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Índice

Resumo

Folhas apresentam grande diversidade morfológica como resultado de diferentes padrões de desenvolvimento. Embora a forma plana das folhas típicas seja eficiente para sua função de fotossíntese, algumas espécies de Aizoaceae também desenvolveram folhas teretes, com simetria radial e lâmina abaxializada. Para entender os mecanismos de desenvolvimento responsáveis pela geração desses padrões, investigamos a ontogênese de folhas com diferentes morfologias no grupo. Neste trabalho, descrevemos o desenvolvimento foliar em espécies de Aizoaceae, incluindo indivíduos com folhas planas e teretes, utilizando as técnicas usuais de anatomia vegetal. Todas as espécies analisadas têm folhas com base bifacial estabelecida em estágios iniciais de desenvolvimento; no entanto, as espécies com folhas de terete possuem lâminas unifaciais. Com interesse no papel do gene *ARP* recente duplicado em Aizoaceae, nós investigamos a possível correlação entre o evento de duplicação e o surgimento da morfologia da folha abaxializada em Aizoaceae. Detectamos, por meio de *hibridização in situ*, que *ARP* é expresso em todo o primórdio foliar, sugerindo que, em Aizoaceae, este gene está envolvido principalmente na determinação do destino das células foliares. Além disso, demonstramos que a expressão dos ortólogos de *ARP* em Aizoaceae são capazes de recuperar o fenótipo selvagem de *Arabidopsis as1*, indicando um alto grau de conservação funcional do gene, mesmo em folhas com uma morfologia tão distinta.

Abstract

Leaves exhibit great morphological diversity as a result of different patterns of development. Although the flat shape of typical leaves is efficient for their photosynthesis function, some Aizoaceae species have also evolved terete leaves, with radial symmetry and abaxialized lamina. In order to understand the development mechanisms responsible for generating such patterns, we investigate the ontogenesis of leaves with different morphologies in the group. In this work, we describe development in Aizoaceae species, including individuals with flat and terete leaves, processed according to usual techniques in plant anatomy. We found that all species have leaves with bifacial base established in early stages of development; however, terete leaved species have unifacial leaf blades. Interested in the role of the recent duplicated *ARP* gene in Aizoaceae, we investigate the possible correlation between the duplication event and the emergence of the abaxialized leaf morphology in Aizoaceae. We detected, by in *situ hybridization* assays, that *ARP* is expressed throughout the leaf primordium, suggesting that in Aizoaceae this gene is involved in the determination of leaf cell fate. In addition, we have shown that the expression of *ARP* orthologs of Aizoaceae is capable of recovering the wild-type phenotype of *Arabidopsis as1*, indicating a high degree of functional conservation of the gene, even in leaves with such a distinct morphology.

INTRODUÇÃO GERAL

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A capacidade de desenvolver uma lâmina foliar ampla, achatada e dorsiventral, como ocorre na maioria das angiospermas, foi um evento chave na evolução das plantas (Gifford & Foster 1989, Nicotra *et al.* 2011). A especialização celular em cada face, somada à extensão da lâmina, aprimorou a captação de luminosidade e trocas gasosas, demandados à realização da fotossíntese, função centralizada nas folhas e ao mesmo tempo minimizam as perdas de água para o ambiente (Eames & McDaniels 1947).

A face adaxial é caracterizada por apresentar epiderme recoberta por cutícula espessa, que protege a folha de dessecação, e o parênquima paliçádico é arranjado de forma a favorecer a distribuição de luz pelo mesofilo (Esau 1977). Na face abaxial, por sua vez, é comum a presença de numerosos estômatos e de parênquima frouxamente arranjado, com amplo espaço intercelular, atributos que permitem a regulação das trocas gasosas e o controle da transpiração (Esau 1977). As células que compõem o sistema vascular nas folhas são arranjadas com xilema em posição adaxial em relação ao floema, e outras características que podem distinguir os domínios adaxial e abaxial das folhas incluem a densidade e tipo de tricomas presentes e a forma e o tamanho das células epidérmicas.

Embora a condição dorsiventral tenha aprimorado a captação de luminosidade e trocas gasosas, demandados à realização da fotossíntese, algumas espécies dispõem de modificações extraordinárias na morfologia foliar, como a ocorrência de folhas cilíndricas e unifaciais, ou em sua função, como as folhas portadoras de reserva, ou aquelas transformadas em gavinhas ou espinhos. Algumas famílias botânicas apresentam pouca variação na morfologia foliar, sendo este um estado de caráter usual em sua identificação; em outras famílias há grande variedade morfológica entre as

espécies, mesmo dentro de um único gênero, como ocorre na família Aizoaceae (Caryophyllales).

Neste trabalho, damos continuidade aos nossos estudos de uma família de plantas com folhas suculentas altamente adaptadas ao ambiente em que vivem. Tal adaptação implica em uma morfologia muito diversa e, ao mesmo tempo, particular de suas folhas, refletindo em inovações em todo o corpo vegetal. A seguir, na introdução geral, apresenta-se a família Aizoaceae, abordando um pouco de sua classificação e ecologia, e as características morfoanatômicas foliares da subfamília Ruschioideae, principal objeto do presente estudo. Um segundo tópico tratará da morfogênese de folhas em angiospermas. Por fim abordaremos o gene *Asymmetric Leaves1/Rough sheath2/Phantastica* (*ARP*), cuja duplicação em Aizoaceae tem sido apontada como uma das possíveis causas para a grande diversificação morfológica ocorrida no grupo.

Embora as folhas representem os órgãos de maior plasticidade no corpo vegetal, todas as espécies compartilham a identidade de dois domínios distintos ao longo do seu eixo adaxial-abaxial (Kaplan 2001; Yamaguchi *et al.* 2012). Essa polaridade pode ser verificada no mesofilo de folhas com arranjo dorsiventral, no sistema vascular (com o xilema ocupando posição adaxial em relação ao floema) e por especializações epidérmicas que podem diferir entre as duas faces da folha.

Mecanismos do desenvolvimento foliar relacionados ao estabelecimento da identidade adaxial-abaxial

Com o advento da biologia molecular alguns dos principais mecanismos de controle do estabelecimento da polaridade adaxial-abaxial nas folhas foram elucidados, a princípio partindo da observação de mutantes de plantas modelo, como *Antirrhinum majus* e *Arabidopsis thaliana*. Um dos primeiros trabalhos a serem realizados nesse

contexto descreveu o papel do gene *PHANTASTICA (PHAN)* em *Antirrhinum*, realizado por Waites & Hudson, em 1995. Em mutantes *phan* regiões com células características da face abaxial diferenciam-se na face adaxial da folha, sendo que as plantas com mutações mais severas desenvolvem folhas cilíndricas com domínio adaxial inteiramente ocupado por células típicas de face abaxial.

Com base nesses resultados, a importância *PHAN* para a diferenciação adequada no domínio adaxial foi melhor elucidada, e elaborou-se o modelo, posteriormente corroborado, no qual a justaposição de células características de ambas as faces se faz necessária para que haja expansão de lâmina foliar, tornando o órgão amplo e planar (Waites & Hudson 1995). Posteriormente foram descritos ortólogos de *PHAN* em outras espécies, como *ROUGHSHEET2* (*RS2*) em *Zea mays* (Timmermans *et al.* 1999) e *ASYMMETRIC LEAVES1* (*AS1*) em *Arabidopsis* (Byrne *et al.* 2000), os quais são referidos conjuntamente como *ARP*, além de outros genes envolvidos na diferenciação de cada uma das faces.

Os genes Class III Homeodomain Zipper (*HD-ZIP III*) *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) e *REVOLUTA* (*REV*) determinam identidade adaxial. Mutantes *phb* de *Arabidopsis* desenvolvem folhas cilíndricas e abaxializadas (McConnell & Barton 1998), fenótipo semelhante àqueles obtidos tanto em phan (Waites & Hudson 1995), quanto nos experimentos de incisão (Sussex 1954; Reinhardt *et al.* 2005). Da mesma forma, a expressão ectópica de *PHB* e *PHV* na face abaxial resulta em adaxialização foliar, assim como ocorre com o ganho de função de alelos de *REV* em *Arabidopsis*, comprovando o papel desses genes na determinação da face adaxial (McConnell & Barton 1998; McConnell *et al.* 2001).

Durante a morfogênese foliar o domínio de expressão de *HD-ZIP III* estende-se desde a região do meristema apical caulinar até a porção adaxial do primórdio foliar,

formando um centro de expressão; perifericamente a esse domínio, na porção abaxial da folha, expressam-se no primórdio foliar os fatores de transcrição abaxiais (Wu *et al.* 2008; Husbands *et al.* 2009). Segundo McConnell *et al.* (2001), as proteínas resultantes da expressão desses genes mediam o sinal vindo do meristema apical caulinar e reprimem a expressão de fatores formadores de tecidos abaxiais na região adaxial das folhas.

Os genes da família *KANADI* (*KAN1-4*) se expressam no domínio abaxial durante o desenvolvimento de órgãos laterais (Eshed *et al.* 2001; Kerstetter *et al.* 2001) com padrão de expressão antagônico a *PHB*, *PHV* e *REV* (Izhaki & Bowman 2007) e *ASYMMETRIC LEAVES2* (*AS2*) (Wu *et al.* 2008), corroborando o modelo proposto por Waites & Hudson (1995). Em mutantes com perda ou ganho de função de *KAN*, a condição bifacial das folhas é comprometida, formando órgãos de simetria radial adaxializados e abaxializados, respectivamente (Izhaki & Bowman 2007; Wu *et al.* 2008). Além de *KAN*, os genes da família *YABBY* também se expressam no domínio abaxial na morfogênese foliar (Siegfried *et al.* 1999) e, segundo Eshed *et al.* (2004), a perda de função de *YABBY* acentua o fenótipo radial das folhas de mutantes que não expressam *KAN*, sendo seu papel importante durante a expansão da lâmina, induzindo a divisão celular na região limítrofe entre os domínios adaxial e abaxial. O *AUXIN RESPONSE FACTOR3* (*ARF3*)/*ETTIN* (*ETT*) também se expressa no domínio abaxial da folha, sendo importante na determinação dessa face (Pekker *et al.* 2005).

Aizoaceae

Pertencente às Caryophyllales suculentas, Aizoaceae tem como centro de origem o sul da África (Ihlenfeldt 1994, Klak *et al.* 2004, Burke 2005). Suas espécies são conhecidas popularmente como *ice plants* (plantas de gelo) e *flowering stones*

(pedras que florescem), em referência às suas folhas suculentas de morfologia muito peculiar, que atraem a atenção de colecionadores e admiradores em todo o mundo.

Muitas das espécies da família Aizoaceae são endêmicas do Succulent karoo, bioma de clima árido que se estende por territórios da Namíbia e sudoeste da África do Sul (Klak *et al.* 2003, Ihlenfeldt 1994, Cowling *et al.* 1999). Considera-se o Succulent karoo como sendo o bioma árido de maior diversidade vegetal do mundo, sendo classificado *hotspot* de biodiversidade (Conservation International). Diversas hipóteses têm sido elaboradas para explicar a tamanha diversidade e endemismo na região do bioma. As principais referem-se ao clima, singular e favorável ao crescimento de plantas suculentas, por ser relativamente úmido, com distribuição de chuvas sazonal e concentrada no inverno (Cowling *et al.* 1999, Desmet & Cowling 1999). Além das características climáticas da região, destaca-se também a heterogeneidade na composição do solo ao longo do bioma (Ellis & Weis 2006) que, somada ao clima, teria proporcionado microclimas distintos e favoráveis ao desenvolvimento e especiação da vegetação (Ihlenfeldt 1994, Schmiedel & Jürgens 1999).

A classificação da família Aizoaceae fora tradicionalmente baseada em caracteres morfológicos de estruturas reprodutivas e na presença de células epidérmicas especiais (*bladder cells*, células-bexiga) nas folhas (Bittrich & Hartmann 1988, Hartmann 1993, 1998, 2001a, 2001b, Chesselet *et al.* 2002), tendo havido diversas circunscrições. A filogenia molecular mais atual (Klak *et al.* 2003), apresenta Aizoaceae dividida em quatro subfamílias: Aizoideae, Sesuvioideae, Mesembryanthemoideae e Ruschioideae, concentrando nesta última o maior número de espécies e a sua maior diversidade morfológica (Klak *et al.* 2003).

Entre as Ruschioideae observam-se arbustos, ervas e diversas geófitas com constituição notavelmente reduzida (Ihlenfeldt 1994). Poucas espécies de Aizoaceae

apresentam folhas achatadas com lâmina expandida lateralmente (fig. 1 A, B); na família folhas trígonas são mais frequentes (fig. C, D), e algumas espécies apresentam folhas cilíndricas (fig. 1 E) (Smith *et al.* 1998). A maior parte das espécies de Ruschioideae e grande parte das Mesembryanthemoideae têm filotaxia oposta e bases foliares conadas, de modo que cada par de folhas forma uma estrutura única, circundando o caule (Smith *et al.* 1998) (fig. 1 B-D, F, G).

Anatomicamente, as folhas de Aizoaceae apresentam parênquima clorofiliano margeando o parênquima armazenador, cujo centro é ocupado pela nervura central da folha, arranjo frequente observado em folhas suculentas (Landrum 2001; Melo-de-Pinna *et al.* 2014, 2016). O diferencial observado em Aizoaceae reside na ocorrência de pequenos feixes vasculares marginais dispostos ao longo da periferia da folha, denominados feixes vasculares periféricos (*peripheral vascular bundles* - PVB, Melo-de-Pinna *et al.* 2014), nos quais o xilema é endoscópico, i.e., sempre direcionado para o centro da folha, com floema voltado para a superfície externa (Melo-de-Pinna *et al.* 2014). Nessa configuração, a posição interna do xilema nos feixes periféricos localizados na região adaxial das folhas de Aizoaceae contraria a polaridade habitual de tecidos vasculares, uma vez que nas folhas o xilema normalmente dispõe-se adaxialmente ao floema (Eames & McDaniels, 1947; Esau 1977, Evert 2006).

A nervura principal da folha apresenta xilema e floema com polaridade convencional, sendo a inversão dos feixes observada apenas na margem da folha (Melo-de-Pinna *et al.*, 2014). Segundo as autoras, células características da face abaxial se diferenciam no domínio adaxial da folha, sugerindo um possível processo de abaxialização foliar envolvido na formação de feixes vasculares com polaridade invertida na face adaxial das folhas de Aizoaceae.

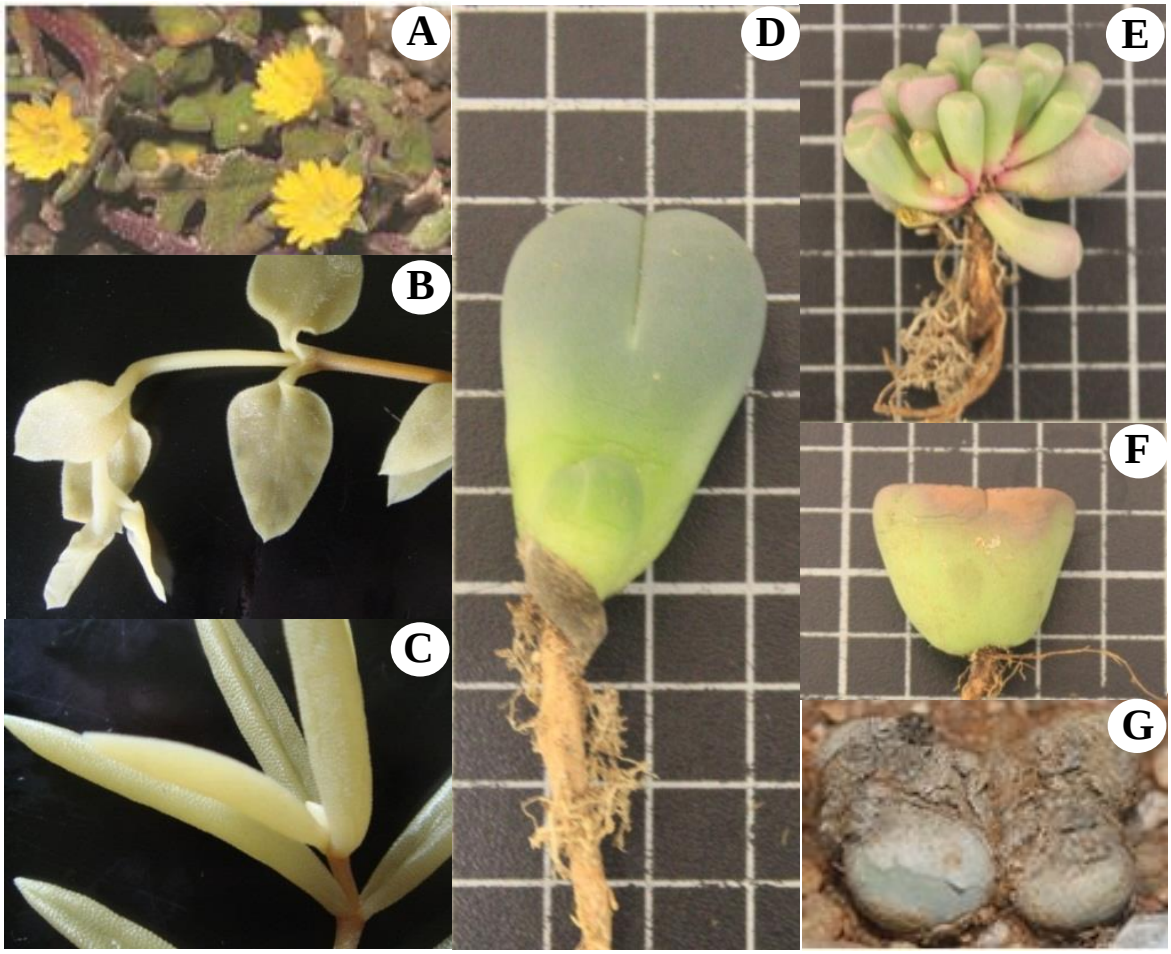

Figura 1. Exemplos de padrões morfológicos de Aizoaceae. A – *Skiatophytum tripolium*, folhas dorsiventrais planas com bases livres; B – *Delosperma tradescantioides,* folhas dorsiventrais planas com bases conadas; C – *D. napiforme*, folhas trígonas com bases conadas; D – *Gibbaeum heathii*, idem a C; E – *Fenestraria rhopalophylla*, folhas teretes com bases conadas; F – *Lithops aucampiae*, folhas quase totalmente conadas; G – *Muiria hortenseae*, folhas completamente conadas.

Ontogênese foliar

Logo após a iniciação do primórdio, a folha em desenvolvimento define seu eixo proximal-distal, e diferencia o cordão de procâmbio da nervura principal (Esau 1977; Evert 2006). A expansão da lâmina foliar ocorre posteriormente, com a atividade da blastozona marginal (Hagemann & Gleissberg 1996; Dengler & Tsukaya 2001). Dessa forma, a abaxialização foliar em Aizoaceae provavelmente se expressa durante a formação da lâmina foliar. Isso se sustenta no fato de que os feixes vasculares

invertidos distribuem-se ao logo do eixo proximal-distal em toda a lâmina, porém estão ausentes na base da folha.

A disposição dos feixes marginais de Aizoaceae assemelha-se ao que se observa em folhas unifaciais, frequentes em monocotiledôneas (Eames & McDaniels 1947). As folhas unifaciais exibem dois domínios morfologicamente distintos ao longo de seu eixo proximal-distal: a lâmina, que compõe a porção unifacial propriamente dita, e a bainha que representa a base da folha e tem estrutura dorsiventral (Kaplan 1973). Em folhas bifaciais, tanto a lâmina quanto a base diferenciam-se mantendo a polaridade adaxial-abaxial, entretanto, nas folhas unifaciais a lâmina foliar é formada apenas por tecidos de face abaxial. Em seção transversal, nota-se a ausência de arranjo dorsiventral dos tecidos, e a presença de feixes vasculares dispostos ao longo de uma elipse, com os polos de xilema sempre voltados ao centro da folha (Kaplan 1973, Yamaguchi & Tsukaya 2010). A partir da ontogênese desse tipo foliar, observa-se uma intensa atividade da blastozona adaxial que proporciona divisões periclinais de células em direção ao meristema (Hagemann & Gleissberg 1999, Kaplan 2001).

Os mecanismos moleculares envolvidos no desenvolvimento de folhas unifaciais foram tema de estudo de Yamaguchi & Tsukaya (2010) e Yamaguchi *et al.* (2010). De acordo com esses trabalhos, a lâmina de *Juncus prismatocarpus* é abaxializada, uma vez que a expressão de um ortólogo de *ARF3* (identidade abaxial) ocorre na margem foliar ao longo de toda a lâmina, ao mesmo tempo em que um ortólogo de *PHB* (identidade adaxial) se expressa apenas no xilema. Na bainha foliar a expressão desses genes é convencional, e por isso essa parte da folha é dorsiventral. Com base em nossos estudos de anatomia do desenvolvimento foliar em Aizoaceae (Melo-de-Pinna *et al.* 2014, 2016), acrescidas do conhecimento disponível sobre desenvolvimento de folhas unifaciais, nos âmbitos morfoanatômico (Kaplan 1970,

1973) e molecular (Yamaguchi & Tsukaya 2010; Yamaguchi *et al.* 2010), nós elaboramos a hipótese de que ocorra processo de abaxialização em algumas espécies desta família, sendo essa a explicação ao fato de tecidos característicos de face abaxial se diferenciarem na região adaxial.

ASYMMETRIC LEAVES1/ ROUGH SHEEAT2/ PHANTASTICA (*ARP*) em Aizoaceae

Segundo o trabalho de Illing *et al.* (2009), durante a diversificação entre Mesembryanthemoideae e Ruschioideae, a duplicação do gene ortólogo de *ASYMMETRIC LEAVES1* (*AS1*), configurou uma importante novidade a partir da qual houve uma importante diversificação morfológica em Aizoaceae. A atividade do gene *ARP* pode estar relacionada à determinação da identidade adaxial, e uma alteração no seu padrão de expressão pode estar associada ao desenvolvimento de células características de face abaxial na face adaxial da folha (Waites & Hudson 1995). A observação do padrão de expressão de *ARPa* e *ARPb* em diferentes tipos foliares de Aizoaceae possibilitaria inferir a influência da atividade desses genes na formação de células abaxiais no domínio adaxial das folhas, e concluir se de fato ocorre abaxialização foliar em Aizoaceae.

Diante deste cenário, propõe-se realizar uma investigação inédita sobre a expressão do ortólogo de *ARP* durante a ontogênese foliar em duas espécies de Aizoaceae, sendo uma de Mesembryanthemoideae e uma de Ruschioideae. O padrão de expressão de *ASYMMETRIC LEAVES1/ROUGH SHEET/PHANTASTICA* (*ARP*) observado pode fornecer evidências que sustentem a hipótese de abaxialização foliar, elaborada com base em estudos de desenvolvimento foliar. Além disso, busca-se discutir se a duplicação do gene (*ARPa* e *ARPb*), ocorrida durante a diversificação de

Mesembryanthemoideae e Ruschioideae, contribuiu para a diversificação na morfologia foliar em Aizoaceae. Para tanto, nossos objetivos são:

§ Descrever a ontogênese foliar de espécies de Ruschioideae com folha dorsiventral e folha unifacial.

§ Verificar se as duas cópias do ortólogo de *ASYMMETRIC LEAVES1/ROUGH SHEEAT/PHANTASTICA* - (*ARPa* e *ARPb*) detectadas no genoma, de fato são expressas durante a ontogênese foliar em Ruschioideae.

§ Descrever o padrão de expressão *in situ* de *ARP* durante o desenvolvimento de folhas em representantes de Mesembryanthemoideae e Ruschioideae.

§ Observar se o gene *ARP* em Aizoaceae tem função homóloga à de seu ortólogo em *Arabidopsis thaliana*.

A presente tese apresenta-se em dois capítulos. No primeiro, abordamos a ontogênese foliar, enfatizando a atividade diferencial de suas regiões de crescimento. No segundo capítulo investigamos o padrão de expressão de *ARP*, a homologia das sequências obtidas, bem como sua possível função do gene durante o desenvolvimento foliar. Em anexo encontram-se a lista de todos os primers utilizados, e as sequências de genes obtidas no presente estudo e o resumo das publicações realizadas ao longo do doutorado.

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CAPÍTULO 1

UNIFACIAL LEAF BLADE IN RUSCHIOIDEAE (AIZOACEAE, CARYOPHYLLALES): ANATOMICAL EVIDENCES OF ABAXIALIZATION PROCESS

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Abstract

Premise of research. Vascularization pattern and anatomy of epidermis and fundamental tissues have been reported in many unifacial leaves, in special the organization of peripheral vascular bundles. In Aizoaceae, peripheral vascular bundles exhibit two patterns regarding the arrangement of its cell types: exoscopic and endoscopic bundles. However, there are no studies including an ontogenetic approach with evidence for a unifacial condition and processes of abaxialization in the group.

Methodology. We investigated ten Ruschioideae species, including individuals with flat and terete leaves processed according to usual techniques in plant anatomy.

Results. All species have leaves with bifacial base established in early stages of development. However, eight of the ten species analyzed have unifacial leaf blades. *Delosperma tradescantioides*, one of species with flat leaves, show a unique anatomical arrangement of peripheral vascular bundles on the midrib region, and lateral vascular bundles with inverted position of xylem cells.

Conclusions. We present anatomical data, as occurrence of bladder-cells and peripheral vascular bundles, supporting the interpretation of unifacial leaf blades in Aizoaceae. Nevertheless, all leaves, present a bifacial leaf base, indicating that probably no leaf is completely unifacial in this group. In addition, the peripheral endoscopic bundles begin their differentiation in abaxial side of leaf primordia, suggesting an abaxialization process during leaf blade development.

Keywords: adaxial-abaxial polarity; endoscopic bundles; peripheral vascular bundles.

Introduction

Aizoaceae (Caryophyllales) consists of about 1750 species, mostly with succulent leaves (Ihlenfeldt 1994; Klak *et al.*, 2003). Among the four subfamilies (Klak *et al.* 2003), Ruschioideae is the richest in both number of species and in life forms (Klak *et al.* 2013). Leaf succulence is highly developed in Ruschioideae and the species present a remarkable morphological diversity, with leaves ranging from flat dorsiventral to more specialized succulent forms with connate trigonous and cylindrical shapes (Hartmann 2002; Melo-de-Pinna *et al.* 2014). The evolution of the terete morphology from a flat-bladed leaf is among the arguments on the mainspring behind the strikingly wide and rapid radiation of this subfamily (Klak *et al.* 2004; Melo-de-Pinna *et al.* 2014).

The evolutionary success of a terete morphology is probably related to the succulence degree it can provide. The water storage capacity in succulent leaves increases by minimizing the leaf surface area to volume ratio, so the most succulent leaves will tend to be terete (Ogburn and Edwards 2013). In some species, photosynthesis and water storage occur in the same tissue ("all cell succulents"); others may exhibit equifacial arrangement, with a sub-epidermal chlorenchyma encircling the inner parenchyma aquifer ("storage succulents") (Ihlenfeldt 1985).

With different arrangements of tissues, the vascular system of leaves may also vary. In the vascular system of bifacial leaves, the xylem poles are usually oriented toward the leaf's adaxial side and phloem poles toward its abaxial side (Eames and McDaniels 1947; Esau 1960; Kaplan 2001). On the other hand, the vascular system in terete leaves frequently exhibits a 3D organization, with small vascular bundles disposed in a radial distribution along leaf periphery (Ogburn and Edwards 2013; Hernandes-Lopes *et al.* 2016). A larger central vascular bundle may be present in some

cases (as seem in Aizoaceae), or the main vascular bundle can appear between the peripheral vascular bundles, occupying an abaxial position (Melo-de-Pinna *et al.* 2016).

In Aizoaceae, peripheral vascular bundles (PVB) exhibit two patterns in regard to arrangement of its cell types (Melo-de-Pinna *et al.* 2014): vascular bundles with xylem oriented either towards the leaf outer surface (exoscopic bundles), or towards the center of the leaf (endoscopic bundles). Endoscopic PVBs occur extensively in Ruschioideae, not directly related to cylindrical morphology, but to leaves that have an expanded or sheathing base (Melo-de-Pinna *et al.* 2014). Exoscopic arrangement of vascular bundles is rare in Aizoaceae (Melo-de-Pinna *et al.* 2014), but has been reported in other Caryophyllales with succulent leaves (Ogburn and Edwards 2013; Hernandes-Lopes *et al.* 2016; Melo-de-Pinna *et al.* 2016). However, few taxa have been studied to date to establish whether there is indeed a strong linkage between leaf shape, leaf base and PVB orientation.

Currently, there are interesting hypothesis relating the presence of endoscopic and exoscopic PVBs to a possible modification of the leaf adaxial/abaxial polarity. The evolution of exoscopic PVBs in terete leaves may be due to a disruption of the abaxial identity of the leaf, making the entire surface homologous to the adaxial side (Ogburn and Edwards 2013). Accordingly, the endoscopic arrangement of the PVB found extensively in Ruschioideae suggests that the leaves may be abaxialized, i.e., that its entire surface may be homologous to the abaxial side (Melo-de-Pinna *et al.* 2014).

Finally, there are evidences for a link between the presence of either endoscopic or exoscopic PVBs and the region of the leaf mesophyll where these bundles first start to differentiate in many succulent plants (Melo-de-Pinna *et al.* 2016). According to authors, while PVBs are initiated in the abaxial domain of leaves with endoscopic bundles, leaves with exoscopic PVBs start their differentiation in the adaxial domain.

In this work, we show anatomical features during the leaf ontogenesis and in mature leaves of different species of Ruschioideae, with structural evidence to process of abaxialization in unifacial leaf blade. Mechanism possibly involved in morphological innovations related to the high diversification of Ruschioideae.

Material and methods

Ten species of Ruschioideae (Aizoaceae) were analyzed, and all material was collected from natural habits in South Africa. Voucher was deposited in the Herbarium at the Universidade de São Paulo (SPB) or Bolus Herbarium (BOL) at University of Cape Town (Table 1).

Three plants of each species were collected including mature leaves and shoot apical region, fixed in formalin-acetic acid-ethanol 50% (FAA) for 48 h and stored in 70% ethanol. The material was submitted to conventional ethanol/tert-butanol gradient (50–100%) dehydration and embedded in paraffin (Ruzin 1999). Transverse and longitudinal sections were made using microtome, stained with safranin-astra blue according to the method of Bukatsch (1972) and mounted permanently on slides with Canada balsam; or sections were stained with toluidine blue (1% in phosphate buffer pH 6.8; O'Brien *et al.* 1964) and then mounted with Entellan.

For scanning electron microscope analyses (SEM), the material was fixed in Karnovsky solution (Karnovsky 1965), dehydrated through a gradient series of ethanol, and critical point dried. Samples were mounted on metal stubs and sputter-coated with gold.

Table 1. List of analyzed species, with respectives leaf characters and voucher. PVB = peripheral vascular bundles. (+) present; (-) absent. Accession numbers from the Bolus Herbarium (BOL) and the herbarium of the University of São Paulo (SPF).

Species	Leaf morphology	Leaf base	Water Storage (Ihlenfeldt, 1985)	PVB	Voucher
Cleretum lyratifolium Ihlenf. and Struck	flat	free	-	absent	Klak 2022 (BOL)
Delosperma herbeum (N.E.Br.) N.E.Br.	terete	connate	storage succulent	endoscopic	ASN 09 (SPF)
Delosperma napiforme Schwantes	terete	connate	storage succulent	endoscopic	ASN 06 (SPF)
Delosperma tradescantioides(A.Berger) L.Bolus	flat	connate	all cell succulent	endoscopic	Klak 331 (BOL)
Fenestraria rhopalophylla (Schltr. and Diels) N.E.Br.	terete	connate	storage succulent	endoscopic	Klak 263 (BOL)
Gibbaeum heathii (N.E.Br.) L.Bolus	terete	connate	all cell succulent	endoscopic	Klak 1125 (BOL)
Gibbaeum petrense (N.E.Br.) Tischer	terete	connate	all cell succulent	endoscopic	ASN 13 (SPF)
Gibbaeum velutinum (L.Bolus) Schwantes	terete	connate	all cell succulent	endoscopic	Melo-de-Pinna 154 (SPF)
Lithops marmorata N.E.Br.	terete	connate	all cell succulent	endoscopic	Melo-de-Pinna 174 (SPF)
Lithops aucampiae L.Bolus	terete	connate	all cell succulent	endoscopic	ASN 08 (SPF)
Muiria hortenseae N.E.Br.	terete	connate	all cell succulent	endoscopic	ASN 12 (SPF)

Results

Morphology of mature leaves

Nine of the ten species analyzed show opposite phyllotaxy and have expanded leaf base, which form a connate region. *Cleretum lyratifolium* is the exception and present alternate leaves without expanded base (Table 1).

Among the 10 species studied, only *Cleretum lyratifolium* (fig. 1*a*) and *Delosperma tradescantioides* (fig. 1*b*, 1*c*) have flat leaf blades, whereas other species present terete leaves (figs. 1*d*-1*l*).

The connate portion may represent less than one-third of leaf length, as in *D. tradescantioides* (fig.1*b*), *Delosperma herbeum* (fig. 1*d*), *D. napiforme* (fig. 1*e*), *Gibbaeum vellutinum* (fig. 1*f*), *G. petrense* (fig. 1*g*), *Fenestraria rhopallophylla* (fig. *1i*), and *Lithops marmorata* (fig. 1*l*); more than two-thirds as in *Gibbaeum heathii* (fig. 1*h*), *Lithops aucampiae* (fig. *1j*) or the opposite leaves may be fully connate, as in *Muiria hortenseae* (fig. 1*k*).

Anatomy of leaf base

In early developmental stages, the adaxial/abaxial domains are marked by more vacuolated cells on the abaxial side (fig. 2*a*, 2*b*), and a procambial strand is visible in the midrib position (fig. 2*b*). Throughout development, mitotic activity in the adaxial region increases primordium thickness by periclinal divisions, forming rows of cells toward the shoot axis (fig. 2*c*, 2*d*).

In mature leaves of all species analyzed, the bifacial anatomy on the leaf base is maintained, with distinction of epidermal and mesophyll tissues between adaxial and abaxial surfaces. Stomata occur only abaxial surfaces (fig. 3*a*, 3*b*), and in species with bladder cells, these cells occur only in the abaxial face (fig. 3*c*, 3*d*).

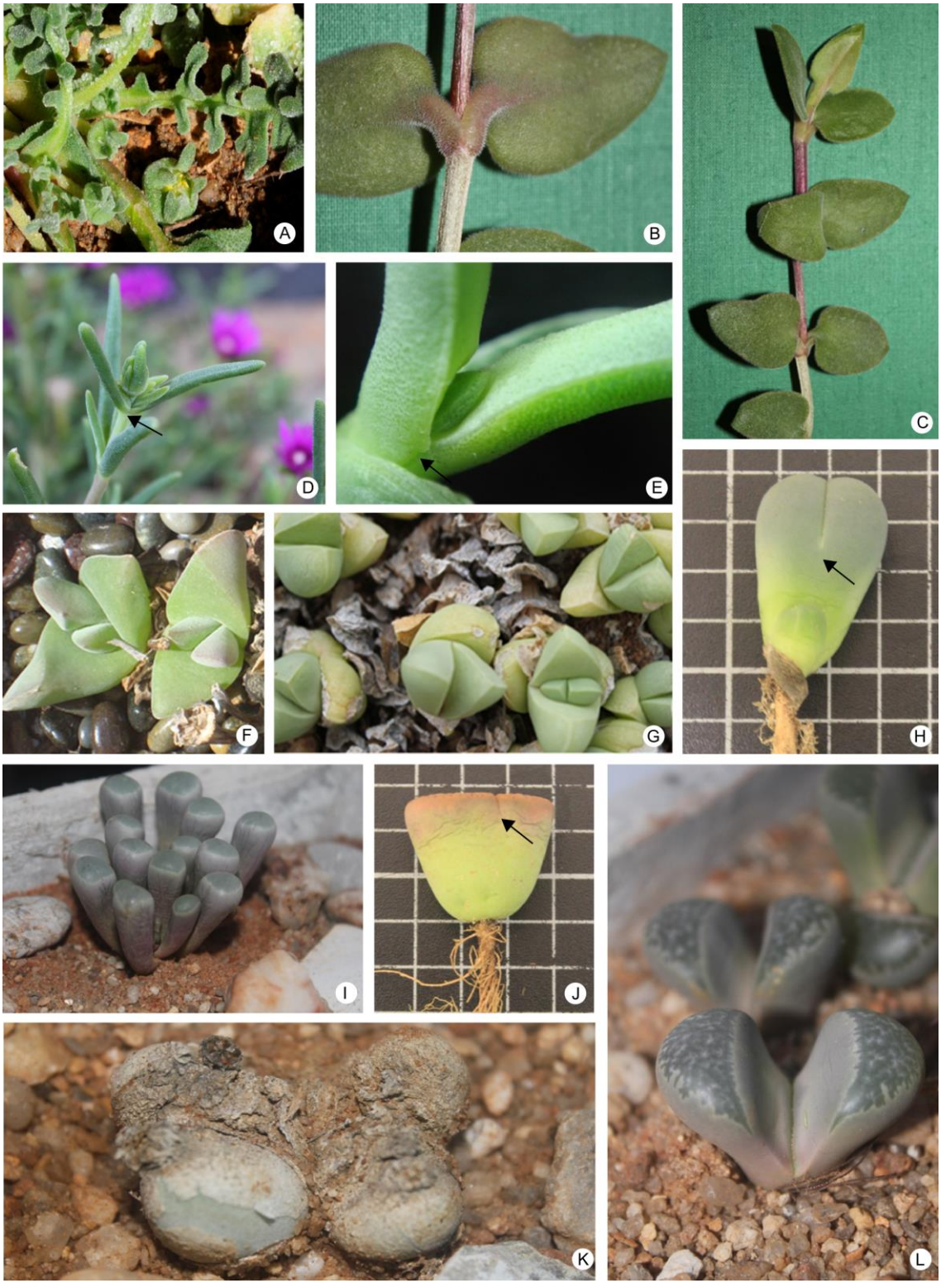

Fig. 1 Leaf morphology of Ruschioideae species. *a* – Flattened blade and unexpanded leaf base in *Cleretum lyratifolium. b, c,* Flattened blade and expanded leaf base in *Delosperma tradescantioides*. *d, e,* Trigonous leaves with expanded base and less than one-third of leaf pair connate (arrows) in *Delosperma herbeum* and *D. napiforme*. *f,* Trigonous leaves with expanded base and less than one-third of leaf pair connate in *Gibbaeum velutinum. g, h,* Trigonous leaves with expanded base and more than two-

thirds of leaf pair connate (arrow) in *Gibbaeum petrense* and *G. heathii*. *i*, *Fenestraria rhopalophylla*, cylindrical leaves with expanded base and less than one-third of the leaf pair connate (in the soil). *j*, Sub-cylindrical opposite leaves almost fully connate in *Lithops aucampiae* (arrow shows a tiny apical region of separation between the leaves). *k*, *Muiria hortenseae* with fully connate leaves. *l*, *Lithops marmorata* with more than two-thirds of leaf pair connate.

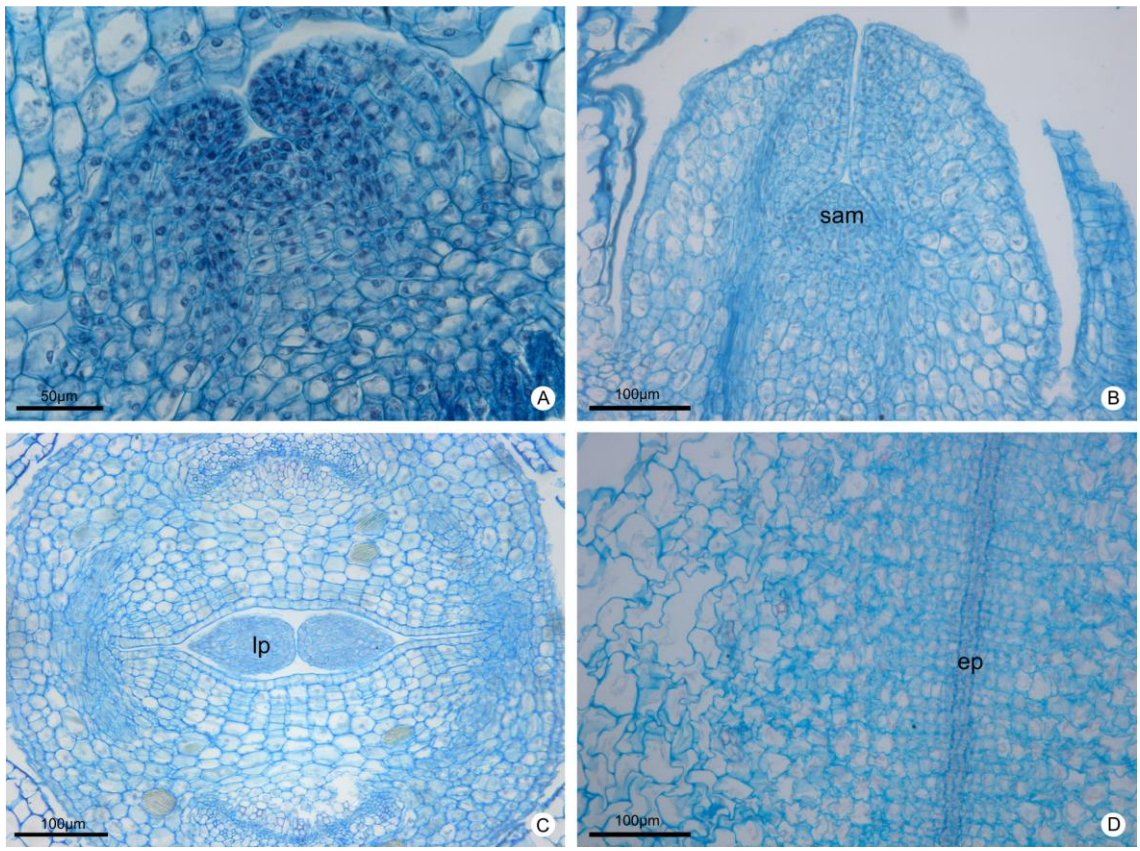

Fig. 2 Leaf anatomy of Ruschioideae. *a*, Longitudinal section of shoot apex of *Delosperma napiforme*. *b*, Longitudinal section of shoot apex of *Delosperma tradescantioides*. *c*, Transection of shoot apex of *D. tradescantioides*. *d*, Transection of two opposite leaves of *Lithops aucampiae*, detailing the adaxial surfaces during development. Ep - epidermis; lp - leaf primordium; sam - shoot apex meristem.

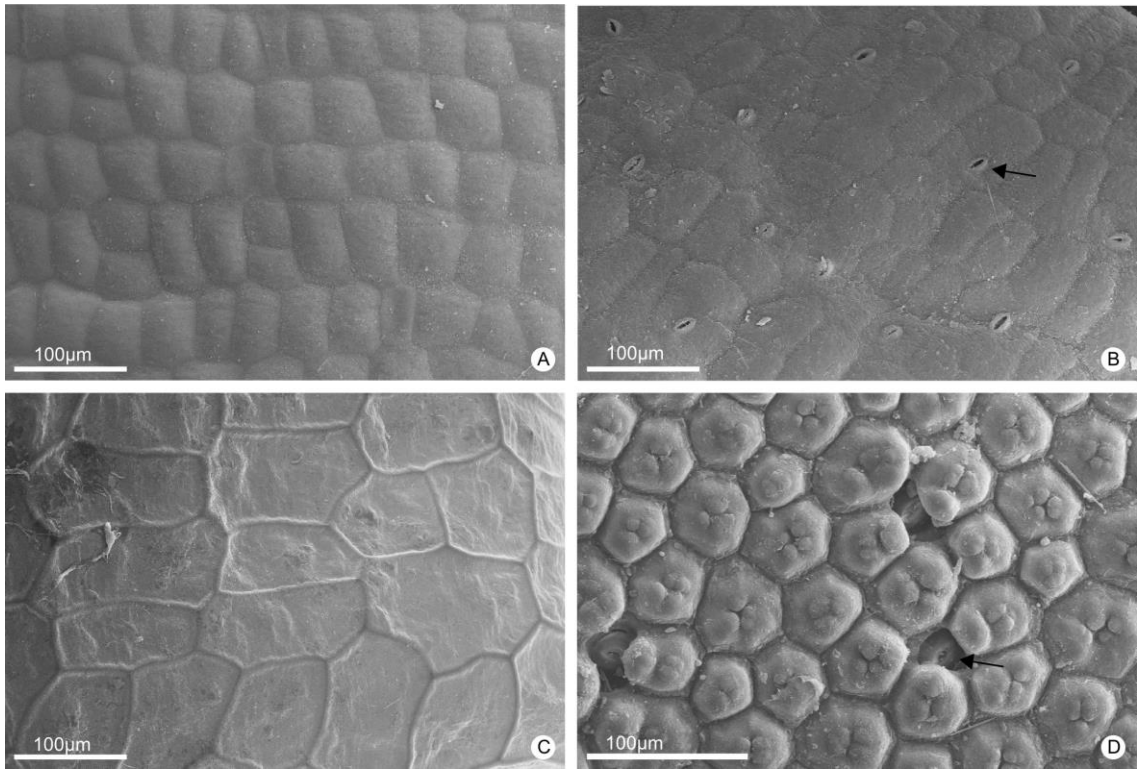

Fig. 3 Scan electron micrographs of leaf base surfaces of Ruschioideae. *a*, Leaf base adaxial side of *Fenestraria rhopallophyla*. *b*, Leaf base abaxial side of *F. rhopallophyla*. *c*, Leaf base adaxial side of *Gibbaeum heathii*. *d*, Base abaxial side of *G. heathii*. Arrows = stomata.

Additionally, on the midrib the vascular bundle is collateral with xylem cells oriented toward the adaxial surface (fig. 4*a*, 4*b*). The peripheral vascular bundles are present in all species with connate leaf base and they are limited to abaxial domain.

In species with connate leaves, the opposite bases form a single structure and from transverse sections it is possible to recognize the epidermal adaxial surface reduced to a narrow gap encircling the shoot apex (figs. 4*c*-4*e*).

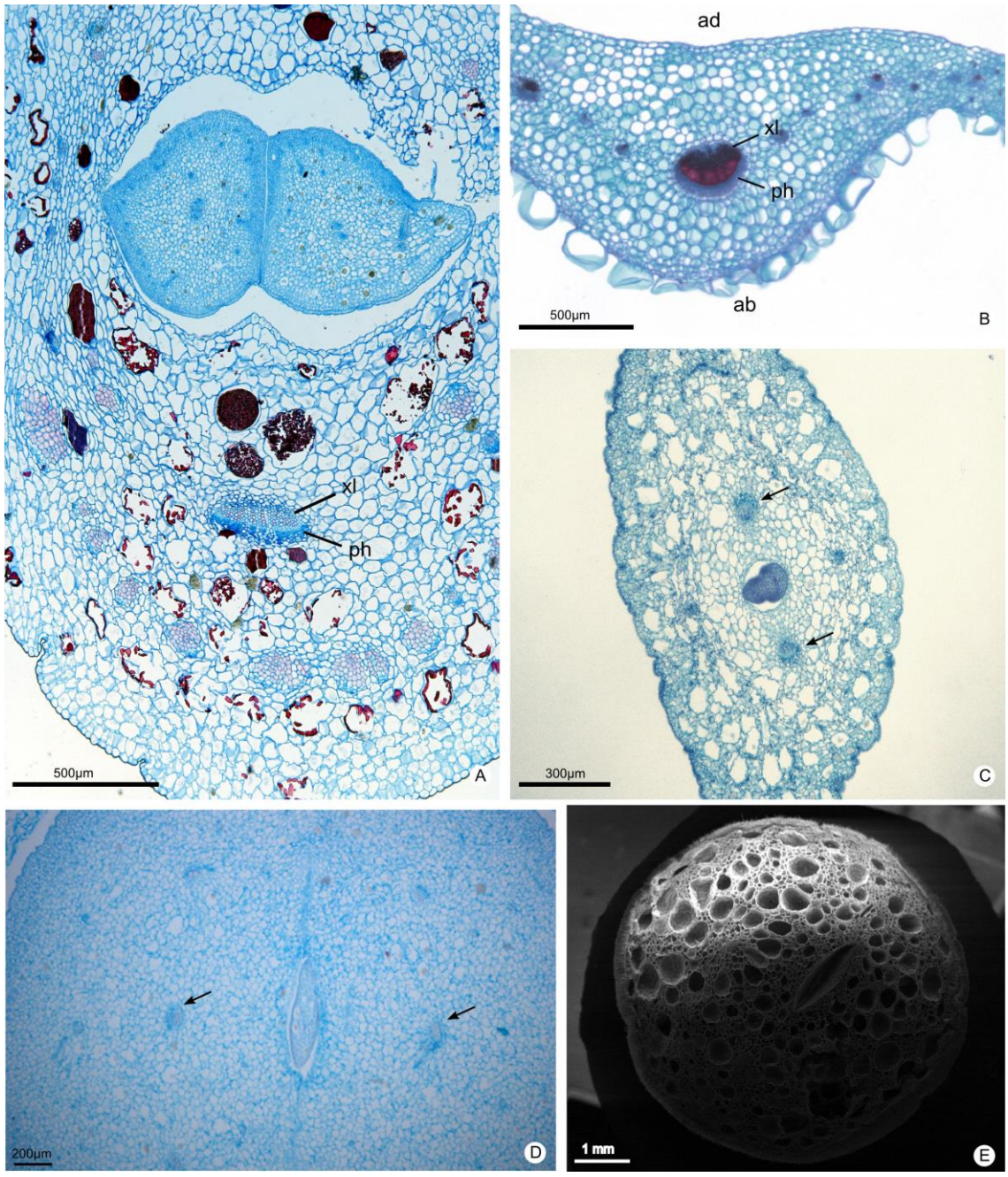

Fig. 4 Transections of leaf base region in Ruschioideae. *a*, *Fenestraria rhopallophylla*. *b*, *Cleretum lyratifolium*. *c*, *Gibbaeum velutinum*. *d*, *Lithops aucampiae*. *e*, *Muiria hortenseae* (scan electron micrography). Arrows = midrib. ad - adaxial leaf surface; ab - abaxial surface; ph - phloem; xl - xylem.

Anatomy of the leaf blade

The epidermis of opposite primordia become juxtapost in adaxial surfaces since early stages of development, enclosing the shoot apical meristem (figs. 5*a*-5*d*), except in *Cleretum lyratifolium*, in which the opposite primordium are not juxtapost (fig. 5*e*). In flat and terete leaves, the epidermis consists of a single layer, with stomata in both adaxial and abaxial surfaces (fig. 6*a*, 6*b*). In the case where on leaf base the bladder-cells were limited to abaxial surface, these cells occur in both adaxial and abaxial surfaces on the leaf blade (fig. 6*c*-6*d*).

In flat leaves, new lateral vascular bundles and mesophyll tissues are originated from expansion of the blade positioned laterally to the midrib (figs. 7*a*-7*c*). However, in terete leaves the differentiation of vascular bundles and parenchyma cells begin from abaxial side (fig. 7*d*). Cells retain meristematic characteristics in the periphery of the organ during leaf development (fig. 7*e*, 7f). Figure 8 summarizes the pattern of vascular bundles arrangement along a terete leaf axis.

In mature leaves of *Cleretum lyratifolium*, a species with flat leaves, the adaxial/abaxial domains of inner tissues display polarity, with palisade chlorenchyma on the adaxial side and spongy parenchyma on the abaxial side (fig. 9*a*, 9*b*). The main and lateral vascular bundles are distributed in a single plane along the mesophyll, with xylem cells orientated toward the adaxial surface and phloem toward the abaxial surface (figs. 9*a*-9*c*).

Delosperma tradescantioides, another species with flat leaves, exhibits a unique condition on the midrib region with endoscopic peripheral vascular bundles with phloem cells oriented toward the surface on both the adaxial and the abaxial sides and xylem cells oriented toward the central region (fig. 9*d*). On the midrib region, chlorenchyma cells occur in both adaxial and abaxial sides and large vacuolated cells in

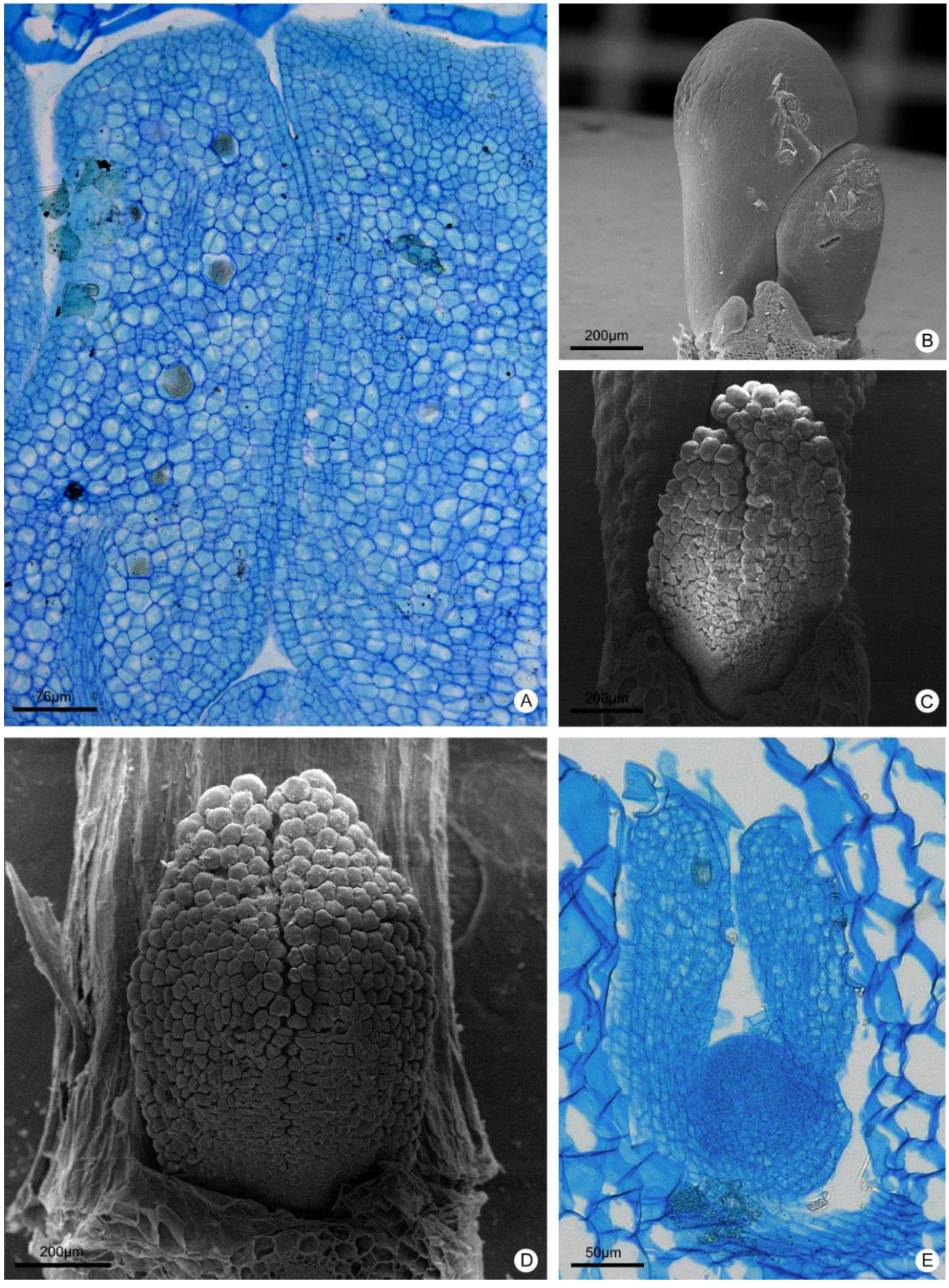

Fig. 5 Leaf primordia of different species of Ruschioideae. *a*, Longitudinal section of shoot apex of *Gibbaeum velutinum*. *b*, Scan electron micrography (SEM) of shoot apex surface of *G. velutinum*. *c* and *d*, SEM of shoot apex surface of *Delosperma herbeum*. *e*, Longitudinal section of shoot apex of *Cleretum lyratifolium*.

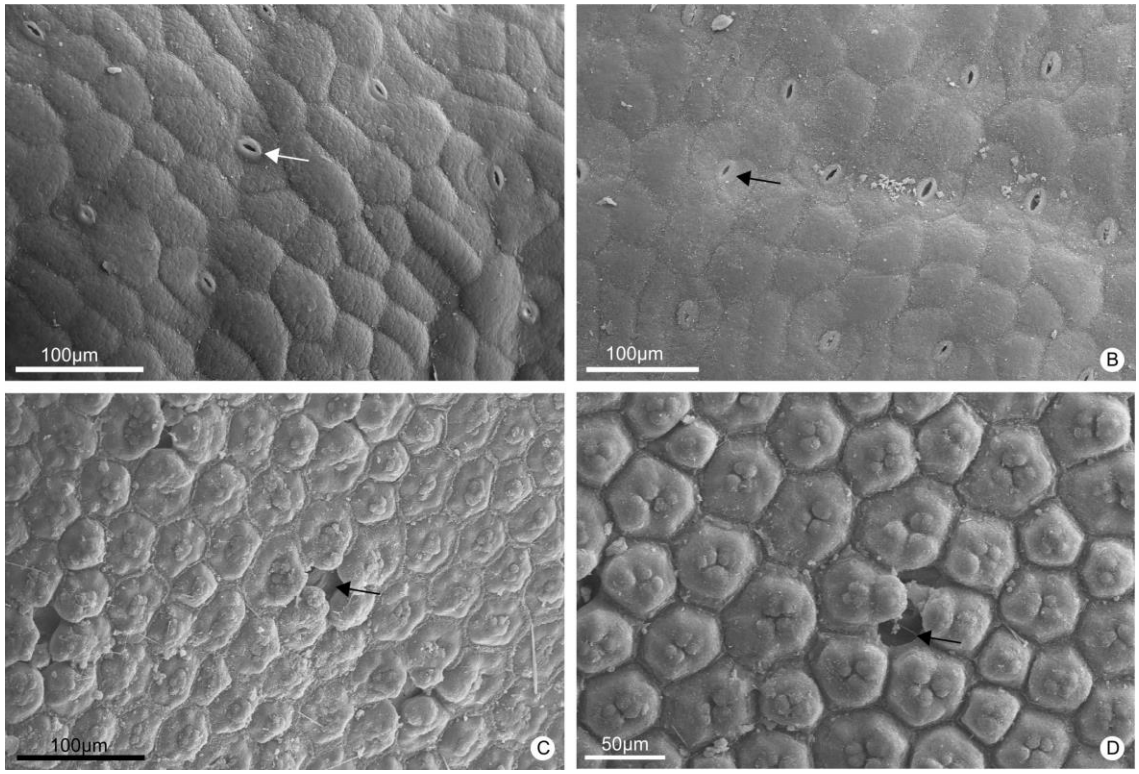

Fig. 6 Scan electron micrographs of leaf blade surfaces of Ruschioideae. *a*, Adaxial side of *Fenestraria rhopallophyla*. *b*, Abaxial side of *F. rhopallophyla*. *c*, Adaxial side of *Gibbaeum heathii*. *d*, Abaxial side of *G. heathii.* Arrows = stomata.

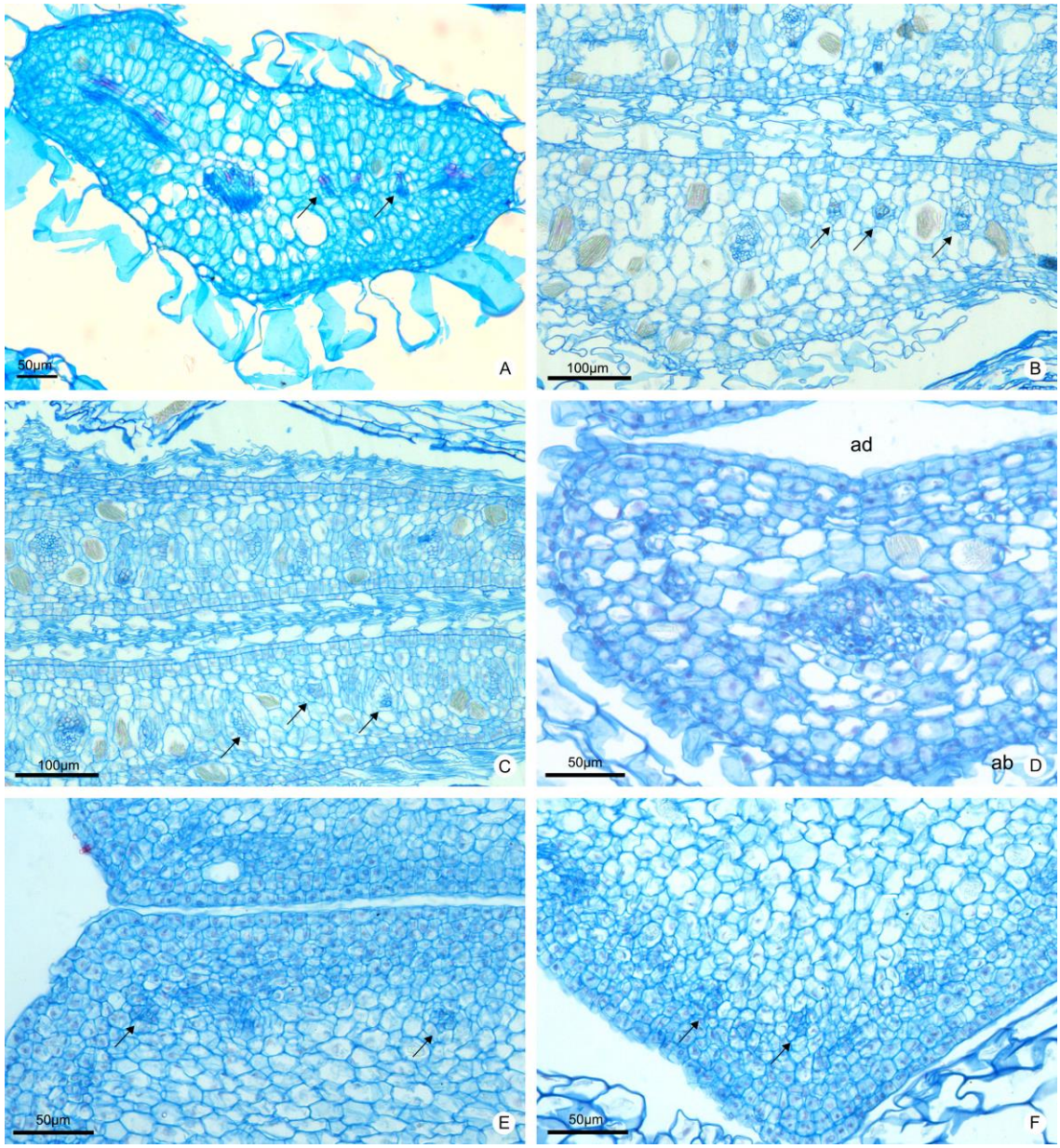

Fig. 7 Leaf transections showing the development of peripheral vascular bundles (arrows) as products of peripheral blastozone during leaf ontogenesis in species of Ruschioideae. *a*, *Cleretum lyratifolium*. *b* and *c*, *D. tradescantioides*, apical and medium leaf regions, respectively. *d*, *Delosperma herbeum*. *e* and *f*, *Fenestraria rhopalophylla*, adaxial and abaxial leaf regions, respectively.

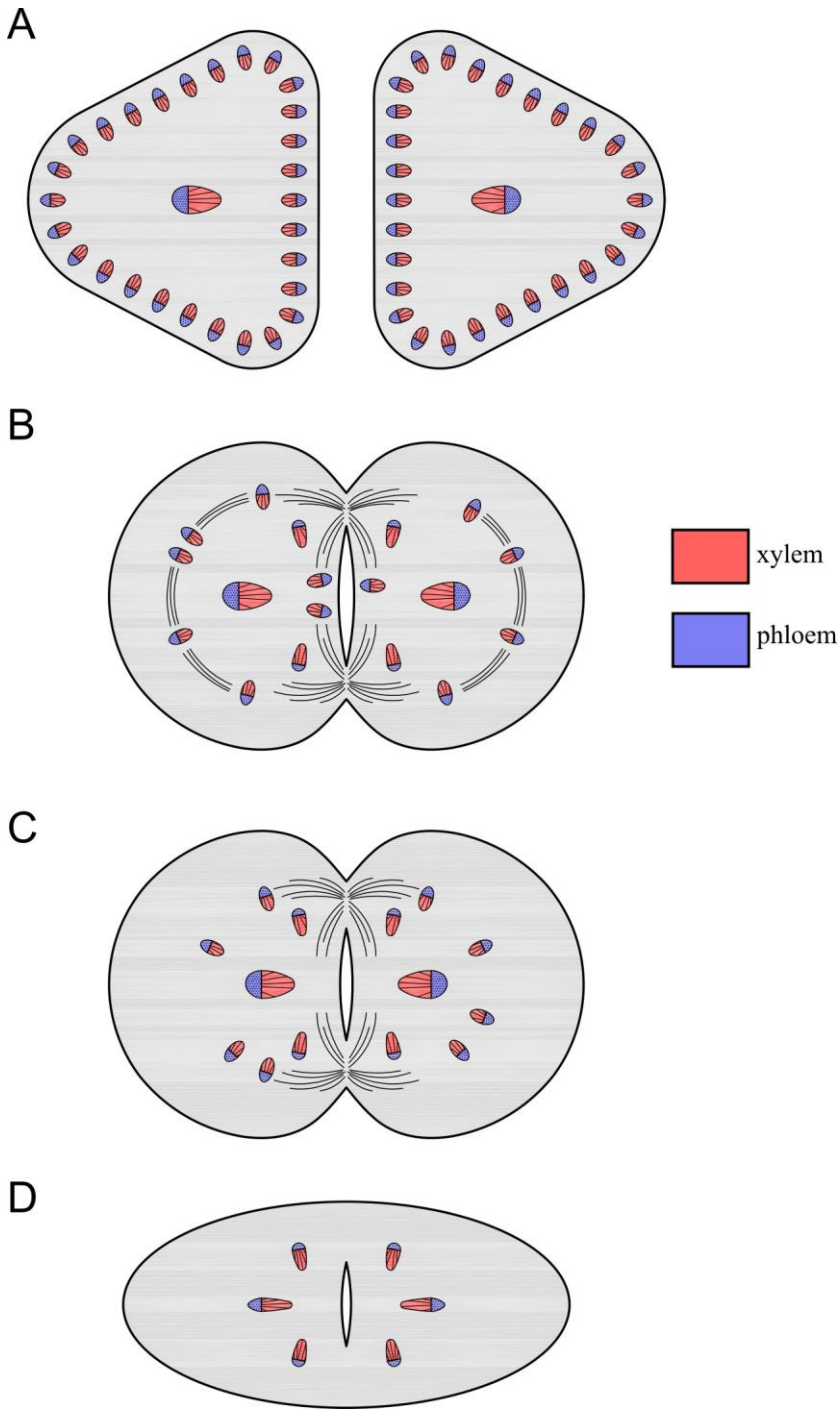

Fig. 8 Schematic representation of peripheral vascular bundles distribution along the proximal-distal axis in unifacial leaves of Ruschioideae. *a*, Distal sector of leaf lamina, showing endoscopic PVB. *b* and *c*, Proximal sector of leaf lamina (*b*) in transition with leaf base (*c*), showing a gradual decrease in the number of PVB in adaxial side. *d*, Totally connate leaf base with three vascular bundles. Illustration by Marcelo Kubo.

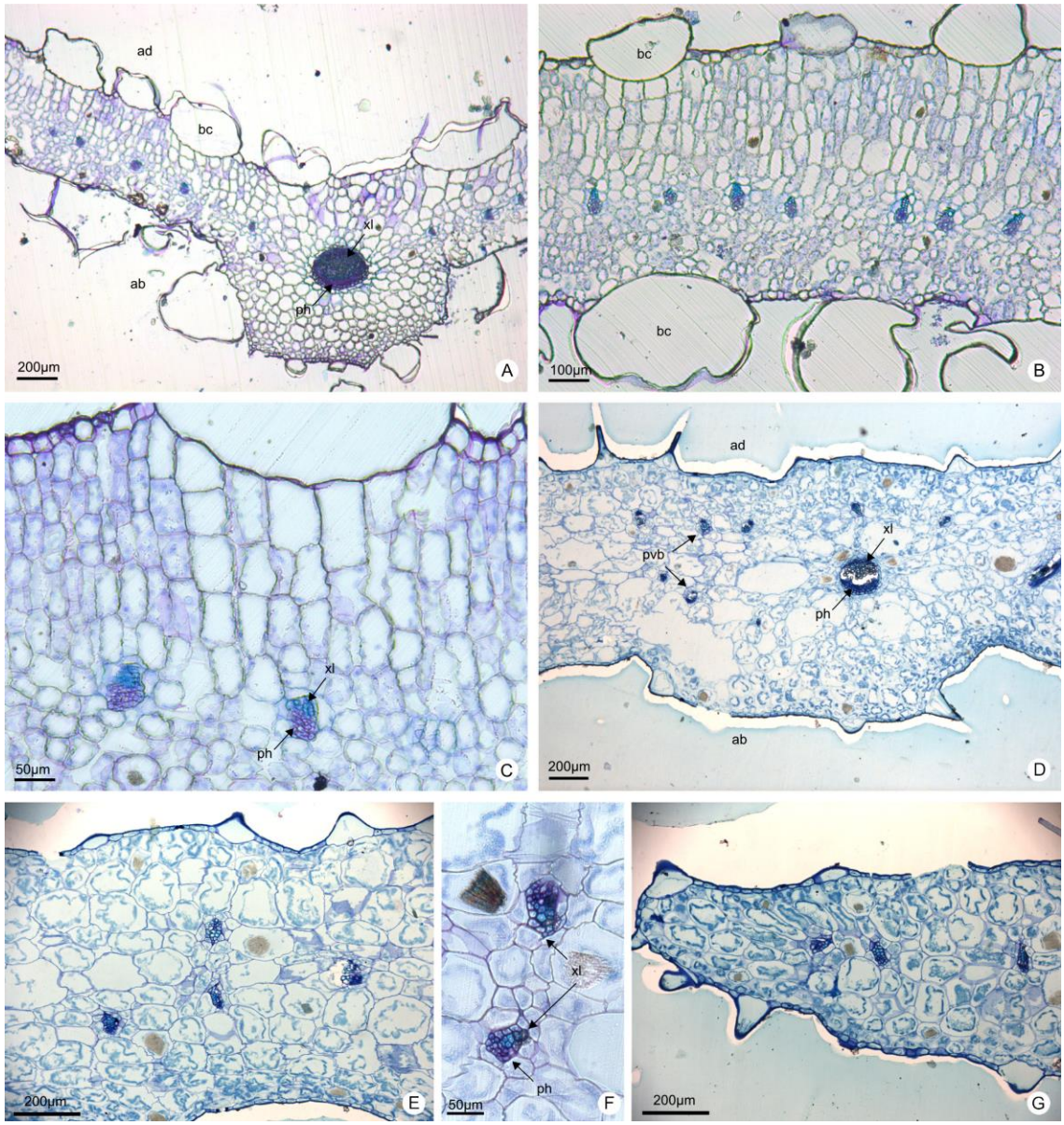

Fig. 9 Transverse sections of the leaf blade of Ruschioideae species. *a-c*, *Cleretum lyratifolium* (flattened blade and unexpanded leaf base). *a*, Midrib region. *b*, Dorsiventral mesophyll with marginal vascular bundles. *c*, Vascular tissue polarity in marginal vascular bundles. *d-h*, *Delosperma tradescantioides* (flattened blade and expanded leaf base). *d*, Midrib region with peripheral vascular bundles. *e*, All succulent mesophyll with peripheral vascular bundles. *f*, Vascular tissue polarity in peripheral vascular bundles. *g*, Leaf border region with marginal vascular bundles. ad - adaxial leaf surface; ab - abaxial surface; bc - epidermal bladder cell; ph - phloem; xl – xylem.

the inner position (fig. 9*d*). Along the leaf blade, the mesophyll cells exhibit no remarkable morphological distinction between adaxial and abaxial sides (fig. 9*e*, 9*f*), with water storage and photosynthesis are carried out in the same tissue. Lateral vascular bundles are distributed along a single plane with collateral arrangement and xylem cells towards the adaxial surface and phloem cells toward the abaxial surface (fig. 9*f*). However, some bundles occur on the adaxial surface with inverted position of vascular tissues, xylem cells toward the abaxial surface and phloem cells toward the adaxial surface (fig. 9*e*, 9*f*).

No remarkable morphological distinction between adaxial and abaxial sides was found in terete leaves. Some leaves present peripheral chlorenchyma and inner water-storage tissue, as in *D. herbeum* (fig. 10*a*, 10*b*), *D. napiforme* (fig. 10*c*, 10*d*), and *Fenestraria rhopallophylla*. In other species, there is not a visible boundary between photosynthetic and water-storage tissues, as in *Lithops marmorata* (fig. 10*e*), *Lithops aucampiae*, *Gibbaeum velutinum* (fig. 10*f*), *G. petrense* and *Muiria hortenseae*.

Peripheral vascular bundles with endoscopic arrangement (figs. 10*b* and 10*e*-10*f*) and the central vascular bundle (midrib) with xylem cells orientated towards the adaxial surface and phloem cells orientated towards the abaxial surface (figs. 10*b*-10*d*) were observed in all species with terete leaves.

Discussion

Terete leaves of Ruschioideae have unifacial lamina and bifacial base

Terete leaves of Ruschioideae have been suggested to arise as an abaxialization process (Melo-de-Pinna *et al.*, 2014) and resemble the unifacial leaves of monocots (Melo-de-Pinna *et al.*, 2016). In the present work, we show anatomical evidence supporting the interpretation of unifaciality in leaves of Ruschioideae.

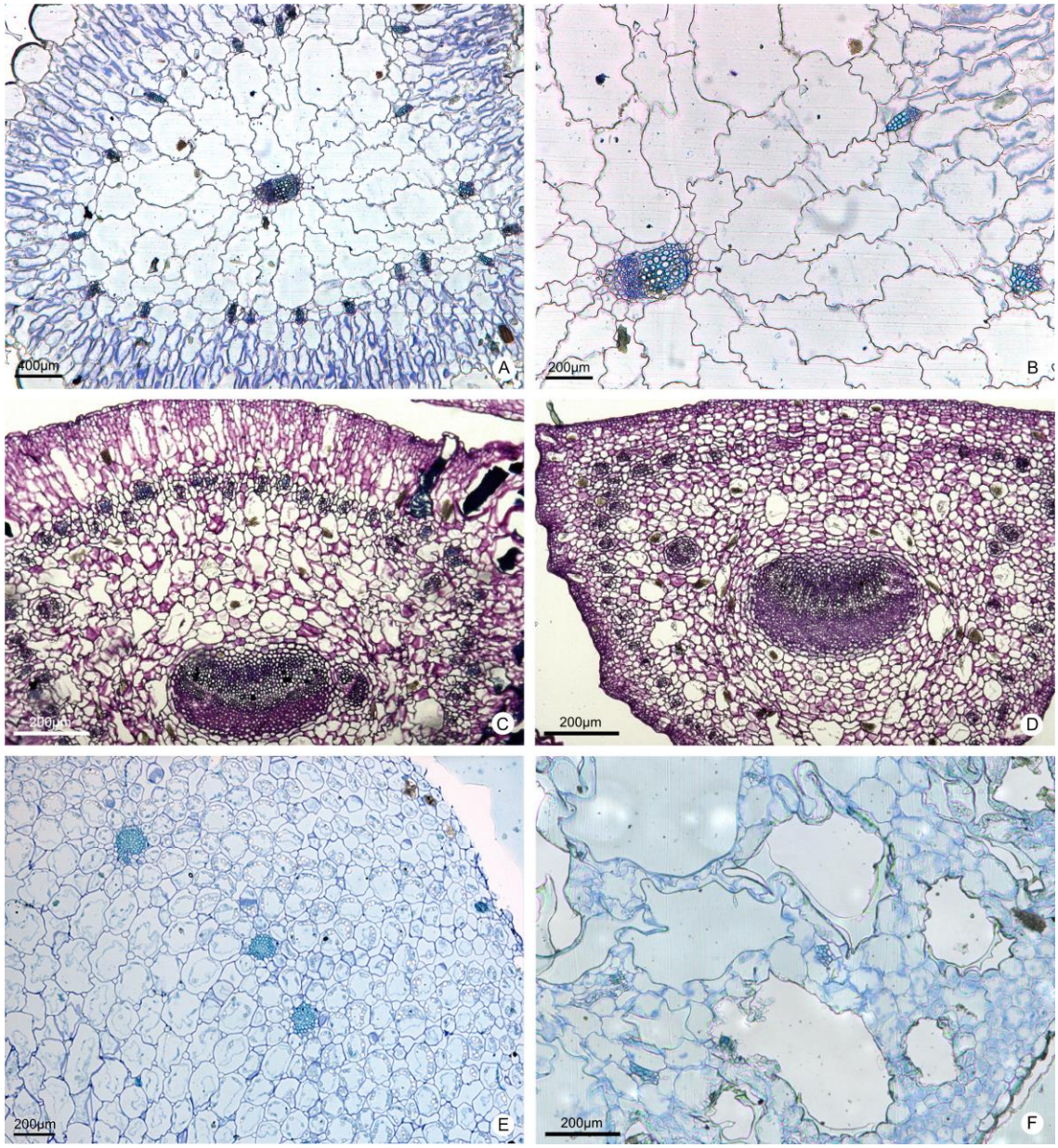

Fig 10 Distribution of the peripheral vascular bundles (PVB) in some species of Ruschioideae. *a* and *b*, Leaf blade of *Delosperma herbeum*, showing detail of endoscopic orientation of xylem (*b*). *c* and *d*, *Delosperma napiforme* showing medium and proximal region of the leaf blade, respectively. *e*, Abaxial region of the leaf blade of *Lithops marmorata*. *f*, Adaxial region of the leaf blade of *Gibbaeum velutinum*.

Leaf initiates as bifacial primordium and it is thought to retain the adaxial/abaxial domains throughout development, even though the adaxial side may become inconspicuous in species with unifacial leaves Hagemann (1970). The main difference between bifacial and unifacial leaves is that, in the first the adaxial and

abaxial sides both grow at similar rates, whereas in the latter the abaxial side grows wider and the adaxial side becomes narrower during leaf development (Hagemann, 1970). In *Senecio* spp. (Asteraceae), Timonin *et al.* (2006) and Ozerova and Timonin (2009) described as subunifacial leaves in which the unifacial region comprise 5% or less of the mature leaf length, and unifacial leaves those with a short bifacial region at their base. Melo-de-Pinna *et al.* (2016) highlights "the fact that considering all leaf parts, including the base, petiole (if present), and blade, leaves like those described above should be considered bifacial, even though the second case represents a leaf blade that has a unifacial condition for most of its length".

In what is classically understood as unifacial leaves of monocots, the bifacial leaf base usually grows around the perimeter of the shoot apex, forming the sheathing base (Kaplan, 1973). In Ruschioideae with unifacial leaves, we observe a similar growth pattern, especially in regard to the connation of opposite leaf bases. In *Mesembryanthemum digitatum* (Mesembryanthemoideae), which have alternate phyllotaxy, the sheathing leaf base may be a consequence of the same mechanism in a species with alternate phyllotaxy (Melo-de-Pinna *et al.*, 2014). According to authors, expanded leaf bases have evolved several times independently and the fused or sheathing base may be an advantage that enables protection of the shoot apical meristem.

Evidence for abaxialization in unifacial leaves

Differently from flat leaves in which a marginal blastozone is associated with the expansion of the leaf blade in only one plane (Esau, 1960; Fahn, 1990; Hagemann and Gleissberg, 1996; Dengler and Tsukaya, 2001), the terete leaves of Ruschioideae exhibit a peripheral blastozone. A very similar condition can be found in other families

of Caryophyllales, such as Cactaceae (Boke, 1944; Freeman, 1970; Gibson, 1977) and Portulacaceae (Hernandes-Lopes *et al.*, 2016), and seems to be one of the different developmental mechanisms responsible for generating succulent leaves with terete morphology (Melo-de-Pinna *et al.*, 2016).

In Ruschioideae species with terete leaves, the differentiation of PVB initiates at the abaxial side of the peripheral blastozone, giving rise to an endoscopic arrangement of vascular bundles. In the particular case of *Delosperma tradescantioides*, the leaf blade is flat and presents vascular bundles with the standard organization found in bifacial leaves, i.e., xylem and phloem oriented towards the adaxial and abaxial sides, respectively. Nevertheless, an interesting arrangement of endoscopic vascular bundles occurs in the midrib region. This conformation of vascular bundles is very similar to what is observed the species with unifacial leaf blade of the same genus.

Flat leaves are rare in Ruschioideae, since the majority of species possess terete or trigonous leaves. Interestingly, the presence of lateral bundles in the outer lamina of leaves of *D. tradescantioides* has been interpreted as a regain of planate venation in the group after the acquisition of endoscopic PVB (Melo-de-Pinna *et al.* 2014). Moreover, this species may represent a transitional form between the bifacial and unifacial development patterns in the group.

In some unifacial leaves the main vascular bundle is a component of the peripheral network, and assumes an abaxial position as result of extensive adaxial growth (Kaplan, 1973; Ozerova and Timonin, 2009; Melo-de-Pinna *et al.*, 2016). Differently, Ruschioideae leaves expand radially due to mitotic activity in the peripheral blastozone. Hence, the main vascular bundle remains at the center of the leaf as have been shown in other succulent groups (Hernandes-Lopes *et al.*, 2016; Melo-de-Pinna *et al.*, 2016). Additionally, vascular cells of the main bundle begin to differentiate

while the primordium is still bifacial, i.e., while adaxial-abaxial identities are still distinguishable. Along most of its length, mitotic activity persists in a subepidermal region throughout the entire leaf periphery, producing only tissues with abaxial-like identity. Conversely, at the leaf base the adaxial and abaxial regions remain distinct throughout development. This condition is clearly noticeable by the distribution of bladder cells and stomata in terete leaves, i.e., only in the abaxial side at the leaf base and surrounding the entire leaf at the blade sector.

The link between the presence of PVB and a process of abaxialization or adaxialization has been recently questioned (Platonova *et al.* 2016). Species of Aquifoliales can show either endoscopic or exoscopic PVB, and Platonova *et al.* (2016) propose a competition between developmental and functional factors directing PVB formation and arrangement.

The acquisition of adaxial/abaxial asymmetry has been the focus of extensive molecular studies in model plants. Mutants lacking either adaxial or abaxial identities may produce abaxialized or adaxialized leaves, which gain radial symmetry in many cases (Fukushima and Hasebe, 2014). In most unifacial leaves, the abaxial surface develops broadly while adaxial surface becomes so narrow that may be unrecognizable in the mature organ (Kaplan, 1973; Ozerova and Timonin, 2009). Thus, unifaciality may arise naturally by the disruption of either the abaxial or adaxial identities of the leaf during development, so that the entire surface is homologous to the adaxial or the abaxial side (Ogburn and Edwards, 2013; Yamaguchi *et al.*, 2010). Also, molecular biology studies provide evidence to support the idea that unifacial leaves are initially bifacial, and that leaf primordia latter undergoes an abaxialization process only in its distal sector, while proximal leaf sector preserves its bifacial condition (Yamaguchi *et al.*, 2010).

In the case of Ruschioideae, adaxial-abaxial polarity seems to be established at the primordia initial developmental phase and maintained during the central vascular bundle differentiation, which forms xylem positioned toward adaxial surface and phloem toward the abaxial direction. We suggest that this polarity is subsequently lost or decreased in the leaf distal sector, especially in regard to adaxial identity expression, resulting in the whole parenchyma periphery assuming abaxial identity. Although abaxialization events inhibit lateral outgrowth of lamina, the flat leaves of *Delosperma tradescantioides* have a small connate base region and presents PVBs with endoscopic bundles only in the midrib region, while the lateral bundles in the mesophyll have xylem oriented to the adaxial surface, characterizing a bifacial mesophyll. These configurations suggest that the adaxial-abaxial expression in leaf primordia of this group provides some variations on the main theme of establishment of adaxial identity. A duplication of *ARP* (*ASYMMETRIC LEAVES/ROUGH SHEAT/PHANTASTICA*, a transcription factor related to adaxial identity, see Machida *et al.*, 2015), occurred during the divergence between Mesembryanthemoideae and Ruschioideae (Illing *et al.*, 2009). The acquisition and maintenance of the two copies of *ARP* in the genome may have contributed for the rapid morphological diversity in Aizoaceae (Illing *et al.*, 2009).

Concluding, anatomical data as occurrence of bladder-cells and peripheral vascular bundles should be utilized to characterization of unifacial condition of leaf blade in Ruschioideae, and the endoscopic bundles differentiated from abaxial side, suggest an abaxialization process of leaf blade development. Our results demonstrate that Aizoaceae is an interesting group to study morphological variation and its relation with molecular mechanism of adaxial-abaxial polarity. Further studies, particularly with a molecular approach, will help to elucidate the adaxial/abaxial mechanisms underlying the leaf morphogenesis of such a diverse group of plants.

Acknowledgements

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CAPÍTULO 2

Expression of *ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA* during leaf ontogenesis in Aizoaceae

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Abstract

One of the important questions in biology is how morphological novelty originated during evolution. Although the flat shape of typical leaves is efficient for their photosynthesis function, succulent plants have also evolved leaves with radial symmetry with abaxial cell types only. Interested in the role of the recent duplicated *ARP* gene in Aizoaceae, we investigate the possible correlation between the *ARP* duplication event and the emergence of a new abaxialized leaf morphology in Aizoaceae. In this work we describe the ontogenesis of bifacial flat leaf and unifacial terete leaf blade in two species of Aizoaceae, as well as the expression pattern of *ARP* orthologs during leaf development by *in situ* hybridization essays. To further determine whether expression of Aizoaceae *ARP* could complement the *as1* phenotype we generated transgenic *Arabidopsis as1* plants expressing Aizoaceae *ARP* orthologues under the 35S overexpression promotor. We found that *ARP* is expressed in the entire developing leaf, suggesting that in Aizoaceae, this gene is mainly involved in the determinacy of leaf cell fate. Addicionally, expression of the Aizoaceae *ARP* genes is able to fully complement the *Arabidopsis as1* mutation, indicating a high degree of functional conservation, even in leaves with such a distinct morphology. Our results corroborate that important processes are maintained throughout the evolution of the groups, and that more studies with non-model plants can bring light and explain how such extraordinary forms develop in nature. How often the abaxialized leaf morphology arose in Aizoaceae is still uncertain. Further studies will help to elucidate the role of duplication in Aizoaceae diversification.

Introduction

Popularly known as ice plants and flowering stones, the Aizoaceae (Caryophyllales) comprises succulent leaved species with center of distribution in the specially rich arid biome referred as Succulent Karoo, located at the winter rainfall region of Southern Africa. In Aizoaceae the succulence is most highly developed in the two subfamilies Mesembryanthemoideae and Ruschioideae in which leaf shapes show a very interesting morphological diversity (Melo-de-Pinna *et al.* 2014, Klak *et al.* 2003).

Ruschioideae is the largest (with 1,585 species in 111 genera) of four subfamilies currently recognized in Aizoaceae (Klak *et al.* 2003). Even if compared with other entire families of succulent plants, the range of life forms in Ruschioideae is unrivaled: true annuals (*Dorotheanthus*); short-lived perennials (*Mesembryanthemum*); shrubs and small trees up to 3 m high (*Ruschia*); highly succulent compact forms (*Conophytum*, *Lithops*); forms sunken in the ground ("window plants," *Fenestraria* spp.); and some not rarely extremely miniaturized (*Oophytum*, *Diplosoma*, *Conophytum*) (Ihlenfeldt 1994, Klak *et al.* 2003, 2004). The species include a wide range of leaf forms - since dorsiventral flat leaves until tiny forms with unifacial terete leaves (Melo-de-Pinna *et al.* 2014, 2016).

Although flat dorsiventral leaves are common in species of the early diverging lineages of the Aizoaceae, they are rarely found in the Ruschioideae (Klak *et al.* 2004, Melo-de-Pinna *et al.* 2014). Most Ruschioideae species exhibit chlorenchyma on both surfaces encircling the specialized water-storage tissue, and vascular system is composed of a midrib with a collateral vascular bundle and smaller vascular bundles disposed all around the periphery. Species with terete to trigonous leaves and expanded leaf base, show peripheral vascular bundles (PVB) with xylem cells orientated towards the center and the phloem cells towards the outer surface (endoscopic pattern), and the

midrib with xylem cells orientated towards the adaxial surface (Melo-de-Pinna *et al.* 2014, 2016). In this group terete abaxialized leaves are more frequent, composing its most diverse clade, the subfamily Ruschioideae.

Diverse leaf morphologies allow species to adapt to their environments. This variation in design results of adjustments in several developmental regulatory pathways which may influence in cell division and expansion patterns, leading to a large range of leaf morphologies. Leaves are made continuously at the periphery of the shoot apical meristem (SAM), and leaf morphology is established along three axes: proximal-distal, adaxial-abaxial and medial-lateral (Machida *et al.* 2013). In flat leaves the establishment of adaxial-abaxial patterning is critical early in leaf development. After the establishment of adaxial–abaxial polarity, the primordium develops into a flat structure partly as a result of active cell division at the marginal region, where the adaxial and abaxial domains are juxtaposed (Waites & Hudson 1995). Interaction between the adaxial and abaxial domains is necessary for the onset of the directed growth that forms leaf blades. Although the mechanism by which directed growth activity is initiated remains largely unknown, analyses of *YABBY* gene family have provided evidence linking adaxial–abaxial polarity and the growth activity (revised by Fukushima & Hasebe 2014).

Many regulators that specify such patterning have been identified. One pathway involved in reprogramming gene expression during leaf development involves the *Arabidopsis thaliana ASYMMETRIC LEAVES1 (AS1*) (Byrne *et al.* 2000), *Zea mays ROUGH SHEATH2 (RS2*) (Timmermans *et al.* 1999), and the *Antirrhinum majus PHANTASTICA (PHAN*) ortholog genes (Waites & Hudson 1995), collectively cited as *ARP*. These orthologs are members of a MYB-related gene family that are required for

repressing expression of certain *KNOX (KNOTTED1*-like homeobox) genes, thus determining the leaf identity (Engstrom *et al.* 2004).

Additionally, *ARP* genes are related with determination of adaxial leaf identity. In *Antirrinum majus phan* mutants leaves fail in develop adaxial cells, resulting in terete abaxialized organs (Waites & Hudson 1995); controversially, *as1 Arabidopsis* mutants don't show obvious signs of any abaxialization process. Although *ARP* genes are conserved, the consequences of its regulation may not be the same.

PHANTASTICA (*PHAN*), which encodes a transcription factor of MYB domain family and was first cloned from *Antirrhinum majus* was the first gene recognized to function in the control leaf adaxial-abaxial polarity (Waites and Hudson 1995). *phan* mutants display a range of phenotypes with weak leaf polarity, including plants with leaves which develops ectopic blade outgrowths sectors where cells have lost adaxial fate and instead have taken on abaxial identity. Some *phan* mutant leaves may completely lack flattened lamina. Leaves may develop with radial symmetry with abaxial cell types only. Together, these phenotypes demonstrate that *PHAN* is necessary to specify adaxial fate in *Antirrhinum majus*, and support the idea that extension of the leaf blade results from the juxtaposition of adaxial and abaxial tissues (Waites and Hudson 1995), a conclusion broadly corroborated in later research.

The role of *ARP* in adaxial–abaxial patterning is conserved in tobacco, tomato, and several other species (Kim *et al.* 2003; McHale & Koning 2004; Hay & Tsiantis 2006). Interestingly, mutations in *Zea mays ROUGH SHEATH2 (RS2)* and *Arabidopsis ASYMMETRIC LEAVES1 (AS1)*, cause no obvious polarity defects (Timmermans *et al.* 1999; Tsiantis *et al.* 1999; Byrne *et al.* 2000). *AS1* and *RS2* interact with *AS2*, a LOB domain transcription factor that localizes to the adaxial-most cell layers of young leaf primordia (Xu *et al.* 2003; Iwakawa *et al.* 2007). Although organ polarity is not

obviously perturbed in *as1 as2* double mutants, *Arabidopsis thaliana* plants that overexpress *AS2* develop an adaxialized leaf phenotype (Lin *et al.* 2003). *AS1, RS2,* and *PHAN* (*ARP*) are expressed uniformly throughout developing primordia, suggesting that any contributions to adaxial–abaxial patterning are regulated by interacting protein partners (Husbands *et al.* 2009).

Studies have indicating a high degree of functional conservation between *ARP* orthologs in other plants consistent with their sequence homology. Additionally, the expression of the *Zea mays RS2* gene is able to fully complement the *Arabidopsis as1* mutation, recovering the wild phenotype (Theodoris *et al.* 2003). These data suggest that some developmental pathways in leaf development are functionally interchangeable between monocots and dicots.

The flat leaf morphology results from precise coordination of cell division and differentiation, such that even slight perturbations in adaxial-abaxial polarity lead to leaf curling and other morphological changes, as cylindrical forms (Husbands *et al.*, 2009, 2015).

Over recent decades, advances in molecular approaches have allowed us to unravel some of the mechanisms of plant development, and significant progress has been made on understanding adaxial-abaxial patterning in model plants. Much less is known on genetic control of leaf development in other plant groups, but comparative studies based on *Arabidopsis*, rice and other species help us to understand plant development in a broad perspective. In this context, analysis of different species will also provide an understanding of how these mechanisms influences phenotype diversity and environmental plasticity.

In this work we investigate the ontogenesis of bifacial flat leaf and unifacial terete leaf blade in Aizoaceae, as well as the expression pattern of *ARP* orthologs during

leaf development. According to Illing *et al.* (2009) a duplication event of *ARP* gene may has contributed to the impressive species radiation in Aizoaceae, a diversification that is supposed to be occurred very rapidly in the group (Klak *et al.* 2004). The argument is sustained by the fact that *ARP* codificates a transcription factor which is involved in leaf architecture building and this may trigger important diversification events (Rensing 2014, Zhang 2003). Also, according to Illing *et al.* (2009), the duplication event coincides with the diversification of Ruschioideae subfamily, the more representative in Aizoaceae, both in number of species and morphological diversity (Illing *et al.* 2009).

Interested in the role of the recent duplicated *ARP* gene in Aizoaceae, we are seeking for more information about the possible correlation between the *ARP* duplication event and the emergence of a new abaxialized leaf morphology in the group.

Material and methods

Plants of *Mesembryanthemum crystallinum* L. and *Delosperma napiforme* Schwantes were cultivated in greenhouse. *Arabidopsis thaliana* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants (wild type Col-0 and *as1* CS3374) were growth under controlled conditions (22°C, 16h light).

RNA isolation and cDNA synthesis

Total RNA of shoot apex, adult leaves and root was extracted using Trizol reagent and treated with TURBO DNA-free kit (Ambion) to remove contaminant DNA. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT). Orthologs of *ARP* were amplified using degenerate primers (see supplementary material) and Phusion High Fidelity polymerase (Thermo

Scientific) under appropriate PCR conditions. The sequence of the 3′-end of the gene in *Delosperma napiforme* was determined with the 3′-RACE system for amplification of cDNA ends (Invitrogen). The amplified products were magnetically purified (Agencourt AMPure XP - Beckman Coulter), cloned into the pJET cloning vector (Thermo Fisher Scientific) and sequenced. Sequences of the *AS1* orthologs in *M. crystallinum* (*McARP*) and *Delosperma napiforme* (*DnARPa* and *DnARPb*) will be deposited in GenBank, and are shown in the supplementary material (S1).

Phylogenetic analysis and assessment of genes homology

We did a translation based alignment using our three cloned coding sequences and the ones available in Illing *et al.* 2009 (following their nomenclature and excluding pseudogenes), plus maize *ROUGH SHEATH2* (*RS2*, accession number AF126489.1) using Geneious 10.2.3 (Kearse *et al.*, 2012). The alignment was trimmed based on the shortest sequence and we made it available as a supplementary material (S2). To define partitions and evolutionary models, we used PartitionFinder 2.1.0 (Lanfear *et al.* 2016). The best scheme was two partitions, one for the 1st+2nd codon position and other for the 3rd position, using the model GTR + GAMMA for both partitions. A maximum likelihood tree with 500 bootstrap replications was produced with RAxML 8 (Stamakis 2014).

Serial section preparation and staining

Shoot apices of soil- grown plants were fixed overnight in 4% paraformaldehyde (pH 7.2). The fixative solution was sequentially replaced with a series of water–ethanol, ethanol–xylene and xylene–Paraplast Plus (Sigma-Aldrich), to prepare paraffin-embedded samples. Serial sections were made using a rotary

microtome and were affixed to glass slides overnight at 42°C. Next, 10µm-thick serial sections were rehydrated through xylene–ethanol and ethanol–water series, and stained with 0.1% toluidine blue in 0.1M phosphate buffer (pH 7.0). The preparations were mounted in Entellan New (Merck Millipore). Section images were taken using a digital camera coupled to a microscope.

RNA *in situ* hybridization

Probes for *in situ hybridization* were design based on the 760bp sequences (Illing *et al.*, 2009). Cloned gene fragments were amplified and Digoxigenin (DIG)-labelled antisense RNA probes were prepared using the DIG RNA Labeling Kit (Roche Applied Science). Next, 10µm-thick serial sections were rehydrated, treated with 0.5 mg/ml Proteinase K for 30 min at 37°C, re-fixed in 4% paraformaldehyde (pH 7.2) for 10 min, and then dehydrated in a water–ethanol series. RNA probe hybridization was performed overnight in a humid chamber at 50°C. After the samples were washed twice with 4X SSC buffer at 50° C for 20 min, the slides were treated with 50 mg/ml RNase A at 37° C for 60 min, washed twice in 0.5X SSC at 50° C for 20 min and then blocked with Blocking Reagent (Roche Applied Science). Signals were detected by incubating the samples in Anti-DIG-AP (Roche Applied Science) for 90 min and NBT/BCIP solution (Roche Applied Science) overnight. After brief dehydration in water–ethanol and ethanol–xylene series, the preparations were mounted in Entellan New (Merck Millipore). Section images were taken using a digital camera coupled to a microscope.

Phenotype rescue experiment

Agrobacterium tumefaciens – mediated transformation is the most widely used protocol for producing transgenic *Arabidopsis* plants. In this method, transformation of

female gametes is accomplished by dipping developing *Arabidopsis thaliana* inflorescences into a 5% sucrose solution containing *Agrobacterium* cells carrying constructions of the genes to be transferred. Treated plants are allowed to set seeds which are then plated on a selective medium to screen for transformants.

For gene construction we used the Gateway System (Thermo Fisher Scientific). Primers containing attB sequences were designed for BP reaction and recombined with pDONR 201 entry vector (Thermo Fisher Scientific). Competent cells (*Escherichia coli* DH10) were transformed by electroporation and plated in selective medium (kanamicin) generating entry clones for *AS1* (*Arabidopsis*, positive control), *Mesembryanthemum crystallinum ARP* (*McARP*) and *Delosperma napiforme ARPa* (*DnARPa*). Colony PCR was performed to confirm the presence of insert in all steps of Gateway protocol. A second recombination reaction (LR) between each entry clone and the destination vector pH7WG2 (containing 35S overexpression sequence cassette) generated the expression clones.

Results

Morphology and anatomy of adult leaves

Mesembryanthemum crystallinum (Figure 1A) has opposite leaves with lamina laterally expanded and is not connate at leaf base. The epidermis is uniseriate, with large globular and translucent cells (bladder-cells) distributed on both sides of the leaf (Figure 1B). The mesophyll is homogeneous, composed of parenchyma that accumulates the functions of photosynthesis and reserve, of the type “all cells succulent”. The midrib has a collateral vascular bundle and in the mesophyll there are

marginal vascular bundles, all with xylem facing the adaxial surface of the leaf and phloem facing the abaxial surface.

Delosperma napiforme exhibits leaves of trigonous morphology and connate leaf bases (Figure 2C). This species exhibits uniseriate epidermis, with a higher degree of succulence and reduced lateral leaf blade expansion in relation to *M. crystallinum*. In *D. napiforme* the delimitation between the fundamental tissues types is more evident, with chlorenchyma arranged in several layers of palisade cells surrounding water-storing parenchyma in the central region (Figure 1D).

Small collateral vascular bundles are seen along the interface between the two fundamental tissues, which exhibit endoscopic xylem (Figures 1D-F). The peripheral bundles gradually become concentrated on the flanks of the adaxial face on the proximal region of the leaf (Figure 1E). At the base of the leaves, the peripheral vascular bundles occur only in the abaxial region and exhibit adaxially positioned xylem. Figure 1F is a detail of the adaxial region of the leaf, and shows PVB endoscopic orientation, i.e. with xylem pointing to the center of the leaf and phloem to the outer surface.

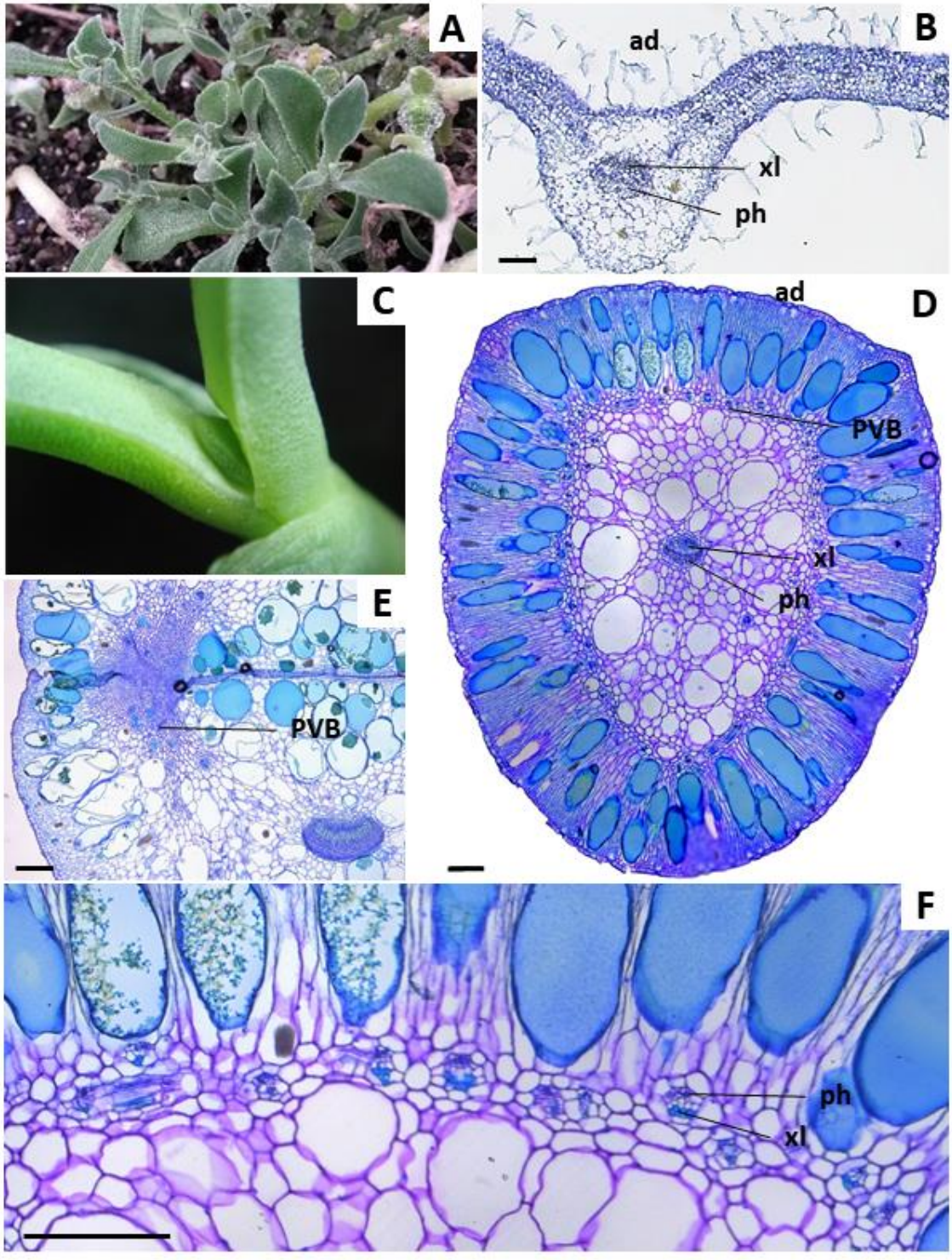

Figure 1. Morphology and anatomy of Aizoaceae leaves. A-B – *Mesembryanthemum crystallinum* L. Morphology (A) and transverse section of leaf blade (B). C, E– *Delosperma napiforme* Schwantes. Morphology (C) and transverse section of leaf blade (D). F – detail of endoscopic peripheral vascular bundles in adaxial region of *D. napiforme* leaf. Ad – adaxial surface; xl – xylem; ph – phloem; PVB – peripheral vascular bundles. Bars = 200µm

Leaf development

After the apical growth of the primordium, the cells of the adaxial face maintain their activity longer, in relation to the cells of the abaxial face of the primordium, which appear more vacuolated (Figures 2A-B). A meristematic region located on two flanking sides of the young leaf primordium at the boundary between the adaxial and abaxial domains was observed in the two leaf primordia (Figure 2C). Continuous activity in this region gives rise to lateral vascular bundles and parenchyma cells, thus promoting the center-lateral growth of the leaf blade in *M. crystallium*. In *D. napiforme* adaxial subepidermal cells divide periclinally to give rise to parenchyma cells, thus contributing to adaxial-abaxial growth (Figure 2C). Also, differentiation of peripheral endoscopic vascular bundles occurs in both sides of the leaf, along the periphery (Figure 2D). At a later stage of development, the adaxial blastozone (growth region responsible for the increase in volume of the leaf primordium) has a marked activity in the proximal portion of the lamina in formation.

Mitotic activity in adaxial portion of primordium results in accentuated growth towards the apical meristem in *D. napiforme*, approaching the epidermis on the adaxial faces of the opposite primordia (Figure 2C). Such prominent growth is not observed in *M. crystallinum* primordia (Figure 2E).

At a posterior stage of development, subepidermal cells with meristematic activity are maintained throughout the margin of the developing leaf. These cells constitute the marginal blastozone, a region of growth that originates all tissues of the leaf margin, by periclinal divisions and expansion of the cells (Figure 2D). In this growing region, procambial strands are formed which will give rise to the peripheral

bundles (Figure 2D), whose differentiation is posterior to the formation of the xylem and phloem cells of the central bundle (Figure 2B).

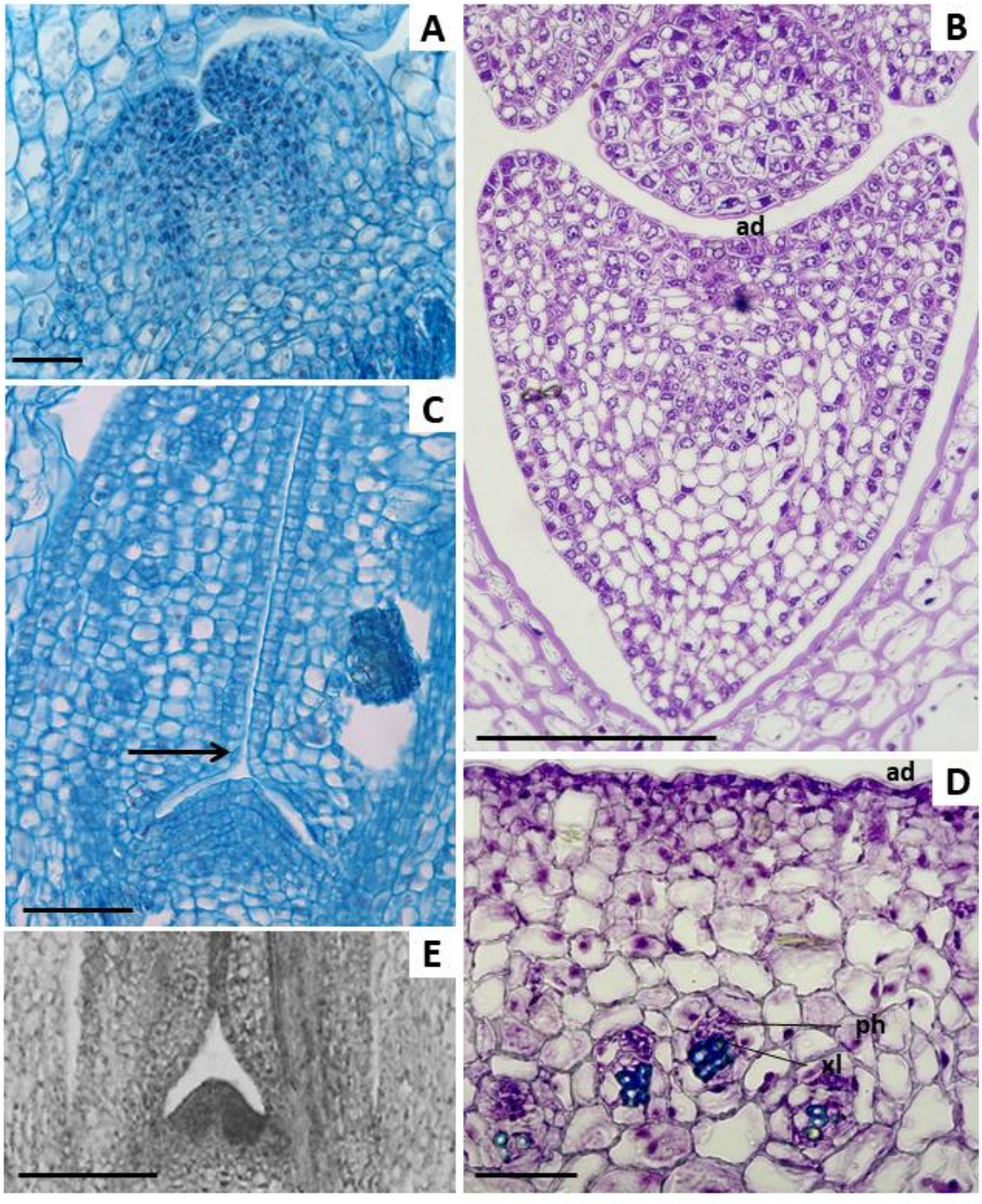

Figure 2. Leaf development in Aizoaceae. A – Longitudinal section of shoot apex in *Delosperma napiforme*. B – Transverse section of shoot apex in *D. napiforme*, showing adaxial blastozone in adaxial region of the leaf primordium. C – Longitudinal section of shoot apex in *D. napiforme*, showing adaxial growth (arrow). D – Marginal blastozone with differentiation of vascular bundles and parenchyma tissue in *D. napiforme*. E – Longitudinal section of shoot apex in *Mesembryanthemum crystallinum*. ad – leaf adaxial surface, ph – phloem; xl – xylem; Bars= 50µm

Expression of *ARP* in *Delosperma napiforme* and *Mesembryanthemum crystallinum*

First, we assessed expression of the *D. napiforme* and *M. crystallinum ARP* orthologs by PCR. Therefore, we amplified a known 760bp genomic sequence using cDNA as template. We observed that *ARP* is expressed in different organs of *Delosperma napiforme* and *Mesembryanthemum crystallinum*. Figure 2 shows agarose gel electrophoresis (0.8%) containing PCR products, where it is possible to identify single bands for each reaction, related to the amplification of the *ARP* gene fragments. We used cDNA rather than genomic DNA as template for PCR and we found evidence that both the *DnARPa* (Figure 3A) and *DnARPb* (Figure 3B) paralogs are expressed in *Delosperma napiforme*.

To further verify the tissue-specific *ARP* expression, we performed *in situ* hybridization essays. We observe a gradient of *ARP* expression in the leaves, from the apex to the base. In transverse sections we observe a strong hybridization signal in the most distal sections (Figures 3A-B), being slightly less intense in the median region (Figures 3C-D) and apparently absent in the leaf base and in the apical meristem (Figures 3E-F). The longitudinal sections tests confirm the gradation, with a stronger hybridization signal obtained at the apical region of the leaves in comparison to the base region (Figure 3G). Figure 3H shows the negative control.

A similar pattern is observed in *Mesembryanthemum crystallinum*, in which it is possible to observe the strong hybridization signal in transverse section on median portion of the young leaves (Figure 4A), as well as in longitudinal section (Figure 4B) all along the organs, but clearly wicker at the leaf base. Figure 4C shows a transverse section at the boundary between the leaf bases and the stem, where it is possible to

observe the lack of hybridization signal in a region immediately under the SAM. Figure 4D is the negative control.

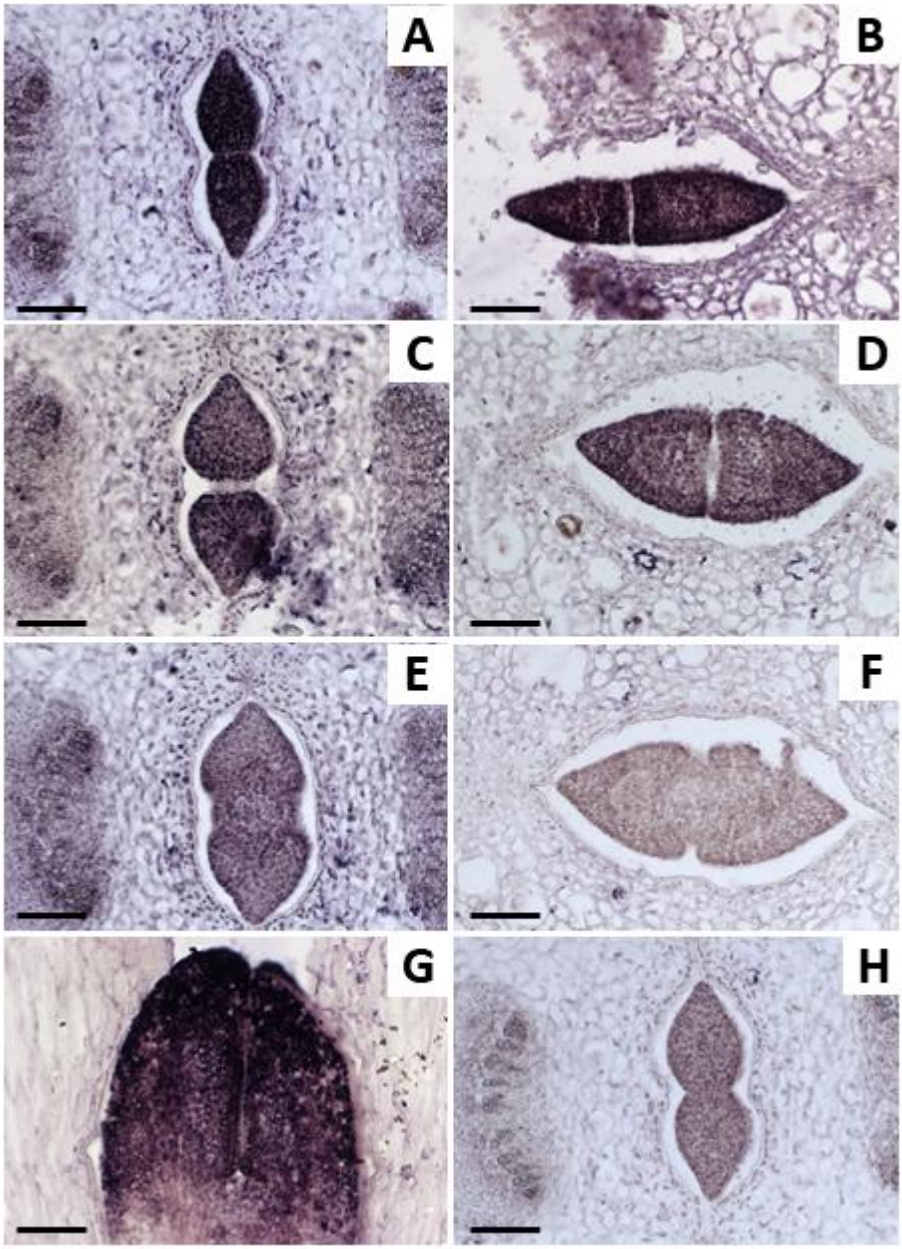

Figure 3. Result of in *situ* hybridization of *DnARPa* and *DnARP*b with anti-Digoxigenin probe in the vegetative shoot apex of *Delosperma napiforme* (Aizoaceae). A – transverse section of distal portion of the young leaves *DnARPa*; B - transverse section of distal portion of the young leaves *DnARPb*; C – transverse section of proximal region of young leaves and shoot apex meristem (SAM) region *DnARPa*; D – transverse section of proximal region of young leaves and SAM region *DnARPb*; E – transverse section of leaf bases and SAM *DnARPa*; F – transverse section of leaf bases

and SAM *DnARPb*; G – longitudinal section of shoot apex *DnARPa*; B – transverse section negative control.

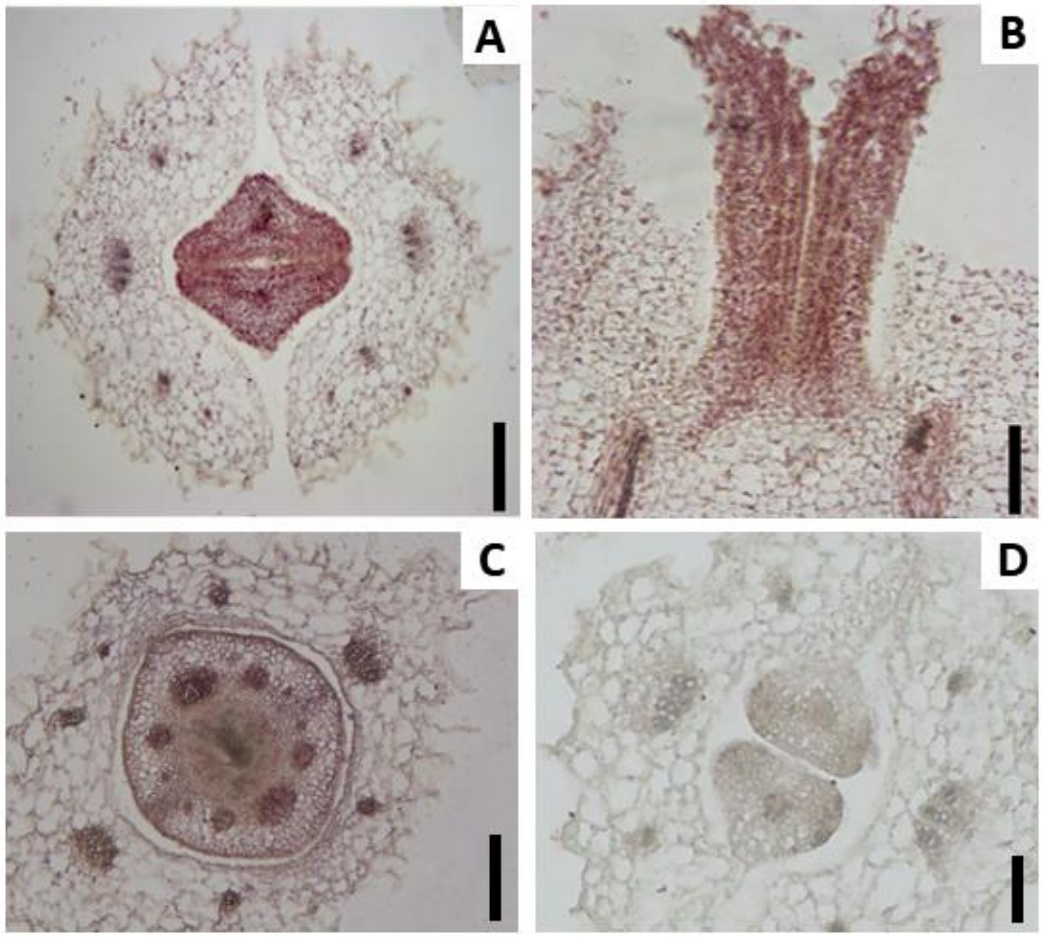

Figure 4. Result of *in situ* hybridization with ARP using anti-Digoxigenin in vegetative shoot apex of *Mesembryanthemum crystallinum* (Aizoaceae). A – Transverse section in median region of the leaf primordium; B – Longitudinal section of the same region of figure A.; C – Transverse section of the shoot apical meristem; D – Negative control. Bars = 200µm

Characterization of the cloned fragments

To further characterize the *ARP* orthologs in both species, we proceeded to clone the whole open reading frame (ORF) of *McARP*, *DnARPa* and *DnARPb*. The 5' portion of the ORF has been shown conserved among *ARP* orthologs, so it was possible to design a degenerate primer for amplification beginning at the start codon (ATG). The use of the 3'RACE kit allowed the PCR amplification of the 3'-terminal portion of *McARP* and one of the existing copies in *D. napiforme* (*DnARPa*). We were not able to

amplify the 3' region of *DnARPb*. Sequences of each gene can be found in supplementary material (S1).

We proceeded to predict the aminoacid sequences encoded by the obtained ORFs. *McARP*, *DnARPa* and *DnARPb* share 72%, 70% and 72% aminoacid identity, respectively, to the Arabidopsis AS1 protein. The putative encoded proteins show a high conservation degree in the MYB domain, as well as in the putative DNA binding motif (Figure 5).

In order to verify the homology of the cloned ORFs and the known *ARP* orthologs, we generated a phylogenetic tree based on the putative aminoacid sequences encoded by the obtained ORFs of *D. napiforme* and *M. crystallinum*, and the Aizoaceae *ARP* partial sequences available in GeneBank (Figure 6). As expected, McARP was grouped with other *Mesembryanthemum* ARP proteins in the maximum likelihood tree, whereas DnARPa and DnARPb were clsutered among the ARPa and ARPb clades respectively, thus suporting the homology between the cloned sequences and the sequences already known for different species of Aizoaceae.

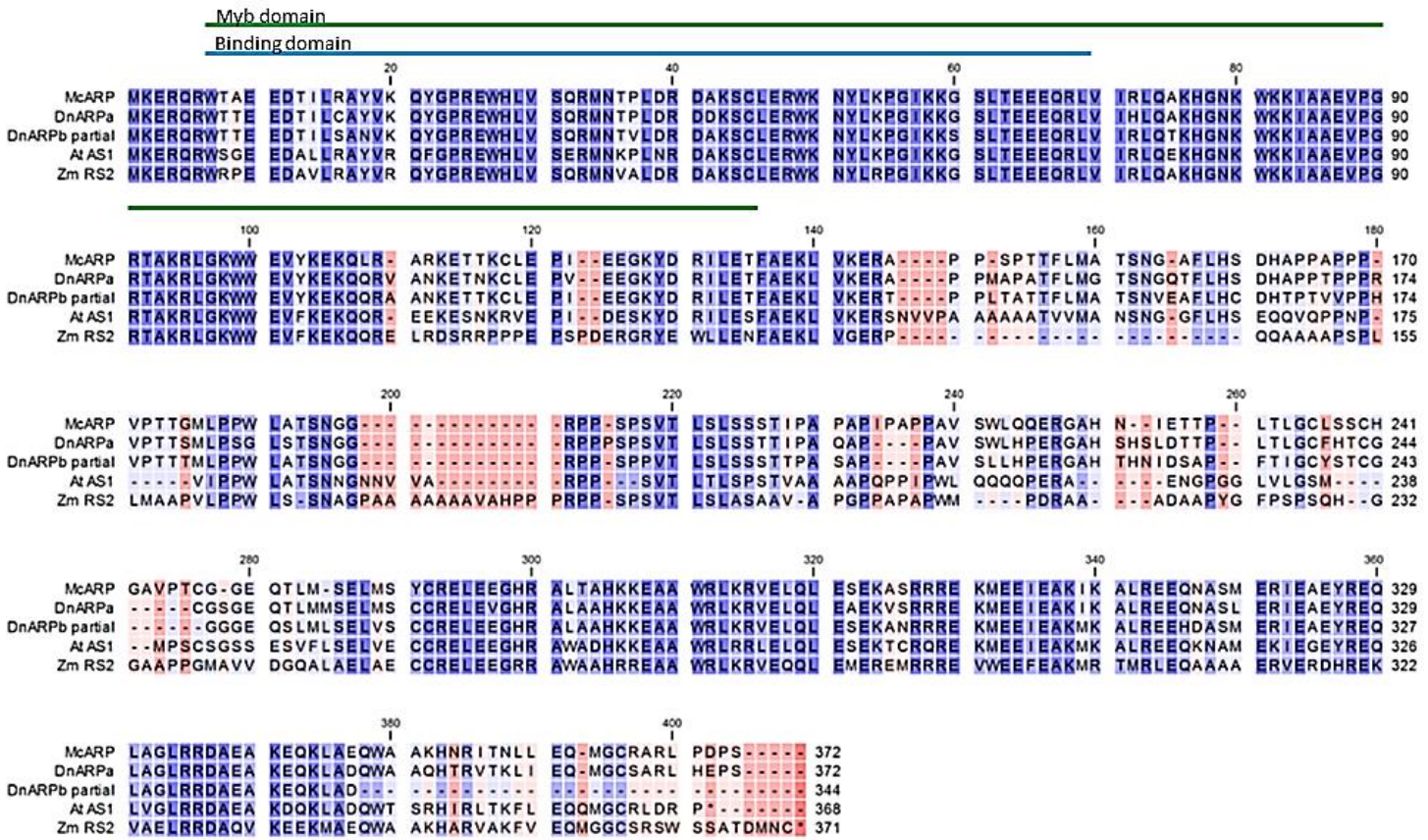

Figure 5. Alignment of the putative protein sequences of Arabidopsis AS1, maize RS2, *McARP*, *DnARPa* and the partial sequence of *DnARPb*. Color indicate conservation degree from high (blue) to low (red).

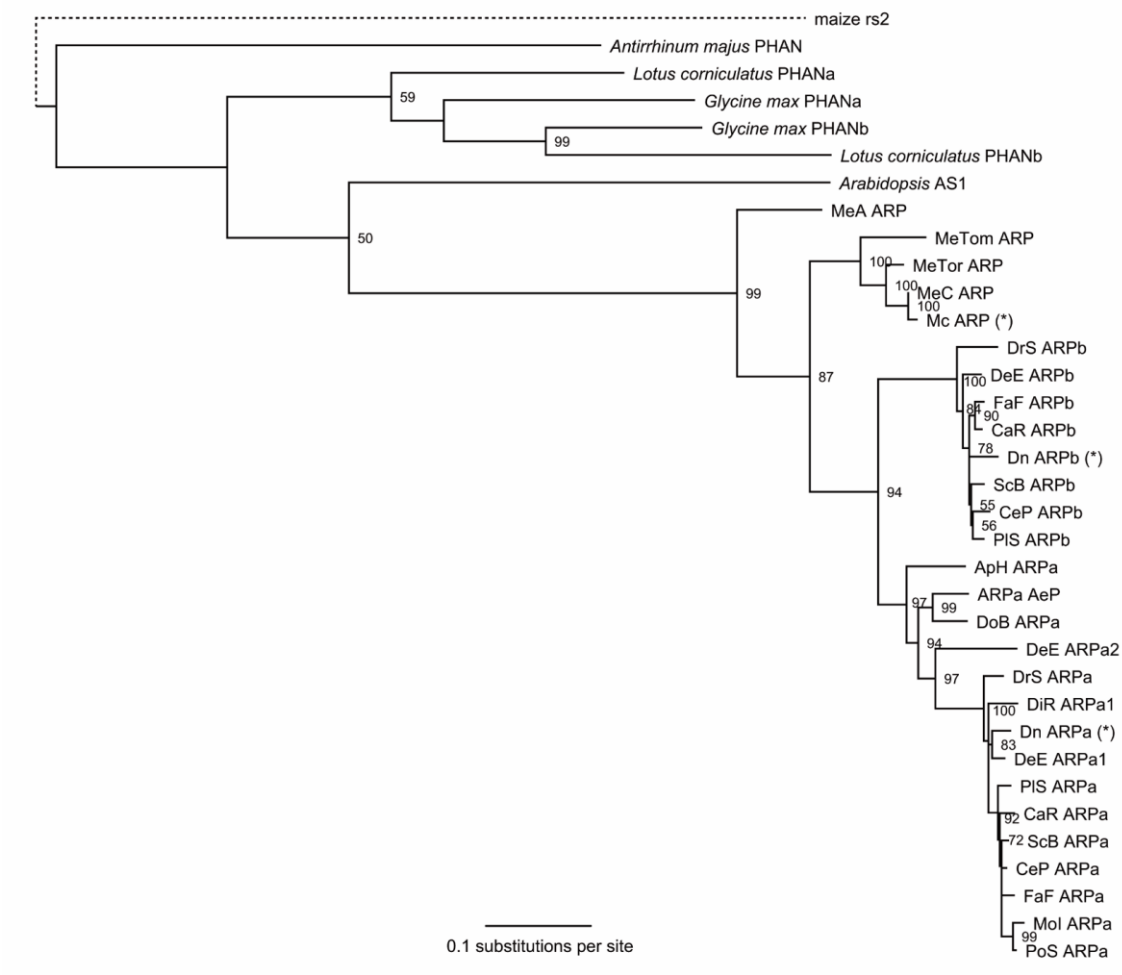

Figure 6. Maximum likelihood tree of ARP protein sequencesgenes focused on Aizoaceae, with genes herein studied marked with asterisks. Bootstrap percentage values below 50 are not shown. Maize rs2 is a long branch (lenght=1,4275) that is shown shortened in the phylogram.

Cloning with Gateway system

With the complete sequences, we designed ATTB primers for Aizoaceae species, according to Gateway protocol. For positive control, ATTB primers were also designed for *Arabidopsis thaliana AS1*, based on the *AS1* gene sequence of the Columbia-0 strain, available from Genebank (accession number NM_129319). Each primer is approximately 50pb (supplementary material S3). The PCR products obtained

were presented as a single band on the electrophoresis gel, for the three species (Figures 7). Miniprep result is shown in Figure 8 and Figure 9 shows the result of the PCR of colonies performed after the cloning procedure, with positive result for six colonies selected from *M. crystallinum* and 33 from *D. napiforme* (all of which generated positive products in the colonies PCR). Apparently, the primer designed for the 3'-terminal region is either inefficient to amplify the *DnARPb*, or this copy is very low expressed, since all 33 sequenced samples of *D. napiforme* contained the *DnARPa* sequence.

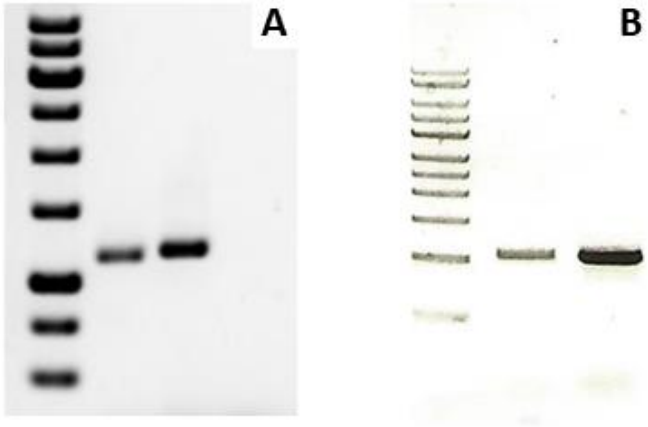

Figure 7. (0.8%) agarose gel electrophoresis of ATTB Gateway PCR products. A - *Mesembryanthemum crystallinum* (left) and *Delosperma napiforme* (right). B - *Arabidopsis thaliana*: mutant as1 CS3374 (left) and Wild-type Col-0 (right). Approximate product size = 1300 bp.

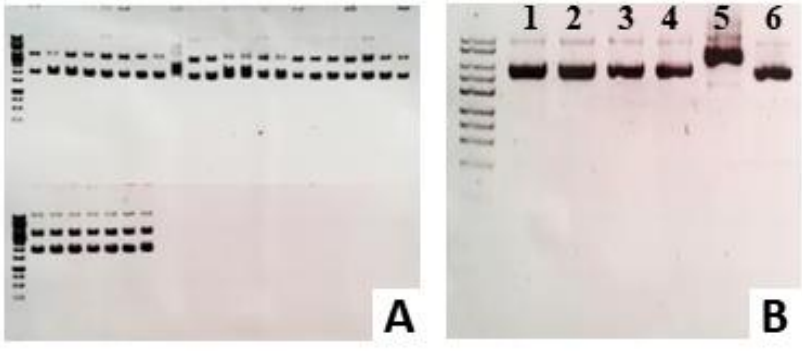

Figure 8. (0.8%) agarose gel electrophoresis of miniprep products from the Gateway System constructs. A - upper row: Construction p-DONR 201 and *DnARPa*; Bottom row: p-DONR 201 and *McARP*. B - Construction pH7WG2 and *DnARPa* (wells 1, 2 and 3); and pH7WG2 and *McARP* (wells 4,5 and 6).

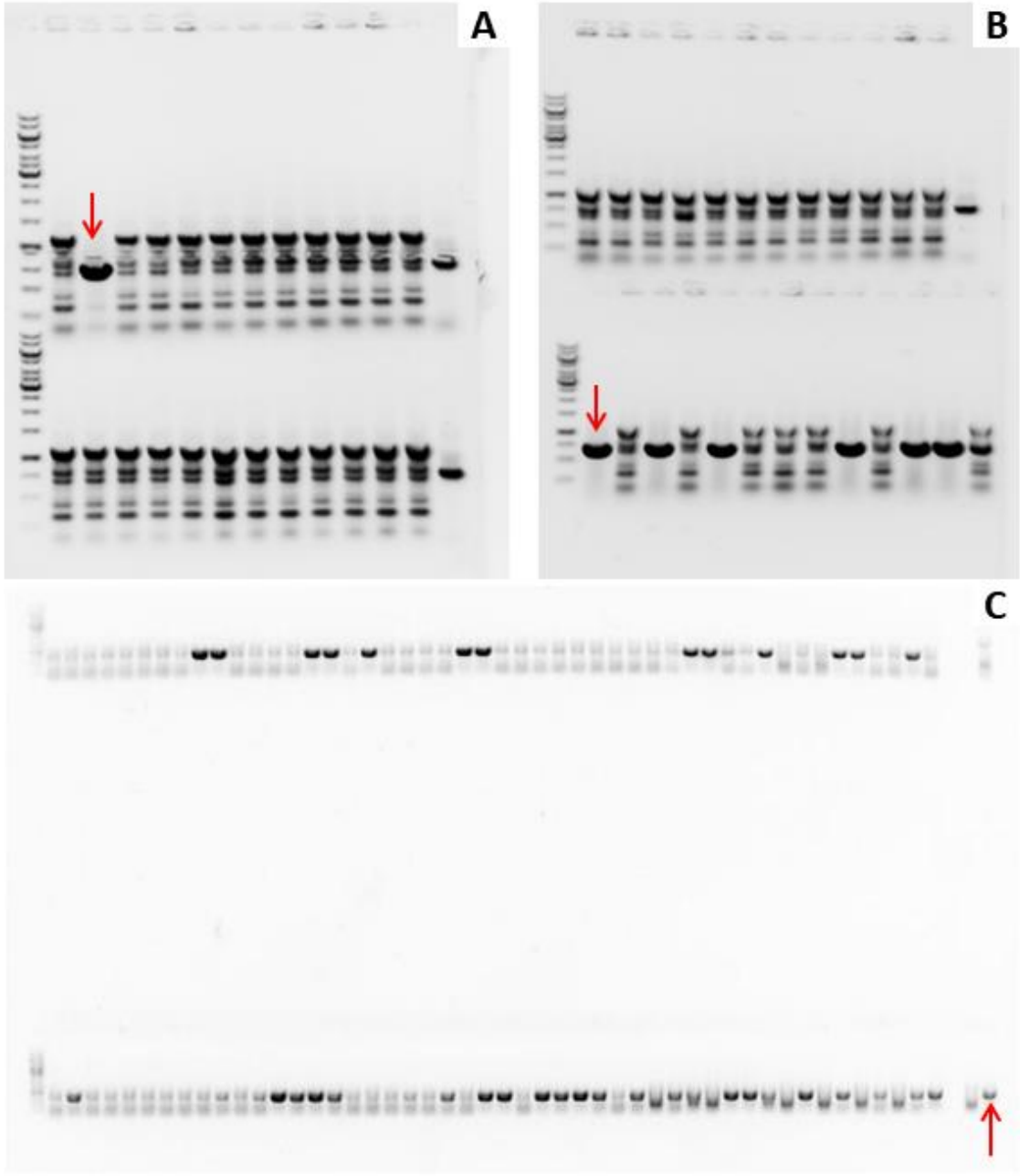

Figure 9. PCR of colonies. Transformation of Escherichia coli with pDONR 201 vector containing *McARP* and *DnARPa*. A - Positive result (arrow) for *DnARPa*; B - six positive for McARP. C - 33 positive results for *DnARPa*. The last wells of each gel contain the positive control. Positive aprox. = 1kb.

Escherichia coli transformation with the construction in the pDONR201 backbone was highly efficient, with a positive result for all samples. Transformation with the expression vector pH7WG2 was also effective. Figure 4 shows the miniprep result in agarose gel electrophoresis for recovery of the constructs. Figure 5 shows solid

medium plates containing positive clones resulting from the constructs in *Agrobacterium tumefasciens*. The positive result of the PCR of colonies confirms the efficiency of the construction (Figure 10).

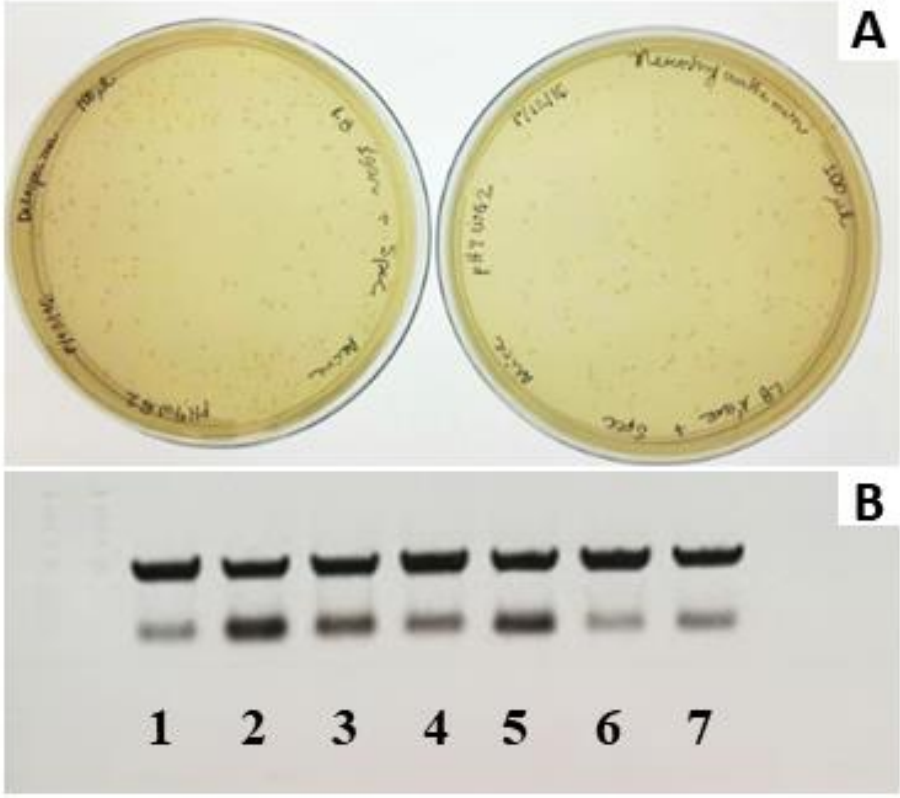

Figure 10. A. Plates of selective YEB medium for spectinomycin, containing *Agrobacterium tumefasciens* constructs. Left plate: construction pH7WG2 and DnARPa; Right plate: construction pH7WG2 and *McARP*. B - agarose gel electrophoresis showing colony PCR products from the above constructs (1-3 *DnARPa*, 4-6 *McARP*).

Phenotype rescue experiment

To determinate whether expression of Aizoaceae *ARP* could complement the *as1* phenotype we generated transgenic *Arabidopsis as1* plants expressing *AS1*, *McARP* and *DnARPa* under the 35S overexpression promotor. 35S::McARP, 35S::DnARPa and 35S::AS1 transformants were recovered, so overexpression is not lethal (Figures 11A-H). In *Arabidopsis*, loss of *AS1* doesn't affect cotyledons morphology (Figures 11A-B). This mutation results in plants with shorter petioles and wider leaf lamina in

comparison with the wild type and this phenotype is observed yet in the first leaf pair developed in the seedlings (Figures 11C-D). The 35S expression of both *DnARPa* (Figures 11E-F) and *McARP* (Figure 11H) resulted in the opposite effect: a long petiole and narrower leaf lamina, recovering the wild type phenotype as observed in 35S:: AS1 (Figure 6G). We didn't observe any range of phenotypes – in solid medium lacking antibiotic we recovered only non transformants (with mutant phenotype) or fully complemented lines (with wild type-like phenotype).

Scanning electron micrographs (SEM) showed differences between the *as1* mutant in comparison to wild-type and transformed *Arabidopsis* leaves. In wild type leaf, stomata are present in both surfaces (Figure 12A-D) and adaxial epidermal cells present sinuous boundaries (Figures 12A and C) with sparsely lobed outlines, constituting a leaf surface with a wavy appearance. Abaxial epidermal cells are sinuous shaped as well, but the cells are disposed in irregular height, then the surface is more uneven (Figures 12B and D). Hairs occur only in the adaxial surface.

In *as1* the epidermal cells shape has a less regular sinuous pattern (Figures 12E-H), but the boundary between the cells is perceptible. In adaxial surface, however, the cells are not well outlined (Figures 12E and G), being possible only the visualization of stomata distributed on a rough surface in abaxial surface (Figures 12F and H).

Both in 35S::McARP *as1* (Figures 13A-D) and 35S::DnARPa *as1* (Figures 13E-H) the characteristics of the leaf surface are similar to that observed in the wild-type. It's interesting to notice that abaxial surface of transformed plants has a regular wavy aspect (Figures 13B, D F and H); slightly different of what is observed in abaxial surface of the wild-type plant (Figure 12B and D). However, it is still very similar to wild type when we compare with the *as1* (Figure 12F and H). Interesting was the

eventual occurrence of hairs on the abaxial surface of transformed plants (not shown), which was not observed in wild-type analyzed specimen.

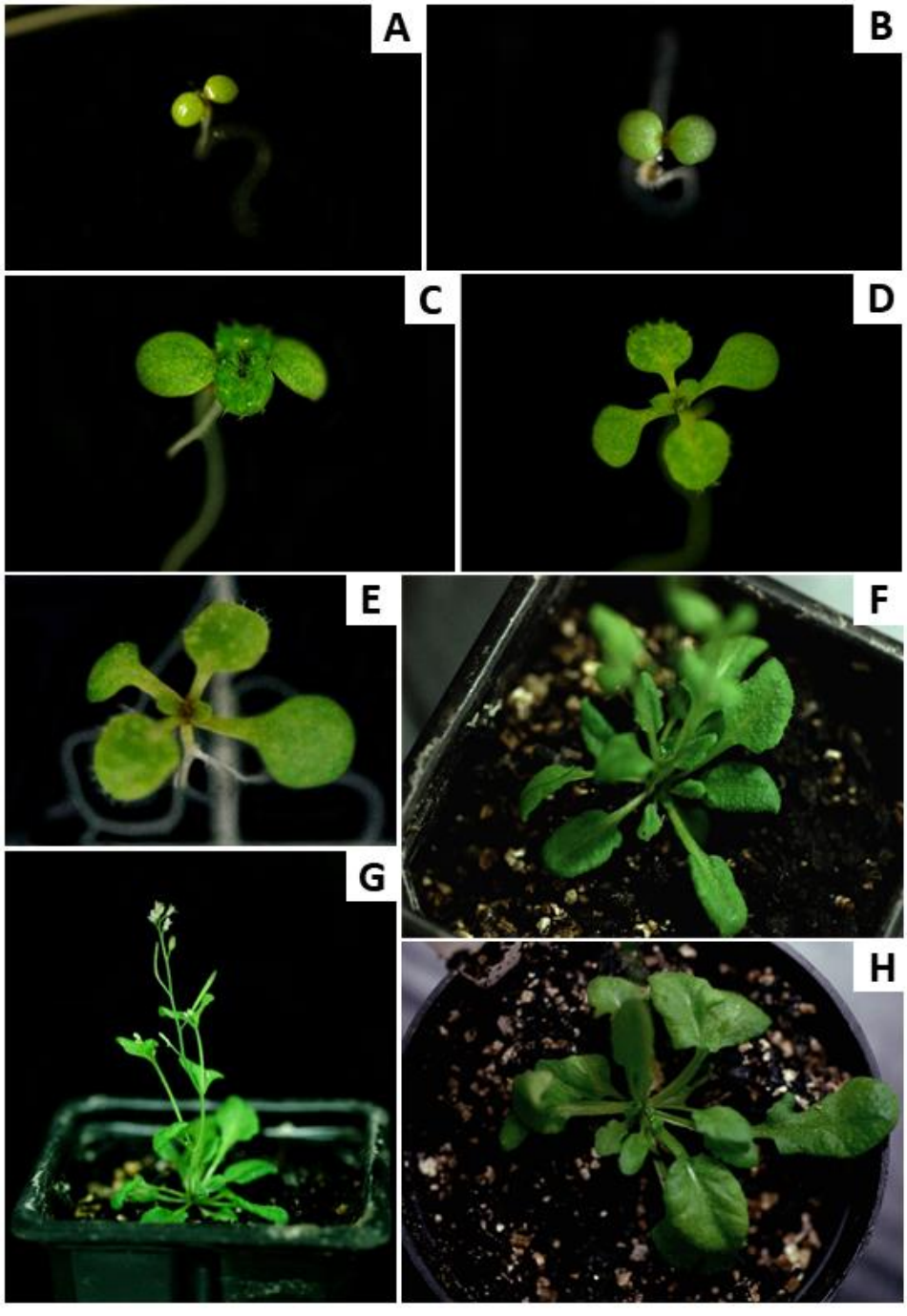

Figure 11. *Arabidopsis thaliana* phenotype. A, B – 5 days seedlings of Col-0 wild type and as1 respectively, exhibiting only the cotyledons; C – 10 days seedling of as1; D - 10 days seedling of Col-0; E – 35S:DnARPa as1 seedling; F - 35S:DnARPa adult; G – 35S:AS1 as1; H – 35S:McARP as1.

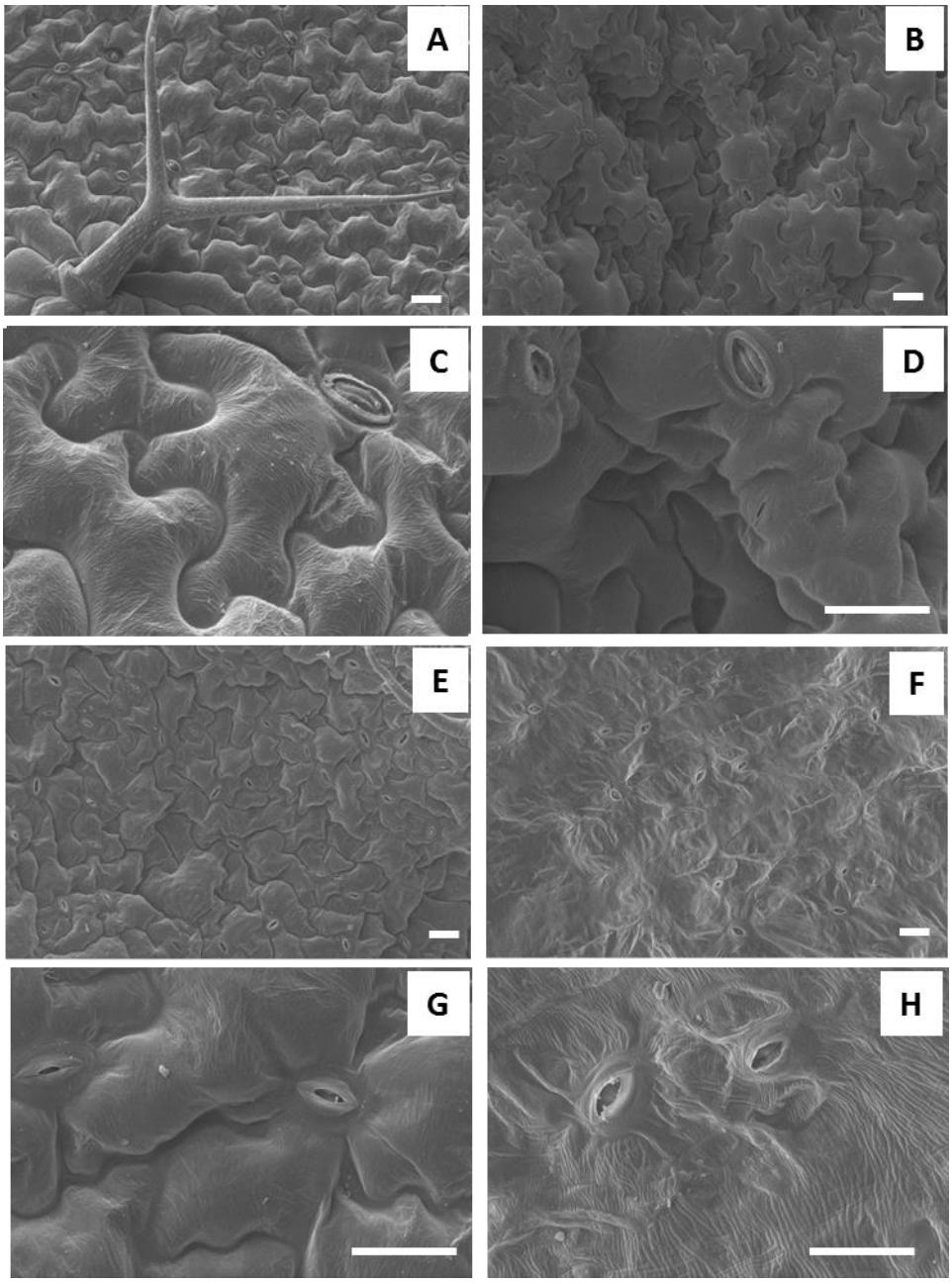

Figure 12. Scanning electron microscopy of leaf surface in wild-type and *as1 Arabidospis*. A – WT adaxial surface; B – WT abaxial surface; C – WT adaxial (cell detail); D – WT abaxial (cell detail). E – *as1* adaxial surface; F - *as1* abaxial surface; G - *as1* adaxial (cell detail); H - *as1* abaxial (cell detail). Bars=20um

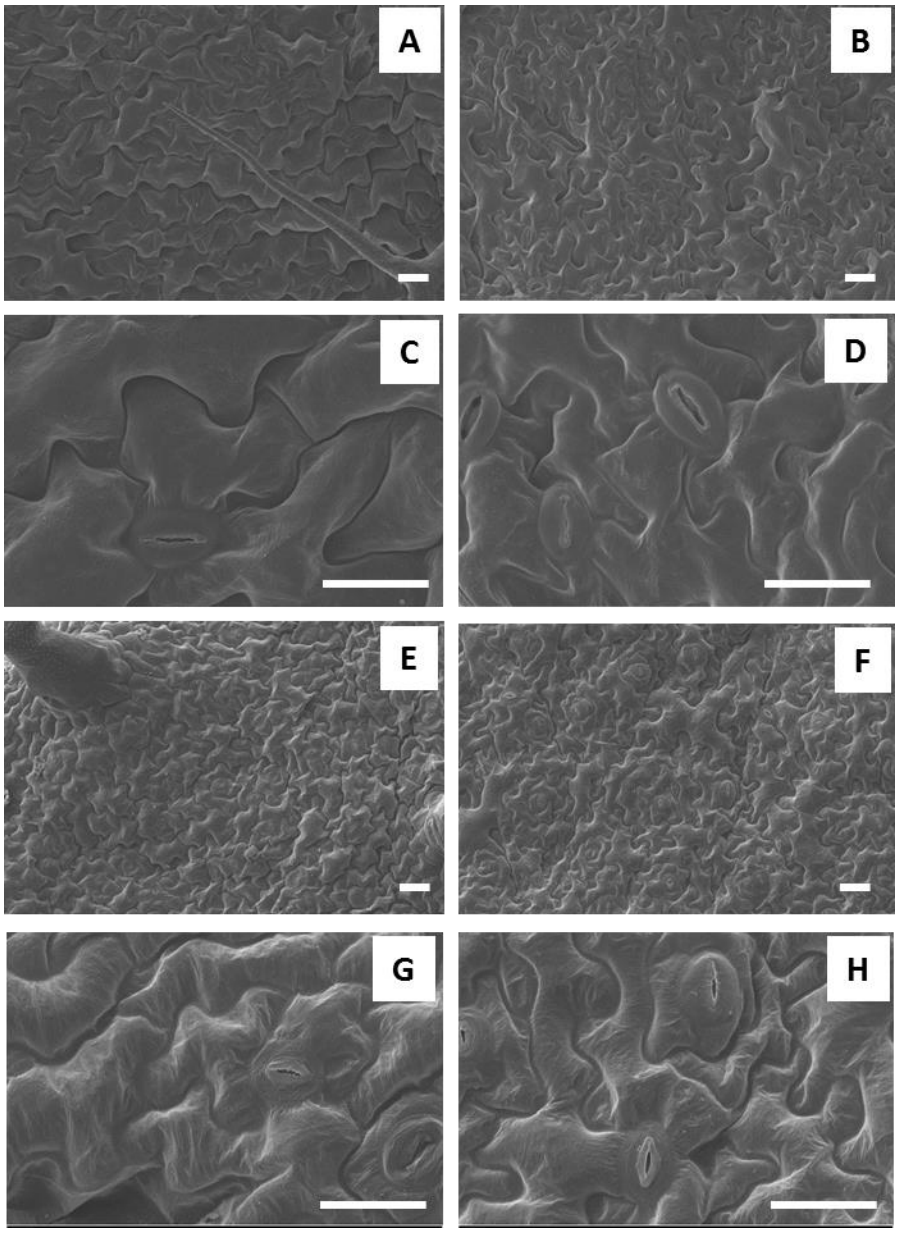

Figure 13. Scanning electron microscopy of leaf surface in *as1 Arabidospis* transformed with Aizoaceae *ARP* orthologs. A – *as1* 35S: McARP adaxial surface; B - *as1* 35S: McARP abaxial surface; C - – *as1* 35S: McARP adaxial (cell detail); D - *as1* 35S: McARP abaxial (cell detail); E - – *as1* 35S: DnARPa adaxial surface; F - *as1* 35S: DnARPa abaxial surface l; G - *as1* 35S: DnARPa adaxial (cell detail); H - *as1* 35S: DnARPa abaxial (cell detail). Bars = 20 um

Discussion

The *ARP* pathway is functionally conserved in plants

Transcription factors play a central role in the regulation of developmental and metabolic programs. Despite the large differences in these programs, the transcription factors are quite conserved between species and most of them can be grouped into families according to the structure of their DNA-binding domains (Romero *et al.* 1998). Functions of MYB transcription factors in plants include regulation of secondary metabolism, control of cellular morphogenesis and regulation of meristem formation (Glover *et al.* 1998). One pathway involved in reprogramming gene expression during leaf development involves the *ARP* gene orthologues which encode nuclear proteins with MYB domain (Theodoris *et al.* 2003, Machida *et al.* 2015).

We found that expression of the Aizoaceae *ARP* genes is able to fully complement the *Arabidopsis thaliana as1* mutation, indicating a high degree of functional conservation between *DnARPa, McARP and AS1*, consistent with their sequence similarity.

The expression patterns of the *ARP* ortholog in the vegetative shoot apex of *Mesembryanthemum crystallinum* and those of the two *ARP* copies in the vegetative apex of *Delosperma napiforme* correspond to those of *AS1* in *Arabidopsis* and to their orthologs in other angiosperms (Mchale and Koning 2004, Timmermans *et al.* 1999, Hay and Tisantis 2006). The conservation in expression pattern suggests that the orthologs of the Aizoaceae play a similar developmental role of other angiosperms *ARP* genes.

In addition, the sequence similarity among *ARP* orthologs suggests a conserved biological role as transcription factor, with evidence pointing to its function as repressor

of *KNOX* genes. Loss of *PHAN* function in *Antirrinum* (Waites and Hudson, 1995), tomato (Kim *et al.* 2003) and *Nicotiana* (Mchale and Koning 2004) inhibits lateral leaf expansion producing radialized leaves due to a loss of adaxial identity (abaxialization). Studies using *Arabidopsis* show that the onset of adaxial identity and competence from axillary bud meristems are correlated events, each bearing a common connection to the expression of HD-ZIP III genes family (*PHABULOSA*, *PHAVOLUTA* (McConnel *et al.* 2001), and *REVOLUTA* (Emery *et al.* 2003).

Our *in situ* hybridization data indicate that *DnARPa*, *DnARPb* and *McARP* are expressed in the entire developing leaf, which is consistent with the descriptions for other species in literature. Thus, suggesting that in Aizoaceae, *ARP* is mainly involved in the determinacy of leaf cell fate, in an opposite fashion as genes involved in the maintenance of indeterminacy. As we have shown, the probe signal is much weaker in the apical meristem region, thus the *in situ* hybridization data corroborates this prediction.

In *Arabidopis thaliana* and some others eudicots, development of adaxial domain requires two classes of genes: those of HD-ZIP Leucine protein family and those of the AS1-AS2 dimer complex (Machida *et al.* 2015). Although genes that are predicted to encode *AS1* orthologs are detected in genome databases of many plant species, genes that might encode amino acid sequences entirely homologous to the *AS2* sequence are not detected so often. The observation that *AS1* is capable to form homodimers in *Arabidopsis* (Theodoris *et al.* 2003), presents a possible mechanism via which Aizoaceae ARP proteins may interact to form complexes. Despite considerable effort, we have been unable to clone the full ORF of *DnARPb.* Therefore, rendering it unfeasible to test whether *DnARPb* is capable of rescue the wild phenotype in

Arabidopsis as1 mutants. Although the initial portion of the *DnARPb* gene is well conserved, there is no data available regarding its 3'-terminal region.

The *DnARPa* and *DnARPb* paralogs

Some synonymous mutations and other particularities are observed along the two copies of *ARP* in *Delosperma napiforme* sequences, but the similarity is recognizable throughout the gene. Additionally, there are no obvious differences between expression patterns of the *D. napiforme ARP* paralogues. *DnARPa* and *DnARPb* signals are both strong along leaf primordia and much weaker in the apical meristem region. This is consistent with the process of shoot versus leaf development in *Arabidopsis* and other angiosperms, in which *KNOX* genes are downregulated in the initiating leaf primordium while *ARP* genes are expressed. This similarity between *DNARPa* and *DnARPb* may be due to a redundant role of the copies during the leaf development.

According to Illing *et al.* (2009), the duplication of *ARP*, occurred during the diversification between Mesembryanthemoideae and Ruschioideae seem to be the source of the main morphological variation event in Aizoaceae. Illing *et al.* 2009 postulate that the duplication and retention of *ARP* paralogues precede the radiation of the Ruschioideae, contributing to the variation in leaf morphology and explosive radiation of the group.

Gene duplications can provide the genetic material for novel developmental mechanisms and are important drivers of evolution (Zhang 2003). These events can occur frequently in eukaryotic genomes but one of the gene pair may be silenced by gene loss (Zhang 2003). According to Illing *et al.* (2009), after the duplication event, the *ARPb* paralogue has been lost in some Ruschioideae species, including *Mossia intervallaris* (as shown in Figure 6, where this species present only the *MoIARPa* copy).

If the endoscopic xylem novelty were correlated to the expression of the two *ARP* copies in Aizoaceae, it would be expected that a loss of the *ARPb* paralogue could provide a reversion of this anatomic character. However, *Mossia intervalaris* present endoscopic PVB although it doesn't present the *ARPb* copy (supplementary material S3), which suggests that this novel anatomy may be not linked to the duplication event.

The presence and orientation of peripheral vascular bundles were the focus of an extensive study in leaves of 81 species representing 72 genera of Aizoaceae (Melo-de-Pinna *et al.* 2014), including a wide range of morphological shapes of leaves sampled from all four subfamilies (see Klak *et al.* 2003). The anatomical analysis revealed that three of the four subfamilies in Aizoaceae have peripheral vascular bundles in their leaves, i.e., Sesuvioideae, Mesembryanthemoideae and Ruschioideae. With exception of *Sesuvium* (Sesuvioideae), all other species with peripheral vascular bundles have an endoscopic orientation (Melo-de-Pinna *et al.* 2014). According to author, the peripheral vascular bundles evolved repeatedly within subfamilies Mesembryanthemoideae and Ruschioideae. How often endoscopic PVBs arose is uncertain, but are likely to have evolved more than once within the succulent Aizoaceae.

Given that *DnARPb* is expressed during leaf development, one mechanism by which this copy could lead to the terete leaf phenotype in Aizoaceae would be the maintenance of its capacity to form dimers with either *DnAPRa* or the *AS2* ortholog (if existent), competing for binding sites and leading to a loss of functionality of the protein complex. We are looking forward cloning this paralogue, with the hypothesis that *DnARPb* may be unable to complement the *as1*. If *DnARPb* could form novel dimers with *DnARPa*, it could be an evidence for the disturbing adaxial side development in Ruschioideae.

Our results show that the two copies behave in a similar way. However, we must consider that the expression signal in the tissue may not correspond to each copy specifically, since the probe may have been unspecific due to the high similarity between the *DnARPa* and *DnARPb* paralogs.

Conclusions

We observed that the pattern of *ARP* gene expression in Aizoaceae is well conserved, even in leaves with such a distinct morphology. This, allied to their ability to rescue wild type phynotype in *Arabidopsis as1* mutant, shows us that important processes are maintained throughout the evolution of the groups, and that more studies with non-model plants can bring light and explain how such extraordinary forms develop in nature. Conversely, one possibility for such morphological variation would be a hypothetical interference between the two *ARP* paralogs in Ruschioideae, modulating its ability to establish the adaxial domain. The molecular studies in non-model species is a challenging and very interesting study field in plant molecular development research today. In the case of *ARP* in Aizoaceae further studies will help to elucidate the role of duplication in Ruschioideae diversification.

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SUPPLEMENTARY MATERIAL

S1 - Primers list

Primer name	bases	degenerations	Sequence 5'--> 3'
AUAP	37	0	GGCCACGCGTCGACTAGTACTTTTTTTTTT TTTTTTT
ARP_R2	21	6	GARTRKAATRCAWACWTAGC
ARP_3'UTR _F	22	0	TGGAGAGGATTGAGGCTGAGTA
ARP_ATG_ F	21	4	ATGAARGARAGRCAACGWTGG
ARP F1 partial	20	2	GTIGGIAAGTGGTGGGAAGT
ARP R1 partial	22	2	GCCATTGATCAGCCA(G/A)(T/C)TTCTG
At AS1 ORF F	20	0	ATGAAAGAGAGACAACGTTG
At AS1 ORF R	20	0	TCAGGGGCGGTCTAATCTGC
ARP R5	19	0	CTGAGTTTGCTCCTCACTG
ARP_ATG_ F2	22	0	ATGAAGGAAAGGCAACGATGG
attB1-ATG-F	50	0	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGAAGGAAAGGCAACGATGG
attB2-R	46	0	GGGGACCACTTTGTACAAGAAAGCTGGGT CTGAGTTTGCTCCTCACTG
At_attB1_A TG_F	49	0	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGAAAGAGAGACAACGTTG
At_attB1_R	48	0	GGGGACCACTTTGTACAAGAAAGCTGGGT TCAGGGGCGGTCTAATCTGC

S2 - Obtained nucleotide sequences of *AS1*, *McARP*, *DnARPa* and *DnARPb*

START CODON – **CDS** – **STOP CODON** – REGION USED AS PROBE

McARP (ORF)

>ATGAAGGAAAGGCAACGATGGACTGCTGAGGAGGACACGATACTACGTGCGTATGTGAAG
CAATATGGTCCAAGAGAATGGCATCTGGTGTCCCAGCGCATGAACACACCCCTTGATAGGG
ACGCCAAGTCGTGTCTAGAGCGGTGGAAGAATTACCTCAAGCCCGGGATCAAAAAAGGCTC
GCTCACTGAGGAGGAGCAGCGTTTAGTTATTCGTCTGCAGGCAAAGCACGGAAACAAATGG
AAGAAAATCGCAGCTGAGGTGCCAGGACGTACAGCTAAGAGGCTCGGAAAGTGGTGGGAA
GTGTACAAGGAGAAGCAACTGAGGGCAAGGAAAGAAACTACCAAGTGCTTGGAGCCAATT
GAGGAGGGGAAGTACGATCGGATTCTGGAGACCTTTGCTGAGAAGCTAGTCAAGGAGCGGG
CTCCCCCCAGCCCCACAACTTTCCTCATGGCTACCTCGAATGGGGCATTTCTACATTCCGATC
ACGCCCCGCCTGCTCCACCTCCTGTTCCAACCACAGGAATGCTTCCTCCCTGGCTTGCCACCT
CAAATGGTGGGAGGCCACCTTCTCCCTCAGTTACCTTGAGTCTGTCGTCGTCGACAATCCCA
GCCCCTGCCCCGATCCCAGCCCCACCAGCAGTGTCTTGGCTTCAGCAGGAAAGGGGGGCCC
ACAACATCGAAACTACCCCTCTTACTTTGGGCTGCTTATCGTCGTGTCATGGGGCGGTTCCTA
CTTGTGGTGGGGAGCAAACACTGATGTCTGAGCTCATGAGTTACTGTAGAGAGCTAGAGGA
AGGGCACCGTGCTTTGACAGCCCACAAAAAGGAAGCTGCTTGGAGGCTAAAGCGAGTAGAG
CTTCAATTGGAGTCAGAAAAGGCTAGTAGGAGGAGAGAAAAAATGGAAGAGATTGAGGCG
AAGATAAAGGCTCTTAGGGAAGAGCAGAATGCTAGCATGGAGAGGATTGAGGCTGAGTATC
GAGAACAGCTGGCTGGGTTGAGAAGGGATGCCGAGGCCAAGGAGCAGAAGTTGGCTGAAC
AATGGGCCGCTAAGCACAATCGCATAACAAACTTGCTTGAGCAGATGGGCTGCAGGGCCCG
GCTTCCCGACCCTAGTTGA

DnARPa (ORF)

>ATGAAGGAAAGGCAACGATGGACTACTGAGGAGGACACGATACTATGTGCGTATGTGAAG
CAATATGGTCCAAGAGAATGGCATCTGGTGTCTCAGCGCATGAACACACCCCTTGATAGGG
ATGACAAGTCGTGTCTAGAGCGGTGGAAGAATTACCTCAAGCCTGGGATCAAGAAAGGCTC
ACTAACTGAGGAGGAGCAGCGTCTTGTTATCCATCTCCAAGCAAAGCACGGCAACAAATGG
AAGAAAATTGCGGCTGAGGTGCCAGGACGTACAGCTAAGAGGCTCGGAAAGTGGTGGGAA
GTGTACAAGGAGAAGCAACAGAGAGTGGCAAATAAAGAAACTAACAAGTGCTTGGAGCCA
GTCGAAGAGGGGAAATACGACCGTATTCTCGAGACCTTTGCTGAGAAGCTAGTCAAGGAGC
GGGCTCCCCCGATGGCCCCCGCTACATTTCTCATGGGAACGTCAAATGGGCAAACTTTTCTA
CATTCCGATCATGCCCCTCCTACTCCACCACCTCGTGTTCCAACCACATCAATGCTTCCTTCC
GGGCTTTCCACCTCAAATGGTGGGAGGCCACCACCATCTCCCTCGGTTACCTTGAGTCTCTC
ATCTACGACAATCCCAGCCCAGGCACCACCAGCAGTATCTTGGCTTCATCCGGAGAGGGGG
GCCCACAGCCACTCTCTCGATACTACCCCTCTTACATTGGGCTGCTTTCATACTTGTGGTTGT
GGAAGTGGGGAGCAGACACTGATGATGTCTGAGCTCATGAGTTGCTGCAGAGAGCTAGAAG

TAGGTCACCGTGCTTTGGCAGCCCACAAGAAGGAAGCTGCCTGGAGGCTAAAGCGGGTAGA
GCTTCAATTAGAAGCAGAGAAGGTAAGTAGGAGGAGAGAAAAAATGGAGGAAATTGAGGC
GAAAATAAAGGCTCTTAGGGAAGAGCAGAATGCTAGCCTGGAGAGGATTGAGGCTGAGTAC
AGGGAGCAGCTGGCTGGGTTAAGAAGGGATGCCGAGGCAAAGGAGCAGAAGTTGGCTGAC
CAATGGGCTGCTCAGCACACTCGTGTTACAAAATTGATTGAACAGATGGGCTGCAGCGCCC
GCCTTCATGAGCCTAGTTGA

DnARPb (Partial)

>ATGAAAGAGAGGCAACGTTGGACAACTGAGGAGGACACGATACTAAGTGCGAATGTGAA
GCAATATGGTCCAAGAGAATGGCATCTGGTTTCCCAGCGCATGAACACGGTCCTTGATAGG
GACGCAAAGTCGTGTCTTGAGCGGTGGAAAAATTACCTCAAACCCGGTATCAAGAAAAGCT
CGCTCACAGAGGAGGAGCAGCGTCTCGTTATTCGTCTCCAGACCAAGCACGGCAATAAATG
GAAGAAAATTGCGGCTGAGGTGCCAGGACGTACAGCTAAGAGGCTTGGAAAGTGGTGGGA
AGTGTACAAGGAGAAGCAACAGAGGGCAGCAAACAAAGAAACTACAAAGTGCTTGGAGCC
TATCGAGGAGGGGAAGTACGACAGGATTCTCGAGACCTTTGCTGAGAAGCTAGTCAAGGAG
CGGACTCCCCCGCTAACCGCCACAACGTTCCTTATGGCCACCTCGAATGTGGAAGCTTTTCT
ACATTGCGATCATACACCTACGGTTGTACCACCTCATGTACCGACCACAACAATGCTTCCTC
CCTGGCTTGCCACCTCAAATGGCGGGAGGCCACCTTCTCCCCCGGTTACCTTGAGTCTCTCA
TCTTCGACAACCCCAGCATCGGCCCCACCAGCAGTGTCTTTGCTTCATCCGGAGAGAGGGGC
CCACACTCACAACATTGATAGTGCCCCTTTTACAATTGGCTGTTATTCTACTTGTGGTGGTGG
TGGGGAGCAATCACTGATGTTGTCTGAGCTTGTGAGTTGCTGTAGAGAGTTGGAGGAAGGG
CACCGTGCTTTGGCAGCCCACAAGAAGGAAGCTGCTTGGAGGCTAAAGCGGGTAGAGCTTC
AGTTAGAATCAGAGAAGGCGAATAGGAGGAGAGAGAAAATGGAGGAGATTGAGGCGAAGA
TGAAGGCTCTTAGGGAAGAGCATGATGCTAGCATGGAGAGGATTGAGGCTGAGTACCGCGA
GCAGCTGGCTGGGTTGCGGAGGGATGCCGAGGCCAAGGAGCAGAAATTGGCTGATC

AS1 (ORF)

>ATGAAAGAGAGACAACGTTGGAGTGGTGAAGAAGATGCATTGTTACGTGCTTACGTTAGA
CAGTTCGGTCCGAGAGAATGGCATCTTGTGTCTGAGCGTATGAACAAACCTTTGAACCGTGA
CGCCAAGTCTTGTTTAGAGAGATGGAAGAATTATCTTAAGCCAGGGATCAAGAAAGGGTCT
TTGACAGAGGAAGAGCAGAGGCTTGTGATCCGTCTTCAGGAGAAACACGGCAACAAGTGGA
AGAAGATTGCTGCTGAGGTTCCCGGGAGGACGGCAAAGCGGTTAGGGAAGTGGTGGGAAGT
GTTTAAGGAGAAGCAACAGAGAGAAGAGAAAGAGAGTAACAAGAGAGTTGAGCCTATTGA
CGAGAGTAAGTACGATCGGATTCTCGAGAGTTTCGCTGAGAAGCTTGTCAAAGAGCGGTCT
AACGTTGTCCCTGCTGCTGCCGCTGCTGCAACGGTTGTGATGGCTAATTCGAATGGAGGGTT
TTTACATTCTGAACAACAAGTTCAGCCTCCTAACCCAGTGATCCCGCCTTGGTTAGCTACTTC
TAACAATGGGAACAATGTTGTTGCAAGGCCTCCCTCGGTAACTTTGACATTATCGCCTTCCA
CAGTGGCTGCAGCTGCGCCTCAACCGCCAATCCCGTGGCTGCAGCAGCAACAGCCTGAGAG

AGCAGAGAACGGTCCAGGGGGACTTGTGTTAGGGAGTATGATGCCGTCTTGTAGTGGGAGT
AGCGAGAGTGTGTTCTTGTCAGAGCTTGTGGAGTGTTGTAGAGAGTTGGAGGAAGGGCACC
GAGCTTGGGCAGACCATAAGAAAGAGGCTGCATGGAGGCTAAGAAGGCTGGAGCTGCAGC
TAGAGTCAGAGAAGACGTGTAGACAAAGGGAGAAGATGGAGGAGATTGAGGCAAAGATGA
AAGCTCTTAGGGAAGAGCAGAAGAACGCAATGGAGAAGATCGAAGGAGAGTACAGAGAAC
AGCTCGTTGGTTTGAGGCGAGACGCAGAGGCCAAAGACCAGAAACTGGCTGATCAATGGAC
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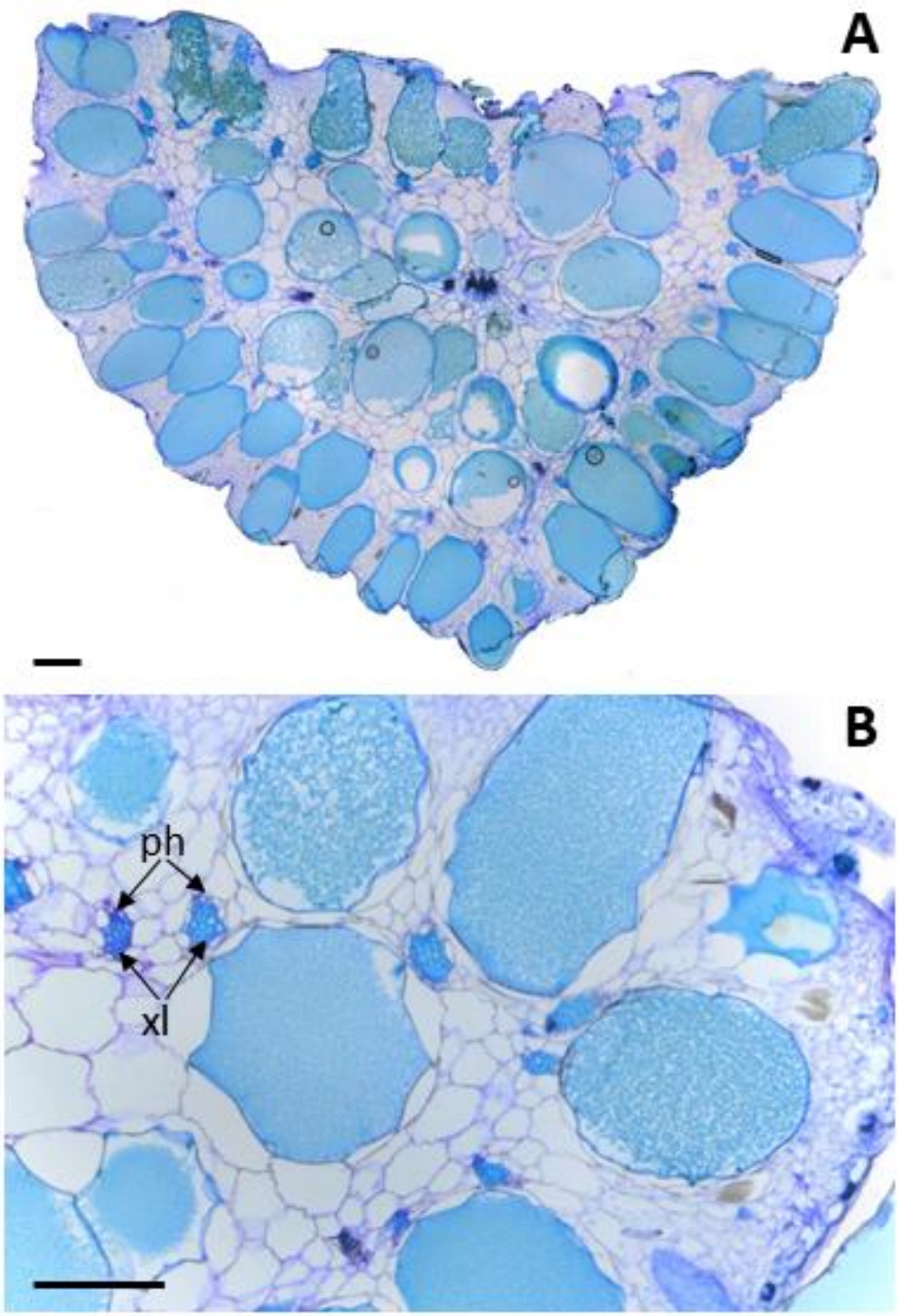
CCTGA



S3. Leaf anatomy of *Mossia intervallaris* (Aizoaceae). A - transverse section of leaf blade B - detail of endoscopic peripheral vascular bundles in adaxial region of *D. napiforme* leaf. xl – xylem; ph – phloem. Bars = 200µm

ANEXO

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GROWTH PATTERNS AND DIFFERENT ARRANGEMENTS OF VASCULAR TISSUES IN SUCCULENT LEAVES

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By their wide morphological diversity, succulent leaves have aroused the interest of many researchers. Nonetheless, comparative anatomical studies of unifacial, bifacial, and subunifacial leaves are scarce. To address this gap, our study examines the growth and differentiation of vascular tissues in succulent leaves of 12 families of angiosperms. Altogether, we identified six arrangements arising as variations of three basic vascularization patterns. In addition, cases of convergent evolution were identified in (1) terete leaves showing both endoscopic peripheral bundles and a central bundle in some Crassulaceae (Saxifragales, eudicots) and Aizoaceae (Caryophyllales, eudicots) and (2) terete leaves showing exclusively endoscopic peripheral bundles in some Asteraceae (eudicots) and Orchidaceae (monocots). Both endoscopic and exoscopic bundles originate from a peripheral region, and in all species with endoscopic peripheral bundles, the differentiation of the vascular bundles begins from abaxial domains, whereas in leaf blades with exoscopic bundles, differentiation begins from adaxial domains. These observations suggest that the molecular mechanism(s) involved in the abaxialization/adaxialization process of the leaf blade could be related to activity of the peripheral region. Therefore, further investigation is being carried out to elucidate peripheral meristematic activity responsible for the differentiation of vascular bundles and the fundamental system.

Keywords: adaxial/abaxial polarity, endoscopic/exoscopic vascular bundles, unifacial leaves.

Online enhancements: appendix figures.

Introduction

Succulent plants are represented by at least 12,500 species from about 690 genera in 83 families (Nyffeler and Eggli 2010), including a wide variety of forms, such as geophytes, prostate creeping perennials, epiphytes, drought deciduous perennials, and annuals (Bobich and North 2009). According to Eggli and Nyffeler (2009, p. 32), "as succulence has not been identified as ancestral character for any of the deeper nodes in the phylogeny of Angiosperm orders, it must have evolved independently at the very least 30 times, i.e. at least once in each order."

Succulent plants may be classified according to (1) an organ or part of an organ that stores water; (2) the location of the water-storage tissue, either outside or inside the chlorenchyma; (3) the nature of the water-storage tissue (e.g., epidermis, parenchyma, or water-storing idioblasts); and (4) water storage combined with other functions in the same tissue or organ (Jürgens 1990). In cases where the same tissue is indicated, Ihlenfeldt (1985) made a further distinction between "all-cell succulents," in which water storage and photosynthesis occur in the same tissue, and "storage-cell succulents," in which they do not.

The presence of peripheral vascular bundles with different distribution patterns is common in succulent leaves (Groom et al. 1994; Freitag and Stichler 2000; Timonin et al. 2006; Hibara et al. 2009; Ozerova and Timonin 2009; Ocampo and Columbus 2010; Koteyeva et al. 2011; Ogburn and Edwards 2013; Melo-de-Pinna et al. 2014). In Portulacineae (Caryophyllales), Ogburn and Edwards (2013) describe the most highly succulent plants as having venation systems that ramify in three dimensions (3D). These plants stand in contrast to those with venation in a single plane (2D). The lower vein density results in longer hydraulic paths between veins and photosynthetic surfaces.

Terete leaves with a 2D vascularization pattern (fig. 1*A*) have a central bundle and small lateral vascular bundles with xylem cells oriented toward the adaxial side and phloem cells oriented toward the abaxial side (Freitag and Stichler 2000; Ocampo and Columbus 2010; Ogburn and Edwards 2013; Melo-de-Pinna et al. 2014; Hernandes-Lopes et al. 2016). However, a second vascularization pattern occurs in succulent leaves with a 3D vascular pattern (fig. 1*B*–1*D*). These leaves exhibit a central

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Repeated evolution of endoscopic peripheral vascular bundles in succulent leaves of Aizoaceae (Caryophyllales)

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Abstract We investigated the presence and orientation of peripheral vascular bundles in leaves of 81 species representing 72 genera of Aizoaceae. Our study included a wide range of morphological shapes of leaves sampled from all four subfamilies of Aizoaceae, with an emphasis on succulent leaves found in Mesembryanthemoideae and Ruschioideae. Our anatomical studies revealed that only three of the four subfamilies in Aizoaceae have peripheral vascular bundles in their leaves, i.e., Sesuvioideae, Mesembryanthemoideae and Ruschioideae. Apart from *Sesuvium* (Sesuvioideae), all other species with peripheral vascular bundles have an endoscopic orientation, i.e., the phloem is positioned closer to the epidermis, whereas the xylem is pointing towards the interior of the leaf. This contrasts the situation in other Caryophyllales, where, with the exception of *Borszczowia* (Amaranthaceae), two different types of three-dimensional venations were observed. The distinct types of three-dimensional venation may in turn provide clues to the different pathways by which the similar leaves evolved. Endoscopic peripheral vascular bundles in Aizoaceae were only found in species with an expanded leaf base and (sub-)cylindrical to trigonous leaves. Although flat-bladed species of Aizoaceae generally have no peripheral vascular bundles, they were present in *Delosperma tradescantioides* (Ruschioideae: Ruschieae). Persistent, flat leaves are very rare within the hyperdiverse Ruschieae, which typically have (sub-)cylindrical to trigonous leaves. Thus, the presence of peripheral vascular bundles in leaves of *D. tradescantioides* indicates that flat-bladed, persistent leaves constitute a reversal within tribe Ruschieae. The additional presence of lateral bundles in the outer lamina of leaves of *D. tradescantioides* can be interpreted as a regain of planate venation. Mapping the evolution of peripheral vascular bundles onto a phylogeny of the Aizoaceae suggests that this character evolved repeatedly within subfamilies Mesembryanthemoideae and Ruschioideae.

Keywords Aizoaceae; leaf anatomy; peripheral vascular bundles; phylogeny; succulence

Supplementary Material The alignment is available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

INTRODUCTION

Leaves occur in a vast array of shapes and sizes, with a distinct adaxial–abaxial polarity necessary for lamina growth (Sarojam & al., 2010; Szakonyi & al., 2010). Establishment of an adaxial–abaxial polarity results in a typical bifacial leaf with vasculature aligned such that the xylem tissue is arranged adaxially and the phloem abaxially. In many plants, the adaxial side of the leaf develops a layer of palisade parenchyma, while on the abaxial side there is spongy parenchyma (Eames & MacDaniels, 1947; Fahn, 1974; Kaplan, 2001). However, the lack of the adaxial identity results in unifacial leaves, which may either be flat-bladed (Tsukaya, 2014) or cylindrical. The cylindrical shape has been reported for monocots (Kaplan 1975; Yamaguchi & al., 2010, 2012; Tsukaya 2014) as well as for eudicots (Timonin & al., 2006; Ozerova & Timonin, 2009). In both cases, there is a sub-epidermal ring of photosynthetic tissue (equifacial) and all vascular bundles exhibit a radial organization, which are referred to as peripheral vascular bundles (PVB, Fig. 1A). Two distinct arrangements of xylem and phloem within the peripheral vascular bundle have been distinguished: endoscopic pattern, with phloem closer to the epidermis and the xylem cells orientated towards the centre (Fig. 1B); and exoscopic pattern, which shows a reversed orientation, i.e., the xylem cells are closer to the epidermis and the phloem cells are orientated towards the interior (Fig. 1C).

Peripheral vascular bundles are common in succulent leaves (Groom & al., 1994; Freitag & Stichler, 2000; Timonin & al., 2006; Ozerova & Timonin, 2009; Ocampo & Columbus, 2010; Koteyera & al., 2011; Ogburn & Edwards, 2013). Recently, two distinct three-dimensional (3D) leaf venation patterns were observed in suborder Portulacineae and in Molluginaceae (Caryophyllales) (Ogburn & Edwards, 2013): One, the exoscopic pattern, termed type I (Fig. 1C) and a second type, termed type II (Fig. 1D), where the veins ramify in three dimensions without altering the orientation of xylem and phloem

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CONSIDERAÇÕES FINAIS

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O primeiro passo na elaboração desta pesquisa surgiu do estudo de ontogênese foliar em espécies de Ruschioideae (Capítulo 1). O desenvolvimento foliar foi observado nas mais diferentes morfologias foliares, entre dorsiventrais e teretes, mostrando a instalação e atividade das regiões de crescimento envolvidas na histogênese da base e da lâmina foliar. Sugerimos que mecanismos moleculares de abaxialização sejam desencadeados durante a formação da lâmina foliar, uma vez demonstrado que o caráter dorsiventral da nervura mediana é definido anteriormente, assim como ocorre na base foliar. A realização de estudos sobre desenvolvimento foliar a partir de análises anatômicas em grupos que apresentam diferentes morfologias foliares - dorsiventrais e teretes- reforça a necessidade de mais estudos sobre os processos envolvidos na formação de folhas cilíndricas dentro das plantas vasculares.

Ferramentas complementares aos estudos do desenvolvimento foliar são de extrema importância quando se aborda aspectos envolvendo os processos de abaxialização e adaxialização. Neste sentido, a partir da análise de expressão gênica e conservação da função de genes relacionados ao estabelecimento da morfologia foliar in Ruschioideae (Capítulo 2), consideramos a hipótese da variação morfológica presente no grupo ser produto de uma possível interferência entre as duas cópias de *ARP* modulando sua capacidade de estabelecer o domínio adaxial.

Em síntese, avanços em técnicas moleculares tem nos permitido desvendar alguns dos mecanismos do desenvolvimento vegetal e um progresso significativo tem sido feito no entendimento da identidade adaxial-abaxial em plantas modelo. Ainda pouco se conhece sobre o controle genético do desenvolvimento folar em outros grupos, mas estudos comparativos com base em *Arabidopsis thaliana*, *Oriza sativa* e outras espécies nos auxiliam a entender o desenvolvimento vegetal em ampla perspectiva.

Análises de espécies diferentes vão ainda fornecer um entendimento sobre como esses mecanismos influenciam na diversidade e plasticidade fenotípica dos mais diferentes grupos de plantas.