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RESEARCH PAPER

Heterotrimeric G-proteins facilitate resistance to plant pathogenic viruses in *Arabidopsis thaliana* (L.) Heynh

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ABSTRACT

Heterotrimeric G-proteins, consisting of Gα, Gβ and Gγ subunits, are important signal transducers in eukaryotes. In plants, G-protein-mediated signaling contributes to defense against a range of fungal and bacterial pathogens. Here we studied response of G-protein-deficient mutants to ssRNA viruses representing 2 different families: *Cucumber mosaic virus* (CMV) (*Bromoviridae*) and *Turnip mosaic virus* (TuMV) (*Potyviridae*). We found that development of spreading necrosis on infected plants was suppressed in the Gβ-deficient mutant (*agb1-2*) compared to wild type and Gα-deficient mutant (*gpa1-4*). In accordance, ion leakage caused by viral infection was also significantly reduced in *agb1-2* compared to wild type and *gpa1-4*. Nevertheless, both viruses replicated better in *agb1-2* plants, while *gpa1-4* was similar to wild type. Analysis of pathogenesis-related genes showed that Gβ negatively regulated salicylic acid, jasmonic acid and abscisic acid marker genes during CMV and TuMV infections. Interestingly, analysis of salicylic acid deficient transgenic plants indicated that salicylic acid did not affect resistance against these viruses and did not influence the Gβ-mediated defense response. We conclude that heterotrimeric G-proteins play a positive role in defense against viral pathogens probably by promoting cell death.

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Introduction

Heterotrimeric G-proteins, consisting of 3 subunits Gα, Gβ and Gγ, are essential eukaryotic signaling molecules, amplifying signals perceived by receptors at the plasma membrane and passing it to cytoplasmic effectors.[1-5] In plants, heterotrimeric G-proteins are involved in multiple biological processes that influence plant development and stress responses.[4-23] Importantly, Gβ and Gγ subunits participate in disease resistance against necrotrophic and hemi-biotrophic pathogens.[5,9,14-18,24-26] To the best of our knowledge G-protein role in defense against viral pathogens never been studied. Most probably, G-proteins mediate several various defense related pathways responding to different types of pathogens.[9,15,18,26] Reasonable amount of experimental evidence supports a hypothesis that G-protein-mediated resistance against hemi-biotrophic fungi and bacteria may be associated with receptor-like kinases (RLKs) and the defense mechanism is probably based on activation of programmed cell death (PCD).[15,16,24,27-29] Recently we established that heterotrimeric G-proteins physically interact with 3 defense-related receptor-like kinases, in particular with BRI1-associated kinase 1, BAK1.[30] It has been reported that viral RNA induces responses that relied on RLKs.[31] BAK1, for instance, has been shown to play a role in resistance against 3 different RNA viruses.[32] We predicted that G-proteins might be a part of this signaling pathway and play a role in defense against viral pathogens.

Salicylic acid (SA)-mediated defense system is a well-known contributor to PCD as well.[33-35] It is highly essential for resistance to biotrophic pathogens.[36,37] Infection by compatible viruses induces SA defense-related gene expression.[32,38-40] In particular, pathogenesis-related 1 (*PR1*) transcript was greatly increased upon treatment with several compatible viruses.[41] Upregulation of this gene reliably indicates activation of SA-mediated defense pathway. However, contribution of this pathway in resistance against viruses remains unclear.[42-46] It has been shown that G-protein mediated defense and PCD responses were independent of SA.[15,17,20,28] Therefore these 2 pathways might contribute additively or synergistically to PCD and/or provide resistance against viruses.

Cucumber mosaic virus (CMV) and *Turnip mosaic virus* (TuMV) belong to 2 different families of positive strand, single stranded (ss) RNA viruses. They both infect multiple plant species, including Arabidopsis and represent the largest group of viral plant pathogens.[47-49] Apart from being a significant agricultural threats, these viruses represent well-studied systems for plant-virus interaction, with optimized experimental conditions.

In this paper we report the positive role of heterotrimeric G-proteins in defense against 2 ssRNA viruses, CMV and TuMV. Our results demonstrate a number of alterations to defense response caused by null mutation in *AGB1* gene. We suggest that G-protein-mediated resistance against viral pathogens involves augment of cell death.

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Results

Differential response of G-protein deficient mutants to TuMV and CMV infection

Since G-proteins have never before been associated with defense against viruses, we chose to start with well characterized viruses – *Cucumber mosaic virus* (CMV) and *Turnip mosaic virus* (TuMV). To establish involvement of G-proteins in resistance against these viruses we inoculated 5-week-old *A. thaliana* null mutants lacking G*α* (*gpa1-4*) or G*β* (*agb1-2*) subunits as well as wild type (WT) Columbia-0 (Col-0) plants. To characterize disease progression and defense response we observed several symptomatic characteristics specific for virus-infected plants: discoloration of rosette leaves, leaf curling and arrest of inflorescence growth. All genotypes were affected by both viruses, and developed visible disease symptoms. However, we observed clear differences in severity and dynamics of disease progression in different genotypes. The onset of disease was indicated by leaf discoloration, which started as obvious chlorotic patches (mottling) on rosette leaves of inoculated plants, while mock-inoculated plants remained uniformly green. The symptoms developed faster and were more severe in CMV-inoculated plants with appearance at 8 days post inoculation (dpi), while TuMV caused first apparent symptoms at 11 dpi. Further observation revealed that chlorosis was progressive and eventually distinct necrosis developed at 16 dpi and 21 dpi on CMV- and TuMV-infected plants, respectively (Fig. 1A). Importantly, chlorotic mottling was perceptibly more developed in WT and *gpa1-4* compared to *agb1-2* (Fig. 1A). The apparent difference between genotypes was observed in respect to necrotic areas, which were not detected upon visual inspection in *agb1-2* plants. To study necrosis development, we stained representative leaves from each genotype with trypan blue. Mock-inoculated WT leaves used as a control displayed no necrotic areas, WT and *gpa1-4* leaves infected with virus showed clear patches of deeply stained dead tissue, while only small separated dots were observed on infected *agb1-2* leaves (Fig. 1B). This demonstrates that G-protein mutant *agb1-2*, but not *gpa1-4*, was impaired in response to viral infection in terms of induced cell death.

It has been reported that plants expressing the salicylate hydroxylase gene, *NahG*, which are deficient in SA, showed wild type-like resistance to CMV,[42,50] indicating that basal resistance to CMV is not SA-dependent. To test the involvement of SA-mediated signaling in resistance against CMV in our experimental system and to study its interaction with AGB1-mediated response, we inoculated SA-depleted, *NahG* transgenic plants, *agb1-2* mutant and *agb1-2/NahG* plants along with WT plants with CMV. The *NahG* plants showed no difference with WT in symptoms (Fig. 1A), in accordance with previous reports.[42,50] At the same time, *agb1/NahG* plants were very similar in symptom development and severity to *agb1-2* plants (Fig. 1A). Analysis of trypan blue staining of infected leaves displayed the WT necrosis development on *NahG* plants and suppressed necrosis on *agb1-2/NahG* leaves (Fig. 1B). Therefore we conclude that endogenous SA does not affect the AGB1-mediated response.

Stunted inflorescence is another distinct symptom caused by infection with CMV or TuMV. Therefore, we evaluated suppression of inflorescence elongation by comparing plant's

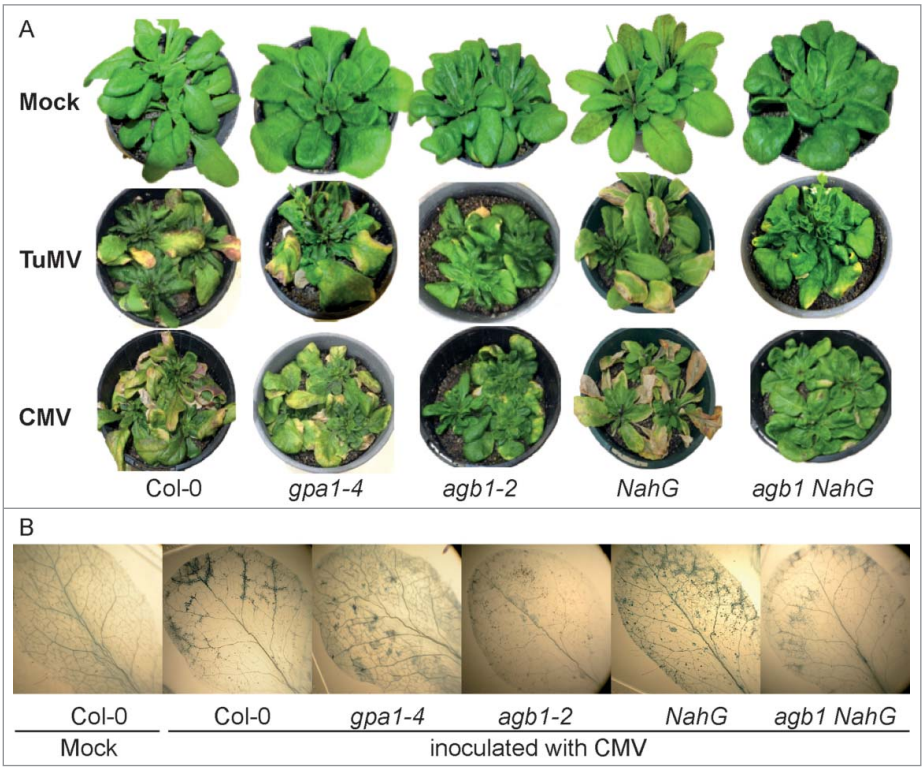

Figure 1. AGB1, but not GPA1, knockout suppresses development of the cell death symptoms induced by CMV or TuMV infection. (A) Development of chlorosis and necrosis on designated genotypes infected with CMV or TuMV 16 and 21 dpi, respectively. Necrosis is obviously less developed in *agb1-2* and *NahG agb1-2* compared to WT (Col-0), *gpa1-4* or *NahG*. (B) Detection of dead cells using trypan blue staining. Lower intensity of blue staining in *agb1-2* and *NahG agb1* compared to WT (Col-0), *gpa1-4* and *NahG* indicates suppression of induced cell death by *agb1* mutation.

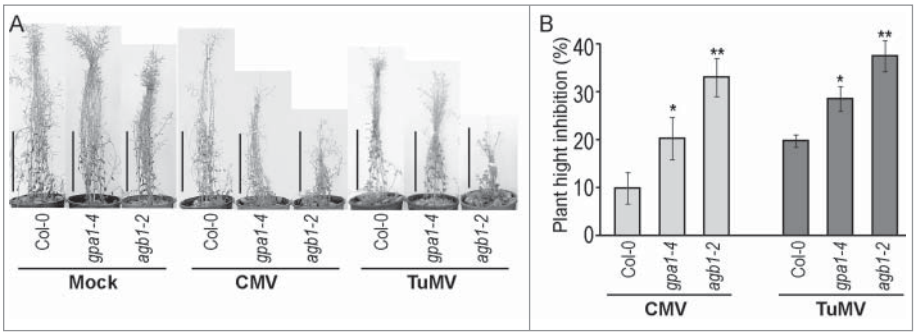

Figure 2. Plant hight inhibition by viral infection was more prominent in *agb1-2* mutants. (A) Several representative plants of listed genotypes were mock-inoculated or infected with CMV or TuMV. Plants were analyzed and photographed 5 weeks after inoculation. (B) Quantification of the inflorescence growth suppression by viral infection. Values indicate mean ratio of (infected plant height) / (average mock-inoculated plant height) ± SE. Asterisks indicate significant differences from wild type Col-0 (Student's t-test, * $P < 0.05$, ** $P < 0.01$). Experiment was repeated 3 times with similar results.

height of mock- and virus-inoculated plants 6 weeks after inoculation. All genotypes displayed shorter inflorescence in infected plants compared to mock-inoculated controls (Fig. 2A). Interestingly, despite an earlier onset of chlorosis and necrosis caused by CMV, the inflorescence growth was stronger inhibited by TuMV. Moreover, both viruses suppressed inflorescence growth more severely in *agb1-2* mutant compared to WT and *gpa1-4*. The effect of the viral infection on inflorescence length expressed as a percentage of mock-inoculated plant height is shown on Fig. 2B. The shown difference between WT and *agb1-2* was statistically significant (Student's t-test $P < 0.001$, $n = 10$). Notably, infected *gpa1-4* plants also exhibited more stunted inflorescence compared to WT, although to a lesser extent than *agb1-2* (Fig. 2B). Student's t-test supported statistical significance of the observed differences between WT and *gpa1-4*, as well as *gpa1-4* and *agb1-2* ($P < 0.05$, $n = 10$).

In addition, non-quantifiable symptoms such as leaf curling and loss of primary inflorescence dominance manifested as multiple stunted secondary inflorescences emerging from the base of the rosette were more pronounced in infected *agb1-2* plants compared to WT or *gpa1-4*. The summary of the symptom analysis is: i) chorosis, and necrosis were less developed in *agb1-2* compared to WT and *gpa1-4*, while leaf curling and inflorescence impairment were significantly more developed in *agb1-2*; ii) inflorescence growth was also inhibited in *gpa1-4* plants, although to a lesser extent than in *agb1-2*; and iii) endogenous SA did not affect resistance to CMV and TuMV and did not interfere with AGB1-mediated responses.

Gβ mutant displayed reduced cell death-related responses

We observed that chlorotic and necrotic lesions caused by CMV and TuMV infection were significantly less developed in the *agb1-2* mutant compared to WT and *gpa1-4* plants. Therefore, we assumed that *agb1-2* mutant may be impaired in other cell death-related responses. One of the well-known method for determining cell death in plants is assessing ion leakage from plant tissue.[51] We quantified the ion leakage in virus-inoculated leaves for the 3 genotypes. Leaf samples from CMV- and TuMV-infected plants were analyzed at 1 and 3 dpi, respectively. Conductivity tests showed higher levels of ion leakage in WT and *gpa1-4* compared to *agb1-2* (Fig. 3A and B), which is consistent with the reduced level of necrotic symptoms observed in *agb1-2* leaves. One of the indicators of cell death at gene expression level is *glutathione S-transferase 1, GST1*. We evaluated *GST1* expression level in CMV-infected plants 24 hours after inoculation and compared it with mock-inoculated control. CMV did not induce *GST1* expression in *agb1-2* plants, while significant almost 2-fold induction was observed

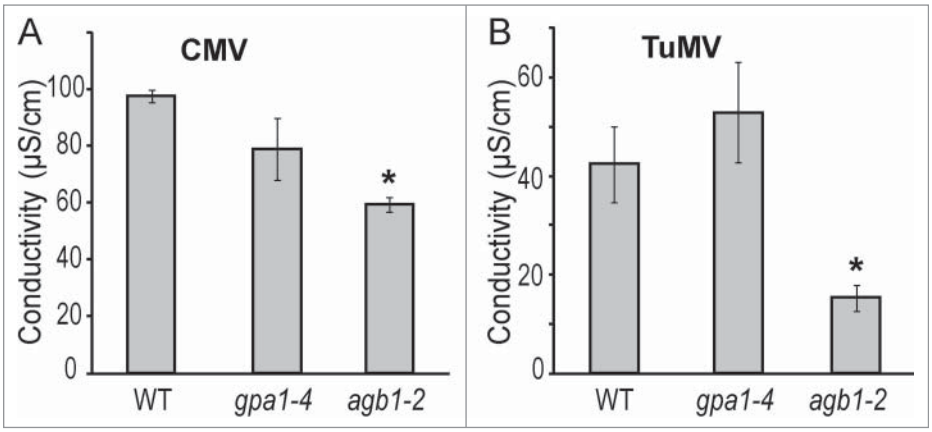

Figure 3. Cell death related ion leakage response to viral infection was compromised in AGB1 deficient plants. Conductivity test was performed on plants of designated genotypes challenged with (A) CMV or (B) TuMV. The ion leakage was evaluated 24 or 72 hours after infection, respectively. Values indicate mean conductivity ± SE. Asterisks indicate significant differences from wild type Col-0 (Student's t-test, * $P < 0.05$).

in WT and *gpa1-4* (Fig. 3C). These experiments confirmed the impairment of cell death responses in *agb1-2* mutant.

Systemic virus accumulation was increased in Gβ deficient mutant

The reduced necrosis and impaired cell death observed in *agb1-2* mutant implies that it may be less restrictive to viral replication and/or systemic movement within the plant. To evaluate viral replication and long-distance movement, we measured accumulation of viral coat protein (CP) mRNA in un-inoculated leaves of CMV- and TuMV-infected plants. Both viral CP and its RNA have been successfully used for quantification of viral particles and genomes in infected plants.[52-54] Two-3 fully expanded leaves of 5-week-old plants of the 3 genotypes: WT, *gpa1-4* and *agb1-2* were inoculated with CMV or TuMV. Seven days after inoculation, total RNA was extracted from adjacent un-inoculated leaves. cDNA was produced by reverse transcription and subjected to quantitative PCR using CP gene primers specific for each virus. As shown in Fig. 4A and B, *agb1-2* mutant accumulated significantly higher levels of CP mRNA than WT or *gpa1-4* plants for both CMV and TuMV.

CMV- and TuMV-induced PR gene expression is enhanced in agb1-2 *mutant*

To test if pathogenesis-related hormonal pathways governed by salicylic acid (SA) and jasmonic acid (JA) or abscisic acid (ABA) are affected by G-protein mediated signaling, we evaluated expression induction of the several marker genes: *PR1* for SA; *PR4* for JA and *RD20* for ABA. Plants of the 3 genotypes were inoculated with CMV, TuMV or mock-inoculated. RNA was extracted one week after inoculation. Relative gene expression was determined by quantitative PCR. Induction levels were calculated as a ratio of virus-inoculated to mock-inoculated gene expression. Distinctively high induction of *PR1* expression was detected in response to both viruses in all 3 genotypes. Importantly, in *agb1-2* the induction was significantly higher compared to WT and *gpa1-4* (Fig. 5A). CMV infection resulted in *PR1* levels increased approximately 300-fold in WT and *gpa1-4*, but about 700-fold in *agb1-2* mutant. Similarly, TuMV induced *PR1* levels 200-fold in WT and *gpa1-4*, while in *agb1-2* the increase was about 650-fold (Fig. 5A). Both viruses were considerably less effective inducing JA and ABA reporter genes, *PR4* and *RD20*, respectively. While *PR4* gene was induced about 5-fold in WT and *gpa1-4* and up to 20-fold in *agb1-2, RD20* gene was not induced in WT and *gpa1-4* and only up to 3-fold induction was observed in *agb1-2* (Fig. 5B and C). All described differences were statistically significant (Student's t-test, $P < 0.05$).

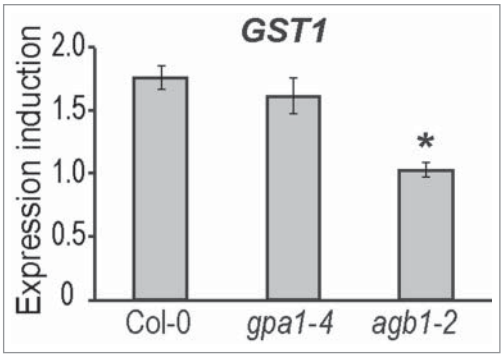

Figure 4. Expression induction of *GST1* in plants infected with CMV. The expression was evaluated with RT-qPCR using *SAND* expression for normalization. Values on the graph represent means of the 3 ratios from 3 independent replicates, error bars show standard error of the mean. Asterisks indicate statistically significant differences from wild type Col-0 (Student's t-test, $P < 0.05$).

Discussion

G-proteins have been shown to play an important role in defense against bacterial and fungal pathogens.[6,9,14,16-18,26] Here we provided the first evidence that G-protein-mediated signaling is also involved in resistance against viral pathogens. We found that *agb1-2* mutant lacking Gβ subunit was more susceptible to 2 ssRNA viruses, CMV and TuMV compared to wild type, Col-0. In contrast, the Gα deficient mutant *gpa1-4* was similar to WT in symptom development and virus accumulation. These observations are reminiscent of previous reports in which Gβ deficient mutant is hypersensitive to fungal and bacterial pathogens, while Gα mutant displays sensitivity similar to wild type.[9,14,18,24,26,28] Therefore, we conclude that the Gα subunit is not involved in the Gβ-mediated defense pathway. Interestingly, recently it was shown that Gβγ actually partnering with extra-large G-proteins (XLG2 and XLG3) to facilitate resistance against fungal and bacterial pathogens.[15] Importantly, the apparent similarity in susceptibility of the *agb1-2* mutant to 3 different groups of pathogens, bacteria, fungi and viruses, suggests a universal defense mechanism controlled by Gβ subunits. Similarly increased virus accumulation was caused by mutations in receptor-like kinase *BAK1* gene.[32] The *bak1-4* and *bak1-5* mutants were found to be more susceptible to 3 different RNA viruses *Oilseed rape mosaic virus* (ORMV), *Turnip crinkle virus* (TCV) and *Tobacco mosaic virus* (TMV).[32] Recently we have demonstrated direct physical interaction between BAK1 and G-protein subunits, Gα, Gγ1 and Gγ2.[30] Here, we observed that Gα did not contribute to the resistance against CMV or TuMV, while Gβ did. Gβ forms compulsory dimers with Gγ1 and Gγ2 subunits and does not function independently.[55,56] Therefore it is reasonable to speculate that Gβγ dimer might function together with BAK1 forming a signal transduction module, which initiates cell death response upon attack of various pathogens. Existence of such a module and its universal defense role has been substantiated by 2 recent reports.[27,31] Liang an co-authors have demonstrated that G-proteins, namely non-canonical Gα, XLG2, the Gβ, AGB1, and the 2 Gγ subunits, AGG1 and AGG2 directly bind Flagellin Sensitive2 (FLS2)-BIK1 receptor complex and are required for flagellin-induced immune responses.[27] This work provided a mechanistic evidence for G-protein role in PTI. While the other group established that viral double stranded RNAs can act as pathogen-associated molecular pattern (PAMP), which induces specific signaling cascades leading to PAMP-triggered immunity PTI induction. In this picture G-proteins represent one of jigsaw-puzzle piece further connecting elements of complex defense network.

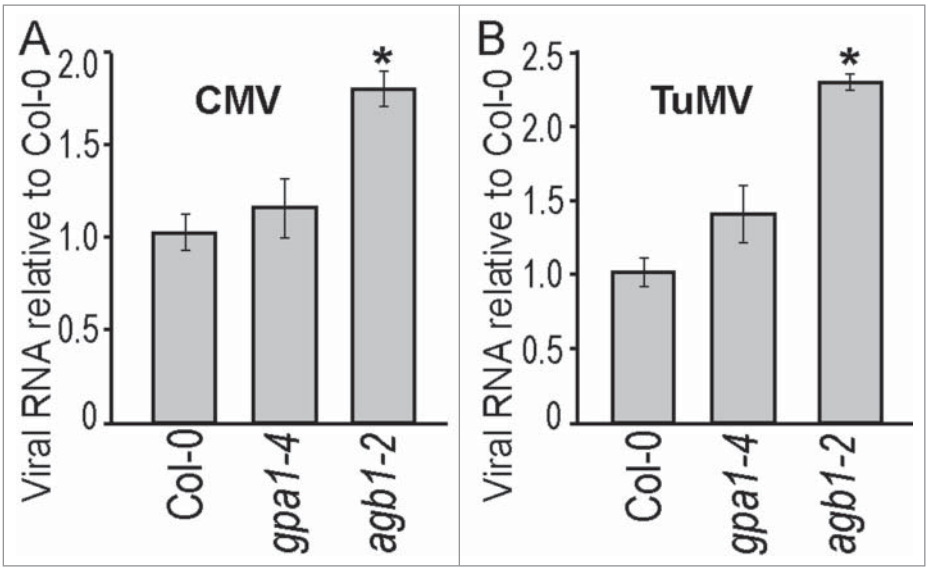

Figure 5. Viral RNA accumulation was higher in AGB1 deficient plants. Five-week-old Arabidopsis plants were inoculated with CMV or TuMV and assessed after 7 days; total RNA was extracted from leaves neighboring the ones used for infection. Viral RNA accumulation was quantified by RT-qPCR targeting the viral coat protein (*CP*) gene and using Arabidopsis *SAND* gene for normalization. (A) CMV *CP* gene accumulation. (B) TuMV *CP* gene accumulation. Values on the graph represent means of 4 independent biological replicates, error bars show standard error of the mean. Asterisks indicate statistically significant differences from wild type Col-0 (Student's t-test, $P < 0.05$).

The first and most striking observation we made was a superficial contradiction of visual symptom severity. On one hand, *agb1-2* rosette leaves showed little chlorosis and negligible amount of spreading necrosis, compared to WT leaves that were covered densely with yellow and brown areas. On the other hand, number of curled leaves and severely stunted inflorescences prevailed on *agb1-2* plants, while WT was significantly less affected in this respect. Usually, symptom severity correlates positively with pathogen progression and advance of the disease. Nevertheless, some symptoms, although indicative of the pathogen presence, are in fact a result of host defense systems activation. For example, formation of the necrotic lesions might be a result of programmed cell death, developed by plants to withstand attacks of obligate biotrophs and viruses.[47,57] These pathogens could not procreate in dead tissue; therefore, infection is usually contained (in case of incompatible interaction) or at least delayed. For these reasons we evaluated virus resistance by assessment of different types of symptoms and confirmed virus accumulation and movement by relative quantification of the viral RNA present in infected plants. Collectively, our data indicate that *agb1-2* mutant was more susceptible to both viruses compared to WT, despite showing less necrosis. The logical explanation for the latter is a participation of AGB1 in cell death development. This hypothesis was supported by reduced ion leakage and *GST1* expression in *agb1-2* mutant. AGB1 involvement in cell death has been suggested through its association with BIR1 (BAK1-interacting receptor like kinase 1).[24,28,58] Arabidopsis *bir1-1* mutant shows constitutive activation of defense response genes and generalized cell death resulting in early lethality at the seedling stage.[58] It was demonstrated that *agb1 bir1* double mutants displayed suppressed cell death observed in *bir1*.[28] Noteworthy, Gα deficiency was unable to reverse the *bir1* phenotype. Considering this data, we concluded that failure of the *agb1-2* mutant to perform cell death initiated by the host plants as a defense mechanism against viruses resulted in increased susceptibility.

Our gene expression analysis signified that *PR1*, a marker gene for SA-regulated immune response, was drastically induced by virus infection in *agb1-2* mutant, suggesting a role for SA in AGB1-mediated resistance. SA plays an important

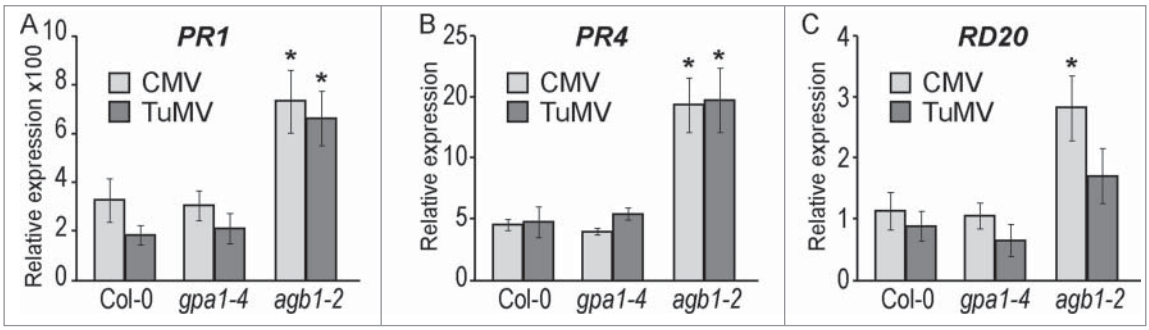

Figure 6. Expression of pathogenesis-related genes upon viral infection. Relative expression of (A) *PR1*, (B) *PR4*, and (C) *RD20* evaluated by RT-qPCR at 7 dpi. Values were normalized with Arabidopsis *SAND* gene. Values on the graph represent means of the 3 independent biological replicates, and error bars show standard error of the mean. Asterisks indicate statistically significant differences from wild type Col-0 (Student's t-test, $P < 0.05$).

role in plant defense and is essential for the initiation of cell death.[34,59-61] Importantly, external application of SA has been shown to increase resistance against TuMV in Chinese cabbage [62] and against CMV in tobacco and Arabidopsis by inhibiting viral replication and viral systemic movement.[45] On the other hand, plants expressing the bacterial *NahG*, and thus, producing negligible levels of endogenous SA, displayed wild-type resistance levels to the virus as reported previously [42,50,63] and confirmed by our experiments. This set of data suggests no role for endogenous SA in resistance against CMV. Our epistasis analysis also showed that endogenous SA did not affect AGB1-mediated response. This conclusion is in agreement with previous observations that AGB1- and SA-mediated pathways contribute independently to complementation of the *bir1-1* phenotype caused by enhanced cell death [28] and to defense against fungal pathogens.[15,17,20] Excessive upregulation of *PR1* expression in *agb1-2* mutant therefore, could be explained as a negative control of SA signaling by G-proteins.

We speculate that upon recognition of a virus, by yet unknown receptor(s) the signal transducing element, consisting of the heterotrimeric G-proteins, initiates the defense mechanism based on cell death. Thereby cell death deficient *agb1-2* plants inevitably become predisposed to faster viral replication and/or movement yielding higher levels of viral particles. The initial failure to contain the viral infection results in the increased severity of secondary disease symptoms (leaf curling and inflorescence inhibition) and stronger induction of the PR genes. We hypothesize that heterotrimeric G-protein signaling mediated through Gβ, but not canonical Gα, subunit contributes in defense response by promoting cell death-associated mechanisms. Future studies will clarify functional interactions between signaling elements of this plant defense pathway and reveal detailed mechanism of action.

Materials and methods

Plant material and experimental conditions

All *A. thaliana* mutant lines (*agb1-2, gpa1-4, NahG, NahG agb1*) were in the Columbia-0 background. All mutants including double knockouts *NahG agb1* had been previously described.[18,20] Plants were grown in University of California potting soil mixture in Percival growth chamber with 10hr-light/14hr-darkness photoperiod at 21°C/23°C with a relative humidity of 75% and a light intensity of 90 μmol $m^{-2}s^{-1}$.

Virus maintenance and plant inoculation

Turnip mosaic virus (TuMV) strain 2080 was originally isolated from wild radish in Victoria (Australia) and subsequently propagated in Pak Choy. *Cucumber mosaic virus* (CMV) strain 207 was originally isolated from tomato in Queensland (Australia) and belongs to subgroup IA, which causes severe symptoms on *Nicotiana* species [64]; it is closely related to the well-characterized isolate Fny (Owen et al., 1990), which causes severe symptoms in *A. thaliana* Col-0 (Zhang et al., 2006; Wang et al., 2010). Freeze-dried leaf samples stored at −20°C were re-activated by mechanical inoculation of 5-weeks-old *Nicotiana benthamiana* leaves. The infected plant was maintained at 23°C with a 12/12 hours light/dark cycle. Arabidopsis inoculation was described previously.[64,65] Briefly, 2-3 *N. benthamiana* leaves with obvious symptoms were removed and grounded in 5 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 20 mM sodium sulfite producing the inoculation solution. Three or 4 fully expanded leaves of 5-week-old Arabidopsis were wounded by gently rubbing with carborundum block, rub-inoculated with the inoculation solution and rinsed briefly with tap water. Control plants were treated with the buffer only. Inoculated and control plants were kept in growth chamber at 21°C/23°C with a 12/12 hours light/dark cycle with light intensity of 90 μmol $m^{-2}s^{-1}$ and 75% relative humidity in a Percival growth chamber.

Extraction and quantitative PCR with reverse transcription analysis

Total RNA was extracted from leaves as described.[66] RNA samples were treated with DNaseI (Invitrogen) as per manufacturer's instruction. Reverse transcription was performed with SuperScript III reverse transcriptase kit (Invitrogen) following manufacturer's instructions. Plant gene expression and viral CP RNA levels were evaluated by quantitative PCR with reverse transcription (RT-qPCR) using FastStart Essentials DNA Green Master (Roche Applied Sciences) following manufacturer's protocol in a 96 Light Cycler system (Roche). Primers for the selected genes used in gene expression analysis and for CMV and TuMV CP genes were: PR4-F TGCTACATCCAAATCCAAGCCT, PR4-R CGGCAAGTGTTTAAGGGTGAAG [20]; PR1-F AAGAGGCAACTGCAGACTCA, PR1-R TCTCGCTA ACCCACATGTTC,[20] RD20-F CCGAAGGAAGGTATGTCCCAG, RD20-R TTCGATTTCCCTCGGTTACATTC [20]; CMV-F TGAGAAAGTACGCCGTCCTC, CMV-R GATGTGGGAA TGCGTTGGTG; TuMV-F AAGACCGACCATACATGCCAC, TuMV-R CCTCTCTCGCACGTATTGGAG; GST1-F TAATAAAAGTGGCGATGACC, GST1-R ACATTCAAATCAAACACTCG.[67] Gene expression was analyzed with Light cycler 96 SW (version 1.1) software and normalized to reference gene, SAND (primers for RT-qPCR: SAND-F GTTGGGTCACACC AGATTTTG, SAND-R GCTCCTTGCAAGAACACTTCA).[68] Data was analyzed using method suggested in.[69]

Necrotic tissue staining

Detection of necrosis caused by cell death was carried out with trypan blue staining as described previously.[70] Briefly, leaves were boiled in lactophenol trypan blue solution (20 ml phenol, 20 ml lactic acid, 40 ml glycerol, 20 ml water and 0.05% trypan blue mix and added 200 ml 96% ethanol) for 2 min. Leaves were distained in chloral hydrate overnight and viewed under a Carl Zeiss Axio Scope A1 microscope equipped with interference or phase-contrast optics.

Quantification of ion leakage

The ion leakage was evaluated as described previously.[71] In brief, 3 days after inoculation, 5 leaves were collected from infected plants in 3 replicates. Six millimeter in diameter leave discs were cut out and placed in a tube with 5 ml of deionized

distilled water and shaken on a rotary shaker at 100 rpm at room temperature for 30 min. Conductivity of the resulted water solution was measured at designated time intervals with conductivity meter Orion 130 (Boston).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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