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HvGCN2 silencing in barley displays enhanced *Blumeria graminis* f. sp. *hordei* susceptibility

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Abstract: Powdery mildew disease, caused by *Blumeria graminis* f. sp. *hordei* (*Bgh*), which belongs to the order Erysiphales, is a major crop disease. The general control nondepressible-2 (*GCN2*) gene of barley was previously found to be overexpressed during the powdery mildew resistance response. Recently, *Arabidopsis thaliana GCN2* (*AtGCN2*) was shown to be involved in disease resistance against biotrophic and necrotrophic pathogens. In order to understand the function of *Hordeum vulgare GCN2* (*HvGCN2*) in the barley powdery mildew resistance response, this gene was silenced by barley stripe mosaic virus (BSMV), mediated by virus-induced gene silencing (VIGS). This is the first study showing the potential importance of *HvGCN2* in powdery mildew disease of barley. Based on our observations, when *HvGCN2* was silenced on average by 53.5%, *Bgh* development was increased by 18.7 to 32.1%, which was determined by primary, secondary and longest hyphae measurements. The number of germinated spores also increased 2.8-fold in *HvGCN2* silenced plants compared to control plants (BSMV:00). On the other hand, under the resistant condition, no difference was observed in *HvGCN2*-silenced plants compared to non-silenced lines although the gene was found to be overexpressed in incompatible interaction.

Key words: general control non-depressible 2 (*GCN2*); barley; *Blumeria graminis* f. sp. *hordei;* powdery mildew; virus-induced gene silencing (VIGS); plant disease resistance

INTRODUCTION

Blumeria graminis is an important disease of barley that decreases the yield and causes serious economic losses [1]. Currently, fungicides are used to limit *Blumeria graminis*, but the fungus quickly becomes resistant to the applied chemicals [2]. *Blumeria graminis f. sp. hordei* (*Bgh*) is the subspecies that can infect *Hordeum vulgare* [3]. Other than *Bgh*, there are subgroups that can infect oat (*f. sp. avenae*), rye (*f. sp. secalis*), poa (*poae*), ryegrass (*lolii*), and bromus (*bromi*) [4]. The genome of *Bgh* was sequenced and found to be larger than 120 Mb [5]. The availability of sequence data will aid developing strategies to cope with this disease in barley.

Plants have a tightly controlled defense system that is specifically designed towards their pathogens. There remains much to investigate in the molecular pathways of this resistance system in plants. The hypersensitive response (HR), a type of programmed cell death, is an important local defense mechanism in plants. This local mechanism results in systemic acquired resistance (SAR) in plants coping with the disease [6]. Activation of phytohormones such as salicylic acid (SA), jasmonate (JA) and ethylene (ET) are well known in plant disease resistance responses [7]. SA signaling is primarily involved in resistance against biotrophs, whereas JA/ET signaling is generally effective against necrotrophs [8]. Activation of the SA-dependent signaling pathway results in expression of pathogenesis-related (PR) proteins [8]. Understanding the signaling pathways for HR and SAR activation is crucial for developing plants resistant to pathogens.

GCN2 is a serine threonine kinase [9] that is responsible for sensing and responding to amino acid starvation in yeast [10]. The expressed sequence tag (EST) and genomic databases were screened with the *AtGCN2* sequence and similar genes and transcripts were found in many plants, including wheat and bar-

ley [11]. It was shown that wheat eukaryotic translation initiation factor 2α (eIF2α) is phosphorylated by yeast GCN2 protein in yeast cells, indicating that this mechanism might be conserved from yeast to plants [12]. *Arabidopsis* eIF2α was not phosphorylated in an *AtGCN2* knockout mutant, indicating that *AtGCN2* is the only kinase phosphorylating eIF2α [13], in contrast to mammals, which also have heme-regulated inhibitor (HRI) kinase, protein kinase R (PKR), and PKR-like ER-localized eIF2α kinase (PERK) in addition to GCN2 [14-16]. Several studies on *Arabidopsis thaliana* have shown that *AtGCN2* was functional during stress responses (biotic and abiotic), normal development of the plant and plant-hormone cross talk [12, 17-20]. *AtGCN2* was shown to be essential for growth under stress conditions, including wounding, starvation, exposure to methyl jasmonate, salicylic acid, or the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) [12]. Furthermore, *AtGCN2* knockout mutants had better heat, drought, and osmotic stress tolerance [17]. *AtGCN2* also had a role in β-aminobutyric acid (BABA)-induced growth suppression, and resistance induced by BABA did not depend on *AtGCN2* [21].

Recently, it was reported that at different developmental stages there are both positive and negative influences of *AtGCN2* on plant immunity against both biotrophic and necrotrophic pathogens. However, the Arabidopsis *AtGCN2* mutant displays enhanced disease susceptibility towards biotrophic pathogens during early developmental stages [18]. It is likely that *AtGCN2* might act as a universal immune regulator in plants by controlling both SA- and JA-mediated defenses [18].

Using yeast two-hybrid analysis, yeast SRP72 was found to physically interact with YR10 protein of wheat [22]. When the response of *HvGCN2* in barley during *Bgh* resistance was tested by qRT-PCR, it was shown that its expression level was increased almost three-fold [22]. The gene was silenced using VIGS in order to confirm its function. The method takes advantage of the plant RNAi mechanism to knock down a gene of interest in order to explore its function [23]. In this technique, a specifically modified complementary DNA (cDNA) of viral origin is introduced to the plant [23]. As the virus replicates, dsRNAs are encoded and cleaved into siRNAs by Dicer enzyme, then siRNA molecules target their complementary mRNA fragments and successfully prevent their translation [24]. VIGS is preferred for its convenience and ability to target specific gene loci [23]. The most crucial advantage of the technique is that the silencing phenotype can be established in 1 to 2 weeks after the application [23]. Since decreased or limited plasmodesmatal activities can result in inefficient silencing [24,25], its efficiency can never reach 100%. Most of the time; it is still sufficient not to knock out the gene completely, especially if the gene is a housekeeping one. Silencing efficiency also differs in different genotypes [26]. Since the understanding of the genes involved in the regulation of disease resistance mechanism can facilitate the development of novel applications in the fight against pathogens, the role of *HvGCN2* in the defense mechanism in barley was studied using BSMV-mediated VIGS. Three control and four silencing treatments were conducted on seedlings, and changes were determined by comparing powdery mildew growth in control and silenced groups, and the level of silencing was confirmed *via* qRT-PCR.

MATERIALS AND METHODS

Plant materials and growth conditions

Barley seeds of Pallas-01 (with the *Mla*1 resistance-gene) were planted in soil and grown under 16 h light and 8 h dark cycles in a Sanyo Versatile Environmental Test Chamber (Model MLR-351H) at 18°C for 10 days. The seedlings were watered with tap water once every 2 days.

HvGCN2 cloning and silencing

BSMV is a tripartite RNA virus with a genome composed of α, β and γ RNAs. All three genomes are necessary for the virus to perform silencing. The BSMV plasmids used for VIGS were pα, and pβΔβa, which contain a deletion of the coat protein of pβ, obtained from Large Scale Biology, whereas the plasmid containing the γ genome and pSL039B-1 was obtained from Steven R. Scofield of Purdue University, USA. The *HvGCN2* target gene fragment was cloned into the γBSMV vector, pSL039B-1, containing the phytoene

desaturase (PDS) gene fragment which was replaced with a 310 bp-long fragment (HO208991.1), after PCR amplification with primers with PacI and NotI sites (TA-HV-GCN2-F-310: 5′-CACTTAATTAAGGAGGAGAGATGCTAGAACT-3′ and TA-HV-GCN2-R-310: 5′-CATGCGGCCGCAACTCCTGCCCAACAAA-3′, corresponding to positions 2542 to 2850 on *TaGCN2* (FR839672.1)). The plasmids (4 μg) were linearized with restriction enzymes: PauI (BssHII) for pBSMVγ:*GCN2*; BcuI (SpeI) for pBSMVβΔβα, and MluI for pBSMVα and pBSMVγ in a 50-μL reaction volume. *In vitro* transcription reactions were carried out on the linearized plasmids according to the manufacturer's protocol (Ambion mMessage mMachine T7 *in vitro* transcription kit; Invitrogen, CA, USA). The silencing procedure was adapted from [27] and [28], with a modifications. The aliquots of BSMV transcripts of each genome were mixed in equal proportions as a 1.5 μL transcription mix (without $LiCl_2$ RNA precipitation) in 27.5 μL of FES (0.2 M glucose, 0.3 M K_2HPO_4, ddH_2O). The inoculation solutions were rub-inoculated from bottom to top on the first leaves of 10-day-old Pallas-01 seedlings. At 14 days post-silencing (dps), the third leaves of the plants were cut into four fragments of which two of the fragments were used for RNA analysis and the other two were used for *Bgh* inoculations.

RNA extraction and cDNA synthesis

RNA extraction from the leaf fragments was carried out using a QIAGEN RNeasy Plant Mini Kit, according to the manufacturer's instructions (QIAGEN, Germany), by pooling leaf fragments from the same leaf into one sample to obtain 100 mg of tissue. Residual DNA in the RNA isolates was removed using Fermentas DNase I (Fermentas, MA, USA) according to the manufacturer's specifications, and lack of any DNA was confirmed by the absence of product in PCR amplification of *HvGAPDH* with primer pairs: Hv-GAPDH-cw1: 5′-CGTTCATCACCACCGACTAC-3′ and Hv-GAPDH-ccw1: 5′- CAGCCTTGTCCTTGTCAGTG-3′. cDNA synthesis was carried out using Invitrogen SuperScript®II Reverse Transcriptase with both random hexamers and oligo-dT primers in one RT reaction. The cDNA products were used in the qPCR analysis.

qRT-PCR

A common strategy using at least one of the primers targeting either upstream or downstream the cloned region, in silencing level determination by qRT-PCR. Both qRT-PCR primers were designed to correspond to the outside of the *GCN2* target region. For qRT-PCR analysis we used the primers GCN2-F-134: 5′-CTGACGCAGATGTGAATGCT-3′ and GCN2-R-134: 5′-CAGTTGTTGGGTCGAAACCT-3′ amplified between nucleotides 500 and 635, which corresponds to positions 615-635 and 500-519 on *TaGCN2* (FR839672.1). Products of expected sizes were confirmed by PCR. Also, we observed a single peak by melting curve analysis. The reactions were carried out as indicated in the manufacturer's protocol (SYBR Green JumpStart Taq Ready Mix; Sigma-Aldrich, MO, USA), on Stratagene Mx3005p. Three PCR technical repeats were performed on equal cDNA concentrations of the leaf fragments from each of the four silenced seedlings and leaf fragments of each of the three naked virus transcript inoculated seedlings.

Five different reference genes were tested as follows: *actin* (AY145451.1), *GAPDH* (M36650.1), *elongation factor* (Z50789.1), *ubiquitin* (M60175.1), and *α-tubulin* (Y08490.1). GeNorm eliminates the candidate with the highest M value and keeps calculating until the last two candidate reference genes are left. The last two candidates are then named as the optimum pair of reference genes [29]. M value calculation is based on the variation of the candidate reference gene with respect to all other candidate genes. As a result, geNorm software provides average expression stability for each candidate reference gene as compared to the rest of the candidate reference genes. The *elongation factor* gene (Z50789.1) with the primer pair 5′-ATGATTCCCACCAAGCCCAT-3′ and 5′-ACACCAACAGCCACAGTTTGC-3′, and the *actin* gene (AY145451.1) with the primer pair 5′-AATGGTCAAGGCTGGTTTCGC-3′ and 5′-CTGCGCCTCATCACCAACATA-3′, were used for normalization of the qRT-PCR experiments. Arithmetic means of relative expression levels were calculated using both reference genes. The calculations for silencing level determination were performed according to Pfaffl [30]. Fold-change data was used to conduct a Student's t-test using JMP®, ver. 7.0 (SAS Institute Inc.,

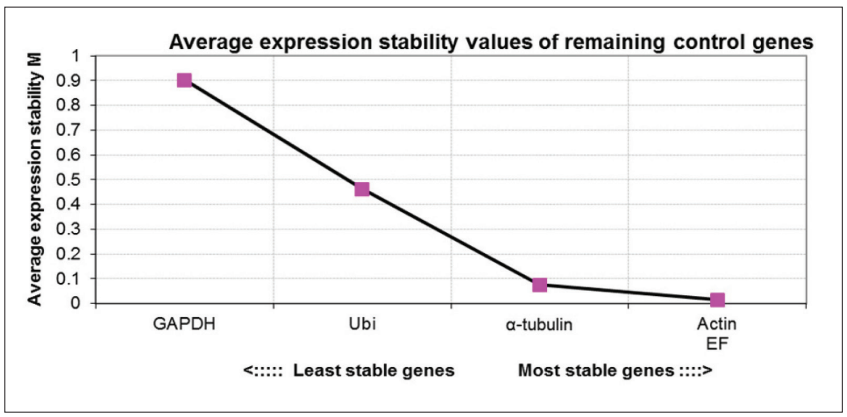

Fig. 1. Average expression stability values of candidate reference genes. GAPDH – glyceraldehyde 3-phosphate dehydrogenase; Ubi – ubiquitin, EF – elongation factor. Actin and EF were selected as the reference genes as they were the most stable genes in BSMV:00-inoculated and *HvGCN2*-silenced leaf samples.

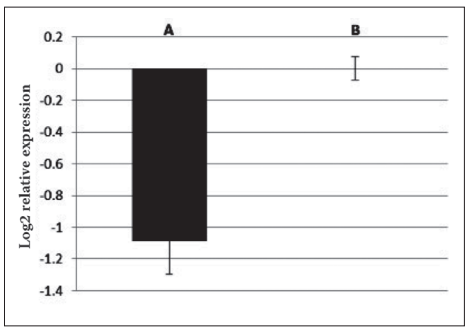

Fig. 2. Relative expression of *HvGCN2* in VIGS-silenced plants (BSMV:*HvGCN2*) with respect to control plants (BSMV:00). Gene expression levels were measured by qRT-PCR and are presented as normalized gene expression ratios. **A** – log 2-fold change of the *HvGCN2* expression level in four biological replicates from silencing experiments. **B** – control *HvGCN2* expression in four biological replicates. All the expression levels of the biological replicates were calculated from triple replicates. The difference between control and silenced groups was statistically significant ($p=0.0167$ according to Student's t-test assuming equal sample sizes and variances).

Cary, NC, 2007). The percent *HvGCN2* expression level after silencing was also calculated by dividing the logarithmic transformation of the fold-change in *HvGCN2* silenced plants to the value in BSMV:00 control plants and multiplying with 100.

Blumeria graminis f. sp. *hordei* inoculations and determination of level of powdery mildew development

Pathogen inoculations were performed using *Blumeria graminis* f. sp. *hordei* races *Bgh-95*(53/01) and *Bgh-103*(64/01), having virulence and avirulence to Pallas-01, respectively. The detached Pallas-01 leaves (post-challenge with BSMV constructs) were positioned onto 1.5% water agar plates with 1% benzimidazole and inoculated by blowing the spores on the leaves in separate chambers to give about 25-30 sporulating colonies per cm^2. Hyphal growth development was determined at 3 days post-inoculation (dpi) and 5 dpi by Trypan blue staining. Trypan blue staining was performed according to Hein et al. [31]. The fungal hyphae were classified as the ones emerging from the germinating spore itself (primary hyphae), as emerging from the primary hyphae (secondary hyphae), and as the longest hyphae. Pictures were taken using a Leica DM4000B microscope/DFC 280 camera, and hyphal lengths were measured using the Leica Application Suite LAS V3.1.

Statistical analysis

Eight control and eight silenced plant detached leaves were used for statistical analysis. For each leaf, the number of germinated spores and the average hyphae metric (longest, primary, secondary hyphae) was calculated and data were used to conduct Student's *t*-test using JMP®, Version 7.0 (SAS Institute Inc., Cary, NC, 2007).

RESULTS

For the selection of best suitable reference genes, geNorm software was used to calculate M-values. *Actin* (AY145451.1) and *elongation factor* (Z50789.1) genes were selected as reference genes for the determination of the *HvGCN2* silencing level, as they were found to be the most stable under the applied experimental condition (Fig. 1).

The same individual silenced plant leaf was used for qRT-PCR and powdery mildew inoculations in order to examine the fungal developmental changes due to silencing. In the VIGS experiments, four individual samples of Pallas-01 seedlings were used for silencing. The qRT-PCR analysis results showed that

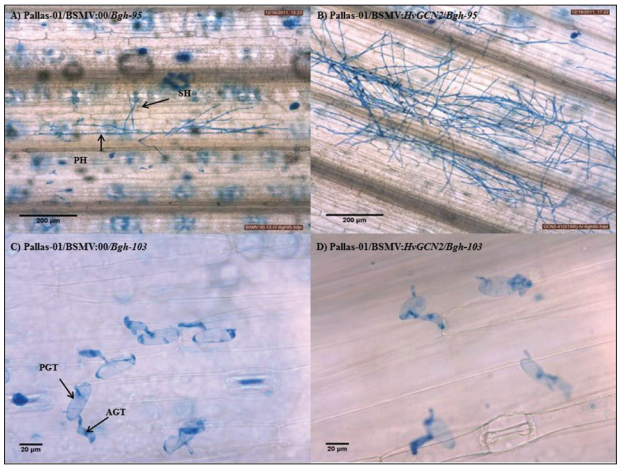

Fig. 3. Pathogen growth at 5 days post inoculation on a control (**A** and **C**; BSMV:00 rub-inoculated Pallas-01) and a *HvGCN2* silenced sample (**B** and **D**; BSMV:*HvGCN2* rub-inoculated Pallas-01). Powdery mildew inoculations with (**A** and **B**) *Bgh-95* (compatible interaction) at 10X magnification and (**C** and **D**) *Bgh-103* (incompatible interaction) at 40X magnification of samples 14 days after silencing of Pallas-01. PH – primary hyphae; SH – secondary hyphae; AGT – appressorium germ tube; PGT – primary germ tube.

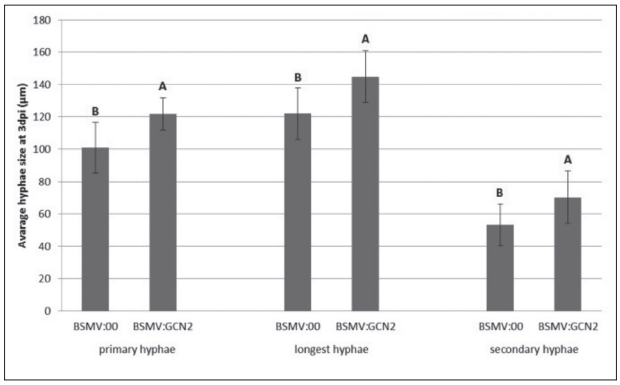

Fig. 4. Powdery mildew growth differences at 3 days post inoculation. Differences in primary, secondary and longest hyphae lengths in *HvGCN2* silenced plants with respect to control plants are shown with A and B (p=0.0078, p=0.0445, p=0.0155, respectively, according to Student's t-test assuming equal sample sizes and variances).

HvGCN2 was silenced in a range of 33-71%, with an average value of 53.5% in the silenced samples as compared to four biological replicates of control samples (BSMV:00, naked viral RNA inoculations), which corresponds to a nearly -1-fold change in log2 (Fig. 2).

HvGCN2 silenced plants were inoculated with a compatible race of *Blumeria graminis* f. sp. *hordei, Bgh-95*. Three and five days after *Bgh* inoculations, leaf fragments were collected for microscopic analysis to determine the number and the length of hyphal formations in *HvGCN2*-silenced samples and compared with the control samples. The intense surface network on the 5 dpi samples prevented accurate quantitative measurements; however, the differences in the amount of hyphal formations were obvious across all silenced samples (Fig. 3). Quantitative evaluations were only conducted on the 3 dpi samples (Fig. 4). The powdery mildew hyphal formation levels were analyzed to correlate with the silencing levels measured via qRT-PCR. For quantitative assessment, a total of 292 germinated *Bgh* spores were analyzed under the compatible interaction condition. Among these 292 fungi, 216 belonged to the *HvGCN2* silenced samples and 76 belonged to the control samples in the same number of leaf pieces and the same total area. A 2.8-fold difference in the germination rates of control samples with *HvGCN2*-silenced samples (p=0.0053) was observed. The average hyphae length within each leaf was calculated by following the classifications of primary and secondary hyphae. Then, the mean values were used to calculate the average lengths of the hyphae used in Fig. 4. They displayed 18.7% difference in longest hyphae lengths between control and silenced groups (p=0.0155), 20.3% difference in primary hyphae lengths between control and silenced groups (p=0.0078) and 32.1% difference in secondary hyphae lengths between control and silenced groups (p=0.0445). This result is consistent with the qualitative analyses presented in Fig. 3. These differences in the germination rates and the size of the hyphae were estimated to be about three-fold increased in silenced plants with respect to control plants after visual inspection at 5 dpi.

When another set of *HvGCN2* silenced and control Pallas-01 (harboring *Mla1* gene) samples was inoculated with the incompatible race of pathogen *Bgh-103*, no differences were observed in the resistance responses (Fig. 3C and D). The number of germinated spores, developmental stages of primary germ tube, appressorial germ tube, and appressorial lobe formations of the avirulent pathogen race were similar at both 3 and 5 dpi after visual inspection.

DISCUSSION

GCN2 is responsible for the phosphorylation of eIF2α. This phosphorylation results in global reduction in protein synthesis, which is important for cell homeostasis [32]. Also, *Arabidopsis* eIF2-α is phosphorylated by GCN2 after treatment with defense hormones SA and JA [12]. Plant GCN2 is believed to function as a sensor and regulator of several stress responsive pathways, including plant defense against insect herbivores [12]. The activation of GCN2 protein is believed to be beneficial for the plant in coping with stress [33]. In *Arabidopsis thaliana*, the presence of GCN2 protein facilitates the action of glyphosate since the effects of the herbicide were minimized in *Gcn2* knock-out mutants [20]. Recently it was found that AtGCN2 protein serves as a general regulator of SA- and JA-mediated immune responses triggered after infection with biotrophic and necrotrophic pathogens [18]. There are examples of both positive and negative influences of *AtGCN2* on plant immunity at different developmental stages, and this is explained by the variable amount of abscisic acid (ABA) accumulation in plants lacking functional *AtGCN2* at early stages of development [18].

In this study, *HvGCN2* was silenced via the BSMV-VIGS system. Using *HvGCN2* mutants rather than gene silencing can affect growth of the plant as *AtGCN2* has also been shown to be involved in growth and developmental processes [18]. For this reason, transient gene silencing was preferred. In order to determine the silencing level of *HvGCN2* accurately, reference genes that were most unaffected by *HvGCN2* silencing and powdery mildew infection had to be found. Under similar conditions to the present study, the ubiquitin [31] or actin [34] genes were used as a reference. We tested five common housekeeping genes (actin, elongation factor, GAPDH, ubiquitin and α-tubulin) as candidate reference genes. As a result of geNorm analysis, actin and elongation factor genes were selected as the reference genes being the most stable gene pair during the applied experiment.

In the course of the experiment, *HvGCN2* was found to be 53.5% silenced on average. Silencing values above 30% are generally considered significant and even 10% was previously reported as a silenced sample [35].

Following gene silencing, the seedlings were inoculated with *Bgh* to determine the importance of *HvGCN2* in barley defense against powdery mildew. Silencing of *HvGCN2* affected both the number and sizes of the germinated spores. The number of germinated spores were 2.8-fold lower in silenced *HvGCN2* samples than in BSMV:00-inoculated samples. On the other hand, hyphae growth measurements at different time points and visual inspection of the powdery mildew spores under the microscope showed that the hyphae in *HvGCN2* silenced samples were up to 32.1% longer when compared to BSMV:00-inoculated samples. These two results showed that the silencing of *HvGCN2* renders *Bgh*-susceptible plants extremely vulnerable to the disease. However, the plant response to the avirulent pathogen was not changed by silencing *HvGCN2*, indicating that *HvGCN2* behaves as a negative regulator of pathogen growth only during susceptibility. As expected, powdery mildew resistance-breaking phenotypes were not observed in *HvGCN2*-silenced *Bgh* resistant barley because *HvGCN2* itself is not responsible for complete resistance. The plants that were used in this study were young seedlings and *Bgh* is a biotrophic pathogen, therefore the results presented here are in accordance with the results of Liu et al. [18] who reported that an Arabidopsis *AtGCN2* mutant displayed enhanced disease susceptibility towards biotrophic pathogens during early developmental stages.

Understanding the role of GCN2 protein in plant disease resistance is of vital importance. As hypothesized by Liu et al. [18], pathogens feed on the host and this may induce amino acid starvation, resulting in GCN2 protein activation. GCN2 phosphorylates eIF2α and the repression of general protein synthesis may initiate plant disease resistance. The gene is not only important for biotic but also for abiotic stresses in plants.

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Author contributions: Study concept and design — FE; MSA; preparation of plant materials — IKO; VIGS methodology — IKO, FE; real time PCR analysis — IKO, FE; interpretation of data — IKO, FE, MSA; draft of the manuscript — FE, MSA; final approval of the version to be published — IKO, FE, MSA.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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