2015

Functional analysis of drought-resistant genes in barley using VIGS

Natalie Catherine Martin
University of Northern Iowa

FUNCTIONAL ANALYSIS OF DROUGHT-RESISTANT GENES IN BARLEY USING VIGS

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Designation

University Honors with Distinction

Natalie Catherine Martin

University of Northern Iowa

May 2015

This Study by: Natalie Martin

Entitled: Functional Analysis of Drought-Resistant Genes in Barley using VIGS

Has been approved as meeting the thesis or project requirement for the Designation, University Honors with Distinction.

_____	_____
Date	Dr. Tilahun Abebe, Department of Biology
_____	_____
Date	Dr. Jessica Moon, Director, University Honors Program

Abstract

The following study analyzed the role of the gamma-aminobutyric acid transaminase (GABAT) gene in the response of barley plants to drought stress. Expression of GABAT in barley was suppressed using virus-induced gene silencing (VIGS). In VIGS, an RNA virus is used to replicate a small portion of the target RNA. In this study, barley stripe mosaic virus (BSMV), that carries a piece of the GABAT gene to infect barley, was used. As the virus replicates inside infected barley plants, it also makes RNA that is complementary to GABAT messenger RNA (mRNA) that is normally made by the host. The presence of this double-stranded RNA triggers the RNA interference (RNAi) pathway, which degrades GABAT, inhibiting its translation. The silenced plants were assessed for their response to drought stress. The amount of GABAT mRNA in the silenced plants was estimated using real-time PCR. The results showed that expression of GABAT was down-regulated by 65-77% in the silenced plants. The silenced plants exposed to drought conditions displayed more drought symptoms than those with a normal amount of GABAT enzyme. This indicates that the GABAT gene plays an important role in the drought tolerance of barley.

Introduction

This study sought to determine the role of selected genes that are induced in barley during drought stress. Barley plants express several genes when exposed to drought conditions. As barley is relatively tolerant to drought conditions, it is speculated that these genes contribute to drought resistance (Abebe, Melmaiee, Berg, & Wise, 2010). However, this has not been confirmed. Dr. Tilahun Abebe's lab in the Department of Biology at the University of Northern Iowa has identified thousands of genes that are induced by drought stress (Abebe et al., 2010). Joshua Mauldin, a former graduate student of Dr. Abebe's, developed a virus-induced gene silencing (VIGS) method to determine the function of selected drought-resistant genes. The present study used a modified version of Mauldin's procedure to examine the response of barley plants to water deprivation when expression of one of these genes (gamma-aminobutyric acid transaminase, GABAT) was silenced via virus-induced gene silencing (VIGS).

Previous studies have evaluated a variety of barley genes for their drought-resistant activity. However, many genes remain uncharacterized, including the GABAT gene. Bray, Bailey-Serres, & Weretilnyk (2000) indicate that only 18% of barley that could be produced each year (under ideal conditions) is actually produced. The majority of this loss (75.4%) is attributed to abiotic stress, primarily drought. Identifying drought-tolerant genes, including GABAT, will aid in the development of crops that can better survive drought conditions. As barley is the fourth most important cereal crop (Sayed, Schumann, Pillen, Naz, & Léon, 2012), research into the genetics of its drought tolerance will improve economic yields as well. The VIGS procedure sought to confirm that the GABAT gene contributes to barley survival in drought conditions.

Literary Analysis

Drought is the primary environmental factor affecting crop yields (Araus, Slafer, Reynolds, & Royo, 2002). The science is simple—without water, plants will not survive. As the world population continues to grow and the environment continues to change, the genetics of drought tolerance requires thorough investigation in order to develop crops that produce higher yields under drought conditions (Araus et al., 2002). One aspect of this strategy is to identify genes responsible for drought tolerance. The project presented here evaluated the role of GABAT in drought tolerance using a procedure modified from Joshua Mauldin's.

VIGS Research

VIGS is commonly used to identify the function of genes by inhibiting their expression (Holzberg, Brosio, Gross, & Pogue, 2002). VIGS uses RNA viruses that carry a segment of the target RNA in a reverse orientation. When the virus replicates inside its host, it also replicates the RNA that matches the host gene's messenger RNA (mRNA). The RNA from the virus then forms a double-stranded RNA with the mRNA made by the host, triggering degradation of the host mRNA via the RNA-induced gene silencing pathway commonly known as RNA-interference (RNAi; see Figure 1). In RNAi, the enzyme, Dicer, cuts the double-stranded RNA into small, 21-24 base, double-stranded RNAs called small-interfering RNAs (siRNA). A complex of proteins, known as RISC (RNA-induced silencing complex), displaces the top strand of the siRNA and uses the lower strand as a guide to identify the complementary target mRNA. When the target is

identified, the RISC cleaves the mRNA, rendering it unusable for protein synthesis (Demircan & Akkaya, 2009). In the absence of mRNA, the target protein cannot be produced, and one can easily deduce the function of the gene by observing the phenotype, or physical characteristics, that develop.

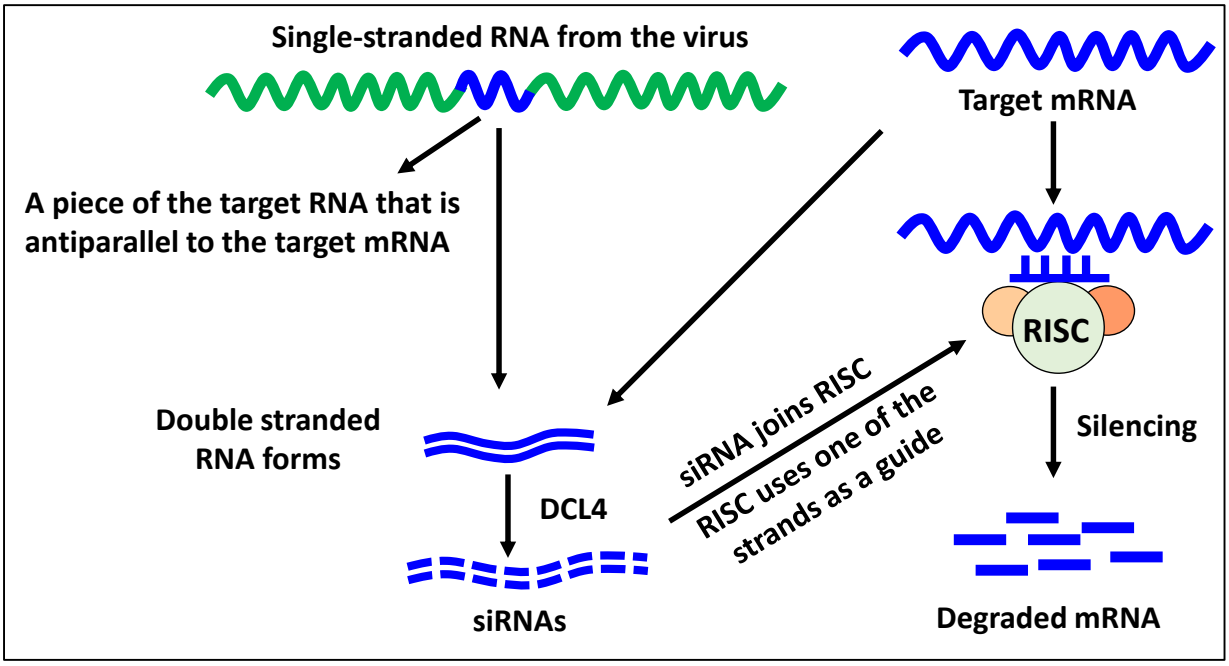

Figure 1. Gene silencing in plants using VIGS. DCL4. Dicer-like 4; siRNA, small interfering RNA; RISC, RNA-induced silencing complex

Ramegowda, Sethil-kuar, Udayakumar, and Mysore (2013) used VIGS to evaluate the function of different genes exposed to multiple sources of environmental stress. They evaluated drought tolerance of plants with silenced genes by measuring water loss. These authors also evaluated the photosynthetic activity of stressed plants. In general, plants decrease their photosynthetic activity during drought stress. Since drought-tolerant plants have the ability to continue photosynthesis during drought, they show very little reduction in photosynthetic activity during stress than plants with their drought-tolerant

genes silenced (Ramegowda et al., 2013). In our study, the response of barley plants expressing GABAT and plants with reduced GABAT expression was evaluated by withholding water. Stress response was visually compared and differences in the expression of GABAT between these groups of plants were evaluated using real-time PCR.

The Virus

The current study uses a modified barley stripe mosaic virus (BSMV, Cui et al., 2012) to suppress expression of the GABAT gene in barley. BSMV is a positive-sense RNA virus (meaning the RNA can be directly translated to proteins) with a tripartite genome consisting of three RNA molecules called α, β, and γ. The three BSMV subgenomes have a methylated 5′-cap and a 3′-poly (A) sequence, followed by a tyrosine accepting structure. The α component encodes an RNA-dependent RNA polymerase (RdRP) to amplify the viral RNA. The β component has four parts: βa codes for a coat protein, while βb, βc, and βd (collectively referred to as the triple gene block proteins) code for movement proteins necessary for the spread of the virus in the host plant. The smallest RNA component (γ) encodes an additional subunit of RNA-dependent RNA polymerase (RdRP) and a multifunctional RNA-binding γb protein. Upon infection, BSMV produces yellow and white streaking and brown, necrotic stripes on the leaves (Mathre, 1985). To use BSMV for VIGS, expression of the coat protein (βa) must be disrupted by mutating the start codon. A fragment of the target gene is inserted at the γb gene in reverse orientation. The three BSMV subgenomes for this project have been cloned into an expression vector driven by the cauliflower mosaic virus 35S promoter

(Meng, Moscou, & Wise, 2009; see Figure 2). The transcripts synthesized from these vectors in the host are self-cleaved at the 3' end by the hepatitis delta virus (HDV) ribozyme, included in the α genome, to generate the RNA subgenomes. A piece of DNA is inserted between the *Not I* and *Pac I* sites of the γ subgenome in reverse orientation to silence a target gene without any effect on the replication of BSMV.

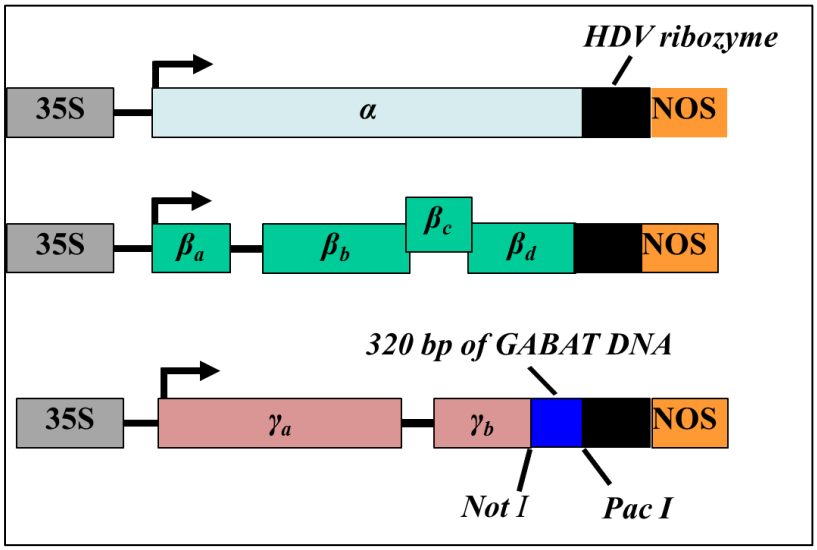

Figure 2. *Schematic representation of the BSMV-VIGS vectors. The three subgenomes of BSMV are under the control of the 35S promoter (35S). Arrows indicate the direction of transcription (Meng et al. 2009).*

GABAT gene

While previous studies have evaluated drought-resistant genes, few have studied the role of the gamma-aminobutyric acid transaminase (GABAT) gene in the response of barley to drought stress. GABAT is a nuclear encoded enzyme that converts the non-protein amino acid, gamma-aminobutyric acid (GABA), to succinic semialdehyde (SSA) in the metabolic pathway known as the gamma-aminobutyric acid (GABA) shunt (Figure 3). The GABA shunt provides an alternative route for the conversion of α-ketoglutarate

to succinate in the tricarboxylic acid (TCA) cycle of respiration in the mitochondria. The GABA shunt plays a role in preventing the buildup of damaging reactive oxygen species (ROS) during stress (Bouché, Fait, Bouchez, Møller, & Fromm, 2003). The exact mechanism of how GABA improves stress tolerance is not well understood, but it might involve a signaling role for GABA stress response (Roberts, 2007).

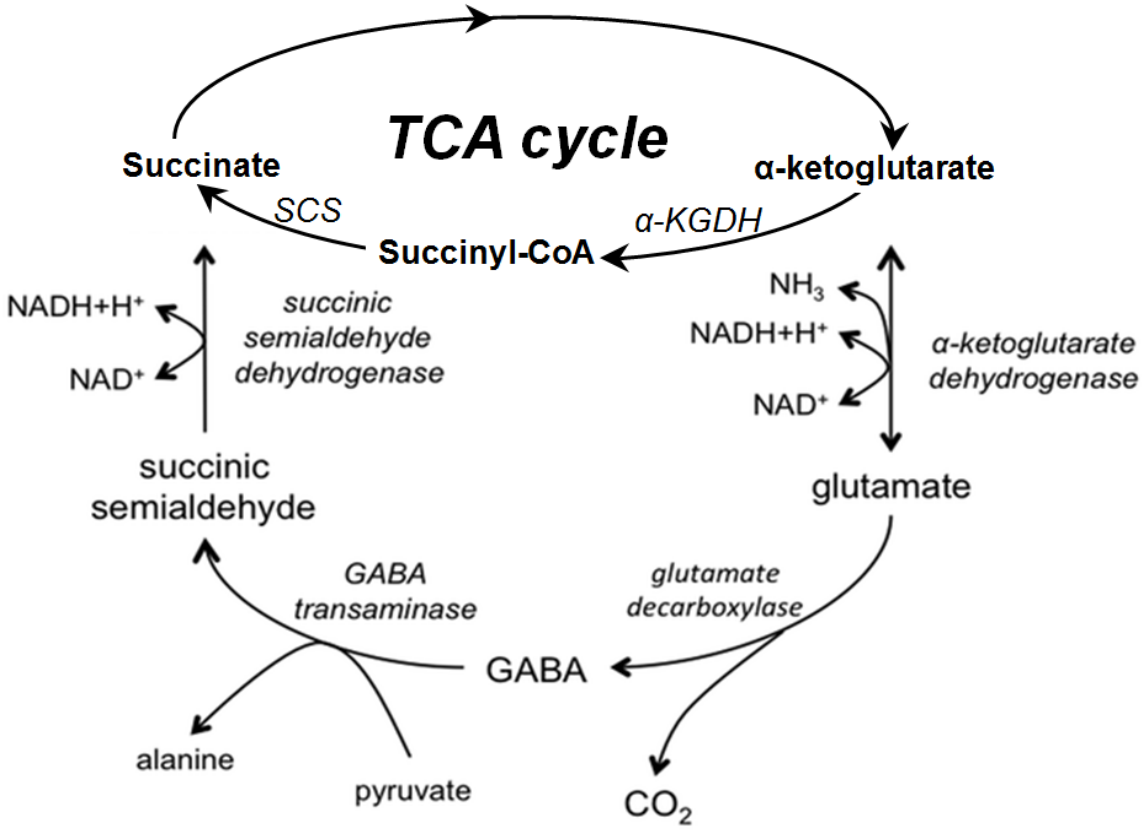

Figure 3. *Schematic representation of the GABA shunt. The GABA shunt is composed of three enzymes: glutamate decarboxylase, GABA transaminase (GABAT), and succinic-semialdehyde dehydrogenase (SSADH). TCA cycle, tricarboxylic acid cycle; SCS, succinyl-CoA synthetase; α-KGDH, α-ketoglutarate dehydrogenase. Modified from Mead, Thynne, Winterberg, & Solomon (2013).*

Methodology

Planting and Infection

The BSMV-sensitive barley, Black Hulless, was used to replicate the virus. A malting barley variety, Morex, was used to silence expression of the target gene (GABAT) and to assess drought stress response. Four Black Hulless barley seeds were planted in six-inch pots with a quarter tablespoon of Osmocote fertilizer (NPK, 19-6-2). Two weeks after planting, Black Hulless was infected with barley-stripe mosaic virus (BSMV). The γ component of the viral genome contained a 320 base pair (bp) region of GABAT in reverse orientation so that, when the viral RNA was replicated, a complementary GABAT RNA was produced. This RNA then matched the native mRNA of GABAT, triggering RNAi. The plants were infected using sap from previously infected Black Hulless leaves. The leaves were ground with a mortar and pestle in about 0.4-0.6 ml of phosphate buffer, depending on the size of the leaf. After grinding, about 2 μl of the sap was injected into the stem of each plant with a pipette. Each plant was injected twice for a total of 4 μl. The remainder of the sap was pipetted onto a strip of parafilm, mixed with carborundum powder, and stroked onto the third leaf of each plant, with each leaf receiving three strokes on each side.

Morex barley was planted one week after Black Hulless. Twelve pots were filled with soil so that each pot had the same weight (about 332 g). The plants were maintained in a growth chamber and watered occasionally to the same weight. A week after planting, 100 ml of Jack's fertilizer was added to each pot. At this time, plants were thinned to two, uniform plants per pot. On the day of infection, the Morex pots were randomly labeled. Six pots were labeled GABAT, after the gene to be silenced, using the virus that carries a

fragment of the GABAT gene (referred to as BSMV-GABAT plants). The other six pots were labeled BSMV as they were infected with the necked (without the coat protein) BSMV virus. This virus did not silence the GABAT gene. The plants were placed in separate plastic containers to avoid cross-contamination. Each group was further divided into two separated subgroups: three pots were randomly assigned to "control" while the other three pots were assigned to "stress" treatment. Morex was infected with the corresponding virus in the same manner as Black Hulless above, using the leaves of infected Black Hulless plants. The plants were kept in a growth chamber with 16h light, 22°C/18°C day/night temperature and 60% relative humidity. All pots were watered daily to the same weight (about 400 g).

Stress Treatment

One week after infection, the stress group was exposed to drought by withholding water for four days. The control group was watered every day to 400 g. In our experience, barley plants showed symptoms of viral infection, or yellow or brown "necrotic stripes" on the leaves (Mathre, 1985). The plants were visually evaluated for stress symptoms as well. Pictures were taken of the plants at the end of the stress period.

Sample Collection and RNA Extraction

On the fourth day of stress, the fourth leaf of each control and drought-stressed plant was collected, flash-frozen in liquid nitrogen, and stored in a -80°C freezer. Total RNA was extracted using RNAZol® RT (Molecular Research Center, Cincinnati, OH). The amount of RNA was quantified using a spectrometer and run on agarose-

formaldehyde gel-electrophoresis in order to confirm its quality. One drought-stressed sample from the silenced group was discarded due to poor RNA quality. Additionally, contaminating DNA was digested with DNase.

Real-time PCR analysis

A small aliquot of each RNA sample was diluted to 20 ng/µl with sterile, RNAse-free water. For each sample, 5 µl of the diluted RNA (100 ng total RNA) was used for real-time PCR analysis using 2 × Verso 1-step qRT-PCR SYBR Green mix (Thermo Scientific). This reagent contains everything needed to perform reverse transcription and relative quantification of the resulting cDNA using SYBR Green I. Twenty-five µl of each sample was loaded into three PCR wells to minimize errors due to pipetting. Real-time PCR analysis was performed using a 7300 Real-time PCR system (Applied Biosystems) with the following thermal profiles:

1. 50°C for 30 minutes—cDNA synthesis step
2. 95°C for 15 minutes—activates the chemically modified DNA polymerase
3. Denaturing—95 °C for 15 seconds
4. Annealing—60°C for 30 seconds
5. Extension—72°C for 30 seconds

× 40 cycles

The reverse transcriptase in the Master Mix forms cDNA, which is then denatured to produce single strands. Primers specific to GABAT bind to the cDNA strands and mark them for polymerization by DNA polymerase. The SYBR Green I dye, included in the real-time PCR reaction to detect amplification of the GABAT DNA, binds to any double-stranded DNA, including non-specific PCR amplification products. A

dissociation (melting) curve was run after the real-time PCR to determine amplification of the desired DNA only. The dissociation curve was run with the following thermal conditions:

1. Denaturation—95°C for 30 seconds (1 cycle)
2. Starting temperature—60°C for 30 seconds (1 cycle)
3. Melting step (increases by 0.5°C per cycle)—60°C for 10 seconds (80 cycles)

Differences in the expression of the GABAT gene (in fold change) between the control and drought-stressed plants were estimated from the cycle threshold (C_t) value, using the delta ($2^{-\Delta\Delta C_t}$) method (Livak & Scmittgen, 2001). The equation for this method is given as follows:

$$Fold\ change = 2^{-\Delta\Delta C_t}$$

Where,

$$\Delta\Delta C_t = \left[\left(C_{t,target\ gene} - C_{t,GAPDH}\right)_{drought}\right] - \left[\left(C_{t,target\ gene} - C_{t,GAPDH}\right)_{control}\right]$$

The delta delta method requires inclusion of a housekeeping gene, which is uniformly expressed in all samples. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene since it does not change its expression in response to drought stress (Abebe et al., 2010). GAPDH was amplified from one control and one drought-stressed plant in which the GABAT gene was functional and one control and one drought-stressed plant in which the GABAT gene was silenced. Then, expression of GABAT between the control and drought-stressed

GABAT-functional plants and control and drought-stressed GABAT-silenced plants was compared relative to the housekeeping GAPDH gene.

Results

BSMV-infection of Morex barley was never 100%. In the final trial, eleven out of the twelve pots showed symptoms of BSMV infection. As there were four different plant treatments (GABAT-functional with stress treatment, GABAT-functional with control treatment, GABAT-silenced with stress treatment, and GABAT-silenced with control treatment), and there were three sets of these treatments, the entire set containing the uninfected plant had to be replaced. For this reason, four out of the total twelve plants evaluated with real-time PCR (GABAT-functional control and stress and GABAT-silenced control and stress) were from a previous trial. This ensures that the plants evaluated in this set were exposed to the same conditions.

Visual Assessments

Eleven of the twelve pots examined showed signs of infection. Figure 4 shows the appearance of infected leaves. Instead of the standard, green leaves, leaves infected with the barley stripe mosaic virus have yellow and brown streaks down the center. Compared with the control plants, drought-stressed plants had more yellow-brown leaves. Figure 5 shows the appearance of a BSMV-GABAT (GABAT-silenced) control plant (on the left) and a BSMV-GABAT drought-stressed plant (on the right). In stressed plants, the stem did not support the whole plant well. Figure 6 shows an example of a BSMV control plant (on the left) and a BSMV drought-stressed plant (on the right). The drought-stressed

plant still showed some symptoms of wilting, but it appeared much healthier than the BSMV-GABAT plant. The drought-stressed BSMV plant was able to support the leaves better and had fewer brown, dying leaves than the drought-stressed BSMV-GABAT plant.

Figure 4: Morex leaves infected with the Barley Stripe Mosaic Virus (BSMV)

Figure 5: Morex plants with a silenced GABAT gene. From left to right: BSMV-GABAT control, BSMV-GABAT stressed.

Figure 6: Morex plants infected with BSMV only. From left to right: control, drought-stressed

Real-Time PCR Results

In real-time polymerase chain reaction (real-time PCR), amplification of a target sequence is performed as the PCR progresses. An increase in the fluorescence of the detection dye (SYBR Green I) that binds double-stranded DNA corresponds to the relative amount of DNA produced throughout the process. The amplification plot of the PCR is displayed in Figure 7. The sigmoidal shape of the graph is typical of real-time

PCR amplification plots. The chart shows the relative fluorescence signal in each of the replicated samples per PCR cycle. The critical part of the graph is the C_t (or threshold cycle), which represents the PCR cycle in which the curve crosses the baseline. This represents the number of cycles it takes for the amount of replicated DNA to surpass the threshold value. The earlier the cycle, the more DNA is available to copy, and the more the gene is expressed. An earlier cycle indicates that little silencing occurred. A silenced gene will produce less RNA and has a higher C_t value. The fold-change also emphasizes the difference between the silenced and the un-silenced samples. The delta delta procedure normalizes expression of GABAT against the housekeeping gene (GAPDH) that does not show significant changes in expression under treatment conditions. Table 2 shows the overall average C_t values and the fold changes.

Figure 8 shows the dissociation curve. The dissociation curve was developed during the separation of the double-stranded DNA (dsDNA). As the temperature increased, the DNA double strands separate. The SYBR Green I dye, added in the PCR reaction, fluoresced when it was in contact with dsDNA. As the DNA double-helix "melted" or separated, this fluorescence decreased. The point of inflection is specific to the DNA sequence. As one peak is shown on the dissociation curve, only one target DNA was amplified. For GABAT, the dissociation peak occurred at about 84°C (Figure 8).

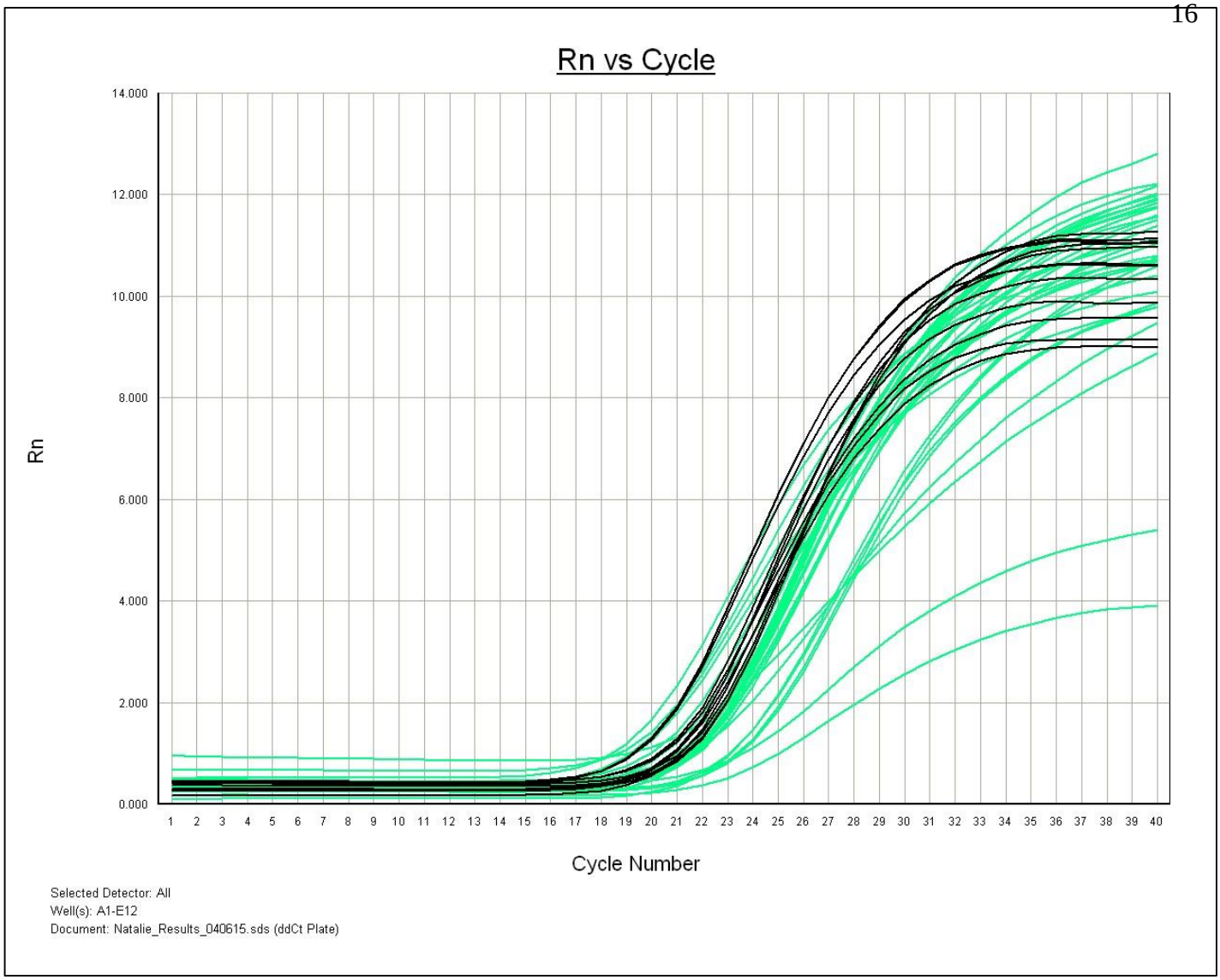

Figure 7: The graph shows the amount of PCR amplified after each amplification cycle. The green lines indicate amplification of GABAT gene while the black lines indicate amplification of GAPDH.

Table 1: Average C_t, ΔC_t, $\Delta\Delta C_t$, *and* $2^{-\Delta\Delta C_t}$ *values.*

Sample Name	Average C_t	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$
Silenced GABAT control 1	21.2824	2.3670	2.3484	0.1964
BSMV GABAT control 1	19.3916	0.0186	0	1
BSMV GABAT drought 1	17.4370	-1.3440	0	1
Silenced GABAT control 2	21.1094	2.1939	1.9201	0.2642
Silenced GABAT drought 2	19.3253	1.5980	2.1047	0.2330
BSMV GABAT control 2	19.6468	0.2738	0	1
BSMV GABAT drought 2	18.2744	-0.5066	0	1
Silenced GABAT control 3	19.3586	0.4431	0.2832	0.8217
Silenced GABAT drought 3	19.5041	1.7768	1.1746	0.4430
BSMV GABAT control 3	19.5328	0.1599	0	1
BSMV GABAT drought 3	19.3832	0.6022	0.4423	0.7359
Silenced GAPDH control	18.9155			
Silenced GAPDH drought	17.7273			
BSMV GAPDH control	19.3730			
BSMV GAPDH drought	18.7810			

**Average values are the average value of the three trials run on each plant sample.*

Table 2: Average C_T Values and Fold Changes for all Samples

	Sample	Average Ct	ΔCt	ΔΔCt	$2^{-\Delta\Delta C_t}$
BSMV	GABAT control	19.5237	0.1508	0	1
	GABAT drought	18.3649	-0.4161	0	1
	GAPDH control	19.3730			
	GAPDH drought	18.7810			
Silenced	GABAT control	20.5835	1.6680	1.5172	0.3494
	GABAT drought	19.4147	1.6874	2.1035	0.2327
	GAPDH control	18.9155			
	GAPDH drought	17.7273			

**Average C_t values were obtained from the averaged values in Table 1.*

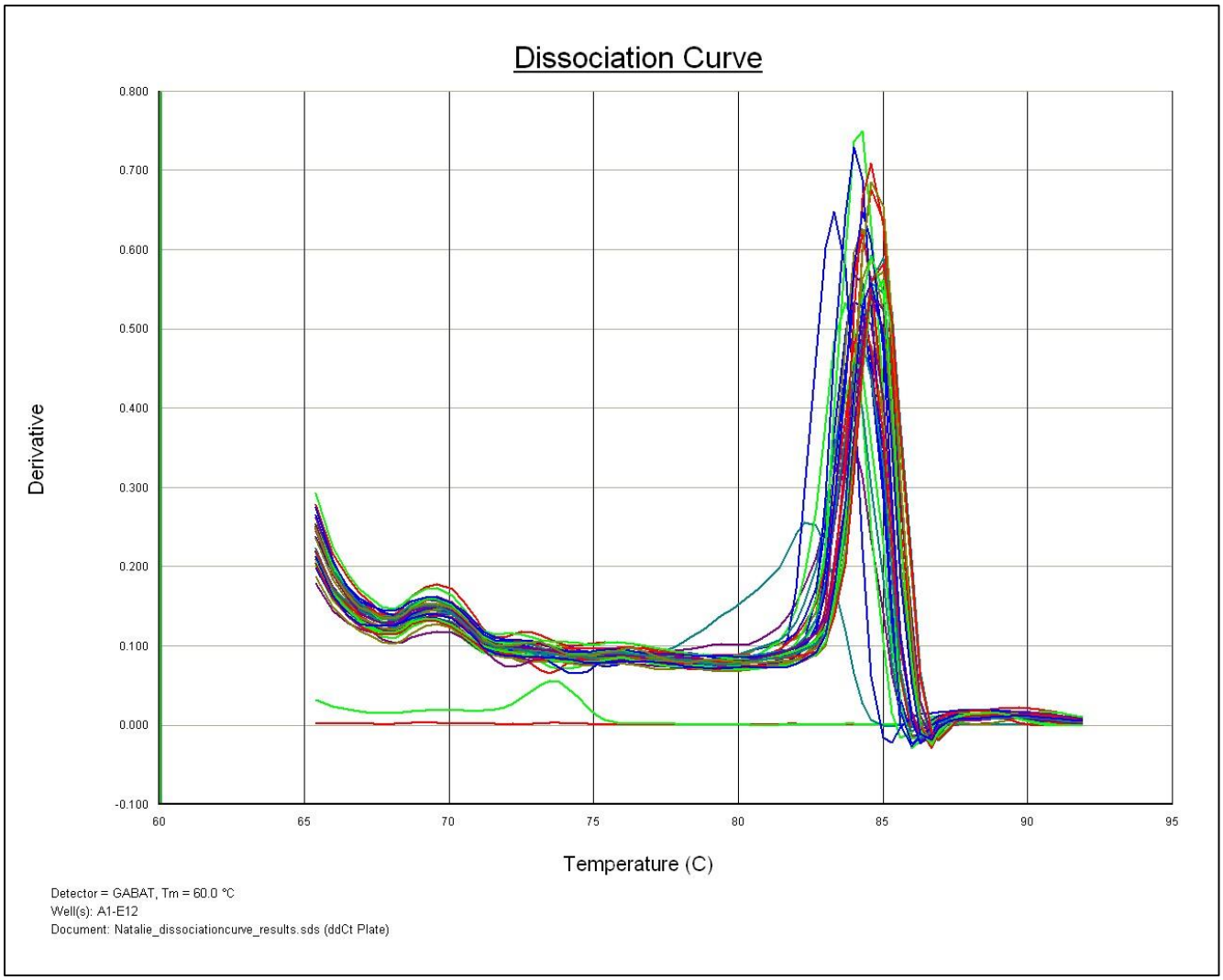

Figure 8: A dissociation curve for GABAT. The colors represent the different samples tested.

Discussion

The visual evidence supports GABAT's role in drought tolerance. In both the BSMV-GABAT and BSMV infected plants, the properly watered, controlled plants were healthier than the stressed plants. Leaves from the control plants were better supported and grew upward. In contrast, the stressed plants displayed wilting. Sayed et al. (2012) discussed how leaf rolling allows a stressed barley plant to reduce water loss and withstand drought conditions. Leaf wilting occurs when the plant does not have this ability and, instead, dries out. The stressed plants also contained more brown leaves. During drought conditions, photosynthetic genes that provide the plant with energy and its green color are down-regulated (Abebe et al., 2010). The observation from the current study also supports the reaction to the drought treatments.

There was also an important difference in the reaction of the plants to drought stress. Both plants in which the GABAT gene was functional and plants in which GABAT was silenced showed stress symptoms, but the symptoms in plants with the GABAT gene silenced were more pronounced. These plants had more discolored, yellow, brown, and wilting leaves. This indicates that the GABAT gene was appropriately silenced. The severe stress symptoms in the plants in which the GABAT gene was silenced support the important role GABAT plays in drought tolerance. The double-stranded GABAT RNA in the BSMV construct lead to the breakdown of the GABAT mRNA, preventing its translation and accumulation of the GABAT enzyme. Any contribution to stress tolerance that the GABAT gene added during drought conditions was abolished in the plants in which GABAT was silenced. Real-time PCR further

clarified the decline in the amount of the GABAT mRNA due to virus-induced gene silencing (VIGS).

The individual results generally show that the BSMV-GABAT (GABAT-silenced) plants in sample sets 1 and 2 had higher C_t values than the BSMV control plants. The C_t value is an indication of the amount of DNA that is present in the sample initially for amplification. Because fewer cycles are needed for the BSMV control plants to reach threshold, more DNA is produced. For example, the silenced GABAT control 1 and 2 samples had a C_t value of 21.824 and 21.1094 respectively, while their respective, untreated control sample, BSMV control 1 and 2 had C_t values of 19.3916 and 19.6468 respectively (Table 1). The average C_t values show this trend as well (Table 2). The dissociation curve (Figure 8) validates these results as it shows that only the target GABAT sequence was amplified.

The fold changes also showed that the GABAT gene was down-regulated in the silenced plants. When normalized with the expression of the housekeeping gene (GAPDH), the average fold change of GABAT in the silenced control sample was 0.3494 (Table 2). This means the GABAT was down-regulated by 65% or that it had a three-fold decrease in expression as compared to the gene in the BSMV control samples.

A comparison between the C_t values of the control and drought-stressed plants also provides information on the function of the GABAT gene. The plants exposed to drought conditions had a lower C_t value, indicating that these plants expressed more of the GABAT gene. For example, in the second sample set, the silenced GABAT control sample's C_t value was 21.1094 while the silenced GABAT drought sample's was 19.3253 (Table 1). Although the GAPDH gene, a gene unaffected by drought conditions,

also shows an increase in expression from the control to drought plants, the GABAT genes have an overall larger increase. This indicates that this gene is up-regulated when exposed to drought conditions and, therefore, plays a role in drought resistance.

Conclusion

This study evaluated the role of the gamma-aminobutyric acid transaminase (GABAT) gene for its role in drought tolerance. The study used virus-induced gene silencing to evaluate the response of barley to drought conditions when the gene is not expressed. The visual evidence supports the conclusion that the GABAT gene aids in the drought resistance of the barley plant. The real-Time PCR C_t values indicate that the gene is up-regulated when exposed to drought conditions. The stressed plants also showed more drought symptoms when the GABAT gene was silenced than when it was functional. The PCR evidence also indicates that the gene was down-regulated in silenced plants, verifying that the increased symptoms occur because of the absent expression of the GABAT gene. With drought being the primary factor affecting barley crop yields (Araus et al., 2002), research into drought-resistant mechanisms is necessary in order to improve crop and economic yields. Research in this area will further contribute to an understanding of drought-tolerant mechanisms and how they can be induced in other plants as well. Positive results could aid in the economic success of farmers and food production.

There were two major problems encountered in this study. One was inconsistent infection of BSMV and the second was the difficulty separating the drought response from the response due to viral infection In the future, the virus should be altered to make

sure it infects consistently and does not overwhelm the plant. In addition, different methods of infection should be evaluated to ensure the infection of every plant in the sample sets. Additional studies should identify a more specific role of the GABA shunt in drought survival, as the role of this pathway is not fully understood. Furthermore, other genes in the GABA shunt should be evaluated to get a clear picture of the role of this pathway in stress tolerance.

References

Abebe, T., Melmaiee, K., Berg, V., Wise, R.P. (2010). Drought response in the spikes of barley: gene expression in the lemma, palea, awn and seed. *Functional & Integrative Genomics*, 10: 191-205.

Araus, J.L., Slafer, G. A., Reynolds, M. P., Royo, C. (2002). Plant Breeding and Drought in C_3 Cereals: What Should We Breed For? *Oxford Journals*, 89(7), 925-940.

Bouché N., Fait, A., Bouchez, D., Møller, S.G., Fromm, H. (2003) Mitochondrial succinic-semialdehyde dehydrogenase of the γ-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. *PNAS,* 100(11), 6843-6848.

Bray, E.A., Bailey-Serres, J., Weretilnyk, E. (2000). Responses to abiotic stresses. *Biochemistry and Molecular Biology of Plants*. B. B. Buchanan, W. Gruissem, R. L. Jones (Ed.) Rockville, MD: American Society of Plant Physiologists.

Cui, Y., Lee, M. Y., Huo, N.; Bragg, J., Yan, L., Yuan, C., et al. (2012). Fine Mapping of the Bsr1 Barley Stripe Mosaic Virus Resistance Gene in the Model Grass *Brachypodium distachyon*. *Public Library of Science One*, 7(6).

Demircan, T., Akkaya, M.S. (2009). Virus induced gene silencing in *Brachypodium distachyen*, a model organism for cereals. *Plant Cell Tiss Organ Cult*, 100, 91-96.

Holzberg, S.; Brosio, P., Gross, C., Pogue, G.P. (2002). Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal*, 30(3), 315-327.

Liang, J. Deng, G., Long, H., Pan, Z., Wang, C., Cai, P et al. (2012). Virus-induced silencing of genes encoding LEA protein in Tibetan hulless barley (Hordeum

vulgare ssp. Vulgare) and their relationship to drought tolerance. *Molecular Breeding* 20(1).

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* 25(4), 402-408.

Mathre, D.E. (Ed.). (1985). Barley Stripe Mosaic. *Compendium of Barley Diseases* (pp. 47-48). St. Paul, MN: The American Phytopathological Society.

Mauldin, J. (2013). Laboratory Notebook.

Mead, O., Thynne, E., Winterberg, B., Solomon, P. S. (2013). Characterizing the role of GABA and its metabolism in the wheat pathogen *Stagonospora nodorum. PLoS ONE* 8(11). Retrieved from <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0078368#pone-0078368-g008>.

Meng, Y., Moscou, M.J., & Wise, R.P. (2009). Blufensin1 negatively impacts basal defense in response to barley powdery mildew. *Plant Physiology*, 149: 271-285.

Ramegowda, V., Senthil-kuar, M. Udayakumar, M. Mysore, K. (2013). A high-throughput virus-induced gene silencing protocol identifies genes involved in multi-stress tolerance. *BMC Plant Biology* 13(1), 1-38.

Roberts, M.R. (2007). Does GABA Act as a Signal in Plants? *Plant Signal Behavior*, 2(5):408-409.

Senthil-Kumar, M., Mysore, K. S. (2011). Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. *Plant Biotechnology Journal* 9(7), 797-806.

Sayed, M., Schumann, H., Pillen, K., Naz, A. A., & Léon, J. (2012). AB-QTL analysis reveals new alleles associated to proline accumulation and leaf wilting under drought stress conditions in barley (*Hordeum vulgare L.). Genetics* 13(1), 61-72.