## CLONAL ANALYSIS BY TRANSPOSON EXCISION OF STOMATAL CLUSTERS IN ARABIDOPSIS

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Most stomatal complexes in Arabidopsis are composed by five cells, in an arrangement characteristic for many species in the Brassicaceae family: the two guard cells that form the stomata proper are surrounded by three subsidiary cells, constituting what is called an **anisocytic stomatal complex**. Early anatomical descriptions on the development of these complexes in some crucifers revealed that both the guard cells and the subsidiary cells that integrate the stomatal complex derive from a single protodermal cell (Paliwal, 1967; Pant and Kidwai, 1967). However, a similar study has not been made in Arabidopsis, while the precise ontogeny of stomatal complexes is a necessary background information to address further developmental questions in the leaf epidermis, such as acquisision of cell identity or pattern formation during stomata development.

While studying the pattern of the different leaf epidermal cells in Arabidopsis plants grown in sterile culture (where gas exchange was limited, and edogenous ethylene could accumulate to high levels) we found that the conserved anisocytic stomata complexes were replaced by stomatal clusters (Serna and Fenoll, 1996; Serna and Fenoll, submitted). Under such growth conditions, paired stomata which shared one of the subsidiary cells (Figure 1A), or formations in which the two stomata were contiguos (Figure 1B) were found. These paired structures looked like phenocopies of the atypical stomata found in the *four lips* (*flp*) mutants (Yang and Sack, 1995). Stomatal clusters integrated by three or more paired guard cells were also often found (Figure 1C thought 1F). These clusters exhibited variable geometries, from linear (Figure 3F) to compact groups (Figure 3D) of stomata, resembling the atypical stomata found in the *too many mouths* (*tmm*) mutant (Yang and Sack, 1995).

We have described the ontogeny of stomatal clusters by following promoter induction for three genes that are markers of competence for cell division (*cdc2a*) (Hemerly et al., 1993), mitotic activity (*cyc1At*) (Ferreira et al., 1994) and guard mother cell and developing guard cell identity (*rha1*) (Terryn et al., 1993). Marker transgenic Arabidopsis lines were used to dissect the expression of the reporter gene *gus* under the control of *cdc2a*, *cyc1At* and *rha1* promoters during the development of stomatal clusters. The patterns for the three molecular markers indicated that each cluster derives from a single protodermal precursor cell through a process that involves assumption of an unscheduled fate by a subset of subsidiary cells (Serna and Fenoll, 1996; Serna and Fenoll, submitted).

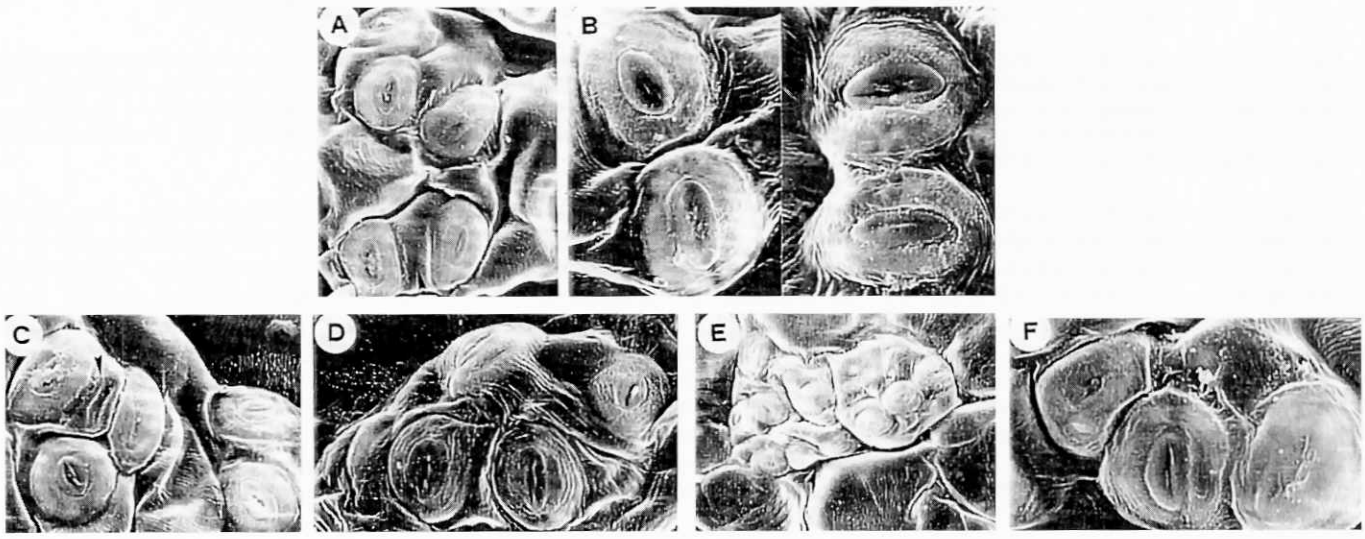


Figure 1. Scanning electron microscopy of stomatal clusters. (A) and (B) Twinned stomata. Arrow in (A) shows a subsidiary cell between two stomata. Note the absence of subsidiary cell between stomata and the diferent orientation of the division planes in (B). (C) thought (F) higher order clusters from the same blade. The arrow shows a collapsed guard cell complex between two stomata in (C). A maduration gradient is clearly visible in (F) (note the diference in size and pore shape).

By a combination of mutant analysis and specific inhibitors, we have shown that the plant hormone ethylene can induce stomatal clusters in Arabidopsis under controlled conditions (see the acompanying report by Serna and Fenoll, this volume). In this work we induced stomatal clusters by treating the plants with 1-aminocyclopropane-1-carboxylic acid (ACC), which is taken up by the plants and converted to ethylene by ACC oxidase (Adams and Yang, 1979). In order to address the ontogeny of these clusters, we have used transgenic plants for a fusion of the cuasi constitutive *35S* promoter to the reporter gene *gus*.. The construct is interrupted at the *promoter-gus* boundary by the *Ac* transposable element (Lawson et al., 1994). Althought the *Ac* element prohibits the *gus* expression in the transgenic plants, random *Ac* excision during development in a given cell leads to the constitutive *gus* gene expression in all descendants of this precursor cell, detectable as blue coloured patches after staining with

the apropiate substrate. As the plant grows, these patches give rise to blue sectors of variable sizes, which can include different tissues, depending on the history of those cells that express *gus* gene.

For this work we selected plants with blue leaf sectors in which *gus* expression was restricted to the epidermal tissue (Figure 2A and 2B). This expression pattern is possible because in Angiosperms cell divisions in the outermost layer of the shoot apical meristem (L1 layer) are restricted to an anticlinal plane. Because of that, the L1 layer gives rise exclusively to the epidermis. Thus, any *Ac* excision in a cell belonging to the L1 layer results in blue sectors which include only epidermal cells (namely L1 sectors). After histochemical GUS staining, transverse sections were used to select leaves showing L1 sectors. Selected leaves were mounted on slides and the borders of sectors generaded by *Ac* excision were examined under bright-field microscopy to detect stomatal clusters. If stomatal clusters arise from indepent cells late in development, then blue and unstained cells should be present in clusters that overlap wih the border line in the sectors. If, on the contary, all cells in a cluster derive from a unique protodermal cell, then any given cluster should have only blue or only unstained cells, even clusters placed along the borderline of L1 sectors. Preliminar analysis indicate that all stomata that integrate most of the stomatal clusters located in borders of L1 sectors are either blue or unstained, supporting a clonal origin from each cluster. Ocasionally, we found in sector borders clusters which express *gus* only in some stomata, or even in one guard cell within the stomata. Two events may explain this finding: 1) the *Ac* element may transpose late during cluster development; 2) some clusters may arise form the fusion between two independent proliferanting clusters. Detailed examination of younger clusters will allow to discriminate between these two possibilities.

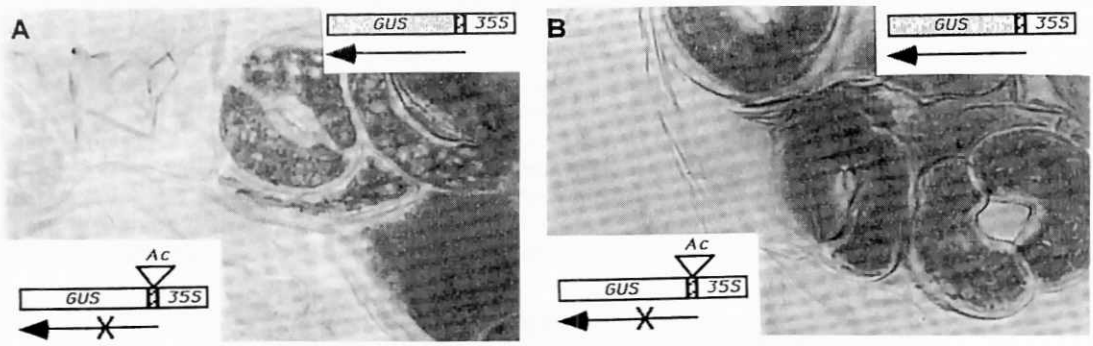


Figure 2. Clonal analysis of cell lineage associated with the stomatal clusters development by transposon excision. Stomatal clusters adjacent to the margen of a sector induced by *Ac* element excision showing *gus* expression from all stomata that integrate them which indicate the importance of cell lineage associate to the development of these complexes. Plants were grown in medium supplemented with 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is the intermediate in the conversion of methionine to ethylene. The transgenic plants containing a *35S-gus* gene interrupted by a *Ac* transposon was generously provided by Caroline Dean (John Innes Center, Norwich, UK).

Our preliminar analysis of borders in sectors induced by *Ac* element excision indicate that stomatal clusters arise from a single protodermal cell. This finding is in agreement with our previous results obtained through the expression analysis of the *cdc2a*, *cyc1At* and *rha1* promoters during development of stomatal clusters (Serna and Fenoll, 7th International Conference on Arabidopis Research. Norwich, UK). In this work we described the development of an initially anisocytic stomatal complex from a single protodermal cell. In these complexes, a subpopulation of subsidiary cells showed *cdc2*, *cyc1At* and *rha1* expression, indicating that such cells can both assume guard mother cell identity and also enter a mitotic cycle. The combination of these two events made possible that a subsidiary cell develop into a stoma inmediatly adjacent to another stoma, determining the appearance of a cluster from a single protodermal cell. The results that we present now gives further support to the hypothesis proposed on the basis of this mechanism, providing the confirmation of the clonal origin of stomatal clusters, which can be traced back to a single protodermal cell. How genetic and environmental factors modulate the actual developmental fate of this cell, determining the formation of a typical stomatal complex or of the anomalous clustered stomata is currently being studied.

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