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Quantitative morphological phenomics of rice G protein mutants portend autoimmunity

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ABSTRACT

The heterotrimeric G protein complex, composed of $G\alpha$, $G\beta$, and $G\gamma$ subunits, plays some role in structural development in plants but this role could be indirect because loss-of-function mutations do not alter the body plan and post-embryonic organs differ only morphologically and not in their identity. This uncertainty has been compounded by the fact that loss of the $G\beta$ subunit in cereals, but not Arabidopsis, is seedling lethal and that loss of maize $G\alpha$ subunit confers prolificacy of a reproductive organ. In this study, we comprehensively profiled the root and shoot structural traits of rice $G\alpha$ -null and viable $G\beta$ -RNAi “knockdown” mutants, and found anomalous morphologies caused by $G\beta$ -RNAi that are distinct from the Arabidopsis orthologue. The rice $G\beta$ -RNAi mutant exhibited reduced radial growth of aerial parts as well as a more compact root architecture, among which smaller root mass seems mainly due to increased necrosis when grown on soil. In addition, three dimensional analyses of rice root system architecture revealed that the smaller root architecture of $G\beta$ -RNAi plant is also due to both reduced root elongation and adventitious root formation. This contrasts to the Arabidopsis $G\beta$ -null mutation that promotes cell proliferation. There is elevated cell senescence activity both visualized by Evans Blue staining and inferred from an expression analysis of cell-death marker genes. We propose that the morphological phenotypes of rice $G\beta$ -RNAi plants are predominantly associated with the mediation of various stresses and cell senescence, consistent with an indirect role for Arabidopsis $G\beta$ in development where the orthologous gene ablation mainly confers altered cell proliferation. We also elaborate our speculative working hypothesis that cell division is a type of stress and as such due to impairment in responding to stress in the G protein mutants, manifests as altered morphology and architecture but not an altered body plan or organ identities.

1. Introduction

The diverse morphologies and terrestrial adaptations in plants are attributed to the coordinated actions of developmental regulators such as transcription factors and signaling proteins that typically operate with a time scale of hours to days. The heterotrimeric guanosine triphosphate (GTP)-binding protein (G protein) is part of a signaling complex operating with a time scale of seconds to minutes (Fu et al., 2014; Liao et al., 2017; Yi et al., 2003) but surprisingly, it looks as if from loss-of-function studies to be involved in slow processes such as cell proliferation, differentiation, and senescence and manifests as structural changes such as leaf and silique morphology but also root architecture as elaborated further below (Urano et al., 2013). Because the physiological functions of

G proteins have been mainly studied in the model plant Arabidopsis, limiting our studies predominantly with this one type of plant habit confines our conclusions on the role of G proteins in regulating plant development. A quantitative comparison between Arabidopsis and rice with orthologous gene knockout and knockdown lines provides a basis for developmental differences and commonalities between these two diverse species, enlightening at times, the mechanism behind the developmental differences. Moreover, utilizing developmental differences in a study enlightens us on direct vs. indirect modes of action of a hypothetical regulator.

The heterotrimeric G protein complex, composed of a $G\alpha$ subunit and an obligate $G\beta\gamma$ dimer, transmits extracellular signals into the plant cells to modulate hormone and stress responses, but also shoot and root

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development (Urano et al., 2013). Loss-of-function mutants for Arabidopsis $G\alpha$ or $G\beta$ genes display comparable developmental defects of aerial tissues characterized by shorter but wider hypocotyl, leaf, silique and seed (Ullah et al., 2001; Urano et al., 2016a). The short-shoot phenotypes are due to a reduced cell cycle rate in the G protein mutants (Chen et al., 2006; Ullah et al., 2001, 2003). In contrast, the $G\alpha$ and $G\beta$ mutant plants demonstrate distinct morphologies of roots (Ullah et al., 2003). In Arabidopsis, the $G\beta$ -null mutation expands root architecture by promoting root elongation and lateral root formation, while the $G\alpha$ -null mutation conversely confers a reduced root mass (Ullah et al., 2003). In maize, loss of the G subunit alters cell proliferation (Bommert et al., 2013; Wu et al., 2018) and is most dramatic in the excessive production of ears but only under pollination stress (Urano et al., 2015). Rice as a monocotyledonous species is another important genetic tool for understanding gene networks regulating structural development (Atkinson et al., 2014; Nelissen et al., 2016). Similar to Arabidopsis, the rice $G\alpha$ -null mutation (*dwarf 1*, *d1*) confers shorter but wider shoot tissues; specifically, leaf, floral bract, seed and panicles (Fujisawa et al., 1999) and confers shorter roots compared with wild type (Izawa et al., 2010). The function of the rice $G\beta$ gene (*RGB1*) was characterized using RNA interference (RNAi) to create weak “knockdown” lines (Utsunomiya et al., 2011; Zhang et al., 2015), because complete ablation of the *RGB1* gene causes embryonic lethality (Utsunomiya et al., 2012). Rice $G\beta$ -RNAi mutants exhibit short and narrow leaf sheaths and blades (Utsunomiya et al., 2011), browning of the lamina joint (Utsunomiya et al., 2011, 2012) and decreased drought tolerance (Zhang et al., 2015).

While the $G\alpha$ -null mutants have comparable developmental defects such as short leaves, flowers and seeds in Arabidopsis and rice, comparison of $G\beta$ mutants in these different plants was limited to qualitative morphological traits and senescence of above-ground tissues (Utsunomiya et al., 2011; Zhang et al., 2015). To provide a basis for developmental differences and commonalities between diverse species that enlighten the mechanism under the control of the common regulator, we quantitated morphological traits of rice $G\alpha$ and $G\beta$ mutants with emphasis on structural root traits. The rice morphological data were further compared with the phenotypic profiles of Arabidopsis orthologous knockout lines (Urano et al., 2016a). Our results, together with expression analyses of cell-death marker genes, suggest that rice $G\beta$ protein is a primary mediator of cell death and other stress-triggered cellular processes that consequently cause morphological defects of

structural roots in rice $G\beta$ mutants. This morphological defect contrasts to Arabidopsis $G\beta$ mutant phenotype which has larger root mass, although our transcriptional meta-analysis suggests that the $G\beta$ function on cell death is present in the dicotyledonous lineages. Taken together, we propose that Arabidopsis and rice conserved $G\beta$ function in cell proliferation associated with longitudinal growth of leaves and seeds, however rice $G\beta$ function in cell senescence produces opposite morphological changes in roots between these angiosperm lineages. We also speculate that the altered root morphologies of rice $G\beta$ mutants is an indirect effect, analogous to autoimmunity.

2. Results

Quantitative root morphological changes reveal distinct roles of $G\alpha$ and $G\beta$ – The Arabidopsis *agb1* mutant exhibits a larger root mass (Ullah et al., 2003) due to increased cell division and branch formation (Chen et al., 2006), whereas the *gpa1* mutant has a compact root especially when grown on soil (Ullah et al., 2003). The $G\alpha$ mutants in rice and maize resemble the Arabidopsis *gpa1* mutant in displaying a small root architecture (Izawa et al., 2010; Urano et al., 2015), however the function of $G\beta$ on root morphology has been scantily analyzed in monocotyledons although a previous study showed images of $G\beta$ -RNAi rice seedlings with compact roots (Zhang et al., 2015). Three dimensional imaging of the rice root system architecture enabled the dissection of the morphological parameters with precision (Fig. 1) (Fang et al., 2013). Some $G\beta$ RNAi seeds generated a coleoptile without a seminal root (Fig. 1A, Supplemental Table 1), possibly related to the lethality and severe cell death phenotypes caused by the complete suppression of $G\beta$ gene expression (Utsunomiya et al., 2012). Images in Fig. 1B are representative roots of wild type Nipponbare (WT), DK22 and $G\beta$ RNAi mutants grown in a transparent nutrient medium. The DK22 and $G\beta$ RNAi mutants demonstrated reduced morphological parameters that represent overall root size, such as total root length, perimeter, surface area and root volume (Fig. 1C and Fig. S1). The $G\alpha$ and $G\beta$ mutations also caused a reduction in the maximum and median number of roots (Fig. 1C, S1N and S1P), while showing a limited effect on the root depth and the root width (Fig. 1C, S1O and S1R). These data suggest that the small root architecture of G protein mutants is primarily attributed to adventitious root formation rather than root length. Fig. 2 illustrates WT, DK22 and $G\beta$ -RNAi (labeled $G\beta$) roots after four weeks of cultivation on soil.

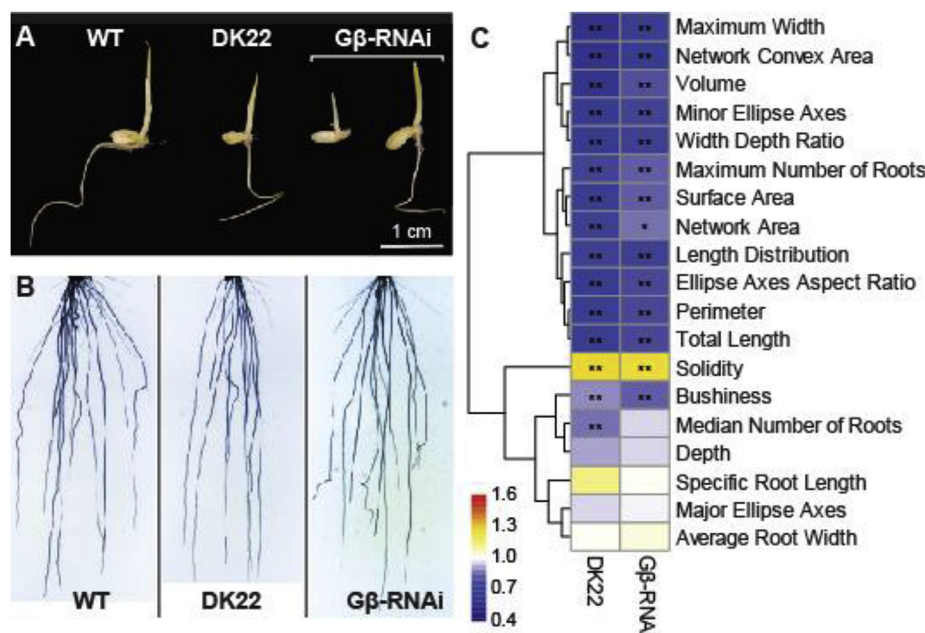


Fig. 1. Three-dimensional architectures of $G\alpha$ -null and $G\beta$ -RNAi roots. (A) Representative coleoptile images of wild type and mutant plants. Less than 40% of $G\beta$ -RNAi seeds were germinated properly with a coleoptile and root (Supplemental Table 1). $G\beta$ -RNAi seedlings with no roots are excluded from the analyses of root architecture in (B, C). Note that there are no $G\beta$ -RNAi seeds growing roots without a coleoptile. (B) Representative images of wild type and mutant roots grown with Yoshida medium for 11 days. (C) Comprehensive morphological profiles of wild type and mutant roots at day 11. The heat map shows fold changes of root morphological traits relative to wild type plants. The statistical differences from wild type values are analyzed by one-way ANOVA followed by the Tukey's post hoc test. The statistical symbols on the heat map, * and **, indicate significant difference from the wild type group at p values smaller than 0.05 and 0.01. The definitions of the root morphological traits are described in detail in previous studies (Galkovskyi et al., 2012; Ingram et al., 2012; Iyer-Pascuzzi et al., 2010). Raw data are presented in Supplementary Fig. 1.

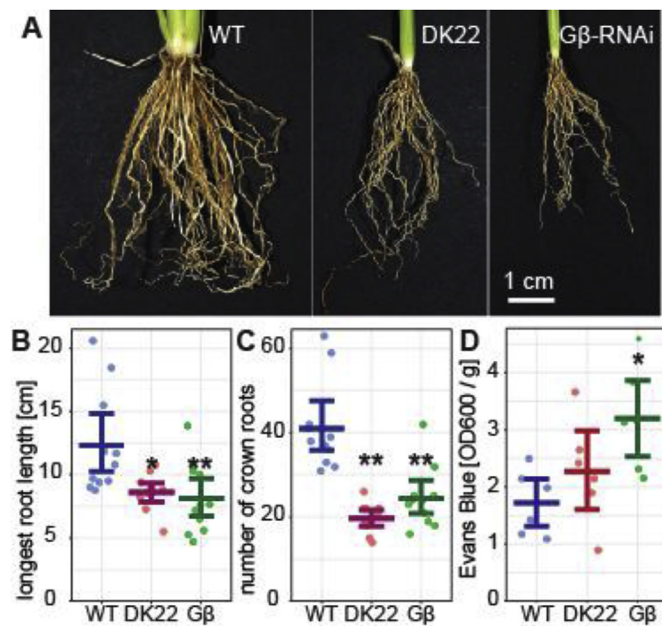


Fig. 2. Root morphological traits of $G\alpha$ -null and $G\beta$ -RNAi plants. The wild type and mutant plants were grown on soil for four weeks before the measurements. Labels: WT, wildtype rice ecotype Nipponbare; DK22, a null allele of the rice $G\alpha$ subunit RGA1 in the Nipponbare landrace; Gβ, $G\beta$ RNAi plants. (A) Representative images of wild type and mutant roots. (B) Length of the longest crown root. (C) Number of crown roots. (D) Amount of Evans blue dye stained and extracted from rice roots. The statistical symbols, * and **, indicate significant difference from the wild type group at p values less than 0.05 and 0.01, respectively, as performed by one-way ANOVA followed by the Tukey's post hoc test.

Observation of root phenotypes grown on soil revealed a more severe reduction in root growth, especially in the $G\beta$ RNAi mutant possibly due to greater stress than when grown in optimized nutrient agar (Fig. 2A) or altered mechano-sensing responses to soil and agar. G protein mutations indeed alter the mechano-sensing of Arabidopsis roots, although not yet researched in rice (Weerasinghe et al., 2009). The $G\alpha$ -null and $G\beta$ RNAi mutants exhibited a shorter root system (WT, 12.3 cm; DK22, 8.64 cm; $G\beta$ RNAi, 8.15 cm, Fig. 2B) with fewer crown roots (WT, 41.0 roots; DK22, 19.6 roots; $G\beta$ RNAi, 24.5 roots, Fig. 2C). Utsunomiya and co-workers (Utsunomiya et al., 2011) showed that $G\beta$ -RNAi rice displayed brownish laminar joints at the mature stage, suggesting enhanced senescence activity in the above-ground tissue. Consistent with that study, our results suggest that the more severe root phenotypes are the consequence of enhanced cell death. To test the hypothesis, we stained soil-grown rice roots with the vital stain Evans Blue, and found that $G\beta$ RNAi roots had quantitatively (Fig. 2D) and qualitatively (Fig. S1T2) more cell death than WT.

Quantitative shoot morphology profiling shows both shared and distinct changes by rice $G\alpha$ knockout or $G\beta$ -RNAi - Loss-of-function mutations in G protein genes result in abnormal morphologies of above-ground tissues including larger shoot apical meristem (SAM) size and greater carpel numbers in Arabidopsis (Ishida et al., 2014; Urano et al., 2016b) and larger inflorescence meristem and ear fasciation in maize (Bommert et al., 2013), however G protein functions in shoot branching have not been investigated. Fig. 3A shows 9-week-old WT, $G\alpha$ -null and $G\beta$ -RNAi plants grown in a greenhouse. Compared with the parental rice cultivar, $G\alpha$ -null and $G\beta$ -RNAi mutant lines were significantly shorter (WT, 83.1 cm; DK22, 54.1 cm; $G\beta$ RNAi, 69.7 cm at week 9, Fig. 3C) and had fewer tillers (WT, 50.2 tillers; DK22 34.1 tillers; $G\beta$ -RNAi, 31.4 tillers at week 9, Fig. 3B), suggesting that both $G\alpha$ and $G\beta$ subunits directly or indirectly regulate plant height and tiller number. In addition, the $G\beta$ -RNAi plant, but not the $G\alpha$ -null mutant, had wider tiller

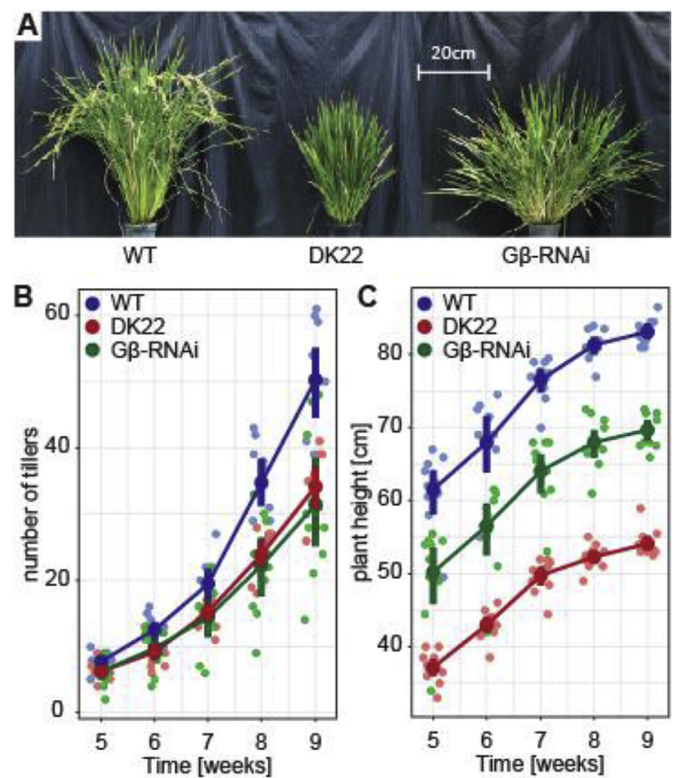


Fig. 3. Plant height and tiller formation of $G\alpha$ -null and $G\beta$ -RNAi plants. (A) Representative shoot images of wild type rice and rice G protein mutants. WT, wild type, DK22, *rga1* ($G\alpha$ subunit); Gβ, $G\beta$ subunit RNAi knock down. Rice plants were grown on soil for 10 weeks. The comparisons of (B) tiller number and (C) plant height among wild type and mutant rice plants at weeks 5 to 9 are shown. Graphs represent raw data (smaller dots), mean value (larger dots) and 95% confidence intervals (bars from mean values).

angles than wild type plants (Fig. 3A).

The shapes of grains and leaves, two other fundamental agricultural traits, are attributed to the coordinated action of longitudinal and radial growth. Rice $G\alpha$ and $G\beta$ protein mutations mediate some of these morphological changes (Utsunomiya et al., 2011). To complete the quantitative collection of shoot structural traits and compare the degree of morphological defects between rice and Arabidopsis, morphological traits of grains, embryos and leaves were quantitated (Figs. 4 and 5). While $G\alpha$ -null and $G\beta$ -RNAi genotypes exhibited 34% and 7% shorter grains than wild type plants (WT, 6.63 mm; DK22, 4.37 mm; $G\beta$ RNAi, 6.16 mm, Fig. 4F), the grain width of two mutants was comparable to WT (WT, 3.02 mm; DK22, 3.01 mm; $G\beta$ RNAi, 3.09 mm, Fig. 4G). In addition, $G\beta$ -RNAi genotype showed a 25% smaller embryo compared to WT (WT, 1.30 mm²; DK22, 1.04 mm²; $G\beta$ RNAi, 0.97 mm², Fig. 4C), which was proportional to the reduction in seed size (Fig. 4D and E).

Similar to Arabidopsis (Lease et al., 2001; Ullah et al., 2001), the wild type, $G\alpha$ -null and $G\beta$ -RNAi rice plants produced leaves and seeds with varying dimensions (Figs. 4 and 5). Further measurements of shoot phenotypes after two weeks of cultivation on soil revealed that $G\beta$ -RNAi plants had shorter and narrower leaf blades compared to the parental line (27% reduction in length and 34% reduction in width, Fig. 5B-D). As reported previously (Fujisawa et al., 1999), $G\alpha$ -null rice plants have qualitatively shorter and wider leaf blades (Fig. 5B and C). The length to width ratio of leaves and seeds revealed that while the $G\alpha$ -null mutation negatively impacted longitudinal growth with a slight increase in radial growth (Figs. 4H and 5D), the $G\beta$ RNAi mutation conferred a decrease in both longitudinal and radial growth of shoot tissues. These data suggest that the morphological changes conferred by $G\beta$ -RNAi contrasts to the morphology of the Arabidopsis *agb1* mutant wherein there are decreases

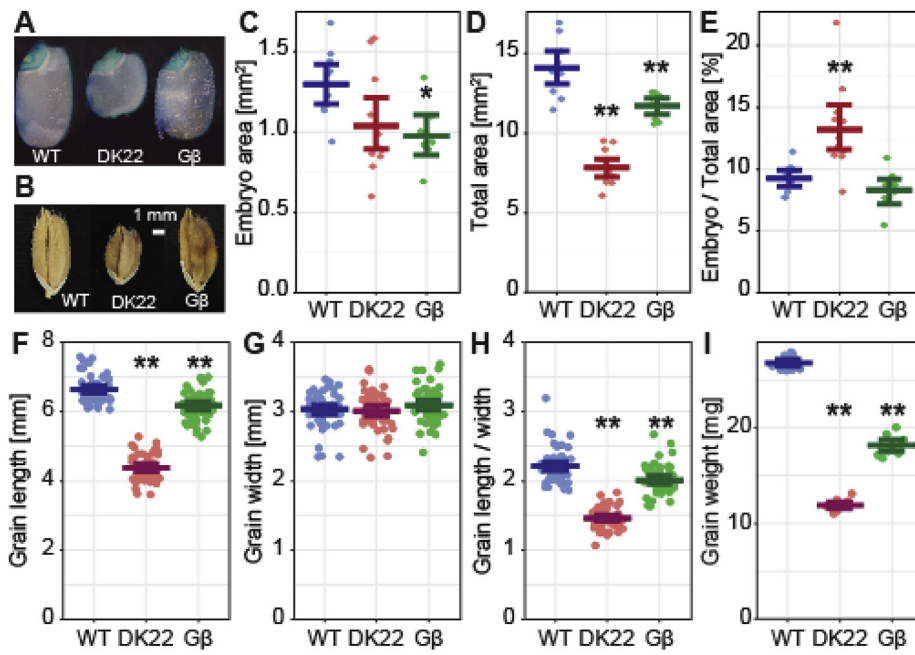


Fig. 4. Embryo and grain morphological traits of $G\alpha$ -null and $G\beta$ -RNAi plants. (A) Representative images of rice embryo of wild type and G protein mutants. WT, wild type, DK22, *rga1* ($G\alpha$ subunit); $G\beta$, $G\beta$ subunit RNAi knock down. (B) Representative seed images of wild type rice and rice G protein mutants. (C) Embryo size, (D) seed size, (E) relative size of embryo of wild type and G protein mutants. (F) grain length, (G) grain width, (H) length-to-width ratio of grain, and (I) grain weight of wild type and G protein mutants. Graphs show raw data and the mean value with the error bars of 95% confidence intervals. The symbols, * and ** indicate a significant difference from the wild type group at p values less than 0.05 and 0.01, respectively, as performed by one-way ANOVA followed by the Tukey's post hoc test.

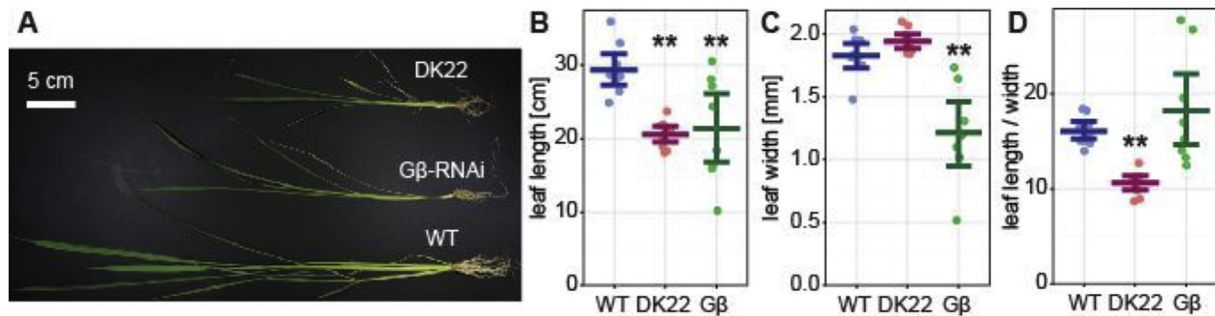


Fig. 5. Leaf morphological traits of $G\alpha$ -null and $G\beta$ -RNAi plants. (A) Representative seedling images of wild type rice (WT) and G protein mutants. The wild type and mutant plants were grown on soil for four weeks before the measurements. WT, wild type, DK22, *rga1* ($G\alpha$ subunit); $G\beta$ -RNAi, $G\beta$ subunit RNAi knock down. (B) Length, (H) width, and length-to-width ratio of second leaf blade. Graphs show raw data and the mean value with the error bars of 95% confidence intervals. The symbols, * and ** indicate a significant difference from the wild type group at p values less than 0.05 and 0.01, respectively, as performed by one-way ANOVA followed by the Tukey's post hoc test.

in longitudinal growth with moderate increases in the radial growth of shoot tissues (Lease et al., 2001; Ullah et al., 2003; Urano et al., 2016a, 2016b). We previously showed that longitudinal and radial growth in Arabidopsis above-ground tissues are separately regulated by two different types of heterotrimer complexes; canonical $G\alpha\beta\gamma$ and an atypical heterotrimer with Extra-Large $G\alpha$ (XLG) and $\beta\gamma$ subunits (Urano et al., 2016a). The quantitative morphological traits of rice above-ground tissues resemble morphological defects observed in the Arabidopsis *xlg* mutant. We speculate the defects in radial shoot growth in rice $G\beta$ mutants may be linked to the result of impairing the atypical heterotrimer G protein pathway similar to what was observed in Arabidopsis (Urano et al., 2016a).

Quantitative comparison of morphological traits in Arabidopsis and rice - The quantitative profiles of shoot and root morphological traits in rice G protein mutants (Figs. 1–5) suggested that $G\alpha$ and $G\beta$ signaling networks modulate longitudinal and radial growth in shoots and roots. As mentioned above, the rice developmental phenotypes caused by the $G\beta$ mutation differ from Arabidopsis (Chen et al., 2006; Lease et al., 2001; Ullah et al., 2003; Urano et al., 2016b). Fig. 6A assesses the similarity of Arabidopsis and rice G protein mutant phenotypes in shoot and root morphology. The quantitative structural profiles obtained from

Arabidopsis (Ullah et al., 2003; Urano et al., 2016a) and rice (Figs. 1–5) $G\alpha$ and $G\beta$ mutant phenotypes are presented in a single heatmap as relative values of each physiological trait compared with the wild type plant (Fig. 6A). Leaf length and shoot branches showed a similar pattern between Arabidopsis and rice mutants, whereas root length and root branches showed a similar defect by the $G\alpha$ -null mutation but opposite in Arabidopsis $G\beta$ -null and rice $G\beta$ -RNAi mutants (Fig. 6A).

The contrasting root morphological defects between Arabidopsis *agb1* and rice $G\beta$ RNAi plants may be attributed to different expression patterns of $G\beta$ and $G\gamma$ genes. To explore this possibility, we compiled and analyzed spatial expression profiles of the $G\alpha$, $G\beta$ and $G\gamma$ subtypes from 284 or 113 RNA-sequencing datasets in rice or Arabidopsis (Fig. 6B and C). The meta-expression analyses suggested that $G\alpha$ and $G\beta$ genes were expressed ubiquitously in rice and Arabidopsis, except in the rice pistil which expresses RGA1 and RGB1 genes at a low level. In contrast, rice RGG2 (type-B $G\gamma$) was abundantly expressed in roots compared to RGG1 (type-A $G\gamma$) and DEP1 (type-C $G\gamma$), while two type-A $G\gamma$ genes AGG1 and AGG2, but not AGG3 (type-C $G\gamma$), were highly expressed in Arabidopsis roots. These results suggest that Arabidopsis and rice roots mainly express different subtypes of $G\gamma$ subunits; namely type-A and type-B $G\gamma$. Because $G\beta$ subunit functions as an obligate dimer with $G\gamma$ subunit in

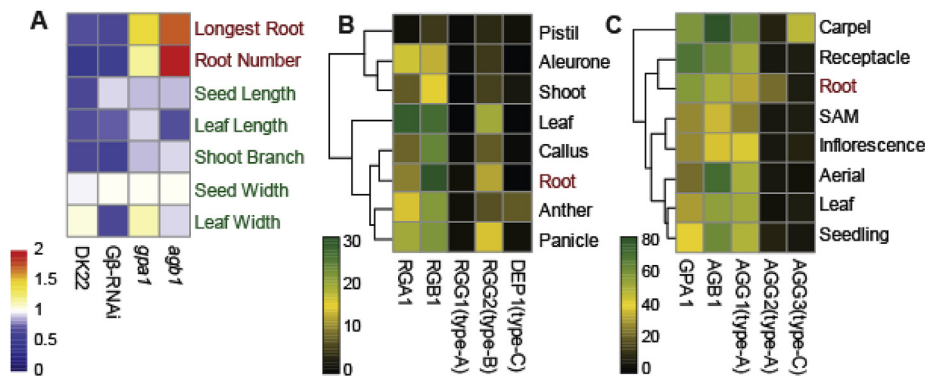


Fig. 6. Comparison of morphological traits and gene expression patterns in Arabidopsis and rice. (A) Quantitative measurements of shoot and root morphology in Arabidopsis and rice G protein mutants. The heat map shows quantitated values relative to those in wild type plants. Arabidopsis quantitative morphological data are obtained from two of our previous publications (Ullah et al., 2003): for shoot branch number and (Urano et al., 2016a) for all other traits. DK22, *rga1* (rice α subunit); Gβ-RNAi, rice Gβ RNAi knock down; *gpa1*, Arabidopsis α subunit null mutant; *agb1*, Arabidopsis Gβ subunit null mutant (B, C) Tissue-specific gene expression profiles of (B) rice and (C) Arabidopsis G protein genes are presented as a heat map of Fragments per Kilobase of transcript per Million mapped reads (FPKM) values. The expression data are obtained from the Information Commons for Rice (IC4R) for rice (B) or the Arabidopsis information portal (Araport) for Arabidopsis (C).

cells, the different G γ subunits expressed in roots may be related to the distinct morphological alterations observed in Arabidopsis *agb1* and rice Gβ RNAi plants.

Elevated cell death activity associated with the Gβ-RNAi mutation – In contrast to the rice cell death phenotypes that indirectly cause small shoot and root structures in the Gβ RNAi mutant, the Arabidopsis *agb1* mutant forms a larger root system without an obvious cell death phenotype under normal growth conditions (Ullah et al., 2003). Therefore, it remains unknown whether the function of Gβ in spontaneous cell death became prominent only in rice or present in Arabidopsis as well. To explore the possible presence of spontaneous cell senescence during root formation in a eudicot and its potential mechanism, we enumerated differentially expressed gene (DEG) sets caused by Arabidopsis *gpa1* or *agb1* mutations and compared the gene list to cell type-specific transcriptomes collected from different areas of Arabidopsis roots (Fig. 7A

and B). The meta-transcriptomic analyses enumerated 168 *agb1*-regulated and 87 *gpa1*-regulated genes that were also highly expressed in meristematic zones (zones 1 to 4), especially in meristematic zones 1 and 2 where cell proliferation occurs (Fig. 7A). Further gene ontology analysis with the 255 genes revealed that biotic stress response (GO group: 0043207) or innate immune response genes (GO group: 0045087) are significantly enriched in the *agb1*-regulated but not *gpa1*-regulated genes (Fig. 7B). This analysis prompted us to hypothesize that the lack of rice Gβ protein causes the elevation of a steady-state autoimmune response; specifically, this autoimmune activity, if enhanced, causes spontaneous cell death even without biotic stresses. This hypothesis was tested in rice by quantitating the expression level of immune marker genes associated with cell death such as *Pathogenesis Related 1a* (*PR1a*) and *PR1b* (Olvera-Carrillo et al., 2015). Under a normal growth condition, expression of *PR1a*, *PR1b* and another immune marker *Chitinase* (*CHI*) was greatly

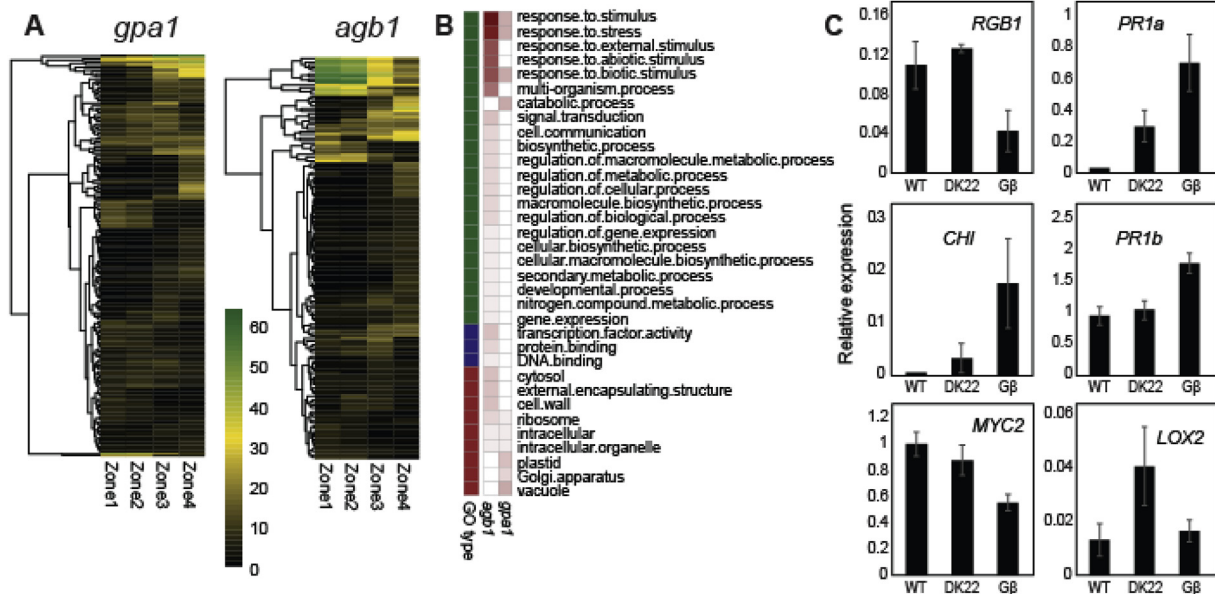


Fig. 7. Expressions of immune maker genes in Gα-null and Gβ-RNAi rice plants. (A) *gpa1*, Arabidopsis α subunit null mutant; *agb1*, Arabidopsis Gβ subunit null mutant Expression pattern of *gpa1*- and *agb1*-regulated genes in four meristematic zones in Arabidopsis roots. The values in heatmaps represent normalized expression levels from a cell-type specific microarray dataset of Arabidopsis roots (Dinneny et al., 2008). The *gpa1*- and *agb1*-regulated genes were selected from publicly-available microarray data using Arabidopsis hypocotyls (See materials and methods). (B) Enriched Gene Ontology (GO) terms and adjusted p-values (FDRs) obtained from GO analysis of *gpa1*- and *agb1*-specific genes were shown as a heatmap. The p-values shown were log-transformed to $-\log_{10}(\text{FDR})$. Note that we tried to analyze gene expression profiles in rice Gβ mutant roots, however failed to extract quality RNA after multiple attempts likely due to the severely enhanced cell death. (C) Expression levels of rice Gβ (*RGB1*) and immune marker genes in wild type and mutant leaf blades. The bars represent the mean value (the expression level of each marker gene relative to the expression level of *Ubiquitin 5*) with S.E.M of four replicates.

upregulated in rice G β -RNAi plants (Fig. 7C), while the expression level of the jasmonate pathway marker genes *MYC2* and *Lipoxygenase 2* (*LOX2*) (Zhai et al., 2013) were slightly or barely altered. The G α -null mutation also affected basal expression level of *PR1a* and *CHI* moderately, although not as high as in G β -RNAi plants. Taken together, we propose that the elevation of a steady-state autoimmune response manifesting at different degrees is the fundamental molecular mechanism to induce senescence in the rice G β -RNAi mutant.

3. Discussion

In animals, development is largely accomplished during embryogenesis involving cell differentiation, growth, death, and migration to complete the final pattern. In plants, the body plan is also established during embryogenesis but considerable development also occurs post-embryonically. Importantly, post embryonic organ formation (Irish, 2010) and branching patterns (Petricka et al., 2012), both fundamental to plant architecture, are subject to environmental signals. Therefore, signal transduction and development intertwine to a large extent in plants (Smyth, 2018; Somssich et al., 2016; Urano et al., 2016b; Wang et al., 2018); consequently, it is more difficult to dissect the primary role of any particular signal transduction pathway in the regulation of pattern from cell behavior.

Clearly, loss-of-function mutations in the subunits of the heterotrimeric G protein complex confer morphological phenotypes in Arabidopsis, rice, and other plants including some alterations in root architecture (Bommert et al., 2013; Fujisawa et al., 1999; Ullah et al., 2001, 2003; Urano et al., 2016b; Utsunomiya et al., 2011) but is this a direct or indirect effect? Our quantitative morphological data here, together with the enhanced expressions of cell death marker genes, posits that the rice G β pathway is not involved in establishing the body plan and this raises the question why the G β knockdown confers morphological phenotypes. We argue that these qualitative phenotypes in rice are mainly indirect effects from the loss of coupling of signals to cellular processes in the G β mutants and that these local changes such as altered cell cycling and programmed cell death manifest iteratively over time as altered morphology.

What are the primary signal pathways? G $\beta\gamma$ dimer mediates stress signals that control the longitudinal growth and cell division in shoot tissues. In addition, decreasing rice G β gene expression causes spontaneous cell death in the root (Fig. 2), shoot and embryo (Utsunomiya et al., 2011, 2012). Similarly, the Arabidopsis *agb1* mutant confers increased cellular senescence induced by various stresses including high salinity, far-red irradiation and fungal infection (Chen and Brandizzi, 2012; Llorente et al., 2005; Trusov et al., 2006; Urano et al., 2016a; Wei et al., 2008; Yu and Assmann, 2015), although no obvious senescence is seen under non-stressed growth conditions. This increased cell death occurrence in rice, conceivably in Arabidopsis as well, could be related to autoimmunity or necroptosis (Coll et al., 2011; Jones, 2001; Utsunomiya et al., 2011, 2012). In this model, the ablation of rice G $\beta\gamma$ signaling elevates the basal level of programmed cell death by inducing autoimmune responses. As shown in Fig. 7C, the cell-death associated immune markers, *PR1a* and *PR1b*, are highly expressed in G β -RNAi plants without pathogen infection (Fig. 7C). These markers are transcribed at a high level in the maize *xlg* mutant as well (Wu et al., 2018). Arabidopsis *agb1* mutants also have increased these marker gene expression compared to WT but only upon exposure to a necrotrophic fungus (Delgado-Cerezo et al., 2012), and exhibit altered defense response and senescence activity upon bacterial and fungal infections (Escudero et al., 2017; Llorente et al., 2005; Trusov et al., 2006).

The Arabidopsis *agb1* mutation down-regulates oxidative burst in response to pathogenic peptides, while expressing salicylic acid (SA)-responsive genes at a high level upon pathogen infection (Delgado-Cerezo et al., 2012; Escudero et al., 2017; Maruta et al., 2015; Trusov et al., 2009; Urano et al., 2016a). Both salicylic acid and reactive oxygen species are associated with programmed cell death in the immune response

as well as regulating plant growth and structural development. Interestingly, rice contains two orders of magnitude higher basal concentration of SA than Arabidopsis does (Silverman et al., 1995), and no further accumulation is necessary to evoke SA signaling pathways (Yang et al., 2004; Yuan et al., 2007). SA and heterotrimeric G protein act separately in immune responses (Trusov et al., 2009), although both pathways serve as upstream signaling pathways for *PR1* gene expression. The high endogenous SA level in rice could explain why the knockdown of rice G β gene increased the expression of cell death marker genes without a pathogen infection (Figs. 2 and 7C). To complete this circle of logic, we argue that development shares signaling pathways with immune system such as reactive oxygen species and immune hormones, and that normal development itself would evoke such shared-signals. For example, embryo formation generates stress-related responses internally and imparts them to neighboring cells and tissues (Joseph et al., 2018; Kurusu and Kuchitsu, 2017). The enhanced autoimmune response could explain the cell death and subsequent morphological defects observed in rice G β RNAi plants, and this suggests that a major role of G β protein is to mediate the shared-signaling pathways in development and immunity.

We speculated previously that plant G protein signaling serves in fine-tuning the balance between cell death, survival, and proliferation (Urano and Jones, 2014). Rice and Arabidopsis have well-conserved developmental regulators and autoimmune response genes (Chen and Ronald, 2011; Smyth, 2018; Somssich et al., 2016; Urano et al., 2016b; Wang et al., 2018), however how these two cellular functions are coordinated at the molecular levels to shape their bodies and to adapt to their living environment remains unknown. We propose here that the cell division that is needed for development itself induces stress signaling and/or that regulation of development and immune responses share signals; in either case, cross-talks with G protein-coupled signaling at the cell level to indirectly control plant architecture. Our proposal should not be confused with the well-studied phenomena of tradeoffs between defense and growth (Huot et al., 2014) which occurs by controlling allocation of plant resources between the use in increasing tissue mass vs. fighting infection. One of the main roles of the G β pathway would be to mediate cellular stress responses during development, and this has an indirect effect on development along with the previously-reported mechanisms through cell proliferation and meristem maintenance (Ishida et al., 2014; Ullah et al., 2001). We acknowledge that the G protein subunit also plays a role in pathogen defense (Llorente et al., 2005; Maruta et al., 2015; Trusov et al., 2006; Urano et al., 2016a) and possibly in the growth vs. defense trade-off response but propose that its role in formation of plant organs and the plant body plan is indirect.

4. Materials and methods

Plant materials and measurement of shoot, root, and seed traits - Seeds of wild-type (*Oryza sativa* ssp. *Japonica*. cv. Nipponbare), G α -null mutant (DK22) and G β -RNAi plants (Fujisawa et al., 1999; Utsunomiya et al., 2011) were surface-sterilized with 70% ethanol, distilled water, 50% bleach, and a drop of Tween-20 at room temperature. The seeds were germinated on half-strength Murashige and Skoog ($\frac{1}{2}$ x MS) medium with 10 μ M gibberellic acid at 28 °C in the dark for three days. Germinated seedlings were transferred to soil pots and grown in the greenhouse with a 12 h day and a 12 h night. The temperature was maintained between 25 – 35 °C. The above-ground traits in Fig. 3 were measured from week 5 to 9. Wild-type plants with at least eight leaves, DK22 plants with at least seven leaves and G β RNAi plants with at least five leaves were selected for measurement of tiller branches and plant height. Because RNAi can vary between individuals, we selected only plants that displayed a previously-characterized G β mutant trait, brown internode, for measurements of the two traits in Fig. 3. Germination, the percentage of seedlings with a coleoptile and radicle, was analyzed after seven days on plates.

Seed, embryo and leaf morphological traits - Seed length and width were measured and calculated using ImageJ software. For

measurement of embryo size, rice seeds were de-husked and soaked in 70% ethanol for more than 24 h, then sliced longitudinally into two halves. The sliced seeds were stained in 0.005% Toluidine blue for 10 s, washed and imaged for measurement of embryo and seed areas with ImageJ. Length and width of second leaf blade were measured from 2 week-old plants.

Three-dimensional analysis of rice root architecture – Sterilized seeds were germinated on Yoshida medium plates for 2 days (Yoshida et al., 1976), and the germinated seedlings were transferred to glass cylinders (approximately 52 cm in height and 8.25 cm in diameter) filled with Yoshida medium and placed in a growth chamber with a 12-h day/night cycle at 28 °C day or 25 °C night. The platform of the imaging system was described in detail previously (Iyer-Pascuzzi et al., 2010). The roots of rice plants were imaged three-dimensionally 11 days post planting. The following 19 root developmental traits were collected: average root width, depth of root network, maximum width of root network, width-depth ratio, volume of root network, total length of root network, specific root length (total length per volume), maximum number of roots, median number of roots, bushiness (maximum to median number of roots), network convex area, solidity, perimeter, length distribution, network area, surface area, major ellipse axes, minor ellipse axes, ellipse axes aspect ratio. The definitions of the root traits are described in detail previously (Ingram et al., 2012; Iyer-Pascuzzi et al., 2010).

Evans blue staining – Approximately 0.2 g of rice roots were collected from approximately 10 mm-long root tips. The roots were weighed and immersed in 0.25% Evans blue solution for 60 min at room temperature. After washing the roots five times in water, the samples were placed in 90% ethanol and 10% glycerol solution for overnight. Evans blue was extracted from the roots with 100% DMF. The relative amount of Evans blue was estimated by measuring the absorbance at 600 nm.

Quantitative PCR – Expression level of *Rgb1* and Immune marker genes *PR1a* (Os07g0129200), *PR1b* (Os01g0382000), *CHI* (Os05g0399300), *MYC2* (Os10g0575000) and *LOX2* (Os08g0508800) was tested with quantitative real-time PCR analysis. RNA samples were prepared from 3-week-old leaf blades, and reverse-transcribed into cDNA with an oligo dT primer. The expression level of each marker gene was presented as a relative value to that of internal control gene *Ubiquitin 5* (Os01g0328400).

Expression profiles of G protein genes – G protein genes expression data were obtained from IC4R for rice and from Araport for Arabidopsis, respectively. For rice, the following tissues/organs were included: pistil, aleurone, shoot, leaf, callus, root, anther and panicle. For Arabidopsis, the following organs were included: carpel, receptacle, root, SAM, inflorescence, aerial, leaf and seedlings were included in Arabidopsis samples. Expression data from the same tissue was averaged and was presented as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). The heatmaps were then generated with the pheatmap package in R for comparison and visualization.

Analyses of publicly available microarray data - Raw data from the affymetrix platform ATH1-121501 (GPL198) from Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) were extracted, which included GSE19520 (*gpa1* and *agb1*), GSE6171 (*gpa1*) and GSE34667 (*gpa1agb1*). Similarly, raw data sets from the same platform (GPL198) E-MTAB-614 (*agb1*) from ArrayExpress (<https://www.ebi.ac.uk/arrayexpress/>) were obtained for further analyses. The data were processed with multiarray averaging normalization using the affy, oligo and limma packages in R (Gautier et al., 2004; Ritchie et al., 2015). Differential expression for each experiment was computed by a linear regression model using log₂ fold change values between wild type and mutant datasets. Differentially expressed gene lists of *gpa1* and *agb1* were then mapped to the Arabidopsis root specific microarray (Dinnyeny et al., 2008) to generate a heatmap from normalized longitudinal zones and cell-type-specific dataset. The gene expression values greater than 20 were then further subjected to GO enriched analysis using the AgriGO

website (Tian et al., 2017) with default settings. The values presented in heatmaps are base-10 logarithms of false discovery rates (FDR).

Author contributions

A.M.J and D.U. designed the project; R.L and D.U. performed experiments. T. W. and D.U. analyzed transcriptional and experimental data. T.W., A.M.J. and D.U. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2019.09.007>.

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