Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in Arabidopsis thaliana

Lingang Zhang 1, Guangzhen Hu 1, Yuxiang Cheng, Jirong Huang *

Institute of Plant Physiology and Ecology Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China

Abstract

Stomata are essential for efficient gas and water-vapor exchange between the atmosphere and plants. Stomatal density and movement are controlled by a series of signal molecules including phytohormones and peptides as well as by environmental stimuli. It is known that heterotrimeric G-proteins play an important role in the ABA-inhibited stomatal opening. In this study, the G-protein signaling pathway was also found to regulate stomatal density on the lower epidermis of Arabidopsis cotyledons. The loss-of-function mutation of the G-protein α-subunit (GPA1) showed a reduction in stomatal density, while overexpression of the constitutively active form of GPA1Q26I increased stomatal density, indicating a positive role of the active form of GPA1 in stomatal development. In contrast, stomatal density increased in the null mutant of the G-protein β-subunit (AGB1) but decreased in transgenic lines that overexpressed the constitutively active form of AGB1Q26I. Taken together, these results suggest that the stomatal density in Arabidopsis is modulated by GPA1 and AGB1 in an antagonistic manner.

Introduction

The stoma, a small pore surrounded by a pair of guard cells, controls exchanges of gas and water-vapor between plants and atmosphere, and thus is critical for photosynthesis and water use efficiency (Hetherington and Woodward, 2003). Stomatal development is characterized by a series of epidermal cell divisions in Arabidopsis (Nadeau and Sack, 2003). First, Undifferentiated epidermal cells, also called meristemoid mother cells (MMC), divide asymmetricaly to produce a small cell, namely meristemoids, and a larger sister cell. This asymmetrical division is named the entry division to initiate the stomatal lineage. Second, meristemoids undergo up to several rounds of asymmetric divisions, namely amplifying divisions, either to increase the number of the total epidermal cells or to convert into guard mother cells (GMC). On the other hand, the larger sister cell can become a pavement cell or undergo spacing divisions, which prevent stomata from direct contact each other, to generate satellite meristemoids. Lastly, GMCS divide symmetrically to form a pair of guard cells. Therefore, stomatal number depends on the frequency of these three asymmetrical cell divisions of the larger sister cells and meristemoids (Bergmann and Sack, 2007).

Recently, a significant progress has been made towards identification of components in the pathway of the stomatal cell lineage. Genetic analyses reveal a main linear pathway initiated by leucine-rich repeat (LRR) receptor-like kinases (Bergmann and Sack, 2007). The subtilisin-like protease STOMATAL DENSITY and DISTRIBUTION 1 (SDD1) may generate a cell–cell signal that is recognized by a LRR receptor-like kinase, TOO MANY MOUTHS (TMM) together with other three ERECTA family receptor-like kinases, ERECTA, ERECTA-like 1 (ERL1) and ERL2 (Berger and Altmann, 2000; Von Groll et al., 2002; Nadeau and Sack, 2002). The signal is transmitted from receptors into the nucleus through a mitogen-activated protein kinase (MAPK) cascade, which is composed of YODA (a MAPKKK), MKK4/MKK5, and MPK3/MPK6 (Bergmann et al., 2004; Wang et al., 2007). Although the direct downstream effectors of this MAPK cascade are unclear, three critical transcription factors containing the basic helix-loop-helix domain have been demonstrated to regulate sequential steps in stomatal differentiation: SPEECHLESS (SPCH) commences the first asymmetric cell division to produce MMCs; MUTE is required for termination of the asymmetric division activity and promotion of differentiation from meristemoids to GMCs; and FAMA regulates the last step of stomatal development to promote guard cell differentiation (MacAlister et al., 2007; Pillitteri et al., 2007; Ohashi-Ito and Bergmann, 2006). In addition to these developmental or genetic factors, stomatal density and distribution on the epidermal layer of cotyledons, stalks and leaves are also influenced by environmental cues (Hetherington and Woodward, 2003; Gray et al., 2000). Many environmental factors such as humidity (Schürmann, 1959), temperature (Srivastava et al., 1995), CO2 partial pressure (Clifford et al., 1995; Gray et al., 2000), and light intensity (Rahim and Fordham, 1991) have been demonstrated to...
modulate stomatal density or stomatal index. In *Arabidopsis*, stomatal number is increased in newly emerging leaves treated by higher CO₂ concentrations or light intensity. Environmental cues can be sensed in mature leaves and then affect stomatal development in the developing leaves through long-distance signaling. One component, designated HIGH CARBON DIOXIDE (HIC) which encodes a putative 3-keto acyl coenzyme A synthase, has been identified to control stomatal number in response to elevated CO₂ ([Gray et al., 2000]). As understanding of stomatal development is largely advanced, it remains unclear how environmental signals are sensed and are subsequently incorporated into the developmental and patterning pathways to finely modulate stomatal number and distribution.

In recent years, much has been learned about the diversity of signal transduction mediated by G-proteins in *Arabidopsis* and rice ([Perfus-Barbeoch et al., 2004]). For many developmental processes, G-proteins play an important role in regulating cell proliferation ([Ullah et al., 2001; 2003; Chen et al., 2003]). Compared to animals, plants have a smaller number of heterotrimeric G-proteins. The *Arabidopsis* genome contains genes encoding only one canonical G-protein α-subunit (Gα), one β-subunit (Gβ), two γ-subunits (Gγ), one Regulator of G-protein Signaling (RGS), and a few putative G-protein-coupled receptors (GPCRs) ([Jones and Assmann, 2004; Offermanns, 2003]). The role of heterotrimeric G-proteins in plant cell division is contingent on cell types. For example, null alleles of *Arabidopsis* Gα subunit (gpa1) exhibit a reduced number of lateral root primordia, whereas null alleles of Gβ subunit (agb1) enhance cell division in roots and produces excessive lateral roots ([Ullah et al., 2003]). In addition, null alleles of RGS1 or overexpression of a constitutively active GPA1 confer an increased cell division in the root apical meristem ([Chen et al., 2003]), indicating that the GTP-bound form of GPA1 plays a positive role in cell proliferation. In contrast, heterotrimeric complex acts as an attenuator of cell proliferation in the root apical meristem ([Chen et al., 2006a, 2006b; 2006c]). In animals, heterotrimeric G-proteins are also crucial for asymmetric cell division to generate cell diversity in addition to their role in cell proliferation ([Gutkind, 1998]). Here we provide a line of genetic evidence that plant G-protein signaling is involved in regulation of the frequency of asymmetrical cell divisions which is required for stomatal development in *Arabidopsis*. GPA1 and AGB1 modulate stomatal density in an opposite direction but have no effect on one-celled spacing. Deletion of GPA1 reduces stomatal density, whereas loss-of-function of AGB1 enhances stomatal density. The results support a proposition that plant G-proteins can accurately adjust cellular activities to maximally adapt environmental changes in multiple ways.

**Materials and methods**

**Plant materials and growth conditions**

Wild type plants used in this study were Columbia-0. All the mutants and transgenic genotypes used were in the Col-0 background. Mutants (gpa1, rgs1, agb1, gpa1 agb1) and transgenic lines (GPA1) and AGBl20) were as described by [Chen et al., 2006]. Seeds were surface-sterilized and grown in sterile culture on half strength Murashige and Skoog agar medium with 1% sucrose. Plates were incubated under light conditions of 70 μmol m⁻² s⁻¹ with 10-h-light/14-h-dark cycles and a constant relative humidity of 40% at 23 °C in the chamber. Cotyledons of 7-day-old seedlings were used in experiments, otherwise indicated specifically.

**Confocal microscopy and stomatal statistics**

For each genotype, stomatal numbers were recorded from 10 cotyledons. To visualize outlines of abaxial epidermal cells, cotyledons were immersed in 1 μM FM4-64 for 30 min, and mounted on slides with the cotyledon abaxial side facing up. Images were taken with a confocal laser-scanning microscope (LSM 510 META, ZEISS). According to the method of Claudia Kutter et al. (2007), the stomatal density, stomatal index, and the proportion of primary and higher-order stomatal complexes were quantified from confocal image of abaxial surface each cotyledon.

**RT-PCR analysis of MUTE and SPCH**

Total RNA was extracted from 7-day-old cotyledons using RNequeous (Ambion) according to the manufacturer’s instructions. After DNase treatment, 1 μg of total RNA was used for reverse transcription. Subsequently, 1 μL of reverse transcription reaction was used as template for PCR amplification. For RT-PCR, the primers for *AMA* were (Forward, 5'-GAGCTGAGCAACTCTTACAAT; Reverse 5'-GAACTCTTGTGCTTGTATCAGT), for *TMM* were (Forward, 5'-CTCTTACCATGAGCAATTA; Reverse 5'-ACGGTACTGTCCTTCTGACT), for *YDA* were (Forward, 5'-CACATGAGATCCTGGACAT; Reverse 5'-GCCATGTTTTAATCCTTTCTGC), for *ERECTA* were (Forward, 5'-GATAATGTCAAAGACGGAAG; Reverse, 5'-GGAAAATCTTTCTACCCAC), for *MUTE* were (Forward, 5'-CATAAAGGGGAGATCAAG; Reverse 5'-CAGAGATGATCTTACGAGC), for *SPCH* was (Forward, 5'-AAAATCTGTCCTGCTTGTAGAAG; Reverse, 5'-AGAAATGAGTACGTACTGC), and for *RBS* were (Forward, 5'-ATGCTGATCTCTTCTCAGA; Reverse, 5'-TTAGAAAAGGAACCCGTCGAAGACAGC). The PCR products were examined on a 1.2% agarose gel stained with ethidium bromide. The same RNA samples and primers were used for real-time PCR analysis. SYBR green was used as the intercalating dye. As an internal control, the RBS transcript was used to quantify the relative transcript level of each target gene. Quantitative RT-PCR analysis was performed using an Opticon-2 real-time PCR machine (MJ Research). The thermal cycling conditions were as follows: 5 min in 96 °C, followed by 30 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Relative levels of each transcript were calculated on the base of transcript levels of wild type. Three replicate biological experiments were conducted.

**GUS staining**

One-day-old *Arabidopsis* seedlings were incubated in GUS staining buffer (10 mM EDTA, 0.1% Triton x-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 100 μg/mL chloramphenicol, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 50 mM sodium phosphate buffer, pH 7.0) for 6 h at 37 °C. The seedlings were then cleared in 20% lactic acid and 20% glycerol and observed on an Olympus IX-70 microscope under Nomarski optics (magnification, ×180). The total number of stomata per cotyledon was quantified from GUS staining images. Counts were made of ten different cotyledons.

**Drought tolerance and water loss**

Seven-day-old seedlings grown on the 1/2 MS medium were air-drought-stressed through opening the cover of plates. The relative humidity decreased from 80% of inside of the plate to 40% of ambient. Picture was taken with Olympus IX-70 microscope after treatment for 45 min. For water loss assay, 50 cotyledons were detached from 7-day-old seedlings, and weighed immediately as fresh weight, then placed in controlled conditions and weighed at indicated time intervals. Water loss was measured and expressed as the percentage of initial fresh weight. In all of the drought tolerance and water loss experiments, seedlings or detached cotyledons were put under continuous 70 μmol m⁻² s⁻¹ fluorescent cool white light at 23 °C and 40% of relative humidity.

**Results**

**GPA1 positively regulates stomatal development**

It was reported that stomatal number increases in the hypocotyl epidermis overexpressing either the wild-type form or the constitutively
active form of the G-protein α subunit GPA1 (Okamoto et al., 2001). To explore how G-protein signaling regulates stomatal development, we examined the number of stomata in the single mutants, gap1 and rgs1. Stomatal density, the number of stomata per unit area, was measured on the abaxial surface of cotyledons of 7-day-old seedlings grown on half MS media under white light (70 μmol m⁻² s⁻¹). As shown in Fig. 1, stomatal density was significantly reduced in gap1 while enhanced in rgs1, which was detected to have an elevated steady-state pool of activated Go (Chen et al. 2003). These data suggest a positive role of the active form of GPA1 in stomatal development. To confirm this hypothesis, we examined stomatal density in cotyledons of transgenic plants that overexpressed the constitutively active form of GPA1QL. Our data showed that overexpressing GPA1QL led to an increase in stomatal density (Figs. 1A, B).

To exclude possibility that difference in stomatal density was caused by change in epidermal cell size, stomatal index (SI), which is defined as the ratio of the number of stomata to the number of epidermal cells plus stomata, was measured in the abaxial surface of cotyledons as described by Salisbury (1927). We found that the stomatal index was also significantly lower in gap1 (23.1%) than in the wild type (32.0%). Likewise, stomatal index of the rgs1 mutant and the GPAQL transgenic plants increased (Fig. 1C). Together, the results suggest that the Go subunit plays a positive role in regulating stomatal development in cotyledons.

**AGB1 counteracts the action of GPA1 on stomatal development**

Since G-protein signaling can be transmitted through the activated Go, the Gβγ dimer, and/or the heterotrimeric complex, we then examined whether Gβγ could influence stomatal development. In contrast to the Go mutant, inactivation of the Gβ subunit (agb1) exhibited a clear increase in stomatal density by ∼24.7% in the cotyledon abaxial surface compared with that of the wild type. To further confirm that the Gβγ is a negative regulator of stomatal development, stomatal density was measured in the transgenic lines overexpressing AGB1. Overexpression of AGB1 (AGB1ox) reduced stomatal density on the abaxial epidermis of cotyledons (Figs. 2A, B). A similar result was observed if stomatal number was evaluated by stomatal index (Fig. 2C). Therefore, we concluded that AGB1 is a negative regulator of stomatal development. Since a phenotype which is opposite in gap1 and agb1 mutants usually indicates that G-protein signaling is mediated by Gβγ in Arabidopsis (Pandey et al., 2008), we tested whether AGB1 is the only signaling branch to regulate stomatal development. Stomatal density and index were examined in cotyledons of the gap1 agb1 double mutant. Stomatal density and index were higher in gap1 agb1 than in the wild type but lower than in agb1 (Figs. 2B, C), suggesting that the genetic relationship between GPA1 and AGB1 in stomatal density regulation can be intricate. They might function antagonistically in the same pathway or in different pathways for stomatal development.

**G-proteins regulate the proportion of the primary and higher-order stomatal complexes in a fine way**

As explained above, stomatal number is mainly dependent on the frequency of asymmetrical cell divisions. Generally, meristemoids are able to undergo up to 3 rounds of asymmetric divisions before differentiating into GMCs (Pillitteri et al., 2007). Each stoma with all its associated subsidiary cells is considered a discrete unit (a stomatal complex). Fig. 3A showed representative confocal microscopy images of individual primary, secondary and tertiary stomatal complexes. To dissect the modified process of stomatal development in G-protein mutants, we traced the cell lineage of individual stomatal complex. The number of the primary, secondary and tertiary stomatal complexes on the abaxial surface was analyzed with at least ten cotyledons for each genotype. Our results showed that loss-of-function of GPA1 conferred a markedly increased number of the primary stomatal complex but lacked the secondary and tertiary stomatal complexes compared with the control. In contrast, the mutation in AGB1 led to produce a higher proportion of the tertiary stomatal complex while a lower proportion of the primary and secondary stomatal complexes than the control.

Fig. 1. GPA1 is a positive regulator of stomatal development. Seedlings were grown on half strength of MS media supplemented with 1% sucrose under light condition of 70 μmol m⁻² s⁻¹ and 10-h-light/14-h-dark cycles at 23 °C. (A) Confocal images of stomata on the abaxial epidermis of cotyledons from 7-day-old seedlings of the wild type (Col), gap1 and rgs1 mutants, and the transgenic line expressing the constitutively active form of GPA1QL. Cell outlines were visualized with FM4-64 staining. Bars = 50 μm in every picture. (B and C) Stomatal density and index calculated from panel A, respectively. The data present average values of ten individual cotyledons ±SD. * and ** denote significant differences between mutant and wild-type plants at the level of P=0.05 and P=0.01 by a t test, respectively.
However, we did not observe that stomata were abnormally clustered or the one-cell spacing rule was affected in G-protein mutants. These results may reflect that G-proteins in influence stomatal development mainly through modulating the frequency of amplifying divisions occurring in meristemoids.

Expression of SPCH and MUTE was up-regulated in agb1 but down-regulated in gpa1

To date, many genes that control the production and spacing of stomata have been identified in Arabidopsis. These genes include putative receptors, a processing protease, a MAP kinase cascade, and transcriptional factors that act at either the early stage or the later stage in stomatal development. In order to understand molecular mechanism underlying the effect of G-proteins on stomatal development, we examined the expression level of genes such as ERECTA, YDA, TMM, FAMA, SPCH and MUTE in cotyledons of 7-day-old seedlings by RT-PCR using gene specific primers. Interestingly, we detected transcriptional difference of SPCH and MUTE, but not ERECTA, YDA, TMM or FAMA among the wild type, gpa1 and agb1 (Fig. 4A). Transcript levels of SPCH and MUTE decreased in gpa1 moderately while increased in agb1. This result was further confirmed by quantitative real-time PCR (Fig. 4B). The data showed that mRNA levels of SPCH and MUTE decreased by 48.3% and 55.2% in gpa1 compared with that of the wild type, respectively. In contrast, mRNAs of SPCH and MUTE accumulated 60.2% and 33.7% more in agb1 than in the wild type, respectively. It has been proposed that the number of stomata is mainly influenced by two factors: entry divisions of postprotodermal cells and later amplifying asymmetric divisions of meristemoids. SPCH is required for the first asymmetric entry division into the stomatal lineage (MacAlister et al., 2007; Pillitteri et al., 2007), while MUTE is a key gene determining the number of satellite meristemoids as well as stomatal differentiation from meristemoids to GMCs (Pillitteri et al., 2007). Thus, our gene expressing data were consistent with changes in stomatal density and patterning in G-protein mutants, suggesting a possible role of SPCH and MUTE in G-protein-mediated stomatal development.

The number of meristemoids is altered in gpa1 and agb1 cotyledons

Because MUTE is expressed strongly in a subset of meristemoids but weakly in GMCs and guard cells (Pillitteri et al., 2007), it is a suitable marker gene to identify meristemoids at the early stage of stomatal development. To further confirm whether alteration in the proportion of different stomatal complexes in G-protein mutants was associated with the number of meristemoids, we introduced the

![Figure 2](image_url)

**Fig. 2.** AGB1 negatively modulates stomatal development in a GPA1-independent manner. Conditions of seedling growth and technique to determine stomatal number was the same as described in Fig. 1. (A) Confocal images of stomata on the abaxial epidermis of cotyledons from 7-day-old seedlings of the wild type (Col), agb1 and gpa1agb1 mutants, and the transgenic line (AGB1ox) overexpressing AGB1. Bars=50 μm in every picture. (B and C) stomatal density and index of Col, agb1, AGB1ox, gpa1 agb1. The data represent average values of ten individual cotyledons±SD. *, significant (P=0.05); **, highly significant (P=0.01).

![Figure 3](image_url)

**Fig. 3.** Effect of GPA1 and AGB1 on the primary and higher-order stomatal complexes. (A) Representative confocal microscopy images of stomatal complexes on the abaxial surfaces of cotyledons. Bars=50 μm. (B) the proportion of primary, secondary, and tertiary stomatal complexes on the abaxial surfaces of the 7-day-old cotyledons of gpa1, Col and agb1. The proportion of stomatal complex types was compared between mutants and the wild type. *, significant (P=0.05); **, highly significant (P=0.01).
construct containing 1.9 kb of the MUTE promoter region driving the expression of β-glucuronidase (MUTEpro:GUS) into gpa1, agb1 and the control plants. The activity of MUTE promoter was examined 24 h after seeds germination through GUS staining. As shown in Figs. 5A and B, the number of cells with GUS staining in a cotyledon was less in gpa1 (49.5) while more in agb1 (69.3) compared with the wild type (60.3), indicating that the number of meristemoids/somata is apparently altered in different genetic backgrounds. These results may indicate the action of G-proteins to the site of meristemoid regeneration in the course of stomatal development.

Water loss of cotyledons is reduced in gpa1 but enhanced in agb1

Transpiration water loss through stomata is a key determinant of drought tolerance [Xiong et al., 2002]. To test if the G-protein-mediated stomatal development was associated with the rate of water loss, 7-day-old seedlings grown on half MS in covered Petri dishes were transferred to 40% of the relative humidity after covers were removed. Cotyledons of agb1 and gpa1 agb1 seedlings exhibited a more severe symptom of water loss than those of the wild type (Fig. 6A). However, under the same condition, gpa1 cotyledons kept water turgor more strongly than wild-type ones (Fig. 6A). These results suggest that stomatal density regulated by G-proteins is proportional to the rate of water transpiration. In order to quantify differences in drought resistance among G-protein mutants and the wild type, transpiration rates of detached cotyledons were measured by their fresh weight changes over every 5 min under the controlled environment. The rate of water loss was significantly faster in agb1 and gpa1 agb1, but slower in gpa1 than the wild type (Fig. 6B). After 30 min, the relative water loss of gpa1 and wild-type cotyledons was 63.7% and 78.9%, respectively. In contrast, as high as 87.4% and 88.5% water loss was detected for agb1 and gpa1 agb1 cotyledons (Fig. 6B). Taken together, these results suggest that G-protein-mediated stomatal development plays a role in controlling water transpiration rate of seedlings.

Discussion

Signaling through G-proteins is highly conserved in all eukaryotes. Despite the presence of a smaller number of G-protein complexes in higher plants, G-protein signaling pathways have been demonstrated to mediate various critical cellular processes. Here, we described another function of G-proteins that engaged in a fine regulation of stomatal development in Arabidopsis cotyledons. Because asymmetrical cell divisions are required for the initiation of the stomatal cell lineage, our findings suggest that G-proteins are involved in regulation of asymmetric divisions. Two G-protein subunits, GPA1 and AGB1, play opposing roles in this process, providing a new insight of G-protein-mediated cell divisions.

Antagonistic regulation of G-protein signaling via GPA1 and AGB1 on stomatal development

In this study, we found that the mutation of GPA1 led to decrease in stomatal density of cotyledons, suggesting that GPA1 is a positive factor adjusting stomatal density in response developmental and/or environmental cues. This phenotype can be attributable to either lack...

Fig. 4. Gene expression of key regulators for stomatal development in gpa1 and agb1. Total RNA was abstracted from cotyledons of 7-day-old seedlings. (A) RT-PCR analysis of transcript levels of ERECTA, YODA, TMM, FAMA, MUTE and SPCH in the wild type, gpa1 and agb1 seedlings. The experiment was repeated three times, and a representative result of RT-PCR product was presented. MUTE and SPCH were up-regulated in agb1 mutant compared with that in the wild type. (B) Transcriptional levels of MUTE and SPCH were subject to be analyzed by quantitative real-time RT-PCR. The transcriptional levels were first normalized to the expression of rubisco small subunit (RBS), and then the value for expression of each gene in the wild type was set to 1. Data are means ± SD of three independent experiments. *, significant (P=0.05); **, highly significant (P<0.01).

Fig. 5. The number of meristemoids analyzed by histochemical localization of MUTEpro:GUS expression 24 h after seed germination in cotyledons of wild-type, gpa1 and agb1 seedlings. (A) MUTEpro:GUS reporter expression in cotyledons. GUS staining is restricted to guard mother cells and developing guard cells. Bars=10 μm. (B) the total number of guard cells and guard mother cells per cotyledon. The data represent average values of ten individual cotyledons ± SD. *, significant (P=0.05); **, highly significant (P<0.01).
of Gα subunit or permanent presence of free Gβγ dimmers. Further analyses of stomatal density in cotyledons of rgsI or transgenic plants that expressed the constitutively active form of GPA1Gβγ verify that the activated form of GPA1 is responsible for promotion of stomatal development. The data are consistent with previous reports showing that the number of stomata increased in hypocotyls of seedlings overexpressing GPA1Gβγ (Okamoto et al. 200), and that leaves overexpressing RGS7 have lower stomatal density compared with the wild type (Chen et al. 2006). G-protein signaling through the GTP-bound form of GPA1 has been shown to confer altered sensitivities to o-glucose (Huang et al., 2006), cell proliferation in the root apical meristem (Chen et al. 2006) and the phytochrome A signaling pathway (Wei et al., 2008). Recent studies of G-protein complexes isolated from the plasma membrane of wild-type plants reveal that about 30% of total Gα is present in large macromolecular complexes of approximately 700 kD associated with Gβγ, whereas the other Gα exists in free Gα (Wang et al., 2008). Considering that plant Gα has its high spontaneous nucleotide exchange coupled with a low GTPase activity, the free form of Gα is probably in the GTP-bound activated form which may directly signal to downstream effectors independently of Gβγ signaling. In addition, the activated form of Gα can also be generated from receptor-coupled dissociation of trimeric G-protein complexes.

On the other hand, our genetic data also revealed involvement of Gβγ signaling in stomatal development. In contrast to decrease in stomatal density in gpa1, loss-of-function of AGB1 results in an obvious increase in stomatal density in cotyledons. Consistently, overexpression of AGB1 led to reduced stomata density compared with the wild type. Thus, AGB1 may act as a negative regulator of stomatal formation. Since stomatal density is higher in agb1 cotyledons than that in rgsI or GPA1Gβγ-overexpressing transgenic cotyledons, the possibility is low that the observed increase in stomatal density in agb1 is resulted from the positive role of the active form of Gα. Thus, the antagonistic action between GPA1 and AGB1 in stomatal density reveals that both Gα and Gβγ can transduce signals to downstream effectors. This prediction is supported by stomatal density in the double gpa1 agb1 mutant which exhibits the mean value of the single gpa1 and agb1 mutants. Such antagonistic effect of GPA1 and AGB1 has been observed in lateral root formation (Ullah et al., 2003; Chen et al., 2006). However, signaling via G-proteins is obviously different between stomatal density and lateral root formation. In the latter case, AGB1 acts downstream of GPA1 and both loss-of-function of rgsI and overexpression of a constitutively active form of GPA1Gβγ have no effect on lateral root formation. In contrast, two branch pathways mediated by GPA1 and AGB1 upon activation of G-proteins are contributable to stomatal development. In animals and yeast, antagonistic or cooperative regulation of signaling pathways by both the Gα and Gβγ subunits has been well documented (Jordan et al., 2000; Slessareva et al., 2006). Our data support the hypothesis that both the Gα- and Gβγ-subunits can simultaneously regulate the same developmental process in plants.

**Effect of G-protein signaling on asymmetrical cell division**

In animals, heterotrimeric G-proteins are crucial for asymmetric cell division, which relies on the spindle being asymmetrically positioned during mitosis (Gönczy, 2002). Although subcellular localization of GPA1 and AGB1 is found in the newly formed cell plane (Chen et al., 2006), implying a possible role of G-proteins in the position of cell plane development, no experiments have been conducted to examine whether G-proteins are involved in plant asymmetrical cell divisions. Stomatal formation is a suitable system to address this question since asymmetrical divisions are required for the generation of meristemoids. It seems that plants own two signaling pathways in control of stomatal development. One is well characterized and composed of a ligand, several receptor-like kinases, and a MAPK cascade. This pathway is likely to respond developmental cues as well as plays an important role in maintaining the one-cell-spacing role. The other one, which is yet to be elucidated probably modulates stomatal density without disrupting the one-cell-spacing role under various environmental conditions. In this study, we found that G-protein-related mutants only exhibit the change in stomatal density, but do not disrupt the rule of one-cell spacing in cotyledons. This finding aids in addressing whether G-protein signaling is involved in regulation of stomatal density under environmental stresses. Meanwhile, gibberellins and ethylene have been shown to promote stomatal formation in hypocotyls (Saibo et al., 2003; Serna and Fenoll, 1997; Gao et al., 2008), it is possible that G-protein-regulated stomatal
development is associated with phytohormone signaling pathways in Arabidopsis.

Detailed examination of the ratio of different stomatal complexes indicated that Go might promote amplification of satellite meristemoids, whereas Gα acts oppositely to Go. This hypothesis is generally in agreement with the well-established role of the G-protein signaling pathway in the modulation of cell division (Ullah et al., 2001 and 2003; Chen et al., 2003) and the strongly meristem-expressed pattern of GPA1, AGB1 and RGS1 (Chen et al., 2006; Anderson and Botella, 2007; Chen et al., 2006). For example, GPA1 mutation often leads to a reduced number of cells in many tissues such as hypocotyls and leaves, whereas AGB1 mutation results in production of more cells in lateral root formation (Ullah et al., 2003). The interesting is that GPA1, AGB1, and RGS1 can all function in regulation of stomatal density. In Arabidopsis, the entry division to initiate the stomatal cell lineage is highly associated with the positive regulator SPCH. Loss-of-function of SPCH has no ability to make any stomata (MacAlister et al., 2007). An important factor regulating asymmetric divisions is MUTE, a negative regulator, by terminating asymmetric divisions and promoting differentiation of stomata (Pillitteri et al., 2007). Expression levels of SPCH and MUTE are moderately down-regulated in gpa1 but upregulated in agb1 at day 7 after seed germination. We reason that the G-proteins may regulate the frequency of entry and/or amplifying divisions to alter stomatal density in the epidermis of Arabidopsis.

A role of G-proteins in fine regulation of water use efficiency

Due to the sessile nature, plants have evolved various highly-ordered sensing systems to dynamically monitor and maximally utilize natural resources through both developmental and physiological processes. Stomatal pores are known to play an important role in balancing CO2 intake with water loss through regulation of stomatal movement and stomatal density. It is well documented that both stomatal development and movement can be regulated by plant hormones, such as GA, ethylene and ABA, and environmental factors including light intensity and CO2 concentrations (Casson and Gray, 2008). Up to date, only the role of the G-protein signaling pathway in stomatal movement has been intensively studied in the presence of ABA. Genetic and physiological results reveal that the Go subunit is required for the inhibition of stomatal opening in ABA-mediated stomatal movement (Wang et al., 2001). Consequently, the gpa1 mutant has a greater rate of water loss than wild-type plants and is prone to be suffered from drought stress. It is interesting that the deletion of GPA1 leads to a reduction in the number of stomata, which can compensate for excessive water loss due to insensitivity of the stomata to ABA signaling. Actually, a similar outcome of GPA1 has been also observed in the epidermal layer (data not shown) where the cell number is significantly reduced while the cell size becomes larger in gpa1 compared with the wild type (Ullah et al., 2001). Since amount of water loss from leaves is related to both stomatal density and movement, the importance of these two factors in drought resistance may vary according to growth conditions and developmental stages. Under our experimental conditions water loss in gpa1 leaves is lower than in wild-type leaves, indicating that stomatal density play a more important role in regulation of water transpiration compared with stomatal movement. This result is consistent with the previous report showing that decrease in stomatal density conferred to higher water use efficiency of Arabidopsis leaves (Chen et al., 2006; Josette et al., 2005; Yu et al., 2008). However, the compensating effect observed in gpa1 is not observed in agb1. Recently, inhibition of inward K+ currents and stomatal opening by ABA is also shown to be impaired in agb1 (Fan et al., 2008). This is consistent with our data that agb1 is more sensitive to drought stress than gpa1. The synergistic regulation of AGB1 in stomatal density and movement might be important in some special surroundings such as high humidity and low levels of carbon dioxide. In summary, our findings reveal another side of G-proteins in fine regulation of inevitable water loss when plants take up the CO2 required for photosynthesis through stomata.

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References


