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ORIGINAL RESEARCH PAPER

Influence of heat stress on leaf morphology and nitrogen–carbohydrate metabolisms in two wucai (*Brassica campestris* L.) genotypes

Lingyun Yuan, Ling Tang, Shidong Zhu, Jinfeng Hou, Guohu Chen, Fan Liu, Shan Liu, Chenggang Wang*

Vegetable Genetics and Breeding Laboratory, College of Horticulture, Anhui Agricultural University, Hefei 230036, China

* Corresponding author. Email: cgwang@ahau.edu.cn

Abstract
Heat stress is a major environmental stress that limits plant growth and yield worldwide. The present study was carried out to explore the physiological mechanism of heat tolerant to provide the theoretical basis for heat-tolerant breeding. The changes of leaf morphology, anatomy, nitrogen assimilation, and carbohydrate metabolism in two wucai genotypes (WS-1, heat tolerant; WS-6, heat sensitive) grown under heat stress (40°C/30°C) for 7 days were investigated. Our results showed that heat stress hampered the plant growth and biomass accumulation in certain extent in WS-1 and WS-6. However, the inhibition extent of WS-1 was significantly smaller than WS-6. Thickness of leaf lamina, upper epidermis, and palisade mesophyll were increased by heat in WS-1, which might be contributed to the higher assimilation of photosynthates. During nitrogen assimilation, WS-1 possessed the higher nitrogen-related metabolic enzyme activities, including nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH), which were reflected by higher photosynthetic nitrogen-use efficiency (PNUE) with respect to WS-6. The total amino acids level had no influence in WS-1, whereas it was reduced in WS-6 by heat. And the proline contents of both wucai genotypes were all increased to respond the heat stress. Additionally, among all treatments, the total soluble sugar content of WS-1 by heat got the highest level, including higher contents of sucrose, fructose, and starch than those of WS-6. Moreover, the metabolism efficiency of sucrose to starch in WS-1 was greater than WS-6 under heat stress, proved by higher activities of sucrose phosphate synthase (SPS), sucrose synthase (SuSy), acid invertase (AI), and amylase. These results demonstrated that leaf anatomical alterations resulted in higher nitrogen and carbon assimilation in heat-tolerant genotype WS-1, which exhibited a greater performance to resist heat stress.

Keywords
wucai; heat stress; leaf morphology; nitrogen assimilation; carbohydrate metabolism

Introduction

Heat stress due to high temperature is a serious threat to crop production and quality worldwide [1]. Transitory or constantly high temperatures could cause an array of morpho-anatomical, physiological, and biochemical changes in plants, which affected plant growth, development, and even phenology and might lead to a drastic decline in economic yield [2,3]. In recent years, this issue has become more urgent due to the global warming. Developing new vegetable cultivars with improved thermotolerance

using various genetic approaches has become the effective way to mitigate the heat stress. For this purpose, the thorough understanding of physiological responses to heat, mechanisms of heat tolerance, and possible strategies are imperative in vegetable production.

Heat stress is now a major concern for crop production and exploiting approaches to sustain high yields of crop under heat stress are important agricultural goals [2]. Plants could alter their metabolisms by various means responding to heat stress, particularly by producing compatible solutes that are able to maintain cell turgor by osmotic adjustment, organize proteins and cellular structures, and modify the antioxidant system to re-establish the cellular redox homeostasis [4–6]. Additionally, disturbance of fundamental processes such as carbon and nitrogen assimilation, respiration, and transpiration may reduce overall metabolic efficiency and result in vegetative developmental defects [7,8].

Wucai (*Brassica campestris* L. ssp. *chinensis* var. *rosularis* Tsen et Lee.) belongs to Chinese cabbage with the beautiful shape and high nutritional value. This crop originated from China and distributed mainly along Yangtze–Huaihe River basin [9]. It grows well in cold weather of late fall and winter [10], but not in the hot summer. The high temperature might inhibit the seedling growth and even cause heat damage. To achieve annual production and meet market demand, it is critical to select and breed heat-tolerant wucai genotype.

The literature on physiological and genetic basis of heat tolerance in wucai is still scarce. Our previous study reported that heat-tolerant genotype WS-1 had higher photosynthetic capacity and photo-chemical activity [11] and stronger antioxidative system than heat-sensitive genotype to protect plant from high temperature [12]. To further reveal the tolerant mechanism, the present study was carried out from leaf morphology and nitrogen–carbon metabolism, which would aid the design of strategies to screen germplasm for heat tolerance traits in wucai.

Material and methods

Plants

Two wucai genotypes, WS-1 (heat tolerant) and WS-6 (heat sensitive), were selected as representative varieties with different sensitivities to heat stress in previous experiment [13]. As reported, the color of WS-1 was darker than WS-6, and WS-1 showed a better quality and higher yield under hot weather than that of WS-6.

The whole experiment was conducted according to Zou et al. [11] at Anhui Agricultural University, Hefei, China. Seeds were sown in soilless substrate [peat/vermiculite (volume) = 2/1] and then moved into a phytotron. The environment was controlled at 25 ±1°C (day) and 18 ±1°C (night) (light/dark 14 h / 10 h) with relative humidity of 70% and 300 μmol m^{-2} s^{-1} photosynthetically active radiation. The uniform size seedlings with four to five true leaves were randomly divided into two groups (50 plants of each group) respectively, which were organized in a complete randomized block design. The four treatments (day/night temperatures) were as follows: Cont-WS-1 (25°C/18°C), Cont-WS-6 (25°C/18°C), HT-WS-1 (40°C/30°C), HT-WS-6 (40°C/30°C). These seedlings were treated under high/normal temperature for 7 days, and then leaves were sampled to determine physiological and biochemistry parameters.

Morphological analyses

The plant height was estimated from cotyledonary node to the growing point with a ruler. The stem diameter was determined at the part of cotyledonary node using a vernier caliper. After the whole plants were washed with distilled water, their fresh weights were measured; and they were dried at 75°C for 72 h to obtain the dry weights. The third leaves from bottom of each treatment were sampled to survey the blade length, blade width, petiole length and width.

Leaf anatomical analyses

Anatomical tissue measurements were performed on the fifth fully expanded leaves (numbered from the center) of control and heat-stressed treatments. Samples of 3 × 4 mm were taken from the middle of the leaves, which were fixed in formalin–acetic acid–alcohol solution for a week, dehydrated, and embedded in paraffin as the procedure of Medina et al. [14]. The thickness of leaf, palisade mesophyll (PM), spongy mesophyll (SM), upper and lower epidermis were taken with an ocular micrometer and exact values were calculated with a factor derived by comparing ocular with stage micrometers. The cell tease ratio (CTR) and spongy ratio (SR) were valued as followed: CTR (%) = $PM/leaf\ thickness \times 100$, and SR (%) = $SM/leaf\ thickness \times 100$.

Analysis of nitrate and ammonium contents

Nitrate and ammonium contents were estimated by taking 0.5 g fresh leaves according to the methods of Cataldo et al. [15] and Solorzano [16], respectively.

Analysis of NR activity

NR activity (EC 1.6.6.1) was determined by the method of Foyer et al. [17] with a slight modification. The reaction mixture contained 50 mM 3-(*N*-Morpholino)propanesulfonic acid–KOH (Mops–KOH) buffer (pH 7.5), 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, and 5 mM EDTA. It was initiated by adding 200 µL of the enzyme protein extract. The mixture was incubated at 30°C for 30 min and terminated by the addition of 0.5 M zinc acetate.

Analysis of glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) activities

The enzymes were extracted by the method of Yuan et al. [18]. The leaves (0.5 g) were homogenized in 10 mM Tris–HCl buffer (pH 7.6) containing 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM 2-mercaptoethanol in ice bath. The homogenate was centrifuged at 4°C and 15 000 *g* for 30 min, and the supernatant was used for determination of enzyme activities. GS (EC 6.3.1.2) and GOGAT (EC 1.4.1.14) activities were determined following the method of Lin and Kao [19]. The activity of GDH (EC 1.4.1.2) was assayed according to Singh and Srivastava [20].

Analysis of free amino acids

Free amino acids levels were determined by the method of Aurisano et al. [21] with slight modifications. The dried leaves powders (0.5 g) were homogenized with 2% sulphosalicylic amino acid at the w/v ratio of 1:5 (pH 2.0). The mixture was centrifuged at 10 000 *g* for 15 min at 4°C. The amino acids levels of supernatant were determined with an amino acid analyzer (Hitachi 835-50, Japan).

Analysis of net photosynthetic rate

The net photosynthetic rate was measured using the portable photosynthesis system (LI-6400, LI-COR Inc., USA) in the third fully expanded leaves. The external CO_2 concentration remained at 380 ±10 µmol mol^{-1} and the light intensity was consistent at 1000 µmol photons m^{-2} s^{-1}.

Analysis of photosynthetic nitrogen-use efficiency (PNUE)

The PNUE was assayed according to the method of Yuan et al. [18]. Fine-ground leave samples of 0.5 g were digested with H_2SO_4–H_2O_2 at 260–270°C, and total N content was determined with Kjeltec 2300 (Foss, Sweden, Germany). Leaf organic N content was obtained through the subtraction of total leaf N and nitrate content. PNUE was calculated as net photosynthetic rate per unit leaf organic N content.

Analysis of carbohydrate contents

The sugars and starch contents in the leaves of wucai were extracted using dry samples (50 mg). Dry samples were boiled in 80% ethanol (v/v) three times. The supernatant was used to determine the contents of total soluble sugar, sucrose, and fructose with a modified phenol–sulphuric acid method [22]. Insoluble residue was washed several times and dried, and then the supernatant was used to determine the starch content.

Analysis of carbohydrate-metabolizing enzyme activities

The third fully expanded wucai leaves were got to determine the enzymes activities involved in carbohydrate metabolism. Sucrose phosphate synthase (SPS; EC 2.4.1.14), sucrose synthase (SuSy; EC 2.4.1.13), and acid invertase (AI; EC 3.2.1.26) were extracted at 4°C according to the method of Lowell et al. [23]. SPS activity for sucrose synthesis was assayed followed the Bird et al. [24]. The reaction mixtures contained 5 mM $MgCl_2$, 7.5 mM UDP-glucose, 7.5 mM fructose-6-phosphate, 50 mM HEPES–NaOH (pH 7.5), and 20 µL of extracted crude enzyme. SuSy activity for sucrose cleavage was measured with the method of Morell and Copeland [25]. Reaction mixtures contained 100 mM HEPES–NaOH (pH 7.5), 100 mM sucrose, 2 mM UDP, 0.025 U UDP-glucose dehydrogenase, 1.5 mM NAD^+, and 20 µL of extracted crude enzyme. The AI activity was measured by the method of Arai et al. [26]. The reaction mixtures contained 100 mM sodium acetate (pH 4.8), 100 mM sucrose, and 100 µL of extracted crude enzyme. The amylase activities were measured using the α-Amylase Assay Kit and β-Amylase Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), respectively, according to the manufacturer's instructions.

Analysis of quantitative real-time PCR

Total RNA was extracted using an RNAprep Pure Plant Kit (Tiangen, China) and isolated RNA was subjected to reverse transcription using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan). qRT-PCR amplification reactions were performed using an iCycler iQ thermo cycler (Bio-Rad, USA) and a SYBR Green kit (Bio-Rad). The specificity of the reactions was verified by melting curve analysis. The relative mRNA level for each gene was calculated as $\Delta\Delta C_T$ values. The actin gene was used as internal control for normalization. The genes sequences were cloned and the primers were designed according to the results. Primers for fragment amplification were as follows: *BccrNR*: 5′-GGGTCGTCTTCCTGATGCTA-3′ and 5'-TCCTTCTCACCGCTCAAGAG-3'; *BccrSPS*: 5'-GACTCATCCTTCAGCGAGGA-3' and 5′-AGCCAACTCCATGTAAGCCT-3′; *BccrSuSy*: 5′-AGCCGTTACTTCGTTGAGGA-3′ and 5′-CTCGGACATGTCAGCTGTTG-3′; *BccrGS*: 5′-TCCAGGATTCCGATTCCACC-3′ and 5′-GCTTTCCAGTAACCCGAACC-3′; *BccrGDH*: 5′-GCAGTAAGCGACATCACAGG-3′ and 5′-TGGATGGTTTGCAGCCTCTA-3′; *BccrAI*: 5′-TGGCTCTCTCGTCATGTTGT-3′ and 5′-CCGATTGTGATCCGCCATTT-3′; *BccrAmy-α*: 5′-GTCGGAATGGATGAACTGGC-3′ and 5′-AGTGTTTAAGACCCGAGCGA-3′; *BccrAmy-β*: 5′-ACGAAGCTCAGGGAAAGACA-3′ and 5′-TCTCGTCGGAAACTGAACCA-3′.

Statistical analysis

The data were statistically analyzed using SAS software (SAS Institute, USA) and Duncan's multiple range test at $p < 0.05$ level of significance.

Results

Plant morphology

In the present study, plant height in WS-1 (heat tolerant) was remarkably decreased, whereas it was increased in WS-6 (heat sensitive) to its control (Tab. 1). Compared to their respective controls, the stem diameter, fresh and dry weight of WS-1 were significantly declined by 10.47%, 8.37%, and 44.29% under heat stress, respectively; the above indexes were decreased by 11.87%, 17.67%, and 57.14% in WS-6. The declined extent of WS-6 was higher than WS-1 by heat. Under normal condition, the plant height of WS-6 was significantly higher than that of WS-1, while the stem diameter, fresh and dry weight had no change between the two genotypes.

As shown in Tab. 1, heat stress resulted in decreases of blade length and width in WS-1, whereas it caused an increase in blade length and a decline in blade width of WS-6. Compared to their respective controls, the petiole length of WS-1 and WS-6 had no changes between normal and heat stress. The petiole width of WS-1 and WS-6 were decreased by 16.88% and 19.4%, respectively, by heat stress to respective controls. And the blade length and width of WS-1 were larger than in WS-6 in normal temperature.

Tab. 1 Effects of heat stress on plant morphology in two wucai genotypes.

	Cont-WS-1	Cont-WS-6	HT-WS-1	HT-WS-6
Plant height (cm)	12.50 ±0.06 ^{c}	13.10 ±0.21 ^{b}	10.27 ±0.62 ^{d}	14.57 ±0.22 ^{a}
Stem diameter (mm)	4.68 ±0.14 ^{a}	4.55 ±0.06 ^{a}	4.19 ±0.06 ^{b}	4.01 ±0.04 ^{b}
Fresh weight (g plant^{-1})	8.96 ±0.15 ^{a}	9.00 ±0.21 ^{a}	8.21 ±0.25 ^{b}	7.41 ±0.13 ^{c}
Dry weight (g plant^{-1})	1.40 ±0.09 ^{a}	1.33 ±0.05 ^{a}	0.78 ±0.01 ^{b}	0.57 ±0.04 ^{c}
Blade length (cm)	8.24 ±0.08 ^{b}	7.83 ±0.15 ^{c}	7.38 ±0.11 ^{d}	8.98 ±0.09 ^{a}
Blade width (cm)	6.38 ±0.06 ^{a}	6.17 ±0.10 ^{b}	6.12 ±0.11 ^{b}	5.67 ±0.08 ^{c}
Petiole length (cm)	5.97 ±0.12 ^{bc}	6.85 ±0.14 ^{a}	5.64 ±0.24 ^{c}	6.70 ±0.35 ^{ab}
Petiole width (cm)	0.77 ±0.04 ^{a}	0.67 ±0.02 ^{b}	0.64 ±0.02 ^{b}	0.54 ±0.03 ^{c}

Values are represented as the mean ±*SE* ($n = 3$). Different letters in the same column indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests. The fourth fully expanded leaves from center were sampled for analyses leaf traits.

Leaf anatomical characteristics

Under normal conditions, the mesophyll of both wucai leaves consisted mainly of elongated columnar palisade mesophyll and a smaller proportion of spongy mesophyll that are irregularly shaped, thereby allowing CO_2 and O_2 to circulate through abundant air spaces (Fig. 1). After heat stress, leaf structure became loose and disordered in two genotypes (Fig. 1c,d). The intercellular spaces among the mesophyll cells were larger and palisade mesophyll was observed to be more separated and thinner relative to control in WS-1. Similar changes were more apparent in WS-6 than WS-1. The boundary between the palisade and spongy tissue became blurred.

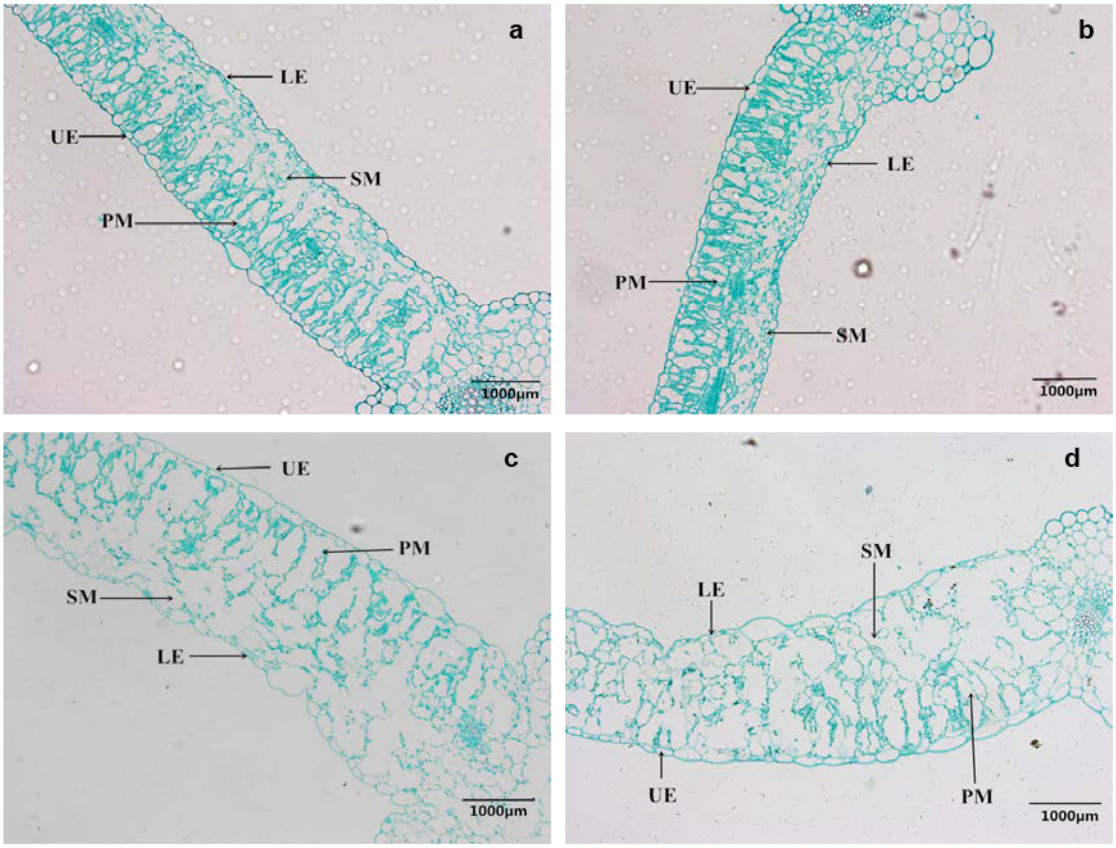

Fig. 1 Effects of heat stress on leaves anatomy of two wucai genotypes exposed to heat stress for 5 days. The pictures were taken using the third fully expanded leaf, numbered basipetally. **a** Cont-WS-1. **b** Cont-WS-6. **c** HT-WS-1. **d** HT-WS-6. UE – upper epidermis; PM – palisade mesophyll; SM – spongy mesophyll; LE – lower epidermis.

Furthermore, in leaf paradermal sections of WS-1 and WS-6 genotypes serially cut from the upper to the lower epidermis, the palisade mesophyll, spongy mesophyll, and entire leaf lamina of the stressed leaves appeared to had undergone a different change trend in thickness (Tab. 2). In WS-1, the increase of thickness was determined to be 24.66% for the upper epidermis, 14.83% for palisade mesophyll, 42.06% for the spongy mesophyll, and 22.31% for entire leaf lamina by heat, respectively, as to control. The thickness of upper epidermis and palisade mesophyll in WS-6 were decreased by

Tab. 2 Effects of heat stress on leaves anatomical characters in two wucai genotypes.

	Cont-WS-1	Cont-WS-6	HT-WS-1	HT-WS-6
Thickness of upper epidermis (μm)	169.73 ±1.75 ^{b}	152.46 ±5.29 ^{b}	211.58 ±9.34 ^{a}	137.01 ±3.32 ^{c}
Thickness of palisade mesophyll (μm)	1319.46 ±45.93 ^{b}	1257.59 ±64.79 ^{b}	1515.12 ±49.60 ^{a}	939.79 ±39.12 ^{c}
Thickness of spongy mesophyll (μm)	766.96 ±34.85 ^{c}	790.02 ±17.79 ^{c}	1089.57 ±28.91 ^{b}	1268.13 ±28.06 ^{a}
Thickness of lower epidermis (μm)	145.42 ±5.05 ^{a}	110.64 ±6.11 ^{bc}	121.10 ±2.41 ^{b}	102.69 ±4.48 ^{c}
Leaf thickness (μm)	2401.57 ±50.85 ^{b}	2310.71 ±51.94 ^{b}	2937.37 ±85.68 ^{a}	2447.63 ±49.23 ^{b}
PM/SM (%)	172.94 ±11.50 ^{a}	159.65 ±11.37 ^{ab}	139.05 ±2.55 ^{b}	74.17 ±3.40 ^{c}
CTR (%)	54.94 ±1.41 ^{a}	54.35 ±1.62 ^{a}	51.57 ±0.50 ^{a}	38.38 ±1.07 ^{b}
SR (%)	31.94 ±1.29 ^{c}	34.25 ±1.42 ^{bc}	37.11 ±0.33 ^{b}	51.82 ±0.91 ^{a}

Values are represented as the mean ±*SE* ($n = 3$). Different letters in the same column indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests. CTR – cell tease ratio; SR – spongy ratio.

10.13% and 25.27%, respectively, while the lower epidermis and leaf thickness were not influenced. Additionally, the wucai plants grown under heat-stress conditions exhibited significant decreases in the PM/SM ratio and WS-6 had a smaller ratio. Compared to control, the CTR of WS-6 was remarkably decreased by 29.40% under heat-stress condition. In contrast, heat stress caused increases of SR in both genotypes, which were 16.17% and 51.31% in WS-1 and WS-6, respectively.

Nitrate and ammonium contents

The wucai plants grown heat-stressed conditions went through remarkable declines of NO_3^- and NH_4^+ contents in WS-1 and WS-6 (Fig. 2). For the NO_3^- content, the decreases by heat were 20.09% and 26.12% in WS-1 and WS-6, respectively. Similar results were found in NH_4^+ content; the decreases of NH_4^+ content in WS-1 and WS-6 were 28.70% and 46.64%, respectively, compared to their controls. The NO_3^- and NH_4^+ contents of WS-1 were significantly higher than that of WS-6 under normal conditions.

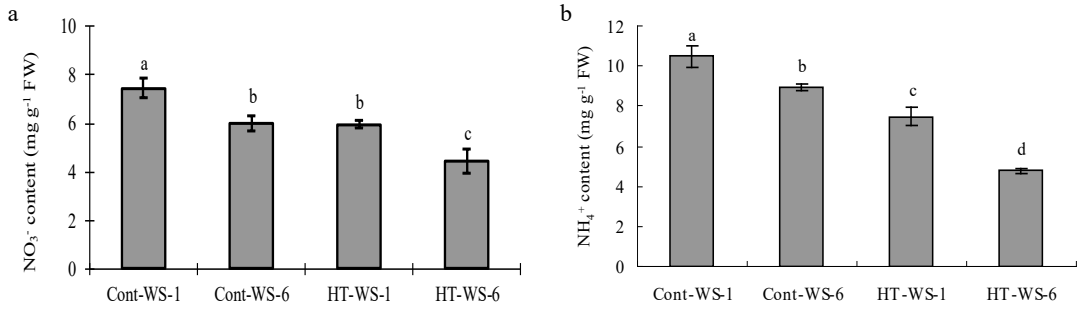

Fig. 2 Effects of heat stress on leaves NO_3^- and NH_4^+ contents of two wucai genotypes exposed to heat stress. The data represent the mean ±*SE* (n = 3). Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

NR activity

Under heat stress, the NR activities showed significant decreases by 16.20% and 33.52% in WS-1 and WS-6, respectively, compared to the controls (Fig. 3). The decrease extent of WS-6 was remarkable higher than WS-1. Under normal conditions, the NR of WS-1 exhibited a higher activity than that of WS-6.

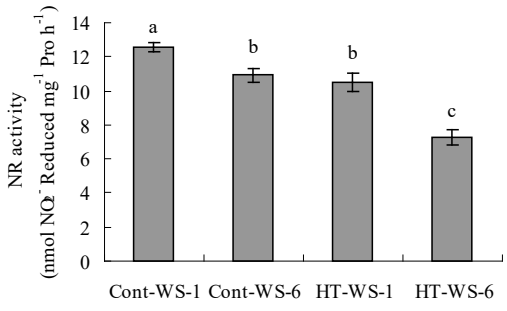

Fig. 3 Effects of heat stress on leaves NR activities of two wucai genotypes exposed to heat stress. The data represent the mean ±*SE* (n = 3). Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

GS, GOGAT, and GDH activities

Significant declines in the GS activities of WS-1 and WS-6 were found in wucai genotypes under heat stress (Fig. 4a). Decreased GS activities in WS-1 and WS-6 were showed to be 29.97% and 38.06% compared to their respective control. Similarly, GOGAT activities of WS-1 and WS-6 were also markedly inhibited by heat stress (Fig. 4b). The reduction of GOGAT in WS-6 was greater than in WS-1.

In contrast, the GDH activities exhibited increase trends in both wucai genotypes under heat stress to the control (Fig. 4c). They were increased by 26.00% and 24.67%, respectively. WS-1 stressed by heat got the highest value. Under normal condition, GS and GDH activities of WS-1 were remarkably higher than WS-6.

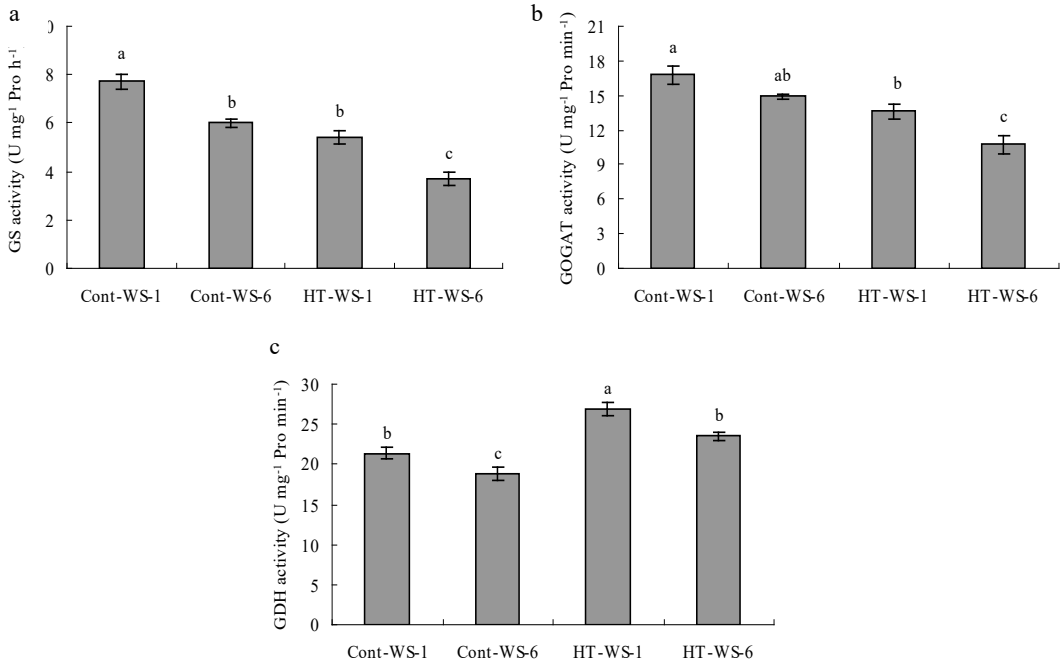

Fig. 4 Effects of heat stress on leaves GS, GOGAT, and GDH activities of two wucai genotypes exposed to heat stress. The data represent the mean ±SE (n = 3). Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

Amino acids levels

The level of total amino acids in leaves of WS-1 and WS-6 showed different changes (Tab. 3). Heat stress had no significant effect on total amino acids level in WS-1, while caused a decrease in WS-6. In WS-1, Ser, Cys, His, and Pro levels were differently increased with respect to control under heat stress, whereas Ala, Met, and Arg exhibited declined levels. Asp, Ser, Ala, Met, Leu, and Arg levels in WS-6 were reduced by heat as compared to the control. Only the Pro level was significantly increased in WS-6. And the rest of amino acids levels had no change under heat stress.

Net photosynthetic rate and PNUE

Heat stress resulted in significant declines in photosynthetic rate as compared to controls in both wucai genotypes (Fig. 5a). The photosynthetic rate was reduced by 27.21% in WS-1 and 43.75% in WS-6 as to its control. The PNUE showed similar trends in WS-1 and WS-6 under heat stress (Fig. 5b). They were significantly decreased by 28.45% and 30.00%, respectively, to the controls in WS-1 and WS-6. Reductions of two indexes in WS-6 under heat stress surpassed that of WS-1. Under normal conditions, there was no significant difference in PN between WS-1 and WS-6, while the PNUE in WS-6 was lower than WS-1.

Carbohydrate contents

The total soluble sugars content was significantly elevated in WS-1 exposed to heat stress compared to control (Fig. 6a), whereas it was not changed in WS-6 under both heat stress and control treatments. Compared to their respective controls, the sucrose content in WS-1 was markedly increased by 18.35% under heat stress; it showed the converse trend in WS-6, decreased by 21.89% (Fig. 6b). Heat stress significantly reduced the fructose content of WS-6 by 15.78% to control; its content was not affect in WS-1 (Fig. 6c). Under heat stress, the starch contents of WS-1 and WS-6 were both reduced, and the reductions were 12.22% and 12.02%, respectively (Fig. 6d). Exception for higher

Tab. 3 Effects of heat stress on leaves amino acids contents in two wucai genotypes.

	Cont-WS-1	Cont-WS-6	HT-WS-1	HT-WS-6
Asp	4.05 ±0.16 [a]	3.60 ±0.11 [a]	3.96 ±0.10 [a]	2.83 ±0.29 [b]
Thr	1.88 ±0.09 [a]	1.40 ±0.11 [b]	1.91 ±0.03 [a]	1.32 ±0.15 [b]
Ser	2.57 ±0.17 [b]	2.40 ±0.13 [b]	3.04 ±0.06 [a]	1.82 ±0.17 [c]
Glu	5.24 ±0.28 [a]	3.97 ±0.28 [b]	5.17 ±0.15 [a]	3.85 ±0.37 [b]
Gly	1.15 ±0.06 [a]	0.82 ±0.07 [b]	1.27 ±0.03 [a]	0.80 ±0.08 [b]
Ala	1.82 ±0.02 [a]	1.31 ±0.06 [c]	1.57 ±0.02 [b]	1.07 ±0.05 [d]
Cys	1.13 ±0.01 [b]	1.03 ±0.01 [c]	1.19 ±0.01 [a]	1.03 ±0.01 [c]
Val	2.08 ±0.07 [a]	1.49 ±0.11 [b]	1.95 ±0.03 [a]	1.40 ±0.14 [b]
Met	1.68 ±0.03 [a]	1.66 ±0.01 [a]	1.31 ±0.14 [b]	0.77 ±0.06 [c]
Ile	1.46 ±0.04 [a]	0.96 ±0.08 [b]	1.40 ±0.01 [a]	0.88 ±0.11 [b]
Leu	3.01 ±0.09 [a]	2.72 ±0.08 [a]	2.86 ±0.03 [a]	2.00 ±0.24 [b]
Tyr	1.45 ±0.01 [a]	1.00 ±0.08 [b]	1.58 ±0.06 [a]	0.96 ±0.11 [b]
Phe	3.19 ±0.05 [a]	2.76 ±0.15 [b]	3.41 ±0.01 [a]	2.66 ±0.17 [b]
Lys	3.11 ±0.06 [a]	2.32 ±0.19 [b]	3.01 ±0.02 [a]	2.16 ±0.24 [b]
His	0.92 ±0.01 [b]	0.72 ±0.05 [c]	1.14 ±0.03 [a]	0.71 ±0.07 [c]
Arg	2.55 ±0.07 [a]	2.15 ±0.08 [b]	2.18 ±0.06 [b]	1.60 ±0.08 [c]
Pro	1.29 ±0.04 [c]	1.29 ±0.01 [c]	1.75 ±0.04 [a]	1.53 ±0.04 [b]
Total	38.59 ±1.12 [a]	31.53 ±0.92 [b]	38.69 ±0.68 [a]	27.41 ±1.09 [c]

Values are represented as the mean ±*SE* ($n = 3$). Different letters in the same column indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

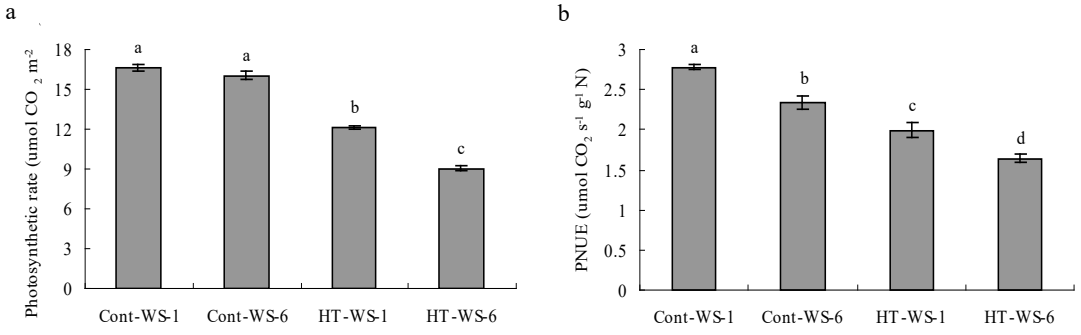

Fig. 5 Effects of heat stress on leaves photosynthetic rate and PNUE of two wucai genotypes exposed to heat stress. The data represent the mean ±*SE* ($n = 3$). Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

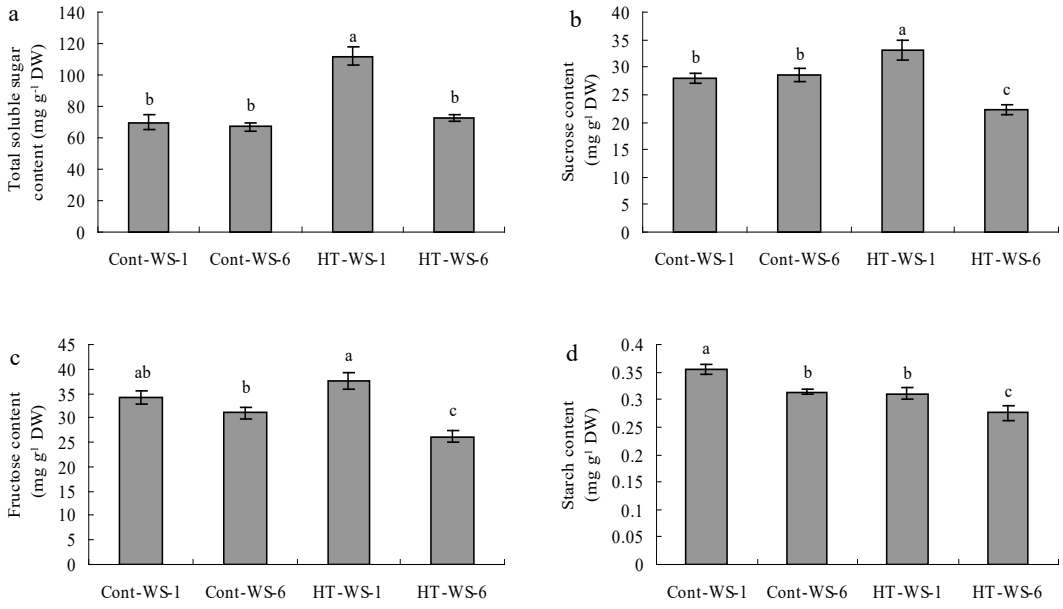

Fig. 6 Effects of heat stress on leaves carbohydrate contents of two wucai genotypes exposed to heat stress. The data represent the mean ±*SE* ($n = 3$). Different letters indicate significant differences at $p < 0.05$ according to Duncan's multiple range tests.

starch content in WS-1, the total soluble sugar, sucrose and fructose contents had no significant change under normal conditions in two genotypes.

Carbohydrate-related enzyme activities

Under heat stress, SPS activity in WS-1 was not affected, whereas it was decreased by 14.07% in WS-6 compared to controls (Fig. 7a). The SPS activity of WS-6 was lower than WS-1 in normal condition. Compared to controls, SuSy activities in WS-1 and WS-6 were significantly inhibited by heat, and the decreases of activities were 16.19% and 25.21%, respectively (Fig. 7b). The amylase activities of both genotypes showed the similar declined trends, i.e., reduction by 10.64% and 24.12%, respectively (Fig. 7d). The reduction of SuSy and amylase in WS-6 was greater than in WS-1. In contrast, heat stress resulted in an increase of AI activity in WS-1 and a decrease in WS-6 (Fig. 7c). Under normal conditions, the SuSy, AI and amylase activities had no difference between WS-1 and WS-6.

Expression of nitrogen–carbon enzymes-related genes

RT-PCR was used to analyze the transcript levels of eight enzymes-related genes involved in the nitrogen–carbon metabolism in two genotypes under heat stress (Fig. 8). Heat stress significantly inhibited the NR genes expression in two genotypes (Fig. 8a). The *GS* and *GDH* genes expression were increased in WS-1 by 26.35% and 42.95%, respectively, compared to control (Fig. 8b,c). In WS-6, *GS* expression was declined and *GDH* was increased with respective to control. *SPS* expression was not affected in WS-1 under heat stress, whereas it was decreased by 32.62% in WS-6 compared to control (Fig. 8d). Heat stress remarkably inhibited *SuSy* genes expression in two wucai, expression level of WS-6 was lowest among four treatments (Fig. 8e). Similar to *GS*, *AI* expression was increased in WS-1 and decreased in WS-6 (Fig. 8f). Expression levels of *Amylase-α* and *β* in two wucai were obviously declined by heat (Fig. 8g,h).

Under normal condition, expression levels of *NR*, *GDH*, *SPS*, and *Amylase-α* were significantly lower in WS-6 compared to WS-1.

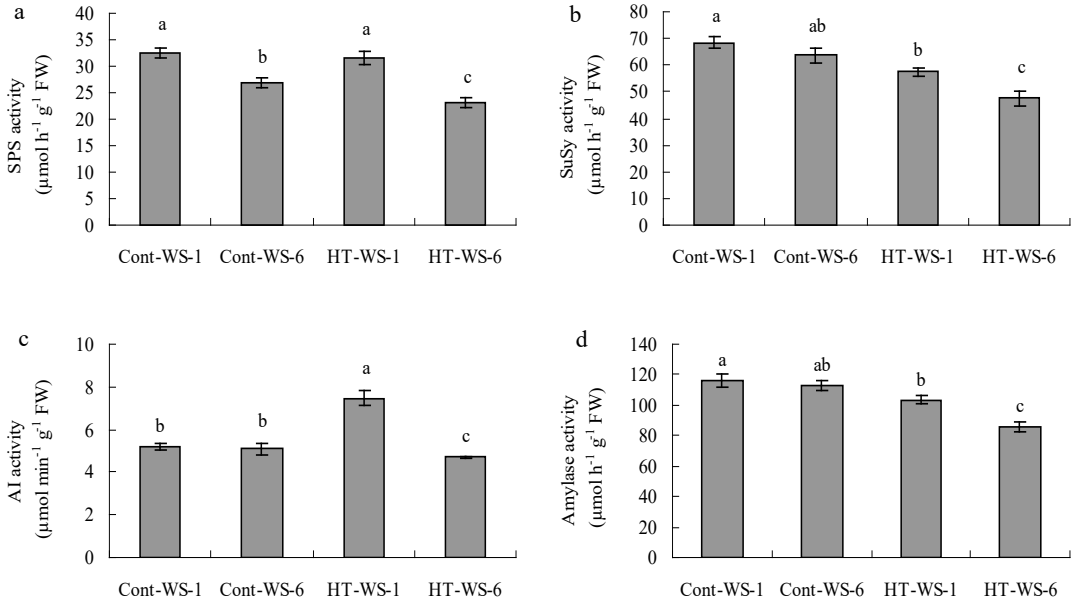

Fig. 7 Effects of heat stress on leaves SPS, SuSy, AI, and amylase activities of two wucai genotypes exposed to heat stress. The data represent the mean ±*SE* ($n = 3$). Different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests.

Discussion

The present study focused on the response of plant morphology, leaves anatomy characteristics, nitrogen and carbohydrate metabolism in wucai exposed to high temperature. For this, we used seedlings of two wucai genotypes with different sensitivity to heat stress: WS-1 (tolerant to heat) and WS-6 (sensitive to heat). Our results showed a greater biomass loss in WS-6 compared to WS-1 and a similar inhibition effect was found at the leaf anatomy and nutrients metabolism under heat stress.

In our study, heat stress significantly hampered the shoot growth and foliage expansion in WS-1 (Tab. 1), which was consistent with the previous report in mung bean and wheat [27,28]. The reduction of WS-1 was apparently lower than in WS-6. The suppression of plant growth might be, at least partly, due to the disorder of nitrogen assimilation and carbohydrate metabolism. However, the plant height and blade length in WS-6 were promoted under heat stress. It might be due to the stress avoidance strategy by decreasing the leaf area to reduce the absorption of solar energy.

Since leaves are the main organs of internal water removal and photosynthates synthesis, leaf structural aspects play a crucial role in acclimation to the external conditions. And heat-stressed wucai undertook leaf anatomical alterations to respond to the temperature stress (Tab. 2). Especially the ratios of PM/SM in two genotypes showed obvious reductions exposed to heat stress. In fact, WS-1 showed a thicker upper epidermis, palisade mesophyll, and leaf lamina than WS-6 under heat stress (Fig. 1). A thicker upper epidermis and palisade mesophyll may enhance survival and growth in WS-1 by improving water relations and providing higher protection for the inner tissues [29]. Since leaf thickness is an indicator of higher assimilation of photosynthates, thicker leaflets under heat stress in WS-1 might indicate a better CO_2 fixation [30]. Under heat stress, CTR of WS-1 was remained relatively consistent to control, indicating the higher cell structure tightness of WS-1 against temperature stress. In our study, the microstructure of the leaf in WS-6 showed the rupture of palisade tissue cells and the larger intercellular spaces were the main reasons for the decreased CTR and increased SR values.

Nitrogen is the most important nutrient for plant growth, and nitrate and ammonium are the major sources of nitrogen in higher plants. In our study, the NO_3^- and NH_4^+ contents were significantly decreased in WS-1 and WS-6 under heat stress, and WS-6 had the lowest contents of NO_3^- and NH_4^+ (Fig. 2). This result was reflected by remarkable declined NR activity (a pivotal enzyme that catalyzes NO_3^- reduction to

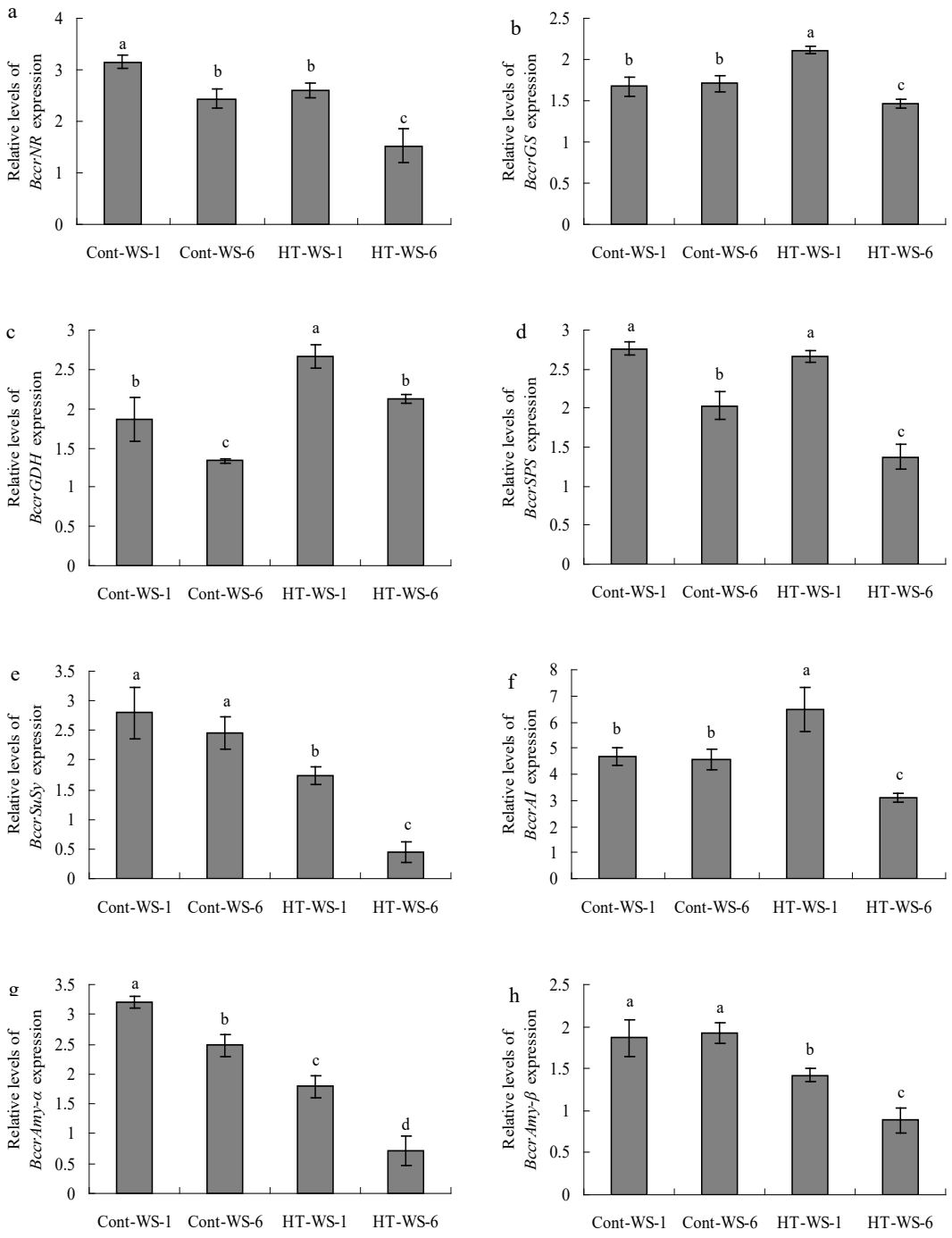

Fig. 8 Effects of heat stress on the key gene expression of nitrogen–carbon metabolism (*BccrNR* nitrate reductase, *BccrGS* glutamine synthetase, *BccrGDH* glutamate dehydrogenase, *BccrSPS* sucrose phosphate synthase, *BccrSusy* sucrose synthase, *BccrAI* acid invertase, *BccrAmy-α* amylase α, *BccrAmy-β* amylase β) in two wucai genotypes. The data represent the mean ±*SE* ($n = 3$). Different letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range tests.

NO_2^-) (Fig. 3), which was due to the decreased transcription level of *NR* (Fig. 8a). The reduced content of NH_4^+ might be associated with the GS, GOGAT, and GDH activities (Fig. 4), which were mainly involved in NH_4^+ assimilation. NH_4^+ is rapidly assimilated into organic N by the GS/GOGAT cycle and GDH alternative pathway. The present work found that when the GS and GOGAT activities were partly inhibited in two wucai genotypes, the GDH activity was markedly enhanced (Fig. 4), consistent with GDH gene expression level (Fig. 8c). This result indicated NH_4^+ assimilation pathway had shift from the normal GS/GOGAT cycle to GDH pathway in two wucai under heat stress. The decreased degree of GS/GOGAT activities in WS-1 was less than WS-6. The

transcript levels of GS in WS-1 were elevated by heat (Fig. 8b), whereas the activity of GS was decreased. The difference between the gene expression and enzyme activity results suggests enzyme activity changes were not only caused by mRNA level, but were also regulated at the post-transcription level and were influenced by cellular metabolism, such as ROS attack [31,32]. These results exhibited the heat-tolerant genotype WS-1 represented a better performance in nitrogen assimilation, which enhanced the nitrogen transformation.

Nitrogen metabolisms are tightly coordinated in some fundamental processes, including nitrogen uptake and photosynthesis [33,34]. Photosynthetic rates were remarkably declined in two wucai leaves (Fig. 5a), which were recognized as sensitive to heat stress. Although heat stress caused a reduction in photosynthetic rate in two wucai, the tolerant WS-1 could still maintain higher photosynthetic rate than the sensitive genotype WS-6. The inhibited photosynthesis may be as a main result of reduced carbon fixation and assimilation [35] and nitrogen absorption. Photosynthetic rate per unit leaf nitrogen of dry weight, considered as photosynthetic nitrogen-use efficiency, namely PNUE, is used to represent the nitrogen use efficiency in leaves [18]. In our study, the PNUE were significantly reduced by heat in WS-1 and WS-6 (Fig. 5b). The reduction of PNUE in WS-6 was greater than in WS-1, which revealed a poorer capacity of nitrogen assimilation to synthesize amino acids in WS-6 (Tab. 3).

According to our results, heat stress markedly decreased total amino acids content in WS-6 with respect to controls, whereas it had no influence in WS-1 (Tab. 3). The amino acids are the main components of osmolytes in higher plant, which are used by cells to maintain turgor pressure, the structural integrity of enzymes and membranes. In WS-1, Pro levels were enhanced by heat stress, which is considered as a carbon and nitrogen source, a membrane stabilizer, and free radical scavenger, and play the pivotal role in stress tolerance [36]. Interestingly, although most of amino acids levels of WS-6 were decreased, the proline level was higher than in control. Thus, the elevated accumulation of proline was attributed to increase the resistance against stress.

Soluble carbohydrates are also considered important factors related to stress in plants. It has recently been proposed that soluble carbohydrates are involved in the ROS balance and response to oxidative stress in plants [37,38]. In our experiment, the total soluble sugar content was significantly increased in WS-1 by heat stress. This response could indicate that accumulation of total soluble carbohydrate was related to tolerance to high temperature in WS-1, whereas in WS-6 no significance was detected. According to present results, the structural changes in leaves of heat-stressed wucai were not conducive to sucrose metabolism, which was evident from remarkable decreases in the enzyme activities that synthesized sucrose, including SPS, SuSy, and AI (Fig. 7a–c). These decreased enzymes activities might be due to reduction of protein synthesis, regulated by decreased transcription levels (Fig. 8d–f). Enzymes such as SPS, SuSy, and AI work in combination to synthesize and hydrolyze sucrose to provide hexoses for various structural and functional requirements such as energy generation or synthesis of macromolecules such as starch [39]. Our observations on these enzymes suggested that sucrose was utilized faster in the leaves (as indicated by high AI activity), compared to its synthesis (as indicated by low SuSy activity), resulting in its rapid depletion to affect the vegetative biomass in WS-1 under heat stress. A decrease in SuSy activity and an increase in AI activity have also been observed in chickpea subjected to a combination of heat stress [40]. In our study, high temperature resulted in drastic reduction in sucrose in WS-6 (Fig. 6b), which might limit the reproductive function and seed development [41,42]. The activities of starch metabolizing enzymes were affected in both wucai genotypes by heat stress, but remarkable differences were found in WS-1 and WS-6 (Fig. 7d). The sharply decreased amylase activities account for decreases in starch content in WS-6. In addition, higher SPS activity under heat stress might contribute to a carbon gradient flux from starch to sucrose [43], causing accumulation of sucrose in WS-1, which was consisted with the report of Phan et al. [44]. Products from degraded starch by SuSy and AI were exported to cytosol and were again converted to sucrose via SPS [45]. The rise in sucrose content in WS-1 might trigger rapid recycling of stored carbon from a source to a sink. In heat-sensitive WS-6, the inactivation of enzymes activities and reduction of mediates inhibited the starch-to-sucrose mobilization in heat stress, which might be from the structural limitation of leaf anatomy and decline in photosynthetic capacity (Fig. 1 and Fig. 5).

Conclusions

Our results demonstrate that under heat stress heat-tolerant WS-1 showed better performance in leaf morphology, i.e., thicker leaf lamina and more integrated cell arrangement which improved the photosynthetic capacity, compared to the heat-sensitive WS-6. The tolerant capacity of WS-1 was attributed to stronger growth, better nutrient absorption capacity and matter accumulation. Additionally, although the nitrogen and carbon assimilation in WS-1 were partly affected by heat, the reduction extent of WS-1 was obviously lower than WS-6, which suggest a higher resistance to heat stress. Thus, WS-1 used in this study might be further utilized for breeding heat stress tolerant cultivars.

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