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Review

Tansley insight

From grasses to succulents – development and function of distinct stomatal subsidiary cells

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Summary

Stomata are breathing pores on leaves that balance photosynthetic carbon dioxide uptake and water vapor loss. Stomatal morphology and complexity are rather diverse when considering stomatal subsidiary cells (SCs). Subsidiary cells are adjacent to the central guard cells (GCs) and are morphologically distinct from other epidermal cells. Yet, how various SCs develop and whether and how they support stomatal gas exchange physiology outside of the grass family is largely unknown. Here, we discuss the development, ontogeny, and putative function of paracytic vs anisocytic SCs, which can be found in grasses and Crassulaceae succulents, respectively. First, we highlight recent advances in understanding how grasses form stomatal SCs. We then summarize novel insights into stomatal development in SC-less *Arabidopsis* to speculate on how this stomatal program might be rewired to enable anisocytic SC formation. Finally, we discuss the functional relevance of paracytic SCs in grasses and the putative roles of anisocytic SCs in succulents.

I. Introduction

The leaf epidermis of land plants is the plant–atmosphere interface that protects the leaf from environmental stress. Several specialized cell types enable contrasting functional requirements in the epidermal barrier; the abundant pavement cells stabilize and seal the leaf, for example, while stomata are interspersed, adjustable breathing pores that facilitate carbon dioxide uptake and transpiration. Stomata usually consist of two guard cells (GCs) that can adjust the central pore's aperture by turgor-driven movements (Lawson & Blatt, 2014; Jezek & Blatt, 2017). Guard cells can be surrounded by additional cells, which differ in size, shape, and/or arrangement from the pavement cells. Based on these purely anatomical and morphological criteria, such GC-associated cells are defined as subsidiary cells (SCs; Box 1; Esau, 2006; Gray *et al.*, 2020). This rather simple definition of SCs lacks any functional implication; it, therefore, includes both physiological 'helper cells' and developmental 'space holders' that merely compensate for differential growth rates among developing epidermal cell types (Box 1; Rudall *et al.*, 2013). When and how often SCs have evolved, however, is unclear. In the moss *Physcomitrium patens*, stomata are directly differentiated from protodermal cells without undergoing divisions (Chater *et al.*, 2016). Therefore, SCs might be an innovation that only occurred once more complex division patterns were required to enable diffuse leaf growth and composite sporophyte development in lycophytes and flowering plants. Here, we (1) highlight different SC arrangements and ontogenies, (2) summarize and speculate on how distinct SCs develop, and finally (3) discuss verified and putative functional roles of different SC arrangements.

Box 1 What are subsidiary cells?

Definition: Subsidiary cells (SCs) are cells that are associated with guard cells (GCs) but differ in size, shape, arrangement, and/or content from pavement cells (Pant, 1965; Esau, 2006). While a functional association is implied, the original definition is purely anatomical and morphological.

Ontogeny: SCs that derive from the same precursor cell as the GCs are called mesogene (e.g. succulents), and those that derive from different precursor cells are perigene (e.g. grasses). If they are of mixed origin, then they are called meso-perigene (for more details see Fryns-Claessens & Van Cotthem, 1973; Rudall *et al*., 2013).

Function: Functional relevance of SCs to stomatal gas exchange was experimentally tested in grasses only. Here, precipitation of stomatal osmolytes (K^+ and Cl^-) in maize leaves showed that these ions primarily reside in SCs when stomata are closed and GCs when stomata are open (Raschke & Fellows, 1971). These early studies concluded that SCs might facilitate fast opening by providing a significant, readily deployable source of osmolytes to rapidly pressurize GCs (Raschke & Fellows, 1971). Thirty years later, pressure probe analyses started to link GC pressure to stomatal aperture in different species (Franks *et al*., 1998; Franks & Farquhar, 2007). Strikingly, applying pressure in GCs did not achieve sufficient stomatal opening in grasses (unlike in other species that lack SCs), but required simultaneous depressurization of the surrounding epidermal cells (Franks & Farquhar, 2007). Therefore, an osmotic 'see-sawing' model was proposed, where reciprocal osmoregulation in GCs and SCs is needed to overcome the mechanical constraints imposed by rigid pavement cells to allow increased opening and closing rates and bigger stomatal pores (Franks & Farquhar, 2007). Indeed, stomata lacking SCs in *bdmute* mutants in *Brachypodium distachyon* showed smaller stomatal opening capacity and a severely decreased speed of stomatal opening and closing (Raissig *et al*., 2017).

II. Subsidiary cell arrangement and ontogeny

Stomatal types are classified according to the arrangement (i.e. 'descriptive') and the ontogeny (i.e. 'developmental') of SCs and other neighboring cells. Many different anatomical and ontogenetic types were described and discussed in great detail (see Rudall *et al*., 2013, and references therein). A complication regarding these definitions, however, is that all GC-surrounding cells are considered (SCs and pavement cells). Here, we want to simplify the complex terminology by focusing on SCs only. In particular, we want to discuss the development and potential function of paracytic and anisocytic SCs found, for example, in Poaceae (grasses) and Crassulaceae (succulents), respectively.

Stomata that lack SCs are called anomocytic and are present in almost all land plant taxa including *Arabidopsis thaliana* (Rudall *et al*., 2013). Paracytic SCs are arranged in parallel to GCs and can vary in number (Rudall *et al*., 2013). Grasses, for example, recruit one paracytic SC on either side of the central GCs (Fig. 1a; Nunes *et al*., 2020). Anisocytic SCs are three to four unequally sized cells that surround the central GCs in a ring-like arrangement (Rudall *et al*., 2013). Succulents of the Crassulaceae family (e.g. *Kalanchoë laxiflora*), for example, form such anisocytic stomata (Fig. 1b; Nunes *et al*., 2020).

Developmentally, the paracytic SCs in grasses are formed by the cells adjacent to the GC precursors and are thus of perigene origin (Fig. 1c; Rudall *et al*., 2013; Gray *et al*., 2020; Nunes *et al*., 2020). The succulents' anisocytic SCs, on the contrary, are mesogene meaning that they stem from the same precursor as the GCs (Fig. 1d). Nonetheless, paracytic SCs can also be of mesogene origin. In horsetails, a single stomatal precursor undergoes two sequential asymmetric cell divisions (ACDs) toward both sides to form two paracytic SCs, before a final symmetric division in the central cell forms the GC pair (Cullen & Rudall, 2016).

III. Development of paracytic subsidiary cells in grasses

The developmental mechanisms of perigene, paracytic SC formation in grasses were extensively studied in recent years. In the grass leaf epidermis, specific protodermal cell files establish stomatal identity through the conserved action of the basic helix–loop–helix (bHLH) transcription factors SPEECHLESS (SPCH) and INDUCER OF CBF EXPRESSION1/SCREAM (ICE1/SCRM; Fig. 1c; Raissig *et al*., 2016; Wu *et al*., 2019). A transverse asymmetric cell division then forms a small guard mother cell (GMC) and a bigger interstomatal pavement cell (Fig. 1c; Stebbins & Shah, 1960; Raissig *et al*., 2016). The GMC then induces subsidiary mother cell (SMC) identity in the neighboring cell file (Fig. 1c) through the action of the grass homologs of the MUTE transcription factor. Translational MUTE reporters in *B. distachyon*, maize, and rice move from GMCs to SMCs suggesting that SMC identity is established noncell-autonomously (Raissig *et al*., 2017; Wang *et al*., 2019). In *B. distachyon*, however, premature or higher *BdFAMA* expression can partially rescue SC formation in the absence of *BdMUTE* (McKown *et al*., 2023).

The SMCs then polarize toward the GMCs and asymmetrically divide to form perigene SCs (Fig. 1c). Mutant analysis in maize and *B. distachyon* revealed a whole battery of polarity and cell division factors that are required to control this extreme asymmetric cell division with its peculiar, lens-shaped cell division orientation (Fig. 1c). Two opposite polarity domains – a proximal domain at the GMC/SMC interface and a distal domain at the apical, basal and distal membrane – guide nuclear migration and specify the cortical division site (Fig. 1c; Zhang *et al*., 2022). SCAR/WAVE regulatory complex proteins (Facette *et al*., 2015), receptor-like kinases of the PANGLOSS (PAN) family (Cartwright *et al*., 2009; Zhang *et al*., 2012), and Rho family GTPases (ROPs; Humphries *et al*., 2011) sequentially accumulate within the proximal domain (Fig. 1c). Together, they guide actin patch formation at the GMC/SMC interface and nuclear migration (Facette *et al*., 2015). WEB1/PMI2-RELATED (WPR) proteins were shown to interact both with PAN receptors and the actin patch and seem to physically connect the proximal BRICK-PANGLOSS-ROP polarity module with the actin patch (Fig. 1c; Nan *et al*., 2023a). In addition, the nuclear envelope protein MAIZE LINC KASH SINE-LIKE2 (MLKS2) likely tethers the SMC nucleus to the cytoskeleton to mediate nuclear migration (Arif Ashraf *et al*., 2022). The distal polarity domain was recently described in *B. distachyon* and is formed by a grass POLAR homolog (Fig. 1c; Zhang *et al*., 2022). POLAR polarization is controlled by the proximal domain and

seems to guide the positioning of the cortical division site rather than nuclear migration (Zhang *et al.*, 2022). Additionally, in maize, two phosphatases (DISCORDIA1 (DCD1) and ALTERNATIVE DISCORDIA1 (ADD1)) are involved in placing the preprophase band and, thus, regulating the cortical division sites in SMCs (Wright *et al.*, 2009). Upon chromosomal segregation, the phragmoplast forms between the two daughter nuclei to form the curved SC wall. During this process, the myosin XI protein OPAQUE1/DCD2 is required for late-stage phragmoplast guidance (Nan *et al.*, 2023b).

Once both SCs are properly formed, the GMC divides symmetrically and differentiates the graminoid, dumbbell-shaped

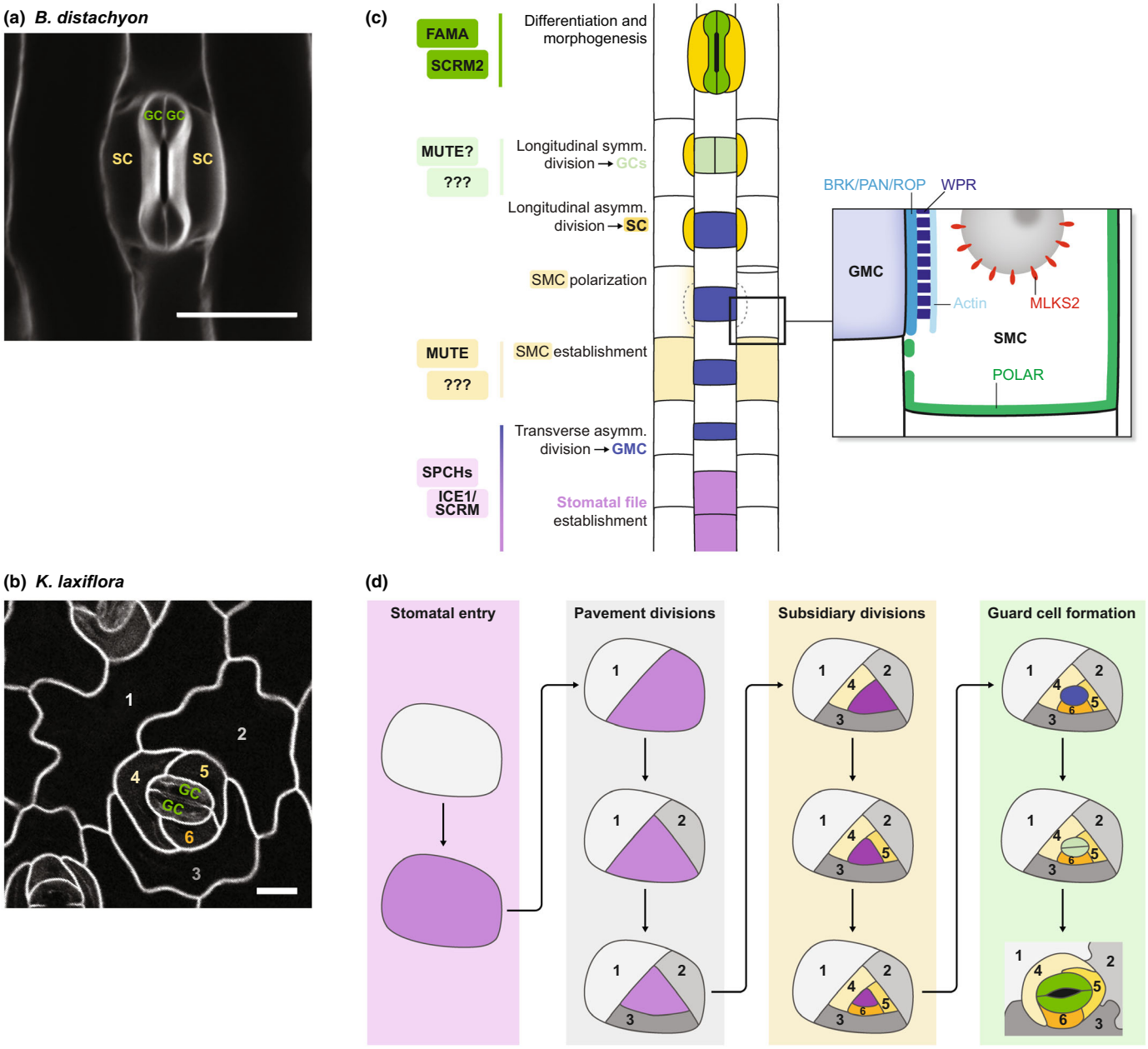

Fig. 1 Subsidiary cell formation in grasses and Crassulaceae succulents. (a) Graminoid stoma of *B. distachyon* featuring two central, dumbbell-shaped guard cells (GCs) and two paracytic, perigene subsidiary cells (SCs). (b) Anisocytic stoma of *Kalanchoë laxiflora* with two central, kidney-shaped GCs and three anisocytic, mesogene SCs (labeled 4, 5, and 6). (c) Paracytic, perigene SC development in grasses. The *SPCH-ICE1/SCRM2* module establishes the stomatal lineage. After a transverse asymmetric division, the guard mother cell (GMC, blue) and *MUTE* establish subsidiary mother cell (SMC) identity in the nonsister, lateral cells (yellow). These cells then polarize to migrate the nucleus and establish cortical division sites (see insert), before an extreme asymmetric division forms the perigene, paracytic SC (yellow). Finally, the GMC divides symmetrically to form the GC pair (green), which then are differentiated by the *FAMA-SCRM2* module. (d) Hypothetical development of anisocytic stomata in *K. laxiflora*. Upon stomatal lineage entry (purple panel), three asymmetric cell divisions generate three pavement cells (gray panel; gray cells labeled 1, 2, and 3), before another three divisions generate three circular SCs (yellow panel; yellow cells labeled 4, 5, and 6). Then, proliferative divisions are ended, the GC lineage is committed (blue cell) and a single symmetric division makes the GC pair (green, green panel). Bars, 20 μm.

GCs (Fig. 1c) – a process that requires the conserved *FAMA-SCRM2* module (Liu *et al.*, 2009; Raissig *et al.*, 2016; Wu *et al.*, 2019; McKown *et al.*, 2023) and complex morphogenetic processes (Spiegelhalder & Raissig, 2021).

IV. Development of anisocytic subsidiary cells

In contrast to paracytic, perigene SC formation, the processes guiding anisocytic SC development remain completely unknown. Morphologically, mature succulent stomata and their surrounding cells (e.g. in *K. laxiflora*; Figs 1b, 2a) suggest that a protodermal cell undergoes 5–6 consecutive, spiraling asymmetric cell divisions that yield 2–3 pavement cells, three (unequally sized) SCs, and one GMC (Fig. 1d). The GMC then divides symmetrically to form the GC pair (Fig. 1d). The spiraling asymmetric divisions seem similar to the amplifying divisions in *Arabidopsis*, where continued asymmetric divisions of the meristemoid 'amplify' pavement cells before differentiating a stoma (Fig. 2b,c; Lau & Bergmann, 2012). In *Arabidopsis*, however, such amplifying divisions seem to occur one to three times rather than five to six times like in the succulents (Fig. 2a,b). Furthermore, the last three stomatal lineage ground cells (SLGCs) that are in contact with the GMC seem to form morphologically distinct, smooth-walled SCs in succulents rather than differentiate into lobed pavement cells like in *A. thaliana* (Fig. 2a,b). In addition, the putative SCs in *K. laxiflora* are differentially stained by toluidine blue compared with the outer

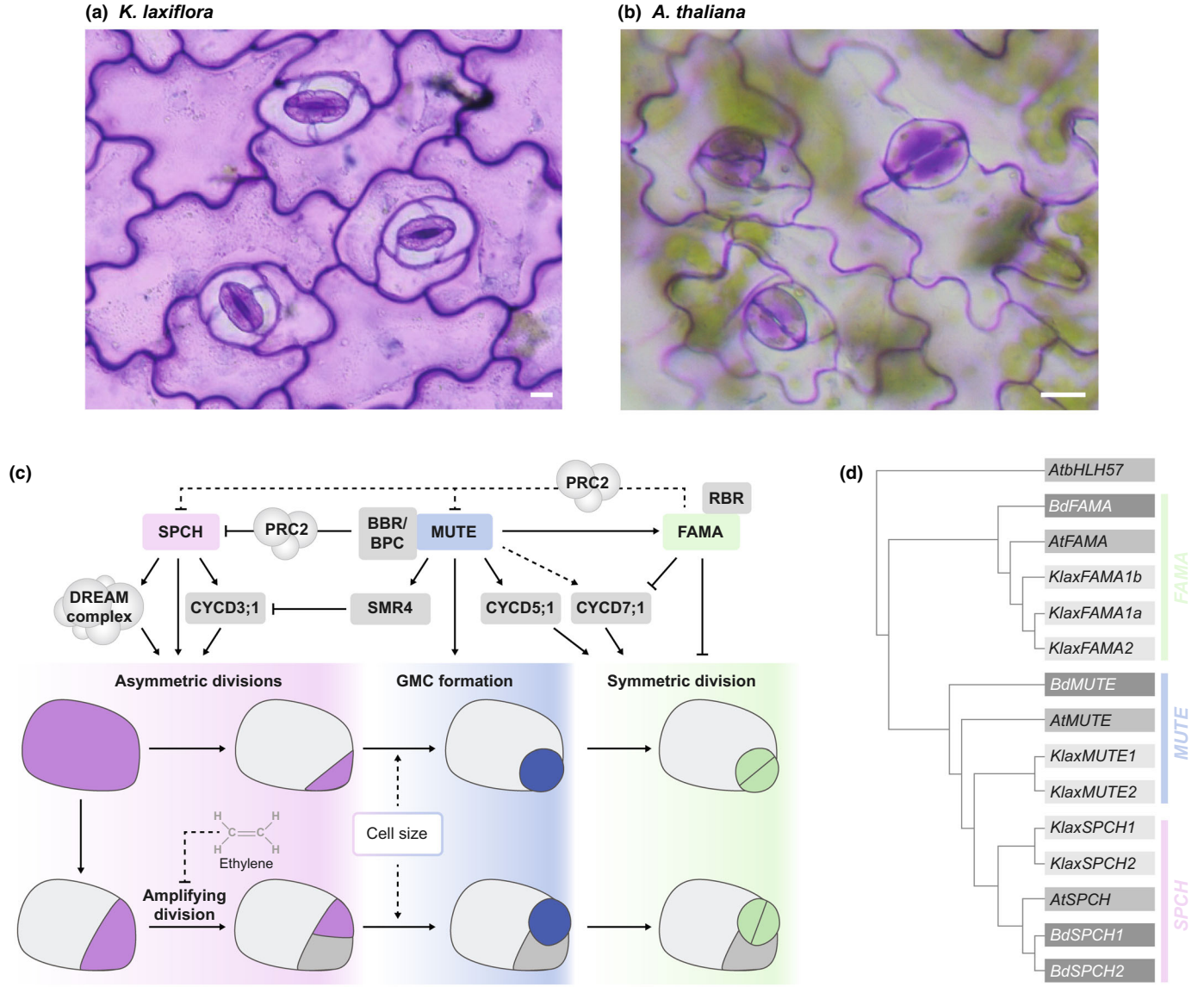

Fig. 2 How is the stomatal formation program of *Arabidopsis thaliana* modified to accommodate anisocytic stomatal development in succulents? (a, b) Toluidine blue-stained epidermal peels of *Kalanchoë laxiflora* (a) and *A. thaliana* (b); note how the guard cell (GC) pairs in *K. laxiflora* are surrounded by differentially stained, smooth, putative subsidiary cells (SCs). In *A. thaliana*, even small neighboring cells are lobed and stained like non-GC-associated pavement cells; Bar, 10 μm. (c) The core bHLH transcription factors (SPCH, MUTE, and FAMA) regulate each other and stage-specific cell cycle genes to control the transition from proliferative, asymmetric divisions to a final symmetric division and guard cell differentiation in *A. thaliana*. Dashed arrows indicate indirect regulation. (d) Phylogenetic gene tree of the core bHLH transcription factors SPCH, MUTE, and FAMA in *A. thaliana* (no SCs), *B. distachyon* (paracytic, perigene SCs), and diploid *K. laxiflora* (anisocytic, mesogene SCs). AtbHLH57 (At4g01460) was used as an outgroup.

pavement cells (Fig. 2a). In *A. thaliana*, however, all pavement cells (GC-neighboring and non-GC-neighboring cells) stain similarly (Fig. 2b). Yet, how succulents induce the higher division capacity of the stomatal precursors (= meristemoids) and establish SC identity and whether the succulents' SCs are physiologically relevant for stomatal function remains enigmatic.

At the core of stomatal development lays a bHLH transcription factor module that is conserved from bryophytes to angiosperms (Lau & Bergmann, 2012; Chater *et al.*, 2017; Nunes *et al.*, 2020). In *Arabidopsis*, SPCH initiates the stomatal lineage and promotes proliferative, asymmetric divisions (Fig. 2c; MacAlister *et al.*, 2007). MUTE terminates these asymmetric divisions, initiates a single symmetric division, and induces FAMA-dependent GC differentiation (Fig. 2c; Pillitteri *et al.*, 2007). Tight control of cell division capacity is crucial to guarantee sufficient formative divisions and a single, final division of GMCs. SPCH directly controls both entry into mitosis by activating *CycD3;1* (Lau *et al.*, 2014; Adrian *et al.*, 2015) and execution of mitosis through the activation of the DREAM complex (Fig. 2c; Simmons *et al.*, 2019). Different plant hormones regulate how stomatal precursors divide; while cytokinin promotes spacing divisions in the SLGC (Vatén *et al.*, 2018), ethylene restricts amplifying divisions of the meristemoid (Fig. 2c; Gong *et al.*, 2021). Furthermore, auxin levels decrease during the meristemoid to GMC transition (Le *et al.*, 2014) and dynamic auxin transport is required for the SLGC to pavement cell transition and pavement cell lobing (Grones *et al.*, 2020).

A cell size threshold robustly commits meristemoids toward the terminal division and differentiation (Fig. 2c; Gong *et al.*, 2022). MUTE then, directly and indirectly, activates a specific set of cell cycle regulators like GMC-specific *CyclinD5;1* and *CyclinD7;1* (Han *et al.*, 2018; Weimer *et al.*, 2018) or the cyclin-dependent kinase inhibitor (CKI) *SIAMESE-RELATED4* (*SMR4*; Fig. 2c; Han *et al.*, 2022). SMR4 interacts with CYCD3;1 to decelerate the cell cycle and potentially facilitate interactions of the GMC-specific Cyclin D factors with the cell cycle machinery (Fig. 2c; Han *et al.*, 2022).

In addition to activating a GMC-specific cell cycle program, MUTE directly represses *SPCH* and the *SPCH* program (Kim *et al.*, 2022) and activates the terminal differentiation factor *FAMA* (Fig. 2c; Han *et al.*, 2018). Chromatin accessibility profiling of the early stomatal lineage identified a combinatorial *cis*-regulatory code, where the MUTE-ICE1/SCRM module (but not the SPCH-ICE1/SCRM module) interacts with the BARLEY B-RECOMBINANT/BASIC PENTACYSTEINE (BBR/BPC) family of plant-specific transcription factors. The BBR/BPCs in turn recruit the polycomb repressive complex 2 (PRC2) to repress *SPCH* and *SPCH*-activated genes (Fig. 2c; Kim *et al.*, 2022). Finally, MUTE-activated FAMA enforces a single GMC division and the terminal GC differentiation by repressing *CyclinD5;1* and *CyclinD7;1* (Han *et al.*, 2018; Weimer *et al.*, 2018) and by associating with RETINABLASTOMA-RELATED 1 (RBR1) and PRC2, respectively (Fig. 2c; Matos *et al.*, 2014).

Currently, we can only speculate on how the stomatal developmental programs are rewired in Crassulaceae to facilitate anisocytic, mesogene SC development. Gene homology analysis revealed that the diploid *K. laxiflora* has amplified the bHLH transcription factors and encodes two *SPCH* homologs, two *MUTE* homologs, and three *FAMA* homologs (Fig. 2d). Proliferation and subfunctionalization of the SPCH-MUTE-FAMA class of transcription factors accompanied more complex sporophyte forms and enabled more intricate stomatal divisions in *Arabidopsis* compared with *P. patens* (Chater *et al.*, 2016). And neofunctionalization of MUTE in grasses allowed grass MUTE to establish the SC lineage rather than the GC lineage (Raissig *et al.*, 2017). Therefore, subfunctionalization, neofunctionalization, or distinct protein regulation of these amplified stomatal bHLHs in Crassulaceae might accommodate meristemoid division capacity or establish SC identity. For example, transcription factor mobility from GCs to neighboring cells might 'retain' stomatal identity and induce SC differentiation like in grasses (Raissig *et al.*, 2017). Alternatively, dynamic auxin transport and inhibition of endoreduplication inherent to pavement cells (Ho *et al.*, 2021) might maintain stomatal identity and suppress pavement cell identity.

The higher division capacity of the meristemoid might simply require adjustments to how the core bHLH transcription factors regulate each other and the cell cycle. Adjustments to the combinatorial *cis*-regulatory code might affect how and when BBR/BPC-like transcription factors interact with MUTE and repress SPCH to end proliferative divisions. Alternatively, different cell cycle regulators could be employed and/or be differently regulated by the stomatal bHLHs. Finally, modifications to plant hormone levels and the hormonal signaling landscape or a different meristemoid cell size threshold could enable more circular meristemoid divisions.

Regardless, careful mutant and reporter gene analysis in Crassulaceae models like *K. laxiflora* (Hartwell *et al.*, 2016) are required to reveal whether and how the amplified stomatal bHLH module and their regulatory interactions with cell cycle factors accommodate anisocytic SC development.

V. Subsidiary cell function

Subsidiary cells are primarily defined according to morphological characteristics and their association with GCs (Pant, 1965; Esau, 2006), although it was suggested to include molecular differences (Gray *et al.*, 2020). Even though Esau implied a 'functional association' of SCs with GCs (Esau, 2006), a functional relevance for SCs was only shown in a few plant species. Whether SCs have general functional relevance for stomatal physiology or whether SCs merely compensate for complex epidermal patterning processes and differential growth rates between epidermal cells is yet to be determined for most plant families (Rudall *et al.*, 2013).

There is strong evidence that SCs are indeed functionally relevant for stomatal gas exchange in grasses, which show some of the fastest stomatal opening and closing kinetics in the plant kingdom (Box 1; Franks & Farquhar, 2007; McAusland *et al.*, 2016), if the mesogene, anisocytic SCs in succulents serve a physiological or rather developmental purpose is unknown. Succulents employ Crassulacean acid metabolism (CAM)

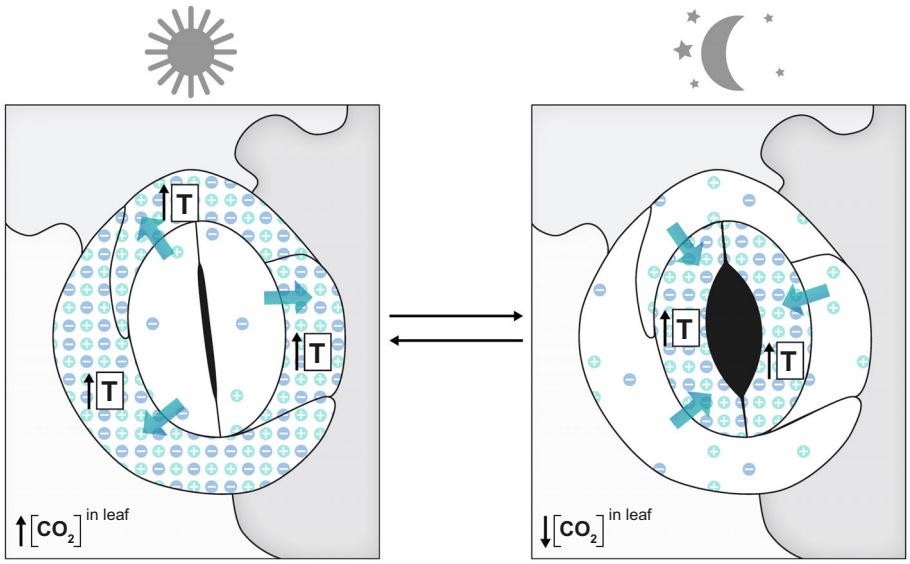

Fig. 3 Are anisocytic, mesogene subsidiary cells 'helper cells'? Stomatal opening and closing in Crassulacean acid metabolism (CAM) plants seem to be regulated by leaf-internal CO_2 concentrations (C_i) rather than light. Much like in grasses, reciprocal ion shuffling and osmoregulation could allow succulent stomata to open quickly for afternoon atmospheric CO_2 fixation when well-watered. In addition, fully turgescent SCs during the day could enforce tight stomatal closure to restrict daytime water loss. Ions (+ and −), ion transport (blue arrows), and high turgor (T) are indicated.

physiology, where carbon is fixed into an organic acid intermediate at night and remobilized during the day for secondary fixation (Dodd *et al.*, 2002). As a consequence, the stomata in CAM plants open during the night and close during the day, which avoids excessive transpiration and significantly improves water-use efficiency (Fig. 3; Males & Griffiths, 2017). Strikingly, all monocot CAM plants form stomatal SCs and SCs are twice as frequent in CAM dicots compared with C_3 dicots (Males & Griffiths, 2017). This suggests a functional relevance of SCs in CAM plants, which seem to primarily regulate stomatal aperture by internal [CO_2] (C_i) rather than light (Fig. 3; Lee, 2010; Males & Griffiths, 2017). Depletion of C_i due to fixation of carbon into malate by the phosphoenolpyruvate (PEP) carboxylase during the night opens stomata, whereas fast release of CO_2 for secondary fixation during the daytime rapidly increases C_i and, consequently, closes stomata (Fig. 3). Subsidiary cells in CAM plants could contribute to sensing C_i and signaling to GCs to regulate stomatal aperture. Furthermore and much like in grasses, an osmotic 'see-sawing' between GCs and SCs might facilitate gas exchange physiology in CAM plants (Fig. 3). This could contribute to fast stomatal opening kinetics during the late afternoon fixation of atmospheric CO_2 in well-watered succulents (Dodd *et al.*, 2002). Alternatively, pressurizing SCs during the day when stomata are closed and GCs are flaccid might actively close the stomatal pore and minimize daytime transpiration. Physiological analysis in emerging succulent models will determine whether and how mesogene, anisocytic SCs contribute to stomatal gas exchange dynamics (Hartwell *et al.*, 2016).

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Competing interests

None declared.

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