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Analysis of RGS1 phosphorylation pattern in response to hormones and abiotic stresses in *Arabidopsis thaliana*

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Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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A mi abuelita Maria, que me guía
y me cuida desde el cielo.

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ABSTRACT

ALCIVAR, Borys Alexander Leon, M.Sc., Universidade Federal de Viçosa, March, 2025. **Analysis of RGS1 phosphorylation pattern in response to hormones and abiotic stresses in *Arabidopsis thaliana***. Adviser: Pedro Augusto Braga dos Reis. Co-advisers: Agustin Zsogon and Elizabeth Pacheco Batista Fontes.

Plants utilize receptor-like kinases (RLKs) and the seven-transmembrane protein RGS1 to regulate G protein activity as a core part of their sophisticated defense mechanisms against various stresses. In this study, we explored the regulatory function of the RGS1 protein, a master regulator in G-protein signaling, with a particular focus on the role of phosphorylation at the serine residues. Our findings indicate that phosphorylation of S278 is crucial for fine-tuning several key plant responses. Water deficit assays revealed that the S278E phosphomimetic mutant exhibited increased tolerance, whereas the S278A phospho-null mutant was more susceptible, strongly suggesting that phosphorylation at Ser278 is a vital component of drought stress tolerance. We also discovered that the role of RGS1 in other hormonal pathways is complex. While our dark germination assays did not yield significant differences between mutants and the wild type, our results with ABA showed that phosphorylation at Ser278 may attenuate the previously reported hypersensitivity effect of the hormone. The results of the response to osmotic and saline stress could not be interpreted. However, protein-protein interaction studies using BiFC assays demonstrated that the RGS1-BAK1 complex dynamically dissociates upon the perception of immune (flg22) and brassinosteroid signals, with evidence suggesting that phosphorylation influences these interactions. These results highlight RGS1 as a sophisticated regulatory hub, whose function is precisely controlled by multi-site phosphorylation to modulate plant responses to a wide array of environmental and hormonal cues.

Keywords: G-protein-mediated signaling, serine, phosphomimetic, ABA, brassinosteroids

RESUMO

ALCIVAR, Borys Alexander Leon, M.Sc., Universidade Federal de Viçosa, março de 2025. **Análise do padrão de fosforilação RGS1 em resposta a hormônios e estresses abióticos em *Arabidopsis thaliana***. Orientador: Pedro Augusto Braga dos Reis. Coorientadores: Agustin Zsogon e Elizabeth Pacheco Batista Fontes.

As plantas utilizam quinases semelhantes a receptores (RLKs) e a proteína de sete transmembranas RGS1 para regular a atividade da proteína G como parte essencial de seus sofisticados mecanismos de defesa contra vários tipos de estresse. Neste estudo, exploramos a função reguladora da proteína RGS1, um regulador mestre na sinalização da proteína G, com foco particular no papel da fosforilação nos resíduos de serina. Nossas descobertas indicam que a fosforilação de S278 é crucial para o ajuste fino de várias respostas importantes das plantas. Testes de déficit hídrico revelaram que o mutante fosfomimético S278E apresentou maior tolerância, enquanto o mutante fosfo-nulo S278A foi mais suscetível, sugerindo fortemente que a fosforilação em Ser278 é um componente vital da tolerância ao estresse hídrico. Também descobrimos que o papel do RGS1 em outras vias hormonais é complexo. Embora nossos ensaios de germinação no escuro não tenham produzido diferenças significativas entre os mutantes e o tipo selvagem, nossos resultados com ABA mostraram que a fosforilação em Ser278 pode atenuar o efeito de hipersensibilidade do hormônio relatado anteriormente. Os resultados da resposta ao estresse osmótico e salino não puderam ser interpretados. No entanto, estudos de interação proteína-proteína usando ensaios BiFC demonstraram que o complexo RGS1-BAK1 se dissocia dinamicamente após a percepção de sinais imunológicos (flg22) e brassinosteroides, com evidências sugerindo que a fosforilação influencia essas interações. Esses resultados destacam o RGS1 como um sofisticado centro regulador, cuja função é controlada com precisão pela fosforilação em vários locais para modular as respostas das plantas a uma ampla gama de estímulos ambientais e hormonais.

Palavras-chave: Sinalização mediada pela proteína G, serina, fosfomimético, ABA, brassinosteroides

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1. INTRODUCTION

Throughout evolutionary history, plants have been subjected to various types of biotic and abiotic stresses, so they developed mechanisms capable of perceiving these signals and being able to survive under these conditions. Plants utilize two main lines of pre-established defense to resist pathogens: physical fortifications provided by the cuticle and the cell wall, and chemical protection from synthesized antimicrobial substances (Bigeard et al., 2015; Yuan et al., 2021). Once they overcome these barriers, pathogens must face the immune system of plants. The first level is pattern-triggered immunity (PTI) which is triggered by the recognition of conserved pathogen-associated molecular patterns (PAMPs) recognized by pattern recognition receptors (PRRs); while the second level is effector-triggered immunity (ETI), triggered by leucine-rich nucleotide-binding repeat receptors (NLRs) that recognize effectors (Nabi et al., 2024; Yuan et al., 2021). Plants use cytoplasmic kinases and receptor-like kinases (RLKs) and receptor-like proteins (RLPs) for stimulus differentiation. RLKs represent one of the largest gene families in plants, with more than 600 genes identified, and in several cases the ligands and signal transduction pathways are already known (Urano et al., 2013). This signal transducer complex is involved in several important biological pathways, controlling of cell division and expansion, regulation of ion channels, modulation of hormonal responses, response to pathogens, and response to various environmental variables such as light, drought and salinity (Zhang et al., 2021).

In this context, one of the most relevant complexes involved in these signaling are the heterotrimeric G proteins, which mediate processes between the perception of signals at the plasma membrane with cellular amplifiers and thus trigger specific responses to stimuli (Pandey, 2019). This complex is conserved in all eukaryotic cells, however, there are some differences between G proteins in animals and plants. The G proteins are composed of α, β and γ subunits, modulated by the nucleotide-binding state (GTP/GDP) (Urano et al., 2013). In animal cells, the exchange of GDP to GTP is carried out by G protein-coupled receptors (GPCRs), which act as transmembrane receptors (Pandey, 2019). The plant Gα subunit exhibits a self-activating property allowing spontaneous GDP–GTP exchange, obviating the need for classical GPCRs for activation. Instead, plants employ a seven-transmembrane protein, known as regulatory G protein signaling (RGS), to accelerate the intrinsic slow GTPase activity of Gα and bringing the G protein complex in an inactive state (Liang et al., 2018; Pandey, 2020). This dynamic between activation and deactivation, where the limiting step is controlled by RGS, enables rapid and tightly regulated responses.

G protein-mediated signaling serves as a crucial hub for integrating diverse environmental information into a plant's intracellular signaling network. Specifically, it translates cues regarding nutrient availability and biotic stress into a cellular response, partly through a process of endocytosis of RGS (J.-G. Chen et al., 2003; Urano et al., 2012). When environmental signals like glucose or its metabolites (mannose, fructose and sucrose) or pathogen patterns such as flg22, phosphorylation occurs at specific RGS residues which facilitates their dissociation from the G-protein complex and subsequent signal propagation (Oliveira, 2022).

One of the best-studied pathways is that activated by the microbial peptide *flg22*, which is recognized by the FLS2 (Flagelin-sensitive 2) receptor, which in turn interacts with BAK1 (Brassinosteroid Insensitive 1) (Chinchilla et al., 2007) and other receptor-like cytoplasmic kinases (RLCKs), such as BIK1 (Botrytis-Induced Kinase 1) (Lu et al., 2010), to amplify the immune signal through phosphorylation-based mechanisms. This active immune complex interacts with G-protein components (both canonical and extra-large proteins) and phosphorylates the RGS1 protein, specifically by BIK1/PBLs (Liang et al., 2018). This results in the release of G proteins from RGS1-mediated repression and allows activation of the immune system. While the ETI process is activated, RGS1 internalization occurs through the formation of the VPS26A/B (β-arrestin-like) heterodimer in the clathrin-mediated endocytosis (CME) pathway (Lou et al., 2024).

In addition to their important role in signaling biotic stress, evidence has been found of the role of some components of the heterotrimeric G-protein subunits in both hormone signaling and abiotic stress response (Jose & Roy Choudhury, 2020; Wu & Urano, 2018). Seed germination and post-germinative growth are inhibited by ABA, and this process is negatively regulated by GPA1(Gα) and AGB1(Gβ). The *gpa1* and *agb1* null mutants exhibit increased susceptibility to ABA-induced inhibition of germination, with AGB1 acting downstream of GPA1 as it inhibits cell division during lateral root development (Liu et al., 2007; Ullah et al., 2003). In addition, they also participate in the regulation of anion and K channels in guard cells, influencing the response to ABA, because *gpa1* and *agb1* are hyposensitive to ABA inhibition of stomata opening (Fan et al., 2008; Wang et al., 2001). On the other hand, *gpa1*, *agb1* and *gcr1* mutants are less sensitive to gibberellic acid (GA) and brassinosteroids (BR) during seed germination (J.-G. Chen et al., 2004). Furthermore, GPA1 and AGB1 positively modulate hypocotyl growth in response to BR, thus these subunits act as positive regulators of BR signaling (Jose & Roy Choudhury, 2020).

The Arabidopsis AtRGS1 protein is composed of conserved domains, which begin with a region of seven transmembrane domains (7TM), a flexible binding region, followed by an RGSbox domain and a disordered C-terminal tail (J.-G. Chen et al., 2003). Within these domains, phosphorylation sites have been identified and are important in various cellular processes. There are *in vitro* and *in vivo* evidence of their phosphorylation caused by RLKs (e.g. WNK8, BRL3, BIK1) (Liang et al., 2018; Tunc-Ozdemir et al., 2017). The serine residue at position 278 (Ser278) is important for the dissociation of RGS1 from the G-protein complex (reducing the interaction with GPA1), a fundamental modulator in the processes of internalization, stability and translocation of nuclei (Simoni, 2024). Likewise, the importance of Ser278 phosphorylation for the phosphorylation of multiple downstream serine groups was evidenced, since there is a high correlation and allows the movement and flexibility of the distal serine group (Oliveira, 2022). These phosphorylation sites of this protein will allow fine-tuning of the signaling responses in which it participates.

Therefore, the present study focuses on analyzing the impact of mutations in specific phosphoserines of RGS1, evaluating the responses generated after exposure to various hormones and abiotic stresses. Taking advantage of complement lines expressing RGS1 and its phosphomimetic or phosphonull mutants, we evaluated the role of specific residues under various treatments with different plant hormones, including ABA, SA, and BR. Additionally, we assessed the impact of these residues on interactions with other membrane proteins, such as FLS2 and BAK1, which are responsible for perceiving and transmitting signals related to hormone responses and bacterial infection. Our findings demonstrate that specific residues are critical for discriminating between stimuli and for modulating protein-protein interactions, although further experiments are needed to understand the signaling mechanism within this new context.

2. MATERIALS AND METHODS

2.1. Plasmid construction

SPYCE and SPYNE vectors, which fused the carboxyl or amino terminus of YFP protein to the RGS1 proteins, respectively, were constructed for each of the target sequences. For this purpose, an LR reaction of the Gateway cloning system was performed, using a donor vector with the sequence of interest (pDONR221/207) and inserted into the SPYCE/NE expression vectors. The plasmids produced were as follows: AtBAK1-SPYCE/NE (pUFV3030/3029), AtFLS2-SPYCE/NE (pUFV3032/3031), AtRGS1-SPYCE/NE

(pUFV3054/3053), AtRGS1^{S278D}-SPYCE/NE (pUFV3502/3501), AtRGS1^{S278A}-SPYCE/NE (pUFV3504/03), AtRGS1$^{S278/428/430/431/435/436A}$-SPYCE/NE (S278A-clusterA; pUFV3584/3583), AtRGS1$^{S278A+S428/431/435E}$- SPYCE/NE (S278A-q2q4q6; pUFV3588/3587), AtRGS1$^{S278E+S428/430/431/435/436A}$-SPYCE/NE (S278E-clusterA; pUFV3586/3585), AtRGS1$^{S278/428/431/435E}$-SPYCE (S278E-q2q4q6; pUFV3590/3589). These vectors were constructed in the Laboratory of Plant Molecular Biology (Bioagro-UFV-BR) in collaboration with the laboratory of Dr. Alan Jones (UNC-US).

2.2. Genetic material

Arabidopsis thaliana ecotype Columbia 0 (Col-0) was used as a control in all experiments. The *rgs1-2* (SALK_074376.55.00), *bak1-3* (SALK_034523), and fls2 (SAIL_691_C4) transferred DNA (T-DNA) mutants were used. The *rgs1-2* complemented lines with AtRGS1wt, also the mutants AtRGS1^{S278E}, AtRGS1^{S278A} and AtRGS1$^{S428/431/435/436A}$ (quadA) fused to YFP tag were provided by the laboratories of Dr. Alan Jones and Dr. Elizabeth Fontes.

2.3. Water deficit assay

Seeds were germinated in soil in a short-day photoperiod growth chamber (8 h/day, 16h/dark). After one week, the seedling was transplanted into individual pots and maintained under constant irrigation for 4 weeks. After that, irrigation was interrupted for 20 days. During this period, leaves were randomly collected from each genotype to calculate relative water content (RWC). For this purpose, fully expanded leaves were collected in a Petri dish with wet paper and their fresh weight (FW) was recorded as soon as possible, to minimize water loss by evaporation. The leaves were then rehydrated to full turgor by placing the petiole in water and constant light for at least 12 hours to obtain the turgid weight (TW). Then, the leaves were dried in an oven at 65°C for 24 hours (or until a constant weight was obtained) to obtain the dry weight (DW). Finally, calculations were made using the following equation: RWC (%) = [(FW - DW) / (TW - DW)] x 100. Samples were taken on days 4, 7, 10, 13, 17 and 20. On the last day of the assay, the plants were rehydrated, and the survival rate was counted.

2.4. Germination rate assay in response to hormones and abiotic stress

Seeds were surface sterilized with a disinfection solution (4:3:1 alcohol:water:bleach) for 10 min, followed by two washes with 96% alcohol and dried until the alcohol had completely evaporated. Sterile seeds were germinated in ½ MS medium with 1% sucrose at pH

5.7 and solidified with 0.8% phytoagar. For the treatments, the media were supplemented with Abscisic Acid (ABA) (1 µM), Salicylic Acid (SA) (250 µM), Mannitol (200 mM) and NaCl (75 mM). For each treatment, a medium without these compounds was used as a control.

The plates were stratified in the dark at 4°C for 24 h. Then, they were transferred to a growth chamber under controlled conditions, with a long-day photoperiod (16 h/light, 8 h/dark) and temperature of 22°C. The germination rate was counted after the second day until the fifth day. Seeds were considered germinated when the radicles completely penetrated the seed coat.

In addition, a treatment with brassinosteroid (24-Epibrassinolide; EBL 2 mM) and a brassinosteroid synthesis inhibitor (brassinazole; Brz 2 mM) were performed. Seeds were germinated under the same conditions and hypocotyl length was measured at day 6. A photographic record was taken and processed with ImageJ software.

2.5. Germination assay in dark

Seeds of the mutants were sterilized and sown in ½ MS medium (same composition as described above). Seeds were stratified at 4°C for 24h, then exposed to 3 hours of light and finally kept in total darkness for 6 days. Afterwards, photos were taken of the etiolated hypocotyls and fully extended roots. The images were processed with ImageJ software.

2.6. Spatial analysis of RGS protein in response to hormones and abiotic stresses

Seeds expressing the RGS1 and the mutant fused to the YFP reporter were sown on solid ½MS medium (described above) for 3-4 days. Seedlings were treated with EBL 2 µM, Brz 2 µM, ABA 1 µM, SA 1 mM and NaCl 100 mM for one hour. Water was used as a control (mock). Images were captured by a Zeiss confocal microscope model LSM 900 Axio Observer. YFP excitation was performed at 514 nm, and the emission detected was in the range of 525-565 nm. The images were analyzed in the manufacturer's software (ZEN Lite 3.10).

2.7. Transient expression in tobacco epithelial cells

The previously mentioned vectors (200 ng) were transformed into *Agrobacterium tumefaciens* (strain GV3101) by electroporation (2.0 kV and 200Ω) and cultured for 48h at 28C. In addition, AtWWP1-SPYNE and WWP1-SPYCE vectors were used as positive control, due to previous interactions results (Calil, 2017). HC-Pro-PVX vector was add to each combination to facilitate the replication of the vectors in tobacco cells. The cell culture was centrifuged at 2500 x g for 5 minutes, followed by washing with infiltration buffer (10 mM MgCl2, 10 mM MES pH5.6 and 100 µM acetosyringone); this step was performed three times. Next, dilutions

were performed to obtain a total OD_{600} of 1 (0.45 for SPYCE/NE vectors and 0.1 for HC-Pro-PVX). *Nicotiana benthamiana* leaves (4-5 weeks old) were infiltrated using sterile syringes and gently pressed on the abaxial surface of the epidermis. Finally, the plants were left to express 72h in constant light (24h/day). Images were captured by a Zeiss confocal microscope model LSM 900 Axio Observer 7. The images were analyzed in the manufacturer's software (ZEN Lite 3.10).

2.8. Statistical analysis

Statistical analyses were conducted using RStudio (version 4.4.2). Continuous data were assessed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) to validate parametric assumptions. For comparisons between the values obtained for each of the mutants and the wild-type control (Col-0), two-tailed independent t-tests were employed. All tests used $\alpha = 0.05$ as the significance threshold. Data are presented as mean ± standard deviation (SD), with exact *p-values* reported where applicable. Analyses utilized core R packages (dplyr, tidyr) and visualization tools (ggplot2).

3. Results

3.1. Phosphorylation of RGS increases tolerance to water stress

RGS1 is involved in growth and development of some organs such as leaves, stems, siliques and hypocotyls, as well as being related to the transduction of drought stress signals (Y. Chen et al., 2006). To determine whether RGS1 protein phosphorylation sites are involved in the regulation of drought tolerance, a soil water deficit assay was performed. For this assay, Col-0 was used as wild-type control, *rgs1* knockout mutant (*rgs1-2*), RGS1 complemented line (hereafter RGS1wt), RGS1^{S278E} phosphomimetic mutants (hereafter S278E) and the RGS1^{S278A} (hereafter S278A) and RGS1$^{S428/431/435/436A}$ (hereafter quadA) phospho-null. For all lines in the control group an RWC of between 80 and 100% was maintained (Figure 1A). Water loss was consistent in all plants subjected to water stress, as there were no significant differences in RWC. A sudden decrease in water content was observed after day 13, which continued until day 20, reaching values of between 25 and 30%. After that, rehydration was carried out and the survival rate was determined.

The survival rate of the wild type was 17%, with similar values for the RGS1wt, quadA and rgs1-2 mutants. The S278A mutant showed extreme sensitivity to water stress, with a survival rate of 0%. In contrast, the S278E mutant showed the highest tolerance to stress,

presenting the highest survival rate (25%) among all genotypes evaluated (Figure 1B). Plant phenotypes reflect these differences in stress response. While the rgs1-2 and S278A mutants began to show signs of yellowing and wilting by day seven, the S278E mutant, despite also experiencing early chlorosis, was able to maintain leaf turgor for a longer period. The quadA, RGS1wt, and Col-0 control lines, however, were notably more resilient, showing no visible signs of stress—such as yellowing or wilting—during the first seven days of the experiment.

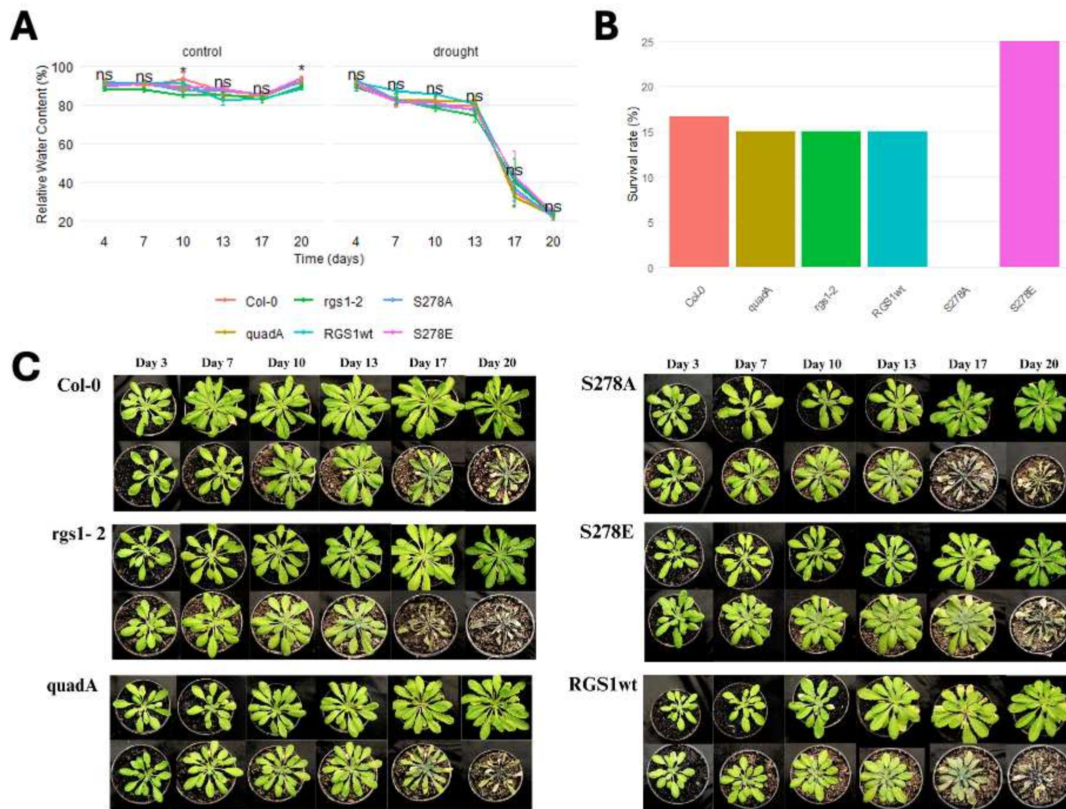

Figure 1. Phenotypic evaluation of lines subjected to water stress. A) Estimation of Relative Water Content (%) during the 20 days of the experiment. B) Survival rate of lines after rehydration. C) Representative photographs of lines during the stages of the experiment. n=20 plants. *$P < 0.05$.

3.2. RGS1 shows differentiated growth in dark germination

A dark germination assay was conducted to determine if the phosphorylation sites are involved in light perception and its connection to hormone signaling. There were not morphological differences between the lines, which presented normal hypocotyl development and apical hook formation (Figure 2A). It was observed that both the RGS1wt, as well as the S278A and S278E mutants, presented a smaller hypocotyl size compared to Col-0 (Figure 2B).

In the case of root development, only the RGS1 and S278A lines showed smaller root size, while S278E did not show significant differences with Col-0.

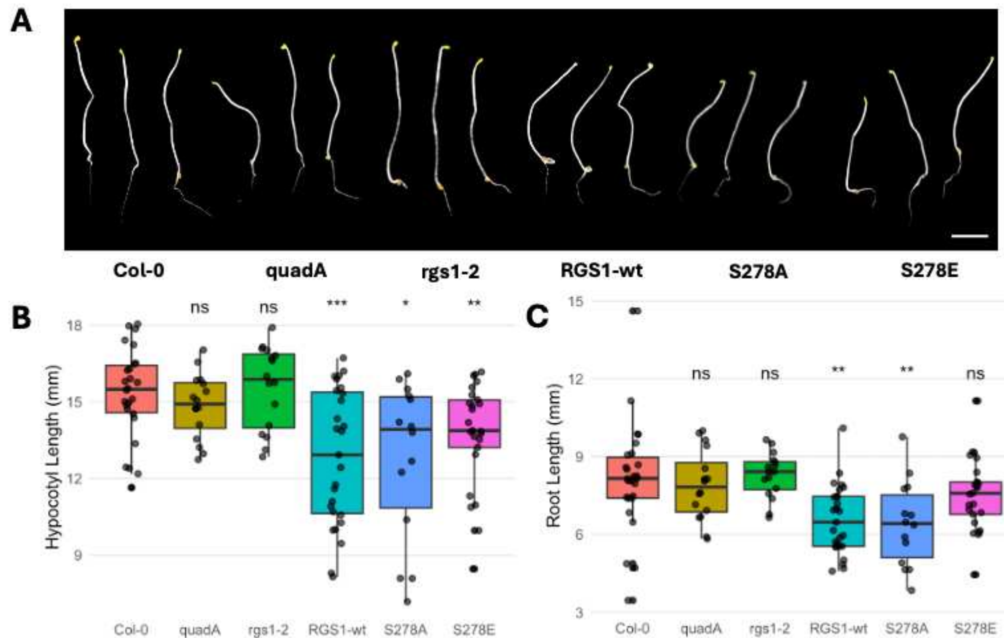

Figure 2. Germination of RGS1 mutants in the dark. A) Phenotype of etiolated seedlings for 6 days. Scale bar 5 mm. B) Length of hypocotyls and roots in dark-germinated seedlings. n=25 seedlings. *$P < 0.05$.

3.3. Brassinosteroids alter germination rate and location of RGS1

The RGS protein, together with G proteins, is involved in the signaling of several plant hormones. To understand the role of RGS1 and its interaction in this complex signaling network, germination assays were carried out. First, a germination assay of RGS mutants was developed with a brassinosteroid (EBL) and a brassinosteroid biosynthesis inhibitor (BRZ). In addition to exhibiting longer hypocotyls, no significant differences were observed in the morphology of mutants germinated on medium with EBL (Figure 3A). On the contrary, a reduction in the size of hypocotyls was observed in plants germinated in BRZ (Figure 3B). Hypocotyl length was quantified in both treatments, and no statistically significant differences were found (Figure 3C). In addition, a germination rate assay was performed over a five-day period to investigate the effects of the hormone on seed viability. Neither the brassinosteroid nor the inhibitor had a significant effect on the germination rate of the lines, except for the S278E mutant (Figure 3D). External addition of the hormone increased the germination rate, while the inhibitor reduced it, with these effects becoming evident from day two onward.

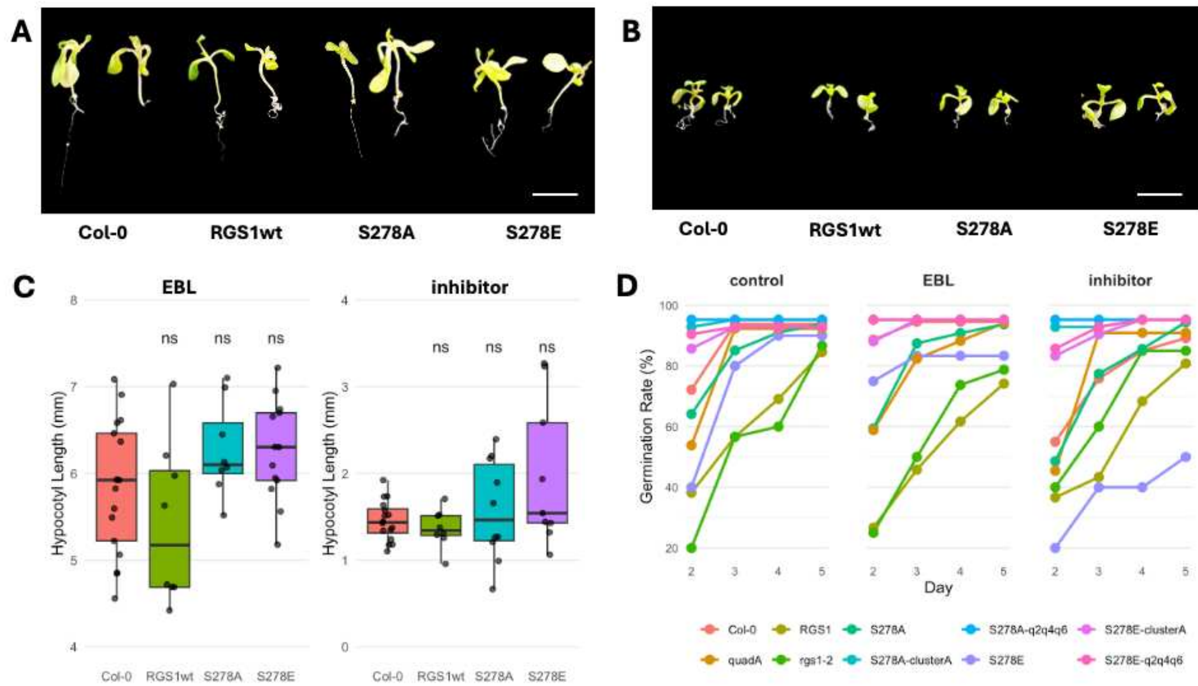

Figure 3. Effect of brassinosteroids on hypocotyl development. 6-day-old seedlings germinated with brassinosteroid (EBL 2 μM) (A) and the inhibitor brassinazole (BRZ 2 μM) (B). Scale bar 5 mm. C) Hypocotyl length of germinated seedlings in both conditions. D) Germination rate over 5 days. n = 10-15 seedlings. ns: not statistically significant ($P < 0.05$).

In addition, roots of these lines (YFP-fused) were observed under a fluorescence microscope. First, a mock treatment with water was performed. It was observed that constitutive expression of RGS occurs on the membrane surface of all mutants (Figure 4). Constant plasma membrane expression and internalization of the RGS protein was observed in the RGS1, S278E, and S278A lines when roots were treated with brassinosteroid. However, the quadA mutant exhibited a drastic drop in fluorescence, which may indicate RGS1 protein degradation under these conditions. Additionally, the S278E mutant showed the formation of protein spots or granules. Finally, treatment with the inhibitor BRZ was performed, resulting in observed changes in expression in all mutants. RGS1 and S278E exhibited greater intracellular scavenging, while quadA and S278A showed a decrease in overall protein expression.

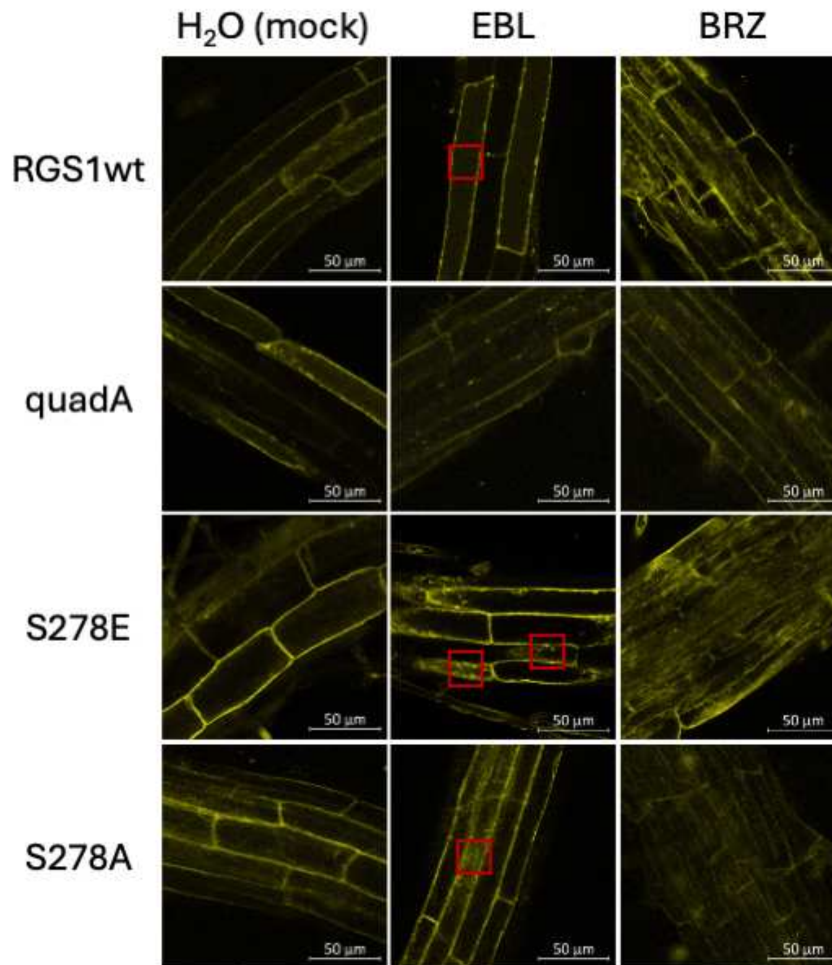

Figure 4. Cellular localization of RGS1 mutants in roots treated with H_2O (mock), EBL (2uM) and BRZ inhibitor (2uM) for one hour. Plants 3-4 days old. Red box: internalization/protein accumulation of RGS1. Scale bar 50 µm.

3.4. Hormones and abiotic stress affect germination rate and location of RGS1

To evaluate how different stress conditions impact germination, seeds were treated with mannitol, NaCl, and abscisic acid (ABA). Under normal conditions, no morphological differences were observed between the lines (Figure 5A). Mannitol treatment caused no change in root morphology but resulted in leaf epinasty (Figure 5B). Exposure to NaCl led to symptoms of nutrient toxicity, characterized by discolored leaves (Figure 5C). ABA had the most severe effect, significantly impairing germination and preventing the development of green seedlings (Figure 5D).

In addition, an evaluation of the germination rate was carried out, where some alterations in seed viability were observed (Figure 5E). The rgs1-2, quadA, S278A, and S278E mutants showed a significant decrease in the initial germination rate (day two) under mannitol

stress conditions. However, the final germination rate on day 5 was like that under normal conditions, meaning that only a delay in the germination rate. Similarly, the initial germination rate decreased in the RGS1, rgs1-2, S278A, S278E, and quadA mutants when treated with NaCl. Over time, the number of seeds in these lines increased until it reached values like the control. However, when these lines were treated with ABA, a drastic reduction in germination was observed in the S278A and RGS1 lines, which persisted until the last day of evaluation.

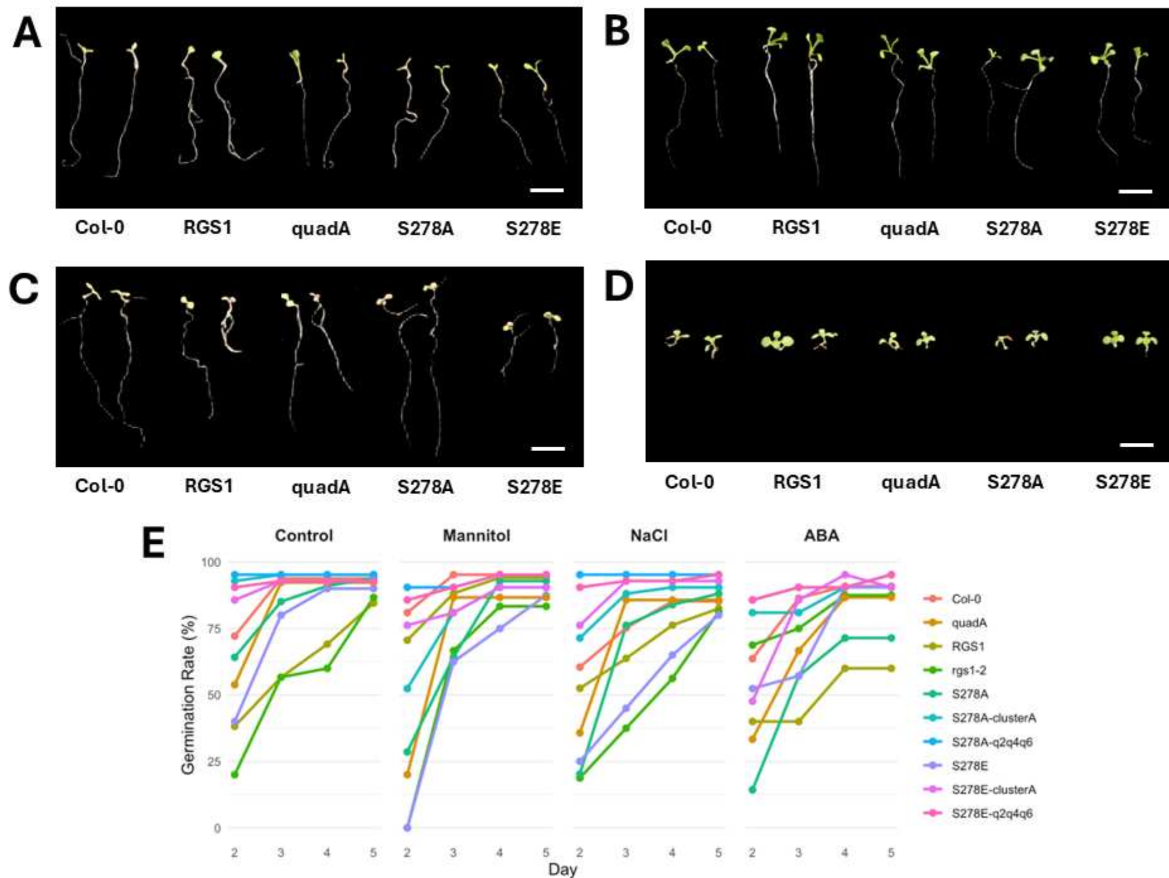

Figure 5. Effect of hormones and abiotic stress on germination of RGS1 mutants. 66-day-old seedlings germinated under normal conditions (A), with mannitol 200 mM (B), NaCl 75 mM (C), and ABA 1 µM (D). Scale bar 5 mm. E) Germination rate of the lines under the same conditions. n=25-20 seedlings.

To complement the results obtained, roots were visualized under a fluorescence microscope with the same treatments described above (Figure 6). Except for mannitol, since no visual phenotypic differences were observed (data not showed), and increasing treatment with salicylic acid (SA). Water-simulated results show constitutive expression of RGS1 at the plasma membrane, in all genotypes analyzed. After one hour of ABA treatment, RGS1wt retained its expression at the membrane, but also showed internalization. Surprisingly, quadA and S278E

lost fluorescence with ABA, showing an apparent process of endocytosis of RGS1 and high sensitivity to this hormone. S278A showed no significant changes with ABA.

Exposure to SA caused cell damage and loss of turgor in all genotypes, although S278E retained expression in the membrane and showed partial internalization in some areas of the cells. In the others genotypes it is not possible to determine whether fluorescence comes from the RGS1 protein or is caused by cell damage. NaCl treatment did not affect RGS1 expression but induced a marked decrease in fluorescence in quadA and S278A, indicating inhibition of RGS1 synthesis or degradation. Again, the S278E mutant maintained its expression with this treatment.

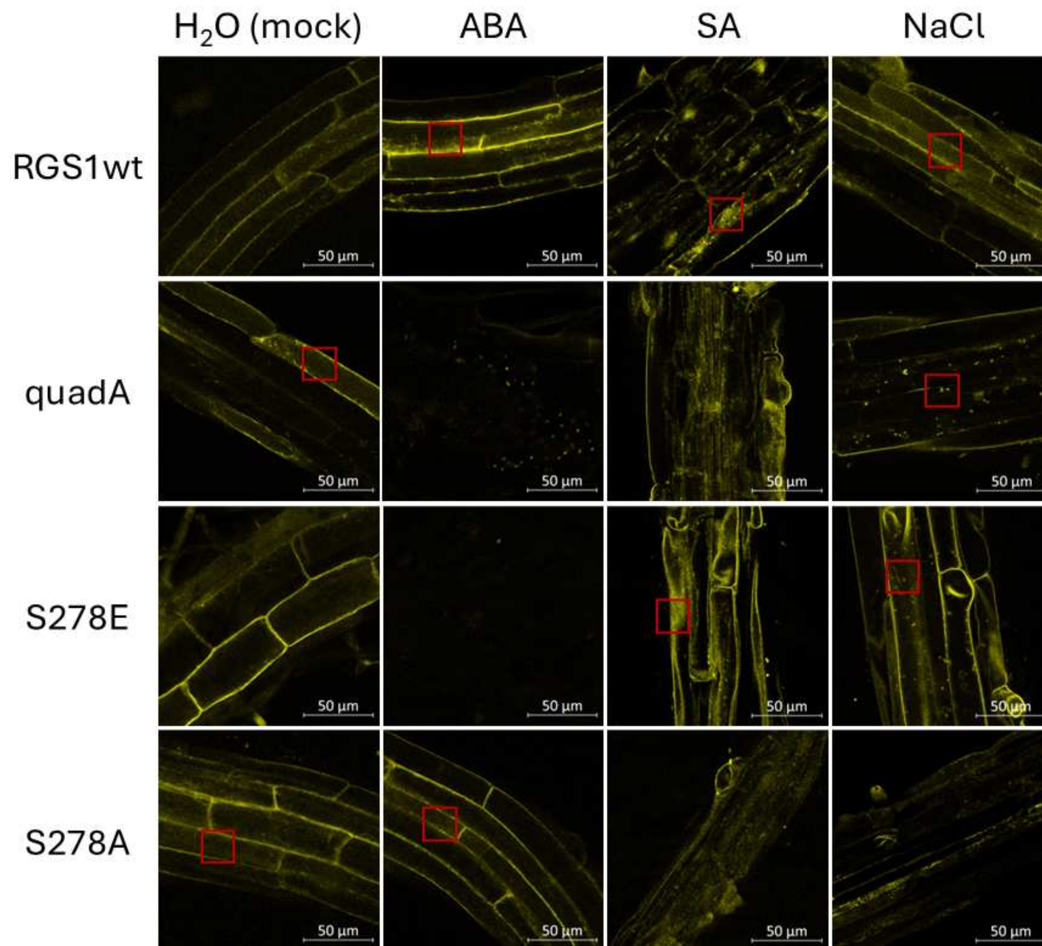

Figure 6. Cellular localization of RGS1 mutants in roots treated with H_2O (mock), ABA (1 µM), SA (1 mM) and NaCl (100 mM) for one hour. Plants 3-4 days old were analyzed under confocal microscope. Red box: internalization of RGS1 protein. Red box: internalization/protein accumulation of RGS1. Scale bar 50 µm.

3.5. Interactions between RGS1 and BAK1 in response to hormones and abiotic factors.

The interaction between the receptor FLS2 and the co-receptor BAK1 was evaluated in the presence and absence of the flg22 peptide. A constitutive interaction between the two proteins was observed under basal conditions, which increased upon the addition of flg22 (Figure 7A). To investigate the connection between the FLS2 receptor and the G-protein signaling pathway, protein-protein interactions with RGS1 were examined. An interaction between FLS2 and RGS1 was observed on the plasma membrane under simulated conditions and disappeared when treated with flg22 (Figure 7B). Another interaction examined was that of BAK1 and RGS1. Under normal conditions, the two proteins were found to interact, and , similarly, treatment with flg22 significantly diminished the interaction and reduced fluorescence (Figure 7C).

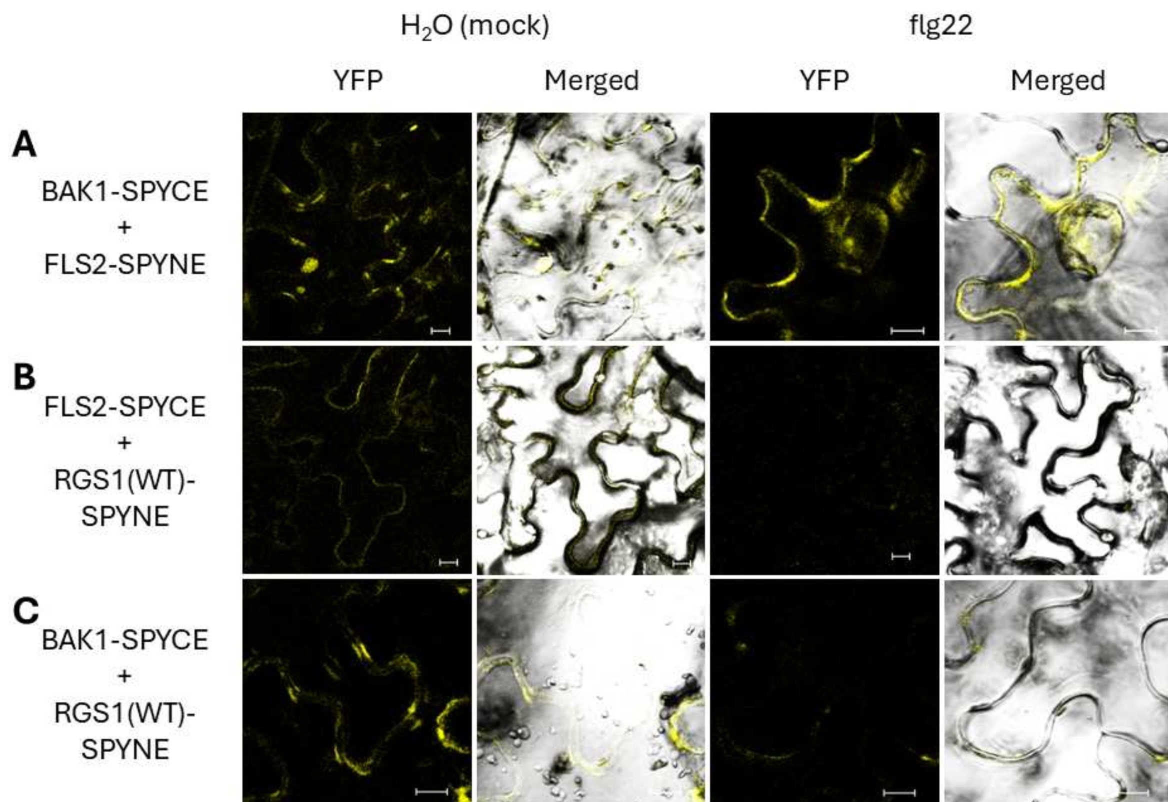

Figure 7. BiFC assay showing the interaction of RGS1 with FLS2 and BAK1 in *Nicotiana benthamiana* cells. Constructs carrying RGS1 fused to N-terminus of YFP (SPYNE) and C-terminus of YFP (SPYCE) fused to either BAK1 or FLS2 were infiltrated in *Nicotiana benthamiana* leaves and observed under confocal microscope after three days of infiltration.

Leaves disks from each infiltration were treated with flg22 (1 µM) for one hour and analyzed at confocal microscope. Scale bar 10 µm.

To investigate the role of RGS1 phosphorylation sites in its interaction with BAK1, a co-localization assay using phosphomimetic and phospho-null mutants was performed. BAK1 was chosen because a more robust interaction was previously observed between RGS1 and BAK1 than between RGS1 and FLS2. The results revealed that all RGS1 mutants interact with BAK1 under normal conditions (Figure 8), suggesting that the phosphorylation of these sites is not a determining factor in the binding of these two proteins. However, a slight decrease in fluorescence intensity was observed with the S278E-clusterA construction (Figure 8C), compared to the others, suggesting subtle modulation of affinity or stability with these mutations. Finally, fluorescence was lost in all cases after treatment with flg22, indicating dissociation of the complex.

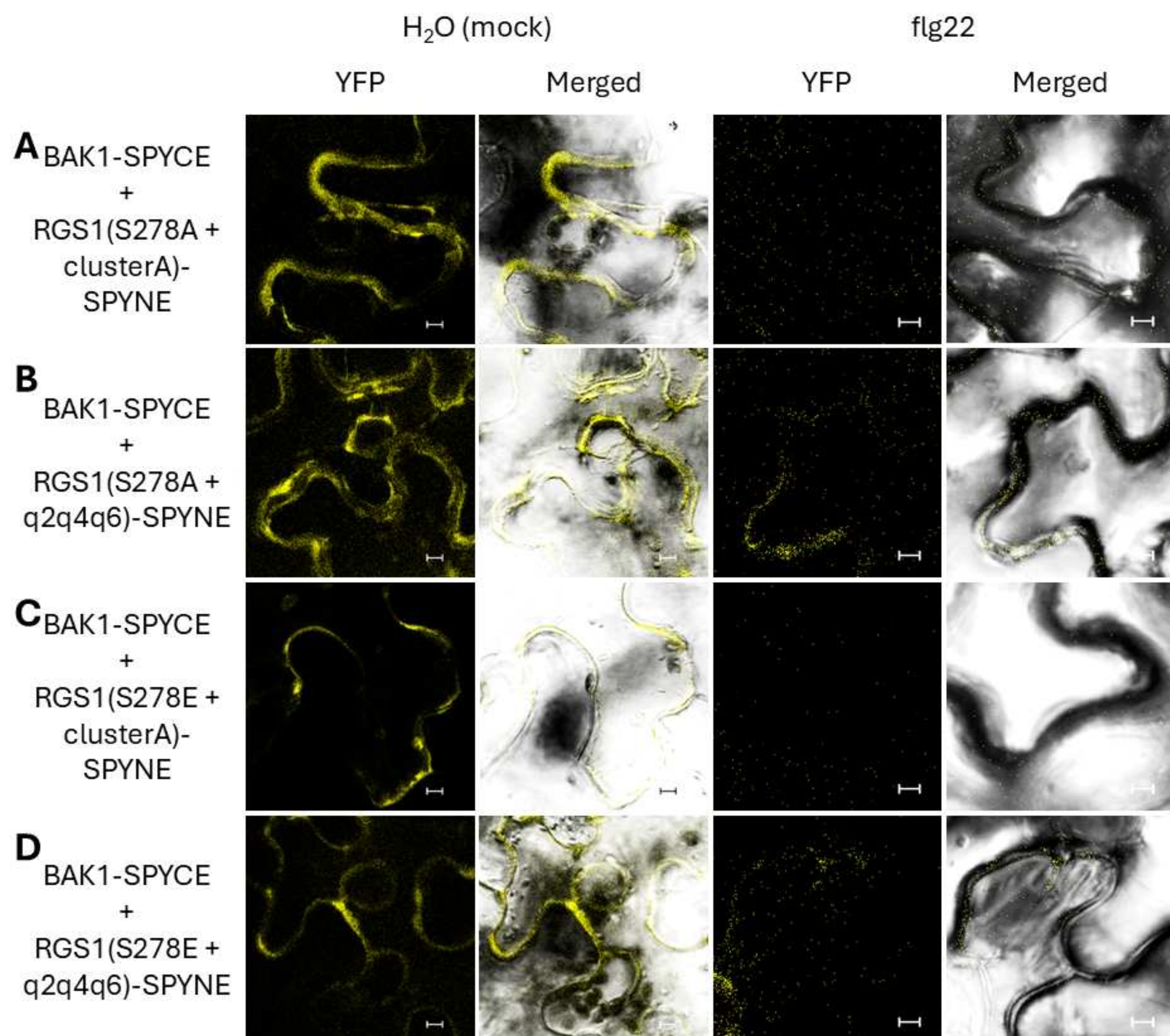

Figure 8. BiFC assay shows the interaction of RGS1 and phosphomutants and phospho null with BAK1 in *Nicotiana benthamiana* cells. Constructs carrying RGS1 or phosphomutants and phosphonull of RGS1 fused to N-terminus of YFP (SPYNE) and C-terminus of YFP (SPYCE) fused to BAK1 were infiltrated in *Nicotiana benthamiana* leaves and observed under confocal microscope after three days of infiltration. Leaves disks from each infiltration were treated with flg22 (1 μM) for one hour and analyzed at confocal microscope. Scale bar 5 μm.

The co-receptor BAK1 is a known regulator of multiple signaling pathways, including the brassinosteroid perception pathway mediated by the BRI1 receptor (He et al., 2013). To investigate if mutations in RGS affect its interaction with BAK1 in the presence of this hormone, a new BiFC assay was performed. The interaction between BAK1 and RGS1 is completely diminished after brassinosteroid treatment (Figure 9A). Interestingly, the S278A-clusterA and S278E-clusterA constructs remained bound to BAK1 after EBL treatment (Figure 9B and 9D), while fluorescence disappeared in the S278A-q2q4q6 and S278E-q2q4q6 constructs (Figure 9C and 9E). This suggests that phosphorylation at the C-terminal tail residues of RGS1 modulates its interaction with BAK1. Specifically, phosphorylation of the serine cluster in this region (q2q4q6) appears to confer greater stability to the RGS1-BAK1 complex.

Furthermore, we evaluated the effect of a brassinosteroid inhibitor (BRZ) on the RGS1-BAK1 interaction. As expected, RGS1 maintained its interaction with BAK1 after BRZ treatment (Figure 9A), suggesting that this signaling pathway is not directly affected by inhibition of brassinosteroid signaling. All other constructs also showed detectable fluorescence. However, a slight decrease in fluorescence intensity at the membrane was observed for the S278A-q2q4a6 and S278E-clusterA constructs (Figure 9C and 9D), although this effect was too subtle to be definitively attributed to BRZ treatment. Controls for the BiFC assay are provided in Supplementary Figure 1 to ensure the reliability of these results.

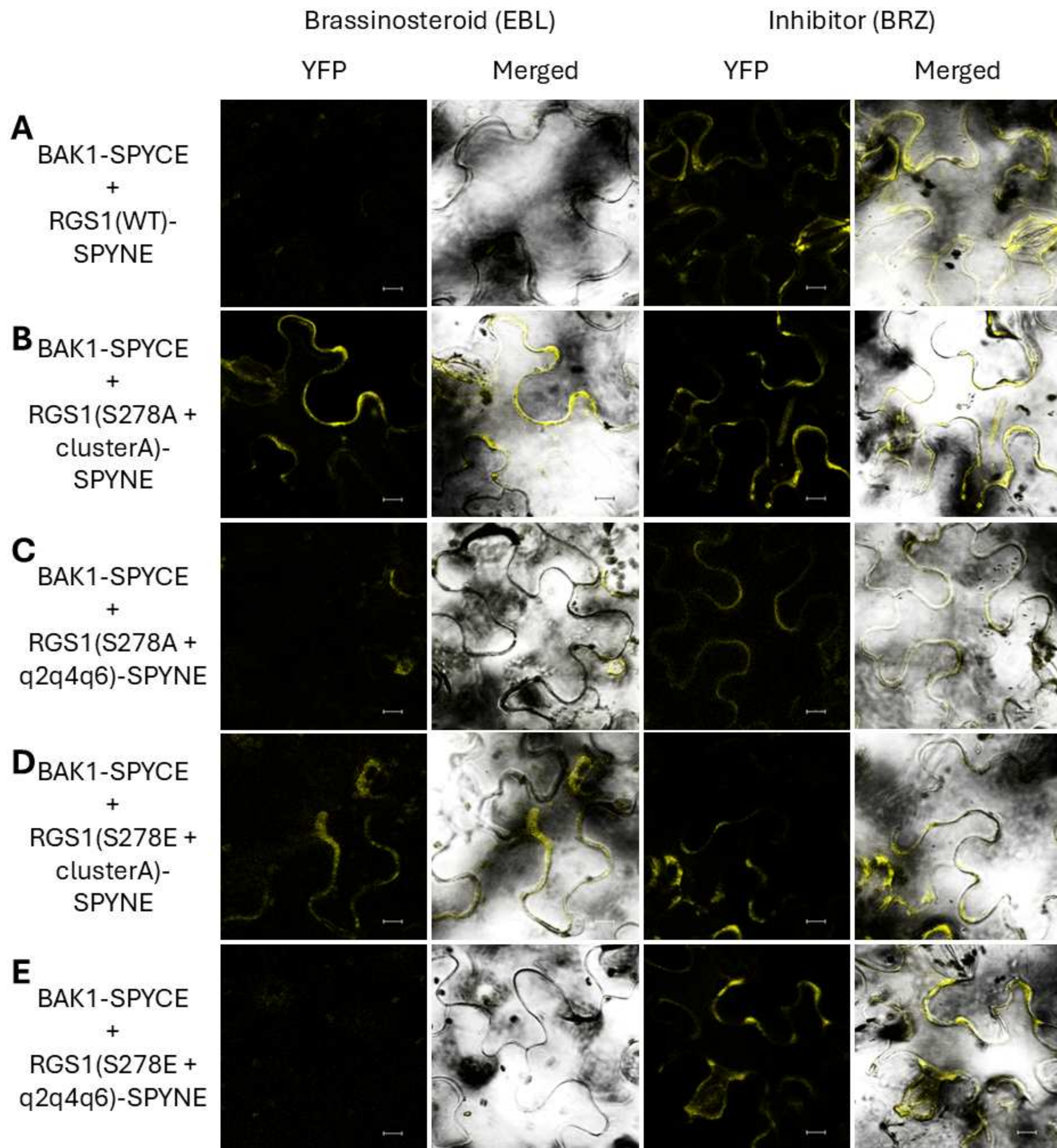

Figure 9. BiFC assay shows the interaction of RGS1 and phosphomutants and phosphor-null with BAK1 in *Nicotiana benthamiana* cells. Constructs carrying RGS1 or phosphomutants and phosphor-null of RGS1 fused to N-terminus of YFP (SPYNE) and C-terminus of YFP (SPYCE) fused to BAK1 were infiltrated in *Nicotiana benthamiana* leaves and observed under confocal microscope after three days of infiltration. Leaves disks from each infiltration were treated with EBL (2 μM) and BRZ (2 μM) for one hour and analyzed at confocal microscope. Scale bar 10 μm.

4. DISCUSSION

RGS1 protein is a master regulator in the G-protein signaling pathway and plays several important roles in the cell regulation. It has been reported that the RGS1 overexpressing line is more tolerant to water deficit, due to an increase in the expression of enzymes involved in ABA biosynthesis (Y. Chen et al., 2006). Our results showed that the line complemented with RGS1 presented a similar survival rate compared to WT. However, the plants tested showed wilt and leaf yellowing symptoms several days later than the other lines. Evaluating the RGS1 phospho mutants, the S278E phosphomimetic lineage showed more tolerant to water deficit, while the S278A phosphonull lineage showed more susceptible to water deficit. This could indicate that phosphorylation of this residue participates in tolerance to water stress, through a yet not identified mechanism. Since RGS1 has been characterized involved in the ABA signaling, once the RGS1 modulate the ABA biosynthesis and the overexpressing lines are hypersensitive to ABA in cotyledon opening and root growth (Y. Chen et al., 2006) it is possible that the S278 residue phosphorylation can participate in finer regulation of ABA metabolism.

To further explore the differences obtained in the assay described above, we chose to perform an assay that allows us to interconnect other known signaling pathways. In this context, dark germination assays allow us to identify if there are alterations in hormone levels, such as auxin, BR, ET and GA (Deepika et al., 2020). Specifically, we evaluated hypocotyl elongation, and we found that all mutants showed smaller size compared to Col-0. Although it has been reported that dark germination of the *rgs1-2* mutant results in a longer hypocotyl (J.-G. Chenet al., 2003; Roy Choudhury & Pandey, 2017), our results show no significant differences between the mutants and WT. When measurements are taken at the roots, no significant differences are observed either. Therefore, it is necessary to increase the number of seedlings and replicates before ruling out the role of these mutations of RGS1 in the hormonal signaling pathway.

On the other hand, a germination test was carried out with different types of hormones, including brasinosteroids. Regarding to BR, no significant differences in hypocotyl length were observed, nor when treated with the BRZ inhibitor. There is no direct evidence so far that RGS1 participates in the BR signaling pathway. A mechanism was described where BRI1-LIKE3 (BRL3), a member of the brassinosteroid receptor family, phosphorylates RGS1 and they cooperate to fine-tune the main glucose response pathway or flg22 (Tunc-Ozdemir & Jones, 2017). However, the authors mention that they did not find sufficient evidence that RGS1 and BRL3 participated in the brassinosteroid perception pathway. Our results corroborate with these findings, as no significant differences in the localization of RGS1 in its native form, when

exposed to BR, were observed either. However, our results also evidenced that the RGS1^{S278E} mutant probably activates some mechanism of endocytosis in the presence of BR. Further assays are needed to determine under what context phosphorylation at this position activates endocytosis in the presence of BR.

Our results showed that ABA germination negatively affected green seedling germination in all RGS1 mutants. This result is consistent with previous reports, where it has been evidenced that RGS1 overexpression delays germination and cotyledon greening (Y. Chen et al., 2006). One of the differences found among the mutants was observed in the S278A lineage, where the effect of the hormone was more drastic. In addition, the microscopy results revealed that RGS1wt and S278A were internalized after one hour of ABA treatment. In the case of the other mutants, fluorescence was completely reduced, so no conclusions can be drawn from them. Therefore, this may indicate that phosphorylation at Ser278 attenuates the hypersensitivity effect of ABA. On the other hand, microscopy results showed a trace of RGS1 endocytosis was observed in both the RGS1 mutant and S278E (Figure 6). So far, no direct mechanism between RGS1 and SA has been evidenced, however, there is evidence to support the interconnection of the G signaling complex in the jasmonic acid (JA)-activated pathogen defense response (Trusov et al., 2006). Due to the cellular damage observed in the root tissue, it is not possible to draw clear conclusions. It is recommended to reduce the concentration of SA in future experiments.

In parallel, the effects of abiotic factors such as mannitol and NaCl on the germination of these lines were explored. It was reported that *rgs1-2* mutants exhibited tolerance to salt stress by exhibiting less attenuated shoot growth and senescence, while *agb1-2* conferred accelerated senescence and aborted development (Colaneri et al., 2014; Yu & Assmann, 2018). Our results showed that the rgs1-2, quadA, S278A, and S278E mutants exhibited sensitivity during the first few days, as evidenced by the low germination rate. However, it increased over time until reaching the levels observed in the control group. In some species, mannitol helps plants to cope with water stress by regulating the osmotic pressure inside the cells, which is why it is a compound used in the evaluation of abiotic stresses (Rathor et al., 2020). A similar effect was observed with the NaCl treatment, affecting the RGS1wt, rgs1-2, S278A, S278E, and quadA lines. The results may seem inconsistent, since a decrease in the initial germination rate for both the RGS1 knockout mutant and the mutant overexpressing. Finally, we cannot conclude that the phosphorylation residues studied are involved in osmotic stress signaling pathways.

BiFC assays allowed us to identify the interactions of RGS1, FLS2 and BAK1, under different conditions. First, the formation of the FLS2-BAK1 heterodimer was observed in the basal state, to later evidence an increase in the formation of these complexes in response to flg22. This process occurs instantaneously after flg22 perception, being one of the best and most studied perception mechanisms in immune signaling (Chinchilla et al., 2007; Koller & Bent, 2014; Sun et al., 2013). FLS2 in its resting state can be in the form of FLS2-FLS2 homodimers (W. Sun *et al*., 2012), however, the predominant state is formed by the binding of FLS2-BAK1-BIK1 complex to heterotrimeric G protein (Pandey, 2020). In addition, the interaction between FLS2 and RGS1 was tested, and co-localization was observed under normal conditions. And when treated with flg22 for one hour, the fluorescence dropped by completely.

This same phenomenon was observed in the BAK1-RGS1 combination, only the fluorescence observed in the resting state was more intense, a result consistent with the literature, where BAK1 has been reported to phosphorylate RGS1 and subsequently lead to endocytosis. Co-immunoprecipitation (Co-IP) and luciferase complementation assays showed that RGS1 interacts with XLG2 and FLS2, then dissociates from these proteins when flg22 is recognized, a process that is facilitated by Ser431 phosphorylation (Liang et al., 2018). However, RGS1 phosphorylation does not occur at a single position, rather it is a multisite phosphorylation caused by both BAK1 and BIK1 (Tunc-Ozdemir et al., 2016). In that sense, some combinations of phosphomimetic and phosphonull mutants of RGS1 were used to understand the interaction dynamics with other proteins. It was possible to evidence that, in the resting state, RGS1 mutants remain tightly bound to BAK1. After one hour of incubation with flg22, dissociation of the RGS1-BAK1 complexes occurred in all cases. However, it is possible that before one hour, differences in the rate of complex cleavage could have been observed, therefore, it is necessary to perform captures at reduced time intervals to reveal the intermediate events of this process.

Moreover, BiFC assay for BR treatment showed that RGS1 dissociates from BAK1 after sensing the signal (Figure 9A). However, S278A+clusterA and S278E+clusterA maintained interaction with BAK1 after treatment. In sense, the Ser 428/340/431/435/436 cluster would be negatively regulating BR pathway activation. Multiple phosphorylation events have already been reported to be required in multiple RGS1 regulatory processes (Oliveira, 2022; Simoni, 2024; Tunc-Ozdemir et al., 2017). However, treatment with the BRZ inhibitor did not produce changes in the obtained fluorescence. Brassinazole inhibits BR biosynthesis upstream of cathasterone (intermediate in the pathway) (Rozhon et al., 2019), so possibly the BAK1-RGS1

complex would not be participating in this biosynthesis pathway, only in the perception of the hormone.

5. CONCLUSION

This study highlights the complex regulatory role of the RGS1 protein, especially with regard to its phosphorylation at critical sites. Our results suggest that Ser278 phosphorylation is a critical control point for ABA-related drought tolerance and hormone hypersensitivity. While some of our findings seem to contradict previous literature, such as the varied responses to dark germination and osmotic stress, they highlight the need for further replication and refinement of experimental conditions. Our BiFC assays confirmed that the interaction between RGS1 and immune receptors such as BAK1 is dynamic and phosphorylation dependent. This interaction reveals complex regulatory nuances in immune and brassinosteroid signaling. Taken together, these findings suggest that RGS1 is a finely tuned molecular hub whose precise function is dictated by a network of phosphorylation events and interactions requiring further investigation.

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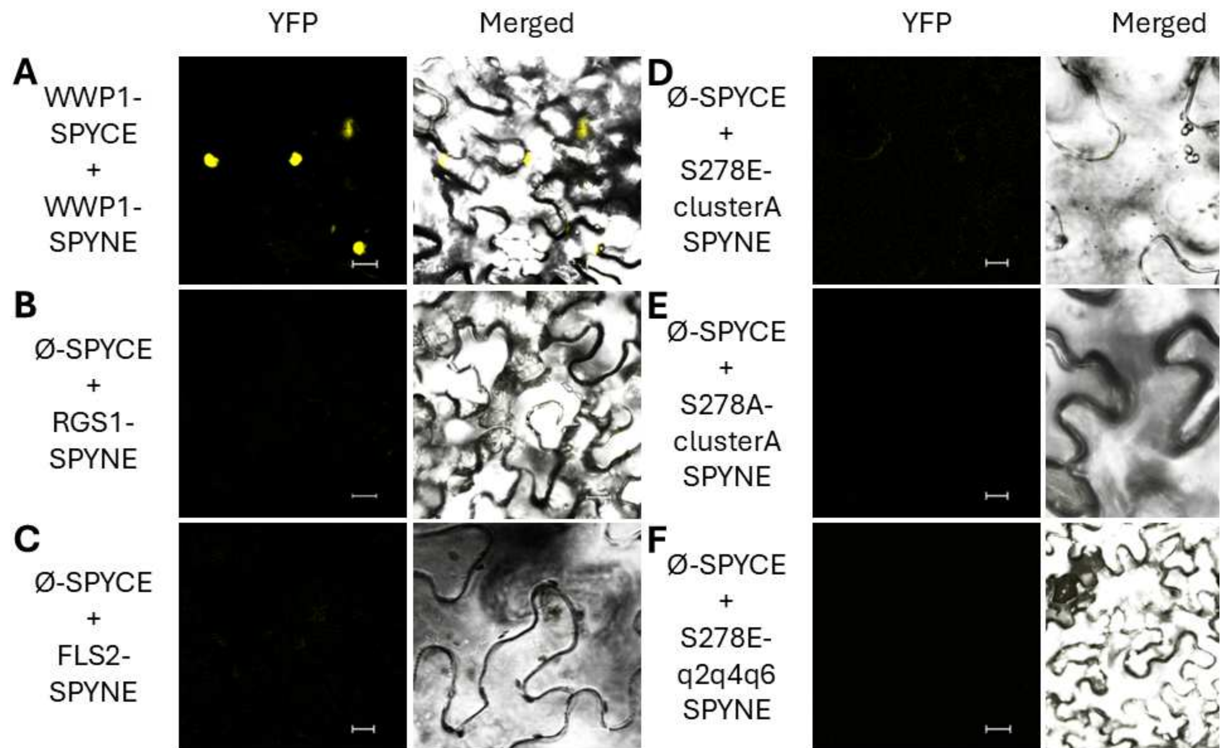
7. SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Internal controls of the BiFC assay. A) Positive control (nuclear localization) of WWP1 fused to N-terminus of YFP (SPYNE) and empty vector C-terminus of YFP (SPYCE). B, C, D, E, F) Control negative, empty vector N-terminus of YFP (SPYNE) and C-terminus of YFP (SPYCE) fused to RGS1, FLS2, S278E-clusterA, S278A-clusterA and S278E-q2q4q6, respectively. *Nicotiana benthamiana* leaves and observed under confocal microscope after three days of infiltration. Scale bar 10 µm.