RUNNING TITLE: Dissecting G-protein signaling

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Title: Dissecting *Arabidopsis* Gβ Signal Transduction on the Protein Surface

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Kun Jiang- wrote the manuscript, characterized the transgenic lines, provided RT-PCR and subcellular localization images, collected data for developmental phenotypes and ABA response  
Arwen Frick-Cheng- made transgenic lines, collected the 2-d old etiolated, glucose data, optimized assays, did many assays for this project but some of that data is not included here (methionine resistance)  
Yuri Trusov- collected the data on *F. oxysporum* resistance  
Magdalena Delgado-Cerezo- collected the data on *P.cucumerina* resistance  
Justine Lorek- Collected the data on fig22-induced ROS production  
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ABSTRACT

The heterotrimeric G protein complex provides signal amplification and target specificity. The Arabidopsis Gβ subunit of this complex (AGB1) interacts with and modulates the activity of target cytoplasmic proteins. This specificity resides in the structure of the interface between AGB1 and its targets. Important surface residues of AGB1, which were deduced from a comparative evolutionary approach, were mutated to dissect AGB1-dependent physiological functions. Analysis of the capacity of these mutants to complement well-established phenotypes of Gβ-null mutants revealed AGB1 residues critical for specific AGB1-mediated biological processes, including growth architecture, pathogen resistance, stomatal mediated leaf-air gas exchange, and possibly photosynthesis. These findings provide promising new avenues to direct finely-tuned engineering of crop yield and traits.

Heterotrimeric G proteins are conserved signaling components across diverse eukaryote species. Upon perception of a cognate ligand by its 7-transmembrane receptor (7TMR) on the cell surface, the 7TMR-associated heterotrimeric G protein complex dissociates to form an activated Gα subunit and an obligate Gβγ dimer, which in turn trigger transient cellular changes by interacting with downstream proteins, typically enzymes, called effectors. In mammals, several Gα and Gβγ interacting effectors were identified, including well-studied adenylyl cyclase 2, phospholipase C β2, and cation channels (Sunahara et al., 1996; Rhee and Bae, 1997; Schneider et al., 1997). None of these mammalian effectors are found in Arabidopsis (Jones and Assmann, 2004), although other potential effectors were proposed or shown (Klopfleisch et al., 2011). For example, physical interactions of the Arabidopsis Gα subunit (AtGPA1) were shown for THF1, an outer membrane protein on the plastid, which works with G proteins in sugar sensing and chloroplast development (Huang et al., 2006). Similarly PLDα1 and the cupin-domain protein Atpirin1 may be important in AtGPA1 modulation of abscisic
acid (ABA) signaling, an important component of plant morphogenesis, and water use efficiency (Lapik and Kaufman, 2003; Mishra et al., 2006). A combination of approaches were exploited to identify physical (Klopffleisch et al., 2011) and genetic (Wang et al., 2006) interactors of Arabidopsis Gβ subunit (AGB1). AGB1 interactors include NDL1 (Mudgil et al., 2009) and ARD1 (Friedman et al., 2011). ARD1 is the only confirmed plant Gβ effector to date; i.e. the only plant protein found so far known whose enzymatic activity is directly modulated by a G protein (Friedman et al., 2011). The NDL1 protein has no known enzymatic function although it resembles a lipase/esterase but lacking the critical catalytic triad. NDL1 and G proteins work concertedly to control polar auxin transport streams in the root (Mudgil et al., 2009) and the inflorescence stem (Mudgil et al., under revision). Mutants lacking NDL1 have reduced basipetal and increased acropetal transport of auxin and consequently lateral root development is altered. Consistent with NDL1 operating in the G protein pathway, seedlings lacking GPA1 have less while those lacking AGB1 have greater root mass than wild-type seedlings, pointing to a role in regulation of cell proliferation (Ullah et al., 2003; Chen et al., 2006). Other cell proliferation defects were characterized in G protein mutants. For example, in the presence of 1% sucrose or glucose, 50-h-old etiolated agb1 seedlings display shorter hypocotyls and more open apical hooks, which are the consequence of reduced epidermal cell numbers in the hypocotyl (Ullah et al., 2003; Wang et al., 2006). agb1 mutants also display an increased stomatal index and altered leaf, silique, and rosette morphologies (Ullah et al., 2003; Zhang et al., 2008; Booker et al., 2011).

From an agricultural perspective, the most significant phenotype of Arabidopsis and rice G protein mutants is the profound difference in innate immune responses. The Arabidopsis agb1 null mutant shows reduced accumulation of reactive oxygen species (ROS) in response to microbial inducers such as bacterial flagellin and EF-Tu (Ishikawa et al., 2009). Moreover, agb1 null mutants are hypersensitive to the necrotrophic fungi, Plectosphaerella cucumerina, Alternaria brassicicola and the hemibiotrophic fungus Fusarium oxysporum (Llorente et al., 2005; Trusov et al., 2006; Trusov et al., 2008; Delgado-Cerezo et al., 2011). Moreover, rice Gα mutants (rgα1)
are more susceptible to the rice blast fungus, *Magnaporthe grisea* (Suharsono et al., 2002). The enhanced susceptibility of *agb1* mutants to *F. oxysporum* may result from altered function of MYC2, a bHLH transcription factor regulating diverse jasmonate-dependent biological processes (Trusov et al., 2008). More recent data also suggest a link between AGB1-mediated *P. cucumerina* resistance and modification of cell wall architecture (Delgado-Cerezo et al., 2011). Another important agricultural trait regulated by the heterotrimeric G protein is transpiration efficiency (TE), which is increased in *gpa1* mutants (Nilson and Assmann, 2010). However, increased TE at high light levels in *gpa1* mutants is simply due to reduced stomatal density (Nilson and Assmann, 2010). GPA1 and AGB1 subunits modulate stomatal density antagonistically (Zhang et al., 2008; Booker et al., 2011).

Finally, and not surprisingly, signal transduction pathways of several phytohormones and environmental stimuli are altered in G protein mutants, including ABA (Wang et al., 2001; Pandey and Assmann, 2004; Pandey et al., 2006), brassinosteroid (Ullah et al., 2002; Gao et al., 2008), auxin (Ullah et al., 2003), and red/far-red light (Wei et al., 2008; Botto et al., 2009), although not all phytochrome responses involve G proteins (Jones et al., 2003).

This myriad of phenotypes may be a manifestation of the complexity of altered activity of targets downstream of G protein activation. AtGPA1 activation occurs when GTP replaces GDP, resulting in a new protein conformation and an exposed surface on the Gβγ subunit. Gβγ dimer activation occurs when it is released from the complex to expose new protein interfaces that were sequestered by Gα and other elements of the G protein complex. We selected a limited but informative set of phenotypes as readouts for AGB1 signaling in a broad range of plant biology including: development, hormone physiology, plant immune responses and disease resistance, as well as CO2 assimilation and transpiration. To identify the active-state protein interfaces for each of these processes, we mutated a cluster of surface-exposed residues to determine which mutations disrupted signaling as interpreted through the inability to complement *agb1* mutant phenotypes upon transgenic expression of the mutant variants. The selection of mutations was
based on phylogenetic and structural analyses for plant-specific surfaces that we originally tested to map the potential interface between an animal Gβ subunit and its cognate target phospholipase β2 (Friedman et al., 2009). In essence, these are residues while conserved in plants have evolved to become uniquely functional in mammals. Our purpose here is to determine what function these residues serve in plants. Specifically, we sought to distinguish different functions of AGB1 among its multiple signaling functions by observing which AGB1-dependent pathways are disrupted when these potential effector interfaces are mutated.

RESULTS

Mutant AGB1 Proteins are Expressed and Properly Folded in the agb1-2 Background

Residues on the AGB1 binding surface for mutagenesis analysis were selected and mutated as described before (Friedman et al., 2009). The residues selected were 1) located on the solvent-exposed surface area of the protein 2) invariant between plants and mammals and 3) not required for structural maintenance of the Gβγ dimer. W109 and S129 are both located in the Gα binding domain, while function of the interfaces containing either residues E248 and R25 or Q120, T188, and R235 is unknown. The conservative residues on the AGB1 protein-binding interface suggest their functional importance in association with downstream effectors. Realizing that one Gβγ effector may share the same Gβ-interacting region with another, we hypothesized that these four mutations may affect the binding of AGB1 to diverse effectors and therefore the corresponding AGB1 mutants will only have partial function as compared to wild type. Each mutant AGB1 variant, either with or without a 10×Myc tag, was expressed in the agb1-2 background and 2-4 independent non-tagged and tagged transgenic lines per construct were selected for characterization (Figure 1) and phenotyping (Figure 2-5). We found that the phenotypes of both the 10×Myc-tagged or non-tagged lines led to the same conclusions. For simplicity and clarity, results of the same set of tagged lines
for each mutation are shown for the subsequent experiments. We found that all four AGB1 mutants code for protein variants were stably expressed (Figure 1, middle panel). In addition, each green fluorescent protein (GFP)-tagged mutant was targeted to the plasma membrane (Figure 1, bottom panel), thus revealing the expected AGB1 subcellular localization. Plasma membrane tethering of AGB1 depends on the formation of a heterodimer with the AGG1 subunit via its N-terminal coiled-coil motif (Obrdlik et al., 2000; Adjobo-Hermans et al., 2006). Therefore, proper plasma membrane localization of mutated AGB1 suggests a functional conformation. Further evidence of authentic protein conformation is provided by the fact that each mutant variant is capable of tightly interacting with its obligate AGG partner in vitro (Friedman et al., 2011). Finally, as described below, each mutant AGB1 was able to genetically complement at least one agb1-2 loss-of-function phenotype. Taken together, these results require that each AGB1 mutant was properly folded and localized in the plant cell.

Dissection of agb1-2 Signaling in Development

Loss-of-function alleles of AGB1 confer morphological alterations in vegetative and reproductive organs of Arabidopsis (Lease et al., 2001; Ullah et al., 2003), some of which are due to aberrant auxin transport (Mudgil et al., 2009). As a validation of our experimental approach, we first tested phenotypic complementation of agb1-2 by the YFP/HA-tagged wild-type AGB1. Our data (Figure S1), together with previous reports (Chen et al., 2006; Chakravorty et al., 2011a), indicate that ectopic expression of AGB1 rescues developmental lesions of the agb1 mutant without causing discernible side effects. We tested functional rescue of the agb1-2 mutant by variant AGB1 proteins of selected developmental phenotypes that represent most stages in the life cycle of Arabidopsis. At 50 h, the etiolated agb1-2 hypocotyls are shorter than wild type (Wang, et al., 2006). None of the mutated AGB1 proteins were able to fully rescue the hypocotyl to the wild-type length, however W109A and S129R mutants partially rescued the hypocotyl phenotype (Figure 2A). At 10-d, lateral-root density of, agb1-2 seedlings is
higher than that of wild type due to increased basipetal auxin transport (Mudgil et al., 2009). S129R AGB1 fully restored the lateral root density to the wild-type level indicating that S129 is not critical for the lateral-root development. The other mutations conferred partial complementation of the lateral root phenotype (Figure 2B). At 3 weeks, agb1-2 leaves have a scorable leaf phenotype; the lamina is compact and rounded and the petiole is shorter (Ullah et al., 2003). Figure 2C shows that among the mutated AGB1 proteins, only plants expressing W109A and S129R had the wild-type leaf morphology. G protein mutants have altered stomatal indices (Zhang et al., 2008; Nilson and Assmann, 2010; Booker et al., 2011). Just as for the lateral root phenotype, only S129R fully rescued the stomatal phenotype while the other transgenic lines either had the agb1-2 phenotype or were partially rescued (Figure 2D). Finally, we scored a late development phenotype. agb1-2 siliques are shorter and wider than wild type siliques and have a blunt tip (Ullah, et al., 2003). The molecular mechanism underpinning this morphological change is unclear, although it is known that the AGB1/AGG3 dimer is an important modulator of reproductive organ shapes (Chakravorty et al., 2011b; Li et al., 2012). We found that W109A and S129R fully rescued this reproductive phenotype while the other two tested AGB1 mutants failed to restore the wild-type morphology (Figure 2E). This indicates that residues R25, Q120, T188, R235, and E248 are critical for signaling underlying several morphologies.

**Dissection of agb1-2 Signaling in Hormone Physiologies**

The agb1-2 mutant is hypersensitive to 6% glucose (Wang et al., 2006), implying a role of the heterotrimeric G protein in mediating sugar signal transduction. We examined functional rescue of the glucose-hypersensitive phenotype in the variant AGB1 transgenic lines. Just as for the hypocotyl length phenotype (Figure 2A), no AGB1 mutant was able fully to restore glucose sensitivity to the wild-type level, however the S129R variant showed a partial rescue (Figure 3A). There was no discernible difference between wild type and the agb1 mutants for the osmotic control treatment (Figure S2).
Abscisic acid, a key water deficiency-related phytohormone, inhibits lateral root development and drastically modulates root morphology under osmotic stress (De Smet et al., 2003; De Smet et al., 2006). The agb1-2 mutant is hypersensitive to ABA-inhibited lateral root development (Pandey et al., 2006). Seedlings were grown vertically on plates with or without ABA (Figure 3B) and ABA-inhibited lateral root development in each transgenic line was compared to that of wild-type and agb1-2 seedlings using two-way ANOVA (Figure 3C). The ABA-hypersensitive phenotype was rescued fully by the S129R and partially by the W109A mutation (Figure 3C). In contrast, neither the double nor the triple mutants complemented this phenotype (Figure 3C).

**Dissection of agb1-2 Signaling in flg22-triggered ROS Production**

The sensing of conserved microbe-associated molecular patterns (MAMPs) such as bacterial flagellin or its elicitor-active peptide component, flg22, results in a series of stereotypic cellular responses that are thought to contribute to the activation of plant innate immune responses (Boller and Felix, 2009). These reactions comprise, amongst others, the influx of calcium ($\text{Ca}^{2+}$) ions from the apoplastic space, the extracellular accumulation of ROS and the intracellular activation of mitogen-associated protein kinase (MAPK) cascades (Boller and Felix, 2009). MAMP-induced formation of hydrogen peroxide ($\text{H}_2\text{O}_2$) is severely compromised in agb1-2 mutant plants (Ishikawa et al., 2009), suggesting a link between AGB1 function and ROS accumulation. We exploited this phenotype to assess the capability of AGB1 mutant variants to complement the agb1 deficiency in ROS production. The W109A mutant completely restored, while the S129R single, the R25D E248K double, and the Q120R T188K R235E triple mutants partially restored flg22-triggered ROS formation in the agb1-2 background (Figure 4). In the case of the W109A, S129R and Q120R T188K R235E variants, these results were corroborated by measurements with additional independent transgenic lines (Figure S3).
Compared to Col-0 plants, \textit{agb1} null mutants are substantially more susceptible to pathogenic fungi \textit{Fusarium oxysporum} (f. sp. \textit{conglutinans}) and \textit{Plectosphaerella cucumerina} (Llorente et al., 2005; Trusov et al., 2006; Trusov et al., 2007). Soil-borne \textit{F. oxysporum} hyphae enter plant roots and then spread through the vascular system. The infection results in root and leaf growth inhibition, anthocyanin accumulation, chlorosis, and finally plant decay (Agrios, 2005). We scored the leaf chlorosis phenotype 8-9 d after inoculation. The lines expressing the variant Q120R T188K R235E were as susceptible to \textit{F. oxysporum} as \textit{agb1-2}, whereas the lines expressing W109A or S129R were similar to wild type (Figure 5A). The lines expressing R25D E248K showed a partial complementation of \textit{F. oxysporum} hyper-susceptibility displayed in \textit{agb1-2} (Figure 5A). These data indicate that W109 and S129 are not critical for AGB1-mediated fungal resistance whereas residues R25, Q120, T188, R235, and E248 are required for defense response to \textit{F. oxysporum}. The same results were obtained for \textit{P. cucumerina} susceptibility (Figure 5B) suggesting that AGB1 may utilize at least overlapping sets of effectors to elicit Arabidopsis defense response against all necrotic fungi.

The heterotrimeric G protein mediates epidermal cell patterning (Zhang et al., 2008) and therefore genetically controls transpiration efficiency at the level of stomatal density (Nilson and Assmann, 2010). We performed gas exchange analysis to determine whether steady-state net photosynthesis and stomatal conductance along a gradient of light intensities are affected by the \textit{agb1} null mutation at ambient CO2 level (~400 \(\mu\text{mol mol}^{-1}\)). We did not detect a significant change in photosynthetic rate or stomatal conductance for the full set of \textit{agb1-2} mutants or wild type leaves under our experimental conditions (Figure S4 and data not shown). Photosynthesis is influenced by multiple environmental factors such as light intensity, CO2 levels, and vapour pressure deficit. As shown in Figure S4 increasing photosynthesis rate and conductance increase with light intensity. Stomatal aperture is affected, in part, by signalling through G proteins. Stomatal aperture is greater in \textit{gpa1} mutants than WT at high light. We speculate that under certain conditions of
[CO$_2$] and light, stomatal aperture is compensating for differences in stomatal index.

**DISCUSSION**

Upon activation, both G$\alpha$ and G$\beta\gamma$ subunits of heterotrimeric G proteins trigger intracellular signal transduction and amplification via physical association with specific effectors. The functional importance of G$\beta\gamma$-effector interaction constrains the evolution of critical residues on the interface of G$\beta$ proteins (Friedman et al., 2009). Through bioinformatic and phylogenetic analysis, we predicted several surface residues are requisite for specific binding of AGB1 to one of its effectors and we validated this prediction using an *in vitro* enzymatic assay (Friedman et al., 2011). Here, we examined functional complementation of G$\beta$-null mutant phenotypes by transforming *agb1-2* with mutated *AGB1* variants with the expectation of mapping candidate surface residues within interfaces between AGB1 and its unknown effectors involved in specific physiological processes. We found that the double and the triple mutants were functionally impaired in rescuing most of the *agb1* phenotypes, indicating residues R$_{25}$, Q$_{120}$, T$_{188}$, R$_{235}$, and E$_{248}$ of AGB1 are critical for overlapping functions. Also, the data suggest that some residues may form common interfaces for the binding of diverse effectors. The W109A mutant rescued some of the loss-of-function phenotypes but failed to complement others (Fig 2,3,4,5), suggesting that a mutation can alter the regulation of some specific effectors without affecting other AGB1-mediated functions. The S129R mutation had a minor effect on AGB1 function and therefore may not be within any effector interface or is within an effector interface that operates in an untested pathway.

An interesting observation is that mutations W109A and S129R made on the G$\alpha$-binding surface had less effect than expected since these amino acids reside in the G$\alpha$-G$\beta$ protein-protein interface. An intensive mutational analysis on the conserved AGB1 surface for G$\alpha$ association demonstrated that this region may not be critical for effector binding in plants (Chakravorty et
al., 2011a). This phenomenon is significantly different from retinal Gβ (Gβ1), in which mutations in the Gα-binding regions cause impaired activation of an array of effectors like phospholipases, adenylyl cyclases, and ion channels (Ford et al., 1998).

The pleiotropic actions of G protein subunits limit their use as complete loss-of-function targets for breeding. For example, the rice d1 mutant, which carries a null mutation of RGA1, the rice Gα subunit, is dwarf and consequently less likely to lodge (Fujisawa et al., 1999). However, the same genetic lesion in RGA1 confers smaller seed size (Fujisawa et al., 1999) and less resistance to the blast fungus Magnaporthe grisea (Suharsono et al., 2002), both dramatically decreasing yield. A decrease in the rice Gβ subunit reduces cell proliferation and confers seed sterility (Utsunomiya et al., 2011). Taken together with the data on AGB1 shown here, genetic ablation of G protein complex proteins confers a wide set of traits, some beneficial and some not beneficial to agriculture.

While the work here is proof of concept, the observations point to a possible means to improve yield in rice. Just as for rga1, loss of the Gβ subunit in rice confers dwarfness (Utsunomiya et al., 2011), an early trait found during the Green Revolution period that doubled rice yield simply by increasing harvest index (Weber and Fehr, 1966). Unfortunately, this Gβ subunit mutant also has smaller seed and increased susceptibility to fungus. In Arabidopsis, the W109A mutant fully restored silique morphology, flg22-triggered ROS production, and fungal pathogen resistance to wild-type levels but this mutation only partially rescued the shorter hypocotyl phenotype (Figure 3 and 5). This indicates that residue W109 of AGB1 is critical for the binding of effectors mediating cell division. We propose that the equivalent residue of rice Gβ could be mutated in order to generate a partially functioning Gβ subunit, one having wild type resistance to rice blast fungus yet, due to reduced cell division, would be dwarf and provide a higher harvest index.

In summary, we identified multiple sites on the AGB1 protein surface for functional significance in G protein signaling. Our work provides fundamental knowledge to direct finely tune engineering of crop traits.
MATERIALS AND METHODS

Plant Material and Growth Conditions
The *Arabidopsis thaliana* ecotype used in this study was Columbia (Col-0). The *agb1-2* mutant was previously described (Ullah et al., 2003). The entire coding sequence of mutated *AGB1* (Friedman et al., 2011) was cloned into the pENTR/D-TOPO™ vector (Invitrogen, Carlsbad, CA) and subsequently shuttled in Gateway-based pGWB2 (no tag) and pGWB21 (N-terminal 10×Myc tag) vectors (Nakagawa et al., 2007) under the control of the CaMV 35S promoter. To generate transgenic lines stably expressing *AGB1* mutants in the Gβ-null background, the binary constructs in the pGWB backbone were introduced into *Agrobacterium tumefaciens* GV3101 pMP90 by electroporation and then into the *agb1-2* mutant by floral dipping (Clough and Bent, 1998). All transgenic plants were selected on half-strength MS medium containing 25 mg L⁻¹ hygromycin. Suitable progeny homozygous for the respective transgene were used for all experiments.

Transcription Analysis
Total RNA was extracted from 10-d-old *Arabidopsis* seedlings using the RNeasy Plant Mini Kit™ (Qiagen, Valencia, CA). The first-strand cDNA was generated by use of the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For each sample, 1 μg of total RNA was reverse transcribed to produce first strand cDNA. Full-length wild-type or mutated *AGB1* transcripts were amplified using primers AGB1 5’ primer (5’-CACCATGTCTGTCTCCGAG-3’) and AGB1 3’ primer (5’-TCAATCATCCTCTCTTGCTCTCC-3’). *ACTIN7* transcripts were amplified as an internal control using primers Act-RT_for (5’-TGTTCCTCAGTATTTGCGTCGTC-3’) and Act-RT_rev (5’-TGCTGGGATGACCAGGTTGATC-3’).

Preparation and Transient Transfection of *Arabidopsis* Protoplasts
To examine the subcellular localization of wild-type and mutated AGB1, the full-length cDNA fragment in the pENTR plasmid was subcloned into the
Gateway-based pK7WGF2 vector (Karimi et al., 2002). The GFP fusion constructs were delivered into Arabidopsis mesophyll protoplasts (approximately $5 \times 10^4$ protoplasts per transfection) as described (Yoo et al., 2007). After incubation in 1 mL of W5 solution (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KCl, 5 mM glucose, and 2 mM MES, pH 5.7) with 1% BSA at RT for 16 h, the subcellular localization of GFP-tagged AGB1 (wild-type or mutant) was visualized using a Zeiss 710 laser-scanning confocal microscope with a ×40 oil-immersion quartz, apochromatic objective.

**Morphologies**

In-plate assays were performed using half-strength MS basal salts, 1% sucrose, and 0.8% phytoagar (Research Products International, IL). Sterilized seeds were sown and stratified for 2 d before moved to the growth chamber at 23°C. For hypocotyl length assays, seeds were pretreated with light (200 $\mu$mol m$^{-2}$ s$^{-1}$) for 2 h and then incubated in the dark for 50 h. Etiolated seedlings on plates were imaged and hypocotyl lengths were measured with the publicly available ImageJ software (http://rsbweb.nih.gov/ij/). Stomatal index was determined as previously described (Zhang et al., 2008) except that seedlings were stained with 1 mg/mL propidium iodine for 15 min and rinsed briefly with ddH$_2$O before visualization under a Zeiss 710 laser-scanning confocal microscope. To examine the development of lateral roots, 10-d-old, vertically-grown seedlings were fixed in 100% FAA (formalin-acetic acid-alcohol) with Eosin Y O/N at 4°C. The fixed seedlings were cleared with 95% ethanol, rinsed with ddH$_2$O and subsequently stained with 100% acetocarmine solution (Carolina Biological Supply, Burlington, NC) as described (Enstone et al., 2001). Red patches represent lateral root primordia.

The morphology of rosette leaves and siliques was determined as described (Boyes et al., 2001; Ullah et al., 2003). Arabidopsis plants were grown in soil under short-day regime (8 h light / 16 h dark). The fully expanded rosette leaves at stage 5.10 and mature siliques at stage 6.50 were detached from the plants for imaging.
Measurements of Responsive Traits

To observe the effect of exogenous glucose on plant growth, seeds were germinated on a low glucose (2%) medium containing nitrogen as KNO₃ (1.9 g L⁻¹) and NH₄NO₃ (1.65 g L⁻¹) as previously described (Cho et al., 2010). Seedlings showing chlorosis were scored as glucose-sensitive. To examine transient glucose signaling in plants, changes in TBL26 transcript level in response to sugar treatment were checked using real-time PCR as previously described (Grigston et al., 2008). To study the inhibitory effect of ABA on lateral root development, seeds were germinated vertically on half-strength MS media solidified with 0.5% (w/v) Phytagel (Sigma-Aldrich, St. Louis, MO). A homogenous subset of four-day-old seedlings was transferred to control (0.1% ethanol, v/v) or treatment (10⁻⁶ μM ABA) plates and grown under the same condition for 5 additional days before the density of visible lateral roots was measured.

Gas-Exchanges Measurements

Photosynthesis (A) and stomatal conductance (gₛ) were measured with an open gas exchange system (LI-6400, LI-COR, Lincoln, NE) as in Rosenthal et. al (2011). Plants were taken out from growth chambers and immediately placed in the cuvette of the gas exchange system and allowed to reach steady state photosynthesis at their growth [CO₂] (~400 μmol mol⁻¹) at the indicated light levels. For measuring photosynthesis, the initial leaf chamber conditions were set to a constant leaf temperature of 23.26 ± 0.24 °C, the leaf vapor pressure deficit was 0.82 ± 0.133 kPa and chamber relative humidity (%RH) was 70.75 ± 5.1%. Leaves in chambers remained under constant conditions for a minimum of 10 min prior to recording steady state net photosynthesis and stomatal conductance. For measuring stomatal conductance, the leaf chamber conditions were set to a constant leaf temperature of 23 ± 0.20 °C, the leaf vapor pressure deficit was 1.19 ± 0.08 kPa and chamber relative humidity (%RH) was 58 ± 1.0%.

Measurement of flg22-induced ROS accumulation
ROS assays were performed as described previously (Gomez-Gomez et al. 1999) with the following modifications: Leaf discs (5 mm diameter) excised from 4- to 5-week old plants were incubated overnight in water then transferred into microtiter plates containing 50 μL water. ROS production was triggered by the addition of 1 μM flg22 peptide (QRLSTGSRINSAKDDAAGLQIA, synthesized by Centic Biotec, Weimar, Germany), applied in a reaction mixture containing 50 μL water, 20 μM luminol (Sigma-Aldrich, Munich, Germany) and 1 μg horseradish peroxidase (Sigma-Aldrich, Munich, Germany). Luminescence was measured in a Centro LB 960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) during a time course of 38 min following flg22 application.

Pathogen Infection Assays

For Fusarium resistance assays, two-week-old Arabidopsis seedlings grown in soil (California University mix) were uprooted, briefly rinsed with water and incubated in Fusarium spore solution for 30-60 s. After inoculation the seedlings were replanted into fresh soil and grown at 28-30 °C. Leaves with yellow veins were counted 8-9 d after inoculation. F. oxysporum spore solution was prepared as described previously (Trusov et al., 2006). To examine susceptibility to the necrotrophic fungus P. cucumerina, 3-week-old soil-grown plants were inoculated with a suspension of $2 \times 10^6$ mL^{-1} spores as previously described (Berrocal-Lobo et al., 2002). Progress of disease symptoms was scored 11 d after infection according to the following rate: 0. plant is completely healthy; 1. some yellow spots can be observed; 2. there are 1 or 2 leaves dead; 3. there are over 3 dead leaves; 4. few tissues remain green; 5. plant completely dead.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5. Experimental data were analyzed with Student t-test, and two-way ANOVA.
**Figure Legends**

**Figure 1.** Site-directed mutagenesis of AGB1. The upper panel shows the top and bottom views of AGB1 (light grey) and the closely associated Gγ subunit (dark grey). Colored residues indicate point mutations generated. From left to right: R25D, E248K, W109A, Q120R, T188K, R235E, and S129R. The middle panel shows results of RT-PCR analysis of Myc-tagged (+) and non-tagged (-) mutated AGB1 expression in the transgenic lines used in this study. Total RNA was extracted from 10-d-old seedlings. PCR was repeated twice for each biological sample and a total of two biological replicates were tested. ACTIN7 was used as the loading control. Ethidium bromide (EB) stained rRNAs (28S rRNA and 18S rRNA) are shown as a quality control for the RNA samples. The lower panel shows plasma membrane localization of mutant AGB1 proteins. Mesophyll protoplasts were isolated from 5-week-old Col-0 plants and subsequently transfected with 20 μg of plasmids that allow transient expression of N-terminally GFP-tagged WT or mutated AGB1. Each image shows a single optical section in the focus at the plasma membrane. GFP signal was not detected in untransfected (ctrl) protoplasts. Bar = 10 μm.

**Figure 2.** Analyses of developmental phenotypes in wild type, agb1-2, and transgenic plants. A, Hypocotyl length of 50-h-old etiolated seedlings (n=15). B, Density of lateral root (emergent lateral roots + lateral root primordia) of 10-d-old seedlings (n=30). C, Morphological comparison of fully expanded rosette leaves and aerial part of plants. D, Stomatal index on abaxial epidermis of cotyledons from 9-d-old seedlings. E, Morphological comparison of mature siliques. The grey scale of bars indicates the extent to which a phenotype was rescued (black = wild type, grey = partially rescued, white = agb1-2 like). Data for wild-type plants are plotted in black and those for agb1-2 mutants are plotted in white. All values are mean ± SE and assays were repeated 2-4 times with similar results. *Significant difference from wild-type plants (**p<0.001; Student’s t-test). Bars in C and E = 1 cm.
**Figure 3.** Analyses of glucose- and ABA-responsive phenotypes in WT, *agb1-2*, and transgenic plants. A. 2% (w/v) Glucose-hypersensitive phenotype of *agb1-2* and of transgenic lines. The percentages of green seedlings were scored after 10 d grown at 23°C. The experiment was repeated three times and data were averaged. The grey scale of bars indicates the extent to which a phenotype was rescued (grey = partially rescued, white = *agb1-2* like). Data for wild-type plants are plotted in black and those for *agb1-2* mutants are plotted in white. The error bars represent SD. *Significant difference from wild-type plants (***, p<0.001; Student’s t-test). B and C, Inhibition of lateral root development by ABA. VLR = visible lateral roots. The experiment was repeated three times and data were averaged and shown in panel B. The error bars represent SD. *-values of ANOVA analyses are plotted in panel C. The hatched bars represent comparison of each transgenic line to WT plants, while the checkered bars represent comparison to *agb1-2* plants.

**Figure 4.** Accumulation of ROS following flg22 treatment. Leaf discs from 4-to 5-week-old plants were treated with 1 µM flg22 and ROS formation was measured as relative light units (RLU) in a chemiluminescence assay during a time course of 38 min after flg22 application. A, Representative response curves of wild type and *agb1-2* mutant plants. Data represent the mean ± SD of at least six leaf discs per genotype. B, flg22-induced ROS production in the indicated genotypes is represented as the integrated area under the ROS curve measured and referred to as Σ RLU. The grey scale of bars indicates the extent to which a phenotype was rescued (black = wild type, grey = partially rescued). Data for wild-type plants are plotted in black and those for *agb1-2* mutants are plotted in white. Results are presented as the mean ± SE of at least four independent experiments with four to twelve leaf discs per genotype. *Significant difference from wild-type plants (*, p<0.05; Student’s t-test).

**Figure 5.** Susceptibility to necrotrophic fungi in WT, *agb1-2*, and transgenic plants. A, Number of yellow-veined leaves per infected plant. A total of 20
plants for each line were inoculated with *F. oxysporum* spores. Chlorotic leaves were counted 8-9 d after infection. B, Disease rating scores 11 d after inoculation with $2 \times 10^6$ spores ml$^{-1}$ of *P. cucumerina*. The grey scale of bars indicates the extent to which a phenotype was rescued (black = wild type, grey = partially rescued to wild type, white = *agb1*-2 like). Data for wild-type plants are plotted in black and those for *agb1*-2 mutants are plotted in white. All values are mean ± SE and data are from one of three independent experiments with the same conclusion. *Significant difference from wild-type plants (*** $p<0.001$; Student’s $t$-test).

Table S1. Confidence levels for statistical differences in genotype-dependent phenotypes. Statistical analyses were performed as described in ‘Materials and Methods’. Phenotypic parameters of each transgenic line were compared to those of WT and *agb1*-2 plants to examine the extent to which a phenotype of *agb1* mutants was rescued. Student $t$-test was adopted for all the phenotypes except ABA responsiveness. The complementation of ABA phenotype was analysed using two-way ANOVA.

Figure S1. Phenotypic analysis on the 35S:AGB1-YFP-HA/*agb1*-2 complementation line. A, Stomatal index on the abaxial epidermis of cotyledons from 9-day-old seedlings ($n=20$). B, Density of visible lateral roots (VLR) of 10-day-old seedlings ($n=30$). C, Comparison of fully expanded rosette leaves and siliques of plants. Values in A and B are mean ±SE. *Significant difference from wild-type plants (*** $p<0.001$). Scale bars in C = 1 cm.

Figure S2. Osmotic control for the glucose-sensing assay. WT, *agb1*-2, and transgenic plants were grown a low 2% mannitol (w/v) medium. The percentages of green seedlings were scored after 10 d grown at 23°C. The experiment was repeated three times and data were averaged. The error bars represent ±SD.
**Figure S3. Accumulation of ROS following flg22 treatment.** Leaf discs from 4- to 5-week old plants were treated with 1 µM flg22 and ROS formation was measured as relative light units (RLU) in a chemiluminescence assay during a time course of 38 min after flg22 application. Flg22-induced ROS production in the indicated genotypes (multiple independent lines for each AGB1 variant) is represented as the integrated area under the ROS curve measured and referred to as Σ RLU. The grey scale of bars indicates the extent to which a phenotype was rescued (black = wild type, grey = partially rescued). Data for wild-type plants are plotted in black and those for agb1-2 mutants are plotted in white. Results are presented as mean ± SE of at least four independent experiments with four to twelve leaf discs per genotype. The asterisk indicates a statistically significant difference (p <0.05; Student’s t-test) compared to Col-0.

**Figure S4. Gas exchange analysis of WT, agb1-2, and the transgenic plants under different light intensities.** A, Photosynthesis obtained at PPFD = 500 μmol m⁻² s⁻¹. Seven plants per genotype were measured in each experiment and data are from one of two independent experiments with same conclusion. No statistically significant difference was found among different genotypes (p >0.05; one-way ANOVA). B, Photosynthesis (upper panel) and stomatal conductance (bottom panel) measured along a stepwise increase of PPFD. Plants were grown at a constant PPFD (400 μmol m⁻² s⁻¹) before use. Leaves were illuminated with target PPFD values for 5 min before each measurement. Three plants per genotype were measured in each experiment. The error bars represent SD. No statistically significant difference was found among different genotypes (p >0.05; two-way ANOVA).

**Supplemental Data**

The following materials are available in the online version of this article.
Supplemental Table S1. Confidence levels for statistical differences in genotype-dependent phenotypes.

Supplemental Figure S1. Phenotypic analysis on the 35S:AGB1-YFP-HA/agb1-2 complementation line.

Supplemental Figure S2. Osmotic control for the glucose-sensing assay.

Supplemental Figure S3. Accumulation of ROS following flg22 treatment.

Supplemental Figure S4. Gas exchange analysis of WT, agb1-2, and the transgenic plants under different light intensities.

ACKNOWLEDGMENTS

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REFERENCES


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## Supplemental Table 1. Confidence levels for statistical differences in genotype-dependent phenotypes

Summary of p-values from statistical analyses

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<td>WT agb1</td>
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<td>Hypocotyl length</td>
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Statistical analyses were performed as described in ‘Materials and Methods’. Phenotypic parameters of each transgenic line expressing the indicated variant AGB1 (R25D, E248; W109A; Q120R, T188K, R235E; S129R either tagged or not tagged to Myc) were compared to those of WT and agb1-2 plants to examine the extent to which a phenotype of agb1 mutants was rescued. Student t-test was adopted for all the phenotypes except ABA responsiveness. The complementation of ABA phenotype was analysed using two-way ANOVA.