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ARTICLE

Effects of Heat Stress during Seed Filling Stage on *Brassica napus* Seed Oil Accumulation and Chlorophyll Fluorescence Characteristics

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ABSTRACT

As global temperature rise, the threat of heat stress to rapeseed production is becoming more obvious. Exploring the response characteristics of two important biological pathways, oil accumulation and photosynthesis, to heat stress during *B. napus* seed filling is helpful in the genetic improvement of heat-tolerant rapeseed. The effects of heat stress on seed oil accumulation and chlorophyll fluorescence characteristics of 29 *B. napus* germplasms with different oil content and environmental sensitivity, including 6 rapeseed varieties which exhibited environment-sensitive/insensitive and with high, medium or low oil content, were tested by whole plant heat stress or the *in vitro* silique culture system. Both assay exhibited similar trend on oil content of the rapeseed germplasms. The heat effect on the chlorophyll fluorescence kinetic parameters F_v/F_m, ETR and Y(II) were also consistent. Heat stress significantly decreased oil content, although there was abundant genetic variation on heat tolerance among the genotypes. Correlation analysis showed that the decrease rate of F_v/F_m of silique heat-stressed *B. napus* developing seed was positive correlative to the decrease rate of mature seed oil content of the whole plant heat-stressed rapeseed ($R = 0.9214$, P-value < 0.01). Overall, the results indicated that heat stress inhibited oil accumulation and photosynthesis in *B. napus* developing seed. The decrease rate of chlorophyll fluorescence parameter F_v/F_m of heat-stressed developing seed could be used as the index of heat tolerant rapeseed identification. Further, two heat insensitive rapeseed varieties with high oil content were identified.

KEYWORDS

Brassica napus L.; heat stress; seed filling stage; oil accumulation; chlorophyll fluorescence characteristics

Nomenclature

F_v/F_m	Maximal quantum yield of PSII photochemistry
ETR	Electron transport rate
Y(II)	Quantum yield of photochemical energy conversion in PSII
$R_{Fv/Fm}$	Decreased rate of F_v/F_m
CV	Coefficient of variation

1 Introduction

Seed development is a critical stage in the life cycle of oil crops. The genetic and environmental factors which involved in this process might directly or indirectly affect the accumulation of rapeseed storage substances [1–4]. As an abiotic stress, heat stress is a major factor limiting crop yield and quality [5–7]. In winter rapeseed production area, especially in the Yangtze River Basin, the temperature rises rapidly during the ripening period of rapeseed, which is easy to cause heat-forced maturity of rapeseed [8]. With the impact of global climatic changes and the frequency of heat wave increased, the negative effects of heat stress on yield and quality of rapeseed are more significant [9,10]. Reduce the harm of heat damage has become an urgent request for the sustainable development of oilseed rape industry [11,12].

The response of plants to heat stress is an extremely complex process. Transcriptome changes resulted from heat stress in the seed of *B. napus* at filling stage had been analyzed by microarray and RNA-seq approaches. It was demonstrated that in addition to a large number HSFs and HSPs, some genes related to lipid degradation were also up-regulated [7,13,14]. A heat-induced *BnGLYI* gene, which encoded a glyoxalase I enzyme, was demonstrated to participate in *B. napus* seed thermal tolerance [15]. High night temperature up-regulated the expression of genes related to fatty acid catabolism, including genes associated with β-oxidation and glyoxylate metabolism, thereby causing the lower seed oil content [6]. The heat induced DNA methylation has been indicated as key factors in *B. napus* adaptation to heat stress [16]. Additionally, the differences in homoeologous gene expression and alternative splicing patterns in allotetraploid plants such as *B. napus* might improve their adaptability to heat stresses [17].

The embryos of rapeseed, soybean and *Arabidopsis* are green during their seed development. Although such “green seeds” are still heterotrophic sink tissues in general, their chloroplasts have thylakoid structure and the same chlorophyll protein complex as leaves [18]. Hence, these green seeds still have certain photosynthetic carbon fixation capacity [19]. In *B. napus* developing seeds, the peak of the photosynthetic capacity coincides with the period of oil accumulation rapidly [20]. *BnWRI1*, the key regulator of *B. napus* seed oil content, synergistically controlled two important biological pathways: oil biosynthesis and photosynthesis during seed development [3].

Photosynthesis is the basis of crop yield formation and one of the most heat vulnerable physiological processes [21–25]. Heat stress at seedling stage resulted in irreversible damage to PSII and decrease of net photosynthetic rate in C3 sunflower and C4 maize leaves [26]. Our previous studies have found that heat stress at seed filling stage down-regulates the expression of *BnWRI1*, a key regulator of oil biosynthesis, and its downstream genes (including those of the *de novo* fatty acid synthesis pathway), thus blocking the carbon flows to triacylglycerol biosynthesis. Furthermore, heat stress induced the photoinhibition pathway, which inhibited photosynthesis, respiration and the maximum photoperiod quantum yield of PSII (F_v/F_m) in *B. napus* developing seeds. Consequently, the inhibition of *BnWRI1* mediated fatty acid biosynthesis pathway and the destruction of PSII might be the main reasons for the decreased oil content in heat-stressed *B. napus* seeds [14]. Here, we examined the effects of heat stress on seed oil content and the chlorophyll fluorescence parameters of *B. napus* germplasms with significant differences in environmental sensitivity, to explore the physiological mechanism of rapeseed yield and quality reduction caused by heat stress, and to seek suitable evaluation index of heat tolerance during *B. napus* seed filling stage. This study will provide useful information about the cultivation of heat-tolerant rapeseed varieties.

2 Materials and Methods

2.1 Plant Materials

The 29 tested *B. napus* accessions were provided by the Zhejiang Academy of Agricultural Sciences, Hunan Agricultural University; Institute of Oil Crops, Chinese Academy of Agricultural Sciences; Southwest University; Yunnan Agricultural University and Zhejiang University (China) (Table S1).

Among them, HS1, HS2, HS3, HS4, HS5 and HS6 are the previously screened *B. napus* germplasm resources of environmentally insensitive or sensitive genotype with different oil content [4,11,27]. The detailed information is given in Table S2.

2.2 Experimental Design

2.2.1 Whole Plant Heat Stress of B. napus

This study was conducted in the Zhejiang Academy of Agricultural Sciences, Hangzhou, China (120°11′E, 30°18′N) for three consecutive years (2015–2017). The *B. napus* accessions were sown on October 8th each year and potted with 1 plant per pot and 10 plants per germplasm. All plants were grown in a greenhouse under the same conditions according to the conventional cultivation management of rapeseed. At the seed filling stage (April 06 every year), six plants of similar size were selected from each tested rapeseed germplasm, and then transferred in pairs into two growth chambers for heat stress (HT) or control treatment (CK), respectively. The parameters of growth chambers were set as described by Huang et al. [14]. Briefly, the growth chambers were set as under a 14 h-light (5:00 to 19:00) photoperiod with light intensity of 300 μmol m^{-2} s^{-1} and relative humidity of 70%. Night temperature was 18°C. For the control chamber, daytime temperature kept constant at 23°C. The heat-stress chamber was set to gradually heat up from 23°C to 35°C at 5:00 to 10:00, maintain at 35°C for 4 h until 14:00, gradually cool down from 35°C to 23°C during 14:00 to 19:00, and then rapidly cool down to 18°C. After 15 days of treatment, all plants were transferred back to the greenhouse and continued to grow under the same conventional conditions until each rapeseed plant matured normally. Mature seeds were harvested by each rapeseed plant individually when siliques desiccation, and their oil contents were measured.

2.2.2 In Vitro Heat Stress of B. napus Silique

Silique heat-stress treatments were performed using the method from Huang with minor modifications [14]. All rapeseed accessions were grown in the experimental field at the Zhejiang Academy of Agricultural Sciences, Hangzhou, China, during the normal growing seasons. At full bloom stage, ten plants of similar size from each rapeseed accession were selected to mark the flowers that open in the day. On the 20 days after flowering (DAF), 180 siliques derived from the marked flowers were excised from the marked plants for each rapeseed accession, and randomly divided into heat-stress and control groups. All siliques were incubated on petri dishes containing Murashige & Skoog medium with 2% sucrose by the pedicels inserted into the medium. Siliques were cultivated in growth chambers under a 14 h-light (5:00 to 19:00) photoperiod with light intensity of 120 μmol m^{-2} s^{-1} and relative humidity of 60%. Night temperature maintained at 18°C. For the control group, daytime temperature remained constant at 23°C. For the heat-stress group, daytime temperature regime was as follows: increased from 18°C to 23°C at 5:00, maintained at 23°C for 3 h, increased from 23°C to 37°C at 8:00, maintained at 37°C for 7 h, decreased from 37°C to 23°C at 15:00, maintained at 23°C for 4 h, decreased from 23°C to 18°C at 19:00 (Fig. S1). After the second heat treatment (15:00), the seeds were separated from each rapeseed sample for analysis of seed chlorophyll fluorescence and oil content.

2.2.3 Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence was determined with an MAXI version Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany) following the manufacturer. For each accession, no less than 20 seeds were used. For the convenience of comparison, the seeds of the same *B. napus* germplasm with or without heat stress were placed adjacent to each other. The chlorophyll fluorescence images and F_v/F_m were collected according to Huang et al. [14]. The experiments were arranged with three biological replicates. The chlorophyll fluorescence parameters at 3 positions were read from each sample, and the average values

were taken as the results. The F_v/F_m decreased rate of the heat-stressed *B. napus* developing seeds ($R_{Fv/Fm}$) was calculated as:

$$R_{Fv/Fm} = [Fv/Fm_{(CK)} - Fv/Fm_{(HT)}]/[Fv/Fm_{(CK)}] \quad (1)$$

where $F_v/F_{m\ (CK)}$ and $F_v/F_{m\ (HT)}$ denote the F_v/F_m of heat-stress *B. napus* developing seeds or the respective control.

2.2.4 Oil Analysis

Oil content of *B. napus* mature seed was determined by Soxhlet extraction method [28]. Oil content and oil per seed of *B. napus* developing seeds were determined according to the method of Wu et al. [3].

2.2.5 Data Analysis

All of the experiments were conducted with three independent biological replicates and two technical replicates unless otherwise specified. The data was statistically analyzed by Student's *t*-test using Microsoft Excel 2010.

3 Results

3.1 Effects of Whole Plant Heat Stress on B. napus Mature Seed Oil Yield

In this study, we investigated the effects of heat stress on rapeseed at seed filling stage. To this aim, we firstly checked the heat effects on the *B. napus* germplasms HS1 to HS6, which have obvious differences in seed oil content and environmental sensitivity (Table S2). As displayed in Figs. 1A and 1C, the seed oil content and oil yield per plant of the tested *B. napus* germplasms decreased after the whole plant heat stress, except for the insignificant change of seed oil content of HS1. Notably, the influence of heat stress on oil yield per plant was more pronounced. These results demonstrated that heat stress is an important environmental factor affecting *B. napus* seed oil accumulation and reducing its economic benefits.

Pairwise comparisons were performed between the environmentally insensitive and sensitive rapeseeds with high, medium or low seed oil content respectively. It was noticed that the decrease rate of mature seed oil content and oil yield per plant of heat-stressed environmental insensitive *B. napus* germplasms HS1, HS3 and HS5 were lower than the decrease rate in corresponding environmental sensitive *B. napus* germplasms HS2, HS4 and HS6 (Figs. 1B and 1D). These results indicated that HS1, HS3 and HS5 were more resistant to heat damage than HS2, HS4 and HS6 respectively at seed filling stage, and the heat tolerance of rapeseed accessions was an important factor affecting their environmental adaptability.

3.2 Effects of in Vitro Silique Heat Stress on B. napus Developing Seed Oil Accumulation

At 20 days after flowering (DAF), rapeseed has entered the stage of rapid accumulation of oil and other storage reserves, which is also a sensitive period to heat stress [14]. The oil content and oil per seed of developing seed collected from siliques subjected to heat stress (HT) decreased significantly compared with those of the corresponding temperature-suitable culture samples (CK) in all tested *B. napus* germplasms (Fig. 2), indicating that even short-term heat stress during seed development had obvious adverse effects on *B. napus* seed oil accumulation.

The environmentally insensitive and sensitive rapeseeds with high, medium or low seed oil content were also pairwisely compared. The results showed that the decrease rate in developing seed oil content and oil per seed of *in vitro* silique heat-stressed environmental insensitive *B. napus* germplasms HS1, HS3 and HS5 were lower than the decrease rate in corresponding environmental sensitive *B. napus* germplasms HS2, HS4 and HS6 (Figs. 2B and 2D), which was consistent with that of the whole plant heat stress to mature seed oil yield in these rapeseed germplasms (Fig. 1). Taken together, the change rate of seed oil accumulation of each *B. napus* accessions exposure to silique heat stress compared with that of the corresponding suitable cultured sample could reflect the heat tolerance of the rapeseed germplasm during seed filling stage.

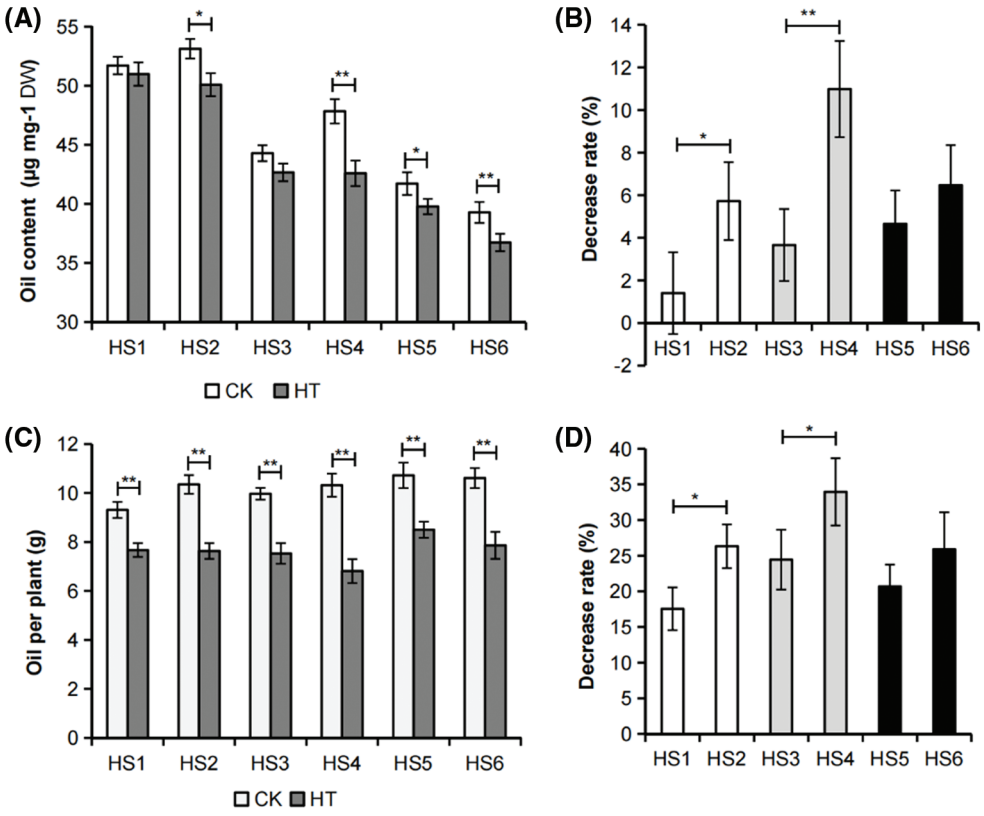

Figure 1: Effects of whole plant heat stress on *B. napus* mature seed oil yield. (A) Mature seed oil content of *B. napus* germplasms with different environmental sensitivity that were exposed to whole plant heat stress (HT) or grown in control (CK) condition. The value represented mean ± SD, $n = 3$ biological replicates (with 2 technical replicates). (B) Decrease rate (%) of mature seed oil content of whole plant heat-stressed *B. napus* plants relative to those of the corresponding control. (C) Oil yield per plant of *B. napus* genotypes with different environmental sensitivity exposure to whole plant heat stress (HT) or grown in control (CK) condition. The value represented mean ± SD, $n = 3$ biological replicates. (D) Decrease rate (%) of oil yield per plant of whole plant heat-stressed *B. napus* plants relative to those of the corresponding control. Asterisks indicate significant differences in the marked comparisons calculated using *t*-test: *, *P*-value < 0.05; **, *P*-value < 0.01

3.3 Effects of in Vitro Silique Heat Stress on Chlorophyll Fluorescence Characteristics of B. napus Developing Seeds

The changes of seed chlorophyll fluorescence parameters caused by silique heat stress in *B. napus* germplasms HS1 to HS6 were measured at the same interface by modulated chlorophyll fluorescence system Imaging-PAM. As shown in Table 1, the effects of heat stress were apparent in the chlorophyll fluorescence characteristics of *B. napus* developing seeds. The *Fv/Fm*, ETR and Y(II) were all decreased in heat-stressed seed of each rapeseed accessions. Again, the chlorophyll fluorescence parameters of the environmentally insensitive rapeseed germplasms HS1, HS3 and HS5 were more stable to heat stress than those of the corresponding environmentally sensitive rapeseed germplasms HS2, HS4 and HS6, implying the changes in these chlorophyll fluorescence parameters were correlated with the heat sensitivity at *B. napus* seed filling stage.

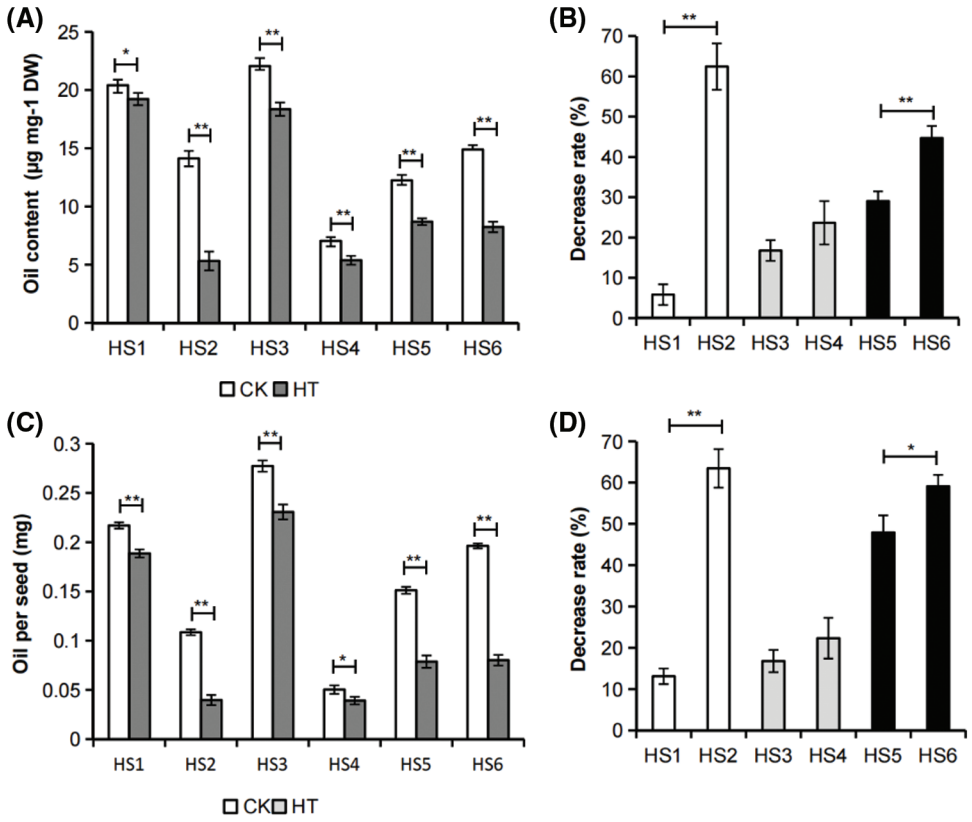

Figure 2: Effects of *in vitro* silique heat stress on *B. napus* developing seed oil accumulation. (A) Effects of silique heat stress on developing seed oil content of *B. napus* germplasms with different environmental sensitivity. The developing seeds were isolated from siliques exposure to heat-stress (HT) or cultured under control condition (CK). The value represented mean ± SD, n = 3 biological replicates (with 2 technical replicates). (B) Decrease rate (%) of oil content in developing seeds isolated from heat-stressed *B. napus* siliques relative to those of the corresponding control. (C) Effects of *in vitro* silique heat stress on oil per seed in developing seeds of *B. napus* germplasms with different environmental sensitivity. The value represented mean ± SD, n = 3 biological replicates. (D) Decrease rate (%) of oil per seed of developing seeds isolated from heat-stressed *B. napus* siliques relative to those of the corresponding control. Asterisks indicate significant differences in the marked comparisons calculated using t-test: *, P-value < 0.05; **, P-value < 0.01

The coefficient of variation (CV) of parameters in *B. napus* accessions were compared. The CVs of seed *Fv/Fm*, ETR and Y(II) in *B. napus* silique cultured under moderate temperatures ranged from 1.12 to 3.10, 7.50 to 18.28 and 6.74 to 24.76, respectively. Meanwhile, the CVs of seed *Fv/Fm*, ETR and Y(II) in heat-stressed *B. napus* silique ranged from 1.95 to 10.74, 14.75 to 34.92 and 11.84 to 49.49, respectively (Table 1). Overall, the CVs of each parameter of the HT seed exhibited an increasing trend compared with the CVs of the corresponding CK seed in the rapeseed accessions, suggesting that heat stress not only inhibited photosynthetic activity of *B. napus* developing seed, but also increased its variation within *B. napus* germplasm. It is noteworthy that, in the same *B. napus* accession, the CVs of *Fv/Fm* were significantly less than the CVs of ETR or Y (II) under both suitable and heat-stress conditions, indicating that the dispersion degree of this photosynthetic index was small within same rapeseed germplasm. Among the chlorophyll fluorescence parameters detected, *Fv/Fm* was relatively uniform among different seeds of the same rapeseed accession, which could be used as an important index for the heat response of rapeseed during seed filling.

Table 1: Effects of *in vitro* silique heat stress on chlorophyll fluorescence characteristics of *B. napus* developing seeds

Parameter	No.	CK		HT		*P*-values	Decrease rate (%)
		Mean ± SD	CV (%)	Mean ± SD	CV (%)		
F_v/F_m	HS1	0.741 ± 0.013	1.75	0.718 ± 0.014	1.95	0.0017	3.10
	HS2	0.714 ± 0.008	1.12	0.658 ± 0.048	7.29	0.0056	7.84
	HS3	0.709 ± 0.021	2.96	0.691 ± 0.024	3.47	0.1055	2.54
	HS4	0.708 ± 0.019	2.68	0.596 ± 0.064	10.74	0.0003	15.82
	HS5	0.709 ± 0.022	3.10	0.705 ± 0.036	5.11	0.7510	0.56
	HS6	0.712 ± 0.008	1.12	0.626 ± 0.041	6.55	1.12378E-05	12.08
ETR	HS1	31.22 ± 2.34	7.50	27.72 ± 4.09	14.75	0.0408	11.21
	HS2	23.74 ± 2.91	12.26	11.28 ± 3.18	28.19	1.90364E-07	52.49
	HS3	19.76 ± 2.17	10.98	19.66 ± 3.69	18.77	0.9456	0.51
	HS4	29.74 ± 3.52	11.84	22.44 ± 5.13	22.86	0.0028	24.55
	HS5	23.96 ± 4.38	18.28	23.94 ± 4.82	20.56	0.9734	0.08
	HS6	26.68 ± 3.21	12.03	15.29 ± 5.34	34.92	4.98655E-05	42.69
Y(II)	HS1	0.403 ± 0.031	7.69	0.358 ± 0.078	21.79	0.1223	11.17
	HS2	0.304 ± 0.037	12.17	0.144 ± 0.071	49.31	1.8083E-05	52.63
	HS3	0.306 ± 0.039	12.75	0.304 ± 0.036	11.84	0.9303	0.65
	HS4	0.381 ± 0.045	11.81	0.287 ± 0.062	21.60	0.0019	24.67
	HS5	0.311 ± 0.077	24.76	0.306 ± 0.095	31.05	0.9164	1.61
	HS6	0.341 ± 0.023	6.74	0.196 ± 0.097	49.49	0.0005	42.52

Note: CK: control, HT: heat stress, CV: coefficient of variation, *P*-values: The significant differences between the control and heat group calculated using *t*-test.

3.4 Response of B. napus Germplasms to Heat Stress at Seed Filling Stage

For further validation of the effects of heat stress on *B. napus* seed photosynthetic characteristic and oil content, we determined the changes of mature seed oil content of whole plant heat-stressed *B. napus* plant and the $R_{Fv/Fm}$ of silique heat-stressed *B. napus* developing seeds in 29 rapeseed accessions with different genetic backgrounds. The seed F_v/F_m of heat-stressed silique decreased compared to that of the corresponding temperature-suitable culture sample (CK) in all the test rapeseed germplasm materials except HS17 (Table 2), showing that heat stress damaged the PSII of *B. napus* developing seeds and disturbed their photosynthetic activity. The changes of F_v/F_m in heat-stressed developing seeds were varied between the rapeseed accessions, indicating that the seed photosystem for each *B. napus* germplasms had different sensitivity to heat stress at filling stage. Similarly, the mature seed oil content of 15 day heat-stressed plants decreased compared with that of the corresponding control grown under suitable temperature (Table 3). However, the decrease rate of mature seed oil content varied with *B. napus* germplasm.

The coefficient of variation of the developing seed $R_{Fv/Fm}$ in silique heat-stress and the coefficient of variation of the decrease rate of the mature seed oil content of whole plant heat-stressed *B. napus* plants were 88.35% and 68.48%, respectively (Table 4), suggesting that there were abundant genetic variations in heat tolerance of seed photosynthesis and oil accumulation of these rapeseed germplasms.

Table 2: Effects of *in vitro* silique heat stress on F_v/F_m in developing seeds of tested *B. napus* germplasms

No.	CK	HT	*P*-values	$R_{Fv/Fm}$ (%)
HS1	0.754 ± 0.017	0.732 ± 0.027	0.0700	2.92
HS2	0.719 ± 0.018	0.619 ± 0.055	9.35061E-05	13.88
HS3	0.674 ± 0.026	0.627 ± 0.021	0.0008	6.92
HS4	0.716 ± 0.033	0.528 ± 0.057	2.231E-07	24.32
HS5	0.698 ± 0.035	0.647 ± 0.039	0.0091	7.31
HS6	0.724 ± 0.032	0.645 ± 0.062	0.0039	11.02
HS7	0.720 ± 0.012	0.695 ± 0.028	0.0219	3.39
HS8	0.718 ± 0.009	0.704 ± 0.019	0.0731	2.03
HS9	0.702 ± 0.022	0.651 ± 0.036	0.0023	7.26
HS10	0.731 ± 0.024	0.660 ± 0.044	0.0006	9.66
HS11	0.681 ± 0.037	0.521 ± 0.054	1.8E-06	23.49
HS12	0.505 ± 0.016	0.471 ± 0.034	0.0173	6.89
HS13	0.704 ± 0.025	0.577 ± 0.045	1.52E-06	17.94
HS14	0.687 ± 0.027	0.632 ± 0.036	0.0020	7.89
HS15	0.707 ± 0.010	0.698 ± 0.015	0.1415	1.24
HS16	0.592 ± 0.026	0.561 ± 0.035	0.0479	5.24
HS17	0.699 ± 0.013	0.721 ± 0.012	0.0017	−3.15
HS18	0.674 ± 0.033	0.602 ± 0.054	0.0038	10.68
HS19	0.722 ± 0.024	0.674 ± 0.043	0.0106	6.62
HS20	0.678 ± 0.016	0.644 ± 0.039	0.0282	4.99
HS21	0.702 ± 0.015	0.499 ± 0.049	2.1E-09	28.92
HS22	0.719 ± 0.031	0.638 ± 0.046	0.0005	11.32
HS23	0.743 ± 0.025	0.714 ± 0.038	0.0711	3.90
HS24	0.631 ± 0.022	0.399 ± 0.044	1.60532E-10	36.72
HS25	0.707 ± 0.019	0.608 ± 0.049	3.43E-05	14.05
HS26	0.752 ± 0.020	0.595 ± 0.051	1.88E-07	20.97
HS27	0.693 ± 0.024	0.667 ± 0.029	0.0525	3.75
HS28	0.734 ± 0.016	0.702 ± 0.036	0.0294	4.28
HS29	0.705 ± 0.011	0.619 ± 0.032	9.2E-07	4.25

Note: CK: control, HT: heat stress, *P*-values: the significant differences between the control and heat group calculated using *t*-test.

The correlation analysis revealed that seed $R_{Fv/Fm}$ of the heat-stressed 20 DAF siliques was significantly positively correlated with the decrease rate of mature seeds oil content of the whole plant heat-stressed rapeseed (R = 0.9214, P-value < 0.01). Therefore, $R_{Fv/Fm}$ could be used as an important reference index for screening and evaluating the *B. napus* heat tolerance during seed filling stage (Fig. 3).

Table 3: Effects of whole plant heat stress on mature seed oil content of tested *B. napus* germplasms

No.	CK	HT	*P*-values	Decrease rate (%)
HS1	50.34 ± 1.35	49.98 ± 1.58	0.6810	0.72
HS2	51.41 ± 1.43	47.69 ± 1.74	0.0023	7.24
HS3	44.65 ± 1.73	42.73 ± 1.68	0.0792	4.30
HS4	47.26 ± 2.05	42.22 ± 1.98	0.0015	10.66
HS5	41.97 ± 1.04	39.92 ± 1.54	0.0218	4.88
HS6	41.43 ± 1.63	38.22 ± 1.38	0.0043	7.75
HS7	50.15 ± 0.97	48.72 ± 1.45	0.0726	2.85
HS8	49.88 ± 2.12	49.49 ± 1.83	0.7443	0.78
HS9	49.35 ± 1.27	46.68 ± 1.57	0.0090	5.41
HS10	52.74 ± 1.65	48.53 ± 1.44	0.0008	7.98
HS11	42.88 ± 1.72	38.02 ± 1.67	0.0006	11.33
HS12	47.33 ± 1.86	44.89 ± 1.54	0.0323	5.16
HS13	46.31 ± 1.38	42.85 ± 1.46	0.0018	7.47
HS14	49.65 ± 0.89	46.93 ± 1.51	0.0034	5.48
HS15	46.59 ± 1.16	45.96 ± 1.32	0.4030	1.35
HS16	50.15 ± 1.78	48.55 ± 1.59	0.1304	3.19
HS17	48.27 ± 1.29	48.17 ± 1.53	0.9080	0.21
HS18	47.46 ± 1.42	43.85 ± 1.47	0.0015	7.61
HS19	51.74 ± 1.57	49.53 ± 1.06	0.0171	4.27
HS20	50.35 ± 0.95	48.88 ± 2.04	0.1392	2.92
HS21	50.47 ± 1.49	44.48 ± 1.63	5.7E-05	11.87
HS22	50.51 ± 2.12	46.69 ± 1.62	1.06E-12	7.56
HS23	48.23 ± 1.59	47.13 ± 1.26	0.2129	2.28
HS24	52.98 ± 1.16	46.48 ± 1.59	1.0731E-05	12.27
HS25	51.99 ± 1.96	48.30 ± 1.47	0.0042	7.10
HS26	52.84 ± 1.67	48.13 ± 1.96	0.0012	8.91
HS27	53.05 ± 1.07	52.98 ± 1.46	0.9300	0.13
HS28	52.39 ± 1.53	51.41 ± 1.09	0.2328	1.87
HS29	54.31 ± 1.77	53.76 ± 1.54	0.5781	1.01

Note: CK: control, HT: heat stress, *P*-values: the significant differences between the control and heat group calculated using *t*-test.

Table 4: Variation of tested *B. napus* germplasms in decrease rate of F_v/F_m and oil content

Trait	Mean	Range	CV (%)
$R_{Fv/Fm}$ (%)	10.30	−3.15~36.72	88.35
Decrease rate of oil content (%)	5.33	0.13~12.27	68.48

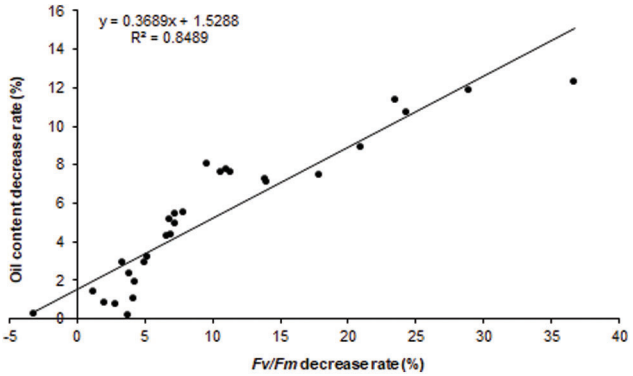

Figure 3: The $R_{Fv/Fm}$ of silique heat-stressed *B. napus* developing seed was positive correlated with the decrease rate of mature seed oil contentin whole plant heat-stressed *B. napus*

Combining the results of Tables 2 and 3, it was found that, ten *B. napus* accessions (HS1, HS7, HS8, HS15, HS17, HS20, HS23, HS27, HS28 and HS29) had $R_{Fv/Fm} < 5\%$ in developing seeds isolated from heat-stressed siliques. After 15 days of whole plant heat-stress at the critical period of oil accumulation, the decrease rate of mature seed oil content of these *B. napus* accessions were all less than 3%, exhibiting stable oil content in response to heat-stress. Among them, the mature seed oil contents of rapeseed germplasms HS27 and HS29 heat-stressed for 15 days were still higher than 52%. These two germplasms with high oil content and heat insensitive could be used as excellent resources in rapeseed breeding for increase heat tolerance.

4 Discussion

The seed oil content of rapeseed is a complex quantitative trait controlled by both genetics and environmental factors [29,30]. The seed filling period is the critical stage in formation of rapeseed yield and quality [3,31,32]. The analysis of thermotolerance of *B. nupus* germplasms with different genetic backgrounds showed that heat stress at seed filling stage generally reduced the seed oil content of the tested accessions (Table 3; Figs. 1A and 1C), which indicated that heat stress was an important environmental factor limiting rapeseed oil yield. It was observed that only 7 h × 2 heat stresses at 37°C had resulted significant decreased oil content (%) of developing seed (Fig. 2). Considering the tiny seed mass of 20 DAF *B. napus* seed [14], this is a reasonable result. The pairwise comparison of the heat responses of environmental insensitive or sensitive *B. nupus* germplasms with high, medium or low oil content respectively showed that, whether the heat-stress were performed with the whole plant treatment or the *in vitro* silique culture system, the thermotolerance of *B. napus* seed oil accumulation was accordant with the insensitive or sensitivity characteristic of seed oil content to the environment in general. These results suggested that the stability of seed oil content to heat stress was an important phenotype of *B. napus* environmental adaptability.

Although the seed of *B. napus* is not a typical photosynthetic organ, the photosynthetic activity of the developing seed itself plays an important role in oil accumulation [18,19]. In *B. napus* developing embryos, the Rubisco bypass metabolic route increased the carbon usage efficiency that yields 20% more acetyl-CoA for oil biosynthesis [33]. In soybean (*Glycine max* L.), broad bean (*Vicia faba* L.) and pea (*Pisum sativum* L.), which are also green seeds, the embryogenic photosynthesis contributed to their oxygen and energy supply and was coupled to oil biosynthetic pathways [34,35]. In order to develop in the limited space inside the seed, the plant embryo must adjust its shape and size, which simultaneously affects the photosynthetic activity of different parts of the embryo and establishes an oil deposition gradient within the *B. napus* embryo [36]. During *B. napus* seed development, the two important biological pathways of oil anabolism and

photosynthesis are co-regulated by the key oil content regulatory gene *BnWRI1* [3]. Heat stress during seed filling stage would irreversibly damage the seed photosystem and up-regulated the genes of photoinhibition pathway, which inhibited the photosynthetic activity of *B. napus* developing seed. Meanwhile, heat stress down-regulated the transcription factor *BnWRI1*, thus inhibiting the oil biosynthesis pathway. Furthermore, seed-specific overexpression of *BnWRI1* in *B. napus* up-regulated the expression of genes related to fatty acid biosynthesis and photosynthesis pathway under both suitable and heat stress conditions, which not only significantly increased seed oil content under normal temperature, but also stabilized *B. napus* seed oil accumulation and photosynthetic activity under heat stress [14]. In *B. napus* exposure to heat stress, the stability of oil content and the maintenance of seed photosynthetic activity was intrinsically related [14].

F_V/F_M was the widely used parameter for determination of the photosystems damage caused by stress. The phenotype by *Fv/Fm* had been used to identification of heat tolerant in wheat [37]. In seed of *B. napus*, the relationship between heat stress and F_v/F_m reduction was also established [14]. In the present survey, the effects of heat stress on chlorophyll fluorescence characteristics of *B. napus* seeds PSII were analyzed. Evidences that the F_v/F_m, ETR and Y(II) for heat-stressed developing seeds of each assayed *B. napus* accessions were significantly reduced,suggested that the processes of light energy capture, the Calvin cycle and the photosynthetic electron transport were all inhibited by heat stress. A similar thermal damage of PSII was reported for leaves of heat stressed C3 sunflower and C4 maize [26]. By pairwise comparison, it was found that the sensitivity of these chlorophyll fluorescence parameters to heat stress was consistent with the heat sensitivity at seed filling stage of the *B. napus* germplasms (Table 1; Fig. 2). The photosynthesis of the developing seed itself provided energy and reductant for fatty acid biosynthesis [17–19]. The results of this study further indicated that the decrease of seed photosynthetic activity might be one of the important reasons of heat stress affecting *B. napus* seed oil content. Investigating the changes of photosynthetic physiology in heat-stressed *B. napus* developing seeds was helpful to improve the accuracy of heat tolerance selection during seed filling stage.

The main production purpose of rapeseed is to harvest seeds. So it is urgent to establish a method for screening and identification of *B. napu*s heat tolerance during seed filling. Due to the tallness and large size of rapeseed plants, how to carry out large-scale and repeatable heat-stress treatment in seed filling stage under controllable conditions has always been an obstacle to the selection of heat-tolerant *B. nupus* germplasms. In this study, the *in vitro* silique culture system, the method for analysis of metabolic flux under defined conditions [14], was used to investigate the effects of heat stress on *B. napus* developing seed oil accumulation. We showed that the decrease rates of seed oil content or oil per seed in developing seeds isolated from heat-stressed siliques could be used to evaluate the *B. napus* heat sensitivity at seed filling stage. The evaluation results are similar to the identification results which using the decrease rate of mature seeds oil content or decrease rate of oil per plant in whole plant heat-stressed *B. napus* as evaluating indicators. This facilitated the efficient and accurate comparison of the *B. napus* heat tolerance during seed filling. Combined with the modulated chlorophyll fluorescence image analysis technology, which allows bulk comparison the changes of photosynthetic characteristics in heat-stressed seed of different *B. napus* accessions at the same interface, and with reference to changes in important traits such as oil content of mature and immature seeds, oil yield per plant, and oil per seed, it was further showed that the decrease rate of seed F_v/F_m, ETR and Y(II) in heat-stressed silique could reflect the sensitivity of the tested rapeseed germplasms to heat stress during seed filling. Moreover, seed $R_{Fv/Fm}$ could be used as a key index for screening and evaluating of *B. napus* heat tolerance at seed filling stage. This newly established system facilitated the comparison and identification of heat tolerance of *B. napus* germplasm.

5 Conclusion

Heat stress during seed filling inhibited oil accumulation and photosynthesis in *B. napus* developing seeds. Particularly, the chlorophyll fluorescence parameters such as F_v/F_m, the maximum photon yield of PSII reaction center, were reduced, and the decrease rates different significant among germplasms, indicating that heat damage of *B. napus* developing seed photosystem to varying degrees. It was found that there were extensive genetic variations in the responses of seed photosynthetic activity and oil content to heat stress at the seed filling stage. The effects of two heat stress methods, silique heat-stressed at seed oil rapidly deposition stage and whole plant heat-stressed during seed filling, had consistent influence trend on *B. napus* seed oil accumulation. Furthermore, the $R_{Fv/Fm}$ of silique heat-stressed *B. napus* developing seed was correlated with the decrease rate of mature seed oil content of the whole plant heat-stressed rapeseed (R = 0.9214, P-value < 0.01), which could be used to predict the heat sensitivity of oil accumulation during *B.napus* seed filling stage. Finally two heat-tolerant *B. napus* germplasms with high oil content were screened out.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Table S1: Tested materials

No.	Variety (line)	Origin
HS1	ZY511	Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China
HS2	ZY036	Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China
HS3	Huayou 8	Yunnan Agriculture University, Kunming, China
HS4	LCD07-4	Southwest University, Chongqing, China
HS5	Xiangyou 15	Hunan Agricultural University, Changsha, China
HS6	Xiang 774-3	Hunan Agricultural University, Changsha, China
HS7	Zheyou 50	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS8	ZS11	Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China
HS9	LC3-9	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS10	SEM2	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS11	Zheshuang 72	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS12	Zheshuang 8	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS13	Zheyou19	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS14	Zheyou 51	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS15	Zheda 619	Zhejiang University, Hangzhou, China
HS16	Head 622	Zhejiang University, Hangzhou, China
HS17	SY-1	Hunan Agricultural University, Changsha, China
HS18	SY-2	Hunan Agricultural University, Changsha, China
HS19	LZS1325	Hunan Agricultural University, Changsha, China
HS20	LZS1327	Hunan Agricultural University, Changsha, China
HS21	LZS1330	Hunan Agricultural University, Changsha, China
HS22	LZS1331	Hunan Agricultural University, Changsha, China
HS23	L8	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS24	SEM3	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS25	SEM6	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS26	W16P1	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS27	W16P2	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS28	W19P2	Zhejiang Academy of Agricultural Sciences, Hangzhou, China
HS29	W27P1	Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Table S2: *Brassica napus* germplasms with different oil content and environmental sensitivity

No.	Variety (line)	Oil content (%)	Characteristics of variety (line)
HS1	ZY511	>50.00	High oil content, Environment-insensitive
HS2	ZY036	>50.00	High oil content, Environment-sensitive
HS3	Huayou 8	43.00~47.00	Medium oil content,Environment-insensitive
HS4	LCD07-4	43.00~48.00	Medium oil content,Environment-sensitive
HS5	Xiangyou 15	36.00~42.00	Low oil content, Environment-insensitive
HS6	Xiang 774-3	36.00~42.00	Low oil content, Environment-sensitive

Note: The oil content (%) is the result of rape varieties planted under conventional field conditions in Zhejiang Province.

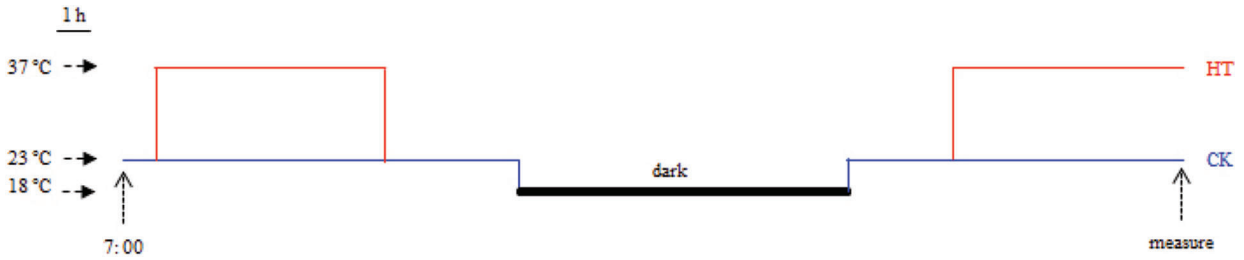

Figure S1: Temperature regime for heat stress of *B. napus* silique