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Physiology and Proteomics of Cabbage Under Heat and Flooding Stress

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Research Article

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ABSTRACT

High temperature and excess rain, which can lead to heat and flooding stresses, seriously impact the yield and quality of cabbages. Two cabbage cultivars were examined in this study: heat- and flooding-tolerant 'Sha-sha-jieu' and heat-tolerant but flooding-sensitive 'Mi-ni'. The goals of this study were to investigate leaf proteomic and physiological changes in plants responding to treatments of high temperature, flooding, and both stresses combined. Forty-five-day-old cabbage plants at temperatures of 22°C or 40°C were treated separately with or without flooding in growth chambers for 0, 6, 12, 24, 48, and 72 h. Treatment at 22°C without flooding was used as the control. Changes in stomatal conductance and chlorophyll fluorescence of stressed leaves indicate that 'Mi-ni' suffered more severely than 'Sha-sha-jieu'. Separated by 2-dimensional liquid phase fractionation, 25 and 26 expressed protein spots were extracted from the stressed leaves of 'Mi-ni' and 'Sha-sha-jieu', respectively. Most of the differentially expressed proteins identified by matrix-assisted laser desorption/ ionization-time of flight mass spectrometry were involved in photosynthesis and ATP synthesis. The most highly expressed proteins were ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), oxygen evolving enhancer (OEE), and chloroplastic ATP synthase. However, both serine/threonine protein phosphatase 7 inactive homolog and entatricopeptide repeat-containing protein At1g79540 exhibited relatively lower expression under high temperature and flooding. In response to high temperature and flooding, proteins such as RubisCO, OEE protein 1, and chloroplastic ATP synthase generally increased, indicating that the regulation of energy production is critical for tolerating heat and flood stressing in cabbages.

INTRODUCTION

Cabbage (*Brassica oleracea* L. var. capitata L.) is a member of the genus bassica and the mustard family, Brassicaceae. It is a multi-layered annual cole crop grown for its dense-leaved heads. Heat and flooding, two of main abiotic stresses for *Brassica*, strongly impact the quality and quantity of cabbage production. Optimal temperatures for cabbages are 20°C in the daytime and 15°C at night; extended periods of higher temperatures result in loose or bolting heads, smaller and tighter heads, and tougher leaf texture [1]. Flooding for 24 h at various growth stages impairs cabbage growth, slows down plant width growth, lowers head quality, and significantly reduces production. The diminished production of flooded cabbages might correspond to slow development of the outer leaves, or whole-plant width, which is considered to be where photosynthetic carbon assimilation mainly occurs once cabbage enters the heading stage [2]. As global warming and climate change progresses, weather extremes in rain and heat are no longer rare, making it important and urgent to develop heat- and flood-tolerant cultivars.

Little is known about how plants respond to multiple co-occurring abiotic stresses at a proteomic level [3]. Multiple simultaneous abiotic stresses, such as heat and water, are often very lethal to crops [4]. Plant physiology is altered along with

the activation of stress proteomes in order to adapt to environmental stresses. The study of stress proteomes can help us to further understand the molecular mechanisms of how plants sustain and cope with stresses. However, most proteomic analyses conducted on various plant species have focused on only one stress at a time. These studies include heat stress on alfalfa, barley, radish, rice, wheat, and Chinese herb, *Pinellia ternata*; and flooding stress on maize, tomato, rice, and soybean [5-13]. Heat stress-related proteins or flood stress-related proteins are involved in a broad range of biological and physiological processes, including ATP synthesis, photosynthesis, disease resistance, protein biosynthesis, redox homeostasis, and antioxidant signaling [14]. Proteomic analysis is a powerful approach for revealing differentially expressed proteins under given conditions. Using two-dimensional liquid phase fractionation (PF2D) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), we identified 85 differentially expressed proteins from three cauliflower cultivars under high temperature and/or flood stressing at 0, 6, and 24 h [15]. We then reported that cauliflower predominantly expressed photosynthesis-related proteins responding to co-occurring high temperature and flood stressing. Overall, these proteomic studies of plant responses to one or multiple abiotic stresses suggest that the regulation of energy production is the common strategy for tolerating abiotic stressing.

Taiwan is located in tropical and subtropical regions where daily temperatures that exceed 30°C during summer (June to September) reduce the quality and quantity of cabbage. Moreover, typhoons always bring heavy rains in summer, so flooding is a major risk to fresh-market cabbage production. The long-term goal of our work is to help breed a competitively higher flood- and heat-tolerant cabbage to be grown in lowlands during summer. Comparative proteomic analyses of cabbage plants subjected to flood and heat conditions allow for the exploration of their various defense-related mechanisms. Therefore, identifying novel proteins and studying their differential expression protein patterns in response to temperature and waterlogging stresses will provide the molecular and physiological bases for improving tolerance to both stresses in cabbages. The objectives of this study were to investigate with MALDI-TOF MS the proteomic and physiological changes in cabbage leaves in response to high temperature and flooding stresses under 0, 6, 12, and 24 h treatments. The differentially expressed proteins that we identified in cabbage leaf treatment responses are discussed, and our results provide a basis for understanding cabbage metabolic pathways and their cross-talk under stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of cabbage (*B. oleracea*) cultivars 'Mi-ni' and 'Sha-sha-jieu' were donated by Mr. L.C. Chung (see Acknowledgments) at AVRDC (Vegetable Research and Development Center)-the World Vegetable Center, Tainan, Taiwan. 'Mi-ni' is a moderately high temperature-tolerant but flood-sensitive cultivar that requires optimum growing temperatures (22°C at day and 18°C at night) for satisfactory production. However, 'Sha-sha-jieu' is a high temperature- and flood-tolerant cultivar suitable especially for warm-subtropical regions like southern Taiwan where average daytime temperatures reach as high as 40°C during the summer. Seeds were immersed in distilled-deionized (dd) water in darkness for 24 h and germinated on wetted Whatman filter papers for three days to ensure uniform germination. Seedlings were then transplanted into 12.7-cm diameter plastic pots containing a mixture of peat and moss (4:1, v:v) and placed in a growth chamber under 300 μmol m^{-2}/s^{-1} light with a 16 h photoperiod provided by fluorescent and incandescent light. The temperature was maintained at 22°C in the light and 18°C in the dark, with a relative humidity of 80%. Plants were watered with a half-strength Hoagland solution [16] three times a week to maintain optimal irrigation and growth for 45 days before imposing heat and flood stresses.

Pots of 'Mi-ni' and 'Sha-sha-jieu' plants were divided into four groups and transferred to 22°C without flooding (C, control), 22°C with flooding (F), 40°C without flooding (H), and 40C with flooding (HF) for periods of 0, 6, 12, 24, 48, and 72 h in each of four growth chambers having a 16 h photoperiod at 300 μmol m^{-2} s^{-1} radiation. Forty degrees was selected as the heat stress temperature in this study because heat-tolerant and heat-sensitive cabbages at 42°C for 3 d demonstrate clear heat stress-associated phenotypes with differing levels of severity [17]. Flood treatment pots were randomly placed in 28 × 14 × 14 cm plastic buckets and subjected to flooding by filling the buckets with tap water to 5 cm above the soil surface. Pots were removed from the buckets at different times following flooding, and plants were removed. The third fully expanded leaves from each plant were clipped, frozen in liquid nitrogen, and stored at -80°C in an ultrafreezer until use. Three replicates from each time interval for the four treatments were randomly placed in growth chambers. The experiment was performed twice independently for a randomized design of growth environment, sampling day, and physiological analysis [18].

Measurement of Stomatal Conductance (SC) and Chlorophyll Fluorescence (CF)

Stomatal conductance (SC) of the third fully expanded leaves were measured with an AP4 potometer (Delta-T Devices, Cambridge, England), with calibration according to manufacturer instructions before measurements were taken. The time interval between measurements was 30 s according to manufacturer instructions. A chlorophyll fluorometer (MINI-PAM, Walz, Germany) was used to measure leaf CF. Measurement of the ratio of the yield of the variable fluorescence (Fv)/maximum fluorescence level (Fm) in the leaves is previously described [19]. Briefly, plants were dark-adapted for 30 min prior to measurements. A red excitation light at <0.1 μmol m^{-2} s^{-1} was applied to measure CF in the dark-adapted state, or minimal fluorescence yield F_o. A saturated light with 6000 μmol m^{-2} s^{-1} was then applied for 0.8 s to measure maximal CF yield after the photosynthesis (PS) II reaction, or F_m. The ratio of F_v/F_m was calculated to determine the potential quantum yield of PS II, where $F_v=F_m-F_o$. Data were collected at 0, 6, 12, 24, 48, and 72 h of incubation.

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The data shown in **Tables 1 and 2** are the means of at least three independent sets of experiments with similar results. Measurements of CF and SC were analyzed by analysis of variance (ANOVA) with a completely randomized design. For significant values, means were separated by the least significant difference (LSD) test at $p \leq 0.05$ using PC SAS 8.2 (SAS Institute, Cary, NC, USA).

Table 1. Stomatal conductance (mmol m^{-2} s^{-1}) changes in 'Mi-ni' and 'Sha-sha-jieu' cabbages under different treatments for 3 days.

Cultivar	Treatment	Duration (h)					
		0	6	12	24	48	72
'Mi-ni'	C	115.7 B	44.9 C	111.8 F	114.9 F	116.2 C	118.6 B
	F	111.2 B	53.1 C	193.1 D	160.9 D	147.0 B	123.7 B
	H	112.3 B	127.6 B	218.0 C	176.0 C	42.8 D	18.8 E
	HF	109.1 B	235.4 A	273.8 A	239.7 A	29.9 D	10.3 E
'Sha-sha-jieu'	C	141.7 A	129.9 B	151.9 E	148.7 E	143.3 B	149.7 A
	F	147.8 A	124.2 B	204.7 C	161.6 D	153.1 B	117.3 B
	H	148.1 A	127.5 B	222.9 BC	215.0 B	161.2 A	68.1 C
	HF	143.2 A	139.0 B	243.3 B	221.1 B	178.2 A	49.7 D

Values represent the means of at least three independent sets of measurements with similar results. Means with the same letters are not significantly different within columns using least significant difference (LSD) tests under ANOVA. (C: non-flooding at 22°C , F: flooding at 22°C, H: non-flooding at 40C , HF: flooding at 40°C).

Table 2. Fv/Fm value changes in 'Mi-ni' and 'Sha-sha-jieu' cabbages under different treatments for 3 days.

Cultivar	Treatment	Duration (h)					
		0	6	12	24	48	72
'Mi-ni'	C	0.813 A	0.821 A	0.822 A	0.814 A	0.813 A	0.807 A
	F	0.824 A	0.819 A	0.821 A	0.810 A	0.791 AB	0.777 B
	H	0.815 A	0.730 C	0.727 C	0.724 C	0.679 D	0.401 E
	HF	0.825 A	0.735 C	0.733 C	0.717 C	0.603 D	0.201 F
'Sha-sha-jieu'	C	0.818 A	0.819 A	0.818 A	0.817 A	0.815 A	0.810 A
	F	0.819 A	0.818 A	0.817 A	0.815 A	0.811 A	0.791 B
	H	0.818 A	0.762 B	0.760 B	0.754 B	0.729 B	0.719 C
	HF	0.823 A	0.749 B	0.744 BC	0.740 B	0.701C	0.528 D

Values represent the means of at least three independent sets of measurements with similar results. Means with the same letters are not significantly different within columns using least significant difference (LSD) tests under ANOVA. (C: non-flooding at 22°C, F: flooding at 22°C, H: non-flooding at 40°C, HF: flooding at 40°C)

Protein Isolation and Quantification

Based on the negative effects on 'Mi-ni' plants at 40°C after 48 h of flood stressing, leaves under 6, 12, and 24 h treatments were chosen for proteomic analysis. Proteins were extracted according to a previous report [20]. Briefly, two grams of the leaves with liquid nitrogen added were ground into powder in a mortar and pestle. The powder was placed in 4.8 mL extraction buffer (5 M urea, 2 M thiourea, 50 mM Tris-HCl, 5 mM Tris (2-carboxyethyl) phosphine hydrochloride, 1 mM protease inhibitor, 10% (v/v) glycerol, 2.5% (w/v) N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfonate, and 2% (w/v) N-octylglucoside), and centrifuged for 30 min at 15,000 g at 4°C. The supernatant was mixed with a 3-fold volume of 100% acetone at -20°C for 30 min and centrifuged at 7,000 g at 4°C for 30 min. The supernatant was then discarded and the pellet washed with 100% acetone and centrifuged at 7,000 g at 4°C for 30 min. The pellet in the centrifuge tube was covered with parafilm, which was punctured with small holes, and left to stand at 4C until the acetone evaporated. The washing procedure with the same centrifugation condition was repeated three times. The final product was kept at -80°C until use. Protein concentration was determined using the Bradford method (Protein Assay, Bio-Rad Laboratories, CA, USA), and bovine serum albumin was used as a protein standard.

Pf2d Analysis and Protein Identification by MALDI-TOF MS and Database Searching

The protocol for PF2D has been published in detail [21]. Briefly, a first-dimension high performance chromatofocusing (HPCF-1D) column (Beckman Coulter, CA, USA) was pre-equilibrated with start buffer (20 mM Tris-HCl, pH 8.5). Samples were injected into the 1D column at a flow rate of 0.2 mL per minute, and after eluting with a pH 4 buffer following 1D analysis, 10% isopropanol was applied to wash out the column. For the second-dimension (2D) analysis, fraction separation was performed using a 4.6 × 30 mm nonporous C18 HPRP column (Beckman Coulter), and the temperature was set to 50°C. The column was washed with 0.08% trifluoroacetic acid (TFA) in acetonitrile for 5 min, then with 0.1% TFA in ddH_2O for 10 min at a flow speed of 0.75 mL per min. Samples were washed with 0.1% TFA in ddH_2O and 0.08% TFA in acetonitrile polar and non-polar elution buffer, thus forming an elution gradient. Proteins in the well were separated by hydrophobicity and hydrophilicity, and then examined under 214 nm UV. After the 2D analysis was done, the column was washed with 100% acetonitrile. The 2D graphs were plotted using ProteoVue software (Eprogen, Darien, IL, USA) to illustrate the proteomic gels separated by isoelectric point (pI) and hydrophobicity **(Supplementary Figure S1).** ProteoVue allowed side-by-side viewing of the 2D runs for two samples and was used to compare and quantify the number of differential expressed protein between them. Each band shown in this chromatogram represents a singly separated protein and the relative intensity of the colors is directly proportional to the difference in protein concentration.

To identify differentially expressed proteins, the protein peaks from F, H, and stressed treatments at 6 h, 12 h, and 24 h time points were compared to controls (C), and the up-regulation and down-regulation of the proteins was identified **(Supplementary Figure S2 and S3).**

The eluents were dried under a vacuum and the pellet dissolved in a reducing solution (50 mM ammonium bicarbonate and 10 mM dithiothreitol) for 60 min at 60°C. After reduction, protein solutions were digested at 37°C for 16 h with 125 µg per mL of trypsin. Trypsin digestion was stopped by adding 1% (v/v) formic acid. Protein samples were mixed with 0.1% TFA in 50% acetonitrile containing 5 mg/ml α-cyano-4-hydroxycinnamic acid and further analyzed by MALDI-TOF-MS (4700 Proteomics Analyzer, Applied Biosystems) to generate a peptide mass fingerprint (PMF). After MALDI-TOF analysis, data from the peaks were collected and used to search for potential proteins using MASCOT (http://www.matrixscience.com). MS values obtained from PF2D were run against the NCBI and SwissProt databases with MASCOT using default search parameters. Additional parameters also included: 1 missed cleavage, fixed modification, and peptide charge ± 1, and variable modifications were carbamidomethyl of cysteine and oxidation of methionine. Trypsin was specified as the proteolytic enzyme. Peptide tolerance and MS mass tolerance were 50 ppm and 0.25 Da, respectively. Peptide mass fingerprinting match confidence was based on the MOWSE score and confirmed by accurate overlapping of matched peptides with MS major peaks. Scores greater than 60 ($p<0.05$) were considered positive. Only significant hits, as defined by MASCOT probability analysis ($p<0.05$), were accepted.

RESULTS AND DISCUSSION

Stomatal Conductance (SC) and Chlorophyll Fluorescence (CF) of Cabbage Plants Treated by Stressing

Comparisons of SC in 'Mi-ni' and 'Sha-sha-jieu' under stressed treatments are shown in **Table 1**, and significant changes in SC were observed between and among genotypes under stresses. SC values in the two cultivars dropped in the 6 h treatment under conditions C and F because it occurred within the dark period in the growth chambers. Zero light and lower temperatures close the stomata and decrease SC, thus reducing leaf evaporation. The 12 h treatment resulted in stomata opening gradually, initiating an increase in SC. SC values reached their maximum at 12 h under all stressed treatments, but then dropped significantly. In addition, the SC of 'Mi-ni' was much lower after 24 h under treatments H and HF than treatment F. When flooded, excess water initially causes plants to over-transpire. After a long period of flooding, plant roots cannot aerobically respire to generate energy, thus causing stomata to gradually shut down and degrade chlorophyll, inhibiting leaf photosynthesis and respiration [22,23]. Therefore, SC values are lowered as flooding continues. Treatments H and HF affected SC in both cabbages more than treatments F and C. High air temperatures raise leaf temperatures, which caused SC to increase significantly after the 6 h treatment. Mass evaporation dissipates heat and thus opens the stomata and increases SC. Because of the increase in leaf water vapor pressure, plant evaporation becomes overly active, causing perennial water shortages. The changes in SC in 'Mi-ni' plants under various treatments for 6 h to 24 h were more dramatic than those of 'Sha-sha-jieu' plants, indicating that the evaporation rate of 'Mi-ni' under stress changed more than in 'Sha-sha-jieu', and stress conditions affected stomata opening and closing more significantly in 'Mi-ni'. This means that stress conditions influenced the evaporation and photosynthetic efficiency of 'Mi-ni' greatly, and transpiration in 'Mi-ni' was increased due to stomata being open (wider aperture and less resistance to flow of the stomata, influence fluid loss) under stress. In particularly, SC values of 'Mi-ni' plants under condition HF from 6 h to 24 h were significantly higher than in 'Sha-sha-jieu' plants, indicating that transpiration in 'Mi-ni' was increased due to stomata being open under flooding at 40°C. Most leaves of 'Mi-ni' were progressively necrotic, epinastic, or wilted over the course of time; however, leaves of 'Sha-sha-jieu' visually were still green after 24 h of stressing. As treatments H and HF progressed, plants became withered and decayed. During the 48 h to 72 h treatment periods, more 'Mi-ni' plants died than 'Sha-sha-jieu' plants **(Supplementary Figure S4).** Stress conditions affected 'Mi-ni' more than 'Sha-sha-jieu', suggesting that 'Sha-sha-jieu' can tolerate stresses better.

The two cultivars displayed different CF responses to various stress treatments, and changes in the F_v/F_m values of both cultivars at 40°C declined at a greater rate than 22°C **(Table 2).** Comparing the same time periods but with different treatments, the F_v/F_m values of 'Mi-ni' and 'Sha-sha-jieu' plants under treatments H and HF after 6 h were significantly lower than under treatments F and C. The F_v/F_m reduction indicates that an important portion of the PSII reaction center was damaged, and the F_v/F_m value in healthy leaves was close to 0.8, which is a typical value for uninhibited plants. 'Mi-ni' and 'Sha-sha-jieu' cabbages under treatments F and C (72 h) kept Fv/Fm values stable; however, under treatments H and HF, Fv/Fm values of both cultivars dropped, demonstrating that the photosynthetic light reactions of both were under stress. Flooding treatments had only minor impacts on the PSII reaction centers of the two cultivars, which indicated that flooding at 22°C had limited influence on the energy transfer rate of their PSII reaction centers [24]. Although the F_v/F_m values of both cultivars began to fall after 6 h of high temperature treatment, those of 'Sha-sha-jieu' dropped more slowly than in 'Mi-ni', suggesting that stress conditions affected the light-dependent photosynthetic reactions of 'Sha-sha-jieu' less. As the treatment went on, F_v/F_m values continued to fall in both cultivars, suggesting that the Fv/Fm values of both were more sensitive to high temperature. High temperature led to a decrease in F_v/F_m values and photosynthetic efficiency, perhaps because the PSII activation energy rate was lowered or the separation of parts of the chlorophyll antenna from the PSII reaction center blocked electron transfer. High temperature causes F_o to rise and F_m to fall. The disappearance of the antenna system causes excess energy under high temperatures to deactivate the PSII reaction center. Dehydration, which results in raising leaf temperature due to a lack of evaporative cooling, also inhibits the PSII reaction center [25]. Reaction center proteins could be modified (conformation changes) and be separated from each other.

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Differentially Expressed Proteins in Cabbage Leaves Among Stress Treatments

After comparing stress conditions (F, H, and HF) to controls (C), 25 and 26 peaks were identified that showed distinct differences in 'Mi-ni' **(Table 3)** and 'Sha-sha-jieu' **(Table 4)**, respectively. The pI, score, coverage %, accession, alteration of expression, and biological functions of these peaks among stress treatments are also represented in **Tables 3 and 4.** Changes in protein accumulation under stress are likely related directly to the physiological response. The different levels of peaks on the graph, which indicated different protein expression levels, were collected for MALDI-TOF-MS analysis. These proteins emerged as key participants in stress tolerance. As stress time increased, additional responsive proteins became evident and were identified. Among 25 identified proteins, only six protein peaks (262, 261, 275, 226, 2315, and 2316) in 'Mi-ni' were down-regulated, whereas the others were up-regulated among stressing treatments. Among them, four (261- 263 and 275) and eight (226, 257, 269, 2610, 2613, 2512, 2614, and 2511) peaks were found after 6 h and 12 h treatments, respectively. Chloroplastic oxygen-evolving enhancer protein 1-1 (OEE1-1, peaks 263, 257, 2610, 2617, and 2532) was identified at all stressing time periods, but ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) large chain was found only at 12 h (peaks 226, 269, and 2511) and 24 h (peaks 2315, 2316, 2218, 2420, 2531, 2733, 2227, and 2429) treatments in 'Mi-ni'. The expression of RubisCO in 'Sha-sha-jieu' rose under stressed treatments at all times, except for peaks 2345 and 2346 dropping in the 12 h treatment **(Table 4).** In addition, the precursor of chloroplastic OEE1-2 was also lower in peaks 2865, 2667, and 2887 in the 24 h treatment. The expression of RubisCO was elevated throughout the treatments; however, comparing controls, OEE decreased after the 24 h treatment. The expression of the RubisCO large chain of 'Sha-sha-jieu' was highly regulated under treatment HF at peaks 2242, 2254, 2762, 2658, 2659, 2679, 2274, 2673, and 2682 in protein abundance. The RubisCO large chain, which was highly expressed in 'Mi-ni' under treatment H (peaks 269, 2218, and 2420), was also frequently found in 'Sha-sha-jieu' at peaks 2738, 2637, 2740, 2248, and 2249. Different peaks of RubisCo large chain were identified between different treatments in same cultivar. These sister peaks may represent close homologues, but this could not be resolved based on mass spectrometric data and might instead be isoforms resulting from differential post-translational modifications [26].

Table 3. Differentially expressed proteins from stressed 'Mi-ni' plants identified by MALDI-TOF-MS.

Peak	Treatment Homologous protein						pI	Score	Cov
	Stress time	(h)	R	Description	Species	Accession			
262	F	6	↓	ATP-dependent Clp protease ATP-binding subunit clpA homolog, chloroplastic (Fragment)	*Brassica napus*	P46523	5.90	62	30%
261	F	6	↓	Probable granule-bound starch synthase 1, chloroplastic/ amyloplastic	*Arabidopsis thaliana*	Q9MAQ	8.76	68	31%
263	H	6	↑	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	P23321	5.55	60	47%
275	HF	6	↓	Histone acetyltransferase GCN5	*Arabidopsis thaliana*	Q9AR19	6.01	62	29%
226	F	12	↓	Ribulose 1,5-bisphosphate carboxylase/oxygenase large chain	*Aethionema cordifolium*	A4QJC3	6.04	75	27%
257	H	12	↑	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	P23321	5.55	72	47%
2610	H	12	↑	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	P23321	5.55	82	47%
269	H	12	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	62	31%
2613	HF	12	↑	ATP synthase subunit alpha, chloroplastic	*Pelargonium hortorum*	Q06FX6	5.67	60	28%
2512	HF	12	↑	Glutamate receptor 3.3	*Arabidopsis thaliana*	Q9C8E7	8.92	63	27%
2614	HF	12	↑	Oxygen-evolving enhancer protein 1-2, chloroplastic	*Arabidopsis thaliana*	Q9S841	5.92	123	36%
2511	HF	12	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	62	32%
2517	F	24	↑	Oxygen-evolving enhancer protein 1-1, chloroplast precursor	*Arabidopsis thaliana*	P23321	5.55	84	38%
2315	F	24	↓	Ribulose bisphosphate carboxylase large chain precursor	*Arabis hirsute*	A4QK26	5.96	75	24%
2316	F	24	↓	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Brassica oleracea var. botrytis*	ABV44287	6.04	80	28%
2626	H	24	↑	Oxygen-evolving enhancer protein 1-2, chloroplastic	*Arabidopsis thaliana*	Q9S841	5.92	69	31%

2218	H	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	74	32%
2420	H	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	92	36%
2219	H	24	↑	Ribulose bisphosphate carboxylase small chain, chloroplastic	*Brassica napus*	P05346	8.23	65	49%
2532	HF	24	↑	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	P23321	5.55	61	46%
2430	HF	24	↑	Pentatricopeptide repeat-containing protein At1g79540	*Arabidopsis thaliana*	Q9SAJ5	8.44	62	20%
2531	HF	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	63	36%
2733	HF	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	69	30%
2227	HF	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	74	32%
2429	HF	24	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	92	36%

R, regulation: proteins differentially expressed compared to the controls (22°C without flooding). ↑, increase; ↓, decrease; Score, probability based molecular weight search (Mowse); score, Cov, sequence coverage percentage.

Table 4. Differentially expressed proteins from stressed 'Sha-sha-jieu' plants identified by MALDI-TOF-MS.

Peak	Treatment			Homologous Protein					
		Stress time (h)	R	Description	Species	Accession	PI	Score	Cov
2236	F	6 h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	60	24%
2738	H	6h	↑	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	P23321	5.55	60	34%
2741	H	6h	↑	ATP synthase subunit beta, chloroplastic	*Oryza sativa subsp. japonica*	P12085	5.38	76	45%
2739	H	6h	↑	Beta-farnesene synthase	*Mentha piperita*	O48935	5.27	60	30%
2637	H	6h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	62	26%
2740	H	6h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	70	30%
2242	HF	6h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	72	23%
2345	F	12h	↓	Ribulose bisphosphate carboxylase large chain precursor	*Olimarabidopsis pumila*	A4QJU1	5.87	94	28%
2346	F	12h	↓	Ribulose bisphosphate carboxylase large chain precursor	*Brassica oleracea*	P48686	5.88	128	27%
2248	H	12h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	82	38%
2249	H	12h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	103	39%
2255	HF	12h	↑	ATP synthase subunit beta, chloroplastic	*Lactuca sativa*	Q332X1	5.13	89	40%
2760	HF	12h	↑	ATP synthase subunit beta, chloroplastic	*Atropa belladonna*	Q8S8W8	5.15	93	32%
2557	HF	12h	↑	Oxygen-evolving enhancer protein 1-2, chloroplastic	*Arabidopsis thaliana*	Q9S841	5.92	63	33%
2254	HF	12h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	60	28%
2762	HF	12h	↑	Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	P48686	5.88	74	30%
2658	HF	12h	↑	Ribulose bisphosphate carboxylase large chain	*Petunia hybrida*	P04992	6.58	68	35%
2659	HF	12h	↑	Ribulose bisphosphate carboxylase large chain	*Carpinus caroliniana*	Q06023	6.26	67	31%

2865	HF	24h	↓	Oxygen-evolving enhancer protein 1-2, chloroplast precursor	*Arabidopsis thaliana*	Q9S841	5.92	88	33%
2667	H	24h	↓	Oxygen-evolving enhancer protein 1-2, chloroplast precursor	*Arabidopsis thaliana*	Q9S841	5.92	97	36%
2770	H	24h	↑	Serine/threonine protein phosphatase 7 inactive homolog	*Arabidopsis thaliana*	Q9LEV0	4.93	70	18%
2887	HF	24h	↓	Oxygen-evolving enhancer protein 1-2, chloroplast precursor	*Arabidopsis thaliana*	Q9S841	5.92	88	33%
2679	HF	24h	↑	Ribulose bisphosphate carboxylase large chain precursor	*Arabidopsis thaliana*	O03042	5.88	92	27%
2274	HF	24h	↑	Ribulose bisphosphate carboxylase large chain precursor	*Brassica oleracea*	P48686	5.88	106	30%
2273	HF	24h	↑	Ribulose bisphosphate carboxylase large chain precursor	*Draba nemorosa*	A4QL27	6.04	96	26%
2682	HF	24h	↑	Ribulose bisphosphate carboxylase large chain	*Calycophyllum candidissimum*	Q31750	5.82	70	22%

R, regulation: proteins differentially expressed compared to the controls (22°C without flooding). ↑, increase; ↓, decrease
Score, probability based molecular weight search (Mowse) score
Cov, sequence coverage percentage

RubisCO catalyzes carbon fixation in the Calvin-Benson cycle by converting ribulose-1,5-bisphosphate (RuBP) to 3-phosphoglycerate. The structure of RubisCO consists of two subunits: the large chain (L, 55 kD) and small chain (S, 13 kD). The active site of enzymatic activity lies within the large chain [27]. The small chain helps the large chain to undergo carboxylation. To increase energy metabolism, the expressions of proteins related to redox homeostasis and response to stimuli were up-regulated, thereby maintaining physiological balance during stress. An up-regulation in RubisCO levels may also indicate an increase in the photorespiration rate [28]. High temperatures decrease the stability and also depress the carboxylation reaction rate of RubisCO. Miller et al. demonstrated that the thermo-tolerant Synechococcus lineage has higher RubisCO stability than other lineages [29]. Photosynthesis is one of the systems that is very sensitive to high-temperature stress. In our study, RubisCO related to photosynthesis was differentially expressed and regulated by combination treatments and among genotypes. 'Mi-ni' and 'Sha-sha-jieu' cabbages at 40°C throughout 24 h flood and non-flood treatments expressed high amounts of large chain RubisCO and its precursor. Treatments H and HF may trigger the physiological heat stress response in plants, which induces stomata closure and lowers the concentration of CO_2 in leaves to decrease carbamylation and promote the interaction of RuBP and the active site of RubisCO. This may be why an increase in the amount of large-chain RubisCO and its precursor were observed. These protein products can be used for either carbon fixation or photorespiration. In addition, under flood or heat stressing, RubisCO can protect protein synthesis in the chloroplast. A previous study showed that under heat stress, cabbage leaf RubisCO transforms into a 41 kDa isoform and interacts with thylakoid-bound ribosomes. It would then behave like a chaperone to protect protein synthesis at high temperatures [30]. These results indicate that heat-tolerant cultivars have a protective mechanism against the thermal degradation of RubisCO. Maintaining a higher RubisCO subunit protein level may help plants survive heat damage.

Oxygen evolving enhancer protein 1 (OEE1) can be found at the electron transport pathway of thylakoid photosystem II. With the assistance of Mn^{2+}, Ca^{2+}, and Cl^- ions, it can release oxygen from water in a light-dependent reaction. Several plant species, such as tobacco, rice, and black mangrove, increase their expression of OEE1 under salinity stressing, and a high expression of OEE1 helps repair the protein damage caused by NaCl [31-33]. Because OEE1 is composed of OEE1 subunit and D1 protein in the PSII, an increase in OEE1 may facilitate photosynthesis. For survival, plants must respond to flood and/or heat stresses differently from the way they regulate protein expression. Photosynthesis is one of the systems that is most sensitive to heat stress [34,35]. OEE expression in 'Mi-ni' and 'Sha-sha-jieu' cabbages was altered under stress. It was clear that 'Mi-ni' upregulated the expression of OEE under treatments H and HF, but this was not obvious in 'Sha-sha-jieu'. However, the general trend in OEE expression in both cabbages was consistent with what occurs in wheat under drought stress [36].

The ATP synthase of the chloroplast is a complex protein oligomer consisting of two elements: CF_1 and CF_2. CF_1 is where ATPs are synthesized. ATP synthase α chain and ATP synthase subunit β are both subunits of CF_1, and the CF_1 α-β complex is the smallest enzymatic subunit of ATP synthase [37]. The electron transport chain of the light-dependent reactions of photosynthesis inside thylakoid results in a proton gradient, thus driving ATP synthase to produce ATPs. The ATPs from light-dependent reactions are then released into the stroma to provide energy for the dark reaction [38]. We discovered that under high temperature, α and β units of ATP synthase increased in 'Mi-ni' with flooding for 12 h and in 'Sha-sha-jieu' without flooding for 6 h and with flooding for

6 h and 12 h. This suggests that these two subunits play an important role in the activity of ATP synthase to synthesize ATPs under stressful conditions. Our findings also support a previous study of *Populus euphratica* leaves, which showed that the expression of the ATP synthase alpha chain and ATP synthase subunit beta increased under 40°C treatment over 3 d. It was suggested that the interactions among subunits α, β, and γ can stabilize ATP synthase CF1 at high temperatures and help plants produce ATPs for tolerating such conditions [39].

Beta-farnesene synthase, which is highly expressed in flowers, can attract pollinators, defend against pathogens, and offer anti-oxidant effects [40]. Beta-farnesene synthase (synonyms include farnese synthase, terpene synthase 10, and terpene synthase 10-B73) is an enzyme that catalyzes the production of beta-farnesene and diphosphate from farnesyl diphosphate [41]. Our study showed that 'Sha-sha-jieu' under high temperature without flooding for 6 h exhibited an increase in beta-farnesene synthase, suggesting that beta-farnesene might help to cope with the heat. Glutamate receptors, originally found in mammals and enabling the flow of cations in response to glutamate binding, are involved in several biological processes in plants such as photosynthesis, response to abiotic stress, and C/N balance [42]. Although glutamate receptors play an important role in mammal neurotransmission, they are still relatively new to plants and not well understood. A recent study indicates that glutamate receptor 3.3, which can be induced by some amino acids, mediates leaf-to-leaf wound signaling [43]. Our study shows that 'Mi-ni' under high temperature with 12 h flooding had an increase in glutamate receptor 3.3, suggesting that the latter in cabbages also responds to abiotic stress.

Serine/threonine phosphatase is associated with the ethylene receptor, an important signal receptor in plants. The tobacco ethylene receptor, *Nicotiana tabacum* histidine kinase (NTHK), not only acts like histidine kinase but also has the activity of serine/threonine phosphatase [44]. A similar finding is seen in Arabidopsis [45]. In addition, NTHK is linked to the salt stress response [46]. Our study shows that 'Sha-sha-jieu' under treatment H increased its serine/threonine phosphatase inactive form. High temperatures might break down ethylene receptors in plants, thus blocking ethylene signaling transduction and inhibiting the production of ethylene to delay aging. ATP-dependent Clp protease may degrade unfolded or abnormal proteins in chloroplasts. ATP-dependent Clp protease consists of two subunits: the regulatory ATPase/chaperone (ClpA, ClpX, ClpY) and proteolytic subunit (ClpP, ClpQ). The ClpA homolog was also expressed in P. *euphratica Oliv* under heat stress (Ferreira et al., 2006). Our study shows that 'Mi-ni' under treatment F for 6 h had a decrease in chloroplastic ATP-dependent Clp protease ATP-binding subunit clpA homolog, which might lead to more abnormal proteins accumulating in 'Mi-ni'. Granule-bound starch synthase 1 is an enzyme that catalyzes the synthesis of amylose. In response to high temperature or drought stress, granule-bound starch synthase 1 was down-regulated in rice [47]. Our study shows that 'Mi-ni' under treatment F for 6 h had a decrease in granule-bound starch synthase 1, indicating that flooding might inhibit the synthesis of amylose in cabbages as drought stress does.

CONCLUSION

Under stressful conditions, chlorophyll fluorescence and evaporation rates in 'Mi-ni' plants declined greatly and degenerated faster than in 'Sha-sha-jieu' plants. The genetic difference was supported by 51 stress-responsive proteins differentially regulated between genotypes under short-term stressing. The early response of cabbages to flooding and high temperature might be an important stress adaptation for survival following not only hypoxia and heat, but also direct damage to cells by flooding and high temperature. Heat- and flooding-tolerant 'Sha-sha-jieu' reacted more rapidly than 'Mi-ni' by the enrichment of photosynthesis-related proteins such as RubisCO and OEE. Along with the surge of photosynthesis-related proteins, the increase in ATP synthase suggests that the regulation of energy production is important. All of the identified proteins likely work cooperatively to reestablish cellular homeostasis under stress and represent stress adaption mechanisms to be exploited in future cabbage breeding efforts. These findings are important for farming in high-temperature areas and wetlands or other areas subject to short and intense rainfall events.

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