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SHORT COMMUNICATION

Reported differences in the flg22 response of the null mutation of AtRGS1 correlates with fixed genetic variation in the background of Col-0 isolates

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ABSTRACT

A role for the heterotrimeric G protein complex in the induction of a transient burst of reactive oxygen species (ROS) by the Microbial-Associated Molecular Pattern, flg22, a 22-amino acid peptide derived from bacterial flagella, is well established. However, the evidence for a negative or positive role for one component of the Arabidopsis G protein complex, namely, Regulator of G Signaling 1 (AtRGS1) leads to opposing conclusions. We show that the reason for this difference is due to the isolate of Col-0 ecotype used as the wildtype control in flg22-induced ROS and our data further support the idea that AtRGS1 is a negative regulator of the flg22-induced ROS response. Whole-genome genotyping led to the identification and validation of polymorphism in five genes between two Col-0 isolates that are candidates for the different ROS response relative to the *rgs1* null mutant.

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A clear role in innate immunity in Arabidopsis for both the canonical and atypical heterotrimeric G protein complex has been demonstrated by several labs.[1–6] In Arabidopsis, the canonical G protein complex contains a Gα subunit (AtGPA1) having a crystal structure nearly identical to the animal Gα subunit[7] but has different nucleotide-binding properties to the animal counterpart. Invertebrates and some yeast Gα subunits exchange GDP for GTP at an intrinsic low rate that is further catalyzed by 7-transmembrane receptors.[8,9] GTP-bound Gα subunits represent the activated state. This GTP is hydrolyzed to GDP with an intrinsic low rate in both plants and animals and this rate is accelerated by Regulators of G Signaling proteins (RGS).[10] The GDP-bound state represents the resting state. The G protein complex also contains a Gβγ obligate dimer that plays a critical role in this pathway as evident by the severe pathogen susceptibility phenotypes.[6,11] Arabidopsis has a set of three atypical Gα subunits called Extra-Large G proteins (XLG) that do not likely bind or hydrolyze guanine nucleotide *in vivo*[12] yet still interact with AtRGS1 and the Gβγ obligate dimer.[12,13] Specifically, XLG2 has an important role in innate immunity.[1,14–16] It is also well established that the Arabidopsis complex contains a 7-transmembrane RGS protein called AtRGS1 shown to accelerate the intrinsic GTP hydrolysis rate of AtGPA1.[17] Because AtRGS1 accelerates GTP hydrolysis, it is considered a repressor of the active state; however, this is not always clear because the loss of AtRGS1 revealed that it has a positive role in G protein-mediated gene expression.[18]

Innate immunity is the first line of detection of and defense against pathogens.[19] Microbial-Associated Molecular Patterns (MAMPs) shed from both pathogenic and nonpathogenic micro-organism are recognized by plasma membrane receptors and recognition by the plant cell initiates a cascade of molecular events that lead to passive defense mechanisms such as strengthening the barrier and creating a less hospitable environment to the invader such as altering the redox of the microenvironment by the production of reactive oxygen species (ROS), a so-called ROS burst. One such MAMP is a 22-amino acid fragment from the bacterial flagellum called flg22.[20] flg22 is recognized by its cognate receptor FLS2. The ligated FLS2 forms a complex with its co-receptor BAK1,[21] triggering a series of signaling events including the phosphorylation and subsequent endocytosis of RGS1.[3]

To determine if AtRGS1 plays a role in the flg22-induced ROS burst, Tunc-Ozdemir[3] genetically ablated AtRGS1 (*rgs1*-2) and found unexpectedly that *rgs1* mutants had less ROS burst, inconsistent with AtRGS1 serving as a negative regulator. Further complicating the phenotype of the *rgs1* mutant is subsequent data from Liang et al.[2] showing that the *rgs1*-2 mutant had a greater flg22-induced ROS burst. The latter report is likely to be correct because this experiment was performed by first backcrossing the *rgs1*-2 mutant into a Col-0 ecotype and using the segregated wild type and mutant AtRGS1 loci for comparison. The former report used a Col-0 that had not been generated through crosses with the *rgs1*-2 mutant. Tunc-Ozdemir used a Col-0 line that was sequenced (Col-0 seq) and broadly distributed to the Arabidopsis research community[22] including our group. However, the original *rgs1*-2 line was generated by a pool of Col-0 seeds originating from the Salk Institute (Col-0 Salk) which we obtained for this study.

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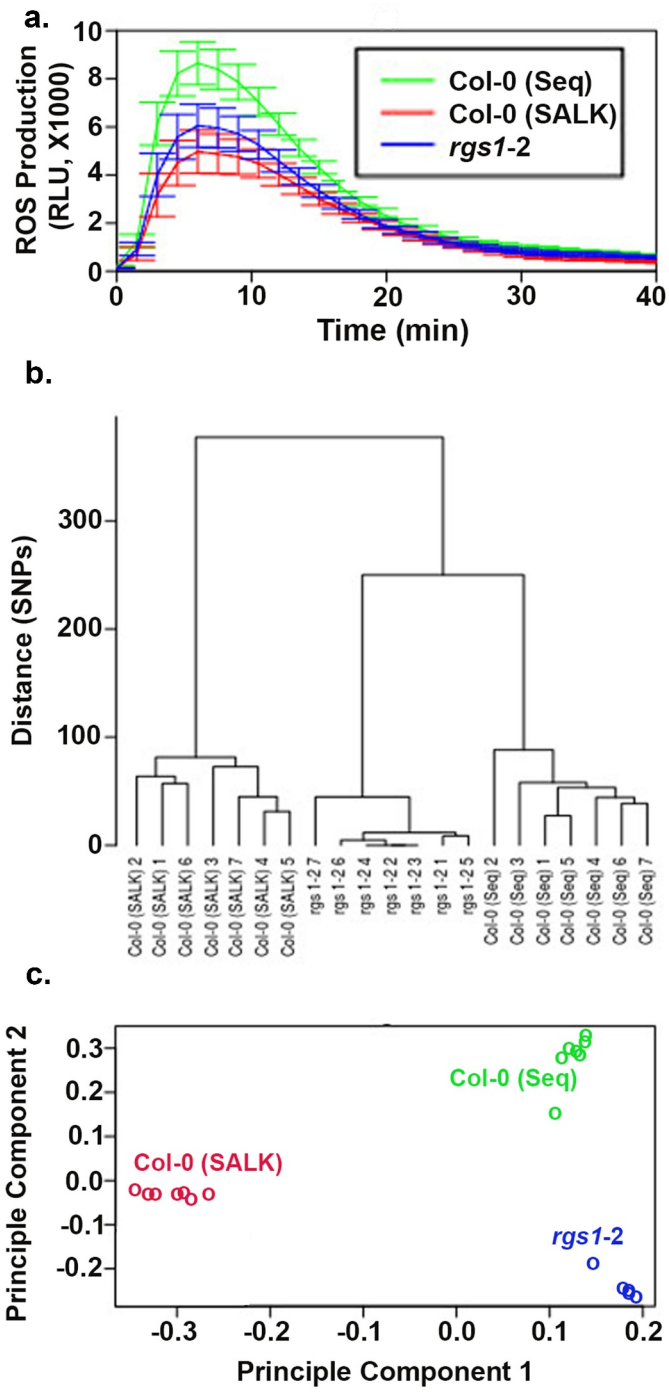

Figure 1. The relative difference in flg22-induced ROS production in the *rgs1*-2 mutant is dependent on the isolate of the Col-0 ecotype. (a). The Col-0 (Seq) isolate was provided by Sally MacKenzie and described in Shao et al.[22] The Col-0 (SALK) isolate was provided by Dr. Jason Reed and described in Alonso et al.[23] ROS production over time was monitored by the method of Chung et al.[24] Flg22 (100 nM) was added at time zero. The solid lines are the means of 16 individual leaf disks, with the shaded area representing the 95% confidence interval (2 × SEM). This experiment was replicate 3 times with the same results. (b). Dendrogram representing distance among isolates built with the set of markers identified in our variant call analysis; (c). PCA analysis using SNPrelate based on same set of markers.

The flg22-induced ROS burst of the *rgs1*-2 mutants in the JonesLab was compared to the two Col-0 pools (Figure 1a). The newer Col-0 seq isolate showed a greater ROS burst than *rgs1*-2 as Tunc-Ozdemir reported whereas the original Col-0 Salk pool showed a lesser ROS burst as reported by Liang et al.

A common approach to analyze the contribution of single genes to quantitative traits is to contrast the values for that trait between a wildtype and a mutant for the gene in the same background as the wildtype. Because *A. thaliana* is self-fertilizing, and because their wildtype ecotypes are maintained by many generations of selfing, it is often assumed that the batches of *WT* are highly homozygous and genetically homogeneous. However, if the propagation of *WT* stocks is not done properly, and because of a *de novo* haploid single nucleotide mutation rate (6.95 10^{-9} per site per generation),[25] it is possible to introduce genetic variation in the *WT* lines. We suspect that genetic variation unintentionally introduced in the Col-0 accession is responsible for these disparate phenotypes.

To measure the extent of genetic divergence in *rgs1*-2 and the two Col-0 isolates from the reference genome and to screen for the genetic variance that may have caused the difference in the ROS burst, we sequenced the genomes of each of seven individuals of *rgs1*-2 and the two Col-0 isolates. We aligned the reads to the *A. thaliana* reference genome (TAIR10, https://www.arabidopsis.org/index.jsp) with the bwa mem (version 07.17) package. We marked and removed PCR duplicates using Picard tools (https://broadinstitute.github.io/picard/) and searched for the variants that better distinguish between the 21 lines using the GATK HaplotypeCaller (version 4.1.2.0). We generated genomic variant calling files (GVCF) on each sample individually. Workflow, logic, filtering, and validation of variants are described in detail in Supplemental Information S1. For the identification of strains, we implemented the SNPmatch package.[26] The results indicate that all the 21 plants belong to the Col-0 accession with no WS-2 ecotype SNPs detected; WS-2 is a frequent source of contamination,[22] Supplemental Information S2. Next, we identified variants that are consistently shared between all the plants of the same line. We found 118 variants (101 SNPs and 17 indels) that were validated with a separate set of data. The validated variants were used for PCA analysis with the SNPrelate package. The first two principal components (PC1 and PC2) accounted for 51.5% and 33.6% of the total variability originated by the segregation of these 118 alleles between the 21 plants analyzed plants (Figure 1c). Clearly, the three lines are separated in the PC1-PC2 space. PC1 separated Col0 (SALK) from Col-0 (seq) and *rgs-1*, while PC2 separated Col-0 (seq) from *rgs-1*. Finally, we applied hierarchical clustering on the validated SNPs to build a dendrogram to represent distances between the different plants, Figure 1b. Thus, in the present report, we identified genetic divergence that separates plants according to their respective lines. It is expected that the presence of genetic polymorphism on *loci* other than the AtRGS1 gene may have a strong effect on ROS production, explaining the observed discrepancy in the data by Tunc-Ozdemir[3] who used Col-0 (Seq) as the wild-type control, and Liang et al.[2] who used an isogenic wild-type control. Most importantly, this extra source of genetic variation between mutant and wildtypes seems to overcome the effects of AtRGS1 on ROS production.

Table 1. Validated variant genes between the two Col-0 isolates. See Table S1 for the filtered list of variant genes.

Locus	Gene	Description	Variant to reference genome	Mutation
AT1G10760	SEX1	Pyruvate phosphate dikinase, PEP/pyruvate binding domain-containing protein	Col-0(Seq)	Missense
AT1G58400		Disease resistance protein (CC-NBS-LRR class) family	Col-0(Salk)	Missense
AT3G53960		Major facilitator superfamily protein	Col-0(Seq)	Missense
AT5G09330	VNI1, ANAC82	transcription factor ANAC family	*rgs1*-2 and Col-0(SALK)	Premature start
AT5G50450		Transcription factor MYND zinc finger family	*rgs1*-2 and Col-0(SALK)	Start codon lost

Thus, whether AtRGS1 is deemed as a negative or positive contributor depends on the Col-0 isolate used as control.

Overall the results (Figure 1a-c) show genetic structure separating the three analyzed lines, indicating that the assumption of population homogeneity between different batches of WT plants or between a single mutant and its WT can lead to inaccurate conclusions. Moreover, we observed that the background of the mutant plant is the one that diverged the most from the reference genome. We do not know how pervasive is this phenomenon with respect to other mutant lines maintained by different laboratories, but we hope our results raise a cautionary red flag when phenotyping mutants and wild-type isolates.

To obtain candidate genes that may be involved in the flg22-induced, AtRGS1-dependent ROS burst, we applied the SNPeffect package to predict phenotypic effects from the detected genetic differences. Supplemental Information S3 lists 44 genes that contain missense and nonsense mutations relative to the Col-0 reference genome for the three genotypes in this study, *rgs1*-2, Col-0 (Seq), and Col-0 (SALK). Five variants are the same in the two Col-0 isolates and 14 of the remaining 44 genes have mutations different than the reference genome in all three of the genotypes leaving only 25 candidate mutations to explain the difference in ROS burst behavior relative to the wild type. The *rgs1*-2 mutant shared the reference Col-0 allele in 19 genes and differed in 6 genes. Validation of the variants using individual genomes prepared with deep sequencing eliminated the problem of poor coverage for some of the 21 sequenced genomes leaving five genes (Table 1), specifically a pyruvate metabolism enzyme, a disease resistance protein, a hexose transporter, and 2 transcription factors. There are a large number of SNPs in noncoding regions including promoters, in mitochondrial DNA, and in transposons that cannot be ruled out as the variant that causes this effect on the amplitude of ROS production by flg22.

Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

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