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Laboratory Exercise

RT-qPCR Demonstrates Light-Dependent AtRBCS1A and AtRBCS3B mRNA Expressions in *Arabidopsis Thaliana* Leaves[S]

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Abstract

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is widely used in diagnosis and research to determine specific mRNA expressions in cells. As RT-qPCR applications increase, it's necessary to provide undergraduates hands-on experience of this modern technique. Here, we report a 3-week laboratory exercise using RT-qPCR to demonstrate the light-dependent expressions of AtRBCS1A and AtRBCS3B genes encoding two *Arabidopsis thaliana* small subunits of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). In the first week, students purified and quantified total RNA from leaves of *A. thaliana* pretreated in the dark for 96 hr and untreated controls. In the second week, RNA samples were separated by formaldehyde gel electrophoresis and used for RT-qPCR. Students calculated expressions of the two genes in dark treated leaves as percentages of those of the controls by using the $2^{-\Delta\Delta C}{}_T$ method and the collected C_Ts. In the third week, class C_Ts, melting curves, students' calculations, and factors affecting the reliability of RT-qPCR results were summarized and discussed. Students' results show that (i) relatively pure and intact RNA samples are obtained; (ii) ACTIN2 is a better reference gene than the 18S rRNA; (iii) the dark treatment reduces both gene expressions to < 1%; (iv) the reduction in the expression of AtRBCS3B is significantly more than that of the AtRBCS1A. Results from pre- and post-lab tests indicate that besides the theory, this exercise helps students learn the applications and associated techniques of RT-qPCR. Future modifications and new experiments that can be developed based on students' learning outcomes and assessment are also discussed.

Keywords: *RT-qPCR; gene expression; cDNA; ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunits*

Introduction

Plants convert carbon dioxide and water to oxygen and carbohydrates via photosynthesis. The light reaction of photosynthesis converts light to chemical energy in the forms of NADPH and ATP to fix the carbon from CO_2. The fixed carbon is reduced to carbohydrates by a process called the "Calvin cycle" or "dark reaction" which requires the enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), to catalyze the carboxylation of ribulose-1,5-bisphosphate (RuBP) [1]. The most common form of Rubisco, found in higher plants and green algae, consists of eight "large" subunits (RBCL) encoded by a single *rbcL* gene and eight "small" subunits (RBCS) encoded by an *rbcS* multigene family. The numbers of expressing members and transcript abundance in the *rbcS* multigene family have been studied in different tissues, under different environments, and during tissue development [2–4]. Both transcription and stability of individual *rbcS* mRNAs are altered in different organs and regulated by the developmental program within these organs as well as by exposure to light [3, 4]. Coruzzi *et al.* have shown that compared with green leaves, the level of *rbcS* mRNAs is reduced to 1-3% in etiolated pea leaves [4]. Also, the pea *pPS-2.4* mRNA accounts for $\sim 30-35\%$ of total *rbcS* transcripts in green leaves but is below detection in etiolated leaves.

In *Arabidopsis thaliana*, the small subunits of Rubisco are encoded by four genes, which are divided into subfamilies A

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and B [5]. The genes in the B subfamily consist of 1B, 2B, and 3B, while the A subfamily consists of 1A. These genes are differentially regulated by light of different qualities [6]. Unlike AtRBCS3B, AtRBCS1A seems to be insensitive to blue light pulses. However, both AtRBCS1A and AtRBCS3B play an important role in controlling photosynthesis. Using reverse transcription quantitative PCR (RT-qPCR), Izumi *et al.* demonstrated that both mRNAs contribute to the accumulation of Rubisco in *Arabidopsis* leaves, and they work additively to produce enough Rubisco for photosynthesis [7].

Quantitative real time polymerase chain reaction (qPCR) is known for its high sensitivity, real time detection of reaction progress, speedy analysis, and precise measurement of the target in the sample [8]. Reverse transcription (RT) of RNA followed by qPCR (so-called RT-qPCR) has become a powerful tool for studying gene expression in cells. Three major steps in RT-qPCR are: (i) the reverse transcriptase-dependent conversion of mRNA into cDNA, (ii) the amplification of the cDNA by qPCR, and (iii) the detection and quantification of amplified products in real time [9]. With the growing number of RT-qPCR applications, it is important to introduce this modern technique to undergraduates. Published laboratory exercises are available for introducing RT-qPCR to undergraduates. Recently, one employed RT-qPCR to analyze four target sequences during the DMSO- differential expression induced differentiation of cultured erythrocytes [10]. Another showed the vernalization-induced reduction of FLOWERING LOCUS C gene expression in *Arabidopsis thaliana* [11]. However, like other qPCR lab exercises, both papers focused on the theory and experimental results but not factors affecting the interpretation/reliability of RT-qPCR data, such as the choice of an appropriate reference gene. For example, if the chosen reference gene shows a large fluctuation in expression, normalization will lead to inappropriate or faulty biological data interpretation. Furthermore, there are no universal reference genes available. It was shown that the widely used reference genes, ACT and GAPDH, are not the most suitable reference genes for RT-qPCR experiments in banana [12].

Therefore, we designed an undergraduate lab exercise to study the effect of 96 hr dark treatment on mRNA expressions of AtRBCS1A and AtRBCS3B encoding the small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in *A. thaliana*. The 3-week lab exercise includes RNA isolation and quantification which were done during the first week, followed by formaldehyde gel electrophoresis of the RNA samples and RT-qPCR in the second week. In the third week, the results and factors affecting the reliability of RT-qPCR experiments were summarized and discussed in class. In addition to addressing the theory of RT-qPCR and demonstrating the 96-hr-dark-induced reduction of both gene expressions in *A. thaliana* leaves, this lab exercise focused on several important factors for the validation of RT-qPCR results, which include: (i) good RNA purity and quality based on O.D. ratios obtained from spectrophotometer and the results of formaldehyde agarose gel electrophoresis, (ii) proper controls, (iii) a reliable reference base by comparing results obtained from two reference genes, ACTIN2 and 18S rRNA, and (iv) high qPCR efficiency by testing the % amplification efficiency of each gene.

Learning Outcomes

After completing the exercise, students should be able to (i) explain RT-qPCR to others and the theory behind it; (ii) carry out total RNA isolation, reverse transcription, and qPCR following the step-by-step protocols provided; (iii) use the $2^{-\Delta\Delta C_T}$ method and collected C_Ts to calculate expressions of target genes; (iv) understand and interpret data obtained from the lab exercise and convert them into tables and figures for writing up a report in the format of a peer-reviewed journal; (v) understand and/or critique scientific research papers on RT-qPCR/qPCR experiments; (vi) set up a similar gene expression study using RT-qPCR with appropriate controls and reference genes.

Materials and Methods

Plant Materials

Arabidopsis (*Arabidopsis thaliana*, accession Col-O) seeds were grown following the protocol provided by the Arabidopsis Biological Resource Center [13]. Briefly, seeds were sown at a rate of 16 – 20 seeds per pot in 10 cm square pots containing mounded potting soil (Miracle-Gro Potting Mix) covered with sterile plastic screening. After sowing, pots were put in covered flats which were then placed in a Conviron E15 growth chamber (Winnepeg, Canada) at 23°C, 60% relative humidity, and 120 μmol/m^2sec light with a long-day photoperiod (14 hr light, 10 hr dark) for ~6 weeks before tissue preparation. Where necessary, the plants were thinned to 3 or 4 per-pot to encourage larger plant size. When plants were 6-week-old, half of them were covered with cardboard lined with double-layered plastic sheets made from 55 gallon black trash bags for 96 hr as the dark treatment. The rest were left under the same photoperiod as the control. Approximately 100 mg leaf tissue was harvested and placed in a 1.5 ml microcentrifuge tube which was quickly frozen in liquid N_2 and then stored at −70°C until ready for RNA isolation.

Week One

Total RNA Isolation and Quantification

Students worked in pairs in the lab. For each lab period, a 30 – 40 min prelab lecture/discussion was given followed by a 3 hr hands-on exercise. At the beginning of Week One, the prelab lecture and discussion focused on specific precautions for working with RNA, such as wearing gloves, the use of RNase free water and RNase inhibitors, etc. Microfuge tubes containing leaf tissue were then retrieved from

the −70°C freezer and immediately placed in liquid N_2. Each student group obtained a tube of leaf tissue. A plastic pestle was used to grind the leaf tissue quickly and thoroughly. Half of the student groups isolated RNA from the control and the other half from the 96 hr dark treatment. The Qiagen RNAeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) was used for RNA isolation by following the "Total RNA Isolation with In-column DNase Treatment" protocol described in the "RNeasy Mini Handbook" provided by the manufacturer. RNA purity and quantity were assessed using a NanoDrop 100 spectrophotometer (Thermo Scientific, Wilmington, DE). The samples were then stored at −70°C until the following week.

Week Two

Formaldehyde Gel Electrophoresis and First Strand cDNA Synthesis

To evaluate the integrity of RNA samples, a 1.2% formaldehyde agarose gel containing GelRed (Phenix Research Products, Candler, NC) was made and gel electrophoresis was carried out by following the procedure described in Qiagen RNeasy Mini Handbook. Due to the time constraint, students set up the reverse transcription (RT) reactions followed by qPCR while waiting for the results of gel electrophoresis. For the first strand cDNA synthesis, each group used its own RNA sample obtained from Week One and carried out the RT reaction by using the Verso cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE) and following the protocol described by the manufacturer. Briefly, a 20 μL aliquot of diluted RNA sample (20 ng/μL) was made. To a 0.5 mL thin wall PCR tube, 11 μL of 20 ng/μL RNA and 1 μL of Primer mix (blend of random hexamers and the anchored oligo-dT [3 : 1] provided in the kit) were added. The tube was heated to 70°C for 5 min and then cooled quickly on ice for 1 min before mixing with the rest of the reaction mix containing 4 μL of 5x cDNA buffer, 2 μL of dNTP mix, 1 μL of RT enhancer, and 1 μL of Verso enzyme mix. "No RT" controls containing everything except the reverse transcriptase were also prepared for both the dark treatment and the control. All tubes were then placed in a MJ Research PTC-100 thermal cycler (MJ Research, Inc. of Waltham, MA) at 42°C for 60 min, and then at 95°C for 2 min to inactivate the enzyme. Finally, 180 μL of sterile water was added to obtain an RNA concentration of 1.1 ng/μL in each reaction tube.

Quantitative PCR

For qPCR, we used GoTaq® qPCR Master Mix (Promega US. Madison, WI). Each group set up eight qPCR reactions with *Arabidopsis* ACTIN2 and 18S rRNA as two reference genes, and AtRBCS1A and AtRBCS3B as the target genes. Since only a single RNA isolation was performed per group, the "control" groups shared their cDNAs with the "treatment" groups and vice versa. The primer pairs and sizes of the corresponding qPCR products are shown in Supporting Information Table S1. A strip of eight 0.2 mL qPCR tubes (BioRad, Hercules, CA) was provided to each group. To each tube, 10 μL of 2x Master Mix, 4 μL of H_20, 4 μL of diluted reverse transcription reaction as described above, and 2 μL of Primer Mix (5 pmole each of Forward and Reverse primers for each gene) were added. The first four tubes were set as follows: cDNA from the untreated control with forward and reverse primers corresponding to "ACTIN2," "18S rRNA," "AtRBCS1A," and "AtRBCS3B," respectively. The same order of primers was used with cDNA from 96-hr dark treatment for the remaining four tubes. Students were reminded to change filter tips for each pipetting. Since qPCR tubes can't be labeled, it is important to remind students to keep everything in the correct order. The tubes were then placed in a Stratagene Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA) or a BioRad CFX Connect™ Real-time PCR Detection System (BioRad USA, Hercules, CA). The PCR cycles were 95°C for 2.5 min, then 40 cycles of 95°C for 20 sec, 50°C for 30 sec, and 72°C for 30 sec and a final cycle of rapid heating to 95°C to denature the DNA followed by cooling to 55°C for the melting curve.

Calculations

Based on the collected threshold cycle (C_T, the number of cycles required for the fluorescent signal to cross the threshold or exceed background level) from the qPCR experiment and the $2^{-\Delta\Delta C}{}_T$ equation [14] shown below, students calculated AtRBCS1A and AtRBCS3B mRNA expressions in leaves of the 96 hr dark-treated plants presented as percentages of those of the controls.

Gene Expression (as % mRNA expression of the control = $2^{-\Delta\Delta C}{}_T \times 100$)

- $C_{T\ GOI\ (control)} - C_{T\ REF\ (control)} = \Delta\ C_{T\ (control)}$
- $C_{T\ GOI\ (dark)} - C_{T\ REF\ (dark)} = \Delta\ C_{T\ (dark)}$
- $\Delta C_{T\ (dark)} - \Delta\ C_{T\ (control)} = \Delta\ \Delta\ C_T$

GOI: gene of interest (AtRBCS1A or AtRBCS3B)
REF: reference gene (ACTIN2 or 18S rRNA)
dark: leaves from plants with 96 hr dark treatment
control: leaves from plants without dark treatment

Hazards

The protocol used in this lab exercise was reviewed and approved by SUNY Geneseo's Institutional Review Board (IRB) and is considered exempt under 45 CFR 46 subpart A, §46.101(b)(1) because it involves research conducted in established or commonly accepted educational settings, involving normal educational practices. Students were required to wear lab coat and closed-toed shoes in lab. Disposable gloves were provided for each student. The liquid N_2 used to freeze leaf tissue needs to be handled with care. For RNA isolation, the Qiagen RNAeasy Plant Mini Kit

TABLE 1 **Average[a] *purity and yield of RNA isolated from* A. thaliana *leaf***

260/280[b]	2.09 ± 0.03
260/230[c]	2.34 ± 0.05
Yield (μg/100 mg leaf tissue)	10.75 ± 3.99

[a]*Average from twelve RNA isolations, six each for the 96-hr dark treatment and the control.*

[b]*Absorbance ratio of nucleic acid to protein. A ratio of ~ 2.0 indicates a pure RNA (14).*

[c]*Absorbance ratio of nucleic acid to carbohydrate, EDTA, and phenol. A ratio of ~ 2.0 - 2.2 indicates a pure RNA (14).*

contain Buffer RLC made of guanidine thiocyanate, Buffer RLT made of guanidine hydrochloride, and Buffer RW1 made of a small amount of guanidine thiocyanate. Guanidine can form a highly active compound when combined with bleach. Thus, all three buffers and materials in contact with them need to be handled carefully. Beta-mercaptoethanol used in RNA extraction is mutagenic and a potential neurotoxin, and should be used in the fume hood. The formaldehyde used for RNA gel electrophoresis can be irritating to the skin, eyes, and respiratory tract and should also be used in the fume hood. SYBR Green I is not known to be mutagenic, but is a DNA intercalating agent and should be used with caution. All these chemicals need to be treated as hazardous waste for special disposal.

Results and Discussion

In this paper, we report the results from three semesters' RT-qPCR exercises performed in Molecular Techniques (Biol 390), a one semester, 2 credit hour lab course required for biochemistry majors. The lab is designed for biochemistry and biology juniors/seniors. All students had previously taken genetics, cell biology, and/or biochemistry and were familiar with DNA, RNA, and protein structures and functions. Most if not all students also take/took the other junior/senior course, Molecular Biology (Biol 322), which does not have a lab component. As such, the molecular techniques course introduces students to basic techniques of DNA, RNA, and protein manipulations commonly used in molecular biology, as well as the use of associated apparatuses. Exercises for DNA and protein manipulations include protein and DNA isolations and quantifications, PCR, agarose gel electrophoresis, DNA cloning and bacterial transformation, restriction digest, web-based sequence analysis, SDS polyacrylamide gel electrophoresis, Western blot, and Immunodetection. The three weeks' RT-qPCR lab exercise corresponds to the part of RNA manipulation.

At the beginning of each lab period, the instructor spent 30 – 40 min going through the background information and protocols, and/or discussing results obtained from the previous week(s). In the first week, students isolated total RNA from leaves of *Arabidopsis thaliana*. As shown in Table 1, relatively pure RNA samples were obtained because both A260/A280 and A260/A230 ratios were ≥ 2. An A260/A280 of 2.0 and an A260/A230 of 2.0 – 2.2 indicate a pure RNA sample [15]. However, the average A260/A230 was 2.34 (> 2.2), suggesting the presence of trace guanidine isothiocyanate used for RNA isolation, which absorbs at ~260 nm [15]. There was no significant difference in RNA purity and yield obtained between leaves of 96 hr-dark treated plants and those of the controls (data not shown). In the second week, formaldehyde agarose gel electrophoresis was performed to evaluate the integrity of RNA samples. Multiple rRNA bands were observed for each RNA sample, suggesting that they were relatively intact and of good quality without smearing (Fig. 1).

In the third week, the C_Ts derived from the amplification curve, necessary controls for the RT-qPCR assay, and melting curves collected were summarized and discussed in class. Based on the collected C_Ts, ACTIN2 is a better reference gene due to having a similar amplification magnitude as those of the two target genes and smaller variations in C_Ts of both the control and treatment (Table 2). Also, for the "No RT" controls, the C_Ts for all genes except 18S rRNA (C_T = ~27) were ~37 (data not shown). An in-class discussion focused on if the presence of C_Ts for the "No RT" controls resulted from our inability to completely remove trace genomic DNA in the RNA samples, which escaped DNase treatment during RNA purification. It might also be the cause for the much lower C_T observed for 18S rRNA (Table 2), a multicopy gene. Additional information on why 18S rRNA was not a suitable reference gene for studying mRNA expressions [16] as well as the significance

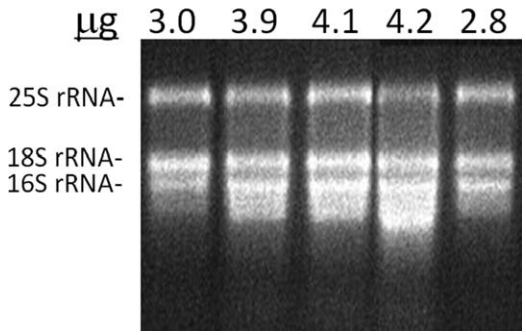

FIG 1 *GelRed stained 1.2% formaldehyde agarose gel showing different rRNA bands in RNA samples from one of lab sections. Fifteen of the 50 μL total RNA obtained by each group was loaded into each well. The number on the top indicates the amount of total RNA loaded. The remainders of the RNA samples were then used for RT-qPCR.*

TABLE 2 *Comparisons of collected CTs of four genes studied in this exercise*

Gene	*CTs**	*Mean ± S.D.*
Actin2 (Control)	25.28, 25.26, 25.07, 25.37	25.23 ± 0.13
Actin2 (Dark)	24.66, 25.53, 24.54, 25.97	25.05 ± 0.45
AtRBC1A (control)	19.40, 20.19, 19.21, 19.74	19.64 ± 0.43
AtRBC1A (Dark)	25.60, 25.62, 26.12, 25.80	25.78 ± 0.24
AtRBC3B (Control)	23.95, 24.14, 23.76, 23.95	23.95 ± 0.16
AtRBC3B (Dark)	32.96, 32.62, 32.78, 32.45	32.70 ± 0.22
18S rRNA (Control)	9.49, 8.18, 9.66, 8.76	8.87 ± 0.68
18S rRNA (Dark)	9.96, 9.19, 8.4, 9.71	9.10 ± 0.69

**Student data from one of the lab sections.*

and qualifications of a good reference gene were also discussed. A brand new unopened qPCR kit was used for the qPCR experiment. Thus, students did not set up the "No template" controls (No TC), but its importance was mentioned. The single peak in the melting curve is often used to confirm the presence of a single qPCR product amplified by a specific set of primers. Students' results show only one qPCR product being amplified by each primer set used (Fig. 2). The 18S rRNA and AtRBCS1A have a similar melt curve peaking at ~76°C. Although not done in this exercise, the amplification efficiencies of the qPCR assay were also addressed (see the Potential New Experiments section below).

For RT-qPCR data analysis, students used the collected C_Ts in combination with the $2^{-\Delta\Delta C_T}$ method to calculate expression of AtRBCS1A and AtRBCS3B in dark treated leaves as percentages of those of the controls. Results are shown in Fig. 3. AtRBCS1A and AtRBCS3B mRNA expressions in the dark were reduced to 0.0075 (0.75%) and 0.0031 (0.31%) of those of the control, respectively. The results are similar to those observed in pea plants, in which *rbcS* mRNA expression is reduced to 1 − 3%, and the pea *pPS-2.4* mRNA is below detection in etiolated leaves [4]. Factors causing the decrease in expressions of both AtRBCS1A and AtRBCS3B were discussed in class. In addition to transcriptional regulation [4], RNA stability may play a role. For example, both transcription and stability of individual *rbcS* mRNAs are altered in different tomato organs and by the developmental program within these organs as well as by exposure to light [3]. The level of the abundant *rbcS* mRNA declined rapidly when potato plants were placed in the dark [17]. Students' results also show that in the dark, the reduction in AtRBCS3B expression was significantly more than that of AtRBCS1A ($t = 2.74$, $p = 0.026$, two-tailed student t test), suggesting possible different regulation mechanisms for the two genes. Previously, it was shown that the expression of AtRBCS1A to light stimulation is regulated differently from that of AtRBCS3B [6]. The variation in the regulation of *Arabidopsis rbcS* gene expressions may have a selective advantage for the organism, and is not yet understood [6].

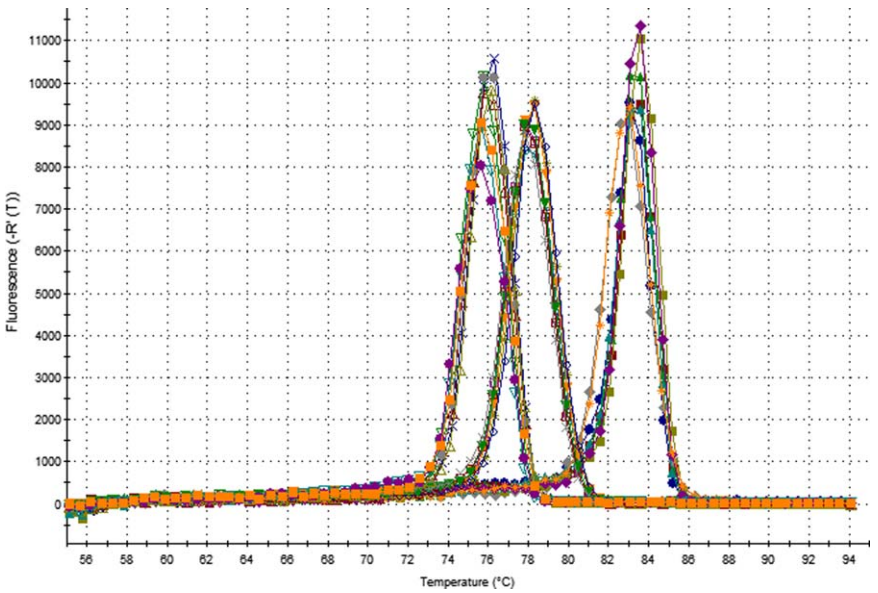

FIG 2 *First derivative plot (-R'(T) versus temperature) of the melting curve from 55°C to 95°C showing three peak temperatures at 76, 78, and 83°C (from left to right) for the 18S rRNA/AtRBCS1A, AtRBCS3B and ACTIN2 target DNAs respectively amplified with the corresponding primers. 18S rRNA and AtRBCS1A have a similar melt curve peaking at ~76°C. Each peak is derived from eight (four 18S rRNA and four AtRBCS1A), AtRBCS3B, or ACTIN2 amplified DNA samples.*

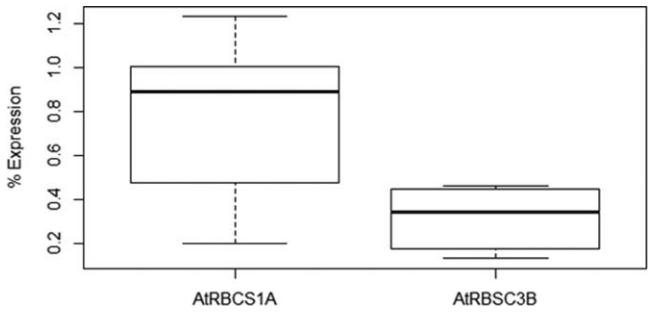

FIG 3

Box-plot showing average AtRBCS1A and AtRBCS3B mRNA expressions in 96 hr-dark-treated A. thaliana *leaves as percentage of those of the controls. The reduction of AtRBCS3B expression (median = 0.34%, mean = 0.31%) is significantly more than that of AtRBCS1A (median = 0.89%, mean = 0.75%). Bars in the boxes indicate the medians. Seven data points per gene from two of lab sections were used. (*t *= 2.74,* p*-value = 0.026, two-tailed student* t *test).*

After completing the experiments, each student group wrote up their results as a report following the format of a peer-reviewed journal. To further strengthen students' understanding of RT-qPCR applications, each group also made a 15 min power point presentation on a primary research paper using RT-qPCR/qPCR techniques in Week Three.

Student Assessment

The main objective of this lab exercise was to teach students the theory of RT-qPCR and its applications and associated techniques by studying AtRBCS1A and AtRBCS3B mRNA expressions in leaves of 96 hr-dark treated *A. thaliana*. To determine if the lab was successful at meeting these goals besides obtaining experimental results, we analyzed the results of pre- and post-lab quizzes in which students had to answer two questions: "What is RT-qPCR?" and "What is its application(s) in research?" We scored the answer for a proper definition of RT-qPCR and for its major application in studying gene expression. On the pre-lab quiz, only 3 out of 21 students provided a correct definition of RT-qPCR although 16 of 21 students knew what qPCR is. On the post-lab quiz, 17 of 21 students could properly define RT-qPCR. The remaining four students still thought RT-qPCR and qPCR being the same by mistaking "RT" as "real time". Extra emphasis should be made to clarify the confusion. For the pre-lab question on the application of RT-qPCR, the same 3 of 21 students provided a correct answer, two of whom were working on student-faculty research projects using RT-qPCR at the time. For the post-lab quiz, 19 of 21 students knew the application of RT-qPCR. Thus, by the end of the exercise, the vast majority of students properly defined RT-qPCR and understood its major application in research.

To see if students understood the overall content of the exercise, the following question was asked in the final exam. "Based on the collected C_Ts from the RT-qPCR experiment that you did, what is your conclusion in terms of the effect of dark treatment on AtRBCS1A and AtRBCS3B mRNA expressions?" Nineteen out of 21 (90%) students gave the correct answer. Most of them also indicated that the RBCS3B mRNA expression was down-regulated more than that of the RBCS1A. To see if students were able to apply what they learned to other experimental settings, the following questions were also asked. "Using RT-qPCR to study the effect of a drug on mRNA expression of a "selfish" gene which is overexpressed to cause cancer in cells, the results are shown in the table below. (a) What is the expression of the "selfish" gene in cancer cells treated with the drug as percentage of that of the 'no drug' control? (b) Does the drug work in treating cancer? Explain. (c) Is actin a good reference gene for this study? Explain."

Genes	*Ct (w/o drug A)*	*Ct (with drug A)*
Selfish	25.8	22.78
Actin	25.71	25.41

Seventeen (81%), 15 (71%), and 13 (62%) out of 21 students gave the correct answers for questions (a), (b), and (c), respectively. Although most students could calculate the mRNA expression in the drug treatment as a percentage of that of the control, some still had difficulty interpreting the data or failed to recognize a good reference gene. More practice questions are needed to improve students' understanding.

One particular challenge for some students working in the laboratory was micro-pipetting, particularly setting up RT-qPCR experiments. A small pipetting variation can result in a significant difference in the quantity of final qPCR product and thus C_Ts and the calculated results. The larger error bars shown in Fig. 3 probably were the result of students' inability to pipette precisely.

Future Modification and Potential New Experiments

The exercise was successful in accomplishing the main objectives. Nevertheless, components can be modified or added into the lab. To ensure good quality RNA samples were obtained, each student group was previously given only one leaf sample (control or dark treatment) for RNA isolation. However, to provide students more hands-on experience and to reduce the experimental variation, one future modification is to let each group isolate RNA from both the control and the dark treatment. Although melting curves confirm a single qPCR product being amplified by each primer set as shown in Fig. 2, an agarose gel electrophoresis can be added to ensure the right sized products were obtained (Supporting Information Figure S1). For

research, the qPCR products obtained need to be confirmed with DNA sequencing. Another potential lab component is to test the qPCR efficiency. The instructor carried out this experiment earlier, and the results were shown to students and discussed in-class. Only the % amplification efficiencies of ACTIN2 and AtRBCS1A were $\geq$90% (Supporting Information Figure S2). Thus, further modification of the protocol is needed to ensure no inhibitors or poor primer pairs to hinder the qPCR assay. To test qPCR efficiency, students can make serial dilutions of the reverse transcription reaction containing the cDNAs followed by qPCR of the diluted cDNAs. The C_Ts collected are then used to make standard curves to find the % amplification efficiency of each gene. Finally, an extended experiment can be added to let students test plants treated in the dark for various durations, that is, 24, 48, and 72 hr to see how the duration of dark treatment affects mRNA expressions of the two genes.

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