Physiological adjustment of pomegranate pericarp responding to sunburn and its underlying molecular mechanisms

Chunyan Liu1,2†, Ying Su1,2†, Jiyu Li1,2, Botao Jia1,2, Zhen Cao1,2 and Gaihua Qin1,2*

Abstract

**Background:** Sunburn is common in pomegranate, and sunburned fruits have poor appearance and low marketability. However, the physiological and metabolic responses to sunburn and their underlying molecular mechanisms in pomegranate fruit are little understood. Fruit of sunburn-sensitive cultivar ‘Hongyushizi’ was used to carry out physiological parameter detection and widely-targeted metabolomics and transcriptome study.

**Results:** Malondialdehyde and relative conductivity increased with the severity of sunburn, which indicated increased membrane injury. Meanwhile, the content of antioxidants (total phenols and flavonoids), which reduce and repair membrane damage, increased and were accompanied by increases in total antioxidant capacity. In sunburned fruits compared with controls, 129 metabolites changed (including naringenin, pelargonidin and kaempferol) and 447 differentially expressed genes including CHI (Pgr25966.1), F3’5’H (Pgr26644.1), and CHS (Pgr005566.1) may have contributed to these changes. Transcription factors, such as NAC 5 (Pgr008725.1), MYB 93 (Pgr001791.1), and MYB 111 (Pgr027973.1) may be involved in phenylpropanoid and flavonoid biosynthesis by regulating the CHI, F3’5’H, and CHS etc.

**Conclusions:** These findings provide insight into the sunburn mechanisms of pomegranate, and also into the genetic improvement of fruit sunburn.

Keywords: Sunburn, Metabolite, Genes, Physiological, Pomegranate

Background

Pomegranate (Punica granatum L.) is an ancient crop that originated from Central Asia and is now widely grown in subtropical and tropical areas [1]. This tree species is well adapted to marginal lands and arid soils. In China, pomegranate is mainly grown in the Henan, Anhui, Shandong, and Shanxi provinces, where extreme summer air temperatures reach 40 °C and fruit sunburn is common [2]. Pomegranate is terminal-bearing plant with thin branches. With the increase of fruit weight, the thin branches gradually bend, and the fruit becomes more vulnerable to sunburn [3]. Because pomegranate fruit grow through summer and mature in autumn, they are exposed to intense solar radiation and high temperatures throughout the summer, leading to sunburn on the exposed surface of the pericarp. Sunburn usually reduces the harvest of pomegranate fruit by 30% [3].

Sunburn is a physiological disorder that frequently occurs in fruit growing in areas with warm climates, resulting from an excess of heat and light irradiance [4]. The damage from sunburn to plants mainly includes inhibition of photosynthesis, cell membrane damage, senescence, and cell death [5, 6]. The main cause of sunburn

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is thought to be an increase in production of reactive oxygen species (ROS), which causes oxidative damage due to the incapacity of the fruit to recover from stress [6]. Plants have evolved efficient regulatory systems for perceiving and responding to high temperature/solar irradiance stress [6, 7]. Antioxidant enzyme and non-enzymatic antioxidants, such as ascorbic acid (AsA), glutathione (GSH), and flavonoids, work in concert with antioxidant enzymes to cope with the intracellular generation of ROS, and strengthen the responses to abiotic/biotic environmental stressors [8, 9].

As sunburn browning occurs or increases in severity, the concentration of antioxidant metabolites increases [6]. Heating apple leaves from 28 to 40 °C increased the content of antioxidant compounds, but after 4 h at 40 °C the antioxidant compounds decreased [10]. Furthermore, in apple peel, antioxidants were up-regulated in response to the increased ROS generated by exposure to high light and temperature [11]. Flavonoids, together with carotenoids and anthocyanins, enhance light absorption in the UV and blue regions of the spectrum and act as scavengers of ROS molecules [12]. Given the detoxifying activity of AsA on O$_3^\cdot$, AsA could be further required for preventing sunburn browning of fruit [13]. However, fruit with severe sunburn showed a lower total phenol content and total antioxidant activity than undamaged fruit [14]. Responses to sunburn have been investigated in sunburn-susceptible fruits, such as apple and grape [15, 16]. However, knowledge of the physiological changes as well as the underlying genetic mechanism response to sunburn in pomegranate is lacking.

To address these issues, physiological changes in pomegranate pericarp with different degrees of sunburn were studied. Then un-targeted metabolome and transcriptome analyses were conducted for severe sunburned pericarp and healthy pericarp. These results provided functional components of pomegranate fruits responding to sunburn and the potential genes underlying the metabolites that showed different accumulations.

**Results**

**Physiological responses of pomegranate pericarp to sunburn**

To investigate the physiological response of pomegranate pericarp to sunburn stress, three grades of sunburned pomegranate pericarp and the control were analyzed. In biomembranes, MDA is one of end products of lipid peroxidation. Pomegranate pericarp suffering from sunburn showed a 47.9–105.8% increase in MDA content compared with control, which reflected increased membrane injury (Fig. 1b). Additionally, under sunburn, pomegranate pericarp showed a 30.4–143.1% increase in relative conductivity, indicating severe membrane integrity impairment (Fig. 1c). Sunburn induced an increase in the content of antioxidants to reduce and repair the damage caused by ROS. Compared with the control, total phenol content increased by 11.6–60.5% and flavonoid content increased by 17.0–114.9%, whereas total phenols and flavonoids were down-regulated significantly in SB-3 compared with SB-2 (Fig. 1d, e). This was accompanied by increased antioxidants, with total antioxidant capacity (FRAP) increasing 42.1–108.3% compared with control, whereas the total antioxidant capacity was down-regulated in SB-3 compared with SB-2 and SB-1 (Fig. 1f). In short, the SB-3 pomegranate had the most severe membrane injury and significantly increased antioxidants and total antioxidant capacity. Thus, pomegranate pericarps with SB-0 (control) and SB-3 (sunburn treatment) were selected for further metabolomic and molecular studies in response to sunburn stress.

**Metabolome of pomegranate pericarp responding to sunburn**

To further investigate the metabolomic response to sunburn, we conducted metabolic profiling analysis on SB-3 and SB-0. A total of 498 metabolites were identified in pomegranate pericarps. We found that 129 differentially accumulated metabolites (DAMs) including flavonoids, lipids, organic acids, amino acids, amino acid derivatives, and vitamins in SB-3 compared to SB-0 (Supplementary Table 1, Fig. 2a).

Then we conducted a KEGG pathway enrichment analysis of DAMs to focus on the pathways responding to sunburn. We found that five pathways were enriched: biosynthesis of secondary metabolites (ko01110), flavonoid biosynthesis pathways (ko00941), biosynthesis of phenylpropanoids pathways (ko01061), flavone and flavonol biosynthesis pathways (ko00944), and isoflavonoid biosynthesis pathways (ko00943) (Fig. 2b). Furthermore, we found that most DAMs decreased in SB-3 compared to SB-0, while nine DAMs including amino acid derivatives (L-γ-glutamylcysteine, L-cysteinyl glycyne, and glutathione reduced form), a flavone (tricin 5-O-hexoside), an organic acid (hydroxy-3-methoxymandelate), a vitamin (L-ascorbate), an anthocyanin (delphinidin 3-O-rutinoside), a coumarin (7-hydroxy-5-methoxycoumarin), and a proanthocyanidin (procyanidin A3) were up-regulated. In the flavonoid biosynthesis pathway, 16 DAMs were down-regulated. In addition to biosynthesis of flavonoids, the phenylpropanoid metabolic branches also contributed to the production of coumarins and the non-flavonoid product scooletin (7-hydroxy-5-methoxycoumarin, pme2993), which is a coumarin and an antioxidant, were markedly up-regulated [17]. Caffeic acid (pme0303) and p-coumaryl alcohol (pme3305) were down-regulated in the sunburned pomegranate pericarp.
Fig. 1  

a The degree of sunburn of ‘Hongyushizi’ pomegranate. SB-0, no sunburn; SB-1, mild sunburn; SB-2, moderate sunburn; SB-3, severe sunburn. 
b MDA content in pomegranate pericarp with four degrees of sunburn. 
c Relative conductivity in pomegranate pericarp with four degrees of sunburn. 
d The total phenolic content in pomegranate pericarp with four degrees of sunburn. 
e The flavonoid content in pomegranate pericarp with four degrees of sunburn. 
f The antioxidant activity in pomegranate pericarp with four degrees of sunburn. The small letters on different columns indicate significant differences.

Fig. 2  

a Volcano plot of DAMs in SB-0 vs. SB-3. Red dots are up-regulated metabolites, green dots are down-regulated metabolites, and black dots are unchanged metabolites. 
b Bubble diagram of KEGG pathway enrichment analysis based on the DAMs in SB-0 vs SB-3.
To understand the genetic mechanisms underlying the different accumulations of metabolites in response to sunburn, we conducted transcriptome analysis on sunburned and control pericarps. A total of 40.29–52.97 million clean reads was obtained through sequencing of the cDNA libraries after stringent quality checks and data cleanup (Supplementary Table 2). The alignment showed that more than 86.47% of clean reads were mapped to the reference genome. Screening of differentially expressed genes (DEGs) showed that 277 genes were up-regulated and 170 were down-regulated with sunburn (Fig. 3a, Supplementary Table 3). Then we conducted GO functional enrichment analysis of all DEGs, and they were divided into three categories: biological processes, cell components, and molecular functions (Fig. 3b). The GO analysis showed that the most DEGs were grouped in the cell wall external encapsulating structure, and oxidoreductase activity, cell wall organization or biogenesis, and secondary metabolic process. To further understand the biological metabolic pathways and the candidate genes contributing to metabolite accumulations, we conducted KEGG pathway enrichment analysis for DEGs. The DEGs were assigned to 85 metabolic pathways ($P < 0.05$), of which the significantly enriched pathways included metabolic pathways (ko01100, 50 genes), biosynthesis of secondary metabolites (ko01110, 37 genes), phenylpropanoid biosynthesis (ko00940, 12 genes), and flavonoid biosynthesis (ko00941, 11 genes) (Fig. 3c, d).

To understand which genes contribute most to sunburn, correlation between DEGs and physiological indexes was carried out (Fig. 4). Seventeen of 23 DEGs in the phenylpropanoid and flavonoid biosynthesis pathways showed correlation with physiological response of pomegranate, particularly Pgr024474.1 encoding HCT, Pgr016268.1 encoding peroxidase, Pgr006021.1 encoding CAD had high positive correlation coefficient ($R > 0.83$ and $P < 0.05$) between its expression pattern and the physiological response. However, the expression pattern of Pgr006318.1 encoding CAD had a negative correlation with the physiological index. These results indicate the phenylpropanoid and flavonoid biosynthesis pathways contribute most to pomegranate sunburn.
**Fig. 4** Correlation analysis for DEGs involved in metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and flavonoid biosynthesis and physiological indexes (total phenols, total flavonoids, relative conductivity, MDA, FRAP). CHS chalcone synthase, CHI chalcone isomerase, FLS flavonol synthase, ANR anthocyanidin reductase, HCT shikimate O-hydroxycinnamoyltransferase, LAR leucoanthocyanidin reductase, DFR bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase, F3′5′H flavonoid 3',5'-hydroxylase, REF1 coniferyl-aldehyde dehydrogenase, CAD cinnamyl-alcohol dehydrogenase, HMGCR hydroxymethylglutaryl-CoA reductase, SQLE squalene monooxygenase, metE 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, PK pyruvate kinase, trpB tryptophan synthase beta chain, ABA2 xanthoxin dehydrogenase, 4M2H isoflavone/4'-methoxyisoflavone 2'-hydroxylase, GPAT glycerol-3-phosphate acyltransferase, ND(-)-neomenthol dehydrogenase, KCS 3-ketoacyl-CoA synthase, CYP92A6 typhasterol/6-deoxotyphasterol 2alpha-hydroxylase, TPS (++)-alpha-terpineol/(4S)-limonene synthase, CYP17A1 steroid 17 alpha-monoxygenase/17 alpha-hydroxyprogesterone deacylase, ppdK pyruvate, orthophosphate dikinase, ISYNA1 myo-inositol-1-phosphate synthase, PLCD phosphatidylinositol phospholipase C, delta, CYPIZ cytochrome P450 family 2 subfamily Z, CYPIA6 cytochrome P450 family 26 subfamily A, HPS2 heparanase, FABF 3-oxoacyl-[acyl-carrier-protein] synthase II, FAT8 fatty acyl-ACP thioesterase B, FAOH fatty acid omega-hydroxylase, POMGNT2 protein O-mannose beta-1,4-N-acetylgalactosaminyltransferase
are shown in the network diagram (Fig. 6a). There was a significant correlation between the expression of the Pgr003851.1 (FLS) and naringenin (pme0376), a naturally occurring flavanone, and pelargonidin (pme1397), a well-known natural anthocyanidin. Kaempferol (pme0200), an anti-inflammatory flavonoid commonly found in

Fig. 5 The phenylpropanoid and flavonoid biosynthesis pathways in pomegranate pericarps under sunburn stress. The biosynthetic pathway of phenylpropanoid and flavonoid referring to the KEGG database (https://www.kegg.jp/kegg-bin/show_pathway?map00940, https://www.kegg.jp/kegg-bin/show_pathway?map00941) [46]. Expression patterns of genes in the phenylpropanoid and flavonoid biosynthesis pathways shown as a heatmap. The three on the left represent healthy fruit, and the three on the right represent sunburned fruit. The red indicates higher and the blue indicates lower. The compounds up-regulated in the metabolome analysis are marked by red arrows, and the compounds down-regulated are marked by green arrows.

Fig. 6 a Association network analysis of structural genes and metabolites of phenylpropanoid and flavonoid biosynthesis pathways. The darker nodes indicate higher reliability. Edge lines represent the relationship between transcripts and compounds. b Co-expression analysis of genes and transcription factors related to the phenylpropanoid and flavonoid pathways. Genes with correlation coefficients > 0.95 are shown. Red circles represent transcription factors and blue circles represent structural genes.
plants, was significantly correlated with the expression of genes Pgr25966.1 (CHI), Pgr13401.1 (CAD), Pgr006021.1 (CAD), and Pgr024473.1 (HCT).

Additionally, there were 29 TFs, including MYB, NAC, bHLH, and HY TFs, showing different expression in SB-3 compared to SB-0 (Supplementary Table 4). For example, Pgr001791.1 encoding MYB93, a gene negatively regulating genes involved in anthocyanin and proanthocyanidin biosynthesis [18], was 20 times up-regulated in sunburned fruits compared to the control. Gene Pgr027973.1 encoding MYB111, which activates the expression of genes involved in flavonol synthesis [19], had high expression following sunburn.

Then, we conducted co-expression analysis of differentially expressed TFs and genes encoding enzymes that participate in phenylpropanoid and flavonoid biosynthesis and found that Pgr027973.1, encoding MYB111, Pgr008725.1, encoding NAC5, and Pgr001791.1, encoding MYB93, were co-expressed with genes involved in phenylpropanoid and flavonoid biosynthesis (R > 0.95) (Fig. 6b). Thus, we conclude that Pgr027973.1, Pgr008725.1, and Pgr0012667.1 may respond to sunburn by regulating genes involved in phenylpropanoid and flavonoid biosynthesis.

**Confirmation of transcriptome data using qRT-PCR**

Five genes involved in phenylpropanoid and flavonoid biosynthesis pathways and four TFs were selected to validate the RNA-seq results using qRT-PCR (Fig. 7). The results showed that the relative expressions of DEGs were
accordant with the transcript accumulations of DEGs in RNA-seq study, indicating the reliability of RNA-seq results.

**Discussion**

High temperature and excess light are a general source of oxidative stress, leading to plant cell metabolism disorders and producing high levels of ROS in the plant. Excessive accumulation of ROS in cells causes oxidative damage to membranes (lipid peroxidation), and triggers the production of free radical scavengers of enzymatic (CAT, APX, SOD, peroxidase) or non-enzymatic systems in response to oxidative damage [6]. In sunburned pomegranate pericarps, the increased MDA content and relative electrical conductivity may be the result of increased membrane injury. Phenols and flavonoids are two important metabolite types in the antioxidant systems of plants. Phenolic compounds accumulated in the skin of apple fruits exposed to sunlight to protect the fruit from stress [21]. Polyphenols possess ideal structural chemistry for free radical scavenging and they have been shown to be more effective antioxidants in vitro than tocopherols and AsA [22]. Flavonoids may be used as scavengers of ROS and can absorb the most energetic short solar wavelengths [23, 24]. Previous studies in Arabidopsis have shown that the accumulation of flavonoids, which results in radical scavenging activity in vitro, leads to the enhancement of oxidative and drought tolerance in vivo [24]. In pomegranate, the total phenol and total flavonoid contents increased with increased sunburn, which may be used to reduce and repair the damage caused by ROS. However, the total phenols and flavonoids decreased significantly in pomegranate pericarps with extremely severe sunburn. This indicated the response of antioxidants could be divided into two phases during the stress. The first stage was associated with protection by ROS scavenging and the inhibition of lipid peroxidation and may be related to moderate stress. In the second phase, pomegranate underwent severe stress, their antioxidant degradation exceeded synthesis and so their content decreased. The total phenolic content and antioxidant activity of pomegranate fruit with severe sunburn were lower than those of undamaged fruit [14]. This mechanism can also be expanded to other stress [25] and antioxidants [26].

In addition to total phenols and flavonoids, AsA and GSH also play a crucial role in plant survival under abiotic stresses [5]. It was demonstrated that the contents of antioxidants AsA and GSH tend to decrease under severe short-term light and/or temperature stress [27]. However, the antioxidant content will gradually increase after long-term exposure to adverse light and temperature conditions. This increase may be due to the extended stress (e.g., days or weeks), leading to acclimated, long-term responses. Acclimation is a process in which plants respond to environmental changes (such as light and temperature conditions) so that they can maintain a steady state under diverse environmental conditions [28]. It was confirmed that the concentrations of AsA and GSH increased with increasing severity of sunburn [29]. We found a similar phenomenon in pomegranate pericarps with severe sunburn.

To cope with sunburn stress, plants have evolved ways to alter their metabolism and reconfigure metabolic networks. This response occurs at the primary metabolic level after short-term sunburn stress, which then trigger elaborate changes in the production of functional secondary metabolites that can protect against sunburn stress [30]. Correlation between DEGs and physiological index indicate sunburn alters the phenylpropanoid and flavonoid pathway most, triggering a marked accumulation of phenols, which may help alleviate the damaging effects of sunburn stress. Similar studies have showed UV-B irradiation alters the phenylpropanoid and flavonoid pathway in Chinese liquorice (Glycyrrhiza uralensis) to protect against UV-B damage [31]. Following sunburn, we found that genes involved in phenylpropanoid and flavonoid biosynthesis were activated and their related metabolite profiles were significantly changed. The DAMs of the phenylpropanoid and flavonoid pathways in SB-3 were down-regulated except for scopoletin. This indicated a higher flux through the coumarin compared with the flavonoid pathway in the sunburned pericarp of pomegranate fruit, which may result in increased products, such as scopoletin, to scavenge ROS [32].

In the present study, key genes encoding the enzymes involved in phenylpropanoid and flavonoid biosynthesis (e.g., CHI, CHS, LAR, and F3’5’H) were up-regulated in the pomegranate pericarp following sunburn. Several TFs, including NAC and MYB TFs, that were activated in pericarp of pomegranate may bind to the promoter regions of key genes encoding the enzymes and play key roles in regulation of DAMs under sunburn. The NAC family is one of the largest gene families in the plant genome and numerous studies have examined the involvement of several types of NACs in abiotic stress response [33, 34]. The ANAC078 protein has been found to be associated with the induction of genes related to flavonoid biosynthesis, leading to the accumulation of anthocyanins in response to high-light stress [34]. The stress-responsive protein OsNAC5 has been reported as the transcriptional activator that enhances stress tolerance by up-regulating the expression of stress-inducible rice genes [35]. In the present study, NAC5 was remarkably accumulated and was associated with the genes (F’3’5’H, LAR, CHI, CHS, DFR, ANR, and CHS) critically
involved in phenylpropanoid and flavonoid biosynthesis, suggesting a vital role in metabolite accumulation with sunburn. The R2R3-MYB subfamily is the largest group of the plant MYB family [36]. Members of the R2R3-MYB family of TFs have been demonstrated to be transcriptional regulators of structural genes of flavonoid biosynthesis [37]. For example, MYB111 in turnip was regulated by different light spectra, suggesting the role of MYB111 in response to light stress [38]. The TF AtMYB111 in Arabidopsis regulates flavonol synthesis and accumulation by activating the expression of genes related to flavonol synthesis (CHS, CHI, F3H, and FLS1) [19]. In the present study, the induced MYB111 promoted expression of CHI, F3’S’H, LAR, REF, and beta-glucosidase involved in phenylpropanoid and flavonoid biosynthesis. MYB57, which has high homology to MYB93, contributes to the negative regulation of anthocyanin and proanthocyanidin biosynthesis in poplar [18]. These findings suggest that MYB93 may act as a transcriptional repressor, inhibiting the genes expression (CHI, LAR, beta-glucosidase, and REF), thereby reducing flavonoid levels in response to sunburn. The exact function of these candidate TFs needs further verification.

Conclusions
A proposed model of sunburn response mechanisms of pomegranate was showed in Fig. 8. Sunburn is a physiological disorder resulting from an excess of heat and/or light irradiance. Sunburn stress induced an increase in the content of antioxidants (total phenols, flavonoids, AsA, and GSH); therefore, the total antioxidant capacity increased to reduce and repair the membrane damage. However, as the severity of sunburn increased, the flavonoids tended to decrease and most metabolites in the phenylpropanoid and flavonoid biosynthesis pathways were down-regulated. The key genes encoding the enzymes involved in phenylpropanoid and flavonoid biosynthesis (e.g., CHI, CHS, LAR, and F3’S’H) and TFs (e.g., NAC5, MYB93, and MYB111) that were activated in pomegranate pericarp may contribute to the DAMs following sunburn. These findings not only provide insight into the sunburn response mechanisms of pomegranate, but also into genetic improvement of fruit sunburn.

Methods
Plant materials
Ripe fruits of P. granatum L. ‘Hongyushizi,’ a sunburn-sensitive cultivar, were selected to study the sunburn response. P. granatum L. ‘Hongyushizi’ is a fruit crop of economic importance and passed the approval of the Anhui Provincial Forest Variety Approval Committee in 2003. Pomegranate ‘Hongyushizi’ were harvested from the Gangji Eco-agricultural Demonstration site, Anhui Academy of Agricultural Sciences, Hefei, China, and the sampling permissions were obtained. We declare that the pomegranate materials in this study comply with institutional, national, and international guidelines for the collection and cultivation of any plant materials. Pomegranate fruit was sorted into four grades according to sunburn browning: no sunburn (SB-0, control); slight sunburn (SB-1, pericarps having dark tan spot); medium sunburn (SB-2, pericarps having sunburn browning); and severe sunburn (SB-3, pericarps having necrosis) (Fig. 1a). Pericarp tissue about 10 mm thick and ±10 g was dissected from fruit with different grades of sunburn, and then immediately frozen in liquid nitrogen and stored at −80 °C until use. Three independently replicated experiments with five fruits each were executed. Pomegranate pericarps of four sunburn degrees were used to study the physiological responses to sunburn. Metabolome and transcriptome analyses were conducted on samples rated SB-0 and SB-3.

Measurements of physiological characteristics and statistical analysis
Malondialdehyde (MDA) levels were determined according to the protocol described by Heath and Packer with modifications [39]. In brief, 7 mL of 10% trichloroacetic acid was added to 0.7 g pomegranate pericarp. The homogenate was centrifuged at 4,000 × g for 10 min. Then, 2 mL of centrifuged supernatant was taken (2 mL of distilled water as control), 2 mL 0.6% thiobarbituric acid (TBA) was added, and the mixture was reacted in a boiling water bath for 15 min. The mixture was then centrifuged after cooling rapidly (10,000 × g for 5 min). The supernatant was used to determine the extinction at 532 and 600 nm (with 0.6% TBA or control as reference). The amount of MDA was calculated by using an extinction coefficient of 155 mM−1 cm−1.

Electrical conductivity was determined according to the soaking method described by Chen et al. [40]. The total phenol content in the pericarp was determined using a modified Folin–Ciocalteu method [41]. In brief, 100 µL of the prepared sample solution was mixed with 0.5 mL of Folin–Ciocalteu reagent and 1.5 mL of 20% sodium carbonate. The volume was adjusted to 10 mL with distilled water, mixed evenly, and incubated in a water bath for 15 min at 100 °C. The absorbance of the solution was measured against a blank at 765 nm with ultraviolet–visible spectrophotometer. Gallic acid was used as a standard and the total phenols were expressed as mg/g gallic acid equivalent.

The flavonoid content was determined according to the method previously described by Jia with slight modifications [42]. Firstly, 200 µL of extract was placed in a 10 mL volumetric flask. Then, 0.3 mL of 5%
NaNO₂ solution was added, followed by the addition of 0.3 mL of 10% AlCl₃ (1:10) 6 min later. After 6 min, 1 mL of 1 mol/L NaOH was added and the total was made up to 10 mL with distilled water. The absorbance of the fully mixed solution was measured at 510 nm. Rutin was used as the standard for the calibration curve. According to the calibration curve, the flavonoids content was calculated.

Fig. 8 A proposed model of sunburn response mechanisms of pomegranate. MDA malondialdehyde, RC relative conductivity, AsA ascorbic acid, GSH glutathione

Antioxidant activity (FRAP) was determined according to a procedure adopted from Benzie and Strain [43]. The FRAP reagent contained 2.5 mL of a 2,4,6-tris (2-pyridyl)-s-triazine solution (10 mmol/L) in hydrochloric acid (40 mmol/L) and 2.5 mL of a FeCl₃ solution (20 mmol/L) blended with 25 mL of an acetate buffer (0.3 mol/L, pH 3.6). FRAP reagent was then warmed to 37 °C. Aliquots of 10 μL diluted sample were mixed with 1.8 mL of FRAP
reagent, and then 200 µL of distilled water was added. The absorbance of the reaction mixture was measured at 593 nm. The results were expressed as the concentration of antioxidants with a ferric reducing ability equivalent to that of 1 mmol/L FeSO₄. Adequate dilution was needed if the FRAP value obtained was greater than the linear range of the standard curve.

Data were analyzed using ANOVA in SPSS Version 16.0 (Chicago, IL, USA). Differences in means were assessed using the least significant difference (LSD) test (P < 0.05).

Metabolites extraction, identification, and quantification
In order to better separate the metabolites, the samples were freeze-dried and ground into fine powder. The freeze-dried pomegranate tissue was crushed using a mixer mill (MM 400, Retsch, Haan, Germany) with a zirconia bead for 1.5 min at 30 Hz. One hundred milligrams of powder were weighed and extracted overnight at 4 °C with 1.0 mL of 70% aqueous methanol. Following centrifugation at 10,000 g for 10 min, the extracted supernatants were absorbed (CNRWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL, Shanghai, China, www.anpel.com.cn/cnw) and filtered (SCAA-104, 0.22 µm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis [44].

Quality control samples (QC) were prepared by mixing sample extracts and used to analyze the repeatability of samples under the same treatment methods. During the instrumental analyses, a quality control sample was inserted into every ten tests and QC samples were analyzed to monitor the repeatability of the analysis. The sample extracts were analyzed using a LC–ESI–MS/MS system (HPLC, Shim-pack UFLC Shimadzu CBM30A system, www.shimadzu.com.cn/; MS, Applied Biosystems 6500 QTRAP, www.appliedbiosystems.com.cn/). The HPLC analytical conditions were as follows: (1) column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm, 2.1 mm × 100 mm); (2) solvent system, water (0.04% acetic acid):acetonitrile (0.04% acetic acid); (3) gradient program, 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 15.0 min; (4) flow rate, 0.40 mL/min; (5) temperature, 40 °C; and (6) injection volume: 2 µL. The effluent was connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

The MS conditions mainly included: electrospray ionization temperature, 500 °C; mass spectrum voltage, 5500 V; curtain gas, 25 psi; collision-activated dissociation, high. In the triple quadrupole, each ion pair was scanned according to the optimized de-clustering potential and collision energy [45].

Metabolomic analysis
Based on the Metware database and the public database of metabolite information, the material was characterized according to the secondary spectrum information. The quantification of metabolites was accomplished using a multiple reaction monitoring analysis by triple quadrupole mass spectrometry and the Analyst 1.6 software (AB Sciex) to process mass spectrometry data. According to the metabolite retention time and peak type, the mass spectrum peaks detected in different samples of each metabolite were corrected to ensure the accuracy of qualitative and quantitative data. A principal component analysis and partial least squares-discriminant analysis (PLS-DA) were applied to the data. Moreover, a hierarchical cluster analysis was performed with R (www.r-project.org/) to visualize changes in metabolic profiles.

With a fold change > 2 or < 0.5, the PLS-DA model indicated a variable importance in project (VIP) value > 1 as the threshold to screen the significantly changed metabolites between SB-0 and SB-3.

RNA extraction, RNA-sequencing (RNA-seq), and differential expression analysis
The pericarps of SB-0 and SB-3 fruit were collected for RNA extraction. Three biological replicates were designed for each treatment. RNA was isolated from pomegranate pericarps using an RNAprep Pure Plant kit (Tiangen, China) according to the manufacturer’s protocol. Sequencing libraries were generated using the NEBNext Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, USA) following the manufacturer’s instructions. After passing the library inspection, the Illumina HiSeq platform was used for sequencing. Unigene expression levels were calculated and normalized to fragments per kilobase of transcript per million mapped reads (FPKM) [46]. Adaptor sequences and low-quality sequence reads (identity ≥ 95% and coverage length ≥ 100 bp) were removed. Furthermore, all differentially expressed genes (DEGs) were analyzed by gene ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes enrichment analyses (KEGG, http://www.genome.jp/kegg) [47]. The correlation matrix between DEGs assigned to significantly enriched pathways (metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, and flavonoid biosynthesis) and physiological data (total phenols, total flavonoids, relative conductivity, MDA, FRAP) was generated using Pearson’s correlation coefficient. Correlation matrices were visualized using the R corplot package (https://github.com/taiyun/corplot).
Integrated transcriptome and metabolome analysis
Based on the gene expression and the metabolite content data, Pearson correlation tests were used to detect associations between DEGs and DAMs. The detected associations with a \( P < 0.05 \) were selected. In addition, DEGs and DAMs were mapped to the KEGG pathway database to obtain information about their common pathways. A co-expression analysis was conducted on genes involved in the phenylpropanoid and flavonoid biosynthesis pathways and on differentially expressed transcription factors (TFs) to identify TFs involved in the regulation of target gene expression. These analyses were performed using Cytoscape 3.7.1 (https://cytoscape.org/).

Quantitative real-time PCR (qRT-PCR) analysis
To verify the accuracy of sequencing results, we evaluated the relative expression patterns of nine DEGs in the transcriptome data. The cDNA was synthesized using a cDNA Synthesis Kit (TaKaRa, Japan; http://www.takara-bio.com/) following the manufacturer’s instructions. Primer Premier 5.0 software (PREMIER Biosoft International, CA, USA) was used to design gene-specific primer pairs. Information on the relevant genes and primer sequences is provided in Supplementary Table 5.

The relative level of gene expression was calculated using the \( 2^{-\Delta\Delta CT} \) method [48]. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for chart preparation. Three biological and technical replicates were performed in qRT-PCR assays. Statistical analysis of variance followed by Duncan’s new multiple range tests were performed with SPSS Version 16.0 (Chicago, IL, USA). Differences were considered significant at \( P < 0.05 \).

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Authors/Contributions
QGH designed and supervised the experiment. LCY and SY conducted experiment, analyzed the data, and drafted the manuscript. LJY revised the manuscript. JBT and CZ help with measurement and statistics. All authors have read and approved the manuscript.

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Availability of data and material
All raw sequence data are available at NCBI project PRJNA752829 and Sequence Read Archive (SRA) with accession number SRR15412367, SRR15412368, SRR15412369, SRR15412370, SRR15412371, and SRR15412372. The addresses are as follows: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA752829. All the data and materials can be shared by contacting the corresponding author, Gaihua Qin (qghaztsu@163.com).

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Sarkhosh A, Yavari AM, Zamani Z. The Pomegranate: Botany, Production and Uses. In: Sarkhosh A, Yavari AM, Zamani Z, editors. Wallingford: CABI; 2020. p. 225–246.
2. Ferrara G, Selahvarzi Y, Ahmadpourmir H, Mazzeo A, Giancaspro A. Production and growing regions. In: Sarkhosh A, Yavari AM, Zamani Z, editors. CABI; 2020. The Pomegranate: Botany, Production and Uses. Wallingford: CABI; 2020. p. 59–93.
26. Munné-Bosch S. The role of α-tocopherol in plant stress tolerance. J Plant Physiol. 2004;161(10):1349–53.

27. Schrader L, Sun J, Felicetti D, Seo J, Jedwol L, Zhang J. Stress-induced disorders: effects on apple fruit quality. In: Proceedings of the Washington Tree Fruit Postharvest Conference. Washington: Wenatchee; 2003. p. 2.

28. Xu S, Li J, Zhang X, Wei H, Cui L. Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. Environ Exp Bot. 2006;56(3):274–85.

29. Munne-Bosch S, Vincent C. Physiological Mechanisms Underlying Fruit Sunburn. Crit Rev Plant Sci. 2019;38(2):140–57.

30. Feng W, Xu Z, Fan X, Zhou Q, Cao J, G. Jing S, Feng B, Wang T. Transcriptome Analysis Reveals Complex Molecular Mechanisms Underlying UV-Tolerance of Wheat (Triticum aestivum, L.). J Agric Food Chem. 2019;67(2):563–77.

31. Almeselmani M, Deshmukh PS, Sairam RK, Kushwaha SR, Singh TP. Protective role of antioxidant enzymes under high temperature stress. Plant Sci. 2006;171(3):382–8.

32. Gupta DK, Palma JM, Corpas FJ. Antioxidants and antioxidant enzymes in higher plants. Switzerland: Springer; 2018. https://doi.org/10.1007/978-3-319-75088-0.

33. Ma YH, Ma FW, Zhang JK, Li MJ, Wang YH, Liang D. Effects of high temperature on activities and gene expression of enzymes involved in ascorbate-glutathione cycle in apple leaves. Plant Sci. 2008;175(6):761–6.

34. Chen L, Li P, Cheung L. Effects of high temperature coupled with high light on the balance between photooxidation and photoprotection in the sun-exposed peel of apple. Plants. 2008;228(5):745–56.

35. Solovchenko A, Merzlyak M. Screening of visible and UV radiation as a photoprotective mechanism in plants. Russ J Plant Physiol. 2008;55(6):719–37.

36. Hideg É, Barta C, Kálai T, Vass I, Hideg K, Asada K. Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photooxidation or UV radiation. Plant Cell Physiol. 2002;43(10):1154–64.

37. Weerakkody P, Jobling L, Infante M, Rogers G. The effect of maturity, sunburn and the application of sunscreens on the internal and external qualities of pomegranate fruit grown in Australia. Sci Hortic. 2010;124(1):57–61.

38. Felicetti DA, Schrader LE. Changes in pigment concentrations associated with the degree of sunburn browning of ‘Fuji’ apple. J Am Soc Hortic Sci. 2008;133(1):127–34.

39. Gambetta JM, Holzapfel BP, Stoll M, Friedel M. Sunburn in Grapes: A Review. Front Plant Sci. 2021;11:2123.

40. Vogt T. Phenylpropanoid biosynthesis. Mol Plant. 2010;3(1):12–20.

41. Wang F, Xu Z, Fan X, Zhou Q, Cao J, G. Jing S, Feng B, Wang T. Transcriptome Analysis Reveals Complex Molecular Mechanisms Underlying UV-Tolerance of Wheat (Triticum aestivum, L.). J Agric Food Chem. 2019;67(2):563–77.

42. Jia Z, Tang MC, Wu JM. The determination of flavonoid contents in mulberry leaves. J Agric Food Chem. 2004;52(12):4039–40.

43. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. Meth Enzymol. 1999;299C(1):152–78.

44. Leprince L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M. Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol. 2006;57:405–30.

45. Wang Y, Zhou B, Sun M, Li Y, Kawabata S. UV-A Light Induces Anthocyanin Biosynthesis in a Manner Distinct from Synergistic Blue plus UV-B Light and UV-A/Blue Light Responses in Different Parts of the Hypocotyls in Tumip Seedlings. Plant Cell Physiol. 2012;53(8):1470–80.

46. Heath RL, Packer L. Photoperoxidation in leaves of spinach exposed to light and oxygen deprivation stress: a review. Ann Bot. 2003;91(2):179–94.

47. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27–30.

48. Salvatierra A, Pimentel P, Moya-Leon MA, Caligari PDS, Herrera R. Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of Fragaria chiloensis ssp. chiloensis. Phytochem. 2010;71(16):1839–47.

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