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ORIGINAL ARTICLE

Combined drought and heat stresses trigger different sets of miRNAs in contrasting potato cultivars

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Abstract

MicroRNAs are small, non-coding RNAs that are responsible for regulation of gene expression during plant growth and development. Although there are many studies on miRNAs in other plants, little work has been done to understand the role of miRNAs in abiotic stress tolerance in potatoes. This study investigates changes in miRNA profiles of two different potato cultivars (tolerant, Unica and susceptible, Russet Burbank) in response to heat, drought and their combination. Transcriptomic studies revealed that miRNA profiles depend on the susceptibility and tolerance of the cultivar and also the stress conditions. Large number of miRNAs were expressed in Unica, whereas Russet Burbank indicated lesser number of changes in miRNA expression. Physiological and transcriptional results clearly supported that Unica cultivar is tolerant to combined drought and heat stress compared to Russet Burbank. Moreover, psRNATarget analysis predicted that major miRNAs identified were targeting genes playing important roles in response to drought and heat stress and their important roles in genetic and post-transcriptional regulation, root development, auxin responses and embryogenesis were also observed. This study focused on eight miRNAs (Novel_8, Novel_9, Novel_105, miR156d-3p, miR160a-5p, miR162a-3p, miR172b-3p and miR398a-5p) and their putative targets where results indicate that they may play a vital role at different post-transcriptional levels against drought and heat stresses. We suggest that miRNA overexpression in plants can lead to increased tolerance against abiotic stresses; furthermore, there should be more emphasis on the studies to investigate the role of miRNAs in combined abiotic stress in plants.

Keywords MicroRNA · Water deficit · High-temperature stress · Combined stress · Gene regulation · sRNA sequencing

Introduction

Potato (*Solanum tuberosum* L.) is an important agricultural crop and ranks fourth among commonly cultivated crops (Djami-Tchatchou and Ntushelo 2017). Its tuber is rich in starch and consumed globally in various ways (Zaheer and Akhtar 2016). Potato is quite important to overcome the challenges of poverty and hunger expected due to adverse effects of global warming and decreasing water availability for agricultural production. However, it is known to be vulnerable to abiotic stresses that ultimately results in poor tuber yield (Hill et al. 2020).

Abiotic stresses include deficient or excessive water, fluctuation in temperature, accumulation of salts and heavy metals in soil. Drought stress (water deficit) is the most common adverse environmental factor due to excessive evaporation, lack of precipitation and deficiency of soil water. High-temperature stress (heat) is another important factor that restricts plant growth, maturation and development, and becoming an important abiotic stress factor to consider due to global warming (Ashraf and Harris 2005). Although individual effects of drought and heat are critical for plant life, combined effects of these stresses are more harmful, and the probability of plants facing concurrent stress conditions in future is getting higher due to climatic changes (Handayani and Wanatabe 2020). Potato is susceptible to drought and heat stresses due to its shallow root system leading to incapability to extract water from deeper soil pockets.

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Tuberization stage is especially critical for stress conditions since it directly affects quality and yield in terms of size and number of tubers (Wagg et al. 2021).

MicroRNAs (miRNAs) are single-stranded, non-coding, endogenously small (20–24 nucleotides) and highly abundant RNAs. They have the ability to form stem-loop structures like hairpins regulating gene expression in post-transcriptional level in physiological and developmental processes such as growth, development, tissue differentiation, signal transduction, hormone secretion and response to environmental stresses, by degrading or repressing target mRNA's translation depending on the complementarity of mRNA and miRNA (Kantar et al. 2010; Kurtoglu et al. 2014; Zhang 2015; Vakilian 2020).

Several miRNAs have been found associated with the gene regulation of single stress-responsive genes in several plants including *Arabidopsis thaliana* (Chen 2005) and *Medicago truncatula* (Wang et al. 2011). However, as the climate change results in not only single stress but a combination of stress conditions during plant development, there is a need to further understand the function of miRNAs and their role in plants against combined stresses. For this purpose, identification of stress-specific or common miRNAs and their target genes to understand regulatory effects in the adaptation of metabolism to stress conditions is required (Devi et al. 2018).

Several studies have been performed on model plants to understand the role of miRNAs, yet there are very limited studies on potatoes aiming to identify miRNAs in response to abiotic stresses and those are mostly relying on in silico identification (Zhang et al. 2014). In addition, to the best of our knowledge, there is no study aiming to identify the change in miRNA profile in response to combined abiotic stress conditions in potatoes. Therefore, here, we selected two potato cultivars with contrasting abiotic stress responses for the identification of conserved or novel miRNAs related with the single or combined abiotic stress treatments. This study, first time in literature, reports novel and conserved miRNAs associated not only to drought or heat stress but also to combined heat and drought stresses in potatoes using sRNA sequencing.

Material and methods

Plant material

Two potato cultivars were selected as plant material for this study. The cultivar Unica (Ramírez et al. 2015; Rolando et al. 2015; Demirel et al. 2020) was reported as tolerant to stress, while cultivar Russet Burbank is known to be sensitive to stress conditions (Stark et al. 2013; Demirel et al. 2017; Demirel et al. 2020).

Plant growth conditions

Potato tubers were planted in 12 L pots containing torf and perlite with a ratio of 2:1 and the pots were irrigated to the soil field capacity. They were grown at 24/16 °C (16 h day/8 h night) in climate-controlled greenhouse during the experiment. Until the stress treatment, irrigation was carried out with 50% diluted Hoagland nutrient solution. Shoot emergence in all plants started at 14 days of planting. The experiment was carried out as a completely randomized design in three replications (five pots per replication).

Stress treatments

Stress treatment started at 40 days after planting at the tuber development stage of both cultivars. Plants were divided into four groups as control (C, control in greenhouse; GC, control in growth chamber), drought stress (D), heat stress (H) and combination of heat and drought stresses (H + D). No stress was applied to the control plants and the plants were allowed to grow under greenhouse with regular irrigation. Drought stress was applied by suspending irrigation water for a total 23 days. All pots were irrigated to soil field capacity before the application of drought and heat stress treatment at 24/16 °C (16 h day/8 h night) in climate-controlled greenhouses and growth chamber. Plants were then shifted to growth chamber for heat and combination of heat and drought stresses; for this purpose, modification of methodology explained by Tang et al. (2018) was used as temperature increased gradually from 24/18 °C to 39/27 °C for 9 days, and then a constant heat of 39/27 °C was applied for 3 days where control plants were kept at 24/18 °C (day/night) of temperature with a 14 h photoperiod and 60–70% relative humidity. Plants were exposed to increased temperature for heat stress with normal irrigation, but for combined stress, water was also withdrawn. Plants were exposed to stress conditions by increasing 1 °C for the first 3 days, 2 °C for the next 4 days until the temperature of the growth chamber reached 38 °C, and finally, plants were kept at 38 °C for 5 days. The treatment was continued for 12 days in total. At the end of the stress period, samples were taken from both control and stress applied plants, frozen in liquid nitrogen and kept at − 80 °C.

Measurement of physiological traits

Gaseous exchange traits

Measurements were taken from uppermost 3rd or 4th leaf with LICOR LI-6400XT portable photosynthesis device having photosynthetically active radiation (PAR) at 1500 µmol m^{-2} s^{-1} and CO_2 concentration was adjusted at 400 µmol mol^{-1}.

Relative water content (RWC)

Leaf samples were harvested and immediately weighed on a precision scale for measuring fresh weight. Leaves were then soaked in pure water at room temperature and kept for overnight to measure turgid weight. Dry weight of the leaf samples was measured by drying in a microwave oven for 10 min at 500 W power, and a complete drying was achieved in oven at 95 °C for 2–3 h. Relative water content was calculated by the equation mentioned below.

$$\mathrm{RWC}(\%) : \left[(\text{Fresh weight} - \text{Dry weight}/(\text{Turgid weight} - \text{Dry weight}) \times 100\right]$$

Proline measurement

Leaf proline content was estimated by following procedure given by Bates et al. (1973). Fresh young potato leaves were collected from each pot in three replicates for each treatment group and directly frozen in liquid nitrogen. Leaf sample (100 mg) was ground in 1 ml of 75% ethanol and left at room temperature overnight for extraction. Centrifugation was done at 14,000 rpm for 20 min and 100 µl of upper phase collected. Supernatant was transferred to a new tube and 900 µl of fresh ninhydrin solution was added (1% ninhydrin, 60% acetic acid, 40% water). The mixture was shaken and kept at 100 °C for 1 h incubation. Reaction was terminated by keeping the samples on ice for 10 min. Three millilitres of toluene was added then, and samples were kept at room temperature for 24 h. The absorbance of resultant red-coloured samples was measured via UV–Vis spectrophotometer (UV-1800 Shimadzu) at 520 nm. Toluene was used as a blank. Proline content is expressed as µmol gFW^{-1}.

Malondialdehyde measurement (MDA)

Leaf samples were collected before termination of stress treatment to calculate the levels of lipid peroxidation by measuring MDA contents following the method of Heath and Packer (1968). The samples (300 mg) were crushed in 0.1% 3 ml (w/v) trichloroacetic acid (TCA) solution and then centrifuged at 10,000 g for 10 min. Upper phase (1.5 ml) was taken and 1.5 ml of 20% (w/v) TCA solution containing 0.5% (w/v) thiobarbituric acid (TBA) was added. Mixture was then incubated at 90 °C in water for 20 min and tubes were kept on ice to stop the reaction. Samples were centrifuged at 10,000 rpm for 5 min, then absorbance was measured at 532 nm and 600 nm. The amount of MDA was calculated using the coefficient value of 155 mM^{-1} cm^{-1}. During absorbance measurement, 20% TCA solution containing 0.5% TBA was used as a blank. MDA content values are expressed as µmol/g fresh weight (FW).

$$\mathrm{MDA}(\mu\mathrm{mol/g\ FW}) = \left[(\mathrm{A532} - \mathrm{A600})/155\right] \times 103\mathrm{x\ dilution\ factor} \times (1/\text{sample weight g})$$

Small RNA library preparation and sequencing

High Pure miRNA Isolation Kit (Rosche, Germany) was used by following manufacturer's instructions to extract small RNA from 100 mg frozen leaf tissues. Extracted RNA was then quantified using NanoDrop-1000 spectrophotometer (Thermo Scientific, USA). RNA samples from the C (control), DS (drought stress), HS (heat stress) and H + D (heat and drought stress) treatment groups were selected for the construction of sRNA libraries. The miRNAs of cultivar/treatment replications were pooled for the formation of small RNA libraries that were later sequenced on an Ilumina HiSeq2500 platform (Novogene, China). cDNA libraries obtained from isolated sRNAs were created by using TruSeq Sample Prep kit (Illumina, San Diego, CA, USA).

Processing and analysis of data

After sequencing, poor quality reads were filtered and Trimmomatic tool was used to trim low-quality terminal bases (Bolger et al. 2014). Adapter sequences were removed by Cutadapt tool (Martin 2011). Additionally, data was processed to eliminate reads that had $sQ \leq 5$ base percentage > 50%, reads containing N > 10%, reads with 5′ primer contaminants, reads without 3′ primer and reads without the insert tag. As small RNA sequencing data also contains reads of other small non-coding RNAs, therefore, read tags were blasted in Rfam database to get rid of matched reads from rRNA, tRNA, snRNA and snoRNA (Kalvari et al. 2018). Reads that were not mapped to Rfam were known repeat sequences such as Repbase by using NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and any tags that were matched with the aforementioned rigorous criteria were excluded for further analysis. The reads that had a length of more than 34 nucleotides were also excluded for analysis. Finally, cleaned putative miRNAs were obtained after filtering length and quality reads, trimming adapter sequences that were used for miRNAs identification.

Mapping of reads to reference potato genome and identification of conserved and novel miRNAs

Reads were mapped to reference potato genome (The Potato Genome Sequencing Consortium 2011). Identification of the precursor sequences was done by tool miRCat. miRNAs that were on stable precursor sequences were reported (Supplementary Fig. 1). For the annotation of novel miRNAs, miRNA prediction software Mireap was used (sourceforge.net/project/mireap/), with the settings of (parameters, a = 19, b = 24, B = 55, d = 200, f = 10, p = 7, s = 100, v = 10, u = 1000 and e = 10 kcal/mol) (Chen et al. 2009). psRNATarget was used for target prediction for all identified miRNAs (Dai et al. 2018).

Differential miRNA expression

After read alignment with potato genome, the expression profiling of the miRNAs was done by read counts. The miRNAs having minimum of 10 reads in the samples were used for differential analysis. For measurement of differential gene expression, DESeq2 (Love et al. 2014) was used. For statistical analysis of the expression of known and novel miRNA in each sample, TPM (Zhou et al. 2010) method was used. To acquire expression of transcripts per million (TPM), miRNA expressions were first normalized. Normalization was done by using the following formula.

$$\text{Normalized expression} = \text{read Count} * 1,000,000)/\text{lib size(lib size : sample miRNA read count).}$$

The TPM values log (base2) were then transformed for the calculation of fold expression. Finally, log2 FC was measure by subtracting values of fold change expression, e.g. if the FC value was more than zero, it was considered upregulated while less than zero was downregulated.

Cluster analysis of miRNA expression difference

Cluster analysis was used to estimate miRNA expression patterns under stress and control conditions. For this purpose, Euclidian distance measure was used. miRNAs were clustered with similar expression patterns to find out unknown function of miRNAs or the function of unknown miRNAs by Heatplus package.

GO enrichment analysis

GO enrichment analysis was performed using the method suggested by (Young et al. 2012, http://www.geneontology.org) by calculating gene numbers for each term and then using Wallenius non-central hyper-geometric distribution to find significantly enriched GO terms in target gene candidates comparing to the reference gene background. Moreover, the Directed Acyclic Graph (DAG) was used to visualize the GO enrichment. Pearson correlation method was used to calculate correlation between treatments.

Validation of results by quantitative real-time PCR (qRT-PCR)

In order to validate the gene expression levels, fresh leaves were collected after stress treatment for total RNA isolation by TRIZOL method. Concentration of the isolated total RNAs was measured using Nanodrop-1000. psRNATarget analysis was used to predict the putative targets of novel and known miRNAs. Stem-loop RT-qPCR method (Chen et al. 2005) was used to determine the gene expression levels of miRNA and a standard qRT-PCR was used for expressional analyses of target genes under stress condition via Qiagen, Rotor-Gene Q. cDNA specific to miRNA from total RNA was synthesized with SL-RT coded primers and then the change in gene expression was investigated by using miRNA-specific primers without stem-loop (Supplementary Table 1). Ef1α (elongation factor 1-α) was used as the reference gene. Expression levels of the genes were calculated by using $2^{-\Delta\Delta Ct}$ proportional calculation method described by Livak and Schmittgen (2001).

Results

Physiological and biochemical parameters

Physiological traits were measured to determine the effect of single and combined stresses on sensitive (Russet Burbank) and tolerant (Unica) potato cultivars. Each observed trait was downgraded with the treatment of single or combined stress conditions, but they were particularly decreased in the sensitive cultivar. Severe stress conditions resulted in significant decrease in gaseous exchange traits of both cultivars with exposure to individual and combined stresses (Fig. 1). Leaf relative water decreased in both cultivars, but the decrease was more pronounced in cultivar Russet Burbank (Fig. 1d). Proline quantification showed higher accumulation in case of only drought stress in both cultivars, while minimal increase was noticed from

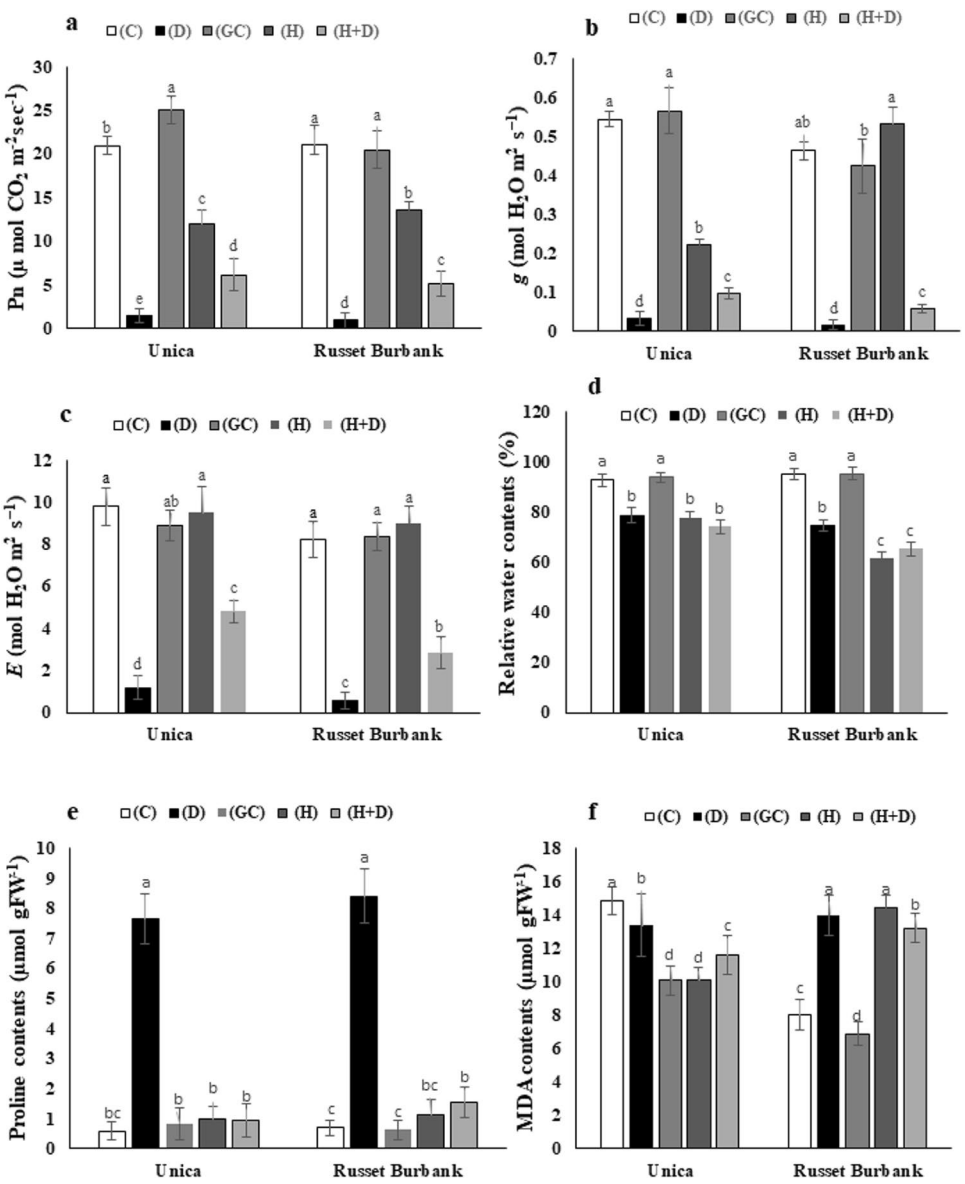

Fig. 1 Effect of stresses on physio-biochemical traits of potato cultivars (Unica and Russet Burbank). C, control; D, drought stress; GC, growth chamber control; H, heat stress; H + D, heat and drought stresses. **a** Photosynthetic rate, **b** stomatal conductance, **c** transpiration rate, **d** relative water contents, **e** proline contents, **f** malondialdehyde contents. Vertical bars represent standard deviation. Letters sharing the same alphabets show non-significant ($P \geq 0.05$) difference, while different letter shows significant ($P \leq 0.05$) difference

remaining stress treatments (Fig. 1e). Malondialdehyde showed higher membrane damage in sensitive cultivar. Higher MDA contents were determined from all stress groups of Russet Burbank cultivar, while Unica showed higher level of MDA contents in plants only under combined heat and drought stresses (Fig. 1f). Physio-biochemical data revealed that Unica cultivar was more tolerant to stress while the Russet Burbank showed sensitivity to stress conditions.

Identification and sequencing of miRNAs

Initially, 447 conserved miRNAs and a total of 315 novel miRNAs were identified from 10 libraries based on both similarity and structural analysis with the next-generation sequencing study. The list of miRNAs detected in this study is given in Supplementary Table 2. The length distribution of miRNA sequences was between 18 and 24 nucleotides. Sequencing results showed varying numbers of reads in each library. In RHD (Russet Burbank heat and drought stresses), the highest number of reads (> 11 million) was observed, whereas in RBH (Russet Burbank heat stress), the lowest number of reads was observed. Data of the total and specific miRNA distributions in the libraries is given in Table 1.

Venn diagrams showing the common reading numbers for specific miRNAs are shown in Fig. 2. The overlapping regions showed the number of miRNAs that were expressed in two or more groups. Multiple comparisons between UNH (Unica heat stress) and UCX (Unica heat and heat and drought stress control) showed that 151 miRNAs were expressed. A total of 156 miRNAs were expressed among RHD in comparison to RCX (Russet

Table 1 Distribution of total and unique reads in miRNA libraries according to next-generation sequencing results

Library	Sample	Total reads	Total bases (bp)	Unique reads	Unique bases (bp)
UNC	UNC	9,991,670	225,323,988	2,972,373	68,764,180
UND	UND	7,222,124	164,189,928	2,106,019	49,065,329
RBC	RBC	4,553,069	105,956,063	1,292,091	30,551,302
RDR	RDR	7,323,990	167,133,452	1,709,192	40,032,783
UCX	UCX	6,396,309	145,267,589	1,941,348	45,193,220
UNH	UNH	6,619,951	148,470,925	1,778,221	41,273,330
UHD	UHD	9,611,232	218,645,115	2,808,248	65,653,718
RCX	RCX	5,954,384	137,814,400	1,444,079	33,993,974
RBH	RBH	2,056,732	46,945,663	784,112	18,322,772
RHD	RHD	11,433,322	257,998,552	2,980,577	69,363,782

RDR Russet Burbank drought stress, *RBC* Russet Burbank drought stress control, *RBH* Russet Burbank heat stress, *RHD* Russet Burbank heat and drought stresses, *RCX* Russet Burbank heat and heat and drought stress control, *UND* Unica drought stress, *UNC* Unica drought stress control, *UNH* Unica heat stress, *UHD* Unica heat and drought stresses, *UCX* Unica heat and heat and drought stress control

Burbank heat and heat and drought stress control). Among UNH and UCX, 151 miRNAs were expressed in response to heat. A total of 145 miRNAs were expressed specifically among UHD (Unica heat and drought stresses) and UCX (Fig. 2a). Comparison among UND (Unica drought stress) and UNC (Unica drought stress control) revealed that a total of 111 miRNAs were expressed where only 22 miRNAs were specifically expressed in response to drought. In RDR (Russet Burbank drought stress) and RBC (Russet Burbank drought stress control), a total of 119 miRNAs showed differential expression. One hundred and forty-nine miRNAs were regulated in RBH compared to RCX, and 12 miRNAs were individually specific (Fig. 2b). Multiple comparisons between RHD and RCX showed that 156 miRNAs showed differential expression, where 22 were specific to heat and drought combined stress. Multiple comparisons between UND and UNC showed that a total of 111 miRNAs were differentially expressed, where 16 miRNAs were specific to drought. Among RDR and RBC, 119 miRNAs showed differential expression, and 17 were only specific for drought. Comparison between RHD and RCX depicted that 31 miRNAs were specific to these treatment groups (Fig. 2c). Details of miRNAs commonly expressed in comparisons of two stress groups are given in Supplementary Table 3.

Target gene prediction and ontology analysis

The regulation of targeted genes was observed from the 14 selected miRNAs which were involved in specific or common regulation against drought and heat stress. The list of targeted genes predicted by computational software psRNA-Target is given in Supplementary Table 4. According to the results (Table 2), predicted target genes took part in different molecular functions, cellular components and biological processes (Fig. 3). RHD and RCX analysis showed that 200 genes were involved in adenyl nucleotide binding, purine nucleotide binding, ribonucleotide binding, nucleotide binding, carbohydrate derivative binding and purine ribonucleotide binding. Around 137 genes were involved in ATP binding; moreover, same number of genes were involved in regulation of cellular and biological processes. Nitrogen compound metabolic process was controlled by 206 genes in Russet Burbank under drought (Fig. 3a). Gene ontology analysis in cultivar Russet Burbank under heat and its control treatment revealed that 213 genes were associated with small molecule binding, anion binding, nucleotide binding and nucleotide phosphate binding (Fig. 3b). Gene annotation results for Unica heat and drought stresses in comparison to its control showed that 156 genes were controlling cellular and biological regulation, whereas 237 genes were involved in anion binding, nucleotide binding and carbohydrate derivative binding. Moreover, maximum number of genes

Table 2 psRNATarget predicted targets of selected responsive miRNAs and previously reported targets in Arabidopsis

miRNA	Predicted target gene in potato	Target length	Regulation
Novel_105	PGSC0003DMT400044357 Zinc finger family protein, MADS-box transcription factor	181	Reverse in drought, reverse in heat & drought
Novel_16	PGSC0003DMT400064010 Zinc ion binding/DNA binding protein/ATP binding	166	Downregulated under all stress
Novel_2	PGSC0003DMT400049895 DNA binding protein	172	Upregulated under all stress
Novel_8	PGSC0003DMT400052092 Mitochondrial transcription termination factor family protein	166	Downregulated under all stress
Novel_9	PGSC0003DMT400081486 Disease resistance protein Gpa2/NBS-LRR resistance protein	179	Upregulated in cultivar Unica & downregulated in cultivar Russet Burbank
stu-miR156a	PGSC0003DMT400029751 Promoter-binding protein SPL9	176	Highly expressed in Russet Burbank under drought stress
stu-miR156d-3p	PGSC0003DMT400001292 Phospholipid-transporting ATPase	188	Highly expressed in Russet Burbank under drought stress
stu-miR160a-5p	PGSC0003DMT400045323 Auxin response factor ARF16	182	Downregulated especially in response to combined stress
stu-miR162a-3p	PGSC0003DMT400029301 Endoribonuclease Dicer homolog 1	211	Downregulated in drought, upregulated in heat and combined stress
stu-miR166a-3p	PGSC0003DMT400054421 PHAVOLUTA-like HD-ZIPIII protein	218	Differential response in both cultivars
stu-miR171a-3p	PGSC0003DMT400066799 GRAS family transcription factor	203	Downregulated specifically in combined stress and Russet Burbank
stu-miR172b-3p	PGSC0003DMT400065313 AP2 transcription factor SlAP2d	150	Highly expressed under drought stress in cultivar Russet Burbank
stu-miR390-3p	PGSC0003DMT400058521 PGR5 1B, chloroplastic	227	Upregulated under all stress treatments
stu-miR398a-5p	PGSC0003DMT400018554 L-ascorbate oxidase	216	Upregulated in response to all stress treatments, especially in combined stress

(316) were associated with ionic binding and organic cycle compound bindings (Fig. 3c). Annotation results in cultivar Unica under heat and drought revealed that more than 200 genes were involved in organic cycle compound metabolic process and heterocycle metabolic process. Additionally, there were genes controlling adenyl nucleotide binding, purine nucleotide binding, purine ribonucleotide binding and carbohydrate derivative binding. More than 340 genes were associated with organic cyclic compound binding and heterocyclic compound binding (Fig. 3d).

TPM distribution

The overall TPM cluster analysis result, clustered by log10(TPM + 1) value, where variation in colour represents the expression of 10 libraries according to log10(TPM + 1) value from lower to higher. The x-axis represents the experimental conditions among libraries and the y-axis represents their relative expression value (Supplementary Fig. 2). As a result of sRNA next-generation sequencing, the distribution of the original miRNA sequences on the basis of the library shows the distribution in terms of TPM (transcripts per kilobase million). In this study, RHD, UHD and UCX groups showed highest peaks of TPM density distribution (Supplementary Fig. 3). As an increase in density distribution represents an increase in gene expression, it suggests that highest number of miRNAs upregulated under drought stress in both cultivars. Three novel miRNAs (Novel_1, Novel_10 and Novel_105) showed higher read counts in normalization procedure as compared to other miRNAs. The highest read count was observed for Novel_1 as 20,864 reads were measured in cultivar Russet Burbank under heat and drought stresses. The highest number of reads for Novel_105 was also observed in heat and drought treatment as 9416 reads.

Pearson correlation

It is known that the square of the Pearson correlation coefficient should be larger than 0.92, under ideal experimental conditions. According to the TPM values of all detected

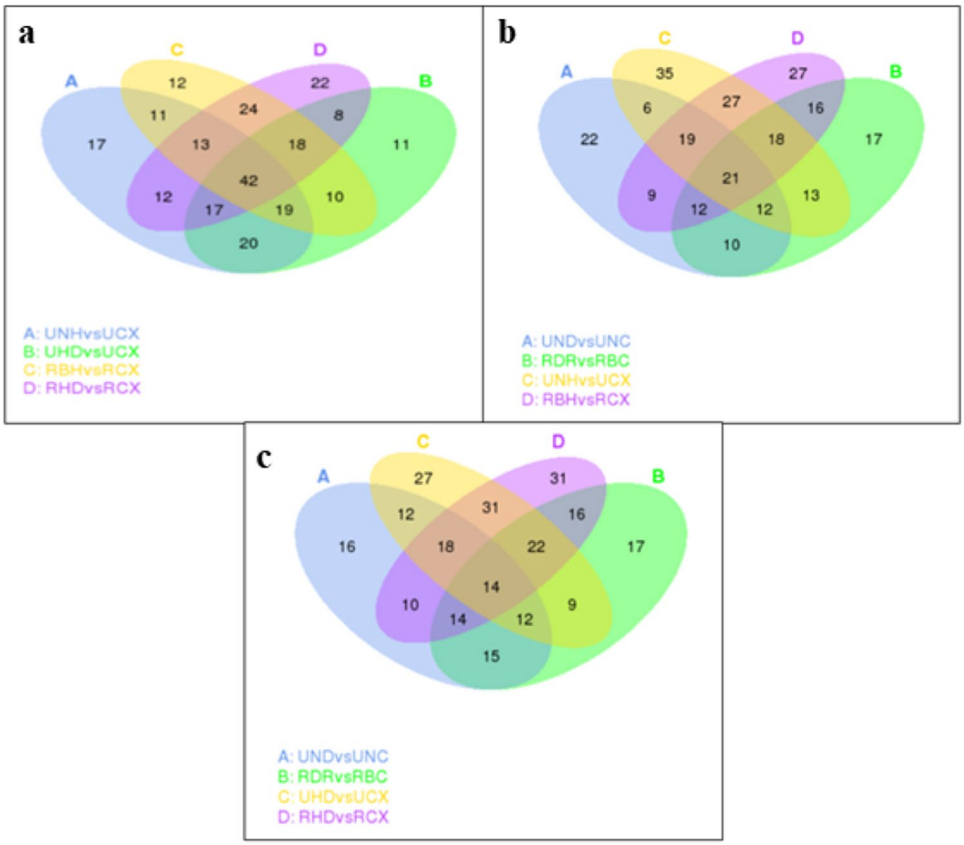

Fig. 2 Venn diagrams of miRNAs created by multiple comparisons. Number of miRNAs expressed in different stress groups, where overlapping regions show those miRNAs which were expressed in more than one stress group: RDR, Russet Burbank drought stress; RBC, Russet Burbank drought stress control; RBH, Russet Burbank heat stress; RHD, Russet Burbank heat and drought stresses; RCX, Russet Burbank heat and heat and drought stress control; UND, Unica drought stress; UNC, Unica drought stress control; UNH, Unica heat stress; UHD, Unica heat and drought stresses; UCX, Unica heat and heat and drought stress control

miRNAs, Pearson's correlation values were calculated for pairwise comparisons among the 10 libraries. The values were > 0.80 among three library comparisons (UNH-UHD, UND-UNC and UNC-UCX) as compared to other treatments (Supplementary Fig. 4).

Cluster analysis

Hierarchal clustering showed differential regulation of miRNAs in 10 different libraries (Supplementary Fig. 5). In the clustering, different area with different colours shows varying expression, i.e. red colour shows higher miRNA expression whereas blue colour represents lower miRNA expression. Results revealed a clear distinctive expression of all miRNAs in both cultivars in response to varying stress conditions. miRNAs in UND, UNH, UHD and RHD groups showed highest expression as compared to other libraries under investigation.

Differential gene expression

sRNA sequencing results were analysed to measure the change in expressional levels of selected miRNAs. Sequencing results revealed that under heat and heat and drought stresses, expression of 6 miRNAs, including Novel_2 and stu-miR398a-5p, downregulated, whereas Novel_16 and Novel_8 showed upregulated expression in both potato cultivars under study. However, in the case of drought stress, 4 miRNAs were downregulated, which includes Novel_105, stu-miR171a-3p and stu-miR172b-3p, while stu-miR156d-3p showed upregulation in both cultivars (Fig. 4).

Validation of miRNA target gene expression

Confirmation of the predicted target genes expression was done by qRT-PCR. The target genes (Table 2) DNA binding protein, PGR5 1B-chloroplastic and Photosystem II core complex proteins psbY-chloroplast for Novel_2, stu-miR390-3p and stu-miR398a-5pq, respectively, were upregulated, whereas zinc ion/DNA binding and mitochondrial transcription termination factor family protein for Novel_16 and Novel_8 were downregulated in both cultivars under all stress conditions (Supplementary Fig. 2). The other target genes showed differential gene expression including Zinc finger family protein, NBS-LRR resistance protein, Phospholipid transporting ATPase, PHAVOLUTA-like HD-ZIPIII protein and AP2 transcription factor SlAP2d for Novel_105, Novel_9 and stu-miR172b-3p, respectively, were upregulated in Unica under all stress treatments, whereas Novel_9 and miR160 were downregulated in cultivar Russet Burbank

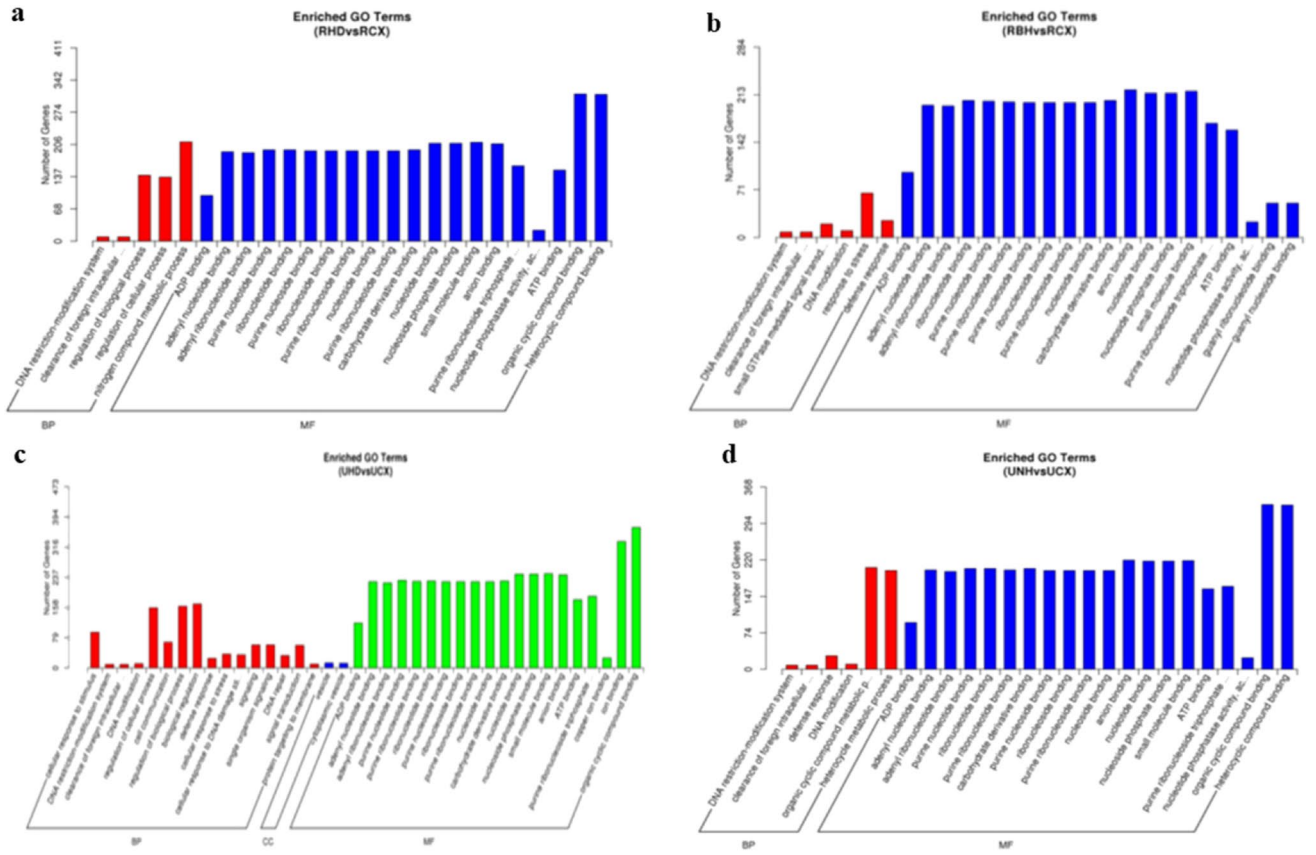

Fig. 3 Gene ontology enrichment analysis of the predicted target genes of differently expressed miRNAs. **a** RHD (Russet Burbank heat and drought stresses) vs RCX (Russet Burbank heat and heat and drought stress control); **b** RBH (Russet Burbank heat stress) vs RCX (Russet Burbank heat and heat and drought stress control); **c** UHD (Unica heat and drought stresses) vs UCX (Unica heat and heat and drought stress control); **d** UNH (Unica heat stress) vs UCX (Unica heat and heat and drought stress control); BP, biological process; CC, cellular components; MF, molecular function

under all given treatments (Fig. 5). The gene expression levels of miRNAs as revealed by sRNA sequencing results with their predicted target genes showed antagonistic changes in qRT-PCR analysis.

Discussion

Abiotic stresses are the major limiting factors which drastically limit potato growth and lead to lower crop yield. Several studies have been performed to unravel potato response to individual stress conditions. However limited studies have explored its behaviour against combined stress conditions (Demirel et al. 2020; Handayani and Watanabe 2020).

Physiological response of the cultivar Russet Burbank confirmed its susceptibility to the applied stresses, while the behaviour of cultivar Unica suggested that it was more tolerant in comparison as reported by earlier studies (Rolando et al. 2015; Demirel et al. 2020). Specifically, the cultivar Russet Burbank showed decreased gaseous exchange trait (stomatal conductance rate, photosynthetic and transpiration rate), reduction in RWC as compared to the cultivar Unica. Physiological results of this study are in accordance with the findings of our earlier report in potato (Demirel et al. 2020).

Plant adaptation to individual or combined stress conditions is related with the regulation of the expression of genes that maintains physiological and metabolic changes. The first physiological response of plant is the closure of stomata to restrict water loss which also results in reduced intake of CO_2. The cultivar Russet Burbank showed reduction in stomatal conductance under drought and heat and drought stress conditions, while it showed higher stomatal conductance rate under heat stress alone as compared to the cultivar Unica. Disrupted gaseous exchange traits due to the imposed stress were evident that is also endorsed by Romero et al. (2017).

Relative water content is widely used for evaluation of stress impact on plant, as it is an indicator of stress tolerance (Gürel et al. 2016). The tolerant cultivar Unica showed higher RWC, while the cultivar Russet Burbank showed

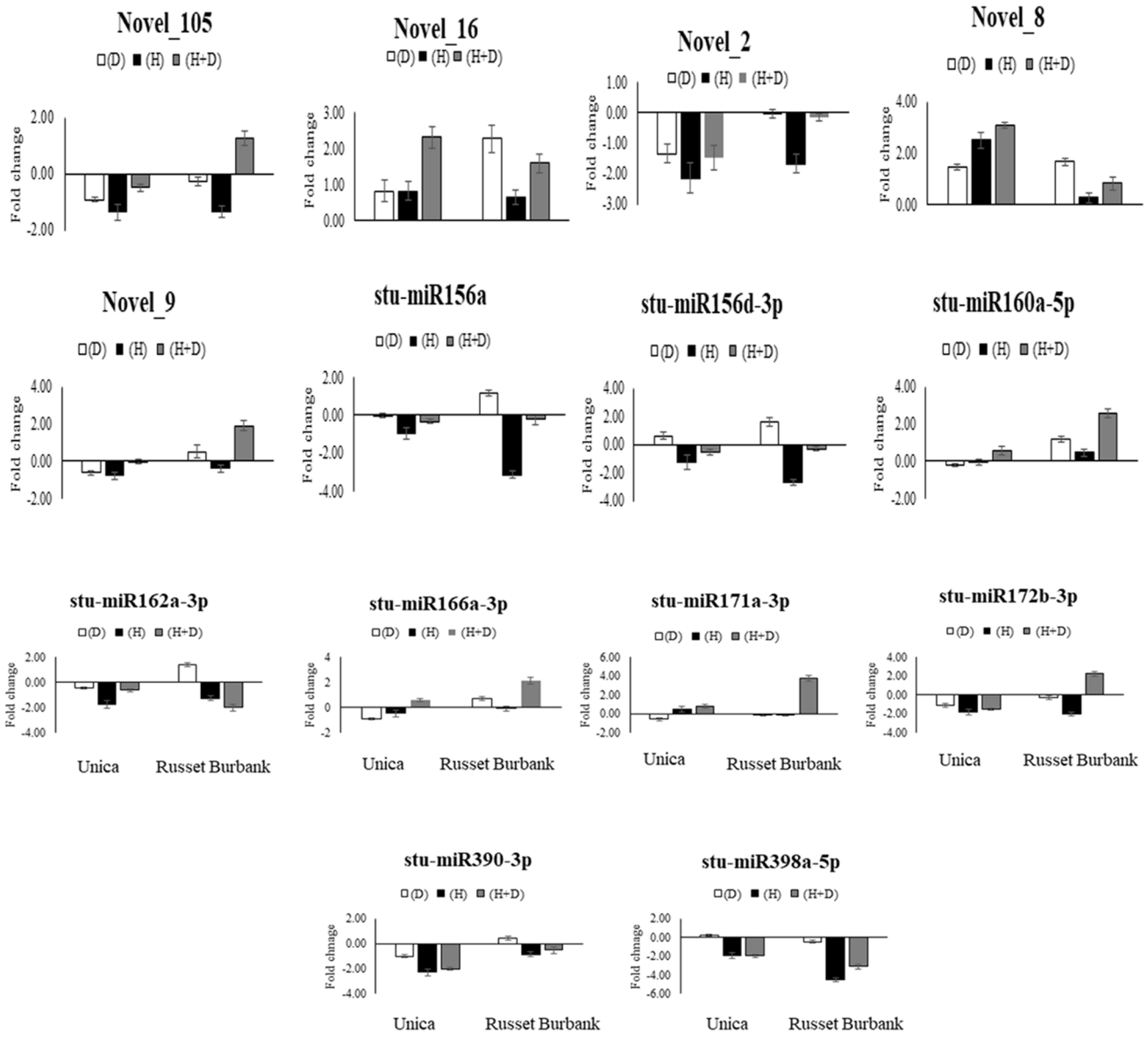

Fig. 4 Fold change in miRNA gene expression according to sequence analysis results in Unica and Russet Burbank cultivars under D (drought stress), H (heat stress) and H + D (heat and drought stress conditions)

minimal RWC in response to stress conditions. Similar results regarding decreased RWC were reported in potatoes under drought stress condition (Banik et al. 2016). The lower RWC in cultivar Russet Burbank depicted its susceptibility to stress which might be due to overproduction of reactive oxygen species (ROS) and triggered oxidative stress (Aksoy et al. 2015). Biochemical response of potatoes with the exposure to stress showed differences in both cultivars. MDA accumulates at higher levels in sensitive cultivar by the higher influence of oxidative stress (Zhang et al. 2018). Thereby, higher MDA contents in cultivar Russet Burbank were measured which indicated higher membrane damage as compared to Unica cultivar. It might be due to weak antioxidant enzymatic activities which favoured higher accumulation of ROS resulting in elevated lipid peroxidation in sensitive cultivar (Batool et al. 2020).

Proline is the main osmo-regulator for cellular homeostasis especially in response to drought stress. Therefore, in our study, higher levels of proline were observed only under drought stress as compared to other applied stresses (Fig. 1e). Our results regarding higher proline accumulation under drought stress were further corroborated by the findings of Batool et al. (2020) and Demirel et al. (2020).

Plant retaliates to abiotic stresses in different ways through different gene regulation responses. Despite miRNAs have become a subject of intensive studies on

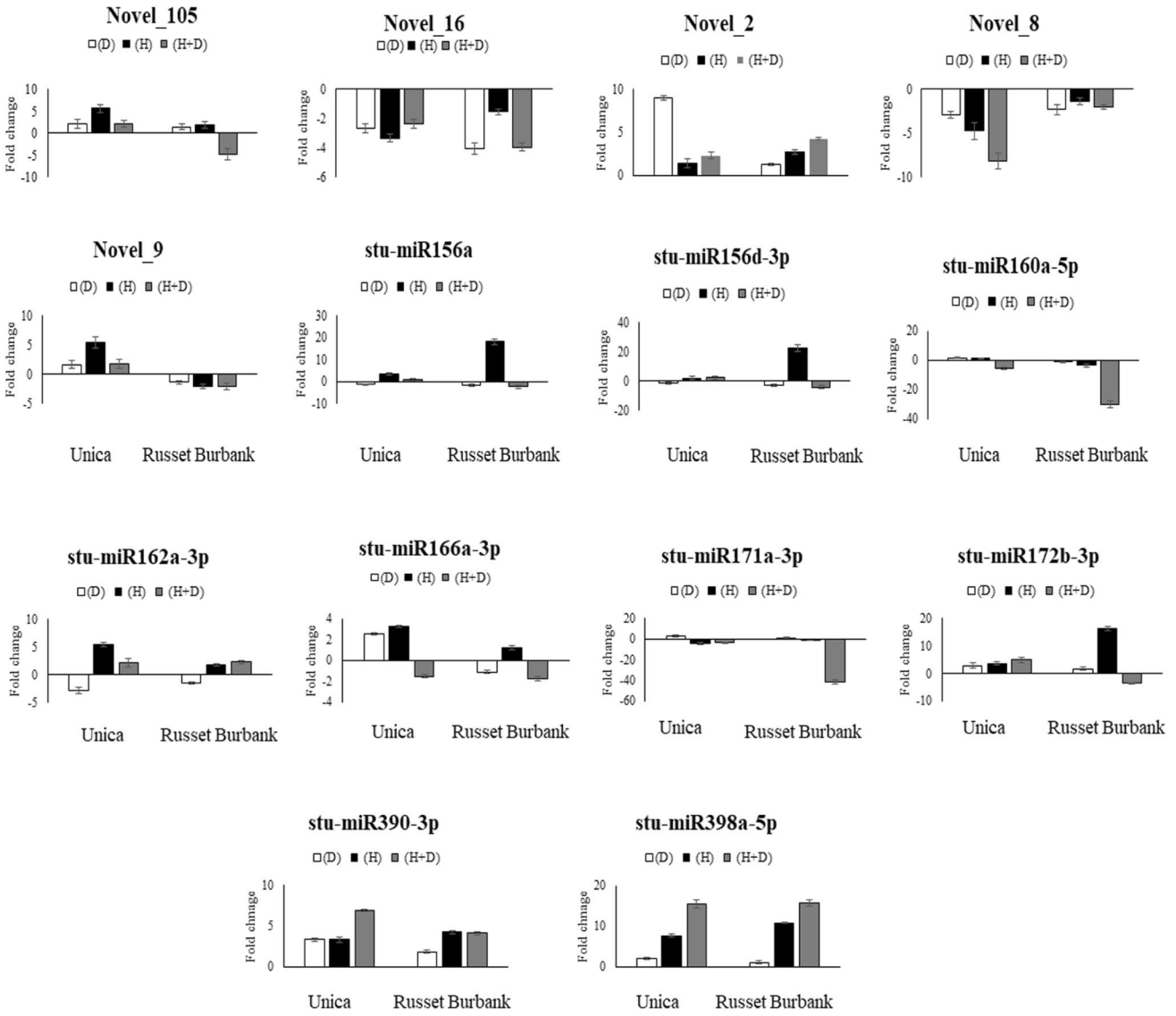

Fig. 5 Fold change in gene expression according to target gene-specific qRT-PCR result in Unica and Russet Burbank cultivars under D (drought stress), H (heat stress) and (H + D heat and drought stress conditions)

increasing stress tolerance in plants recently, there are only a few computationally detected potato miRNAs (Dryanova et al. 2008) and in most cases without experimental confirmation. In this study, we aimed both to identify conserved potato miRNAs associated with abiotic stress tolerance to better understand the function of plant miRNAs in stress adaptation and to detect differences and similarities in miRNA profiles obtained in contrasting cultivars in response to single or combined abiotic stress conditions. miRNAs play a key role in post-transcriptional regulation, and in order to completely understand gene expressional regulation, deep characterization of miRNAs is important (Jones-Rhoades et al. 2006). Identification of miRNAs is often a difficult procedure as plants have large RNA pools. An efficient way to understand the role of miRNAs in gene regulation is to estimate their target genes (Marmisolle et al. 2020). Bioinformatics studies in plants are fruitful under many circumstances to find target genes of major miRNAs (Rhoades et al. 2002).

stu-miR160a-5p was found to be conserved in this study that is associated with ARF-16 (auxin response factor) regulation which is known to be responsible for drought response in potatoes. In cultivar Russet Burbank, ARF-16 decreased significantly which depicted its susceptibility to applied stresses. In an earlier study, miR167 was linked with the downregulation of ARF-16 in rice (Liu et al. 2009). Literature suggests that miR160a also targets other ARF factors, including ARF- 10 and -17. The ARF-16 also plays a key role in basal embryonic regions, root caps, leaves and vascular tissues (Rhoades et al. 2002; Wang et al. 2005). In

drought stress, it was reported to be upregulated in maize, wheat and Arabidopsis (Liu et al. 2016). Moreover, in another study, miR167d in drought conditions was downregulated in maize and inhibited stress response by enhancing phospholipase (Wei et al. 2009).

The other miRNA identified in our study Novel_105 was targeting Zinc finger family protein and MADS-box transcription factor according to in silico prediction. In cultivar Unica, Novel_105 downregulated which means that its target genes upregulated. In cultivar Russet Burbank, Novel_105 downregulated in heat and drought combined stress. This suggests that Novel_105 downregulation is important for drought stress regulation in potatoes. Recent studies also suggest that miRNA regulation is associated with zinc finger family proteins that are involved in stress tolerance to abiotic stresses. Moreover, they are also involved in senescence regulation (Kong et al. 2006; Pomeranz et al. 2010). Novel_105 was also found to be linked with MADS-box transcription factor which is induced under various stress conditions. It was suggested that downregulation of MADS-box gene in pepper caused susceptibility to abiotic stresses in plants (Chen et al. 2019). Moreover, under abiotic stress, MADS-box genes were expressed in roots of Arabidopsis; this might show their influence in abiotic stress (Rounsley et al. 1995). In short, MADS-box genes are key components of genetic regulatory networks involved in abiotic stress and plastic developmental responses in plant (Castelán-Muñoz et al. 2019).

The expression of Novel_8 was observed to be upregulated in both cultivars. However, upregulation of Novel_8 was much higher in the cultivar Unica with the exposure to heat and heat and drought stresses. Contrarily, in cultivar Russet Burbank, upregulation was minimal under heat and heat and drought stress treatments. Target gene for Novel_8 was in silico predicted in this study as mitochondrial transcription termination factor family protein. This protein family takes part in post-transcriptional regulation. It also responses to environmental factors and helps plant to adapt to environmental changes; furthermore, it also connects to regulate gene expression in mitochondria and chloroplast (Quesada 2016). The difference observed between potato cultivars used in this study, therefore, can also be explained with the differential regulation of Novel_8 observed.

The target of miRNA166 was identified as PHAVOLUTA-like HD-ZIPIII protein, a member of HD-Zip family which is known to play an important role in plant growth and against abiotic stresses (Li et al. 2020). In cultivar Unica, downregulation of miRNA166 was seen under heat and drought condition, whereas a slight upregulation was seen under combined heat and drought stress combination. In cultivar Russet Burbank, this miRNA showed significant upregulation in response to drought and heat and drought stresses. This suggested that Unica cultivar accumulated higher HD-ZIP proteins under heat and drought stress. This might be related with higher tolerance of Unica to heat and drought as compared to Russet Burbank. Members of this protein family are involved in organ growth regulation, de-etiolation and blue light signalling. Recent studies showed that HD-ZIP III proteins also controlled embryogenesis, apical meristem development, morpho-physiological changes in roots, leaf polarity and vascular bundle development (Robischon et al. 2011; Baima et al. 2001; Kim et al. 2005). This protein family also collaborates with other proteins for several other cellular functions and root development (Hawker and Bowman 2004; Landau et al. 2015).

Among all the identified novel and conserved miRNAs, it was noticed that in cultivar Unica, Novel_16 and Novel_8 showed upregulation in both cultivars under all imposed stresses, whereas stu-miR156d-3p showed upregulation in only drought stress in both cultivars. It targets phospholipid transporting ATPases. These phospholipid flippases are important regulators of transbilayer lipid asymmetry in eukaryotic cell membranes and play key roles in many signalling pathways. P4-ATPases, especially, are responsible for the uphill transport of phospholipids to the cytosolic leaflet of the plasma membrane, as well as membranes of the late secretory/endocytic pathways construct transbilayer asymmetry (Montigny et al. 2016).

L-ascorbate oxidase is an enzyme that is important in oxidation process and it plays a key role in plant in response to abiotic stress (Akram et al. 2017). Downregulation of miR398a was observed under heat and combined stress conditions. However, in the case of cultivar Unica, a slight overexpression of this miRNA under drought stress was noticed. According to earlier reports, this enzyme is involved in cellular environmental signalling, hormonal functioning and stress tolerance supporting the findings of our study (Stevens et al. 2017).

In summary, increasing abiotic stress tolerance of agriculturally important plants is an ongoing debate in the hearth of climate change. It is obvious that the changes in environmental conditions lead plants to face the problems of combined stresses but not single ones as most of the studies are based on. Keeping in mind that miRNAs are a very good targets to change the tolerance level of plants in response to changes in their environment, in this study, we investigated the changes in miRNA profiles of two contrasting potato cultivars in response to single or combined heat and drought stresses. Results indicated that as in the case of comparative transcriptomic studies, miRNA profiles also depend both on the tolerance or susceptibility of the cultivar and stress conditions and showed common or specific regulation of miRNA expression. Higher number of miRNAs were expressed in cultivar Unica as compared to cultivar Russet Burbank, which might indicate the

regulatory role of expressional changes in miRNA in tolerance to abiotic stresses. It is important to note that most of the identified miRNAs were related with components of genetic regulatory networks, post-transcriptional regulation, root development, auxin response and embryogenesis and found to be targeting important genes related with heat and drought tolerance. Our findings recommended that studies should more focus on miRNA profile comparisons of contrasting cultivars of several plant specific to create more general background in order to find key factors for abiotic stress tolerance. This study only focused on eight miRNAs (Novel_8, Novel_9, Novel_105, miR156d-3p, miR160a-5p, miR162a-3p, miR172b-3p and miR398a-5p) and results indicated and supported the possible use of those miRNAs in increasing tolerance levels of sensitive plant species which requires further studies by transgenic approaches.

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Author contribution ZNOG and UD designed and performed the experiment. SÇ and MEÇ provided potato cultivars and contributed to the design and follow-up of the experiment. ZNOG, UD, EA and AB analysed the data obtained. ZNOG wrote the manuscript.

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Data availability The datasets supporting the conclusions of this article are included within the article as Supplementary Material.

Declarations

Conflict of interest The authors declare no competing interests.

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