUC3 in boundary specification and for ZmNAM1 and ZmNAM2 in SAM establishment, similar to CUC gene function in Arabidopsis. Demonstrating the orthologous function of Arabidopsis genes involved in cotyledon development in monocots is difficult, due to morphological differences. However, orthologues of STM exist in maize (Knotted1; Long et al., 1996) and rice (Sentoku et al., 1999) and the maize AS1 homologue is rough sheath2 (RS2; Timmermans et al., 1999; Tsiantis et al., 1999). barren inflorescence2 (bif2) in maize is an orthologue of Arabidopsis PINOID (McSteen et al., 2007) and bif2 homologues from other grass species and their comparison with PINOID shows that the sequence, expression, and function is conserved between monocots and eudicots. PIN1 orthologues have also been isolated from maize (Carraro et al., 2006), but as yet, not subject to mutagenesis. The DRN homologue in maize, ZmDRN is expressed in the prospective scutellum (Zimmermann and Werr, 2007), thereby showing an analogous expression pattern to that in Arabidopsis, where DRN expression pre-patterns cotyledon development in the Arabidopsis globular embryo, and suggesting an orthologous function.

There is, therefore, clear conservation of many transcription factor hierarchies controlling cotyledon development in Arabidopsis in divergent plant species, and involving auxin (Table 1). The function of these genes in monocot cotyledon development is often hard to elucidate, and even if they have no role in cotyledon development, their further characterization by heterologous complementation, together with the isolation of more heterologous genes will reveal the repertoire of ancestral pathways affecting cotyledon development and those which have sub-functionalized or diverged. An evolutionary developmental biology perspective should help to address this question.

### Cotyledon development in gymnosperms

Studies on cotyledon development in gymnosperms are few and are mainly related to cotyledon number (Harrison and Von Aderkas, 2004) and limited to somatic embryos and morphological descriptions. However, a comparison of expressed sequence tags from loblolly pine embryos with those of Arabidopsis revealed a large conservation with angiosperm embryogenesis-related genes (Cairney et al., 2006). The variation in cotyledon number within a given gymnosperm species correlates with embryo size, which alters from year to year (Butts and Buchholz, 1940). There is a greater degree of variation in cotyledon number in somatic embryos compared with zygotic embryos. The ring-shaped initiation of multiple cotyledons in gymnosperms means that multiple axes of bilateral symmetry are established by cotyledon initiation and not just two axes as in eudicots.

### Pleiocotyly and natural variation in cotyledon number

An abnormally large number of cotyledons within a species has been referred to in the literature as pleiocotyly or polycotyly. Pleiocotyly in natural populations has not been extensively studied, although widespread examples of spontaneous tricotyledony have been reviewed by Holtorp (1944) and Haskell (1954) in Populus (Rajora and Zsuffa, 1986), Prosopis cineraria (Nagesh et al., 2001a), Acacia melliflora (Nagesh et al., 2001b), Tectona grandis (Nagesh and Kardam, 2004), Sapindus emarginatus (Nagesh et al., 2004), and sunflower (Hu et al., 2005). The genetic basis for some of these examples is not known, but a tricotyledonous mutant of Catharanthus roseus was found to be controlled by a single recessive mutation (Rai and Kumar, 2001). In tomato, two independent loci causing polycotyledony were characterized: the polycotyledon (poc) mutation was mapped (Madishetty et al., 2006) and the mutant showed increased levels of polar auxin transport, suggesting that the cotyledon phenotype is a result of altered auxin distribution (Al-Hammadi et al., 2003). The defective embryo and meristems (dem) mutant also lacks a functional SAM and is caused by the mutation of a gene encoding a novel protein of unknown function (Keddie et al., 1998). In pea, three independent single cotyledon (sic) loci when mutated, give rise to single or fused cotyledons (Liu et al., 1999). The frequency of pleiocotyly has been studied as a complex trait in wild radish populations: Raphanus raphanistrum and Raphanus sativus show either more or fewer than two cotyledons at a frequency of between 0% and 5% (Conner and Agrawal, 2005). Paternal half-sibling families were compared to assess whether the low phenotypic penetrance was due to stabilizing selection or a lack of genetic variance for the trait. These alternatives could not be distinguished due to the small samples and low number of phenotypic plants. However, artificial selection has shown that genetic variance for pleiocotyly is not lacking: the frequency of tricotyledonous seedlings resulting from a cross between wild and cultivated sunflower lines increased from 2% in the F2 generation, to about 50% in the F5 generation (Hu et al., 2005), showing that additive genetic variation for polycotyly exists. Similarly, artificial selection for pleiocotyly in Antirrhinum majus was able to increase its frequency
from 5% to 64% (Haskell, 1954), which suggests that lack of genetic variation is not the reason for the usually invariant number of cotyledons. The variable numbers of cotyledons in gymnosperms has been addressed in clonal populations of somatic embryos of hybrid larch and was found to correlate directly with the diameter of the apical embryo surface (Harrison and Von Aderkas, 2004).

**Breeding for cotyledon number**

The possibility exists that additive variance in natural populations of some plants could allow the selection of pleiocotyly as a trait, although the potential adaptive value of variable cotyledon numbers has not been well studied and is dependent on understanding the genetic basis of natural variation in cotyledon number. The possible agricultural benefits of pleiocotyly or manipulating cotyledon size are greater yield and quality of oils in oil seed plants such as rape or sunflower, where oil is stored in the cotyledons rather than the endosperm. Additional cotyledons may even confer an enhancement in seedling establishment; a tricotyledonous sunflower mutation had no detrimental effect on growth and development and led to three leaves per node, increasing photosynthetic surface area and therefore potential productivity (Hu et al., 2005).

**Conclusions**

The *Arabidopsis* developmental paradigm has elucidated many gene hierarchies regulating cotyledon development and has demonstrated the key involvement of auxin. A further feature of the control of cotyledon development is redundancy amongst gene families such as the *PIN* (Friml, 2003), class III HD-ZIP (Pigge et al., 2005), YUCCA (Cheng et al., 2007a) or TIR/AFB (Dharmasiri et al., 2005b). Redundancy also exists between transcription factor pairs such as DRN/DRNL (Chandler et al., 2007) and TOAD2/RPK1 (Nodine et al., 2007). This genetic robustness and plethora of parallel pathways presumably reflects evolutionary adaptation to ensure buffering against negative mutations at a critical point in plant survival, thereby ensuring that the embryonic patterning programme proceeds successfully. It will be interesting to discover how conserved this redundancy is amongst other eudicots for which *Arabidopsis* is not representative. Despite the enormous diversity of cotyledon morphology and development within and between the monocots and eudicots, the existence of many cotyledon development genes and regulatory pathways are conserved, such as those involving *CUC* genes, *STM*, pathways involving auxin, although in many cases no monocot mutants are available to demonstrate how they affect cotyledon development. The increased ease of isolating gene homologues by heterologous cloning and applying the information to evolutionary developmental biology will increasingly reveal the extent of this conservation across the plant kingdom and distinguish pathways which have divergently evolved. It will also inform the debate on the evolution of cotyledon states.

The similarities in the genetic regulation and auxin-dependent initiation and outgrowth of cotyledons and leaves demonstrates the homology between the structures. However, open questions such as what distinguishes leaf founder cells from cotyledon precursor cells and how cotyledon and leaf development differ, including determinants of phase change and the relationship of cotyledon initiation to the SAM, remain tantalizing foci of research.

Natural variation in cotyledon number is under-researched and understanding the genetic basis of pleiocotyly could enable breeding agronomically important seed oil crops for increased productivity based on this trait.

**Acknowledgements**

JWC thanks Melanie Cole for PIN1 and DRS *Arabidopsis* embryo pictures, Judith Nardmann for maize embryo photos, Siegfried Menrath and Jürgen Hintzsche for providing plant material, and Wolfgang Werr for critical reading of this manuscript. JWC also gratefully acknowledges funding for this work from the Deutsche Forschungsgemeinschaft via SFB572.

**References**


