Signaling Specificity Provided by the *Arabidopsis thaliana* Heterotrimeric G-Protein γ Subunits AGG1 and AGG2 Is Partially but Not Exclusively Provided through Transcriptional Regulation

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Abstract

The heterotrimeric G-protein complex in *Arabidopsis thaliana* consists of one α, one β and three γ subunits. While two of the γ subunits, AGG1 and AGG2 have been shown to provide functional selectivity to the Gβγ dimer in Arabidopsis, it is unclear if such selectivity is embedded in their molecular structures or conferred by the different expression patterns observed in both subunits. In order to study the molecular basis for such selectivity we tested genetic complementation of AGG1- and AGG2 driven by the respectively swapped gene promoters. When expressed in the same tissues as AGG1, AGG2 rescues some *agg1* mutant phenotypes such as the hypersensitivity to *Fusarium oxysporum* and D-mannitol as well as the altered levels of lateral roots, but does not rescue the early flowering phenotype. Similarly, AGG1 when expressed in the same tissues as AGG2 rescues the osmotic stress and lateral-root phenotypes observed in *agg2* mutants but failed to rescue the heat-stress induction of flowering. The fact that AGG1 and AGG2 are functionally interchangeable in some pathways implies that, at least for those pathways, signaling specificity resides in the distinctive spatiotemporal expression patterns exhibited by each γ subunit. On the other hand, the lack of complementation for some phenotypes indicates that there are pathways in which signaling specificity is provided by differences in the primary AGG1 and AGG2 amino acid sequences.

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Introduction

Heterotrimeric G-proteins (G-proteins), consisting of three subunits Gα, Gβ and Gγ, are involved in a diverse range of vital biological processes including hormone regulation, neurotransmission, light perception and cell proliferation [1,2,3]. In animal systems, G-proteins mediate signaling initiated by seven-transmembrane (7 TM) spanning G-protein coupled receptors (GPCRs) after activation by an external stimulus. Activation of the GPCR promotes the exchange of GDP for GTP in the Gα subunit and as a result Gα-GTP dissociates from the Gβγ dimer, allowing Gα-GTP and Gβγ to activate their respective downstream effectors. Termination of signaling occurs when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Gα and the inactive heterotrimer re-associates back at the receptor [4]. In plants, the G protein complex is self-activating [5,6,7,8] and therefore does not need a GPCR [9]. Instead, many plants utilize a 7 TM Regulator of G Signaling (RGS) protein, that regulates the GTP hydrolysis reaction of the Gα subunit [Urano. unpublished].

In humans, there are 23 Gα, 5 Gβ and 12 Gγ subunits, allowing a large number of different heterotrimer combinations Gαβγ [10,11,12,13,14]. It is generally believed that the diversity of heterotrimer combinations provides the required coupling specificity to allow signaling by over 800 GPCRs [15]. It was initially thought Gα was the only subunit active in signaling relegating the role of the Gβγ dimer to Gα inactivation and escort to the receptor. It was subsequently established that different Gβγ dimers interact with specific effectors, proving that the Gβγ dimer contributes to both active signaling and heterotrimer specificity [16,17]. Signaling specificity by the different Gβγ dimers can be a consequence of the dimer’s intrinsic structural properties but it can also be dictated by the tissue specificity of their expression patterns [18,19,20,21,22].

Plant G-proteins are also involved in numerous signaling processes including interactions with rhizobia [23], defense against pathogens [24,25,26,27,28,29,30], morphological development and growth [31,32,33], cell proliferation [34,35], ion-channel regulation [36], stomatal control [37,38], light perception [32,39,40,41,42], abiotic stress [43,44,45,46] and hormonal
responses including glucose, brassinosteroid, abscisic acid and jasmonate [47,48,49,50,51,52,53]. G-proteins have also been linked to yield related quantitative trait loci in important crops such as rice [54,55]. Unlike animals, where G-proteins underwent extensive subunit duplication and divergence of function, the plant G-protein repertoire is much simpler. Only one Gα (GPA1), one Gβ (AGB1) and three Gγ subunits (AGG1, AGG2 and AGG3) are encoded in the Arabidopsis thaliana genome [54,55,56,57,58,59].

Among the three Arabidopsis Gγ subunits, AGG1 (Gγ1) and AGG2 (Gγ2) strongly resemble the canonical mammalian Gγ [50,51,52,53]. AGG3 (Gγ3) on the other hand is quite different from AGG1 and AGG2 being more than twice the size (253 a.a.) and exhibiting a modular structure with a γ-like domain at its N terminus, followed possibly by a transmembrane domain and a long cysteine rich C-terminal region [29,54,58,59]. Despite AGG1 and AGG2 sharing extensive sequence conservation (48% amino acid identity and 65% similarity considering conservative substitutions), Trusov et al. [61] reported that Arabidopsis agg1 and agg2 mutants exhibit distinct phenotypes, prompting the hypothesis that the different Gγ subunits confer specificity to the Gβγ dimer in plants. An important and still unanswered question is the molecular basis for such specificity. In normal circumstances it would be fair to assume that the basis for the specificity resides in the molecular structure of the two Gγ subunits (and ultimately in their amino acid composition). Nevertheless, promoter studies showed that the two closely related AGG1 and AGG2 subunits have tissue and developmental expression patterns that rarely overlap [61,62], raising the possibility that the basis for the specificity could be either partially or totally provided by their mutually-exclusive expression patterns. In leaves, AGG1 expression was restricted to veins, while AGG2 expression was observed primarily in guard cells. In roots AGG1 expression was restricted to veins, while AGG2 expression was observed primarily in epidermis. This would provide a transcriptional means to control the level of the Gβγ subunit on the plasma membrane and therefore the capacity for signal output.

A number of hypothetical scenarios can be envisaged including (i) Gβγ1 and Gβγ2 may activate specific sets of effectors, therefore mediating different signaling processes; (ii) Gβγ1 and Gβγ2 may activate common sets of effectors, with their presence or absence in an individual tissue dictating their involvement in signaling and (iii) an intermediate case in which spatio-temporal separation of expression and a degree of effector specificity contribute to the final response.

In order to determine if the specificity observed for Gγ function resides in transcriptional control of the AGG1 and AGG2 genes we swapped gene promoters and tested for genetic complementation in the respective Arabidopsis agg1 and agg2 mutants. Phenotypic analyses revealed that AGG1 and AGG2 are able to complement some but not all mutant phenotypes, indicating the existence of both transcriptional spatial and temporal regulation of the Gβγ activity but also suggesting that some signaling specificity information resides in the primary amino acid composition of both subunits.

### Materials and Methods

**Plant Material**

The agg1-1c mutant allele of AGG1 (At3g63420) and the agg2-1 mutant allele of AGG2 (At3g22942) in the Col-0 background, were described previously [61].

To generate the agg1-1c AGG1::AGG1 complementation lines, an AGG1 fragment from ~2 kb 5’ of the start codon to ~0.8 kb 3’ of the stop codon was amplified from wild-type genomic DNA using Elongase (Invitrogen). The primers used were: 5’-GAAAGAGAGGATTCGTGTTAGCTAGCC-3’ and 5’-GAAGGGACCTCCTAATGAGTCATCAA-3’. The resulting 3.8 kb fragment was cloned into the pGEM-T Easy vector (Promega) and transferred using EsEaRI sites into the binary vector pCAMBIA1300. Subsequently, the construct was transformed into Arabidopsis agg1-1c plants by Agrobacterium tumefaciens-mediated transformation [63]. Primary transformants were selected with hygromycin B. At least ten independent homozygous transgenic lines were obtained.

The agg1-1c AGG1::AGG2 transgenic Arabidopsis were generated as follows. Elongase (Invitrogen) was used to amplify sequences from wild-type Arabidopsis genomic DNA. The following primers were used: for the AGG1 promoter region, 5’-GGGGTACCGGGGCGTCTAGATGAAGACACAATCAACC-3’ and 5’-GGCTCGAGTCTCCTAGAGGTCGCA-3’; for the coding region and terminator of AGG2, 5’-GGGTTCTAGATGAAGACACCACAAC-3’ and 5’-GGGCCGCGCTTTGTGGTTCTGATGTTTTCCT-3’. Restriction sequences (underlined) were incorporated on the ends of fragment for cloning purposes. The PCR products were ligated into pGEM-T Easy vector (Promega). The AGG2 fragment was transferred into the pBluescript SK+ vector using XhoI and NotI restriction sites. AGG1 promoter fragment was inserted in front of AGG2 fragment in the pBluescript SK+ using XhoI and NotI restriction sites. PCR was then performed on the AGG1 promoter-AGG2-pBluescript SK+ construct with XhoI and NotI restriction sites. The construct was transformed into the pGEM-T Easy vector (Promega). The AGG2 fragment was then transferred into the pBluescript SK+ vector using XhoI and NotI restriction sites. The construct was transformed into Arabidopsis agg1-1c plants using the binary vector pMDC99. The construct was again transformed into agg1-1c by the floral dip method. Transformants were selected based on resistance to hygromycin B.

agg2-1 AGG2::AGG2 transgenic lines were generated by transformation of a 4.7 kb AGG2 fragment, from 1.6 kb 5’ of the start codon to 1.8 kb 3’ of the stop codon, into agg2-1. The primers used to amplify the 4.7 kb fragment were 5’-GGTACGGGCGGCATTGCCAGCGATTTTGGC-3’ and 5’-GGGGACCACTTTGTACAAAGCGC-3’. The resulting fragment was cloned into pGEM-T Easy vector (Promega), and transferred to the binary vector pUQ477 using terminal NotI restriction sites. The final construct was transformed into agg2-1 by floral dip, and transformants were selected using BASTA as resistance to hygromycin B. Agrobacterium tumefaciens was used to transfer the construct into the binary vector pUQ477 using terminal NotI restriction sites. The resulting transgenic lines were then transformed into pBluescript SK+ vector using XhoI and NotI restriction sites. The AGG2 promoter fragment was inserted in front of the AGG1 fragment using XhoI and NotI restriction sites. The pBluescript SK+ vector was then ligated into pGEMT-Easy (Promega). The AGG1 coding region and terminator fragment was then transferred into pBluescript SK+ vector using XhoI and NotI restriction sites. The AGG1 promoter fragment was inserted in front of the AGG2 fragment using XhoI and NotI restriction sites. The pBluescript SK+ vector was then transformed into the pUQ4227 vector using terminal NotI restriction sites. The final
Quantitative Real-time RT-PCR Analysis

Total RNA was extracted from two-week-old seedlings as described previously [56]. First strand cDNA synthesis was conducted using the SuperScript III RT kit (Invitrogen) according to the manufacturer’s instructions. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7900 HT Sequence Detection System (Applied Biosystems). The following primer pairs, designed using Primer Express software (Applied Biosystem), were used in the qRT-PCR: AGG1, 5'-UTR, forward, 5'-GGATGGAGACCCGTCGGAGAACTTCA-3', reverse, 5'-CTGGCTAGAAGGTCGCAGAT-3'; AGG1 exon2, forward, 5'-GGAGGTCCGAGAACAGATATTG-3', reverse, 5'-GGAGGTCGAGAACACAGATATTG-3'. Twenty plants from each of the wild-type and transgenic lines were used for the assay. Before sowing onto solid media, all seeds were stratified for 48 h at 4°C in darkness. 6% w/v D-mannitol was added to the plates used for the osmotic stress germination assay. Germination was determined as an obvious protrusion of the radicle. For root assays, seedlings were grown at 26°C on vertical plates supplemented with 1% sucrose for 14 days, and the number of lateral roots per seedling was counted using a dissecting microscope. For adventitious root development, media containing 3% sucrose was autoclaved. Once cooled to 55°C, NAA was added to a final concentration of 1 μM, from a stock solution of 10 mM. Hypocots from 5-day-old etiolated seedlings were aseptically excised and transferred onto the NAA supplemented media. Adventitious root development on the plate was photographed after 10 days incubation at 26°C.

Results

Complementation Constructs and Transgene Expression

To determine whether Gy1 and Gy2 are functionally interchangeable we designed a cross-complementation strategy. Since the expression profiles of AGG1 and AGG2 are non-overlapping we carefully designed the complementation constructs trying to reproduce as much as possible the genomic environment for each of the two genes. In order to complement the agg1 mutation, we fused 1 kb of the promoter region of AGG1 (including the 5' UTR) to an AGG2 genomic fragment containing the entire gene, starting at the start ATG codon and ending 1.8 kb downstream of the 3' UTR to include the terminator sequences (AGG1p::AGG2; Fig. 1A). To complement the agg2 mutation we fused 2 kb of the AGG2 promoter region, including the AGG2 5' UTR, to a genomic fragment containing the entire AGG1 gene from the translational start codon and extending 1.8 kb downstream of the 3' UTR (AGG2p::AGG1; Fig. 1C). As positive controls we prepared constructs containing the entire AGG1 and AGG2 genes, including promoter and terminator regions (AGG1p::AGG1 and AGG2p::AGG2 respectively in Fig. 1A and 1C).

The AGG1p::AGG2 and AGG1p::AGG1 constructs were used to obtain transgenic Arabidopsis lines in the agg1-1c T-DNA mutant background (designated agg1 AGG1p::AGG2 and agg1 AGG1p::AGG1 respectively in this work) while the AGG2p::AGG1 and AGG2p::AGG2 were introduced into an agg2-1 background (designated agg2 AGG2p::AGG1 and agg2 AGG2p::AGG2 respectively, in this work) [61]. At least ten homozygous transgenic lines were generated from each of the constructs. Expression of the transgenes was analyzed in all transgenic lines and those with silencing or aberrant expression, as well as those with obvious insential effects were discarded. Three lines for each of the complementation constructs and one line for each control were further characterized. The length of the promoter regions used in this study, especially in the case of AGG1, was chosen to maximize

Flowering time Analysis

Seeds were sown on soil and stratified for 48 hours at 4°C in darkness. Thirty plants per line were grown at 22°C. Where flowering induction is required, seedlings were initially grown at 22°C for two weeks before being transferred to a 29°C growth room. Flowering time was determined by the age of the plant in days when the inflorescence reached approximately 1 cm in height from the rosette.
Figure 1. AGG1 and AGG2 complementation constructs and expression analysis in transgenic lines. (A) AGG1 complementation constructs. The AGG1p::AGG1 construct contains the entire AGG1 gene starting 1 kb upstream of the 3′ untranslated region (UTR) and extending 1.8 kb downstream of the 3′ UTR region to include the native AGG1 termination sequence. The AGG1p::AGG2 construct contains the AGG1 promoter region as well as the AGG1 5′ UTR, fused to an AGG2 genomic fragment, starting at the ATG start codon and including 1.8 kb downstream of the 3′ UTR to include the native AGG2 termination sequence. Expression of the AGG1p::AGG2 construct will result in a ‘hybrid’ mRNA molecule containing the 5′ UTR from AGG1 fused to the coding region from AGG2. (B) Relative mRNA expression levels. Solid black bars show the relative abundance of the AGG1 mRNA in WT (Col-0) and agg1-1c mutant plants. Self-complementation of the agg1-1c mutant with the AGG1p::AGG1 construct results in similar levels of AGG1 mRNA to those observed in WT plants. Cross-complementation of the agg1-1c mutant with the AGG1p::AGG2 construct results in similar levels of expression of the AGG2 hybrid mRNA (dashed bars) to those observed for AGG1 mRNA in WT and self-complementation lines. (C) AGG2 complementation constructs. The AGG2p::AGG2 construct contains the entire AGG2 gene starting 2 kb upstream of the 3′ untranslated region (UTR) and extending 1.8 kb downstream of the 3′ UTR region to include the native AGG2 termination sequence. The AGG2p::AGG1 construct contains...
the AGG2 promoter region as well as the AGG2 5' UTR, fused to an AGG1 genomic fragment, starting at the ATG start codon and including 1.8 kb downstream of the 3' UTR to include the native AGG1 termination sequence. Expression of the AGG2p:AGG1 construct will result in a hybrid mRNA molecule containing the 5'UTR from AGG2 fused to the coding region from AGG1. Relative mRNA expression levels. Solid grey bars show the relative abundance of the AGG2 mRNA in WT (Col-0) and agg2-1 mutant plants. Self-complementation of the agg2-1 mutant with the AGG2p:AGG2 construct results in similar or higher levels of AGG2 mRNA to those observed in WT plants. Cross-complementation of the agg2-1 mutant with the AGG2p:AGG1 construct results in equal or higher levels of expression of the AGG1 hybrid mRNA (dashed bars) to those observed for AGG2 mRNA in WT and self-complementation lines. In (A) and (C), AGG1 genomic sequences are represented in black while AGG2 sequences are in grey. Regions upstream of the 5'UTRs (promoters) and downstream of the 3'UTRs (terminators) are represented in solid lines; 5' and 3' UTRs are represented in dashed boxes, exons are represented in solid boxes and introns are represented in white boxes. Arrows represent the position of the primers used for real-time quantitative PCR (RT-qPCR). The solid triangles show the position of the T-DNA insertions in the agg1-1c and agg2-1 mutants. In (B) and (D), transcript levels are shown as relative to ACTIN genes expression, mean ± SE of three replicates.

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the length of the upstream region for each gene without including the full coding region of the neighboring genes which may lead to unwanted ectopic effects. The ultimate proof that we captured the entire promoter is the observation that the promoter segments chosen were able to drive genetic complementation of the cognate coding sequence. For example, the chosen AGG1 promoter segment driving expression of the AGG1 coding region genetically complemented the agg1 mutant (Figure 2).

To test the hypothesis that transcriptional regulation imparted at least part of the functional specificity of the Gγ subunits we expressed a hybrid messenger RNA containing the 5'UTR of AGG1 fused to the coding region and 3'UTR of AGG2. This was intentionally designed to include possible AGG1 regulatory elements present in this region, which could influence transcription or translation rates, and to account for any influence of this region in the stability of the mRNA. This strategy also provided a robust and reliable method to quantify the expression of the transgene using quantitative real time PCR with a forward primer located in the 5'UTR of AGG1 and a reverse primer located in the coding region of AGG2. This combination of primers detects only hybrid RNA molecules avoiding detection of the native AGG2 mRNA and any incomplete or aberrant AGG1 mRNA present in the agg1-1c T-DNA mutant.

A critical pre-requisite for the success of our approach is to obtain transgenic lines with transgene expression levels at least equal to those observed for the native gene in wild type plants. Fig. 1 shows the relative expression levels determined using Quantitative RT-PCR for all genotypes used in this study (wild-type, mutant and transgenic complementation lines). The agg1 AGG1p:AGG1 complementation line produced similar AGG1 transcript levels to wild-type plants while the levels of the AGG2 hybrid transcript in the AGG1p:AGG2 transgenic lines were also similar to wild-type AGG1 levels (Fig. 1B). As expected, no AGG1 transcript was detected in the agg1-1c mutant. Conversely, the AGG2p:AGG1 construct generates a hybrid messenger RNA containing the 5'UTR of AGG2 fused to the coding region and 3'UTR of AGG1. Analysis of the AGG2 self- and cross-complementation lines showed transcript levels equal or greater to wild-type levels (Fig. 1D). In order for the cross-complementation strategy to work it was important to achieve at least the same levels of expression present in wild type plants, therefore higher expression levels in the transgenic lines, compared to wild type plants was still useful to determine if AGG1 complements agg2 mutants and vice versa.

AGG2 can Complement Some but not all agg1 Mutant Phenotypes

The agg1 AGG1p:AGG1 self-complementation and agg1 AGG1p:AGG2 cross-complementation lines were characterized to determine their ability to revert several phenotypes observed in the agg1-1c mutant. In most assays, the control AGG1p:AGG1 construct was able to restore the agg1-1c mutant phenotype to wild-type, suggesting that the promoter region used in the constructs was sufficient to drive enough expression in the correct tissues to restore the function of the native AGG1 protein. F. oxysporum is a soil borne fungal pathogen which colonizes the vascular system of plants such as Arabidopsis. Symptoms of infection manifest in yellow chlorotic leaves hence disease progression can be quantified by counting the percentage of chlorotic rosette leaves per plant [69,70]. As previously reported by Trusov et al. [61], agg1-1c mutants exhibit hypersensitivity to F. oxysporum as evidenced by the faster development of leaf chlorosis, being twice the ratio of wild-type nine days after inoculation (Fig. 2A). The hypersensitivity to F. oxysporum was restored to wild-type levels in the self-complementation line (agg1 AGG1p:AGG1) (Fig. 2A, P<0.05, one way ANOVA). Similarly, in all three cross-complementation lines assayed the ratios of chlorotic leaves were comparable to wild-type (Fig. 2A, P<0.05, one-way ANOVA), indicating that AGG2 fully rescued the agg1-1c F. oxysporum susceptibility phenotype.

It has been proven that G8 attenuates auxin-induced cell division leading to lateral root proliferation, although it does not directly couple auxin signaling [32,33]. Initial characterization revealed that the agg1-1c mutant also contains a larger number of lateral roots than wild-type. This fact, combined with the tissue-specific expression pattern observed for AGG1 led to the hypothesis that AGG1 combines with AGB1 as a negative regulator of auxin-induced cell division with a possible role in the acropetal auxin stream [61]. Our assays confirmed that 14-days-old mutant agg1-1c seedlings produce on average ~25% more lateral roots and root primordia than wild-type plants (Fig. 2B) (P<0.05, one way ANOVA). The self-complementation line restored the number of lateral roots to wild-type levels. Likewise, all cross-complementation lines rescued the phenotype with the numbers of lateral roots showing no significant differences with wild-type, indicating that AGG2 is able to restore the lateral root phenotype of agg1-1c (Fig. 2B). In addition, agg1-1c hypocotyls incubated with exogenous 1-naphthaleneacetic acid (NAA) display increased adventitious root formation [61] (Fig. 2C). Our results confirm this observation and also show that either AGG1 or AGG2 can complement this phenotype returning the number of adventitious roots to wild-type levels. Therefore, for both auxin-related responses, AGG2 can successfully complement AGG1 in Arabidopsis.

A number of studies established the involvement of G proteins in germination [52,71,72,73,74,75]. In particular, Gγ subunits have a role in the response to osmotic stress during germination with agg1-1c seeds being hypersensitive to mannitol [61]. To determine if AGG2 is able to rescue this agg1-1c mutant phenotype, relevant seed lines were sown on a single plate containing media supplemented with 6% D-mannitol. The seeds used in this assay were obtained from simultaneously grown plants to ensure synchronized germination and all the experiments were performed in triplicate. The germination percentage of each line was then scored on each plate and averaged between replicates.
Our results confirmed the hypersensitivity to D-mannitol in the agg1-1c mutant (Fig. 2D and E). The difference with wild-type was most dramatic on day 8 when 80% of wild-type seeds had germinated, compared to 50% of the agg1-1c seeds. Interestingly the agg1 AGG1p::AGG1 self-complementation line did not completely restore germination levels, showing a small but statistically significant difference in number of lateral roots (P<0.05, one-way ANOVA). The self-complementation line did not completely restore germination levels, showing a small but statistically significant difference with wild-type plants (Fig. 2E). Two of the three cross-complementation lines showed similar germination dynamics to the self-complementation line while the third did not show any restoration of the germination levels, perhaps as a result of expression differences due to transgene positional effects (Fig. 2E).

AGG1 can Complement some but not all agg2 Mutant Phenotypes

As observed for agg1-1c mutants, agg2-1 mutants are also hypersensitive to D-mannitol during germination [62]. When we tested sensitivity to D-mannitol in wild-type, agg1-1c mutants, agg2 AGG2p::AGG2 self-complementation line and three different agg2 AGG2p::AGG1 cross-complementation lines, the largest differences were observed in day 3 (Fig. 3A). On that time point, the germination percentage of agg2-1 seeds was significantly lower than wild-type (Fig. 3B; P<0.05, one way ANOVA). The self-complementation line restored germination to wild-type levels as did all three cross-complementation lines (Fig. 3B), suggesting that
AGG1 can perform a similar function to AGG2 in the control of osmotic stress. This finding also supports the notion that Gc1 and Gc2 have a synergistic role in regulating the osmotic stress response component of germination.

Like agg1-1c mutants, agg2 mutants also produce more lateral roots than wild-type plants. Since the expression of AGG2 in roots is restricted to the cortex, it was hypothesized that this phenotype was due to defects in basipetal auxin transport or signaling [61]. We therefore investigated whether expressing AGG1 in the cortex region of agg2 mutants would restore the root phenotype. As expected, agg2-1 displayed significantly more lateral roots (including root primordia) than wild-type (P<0.05, one way ANOVA; Fig. 3C). The self- and cross-complementation lines restored the root numbers to wild-type levels indicating that AGG1 and AGG2 are functionally interchangeable in the control of lateral root formation (Fig. 3C).

The early flowering phenotype observed in agg1-1c mutants is not observed in agg2 mutants, at least when grown under long day conditions at 23°C. However, we observed early flowering in agg2-1 when grown at 29°C compared to wild-type plants (Fig. 3D). To confirm the initial observations, we simultaneously grew at least 30 plants on soil for each of the studied genotypes; i.e. wild-type, agg2-1 mutant, self-complementation line and three cross-complementation lines. Two trays were initially grown at 23°C under long day regime for each of the lines. After two weeks, one of the trays was moved to 29°C, with similar light conditions and plant age at bolting was recorded for both trays. While no differences in flowering time between wild-type and agg2-1 were apparent at

Figure 3. AGG1 complements some but not all agg2 mutant phenotypes. (A) Germination dynamics of wild-type, mutant and complementation lines on 0.5x MS supplemented with 6% D-mannitol during 9 days after stratification. Each genotype was analyzed in three replica plates with more than 100 seeds. Insert shows control germination without D-mannitol. (B) Percentage of germinated seeds at day 3 from panel (A) demonstrating highest difference between genotypes. Bars represent average value of three replicates (more than 100 seeds each). Error bars show standard errors. Letters indicate groups with statistically significant differences in seed germination (P<0.05, one-way ANOVA). (C) Total number of lateral roots was scored in two-week-old seedlings grown vertically on 0.5x MS supplemented with 1% sucrose. Bars represent average values SE of 15 plants per genotype. Letters indicate groups with statistically significant differences in number of lateral roots (P<0.05, one-way ANOVA). (D, E) AGG1 failed to complement the agg2-1 mutant on high temperature-induced flowering. (D) Effect of the agg2-1 mutation on high temperature-induced flowering. Col-0 and agg2-1 plants were initially grown at 22°C for two weeks and then divided into two groups: the first group was kept at 22°C, while the second group was transferred to 29°C. Day of inflorescence appearance was recorded for at least 30 plants of each genotype. Bars represent the average number of days from germination till inflorescence appearance ± SE. Letters indicate groups with statistically significant differences (P<0.05, one-way ANOVA). (E) Average number of days from germination till inflorescence appearance in at least 30 plants of each genotype induced at 29°C. Bars represent the average number of days from germination till inflorescence appearance ± SE. Letters indicate groups with statistically significant differences (P<0.05, one-way ANOVA). doi:10.1371/journal.pone.0058503.g003
23°C, flowering time for agg2-1 mutants grown at 29°C was significantly shorter than wild-type (Fig. 3E; P<0.05, one way ANOVA). The self-complementation line restored flowering time at high temperature to wild-type levels but none of the three cross-complementation lines successfully rescued the agg2-1 mutation (Fig. 3E).

Discussion

Two proteins are functionally redundant when the absence of one can be compensated in vivo by the second one. Consequently, a null mutation in one of two redundant proteins will not result in phenotypic alteration (i.e. the mutant will have a wild-type phenotype). Two proteins are functionally interchangeable if they can perform the same biological functions, even if they do not belong to the same species. The Arabidopsis and rice Gγ subunits are obviously not redundant but can be functionally interchangeable if they can complement each other. The fact that agg1-1 and agg2-deficient mutants display distinct phenotypic alterations clearly established that AGG1 and AGG2 are not functionally redundant in planta and confer specificity to the Gß dimer in vivo [61]. The first and most obvious explanation for the observed specificity provided by AGG1 and AGG2 is that the Gß1 and Gß2 dimers activate different sets of effectors in response to diverse signals. However, with further consideration, the non-overlapping expression patterns observed for AGG1 and AGG2 raise the possibility that the observed specificity could be due to the spatiotemporal separation observed for both proteins [61,62]. It is therefore important to determine whether artificially expressing AGG2 in the same tissues and developmental stages in which AGG1 is normally present will rescue the agg1 mutant phenotypes. The same logic applies to the reverse. We hypothesized that, given the high degree of similarity between both proteins, AGG1 and AGG2 should be functionally interchangeable.

Our results summarized in Table 1 show that in most cases AGG1 and AGG2 are functionally interchangeable. Four out of five agg1-1c phenotypes tested were fully rescued by AGG2, demonstrating that AGG2 is able to functionally replace AGG1 in response to pathogen attack, auxin control of root development and osmotic stress during germination. Similarly, two out of three agg2-1 phenotypes were rescued by AGG1, proving that AGG1 is able to replace AGG2 in responses linked to auxin in root development and osmotic stress during germination. Collectively, the reciprocal complementation achieved by the two Gγ subunits suggests that both subunits are able to perform many of the same biochemical activities. However, both Gγ subunits are not always functionally interchangeable. AGG2 did not fully complement the agg1 early flowering phenotype, and AGG1 did not restore the thermosensitive flowering phenotype of agg2.

The fact that AGG1 and AGG2 complement each other in pathways responding to osmotic stress, auxin and defense suggests that the Gß1 and Gß2 dimers activate a number of common effectors, although the identity of those effectors is still unknown. While hypothetical, it is also possible that G-protein involvement could be somewhat indirect resulting from crosstalk among several pathways. Interestingly, there is accumulating evidence suggesting that osmotic stress, defense and auxin pathways modulate each other. Abiotic stress and wounding affect auxin responses [76,77,78]. While auxin is implicated in the regulation of plant defense [79,80]. It is therefore tempting to speculate that Gß1 and Gß2 may modulate pathways responding to auxin, osmotic stress and pathogen attack at a point, or points of cross talk, using similar signaling mechanisms. This explains why a simplistic repertoire of G proteins functions in such divergent signaling processes.

Effectors Activation by Gß

Alternatively, the fact that AGG1 and AGG2 are functionally interchangeable may indicate that the binding and activation of effectors resides on recognition sites predominantly or exclusively located on the surface of AGB1. Three recent studies revealed that several amino acid residues on AGB1 are essential for effector activation. The first identified acireductone dioxygenase 1 (ARD1) as an AGB1 interactor [81]. Physical interaction was proven in yeast 3-hybrid experiments, while genetic interaction was demonstrated by the rescue of the agb1-2 short hypocotyl and open apical hook phenotypes of etiolated two-day-old seedlings by ARD1 overexpression. ARD1 was shown to modulate cell division to control hypocotyl length and AGB1 was able to stimulate ARD1 enzymatic activity in vitro. The ability to stimulate ARD1 activity was abolished by several point mutations in AGB1, either single W109, double E248/R25 or triple Q120/T188/R235, suggesting that these residues are essential for ARD1 stimulation. This study proves that AGB1 contains key contact residues for some effectors, such as ARD1.

In the second study, site directed mutagenesis of Arabidopsis AGB1 and the ability of the different mutations to rescue agb1 phenotypes was tested [82]. Substitution of T65 for alanine rendered AGB1 unable to complement the hypersensitivity of the agb1 mutant to D-mannitol during germination. In addition, mutation at D250 failed to restore lateral root numbers in the agb1 mutant to wild-type levels. These observations highlight the importance of individual AGB1 residues in the activation of the effectors involved in osmotic response and lateral root formation.

| Mutant background | phenotype | Complementation |
|-------------------|-----------|-----------------|
| agg1-1c           | Reduced resistance to Fusarium | Yes |
|                   | Increased adventitious root growth under auxin induction | Yes |
|                   | Increased lateral root growth | Yes |
|                   | Increased sensitivity to osmotic stress during germination | Yes |
| agg2-1            | Early flowering time | Partial |
|                   | Increased lateral root growth | Yes |
|                   | Increased sensitivity to osmotic stress during germination | Yes |

Table 1. Summary of Gγ1 and Gγ2 complementation studies.

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Our results showing that AGG1 and AGG2 are both able to restore D-mannitol sensitivity at germination and lateral root numbers are consistent with the above observations and may indicate that the effectors involved in these two responses form a direct contact with AGB1 residues for activation independently of the AGG subunit attached to AGB1 in the Gß dimer.

In a third study, a comparative approach was used to identify a set of residues on the AGB1 surface implicated in protein-protein interfaces [83]. The assumption was that these residues are critical in specific AGB1-effector contacts. Mutation of these residues in combination with genetic complementation assays enabled dissection of the AGB1 protein surface for a variety of ABG1-mediated physiologies (developmental, hormone responses, pathogen defense, and photosynthesis). Interestingly, residues R25 and E248 lie along the AGB-binding tract. Unfortunately, the AGG1-specific differences in flowering reported here were not tested in that study.

Gγ1 and Gγ2 are not Functionally Interchangeable in the Control of Flowering Time

Our results showed that AGG2 failed to complement the early flowering phenotype of aggl-1c, while AGG1 was unable to complement the thermo-sensitive flowering phenotype of aggl-2. Although both are flowering time phenotypes, our results suggest that Gß1 and Gß2 act in separate signaling pathways suggesting that they signal to different downstream effectors and are therefore not functionally interchangeable.

Flowering is a complex process whereby plants go through a transition between vegetative and reproductive phases and is influenced by many environmental factors including photoperiod, temperature, humidity and nutrient availability [84]. Endogenous factors such as carbohydrate reserves and genetic make-up also play a role during the transition phase [85,86,87]. Without a comprehensive study of G protein involvement in flowering induction, it is dangerous to speculate as to the specific roles of AGG1 and AGG2 with any confidence. Although speculative, it is interesting to note that G-proteins are implicated in modulating responses to gibberellins (GA) and brassinosteroids (BR), both of which promote flowering [88,89,90,91]. It was recently suggested that G-proteins mediate the cross talk between auxin and BR signaling [91]. AGG1 is clearly implicated in auxin signaling as evidenced by the auxin sensitive traits of aggl-1c, opening the door to its involvement in cross talk with BR, therefore having an effect on flowering time through BR-mediated inhibition of FLC the potent flowering suppressor [89]. On the other hand, thermal induction of flowering is dependent on GA, suggesting that the thermosensitive flowering phenotype of aggl-2 may be due to a role of Gß2 in GA signaling [92].

Differential post-translational modification may contribute to the selective functions of AGG1 and AGG2 as has been proven in Cdc42, a GTPase with an important role in the regulation of cell polarity and the actin cytoskeleton [93]. Both AGG1 and AGG2 undergo prenylation, but AGG2 undergoes additional S-acylation, most probably by addition of a palmitoyl group. This second lipid modification was suggested to be the reason AGG2 is able to localize to the membrane more efficiently than AGG1 [94,95]. Differential membrane affinity plays a major role in mammalian Gγ specialization [96]. It is possible palmitoylation of AGG2 could provide a defining functional difference between the two proteins due to altered membrane affinity. Additionally, lipid moieties are able to form direct contact with effectors and differential lipidation within a protein family can result in conformational variation, allowing different interaction surfaces to be available to different effector subsets [97].

Conclusions

There is ample proof that the Gß plays a crucial role in the physical interaction with effectors in Gßγ dimer-mediated signaling in plants and animals [3,29,81,90,99]. It is also known that, in animal systems, interaction with effectors reside in different Gß residues [100,101]. This fact together with the existence of multiple Gß subunits with divergent sequences can easily provide specificity for the multiple signaling pathways mediated by the Gßγ dimer. However, it is apparent that plants present a very different picture. The openly different phenotypes shown by the AGG1-, AGG2- and AGG3-deficient mutants together with the fact that there are single alpha and beta subunits clearly indicates that γ subunits provide functional selectivity in Gßγ dimer signaling in Arabidopsis. This study investigated the molecular basis for such selectivity in the two prototypical γ subunits, AGG1 and AGG2. AGG3 was not included in the study due to its atypical structural characteristics and strong differences with AGG1 and AGG2, making it highly unlikely to share effectors with the other two γ subunits. Our results show that for some pathways the selectivity is not embedded in their molecular structure, as proven by the ability of AGG2 and AGG1 to complement aggl and aggg2 mutants respectively. Effector contact points reside in Gß and possibly in conserved residues between AGG1 and AGG2. In these cases, specificity is provided by the spatiotemporal differences in AGG1 and AGG2 expression patterns. Nevertheless this is not the case for all phenotypes, implicating that there are some pathways for which signaling specificity is at least partially provided by non-conserved amino acid residues in AGG1 and AGG2. In these cases, specificity is embedded in the molecular structure of AGG1 and AGG2, although differences in expression patterns could also contribute. Contact point/s between effectors and the Gßγ dimer are crucial for effector activation and there are a number of studies that have identified important amino acid residues in Arabidopsis Gß [81,82,102]. The next obvious step is to perform similar mutagenesis studies in AGG1 and AGG2.

Author Contributions

Conceived and designed the experiments: LT DC YT AMJ JB. Performed the experiments: LT DC YT AMJ. Analyzed the data: LT DC YT AMJ JB. Contributed reagents/materials/analysis tools: AMJ JB. Wrote the paper: LT JB.

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