EHTYLENE INDUCES STOMATA DIFFERENTIATION IN ARABIDOPSIS

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A common feature to multicellular organisms is the acquisition of different cell fates during development. In this process, from an apparently homogeneous cell layer or cell mass, an array of different cell types is built into a differentiated tissue. The presence of root-hair cells and hairless cells in the root epidermis, and of trichomes, guard cells and pavement cells in the leaf epidermal layer of Arabidopsis is a clear examples that show the wide diversity of cell fates that can be generated during the development of the specialized epidermal tissue. It is not a new observation that the distribution of these specialised cell types in the epidermis is not at random, but that they follow instead an ordered arrangement which is remarkably conserved. During the last years, a tremendous progress has been made in the genetic control of the developmental pathways that lead to trichome and root hair patterning and differentiation in Arabidopsis. In contrast, very little is known about the mechanisms that regulate stomata differentiation and spatial distribution in the leaf blade.

Stomatal complexes in many Brassicaceae species (the family which includes Arabidopsis) are anisocytic. This term applies to complexes of epidermal cells which include the two guard cells (the stoma proper), surrounded by three subsidiary cells. Both the guard cells and the subsidiary cells that integrate these complexes derive from a single protodermal cell (Paliwal, 1967; Pant and Kidwai, 1967). Equivalent information for the model plant Arabidopsis has been lacking. The presence of similar complexes in Arabidopsis leaves, together with the expression analysis of several genes which are markers for cell division and cell identity, strongly suggests that a cell lineage may be associated with the development of stomatal complexes (Serna and Fenoll, in preparation). This fact imposes that stomata should always be separated by at least two non-guard cells, and could explain why stomatal complexes are regularly spaced in the epidermis. Recently, two mutants exhibiting clustered stomata in the cotyledons have been described with the self-explanatory names of *too many mbuths (ttm) and four lips* (*fls*) by Yang and Sack (1995). These mutants have opened the possibility for genetic dissection of stomata spacing. In a previous work, we described the induction by growth conditions of phenocopies of these mutants in wild-type Arabidopsis plants, and traced the ontogeny of stomatal clusters with molecular markers (Serna and Fenoll, 1996). Because these stomatal clusters were induced in plants grown in different types of sterile cultures whereever gas exchange was limited, we suggested that endogenously produced ethylene could accumulate to high levels under these conditions and could be related to cluster formation.

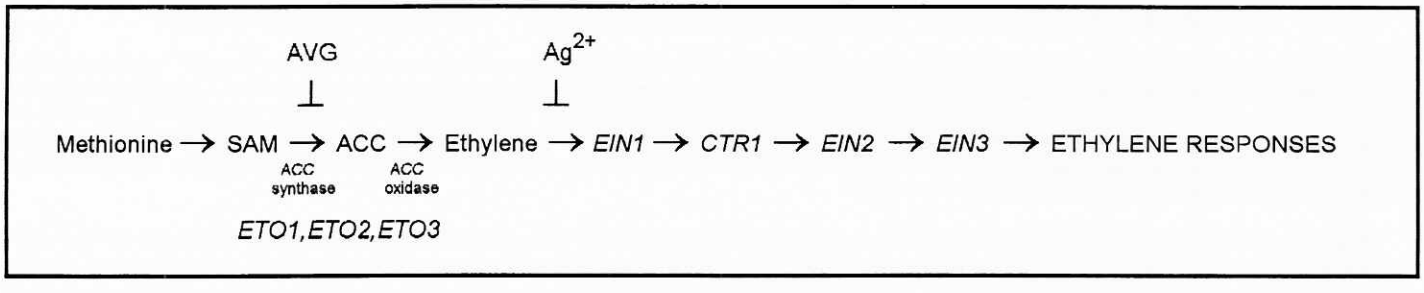

Figure 1. Ethylene biosynthetic and signal transduction pathway. The biosynthetic pathway described by Yang and Hoffman (1984). The order of gene action in the signal transduction pathway is based on double-mutant analysis (reviewed by Zarembinski and Theologis, 1994). SAM: S-adenosyl-L-methionine. ACC: 1-aminocyclopropane-1-carboxylic acid. AVG: aminoethoxyvinylglycine.

Plant growth and development are regulated by complex interactions between endogenous signals and environmental cues. The gaseous hormone ethylene (C_2H_4) is involved in many developmental processes including seed germination, seedling growth, leaf abscision, flower and fruit senescence, sex determination and responses to pathogen attack. The ethylene biosynthetic pathway from Methionine takes place in only two steps, catalyzed by two well-known enzymes, ACC Synthase and ACC Oxidase (see Figure 1). Experimental interference with this pathway can be carried out by specific inhibitors of these enzymes, by gene inactivation in transgenic plants or by genetic mutations such as those present in several ethylene overproducer (*eto*) mutants. Similarly, the pathway of ethylene perception and ethylene action has also been defined in genetic terms by a collection of mutants which can be ordered in a linear array. In addition, hormone perception by the proposed ethylene receptor (EIN1) can be blocked by silver ions. Midway in the signal transduction path lies a repressor protein (CTR); mutations that inactivate this protein constitutively promote the ethylene responses, in the absence of the hormone (*constitutive triple response* mutant, *ctr1* (Kieber et al., 1993)).

This plant growth regulator has also been shown to alter the cell patterning in the root epidermis of Arabidopsis inducing 'ectopic' root hair differentiation (Dolan et al., 1994; Tanimoto et al., 1995), but no effect on aereal tissues has been reported. We have taken a pharmacological approach using the hormone precursor 1-aminocyclopropane-1-carboxylic acid together with inhibitors of ethylene synthesis and ethylene perception, in parallel with a genetic approach using the *ctr1* mutant which has constitutive activation of the ethylene response. Our results provide the first evidence that ethylene modulates stomata patterning in the leaf epidermis of Arabidopsis, perhaps by acting on cell differentiation during leaf development.

To test our hypothesis on the ethylene effect upon formation of stomatal clusters, we have compared stomata spacing in wild-type plants grown in the presence of an ethylene precursor. When Arabidopsis plants were grown in medium supplemented with exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) which is converted to ethylene by ACC oxidase (Adams and Yang, 1979), a higher than normal density of stomata was observed; often two stomata shared a subsidiary cell, (Figure 2A) contrary to the typical anisocytic complexes described above. In contrast, when aminovinylglycine (AVG) was added, preventing ethylene synthesis by ACC synthase inhibition (Yang and Hoffman, 1984), stomata were separated by at least two epidermal cells (Figure 2B). This stomata spacing might be a direct consequence of the possible cell lineage associated to stomata development. Similary, supplementing the growth medium with silver ions (Ag^{2+}) which block ethylene percepcion (Beyer, 1976), the normal stomata pattern exhibited in the presence of AVG was reproduced again (data not shown). These results show that exogenous ethylene induces stomatal clusters, while inhibition of either ethylene synthesis or perception prevents cluster formation in the presence of hormone precursor. Therefore, differentiation of stomata in the leaf epidermis is positivey regulated by ethylene.

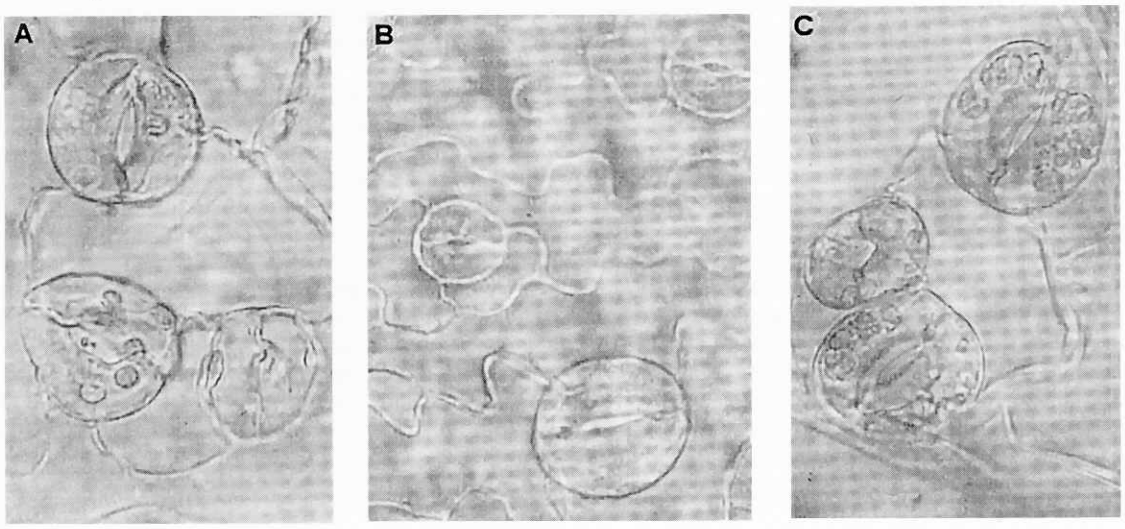

Figure 2. Effects of ethylene on the stomata patterning in the adaxial surface of fully expanded first rossete leaves of wild-type and *ctr1* mutant (provided by the Nottingham Arabidopsis Stock Center). (A) Three-week-old wild-type Arabidopsis germinated and grown in the presence of 1-aminocyclopropane-1-carboxylic acid (ACC) or (B) in medium supplemented with aminovinylglycine (AVG). (C) Three-week-old *ctr1* mutant grown in the presence of aminovinylglycine (AVG). The stomata clustered are clearly visible in (A) and (C); in (B) stomata are separaded by at least two cells which may be a direct consequence of the possible cell lineage associated to the development of these complexes. ACC is the intermediate in the conversion of methionine to ethylene. AVG inhibits the conversion of methionine to ACC.

If ethylene induces stomata clusters because it somehow interferes with pre-established differentiation pathways in the leaf epidermal cells, then *ctr1* mutant plants which are phenocopies of wild-type plants treated with exogenous ethylene should also show disruption of normal stomata spacing . As expected, *ctr1* mutant plants present a high frequency of stomata in which the minimal distance between stomata -two cells- has been reduced, resulting in stomata sharing subsidiary cells, a characteristic of ethylene treatment. Twinned stomata resembling the four lips phenotype (see Serna and Fenoll, this volume) were also found at a lower frequency. When *ctr1* mutant plants were grown in medium supplemented with AVG this atypical stomata spacing was not reverted (Figure 2C), a prediction from the model depicted in Figure 1. The analysis of additional ethylene mutants, which is being performed, will further demonstrate that ethylene induces cell differentiation in the leaf epidermis. The combined use of the ethylene inhibitors and specific Arabidopsis epidermal-cell marker lines will allow to address the question of which particular cells in the developing epidermis are responding to this morphogen.

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