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

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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 $GPA1^{QL}$ 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 *AGB1*. Stomatal analysis of the *gpa1 agb1* double mutants displayed an average value of stomatal density compared to the single mutants. 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 asymmetrically 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), CO_2 partial pressure (Clifford et al., 1995; Gray et al., 2000), and light intensity (Rahim and Fordham, 1991) have been demonstrated to

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modulate stomatal density or stomatal index. In *Arabidopsis*, stomatal number is increased in newly emerging leaves treated by higher CO_2 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 CO_2 (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 plant 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 primordial, 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 lines used were in the Col-0 background. Mutants (*gpa1*, *rgs1*, *agb1*, *gpa1 agb1*) and transgenic lines (GPA^{QL}, AGB1ox) 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 RNAqueous (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 *FAMA* were (Forward, 5′-GAGCTCGAGCAACTCCTACAAT; Reverse 5′-GAAGTCGTTGTCGTTGTCATGT), for *TMM* were (Forward, 5′-TCCTTCACCTAGAGGGCAATAA; Reverse, 5′-ACGGTACTGGTCCTGTCAGACT), for *YDA* were (Forward, 5′-CACCATGAGATCACTGGACATT; Reverse 5′-GCCATGTTTTAATCCTTTCTGC), for ERECTA were (Forward, 5′-GATAATGTCAAAGACGGGGAAC; Reverse, 5′-GGAAAACTTTCTTCACCACACC), for MUTE were (Forward, 5′-CATCAAAAGGGGAGATCAAG; Reverse 5′-CAGAGATGATCTTTACGAGC), for *SPCH* were (Forward, 5′-AAAATCGGCTTTGGCTGATGTGAAG; Reverse, 5′-AGAAAGTGAGTACGTACTGC), and for *RBS* were (Forward, 5′-ATGGTGATGGCTGGTGCTTCTTCTTTGGA; Reverse, 5′-TTAGAGAGGAACGCTGTGCAAGACGACT). 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 Optican-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-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