The α subunit of the heterotrimeric G protein regulates mesophyll CO₂ conductance and drought tolerance in rice

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Summary

- Mesophyll conductance, gₘ, determines CO₂ diffusion rates from mesophyll intercellular air spaces to the chloroplasts and is an important factor limiting photosynthesis. Increasing gₘ in cultivated plants is a potential strategy to increase photosynthesis and intrinsic water use efficiency (WUE). The anatomy of the leaf and metabolic factors such as aquaporins and carbonic anhydrases have been identified as important determinants of gₘ. However, genes involved in the regulation and modulation of gₘ remain largely unknown.
- In this work, we investigated the role of heterotrimeric G proteins in gₘ and drought tolerance in rice d1 mutants, which harbor a null mutation in the Gα subunit gene, RGA1.
- d1 mutants in both cv Nipponbare and cv Taichung 65 exhibited increased gₘ, fostering improvement in photosynthesis, WUE, and drought tolerance compared with wild-type. The increased surface area of mesophyll cells and chloroplasts exposed to intercellular airspaces and the reduced cell wall and chloroplast thickness in the d1 mutant are evident contributors to the increase in gₘ.
- Our results indicate that manipulation of heterotrimeric G protein signaling has the potential to improve crop WUE, and productivity under drought.

Introduction

Drought is one of the most important environmental variables affecting crops, causing more annual loss in crop yield than all pathogens combined (Gupta et al., 2020). This constraint on global crop production will be further exacerbated by climate change (Wassmann et al., 2009). Drought commonly compromises paddy-grown rice and is the single biggest constraint on yield production in rain-fed rice (Tuong & Bouman, 2003). Because rice is the staple food for more than half of the world’s population, the development of varieties with improved photosynthesis and water (H₂O) use efficiency in the face of suboptimal conditions is of enormous importance for both present-day and future food security (Long et al., 2015).

During photosynthesis, the provision of CO₂ to the sites of carboxylation in the chloroplast stroma first requires the diffusion of CO₂ from the atmosphere through stomata into the leaf intercellular air spaces, and is thus dependent on stomatal conductance, gₛ. In the next phase, CO₂ flows from intercellular air spaces to the fixation site through several resistances: cell wall, cell membrane, cytoplasm, chloroplast envelope, and finally the stroma, where CO₂ encounters Rubisco. These resistances in series determine the mesophyll conductance to CO₂, gₘ, which is calculated as the photosynthetic rate Aₚ divided by the CO₂ drawdown from its concentration in the intercellular air spaces Cᵢ, to the site of CO₂ fixation Cᵣ. High mesophyll conductance ensures sufficient CO₂ concentration at the fixation site and is, therefore, strongly correlated with photosynthesis (Harley et al., 1992; Gago et al., 2020). The intrinsic water use efficiency WUE, (the photosynthesis to stomatal H₂O conductance ratio) can be improved by limiting transpiration via reducing stomatal conductance to H₂O vapor gₛ and/or by increasing photosynthesis (Barbour et al., 2010). Enhancing or maintaining gₛ when stomatal conductance to H₂O decreases is another strategy to improve WUE (Barbour et al., 2010; Tomeo & Rosenthal, 2017).

Two types of components contribute to mesophyll conductance. First, at the structural level, anatomical characteristics, most notably mesophyll surface area exposed to intercellular air spaces (Evans et al., 1994), chloroplast distribution in cells (Tholen et al., 2008), and cell wall thickness (Terashima et al., 2011; Ellsworth et al., 2018), impact gₛ. Second, at the biochemical level, gₛ may be regulated by cooporins (aquaporins that are permeable to CO₂), facilitating CO₂ diffusion through the cell membrane and chloroplast envelope (Evans et al., 2009), and by the efficiency of the conversion of CO₂ to bicarbonate (HCO₃⁻) by carbonic anhydrases (CAs) (Gillon & Yakir, 2000).

Heterotrimeric G proteins are interesting candidates involved in the regulation of stomatal conductance (Ferrero-Serrano & Assmann, 2016), yet their potential role in the regulation of gₛ remains unexplored. Heterotrimeric G proteins are GDP-GTP binding proteins composed of Gα, Gβ, and Gγ subunits that...
work with G protein-coupled receptors to transduce extracellular signals into intracellular responses. G proteins impact numerous aspects of plant morphology and modulate responses to many environmental stresses (Wang et al., 2001; Fan et al., 2008; Zhang et al., 2008; Nilson & Assmann, 2010a; Chakravorty et al., 2011), including drought stress (Nilson & Assmann, 2010b; Ferrero-Serrano & Assmann, 2016).

The rice genome contains a single canonical Gα (RGA1) and four extra-large Gα subunits (XLG), one Gβ (RGB1), and four Gγ (RGGL1, RGG2, GS3, and DEPI) genes, with a fifth related Gγ gene or pseudogene, OsGGC2 (Perfus-Barbeoch et al., 2004). Mutations in rice G protein subunits have been found to affect numerous agronomically related phenotypes (Botella, 2012; Cui et al., 2020). Multiple RGA1 mutants have been identified that result in dwarf plants with short and erect leaves compared with wild-type (WT) despite having the same et al d1 identified that result in dwarf plants with short and erect leaves (Nilson & Assmann, 2010b; Ferrero-Serrano & Assmann, 2016).

Gas exchange measurements

Gas exchange and fluorescence measurements were conducted using the LI-6800 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) equipped with a 2 cm² fluorescence leaf chamber. Measurements were conducted at light intensities of 1500 μmol m⁻² s⁻¹ (determined as saturating light from Aₙ - photosynthetic photon flux density (PPFD) curves) and an air flow rate of 300 μmol s⁻¹. The block temperature was set to 32°C, and the relative humidity was controlled at 50% using the LI-6800 humidifier and desiccant. All gas exchange measurements were made between 09:00 h and 13:00 h. We chose the center of the newly expanded leaf, 5–10 cm from the tip of the leaf lamina, for measurement. After a leaf was clamped in the LI-6800 chamber, we allowed 10 min of acclimation to a fixed reference CO² concentration of 400 μmol mol⁻¹ air. Then, CO₂ response curves for gas exchange combined with Chl fluorescence were measured. The initial CO₂ concentration was set to ambient CO₂ concentration (400 μmol mol⁻¹ air), which was then reduced to 300, 200, 150, 100, and 50 ppm. Then, the CO₂ concentration was returned to 400 μmol mol⁻¹ air and then increased to 600, 800, 1000, 1200, and 1600 μmol mol⁻¹ air. Mesophyll conductance gₘ was estimated using the variable J method (Harley et al., 1992; Loreto et al., 1992):

\[
g_m = \frac{A_n}{C_i - C_c}
\]

(Aₙ, net photosynthesis rate; Cᵢ, CO₂ concentration in the leaf intercellular airspaces; Cᵦ, CO₂ concentration in the chloroplast stroma); Aₙ and Cᵢ were both determined from gas exchange measurements, and Cᵦ is estimated from combined measurements of gas exchange and Chl fluorescence as:

\[
C_a = \frac{\Gamma^* [J + 8(A_N + R_d)]}{J - 4(A_N + R_d)}
\]

(J, electron-transport rate, calculated based on Chl fluorescence; R₉, nonphotorespiratory respiration in light; Γ*, apparent photocompensation point, estimated according to Laisk & Loreto (1996)). CO₂ response curves were measured under three different light intensities (100, 200, and 400 μmol m⁻² s⁻¹) and at 21% oxygen (O₂) and fitted linearly. The y and x values of the average of three CO₂ response intersections were taken as R₉ and Cₗ*(Supporting Information Fig. S1).

The Γ* is dependent on gₘ and R₉ as follows (von Caemmerer et al., 1994):

\[
\Gamma^* = C_{\Gamma} + \frac{R_d}{g_m}
\]

However, since no gₘ value was available before the measurements, the conversion was not completed and the Cₗ* was taken as a representation of Γ* (Qiu et al., 2017). We have also tested constant Γ* values obtained from Bernacchi et al. (2002) (Fig. S2).
The electron transport rate $J$ is calculated as follows:

$$J = \Phi_{\text{PSII}} \times \text{PPFD} \times \alpha \times \beta$$

($\alpha$, coefficient of leaf absorbance; $\beta$, fraction of absorbed quanta that reaches photosystem II (PSII)). The quantum yield of $\Phi_{\text{PSII}}$ was calculated according to Genty et al. (1989):

$$\Phi_{\text{PSII}} = \frac{F'_m - F_t}{F'_m}$$

($F_t$, steady-state fluorescence; $F'_m$, maximal fluorescence in the light-adapted state, after a light-saturating pulse of the multiphase flash fluorometer of the LI-6800). The quantum yield of CO$_2$ fixation was defined as:

$$\Phi_{\text{CO}_2} = \frac{A_n + R_d}{\text{PPFD}}$$

The combined use of gas exchange and Chl fluorescence relies on the assumption that there is a linear relationship between $\Phi_{\text{PSII}}$ and $\Phi_{\text{CO}_2}$ under nonphotorespiratory conditions, since CO$_2$ fixation is the only sink for electrons (Valentini et al., 1995). From the response curve measured at 1% O$_2$, we performed a linear regression of actual quantum efficiency of PSII $\Phi_{\text{PSII}}$ and the quantum yield of CO$_2$ fixation $\Phi_{\text{CO}_2}$ to obtain a regression coefficient $k$. The theoretical model and experimental observations were limited to the linear region of $\Phi_{\text{CO}_2} < 0.05$ and $\Phi_{\text{PSII}} < 0.5$ (Fig. S3). The slopes of the linear regression $k$ and the $y$-axis intercept $\beta$ were used to recalculate the $\alpha \times \beta$ for the electron-transport rate $J$ based on Chl fluorescence measured at 21% O$_2$ as:

$$J_{\text{cal}} = \left(\frac{4(\Phi_{\text{PSII}} - b)}{k}\right) \times \text{PPFD}$$

The $C_s$ values were corrected for cuticular diffusivities to H$_2$O and CO$_2$ according to Boyer et al. (1997):

$$C_i = C_s - 1.6 \frac{A_n}{E_l - 2E_c} (W_i - W_a)$$

($C_s$, CO$_2$ concentration of the air surrounding the leaf (taken as the Li-Cor CO$_2$ reference); $W_i$ and $W_a$, mole fractions of H$_2$O in the leaf intercellular air spaces and in the air surrounding the leaf, respectively; $E_l$, leaf transpiration; $E_c$, cuticular transpiration (assumed to be constant at 5 mmol H$_2$O m$^{-2}$ s$^{-1}$) (Flexas & Medrano, 2002).

CO$_2$ concentration in the chloroplast stroma $C_c$ was then calculated with the observed $g_m$ value according to Fick’s first law of diffusion:

$$C_c = C_i - \frac{A_n}{g_m}$$

The $A_n$-$C_i$ curves were converted into $A_n$-$C_c$ curves and analyzed using the plantecophysics R package for analyzing and modeling leaf gas exchange data (Duursma, 2015). The maximum carboxylation rate $V_{\text{max}}$ and rate of electron transport $J_{\text{max}}$ were computed.

**Temperature dependence of photosynthesis and $g_m$**

Leaf temperatures were controlled with the LI-6800 thermocouple by fixing the block temperature to the desired temperature. Well-watered plants were initially measured at 20°C and then at 25, 30, 35, and 40°C. We allowed at least 30 min at each temperature after the leaf temperature had stabilized before measurements were taken. After $A_n$ and $g_m$ reached steady state, a new $A_n$-$C_i$ curve was performed as described earlier herein.

**Leaf anatomy**

At the conclusion of the gas exchange measurements, leaf samples from T65 WT, and $dl$ plants were cut from the exact leaf patches that were measured for physiology and immediately fixed in a buffer containing 2.8% glutaraldehyde in 0.1 M HEPES buffer, pH 7.2 (with 0.02% v/v Triton X-100) at room temperature for 2 h, and then overnight at 4°C. Samples were then postfixed in 1% osmium tetroxide overnight in a light-proof container and then dehydrated in a graded acetone series (20–100%). The dehydrated segments were subsequently embedded in Spurr’s resin and polymerized at 60°C for 2 d. Transverse sections, 1000 μm thick, were prepared using an ultramicrotome (Leica EM UC6; Leica Mikrosysteme GmbH, Vienna, Austria), stained with 1% toluidine blue in 1% borax (pH 9.0) and observed under a light microscope (Olympus BX51; Olympus, Tokyo, Japan). Ultrathin cross-sections for transmission electron microscopy (TEM) were cut with glass knives on an ultramicrotome and the sections were stained with uranyl acetate and lead citrate before viewing with an FEI Tecnai G2 Spirit BioTwin (FEI; Hillsboro, OR, USA). Sections were imaged and the mesophyll surface area exposed to intercellular air spaces $S_m$ and the chloroplast surface area exposed to intercellular air spaces $S_i$ were measured using ImageJ software (https://imagej.nih.gov/ij/; US National Institute of Health, Bethesda, MD, USA), and calculated as described in Evans et al. (1994) and Syvertsen et al. (1995): $S = (I/W) \times 1.42$ (Scelfaro et al., 2011; $I$ is $S_m$, or $S_i$; $W$, total perimeter of mesophyll cells or chloroplasts facing the intercellular air space; $I$, the analyzed cross-section width; 1.42, curvature correction factor for the ellipsoidal mesophyll cell-shape (Evans et al., 1994)). The volume fraction of intercellular air space was calculated from the optical cross-sections as $f_{\text{ias}} = S_{\text{ias}}/(S_m + S_{\text{ias}}$) (Xiong et al., 2016; $S_{\text{ias}}$, cross-sectional area of the intercellular air space measured with ImageJ). Mesophyll conductance modeled with the anatomic parameters was calculated according to the one-dimensional model of Tomás et al. (2013):

$$g_m = \frac{1}{\frac{1}{f_{\text{ias}}} + \frac{RT_c}{H_{\text{gas}}}}$$

($g_{\text{ias}}$, CO$_2$ conductance in the gas phase from the substomatal cavities to the outer surface of the cell wall surface; $H_{\text{gas}}$, CO$_2$
liquid-phase conductance from the outer surface of cell wall surface to the chloroplasts; \(R\) gas constant (Pa m\(^3\) mol\(^{-1}\) K\(^{-1}\)); \(T_a\), absolute temperature (kelvin); \(H\), Henry’s law constant (atm m\(^3\) mol\(^{-1}\)). The gas-phase conductance to CO\(_2\) was calculated as:

\[
g_{\text{gas}} = \frac{D_{S_i} f_{L_{\text{eff}}}}{\Delta L_{\text{eff}i} r}
\]

(\(D_{S_i}\) diffusivity of CO\(_2\) in air at 25°C (0.000151 m\(^2\) s\(^{-1}\)); \(f_{L_{\text{eff}}}\) fraction of leaf air space; \(\Delta L_{\text{eff}i}\), effective diffusion path length in the gas phase, estimated as half the mesophyll thickness; \(r\), tortuosity of the diffusion path (1.57 mm\(^{-1}\)); Tomás et al., 2013). The CO\(_2\) liquid-phase diffusion conductance through cell wall (\(g_{cw}\)), cytosol (\(g_{cyt}\)), and stroma (\(g_{st}\)) were calculated according to a general formula:

\[
g_i = \frac{D_{S_i} r_{i,p} p_i}{\Delta L_i}
\]

\((g_i (i = \text{cw, cyt, or st}), \text{individual component of the liquid-phase CO}_2 \text{ conductance; } D_{S_i}, \text{CO}_2 \text{ aqueous diffusion coefficient at } 25^\circ\text{C} (1.79 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}); p_i, \text{effective porosity (1 m}^3 \text{ m}^{-3} \text{ for cytosol and stroma; for the cell wall we assumed identical } p_i \text{ for the } d1 \text{ and WT of } 0.3 \text{ m}^3 \text{ m}^{-3}; r_{i,p} \text{ factor accounting for the reduction in diffusion due to the presence of solutes and macromolecules (dimensionless, 1.0 for cell wall and 0.3 for cytosol and stroma; Niinemets & Reichstein, 2003); } \Delta L_i \text{, diffusion path length (meters) for each component along the CO}_2 \text{ diffusion pathway}).\)

Next, the liquid-phase conductance \(g_{liq}\) was calculated as the sum of the inverse of the individual component’s conductances, in series:

\[
g_{liq} = \frac{1}{g_{cw}} + \frac{1}{g_{cyt}} + \frac{1}{g_{st}} + \frac{1}{g_{s}}
\]

The plasma membrane conductance \(g_{pm}\) and the chloroplast envelope conductance \(g_{envelope}\) were each considered to be 0.0035 m s\(^{-1}\), as described in Evans et al. (1994).

Transcriptome analysis

We reanalyzed RNA-sequencing (RNA-seq) data from a previously published study of 2-month-old WT and \(d1\) T65 plants that were grown under well-watered conditions equivalent to those described here (Ferrero-Serrano et al., 2018). These data are publicly available in the Gene Expression Omnibus (GSE103747). The data analyzed were originally collected from the flag leaf at the region of maximum width; that is, the same region as sampled in this study for gas exchange and leaf anatomy. Statistically different expression levels between T65 and \(d1\) were considered to occur when \(q < 0.05\) and fragments per kilobase of exon per million mapped fragments FPKM > 1 in at least one genotype.

Results

We compared the physiological performance of rice WT and \(d1\) mutants in the T65 and NB cultivars under well-watered and water-limited conditions, with the goal of testing whether the ability to tolerate drought in \(d1\) (Ferrero-Serrano & Assmann, 2016) is associated with higher mesophyll conductance \(g_m\) and WUEi.

Fig. 1 shows the order of plant wilting from the beginning of the drought experiment (day 0), indicating higher drought sensitivity for the NB genotypes than the T65 genotypes. NB plants wilted after 10 d, T65 after 16 d, \(d1\) (NB) after 20 d, and \(d1\) (T65) was the most drought tolerant and wilted only after 24 d.

For both WT genotypes and their \(d1\) respective mutants, the initial \(g_m\) value was significantly higher in the \(d1\) mutants (Fig. 2a). The \(g_m\) values reached nearly zero (i.e. full stomatal closure) after 12 d in WT and 15 d in \(d1\) in the NB genotype, and after 15 d in WT and 20 d in the \(d1\) mutant in the T65 genotype. By the time of zero \(g_m\), leaves were starting to show signs of wilting in the WT plants, but the \(d1\) mutant leaves remained green and turgid in both genotypic backgrounds. Accordingly, drought stress levels were defined not by days since watering was withheld but by three intervals of RSWC (Fig. 2b), with well-watered defined as 100% > RSWC > 50%, moderate drought as 50% > RSWC > 35%, and severe drought as RSWC < 35%.

Under well-watered conditions, all gas exchange parameters quantified for the \(d1\) mutants were greater than for the corresponding WT, and differences were larger in NB than in T65. The photosynthetic rate was significantly higher in the \(d1\) mutants than in T65 and NB WT (Fig. 3a). The \(g_m\) values observed in \(d1\) under these conditions can be explained by its significantly higher \(g_{fw}\) (Fig. 3b) and, as we hypothesized, significantly higher \(g_{sw}\) (Fig. 3c). It is important to note here that the higher \(g_{sw}\) leads to slight but statistically significant lower intrinsic leaf-level water use efficiency in \(d1\) vs WT under well-watered conditions (Fig. 3d). The photosynthetic rates in \(d1\) plants decreased more gradually and were significantly higher than in WT under moderate stress (Fig. 3a). Under severe water stress, the photosynthetic rate was almost twice as high in \(d1\) than in WT. With regard to \(g_{sw}\), under severe drought conditions, there was no significant difference in \(g_{sw}\) between \(d1\) mutant and WT in either genetic background (Fig. 3b). A higher photosynthetic rate with the same \(g_{fw}\) led to 50% and 33% greater WUEi, for the \(d1\) plants compared with WT T65 and NB, respectively (Fig. 3d). The \(d1\) mutants in both genetic backgrounds have higher \(g_m\) relative to the corresponding WT under both moderate and severe water stress (Fig. 3c). In both genotypic backgrounds, the \(g_m\) of the \(d1\) was 30–45% higher than in WT, under well-watered conditions and throughout the drought time course.

Plotting \(g_m\) as a function of stomatal conductance to CO\(_2\) \(g_{sc}\) (\(g_{sw}/1.6\)) interestingly shows a steeper decrease in \(g_m\) for a given decrease in \(g_{sc}\) in the WT plants compared with the \(d1\) mutants in both T65 and NB backgrounds (Fig. 4).
Temperature dependence of photosynthesis and $g_m$

We evaluated the effect of leaf temperature on the CO$_2$ assimilation $A_e$, Rubisco capacity for carboxylation $V_{cmax}$, and mesophyll conductance $g_m$ (Fig. 5). The thermal optimum for CO$_2$ assimilation rate $V_{cmax}$ and mesophyll conductance was 30–35°C for both WT and $d1$ (Fig. 5a–c). The CO$_2$ assimilation was significantly higher in $d1$ than in WT in the temperature range 30–35°C and was not significantly different under lower (20–25°C) and higher (40°C) leaf temperatures (Fig. 5a). The mesophyll conductance was significantly higher in $d1$ than in WT in the temperature range 30–40°C and was not significantly different...
under lower (20–25°C) leaf temperatures (Fig. 5b). The $V_{\text{max}}$ increased with temperature and was higher in the $d1$ mutant than in WT in the temperature range 25–30°C (Fig. 5c).

**Relationship between mesophyll conductance and leaf anatomical traits**

The leaf thickness $T_{\text{leaf}}$, the mesophyll surface area exposed to intercellular air space per unit leaf area $S_{\text{m}}$, the mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per unit leaf area $S_{\text{c}}$, the mesophyll cell wall thickness $T_{\text{cw}}$, and the chloroplast thickness $T_{\text{chl}}$ were significantly different between WT and $d1$ (Fig. 6; Table 1). Modeling of the diffusion resistance along the CO$_2$ diffusion path from intercellular air spaces to the chloroplast stroma indicated significant differences between WT and the $d1$ mutant in $g_{\text{m}}$ (Fig. 7a). Accordingly, the higher $g_{\text{m}}$ in $d1$ mutants compared with WT can be explained at least in part by the anatomy of the mesophyll. The resistance of the liquid phase, requiring diffusion across the plasma membrane, cytosol, chloroplast membranes, and chloroplast stroma, was 53% lower in the $d1$ mutants than in WT (Fig. 7b). The cell wall resistance and stroma resistance were 18% and 44% lower, respectively, in $d1$ than in WT, whereas the cytosolic resistance was statistically similar (Fig. 7c).

**$A_{\text{n}}$–$C_{\text{i}}$ curve analysis**

We also aimed to determine in these genotypes the contributions to the photosynthetic limitation of CO$_2$ diffusion (stomatal and mesophyll conductance) vs the biochemical carboxylation capacity of the enzyme Rubisco ($V_{\text{max}}$). As observed from $A_{\text{n}}$–$C_{\text{i}}$ curves (Fig. 8a–c), $d1$ also exhibited greater photosynthetic capacity: at saturating CO$_2$, maximum CO$_2$ assimilation $A_{\text{max}}$ was greater in $d1$. Analysis of the $A$–$C_{\text{i}}$ curves revealed that

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**Fig. 3** Rice $d1$ mutants exhibit improved CO$_2$ assimilation rate and mesophyll conductance under well-watered and drought conditions compared with wild-type (WT). (a–f) Boxplots of physiological parameters of the different rice cultivars: WT ‘Taichung 65’ (T65) and ‘Nipponbare’ (NB), and $d1$ in the T65 background ‘$d1$ (T65)’ and $d1$ in the NB background ‘$d1$ (NB)’, under well-watered conditions (relative soil water content (RSWC) 100–50%), moderate drought stress (RSWC 50–35%) and severe drought stress (RSWC < 35%), for (a) CO$_2$ assimilation $A_{\text{n}}$, (b) stomatal conductance to water vapor $g_{\text{sw}}$, (c) mesophyll conductance to CO$_2$ $g_{\text{m}}$, (d) leaf intrinsic water use efficiency WUE$_i$, (e) the air to intercellular airspace CO$_2$ drawdown ($C_a - C_c$), and (f) the intercellular airspace to mesophyll CO$_2$ drawdown ($C_i - C_c$). The upper and lower parts of the boxes represent the 25th and 75th percentiles, respectively. The horizontal line within the boxes marks the median. The whiskers reach the largest and smallest values. Points are individual measurements. Connecting group lines indicate the P-value score of the means between WT and $d1$ according to Student’s t-test.
Rubisco capacity for carboxylation normalized to 25°C ($V_{cmax}$), and electron transport capacity normalized to 25°C ($J_{max}$) were significantly higher in $d1$ than in WT (Fig. 8d–f) under both well-watered and drought conditions for both T65 and NB backgrounds.

RNA-sequencing analysis

We conducted a reanalysis of RNA-seq data from T65 and $d1$ seedlings under well-watered conditions (Ferrero-Serrano et al., 2018) to explore genotypic differences in the expression of genes implicated in the regulation and modulation of $g_m$. Our analysis revealed differential expression of several plasma membrane intrinsic proteins (PIP) aquaporin genes (Table S1) and different alpha and beta CAs (Table S2). However, we did not find a consistent upregulation of these genes in one genotype vs the other that would infer at the transcriptional level a role of these gene families in the regulation of $g_m$. However, our reanalysis of this RNA-seq data set reveals a consistent upregulation in $d1$ of key regulatory enzymes of the Calvin–Benson cycle (Fig. 9; Table S3).

Discussion

Rice RGA1 mutant $d1$ exhibits increased mesophyll conductance to CO$_2$

Improving mesophyll conductance has the potential to boost photosynthesis and crop water use efficiency, especially under drought conditions. However, any goal to manipulate $g_m$ for crop improvement first requires an understanding of its genetic and physiological basis. We discovered that the canonical $\alpha$ subunit of the rice heterotrimeric G protein, RGA1, is a regulator of mesophyll conductance. Our results confirm previously reported enhanced drought tolerance of $d1$ plants in the T65 background (Ferrero-Serrano & Assmann, 2016; Cui et al., 2020), but additionally show cultivar independence, as $d1$ is also more drought tolerant in the NB background. Such cultivar independence is essential toward incorporating the dwarfing and drought tolerance phenotypes of $d1$ into elite cultivars. Mechanistically, our results reveal that this drought tolerance is related to higher $g_m$ in $d1$, which confers higher CO$_2$ assimilation rates over the drought time course. The greater $g_m$ in the $d1$ mutants for both genotypic backgrounds (T65 and NB) allows greater CO$_2$ concentration at the sites of carboxylation ($C_c$), leading to greater CO$_2$ assimilation rates without any increase in stomatal conductance and transpiration, thus resulting in an increase in WUEi under drought conditions (Figs 2, 3).
global warming. Indeed, we previously observed lower leaf temperatures in \( d1 \) than in WT, which may also be conferred by the more erect stature of \( d1 \) as opposed to WT (Ferrero-Serrano et al., 2018). However, despite its lower WUE\(_i\), under well-watered conditions, \( d1 \) mutants under drought increased WUE\(_i\) via stomatal closure while maintaining high \( g_m \) (Figs 2, 3). In other words, the dynamic response of \( g_s \) changes the relative importance of \( g_s \) and \( g_m \) during drought: \( d1 \) exhibited a delayed decline in \( g_m \) relative to that of \( g_s \) (Fig. 4), which led to higher \( A_n \) under drought. A higher \( g_m : g_s \) ratio under limited water conditions has been previously documented for other highly drought-tolerant species, such as eucalyptus, poplar, and Ziziphus spinachristi trees (Cano et al., 2014; Théroux-Rancourt et al., 2015; Zait et al., 2019) and could be a key trait for selection and breeding for WUE\(_i\) improvement in crops (Barbour et al., 2010; Barbour & Kaiser, 2016; Flexas et al., 2016; Tomeo & Rosenthal, 2017).

Role of \( RGA1 \) in mesophyll anatomy and diffusive conductance to CO\(_2\)

Leaf anatomy is an important determinant of \( g_m \) from the substomatal cavities through the intercellular air space to the carbon fixation sites in the chloroplast (Nobel, 1991; Parkhurst, 1994). G protein signaling has been shown to play a crucial role in developmental processes in a number of plant species, including rice (Perfus-Barbeoch et al., 2004; Urano et al., 2020). For instance, loss-of-function mutations in the \( \alpha \) subunit of the heterotrimeric G protein mediate morphological changes in cell proliferation in Arabidopsis and maize (Wang et al., 2001; Urano et al., 2016), shoot meristem size in maize (Bommert et al., 2013), Arabidopsis internode length in Arabidopsis (Ueguchi-Tanaka et al., 2000), and root morphology (Chen et al., 2006; Ding et al., 2008) in Arabidopsis.

Our results showed that \( d1 \) mutants display altered mesophyll \( (S_m) \) and chloroplast \( (S_c) \) area exposed to intercellular airspaces, parameters that have previously been shown to positively correlate with the increase of \( g_m \), WUE\(_i\), and \( A_{\text{max}} \) in rice (Giuliani et al., 2013; Xiong et al., 2016; Ouyang et al., 2017). The WT values of the anatomical parameters obtained in this study are within the ranges described in the literature for rice (Giuliani et al., 2013; Xiong et al., 2015b, 2017; Ouyang et al., 2017; Ellsworth et al., 2018). The \( d1 \) mutants were found to display reductions in cell

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**Table 1** Structural and anatomical traits of well-watered rice ‘Taichung 65’ (T65) and the \( d1 \) mutants.

|          | \( T_{\text{leaf}} \)(μm) | LMA (g m\(^{-2}\)) | \( S_m \)(m\(^2\) m\(^{-2}\)) | \( S_c \)(m\(^2\) m\(^{-2}\)) | \( T_{\text{cw}} \)(μm) | \( T_{\text{cyt}} \)(μm) | \( L_{\text{cxt}} \)(μm) | \( T_{\text{ch}} \)(μm) |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| T65      | 68.5 ± 1.7 a    | 62.9 ± 3.9 a    | 17.5 ± 0.8 a    | 13.9 ± 0.7 a    | 0.128 ± 0.004 a | 0.05 ± 0.0013 a | 4.3 ± 0.4 a     | 2.6 ± 0.25 a    |
| \( d1 \) | 74.9 ± 2.8 b    | 66.5 ± 2.7 a    | 22.5 ± 1.3 b    | 19.5 ± 1.6 b    | 0.106 ± 0.004 b | 0.03 ± 0.007 a  | 4.6 ± 0.4 a     | 1.48 ± 0.26 b   |
| \( P \) value | 0.016         | 0.4             | 0.004           | 0.004           | 0.00433         | 0.216           | 0.55            | 0.0084         |

\( T_{\text{leaf}} \), leaf thickness; LMA, leaf mass per area; \( S_m \), mesophyll surface area exposed to intercellular air space per unit leaf area; \( S_c \), mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per unit leaf area; \( T_{\text{cw}} \), mesophyll cell wall thickness; \( T_{\text{cyt}} \), distance between cell wall surface and chloroplasts; \( L_{\text{cxt}} \), chloroplast length; \( T_{\text{ch}} \), chloroplast thickness.

Values are mean ± SE, \( n = 4 \) plants per genotype; four to seven images analyzed per plant. Letters indicate statistical difference according to Student’s \( t \)-test.
Fig. 7 Rice d1 mutants’ anatomical properties are associated with a reduction in CO₂ liquid-phase resistance compared with wild-type (WT). (a) Mesophyll conductance $g_m$ modeled with anatomical parameters of the ‘Taichung 65’ (T65) and d1 mutant. (b) The overall resistance of CO₂ diffusion imposed by the intercellular air spaces in the gas phase and by the CO₂ diffusion in the liquid phase in the T65 cultivar and corresponding d1 mutant. (c) The CO₂ transfer resistance of the cell wall ($r_{cw}$), cytosol ($r_{cyt}$) and the chloroplast stoma ($r_{st}$) of the T65 cultivar and the corresponding d1 mutant (c). The upper and lower parts of the boxes represent the 25th and 75th percentiles, respectively. The horizontal line within the boxes marks the median. The whiskers reach the largest and smallest values. Points are individual measurements. Connecting group lines indicate the P-value score of the means between WT and d1 according to Student’s t-test.

Fig. 8 Rice d1 mutants exhibit greater photosynthetic capacity relative to wild-type (WT). (a–c) CO₂ assimilation in response to changes in CO₂ concentration in the leaf intercellular air spaces $C_i$ in the different rice genotypes: WT ‘Taichung 65’ (T65) and ‘Nipponbare’ (NB), and d1 in T65 background ‘d1(T65)’ and d1 in NB background ‘d1(NB)’ under (a) well-watered (WW) conditions (relative soil water content (RSWC) 100–50%), (b) moderate drought (MD) stress (RSWC 50–35%), and (c) severe drought (SD) stress (RSWC < 35%). Data are means ± SEs of WT (triangles) and d1 (circles). Black lines represent the cubic curve fit for the data of the WT rice genotypes, and grey lines represent the cubic curve fit for the data of the d1 mutant. The shaded areas show the 95% confidence interval of the regression lines. (d–f) Box plots of the physiological parameters calculated from the CO₂ response curves of the different rice cultivars: WT ‘Taichung 65’ (T65) and ‘Nipponbare’ (NB), and d1 in T65 background ‘d1(T65)’ and d1 in NB background ‘d1(NB)’, under WW conditions (RSWC 100–50%), MD stress (RSWC 50–35%) and SD stress (RSWC < 35%), for (d) maximum photosynthetic rate $A_{max}$, (e) maximum capacity for Rubisco carboxylation normalized to 25°C $V_{cmax}$, and (f) maximum electron transport rate normalized to 25°C $J_{max}$. The upper and lower parts of the boxes represent the 25th and 75th percentiles, respectively. The horizontal line within the boxes marks the median. The whiskers reach the largest and smallest values. Points are individual measurements. Connecting group lines indicate the P-value score of the means between WT and d1 according to Student’s t-test.
wall thickness and chloroplast thickness (Fig. 6; Table 1), which together accounted for a reduction of 53% in the CO₂ liquid-phase resistance compared with WT (Fig. 7). Reduced cell wall thickness in the \textit{d1} mutant is supported by the latest finding that \textit{RGA1} modulates genes involved in cell wall composition in rice (Pathak \textit{et al.}, 2021). Moreover, in Arabidopsis, G proteins have been shown to interact with proteins involved in cell wall modification (Klopfleisch \textit{et al.}, 2011) and to regulate trafficking of

\textbf{Fig. 9} Rice \textit{d1} mutant exhibits an upregulation in messenger RNAs (mRNAs) encoding key Calvin cycle enzymes. The Calvin cycle illustrating genotypic differences in the expression of photosynthesis-related genes (Supporting Information Table S3). Statistically different expression levels between ‘Taichung 65’ and \textit{d1} were designated as those where \textit{q}-values were less than 0.05 and fragments per kilobase of exon per million mapped fragments was greater than one in at least one genotype. Orange boxes represent genes exhibiting statistically significant up-regulation of mRNA in \textit{d1}. White boxes represent nonstatistically significant different regulation of RNA between \textit{d1} and wild-type (WT). Values inside orange and white boxes indicate the differential regulation (Log2 fold-change) of mRNA in \textit{d1} relative to WT. 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; FBA, fructose-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; R5P, ribose 5-phosphate; RBCS, Rubisco small subunit; RPE, ribulose-phosphate 3-epimerase; RPI, ribose-5-phosphate isomerase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; SBPase, sedoheptulose 1,7-bisphosphatase; SBPase, sedoheptulose 1,7-bisphosphatase; TPI, transketolase; TKL, transketolase; TPK, triosephosphate isomerase; X5P, xylulose-5-phosphate.
cellulose synthase to the plasma membrane (McFarlane et al., 2021). Indeed, differences in cell wall composition of G protein mutants have been identified by Fourier transform infrared spectroscopy (Delgado-Cerezo et al., 2012).

The anatomical characteristics of the d1 mutant leaf can explain the higher $g_{\text{in}}$ in d1 over the WT under well-watered conditions. However, an interesting question is how leaf anatomy contributes to any changes in $g_{\text{in}}$ and photosynthesis under drought. $g_{\text{in}}$ is known to be affected by light transmittance through mesophyll layers (Théroux-Rancourt & Gilbert, 2017) (Fig. S4). The erect architecture of the d1 leaves reduces the amount of incident light intercepted by the leaves (Setter et al., 1996), which enables more efficient light capture and protects the photosynthesis machinery from photoinhibition (Ferrero-Serrano et al., 2018). Moreover, drought stress exacerbates photoinhibition and increases the need for effective photoprotection (Cornic & Fresneau, 2002; Takahashi & Badger, 2011). We therefore suggest that d1 mutants increase light use efficiency and photosynthesis by architectural avoidance of high light intensities, which redistributes chloroplasts for maximal light interception and, thus, reduces the diffusional pathlength from the cell wall to the chloroplast envelope, and by increased chloroplast surface area, both of which consequently increase $g_{\text{in}}$.

Role of metabolic factors in the regulation of mesophyll conductance in the d1 mutant

Increases in leaf temperature occur alongside drought due to stomatal closure. We showed that $g_{\text{in}}$ dynamically changes in response to leaf temperature (Fig. 5) and CO$_2$ concentration (Fig. S5). Because these changes occur on a rapid timescale, they are unlikely to involve anatomical changes (Bernacchi et al., 2002; Carriquí et al., 2019). We accordingly hypothesize that metabolic processes could contribute to the differential reduction in $g_{\text{in}}$ we observe between WT and d1 mutants over the drought time course. The erect architecture of d1, together with its higher values of $g_{\text{in}}$ under well-watered conditions (Fig. 2), reduces leaf temperature compared with WT (Ferrero-Serrano & Assmann, 2016; Ferrero-Serrano et al., 2018). Our results suggest that, at extreme ambient temperatures, d1 architecture could help maintain the leaves at the optimal temperature for maximum $g_{\text{in}}$ (Fig. 5c). Changes in leaf temperature can rapidly affect $g_{\text{in}}$, especially in crops typical of warm and tropical habitats, such as rice (von Caemmerer & Evans, 2015). Though the mechanism is yet to be determined, previous studies have suggested that temperature exerts a direct effect on membrane permeability to CO$_2$ by activating and increasing expression of aquaporins (Bernacchi et al., 2002; Groszmann et al., 2017; Qiu et al., 2017). It has been previously shown that overexpression of the aquaporin PIP2 increased $g_{\text{in}}$ by 40% in transgenic rice plants, highlighting the role of aquaporins in the diffusion of CO$_2$ through the mesophyll cell membrane (Hanba et al., 2004). This contrasts with the lack of changes in $g_{\text{in}}$ observed in Arabidopsis aquaporin PIP1 and PIP2 knockout lines relative to Col-0 (Kromdijk et al., 2020). In cucumber, suppression of the Go subunit results in the overexpression of several aquaporins under well-watered conditions (Yan et al., 2020). Our RNA-seq analysis shows that some PIP aquaporin genes are differentially expressed in d1 mutants (Table S1), but these do not exhibit a consistent response: PIP1.3, PIP2.4, and PIP2.1 are downregulated and PIP2-7 is upregulated in d1. It is important to note that leaves sampled for the RNA-seq reanalysis that we report here were harvested under well-watered conditions. Thus, it remains possible that aquaporins actively change expression or activity during drought (Huang et al., 2021), with consequent impacts on $g_{\text{in}}$. In rice, OsPIP1.1 and OsPIP2.1 were found to be upregulated in both roots and leaves in response to water depletion (Guo et al., 2006). Further studies on rice aquaporins, including their CO$_2$ permeability and whether their expression and activity are drought regulated, are needed to assess their relative importance to the metabolic component of $g_{\text{in}}$ in WT vs d1.

Another factor affecting resistance along the CO$_2$ diffusion pathways is CA (Flexas et al., 2012). CA catalyzes the interconversion of CO$_2$ and HCO$_3^−$ (Momayyезi et al., 2020). In rice, the downregulation of CA activity by a deficiency in zinc (a cofactor of CA) results in a 2.3-fold reduction in $g_{\text{in}}$, emphasizing the importance of CA (Sasaki et al., 1998). Our RNA-seq analysis under well-watered conditions shows upregulation in d1 relative to WT of the genes encoding the chloroplast-abundant αCA1 (Table S2) and the βCA1 (Table S2). However, CA enzyme activity assays show no statistically significant differences between the d1 mutant and WT (Fig. S6).

It is important to point out that, in Arabidopsis, the G protein α subunit knockout mutant, gpa1-3, can accumulate more biomass, with less water loss (Nilson & Assmann, 2010b). The gpa1-3 mutant exhibits reduced discrimination to $^{13}$C in the absence and presence of ABA, which strongly correlates with high water use efficiency (Nilson & Assmann, 2010b). The gpa1-3 mutant is hypersensitive to ABA inhibition of stomatal opening (Wang et al., 2001), which could negatively affect WUE under drought. However, recent evidence shows that ABA has an essential role in the regulation of $g_{\text{in}}$. Higher exogenous ABA in the leaves decreases $g_{\text{in}}$ (Mizokami et al., 2015), presumably by reducing the aquaporins’ plasma membrane CO$_2$ conductivity (Shatil-Cohen et al., 2011; Sorrentino et al., 2016). Thus, a higher $g_{\text{in}}$ in d1 in the presence of drought may arise from lower sensitivity to ABA in the mesophyll cells. Accordingly, the role of RGA1 in specific tissues requires further investigation.

Improved photosynthetic capacity in the d1 mutant

Our results show that d1 exhibits a higher maximum capacity for Rubisco carboxylation $V_{\text{cmax}}$, maximum electron transport rate $J_{\text{max}}$, and maximum CO$_2$ assimilation $A_{\text{max}}$ relative to WT (Fig. 8). Photosynthetic capacity is related to the nitrogen (N) content because the proteins of the Calvin cycle and thylakoids contain the majority of leaf N (Evans, 1989). The majority of N in the thylakoids is found in the photosystem reaction centers (Evans, 1989). As shown in a previous study, photosynthetic reaction center genes are upregulated in d1 plants (Ferrero-Serrano et al., 2018). Our reanalysis of the RNA-seq data from that study also reveals the upregulation in d1 of key enzymes of the
Calvin–Benson cycle (Fig. 9; Table S3). We hypothesize that the upregulation of the biochemical factors of photosynthesis in the d1 mutant may arise in part from allocation of a larger fraction of leaf N to photosynthesis processes, resulting in an increase of photosynthetic N use efficiency (PNUE) (Gao et al., 2020). This hypothesis is also supported by the typical dark-green leaf phenotype (Fujisawa et al., 1999), higher Chl content, and upregulation of key enzymes in Chl biosynthesis observed in the d1 mutant (Ferrero-Serrano et al., 2018). A larger PNUE in rice is associated with a higher $S$ and thinner cell walls, leading to a higher $g_{mn}$ (Li et al., 2009; Gao et al., 2020; Ye et al., 2020). Additionally, we observed that $g_{mn}$ was more sensitive to elevated CO$_2$ in d1 relative to WT, under both well-watered and drought conditions (Fig. S5). This is consistent with the finding that N deficiency in rice leads to hyposensitivity of $g_{mn}$ to changes in light and CO$_2$ (Xiong et al., 2015).

Conclusion

The rice G protein $G\alpha$ subunit, RGA1, regulates mesophyll conductance in large part by altering mesophyll anatomy. This is the first identification of a gene that pleiotropically affects multiple aspects of mesophyll anatomy related to $g_{mn}$. In future research, it will be important to determine the cell signaling mechanisms whereby RGA1 controls such disparate anatomical properties as cell wall thickness, mesophyll cell size, and chloroplast surface area. Moreover, the d1 mutants also have elevated photosynthetic capacity compared with the WT congeners. Accordingly, the results of this study demonstrate that genetic modulation of heterotrimeric G protein activity in rice provides a novel strategy to increase $g_{mn}$ and WUE$_i$ under drought. In rice, the introduction of dwarfism was a crucial agronomic advance (Hargrove & Cabanilla, 1979; Hedden, 2003; Ferrero-Serrano et al., 2019), but it currently has a very narrow genetic basis (Chang & Vergara, 1972; Hargrove et al., 1980). Current elite varieties display drought sensitivity, which is associated with introgression of the chromosomal region containing the Green Revolution dwarfing gene $sd1$ (Vikram et al., 2015). We propose that the manipulation of G protein signaling in rice offers a novel strategy not only to confer the desired semi-dwarf phenotype but also to improve photosynthesis and water use efficiency and increase drought tolerance.

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Author contributions

YZ planned and performed the experiments, analyzed the data, and wrote the text; AF-S performed preliminary experiments, analyzed the RNA-seq data (Fig. 9; supporting information tables) and wrote the text. SMA planned and designed the research and wrote the text.

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Data availability

All TEM images have been uploaded to GitHub and are publicly accessible at https://github.com/AssmannLab/rice_mesophyll_conductance. All other data are available upon request from the authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Estimation of mitochondrial respiration in the light (R_l) and the intercellular CO₂ compensation point (C_C) in WT rice and the ΔI mutant.
Fig. S2 Different estimation methods for $g_m$ all maintain the difference in $g_m$ observed between WT rice and $d1$ mutants.

Fig. S3 Relationship between photochemical efficiency of PSII ($\Phi$PSII) and apparent quantum yield of CO$_2$ assimilation ($\Phi$CO$_2$) under non-photorespiratory conditions (1% O$_2$).

Fig. S4 Light responses of CO$_2$ assimilation rate ($A_n$) (a), mesophyll conductance ($g_m$) (b), and stomatal conductance to water vapor ($g_{sw}$) (c), in wild-type rice Taichung 65 and the $d1$ mutant.

Fig. S5 The rapid response of mesophyll conductance ($g_m$) to changes in CO$_2$ concentration in the leaf intercellular air spaces ($C_i$) in different rice genotypes.

Fig. S6 WT rice and the $d1$ mutant do not differ in carbonic anhydrase activity.

Table S1 Expression levels of plasma membrane intrinsic proteins (PIPs) in Taichung 65 wild-type rice (WT) vs. the Taichung 65 $d1$ mutant.

Table S2 Expression levels of carbonic anhydrases in Taichung 65 wild-type rice (WT) vs the Taichung 65 $d1$ mutant.

Table S3 Expression levels of Calvin cycle enzymes in Taichung 65 wild-type rice (WT) vs the Taichung 65 $d1$ mutant.

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