Stomatal cell biology

Uritza von Groll and Thomas Altmann*

In the past two years, major advances in our knowledge of the cellular events involved in the formation of stomatal complexes, particularly those responsible for the establishment of the stomatal pattern, have been made. These events are altered in the *Arabidopsis* mutants *sdd1-1*, *tmm* and *flp*. Molecular cloning of the *SDD1* gene initiated the elucidation of the underlying molecular mechanisms. The proposed function of SDD1 as a processing protease provides a hint towards the involvement of a proteinaceous signal in the formation of stomatal complexes.

Addresses
*Max-Planck-Institute of Molecular Plant Physiology, Dept. Willmitzer, Am Mühlenberg 1, 14476 Golm, Germany;
e-mail: altmann@mpimp-golm.mpg.de

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Abbreviations

cer	*eceriferum*
cop1–10	*constitutive photomorphogenic1–10*
erh3	*ectopic root hair3*
flp	*four lips*
GC	guard cell
GCPEPC	*GUARD CELL PHOSPHOENOLPYRUVATE CARBOXYLASE*
GCPRP	*GUARD CELL PROLINE-RICH PROTEIN*
GMC	guard mother cell
HIC	*HIGH CARBON DIOXIDE*
KAT1	K^+_{in}-channel gene from *Arabidopsis thaliana*
KST1	K^+_{in}-channel gene from *Solanum tuberosum*
MMC	meristemoid mother cell
RHA1	ras-related GTP-binding protein gene in *Arabidopsis thaliana*
sdd1-1	*stomatal density and distribution1-1*
SM	satellite meristemoid
tmm	*too many mouths*

Introduction

Stomata are specialised cellular structures in the epidermis of aerial plant organs that control gas exchange (i.e. H_2O release and CO_2 uptake) between the plant organs and the atmosphere by modulating the aperture of the stomatal pore flanked by two guard cells (GCs). For the establishment of an optimal flow of gases, the positioning of the stomatal pores in the epidermis is just as important as the ability to open and close the pores [1]. In almost all plant species, stomata are separated from each other by at least one epidermal cell, providing an intermediate level of order to the distribution pattern [2]. In addition to the spacing control, stomatal density is modulated in response to endogenous and exogenous factors.

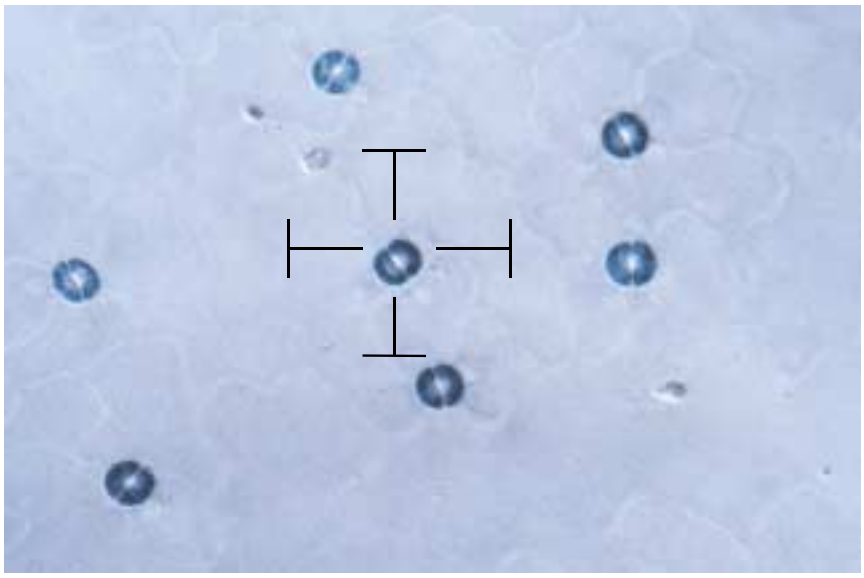
In this review, we focus on the mechanisms regulating stomatal pattern formation and present an overview on stomatal development and differentiation. Recent studies of the *Arabidopsis* stomata mutants *stomatal density and distribution1-1* (*sdd1-1*) and *too many mouths* (*tmm*) have uncovered the cellular events that occur during stomatal development, and provide a doorway to the elucidation of the molecular mechanisms responsible for stomatal patterning [3••,4•].

Stomatal development and differentiation

The development and differentiation of stomatal complexes have been studied in detail in several different plant species [5,6,7•]. In both monocotyledons and dicotyledons, the first visible event in this process is the formation of a meristemoid [8]. These meristemoids continue to divide while division of most of the surrounding cells stops.

In dicotyledons such as *Arabidopsis*, the formation of the stomatal cell lineage involves several cell-fate choices during the cell divisions preceding the differentiation into GCs. A protodermal cell (i.e. a stomatal initial or meristemoid mother cell [MMC]) that enters the pathway leading to stomatal complex formation undergoes an unequal division to form a meristemoid, the smaller daughter cell (which is usually triangular), and a neighbouring cell. The meristemoid undergoes further asymmetric divisions that are oriented in such a way that the smaller daughter cell of the final division is located at the centre of the resulting cell complex. Prior to an asymmetric division, the meristemoid enlarges and positions its nucleus and a preprophase band of microtubules to one side of the cell, while its vacuole locates to the other. After the final asymmetric division, the meristemoids have evenly thickened cell walls and are often triangular in shape. The meristemoids develop into guard mother cells (GMCs), which are characterised by a more oval shape, wall thickenings at opposite ends of the cell, and increased starch accumulation [7•]. In monocotyledons, such as maize or *Tradescantia*, the rectangular-shaped stomatal initial cells, the products of an asymmetric division, develop into GMCs without any intervening cell divisions. In both monocotyledons and dicotyledons, the GMC divides symmetrically into the two GCs, which then differentiate to acquire their unique structural and biochemical features [9]. In *Arabidopsis*, wall thickening marks the symmetric division sites of the GMC because they occur where the cell plate fuses with the parent cell wall. The GC walls are especially thick at the outer edge that faces the atmosphere, along the pore, at the region facing the substomatal cavity and where the outer periclinal wall joins with the neighbour cell. In mature GCs, starch accumulation is increased, large vacuoles are located at both ends of the GC, and the nucleus is usually located opposite the pore and closer to the inner paradermal wall of the GC [7•]. Mechanisms that accompany the development of stomatal complexes, such as the unequal distribution of cell-fate determinants during asymmetric divisions, have been suggested to explain the different cell fates that arise during the formation of stomata [7•].

Figure 1



Current Opinion in Plant Biology

The presence of a stomata-free region surrounding each stoma is the universal principle in stomatal patterning. In wild-type plants (*Arabidopsis thaliana* variation C24), stomata are separated by at least one epidermal cell. For better visibility, GCs expressing β-glucuronidase (GUS) were histochemically stained with X-gluc.

In some dicotyledons, such as *Arabidopsis*, any of the subsidiary cells have the potential to divide asymmetrically to form a satellite meristemoid (SM), thereby producing secondary stomatal complexes through re-iteration of the process described above. In the same manner, tertiary complexes or complexes of even higher order can be generated [3••,4•,10].

GCs perform a highly specialised function, that is, they undergo turgor-driven movements that cause the opening and closure of the central pore of the stomata. Accordingly, it can be expected that these cells exhibit a specific gene expression pattern. The *RHA1* (encoding a ras-related GTP-binding protein in *Arabidopsis thaliana*), *KAT1* (encoding a K^+_{in}-channel from *Arabidopsis thaliana*) and *HIC* (*HIGH CARBON DIOXIDE*) genes in *Arabidopsis* and the *KST1* (encoding a K^+_{in}-channel from *Solanum tuberosum*), *GCPEPC* (*GUARD CELL PHOSPHOENOLPYRUVATE CARBOXYLASE*) and *GCPRP* (*GUARD CELL PROLINE-RICH PROTEIN*) genes from potato are predominantly expressed in GCs [11,12,13••,14–16]. *KAT1* and *KST1* encode inwardly rectifying potassium channels, which are critical components for stomatal opening and which contribute to the cell-type-specific function. PEPC is believed to be one of the key enzymes in the malate-production pathway acting during stomatal opening. The *HIC* gene encodes a putative 3-keto acyl coenzyme A synthase, an enzyme involved in the synthesis of very-long-chain fatty acids. Under elevated CO_2, *hic* mutants show increases in both stomatal index and density, and so it was suggested that *HIC* is a negative regulator of stomatal development that is involved in the response to high CO_2 concentrations. The *RHA1* gene encodes a GTP-binding protein of the YPT/Rab-family, whose specific function in GCs is unknown. *GCPRP* encodes a repetitive proline-rich protein (RPRP). RPRPs are a subgroup of the Hyp-rich glycoproteins, a major class of structural proteins present in the primary cell wall of higher plants. The strong expression of the *StGCPRP* gene in GCs indicates that its encoded protein has an important function in the specialised stomatal cell wall. Two proteins similar to StGCPRP are known to be encoded by the *Arabidopsis* genome.

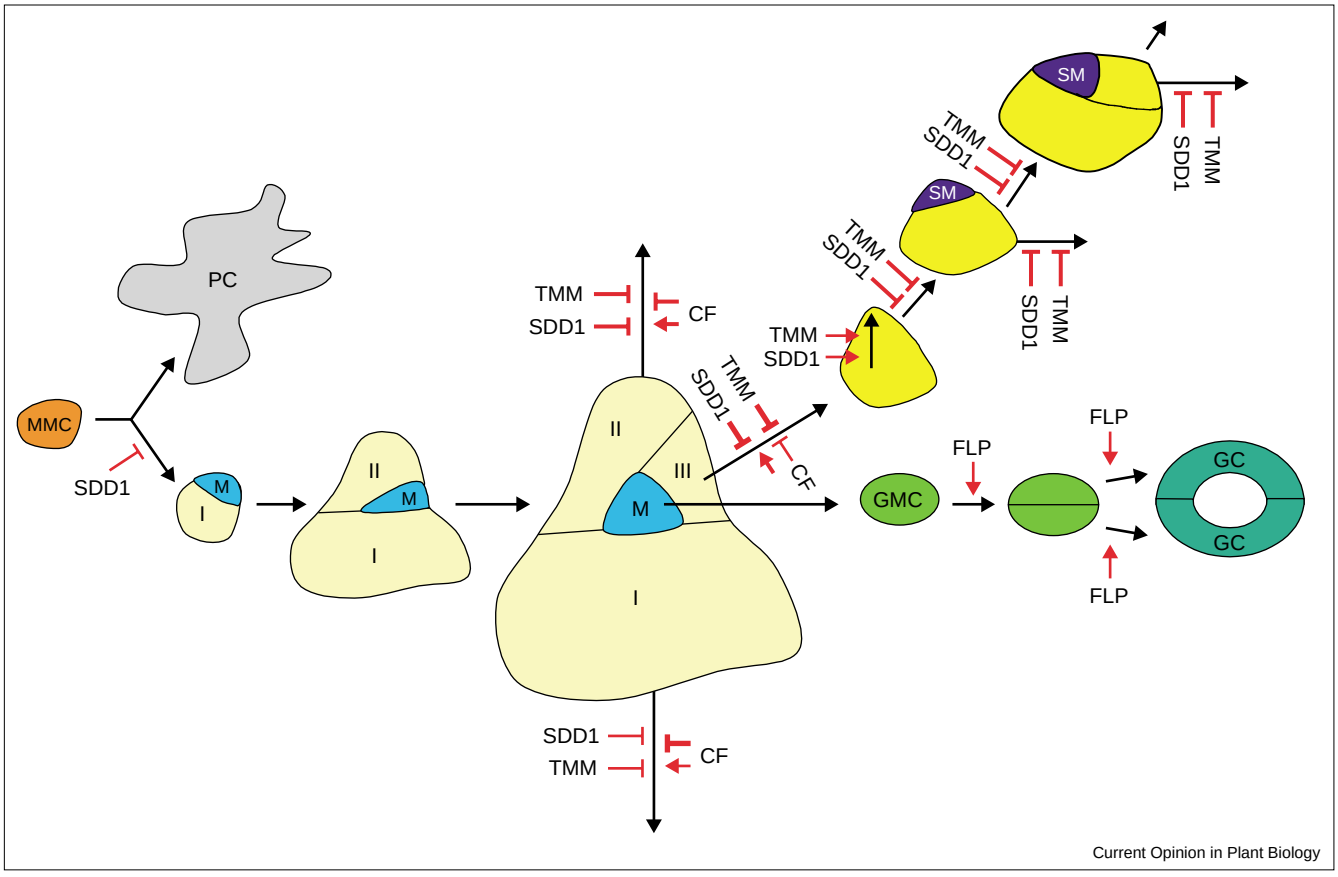
Although *KST1*, *KAT1*, *RHA1* and *GCPRP* are predominantly expressed in GCs, *KST1*-promoter activity was also observed in flowers; in addition, the KAT1 promoter is active in root vasculature. *RHA1* is expressed in roots and stipules, and *GCPRP* is highly expressed in mesophyll cells of young leaves. Of the genes mentioned, only the *HIC* and *GCPEPC* genes appear to be exclusively expressed in GCs.

Stomatal pattern formation

Under natural growth conditions, stomata are not randomly distributed [1]. The established stomatal pattern can be evaluated by determination of the 'R value', a parameter defined as the ratio of the measured average distance between nearest neighbours and the average nearest neighbour distance expected for a random pattern with the same (average) density [17]. A completely random distribution would have an R value of 1, whereas a completely ordered pattern would have a value of R=2.15. For stomatal patterning, R values of about 1.4 were obtained [2,18], indicating a limited degree of order.

The major and universal principle of order in stomatal patterning is the presence of a stomata-free region surrounding each GC pair, this prevents immediate contact between the GCs of neighbouring stomata [19]. In wild-type plants,

Figure 2



A model of stomatal development and pattern formation in *Arabidopsis thaliana*. A stomatal cell lineage is initiated by an unequal division of a protodermal cell (the stomatal initial or MMC; orange), which produces a meristemoid (M; light blue) and a neighbouring cell (the subsidiary cell; yellow). The meristemoid successively undergoes further asymmetric divisions, finally resulting in the formation of a centrally located cell, which converts into a GMC (light green). The GMC divides symmetrically to produce two GCs (dark green). The neighbouring cells (I, II and III), predominantly the most recently formed (i.e. III), have the potential to divide asymmetrically to form SMs (dark blue). SMs may undergo up to two further asymmetric divisions, thereby producing secondary stomatal complexes. In the same manner, tertiary or even higher order stomatal complexes can be generated. Alternatively, the protodermal cell can differentiate into a pavement cell (PC; grey). The gene products of *SDD1*, *TMM* and *FLP* and putative 'competence factors' (CF) are involved in the control of stomatal development and pattern formation. First, SDD1 negatively controls the number of cells entering the stomatal pathway. Second, the frequency at which neighbouring cells initiate SM formation by undergoing asymmetric divisions is negatively controlled by both SDD1 and TMM. Putative CFs control which of the neighbouring cells are competent to form SMs. They may act either positively, with strongest expression in the youngest neighbouring cell (i.e. III), or negatively, with the most severe effect on the oldest neighbouring cell (i.e. I). As a result of their action, it is predominantly the youngest neighbouring cell that initiates the formation of satellite stomata. Third, in addition to the frequency, the orientation of the asymmetric division of the neighbouring cell is regulated by SDD1 and TMM. Fourth, both SDD1 and TMM negatively control the number of successive asymmetric divisions that the SMs undergo during the formation of satellite stomata. It is proposed that FLP is involved in regulating GC/GMC identity. The size of the arrow or T-bar represents the strength of induction or inhibition by SDD1, TMM, CF or FLP.

stomata are separated by a minimum distance of at least one intervening epidermal cell (Figure 1).

Recent analysis of serial imprints from primary leaves of *Arabidopsis thaliana* variety C24 implies that different mechanisms acting in combination are involved in the regulation of stomatal pattern formation. It has been shown that most of the stomatal complexes (i.e. primary stomatal complexes consisting of the central GC pair and surrounding neighbouring cells) in *Arabidopsis* primary leaves develop from single precursor cells (stomatal initials or MMCs), as a result of three asymmetric cell divisions and hence are clonally related [3••]. These findings support the cell-lineage hypothesis in which an ordered succession of cell divisions leading to the formation of stomatal complexes is responsible for the establishment of the stomatal distribution pattern [20]. Studies using transposon-induced sectors in which the majority (77–87%) of stomatal complexes exhibit staining patterns consistent with a clonal origin [18,21•] also support this theory. Nevertheless, a different conclusion has been drawn from the analysis of serial imprints from cotyledons and primary

leaves of *Arabidopsis thaliana* variety Columbia. According to this analysis, two-thirds of all stomatal complexes are nonclonal [4•]. The interpretation of this analysis, however, is strongly influenced by the definition of the meristemoid/MMC, because only the descendants of these cells were defined as clonal or nonclonal.

Serial imprint analysis also showed that the smaller daughter cell from the first asymmetric division of a neighbouring cell — the SM, which gives rise to a higher order stomata — was always positioned opposite the pre-established (primary or secondary) GC pair [3••,4•]. Like the primary stomata, secondary and tertiary stomata are also formed through a succession of asymmetric divisions, although these divisions may vary from one to three in number [3••]. Satellite stomata derive predominantly from the youngest neighbouring cell (77%) and less frequently from the youngest-but-one neighbouring cell [3••].

The formation of a stomata-free region surrounding each stoma in the abaxial side of *Arabidopsis* primary leaves therefore depends on: first, the number of asymmetric divisions of a (primary) meristemoid, resulting in a stomatal cell lineage; second, the strict placement of the SMs opposite the previously formed GMCs or GC pairs within the cell lineages; third, the number of asymmetric divisions of SMs; and fourth, which of the neighbouring cells undergo asymmetric divisions to produce SMs and with what frequencies.

Fifty-two percent of the epidermis of *Arabidopsis* primary leaves consists of pavement cells, which do not derive from stomatal cell lineages [4•]. The existence of these pavement cells and the control of the number of protodermal cells entering the pathway of stomatal complex formation may contribute to the avoidance of stomatal cluster formation (i.e. the positioning of stomata in direct contact with each other).

Furthermore, it is likely that cell–cell interactions are involved in the establishment of the final stomatal pattern. Thus, adjacent meristemoids (resulting from 'patterning mistakes') are corrected either by oriented asymmetric divisions or by an apparent change in the cell fate of one meristemoid, which de-differentiates into a pavement cell [4•]. In both monocotyledons and dicotyledons, the appearance of immature stomata has been observed [22–27], indicating that improperly positioned cells that initiated the developmental process towards stomatal formation may be prematurely arrested and may even de/re-differentiate into epidermal cells.

Several mutants affected in stomatal patterning and differentiation have been isolated in *Arabidopsis* [3••,28–30] and barley [31]. The mutants *sdd1-1*, *four lips* (*flp*) and *tmm* show specific alterations in stomatal patterning. Stomatal clusters occur in all three mutants, but the characteristics of the clusters formed in these mutants differ. In *sdd1-1*, GC clusters are exclusively 'even numbered' (most frequently composed of four GCs), whereas in *flp*, clusters are either even or odd numbered. In *tmm*, stomatal clusters are found predominantly in cotyledons and primary leaves; they are even numbered but consist of many more GCs (on average, eight GCs per cluster) than are found in *sdd1-1* and *flp*. The *tmm* mutation almost eliminates stomatal formation on the abaxial side of the sepal, in the inflorescence stem and in the hypocotyls; the number of stomatal units in cauline leaves, flower stalks, and siliques is also reduced by *tmm* [32]. The *sdd1-1* mutant exhibits a 2–4-fold increase in stomatal density in all parts of the plant, but only a minor fraction of the additional stomata occur in clusters. In *tmm*, as in *sdd1-1*, the number of GCs is greatly elevated, but the majority of additional stomata are arranged in large clusters. In *flp*, the number of GCs is only moderately increased.

Analysis of dental resin imprint series from cotyledons and primary leaves revealed that the number of protodermal cells entering a stomatal-formation pathway is significantly greater in the *sdd1-1* mutant than in wild-type plants. In addition, more secondary and higher-order satellite stomata are formed in this mutant than in wild-type plants, reflecting an extension of the stomatal cell lineages and an increase in the number of stomata produced by an individual cell lineage. The number of cells entering a stomatal cell lineage, and the frequency with which neighbouring cells undergo asymmetric divisions to produce SMs, is negatively controlled by *SDD1* [3••]. In cotyledons and primary leaves, the frequency with which neighbouring cells undergo asymmetric divisions is also negatively controlled by *TMM*; in the *tmm* mutant, an overproduction of SMs was observed in these organs [4•].

The activity (in terms of development) of the neighbouring cells is probably controlled not only by the action of SDD1 and TMM but also by 'competence factors' that determine which of the neighbouring cells may extend the cell lineage through formation of SMs. The existence of such competence factors is supported by the observation of gene activities that mark the competence of cells for cell division (At*CDC2a*) and mitotic activity (At*CDC1a*). These genes show promoter activity in some small neighbouring (i.e. subsidiary) cells [33]. In contrast to wild-type plants, in which most higher-order stomata are formed through three (56%) or two (37%) cell divisions, most of the secondary and higher-order stomata develop through two (76%) or one (18%) cell division(s) in the *sdd1-1* mutant [3••]. *SDD1* thus negatively controls the number of asymmetric divisions of SMs. The formation of stomatal clusters is caused in the *sdd1-1* and *tmm* mutants by a relaxed control of SM placement; the SM is found strictly opposite the previously formed stomata in the wildtype. Thus, *SDD1* and *TMM* regulate the orientation of asymmetric divisions that produce satellite stomata, an important aspect of stomatal pattern formation.

The appearance of clusters comprising odd numbers of GCs in *flp* indicates a function of FLP in the control of

GC/GMC identity (Figure 2). The occurrence of paired stomata has also been described in leaves of the barley mutant *eceriferum* (*cer*) and the *Arabidopsis* mutant *ectopic root hair3* (*erh3*), and in the cotyledons of the *Arabidopsis* mutant *constitutive photomorphogenic1–10* (*cop1–10*). Abnormal stomatal patterning and differentiation are some of the multiple defects of the *cer*, *erh3* and *cop1–10* mutants. In the *cer* mutant, alterations in the pattern of stomatal complexes coincide with wax deficiencies in several organs, the character that led to the isolation of the mutant. The *cop1–10* mutant was identified by its constitutive light-grown phenotype when grown in the dark, and the *erh3* mutant was identified through its formation of ectopic root hairs. The way in which these genes are directly or indirectly involved in stomatal patterning remains to be elucidated.

The *TRANSPARENT TESTA GLABRA1* (*TTG1*) gene from *Arabidopsis thaliana*, which is proposed to be a negative regulator of root-hair development, has some effect on stomatal spacing. It encodes a WD40-repeat protein that prevents stomatal development in hypocotyl epidermal cells by altering epidermal cell-file specification, but does not appear to play a role in cotyledon and leaf stomatal development [34,35].

Of the genes specifically involved in the control of stomatal spacing, hitherto only *SDD1* has been identified molecularly [3••]. *SDD1* encodes a subtilisin-like serine protease related to eukaryotic and prokaryotic proteins. Expression analysis through mRNA *in situ* hybridisation revealed activity of the *SDD1* gene in meristemoids/GMCs (U v. Bieberstein, D Berger, T Altmann, Abstract S 09-5, 6th International Congress of Plant Molecular Biology, Québec, Canada, June 2000). Like other eukaryotic subtilases, SDD1 is thought to act as a processing protease involved in the mediation of a signal emanating from GMCs, which mediates a signalling cascade involved in the induction of stomatal complex formation.

Conclusions

Significant progress has been made towards the elucidation of the cellular and molecular processes involved in GC formation and stomatal patterning. It has been firmly demonstrated that the distribution pattern of stomata in *Arabidopsis* is determined by the frequency and orientation of the divisions of meristemoids/MMCs. These processes are affected in the *sdd1-1* and *tmm* mutants. Analysis of these mutants revealed that *SDD1* and *TMM* negatively regulate the frequency of meristemoid/MMC divisions, and indicated that further 'competence factors' that act in different regulatory pathways are probably involved in the control of this process. Furthermore, both *SDD1* and *TMM* are required for the proper control of the orientation of the first asymmetric division of a neighbouring cell, which forms a SM. It remains to be tested whether *SDD1* and *TMM* act in the same pathway(s).

The molecular identification of the *SDD1* gene marked the transition towards the elucidation of the molecular mechanisms underlying the observed stomatal patterning processes. The similarity of SDD1 to processing proteases (i.e. subtilisin-like serine proteases) indicates the potential involvement in stomatal patterning of pro-proteins, which may require processing for activation and that may act as signal molecules or as receptors triggering a range of cellular responses. Further genetic, molecular biological and biochemical analyses will be required to identify other components of the signal transduction pathway(s) and the corresponding effectors that are involved in these responses.

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