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Heterotrimeric G proteins control stem cell proliferation through CLAVATA signaling in *Arabidopsis*

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Abstract

Cell-to-cell communication is a fundamental mechanism for coordinating developmental and physiological events in multicellular organisms. Heterotrimeric G proteins are key molecules that transmit extracellular signals; similarly, CLAVATA signaling is a crucial regulator in plant development. Here, we show that *Arabidopsis thaliana* Gβ mutants exhibit an enlarged stem cell region, which is similar to that of *clavata* mutants. Our genetic and cell biological analyses suggest that the G protein beta-subunit1 AGB1 and RPK2, one of the major CLV3 peptide hormone receptors, work synergistically in stem cell homeostasis through their physical interactions. We propose that AGB1 and RPK2 compose a signaling module to facilitate meristem development.

Keywords *Arabidopsis thaliana*; heterotrimeric G protein; peptide hormone; RECEPTOR-LIKE PROTEIN KINASE 2; stem cell homeostasis
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Introduction

Coordinated cell proliferation and cell differentiation are essential processes in multicellular organisms. To achieve these functions, organisms have developed scrupulously designed cell-to-cell communication systems over the course of evolution. Plants have established unique ligand-receptor-based signaling modules, such as the CLAVATA (CLV) pathway, which comprises the CLV3 peptide hormone and the extracellular leucine-rich repeat (LRR) domain-containing receptors CLV1, CLV2-CORYNE (CRN)/SUPPRESSOR OF LLP1 2 (SOL2), and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) [1,2]. In the shoot apical meristem (SAM) of *Arabidopsis thaliana* (*Arabidopsis*), CLV signaling restricts the expression of the homeobox-containing transcription factor WUSCHEL (WUS) [3–5]. Conversely, WUS promotes the expression of *CLV3*, forming a negative feedback loop that controls the number of stem cells [3–5]. While the peptide-binding plasma membrane components have been well studied, the molecules that mediate intracellular signaling by these receptors are largely unknown. The protein phosphatase KAPP and a Rho GTPase-related protein have been shown to physically interact with the CLV1 receptor [6], and the protein phosphatase 2Cs POLTERGEIST (POL) and POL-LIKE 1 (PLL1) are also known to be signaling mediators [7]. However, further analyses are needed to trace the signaling pathway from the receptor to cellular processes.

On the other hand, heterotrimeric G proteins, composed of alpha (Gα), beta (Gβ), and gamma (Gγ) subunits, are important signaling molecules that link extracellular signals to intracellular mechanisms in eukaryotes [8,9]. The basic components and mechanisms of G protein signaling have been studied extensively in mammalian cells: G protein-coupled receptors (GPCRs) sense extracellular ligands and stimulate G proteins, whereupon Gα and Gβγ dissociate and provoke variable cellular events [8]. In resting cells, GDP-bound Gα associates with Gβγ, and ligand-stimulated GPCRs promote the exchange of GDP for GTP, causing Gα and Gβγ to dissociate. Although land plants express similar G protein components, they are controlled by slightly different systems compared with canonical G proteins. In plants, Gα can spontaneously exchange GDP for GTP, while the seven-pass transmembrane domain-containing protein RGS1 inhibits G signaling through the formation of an inactive

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complex [10–12]. Extracellular ligands bound to RGS1 stimulate the release of Gα to activate signaling. This self-activating ability helps explain the absence of clear GPCR homologues in plant genomes [13]. Several transmembrane proteins have been annotated as plant GPCRs based on their sequence; however, clear evidence that these candidates function as GPCRs has not been reported [9,13]. These facts provide possibilities for the mode of G signaling which is the presence of alternative, non-canonical GPCRs and GPCR-independent function of G proteins. Despite their unique regulatory mechanisms, plant G proteins are involved in various aspects of morphological and physiological processes, much like their mammalian counterparts [12,14–18].

Recently, Bommert *et al* [19] reported genetic evidence that maize Gα modulates CLV signaling in the control of shoot meristem size. However, the biochemical and cell biological processes underlying the cross-talk between the CLV pathway and G proteins remain unclear, as these extraordinary phenotypes have only been reported for Gα mutants in maize [19]. Here, we show that the *Arabidopsis G protein beta-subunit1* (*agb1*) mutant exhibits an enlarged SAM, similar to that of *clv* mutants. Genetic analysis suggests that AGB1 works together with RPK2, a leucine-rich repeat-receptor-like kinase (LRR-RLK), in stem cell homeostasis. Bimolecular fluorescence complementation (BiFC) assays and co-immunoprecipitation (co-IP) analyses indicate that AGB1 associates with RPK2. These results establish the involvement of AGB1 in meristem development in the RPK2-dependent signaling pathway and indicate the diversity of CLV signaling in plants.

Results and discussion

Identification of mutations in a gene encoding a heterotrimeric G protein β subunit in *clv2 enhancer 1* mutants

To decipher the molecular mechanisms underlying the CLV signaling pathway, we conducted a genetic screen to search for mutations that enhance the phenotypes of *clv2* mutants. As a result, we isolated 48 mutants with obviously enlarged SAMs, which have been designated *clv2 enhancer* (*clen*) mutants. From these mutants, *clv2 enhancer 1* (*clen1*) was selected for further study. *clv2-101*, a null allele of *clv2* mutation, displays approximately twofold (21.25 μm) larger SAMs than wild-type plants, in which SAM height is 10.18 μm on average, and the *clv2 clen1* double mutant exhibits sevenfold (71.73 μm) larger SAMs (Fig 1A–C and G). Similarly, the pistils of wild-type flowers have 2 carpels, whereas *clv2-101* and double-mutant plants present 2.5 and 3.8 carpels on average, respectively (Fig 1H–J and N). The enlarged SAMs and increased carpel numbers observed in *clv2 clen1* mutants relative to wild-type or *clv2* mutant plants suggest decreased CLV3 signaling activity [3]. Using a positional cloning approach, the *clen1* mutation was roughly mapped to near the nga1139 marker (33/34 chromosomes) on chromosome 4, and the genomic DNA sequence was analyzed via the SOLiD system to identify mutations [20]. Thus, we detected a nucleotide substitution in the *AGB1* gene that converts a Trp residue into a stop codon (Supplementary Fig S1). In addition to the *clen1* mutant, we have identified this mutation in 8 *clen* mutants and have found other four point mutations in five additional *clen* mutants (Supplementary Fig S1).

The nonsense mutation in the *AGB1* gene is expected to be responsible for the observed *clen1* phenotypes. We therefore examined SAM size and carpel number in the previously isolated *agb1-2* and *clv2 agb1-2* mutants [17]. *agb1-2* produced 1.4-fold larger SAMs than wild-type plants, while the carpel number was still 2 (Fig 1D, G, K and N), suggesting that the single mutation in *AGB1* is sufficient to affect SAM height. Moreover, the *clv2 agb1-2* double mutant exhibited a *clv2 clen1*-like phenotype, showing 6.5-fold larger SAMs and 1.9 times the number of carpels compared with wild-type plants (Fig 1E, G, L and N). Although overexpression of *AGB1* did not affect plant architecture in the wild type (Supplementary Fig S2), it suppressed the enhanced abnormalities of the *clv2 clen1* mutant, resulting in a *clv2*-like phenotype (Fig 1F, G, M and N). These plants also resembled a *clv2 clen1* mutant that harbors a genomic fragment of the *AGB1* gene (Supplementary Fig S3). These results show that a mutation in *AGB1* enhances the abnormalities of the *clv2* mutant and suggest that AGB1 regulates SAM height and carpel number.

AGB1 is involved in SAM maintenance through the CLV3 signaling pathway

CLV3 restricts cell proliferation in the SAM, and synthetic CLV3 peptide treatment induces SAM consumption due to diminished cell proliferation [5,21]. To investigate whether the enlarged SAM phenotype observed in *agb1* is a consequence of a disturbance of CLV3 signaling, we examined the sensitivity of the *agb1-2* mutant to the CLV3 peptide (Fig 2). Wild-type seedlings grown on MS media containing 5 μM CLV3 did not develop stems under these conditions, even at 20 days after germination (Fig 2E, M and Q). Conversely, 10% of *agb1-2* mutants developed a stem at the same stage (Fig 2G, O and, Q). Furthermore, *clv2 agb1-2* double mutants showed strong resistance compared with *clv2* or *agb1-2* mutants (Fig 2H, P and Q).

Next, we examined the genetic relationship between *AGB1* and *WUS*, which is known to function downstream of CLV signaling [4,5]. Similar to the *wus-101* single mutant, the SAM was terminated in the *wus-101 agb1-2* double mutant (Supplementary Fig S6), indicating that *WUS* is epistatic to *AGB1*. Taken together, these findings suggest that *AGB1* regulates the SAM activities through a CLV3-related pathway.

Heterotrimeric G proteins are expressed in the inflorescence meristem

Given that AGB1 is a heterotrimeric G protein subunit, the involvement of other G protein components was predicted. To examine whether G proteins are expressed in SAMs, we performed *in situ* mRNA hybridization experiments. Expression of both *GPA1* and *AGB1* was observed in the inflorescence meristem, floral meristem, and floral organ primordium (Supplementary Fig S7A and B). Conversely, weak *AGG1* expression signals were detected, whereas *AGG2* was not (Supplementary Fig S7C and D). The expression of these genes in vegetative SAMs and inflorescences was supported by the *Arabidopsis* eFP Browser database (Supplementary Fig S7E–G). Thus, the fact that not only *AGB1* but also other G protein components were expressed in SAMs highlighted the possibility that the G protein signaling complex is involved in the CLV signaling pathways.

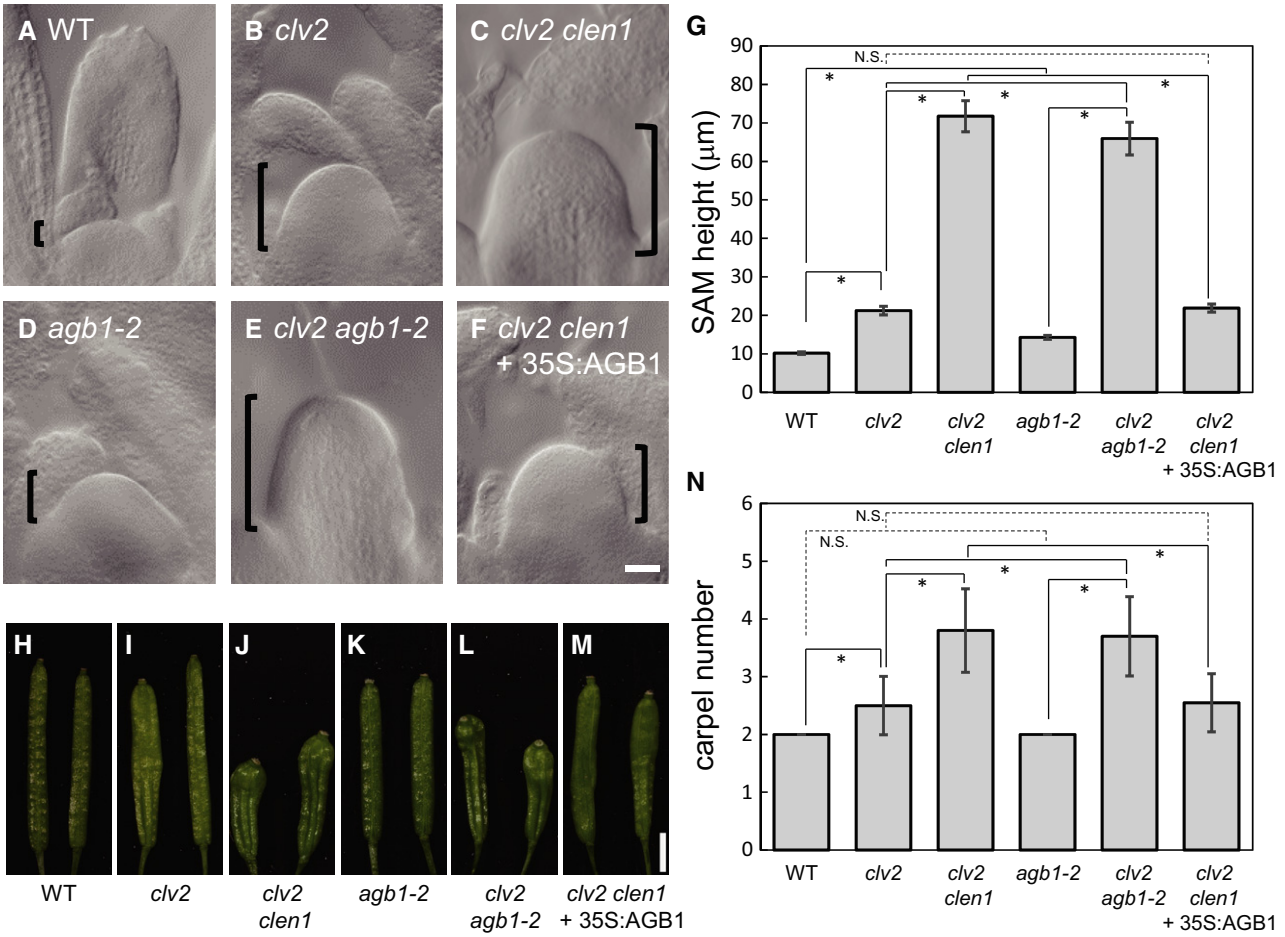

Figure 1. The G protein β-subunit regulates SAM maintenance.

A–F DIC images of SAMs. Wild-type (A), *clv2* (B), *clv2 clen1* (C), *agb1-2* (D), *clv2 agb1-2* (E), and *clv2 clen1* plants transformed with *35S:AGB1* (F) are shown. Brackets indicate height of SAM.

G Quantitative analysis of SAM height.

H–M Dissecting microscope images of mature siliques. Wild-type (H), *clv2* (I), *clv2 clen1* (J), *agb1-2* (K), *clv2 agb1-2* (L), and *clv2 clen1* plants transformed with *35S:AGB1* (M) are shown.

N Quantitative analysis of carpel number.

Data information: Scale bars = 25 μm in (A–F), 1 mm in (H–M). Error bars in (G) and (N) represent SD. The histograms and complete data are shown in Supplementary Figs S4 and S5 and Supplementary Table S1. An asterisk indicates a statistically significant difference from the neighboring value (*$P < 0.05$). N.S. indicates not significant.

The heterotrimeric G protein γ subunit, but not Gα, is also involved in CLV3 signaling

To investigate the possibility that the Gα and Gγ subunits are involved in CLV signaling, we examined both SAM height and CLV3 peptide sensitivity in Gα and Gγ null mutants, designated *gpa1-4* and *agg1-1c agg2-1*, respectively [22,23]. A recent report showed that a maize Gα mutant exhibited a very large meristem phenotype [19]. In contrast, *gpa1-4* plants did not show obvious SAM or carpel abnormalities, and the mutation did not affect the degree of resistance to CLV3 compared with the wild type (Supplementary Table S1, Supplementary Fig S8A). Furthermore, the additional mutation of *GPA1* did not affect the *clv2* mutant phenotype (Supplementary Table S1). Conversely, the *agg1-1c agg2-1* double mutant produced 1.4-fold larger SAMs than the wild type as well as 2-carpel siliques, and 10% of the Gγ mutants maintained a SAM even on CLV3-containing media (Supplementary Table S1, Supplementary Fig S8A), similar to what was observed in the *agb1-2* mutants. Furthermore, *clv2 agg1-1c agg2-1* triple mutants showed enhanced abnormalities and SAMs were maintained in the triple mutant at the similar frequency as in the *clv2 agb1* double mutants when treated with CLV3 (Supplementary Table S1, Supplementary Fig S8A).

In mammals and plants, Gβ and Gγ are known to form a heterodimer [8,9,24]. *Arabidopsis* Gγ appears to act with Gβ during CLV signaling to regulate SAM height and carpel number. This idea is also supported by the results of the examination of carpel number phenotypes at higher temperatures. Morphological abnormalities in flowers are occasionally strengthened at higher temperatures [25]. Accordingly, the *agb1-2* and *agg1-1c agg2-1* mutants both exhibited 3-carpel pistils, whereas wild-type and *gpa1-4* plants all presented 2

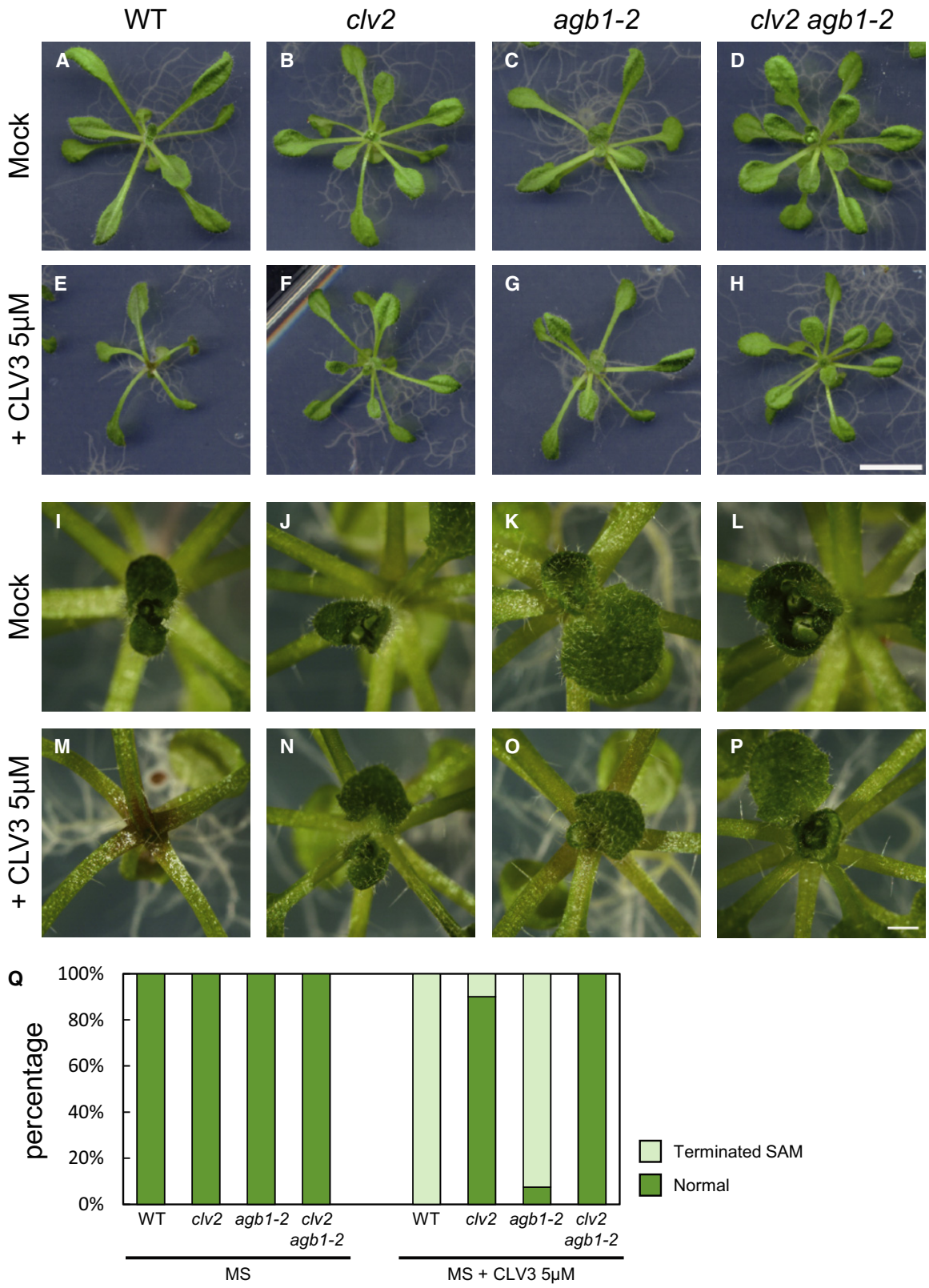

Figure 2. AGB1 is involved in SAM maintenance through CLV3 signaling.

A–P Eighteen-day-old seedlings of wild-type (A, E, I, and M), *clv2* (B, F, J, and N), *agb1-2* (C, G, K, and O), and *clv2 agb1-2* (D, H, L, and P) plants. The plants were grown on agar medium with (E–F, M–P) or without (A–D, I–L) 5 μM CLV3 peptide. (I–P) represent closer views of (A–H). Scale bars = 1 cm in (A–H), 1 mm in (I–P).

Q Quantification of the seedlings showing terminated SAMs observed 20 days after germination.

carpels (Supplementary Fig S8B). The difference between the Gα mutant phenotypes and those of the Gβ and Gγ mutants is not unexpected, as Gα mutations are often reported as the exception among G protein mutants [13].

Gβγ controls SAM maintenance in the RPK2 pathway

The fact that the *clv2-101 agb1-2* double mutant showed detectable additive phenotypes suggests that AGB1 mediates CLV3 signaling in a CLV2-independent manner. In contrast to *clv2 agb1-2* mutants, which exhibit 3.1-fold larger SAMs than *clv2* mutants, the *clv1 agb1-2* and *rpk2 agb1-2* mutants exhibited 2-fold and 1.5-fold larger SAMs than the corresponding single mutants (Fig 3A). Furthermore, comparing *rpk2* and *rpk2 agb1-2* plants revealed similar carpel numbers, whereas the *clv2 agb1-2* and *clv1 agb1-2* mutants showed a significantly increased carpel number relative to the single mutants (Fig 3B). All of the double mutants showed a significantly enhanced phenotype with the corresponding single mutants. However, the degree of enhancement was smaller in *rpk2 agb1* for SAM height and only the *rpk2 agb1* produced a similar number of carpels when compared with the *rpk2* single mutant. These results lead us to hypothesize that AGB1 is at least partially involved in the RPK2-dependent CLV signaling pathway.

AGB1 associates with RPK2 *in planta*

Based on the results of the genetic analyses, AGB1 is expected to interact with CLV signaling components either directly or indirectly. AGB1 has been observed to localize to the plasma membrane, nucleus, cytoplasm, and Golgi apparatus [24,26–28]. Our expression analysis confirmed the presence of AGB1-GFP signals at the plasma membrane (Supplementary Fig S9). Because LRR-containing receptor complexes also localize to the plasma membrane, any complex that these proteins form is likely to be present here.

We next tested whether AGB1 associates with CLV1, RPK2, and CLV2 using BiFC assays. Protoplasts transformed with AGB1 and RPK2 exhibited a positive BiFC signal when a CLV3-expressing vector was co-transformed, and the signal was localized to the plasma membrane (Fig 4A and C). However, we did not detect an interaction between AGB1 and either CLV1 or CLV2 (Fig 4A and C). AGB1 is therefore predicted to receive CLV signals through RPK2, though it is unclear how CLV3 facilitates this interaction. Furthermore, we performed a co-IP assay to confirm the physical interaction between AGB1 and RPK2. FLAG-tagged AGB1 was pulled down with Venus-tagged RPK2c, which contains the C-terminal intracellular domain, whereas most of the AGB1-FLAG disappeared after IP when expressed alone or with mCherry-Venus (Fig 4D). These results suggest that RPK2 is capable of interacting with AGB1. Taking these results together with the genetic data, we propose that AGB1 functions preferentially with RPK2 on the CLV signaling to regulate cell proliferation activities in SAMs.

Conclusion

Heterotrimeric G proteins are evolutionarily conserved signaling molecules that mediate the transduction of extracellular cues into intracellular signals, in combination with transmembrane GPCRs. In plants, several transmembrane proteins have been reported as GPCRs. In this study, we have shown that an LRR-RLK receptor, RPK2, is able to interact with G proteins. Surprisingly, among the examined G protein mutants, only the Gα mutant did not exhibit any abnormalities in CLV signaling-related processes, suggesting that mutations in *GPA1* did not disrupt CLV signaling. However, we cannot exclude the possibility that Gα or related proteins serve as a bridge between RPK2 and Gβγ dimers, as in canonical GPCR and G protein interactions. The *Arabidopsis* genome encodes three extra-large G proteins (XLGs), which contain a Gα-like domain

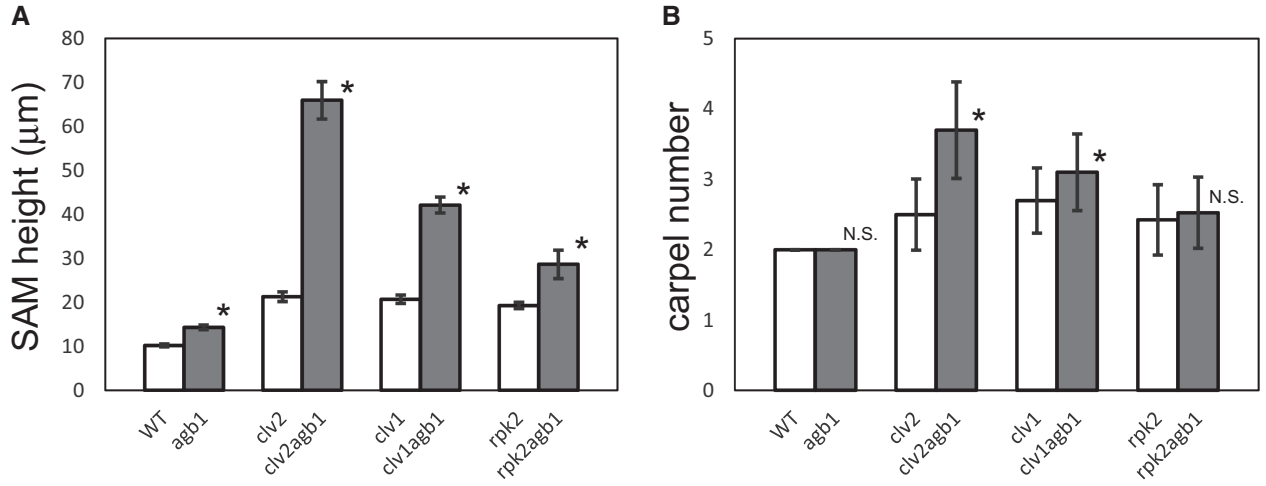

Figure 3. Genetic relationship between the AGB1 and CLV3 receptors.

A, B Quantitative analyses of SAM height (A) and carpel number (B) are shown. White bars correspond to the wild-type plants or single mutants for the indicated receptors, whereas gray bars represent *agb1-2* single mutants or double mutants for AGB1 and the indicated receptors. Error bars represent SD. Note that the carpel number observed in all wild-type and *agb1-2* plants was 2, and the corresponding SD was therefore 0. An asterisk indicates a statistically significant difference from the neighboring value. N.S. indicates not significant (*$P < 0.05$). The histograms and complete data are shown in Supplementary Figs S4 and S5 and Supplementary Table S1.

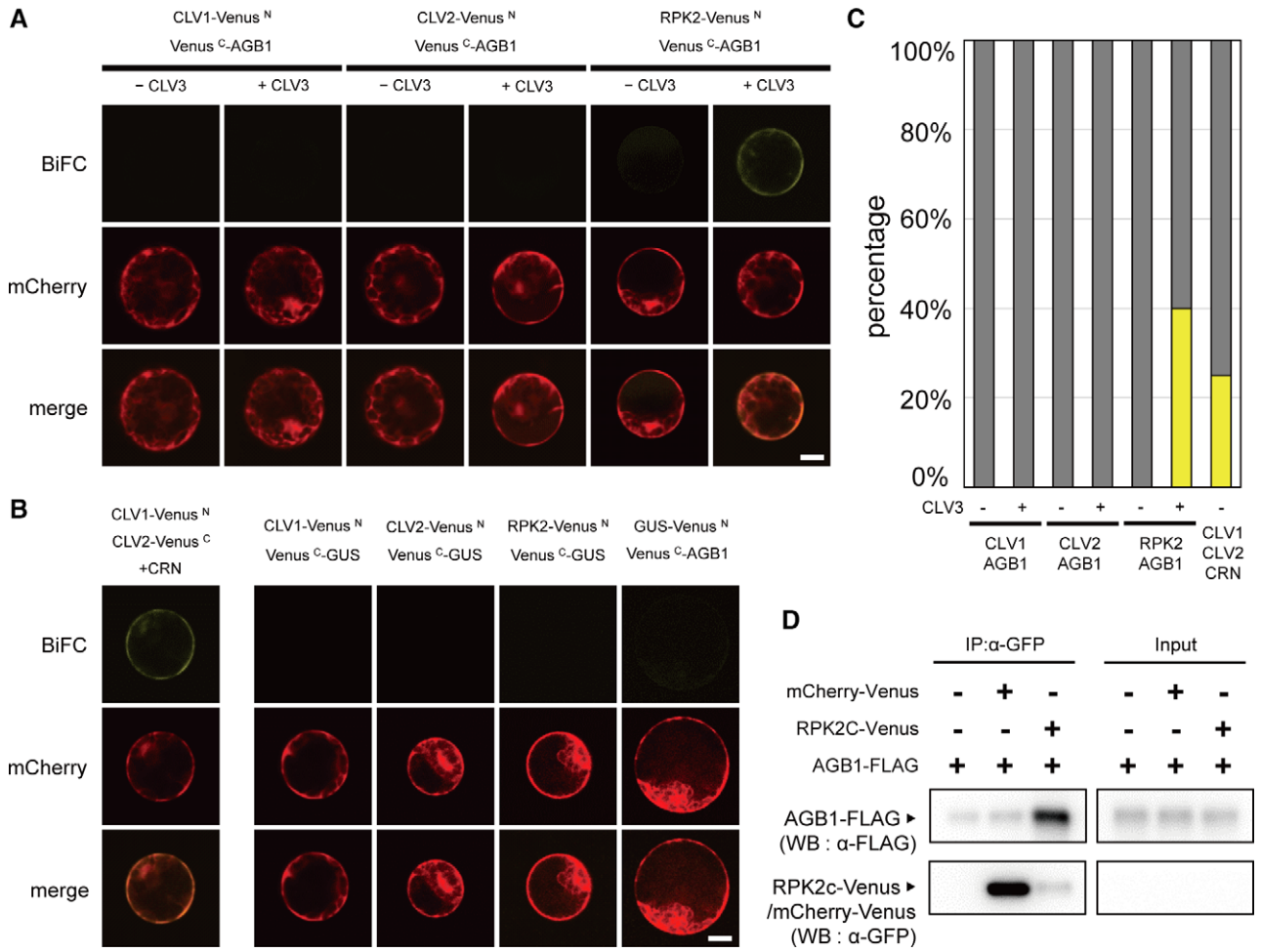

Figure 4. AGB1 is able to interact with RPK2.

A Protoplasts expressing the indicated proteins tagged with the N- or C-terminal halves of Venus.

B Positive and negative controls for the BiFC analysis. BiFC signals (upper), mCherry fluorescence (middle), and merged (bottom) images are shown.

C Quantification of the BiFC assays. The results for the positive control and the experiments for CLV1, CLV2, RPK2, and AGB1 are shown. BiFC signals were measured as described in Materials and Methods. The percentages of cells with BiFC signals are indicated by yellow bars (n = 20).

D Co-IP assay showing the physical interaction between AGB1 and RPK2. AGB1-FLAG alone or with mCherry-Venus or RPK2c-Venus was transiently co-expressed in protoplasts. Total protein extracts were subjected to IP with an anti-GFP antibody. The presence of AGB1-FLAG (upper) and Venus-tagged proteins (bottom) was determined by Western blotting. Note that the AGB1-FLAG co-expressed with RPK2c-Venus was condensed after IP. The co-IP experiments were repeated three times, with similar results.

Data information: Scale bars = 10 μm in (A) and (B).

[9,29]. As any potential overlapping functions of XLGs were not addressed in this study, further analyses will be needed to evaluate the biological relevance of XLGs not only in CLV signaling but also in G protein function. In fact, a recent report showed that maize Gα mutants exhibit enlarged meristem phenotype leading the authors to infer a function of Gα in SAM maintenance [19]. Despite clear evidence of the involvement of Gα in the maize CLV-like pathway, further research is required before any generalizations can be made because severe phenotypes, such as those observed in the maize Gα mutant, have not been reported in either *Arabidopsis* or rice Gα mutants [13,19,30]. The critical amino acids for Gα function have been reported. In particular, the Thr residue in the switch I region of GPA1 is important for the interaction between regulatory proteins for activation [31]. Although *Arabidopsis*, rice, and other species, including dicots, gymnosperms, and animals, harbor the conserved amino acid in the Gα subunit, maize and some other monocots do not exhibit this residue [12]. This evolutionarily distinct background of heterotrimeric G proteins could be another explanation for the differing meristem phenotypes observed in maize compared with *Arabidopsis* (Fig 1 and [12,19]).

Taken together, our results suggest the hypothesis that CLV3-RPK2 signaling activates a heterotrimeric G protein through an interaction, at least in *Arabidopsis*. Thus, these results support the notion that LRR-RLK-type receptor RPK2 acts as an alternative GPCR, similar to canonical GPCR-G protein systems. This situation contrasts with that in maize, where FEA2, an LRR type receptor, mediates CLV-like signaling and G proteins [19]. Although

Arabidopsis and maize utilize common G proteins for meristem maintenance, different systems consisting of various combinations of receptors and G protein subunits are employed. Therefore, these differences might contribute to the diversity of the signaling pathways in plant development.

Materials and Methods

Plant materials

The following *Arabidopsis* wild-type and mutant lines were obtained: wild-type Columbia-0 (Col-0); *clv1-101* (CS858348) in the Col-2 background; *clv3-8* ER in an unknown background (CS3604) [32]; *rpk2-2* [5], *clv2-101* (GK686A09), *gpa1-4* (SALK_001846), *agb1-2* (CS6536), and *wus-101* (GK870H12) in the Col-0 background; and *agg1-1c agg2-1* (kindly provided by Jimmy Botella) in a mix of Col-0 and Wassilewskija (Ws).

SAM measurement

Seven-day-old seedlings were fixed with 70% ethanol, cleared in a mixture of chloral hydrate, glycerol, and water (8:1:2; w/v/v), and observed using a microscope (ZEISS AXIO Imager M1) that was equipped with Nomarski optics. The base of the SAM was defined as the location of the leaf primordium, and the height was measured between the top and base of the SAM, as described [5].

Peptide assay

The CLV3 peptide was synthesized as described previously [33]. Seedlings were grown on MS plates containing CLV3 peptides until 18–20 days after germination.

Protoplast transformation

Arabidopsis leaf mesophyll protoplast transformation was performed as described previously [34]. True leaves of 3-week-old seedlings were collected and chopped in an enzyme solution containing 0.6% Cellulase 'ONOZUKA' RS (Yakult Pharmaceutical Industry) and 0.6% Macerozyme R10 (Yakult Pharmaceutical Industry). Isolated protoplasts were washed and re-suspended at a concentration of 2×10^7 protoplasts per ml for polyethylene glycol (PEG)-mediated transformation. Vectors for transient expression were mixed with the protoplasts in transformation buffer [0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, and 40% PEG (w/v) (Sigma)]. After washing, the protoplasts were incubated in liquid culture medium containing 0.4 M mannitol for 12–24 h at 23°C.

Further experimental details are provided in Supplementary Methods.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

MH, HT, KS, MH, HF, and SS conceived or designed the experiments. TI, RT, MY, MA, KM, MF, KY, and SS performed the experiments. KY, SS, and MH analyzed the data. TI, RT, MY, MA, and SS wrote the manuscript. KS was deceased on September 28, 2013.

Conflict of interest

The authors declare that they have no conflict of interest.

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