Physiological and molecular changes to short and prolonged heat in highlands China potato genotype

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Abstract

**Background:** Potato is the fourth world’s most important crops. Global warming has heavily constrained potato production. Although some work has been undertaken regarding the response of potato to moderately increased temperature (25-30°C), few studies have examined the extreme high temperature above 35°C and sustaining high temperature impact on physiological, biochemical and molecular responses of potato.

**Methods:** Potato plants were subjected to high temperature (35°C/33 °C day/night) treatments for 6 h (short) and 3 days (long), physiological and biochemical response of electrolyte leakage and photosynthetic performance were measured, transcriptome and metabolome profiles of leaves were examined. Expression profiles of 20 DEGs were verified by RT-qPCR, heat induced conserved genes were transient expressed in *Nicotiana benthamiana*.

**Results:** Growth at short heat stress induced stomata open and lower membrane stability. Prolonged heat stress decreased the photosynthetic parameters and increased photosynthetic pigments. Integration of transcriptomics and metabolomics methods demonstrated that 448 heat upregulated and 918 heat downregulated genes as well as 325 and 219 compounds in the positive and negative ionization modes, respectively, that were up- or down-regulated in leaves detected in responsive to short and prolonged heat stress. Global transcripts changes were mainly induced by short heat stress, where metabolites changes were mainly activated by prolonged heat stress. General responses to heat stress in gene expression and metabolite accumulation enriched in amino acid metabolism and secondary metabolism pathway. Metabolite and transcript abundances for the up-
regulation of flavone and flavonol biosynthesis under the prolonged heat stress were closely correlated. Both conserved and heat- and potato-specific stress responsive genes were identified by comparing heat and drought stress in potato as well as heat stress in potato and Arabidopsis shoots, transient expression of four heat induced genes in *Nicotiana benthamiana* exhibited heat tolerance to higher temperature.

**Conclusions:** A new potato leaf transcriptomes and metabolomes revealed a widely adaptive response to high temperature by mainly generation and accumulation of heat shock proteins.

**Background**

Global warming has been significantly affecting on the growth and yield of crops, which will be a major threaten to agricultural food production and to food security [1]. It has been often noted that the extreme annual daily maximum temperature is predicted to increase by about 1 to 3ºC by the mid twenty-first century [2]. Temperatures above the normal optimum are perceived as heat stress (HS) by all living organisms. HS has an impact on development of different crop species [3]. Heat stress disrupts cellular homeostasis and leads to severe changes in structure and metabolic function as well as physiological process of plant [4]. Notably, heat stress is often accompanied by drought stress or other stresses that can cause extensive agricultural losses [5]. Therefore, it is urgent to understand the molecular thermos tolerance mechanisms to breed and select of heat tolerant plant lines than ever before.

Considering the importance of effect of global warming on crops yield, the research topic on thermal stress in potato is currently focused. Potato, one of the major
staple crops in the world, is a cool-season and highly heat-sensitive crop with an optimal growth temperature of approximately 20–25°C and a tuber formation temperature of 15–25°C [6]. The potato is the fourth most widely planted crops and is used as a major food crop in China, where most of potato is grown in summer, however, the seasonal extreme high temperature in main potato producing area in the arid zones in north China have negative effect on potato growth and subsequent potato production. High temperature stress is considered as a main environmental factor affecting growth, tuber yield and quality of potato [7]. The tuberization signal is inhibited by heat exposure [8], StSP6A, a tuberization signal, was shown to be decreased expression when temperature increased. The capacity of photosynthesis was decreased because the inhibition of CO₂ fixation and chlorophyll loss [9], carbon transport is also sensitive to high temperature which results in starch accumulation decreased [10], elevated temperature also have negative impacts on potato skin finish [11]. Additionally, high temperatures during tuber maturation have a negative effect on tuber dormancy and can cause the potatoes to start sprouting [12]. Due to the global climate warming resulting in temperature rising, it is strongly necessary to raise heat-tolerant potato varieties to cope with elevated temperature that sustain high yields under these conditions [7].

To assess the effect of heat stress on potato growth performance, several research groups have identified differential expression patterns of genes in response to heat in different tissues using microarray analysis, results from Rensink et al [13] identified 416 heat specific response genes using cDNA microarray from potato root and leaf, 95 thermo-tolerance genes in potato were identified from cDNA libraries using a yeast-based functional screening method [14]. A subset of expressed genes were identified in the periderm exposed to high soil temperatures (33°C) using a
potato cDNA array [15], the elevated temperature had a significant effect on the gene expression of leaf and tuber with large amounts of transcripts exhibiting a rhythmic oscillation at higher temperature [8], results from Hastilestari [16] showed that 671 differentially expressed transcripts were induced by moderately increased temperature. The differentially expressed genes identified mainly involved in many biological process and molecular function as well as metabolite accumulation. Although above-mentioned studies have examined heat stress responses in potato, they have primarily focused on responses to mildly elevated temperature (30 °C) or the immediate heat shock. Recently, the extreme day temperature above 35 °C and sustaining high temperature occurred during summer season. In addition, compared to microarray analysis, RNA sequencing (RNA-Seq), as a high-throughput and low-cost sequencing method, can be used to investigate the transcriptome changes in genome wide level, therefore we combined physiology, metabolism, and transcriptome to analysis the transcriptomic and metabolic response of short (6 h) and prolonged heat exposure (35/33 °C, day/night) on potato plants development and to identify putative regulators.

Methods

Plant material
Sprouted microtubers of the potato cultivar “Hezuo 88” were planted to 1.0 L pot filled with vermiculite and cultivated in the glasshouse at 23°C/21°C during 16/8 hours day/night periods, respectively. The light intensity was approximately 10000 Lx. After 8 weeks cultivation, the plant with the same growth status were selected and transferred into growth chamber for a three day adaption period with 14/10 day and darkness at 23°C, and then subjected to heat treatment. For high temperature
treatment, 24 plants were kept at 35°C during light and at 33°C during the dark phase, samples were taken before the treatment and after 6 hours (6 h) and 3 days (3 d) stress application. The third leaves were collected, and immediately frozen in liquid nitrogen, and stored at −80°C until further assays.

**Physiological indices and metabolic assays**

Electrolyte leakage was measured according to methods described by Dionisio-Sese [17], fresh leaf samples with a mass of approximately 100 mg were cut into strips about 5 mm in length, the cuttings were put into test tube with 10 mL deionized water. The tubes were capped and placed in a water bath at 32°C. The initial electrical conductivity (EC1) was recorded by the electrical conductivity meter (DDS-307A, Shanghai China) after samples were incubated at 32°C for 2 h, the final electrical conductivity (EC2) was measured for all released electrolytes after samples were autoclaved at 121°C for 20 min. The electrolyte leakage (EL) was calculated according to the formula: \( EL = \frac{EC1}{EC2} \times 100 \). The photosynthesis system I and II (PSI and PSII) parameters were measured as reported previously [18].

**RNA samples preparation and high-throughput sequencing**

The total RNA was isolated using Trizol Reagent (TIANGEN, Beijing, China) following the manufacturer’s instructions. RNA integrity and concentration was measured by electrophoresis on 1.0% agarose gels and the NanoPhotometer® spectrophotometer. Paired end sequencing libraries with average insert size of 300-400 bp were generated with AMPure XP beads and sequenced on HiSeq4000 (Illumina, San Diego, USA) following manufacturers’ guidelines. Raw data was processed and filtered by Illumina pipeline to generate FastQ files (http://www.Illumina.com). After that, approximately 150-bp clean reads were obtained from 6 sequenced libraries. The genome sequence of potato retrieved from
the Potato Genomics Resource (PGSC) acts as the reference. All the clean reads were then mapped onto the PGSC by STAR (v2.5.1b). The number of reads mapped to each gene was counted using HTSeq v0.6.0, then the abundance of transcripts was normalized by the FPKM. Gene abundance difference of two treatments with one biological replicates each was conducted using the negative binomial distribution model by the DESeq2 R package (1.10.1), and false discovery rate (FDR) was calculated by adjusting the P-values using Benjamini and Hochberg’s approach.

**Metabolite extraction, measurement, and analysis**

One hundred milligrams of powdered leaf material was extracted with 400 µL of 80% methanol at -20°C for 1 h. The solution was centrifuged at 14,000g for 20 min at 4°C, and the extraction solvent was removed in a Speed-Vac, the dried samples were resolved in 100 µL of 80% methanol in water and centrifuged at 14,000g at 4°C for 15 min. LC-MS measurements were performed on the QE HF-X coupled to the Vanquish UHPLC (Thermo). Chromatographic separation was achieved on a Accucore HILIC column. Eluent A was 95% acetonitrile in water with 0.1% formic acid, and eluent B was 50% acetonitrile with 0.1% formic acid. The flow rate was 300 mL /min, and the column temperature was set at 40°C. Mass calibration was achieved with low-concentration ESI Tuning Mix (Agilent). The mass spectrometer was operated as follows: Spray voltage: 3.2kV, Sheath gas flow rate: 35arb, Aux gas flow rate: 10arb, Capillary temp: 320 °C. Polarity: positive; negative. Mass spectra were acquired in a mass range of 100 to 1,500 m/z.

The raw data files were separately imported into Compound Discoverer 3.0 to carry out peak detection. The resulting peaks were normalized and used for molecular formula prediction. The molecular formula were further imported into mzCloud and ChemSpider database for accurate relative quantification. The discriminant
metabolites were determined by OPLS-DA.

**Transient expression in *Nicotiana benthamiana***

Plasmid containing target sequences were transferred to Agrobacterium strain LBA4404. Agrobacterium mediated transformation of *Nicotiana benthamiana* was conducted as previously described [19]. Leaves of *Nicotiana benthamiana* plants were agro-infiltrated by using a needleless syringe. After 12 hours incubation at 22 °C, the agro-infiltrated plants were transferred to growth chamber at 45 °C with 12 h day and 8 h night under light intensity of 10000 lx, and then leaves were harvested for electrolyte leakage measurement as mentioned above.

**Real-time qRT-PCR analysis**

First-strand cDNA was synthesized from 0.5 μg of total RNA, using the Prime Script™RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturers’ instructions. Real-time PCR was carried out on a CFX96™ Real-Time System (BIO-RAD, California, USA). Each reaction includes 5 μl 2×Plus SYBR real-time PCR mixture (BioTeke, Beijing, China), 1 μl of potato cDNA, 0.5 μl each forward and reverse primer, and 8 μl sterile H₂O, following the manufacturer’s protocol. Gene-specific primers were designed based on the corresponding potato gene DNA sequences using Primer 3 (version 0.4.0) (Supplementary Table 1). Each reaction was performed in triplicate. Fold change was calculated using the \(2^{-\Delta\Delta CT}\) method in comparison with the *ubi3* as an internal control gene [20].

**Results**

**Phenotypic responses to heat stress in potato**

Heat stress induces several alterations on plant growth, cell division, and enzymes,
hormones, membranes, among others. Plant cuticle out of the epidermis of leaves have protective effect on abiotic stress, a large number of waxy crystals in epidermis of potato leaves were formed after 3 days heat treatment, we also found that most of stomata were opened after 6 hours heat treatment, but this was not obvious for control and 3 days heat treated leaves, indicating potato leaf responsive to short heat stress by regulating stomata activity (Fig. 1). Electrolyte leakage assay has been widely used to examine the level of plant tolerance to various stresses including heat stress. Percentage electrolyte leakage from 3 days heat treatment leaf tissue was significantly high than that from control and 6 hours heat treated leaf tissue (Fig. 2).

High temperature seriously affects plant leaf photosynthetic performance. In potato, compared to control, we noted that prolonged heat stress caused a significant decrease in Fv/Fm, Yield (I) and Yield (II), and non-photochemical quenching (qN), but these photosynthetic fluorescence parameters had no obvious response to short heat exposure except for qN (Fig. 3), suggesting that the photochemical yield of PSI and PSII, the maximum photosynthetic efficiency, and the photoprotective capacity had been negatively affected over time in potato leaves. However, the changes of photosynthetic pigments including chlorophyll a, chlorophyll b and total chlorophyll were not significant during heat exposure.

**HS-induced transcriptional changes in potato leaf**

For molecular understanding of potato leaf response to HS, the totally expanded third leaves of cultivar Hezuo 88 were isolated from 6 hours and 3 days heat-treated (35/33°C) plant. The same type of leaves taken from normal growing temperature (24°C) was used as controls. Illumina RNA sequencing experiments was conducted on total RNA from these tissues consisting of two biological replicates. Approximate
45 million valid reads were generated from each of the six samples and aligned to the Genome Sequencing Consortium (PGSC) using STAR (v2.5.1b). Gene expression was quantified as FPKM values. We totally identified 18052 genes to be expressed in leaf at one or three different time points investigated, namely control, after 6 hours and 3 days, in potato leaf exposed to temperature at 35°C. Differentially expressed genes were identified using the DESeq2 package (1.10.1) in R based on a negative binomial distribution, a total of 448 heat upregulated genes and 918 heat downregulated genes were detected in heat treatment compared to control. By setting the fold change value (log$_2$FC) >2 with FDR < 0.05 as cut-offs (Supplementary Table 2), we identified 160 and 130 genes were specifically up regulated, and 538 and 94 genes were specifically downregulated in responsive to short and prolonged heat stress respectively. Among them, 35.3% of the genes (158 out of 448) were upregulated and 31.2% (286 out of 918) were downregulated in common (Fig. 4A). These common expressed genes might take part in both transient stresses signaling sensing after the starting of heat stress exposure and adaptive response to sustained stress. Interestingly, the number of significantly up- and down-regulated was reduced after 3 days high temperature exposure.

**Functional category enrichment**

To understand the functional significance of short and prolonged heat induced gene expression data better, the MapMan software$^{21}$ was used to group the DEGs into functional groups or ‘bins’. As illustrated by the ‘metabolism overview’(Figure S1), the result of comparing 0 and 6 hours heat exposure showed that genes involved in major CHO metabolism (susy degradation, beta-amylase), cell wall, amino acid metabolism and secondary metabolism were strongly induced, whereas most of
genes associated with the photosynthetic machinery (light reaction), major CHO metabolism (starch synthesis and invertase degradation), nucleotide metabolism and lipid metabolism was highly reduced, these deregulated genes are mainly involved in transient response to heat stress. The transient response indicates heat was sensed and the adverse impact of heat exhibited on different aspects of primary metabolism. The global expression changes of genes specifically expressed at 3 days after heat stress related to metabolism were comparably weak, highly induced genes associated with secondary metabolism and cell wall persisted and was further expanded to nucleotide metabolism. Short and prolonged heat stress in common results in an increased present of transcripts mainly involved in the process of photosynthesis and secondary metabolism, whereas an decreased present of transcripts mainly involved in the process of cell wall, these consistently up- or down-regulated genes probably participate in an adaptive response to heat exposure or inhibitor effects on potato growth.

PageMan program was further used to enrich functional categories to identify affected process by heat process. DEGs induced at 6 hours after heat stress was enriched for amino acid metabolism, cell wall metabolism, hormone metabolism, secondary metabolism, stress, and protein process. DEGs induced at 3 days after heat stress are enriched for misc process. The impact of high temperature on DEGs induced at 6 h and 3 d after heat stress in common was mainly enriched for photosynthesis, cell wall, stress, RNA and protein (Fig. 5). In combination the changes of stomata phenotype and photosynthetic parameters with enrichment in significantly expressed genes related to aspects of photosynthesis, showing short heat stress suppressed photosynthesis and potato growth during development. The majority of significantly expressed genes were not enriched under prolonged heat
stress, suggesting that potato adaption to sustained heat stress without global changes in gene expression profiles.

**Short and prolonged heat stress repressed transcription factors (TFs) in heat signaling**

The effects of heat on the expression of transcription factor genes were also examined. Fig. 4B shows the expression of 70 transcription factors (TFs, 14 induced or 56 repressed) that were significantly (P>0.05) expressed by short heat stress, whereas 21 (7 induced or 14 repressed) TFs was expressed by prolonged heat stress, in addition, 36 TFs (6 induced or 30 repressed) was overlapped expressed by short and prolonged heat (Supplementary Table 3). However for these transcription factors, the minority genes increase whereas the majority decrease, we found short heat stress induced TF genes is enriched for homeobox, G2-like, C2C2-GATA, MYB, C2C2-CO-like, Psudo ARR, AP2/EREBP and bHLH; Prolonged heat stress induced TF genes are enriched for PHD, MYB-related, bHLH, GRAS, AP2/EREBP, and Aux/IAA; Short and prolonged heat stress in common induced TF genes are enriched for nucleosome/chromatin assembly, bHLH, MYB and C2C2-DOF (Figure S2). A large number of TF-encoding genes were repressed after heat treatment. WRKY-type TFs have been shown to act as regulators either repressing or activating gene expression at transcriptional level, and numerous members of the large family play roles in regulating many stress reactions in plants [22]. In our study, 13 WRKY genes were found to be repressed by short and prolonged heat treatment. Several of WRKY genes also found to be repressed in response to heat in rice [23]. Analysis of AtWRKY25 mutants indicated that it plays roles in the response to heat stress. However, there is little evidence about the potential function of these WRKY genes as regulator in heat stress response. We also found heat stress repressed the
expression of three bZIP genes, and the potato bZIP gene family has been characterized in response to several abiotic stresses including heat [24]. Some of other TF family members, such as PHOR1, ARR, C2H2 zinc finger family and MYB showed decreased expression after heat treatment, providing the evidence that whether these TFs may function as general repressors of heat responses.

**Comparison of heat stress-responsive in potato with heat stress-related genes in Arabidopsis shoots and drought stress-related genes in potato**

To investigate leaf-specific and conserved heat stress response gene expression between different species and plant organs, we compared DEGs in heat treated potato leaf with the DEGs in heat-exposed Arabidopsis shoots [25]. Even though the time point of heat treatment between the two experiments differed for potato and Arabidopsis, the number of DEGs in potato leaf was of a similar magnitude to the number of DEGs in Arabidopsis shoots (776 up-regulated; 1143 down-regulated). By comparing the HarvEST-based, Arabidopsis best BLAST hits of the DEGs in potato leaf with the DEGs in Arabidopsis shoots showed an overlap of 45 up-regulated and 102 down-regulated genes between potato leaf and Arabidopsis shoots (Fig. 6 and Supplementary Table 4).

To further identify the common expressed genes between heat stress and other abiotic stresses, these heat response genes in our study were compared to previous released potato drought-responsive DEGs in leaf [26]. We identified an overlap of 33 heat-repressed and 18 heat-induced genes between heat- and drought-responsive DEGs (Fig. 6 and Supplementary Table 5), among them, fifteen induced genes were associated with heat shock protein, one gene with function of peptidylprolyl isomerase, two genes as conserved gene of unknown function.

Two experiments have been performed using microarray to identify heat-responsive
genes from potato leaves. Comparing of our data sets with Hancock et al [8] and Hastilestari et al [16] shared number of 30 and 62 induced and 37 and 44 repressed genes respectively (Fig. 7, Supplementary Table 6). We noticed that only a small overlap of 15 up regulated genes and 1 down regulated genes (fold change more than 2) among three studies, suggesting these common induced genes were likely to take part in heat tolerance and/or heat response in potato leaves. Some of these are well-known gene members associated with the plant constitutive heat stress response, including small heat shock protein (HSP) genes and cytosolic ascorbate peroxidase genes. However, this overlapped group also includes other as yet uncharacterized genes such as BCL-2-associated athanogene 6 and non-specific lipid-transfer protein. Further functional analysis showed that 15 DEGs take part in photosystem II, the remaining genes were involved in lipid metabolism, heat stress and misc (glutathione S transferases), indicating they are potential candidates for maintaining development of potato cultivars under elevated temperatures.

**Transient expression of heat induced genes in *Nicotiana benthamiana* leaves**

To further characterized the function of heat induced common expressed genes from different studies. A binary construct containing sequence of the ATG start codon from four selected genes driven by a 35S promoter was constructed and transferred into *Nicotiana benthamiana* leaves by agro-infiltration. plants were placed in a growth chamber at 45 °C for 24 h under 12 h light conditions. In compare with control, we found the transgenic plants expressing the transgenes exhibited evident lower levels of cell membrane damage (Figure S3).

**RNA-seq validation by RT-qPCR**

We examined the expression pattern of 20 randomly selected DEGs using RT-qPCR
in potato leaf after 6 hours and 3 days heat exposure. The results indicate that 18 out of the 20 genes expression between RT-qPCR and RNA-seq are very concordant, excluding two genes PGSC0003DMG400021877 and PGSC0003DMG400012436, their expression was transient increased after 6 h heat exposure but decreased to control level after sustained heat stress by RT-qPCR, while their expression maintained decreasing after starting of heat exposure and prolonged heat stress by RNA-seq (Fig. 8). This result indicates that the DEGs identified by RNA sequencing in response to heat in potato are reliable.

**Short and prolonged heat stress cause metabolic alterations in potato leaf**

Potato leaves contained a huge number of soluble metabolites, most of which have not yet been identified, the nonbiased LC-MS global metabolomics approach detected up to 325 and 219 compounds in the positive and negative ionization modes, respectively, that were up- or down-regulated in leaves, unlike the different expressed genes, prolonged heat stress resulted in drastically higher numbers of responsive metabolites, this was indicative for potato leaves adaptation to the high temperature in the metabolome (Table 1, Supplementary table 7). Since many compounds are still unknown, to obtain further information on discriminant metabolite, 4 compounds in 6 hours stress compared to control, and 16 compounds in 3 days stress compared to control were annotated with KEGG (Table 2). Short heat stress suppressed the levels of secondary metabolites biosynthesis such as stevioside, prolonged heat stress decreased levels of biosynthesis of amino acids such as histidine, and plant hormone such as jasmonic acid. Both short and prolonged heat stress caused the upregulation of flavone and flavonol biosynthesis precursors (quercetin, apigenin ) and amino acids biosynthesis (L-Proline, tyrosine), which indicates the higher accumulation of these metabolites under the heat stress
condition providing evidence for a rapid impairment of potato leaf under short heat stress.

To find patterns linking the transcriptome and metabolome, correlations on KEGG pathways were calculated based on fold changes of metabolites and transcripts. At least one metabolite and more than 100 transcripts for biosynthesis of secondary metabolites and biosynthesis of amino acids under short heat stress were closely matched. After sustained heat stress, several pathways including fatty acid biosynthesis, fructose and mannose metabolism, carbon fixation in photosynthetic organisms and plant hormone signal transduction and others were also active and merged together at the metabolic and transcriptomic levels, indicating global changes on metabolic level induced by prolonged heat stress to adapt elevated temperature (Supplementary table 8).

Discussion

Elevated temperature had negative effects on plant growth and development, plant have evolved adaptive mechanism to response heat shock. Previous gene expression studies mainly focused on potato responses to short heat treatment, but the effect of sustained heat treatment on potato responses and their difference are poorly understood. Here we treated potato with heat for 6 h or 3 d to review a comprehensively new transcriptome and metabolism changes underlying short and prolonged HS responses in potato.

Short heat stress maintaining the stomata open and continued stress affect photosynthetic parameters

Stomata play a critical role in supporting fluxes of water and carbon dioxide in the soil-plant-atmosphere-climate system, meanwhile it also drive both plant mass
transport and energy exchange. The result of our study showed short heat treatment maintained open stomata, this probably could lead to a greater water loss from its leaves, at the same time, allow the plant to maintain the efficient photosynthesis, the continued stress triggered stomatal closure and induced a decline in the photosynthetic parameters of Fv/Fm, Yield (I) and Yield (II), which related to maximum photosynthetic potential of the leaves, and the real-time quantum yields of PSI and PSII, respectively [27]. Conflicting results that temperature had no effect on stomata [28, 29], or that increased temperature triggered stomatal closure have often been reported [30, 31]. Similarly, elevated temperatures about 30 °C have no effect on photosynthesis, or that severely inhibited photosynthesis for some heat susceptible or heat tolerant potato cultivars have also been published [32, 33]. Different from Hancock et al [8] study that the content of photosynthetic pigments, chlorophyll a and chlorophyll b, significantly decreased up to 20% after moderately elevated temperatures with 30 °C at day and 20 °C at night up to 5 weeks, our results of potato exposure to heat up to 3 days in agreement with previous studies [4] that leaf chlorophyll a and chlorophyll b increased slightly at the higher temperature. Gene enrichment analysis revealed enhanced expression of transcripts associated with aspects of PSII polypeptide subunits and ferredoxin by short and continued heat treatment. Despite the slight increase in the levels of photosynthetic pigments at continued heat treatment, all of transcripts encoding CHLOROPHYLL A/B BINDING PROTEIN associated photosystem II were significant deceased. Up-regulated protective proteins are characterized in response to heat stress Heat-shock protein (HSP) synthesis is involved in adaptive response to heat exposure. The potato genome encodes 20 HSP70 and 48 sHSP (sHSP, HSP70, HSP90,
HSP100) families [34, 35]. In our study, 35 out of 37 heat stress-related transcripts and heat shock proteins was induced by short heat, among them, 26 heat shock proteins was maintained up regulation by continued heat treatment. These HSPs includes 21 smHSP and 8 HSP with molecular mass from 70 and 130 kDa. smHSP accumulation known to be taking parting in thermos tolerance in plants and represent a major group of genes involved in heat response in higher plant [36]. HSP17.6 and HSP101 proteins were strongly accumulated in the shoots and microtubers of the heat-sensitive potato cultivar Désirée and the heat-tolerant potato cultivar Festival to response heat exposure [37]. In potato, smHSPs were synthesized and accumulated at 35 and 40 °C. Previous study showed that the expression of a 18 kDa protein was significantly over-presented when potato leaves were treated at 35 °C for 1 h; The HSPs with molecular mass from 70 and 130 kDa increased their expression after heat exposure at 40 °C for 2 h [37]. Introduction of the heat shock protein gene encoding HSP17.7 into potato enhanced heat tolerance [38], a potato HSC70 gene has been recently demonstrated to protect against elevated temperature in potato [19], we therefore conclude that a moderate amount of HSPs were synthesized after the first moment of heat exposure, the majority of HSPs were further induced to answer prolonged heat when elevated temperature stress sustained. The present work also showed the two DNAJ heat shock protein was decreased after 6 hours heat and one after 3 days heat treatment, DnaJ proteins, either alone or combing with heat-shock protein 70, act as molecular chaperones and take part in various important cellular processes, including protein assembly/disassembly, folding/unfolding, and degradation [39, 40], meanwhile, it also act as extremely important regulator for cellular protein homeostasis under stress conditions [41], therefore, DnaJ proteins decreased their expression level in
potato provide evidence that these proteins were involved in heat response.

General and specific responses to heat-stress in potato

By comparison of heat-treated transcriptomes in potato leaf during development among three cultivars, 1364 genes were differentially expressed in “Hezuo 88”, whereas 2190 genes were deregulated in “Desirée” from Hancock et al’s research [8], and 2949 genes were deregulated in “Agria” from Hastilestari et al’s research [16]. We found that the number of DEGs in our study was lower than microarray data conducted by Hancock et al [8] and Hastilestari et al [16], despite RNA-seq was employed. This is because the two methods fundamentally are different. In addition, we used the third leaves counted from the top of plant, but probably in these tissues heat treatment does not cause large number of temperature associated changes in gene expression, however, Hancock et al [8] report the whole-compound leaves including secondary leaflets were used for DEGs identification; the research of Hastilestari et al [16] did not present which leaves were excised. Another explanation is that the heat treatments of different temperature and durations applied in these studies. Hancock et al [8] reported short/middle-term heat responses for 48 hours treatment, and Hastilestari et al [16] applied the heat for 10 days durations, whereas we employed heat response by a short (6 hours) and continued (3 days) treatment.

Comparison of our transcriptomes presented a higher overlap of differentially expressed genes with those reported by Hastilestari et al [16] than those published by Hancock et al [8], this is the fact that prolonged heat treatment was applied in both studies, meanwhile cv. Agria is more sensitive to elevated temperature than cv. Desirée [42], we presumed that these genes are mostly involving in an adaptive response to high temperature. Another study using a yeast-based functional
screening method identified 95 potential candidate genes after 2 and 48 hours of treatment in Desiree. We found 10 out of those 95 genes also showed increased expression in current RNA-seq data. The result of comparing our RNA-Seq data and previous microarray data showed that only 16 genes were detected as common expressed gene, while 370 up regulated and 837 down regulated genes are specifically expressed by RNA-seq methods, indicating a number of new candidate genes that had not been linked to heat response previously. These common expressed genes catch our attention that a mainly adaptive response to heat is the generation of heat shock proteins. Therefore, three class I heat shock protein genes and one non-specific lipid-transfer protein gene were found among these studies might be act as molecular markers to assess heat tolerance in potato at development process. Indeed, transient expression of these heat induced genes in common in Nicotiana benthamiana resulted in a great membrane stability to heat shock than controls.

Secondary metabolite involved in heat stress

The accumulation of secondary metabolites such as flavonoids and phenylpropanoids is correlated with heat stress response and tolerance\textsuperscript{43}. Secondary metabolite is involved in resistance against heat shock\textsuperscript{44}. Short heat specifically induced isoprenoids, simple phenols as well as of the transcript levels for their respective biosynthetic enzymes’ transcripts, prolonged heat stress specifically induced the upregulation of phenylpropanoids’ transcripts. A sustained up-regulation of six phenylpropanoids metabolism related genes was found in potato leaves respond to heat stress (Figure S4). Phenylpropanoids is believed to play a critical role in the protection of plants against biotic and abiotic stress, in many
cases by inhibiting the formation of ROS via a range of different mechanisms [45]. The increase of the activity of phenylalanine ammonia-lyase (PAL), the key enzyme of the phenylpropanoid pathway, was reported as a major mechanism of acclimation of cells to HS [46]. Flavone and flavonol biosynthesis precursors in the metabolome showed increased levels of accumulation in the leaves of heat-stressed potatoes. The accumulation of various flavonols was identified in a detailed metabolomics analysis of heat stressed tomato [47]. The accumulation of anthocyanins, a class of flavonoids, was also induced by HS in vegetative tissues [48]. The downregulation of secondary metabolites biosynthesis such as stevioside after the starting of heat stress exposure might be explained as transient reactions avoiding from adverse environmental conditions. The accumulation of some secondary metabolites under drought stress has also been reported in potato [49], although few reports have considered the role of individual metabolites, we have demonstrated a role of these secondary metabolite in response of heat-stress in transcriptomic and metabolic level, and we provide evidence that certain metabolites (flavone and flavonol) might play a specific protective role.

**Conclusion**

In summary, short and prolonged heat treatment on potato leaves resulted in lower membrane stability and decreased photosynthetic parameters, and this is accompanied by transcriptomics and metabolomics changes. Transcripts profiles were mainly induced by short heat stress, where metabolites profiles were mainly activated by prolonged heat stress. General responses to heat stress in gene expression and metabolite accumulation enriched in amino acid metabolism and secondary metabolism pathway. The conserved and potato-specific stress-
responsive genes by comparison heat stress with drought stress responses in potato, and heat stress responses in Arabidopsis shoots, as well as heat stress responses in different potato varieties under different high temperature exposure were identified. These datasets provides evidence that transient reactions in the transcriptome under short term heat exposure and adaptive changes in the metabolome after sustained heat exposure.

Declarations

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Author contributions

Y.L., and B.L. designed the experiment. B.L., M.C., and L.K. performed the experiments. B.L. wrote the manuscript, Y.L., and Q.C. revised and edited the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The sequence data generated in this study have been deposited at NCBI in the Short Read Archive database under the BioProject accession number PRJNA588378. All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.
Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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### Tables

**Table 1** Number of differentially expressed metabolites in response to 6 hours and 3 days heat stress.

|                      | H6h | Overlap | H3d |
|----------------------|-----|---------|-----|
| Metabolome (-)LC-MS  |     |         |     |
| Compound up          | 24  | 12      | 86  |
| Compound down        | 15  | 10      | 94  |
| Metabolome (+)LC-MS  |     |         |     |
| Compound up          | 10  | 3       | 115 |
| Compound down        | 44  | 32      | 156 |

**Table 2** Important metabolites involved in heat stress identified by partial least square discriminant analysis and significant analysis of metabolites.
| ID          | formula     | Kegg_ID   | Pathway name                                      |
|------------|-------------|-----------|---------------------------------------------------|
| H6h vs HC  |             |           |                                                   |
| Com_99_neg | C38H60O18   | cpd:C09189| Biosynthesis of secondary metabolites             |
| Com_224_neg| C26H28O16   | cpd:C12637| Flavone and flavonol biosynthesis                 |
| Com_232_neg| C8H9NO3     | cpd:C00250| Metabolic pathways                                |
| Com_608_neg| C5H9NO2     | cpd:C00148| Biosynthesis of amino acids                       |

| H3d vs HC  |             |           |                                                   |
| Com_330_neg| C16H30O2    | cpd:C08362| Fatty acid biosynthesis                           |
| Com_1053_neg| C27H44O3   | cpd:C01673| Steroid biosynthesis                              |
| Com_22_neg | C8H10O2     | cpd:C06044| Tyrosine metabolism                              |
| Com_605_neg| C28H44N2O8S | cpd:C06462| Arachidonic acid metabolism                      |
| Com_224_neg| C26H28O16   | cpd:C12637| Flavone and flavonol biosynthesis                 |
| Com_1186_neg| C26H28O14  | cpd:C04858| Flavone and flavonol biosynthesis                 |
| Com_1450_pos| C3H7O6P    | cpd:C00118| Glycolysis / Gluconeogenesis                      |
| Com_1193_pos| C6H9N3O2   | cpd:C00135| Biosynthesis of amino acids                       |
| Com_566_pos | C2H5O4P    | cpd:C03167| Phosphonate and phosphinate metabolism            |
| Com_2095_pos| C30H54N10O10S2 | cpd:C16564| Glutathione metabolism                           |
| Com_3261_pos| C18H32O4   | cpd:C04717| Linoleic acid metabolism                         |
| Com_1277_pos| C12H18O3   | cpd:C08491| Plant hormone signal transduction                |
| Com_1814_pos| C24H29NO10 | cpd:C11813| Isoquinoline alkaloid biosynthesis               |
| Com_1804_pos| C21H30O3   | cpd:C03205| Metabolic pathways                               |
| Com_150_pos | C7H8       | cpd:C01455| Metabolic pathways                               |
| Com_517_pos | C21H30O2   | cpd:C00410| Metabolic pathways                               |

VIP = variable importance in projection.

Figures
Figure 1

The characteristics of stomatal and leaf cuticle under heat stress in potato. A and

Figure 2

Membrane damage in development potato leaf after 6 hours and 3 days incubation
Photosynthetic activity after 6 hours and 3 days incubation at 35°C. A. The content of total chlorophyll, ChlA, and ChlB.

Figure 3

Number of up- and down-regulated genes at two different time points of heat stress.

Figure 4

A. Venn diagrams depicting the common and specific heat responsiveness of transcription factors after short and prolonged heat stress.

B. Venn diagrams showing the number of up-regulated and down-regulated genes at different time points.
Enriched functional categories of induced gene expression in heat-stressed samples.
Common and specific elements in heat-stressed potato, Heat-stressed arabidopsis:

Number of common elements in the heat stress and published datasets. Venn dia...
Figure 8

Analysis of mRNA level by RT-qPCR. The mRNA level of 20 randomly selected genes was analysed by RT–qPCR...

Supplementary Files

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