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Review article

# Stomatal development and genetic expression in *Arabidopsis thaliana* L.

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ABSTRACT

Stomata are turgor-driven microscopic epidermal valves of land plants. The controlled opening and closing of the valves are essential for regulating the gas exchange and minimizing the water loss and eventually regulating the internal temperatures. Stomata are also a major site of pathogen/microbe entry and plant defense system. Maintaining proper stomatal density, distribution, and development are pivotal for plant survival. *Arabidopsis* is a model plant to study molecular basis including signaling pathways, transcription factors, and key components for the growth and development of specific organs as well as the whole plant. It has intensively been studied and found out the driver for the development and patterning of stomata. In this review, we have explained how the MAPK signaling cascade is controlled by TOO MANY MOUTHS (TMM) receptor-like protein and the Erecta (ER) receptor-like kinase family. We have also summarized how this MAPK cascade affects primary transcriptional regulators to finally activate the main three basic Helix-Loop-Helix (*bHLH*) principal transcription factors, which are required for the development and patterning of stomata. Moreover, regulatory activity and cellular connections of polar proteins and environmentally mediated ligand–receptor interactions in the stomatal developmental pathways have extensively been discussed in this review.

## 1. Introduction

Stomata are a kind of leaf epidermal microscopic pores through which gas and vapor are exchanged between plants and atmosphere that regulate two vital physiological processes like photosynthesis and transpiration. During stomatal lineage, epidermal stomata couple with mesophyll cells to match leaf photosynthetic potential with gas exchange capacity (Dow et al., 2017). Stomata are considered as a key element for plant productivity, which represent as an open model for identifying cell patterning and development (Nadeau and Sack, 2002). Stomata are available in all terrestrial plants in species-specific patterns and play a vital role in allowing atmospheric carbon dioxide consumption while reducing water loss of plants as well as plant development and survival.

Each stomatal pore comprises two specialized guard cells encasing one pore and associated with neighboring subsidiary cells. The stomata, together with its subsidiary cells, are called the "stomatal apparatus" which is a triangular shape-like structure. However, dome-shaped structures have also been found (Inamdar et al., 1971). Shape, size, and location of stomata on the leaf surface may vary based on plants adaptation to different environmental conditions (Kirkham, 2014).

Plant growth and development along with functions can be influenced by stomatal morphological features. Stomatal density and stomatal index are commonly used to specify leaves development and plant growth (Kusumi, 2013). Besides, plant photosynthesis and transpiration can also be directly influenced by the density of stomata as well as the volume of stomata (Flexas and Medrano, 2002). If the stomatal density is increased, releasing of vapor from leaves is increased proportionally (Flexas and Medrano, 2002). Stomatal development gives a scheme for analyzing and studying the basic processes of plants at different stages (Lau and Bergmann, 2012). Therefore, diverse researches have been performed on stomata in a huge number of plants including rice (Liu et al., 2009), maize (Wang et al., 2019), and tomato (Ortega et al., 2019). Recent studies describe an intrageneric diversity in the patterns of meristemoid division within the ecologically diverse genus *Callitriche* (Doll et al., 2021).

In model plant *Arabidopsis thaliana*, stomata development is rigorously organized and controlled by numerous internal genes and

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environmental factors. Stomata developmental pathway is greatly regulated by a number of genetic components, including peptide ligands, membrane-associated receptors, and receptor-like kinases, MAP kinase, and a chain of transcription factors. Intensive study on the *Arabidopsis* revealed those critical peptide signaling paths, transcription factors, and polarity components that together determine stomatal development and patterning (Herrmann and Torii, 2021).

This review will elaborate the genetic mechanisms and environmental factors underlying stomatal development and patterning, which might be useful for understanding basic molecular mechanisms like intra and intercellular communications, cell fate and polarity determination. Moreover, relationship between peptide ligands and receptor-like proteins, specific stomata cells generating transcription factors and their roles have been intensively discussed. Our study on stomata development in *A. thaliana* will give light on the understanding about this intensive pathway as well as create a new window for studying details about several genes involved in this developmental pathway.

## 2. Stomatal complexes and patterning

Stomatal complex is a portion of the epidermis containing two guard cells, a stomatal pore and sometimes surrounded by neighboring three subsidiary cells (Fryns-Claessens and Van Cotthem, 1973), called anisocytic stomatal complex, and known as a functional unit for stomatal movements (Serna and Fenoll, 2000b). As part of the stomatal complex, subsidiary cells may support guard cell function which likely to be varied due to biochemical, mechanical or anatomical structure (Gray et al., 2020). The number of epidermal stomata restrains the maximum potential diffusive capacity, which is a consequence of the genetic and cellular machinery that regulates the development and patterning of the stomata (Lau and Bergmann, 2012). The anisocytic stomatal complex is present in *Arabidopsis* leaves which are the fundamental structural unit formed in *A. thaliana* during leaf development (Serna and Fenoll, 2000b; Berger and Altmann, 2000). A series of predictable divisions are involved in this complex formation (Serna and Fenoll, 2000a). Three stomatal precursors, meristemoid mother cells (MMCs), meristemoids, and guard mother cells (GMCs) are continuously involved in this formation. The first two are divide asymmetrically and the latter symmetrically. Unequal cell division occurs in the protodermal cell, as a result, at first one subsidiary cell and then the meristemoid is formed leading to the beginning of stomatal complex formation. The meristemoid continues up to two more asymmetric divisions, resulting in a central meristemoid being created that acquired a new form to obtain GMC identity (Zhao and Sack, 1999). Finally, the GMC undergoes symmetrical division, as a result of this division; GMC produces two GCs of similar size. Then the most recent neighboring subsidiary cells divide asymmetrically to form satellite meristemoids that further asymmetrically divide, and produce secondary anisocytic stomatal complexes (Serna et al., 2002). Meristemoids, MMCs, and GMCs are successively involved in stomatal complex formation.

Meristemoids might have ability to self-renew that yield in a larger cell and a smaller meristemoid by amplifying division. At the terminal stage, meristemoid loses its self-renewal activity to form rounded shape GMC, which after symmetric cell division generates two guard cells as a pair. MMCs or meristemoids are gone through unequal divisions that form larger cells, and when some of the larger cells come into contact with the stoma that can control the MMCs to initiate cell division pattern, and finally stomata are form (Serna, 2013).

## 3. Stomatal development in *A. thaliana*

In *Arabidopsis* the developmental process of stomata starts with converting the specialized protodermal cell into an MMC, which produces a smaller triangular meristemoid and a larger sister cell by an asymmetric division (Geisler et al., 2000, Figure 1). This type of asymmetric division is known as entries division. A larger ground daughter cell and a meristemoid are generated through additional asymmetric amplifying divisions. The amplifying divisions are raised from asymmetric divisions that form guard cells. The stereotypical asymmetric division of MMC gives rise to a meristemoid cell and a sister cell (Pierre-Jerome et al., 2018). At the end of the division, meristemoid turned into GMC, and then again symmetrically divided to form a paired guard cell. Then, via several morphological changes, guard cell walls become thick and separate and pore is formed. New satellite meristemoids are produced by the asymmetric spacing divisions of both of the daughter cells from where most of the stomata are originated (Geisler et al., 2000). On the other hand, the entry division in the MMC is randomly directed. In case of the formation of beneath MMC, their asymmetric entry divisions formed two adjacent

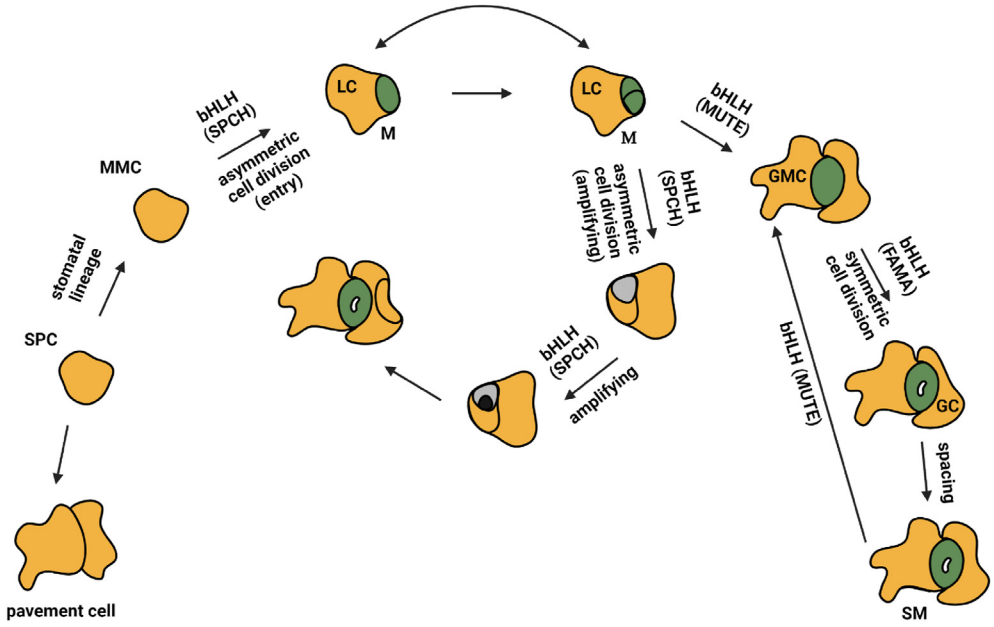


**Figure 1.** The schematic presentation of cell division during stomatal development in *Arabidopsis*. A specialized protodermal cell (SPC) initiates the stomatal lineage becoming a meristemoid mother cell (MMC). MMCs are subjected to asymmetric division (Entry) with the influence of a *bHLH* transcription factor *SPCH* that produces a larger sister cell (LC) and a smaller meristemoid (M; dark). Meristemoid then differentiate into a GMC (darker shading) with the regulation of *MUTE* expression or undergo further asymmetric cell division (Amplifying) by *SPCH* to complete additional stomatal development. Finally, a GMC divides symmetrically and results mature guard cell pair (GCs) by FAMA transcription factor that surround the stomatal pores. LC differentiate into pavement cells or adopt MMC fate and undergo spacing division (asymmetric) and generate secondary meristemoid (SM). The spacing division is oriented, therefore, SM do not form adjacent to the stomatal lineage. *SPCH* regulates the entry and amplifying divisions, *MUTE* directs the development from meristemoid to GMC, and *FAMA*, *FLP* and *MYB88* regulate guard cell division and differentiation.

meristemoids, where isolated MMC remain unchanged (Lucas et al., 2006), and the adjacent meristemoids undergoes spacing division to form a daughter cell between them, or it may divide into a pavement cell (Lucas et al., 2006; Geisler et al., 2000, Figure 1). The core mechanisms of stomatal development in the context of epidermal tissues in *Arabidopsis* have extensively been reviewed very recently by Torii (2021).

Gene expression in the organisms is regulated by large protein complexes containing transcription factors and co-regulators that act in a combinatorial manner and are organized into gene regulatory grids, which contain all possible connections between transcription factors and their corresponding target genes in the protein–DNA interaction space (MacAlister et al., 2007; McKown and Bergmann, 2020). The gene regulatory grid has a temporal and/or geographical expression in the form of gene regulatory networks (Mejia-Guerra et al., 2012). Many genes have been implicated in the signaling network regulating cell fate determination of the epidermis and stomatal patterning (Pillitteri and Torii, 2012; McKown and Bergmann, 2020).

Three *bHLH* transcription factors, *SPEECHLESS* (*SPCH*), *MUTE* and *FAMA* are involved in the beginning of stomatal lineage, termination of meristemoid fate, and the transition of GMC to immature GC (Macalister and Bergmann, 2001; MacAlister et al., 2007; Ohashi-Ito and Bergmann, 2006; Pillitteri and Torii, 2007). Those genes form dimers with functionally redundant *INDUCER OF CBF EXPRESSION1* (*ICE1*) and *SCREAM2* (*SCRM2*), and play their core regulatory actions in stomata development (Kanaoka et al., 2008). Those are individually specific in the developmental window, and each of them is unconditionally necessary for stomata formation (Pillitteri et al., 2007; Ohashi-Ito and Bergmann, 2006). *SPCH* expression is dynamic and found in a subset of two young neighboring epidermal cells (Pillitteri and Torii, 2007). *SPCH* undergoes a successful asymmetric division, then remaining in the meristemoid, disappears from the stomatal lineage ground cell, and carries on asymmetric divisions (Robinson et al., 2011).

## 4. Genetics of stomata development

The function of TMM was first characterized as a required gene for stomatal patterning and development (Nadeau and Sack, 2002; Yang and Sack, 1995). TMM can positively or negatively regulate stomatal development. Failure to properly positioning of the spacing and amplifying divisions as well as stop entry divisions in adjacent stomata cells may result in additional and clustered stomata (Nadeau and Sack, 2002; Geisler et al., 2000). TMM is a receptor-like protein, containing a leucine-rich repeat (LRR) sequence that is mostly expressed throughout the stomata developmental process including entry division, as well as, it is frequently found in stomata adjacent cells or their initials (Nadeau and Sack, 2002). The presence of both ligands and a downstream signaling cascade is indicated by a receptor-based signaling system regulating stomatal lineage divisions (Figure 2) (Casson and Gray, 2008). The *stomatal density and distribution-1* (*sdd1*) mutant regulate the quantity of entry and amplification division and positioning of spacing division (Von Groll et al., 2002; Berger and Altmann, 2000). *SDD1* encodes a putative subtilisin-like serine protease, and is proposed to activate signaling components via the proteolytic processing of their precursors (Schaller, 2004). *SDD1*also generates extracellular environmental signal peptide that interacts with the TMM receptor (Von Groll et al., 2002). ERECTA (ER) family is also another LRR receptor-like kinase that is involved in the stomatal lineage. Signal perceiving by TMM forms a heterodimer receptor with ER that transmits the signal into the cell (Shpak et al., 2005).

The ligand *epidermal patterning factor-1* and *epidermal patterning factor-2* (*epf1* and *epf2*) are the substrate of *SDD1*, which negatively regulates the stomatal development and patterning by activating the SERKs (Zoulias et al., 2018; Hara et al., 2007; Vandepoele et al., 2002). Genetic research reveals that *epf1* is working in a similar direction as TMM and also the ER family, which is a good candidate for *TMM* ligand. The *epf1*

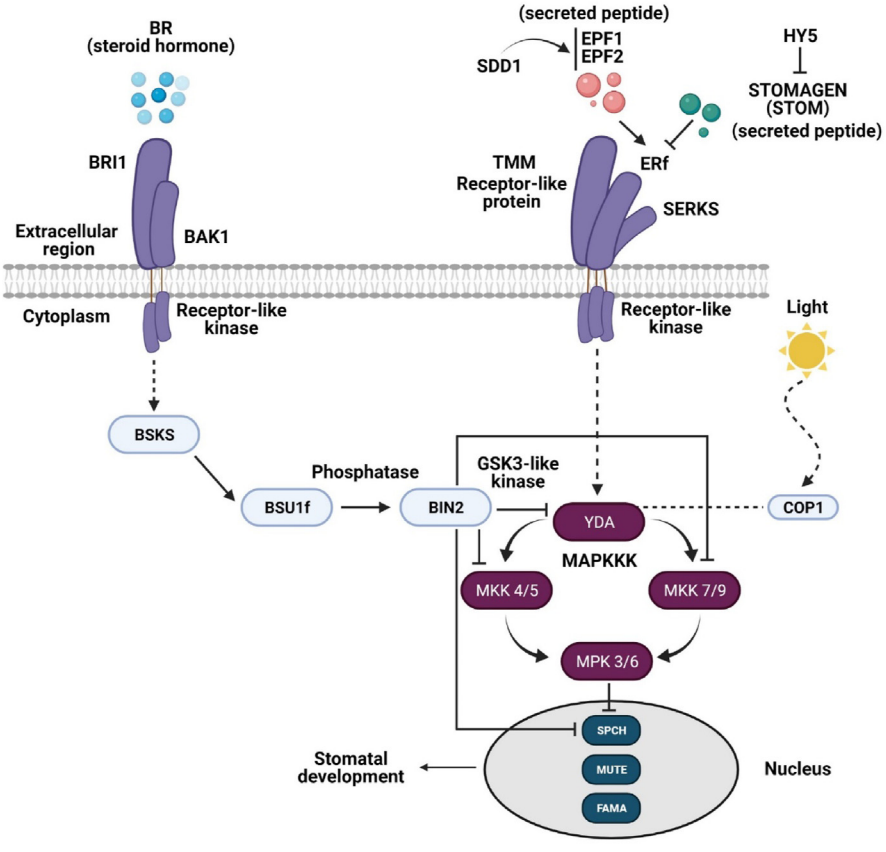


**Figure 2.** Signaling pathway of receptor-ligand interactions integrate intrinsic and environmental signals governing *SPCH* and stomatal development. The MAPKKK YDA, MKK4/5, MKK7/9, and MPK3/6 signaling cascade is employed to repress stomatal production. TMM is associated with the ER family receptor kinases at the cytoplasm. Stomata cell divisions are regulated upstream by *EPF1/2* processed by *SDD1*. The binding of *EPF1/2* ligand by the ERf/TMM/SERK receptor complex and activation (including the antagonistic *STOMAGEN* that competes with EPF for the binding sites of the ERf/TMM/SERK complex) stimulates upstream of MAPK signaling cascade *YDA* and thereby activates MKK4/5 and MKK7/9, and then MPK3/6. This MAPK cascade interacts with *SPCH*, *MUTE* and *FAMA*. In the brassinosteroids (BR) signaling pathway, an intermediate signaling receptor GSK3-like kinase, *BIN2*, is activated by the BRI1-BAK1 complex, which negatively regulates *YDA* and *SPCH*. *BIN2* suppresses *SPCH* by directly phosphorylating or indirectly through *YDA* or MKK4/5. The influence of light is observed to regulate the stomatal quantity through the ubiquitin E3 ligase *COP1*. Solid lines indicate established biochemical interactions and dashed lines reveal indirect interactions. *BAK1*, BRI1ASSOCIATED RECEPTOR KINASE 1; *BRI1*, BR INSENSITIVE 1; *BSU1f*, family of BRI1-SUPPRESSOR 1. Blocked lines indicate negative control, and arrows are positive regulation (modified from Zoulias et al., 2018; Torii, 2021).

has a secretory N-terminal signal which released mature peptides after cleaving (Casson and Gray, 2008). However, genetic evidence is consistent with *epf1* as well as *sdd1* behaving the same way as TMM (Hara et al., 2009; Von Groll et al., 2002).

The other positive regulator STOMAGEN (*STOM*) peptides also underpins the peptide signaling (Sugano et al., 2010), which helps to bind receptor kinase with the EPF peptides. *STOM* has stomata-inducing activity in a dose-dependent manner, thus potentially increases the stomatal density to form stomata clusters. Another transcription component, *ELONGATED HYPOCOTYL 5* (*HY5*), potentially regulates the *STOM* expression. In downstream of photoreceptors, *HY5* regulates the stomatal development in response to light signals by binding with *STOM* (Zoulias et al., 2020). *STOM* belongs to the *EPF1/2* family and positively regulates stomatal development by binding with the ERf receptor, and switch off the MAPK pathway (Sugano et al., 2010). *EPF1/2* binds to ERf, TMM and SERK receptor kinases that activate a MAP kinase that phosphorylates *SPCH* (Figure 2) (Meng et al., 2015; Sugano et al., 2010).

In the receptor-ligand downstream interactions, a MAPK cascade negatively controls the developmental pathway (Wang et al., 2007; Bergmann et al., 2004). A number of kinases regulators have been identified in this pathway including MAPKKK YODA (*YDA*), MKK4/5, MKK7/9, MPK3/6 (Lampard et al., 2009; Wang et al., 2007; Bergmann et al., 2004, Figure 2). Genetic evidence places the *YDA* in the downstream of *ERf*, and TMM signaling, as well as connects the MAPKs to *bHLH* transcription factors, meanwhile, MPK3/6 directly phosphorylated to regulate *SPCH* (Lampard et al., 2009). The kinase cascades are preserved with MAPKKK signaling to downstream MAPKKs and MAPKs by serine/threonine phosphorylation (Mishra and Joy, 2006), and with MPK3/6, controls stomatal patterning and development, whereas MKK4/5 also act in the same pathway with *YDA* and regulates the development (Wang et al., 2007; Casson and Gray, 2008). Both MPKs are activated in response to stresses (Asai et al., 2002; Kovtun et al., 2000), however, activation by *YDA* tends to confer specificity to stomatal development.

Besides MAPK cascade signaling for stomatal development, stomatal lineage is also regulated by nutrition, hormones, and/or environmental signals, thereby regulates the stomatal number to balance between plant and environment (Gong et al., 2021; Han et al., 2020; Lau et al., 2018; Wang et al., 2007). Recent research found brassinosteroid (*BR*) signaling pathways also regulate *SPCH* function to phosphorylate *BR* genes (Gudesblat et al., 2012; Kim et al., 2012). BR regulators are perceived by the kinase *BRASSINOSTEROID INSENSITIVE1* (*BRI1*; He et al., 2000). *BRs* to *BRI1* binding activated *BRASSINOSTEROID SIGNALING KINASEs* (*BSks*) like family kinases (Tang et al., 2008), which appears to both phosphorylate and negatively regulate *SPCH* by the glycogen synthase kinase 3 (*GSK3*)-like kinase BRASSINOSTEROID INSENSITIVE2 (*BIN2*) kinase (Li and Nam, 2002) and *BRI1 SUPPRESSOR1* (*BSU1*; Mora-García et al., 2004; Figure 2). *BIN2* inactivation suppresses stomatal formation and mutation in *BSU1* composed stomata. The *SOMATIC EMBRYOGENESIS RECEPTOR KINASE3* (*SERK3* also called *BAK1*), characterized as a serine-proline-rich extracellular domain, and is necessary to BR signaling in stomatal development that compiles intracellular signaling and extracellular ligand recognition (Meng et al., 2015).

Light quantity and quality are also important factors for stomata development on the new leaves. An E3 ubiquitin ligase *CONSTITUTIVE PHOTOMORPHOGENIC1* (*COP1*) transduces light signals perceived by photoreceptors, and regulates the activity of *COP1*, which targets positive regulators of photomorphogenesis (Lau and Deng, 2012; Kang et al., 2009, Figure 2).

## 5. *bHLH* genes involved in stomatal development

The transcription factor superfamily *bHLH* combines numerous proteins which are found in different types of organisms including plants, fungi and animals (Schiefelbein et al., 2009). In *A. thaliana*, besides stomatal patterning, the members of *bHLH* are engaged in a wide range of growth and developmental signaling such as abiotic stress responses, light signaling, trichome and root hair formation, and flowering time control (Zhou et al., 2009; Masucci and Schiefelbein, 1994; MacAlister et al., 2007). The *bHLH* family is the largest family consisting of 147 members of transcription factors in *Arabidopsis thaliana* (Zhao et al., 2012). The domain of *bHLH* contains around 60 amino acids, with functionally distinguishing elementary and *bHLH* regions. The *N terminus* elementary region of *bHLH* domains acts as a DNA-binding motif (Li et al., 2006). *Arabidopsis* recognizes a triangle of related transcription factors called *bHLH* type: *SPCH*, *MUTE* and *FAMA* that are important for differentiation of guard cells and production of stomata (MacAlister and Bergmann, 2011). The sequential activities of these three genes are required to enhance cellular transition during stomata development in *A. thaliana* (Ohashi-Ito and Bergmann, 2006).

These three types of transcription factors are involved in three essential functions such as, *SPCH* functioned in asymmetric divisions where *MUTE* specifies GMC fate and *FAMA* needed for the variation of the GCs (MacAlister et al., 2007). The transition of stomatal development starts with the action of *SPCH*, which is expressed in MMCs and is confined to meristemoid after a series of asymmetric divisions (Pillitteri and Dong, 2013, Figure 3). Furthermore, the mutation in *SPCH* hampers the initiation of stomatal lineage. An epidermis which is composed of abnormal pavement cells is produced due to the mutation in *SPCH*. In addition, over expression and inhibition of the *SPCH* function govern the asymmetric division pattern of MMCs and meristemoid (Robinson et al., 2011). Therefore, *SPCH* is very crucial for the initiation of stomata lineage through asymmetric cell division. Moreover, this stomatal-specific regulator has further developmental effects on the leaf, leading to modify mesophyll structure (Dow et al., 2017).

On the other hand, another *bHLH* gene *MUTE* is functional at the termination of asymmetric division of meristemoid. Mutation in *MUTE* results in multiple divisions of meristemoid, which ultimately leads to inappropriate transition of GMC or GC. The *FAMA* controls the ultimate final transition of GMC to GC (Figure 3). Several spatial, temporal and mutant expression analyses show that SPCH is associated with MMCs to meristemoids transition, while MUTE drives meristemoids to GMCs transition, and FAMA controls the final proliferation from GMCs to GC (Pillitteri et al., 2008). These three principal transcription factors are the genetic toolbox that tightly controls stomatal development, patterning and stomata formation (Chater et al., 2017). Thus, three closely related genes function sequentially during stomata development *in A. thaliana*.

## 6. Four lips (FLP) negatively regulate the cell proliferation in stomata development

The structure and function of stomata are closely associated with *FLP* and *MYB88* like transcription components that control stomatal development; also take part in megasporogenesis in *Arabidopsis* (Lei et al., 2015, Figure 4). *FLP* and *MYB88* negatively regulate cell proliferation in the initial developmental pathway of *Arabidopsis*. The *R2R3 MYB* transcription factor, encoded by the *FLP* gene, screens the mutant that displays abnormal stomatal development. The extra symmetric divisions and ectopic conditions can be induced by the mutation in the *FLP* gene. On the other hand, *FLP* and *MYB88* have the same function as *FAMA* genes and redundant functions in restricting the lethal division occurring in the cell lineage of stomata. The function of the *FLP* is to enforce the GMC division (Lei et al., 2015). During single stomata development, some *CDKB1* and *CYCA2* genes promote symmetrical division of precursor GMC. Loss of function mutations in *FLP* induces extra symmetric and ectopic divisions.

The central divisions in the lineage of stomata are overly restricted by *MYB* like transcription factor called *FLP; MYB124* and *MYB88*. Though mutation in *myb88* shows normal stomata, *flp-1 myb88* double mutants exhibit severe defects in stomata formation than *flp* single mutants (Wang et al., 2015). *FLP* is a typical two-repeat (R2R3) gene and express stomatal cell lineage at the terminal division.

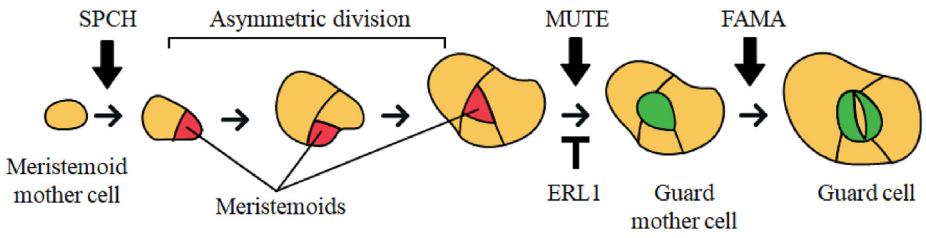


**Figure 3.** Three *bHLH* genes are responsible for consecutive actions of stomatal development. *SPCH* efforts the initial asymmetric division that starts stomatal development. *MUTE* switches the terminating stage of the unequal meristemoid division into GMCs. The separation between mature guard cells and GMCs is regulated by *FAMA* (modified from Negi et al., 2013).

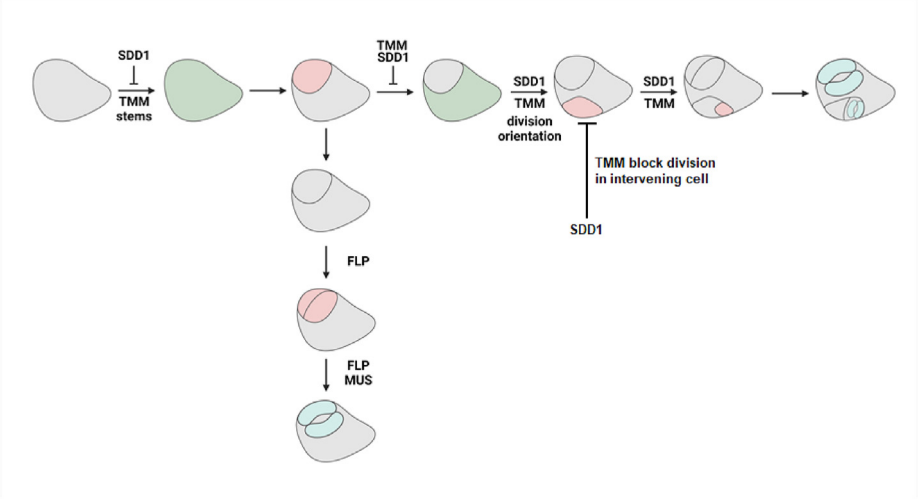


**Figure 4.** A schematic model for gene products acting in stomata structuring. Summarization of the main events that have been disrupted by various mutations. Greenish resembles the selection of an MMC fate. Meristemoids are orange in color. "T"-shaped lines indicated negative regulation, arrows indicated positive regulation.

*FLP* mutation induces the formation of a group of four or more of GCs in direct contact (Lee et al., 2014b). *FLP* and *FAMA* are functionally redundant to ensure a symmetrical GMC division (Lee et al., 2014a). *FAMA* is responsible for GC fate generation, while *FLP* exhibits a SIS phenotype, even *FLP* does not confer GC fate (Lee et al., 2014a). The *flp* mutants have groups of laterally spaced stomata in close interaction with each other. Clusters are present in the plant in all areas where stomata are contained in the wild form (Nadeau and Sack, 2002). In *flp-1*, cluster is less frequent in stems, flower stalks, and siliques, rather than in a dorsoventral like a leaf rosette.

## 7. Involvement of *SCAP1* in functional stomata development

The *stomatal carpenter1* (*scap1*) mutant in *Arabidopsis* has irregular shaped guard cells and is unable to control stomatal aperture, which encodes DNA binding with one finger type transcription factor, Dof, that expressed during the development of GCs, excepting GMCs, mostly regulates the $K^+$ ion, MYB60 transcriptional elements, and pectin methylesterase expression in stomatal morphogenesis (Negi et al., 2013; Yanagisawa, 2002). *SCAP1* acts as a key transcription factor at the final stage in guard cell differentiation, but no action in the early stages, which also regulates essential guard cell maturation processes (Negi et al., 2013, Figure 5). Differential cell wall thickenings in guard cells allow them to change shape in response to turgor pressure changes, allowing them to function as valves (Bergmann and Sack 2007). The uneven thickening pattern of the walls is produced by young guard cells. At the same time, in order to gain control of the ion balance, they seem to undergo significant changes to gene expression, which is required for stomatal movement. As a whole, *SCAP1* transcription factor mainly controls guard cell maturation and the achievement of full functionality at the final stage of stomata formation (Negi et al., 2013 and 2014, Figure 5).

Antagonistically, Castorina et al. (2016) performed comprehensive analysis of the control of *SCAP1* expression during leaf development and found that the transcriptional component *SCAP1* is essential in the genetic pathway of stomatal production and might be a key regulator involving *SCAP1* mediated down regulation of *EPF2* activation. *SCAP1* plays a spatially distinct role in the GC maturation, as well as has the fundamental role in GC patterning and function. Premature activation of *SCAP1* expression in leaf primordia is similar with the expression of other regulators of stomatal development and independently regulates the early stage of GC lineage differentiation.

## 8. *KIN10* stabilizes *SPCH* and positively regulates stomatal lineage

The plants conserved domain of a central energy sensor kinase, sucrose non-fermenting1 (SNF1)-related kinase 1 (SnRK1), stimulates stomatal lineage at the short-day condition which is evolutionarily conserved with SNF1 in yeast and animals (Crepin and Rolland, 2019; Baena-González et al., 2007). *KIN10* is a catalytic α-subunit of SnRK1, and its mutation results in a lower number of stomatal indexes, however, its overexpression dramatically increased stomatal formation and development (Han et al., 2020, Figure 5). Nuclear *KIN10*s are highly active to phosphorylate and stabilize *SPCH* in the stomatal lineage, thereby promoting stomatal development (Han et al., 2020). Recently *KIN10*'s location was found in the endoplasmic reticulum and nucleus that reprograms gene expression through translocation under metabolic stress situations (Blanco et al., 2019; Ramon et al., 2019). *KIN10* is

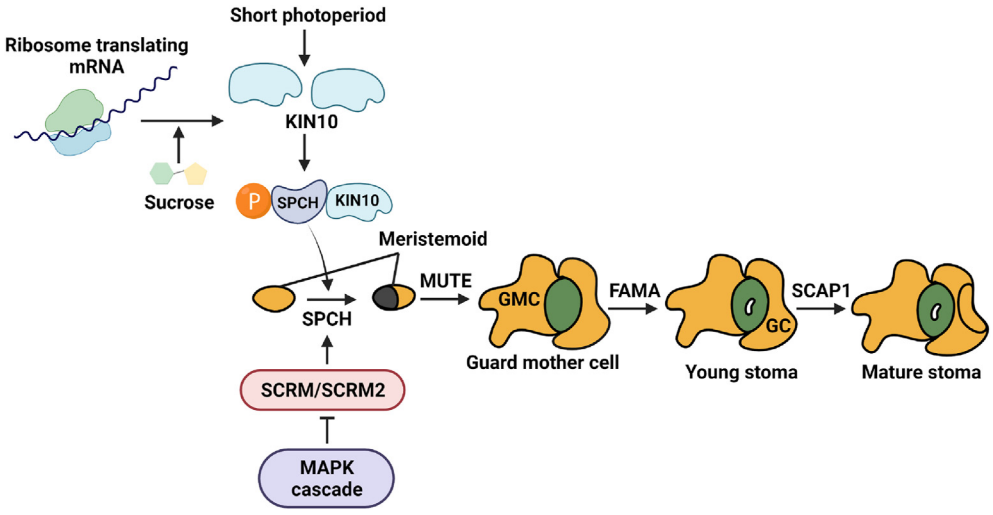


**Figure 5.** Model of *KIN10*, *SCAP1*, and *SCRM* regulating stomatal development. Plants grown in short photoperiod *KIN10* protein is induced and accumulated by increasing its translation by sucrose supply. *KIN10* phosphorylated and stabilized *SPCH* that regulate stomatal development. SCRM works as a structural subunit to recruit MAPK to interact with *SPCH* and thereby regulate stomatal development. *SPCH*, *MUTE* and *FAMA* are related to early stomatal development. *SCAP1* directs the final stages of stomatal development and generates mature stomata. GMC, guard mother cell; GC, guard cell.

involved in the sugar-promoted developmental program and its tuning activity optimizes stomatal development by environmental signals. Even *SPCH* is a downstream core regulator of environmental and hormonal signals for stomata development (Gudesblat et al., 2012; Lau et al., 2018), whereas Han et al., (2020) found that *KIN10* could increase the levels of *SPCH*, which positively regulates stomatal development. They analyzed the interaction by two-hybrid system and found that *KIN10* interacts only with *SPCH* and its mutation greatly reduces the stability of *SPCH*.

## 9. SCREAM promotes stomatal lineage by coupling MAPKs to *SPCH*

The *bHLH* transcription factor SCREAM scaffold and recruits MPK3/6 to down-regulate *SPCH* to initiates cellular lineages by directly binding to MPK3/6 through a bipartite conserved motif KRAAM. Kinase docking and KRAAM motifs of SCRM both are involved in MPK6 binding. *SCRM* and *SCRM2* function for *SPCH* at early stage, whereas function for *MUTE* and *FAMA* at later stage that substantially bridges MAPKs and *SPCH* and enforce entry division of the stomatal lineage (Putarjunan et al., 2019). Results of Putarjunan et al., (2019) revealed a mechanistic basis that the SCRM integrates upstream repressive signals and downstream activators during stomatal formation, and influences the recruitment of MAPK regulatory cascade. On the other hand, mutations in the KRAAM motif abrogate phosphorylation and degradation of SCREAM that produced unrestrained stomatal differentiation (Putarjunan et al., 2019). The SCRM gain-of-function mutant displayed constitutive stomatal differentiation (Li et al., 2017), and contrariwise, successive reduction of SCRM and SCRM2 recapitulated the *FAMA*, *MUTE*, and *SPCH* phenotypes and determine initiation, proliferation, and termination of stomatal differentiation pathway (Kanaoka et al., 2008; Putarjunan et al., 2019, Figure 5). SCRM mutation produced weak phenotype for *fama* due to GMCs alignment, whereas mutation in SCRM2 produced *mute* type phenotypes, and *scrm scrm2* double mutant revealed the *SPCH* phenotype. Various interactions of SCRM with MPK6/3 revealed the non-redundancy of active MPK3/6 in stomatal lineage, and maybe other MPK3/6 regulated pathways (Wang et al., 2007; Putarjunan et al., 2019).

## 10. *COP1* stimulate stomatal development under light mediated-regulation

Besides the functions of *bHLH* transcription factors, stomatal development is also found to be influenced by light intensity. In the absence of light, *CONSTITUTIVE PHOTOMORPHOGENIC1* (*COP1*) degrades ICE proteins through ubiquitination. Accordingly, the ICE peptides aggregate in the nuclei of *COP1* mutants that comprehensively produce stomata. Light in various wavelength ranges suppresses the ICE degradation by *COP1*-mediated regulation that induced stomata formation (Lee et al., 2017). *COP1* acts with the TMM in downstream of *CRY, phyA,* and *phyB* but upstream of *YDA*, *SPCH*, *MUTE*, and *FAMA* (Kang et al., 2009). *COP1* specifically and directly targets transcriptional regulator *HY5* for degradation through their direct physical interactions. The *HY5* responds to light signals and functions downstream of the photoreceptors (Ang et al., 1998; Holm et al., 2002).

Meng et al., (2018), demonstrated that *AN3* is a positive regulator that interacts with the *COP1* to regulate stomatal development in light-induced conditions. *AN3* integrates light signaling into the production and spacing of stomata as well as points the connection between light signaling and stomatal patterning. It has been revealed that *AN3* acts upstream of *COP1* and downstream of *TMM*.

## 11. MASS positively regulates stomatal development

Xue et al. (2020) found that *Arabidopsis* gene At1g80180, At1g15400, and At5g20100 are the substrate of MAPK and performed in-depth functional genetic characterization of these three MAPK SUBSTRATES IN THE STOMATAL LINEAGE (MASS) proteins, and hypothesized that being a member of plasma membrane, it promotes the stomatal formation and regulate patterning. MAPKs phosphorylate MASS proteins, and its putative substrates control the MASS function and its subcellular localization, thereby MASS suppresses *YDA* function. Therefore, MASS and YDA functional MAPK cascade provides insides how MAPK signals fine-tune stomatal development. MASS proteins are strongly linked to kinase signaling to fine-tune stomatal synthesis and patterning, providing a potential divergence of the *YDA*-MPK3/6 cascade in the developmental regulation in plants (Xue et al., 2020).

## 12. Jasmonate regulation in stomatal development

Although jasmonate (*JASMONATE ASSOCIATED MYC2 LIKE 2, JAM2*) is an important signal in plants that regulates a variety of physiological activities, stomatal development of *Arabidopsis* cotyledons is negatively regulated by jasmonate. Cotyledons stomata quantity in mutant and control, treated with methyl jasmonate, was significantly reduced, whereas stomatal development was enhanced by blocking endogenous jasmonate biosynthesis (Han et al., 2018). MYC 2, 3 and 4 transcriptional components are involved in jasmonate signaling by acting upstream of the *SPCH* and *FAMA*, and are found to redundantly modulate jasmonate-inhibited stomatal formation. Jasmonate repression is dependent on MYC (Han et al., 2018). Jasmonate signaling repressor

**Table 1.** Genes involved in stomatal development in *Arabidopsis*.

| Gene Name | Gene Locus | Uniport ID | Sub cellular Localization | Molecular Function | Expression | Description | References |
|---|---|---|---|---|---|---|---|
| *SPCH* | AT5G53210 | 835402 | Nucleus | DNA binding protein dimerization, Activated as transcription factor *SPEECHLESS* | Collective leaf structure, guard cell, leaf epidermis | Encodes a basic helix-loop-helix (*bHLH*) transcription factor that act in asymmetric divisions of the stomatal complex | (MacAlister et al., 2007) |
| *MUTE* | AT3G06120 | 819785 | Nucleus | DNA-binding transcription factor activity, transcription cis-regulatory region binding | Guard cell, guard mother cell, initial cell | Encodes a basic helix-loop-helix (*bHLH*) protein that controls meristemoid differentiation during stomatal development | (Heim et al., 2003) |
| *FAMA* | AT3G24140 | 822000 | Nucleus | protein dimerization activity, DNA binding transcription factor activity | Cauline leaf, collective leaf structure, cotyledon, flower pedicel, guard cell, guard mother cell | Guard cell differentiation and stomatal complex development | (Heim et al., 2003) |
| *FOUR LIPS* | AT1G14350 | 837997 | Nucleus | transcription regulatory region sequence-specific DNA binding | Guard cell, guard mother cell, hypocotyl | Encodes a putative *MYB* transcription factor involved in stomata development | (Lai et al., 2005) |
| *MYB88* | AT2G02820 | 814812 | Nucleus | transcription co-regulator activity | Low levels in roots, leaves, hypocotyl stems, flowers, siliques and buds | Encodes a putative transcription factor (*MYB88*), involved in stomata development | (Lei et al., 2015) |
| *MYB124* | AT1G14350 | 837997 | Nucleus | DNA-binding transcription factor activity, protein binding, sequence-specific DNA binding | High level in all shoot organs specially leaves, stems, flowers, siliques and floral buds. Often found in tips of roots. | Encodes a putative *MYB* transcription factor involved in stomata development | (Lai et al., 2005) |
| *CDKB1* | AT2G38620 | 824585 | Nucleus | ATP binding, cyclin-dependent protein serine/ threonine kinase activity, protein binding | Extremely present in guard cells and cotyledon precursor cells | Encodes a member of a plant specific family of cyclin dependent kinases | (Segers et al., 1996) |
| *CYCA2* | AT1G15570 | 838127 | Nucleus | Involved in regulatory activity like cyclin-dependent protein serine/threonine kinase | *MYB88* and *MYB124*Suppressed by newly formed guard cells, leaf apex, leaf lamina base | A2-type cyclin. Negatively regulates endocycles and acts as a key regulator of ploidy levels in *Arabidopsis* endo reduplication | (Vanneste et al., 2011) |
| *TMM* | AT1G80080 | 844348 | Plasma membrane | Peptide binding, protein binding | Presented in cell lineage evolved stomata in the epidermis | Encodes a transmembrane leucine-repeat containing receptor-like protein, expressed in proliferative post protodermal cells. Recessive mutation leads to disruption of asymmetric cell division during stomata development. | (Horst et al., 2015) |
| *YODA* | AT1G63700 | 842674 | Plasma membrane | protein serine/threonine kinase activity, MAP kinase kinase kinase activity | Copolarizes with *BASL* and *MPK3/MPK6* in stomatic asymmetric cell division (ACD) cells | Member of MEKK subfamily, a component of the stomatal development regulatory pathway | (Zhang et al., 2016) |
| *MAPK3* | AT3G45640 | 818982 | Nucleus | Entire epidermis converted to stomata, MAP kinase activity | Expressed in epidermal cell | Encodes a mitogen-activated kinase whose mRNA levels increase in response to touch, cold, salinity stress and chitin oligomers | (Pillitteri and Torii, 2007) |
| *KIN10* | AT3G01090 | 821259 | Nucleus | kinase activity, kinase binding, phosphatase binding, protein binding | carpel, cauline leaf, collective leaf structure, cotyledon | Encodes a SNF1-related protein kinase, interacts with SCF subunit SKP1/ASK1 and 20S proteosome subunit PAD,1 also interact with PRL1 DWD-containing protein | (Han et al., 2020) |
| *COP1* | AT2G32950 | 817857 | Nucleus | identical protein binding, ubiquitin protein ligase activity | carpel, cauline leaf, collective leaf structure, cotyledon | Represses photomorphogenesis and induces skotomorphogenesis | (Lee et al., 2017) |
| *SCREAM* | AT3G26744 | 822287 | Nucleus | DNA binding, DNA-binding transcription factor activity | carpel, cauline leaf, collective leaf structure, cotyledon | Encodes a MYC-like *bHLH* transcriptional activator that binds specifically to the MYC recognition sequences in the CBF3 promoter and inhibits the expression of ABI3 | (Putarjunan et al., 2019) |
| *MASS* | AT1G15400 | | Plasma membrane | protein binding | carpel, cauline leaf, collective leaf structure, cotyledon | Tightly connected with MAPK signaling to fine-tune stomatal production and patterning. | (Xue et al., 2020) |
| *JAM2* | AT1G01260 | 819244 | Cytoplasm and Cytosol | DNA-binding transcription factor activity, protein binding, protein dimerization activity | carpel, cauline leaf, collective leaf structure, cotyledon | *bHLH*13 interacts with JAZ proteins, and functions redundantly with *bHLH*3, *bHLH*14 and *bHLH*17 to negatively regulate jasmonate responses. | (Thines et al., 2007) |
| *SCAP1* | AT5G65590 | 78473 | Nucleus | DNA-binding transcription factor activity, sequence-specific DNA binding | endosperm, guard cell | Encodes a plant-specific Dof-type transcription factor expressed in maturing guard cells, but not in guard mother cells | (Negi et al. 2013) |

*JASMONATE ZIM-DOMAIN (JAZ)* alleviates the functions of downstream transcription factors. JAZ proteins activate JA responses, releasing MYC2 to increase JA-induced gene expression in stomatal development (Thines et al., 2007; Chini et al., 2007).

## 13. Polarity protein is pivotal for stomatal development

Cell polarity is a cellular process required to develop two daughter cells that differ in cell fates and is essential for the formation of multicellularity in plants. Cell polarity also plays an important role in the regulation of asymmetric cell division (Muroyama and Bergmann 2019). In recent times tremendous research has been done on cell polarity and stomatal development in *Arabidopsis*. During cellular development, plants deploy several polarity proteins to orchestrate cell polarity. Several exclusive studies unraveled how polarity proteins and their regulators polarize during stomatal asymmetric cell division and their linkage in the fate asymmetries. Some of the such polar protein, BASL (Gong et al., 2021; Dong et al., 2009), BREVIS RADIX family (BRXf), BREVIS RADIX-LIKE 2 (BRXL2) (Rowe et al., 2019), POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION (POLAR) (Pillitteri et al., 2011), as well as CONSTITUTIVE TRIPLE RESPONSE (CTR1) (Gong et al., 2021), have intensively been studied, and found that they directly interact with signaling kinases, and act as scaffolds.

Using combination of quantitative imaging and lineage tracking, Gong et al. (2021) untangled the contributions of a peptide signaling effector BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) in *Arabidopsis* stomatal lineage and found that BASL regulates division plane placement and cell fate enforcement of asymmetric cell divisions in the lineage. More specifically, they found that pre-division BASL plays a pivotal role in division orientation, whereas post-division BASL confirms proper daughter cell fate asymmetry.

In recent studies, enriched accumulation and differential expression of RNA polymerase II-related mediator DEK and the transcription factor *MYB16* have been found in the stomatal lineage ground cell (SLGCs) after asymmetric division and suggest that cell cycle machinery balanced the SLGC potential (Ho et al., 2021). Nucleolar localized DEK was highly expressed in newly born SLGCs and helped to exit from self-renewal at an early stage and shifted toward differentiation into pavement cell (Ho et al., 2021). Moreover, transcriptome profiling of stomatal lineage cells revealed enrichment and preferential localization of MYB16 in SLGCs, which gives clues to why MYB16 prefers SLGCs but not meristemoids (Ho et al., 2021). Using stage-specific miRNA expression profiles from stomatal lineage cells, it was demonstrated that stomatal formation and patterning are remarkably regulated by stomatal lineage miRNAs that avoid clustered stomata formation, which revealed that miR399-mediated *PHO2* regulation contributes to the control of stomatal development (Zhu et al., 2020). The polar protein CTR1, a raf-like kinase which couples with ethylene receptor, has been identified functional as the core component in the ethylene and glucose signaling pathway, resulting in overall depolarization of BRXL2, and antagonistically regulate the balance of asymmetric and symmetric cell divisions in the stomatal lineage (Gong et al., 2021). The MYB16 is involved in the stomatal cluster formation by disrupting polarity in asymmetric cell division (Yang et al., 2021). BASL regulates nuclear migrations to orient division planes and scaffolds intracellular signaling cascades that determine cell fates in the stomatal lineage (Muroyama et al., 2020).

Other BASL data suggested that the temporal activity of polarized BASL is not necessarily to be inherited through the division but can function after asymmetric cell division (Gong et al., 2021). By using time-lapse microscopy, Muroyama et al. (2020) identified two oppositely oriented nuclear migrations that lead to epidermal patterning. *MYOXI-I* as a myosin required for the second migration and functions in the controlling of post-division nuclear migration, whereas loss of *MYOXI-I* decreases the stomatal density due to inability to orient a specific subset of asymmetric cell division, on the other hand, myoxi-i stomatal phenotypes revealed post-division nuclear migration is necessary for the orientation of successive asymmetric cell division to enforce the one-cell spacing rule (Muroyama et al., 2020). Besides our discussion, a huge number of genes have been identified those are involved in stomatal development. In the Table 1, we have summarized a list of genes with their molecular functions involved in stomatal development in *Arabidopsis*.

## 14. Conclusion and future perspectives

Stomata play an important role in terrestrial plants to balance water evaporation with photosynthesis. Stomata is the best ever identified model system for studying developmental and signaling processes due to its easy accessibility of stomata on the plant exterior surface. Therefore, significant fundamental discoveries on stomata maximize the improvement of crop performance and sustainability through conventional breeding to biotechnology (Bergmann, 2004; Torii, 2015; McKown and Bergmann 2020). *Arabidopsis* has been used as a model genetic system in stomata development that provides insight of dicot stomata development. Research with *Arabidopsis* has geared up our understanding of both gene expression and the development of stomata. Determination of functional mechanisms of TMM and ER family in *MAPK* signaling cascade and how they influence transcriptional regulators is very much necessary. Further study of components like *SCAP1*, *KIN10*, and *COP1* might cover the better understanding of processes essential for stomatal development and function.

Plants are always under threatening conditions due to drastic global environmental changes. Therefore, plants need to enhance their ability to tune their physiological programs that might enable them to cope with the changing environments and it is crucial for plants' success. The extensive research aspect of such developmental regulation is that of stomatal development. The understanding of cellular interaction of components like LRR-RLKs, LRR-RLPs, SERKs, TMM, and kinase cascades, and secreted peptides, receptors and ligands, as well as specific antagonistic relationship throughout the regulatory pathway in stomata development might shade this goal to be achieved.

Research with *Arabidopsis* has greatly improved understanding of stomatal development that might be useful for other organisms. In this review, the morphology and physiology regarding stomata formation and development have been discussed. The MMCs, and GMCs are continuously involved in stomata formation through asymmetric and symmetric division and produce two GCs of similar size, then form satellite meristemoids and in progression, produce stomatal complexes. Moreover, many of the recent molecular insights regarding stomatal complex formation have been elucidate. The function of core transcriptional elements, *SPCH*, *MUTE* and *FAMA*, with their regulatory mechanisms have been discussed, involved in the beginning of stomatal lineage, termination of meristemoid fate, and the transition of GMC to GC. Receptor-like protein, TMM, functionally expressed at entry division, and *SDD1* interact with the TMM receptor. Kinases regulators MAPKKK YODA (*YDA*), MKK4/5, MKK7/9, and MPK3/6 negatively control the developmental pathway. Nutrition, hormones, and/or environmental signals, like *BRI1*, *BSks*, *BIN2*, *BSU1*, *BAK1*, and *COP1* have been identified regulate the stomatal number to balance between plant and environment. Cell polarity protein is essential for the formation of multicellularity in plants and plays an important role in the regulation of asymmetric cell division. Polarity proteins BASL, BRXf, BRXL2, POLAR, and CTR1, have intensively been studied and found that they directly interact with signaling kinases, and act as scaffolds. The DEK and MYB16 have been found in SLGCs after asymmetric division and suggest that cell cycle machinery might balance the SLGC potential in stomatal lineage.

Even some specific inducible promoters have been identified; however identification of the target transcription factor, binding site, transcriptional activator as well as effector of a promoter controlling the core point of the regulatory pathway in stomatal development might give valuable insights for the molecular basis of the stomatal patterning in

*Arabidopsis* as well as other plants. Moreover, deciphering the molecular pathways underlying the developmental cues might open a promising attributes for agricultural implication. Therefore, it is a burning need to continue fundamental research for intensive understanding of the developmental mechanisms, and integration of plants and signals to optimize stomatal formation throughout the plant kingdom.

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