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Individual and combined effect of drought and heat stresses in contrasting potato cultivars overexpressing miR172b-3p

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Abstract: MicroRNAs (miRNAs) are essential players of plant defence systems because of their involvement in reprogramming gene expression under adverse environmental conditions including drought and heat, which are considered major players in limiting crop productivity. miR172b-3p was previously determined as a remarkable stress-responsive miRNA in our next-generation sequencing (NGS) analysis in potato. This study aims to understand the functions of miR172b-3p and its target (*ERTF RAP2-7-like*) under drought, heat, and combined treatments by overexpressing the miR172b-3p in stress-tolerant (Unica) and sensitive (Russet Burbank) potato cultivars. miR172b-3p overexpression in transgenic lines suppressed the *ERTF RAP2-7-like* expression leading to enhanced carbon fixation efficiency. Meanwhile, the accumulation of hydrogen peroxide (H_2O_2) was reduced in both cultivars, proving that it is involved in the front-line tolerance mechanism against individual drought, heat, and their combination. In conclusion, our results prove that the stress tolerance could be enhanced by miR172b-3p-mediated negative regulation of *ERTF RAP2-7-like* gene in potato under drought, heat, and their combination. Our findings represent the first step towards the improvement of tolerance against multiple abiotic stresses in potato.

Key words: Abiotic stress, *ERTF RAP2-7-like*, miR172b-3p, overexpression, *Solanum tuberosum*

1. Introduction

Potato ranks as the third most important crop after wheat and rice (Muleta and Aga, 2019). In developing countries, about 50% of the potato crop is being grown by resource-poor farmers (Monneveux et al., 2013). Potato tubers are classified as a valuable food source due to their potential high caloric value and possessed vitamins and antioxidant properties (Pihlanto et al., 2008). Potato tubers are consumed directly or as processed products and animal feed as well as used in industrial production (Kirkman, 2007).

Globally out of all abiotic stress factors, drought, and heat stresses are declared as the most complex ones affecting potato growth, survival, and crop productivity (Slater, 1968; Monneveux et al., 2013). Major research has been focused on the effects of individual abiotic stresses in potato, but the plants may frequently experience a combination of drought and heat stresses in the field (Voesenik and Pierik, 2008), and unfortunately, our understanding of the combined stress effect is limited (Beetge et al., 2019). The effect of individual stresses is quite different from the combined effects (Tahmasebi et al., 2014). Crop yield can be increased especially in low-yielding environments if heat and drought-adaptive traits are combined in a genotype (Lopes et al., 2013).

Molecular regulation at the posttranscriptional level possesses a vital role for development, growth, nutrient allocation, and defensive mechanism in plants. MicroRNAs (miRNAs) are essential regulators of growth and stress tolerance by having a multidimensional interaction with their target genes at both transcriptional and posttranscriptional levels in plants and animals

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(Voinnet, 2009; Ebert and Sharp, 2012). miRNAs have been successfully used as potential targets to genetically engineer abiotic stress tolerance in plants. An alteration in a miRNA's expression may cause enhancement in plant stress tolerance (Shriram et al., 2016). Functional characterization of stress-responsive miRNAs in potato under individual abiotic stresses such as drought and heat and their combination may help us to modify pathways regulating the abiotic stress tolerance/resilience in potato. Therefore, potato cultivars resilient to multiple stresses could be developed by a miRNA-based modification.

According to our previous study (Kaplan, 2017) as well as others (Gupta and Sharma, 2013; Barah et al., 2016) miR172b-3p targets the ethylene-responsive transcription factor *RAP2-7-like* (*ERTF RAP2-7-like)* gene related to stress tolerance in plants. *ERTF RAP2-7-like* is not only involved in the transitions between developmental stages of the shoot apical meristem (Moldovan et al., 2010), but it also regulates DNA-binding transcription factor activity during single and combined abiotic stresses (Marmisolle et al., 2020). *ERTF RAP2-7-like* is a part of the AP2/ERF transcription factor family and the family members have already been extensively reported for their advantageous roles in enhancing tolerance against biotic and abiotic stresses in various plant species including Arabidopsis and tobacco (Sakuma et al., 2006; Agarwal et al., 2010; Hoang et al., 2014; Kavas et al., 2020). Later, it was reported to be directly regulated by miR172 (Zhu et al., 2009; Zhu and Helliwell, 2011; Wu et al., 2009; Mathieu et al., 2009), and by controlling developmental timing, this pathway is involved in stress-responsive mechanisms (Han et al., 2013; Hwang et al., 2011; Díaz-Manzano et al., 2018). miR172b is reported to be involved in abiotic stress-induced development and tolerance mechanisms against drought, salt, and heat stress conditions at post germinative phases in several different plant species including Arabidopsis, tobacco, barley, tomato, potato, rice, and Chinese cabbage (Zou et al., 2013; Yin et al., 2014; Sailaja et al., 2014; Zhao et al., 2017; Kuang et al., 2019; Ahmed et al., 2019). There is a lack of knowledge in miRNA signal transduction pathways, responsive mechanisms, and nomenclature in the literature (Zhang and Unver, 2018). Studies about variation in accumulation level and role of miR172b-3p in plant species, especially in potato, under single or combined abiotic stress conditions are very limited. It is well known that miRNAs' response to abiotic stress varies among genotypes of the same species and miRNA-mediated gene regulation may differ according to their capacity to respond to abiotic stresses (Zhang, 2015). Therefore, an important objective behind this research was to unveil the consequences of the overexpression of miR172b-3p on tolerant and susceptible potato cultivars facing abiotic stress conditions.

We hypothesized that the potato resilience against adverse environmental conditions could be reduced by the suppression of *ERTF RAP2-7-like* expression due to overexpression of miR172b-3p. This hypothesis was tested by using two contrasting (sensitive vs tolerant) potato cultivars overexpressing miR172b-3p. The expression patterns of miR172b-3p and *ERTF RAP2-7-like* were investigated after application of drought, heat, and combination of heat and drought stress treatments along with physiological and biochemical measurements of transgenics compared to their nontransgenic counterparts. Abiotic stress-related biochemical and physiological indicators and expression of miR172b-3p and its target gene showed an alteration in their levels in transgenic plants after stress application. These results suggest that miR172b-3p may be involved in *Solanum tuberosum* defence responses under abiotic stresses.

2. Materials and methods

2.1. Plant materials

To investigate the function of miR172b-3p in potato under abiotic stress conditions, two potato cultivars with contrasting abiotic stress tolerance and their transgenics were used as plant materials in the study. While Unica is considered a tolerant cultivar (Basu and Minhas, 1991; Gutiérrez-Rosales et al., 2007; Rolando et al., 2015; Demirel et al., 2020), Russet Burbank is classified as sensitive (Stark et al., 2013; Demirel et al., 2017, 2020). The transgenic genotypes were miR172b-3p overexpressing lines of the wild-type cultivars, Unica and Russet Burbank.

2.2. Construction of transformation vector and production of miR172b-3p overexpressing transgenic lines

For the amplification of pre-miR172b cDNA, total RNA was first extracted from Russet Burbank using Trizol solution (Invitrogen, Catalog number: 155926) with minor changes in the manufacturer's protocol. Then, cDNA of pre-miR172b was synthesized from the total RNA sample using a specific stem-loop primer, miR172b-3p-SL-RT (Table). Pre-miR172b-3p cDNA was amplified by PCR with pre-miR172b-3p specific primers, miR172b-3p-S-F, and miR172b-3p-S-R (Table) containing the overhangs of *Nco*I and *BstE*II restriction enzymes, respectively. The binary vector, pCAMBIA-1301, was digested with the same restriction enzymes to replace the *gusA*. The predigested vector and the insert were further ligated and transformed in *E. coli DH5α*. Once confirmed by standard molecular assays, the final vector was named pCAMBIA-miR172-3b (Figure 1). The developed construct was electroporated to *Agrobacterium tumefaciens* LBA4404 using Gene Pulser Xcell Electroporation System (BioRad, Cat. No. 1652660). All DNA manipulations were performed according to the standard protocols (Sambrook and Russell, 2001)

Table. Primers used in this study.

Primer name	Target	Usage	Primer sequence
miR172b-3p-SL-RT (stem loop)	miR172b-3p pre-miRNA miR172b-3p mature miRNA	cDNA synthesis	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACATGCAG- 3’
miR172b-3p-F	miR172b-3p cDNA	RT-qPCR for stu-miR172b-3p	5’- GGCGGAGAATCTTGATGATGCTGC-3’
Universal reverse primer	miR172b-3p cDNA	RT-qPCR for stu-miR172b-3p	5′-CCAGTGCAGGGTCCGAGGTA-3′
EF1 α _F	EF1-α	RT-qPCR	5’- GGACCCAACTGGTGCCAAAG-3′
EF1 α _R	EF1-α	RT-qPCR	5’- CTCGCCACCGCCTATCAAGT-3′
St ERTF RAP2-7_F	*ERTF RAP2-7-like*	RT-qPCR	5’-TAGGTACCTCCCACGGACAC-3′
St ERTF RAP2-7_R	*ERTF RAP2-7-like*	RT-qPCR	5’- TGTTGGGACCAGACATTTGA-3′
35S promoter _F	35S Promotor	PCR	5’-CATGGAGTCAAAGATTCAAATAG-3′
NOS Poly-A tail terminator _R	NOS Terminator	PCR	5’-AACCCGGGCCCGATCTAGTAACATA-3′
miR172b-3p-S-F	Pre-miRNA	Ligation control	5’-GCACCATGGAGATCTGCAGCACCATCAAG ATTCA-3’
miR172b-3p-S-R	Pre-miRNA	Ligation control	5’-GCAGCTAGCGGTNACCATGCAGC ATCATCAAGATTC-3’

The sprouts of Unica and Russet Burbank were subjected to surface sterilization and multiplied by monthly subculturing of single-node stem explants on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The potato plantlets were then maintained in a growth room with 16/8 h of light/dark photoperiod and a temperature of 25 ± 2 °C. The leaf and internodal explants of both cultivars were inoculated with Agrobacterium suspension (A600 nm = 0.7) for 10 min with gentle shaking in a liquid medium without antibiotics followed by incubation on cocultivation medium for approximately three days. Overall, the transformation procedure was followed as described by Bakhsh et al. (2020) with some modifications.

Following the cocultivation, the explants were subcultured to callus and shoot induction medium (MS salts and vitamins, 30 g/L sucrose, 0.2 mg/L NAA, 2 mg/L BAP, 4 mg/L hygromycin, kinetin 1 mg/L, and trans-zeatin 2 mg/L). Developed shoots from calli were confirmed for their transgenicity by PCR technique using primers of 35S promoter and NOS poly-A tail terminator region along with forward and reverse primers of hygromycin (Table). After confirmation of the integration of pre-miR172b-3p gene into the host genome, transgenic plants were grown under the same incubation conditions used for callus and shoot regeneration as previously mentioned.

2.3. Sequence and bioinformatics analysis

Clustal Omega (Sievers and Higgins, 2014) software was used for multiple sequence alignment of the target sequence of miR172b-3p on *RAP2-7-like* mRNA, *RAP1* mRNA, *AP2/ERF TOE3* transcript variant X2 mRNA, *TOE3* mRNA, and *APETALA2* mRNA in potato. National Center for Biotechnology Information (NCBI) BLAST network server was used for the identity search of nucleotides.

2.4. Individual or combined drought and heat stress applications

Plantlets with 5–6 nodes of wild-types (nontransgenic) and their miR172b-3p overexpressing transgenic lines originated from tissue culture conditions were transplanted to compost (Klasmann TS1) and perlite (2:1) containing 5 L pots (22.5 cm diameter top, 16.5 cm diameter base, and 18 cm depth). Plants were grown and watered regularly until the initiation of stress treatment in a net house. Plants were fertilized with N-P-K (18%-18%-18%) once during the growth phase. Three different stress experiments for drought (D), heat (H), and combined heat-drought (HD) treatments with two wild-type cultivars (Unica and Russet Burbank) and two transgenic genotypes (miR172-3p overexpressing transgenics of Unica and Russet Burbank) were set up 40 days after transplanting. Each experiment had its specific control (nonstress) and stress treatment. Each experiment was conducted with four replications, and each pot containing two plants represented one replication. Drought (D) experiment was carried out in the net house (temperature was 24–26 °C day/16–18 °C night, the humidity was 55%–65% with no artificial

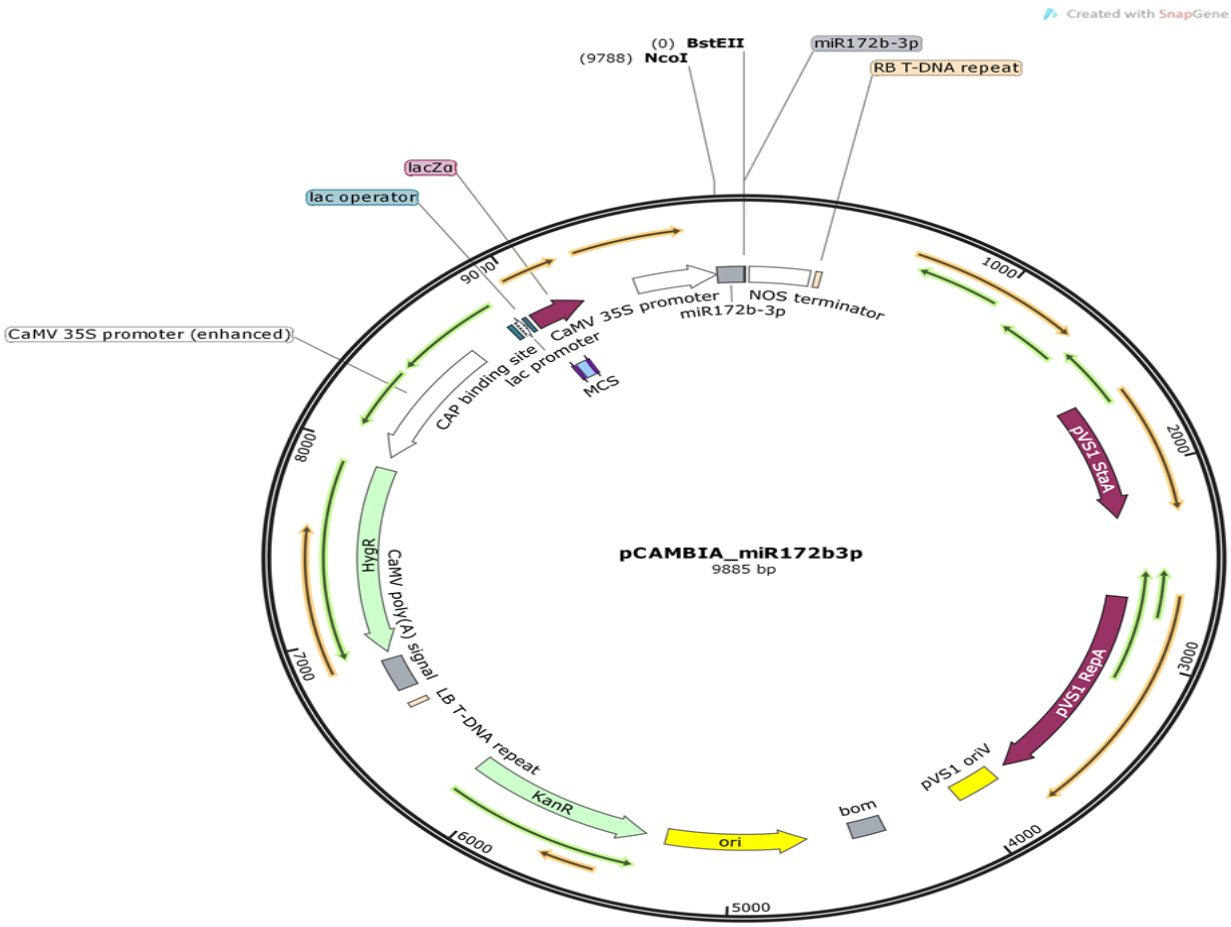

Figure 1. Schematic image of pCAMBIA_miR172b3p vector (generated by SnapGene). Pre- miR172b-3p was cloned upstream to NOS poly-A tail Terminator with overhangs of *Nco*I and *BstE*II. The vector contains *Hygromycin phosphotransferase (hptII)* encoding resistance against hygromycin for a plant selectable marker whereas it contained kanamycin for bacterial selection.

light conditions), while the heat (H) and combined heat-drought (HD) experiments were executed in the environmentally controlled walk-in growth chambers. In the drought experiment, drought was given to one group of plants by withdrawing the watering for 20 days whereas the plants in the control group were irrigated regularly under net house conditions. For heat and combined heat-drought experiments, two identical growth chambers were used side-by-side, one for the control and the other for the stress treatments. Control conditions of heat and combined drought-heat experiments were set up as 24/18 °C (day/night) of temperature with a 14 h photoperiod and 60%–70% relative humidity. In the other chamber, the temperature was gradually increased for heat treatment to plants. The gradual temperature increase was from 24/18 °C to 39/27 °C for 9 days, then; a constant heat of 39/27 °C was applied for 3 days. Therefore, plants were exposed to heat for 12 days. The plants in the heat treatment group were irrigated regularly while the plants in the combined heat-drought treatment group were exposed to drought by withdrawing the irrigation for 12 days. Physiological traits were measured on plants at some intervals during stress treatments. For further biochemical and molecular analyses, the upper third and fourth leaves on the plants were harvested and immediately frozen in liquid nitrogen and stored at –80 °C.

2.5. Measurements of physiological traits

The terminal leaflet of a fully expanded upper third or fourth leaf was used for the measurement of physiological traits throughout stress treatments in different time intervals (0, 6, 10, 12, 16, 20th day of stress treatment) to monitor stress severity and define the suitable interval for successive analysis of biochemical, molecular and physiological responses to single and combined stresses. Standard error and mean value were analyzed by considering three biological replicates.

2.5.1. Leaf gas exchange and photosynthesis rate

The top third or fourth fully expanded leaves were considered to measure the stomatal conductance (Gs), transpiration rate (E) and photosynthetic rate (Pn) between

9:00 and 11:00 am by using a portable LICOR LI-6400XT photosynthesis system. CO_2 concentration was kept at 400 µmol mol^{-1} and photosynthetically active radiation (PAR) was kept at 1500 µ$molm^{-2}s^{-1}$. For each cultivar, one plant from each of three biological replicates was considered to measure stomatal conductance, transpiration rate, and Pn. These measurements were recorded at abovementioned time intervals during the stress period during the stress treatment period and finally performed on the last day of stress treatment. Measurements were taken from transgenic and wild-type plants of both cultivars in different time intervals (0, 6, 10, 12, 16, 20th day of stress treatment) to monitor stress severity and defining suitable interval for successive analysis of biochemical, molecular, and physiological responses to single and combined stresses.

2.5.2. Chlorophyll content

A chlorophyll meter (Minolta SPAD 502, USA) was used to estimate chlorophyll content on upper fully expanded leaves. Chlorophyll measurement was performed at abovementioned time intervals during the stress period and finally on the last day of stress. Five leaves of two individual plants were used to conduct chlorophyll content measurement and the average value was supposed as one biological replicate.

2.5.3. Leaf temperature

The leaf temperature of fully expanded upper leaves was measured by an infrared thermometer (Sinometer BM380, China) at abovementioned time intervals during the stress period. Three leaves of two individual plants were used to record their leaf temperature and the average value was supposed as one biological replicate. Leaf temperature was measured several times during the stress treatment period and the final measurement was done on the last day of stress treatment.

2.5.4. Relative water content

Relative water contents (RWC) of transgenic and control plants were measured at abovementioned time intervals during the stress treatment period and finally on the last day of stress treatments. The terminal leaflet of a fully expanded upper third or fourth leaf was used to measure the RWC during the stress treatment period. Three biological replicates were taken from separate plants. The procedure reported by Demirel et al. (2020) was practiced and the following equation was used to calculate the final RWC value of cultivars.

RWC% = [(fresh weight–dry weight) / (turgor weight–dry weight)] × 100

2.6. Biochemical analysis

2.6.1. Lipid peroxidation

The lipid peroxidation level was measured by calculating the accumulation of MDA. The average value of the top two expanded leaves from separately analyzed plants was taken as one biological replicate, whereas standard error and mean values were analyzed from three biological replicates. MDA was measured by following the thiobarbituric acid (TBA) reaction protocol developed by Heath and Packer (1968). The following equation was used to calculate MDA content as µmol/g fresh weight (FW) with the extinction coefficient of 155 M^{-1} cm^{-1}.

MDA (µmol/g FW) = [(A532 – A600) /155] × 10^3 × dilution factor × (1/tissue weight g)

2.6.2. Hydrogen peroxide (H_2O_2) content

To determine the H_2O_2 activity, leaves were collected on the last day of stress treatment according to Loreto and Velikova (2001). One biological replicate consisted of the average value of the top two expanded leaves from separately analyzed plants, whereas H_2O_2 content was measured after harvesting leaves from three independent plants of each treatment by practicing calibration against a standard curve.

2.6.3. Proline content

Proline content was measured on the last day of stress treatment by following the procedure of Bates et al. (1973) with minor modifications. The average value of the top two expanded leaves from separately analyzed plants was taken as one biological replicate, whereas leaves used for measurement of proline were collected from three independent plants of each treatment by practicing calibration against a standard curve.

2.7. Gene expression analysis of miR172b-3p and *ERTF RAP2-7-like*

Total RNA was extracted from leaf samples by using Trizol reagent (InvitrogenTM, catalog number:155926). For removing potential gDNA contamination, the total RNA was treated with DNase I (ThermoFisher Scientific). The RNA quality and quantity were accessed by agarose gel electrophoresis and a spectrophotometer (BioSpec UV-vis Spectrophotometer), respectively. cDNA of the miRNA and the target gene was constructed from 1 µg of total RNA using Omniscript Reverse Transcriptase (Omniscript RT Kit, Catalog No: 201511). *stu*-miR172b-3p-SL-RT stem-loop primer and the oligo-dT primer (Table) were used for the cDNA synthesis of miR172b-3p or *ERTF RAP2-7-like*, respectively. The expression analysis of the miRNA and the target gene was done from three biological and three technical replications by Real-Time quantitative PCR (RT-qPCR) in 10 µL of final volume including 100 ng of cDNA, 0.8 µM of each primer, and 1× SYBR Green PCR Master Mix (Applied Biosystem). The RT-qPCR mixture was incubated at 94 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s using a real-time PCR cycler (Qiagen, Rotor-Gene Q). After RT-qPCR, melting curve analysis was done to detect specific

amplicon amplification by incubating at 99 °C to 70 °C with a transition rate of 1.0 °C/min. Relative expression levels of miR172b-3p and *ERTF RAP2-7-like* were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *EF1α* (Nicot et al., 2005) was used for normalization. Primers used in the expression analysis were presented in Table.

2.8. Statistical analysis

ANOVA was performed on the physiological dataset based on the randomized complete block design considering stress treatments (control, D, control of H or HD, H, and HD) and genotypes (Unica, Russet Burbank, transgenic Unica, and transgenic Russet Burbank) as the main factors. Since genotypes consist of transgenics and nontransgenics with their control groups, ANOVA was performed on each genotype with a complete set of stress treatments. Gene expression values were also analyzed by ANOVA, in this case, a comparison of genotypes (transgenic, wild-type), samples of control, heat, and drought treatments, and the interaction between the two was practiced. Statistical differences among treatments were estimated by the least significant difference test (LSD) at a probability threshold level of $p < 0.05$. All statistical analyses were done using Statistix 8.1 software. Paired t-test was conducted to calculate the significant difference ($p < 0.05$) between transgenics and nontransgenics at each stress treatment using SPSS software. XLSTAT was used for principal components analysis (PCA), to summarize comprehensive variation among the samples; a sample correlation matrix was used to assure equal weight distribution to all biochemical variables.

3. Results

Agrobacterium-mediated transformation of both cultivars was performed as described by Bakhsh et al. (2020) with minor modifications. Since the main objective of the study was to determine the role of stu-miR172b-3p in potato during heat and drought stresses, the transgenic plants of both drought-tolerant (Unica) and sensitive (Russet Burbank) cultivars were obtained and confirmed by PCR analysis (Figure S1). Based on the PCR results, one transgenic line with high miRNA expression from each cultivar was selected and multiplied in tissue culture conditions to obtain all identical transgenic plants for further abiotic stress assays.

3.1. Target sequence analysis of miR172b-3p in potato

Multiple sequence alignment of nucleotide sequences of the target site (CTGCAGCATCATC AGGATTCG) of miR172b-3p with *ERTF RAP2-7-like* and other genes in potato was carried out to classify the genes carrying target sequences of stu-miR172b-3p. As shown in Figure 2, the target sequences of miR172b-3p were showing high homology to *ERTF RAP2-7-like* mRNA and other genes in potato such as *RAP1* mRNA, *AP2/ERF TOE3* (LOC102589826) transcript variant X1 mRNA, *TOE3* (LOC102582479) mRNA, and *APETALA 2* mRNA. This high homology of the target sequence in other genes suggests that miR172b-3p overexpression can also suppress the hierarchy of the abovementioned genes to encounter stress conditions.

3.2. Physiological responses to the individual or combined heat and drought stresses

Physiological responses were primarily measured to spot the exact period at which both cultivars encountered stress conditions, so that variability in stress-induced responses between stress-sensitive and tolerant cultivars could be distinguished.

Generally, stress treatments caused a gradual reduction in photosynthetic rates in wild-type and 35S:miR-172b-3p transgenic plants of both cultivars from the 10th day to the end of treatments, however, no reduction in Pn was observed in either wild-type Unica's or transgenic Unica's under heat treatment (Figure 3). A reduction of 71.2% (from 32.6 to 9.4 μmol m^{-2} s^{-1}) and 77.0% (from 38.7 to 8.9 μmol m^{-2} s^{-1}) was observed in Pn of wild-type and transgenic plants of Unica, respectively. Besides, the Pn of wild-type and transgenic plants of Russet Burbank reduced by 71.1% (from 24.9 to 7.2 μmol m^{-2} s^{-1}) and 73.5% (from 37.3 to 9.9 μmol m^{-2} s^{-1}), respectively. While there was no significant change between Pn of wild-type and transgenic of Unica under

```
AP2          ttcctcctcagatatttgcagctgctgcagcatcatcaggattccctcagcagatagtaa 1707
TOE3         ctaattatgagataattacaactgctgcagcatcatcaggattcccccagcagataataa 1657
ERF          ctcctcctcacctatttgcaactgctgcagcatcatcaggattccctcagcagatatt-- 1796
RAP1         gtccttcagtgccactcttctctactgcagcatcatcaggattcgctaatacaactacag 1488
RAP2-7       gtcctacattgccactcttctctactgcagcatcatcaggattcggaaattcagcaaaca 1668
stu-miR172b-3p     ------------------------ctgcagcatcatcaggattcg----- 21
                                            ********************
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Figure 2. Alignment analysis of the predicted nucleic acid sequence of the target site of miR172b-3p with *ERTF RAP2-7-like* and other genes in potato. APETALA2 is *APETALA2* LOC102582568 mRNA (XM_006339808.2), TOE3 is *TOE3* (LOC102582479) mRNA (XM_006343131.2), ERF is *AP2/ERF TOE3* (LOC102589826) transcript variant X1 mRNA (XM_006360152.2), RAP1 is *RAP1* mRNA (FM246879.2), *RAP2-7* is *ERTF RAP2-7-like* (LOC102605336) mRNA (XM_006338995.2) and stu- miR172b-3p is target site of miR172b-3p. Where * shows consensus in all nucleotide sequences.

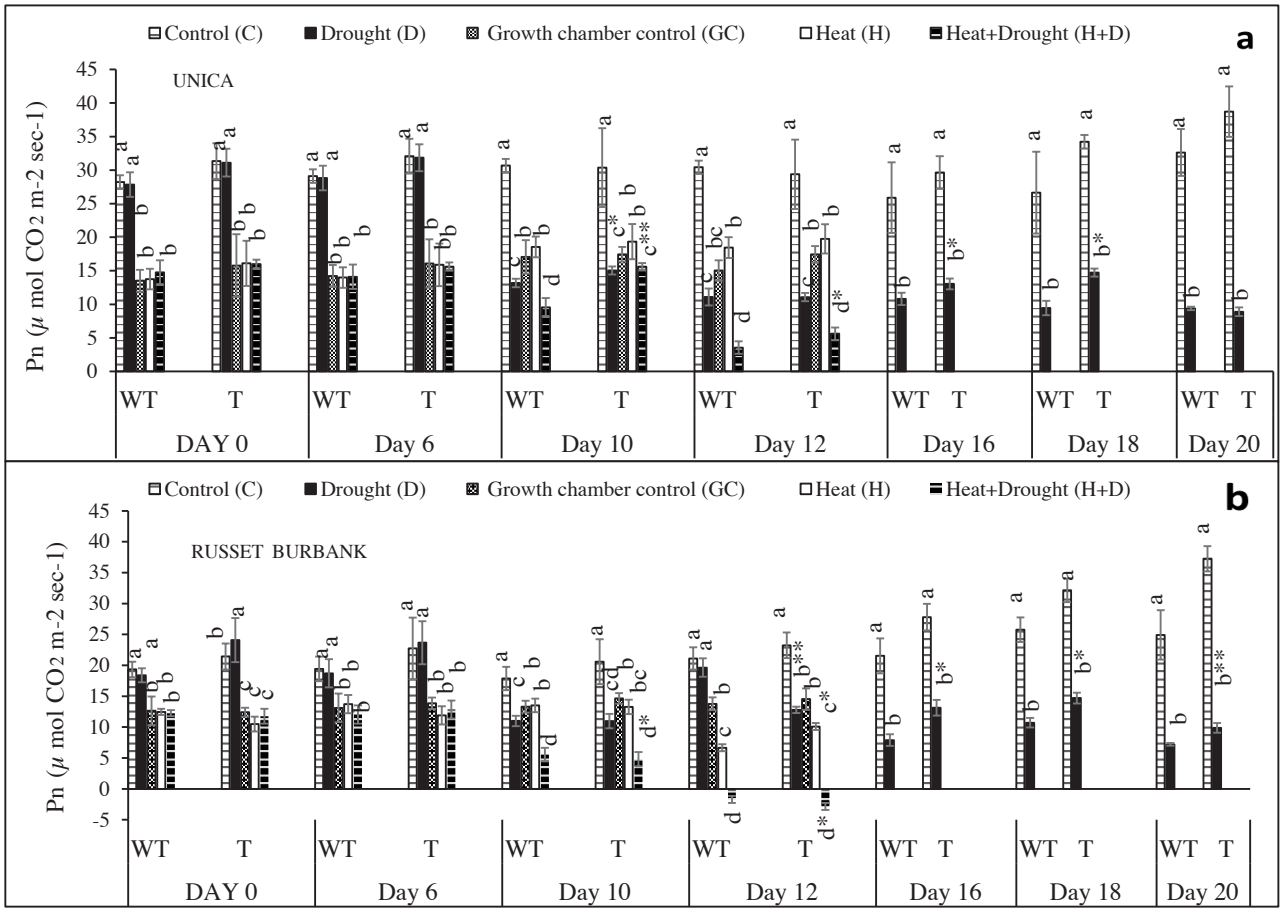

Figure 3. Effect of abiotic stress on the photosynthetic rate of potato genotypes. Transgenic (T) and wild-type (WT) plants were classified as control (▤), drought (■), control for heat and combined heat-drought stresses (▩), heat (□), and combined heat-drought (▬). The photosynthetic rate was measured on Unica (a) and Russet Burbank (RBB) (b). Data are shown as mean ± SD of four independent biological replicates. The significant differences among the treatments were estimated by one-way ANOVA with the least significant difference test (LSD) at a probability threshold level of $p < 0.05$. and represented with different letters. Asterisks (*,**) showed the significant difference of transgenics from wild-type plants at $p < 0.05$ and $p < 0.01$ respectively among each stress treatment calculated through paired t-test.

drought conditions, contrarily, the Pn of transgenic Russet Burbank was significantly higher than wild-type under drought. For heat treatment, no significant change was observed in Pn of both wild-type and transgenic of resistant Unica, whereas Pn of wild-type and transgenic of sensitive Russet Burbank significantly reduced on the 12th day of the treatment. Transgenic Russet Burbank showed a significantly higher Pn ratio than its wild-type plants under the heat treatment whereas, Unica exhibited no significant difference between wild-type and transgenic plants. However, the first significant decrease in Pn under the combined heat and drought treatment was observed on the 10th day of the treatment for wild-type Unica and both wild-type and transgenic plants of Russet Burbank. While both wild-type and transgenic plants of resistant cultivar Unica kept on fixing carbon on the 12th day of combined stress treatment (Figure 3a), both wild-type and transgenic plants of the sensitive cultivar Russet Burbank showed clear respiration on the 12th day of combined heat-drought stress (Figure 3b). The photosynthetic rate of transgenic and wild-type Unica plants reduced by 67.8% (from 17.4 to 5.6 μmol m^{-2} s^{-1}) and by 76.4% (from 15.1 to 3.6 μmol m^{-2} s^{-1}), respectively, compared to their control plants on the 12th day of the combined stress treatment (Figure 3a). Since the lowest and the significant decrease in Pn was observed on the 20th day for drought treatment and the 12th day for heat only and combined heat-drought, we decided to consider these days as the last day of the stress treatments and further investigation was performed on the plants at the last days of the treatments.

The stomatal conductance and transpiration rate of the potato genotypes showed a similar response to the same stress treatments (Figures 4a and 4b). The stomatal conductance and transpiration rate of both wild-type and

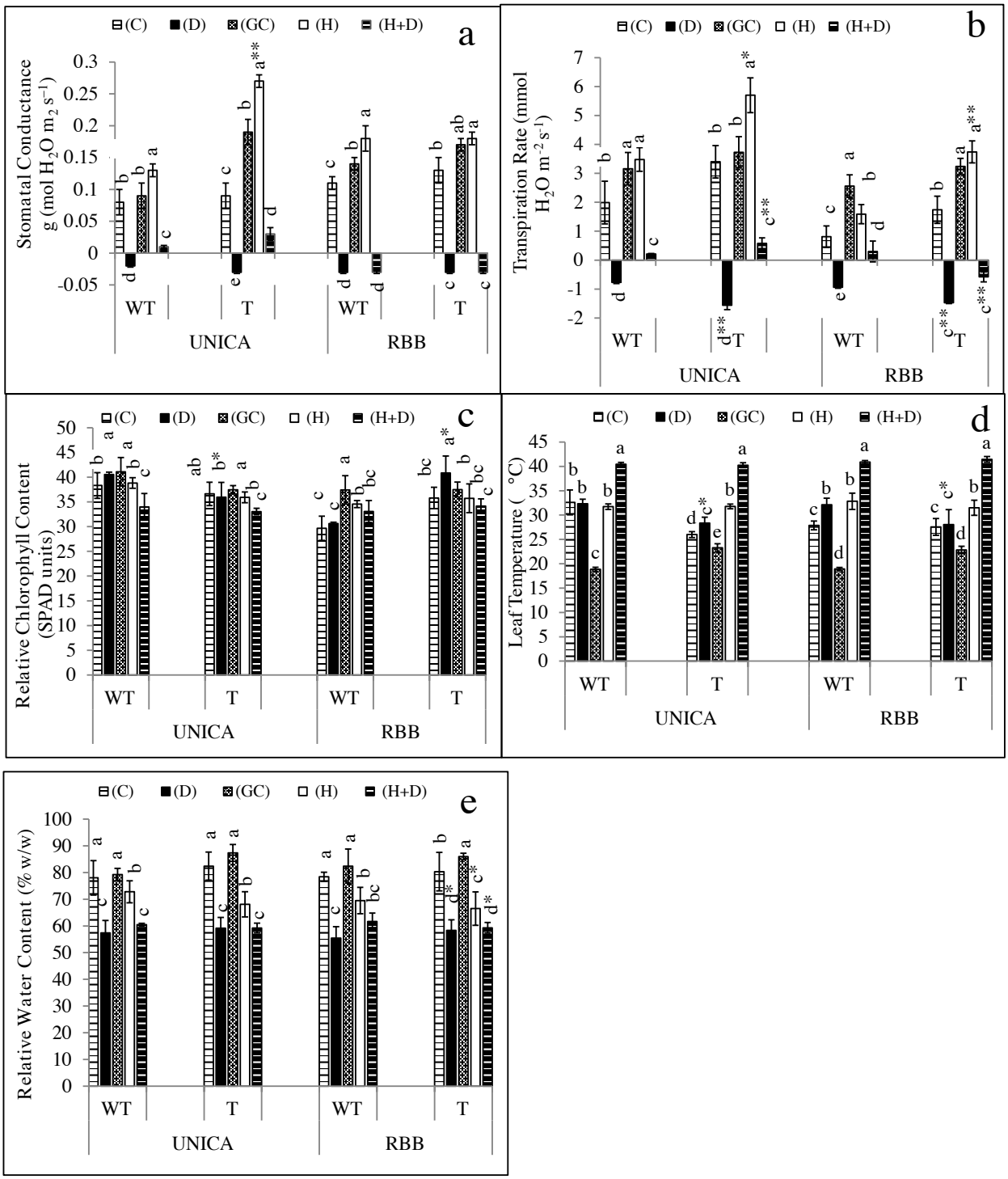

Figure 4. Effect of abiotic stress on physiological traits of potato genotypes. Transgenic (T) and wild-type (WT) plants of Unica and Russet Burbank (RBB) were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, ▬) stresses. Stomatal conductance (a), transpiration rate (b), relative chlorophyll content (c), leaf temperature (d), and relative water content (e) were measured as outlined in the text. Data are shown as mean ± SD of four independent biological replicates. The significant differences among the treatments were estimated by one-way ANOVA with the least significant difference test (LSD) at a probability threshold level of $p < 0.05$. Differences between means with different letters are significant at $p < 0.05$. Asterisks (*,**) showed the significant difference of transgenics from wild-type plants at $p < 0.05$ and $p < 0.01$ respectively among each stress treatment calculated through paired t-test.

transgenic plants of both cultivars exhibited a significant reduction under drought and combined heat and drought conditions (Figure 4a). Despite that, stomatal conductance was significantly higher in both wild-type and transgenic plants of Unica and wild-type Russet Burbank under heat treatment than in control plants. While transpiration rates of wild-type Unica and transgenic Russet Burbank did not significantly change under heat, the transpiration rate of transgenic Unica increased contrarily it showed a decreasing trend in wild-type Russet Burbank (Figure 4b).

Leaf chlorophyll content of wild-type Unica and transgenic Russet Burbank increased under drought, whereas a decrease was observed in transgenic Unica (Figure 4c). On the other hand, the chlorophyll content of wild-type and transgenic plants of both cultivars showed a decline under heat and combined heat and drought conditions. While transgenic Unica accumulated significantly less chlorophyll than wild-type under drought, it was higher in transgenic Russet Burbank under drought conditions. A significant increase was observed in the leaf temperature of transgenic Unica under drought, however, there was no significant change in the leaf temperature of transgenic Russet Burbank (Figure 4d). Besides, both transgenic Unica and Russet Burbank exhibited significantly fewer leaf temperatures than wild-type under drought conditions. The wild-type and transgenic plants of both cultivars significantly enhanced their leaf temperature under heat and combined heat and drought conditions. However, there was no significant difference in the leaf temperature of transgenic plants of both cultivars as compared to their wild-types under heat and combined heat and drought conditions. Under all stress conditions imposed, a significant reduction was recorded in leaf water content of wild-type and transgenic plants of both cultivars as compared to their controls (Figure 4e). However, only transgenic Russet Burbank plants showed a significant difference ($p < 0.05$) compared to their respective wild-type plants under all stress conditions.

Overall, the data proved that wild-type and transgenic plants of both cultivars significantly altered their response to the drought, heat, and their combination. However, the transgenic and wild-type plants of tolerant Unica were able to carry on photosynthesis over an extended period of stress treatments in contrast to the susceptible Russet Burbank. The change in photosynthetic rate and transpiration rate were in agreement with the stomatal conductance during the stress period showing that photosynthesis and transpiration activities were dependent on the stomatal conductance.

3.3. Biochemical responses to the individual or combined heat and drought stresses

Transgenic and wild-type plants of resistant cultivar Unica did not show any significant difference in leaf MDA content under drought conditions (Figure 5a). However, the MDA content of wild-type Unica significantly raised under heat and combined heat and drought conditions, whereas a significant reduction was observed in transgenic Unica under heat conditions. On the other hand, no significant change was observed for MDA content between wild-type and transgenic Unica under all stress conditions. The MDA content in wild-type plants of susceptible cultivar Russet Burbank showed a significant increase in leaf MDA in plants exposed to all stress conditions, whereas in transgenic Russet Burbank, there was a significant decrease in drought and increase in combined heat and drought conditions. The MDA content of transgenic Russet Burbank comparing to the respective wild-type showed a significant decrease of 53.3% and 45.8% under drought only and heat only conditions, respectively, however, no significant difference was observed under combined heat and drought conditions.

In both wild-type and transgenic Unica, we observed an elevating trend in H_2O_2 accumulation under drought conditions, whereas decreasing trend was observed under heat and combined heat and drought conditions (Figure 5b). However, wild-type and transgenic plants of Russet Burbank exhibited a reduction in almost all applied stress conditions. Transgenic plants of both cultivars showed significantly lower H_2O_2 accumulation under drought only and combined stress treatment in contrast to their wild-type plants. The transgenic plants of both cultivars showed a similar amount of H_2O_2 accumulation to the wild-type plants under heat conditions.

Proline accumulation significantly increased in both wild-type and transgenic plants of both cultivars under all applied stress conditions (Figure 5c). Drought conditions caused more than 7 times accumulation of proline in all potato plants. Besides, proline accumulation of transgenic plants of both cultivars was significantly higher than respective wild-type plants under drought conditions. Similarly, transgenic plants of Unica under heat and combined heat and drought conditions produced significantly higher proline than the wild-type plants.

3.4. Principal component and correlation analysis of biochemical variables

An overview of the impact of stress treatments in transgenic and wild-type genotypes was attained by conducting principal component analysis (PCA).

PCA analysis was conducted for transgenic and wild-type plants of both potato cultivars by considering all biochemical variables under study. PCA biplot of the first two components (PC1 and PC2) explained 89.40% and 86.92% variance for the biochemical variables tested among the five treatments of Unica and Russet Burbank cultivars, respectively (Figures 6a and 6b). The first principal component (PC1) can be identified as the

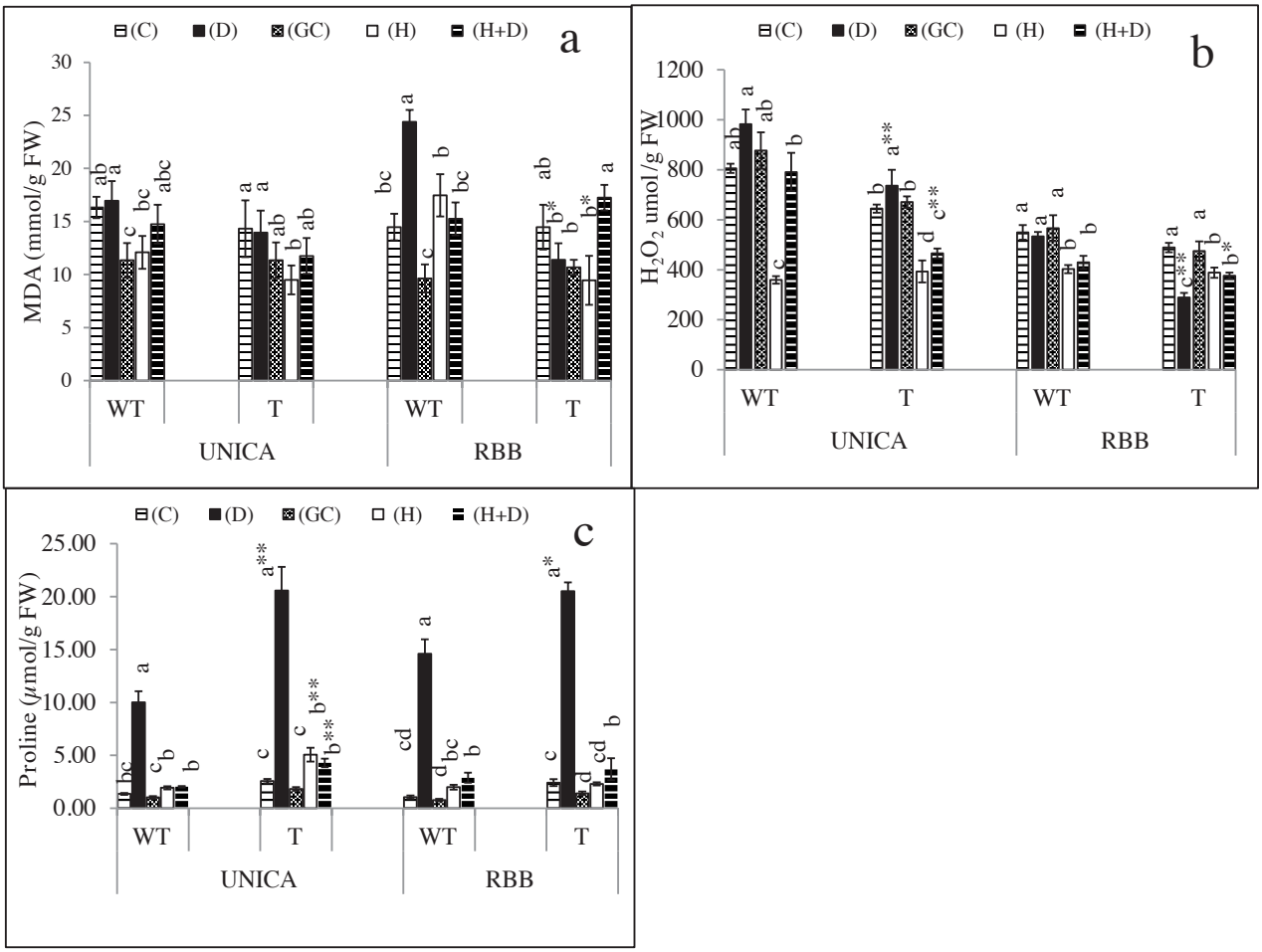

Figure 5. Effect of abiotic stress on biochemical traits and relative chlorophyll content of potato genotypes. Transgenic(T) and wild-type (WT) plants of Unica and Russet Burbank (RBB) were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. Malondialdehyde (MDA) (a), H_2O_2 (b), and proline (c) contents were measured as outlined in the text. Data are shown as mean ± SD of four independent biological replicates. The significant differences among the treatments were estimated by one-way ANOVA with the least significant difference test (LSD) at a probability threshold level of $p < 0.05$. Differences between means with different letters are significant at $p < 0.05$. Asterisks (*,**) showed the significant difference of transgenics from wild-type plants at $p < 0.05$ and $p < 0.01$ respectively among each stress treatment calculated through paired t-test.

variation in five different treatments for transgenic and wild-type plants of both cultivars under control and stress conditions. The second principal component (PC2) was considered for the distribution of three biochemical variables in response to control and stress conditions. In wild-type Unica, control, drought, and combined heat and drought treatments appeared to have positive PC1 values and have a similar response to MDA and H_2O_2. It showed that these treatments were more responsive to MDA and H_2O_2 while the case was opposite for proline. Contrarily transgenic control, heat, and combined heat and drought treatments were less responsive to MDA and H_2O_2 as compared to their respective wild-type plants. In the case of proline accumulation, transgenic plants under drought treatment were highly responsive in contrast to wild-types in drought and all other treatments. Russet Burbank control plants of transgenic and wild type showed an average response to H_2O_2. Wild-type plants of Russet Burbank under drought conditions were highly responsive to MDA as compared to all other treatments. In the case of proline accumulation, transgenic Russet Burbank was more responsive to combined stress treatment as compared to all other treatments. Whereas wild-type Russet Burbank under heat and combined stress treatments showed an average response to proline accumulation. The correlation analysis showed a significant positive correlation between MDA and H_2O_2 ($r = 0.534$ ***) in Unica, however, proline showed no significant correlation with MDA ($r = 0.163$)

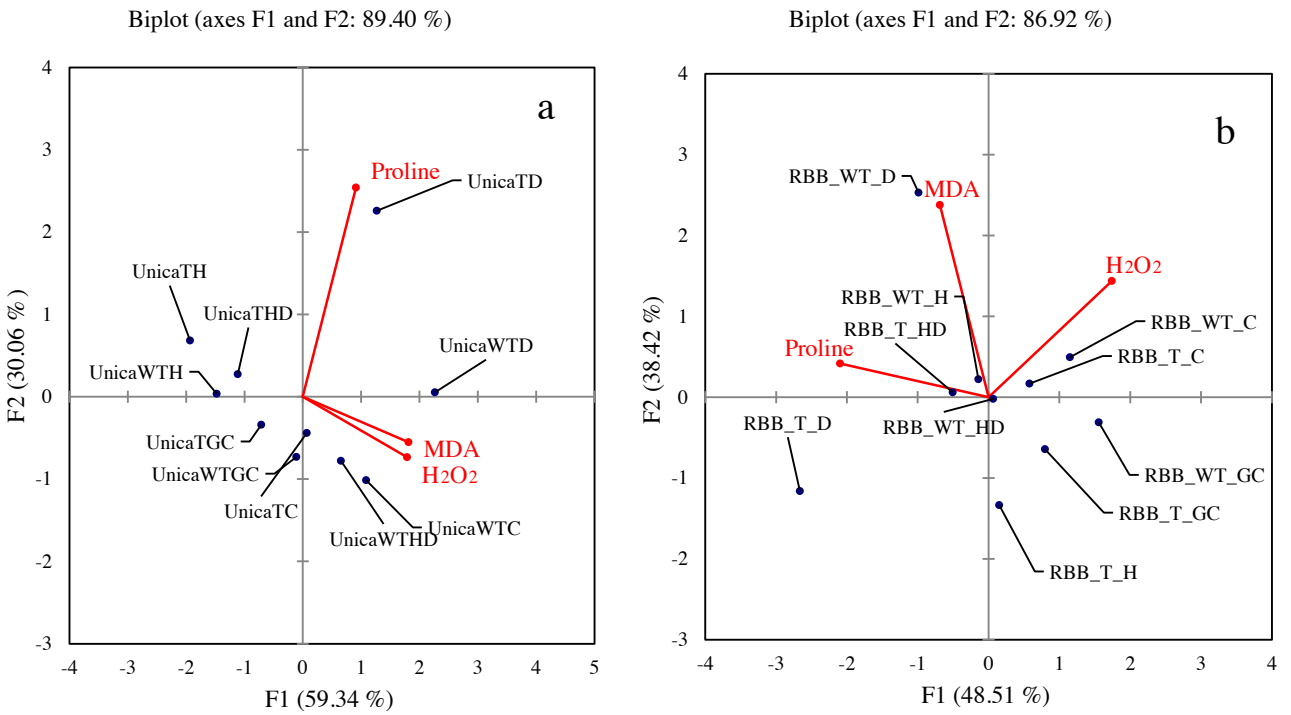

Figure 6. PCA biplot of the first two principal components (89.40%, 86.92%) for biochemical variables of Unica (a) and Russet Burbank (b) cultivars grown under five different treatments. PCA biplot is a combination of a score plot of treatments (represented as dots) and a loading plot of biochemical variables (represented as vectors). Where RBB is Russet Burbank, WT is wild-type plants, T is transgenic plants, C is the control for plants under drought (D) stress, GC is the control for plants under only heat (H) and combined heat and drought (HD) stress.

and H_2O_2 (r = 0.174). On the other hand, a significant negative correlation was observed between proline and H_2O_2 (r = –0.41**) in transgenic and wild-type plants of Russet Burbank grown under control and various stress conditions. Interestingly, MDA showed no significant correlation with H_2O_2 (r = 0.13) and proline (r = 0.24) in Russet Burbank.

3.5. Changes in transcript levels of miR172b-3p and *ERTF RAP2-7-like* under the individual or combined drought and heat stresses

Significant differences in CO_2 fixation capacity along with stomatal conductance and transpiration rate were observed between stress-sensitive and stress-tolerant cultivars after 12 days of heat and combined heat-drought treatments, and 20 days of drought treatment. We, therefore, selected 12 days of heat and combined heat-drought treatments, and 20 days of drought treatment as the last day of stress application for analyzing the leaf transcriptional profiles of miR172b-3p and *ERTF RAP2-7-like* from both cultivars. Expressional differences of miR172b-3p and *ERTF RAP2-7-like* on the last day of stress application can be a key to associate an expressional profile with the effectiveness of stress tolerance and susceptibility in Unica and Russet Burbank, respectively. The expression value of miR172b-3p in wild-type plants of Unica showed nonsignificant change under drought while a significant decrease was observed under heat and combined stress conditions (Figure 7a). Wild-type Russet Burbank showed a significant decline in miR172b-3p expression under all stress conditions as compared to their controls (Figure 7a). Transgenic plants of Unica and Russet Burbank showed a significant increase in the expression value of miR172b-3p under control and all stress conditions in comparison to their respective wild-type plants. Transgenic plants of Unica showed an increase of 1.53 and 2.70 folds in miR172b-3p expression under control and drought conditions, respectively, compared to the wild-type plants under the same conditions, whereas 3.18, 3.23, and 8.68 folds of increase was observed under control, heat and, combined stress, respectively. Similarly, transgenic Russet Burbank showed an increase of 2.66 and 4.07 folds in miR172b-3p expression under control and drought, respectively, whereas it exhibited an increase of 4.06, 6.27, and 5.98 folds under control, heat, and combined stress conditions, respectively. Wild-type plants of both Unica and Russet Burbank depicted a significant increase in the expressional values of *ERTF RAP2-7-like* under all applied stress conditions (Figure 7b). Despite this, the expression of *ERTF RAP2-7-like* in the transgenic plants

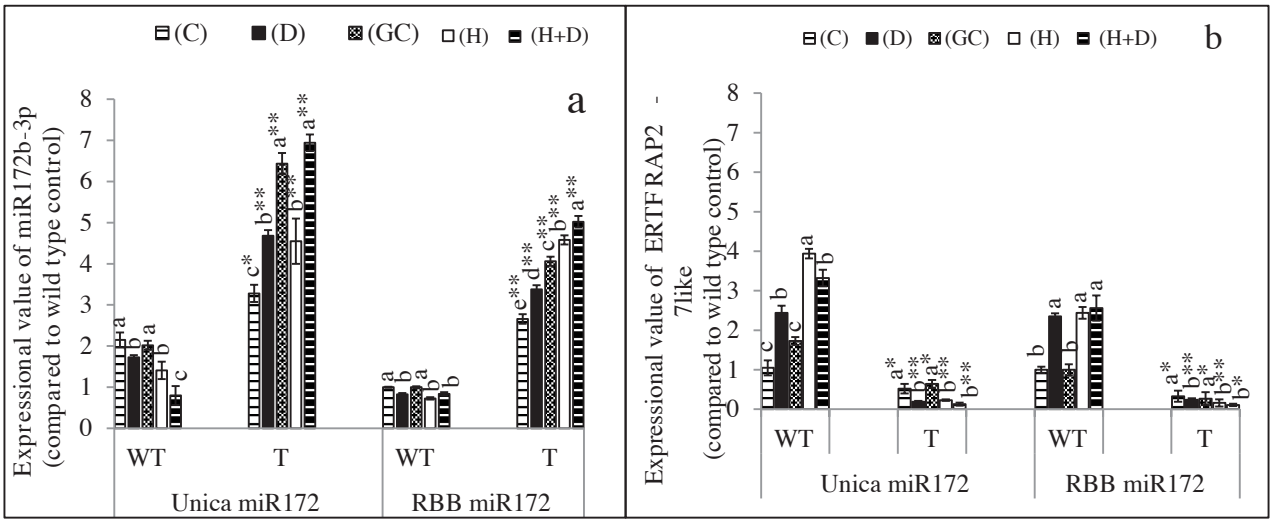

Figure 7. Relative transcript level (compared to wild-type Russet Burbank control) of miRNA (miR-172b-3p) and its target gene (*ERTF RAP2-7-like*). Transgenic (T) and wild-type (WT) plants of Unica and Russet Burbank (RBB) were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬). Relative expression levels of *ERTF RAP2-7-like* (a) and miR-172b-3p (b) were quantified by the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SD of three independent biological replicates. The significant differences among the treatments were estimated by one-way ANOVA with the least significant difference test (LSD) at a probability threshold level of $p < 0.05$. Differences between means with different letters are significant at $p < 0.05$. Asterisks (*,**) showed the significant difference of transgenics from wild-type plants at $p < 0.05$ and $p < 0.01$ respectively among each stress treatment calculated through paired t-test.

of Unica and Russet Burbank dramatically downregulated under all stress conditions compared to their transgenic and wild-type controls. Transgenic Unica showed a decline of 2.02 and 12.84 folds in the expression of *ERTF RAP2-7-like* under control and drought, respectively, compared to wild-type plants under the same conditions, whereas a 2.70, 17.13, and 25.54 folds downregulation was observed under the control, heat, and combined stress conditions in comparison to their wild-type plants under the same conditions. Following the same pattern, the expression of *ERTF RAP2-7-like* was downregulated in transgenic Russet Burbank by 3.03 and 9.79 folds under control and drought, respectively, whereas a downregulation of 3.70, 15.25, and 23.27 folds were observed under control, heat, and combined stress conditions. A comparison of wild-type controls to the stress treatments of wild-type depicted that *ERTF RAP2-7-like* expression in wild-type Unica and Russet Burbank showed an increase of 2.32 and 2.35 folds under drought, respectively. Besides, wild-type Unica exhibited an increase in the expression by 2.27 and 1.91 under heat and combined heat and drought conditions, respectively, whereas the expression was upregulated by 2.44 and 2.56 folds in the wild-type Russet Burbank. Conversely, transgenic plants of Unica and Russet Burbank showed a decline of 1.24 and 1.20 folds under drought, 1.43 and 1.36 folds under heat, 2.52 and 1.19 folds changes under combined stress conditions, respectively, compared to their transgenic control plants. Overall, a negative correlation was observed between the expression of miR172b-3p and *ERTF RAP2-7-like* in potato under control and different abiotic stress conditions.

4. Discussion

Plant miRNAs commonly showed a negative correlation with their target genes (Bagga et al., 2005). Some miRNAs and their target genes function in the tolerance mechanisms under stress conditions. An imperative way to understand the biological function of miRNAs is to identify the target genes they regulate (Marmisolle et al., 2020). In plants, bioinformatics is successful and helpful to identify high complementary targets of most of the known miRNAs (Jones-Rhoades and Bartel, 2004). Individual drought, heat, and their combination are among the abiotic stress factors that are difficult to control because of their complex and poorly understood mechanisms and interactions in potato. In our previous work, many miRNAs were identified in response to the individual and combined drought and heat stress factors by high-throughput sequencing in two contrasting (abiotic stress susceptible vs. tolerant) cultivars of potato (Kaplan, 2017). Among 24 potential miRNAs, miR172b-3p become prominent as its expression was suppressed after stress application. Transgenic plants overexpressing pre-miR172b-3p from both contrasting cultivars (Unica-tolerant and Russet Burbank-sensitive) were generated to further characterize the functions of miR172b-3p in

potato. Through analysis of the expression level of mature miR172b-3p and target gene together with the various physiological and biochemical indicators suggested that miR172b-3p was involved in potato defense response to abiotic stress factors.

miR172b has been abundantly studied in the development and control of flowering time (Lauter et al., 2005; Nair et al., 2010), but recent studies have suggested its roles in biotic (Li et al., 2014; Gai et al., 2014) and abiotic stress tolerance (Kuang et al., 2019). The results of our study revealed that expression levels of miR172b-3p and its target gene *ERTF RAP2-7-like* were changed and negatively correlated with each other after the application of abiotic stress conditions. The results of other studies also showed that the expression levels of miR172b-3p were affected by stress applications (Gai et al., 2014; Luan et al., 2018; Kuang et al., 2019) while the expression levels of target genes were downregulated after stress application (Luan et al., 2018). *RAP2-7-like* protein, being a part of the AP2/ERF transcription family, plays a major role in the regulation of transcription directly by binding to the TBSF motif in the promoter of immune receptor gene *FLAGELLIN-SENSING2* (*FLS2*) and inhibits its activity (Sreenivasulu et al., 2007). This model elaborates that overexpressing miR172b causes suppression of *TOE1* (*ERTF RAP2-7*), ultimately enhancing the activity of FLS2-mediated immunity during plant development in Arabidopsis (Zou et al., 2018). The miR172b-*TOE1* module has been reported as a major integrator to coordinate plant development, immunity, and timing of flowering against environmental factors (Zou et al., 2018). Therefore, its regulation under abiotic stresses is essential for the survival of the plants and eliminating the yield losses in crops.

Physiologically, in the case of individual drought treatment, an improvement in carbon assimilation was observed in transgenic plants of sensitive RBB but not in resistant Unica as compared to their wild-types, indicating an enhancement in the photosynthetic capacity due to greater tolerance. Transgenic Unica showed a significantly high photosynthetic rate (Pn) and transpiration rate (E) as compared to their wild-type plants under combined stress, whereas a clear destructive effect of drought and heat interaction was observed on Pn and E in transgenic and wild-type RBB. The heat treatment showed no significant change in Pn between transgenic and wild-type plants of Unica, whereas transgenic Russet Burbank showed a significant increase as compared to its wild-type plants. The reason for this observation may be the maintenance of functional stomatal activity due to continuous water availability to support transpiration rate (E) and photosynthetic rate (Pn). A lower leaf temperature was detected in the plants under single heat stress as compared to the plants kept under combined stress conditions. This feature might be attributed to the continuity of E under heat-only conditions. Similar results were also concluded in Arabidopsis and tobacco studies (Rizhsky et al., 2002, 2004).

In the cells, membrane damage can be determined by measuring the MDA levels, which is a marker of the oxidized product of lipid peroxidation. In our study, we observed that transgenic RBB and Unica under heat-only stress resulted in significantly less membrane damage in the former genotype whereas the latter showed stable lipid peroxidation as compared to their respective wild-type plants. Interestingly in contrast to wild-types, Pn activity conformed with the MDA levels showing stability and significant improvement in transgenic Unica and RBB, respectively. In the case of H_2O_2 accumulation, no significant difference was observed among wild-type and transgenic plants of both genotypes under heat stress. This phenomenon suggested that under heat conditions, potato plants improve water use efficiency to stabilize cellular membrane and balance photosynthesis and transpiration activity for their survival but under combined stress conditions unavailability of water with high heat may cause more adverse effects at physiological, and biochemical level (Zhao et al., 2014). We also observed that in contrast to wild-type plants, transgenic RBB showed no significant difference in MDA level under combined stress, whereas a significant decrease under drought stress was observed. Although transgenic Unica did not show a significant difference from wild-type plants, however, it showed a change of equal MDA accumulation according to their respective controls. On the other hand, we found a significant decrease in H_2O_2 accumulation in transgenic Unica and RBB than wild-type plants under drought and combined stress conditions. In transgenic Unica, a decrease in H_2O_2 accumulation is in agreement with improved Pn under combined stress whereas, in transgenic RBB, less MDA and H_2O_2 accumulation are in agreement with an increase in Pn under drought stress, when compared to their corresponding wild-types. This data suggests that combined stress causes activation of drought protective mechanisms in plants of both genotypes. Moreover, an overexpression of miR172b-3p may cause transgenic plants to acclimatize the stress by enhancing the capacity of the light-harvesting complex thereby reducing the cellular damage caused by the overproduction of reactive oxygen species (ROS) in the photosynthetic electron transport chain (Ruban, 2016). Similar results of less MDA and H_2O_2 accumulation after stress application were reported in transgenic *S. lycopersicum* having overexpression of miR172a and miR172b (Luan et al., 2018) and in transgenic Arabidopsis having overexpression of soybean miR172c (Li et al., 2016). In contrast to heat and drought

individual treatments, under combined stress-specific expression of few transcripts and high expression with synergistic interaction of many transcripts (mainly related to drought) related to acute or acclamatory stress responses is also a reason for more adverse effects of combined heat and drought stress observed in potato, tobacco, and Arabidopsis (Rizhsky et al., 2002, 2004; Demirel et al., 2020). One possible reason can be the affection of ABA-responsive genes, as it has already been reported that overexpression of soybean miR172c increased ABA sensitivity in Arabidopsis (Li et al., 2016). However, yet it needs to be unveiled that under combined stress conditions, how come the interaction of transcripts undergo modification depending upon the potential of genotype and alters their role in complex defensive networking. Taken together, these observations indicate that drought and heat individual and combined stress-responsive genes regulated by miR172b-3p may involve in defense mechanisms to enhance stress tolerance.

The involvement of miR172 in the regulation of drought tolerance in tobacco and rice has also been reported (Frazier et al., 2011; Zhou et al., 2010; Ferdous et al., 2015). Suppression of *TOE1* and *WRKY44* due to overexpression of miR172b-3p is one of the reasons behind this tolerance behavior against drought stress. Han et al. (2013) reported that *WRKY44* is involved in sugar metabolism and signaling, they performed yeast two-hybrid screening to detect the interaction of *WRKY44* and *TOE1* and resulted that miR172 can suppress *WRKY44* and *TOE1*, which encodes interactive proteins. This suppression leads to drought escape and tolerance by affecting sugar signaling in Arabidopsis having overexpression of miR172, which is in agreement with the behavior of potato plants observed in the present study. Heat stress-causing downregulation of miR172 (Xin et al., 2010; May et al., 2013; Khaksefidi et al., 2015) and upregulation of *TOE1* (May et al., 2013; Zhao et al., 2016) have already been reported and the same results were observed in potato in our previous study (Kaplan, 2017). High temperature causing *TOE1* upregulation due to downregulation of miR172 is reported to have an association with miR156-regulated *Squamosa Promoter-Binding Protein-Like (SPL)* genes to modulate developmental transitions by regulating their expression (Wu et al., 2009; Nonogaki, 2010; Jung et al., 2011; Stief et al., 2014; Zhao et al., 2016; Matthews et al., 2019). Downregulation of *SPL* genes due to miR156 causes phenotypic and biochemical advantages and improvements in tolerance of heat (Matthews et al., 2019) and abiotic stress (Cui et al., 2014). High temperature causes alteration in expressions of all components in miR156-*SPL*-miR172-*AP2* (*TOE1*) (Zhao et al., 2016). According to this module and our findings, overexpression of miR172b-3p and downregulation of *TOE1* may ultimately cause downstream of *SPL* genes, which is in agreement with the working pattern of miR156 and results in an improvement in tolerance against high temperature.

As opposed to H_2O_2, the accumulation of proline was high in wild-type plants under drought stress but it was strongly enhanced in transgenic plants of both cultivars as compared to their wild-types. Our results confirmed the previous findings in potato (Knipp et al., 2006; Demirel et al., 2020) and Arabidopsis (Rizhsky et al., 2004). It has already been proved that apart from being an osmoprotectant, proline also acts as a potent nonenzymatic antioxidant (Smirnoff and Cumbes, 1989; Mohanty and Matysik, 2001; Szabados and Savouré, 2010; Giberti et al., 2014; Rejeb et al., 2014; Chen et al., 2020) and downturns MDA, H_2O_2 (Ozden et al., 2009; Sobahan et al., 2016; Margutti et al., 2017) and free radicals in potato (Knipp et al., 2006). A comparison of proline content with H_2O_2 under all stresses in transgenic plants also indicates that an increase in proline content is also the reason for the lower accumulation of H_2O_2. Compared with wild type, the same results of maximum increase in proline in transgenic Arabidopsis plants having overexpression of miR172c resulted in a downturn of H_2O_2 and this increase causes an improvement in tolerance against drought stress (Li et al., 2016). Overall, these results imply that in *Solanum tuberosum* the mir172b-3p-*ERTF RAP2-7-like* acts as a module, which may regulate antioxidants to reduce the ROS accumulation and prevent cell membrane damage during drought and heat stresses.

In summary, unique changes were identified at physiological, and biochemical levels under individual heat and drought stresses and in their combination. We observed a negative feedback loop of *ERTF RAP2-7-like* in the result of overexpression of stu-miR-172b-3p in transgenic plants. Our experiments stipulated that stress-tolerant and stress-sensitive genotypes having overexpression of miR172b-3p countered to stress by i) enhancing the light-harvesting capacity and limiting nonphotochemical quenching, and ii) compensating the photosynthesis reduction by deflection in metabolism to maintain development and growth phases, and iii) by reducing ROS, MDA and increasing proline accumulation under different stress conditions. We observed and confirmed that the downregulation of *ERTF RAP2-7-like* may cause behavioral variation in stress-sensitive and tolerant varieties as a result of combined and single stresses. Under adverse environmental conditions, developmental remodeling can be fine-tuned by miR172b-3p and *ERTF RAP2-7-like*. To the best of our knowledge, this research presents the only effects of miR172b-3p overexpression under individual and combined stress in potato. Hence along with this provided information, many questions are yet to be answered. For example, how

the postgerminative development and adaptation are fine-tuned by various target genes of miR172b-3p? What are the regulatory networks modulated by ABA signals after their transduction to miR172b-3p in potato under single or combined stress conditions? In future research, answering these questions will provide a novel vision into the miR172b-3p-mediated signal transduction during different developmental stages in response to adverse abiotic conditions and will ultimately enhance multistress tolerance in potato.

5. Conclusion

In conclusion, although we observed a negative correlation in the expression levels of miR172b-3p and *ERTF RAP2-7-like*, there was not a uniform increase or decrease in their expressional patterns, respectively, under stress conditions, indicating the *ERTF RAP2-7-like* are being regulated by other miRNA families as well. As opposed to transgenic RBB, transgenic Unica showed less MDA accumulation under combined stress conditions. While transgenic RBB showed a decrease in H_2O_2 content under drought only as opposed to transgenic Unica. Through indicators of physiological (Pn, E, RWC), biochemical (MDA, H_2O_2, Proline), and molecular analysis, this study showed that miR172b-3p probably is involved in individual and combined stress tolerance in potato. Our results will contribute to plant abiotic stress interaction studies which can guide molecular breeding of potato to improve tolerance against abiotic stress in the future.

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Contribution of authors

AA performed the research and experimentation in all subjects of the study, statistical analysis, and prepared the draft manuscript. AB and IT performed laboratory molecular analysis. ZNÖG, UD, AB, EA MEC, and SC conceived and designed the research, analyzed the results of the study, produced solutions to the problems in the study. All authors read and developed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This article lacks any study related to human or animal participants performed by any of the authors.

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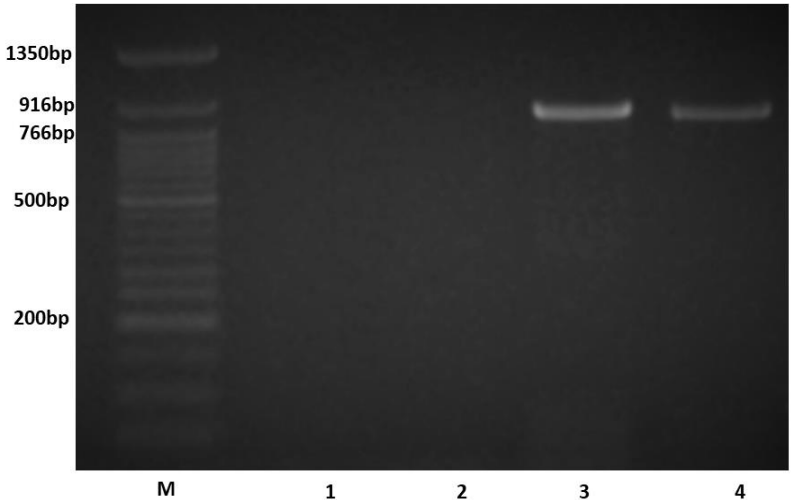

Figure S1.