Acta Pharmacologica Sinica 2006 Jul; 27 (7): 950–958

Blackwell Publishing

Full-length article

Network regulation of calcium signal in stomatal development[1]

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Key words

stomatal index; stomatal development; signal transduction pathways; signal network; calcium

[1] Project supported by the National Natural Science Foundation of China (No 3017016, No 90102015) and by a grant of doctoral discipline from the Ministry of Education of China (No 20030335043).
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Received 2006-04-23
Accepted 2006-05-30

doi: 10.1111/j.1745-7254.2006.00400.x

Abstract

Aim: Each cell is the production of multiple signal transduction programs involving the expression of thousands of genes. This study aims to gain insights into the gene regulation mechanisms of stomatal development and will investigate the relationships among some signaling transduction pathways. **Methods:** Nail enamel printing was conducted to observe the stomatal indices of wild type and 10 mutants (plant hormone mutants, Pi-starvation induced CaM mutants and Pi-starvation-response mutant) in *Arabidopsis*, and their stomatal indices were analyzed by ANOVA. We analyzed the stomatal indices of 10 *Arabidopsis* mutants were analyzed by a model PRGE (potential relative effect of genes) to research relations among these genes. **Results:** In wild type and 10 mutants, the stomatal index didn't differ with respect to location on the lower epidermis. Compared with wild type, the stomatal indices of 10 mutants all decreased significantly. Moreover, significant changes and interactions might exist between some mutant genes. **Conclusion:** It was the stomatal intensity in *Arabidopsis* might be highly sensitive to most mutations in genome. While the effect of many gene mutations on the stomatal index might be negative, we also could assume the stomatal development was regulated by a signal network in which one signal transduction change might influence the stomatal development more or less, and the architecture might be reticulate. Furthermore, we could speculate that calcium was a hub in stomatal development signal regulation network, and other signal transduction pathways regulated stomtal development by influencing or being influenced by calcium signal transduction pathways.

Introduction

Stomata plays an important role in signal sensing, transduction and driving environmental change. Stomatal morphology, development, distribution and behavior respond to a spectrum of signals, from intracellular signaling to global climatic change[1]. In addition to regulating stomatal movements, environmental signals also alter the number and density of stomata formed during the development of the leaf[2]. Drought stress could reduce stomatal density and stomatal conductance[3]. When field water capacity reduced, stomatal density decreased, while the concentration of abscisic acid (ABA) accumulation increased[4]. Some soybean cultivars might respond to the increased levels of ultraviolet-B radiation by increasing water-use efficiency and this response could be manifested through changes in stomatal development and functioning[5]. Moreover, many signal transduction pathways are involved in stomatal acclimation process. It has been described as a negative efficient between CO_2 concentration and stomatal density[6–8]. If the effects of CO_2 on stomatal aperture are brought about through a signaling network, then alterations in sensitivity to this signal should have effects on other pathways[1]. Besides, stomata are influenced by rhythms, that either control stomatal aperture directly, or modulate the response of stomata to other signals[1]. It has also been found that the complex signal network existed in the regulation of stomatal movements[9]. Many environmental factors (eg, light, CO_2, soil

water content, atmospheric water vapor pressure, temperature and wind) can induce and regulate stomatal movements. Besides, the stomatal movements are regulated by many factors including, vacuolar ion channels in guard cells, Ca^{2+} ($[Ca^{2+}]_{cyt}$ oscillation), CaM, K^+, Mg^{2+}, ABA, protein kinases, phosphatases and so on[5,9,10–25].

The phytohormones play an important role in plant physiological processes. Although there is abundant evidence that ABA closes stomata, ethylene and cytokinin are also both responsible for stomatal response[26–30]. Stomatal opening is induced by cytokinins[30]. Stomatal sensitivity to applied cytokinin varies widely according to the species the cytokinin applied[27,28] and leaf age[26]. Epidermal strip experiments showed that increasing ethylene synthesis (via application of Ethrel or 1-aminocyclopropane-1-carboxylic acid (ACC) had variable effects on stomatal response of *Vicia faba*)[26]. Phosphorus is not only a constituent of such key cell molecules as ATP, nucleic acids, and phospholipids, but also a pivotal regulator in many metabolisms, including energy transfer, protein activation, and carbon and amino acid metabolic processes[31]. In higher plants, Pi limitation enhanced Pi use[32–34]. In plant cells, all the elements for a calcium-based messenger system (includ-ing a highly regulated low level of $[Ca^{2+}]_{cyt}$, plasma membrane and endomembrane calcium pumps and channels, and spatially controlled calcium-dependent regulatory proteins and kinases) are contained, which could couple the external stimuli of hormones to their physiological response[35]. Calcium signaling in guard cells is one of the major pathways regulating stomatal movement in which Ca^{2+} may act as a second messenger[2,9,14–18,36]. $[Ca^{2+}]_{cyt}$ oscillation signals of the guard cell are induced by many external stimuli, such as ABA, calcium, H_2O_2, membrane voltage, drought and so on, and the changes in $[Ca^{2+}]_{cyt}$ regulate stomatal opening and closing[8,13,14,19,37–47]. Moreover, CaM plays an important role in cell signal transduction[36]. On the basis of our current knowledge of guard cell signaling, perhaps the best explanation for a hub in stomatal development is the change in the concentration of guard cells $[Ca^{2+}]_{cyt}$ that have been induced by ABA, extracellular calcium ion, and so on.

The known stomatal pattern mutants include the recessive mutations *too many mouths* (*tmm*), *four lips* (*ftp*), and R-558 in *Arabidopsis*[48,49]. TMM controls stomatal initiation and spacing, *FLP* may regulate guard mother cell fate, and the *R-558* gene product regulates stomatal density. The complexity of the mechanisms that regulate stomatal development was beginning to be revealed by analyzing these mutant phenotypes[49,50]. If guard cell signaling is organized as a network, then a striking property of the network is that it acclimates to external signals[1]. Acclimation to one signal leads to alterations in sensitivity to another signal and are consistent with a network-based organization[1]. Further efforts and more suitable models from all research disciplines should be used to elucidate this topic. On the foundation of previous researches, we considered that guard cell signals were recognized and organised by a system or a network in plants. We hypothesized that stomatal index was controlled by multiple genes and these genes interacted with each other in a network. Therefore, we also presumed that calcium signaling in guard cells might play a central and primary role in regulating stomatal development. The purpose of the present study was to try to describe the regulation of signal network on stomatal developments in plants.

Materials and methods

Plant material *Arabidopsis thaliana* lines used in this study were Columbia wild-type ((Nottingham Arabidopsis Stock Centre (NASC)) and 10 mutants (cin3-1, ein3-1, ein4, era1-2, gca2, E1, E2, E3, PG1, RW1, phr1) (Table 1). The wild-type background of these mutants was Columbia.

Growth conditions The 1/2 strength Murashige and Skoog medium[60] supplemented with 10 g/L sucrose, was used for seed germination and as basal medium. The pH of the medium was adjusted to 5.7 before agar (Difco, 0.8% agar) was added. All media were autoclaved for 20 min at 121 °C. Seeds were surface sterilized by soaking in 75% alcohol for 30 s and followed by 15% Clorox for 15 min. The seeds were then rinsed five times in sterilized water prior to transfer into prepared culture medium. Then the *Arabidopsis* seeds, after germinating in the culture medium for seedling development at 4 °C in the dark for 48 h, were placed under 15 h photoperiod [125 $\mu mol \cdot m^{-2} \cdot s^{-1}$, provided by tissue culture chamber (CU-36L5, Percival Scientific, Iowa, USA)] at 20 °C. After about two weeks, samples were transfered into 11-cm diameter pots with perlite, which has a vermiculite base. Vernalized seeds of *Arabidopsis thaliana*[34] were grown hydroponically in nutrient solution containing 5 mmol/L KNO_3, 2.5 mmol/L KH_2PO_4, 2 mmol/L $MgSO_4$, 2 mmol/L $Ca(NO_3)_2$, 50 μmol/L Fe-EDTA, 70 μmol/L H_3BO_4, 14 μmol/L $MnCl_2$, 0.5 μmol/L $CuSO_4$, 1μmol/L $ZnSO_4$, 0.2 μmol/L Na_2MoO_4 and 0.01 μmol/L $CoCl_2$, pH 5.7[61]. Plants were grown in growth chambers (AR-75L, Percival Scientific, Boone, IA) under the light of a photosynthetic photon flux density of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 15-h light/9-h dark photoperiod. The temperature and humidity were controlled at 22 °C and 70%, respectively. After the pots were covered with plastic film for 3 d, plants were watered with nutrient solution thoroughly from below, twice each week.

Determination of stomatal index for 20 plants of each

Table 1. Material type and description of partial characteristic.

Material type	Description of partial characteristic
Wild type	Columbia
cin 3-1	Cytokinin-insensitive mutant, is defective in the induction of ethylene biosynthesis by cytokinin, do not display the triple response in the presence of low concentrations of cytokinin; but were indistinguishable from wild-type seedlings in their response to ethylene[51].
ein3-1, ein4	Ethylene insensitive mutant, don't have the triple response to the plant hormone ethylene[36], are full or partial insensitive to ethylene, the most obvious insensitive is EIN3[52]. They are belong to the ethylene-response pathway[36,52,53]: EIN4[36,42,44], is in the ETR receptor family, EIN3[54,55] is a key transcriptional regulator in ethylene signaling.
era1-2	Enhanced response to ABA 1, a protein farnesyl-transferase mutant. Hypersensitivity of ABA–induced cytosolic calcium increases[56]. Farnesylation has a role in embryonic ABA signaling[57,58]. A negative regulator of ABA sensitivity must be acted by a farnesyl transferase to function[57].
35S: *AtPsiCaM*	mutants (35 s over-expression of *Arabidopsis* Pi-starvation induced CaM).
E1, E2, E3	Constructed 35 s over-expression vector, the over-expression plants was obtained with *Arabidopsis* soakage transgene method[59] The change in $[Ca^{2+}]_{cyt}$ is almost promoted by many stimulation to the plant cell at first. CaM plays an important role in cell signal transduction, Ca^{2+}-ATPase regulated by CaM distributes widely in plant cell, plays an important role in controlling $[Ca^{2+}]_{cyt}$ [36]
PG1	Transducted promotor and GUS into CaM gene[59].
RW1	35s intervention of *Arabidopsis* Pi-starvation induced CaM mutant 3[59].
phr1	*Phosphate tarvation response 1*, weakly responsive to phosphate starvation. PHR1 encodes a member of the MYB superfamily conserved between *A thaliana and C reinhardtii*, phr1 mutant alleles are impaired in different Pi starvation responses.

mutant and the wild type, all rosette mature leaves were removed from each plant. After lower epidermis surface of mature rosette leaves was dealt with the methods of nail enamel printing[62], stomatal density (number of stomata per unit area) and epidermal cell density (number of epidermal cell per unit area) were measured with microscope eclipse E600W (Japan, NIKON). We measured the stomatal density and epidermal cell density in five positions per leaf, including tip, middle, and base, the part near to tip, the part near to base.

The epidermal cell density (non-stomatal cells) and stomatal density enabled calculation of the stomatal index as follows[63]:

Stomatal index=100×stomatal density/(stomatal density+epidermal cell density)

Statistical analyses of data Means of the stomatal indices in wild type and 10 mutants were calculated and stomatal indices were comparable among different leaf locations (tip, middle, base, the part near to tip, the part near to base) on lower epidermis surface within each plant type by using ANOVA. Moreover, stomatal indices in wild type and 10 mutants were compared using ANOVA among the different plant types. LSD (least significant difference) test at the 0.05 significance level was used to determine differences between mutants (n=100).

Potential relative effect of genes Consequently, on the basis of the hypothesis that stomatal development is controlled by a signal network, we used potential relative effect of genes (PREG) to describe the difference between two mutants. The calculation of the PREG was as follows:

PREG=–(Im–Imi)/Im

Where Im is the stomatal index of certain type, and Imi is the stomatal index of relative types. PREG is negative (when Imi<Im) or positive (when Imi>Im).

Results

Stomatal distribution and stomatal index In the wild type and 10 mutants, stomatal indices did not differ with respect to location (tip, middle, base, the part near to tip, the part near to base) on the lower epidermis (LSD test, P>0.05). The distribution pattern of stomata on the lower epidermis was not affected significantly in all types of mutants used in our research. Compared mutants with wild type, the stomatal indices of 10 mutants decreased significantly (LSD test, P <0.05) (Figure1), that of ein4 (ethylene-insensitive mutant 4)

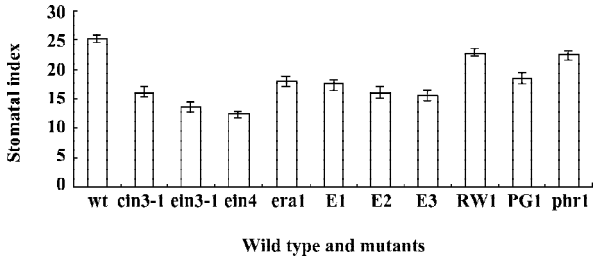

Figure 1. Stomatal index on the lower epidermis of 10 mutants and wild type plants of *Arabidopsis thaliana*. Mean±SEM. Values are means for at least 100 views of lower epidermis surface per type. Compared mutants with wild type, significant differences in stomatal index were detected for the 10 mutants (LSD test, $P<0.05$).

was the most obvious change in 10 mutants, and that of RW1 (35s intervention of *Arabidopsis* Pi-starvation induced CaM mutant 3) was the least obvious change in 10 mutants.

Potential relative effect of genes We found that significant change existed between some mutants by ANOVA (LSD test, $P<0.05$) (Figure 2). PREG describing the difference between two mutants is shown in Figure 2 and Figure 3. PG1 had a positive effect on ein and cin, while a negative effect on RW1 and phr1; phr1 had a positive effect on ein, cin, era1-2, E, PG1; ein had a negative effect on phr1, PG1, RW1, E, era1-2, and cin; cin had a positive effect on ein, while a negative effect on phr1, PG1, and RW1; era1-2 had a positive effect on ein, while a negative effect on phr1 and RW1; E had a positive effect on ein, while a negative effect on phr1, PG1, and RW1; RW1 had a positive effect on PG1, ein, cin, era1, E. Therefore, significant changes and interactions might exist between some mutant genes.

Discussion

Stomatal distribution In the wild type and 10 mutants, stomatal indices did not differ with respect to locations. In endogen, there was regularity in the distribution of stomata on the surface of rice leaves; generally, the stomata arranged vertically in rows between veins, and were also well-distributed near the veins, the edge and the tip of a leaf[64]. We conferred that dissimilarity might exist between dicot and endogen, and these mutations in *Arabidopsis* did not influence stomatal distribution.

Stomatal index: the negative effect of these mutants on stomatal index in *Arabidopsis thaliana* Comparing mutants with wild type, we found that the stomatal indices of 10 mutants decreased obviously, and significant difference existed between wild type and 10 mutants by ANOVA (LSD test, $P<0.05$; Figure 1). The results were consistent with evolutionism that these mutants had the negative effects on stomatal

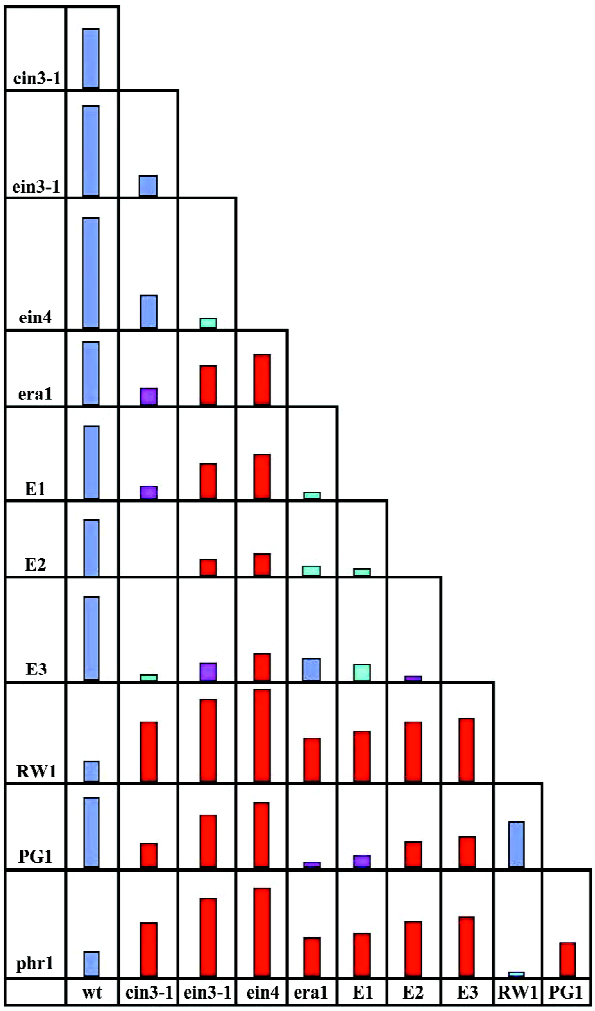

Figure 2. Potential Relative Effect of Genes (PREG). PREG may be negative (red, purple) or positive (blue, green). Within rows, red and blue strip mean that significant difference exists between two types of *Arabidopsis thaliana*, and purple and green strip means that no significant difference exists between two types of *Arabidopsis*. Blank means that no difference exists between two types of *Arabidopsis* (LSD test, $P<0.05$). The longer strip expresses a higher PREG.

index (SI) in *Arabidopsis thaliana*. From an evolutional point of view, most mutations have negative effects on plant growth. For newly arisen mutations, these effects will most likely be harmful because prevailing genotypes are generally well adapted for their particular environments, and most changes are unlikely to improve them further[65]. Therefore, we could consider that ethylene, cytokinin, ABA and *AtPsiCaM* (Pi-starvation induced CaM) and Pi-starvation-response had something to do with regulating stomatal development; different signal transduction pathway could influence the same plant process.

Why did the stomatal indices in all of the tested mutants

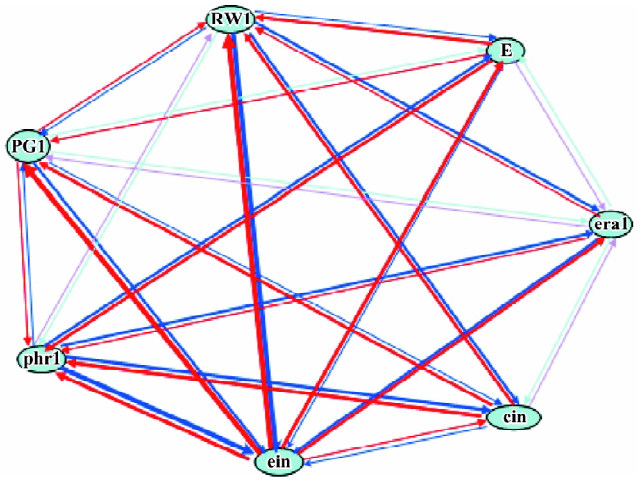

Figure 3. A simplified possible model for the *Arabidopsis* stomatal development regulatory network based on the data in Figure 2. We hypothesized the relation existed between mutants. In this model, arrow point at the mutant which is regulated. Red lines indicate significantly negative regulation, blue lines indicate significantly positive regulation. The thicker line represents a more obvious relative. Purple lines indicate significantly negative regulation and Green lines indicate positive regulation.

change so significantly? The genome of *Arabidopsis* is nearly the smallest one in high plants, and it is haploidy[54], furthermore, it is easy to get an *Arabidopsis* mutant. Thus, combining the results, we could speculate that the stomata intensity in *Arabidopsis* might be highly sensitive to most mutations in genome, while the effects of many gene mutations on the stomatal index might be negative. Moreover, we also could presume that the stomatal development was regulated by a signal network in which one signal transduction pathway change might influence the stomatal development more or less; and the stomata intensity could be used as an index of the relative regulation in the genome transcription signal and metabolic networks.

According to the result, regardless of natural over-expression or intervention in CaM mutant, stomatal indices all decrease compared with wild type (Figure 1). Whether the expression of CaM was enhanced or intervened, both induced the decreased change of stomatal index. These showed that CaM was very sensitive to stomatal development. Therefore, we considered that whether the expression of CaM was enhanced or intervened, both influence the calcium signal transduction. Therefore, the calcium signal transduction was possibly a hub in the stomatal development regulation network.

The decrease of the stomatal indices in ethylene insensitive mutants is most obvious compared with that of wild type (Figure 1). The results showed that ethylene signal made the change of stomatal index decrease and ethylene signal transduction was sensitive to stomatal development, which could be influenced by calcium signal transduction.

The gene network The network of gene interactions may be obtained among that of calcium signal transduction, Pi signaling pathway, ETH signaling pathway, CK signaling pathway and ABA signaling pathway based on our findings (Figure 4).

Calcium signal transduction The role of calcium in various signal transduction pathways is well known[66,67]. Several studies have implicated Ca^{2+} in ethylene signal transduction[68]. Evidence was presented earlier that Ca^{2+} participates in cytokinin signaling in Amaranthus[67,69]. Organic

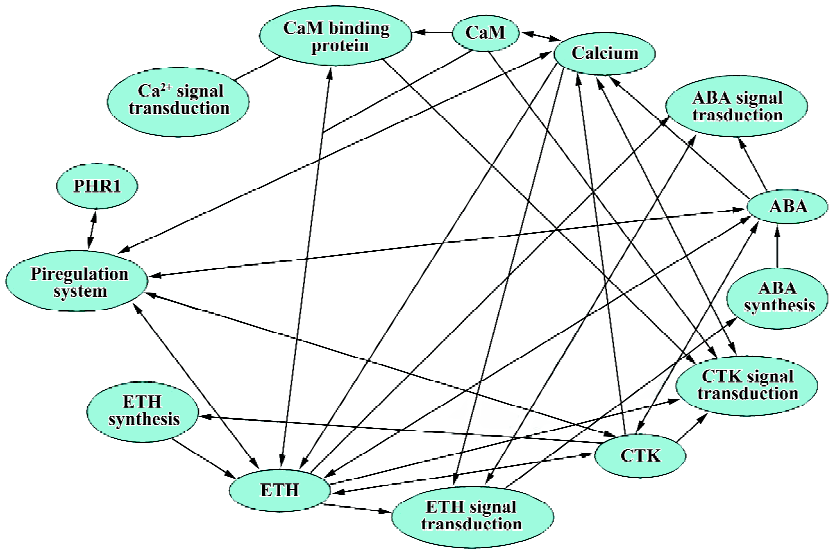

Figure 4. A simplified model for the known interaction network in *Arabidopsis*. Arrow means the action orientation.

acids are secreted to calcium cations, which increases mobilization of Pi from both acidic and calcareous soils[70]. CaM, a key calcium sensor in all eukaryotes, regulates diverse cellular processes by interacting with other proteins[36,68]. CaM join in the process of controlling Ca^{2+} signal speciality[36]. Calcium, through CaM, could regulate the activity of EICBP (ethylene-induced CaM-binding protein), which is an ethylene inducible gene[68]. The proposed cytokinin-induced rise in intracellular calcium may be affected in part by the activation of CaM[71]. Cytokinin-regulated responses is inhibited by CaM-binding compounds[72]. CaM-binding proteins are also involved in ethylene signal transduction[68] .

Pi signaling pathway Vicente Rubio *et al* considered that this protein PHR1 acted downstream in the Pi starvation signaling pathway[33]. Pi starvation-responsive genes appear to be involved in multiple metabolic pathways, implying a complex Pi regulation system in plants[34]. Phosphorus regulated almost every signal transduction pathway by a constituent of such key cell molecules and a pivotal regulator in many metabolisms (including energy transfer, protein activation, and carbon and amino acid metabolic processes)[35,36,67,73,74].

Ethylene signaling pathway Ethylene could negatively regulate ABA synthesis[75]. A calmodulin binding protein from *Arabidopsis* is induced by ethylene[68]. It is likely that ethylene mediates specific aspects of Pi signaling in vascular plants[70].

Cytokinin signaling pathway Cytokinin, which may involve different classes of Ca^{2+} channel[76], increases intracellular Ca^{2+} in Funaria[77] and in moss protoplasts[78]. Cytokinins can elevate ethylene biosynthesis in etiolated *Arabidopsis* seedlings via ACC synthase[51]. It is likely that cytokinin mediates specific aspects of Pi signaling in vascular plants[70,79]. Under Pi-starvation conditions, Pi regulation system is regulated by cytokinin[80].

Abscisic acid signaling pathway ABA has been shown to increase the probability of the opening of hyperpolarization-activated Ca^{2+}-permeable channels. It has also been established that ABA induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells[74]. Under Pi-starvation conditions, Pi regulation system is regulated by ABA[80].

Two signaling pathways interaction Chang *et al* have provided evidence that the regulation of flower senescence involves the interaction of cytokinins, ethylene, and ABA[81]. Ethylene-mediated cross-talk between calcium-dependent protein kinase and mitogen-activated protein kinases (MAPK) signaling controls stress responses in plants[82]. Ethylene is involved in the cytokinin signal transduction, or that ethylene and cytokinins both participate a conjunct approach or composition[54,83]. ABA signaling is regulated by the ethylene response pathway in Arabidopsis[75]. Unlike several other hormone interactions involving ethylene, cross-talk between ABA and ethylene appears to occur at many levels and is dependent on the tissue and the process being assayed[75,84–86]. Reducing the ethylene response could induce ABA synthesis, which in turn could increase the dormancy of the seed[75].

The hypothesis: a network regulate stomatal development Each cell is a production of multiple signal transduction programs involving expression of thousands of genes[87]. In addition, the presence of intracellular signaling components that feature in multiple signal pathways suggests that the existence of truly stand-alone pathways are highly unlikely, and the architecture must become increasingly reticulate[1,87]. The eukaryotic cellular functions are highly connected through networks[87], the hub of signal transduction that regulate other signal transduction. It is possible that these signal transduction pathways are modified as guard cells progress through the cell cycle, in response to changes in environment, and during stomatal development.

Is there any evidence that guard cell signaling is organized as a network and specifically as a type of network known as a scale-free network? The multiple transcriptional regulators within each category were able to bind to genes encoding regulators that are responsible for control of other cellular processes[1]. Recent works show that the control of stomatal aperture by environmental signals depends on coordinated alterations to guard cell turgor (ionic fluxes and sugars), cytoskeleton organization, membrane transport, and gene expression and multiple cellular processes[1]. The action of hormones (auxin and abscisic acid) on guard cells and the organic anions enhanced by changes in apoplastic K^+, $Cl^-$and Ca^{2+} can alter their response to light to modulate stomatal opening[25]. It has also been revealed that a bifurcating signaling pathway directs ABA effects on stomatal movement[88]. If the effects of CO_2 on stomatal aperture and development might be brought about through a signaling network, then alterations in sensitivity to this signal should have effects on other pathways[1,89].

Moreover, during the process of evolution, a plant repairs the original signal transduction pathway to acclimatize oneself to new environmental change or something else. We speculated that the signal transduction pathway interaction, which could regulate other morphology, distribution and behavior in plants (eg, roots) responds to a spectrum of signal pathways in plants, but were also able to regulate stomatal development. In this network, one signal transduction pathway could regulate stomatal development indirectly,

that is, it could act on other signal transductions that might influence stomatal development directly by an existing pathway. The stand-alone, stimulus-specific signaling pathways might be an inadequate means of controlling stomatal development. We speculated that the existing regulation network was also able to regulate stomatal development. Moreover, we presumed the relation existed between mutants; consequently, we constructed a simplified model (Figure 2, 3) for the *Arabidopsis* stomatal development regulatory network upon these data in this paper. Comparing the two figures (Figure 3, Figure 4), we found similarity between them. We could hypothesize that calcium as a hub, play an important role in regulating stomatal development in the network; calcium signaling exerted more influence on regulating stomatal development than ethylene, cytokinin, ABA and Pi signal transduction pathway; the other signal transduction pathways all regulated stomtal development by influencing calcium signal transduction pathways or being influenced by calcium signal transduction pathways. Therefore, the presence of multiple cellular processes might interact to regulate stomatal development, and the architecture might be reticulate.

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