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Investigation of the effects of overexpression of Novel_105 miRNA in contrasting potato cultivars during separate and combined drought and heat stresses

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Abstract: Potato (*Solanum tuberosum* L.) growth, development and production are sensitive to abiotic stress conditions, yet studies about investigation of stress response of potato plants in regulation of gene expression level are limited. The aim of this study was to identify the role of newly identified Novel_105 miRNA in contrasting potato cultivars (tolerant Unica and sensitive Russet Burbank) in response to heat, drought and combined heat and drought stresses using transgenic approach. Transgenic plants of Unica and Russet Burbank were generated with overexpression of pre-miRNA of Novel_105 and both wild type and transgenic T0 lines were subjected to stress. Physiological parameters (gaseous exchange, leaf temperature, chlorophyll content and relative water content) were observed along with biochemical parameters including proline, malondialdehyde and hydrogen peroxide. The overexpression of Novel_105 improved physiobiochemical functions of the both cultivars under single or combined stress conditions. The increase in Novel_105 miRNA expression was observed along with a decreased expression in its predicted target E3 ubiquitin-protein ligase XBAT35, that is responsible for cell death in potato. This is a preliminary study for the development of abiotic stress resilient potato cultivars proving the overexpression of Novel_105 miRNA increased abiotic stress tolerance presumably by repressing the expression of XBAT35 responsible from promoting cell death.

Key words: Drought, heat, combined heat and drought stresses, Novel_105, transgenic overexpression, potato

1. Introduction

Potato is a globally used vegetable and a widely cultivated crop after wheat, maize and rice (Djami-Tchatchou et al., 2017). It is perennial in nature and belongs to Solanaceae family. It can grow at optimum temperatures ranging between 17 and 21 °C, and it is quite sensitive to environmental factors such as high temperature and drought (Shriram et al., 2016). Different abiotic stresses affect potato productivity, growth, yield and quality depending on duration, severity and timing of the stress. Drought stress is the most common environmental factor that negatively affects potato productivity and yield (Aksoy et al., 2015). Water scarcity hinders potato tuber development especially in areas with insufficient irrigation and inconsistent rainfall patterns (Monneveux et al., 2013). Major potato cultivars have shallow root system, which makes the plant unable to fulfil their moisture requirement under drought stress. During potato growth cycle, tuberization and emergence are considered as the most decisive periods which are directly related with final yield (Obidiegwu et al., 2015). Drought causes reduction in tuber number and size and hence decreases plant productivity (Deblonde and Ledent, 2001). High temperature stress is also a major factor that restricts plant growth, maturation and development. Plants can face heat stress because of seasonal variations and geographical conditions (Shriram et al., 2016; Sunkar and Jagadeeswaran, 2012). Drought and heat stress individually can cause serious damage to plant, but in combination they even pose severe threat to plant production. Moreover, combined effect of drought and high temperature was shown to be more harmful compared to individual stress treatment in potato (Demirel et al., 2020).

MicroRNAs (miRNAs) are endogenously small (20–24 nt), single-stranded and noncoding RNAs. They can form stem-loop structure and are found in most eukaryotes (Yu et al., 2017). They have an important role in gene regulation at posttranscriptional level as they degrade and repress target mRNA's translation depending on complementarity (Din et al., 2014). MiRNAs are found abundantly and are well-studied in several plant species (Rajwanshi et al., 2014). According to recent studies, it was observed that miRNAs also function in growth, development, tissue differentiation, signal transduction, hormone secretion and play an important role in plant's response to environmental stress (Din et al., 2014; Zheng and Qu, 2015). Several successful studies have been reported about the role of miRNAs in stress tolerance in potato (Li et al., 2020; Saminathan et al., 2019).

In response to abiotic stresses including heat and drought, plants adopt different mechanisms to cope against them (Xiong and Zhu, 2002). As abiotic stresses disrupt the plants at transcriptional and posttranscriptional levels, it is necessary to understand these phenomena. Abiotic stress response is controlled by number of genes which interact with each other and they are under influence of transcription factors (Yamaguchi-Shinozaki and Shinozaki, 2006). Since the beginning of the discovery of miRNA's involvement in

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development of plants, a large number of stress related miRNAs have been identified (Jones-Rhoades et al., 2006).

In potato, few miRNAs associated with abiotic stress response or tolerance have been identified (Hwang et al., 2011). Yang et al. (2014) illustrated that stu-miRNAs play a key role in drought stress in potato as they regulate the expression of MBY transcription factor-like (*GAMYB-like*) genes. Moreover, recent studies on potato have also explained the potential role of miRNAs in the regulation of proline accumulation during water scarcity (Yang et al., 2013). A previous study also depicts that proline contents under drought increases in transgenic potato (Li et al., 2020).

Although mature miRNAs have been used to understand the stress tolerance mechanism in different plants, there are limited studies in potato on stress related miRNAs. In addition, there has been lack of information on stress tolerance mechanism in potato under high temperature stress and there has been limited number of studies on combined stresses that plants encounter during their life cycle in the literature (Handayani and Watanabe, 2020; Demirel et al., 2020). Therefore, the aim of this study was to explore the role of new Novel_105 miRNA in Unica and Russet Burbank cultivars in abiotic stress tolerance, in response to drought, high temperature and combined heat and drought stress conditions through transgenic approach by overexpression of pre-miRNA of Novel_105.

2. Materials and methods

2.1. Selection of plant material and Novel_105 miRNA

Two potato cultivars were selected based on their varying tolerance to abiotic stresses. The cultivar Unica was selected as the tolerant, whereas the cultivar Russet Burbank was reported to be sensitive to abiotic stresses based on earlier reports (Rolando et al., 2015; Demirel et al., 2017; Demirel et al., 2020).

According to previous study done by Kaplan (2017) regarding the identification of miRNAs in response to drought and high temperature stresses in potato cultivars Unica and Russet Burbank by next generation sequencing (NGS) technique, a total 314 of miRNAs were identified and it consisted of 104 novel miRNAs and 210 conserved miRNAs. Among 104 novel miRNAs, Novel_105 miRNA was selected for this study since psRobot (plant small RNA analysis toolbox) analysis predicted its putative target as E3 ubiquitin-protein ligase XBAT35 which is known to be functional in abiotic stress response (Kaplan, 2017).

2.2. Vector modification and pre-miRNA cloning to vector

Third and fourth leaf samples from potato plants were collected and pooled for total RNA isolation from both Unica and Russet Burbank cultivars using Trizol reagent (Thermo Fisher Scientific). cDNA synthesis was performed with *Pfu* polymerase (Promega) using Oligo dT primer (Thermo Scientific) for the target gene and specific stem loop primer for pre-mir_Novel_105 amplifications (Novel_105-RT, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCATCAA-3′). Amplification of pre-mir_Novel_105 cDNA was done by following the PCR conditions of denaturation at 94 °C for 2 min, annealing at 58.4 °C for 15 s, extension at 72 °C for 20 s in 34 cycles, and final extension at 72 °C for 10 min with forward primer Novel-105F (5′-GCACCATGGAGATCTTGGGTTGCTGTAGGATTG-3′) and reverse primer Novel-105R (5′-GCAGCTAGCGGTNACCGTTATGGATTGCTGAAGGAT -3′) having *Nco*I and *BstE*II restriction enzyme sites. The amplified fragment of Novel_105 miRNA were cut from gel, purified with GeneJET Gel Extraction kit (Thermo Scientific) and digested with the *Nco*I and *BstE*II restriction enzymes. Binary vector pCAMBIA-1301 was also modified by removal of *gusA* site (Figure S1) and digested with the *Nco*I and *BstE*II restriction enzymes. The ligation of gel extracted and purified vector and Novel_105 fragment was performed using 0.02 pM cDNA, 0.06 pM vector, 5 U T4 ligase enzyme (Promega), and 1X ligase buffer at 22 °C overnight. The reaction was terminated at 70 °C for 5 min to inactivate T4 ligase enzyme. Ligation of digested vector and Novel_105 pre-miRNA insert was verified by PPB primers after transformation to *E. coli* strain *DH5α* (Table). The PCR conditions were denaturation at 94 °C for 3 min, annealing at 55 °C for 15 s, extension at 72 °C for 15 s in 34 cycles, and final extension at 72 °C for 7 min. The developed construct was electroporated at 100V for 30 s to *Agrobacterium tumefaciens* LBA4404 using Gene Pulser Xcell Electroporation System.

2.3. Potato tubers sterilization and transformation

Transgenic potato plants were generated to identify the function of Novel_105 miRNA under drought and heat tolerance of potato cultivars. Firstly, sprouts of Unica and Russet Burbank tubers were washed with Tween-20. They were put into 70% ethanol for 5 min. Then, they were put into Mancozeb antifungal solution (2 mg/L) for 10–15 min and were washed with 70% ethanol. After, washing with distilled water thrice, sprouts were put into solution of 1:20 diluted Sulcid antibiotic (1 g ampicillin + 0.5 g sulbactam) for 10 min. After antibiotic treatment, they were put into 1% hydrogen peroxide solution for 1 min. Sprouts were then cultured on

Table. List of primers used in the study.

Primer name	Primers sequence (5′ to 3′)	Length
PBB-Forward	GAGAACACGGGGGACTCTTG	20
PBB-Reverse	TAATCATCGCAAGACCGGCA	20
Hygromycin Forward Primer	TTGGGAATCCCCGAACATCG	20
Hygromycin Reverse Primer	CTCTCGGAGGGCGAAGAATC	20
35S Forward Primer	CATGGAGTCAAAGATTCAAATAG	23
NOS Poly-A tail Reverse Primer	AACCCGGGCCCGATCTAGTAACATA	25
ChvA gene Forward Primer	CGAAACGCTGTTCGGCCTGTGG	22
ChvA gene Reverse Primer	GTTCAGCAGGCCGGCATCCTGG	22

Murashige and Skoog (MS) medium containing 4.4 g MS salt (Duchefa Biochemie), 7.5 g agar and 30 g sucrose per 1 L medium at pH 5.7 in Magenta GA-7 culture boxes. Boxes were incubated in growth chamber for 16 h of photoperiod at temperature of 22 °C. A single node cut was made every 4 weeks from regenerated shoots and these nodes were subcultured for micropropagation.

Transformed Agrobacterium LBA4404 cells were inoculated with internode explants of four-week-old plants grown in tissue culture. Transformed bacteria was grown in LB liquid media with 1 mL/L kanamycin (from 50 mg/mL stock) before inoculation. The internodes were cut from Unica and Russet Burbank, wounded, and incubated in 20–30 mL water containing 400 µL culture solution for 2–3 min. The inoculated explants were incubated in growth chamber for 2 days in solid MS-0 (hormone and antibiotic-free) medium containing acetosyringone 1 mg/L. After that, the explants were transferred to callus-forming medium and the culture was incubated at 27 °C under 16 h photoperiod conditions. The callus medium consisted of 4.4 g/L MS salts and vitamins (Duchefa Biochemie), 30 g/L sucrose, 7.8 g/L agar, 0.1 mg/L NAA (A-Naphtalene acetic acid) (Duchefa Biochemie), 1 mg/L BAP (6-Benzylaminopurine) (Duchefa Biochemie), 2 mg/L zeatin riboside (Duchefa Biochemie), 0.1 mg/L GA3 (Giberellin) (Sigma-Aldrich), 100 mg/L ascorbic acid, 4 mg/L hygromycin (Alfa Aesar) and 500 mg/L Sulcid antibiotic (1g ampicillin + 0.5 g sulbactam). Developed calli was transferred to shoot formation medium containing 4.4 g/L MS salts and vitamins, 30 g/L sucrose, 7.8 g/L agar, 0.1 mg/L NAA, 1 mg/L BAP, 0.1 mg/L GA3, 2 mg/L hygromycin and 300 mg/L Sulcid antibiotic. When putative transgenic sprouts were approximately 1.5 cm long, they were transferred to MS-0 media without antibiotic and placed in growth chamber at 16 h of photoperiod and 25 °C.

2.4. Confirmation and growth of transgenic plants

When plants in growth chamber were grown enough, leaves were cut from putative transgenic plants. DNA isolation was done by GeneJet Plant Genomic DNA Purification Kit (Thermo Fischer Scientific). Validation of transgenic plants was done with a PCR conditions of denaturation at 94 °C for 4 min, annealing at 58.4 °C for 20 s, extension at 72 °C for 45 s in 34 cycles, and final extension at 72 °C for 7 min by Hygromycin primers (Table 1), whereas PCR conditions were denaturation at 94 °C for 2 min, annealing at 58.4 °C for 15 s, extension at 72 °C for 20 s in 34 cycles, and final extension at 72 °C for 10 min for 35S primers (Table 1). The transgenic plants were also tested for the presence of Agrobacterium contamination by observing an amplicon of 898 bp length as a result of analysis with primers specific to the *ChvA* gene (Table 1).

When hygromycin and 35S confirmed transgenic T0 plants were about 15 cm long, they were transferred to soil with 2:1 ratio of peat moss and perlite to allow acclimation to soil conditions. Four-week-old transgenic plants were transferred to 12 L pots as a one plant per pot containing a 2:1 ratio of peat moss and perlite into greenhouse and fully controlled growth chamber. All pots were irrigated to soil field capacity until stress treatment and maintained at 24 °C/16 °C (16 h day/8 h night) in climate-controlled greenhouse or growth chamber.

2.5. Stress treatment

Wild type and transgenic potato cultivars (Unica and Russet Burbank) were exposed to stress conditions at tuber development stage. Potato plants were divided into four groups i.e. control, drought, high temperature, combined drought and high temperature stresses. For heat and heat and drought stress, plants were kept in growth chamber, whereas for drought stress plants were kept in greenhouse. The control groups for heat and heat and drought stress, and drought stress were also maintained at the same conditions (growth chamber and greenhouse, respectively). All plants in the control group were irrigated regularly. For drought stress treatment, plants in greenhouse were deprived of water for total 20 days. For heat stress, temperature increased gradually from 24/18 °C to 39/27 °C in 9 days, then a constant heat of 39/27 °C was applied for 3 days (14 h photoperiod and 60%–70% relative humidity), where control plants were kept at a second growth chamber maintained at 24/18 °C (day/night) of temperature with a 14 h photoperiod and 60-70% relative humidity. Pots were kept fully irrigated during heat stress treatment. Combined stress of heat and drought was applied similar to heat stress, except that the plants were also subjected to drought stress by terminating irrigation for 12 days (Yalçın, 2020).

2.6. Physiological measurements

Fully-expanded upper 3rd and 4th leaves were collected from stress treated and control plants, pooled together and was used for the measurement of physiological traits during the course of stress application at 0, 7, 9, 12, 16, 18 and 20th day of stress treatment in four replications.

2.6.1. Gaseous exchange traits

Stomatal conductance, photosynthetic rate and transpiration rate was measured between 9:00–11:00 am by constant light intensity of photosynthetic device (1500 µmol/m^2s), CO_2 amount (400 µmol) and airflow (500 µmol/s). The measurement was recorded using a portable gas exchange system LiCor 6400 XT (Li–COR Biosciences, USA). Measurements were recorded from both wild type and transgenic plants at 0, 7, 9, 12, 16, 18 and 20th day to evaluate severity of stress at biochemical and molecular levels.

2.6.2. Chlorophyll and leaf temperature measurements

Leaf chlorophyll contents were measured between 9:00–11:00 am with Chlorophyll-Meter (Konica Minolta SPAD-502 Plus Chlorophyllometer) from all control and stress treated plants in four replications. Leaf temperature was measured between 9:00–11:00 am using IRT instrument (MASTECH BM380). The apical leaflet of the second and third leaf of the potato plants were used for leaf temperature measurements. The data was collected at the last day of stress treatments and given as mean of second and third leaf measurements.

2.6.3. Relative water content

Relative water content (RWC) of transgenic and control plants was measured on the last day of stress treatments. Samples were collected from four different pots for each stress treatment separately. Fresh weight of potato leaves was

measured and leaves were putted in distilled water overnight for the measurement of turgid weight. Leaves were dried in oven for measuring dry weight of leaves. RWC was calculated according to the following equation;

$$\text{RWC}\ (\%) = [(\text{Fresh weight} - \text{Dry weight})/(\text{Turgor weight} - \text{Dry weight})] \times 100\,.$$

2.7. Biochemical parameters

All samples for biochemical experiments were taken on the last day of stress as four replicates. Fully-expanded upper 3rd and 4th leaves were collected from stress treated and control wild type and transgenic plants, pooled together and directly frozen in liquid nitrogen for storage at –80 °C until further use. All biochemical measurements were measured as three replicated and data were presented as mean values.

2.7.1. Proline content

Proline content was determined by using method developed by Bates et al. (1973) with minor modifications. Briefly, leaf samples were ground in 2 mL of 3% sulfosalicyclic acid and then supernatant was mixed with 200 µL ninhydrin solution. Samples were shaken gently and then incubated at 90 °C for an hour. The reaction was terminated by placing samples on ice. A total of 2 mL toluene was added after cooling. The concentration of proline was calculated from a standard curve using the following equation (µg proline in extract/115.5)/g sample = µmol/gFW.

2.7.2. Malondialdehyde content

Lipid peroxidation level was measured from leaf samples through measuring accumulation of malondialdehyde (MDA). MDA content was determined following the thiobarbituric acid (TBA) reaction protocol given by Heath and Packer (1968). MDA contents were calculated by using the following equation given below.

$$\text{MDA}\ (\mu\text{mol/g FW}) = [(\text{A532} - \text{A600})/155] \times 10^3 \times df \times \left(\frac{1}{tissue}\, weight\ (g)\right)$$

2.7.3. Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was measured from potato leaves by following protocol Loreto and Velikova (2001).

2.8. Gene expression analysis of Novel_105 and E3 ubiquitin-protein ligase *XBAT35*

Fully-expanded upper 4th leaves were collected from stress treated and control wild type and transgenic plants and directly frozen in liquid nitrogen for storage at –80 °C until further use. RNA extraction was performed by Trizol reagent. Quality and quantity of RNA was checked by agarose gel electrophoresis and a nanodrop spectrophotometer. cDNA synthesis was done by using Omniscript Reverse Transciption Kit (Omniscript RT Kit) to construct cDNA from miRNA samples. Expression analysis of the miRNA and its putative target gene was performed separately from three biological and three technical replications. The putative Novel_105 target, E3 ubiquitin-protein ligase *XBAT35* was amplified using forward (5′-GCAGTAAACGCATACGCAGA-3′) and reverse primers (5′-CAGTTTCACTTCTCCGCACA-3′), whereas mature miRNA Novel_105 specific primer (5′-GCCTGGGTTGCTGTAGGATTGATG-3′) was used with miRNA-R-Universal primer (5′-CCAGTGCAGGGTCCGAGGTA-3′) for amplification. Real-time quantitative PCR (qRT-PCR) was performed separately in 10 µL of final volume having 100 ng of cDNA, 0.8 µM of each primer, and 1 × SYBR Green PCR Master Mix (Applied Biosystem). The qRT-PCR 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). Melting curve analysis was performed to detect specific amplification by incubating at 99 °C to 70 °C with a transition rate of 1.0 °C/min. Relative expression levels of Novel_105 and E3 ubiquitin-protein ligase *XBAT35* were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The housekeeping gene EF1α (elongation factor 1-α) forward (5′- GGACCCAACTGGTGCCAAAG-3′) and reverse (5′-CTCGCCACCGCCTATCAAGT-3′) primers were used for normalization.

2.9. Statistical analysis

Data were analyzed statistically by the least significant difference test (LSD) to compute mean values by Statistical Package Statistix 8.1. All data were compared with their respective controls at the same time points.

3. Results

3.1. Morphological response of potato

Morphological differences among the wild type and transgenic Novel_105 Unica and Russet Burbank potato cultivars were observed after 20 days of drought stress (Figure 1) and 12 days of heat and combined heat and drought stress (Figure 2). Transgenic Unica Novel_105 showed better growth with more leaves and branches as compared to its counterpart Unica wild type in response to given stresses. In comparison of transgenic Unica and Russet Burbank cultivars after drought stress, Russet Burbank transgenic cultivars appeared physically better than wild type Unica cultivar, whereas Russet Burbank Novel_105 transgenic plant appeared to have greener and more alive leaves (Figure 1). Furthermore, Unica wild type and transgenic cultivars were less affected by drought stress than Russet Burbank cultivars for both wild type and transgenic plants in terms of leave aliveness and color. It is important to note that heat stress affected transgenic plants less than combined heat and drought stress. Under combined stress, plants nearly died and there were no leaves (Figure 2). Additionally, the impact of drought stress was less on Unica plants as compared to heat or heat and drought combined stress. Similar results were observed for Russet Burbank cultivar that combined stress affected plants more than drought stress. However, transgenic Russet Burbank plants were not influenced by heat and combined stress like transgenic Unica cultivars. Combined stress had adverse effect on plants more than individual stresses based on initial morphological comparison.

3.2. Gaseous exchange parameters in response to stress

Photosynthetic rate of potato plants was measured at different time intervals during the stress period (Figure 3). The photosynthetic rate of both wild type and transgenic plants showed grouping together in Unica and Russet Burbank cultivars, indicating overexpression of Novel_105 had no affect on steady state photosynthesis. Drought stress of 20 days resulted in significant ($p < 0.05$) decrease of 63% in

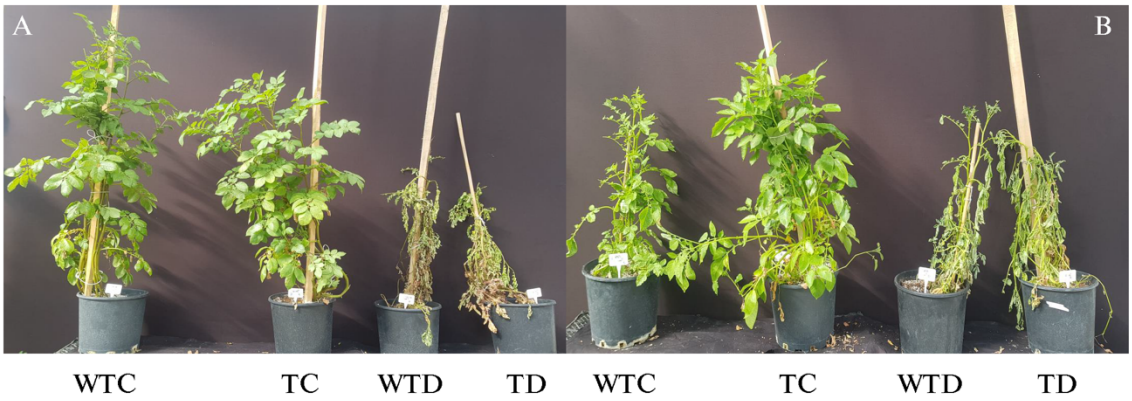

Figure 1. Phenotypic response of wild type and transgenic plants of Unica (A) and Russet Burbank (B) genotypes under drought stress. WTC: wild type control, TC: transgenic control, WTD: wild type under drought stress, TD: transgenic under drought stress.

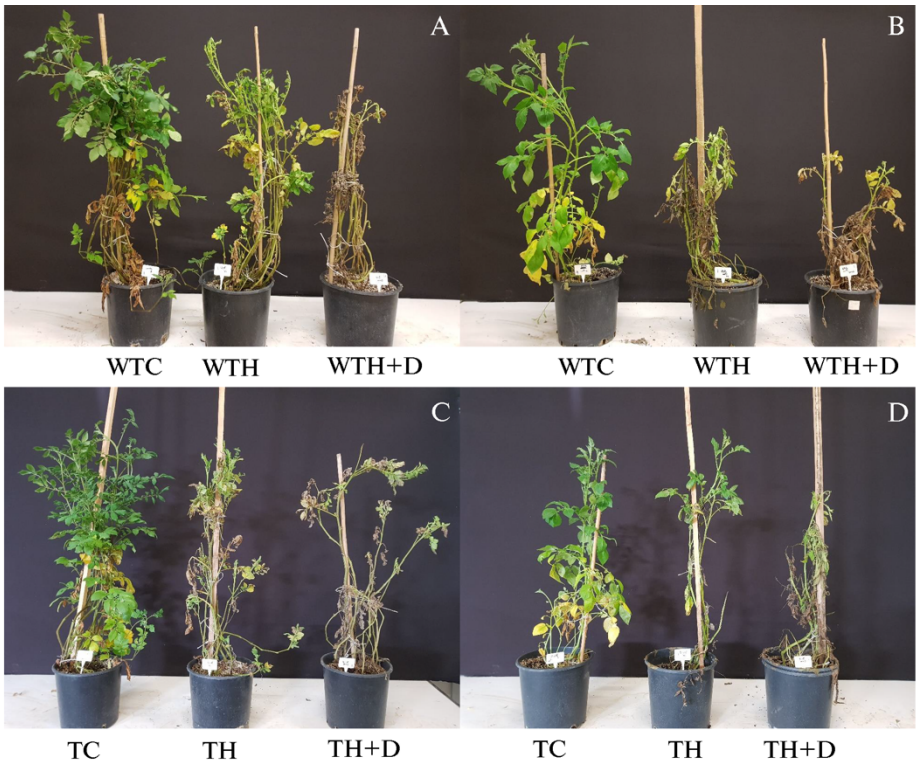

Figure 2. Phenotypic response of wild type Unica (A), wild type Russet Burbank (B), transgenic Unica (C) and transgenic Russet Burbank (D) plants under heat and heat and drought stresses. WTC: wild type control, WTH: wild type under heat stress, WTH+D: wild type under heat and drought stress, TC: transgenic control, TH: transgenic under heat stress, TH+D: transgenic under heat and drought stress.

photosynthetic rate of Unica wild type plants compared their control plants at the same time point, whereas the transgenic plants showed 61% decrease in the same comparison, and in most cases they grouped together indicating similar changes in photosynthetic rate in response to drought. In case of heat stress, photosynthetic rate of wild type Unica plants showed significant ($p < 0.05$) increase up to 34% after 12 days as compared their control plants and a 56% decrease in heat and drought stress was observed in the same comparison, while transgenic Unica Novel_105 showed 58% increase in response to heat and a 47% decrease with the exposure of combined heat and drought stress conditions as compared to their control plants at 12 days. The wild type and transgenic Unica plants were grouped separately in their response to heat and combined heat and drought stress. The cultivar Russet Burbank wild type showed a 61% decrease in photosynthetic rate after 20 days of drought stress in comparison to its control, whereas the transgenic plants showed only a 32% decrease in the same comparison. In case of heat stress photosynthetic rate of Russet Burbank wild type plants decreased up to 39% and a 70% decrease of heat and drought stress after 12 days compared to their control plants at the same time point, while transgenic Novel_105 Russet Burbank plants showed a 13% increase in response to heat stress and a 55% decrease with the exposure of combined heat and drought stress conditions in the same comparison. The grouping of Russet Burbank plants in response to drought,

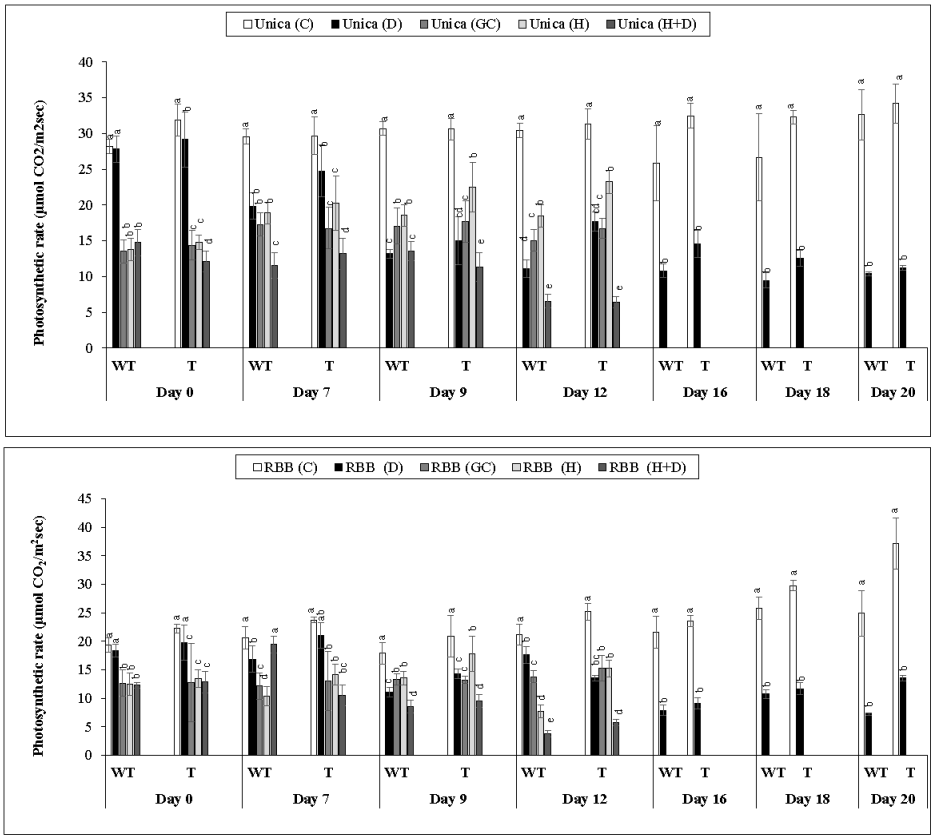

Figure 3. Effect of abiotic stress on the photosynthetic rate of transgenic (T) and wild type (WT) Unica and Russet Burbank (RBB) potato cultivars. X axis shows data taken at different time intervals of stress treatment. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

heat and combined heat and drought stress were separate in wild type and transgenic plants.

The cultivar Unica performed better than the cultivar Russet Burbank as it showed higher stomatal conductance in response to stress conditions (Figure 4). The grouping of the changes in stomatal conductance in wild type and transgenic Unica and Russet Burbank plants indicated similar responses. The cultivar Unica wild type showed significant ($p < 0.05$) reduction in stomatal conductance of 103% after exposure of 20 days of drought stress as compared to its control, whereas the transgenic Unica Novel_105 depicted a reduction of 98% in the same comparsion. The cultivar Russet Burbank wild type and its transgenic showed a reduction of 110% in case of drought stress of 20 days as compared to their respective control plants. Unica wild type showed a reduction of 19% under heat stress and 94% under combined heat and drought stress after 12 days of stress as compared their control plants, while its transgenic plants showed an elevated stomatal conductance rate.

Transpiration rate measurement showed significant ($p < 0.05$) differences in wild type and transgenic plants while compared after 20 days of drought and 12 days of heat and heat and drought combined stresses (Figure 5). The transpiration rate of both cultivars, whether transgenic or wild type decreased to minus values in 20 days of drought stress. The overall performance of cultivar Unica was better in response to applied stresses than the cultivar Russet Burbank as it showed higher transpiration rates. The cultivar Unica wild type showed a reduction of 17% under heat stress and 65% under combined heat and drought stress after 12 days as compared to their respective control plants, while its transgenic plants showed a higher transpiration rate with the increase of 163% and a decrease of 28% under heat and drought stress in the same comparison. The cultivar Russet Burbank wild type showed a reduction of 54% under heat stress and 91% under combined heat and drought stress after 12 days as compared to their control plants, while its transgenic plants showed a higher transpiration rate with the increase of 162% and a decrease pf 65% under heat and drought stress in the same comparison. The comparison of grouping of wild type and transgenic Unica and wild type and transgenic Russet Burbank plants indicated similar response in drought, heat and combined heat and drought stress.

3.3. Changes in chlorophyll contents and leaf temperature

Leaf chlorophyll content was also quantified after 20 days of drought and 12 days of heat and combined heat and drought stress from wild type and transgenic plants (Figure 6a). The leaf chlorophyll content as measured by SPAD units were

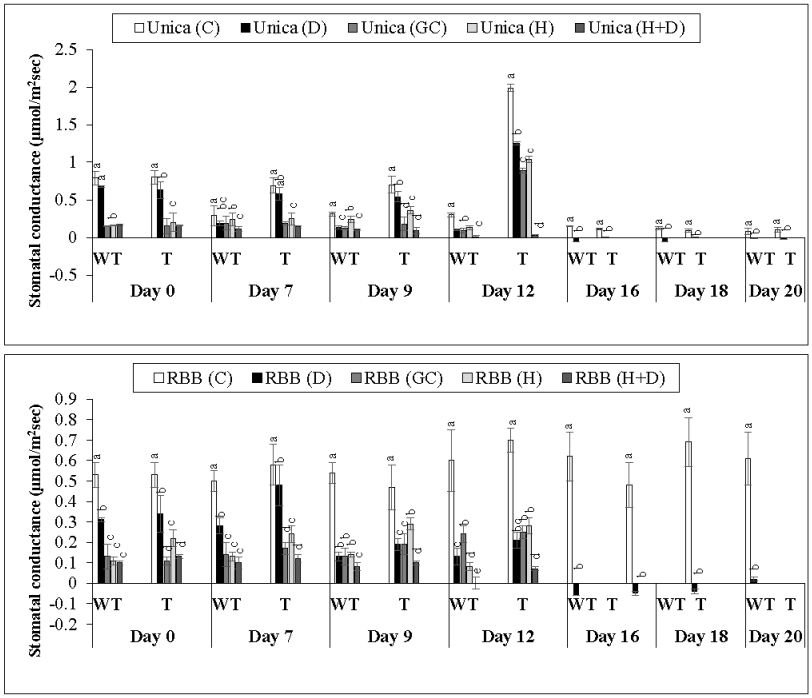

Figure 4. Effect of abiotic stress on the stomatal conductance of transgenic (T) and wild type (WT) Unica and Russet Burbank (RBB) potato cultivars. X axis shows data taken at different time intervals of stress treatment. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

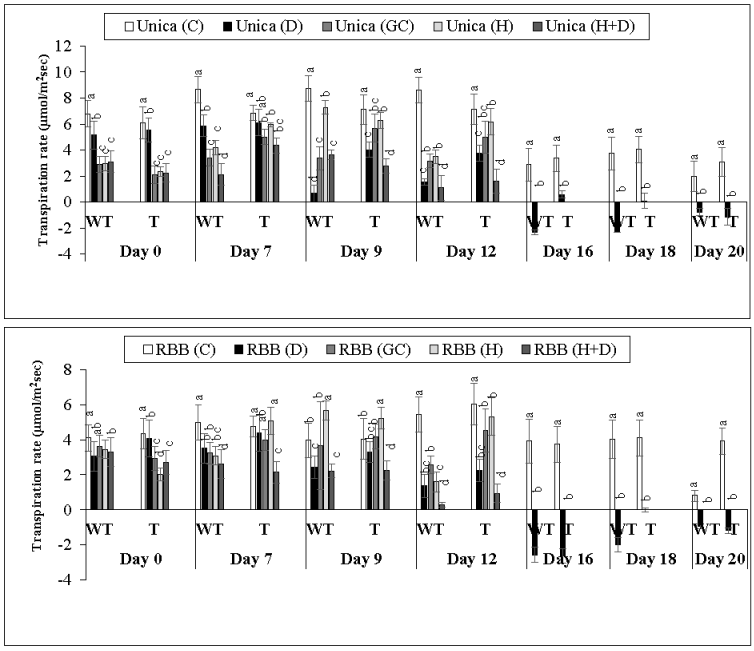

Figure 5. Effect of abiotic stress on the transpiration rate of transgenic (T) and wild type (WT) Unica and Russet Burbank (RBB) potato cultivars. X axis shows data taken at different time intervals of stress treatment. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

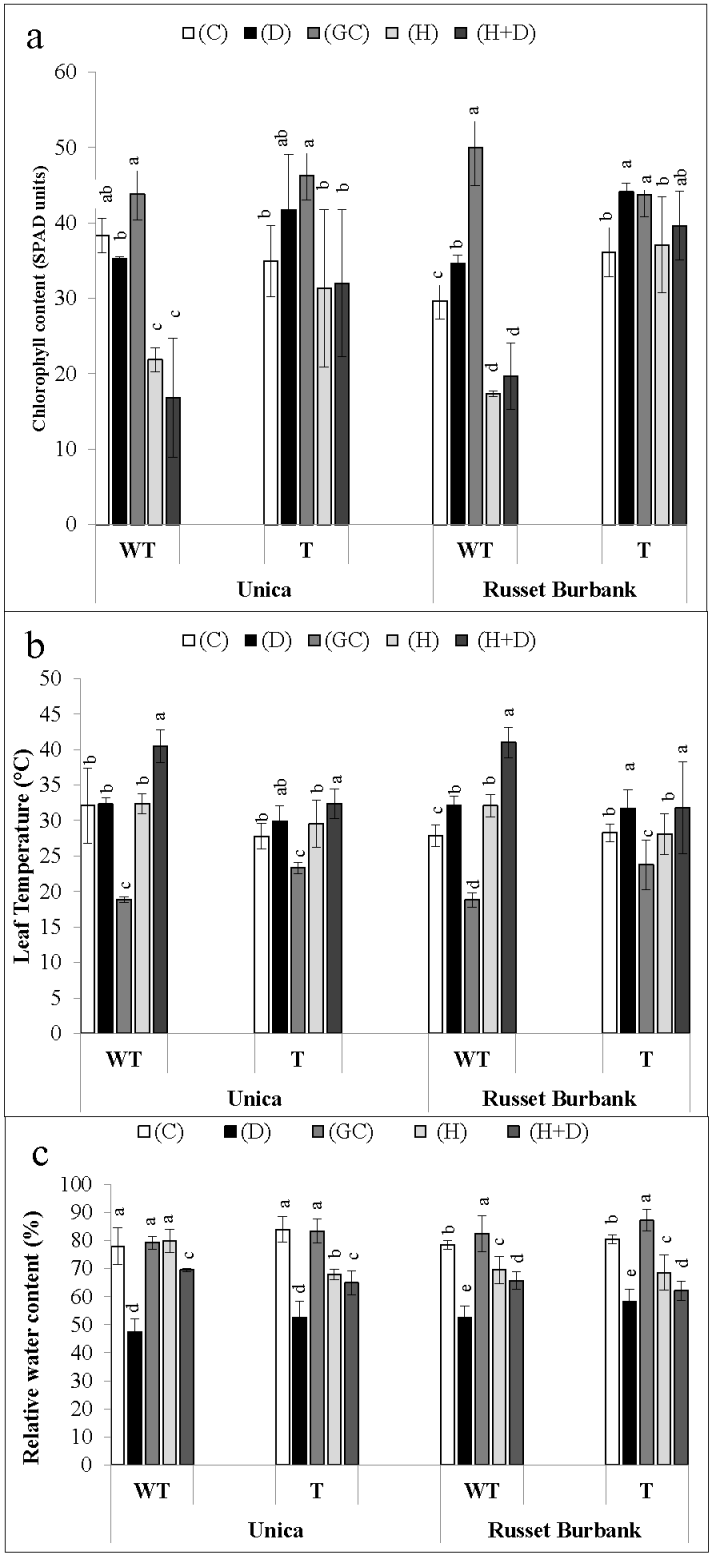

Figure 6. Effect of abiotic stress on the chlorophyll content (a), leaf temperature (b) and relative water content (c) of transgenic (T) and wild type (WT) Unica and Russet Burbank potato cultivars. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

different in wild type and transgenic Unica and Russet Burbank plants as indicated by separate grouping in Figure 7a. Upon stress treatment, Unica wild type showed a reduction of 8% as compared to its respective control, while its transgenic plant showed significant ($p < 0.05$) increase of 19% in chlorophyll content in the same comparison performed under

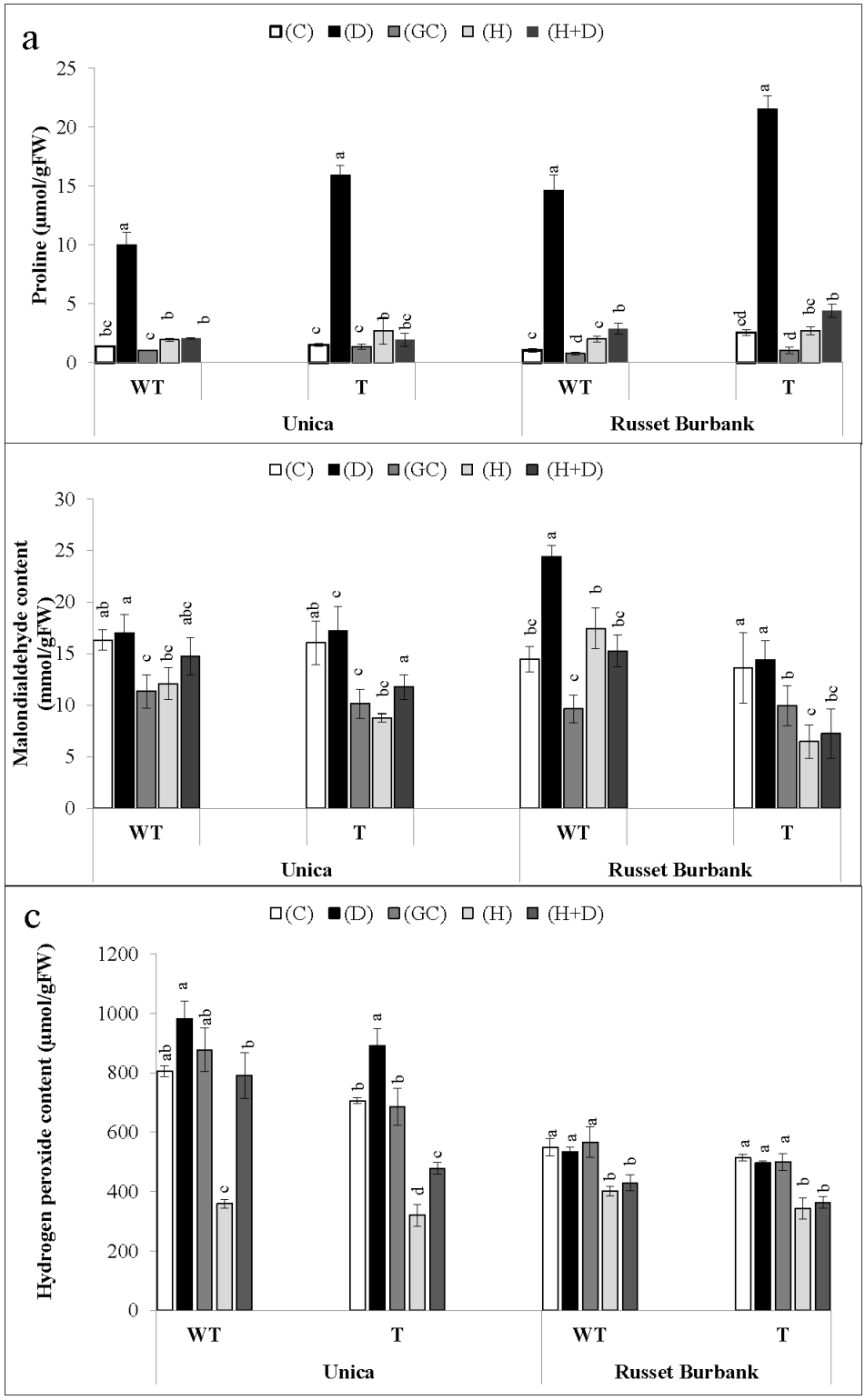

Figure 7. Effect of abiotic stress on the proline (a), malondialdehyde (b) and hydrogen peroxide (c) contents of transgenic (T) and wild type (WT) Unica and Russet Burbank potato cultivars. Transgenic (T) and wild type (WT) plants of potato. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

drought stress. When chlorophyll contents were measured from sensitive Russet Burbank cultivar under drought stress, its wild type showed a 17% increase as compared to its control, likewise the transgenic Russet Burbank Novel_105 also

exhibited a 22% higher chlorophyll content in the same comparison. Heat and combined stress treatments resulted in more damage to chlorophyll content as compared to drought stress group. The cultivar Unica wild type depicted a 50% and a 62% reduction in chlorophyll content in comparison to their respective control plants in response to heat and combined heat and drought stress, respectively. Unica Novel_105 transgenic plants showed increase in chlorophyll contents by 19% with the exposure to drought stress as compared to their control plants, while a decrease of 32% was observed in response to both heat and combined heat and drought stress treatment in comparison of 0th and 12th day measurements with their respective controls. The cultivar Russet Burbank wild type depicted a 65% and a 61% reduction in comparison to its respective control in chlorophyll content in response to heat and combined heat and drought stress, respectively. Russet Burbank Novel_105 transgenic plants, on the other hand, showed increase in chlorophyll content by 19% with the exposure to drought stress as compared to their respective control, while a decrease in chlorophyll contents of 15% and 9% was observed in comparison of 0 and 12 day measurements of respective control plants in response to heat and combined heat and drought stress, respectively. In all cases, the chlorophyll content of transgenic plants were higher than its wild type counterparts.

The changes in leaf temperature of wild type and Novel_105 transgenic Unica and Russet Burbank cultivars as compared to their control plants grown under the same conditions were given in Figure 6b. The cultivar Unica wild type showed no significant ($p > 0.05$) difference as compared to its control plant as indicated by similar grouping, whereas it had higher leaf temperature under heat and combined heat and drought stress with an increase of 72% and 115%, respectively (Figure 6b). Interestingly, transgenic Unica plants had higher leaf temperature under drought stress as compared to its own wild type, whereas the increase in leaf temperature under heat and combined heat and drought was lower than its wild type. The cultivar Russet Burbank showed a 15% increase in leaf temperature in response to drought stress, however no significant difference was observed as compared to Unica wild type. Both cultivars showed an increase in leaf temperature of about 72% and 115% when compared to their respective controls under heat and combined heat and drought, respectively.

3.4. Relative water contents

The relative water content of both transgenic and wild type Unica and Russet Burbank cultivars were similar as expected as shown in the same group (Figure 6c). The cultivar Unica wild type and its transgenic showed a nonsignificant ($p > 0.05$) difference with the imposition of drought stress, whereas a significant ($p < 0.05$) change was noticed in Russet Burbank, as wild type showed 33% decrease in RWC and transgenic plant exhibited 28% decrease indicating better performance of transgenic Novel_105 Russet Burbank plants compared to their sensitive wild types. The response of transgenic Unica and Russet Burbank plants was almost the same since both cultivars resulted in more decrease in RWC as compared to wild type under heat and combined heat and drought stresses.

3.5. Biochemical changes with the exposure to stress

Proline accumulation increased in all the stress groups irrespective of wild type and transgenic plants (Figure 7a). The significant ($p < 0.05$) accumulation of proline was observed in response to drought stress in both cultivars as compared to other stress groups, and interestingly this accumulation appeared in the same group both in wild type and transgenic plants. The transgenic Unica showed higher accumulation under all stress groups except the combined heat and drought stress. Differential responses by wild type and transgenic Russet Burbank cultivars were observed as indicated by separate grouping. The transgenic Russet Burbank plants had significantly ($p < 0.05$) higher proline amount than its wild type plants under all stress groups. Besides this, proline amount was higher in sensitive Russet Burbank than tolerant Unica cultivar both in wild type and Novel_105 transgenic plants. The changes in MDA content in transgenic and wild type Unica plants were different than the changes observed in transgenic and wild type Russet Burbank plants as indicated by separate grouping in Figure 8b. MDA contents showed nonsignificant ($p < 0.05$) difference in Unica wild type and transgenic plants after exposure to stress conditions. The change in MDA content upon heat stress in transgenic Unica plants showed a 13% decrease compared to its wild type control. Russet Burbank showed increased MDA contents in wild type plants, whereas it exhibited a 35% and a 27% decline in MDA contents when compared to their respective controls in response to heat and combined heat and drought stress, respectively. Hydrogen peroxide (H_2O_2) accumulation in wild type and transgenic Unica plants were more pronounced as compared to that of both wild type and Novel_105 transgenic Russet Burbank plants as indicated by separate grouping in Figure 8c. H_2O_2 accumulation increased significantly ($p \leq 0.05$) under drought stress in both wild type and transgenic Novel_105 Unica plants as compared to their respective controls. Russet Burbank, on the other hand, showed minimal reduction in H_2O_2 in both wild type and transgenic potato cultivars under all stress conditions.

3.6. Transcriptional profiles of Novel_105 and its putative

Mean normalized gene expression of miRNA Novel_105 and its putative target *XBAT35* was quantified before termination of stress for the evaluation of transcript changes in both sensitive and tolerant potato cultivars (Figure 8). The comparison of gene expression of Novel_105 under control conditions indicated separate grouping of wild type and transgenic lines implying the increased expression of Novel_105 in transgenic lines as expected. The expression levels of Novel_105 was slightly higher in transgenic Unica cultivar plants as compared to transgenic Russet Burbank cultivar under drought stress as implied by separate grouping. The transgenic Unica cultivar showed lower gene expression of Novel_105 level than transgenic Russet Burbank cultivar under heat stress, as they appeared in different groups. It is interesting to note that wild type Unica and Russet Burbank cultivars accumulated lower transcript levels of Novel_105 as compared to its transgenic plants. The elevated gene expression level was measured with a significant ($p \leq 0.05$) difference from its control plant with the exposure to all the

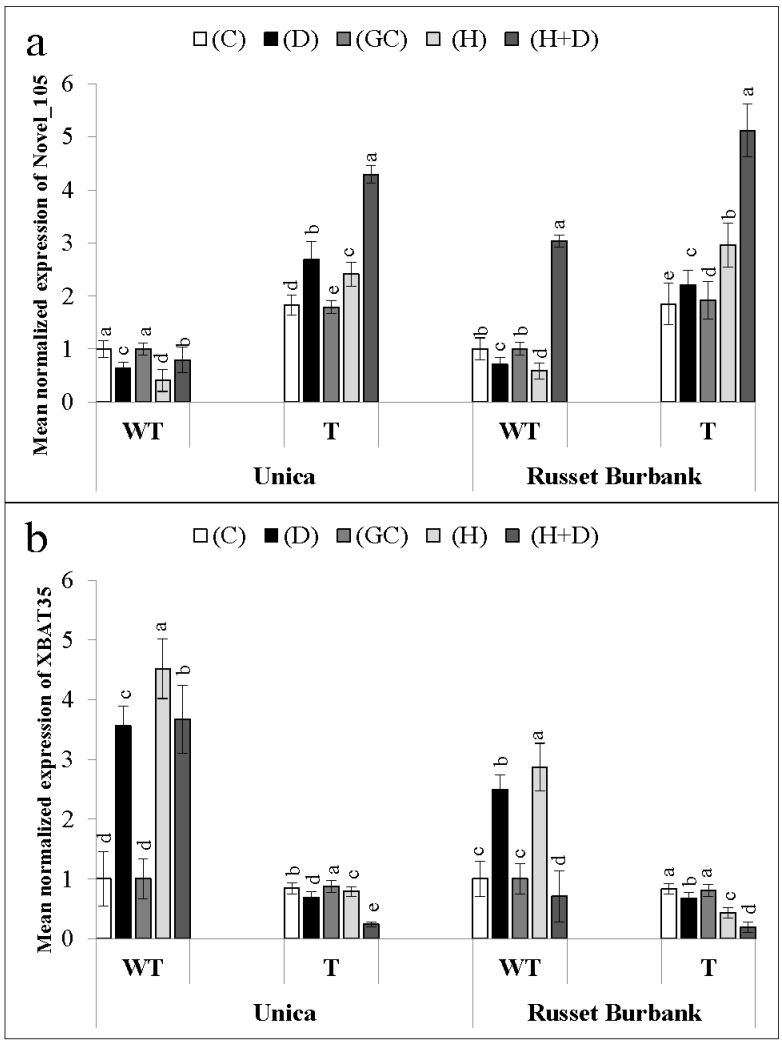

Figure 8. Effect of abiotic stress on the mean normalized gene expression levels of Novel_105 (a) and its putative target gene E3 ubiquitin-protein ligase *XBAT35* (b) in transgenic (T) and wild type (WT) Unica and Russet Burbank potato cultivars. Transgenic (T) and wild type (WT) plants of potato. Data shown as mean ± SD of four independent biological replicates. C: control, D: drought stress, GC: growth chamber control, H: heat stress, H+D: heat and drought stress. Letters sharing the same alphabets shows nonsignificant ($p \geq 0.05$) difference, whereas different letter depicts significant ($p \leq 0.05$) difference when compared with their respective controls.

stresses, whereas the higher gene expression was quantified in response to combined heat and drought stress conditions (Figure 8a). Figure 9b shows the mean normalized change in gene expression of E3 ubiquitin-protein ligase *XBAT35*, the psRobot based putative target of Novel_105. The antagonistic change between expression levels of Novel_105 and its putative target *XBAT35* is implied in comparison of Figures 8a and 8b, as the increase in the expression of Novel_105 is followed by a decrease in the expression of *XBAT35*. The gene expression of putative target gene *XBAT35* appeared to be significantly ($p \leq 0.05$) higher than in wild type plants compared to its transgenic plants as implied by separate grouping. These data showed that the overexpression of Novel_105 resulted in suppression of *XBAT35* gene expression in transgenic plants supporting the possibility of Novel_105 targeting *XBAT35* in posttranscriptional regulation (Figure 8b).

4. Discussion

This study provides the first investigation of Novel_105 miRNA and its putative target gene (*XBAT35*) in response to alone and combined stress conditions. The comparison was based on changes upon drought, heat and heat and drought stress responses of a tolerant (Unica) and a sensitive (Russet Burbank) potato cultivar with the overexpression of miRNA Novel_105. Current study revealed the beneficial role of Novel_105 to confer stress tolerance in potato with the suppression of its target gene.

The overexpression of Novel_105 affected transgenic Unica and Russet Burbank plants both morphologically and in response to stress treatments used in the study (drought, heat, combined heat and drought) (Figures 1 and 2). Overall, Unica Novel_105 transgenic plants were more affected by drought stress compared to Novel_105 transgenic Russet

Burbank plants. Similar results were observed under combined heat and drought stress conditions, and it affected Russet Burbank plants more than drought stress. Transgenic Unica plants exhibited more resistance to heat and combined heat and drought stress as compared to its wild plants. It is obvious from literature that combined stress has an adverse effect on plants than individual stresses. According to Djami-Tchatchou et al. (2017) drought stress reduces plant growth and crop yield. It can be assumed that Novel_105 miRNA might have relation with drought-responsive genes as in this study transgenic Unica and Russet Burbank plants showed more resistance against drought as compared to wild type plants.

Potato tuber yield is majorly affected due to damaged photosynthetic activity (Hancock et al., 2014). Abiotic stress decreases photosynthetic rate of plants as it affects protein thylakoid membranes, pigments and soluble proteins, photophosphorylation and disturbs electron transport chain (Morales et al., 2020). In this study, Novel_105 transgenic Unica and Russet Burbank plants showed higher photosynthesis rate under abiotic stress as compared to wild plants as observed in a time course manner (Figure 3). In a previous study, greater reduction in photosynthetic rate was observed under drought than heat stress (Sehgal et al., 2017). Our study showed that transgenic plants showed higher photosynthetic rate than wild type plants even though there was also a significant difference in photosynthetic rate of both transgenic plant cultivars. Decreased photosynthetic activity was attributed to closure of stomata that accumulated lower internal CO_2 concentration (Chaudhry et al., 2020), which was observed in both wild type and Novel_105 transgenic Unica and Russet Burbank plants as the decrease in stomatal conductance through the time dependent manner in response to drought, heat and combined heat and drought stresses (Figure 5). Overall stomatal conductance was higher in Novel_105 overexpressing transgenic Unica and Russet Burbank plants and decreased more progressively in the time course of stress treatments as compared to their counterpart wild types (Figure 5). Fluctuations in CO_2 concentration triggers excessive production of reactive oxygen species that caused oxidative stress and resulted in declined photosynthetic rate (Choudhury et al., 2017). Moreover, the other prime cause of decreased gaseous exchange traits under drought stress is due to limited water supply, as significant reduction in RWC was noticed under drought stress this study (Figure 6c). Reduction in internal water contents also decreased the transpiration rate under drought stress since potato plants mediate their water status by closing stomatal apertures (Dahal et al., 2019). Transpiration rate in wild type and Novel_105 transgenic Unica and Russet Burbank plants, on the other hand, increased under heat stress (Figure 4) which can be explained as an adaptation strategy to elevated temperature (Dahal et al., 2019). Overall, stress treatments disturbed the gaseous exchange traits of both wild type and Novel_105 transgenic potato cultivars used in the study. It can be explained as due to the closure of stomata that resulted in declined transpiration rate. Under stress, plants undergo stomatal closure and reduce stomatal size as a strategy to reduce water losses via transpiration (Zhang, 2007). It is an osmotic stress avoidance strategy and also associated with turgor potential of plant (Jensen, 1981). Moreover, significant decrease in RWC resulted in lower internal water contents that also caused reduction in transpiration rate (Dahal et al., 2019). Our results are also in agreement with our earlier report Demirel et al. (2020). In this study, Novel_105 transgenic Unica and Russet Burbank plants was observed to be less affected by drought stress than their wild types. Unica Novel_105 transgenic plants showed higher stomatal conductance under heat and combined heat and drought stress than its wild type plants. Stomatal conductance in Russet Burbank transgenic and wild type plants was equally affected. Decrease in conductance is directly associated with decreased photosynthetic rate, and under drought stress reduced stomatal conductance resulted in lowered photosynthetic activity.

Chlorophyll contents observed to be higher in Novel_105 transgenic Unica and Russet Burbank plants can be explained by the overexpression of miRNA Novel_105 that might help in restoration of the function of chlorophyll binding proteins (Barozai and Wahid, 2012). It was evident from the comparison of drought and control in Unica wild type plants, chlorophyll index was higher than transgenic Unica plants (Figure 6a). The transgenic Unica plants also performed better than wild plants under heat and combined heat and drought stress in terms of chlorophyll content. A similar pattern was also observed for Russet Burbank transgenic and wild type plants. The transgenic Novel_105 plants of Russet Burbank performed better than transgenic Unica plants. Chlorophyll is necessary for light harvesting and it was negatively affected by heat (Efeoglu and Terzioglu, 2009). Heat stress also causes degradation and restricted chlorophyll molecules in plants (Ashraf and Harris, 2013). Loss in chlorophyll may be due to the chloroplast disfunction and leaf senescence, previous studies also reported similar results in potato (Li et al., 2019; Katsoulas et al., 2016).

The wild type Unica plants exhibited higher leaf temperature under drought and heat stress (Figure 6b). Similar study was devised on potato where only stress tolerant genotypes showed higher leaf temperatures under heat and drought stress (Demirel et al., 2020). This might be due to nonphotochemical quenching of chlorophyll fluorescence and light absorption in the form of heat (Ruban, 2016). Under drought stress, both wild type and transgenic Russet Burbank plants showed higher leaf temperature than those of Unica plants, but under heat and combined heat and drought stress, Unica plants showed higher leaf temperature than Russet Burbank plants. This may be due the effect of stomatal conductance on leaf temperature as reported earlier that showed the lower stomatal conductance increased leaf temperature in potato and vice versa (Urban et al., 2012). Delazari et al. (2018) also reported that potato showed higher leaf temperature under drought stress.

Relative water content decreased both in wild type and Novel_105 transgenic plants under drought stress (Figure 6c). As in the case of other plants under stress conditions, it was reported that stress conditions including heat and drought

stress resulted in reduced RWC in potato (Liu et al., 2005; Asthir, 2015; Handayani and Watanabe, 2020). It is important to note that the Novel_105 transgenic Unica plants showed higher relative water content than Unica wild type plants supporting the beneficial role of Novel_105 overexpression in overall stress tolerance and the transgenic plants of Russet Burbank exhibited least decline in RWC compared to cultivar Unica wild type.

Proline is an important trait as it plays important role in drought stress (Ashraf and Foolad, 2007). Proline accumulation under drought and heat stress increased in Novel_105 transgenic Unica and Russet Burbank plants as compared to wild plants which can be explained by the cultivar differences (Figure 7a). No change was observed for proline accumulation under combined heat and drought stress. The results of this study showed accordance with an observation in Arabidopsis where proline accumulation was seen to be minimized under combined heat and drought stress (Rizhsky et al., 2004). In cultivar Unica wild type proline contents were lower than cultivar Russet Burbank wild type, where similar outcome had also been reported by Jerez et al. (1993) where lower proline contents were estimated in drought resistant potato cultivar. It may suggest that proline accumulation is an abiotic stress adaptation mechanism in resistant plants and has role in normalization of osmotic pressure (Koroban et al., 2016; Naz et al., 2018). Furthermore, in some plants, cells undergo beneficial stress injury by increasing proline content as a response to stress (Mattioli et al., 2009). Proline content in wild type plants was lower as compared to Novel_105 overexpressing transgenic plants, which may suggest that in transgenic plants overexpression indirectly changes the expression of genes associated with proline synthesis which ultimately promote proline accumulation as previously explained by Megha et al. (2018).

In this study overexpression of Novel_105 and suppression of its corresponding putative target gene (E3 ubiquitin-protein ligase *XBAT35*) was noticed in response to all stress treatments in transgenic Unica and Russet Burbank plants (Figure 8), whereas wild type plants failed to accumulate higher transcript levels as expected. Level of mean normalized Novel_105 gene expression was higher in Unica wild type cultivar than Russet Burbank wild type cultivar under drought stress. Contrarily, Unica wild type cultivar had lower mean normalized gene expression level than Russet Burbank wild type cultivar under heat stress, which can be explained as the differential response of tolerant and sensitive cultivars to the applied stresses. The negative role of *XBAT35* had been observed through promoting cell death upon abiotic stresses (Liu et al., 2017), thereby repression of this gene, that is the lower gene expression of *XBAT35,* in posttranscriptional level by overexpression of Novel_105 improved potato growth. The changes in several parameters including gaseous exchange, RWC, leaf temperature, prolin accumulation and MDA between wild type and transgenic Unica and Russet Burbank cultivars, further supported that the decreased expression of *XBAT35* is essential to enhance stress tolerance (Li et al., 2020). Similar results were reported by exploiting miRNA approach to engineer drought tolerant potato (Pieczynski et al., 2013). Different miRNAs had been identified in recent past, which are associated with the regulation of the genes involved in drought tolerance, playing important roles in signaling pathways, heat shock proteins HSPs, thermotolerance, regulation of cell organs development (Pieczynski et al., 2013; Yang et al., 2019). In potato, 4 novel miRNAs (miR811, miR814, miR835 and miR4398) were identified potentially responsible to confer tolerance against abiotic stress (Zhang et al., 2014). This study suggests the role of Novel_105 miRNA in the regulation of its psRobot predicted putative target *XBAT35* gene, which is a major player in ethylene signaling and also involved in regulation of apical hook curvature which also targets E3 ubiquitin ligase (Carvalho et al., 2012).

5. Conclusion

This study pondered that Novel_105 transgenic Unica and Russet Burbank plants had better morphological traits than their wild type plants under abiotic stress conditions. Beside this, improved gaseous exchange traits along with lower leaf temperature, least damage to chlorophyll contents and higher RWC was also noticed in transgenic Novel_105 Unica and Russet Burbank plants compared to their wild types. Biochemical traits such as minimal membrane damage (MDA) and higher proline contents further corroborated that the tolerance mechanism was activated in transgenic plants with the overexpression of Novel_105 miRNA. Its overexpression suppressed the expression of putative target *XBAT35* which is believed to have a role in stress tolerance. These findings will enable plant breeders to develop stress tolerant potato cultivars through exploring the function of Novel_105 in regulating stress response, which ultimately help in development of abiotic stress resistant cultivars. It is important to note that these findings are preliminary and should be further validated in field trials.

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Appendix

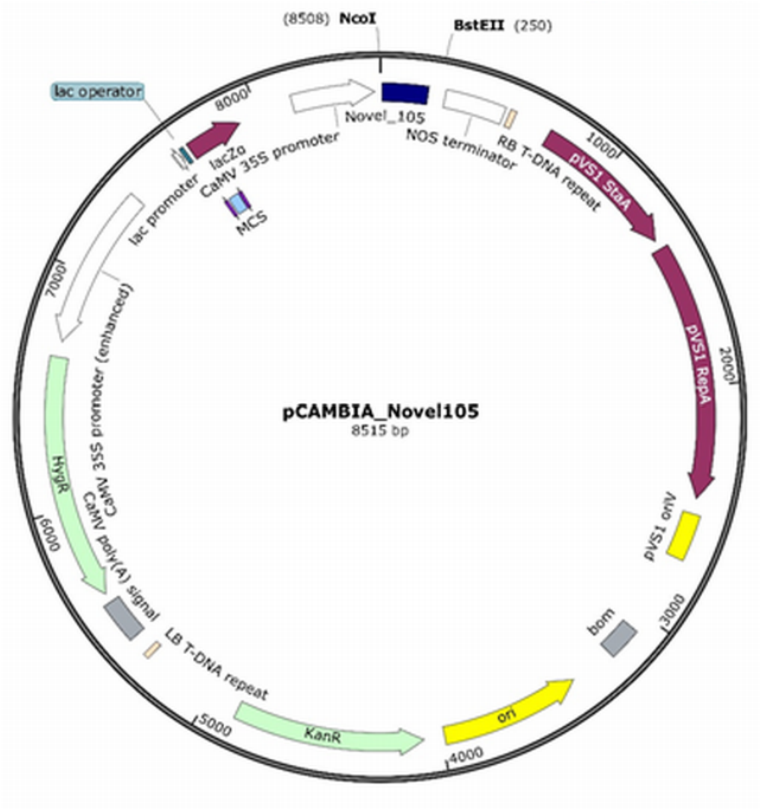

Figure S1. Schematic diagram of pCAMBIA_Novel_105_pre_miRNA vector used in the study. Novel_105 was cloned upstream to NOS poly-A tail terminator with overhangs of *Nco*I and *Bst*EII. Vector contained *Hygromycin phosphotransferase (hptII)* resistance against hygromycin for a plant selectable marker while it also harbored kanamycin for bacterial selection.

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