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Roles of heterotrimeric G proteins in guard cell ion channel regulation

Wei Zhang
The Key Laboratory of Plant Cell Engineering and Germplasm Innovation; Ministry of Education; College of Life Science; Shandong University; Jinan, Shandong, China.

Stomata are formed by pairs of surrounding guard cells and perform important roles in photosynthesis, transpiration and innate immunity of terrestrial plants. Ionic solutes in the cytosol of guard cells are important for cell turgor and volume change. Consequently, transmembrane flux of ions such as K^+, Cl^-, and malate2$^-$ through K^+ channels and anion channels of guard cells are a direct driving force for turgor change, while the opening of calcium permeable channels can serve as a trigger of cytosolic free calcium concentration elevations or oscillations, which play second messenger roles. In plants, heterotrimeric G proteins have fewer members than in animals, but they are well investigated and found to regulate these channels and to play fundamental roles in guard cell function. This mini-review focuses on the recent understanding of G-protein regulation of ion channels on the plasma membrane of guard cells and their participation in stomatal movements.

Key words: guard cell, heterotrimeric G protein, ion channel, *arabidopsis thaliana*, stomata, plasma membrane, patch clamp

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*Correspondence to: Wei Zhang;
Email: weizhang@sdu.edu.cn

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Heterotrimeric G proteins, composed of Gα, Gβ and Gγ subunits, are key elements of cellular signal transduction networks. In plant species, fewer members of G proteins are present than in animals. For example, only one Gα subunit (GPA1), one Gβ subunit (AGB1) and two Gγ subunits (AGG1 and AGG2) are reported in Arabidopsis while 23 Gα, 5 Gβ and 12 Gγ subunits have been identified in human.[1] All three kinds of subunits are expressed in guard cells. Ubiquitous expression of *GPA1* throughout plant was ascertained by northern and promoter::GUS analyses and RT-PCR results also indicate guard cell expression.[2-4] *AGB1* is ubiquitously expressed throughout the plant and its promoter::GUS transgenic lines show strong expression in guard cells.[5-7] For Gγ subunits, RNA blots show *AGG1* and *AGG2* expression throughout the plant, however, reporter gene analysis shows guard cell expression of *AGG2* but not *AGG1*.[7-9] The guard cell expression of G protein subunits implies the function of G protein in guard cell signaling and stomatal movement regulation.

Stomata are microscopic pores in the epidermis of terrestrial plants, which serve as the mouths of plants for gas change since through them CO_2 enters leaves for photosynthesis and water vapor is lost as transpiration.[10-13] In addition, stomatal movements induced by pathogen and pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) are a component of the plant innate immunity system.[14-16] Biotic and abiotic stresses (e.g. water deficiency, cold, pathogens) and their induced phytohormone changes (e.g. abscisic acid [ABA], ethylene) have been widely investigated in stomatal movement regulation, and stomatal apertures are directly regulated by volume change of the surrounding guard cell pairs. The accumulation/release of ionic solutes through ion channels on the guard-cell plasma membrane together with malate production/metabolism induces water influx/efflux driving increase/decrease of cell turgor and volume which co-operates with the radial reinforcement of the guard cell walls to widen/shrink stomatal aperture.[10,17] Given that mature guard cells

lack plasmodesmata with neighboring cells, all ion uptake and efflux must pass through ion channels and ion transporters on the plasma membrane.

In Arabidopsis guard cells, the model cell type for cell signaling of the model plant species, all three kinds of ion channels (K^+ channels, anion channels and Ca^{2+}-permeable channels) have been investigated and found to be regulated by heterotrimeric G proteins.[10,17] Their ion channel activities can be measured in intact guard cells, guard cell protoplasts, or cell membrane patches using the patch clamp technique.[15,18,19] Patch clamping can be used to measure ion fluxes in whole cells or even through a single ion channel.[20,21] The patch clamp technique under the whole-cell recording configuration can measure the currents through hyperpolarization-activated inward K^+ channels which account for K^+ accumulation during stomatal opening, and the depolarization-activated outward K^+ channels which, together with R-type and S-type anion channels, mediate solute removal during stomatal closure. Besides these ionic fluxes which directly elicit changes in turgor, Ca^{2+}-permeable channels which participate in Ca^{2+} signaling are also regulated by G proteins. For better visualization of the currents through K^+, anion and Ca^{2+}-permeable channels, real current traces and their idealized current/voltage relationships are indicated in **Figure 1**. The G-protein regulation of inward and outward K^+ channels, S-type anion channels, and Ca^{2+}-permeable channels and their significance for stomatal movements will be discussed below, and the genes encoding them which have been explored up to now also will be discussed.

G-protein Regulation of Guard Cell Ion Channels During Stomatal Movements

Evidence suggesting that G proteins regulate stomatal movements and ion channel activities was first obtained in the early 1990s using electrophysiological and pharamacological methods applied mainly to guard cells of the broad bean, *Vica faba*. With the sequencing of the Arabidopsis genome and identification of G protein encoding genes, Arabidopsis quickly became the model system for the study of G protein function in plants. The acquisition and characterization of mutants lacking functional heterotrimeric G proteins facilitated direct examination of the roles of heterotrimeric G proteins in the regulation of ion channels and stomatal movements.

ABA is the best studied regulator of stomatal movements. ABA inhibits stomatal opening and promotes stomatal closure, reducing transpirational water loss; in addition, guard cell ABA signaling is one of the best-defined cellular signaling networks in plants.[13,22,23] Most of the roles of G proteins in regulation of ion channels during stomatal opening are associated with ABA signaling. Electrophysiological experiments using G protein modulators, phytohormones and G protein mutants have greatly contributed to our understanding of the G-protein signaling network of stomatal movements.

G-protein regulation of K^+ channels. Guard cell plasma membrane K^+ channels mediate K^+ uptake/release and thus control changes in guard cell turgor change.[24,25] Since G proteins can be constitutively activated by GTPγS or cholera toxin, and inactivated by binding to GDPβS or pertussis toxin, a combination of pharmacological and electrophysiological methods was once widely used to study G-protein regulation of guard cell ion channels and stomatal movements. G protein regulation of ion channels was first demonstrated in *Vicia faba* guard cells, in which the inward-rectifying K^+ channels were found to be activated by GDPβS and inhibited by GTPγS, cholera and pertussis toxins.[26] That finding was supported by further single-channel recordings from isolated membrane patches, showing the G-protein regulation of ion channels can occur via a membrane delimited mechanism.[27] Besides electrophysiology, light-induced stomatal opening could be promoted by microinjection of GTPγS into guard cells.[28] These early studies strongly suggested the involvement of heterotrimeric G proteins in the regulation of ion channels and stomatal movements.

Arabidopsis genes encoding Gα and Gβ subunits of the G protein were identified even before the Arabidiopsis genome was completely sequenced.[5,29] Completion of whole genome sequencing, together with the development of guard cell protoplast isolation and patch clamping techniques for Arabidopsis, resulted in this species becoming the model plant system for study of G protein regulation of ion channels at molecular level. T-DNA insertional mutants lacking functional genes for heterotrimeric G proteins could be used directly for the study of the involvement of heterotrimeric G proteins in regulation of guard cell ion channels and G protein effects on stomatal movements.[1] In guard cells of null mutants of the sole Gα gene, *GPA1*, ABA inhibition of inward K^+ channels was abolished, consistent with the observation that light-induced stomatal opening is hyposensitive to inhibition by ABA in these *gpa1* mutants.[4] Interestingly, consistent ABA hyposensitive phenotypes were observed in *agb1*, and *gpa1abg1* mutants: without ABA, there is no alteration in K^+ currents and with ABA, inhibition of inward K^+ currents and light-induced stomatal opening is impaired.[30] Similar experiments have also been conducted in mutants of the two identified Gγ genes, *agg1* and *agg2*,[8,9] unexpectedly, neither single mutants nor double mutants of these two Gγ subunits showed similar phenotypes to that of *gpa1* or *agb1*; rather, these mutants showed wild-type ABA inhibition of inward K^+ channels and stomatal opening.[31] So, it is reasonable to speculate that there exist unidentified, additional Arabidopsis Gγ(s) which work(s) in guard cells together with GPA1 and AGB1. Besides the phytohormone ABA, loss of function of GPA1 also blocks flg22 inhibition of inward K^+ channels and stomatal opening, indicating that plant G proteins are common elements for crosstalk of ABA and elicitors in guard cells.[15]

To date, nine genes encoding K^+-channels have been identified in Arabidopsis: *KAT1*, *KAT2*, *AKT1*, *AKT5*, *SPIK*, *AKT2/3*, *AtKC1*, *SKOR* and *GORK*.[10,32,33] In guard cells, the inward K^+ channel members or subunits *KAT1*, *KAT2*, *AKT1*, *AKT2/3*, *AtKC1*, and the outward K^+ channel, *GORK*, are expressed.[32-34] Evidence accumulating from heterologous expression and functional analysis of heteromeric inward K^+ subunits (e.g. *At*KC1 and AKT1) indicates

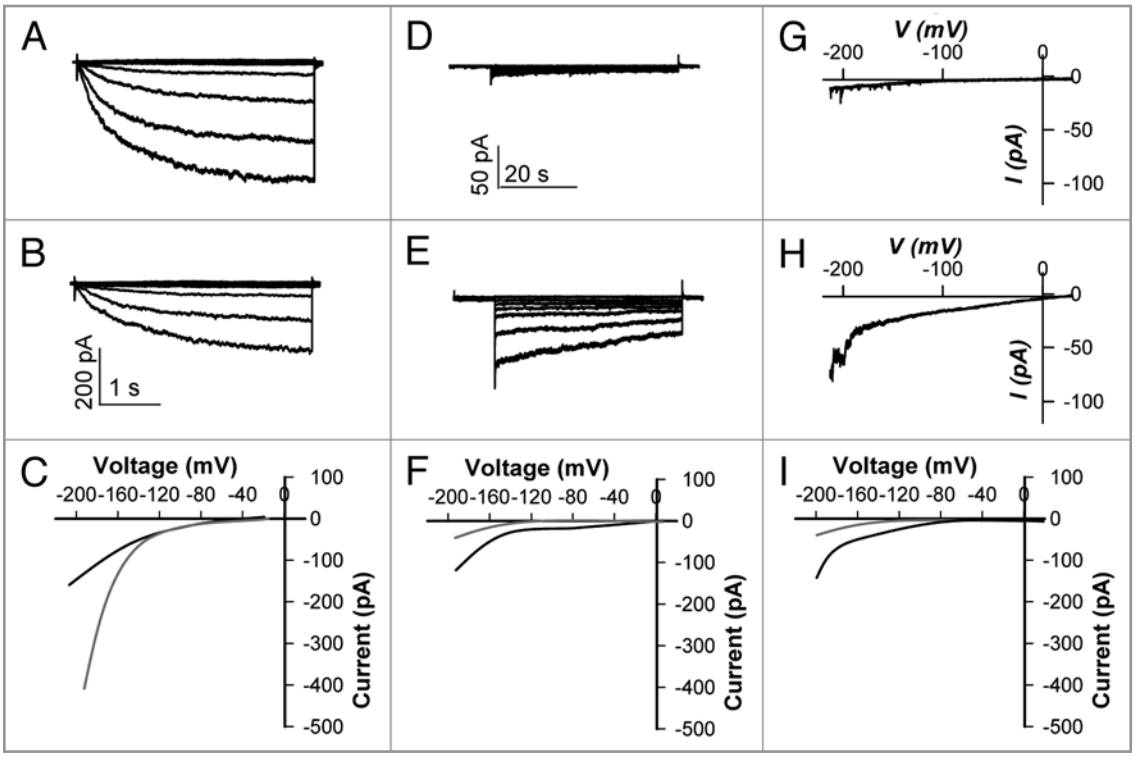

Figure 1. Current traces and idealized current/voltage relationships of wild type guard cell plasma membrane ion channels involved in G-protein regulation (A–C), ABA inhibition of whole-cell inward K^+ currents. (A) indicates inward K^+ currents of wild type guard cell protoplasts in response to hyperpolarizing voltages under control conditions [Scale bar is shown in (B)]; (B) indicates inward K+ currents of wild type guard cell protoplasts with ABA treatment; (C) indicates the idealized current/voltage relationship of inward K^+ currents for control (gray) and ABA treatments (black). (D–F), ABA activation of slow anion currents. (D) indicates anion currents of wild type under control condition and (E) shows current after ABA treatment; (F) indicates the idealized current/voltage relationship of anion currents for control (gray) and ABA treatments (black). (G–I), ABA activation of currents through Ca^{2+}-permeable channels. (G) indicates currents through Ca^{2+}-permeable channels of wild type under control condition and (H) shows current after ABA treatments; (I) indicates the idealized current/voltage relationship of currents through Ca^{2+}-permeable channels for control (gray) and ABA treatments (black).

that functional inward K^+ channels are heteromers,[35] and since ABA could not totally inhibit the inward K^+ currents, further work is needed to clarify which kind of specific inward K^+ channels are regulated by G proteins. Furthermore, even though there is no significant difference before and after ABA application in outward K^+ currents of guard cells in wild type and the mutants of G-protein subunits,[4,30,31] a gradual inhibitory effect of flg22 on outward K^+ channels observed in wild type guard cells was absent from *gpa1* mutants, indicating the involvement of G-protein in regulation of outward K^+ channels and thereby stomatal closing.[15]

G-protein regulation of anion channels. Anion channels on the plasma membrane of guard cells which mediate anion efflux during stomatal closure are categorized as R-type (rapid) and S-type (slow) according to their electrophysiological characteristics.[11] Since the S-type channels could be activated by a large range of voltages and exhibit slow deactivation allowing export of a large amount of anions, they are a major component of the membrane depolarization mechanisms that drive stomatal closure (**Fig. 1**).[36] ABA activates both S-type and R-type anion channels;[36] G-proteins involvement in ABA regulation of S-type channels has been observed, while R-type channels have not yet been assessed for such regulation.[4,30] *gpa1* and *agb1* mutants show reduced ABA activation of outward anion channels under strong cytosolic pH buffer, however, under weak pH buffering, ABA activation of outward anion channels is identical in wild type and in mutants of *gpa1* or *agb1*.[4,30] These results demonstrate the existence of two parallel pathways that mediate ABA activation of S-type anion channels: one through cytosolic pH and another dependent on G proteins. The genes encoding anion channels have been identified recently; *SLAC1* encodes S-type anion channels and R-type channels are likely encoded by *AtALMT12*, but other reports showed the existence of R-type anion currents in *atalmt12*, so its function needs further confirmation.[37-39] GPA1 and AGB1 mediate ABA activation of S-type anion channels implies indirect or direct regulation of SLAC1 by G proteins.

G-protein regulation of Ca^{2+}-permeable channels. Hyperpolarization-activated Ca^{2+}-permeable channels on the plasma membrane of guard cells trigger Ca^{2+}_{cyt} elevation, and such elevation inhibits inward K^+ channels and activates both S-type and R-type anion channels, facilitating net solute removal during stomatal closure.[12,40-42] ABA promotes the production of reactive oxygen species (ROS) such as H_2O_2 which activate plasma membrane

Ca^{2+} channels resulting in an increase in Ca^{2+}_{cyt} levels and stomatal closure.[42] Since ABA promotes Ca^{2+}_{cyt} elevation and Ca^{2+}_{cyt} elevation in turn activates S-type anion channels,[4,12,30] it is reasonable to hypothesize that G proteins are involved in ABA regulation of Ca^{2+}-permeable channels and Ca^{2+}_{cyt} generation. This hypothesis was directly supported by recent electrophysiology experiments.[43] In that study, ABA activation of plasma membrane Ca^{2+}-permeable channels and ROS elevation in guard cells was minimal in *gpa1* mutants, while H_2O_2 activation of these channels and of ROS elevation could be observed in guard cells of both wild type and *gpa1*. These data suggest that G proteins function upstream of ROS production but downstream of ABA perception in guard cells.[43] To date, the specific genes encoding guard-cell voltage-dependent Ca^{2+}-channels have not been identified. Three gene families, comprising 41 genes total have been proposed as the best candidates: glutamate receptor family (20 members), cyclic nucleotide gated channel family (CNGCs, 20 members) and the two pore calcium channel, *TPC1*.[32] TPC1 was identified as vacuolar Ca^{2+} activated channel, but its function still needs further confirmation.[44] Ca^{2+} currents could be activated by cAMP, and heterologous expression and functional analyses of CNGC2 showing CNGC members mediate Ca^{2+} influx in response to Ca^{2+} needed stresses, but Ca^{2+} current could also be seen without cAMP indicating the existence of other voltage-dependent Ca^{2+}-permeable channels.[45-49] The uncertainty of molecular identification of the Ca^{2+} channels that mediate ABA activation of guard cell Ca^{2+} currents make it impossible to address which gene(s) encode(s) the Ca^{2+} channels targeted by G-proteins.

Conclusion and Perspectives

To summarize, with the aid of the patch clamp technique and G protein subunit mutants, accumulating evidence firmly supports the G protein regulation of K^+, anion and Ca^{2+} channels. With the collective assistance of ion channel cloning and in vivo functional analyses, soon we may know the specific molecular mechanisms and channel(s) targeted by the G protein.

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