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Evolution of petal epidermal micromorphology in Leguminosae and its use as a marker of petal identity

Isidro Ojeda[1,3,*], Javier Francisco-Ortega[2,4] and Quentin C. B. Cronk[1,3]

[1]*Department of Botany, University of British Columbia, 3529-6270 University Blvd, Vancouver, BC, Canada V6T 1Z4,* [2]*Department of Biological Sciences, Florida International University, Miami, FL 33199, USA,* [3]*Centre for Plant Research, University of British Columbia, Macmillan Building, 6683 Main Mall, Vancouver, BC, Canada V6T 1Z4 and* [4]*Centre for Tropical Plant Conservation, Fairchild Tropical Botanic Garden, Coral Gables, Miami, FL 33156, USA*

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• *Background and Aims* The legume flower is highly variable in symmetry and differentiation of petal types. Most papilionoid flowers are zygomorphic with three types of petals: one dorsal, two lateral and two ventral petals. Mimosoids have radial flowers with reduced petals while caesalpinioids display a range from strongly zygomorphic to nearly radial symmetry. The aims are to characterize the petal micromorphology relative to flower morphology and evolution within the family and assess its use as a marker of petal identity (whether dorsal, lateral or ventral) as determined by the expression of developmental genes.
• *Methods* Petals were analysed using the scanning electron microscope and light microscope. A total of 175 species were studied representing 26 tribes and 89 genera in all three subfamilies of the Leguminosae.
• *Key Results* The papilionoids have the highest degree of variation of epidermal types along the dorsiventral axis within the flower. In Loteae and genistoids, in particular, it is common for each petal type to have a different major epidermal micromorphology. Papillose conical cells are mainly found on dorsal and lateral petals. Tabular rugose cells are mainly found on lateral petals and tabular flat cells are found only in ventral petals. Caesalpinioids lack strong micromorphological variation along this axis and usually have only a single major epidermal type within a flower, although the type maybe either tabular rugose cells, papillose conical cells or papillose knobby rugose cells, depending on the species.
• *Conclusions* Strong micromorphological variation between different petals in the flower is exclusive to the subfamily Papilionoideae. Both major and minor epidermal types can be used as micromorphological markers of petal identity, at least in papilionoids, and they are important characters of flower evolution in the whole family. The molecular developmental pathway between specific epidermal micromorphology and the expression of petal identity genes has yet to be established.

Key words: Epidermis, Fabaceae, Papilionoideae, Caesalpinioideae, Mimosoideae, petal surface, scanning electron microscopy, papillose conical cells, tabular rugose cells, tabular flat cells, organ identity.

INTRODUCTION

The petal epidermal surface is important in pollination, as it influences the way in which pollinators perceive and interact with the flower. There is evidence that petal epidermal type and its surface affect colour depth (Kay *et al.*, 1981; Kay, 1988; Gorton and Vogelmann, 1996; Dyer *et al.*, 2007), iridescence (Whitney *et al.*, 2009), scent production (Kolossova *et al.*, 2001), temperature (Comba *et al.*, 2000; Dyer *et al.*, 2006) and provides tactile cues (Stirton, 1981; Kevan and Lane, 1985; Comba *et al.*, 2000; Whitney *et al.*, 2009).

Previous surveys have classified and analysed the distribution of the epidermal surface of petals within the angiosperms (Barthlott and Ehler, 1977; Kay *et al.*, 1981). Extensive and detailed analyses have been provided for a few groups, such as Asteraceae (Baagøe, 1977; Baagøe, 1980; Hansen, 1991) and Leguminosae (Stirton, 1981), in which the characteristics of these epidermal types have been used for taxonomic and phylogenetic analyses. Among the epidermal types described in these studies, papillose conical cells (PCS) are frequently reported. Between 60 and 80 % of the angiosperm species analysed have at least one petal with this epidermal type on the adaxial surface (or upper side, towards the floral axis) of the petals (Kay *et al.*, 1981; Christensen and Hansen, 1998). The frequency of this cell type, coupled with other evidence, suggests that it has an adaptive value (Glover and Martin, 2002).

Several recent studies have elucidated the molecular genetics of the papillose conical cell type. The *MIXTA* gene, an MYB transcription factor, is required for the development of PCS in *Antirrhinum majus* (Noda *et al.*, 1994). Further evidence suggests that this gene is sufficient to produce PCS in other groups within the asterid clade (Solanaceae and Scrophulariaceae). The developmental programme leading to this epidermal pattern is therefore quite conserved, at least in these groups (Glover and Martin, 2002). However, it seems that a different developmental pathway may operate in the Brassicaceae (rosid clade) (Glover *et al.*, 1998; Payne *et al.*, 1998).

* For correspondence. Email isidro@interchange.ubc.ca

Petal identity, whether dorsal (adaxial), lateral or ventral (abaxial), is set at an early developmental stage by the expression of identity genes that induce transcription of other genes responsible for the shape, size, colour and epidermal type characteristics of individual petals (Cronk, 2006). A change of petal identity may therefore change features of the epidermal surface. The functions of two petal identity genes have so far been described in the Leguminosae (Feng *et al.*, 2006). One of these genes, *Lotus japonicus CYCLOIDEA 2* (*LjCyc2*) is a transcription factor in the TCP gene family (named after its first three characterized members, TB1 in maize, CYC in snapdragon and PCF in rice) with a dorsalizing action. This gene is associated with dorsal petal identity and hence papillose conical cell formation, as PCS are characteristic of standard petals in *L. japonicus*. The second gene, *LjCyc3*, is a lateralizing factor. This gene is responsible for the lateral petal identity and hence the formation of tabular rugose cells (TRS) with a jigsaw puzzle-shape that are characteristic of wing petals. The molecular basis of jigsaw puzzle-shape cells has recently been elucidated (Fu *et al.*, 2005).

These results suggest that each petal in the papilionoid legume flower has a distinct molecular identity, that may be marked by epidermal type. In transgenic plants that overexpress *LjCyc2* all petals have PCS, indicating that all petals have been converted to a dorsal identity. Furthermore, natural evolution in legume flowers may occur by shifts in petal identity (Citerne *et al.*, 2006).

Many legume species have a specialized flower morphology that promotes pollinator specificity (Fig. 1). Papilionoid legumes generally have three kinds of petals: a dorsal petal, called a 'standard', two lateral petals or 'wings', and two ventral petals forming the 'keel'. The distribution of the epidermal types within this family has not been analysed in detail. A few studies have included some legume species (Kay *et al.*, 1981; Christensen and Hansen, 1998), but in the second case only the dorsal petal was analysed (C. I. Christensen, University of Copenhagen, Denmark, pers. comm.). A more extensive survey (Stirton, 1981) focused only on the papilionoids and was restricted to lateral petals. Another study has reported the epidermal type of two

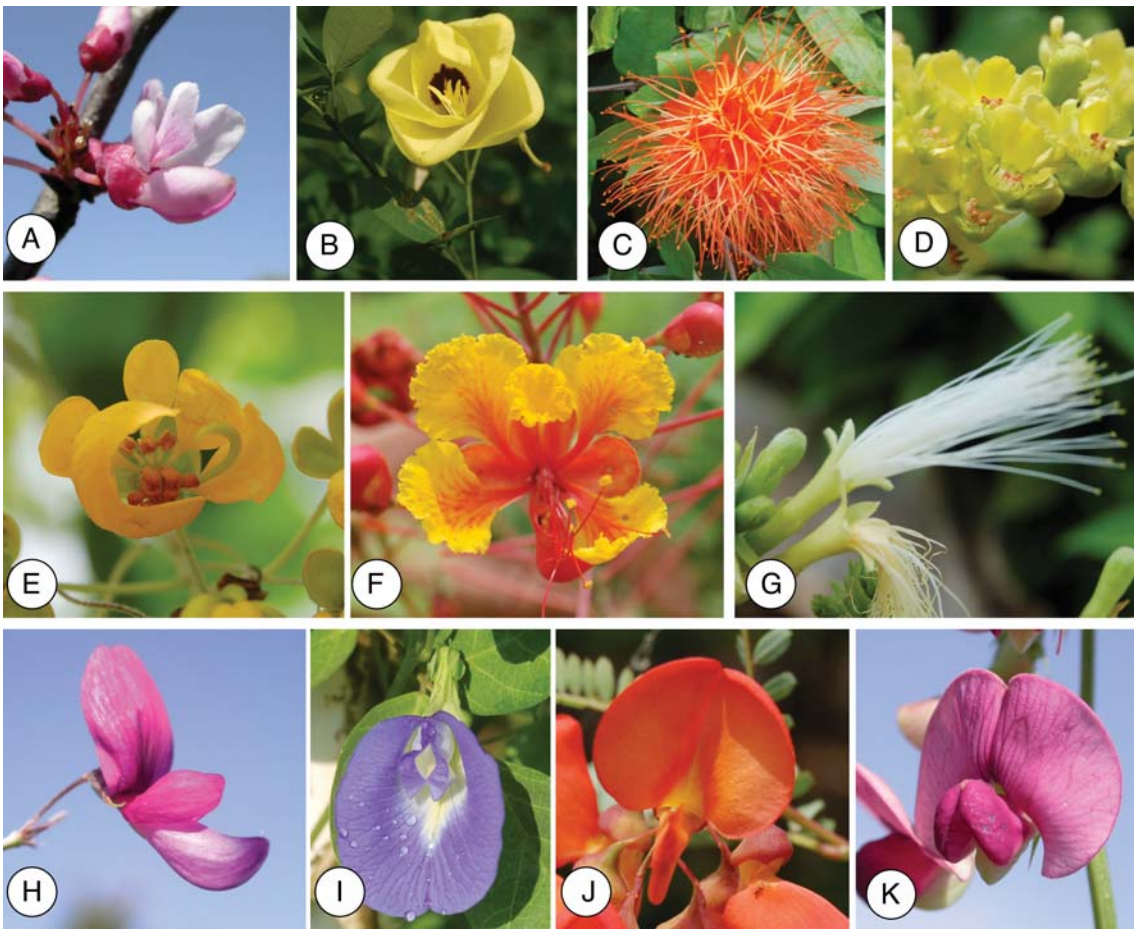

FIG. 1. Diversity of flower symmetry in the Leguminosae: (A) zygomorphic flowers of *Cercis canadensis* (Caesalpinioideae); (B) flowers of *Bauhinia tomentosa* (Caesalpinioideae); (C) radially symmetric flower of *Brownea capitella* (Caesalpinioideae); (D) zygomorphic flowers of *Tara cacalao* (Caesalpinioideae); (E) asymmetric enantiostylous flower in *Cassia emarginata* (Caesalpinioideae); (F) zygomorphic flower with the dorsal petal differentiated with respect of the other petals in *Caesalpinia pulcherrima* (Caesalpinioideae); (G) radial flower with reduced petals in *Inga paterno* (Mimosoideae); (H) zygomorphic flowers in *Lespedeza thunbergii* (Papilionoideae; with the ventral petal more exposed); (I) *Clitoria ternatea* (Papilionoideae; the dorsal petal is pointing downwards); (J) *Sesbania punicea* (Papilionoideae); (K) *Lathyrus sylvestris* (Papilionoideae; ventral petals enclosed).

Lathyrus species, but only the dorsal and lateral petals were analysed (Hammett *et al.*, 1994). There is therefore a lack of detailed information about the epidermal types within the family. Knowledge of the molecular developmental basis of petal identity has therefore not been coupled with a systematic analysis of the epidermal types and their distribution within the Leguminosae, even though epidermal types could be useful micromorphological markers of petal identity in developmental studies, for instance in the analysis of developmental mutants. In order to address this lack of information, a survey of the epidermal surface of the various types of petals within this family was conducted.

The aims of the present study were to (*a*) characterize the epidermal cell types of petals in the major clades of the Leguminosae, (*b*) determine their distribution along four axes of distribution, one within the flower (dorsiventral) and three within each petal (abaxial–adaxial surfaces, proximo-distal and medio-lateral) and (*c*) relate these patterns to our current understanding of the molecular genetic basis of petal identity and flower evolution within the family.

MATERIALS AND METHODS

Taxon sampling

Representative species were chosen from each of the three subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) and all 12 major clades currently recognized in the Leguminosae following recent phylogenetic analyses and taxonomic treatments (Wojciechowski *et al.*, 2004; Lavin *et al.*, 2005; Lewis *et al.*, 2005). In total the sampling included 175 species representing 26 of the 37 tribes of the family, and 89 genera (Table S1 in Supplementary Data, available online; Lewis *et al.*, 2005). For an outgroup comparison four species of the closely related family Polygalaceae were analysed. Polygalaceae also has zygomorphic flowers but of a different constitution (Prenner, 2004*a*; Prenner and Klitgaard, 2008). Comparison with Polygalaceae is problematic as in this family the two adaxial petaloid organs are sepals. The lateral petals are reduced and the two adaxial petals may be considered to have closest functional equivalence to the lateral petals in legumes. The single abaxial petal forms the keel-like structure. We have therefore chosen to compare functionally equivalent structures (Table S2 in Supplementary Data).

Mimosoid legume flowers also have a contrasting morphology (Prenner, 2004*b*) with an abaxial median petal rather than an adaxial median petal. In this case the dorsal petals are compared with the dorsal petal in other legumes and the ventral petal is compared with the ventral petals in other legumes.

To explore the variation within species several individuals were studied in two taxa (*Lotus japonicus*, four individuals; *Trifolium repens*, three individuals) but subsequent sampling used only one individual to represent each species as intra-specific variation was found to be negligible.

The variation among species within the same genus was explored in 11 genera, in all of which four or more species were analysed. In *Senna*, 12 species were included representing three of the six recognized sections (*Chamaefistula*, *Senna*, *Peiranisia*) (Irwin and Barneby, 1982). These species represent all major clades currently recognized in this genus and included the entire range of flower symmetry within the group (Marazzi *et al.*, 2006; Marazzi and Endress, 2008). Seven species of *Lathyrus* belonging to two of the nine sections of this genus (*Lathyrus* and *Orobus*) were studied (Kenicer *et al.*, 2005). In *Lotus* seven species from three of its 14 sections (*Bonjeana*, *Lotus* and *Pedrosia*) were included (Degtjareva *et al.*, 2006). In *Bauhinia*, six species with diverse flower morphology were analysed. In *Cassia*, six species were included with a range of flower morphology and petal differentiation. Six species of *Dalbergia* were analysed. Within *Erythrina*, five species with diverse flower morphology were included. Within *Vicia*, five species were sampled representing the two subgenera *Cracca* (sections *Cracca* and *Cassubicae*) and *Vicia* (sections *Vicia* and *Faba*) currently recognized within this genus (Choi *et al.*, 2006). In *Trifolium*, five species belonging to two of its subgenera, *Chronosemium* and *Trifolium*, and including sections *Trifoliastrum*, *Involucrarium*, *Trifolium* and *Vesicastrum*, were analysed (Ellison *et al.*, 2006). In *Genista*, four species representating the subgenera *Spartocarpus* (section *Spartocarpus*) and *Genista* (section *Spartioides*) were sampled, together with the species *Ulex europaeus* and *Chamaespartium sagittale*, currently considered to be nested within this group (Pardo *et al.*, 2004). Finally, four species of *Dalea* were included.

Microscopy and cell type classification

Petals of fully open mature flowers were the subject of micromorphological studies using either a Hitachi S-2600N or a JEOL JSM-5900 LV scanning electron microscope at an acceleration voltage of 10–15 kV. Either high-vacuum or low-vacuum conditions were used without significant differences on the structures observed. Some species were analysed using a light microscope (Motic B Series) from preserved flowers in 70 % ethanol or from herbarium specimens. In the latter case the flowers were first rehydrated, fixed in FAA, preserved in 70 % ethanol and then analysed. One species, *Lotus japonicus*, was tested under all different conditions to rule out treatment effects or artefacts. Entire or partial petals were mounted on scanning electron microscope stubs with double-sided adhesive tape. The distribution of the epidermal types was recorded using the following four axes (Fig. S1 in Supplementary Data, available online) along which differentiation may occur, as listed below.

(1) A dorsiventral axis within the flower. In the case of zygomorphic (monosymmetric) flowers (Endress, 2001), the dorsal, one lateral and one ventral petal were analysed (Fig. S1(A, C) in Supplementary Data). In actinomorphic (or polysymmetric) flowers all five petals were analysed. In some caesalpinioids, with asymmetric flowers, all five petals were also analysed separately.
(2) The abaxial–adaxial axis within the petal, i.e. upper and lower surfaces.
(3) A proximo-distal axis within each petal, i.e. base to tip (Fig. S1(D–F) in Supplementary Data).
(4) A medio-lateral axis within the petal, i.e. middle to edge (Fig. S1(D–F) in Supplementary Data).

TABLE 1. *Classification of the epidermal types observed in Leguminosae*

Major type group	Major epidermal type	Abbreviation	Figure	Example
Papillose	Papillose conical cells with striations	PCS	2D, J and P	*Lotus japonicus* (standard)
	Papillose knobby cells with a rugose sculpture	PKR	2E, K and Q	*Robinia pseudoacacia* (standard and lateral)
	Papillose lobular cells with striations	PLS	2F, L and R	*Lathyrus venetus* (standard)
Tabular	Tabular rugose cells with longitudinal striations	TRS*	2B, H and N	*Wisteria sinensis* (wings)
	Tabular rugose cells with a granulose sculpture	TRG	2A, G and M	*Calliandra haematocephala* (all petals)
	Tabular flat cells with longitudinal striations	TFS	2C, I and O	*Lotus japonicus* (keel)

* TRS is the most variable type. The main subtypes may be distinguished as follows: (i) cells elongated with dense striation; (ii) cells more or less isodiametric with dense striation; (iii) isodiametric or elongated cells with less-dense striations.

The epidermal types were classified based on cell-shape traits (the primary sculpture) and on the fine relief of the cell wall (or secondary sculpture; Barthlott, 1981, 1990), using the standard terminology regularly applied in similar studies within angiosperms (Kay *et al.*, 1981). The epidermal types were classified into two main types: papillose and tabular (Table 1). These two types were further subdivided based on cell shape and sculpture of the outer surface. The distribution of these epidermal types was then recorded in relation to the four axes described above, either on entire petals (in the case of small flowers) or portions (representing each one of the sections) in the case of species with large flowers.

Reconstruction of evolutionary changes

Character evolution was studied using parsimony (DELTRAN) as implemented in MacClade 4·0 (Maddison and Maddison, 2000) and maximum likelihood using Mesquite (Maddison and Maddison, 2009). No major differences were found using the different methods. Traits were coded as binary characters (absence and presence). The distribution of these traits was mapped on a tree built according to recent phylogenetic analyses of the legumes (Wojciechowski *et al.*, 2004; Lavin *et al.*, 2005). Additional phylogenetic studies of individual genera or tribes were consulted in order to have a fully resolved phylogeny (McMahon and Hufford, 2004; Pardo *et al.*, 2004; Kenicer *et al.*, 2005; Choi *et al.*, 2006; Degtjareva *et al.*, 2006; Ellison *et al.*, 2006; Marazzi *et al.*, 2006). In a few cases where there was a lack of information on specific groups, the polytomies were resolved randomly using MacClade.

RESULTS

Epidermal types within Leguminosae

A total of six major epidermal types were recorded; five of them occurred in the papilionoids, three were found in the caesalpinioids and only one type was detected in the mimosoids (Table 1 and Fig. 2).

Of the major categories only the TRS with striations had marked variation in cell shape, size and in the fine relief of the cell wall. Therefore, this epidermal type was further subdivided into three minor subtypes (i, ii and iii) as described in footnote to Table 1. Some papilionoid species had only TRS on all three types of petals, but these minor differences (as marked by the TRS subtypes) can potentially be used to distinguish petal types in at least some species (Table 1 and Fig. 3).

Strong micromorphological variation in petals is exclusive to papilionoid legumes

The three distinct types of petals found in papilionoids generally showed clear micromorphological differences at the epidermal level (Fig. 4A). Character analysis using parsimony and maximum likelihood indicates that this represents a single character state gain (Fig. 5). The early divergent *Cladrastis* branch of the papilionoids has only two major epidermal types, one occurring on the dorsal and lateral petals, and the other found on the ventral petal. Within papilionoids two groups, the tribe Loteae and the genistoid clade, commonly have the greatest micromorphological differentiation; their standards, wings and keels are each characterized by specific epidermal features. Similarly, all of the five epidermal types that occur in papilionoids tend to be associated with a particular petal type (Table 2). For instance, tabular flat striate cells (TFS) are restricted to keel petals and PCS are generally characteristic of the standard petal. Further details of the association between epidermal types and petal types are given elsewere (Table S2 in Supplementary Data available online).

An interesting feature of papilionoid legumes is the occasional presence of more than one epidermal type on the same petal. In general, the base of each petal has poorly differentiated cells (cells of simple shape, without prominent surface features and characteristics of early developmental stages), while cells in the middle and distal part are more strongly differentiated (Fig. 6A–J). However, in some species, two strongly differentiated but quite different epidermal types were observed in the same petal (Fig. 6). For example, in *Lathyrus* the dorsal petal has mainly TRS but has a border with papillose lobular cells (PLS; Fig. 6K–P).

Loss of micromorphological variation in Indigofera*, Amorpheae and the inverted repeat-lacking clade (IRLC)*

Although most papilionoids have high micromorphological variation along their floral dorsiventral axis, three papilionoid groups do not exhibit this pattern and have only one major epidermal type on all petals. The *Indigofera* clade, the Amorpheae and most species of the IRLC have TRS covering most of their petals. In these groups, PCS are absent from the dorsal petal (although there are numerous exceptions, as noted in Table S2 in Supplementary Data; Fig. 5) and the area

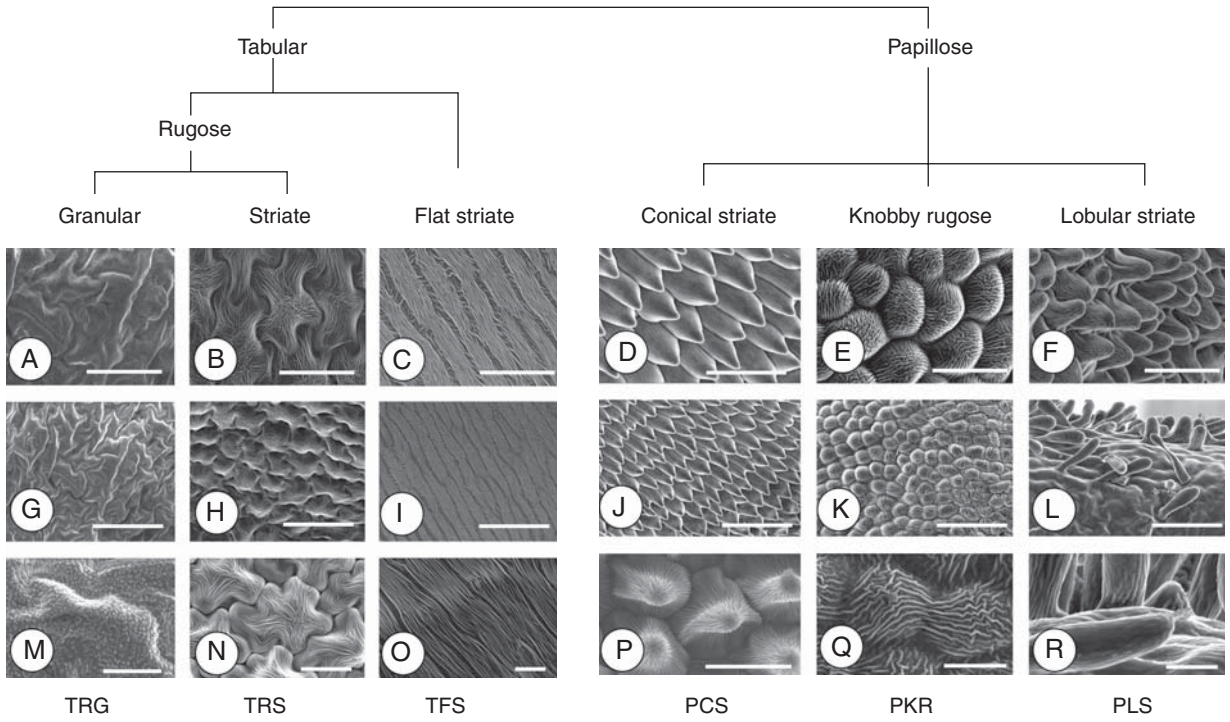

FIG. 2. Classification of the major epidermal types in Leguminosae: (A, G, M) tabular rugose cells with granulose sculpture (TRG) in *Calliandra haematocephala* (Mimosoideae); (B, H) tabular rugose cells with striation (TRS) in *Wisteria sinensis* (Papilionoideae) and (N) in *Lotus japonicus* (Papilionoideae); (C, I) tabular flat cells (TFS) with striations in *W. sinensis* and (O) in *Lotus japonicus*; (D, J, P) papillose conical cells (PCS) in the dorsal petal of *Lotus japonicus*; (E, K, Q) papillose knobby cells (PKR) in the dorsal and lateral petals of *Robinia pseudoacacia* (Papilionoideae); (F, L) papillose lobular cells (PLS) in the dorsal petal of *Lathyrus venetus* and (R) in *Lathyrus sylvestris* (Papilionoideae). This last epidermal type was only observed in these two species. All images correspond to the adaxial side of the petal. Scale bars: (A–F) = 50 μm; (G–L) = 100 μm; (M–R) = 20 μm.

covered by TFS on the ventral petal is commonly reduced to a small region at the tip.

In the Amorpheae, all species analysed have TRS on the three types of petals, with the exception of *Dalea leporina*, which has TRS on ventral and PCS on dorsal and lateral petals. The lack of micromorphological variation is evident both in species with a typical papilionaceous corolla, with three types of petals, such as in *Psorothamnus arborescens* and *Marina* spp. and in species with two petal types, such as *Apoplanesia paniculata*.

It is noteworthy that, although TRS is the dominant epidermal type in all three petals, the ventral petal of these groups may still have a small amount of the TFS epidermal type that characterizes this petal type in other papilionoids. Furthermore, although these groups have the same major epidermal type (TRS) on the dorsal petal and on the wings, in some cases minor differences in cell size and shape were detected that made it possible to distinguish between them easily.

This was particularly clear in the IRLC. Most of the species in this clade have lost the diversity of major epidermal types, as they have only TRS with striations; however, in some instances, striking differences were found between the cell morphology of TRS on different petals within the flower. Variation in several features, such as cell size, shape of the cells (whether elongated or isodiametric), marginal features (such as the waviness of the cell margin) and features of the surface (such as density of striations), allows clear micromorphological identification of each petal type.

Therefore the TRS cell type were further subdivided into three subtypes (i, ii and iii), in which subtype i (TRS^{i}) has elongated cells and subtype ii (TRS^{ii}) has isodiametric cells. In these two subtypes, the cell wall is usually well delimitated with dense striations. In contrast, TRS subtype iii (TRS^{iii}) has elongated cells that tend to have weak cell wall delimitation and a lower density of striations. TRS^{i} and TRS^{ii} were mainly observed on the dorsal and lateral petals, respectively, while TRS^{iii} was found exclusively in the ventral petal (Fig. 3).

In some of the IRLC species, e.g. *Pisum sativum* (Fig. 3A–C), this variation allows all three types of petals to be differentiated, or at least for the ventral (keel petal) to be differentiated from the dorsal and lateral petals, as in *Vicia hirsuta* (Fig. 3D–F) or *Trifolium repens* (Fig. 3G–I). However, in some IRLC species, such as *Melilotus* spp. (Fig. 3J–L), there are no obvious differences between the different petals (Table S2 in Supplementary Data).

Zygomorphy in caesalpinioids is not associated with strong micromorphological variation

The caesalpinioids show micromorphological differences of major epidermal types between species (Fig. 4B–D and Fig. 3M–O). Despite the variation in petal size and shape within flowers of many of the caesalpinioids surveyed

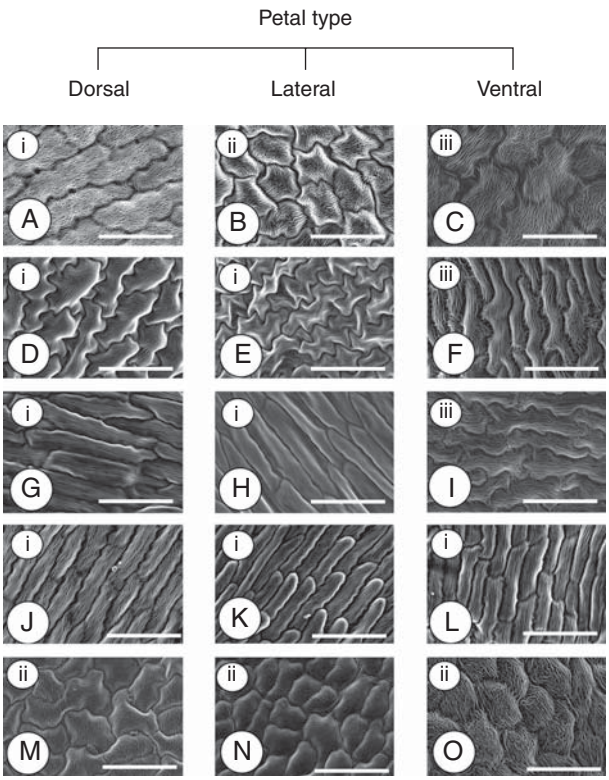

FIG. 3. Minor epidermal types within the tabular rugose cells with striations (TRS). Minor epidermal variants within TRS are designated as i, ii and iii. (A–C) Variation among minor epidermal types in *Pisum sativum* (Papilionoideae) enables each petal type to be distinguished. Variation within minor epidermal types distinguished the ventral but does not distinguish between the lateral and dorsal petals in (D–F) *Vicia hirsuta* (Papilionoideae), (G–I) *Trifolium repens* (Papilionoideae). Variation among minor epidermal types does not allow clear characterization of the three petal types in (J–L) *Melilotus officinalis* (Papilionoideae) and (M–O) *Cercis canadensis* (Caesalpinioideae). All illustrations correspond to the adaxial side of the petal, except (H, I, K, L, N, O), which correspond to the abaxial side. All scale bars = 50 μm.

(Fig. 1A–F), the major epidermal type of each petal within a flower is uniform, usually TRS (64 %) while PCS is less common (Table 3). Character state reconstruction indicates that TRS is the ancestral petal cell type in caesalpinioids and in legumes as a whole.

There is therefore no association of a particular major epidermal type with any specific petal, although some very minor variations in cell size and form were found. Interestingly, even *Cercis*, a caesalpinioid with strongly zygomorphic flowers that superficially resemble papilionoid flowers has all its petals as TRS with only minor differences distinguishing them (Fig. 3M–O).

Species representing all the main variation of flower symmetry within the genus *Senna*, which ranges from radial to asymmetric flowers (enantiostyly) (Marazzi *et al.*, 2006; Marazzi and Endress, 2008) were included. All the petals in individual flowers of this genus have the same major epidermal type (TRS, PCS or PKR), even in species such as *Senna mucronifera* or *Cassia emarginata* (Fig. 1E), where each of the five petals are different in size and shape.

The occurrence of papillose cells in the Leguminosae

Papillose cells (of all types) are particularly characteristic of papilionoids, but also appear to have evolved independently in some caesalpinioids (Fig. 5). Papillose cells (PCS and PKR) are mainly found in the dorsal part of the papilionoid flower (especially the standard). However, some species have papillose cells on the wings. More rarely papillose cells are found on the keel. For example, in *Lespedeza thunbergii* (Fig. 1H) and *Desmodium incanum*, the keel petals are mainly covered by TFS, as in the majority of papilionoids. However, the tip of the keel has some cells intermediate between tabular flat cells (TFS) and PCS in the most exposed area.

In the 175 species analysed in this study, only seven species (*Dalbergia brownei*, *Canavalia rosea* and five species of *Erythrina*) had well-developed PCS on the keel. *Dalbergia brownei* and *Canavalia rosea* display PCS only in small patches. The genus *Erythrina* is notable for having papillose cells (PCS and PKR) on all three types of petal.

DISCUSSION

Functional significance of papillose cell types

Papillose cells are characteristic of many papilionoid lineages and this group of cell types may have evolved in papilionoids due to possible functional advantages. Papillose cells may increase petal brightness and therefore increase pollinator visitation rates (Glover and Martin, 1998; Comba *et al.*, 2000; Dyer *et al.*, 2006).

It is interesting to consider why papillose cells (PCS and PKR) tend to be characteristic of the dorsal and lateral petals in papilionoids (Fig. 4A), while these cell types are virtually absent in the ventral petals of the papilionoid subfamily (Table 2). Functionally, this may be because keel petals are in many cases covered by the lateral petals and are not usually prominent in pollinator attraction. This explanation is supported by the fact that species with papillose cells on the keel generally have an exposed keel which probably functions in pollinator attraction (e.g. *Erythrina*, *Crotalaria* and *Lespedeza*).

Lack of micromorphological variation in the Caesalpinioideae

In contrast to papilionoids, caesalpinioids have relatively little micromorphological variation. Differences between dorsal, lateral and ventral petals do occur but are small and never involve major epidermal types (Fig. 4). This implies that a strong connection between the dorsiventral patterning of the flower and the developmental patterning of cell form, that is so evident in papilionoids, never evolved in caesalpinioids. Within the legumes, this connection between symmetry and cell type differentiation is therefore a unique derived character of papilionoids.

This lack of variation in caesalpinioids is independent of floral patterning, from nearly radial, as in *Bauhinia natalensis* and *B. petersiana*, to strongly zygomorphic flowers, as in *Tara cacalao* or *Cercis* spp. (Fig. 1D). For instance, *Cercis* has highly zygomorphic flowers (Fig. 1A), and still displays much less micromorphological variation (Fig. 3M–O) than a typical papilionoid (Fig. 4A). These results support previous

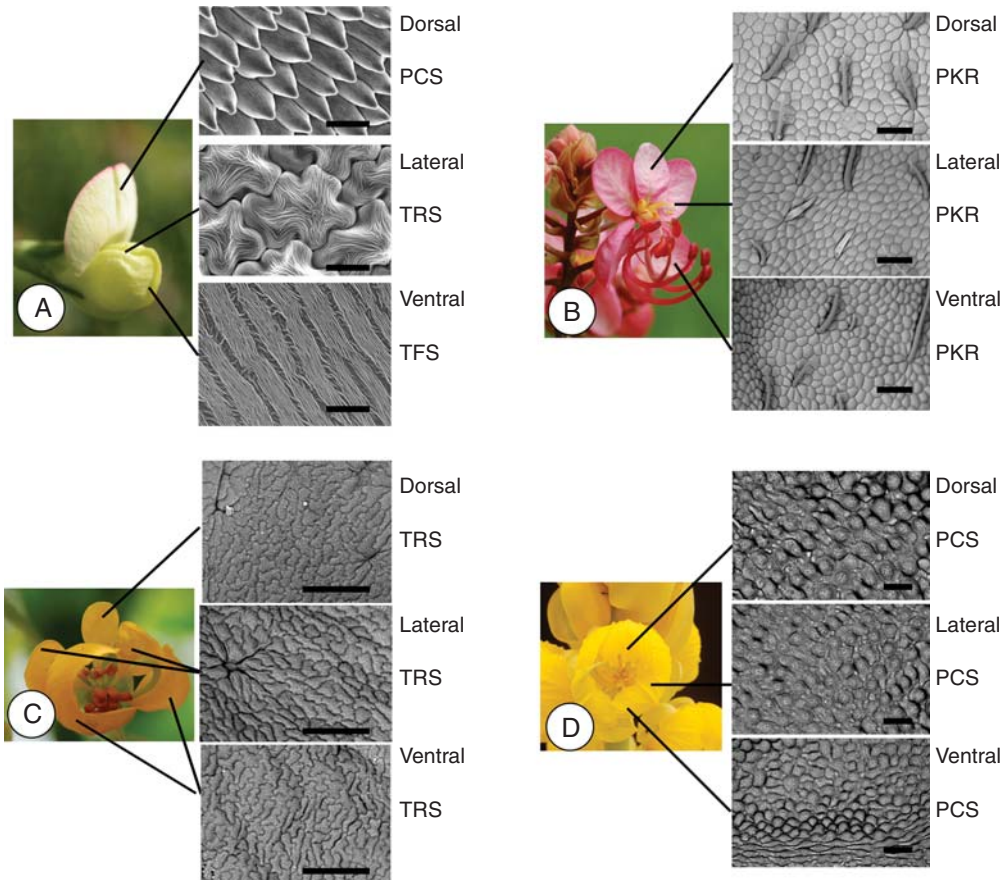

FIG. 4. Distribution of the epidermal types along the dorsiventral (adaxial–abaxial) axis within the flower: (A) micromorphological variation in *Lotus burttii* (as in almost all Loteae) (Papilionoideae); (B) lack of micromorphological variation of major epidermal types in *Cassia roxburghii* (Caesalpinioideae) with only PKR; (C) *Cassia emarginata* (Caesalpinioideae) with only TRS; (D) *Senna alata* (Caesalpinioideae) with only PCS on all petals. All petals have the adaxial side shown, except *L. burttii*, where the abaxial side is presented on lateral and ventral petals and *Senna alata* where images shown the abaxial side. Scale bars: (A) = 20 μm; (B, D) = 50 μm; (C) = 100 μm.

studies suggesting that the floral morphology of this genus, although superficially similar to a papilionoid, is only a weak convergence at the anatomical level to the papilionoid flower (Tucker, 2002).

In most caesalpinioids the adaxial and abaxial surface of the petal have the same major epidermal type. However, this is not the case in *Senna alata* (with all petals having PCS on the abaxial side and PKR on the adaxial one) and *Bauhinia tomentosa* (with PCS on the abaxial side and TRS on the adaxial side of all petals; Table S2 in Supplementary Data). These two species have flowers which do not fully open and it is the abaxial (exposed) (Fig. 1B) surface that has papillose cells, which may enhance brightness and therefore the pollinator attractiveness of the flowers.

Another interesting feature of the caesalpinioids is the presence of trichomes in about 29 % (11 species of 39) of the species analysed and stomata in 15 % (6 out of 39). In most species the distribution of trichomes was homogeneous on all the petals within the flower (all petals having trichomes). This feature is shown in *Cassia emarginata* (Fig. 4B). However, in six species, trichomes were localized on a specific petal and, hence, the distribution of the trichomes could be used as an indicator of petal identity in these species. In Table S2 in Supplementary Data, species with trichomes and stomata are denoted by a superscript 't' and 's', respectively. Trichomes and stomata are also occasionally found in papilionoids but more rarely.

Mixing of epidermal types in the same petal surface

A unique feature of papilionoids is the occasional occurrence of more than one major epidermal type within a single petal. Between the two major epidermal types there is a transition zone or morphocline (Baagøe, 1977; Hansen, 1991), which has been reported previously in other groups of angiosperms (Barthlott and Ehler, 1977; Hansen, 1991, Hansen, 1992; Christensen and Hansen, 1998). The shifts observed involve changes from TRS to PLS (Fig. 6K–P), TRS to PCS (Fig. 6Q–V) or TRS to PKR in the dorsal and lateral petals, and from TRS to TFS in the ventral petal. The transition zone, with intermediate cell morphology between the two major epidermal types, is always relatively narrow (Fig. 6).

If the epidermal cell type is responding to the expression of underlying petal identity genes, as seems to be the case in at least some legumes (Feng *et al.*, 2006; Wang *et al.*, 2008), these morphoclines may then indicate gene expression

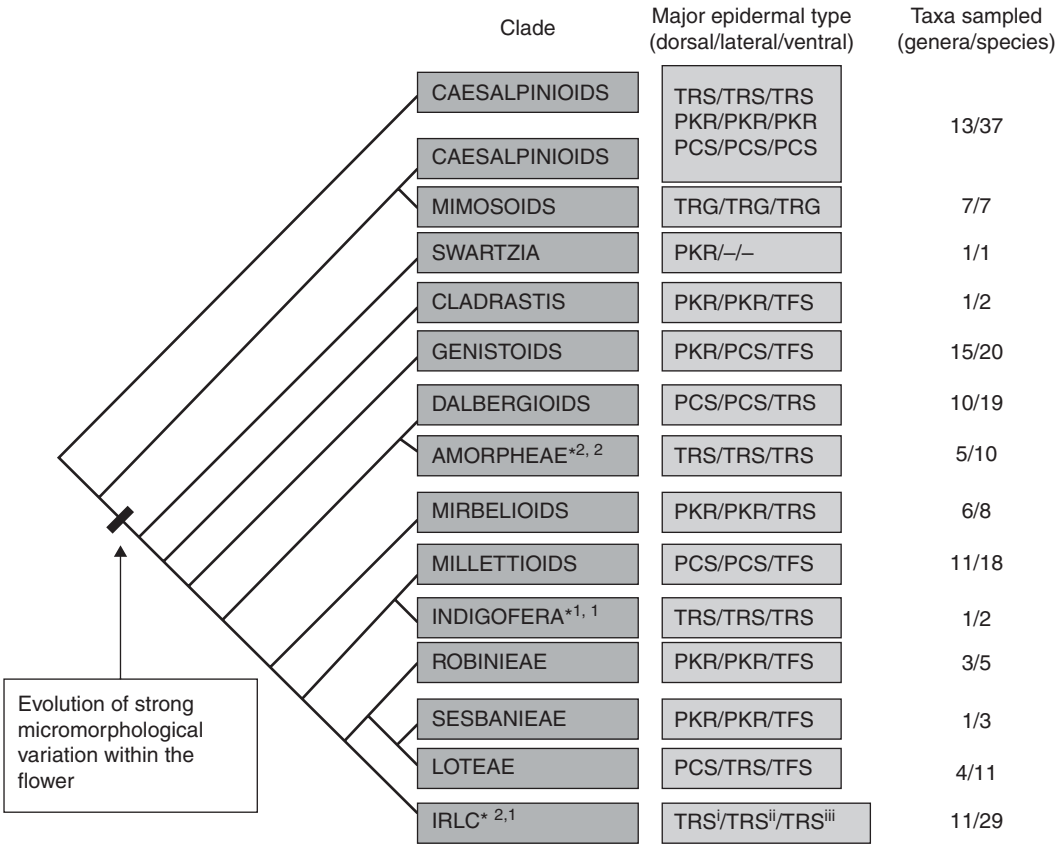

FIG. 5. Schematic representation of the phylogenetic relationships within Leguminosae showing the typical distribution of the major epidermal types observed. This figure is intended to summarize the main patterns but it should be noted that rare variant patterns may occur in clades as well as those listed. The general epidermal surface observed within the flower is given in relation to the dorsiventral axis within the flower, using the representation: dorsal/lateral/ventral petal. Clades with an asterisk contain lineages with loss of papillose cells (PCS and PKR). The numbers following the asterisk indicate the number of losses under parsimony and ML, respectively. A dash indicates lack of this type of petal. [Tree according to Wojciechowski *et al.* (2004); Lavin *et al.* (2005) and Lewis *et al.* (2005).]

TABLE 2. *Distribution of the major epidermal cell types within sampled Papilionoideae*

	PCS		PKR		TRS		TFS	
Petal	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
Dorsal	38	45	42	40	53	48	0	0
Lateral	44	33	38	32	48	66	0	0
Ventral	1	2	4	3	61	61	63	64

PCS, Papillose conical cells; PKR, papillose knobby cells, TRS, tabular rugose cells with striations, TFS, tabular flat cells with striations.

boundaries within organs. It might even suggest that such petals are of 'mixed identity' and hence developmentally composite organs.

Contrasting loss of micromorphological variation within flowers of the Amorpheae and IRLC

Despite the striking micromorphological variation observed within the papilionoid flower, some species lack this variation. This is especially evident in the Amorpheae. It is interesting to note that the Amorpheae is often characterized by flowers of altered zygomorphy. They vary from zygomorphic (flowers with corollas of three types of petals as in *Psorothamnus*, *Marina* and some *Dalea* species) to subactinomorphic (five petals poorly differentiated into two types as in *Apoplanesia* and *Eysenhardtia*), while some species have only one petal (*Amorpha*) and other species lack petals altogether (*Errazurizia* and *Parryella*) (McMahon and Hufford, 2004, 2005; McMahon, 2005).

The tendency to weak dorsiventrality and subactinomorphy in this group is therefore associated with a lack of micromorphological diversity in the major epidermal types. Other dalbergioids, the group to which Amorpheae belongs, generally have flowers typical of other papilionoids.

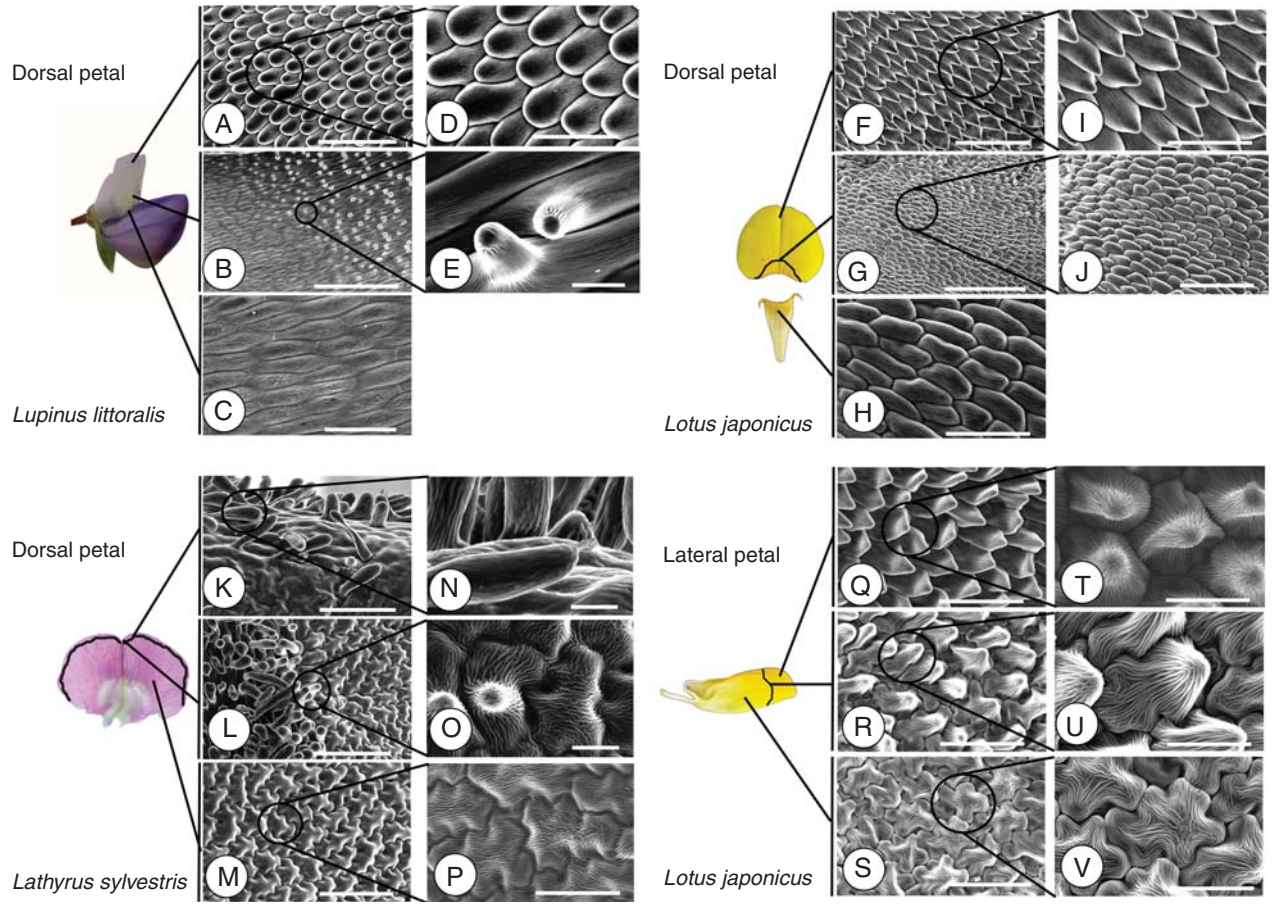

FIG. 6. Distribution of epidermal types along a proximo-distal axis within the same petal. (A–E) *Lupinus littoralis* with transitions from poorly differentiated cells at the base of the adaxial side in the dorsal petal (C), to papillose cells (PCS) on the central part and apex of the petal (A, B). (F–J) the abaxial side of the dorsal petal in *Lotus japonicus* with transitions from poorly differentiated cells at the base of the petal (H) to PCS in the central and distal regions (G, H). A photograph of the standard petal of *Lotus japonicus* is shown besides the images with the claw separated and shown below. (K–P) The adaxial side of the dorsal petal in *Lathyrus sylvestris* has a transition zone from TRS to PLS on the borders of the petal where cells have a mixture of morphological features of both epidermal types. (Q–V) The abaxial side of the lateral petal in *Lotus japonicus* where TRS is mainly observed at the base and in the central part of the petal, and there is a transition zone from TRS to PCS where the cells have a mixture of both epidermal types. Scale bars: (A, C, F, J–M) = 100 μm; (B) = 500 μm; (D, H, I, O–S) = 50 μm; (G) = 200 μm; (E, N, T–V) = 20 μm.

TABLE 3. *Distribution of the major epidermal types within sampled Caesalpinioideae*

	PCS		PKR		TRS	
Petal	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
Dorsal	5	3	10	10	21	23
Lateral	5	3	11	12	22	22
Ventral	5	3	11	12	22	23

PCS, Papillose conical cells; PKR, papillose knobby rugose cells; TRS, tabular rugose cells with striations.

The IRLC has also lost diversity of major epidermal types (TRS being the most common type), but in this case it is not accompanied by any loss of dorsiventral patterning as the flowers are highly zygomorphic and some species show differentiation in subtypes of TRS (Fig. 3). This indicates that the loss of epidermal diversity in Amorpheae and IRLC may be different in mechanism with different underlying genetic control in each group.

Genetic control of petal micromorphology and petal identity

Most studies of genetic control of petal micromorphology have focused on PCS. *MIXTA*, a transcription factor of the MYB family, has been associated with the differentiation of PCS in *Anthirrinum majus*, and in some species of Solanaceae (Noda *et al.*, 1994; Glover and Martin, 2002). In *Petunia hybrida*, an orthologue of *MIXTA*, *mybPh1*, is associated with conical cells (van Houwelingen *et al.*, 1998; Avila

et al., 1993). However, there is not yet any evidence that *MIXTA* homologues play a role in PCS differentiation in legumes.

An additional network of genes has been associated with the jigsaw puzzle shape of *Arabidopsis* leaf pavement cells. Pavement cell morphogenesis is controlled by two antagonistic pathways, Rho GTPase (*ROP*) and *ROP* effector protein (*RIC*) pairs with opposing action. The countersignalling of these two pathways has been associated with the interdigitation and final shape observed in these epidermal cells, with lobes and indentations. The pair *ROP2*/*RIC4* promotes cell growth on lobes, and the gene pair *ROP2*/*RIC1* restrains outgrowth, hence producing the indentations (Fu *et al.*, 2005; Mathur, 2006; Guimil and Dunand, 2007).

There is at present no information as to the precise pathway by which petal identity genes promote the differentiation of the epidermal types described in this survey.

Lotus japonicus CYCLOIDEA 2 (*LjCyc2*), a transcription factor of the TCP family, promotes dorsal petal identity, i.e. the expression of this gene activates other gene networks necessary for dorsal petal traits and it therefore confers specific organ fate on the primordium in which it is expressed. *LjCyc2* expression is therefore necessary for the differentiation of PCS in this petal (Feng *et al.*, 2006). Overexpression of this gene in transgenic plants promotes a dorsalization of all petals, with the consequent production of PCS cells in all petals.

In *Lotus japonicus*, *LjCyc2* is exclusively expressed on the adaxial side of the flower (dorsal petal) and thus affects dorsal petal identity (Feng *et al.*, 2006). It has been demonstrated that in species with a gain of dorsal identity, such as *Cadia purpurea*, this gene is expressed throughout the flower, and all petals have the same shape, symmetry and identity (Citerne *et al.*, 2006). Studies of the petal micromorphology of *Cadia* and related species would therefore be of great interest.

Another gene, *Lotus japonicus KEELED WING 1* (*KEW1* or *LjCyc3*), is associated with lateral petal identity. *LjCyc3* is required for normal lateral petal development, which includes TRS with a jigsaw puzzle shape. As in *LjCyc2*, mutations that knockout the activity of *LjCyc3* causes a ventralization of the lateral petal, with a subsequent lack of TRS and the presence of TFS (Feng *et al.*, 2006).

To date, the role of *LjCyc2* has only been explored in detail in *Lotus japonicus* (Feng *et al.*, 2006) and *Pisum sativum* (Wang *et al.*, 2008). Orthologues of *LjCyc2* (*PsCyc2*) and of *LjCyc3* (*PsCyc3*) have recently been cloned in *Pisum sativum* (Wang *et al.*, 2008). These two genes are required for normal zygomorphic development. *PsCyc2* is associated with dorsal identity and *PsCyc3* with lateral identity. But unlike *L. japonicus*, all petals have the same major micromorphology (TRS) and the identity of each petal (dorsal, lateral and ventral) is associated with the variation of features within this major epidermal type (Wang *et al.*, 2008). Homologues of *CYCLOIDEA* have also been explored in Genisteae (Citerne *et al.*, 2003, 2006; Ree *et al.*, 2004).

Therefore, the activation of the *ROP*/*RIC* pathway, essential for jigsaw puzzle cell shape, must be downstream of the *LjCyc3* identity gene.

No ventral identity gene has yet been found, and the cause of ventral petal identity (and hence TFS differentiation) in legumes is still unknown. However, in both *Lotus japonicus* and *Pisum sativum*, the double knockout of the dorsal and lateral identity genes causes a ventralization of all the petals, suggesting that ventral identity and TFS are perhaps the default states in these groups.

Evolution of petal micromorphology

In the present study it has been shown that in the Caesalpiniaceae different petals within a flower are not strongly distinguished micromorphologically and the most common pattern is TRS/TRS/TRS (in dorsal, lateral and ventral petals, respectively). The ancestral state reconstruction analysis suggests that this pattern probably represents the ancestral condition in the Leguminosae (Fig. 5). More or less papillose cells have independently evolved at least six times in the Caesalpinioideae and, when they occur, species have either PKR (in all petals) or PCS (in all the petals). Papillose cells appear to be absent in Mimosoideae, and their very small petals all have TRG.

In addition, the present study suggests that the strong micromorphological differentiation within the flower is an advanced condition of the family that has evolved only within the papilionoid clade. Dorsiventral differentiation of major epidermal types appears to have evolved at the base of the papilionoid clade (with the most primitive papilionoids state being PKR/PKR/TFS), reaching maximum differentiation independently in Loteae (PCS/TRS/TFS) and Genistoids (PKR/PCS/TFS).

Papillose cell types appear to have evolved many times in legumes but may have evolved only once in papilionoid legumes (at the base of that clade) and this character has apparently been lost at least four times in the subfamily (Fig. 5). However, most of the papilionoid lineages that have lost papillose cells (groups predominantly with TRS/TRS/TRS as a character reversal), still display dorsiventral differentiation between petals by means of different TRS subtypes (e.g. TRS^{i}, TRS^{ii} and TRS^{iii}).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following files. Table S1: List of species sampled during this study. Table S2: Distribution of the major epidermal types in each of the three types of petal in the Leguminosae. Fig. S1: The four axes of variation considered in the study of epidermal types and their distribution on each petal.

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