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Efficacy of a Novel Molecular Tool in Silencing Arabidopsis Genes

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ABSTRACT

RNA interference (RNAi), a pathway capable of silencing genes, has until recently only been achievable in the laboratory by the use of one method, expression of inverted repeat sequences of DNA. These constructs generate a double-stranded RNA, which in turn induce post-transcriptional silencing of other genes that bear sequence homology with the transgene. This approach of targeted gene silencing is extremely useful for studying the function of genes and engineering new traits in both plants and animals. It has recently been discovered that a transgene lacking the polyadenylation signal, called a truncated transgene, is also capable of inducing RNAi in plant cells. This technique was used in efficiently silencing two genes of *Arabidopsis thaliana*, the Phytochrome A (PHYA) and Phytochrome B (PHY B) genes; however, the effectiveness of this method on a broader range of genes is unknown. The purpose of this study is to analyze the effect of truncated-transgene expression on the homologous native genes in the Arabidopsis genome. More specifically, the rate of silencing of three genes, Variegated 2 (VAR2), Brassinosteroid Insensitive 1 (BRI1) and Apetala 1 (AP1) due to the expression of truncated VAR2, BRI1, and AP1 transgenes, respectively, in *Arabidopsis thaliana* was examined. This experiment provided important data for assessing the efficacy of truncated transgene based gene silencing system for plants.

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MEET THE STUDENT-AUTHOR

Chase Purnell

I grew up in Hot Springs, Ark., and graduated from Lakeside High School in 2008. Later that year, I began pursuing a biology degree at the University of Arkansas after receiving the Chancellor's Scholarship and the Honors College Academy Scholarship. I soon became a member of the premedical honor society, Alpha Epsilon Delta, as well as Sigma Alpha Epsilon fraternity. As a commercial pilot, I enjoy flying on a regular basis, and I also like spending time outdoors.

In the Spring of 2011, I began undergraduate research in the department of Crop, Soil and Environmental Science under the direction of Vibha Srivastava. With her help and that of Aydin Akbudak, I completed an undergraduate honors research project funded by the Arkansas Science and Technology Authority and the University of Arkansas Honors College. After graduation, I will be attending medical school at the University of Arkansas for Medical Sciences in Little Rock. I would like to thank Vibha Srivastava's entire research team for their help and support throughout the duration of my project, as well as my family and friends who have helped me every step of the way.

INTRODUCTION

Nearly all industries ranging from healthcare to manufacturing and even agriculture benefit in some way from research performed in the field of biotechnology. This dynamic field is focused on improving or modifying products through the use of biological systems of any sort. The economically important crop, cotton, is of unique interest to many scientists due to the high level of plant toxins contained in the seeds. If somehow these levels could be lowered or eliminated, a previously toxic part of the plant would be available for human consumption (Perrimon et al., 2010). While this is just one of many examples, a promising approach towards finding a solution involves gene silencing (Cogoni and Macino, 2000).

Gene silencing can be defined as the interruption or suppression of the expression of a gene at either transcriptional or post-transcriptional levels. Often times, completely knocking out a gene may be advantageous, such as silencing the gene that produces toxins in cotton. Sometimes, however, complete silencing is not desired and only knocking down the gene would be a successful outcome, as in the case of lignin synthesis. Because lignin provides stability to plants, its complete silencing would be undesirable, but levels need to be reduced to simplify the production of biofuels (Beavis, 2007). RNA interference (RNAi) is a pathway capable of silencing a specific gene through the degradation of the targeted gene transcript, the messenger RNA (mRNA); and thus, RNAi is also referred to as post-transcriptional gene silencing (PTGS) (Vaucheret et al., 2001).

Recently, Nicholson and Srivastava (2009) described the use of truncated gene constructs (genes lacking a transcription termination signal) to induce silencing of two different genes in Arabidopsis. Expression of such gene constructs is expected to produce improperly terminated gene transcripts, which are marked for degradation by RNAi mechanism. The resulting siRNA then targets the degradation of homologous mRNA leading to the silencing of the specific gene. The distinction of silencing induced by truncated gene constructs is that it leads to gene knock down rather than knock out. However, it is important to test the utility of this new method on other Arabidopsis genes. Therefore, the purpose of the present study is to examine the effectiveness of this new method when targeting the Variegated 2 (VAR2), Brassinosteroid Insensitive 1 (BRI1) and Apetala 1 (AP1) genes in *Arabidopsis thaliana*.

Three genes, VAR2, BRI1, and AP1, will be the genes of interest in this study. VAR2, also known as Variegated 2, functions in thylakoid membrane biogenesis (TAIR, 2011). As a result of gene mutation, variegation of leaves occurs because a chloroplast thylakoid membrane protein is lost. Variegated plants can be easily identified because their leaves have white sectors, areas that contain non-pigmented plastids, while normal chloroplasts are found in the green areas (Chen et al., 2000). The next gene of interest is the BRI1 gene, also known as Brassinosteroid Insensitive 1, which

regulates leaf and flower development and response to UV-B (TAIR, 2011). The gene functions as a transmembrane receptor kinase and is important because it transduces brassinosteroid signals (Wang et al., 2001). A mutation of this gene results in a dwarf phenotype (TAIR, 2011). Finally, the last gene being investigated is the Apetala-1 or AP1 gene. This gene is responsible for determining flower meristem identity during early plant development, and later is responsible for initiation and development of floral organs. More specifically, the resulting AP1 protein determines where floral organ primordial will develop and it is also important for differentiation of cell types. Thus, a mutation in the gene causes incomplete flower development resulting in irregular flowers (Irish and Sussex, 1990).

Overall, the goal is to test the effectiveness of 'truncated gene constructs' in silencing three new genes in Arabidopsis—VAR2, BRI1, and AP1. The truncated constructs of each of these genes contains 500-700 base pair gene coding region without a transcription terminator under the control of a strong promoter (cauliflower mosaic virus 35S promoter) (Figs. 1-3). Arabidopsis lines transformed with these constructs were generated in the laboratory in an ongoing project.

To fulfill the above goal, the following objectives have been developed: (1) to study the phenotype of Arabidopsis plants expressing truncated VAR2, BRI1, and AP1 gene constructs; and (2) to determine the respective transcript levels using real-time polymerase chain reaction (RT-qPCR) based assay.

MATERIALS AND METHODS

Selection of Transgenic Plants. Using the laboratory pool of transgenic Arabidopsis seeds (T_1 lines) expressing truncated VAR2, BRI1, or AP1 fragments, each individual line was plated separately on MS (Murashige-Skoog) media containing the antibiotic kanamycin (50 mg/L). Because each transgene was coupled with NPT II, the kanamycin resistance gene, only those lines in which successful transgene integration had occurred would grow. All plants were grown for 16 hours of light and 8 hours of dark at 23 °C for a 2-week period. Following selection, the transformed seedlings were transplanted to small pots containing an even mixture of soil and vermiculite to continue growth. After several weeks, these T_1 lines produced seeds (T_2 generation), which were collected and sterilized using 50% bleach. Following vernalization for three days, these seeds were selected and grown in the same manner for phenotype analysis.

RNA Isolation. Isolating RNA from T_2 lines varied depending on the gene being studied. Since the VAR2 gene is highly expressed in cauline leaves, they were collected for RNA isolation. Similarly, cauline leaves were collected for BRI1 analysis. For AP1, because its primary function resides in flower development, only the floral tissue was collected for analysis (TAIR, 2011). After tissue collection, liquid nitrogen was used to freeze the target tissues and later ground to fine dust using mortars and pestles. Then Qiagen's RNeasy kit was used for isolating the RNA from each sample (Qiagen, Valencia, Calif.). Next, these samples were treated with RQ1 RNase-Free DNase to rid the samples of any DNA they may contain. Finally, each sample of RNA was examined for quality and was quantified using ND-1000 spectrophotometer (Nanodrop Products, Wilmington, Del.).

RT-qPCR Analysis. The final experimental step was to determine the effect of truncated constructs on the expression of the corresponding genes. To accomplish this goal, the first step was to design the primers to be used in RT-qPCR. Recalling the experimental design, it is crucial to amplify an area of the endogenous gene that is outside the transgene. Using the nucleotide sequence of each gene and the nucleotide sequence of each transgene construct, a suitable area for each gene was selected for amplification by using the Primer3 program (Fig. 4). Several sequences were examined before a final set of primers was selected based on length, melting temperatures and proximity to the transgenic construct.

Next, using a CFX96 Real-Time PCR Detection System (Bio-Rad Inc., Hercules, Calif.), real time quantitative polymerase chain reaction (RT-qPCR) was performed. VAR2, BRI1, and AP1 expressions were quantified in 25 ng DNase-treated RNA samples using SuperScript III Platinum SYBR Green One-Step RT-qPCR Kit (Invitrogen, Grand Island, N.Y.). Each sample was replicated three times and the resulting average Ct value was used for the analysis. The annealing temperature for each gene, as determined by a PCR gradient, is as follows: VAR2, 62 °C; BRI, 58.5 °C; and AP1, 62 °C.

To determine the expression level of each gene in their respective lines, the $\Delta\Delta C_t$ method was utilized. To serve as a reference, the genes Prefoldin 2 (PDF2) and Actin 2 (ACT2) were used as internal reference genes to normalize the PCRs for the amount of RNA added to each reaction (Livak et al., 2001). The C_t value of these reference genes were subtracted from the Ct value of the target genes, thus yielding the first ΔC_t. Then the ΔC_t value of the non transgenic line, Columbia, was subtracted from the ΔC_t value of each transgenic/mutant line, thus yielding $\Delta\Delta C_t$. To calculate the fold change for each transgenic line as compared to the non-transgenic Columbia, the following equation was used:

$$\text{Fold Change} = 2^{-(\Delta\Delta C_T)}$$

RESULTS AND DISCUSSION

RT-qPCR was performed to determine the extent to which the plants had been silenced. Those plants with the truncated VAR2 gene, pVAR2F, showed silencing in 60%

(3/5) of the lines ranging from 2% to 82% suppression with a mean of 12.4% suppression when measuring against ACT as the housekeeping gene (Fig. 5). The housekeeping gene ACT was used because the melting curves obtained using PDF gene were incorrect, most likely because of a problem with the PDF primers.

The result of this new method when tested using the BRI1 gene showed tremendous potential as well. Using eight separate lines of Arabidopsis transformed with the transgene pBRI1, the data generated using RT-qPCR indicated silencing was present in 100% (8/8) of the lines ranging from 4% to 58% suppression in BRI1 transcript abundance with a mean of 42% suppression when measuring against PDF as the housekeeping gene (Fig. 6).

As for the AP1 gene, silencing was observed in 100% (3/3) of the lines ranging from 46% to 77% suppression with a mean of 64.6% when using the transgene pAP1 and averaging the results when using ACT & PDF as the housekeeping genes (Fig. 7).

The results obtained from the phenotype and RT-qPCR analyses supported the hypothesis that truncated transcripts induce silencing of the target gene. Beginning with the VAR2 plants, the phenotypic analysis showed some variegation during the early stages of development, but once maturation had been reached, the leaves with variegation had recovered and thus the mutant phenotype was no longer visible (Fig. 8). As for the BRI1 gene, the expected phenotype as described by TAIR (Arabidopsis.org) was observed. The plants were bushy and short relative to the control. In addition, all were infertile as observed by the lack of formation of seedpods (Fig. 9). Finally, for the AP1 gene, the expected phenotype was harder to analyze because it only appears in floral tissue. According to Arabidopsis.org, silencing of this gene will result in irregular flowers; however, this was not observed during this trial. Some research suggests that the mutant floral phenotype will only be observed when the gene is fully silenced, in other words, a knock out. The lack of phenotype may also be attributed to the tissue specificity. It may be possible that silencing did not penetrate the cell layer where the AP1 gene is expressed.

One common problem encountered during experimentation across all transformed Arabidopsis lines occurred after the seeds were plated on media. After Kanamycin selection killed the untransformed seedlings, the healthiest of those still growing were chosen for transplantation. It is hypothesized that those that appear very unhealthy may be silenced to a greater extent than the healthier plants chosen, but because they almost never survived transplantation, a definite conclusion is hard to determine. However, the unhealthy appearance may just be a result of the seedlings susceptibility to kanamycin. Another problem encountered during the project stemmed from the MS media. For unknown reasons, two separate mixtures of MS media were faulty and caused all seedlings to die, thus altering the experimental timeline. Finally, data obtained from the VAR2 analysis indicated that two lines expressed the target gene at higher levels than the control. The cause of this finding most probably is the result of either low/degraded RNA, primer binding problems, and/or experimental procedure errors.

The study of RNAi is a rapidly growing field due to its ability to provide a reverse approach to challenging genetics problems. The driving force behind RNAi was shown to be double stranded RNA (dsRNA) when gene expression in the nematode *Caenorhabditis elegans* was significantly manipulated (Fire et al., 1998) by injecting dsRNA into cells rather than when two single stranded RNA (ssRNA) were injected. Researchers found this introduction interfered with the expression of an endogenous gene that bears sequence homology with the dsRNA, hence the name RNA interference. In addition, it was found that RNAi was systemic, meaning that injection of dsRNA in one tissue can cause silencing in distant tissues (Fire et al., 1998). The applications of RNAi have become apparent with the completed genome sequencing of several organisms (Perrimon et al., 2010).

RNAi, as previously stated, is initiated by dsRNA introduction into a cell. Enzymes cleave the dsRNA into small fragments called short interfering RNA (siRNA) ranging from 21 to 30 nucleotides in length (Zamore et al., 2000). These siRNAs are separated into ssRNA and aid in guiding the RNA-induced silencing complex (RISC) to trap the homologous transcripts produced by the native genes (mRNA) (Chakraborty et al., 2004). Within the RISC, the ssRNA pairs with mRNA due to base complementarity, creating a dsRNA structure. As a result, the mRNA cannot be used by the cell for producing a functional protein (Baulcombe et al., 1999). This new dsRNA suffers the same fate as other dsRNA and is cleaved into small fragments, thus repeating this process.

While dsRNA is key to initiating RNAi, the methods by which dsRNA are produced vary. The most popular method involves transcribing dsRNA, which is homologous to the target gene, from an inverted repeat structure of DNA introduced into an organism's genome (Wesley et al., 2001). The dsRNA can also be injected as done in the work performed by Fire et al., 1998 on *C. elegans*. While the inverted repeat method is effective in targeted gene knock out, additional methods may be needed that may result in gene knock down more reliably and reproducibly.

In this study, this new silencing tool proved to be an effective method for gene knock down across multiple lines of *Arabidopsis thaliana* when testing the VAR2, BRI1, and AP1 native genes. The plant *Arabidopsis thaliana* was chosen for gene silencing work because of its small size, large number of offspring, and a small nuclear genome (The Arabidopsis Genome Initiative, 2000). While the level of suppression varied from gene to gene, it is important to

note that this method never knocked out gene expression. The data obtained from the RT-qPCR were consistent with the phenotypic analysis, except in the case of AP1. Based on these results, the method of inserting truncated transgenes as a method for knocking down gene expression is a successful and promising tool for future research.

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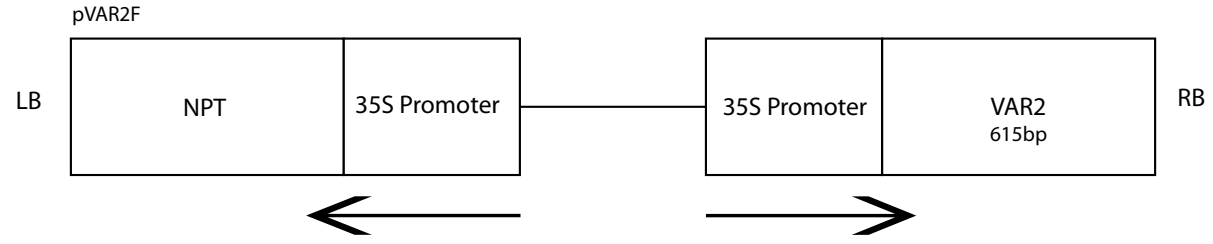

Fig. 1. Transgene construct in binary vector pPZP200 (*Agrobacterium tumefaciens* vector) backbone used to generate transgenic Arabidopsis lines. Fragment of Variegated 2 (VAR2) gene expressed by 35S promoter and oriented oppositely to the selection marker (NPT) conferring resistance to kanamycin. LB and RB refer to left and right borders of T-DNA.

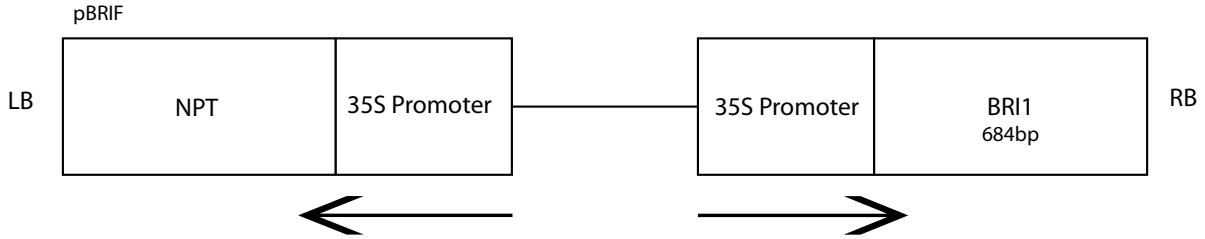

Fig. 2. Transgene construct in binary vector pPZP200 (*Agrobacterium tumefaciens* vector) backbone used to generate transgenic Arabidopsis lines. Fragment of Brassinosteroid Insensitive 1 (BRI1) gene expressed by 35S promoter and oriented oppositely to the selection marker (NPT) conferring resistance to kanamycin. LB and RB refer to left and right borders of T-DNA.

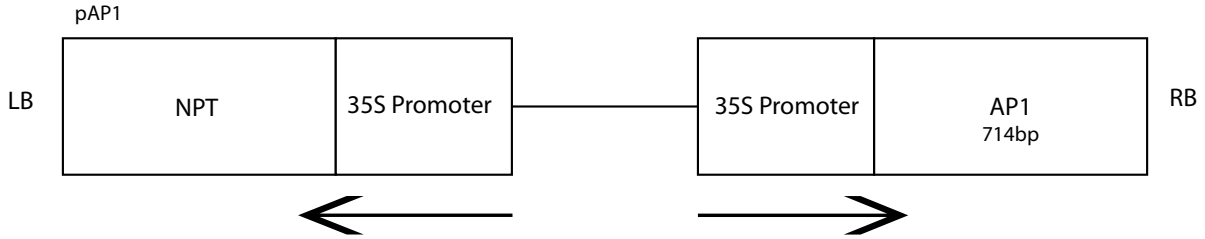

Fig. 3. Transgene construct in binary vector pPZP200 (*Agrobacterium tumefaciens* vector) backbone used to generate transgenic Arabidopsis lines. Fragment of Apetala 1 (AP1) gene expressed by 35S promoter and oriented oppositely to the selection marker (NPT) conferring resistance to kanamycin. LB and RB refer to left and right borders of T-DNA.

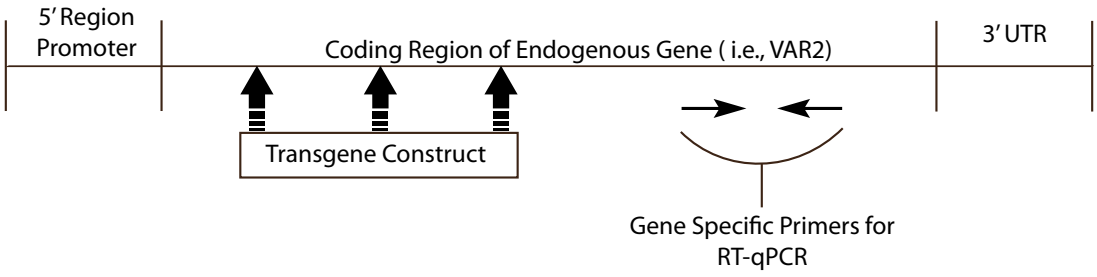

Fig. 4. Strategy for gene expression analysis in transgenic Arabidopsis lines expressing Variegated 2 (VAR2), Brassinosteroid Insensitive 1 (BRI1) and Apetala 1 (AP1) gene constructs (see Figs. 1,2, and 3). The top line represents endogenous genes. Rectangle below represents the fragment cloned into the transgene construct. For gene expression analysis, gene specific primers (opposing arrow heads) were used in real time quantitative PCR (RT-qPCR) using total RNA in the reaction. The 5' region represents the gene promoter and 3'UTR is an untranslated region.

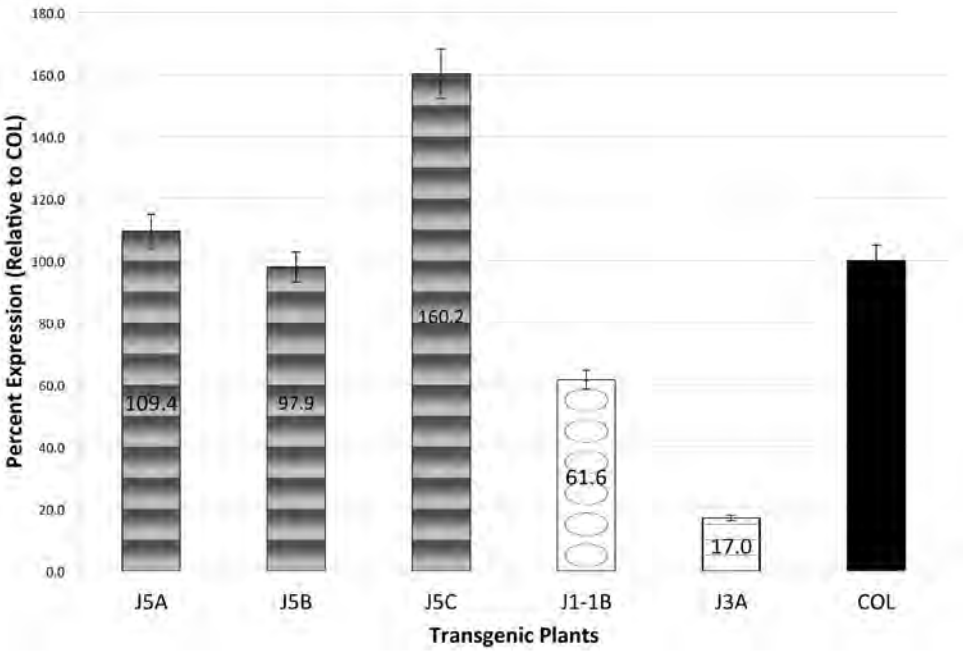

Fig. 5. RT-qPCR data analysis of Variegated 2 (VAR2) gene when using ACT as the housekeeping gene showing standard error bars for alpha 0.05. Samples with the same texture indicate plants sharing a common parent, i.e. J5A, B, and C all share a single parent. Columbia (COL) is the non-transgenic control.

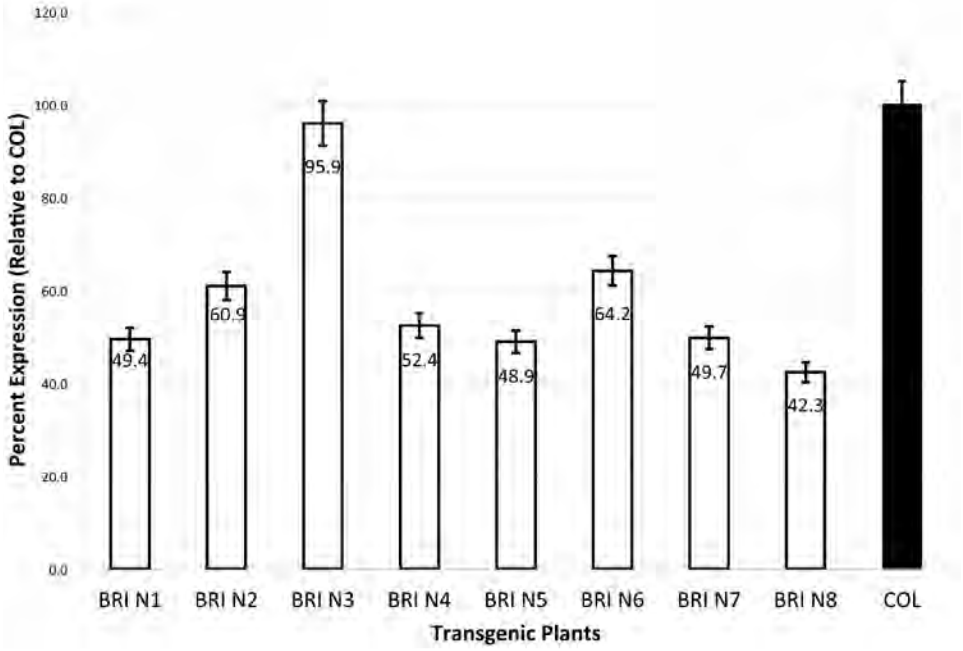

Fig. 6. RT-qPCR data analysis of Brassinosteroid Insensitive 1 (BRI1) gene when using PDF as the housekeeping gene showing standard error bars for alpha 0.05. Each BRI1 sample represents the progeny of a different T1 parent. Columbia (COL) is the non-transgenic control.

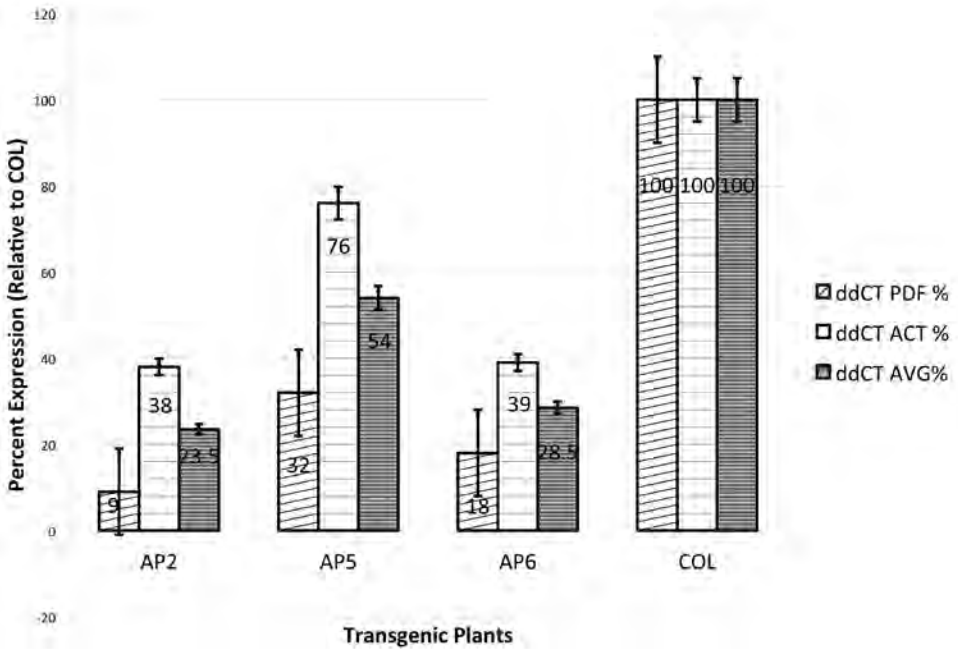

Fig. 7. RT-qPCR data analysis of Apetala 1 (AP1) gene showing standard error bars for alpha 0.05. All samples are derived from different parents. Columbia (COL) is the non-transgenic control, and both ACT and PDF were used as the housekeeping genes as shown by ddCT PDF% and ddCT ACT%, respectively. The results of these two reference genes were averaged as shown by ddCT AVG%.

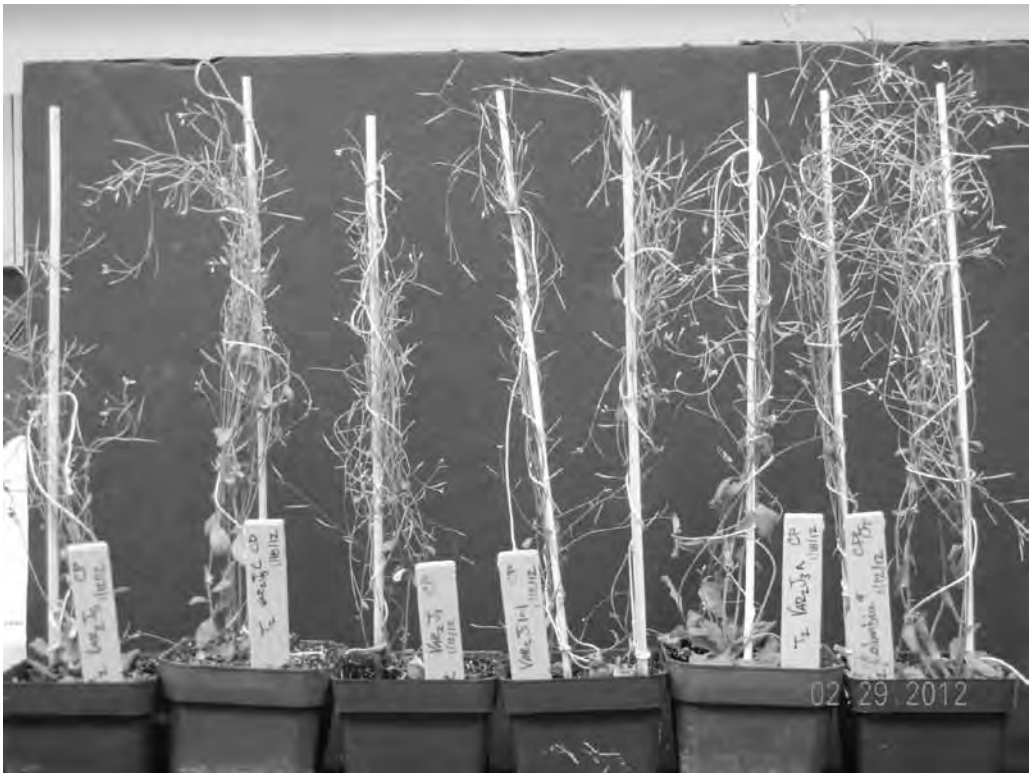

Fig. 8. Phenotypic analysis of mature Variegated 2 (VAR2) plants. At this stage, the mutant phenotype, once visible in young seedlings, is no longer visible.

Fig. 9. Phenotypic analysis of mature Brassinosteroid Insensitive 1 (BRI1) plants. At this stage, the mutant phenotype is visible.