

Article

Evaluating Heat Tolerance in Leaf Mustard (*Brassica juncea*) Using Cell Membrane Thermostability Across Multiple Environments with AMMI and GGE Biplot Analyses

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Abstract

Heat stress is an increasing constraint on leaf mustard production (*Brassica juncea*); however, methods for screening heat tolerance and evaluating genotype stability across environments remain limited. This study established a rapid screening method for heat tolerance based on relative injury (RI, %), expressed as cell membrane thermostability (CMT) under heat treatments. A preliminary temperature-response experiment showed a sigmoidal increase in RI, with 45 °C providing the greatest discrimination among genotypes; therefore, it was selected as the evaluation temperature for heat tolerance. Six leaf mustard accessions from Myanmar and Japan were evaluated under five sowing conditions. The combined ANOVA revealed significant effects of genotype, environment and genotype-by-environment interaction, indicating that heat tolerance is genetically controlled but is also influenced by environmental conditions. Genotype stability across environments was evaluated using the additive main effects and multiplicative interaction (AMMI) and genotype plus genotype-by-environment (GGE) biplot. The analysis showed that JP256550 (M3) had the lowest and most favorable genotype selection index (GSI) for CMT, whereas Yamagata seisai and Miike takana, with the lowest AMMI stability value (ASV), showed the most stable CMT across environments. In contrast, JP256560 (M1) and JP256554 (M4) exhibited greater environmental sensitivity and specific adaptation. Different sowing times also accelerated plant development and flowering, with the Myanmar accessions bolting earlier than the Japanese accessions. The results indicated that CMT measured at 45 °C is an effective phenotyping tool for heat tolerance screening in combination with multi-environment stability analysis, providing a practical approach to identify stable genotypes and suggesting that M3 is a promising candidate for future heat-tolerance breeding.

Keywords: heat tolerance; leaf mustard; cell membrane thermostability; AMMI; GGE biplot; flowering time



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1. Introduction

Leaf mustard (*Brassica juncea* (L.) Czern. & Coss.) is an important and highly diverse species of the genus *Brassica*. Its seeds are cultivated worldwide for their pungent flavor and high oil content ranging from 30 to 40% [1]. Leaf types are widely cultivated for

pickling in Southeast Asian countries because of their strong pungency and better postharvest freshness [2,3]. Temperature is a major abiotic stress that affects the timing of seed germination and seedling establishment. Temperatures between 25 and 35 °C promote water uptake and accelerate germination in *Brassica* species [4,5]. However, prolonged thermal stress (27–35 °C) during the seedling stage can reduce the marketable fresh weight of *B. juncea* by up to 58% [6]. Moreover, the long photoperiods during summer cause early flowering, shorten the vegetative period and reduce leaf yield [7,8].

As a cool-season crop, leaf mustard is typically cultivated during the early spring (April) or autumn–winter (September–October) in Tokyo, Japan. However, the increasing and unpredictable temperature conditions have recently begun to challenge these traditional cultivation periods. Therefore, developing a reliable method to evaluate heat tolerance and identify heat-tolerant genotypes is essential for breeding heat-resilient leaf mustard. Cell membrane thermostability (CMT), determined by measuring electrolyte leakage, is a widely used physiological indicator of heat tolerance in plants [9–11]. Membrane injury is quantified based on electrolyte leakage after heat treatment and the temperature causing approximately 50% membrane injury (lethal temperature—LT50) is commonly used to establish suitable conditions for CMT evaluation; however, the optimal temperature for evaluating leaf mustard has not yet been identified.

Wild relatives and traditional accessions are important genetic resources that provide diversity for the improvement of tolerance to abiotic stresses [12]. The materials for heat tolerance evaluation were local *B. juncea* leaf mustard accessions collected from Myanmar, a tropical region with high temperatures and humidity [13–15]. Heat tolerance is a complex quantitative trait controlled by multiple genes and strongly influenced by environmental factors; thus, a multi-environment trial (MET) is necessary for its evaluation. To identify superior lines with wide adaptability across environments, it is important to understand the genotype-by-environment interaction (GEI) [16]. In this regard, analysis of variance (ANOVA), the additive main effects and multiplicative interaction (AMMI) model [17–19] and the genotype plus genotype-by-environment (GGE) model [20] are effective methods.

Therefore, this study aimed for:

- Development and validation of a CMT-based method for evaluating heat tolerance in *Brassica juncea* leaf mustards;
- Identification of superior, stable and broadly adapted genotypes across multiple environments in Japan by AMMI and GGE biplot analyses.

2. Results

2.1. Preliminary Experiment: Heat Treatment Optimization for CMT Evaluation

Relative injury (RI, %) increased progressively with increasing temperature across all cultivars/accessions, following a sigmoidal response curve (Figure 1). Estimated LT50 values clustered around 45 and 50 °C, with 45 °C providing the clearest discrimination among cultivars/accessions. Accordingly, 45 °C was selected as the evaluation temperature for the subsequent heat-tolerance assay.

At 45 °C, RI (%) differed significantly among the ten cultivars/accessions of *B. juncea* leaf mustard ($p < 0.05$; Table 1). Murasakinaga obatakana showed the lowest RI (14.9%), indicating the least membrane injury, whereas the unknown accession had the highest RI (74.6%).

Field growth evaluation classified the tested *B. juncea* cultivars/accessions into three groups based on final plant height (Table A1). Within each group, a negative correlation was observed between RI (%) at 45 °C and plant height during the high-temperature period, with r ranging from -0.61 to -0.99 . It indicated that cultivars/accessions with lower RI values tended to maintain greater plant height under elevated-temperature conditions.

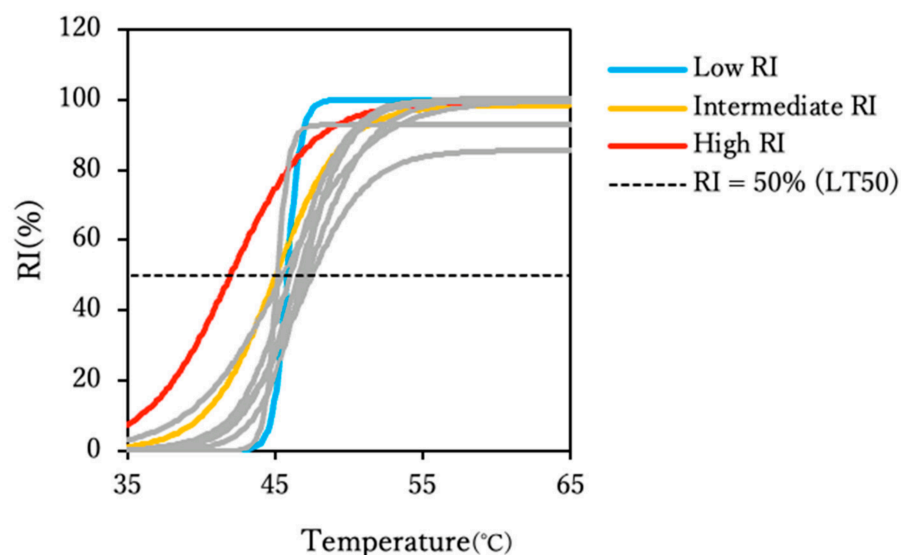


Figure 1. Sigmoid response curves of relative injury under four different heat stresses; RI% = relative injury; LT50 = lethal temperature at 50%, where 50% of the cell membrane was damaged; the grey lines represent the RI values of the remaining accessions.

Table 1. Relative injury and lethal temperature at 50% of ten leaf mustard genotypes.

Cultivars/Accessions	Accession Number	LT50	RI (%) at 45 °C
Sendai basyona	JP26129	46.0	35.9 bc
Murasakinaga obatakana	JP26116	45.9	14.9 c
Yamashiona	JP26134	47.2	26.6 bc
Okinawa karashina	JP26137	46.6	28.8 bc
Yamagata seisai	JP26108	47.0	21.6 bc
Unknown	JP90743	42.0	74.6 a
PAK-85870	JP88061	45.1	49.3 ab
Chinese mustard	JP73386	47.5	26.6 bc
BGR 413	JP80937	45.2	40.5 bc
INT/TAINAN	JP37393	45.5	46.0 ab

LT50 = lethal temperature at 50%. RI (%) = relative injury. Means followed by different letters within the RI column are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test.

2.2. Main Experiment: Multi-Environment Evaluation of Heat Tolerance

2.2.1. Monthly Variations in Cell Membrane Thermostability Across Different Environments

Similar to RI (%), cell membrane thermostability (CMT) values quantify the extend of cell membrane damage; consequently, lower CMT values reflect reduced membrane injury and therefore greater heat tolerance of the genotype. Significant differences in mean CMT among accessions were observed in four environments, E1, E2, E3 and E4, whereas no significant difference was detected in E5 (Table 2, Figure A1). The lowest mean CMT was recorded in E3 (May 2023) and E2 (August 2023), at approximately 51%, whereas the highest value was observed in E4 (September 2022), reaching 62.10%. Significant variations in mean CMT were also detected between accessions. M3 exhibited the lowest mean CMT (44.10%), followed by Miike takana (49.86%). In contrast, M1 and M4 recorded the highest CMT values of 59.58% and 60.78%, respectively.

Table 2. Cell membrane thermostability of six genotypes across five environments.

Genotypes/Environments	E1	E2	E3	E4	E5	Mean
M1	65.58 a	49.99 ab	51.06 ab	80.65 a	50.59 a	59.58 ab
M3	46.91 ab	43.35 b	36.30 b	43.36 c	50.57 a	44.10 c
M4	50.22 ab	64.24 a	56.40 a	67.61 ab	65.44 a	60.78 a
Miike	41.12 b	50.12 ab	48.83 ab	58.08 bc	51.13 a	49.86 bc
Mura	59.90 ab	40.90 b	52.03 a	56.18 bc	53.26 a	52.45 abc
Yama	55.25 ab	56.67 ab	58.99 a	66.70 ab	57.05 a	58.93 ab
Mean	53.16 ab	50.88 b	50.60 b	62.10 a	54.67 ab	

Means followed by different letters within each column are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test. Values are means of three plants per accession, with three samples for each plant.

M3 consistently exhibited lower CMT values in E2, E3 and E4 of 43.35%, 36.30% and 43.36%, respectively. M1 recorded its lowest CMT in the E2 and E5 environments (50.01% and 49.41%, respectively), while M4 produced the lowest CMT in E1 and E3 (50.22% and 56.40%, respectively). Genotypes showed contrasting environmental responses, with M1 exhibiting a marked decrease in CMT from 65.58% in E1 to 49.99% in E2, while M4 showed the opposite trend, increasing from 50.22% to 64.24%.

2.2.2. Combined Analysis of Variance for Cell Membrane Thermostability and Agronomic Traits

The combined ANOVA revealed a highly significant effect of environment (E), genotype (G) and genotype $\times$ environment interaction (GEI) on CMT, plant height and number of leaves ($p < 0.001$; Table 3). Genotype accounted for 31.15%, 23.83% and 45.90% of the total variation in CMT, plant height and number of leaves, respectively. Environmental effects were particularly pronounced for plant height and number of leaves, accounting for 65.21% and 45.23%, respectively; however, they contributed less to CMT (14.99%). In contrast, the GEI accounted for 27.24% of the variation in CMT, compared with 9.92% and 7.34% for plant height and number of leaves, respectively.

Table 3. Combined variance analysis for ANOVA of cell membrane thermostability and other development traits.

Sources	CMT			PH			NOL		
	df	MS	SS (%)	df	MS	SS (%)	df	MS	SS (%)
Genotype	5	655.69 ***	31.15	5	2036.62 ***	23.83	5	405.05 ***	45.90
Environment	4	394.23 ***	14.99	4	5573.23 ***	65.21	4	399.14 ***	45.23
Interactions	20	143.35 ***	27.24	20	847.89 ***	9.92	20	64.78 ***	7.34
Residuals	60	46.68	26.62	210	89.26	1.04	210	13.55	1.54

*** Refers to significance if $p < 0.001$; df = degrees of freedom; MS = mean of squares and SS (%) = % explained of sum squares. CMT = cell membrane thermostability. PH = Plant height (cm). NOL = number of leaves. CMT was measured from three plants per accession, with three subsamples for each plant. PH and NOL were recorded for eight plants per accession.

2.2.3. Additive Main Effects and Multiplicative Interaction (AMMI) Analysis of Cell Membrane Thermostability

AMMI analysis was performed to further evaluate the genotype stability and interaction patterns for CMT across five environments. The results showed that G, E and GEI effects were all highly significant ($p < 0.001$) (Table 4). Partitioning of the GEI revealed that the first two interaction principal component axes (IPCA1 and IPCA2) explained most interaction patterns (90.34%). IPCA1 accounted for 59.09% of the GEI sum of squares, whereas

IPCA2 explained an additional 31.25%. The GEI pattern showed a distinct response of genotypes to the environments.

Table 4. The AMMI analysis of variance for cell membrane thermostability.

Source	df	MS	SS (%)
Genotype	5	655.69 ***	31.15
Environment	4	394.23 ***	14.99
Interactions	20	143.35 ***	27.24
IPCA1	8	211.79 ***	59.09
IPCA2	6	149.31 **	31.25
IPCA3	4	63.86 ns	8.91
IPCA4	2	10.76 ns	0.75
Residuals	60		26.62

***, ** refer to significant if $p < 0.001$ and $p < 0.01$, respectively; ns refers to not significant; df = degrees of freedom; MS = mean of squares; SS = sum of squares; SS (%) = explained sum of squares (%); IPCA = interaction principal component axis.

In the AMMI biplot, environments and genotypes located close to the origin or at the center generally displayed lower $G \times E$ interactions and, consequently, greater stability across environments [21]. Based on this criterion, E3 showed the lowest interaction, whereas E2 and E5 were relatively stable environments (Figure 2). In contrast, E1 and E4 showed stronger environmental interaction. Among genotypes, Yama showed the greatest stability, followed by Miike and M3.

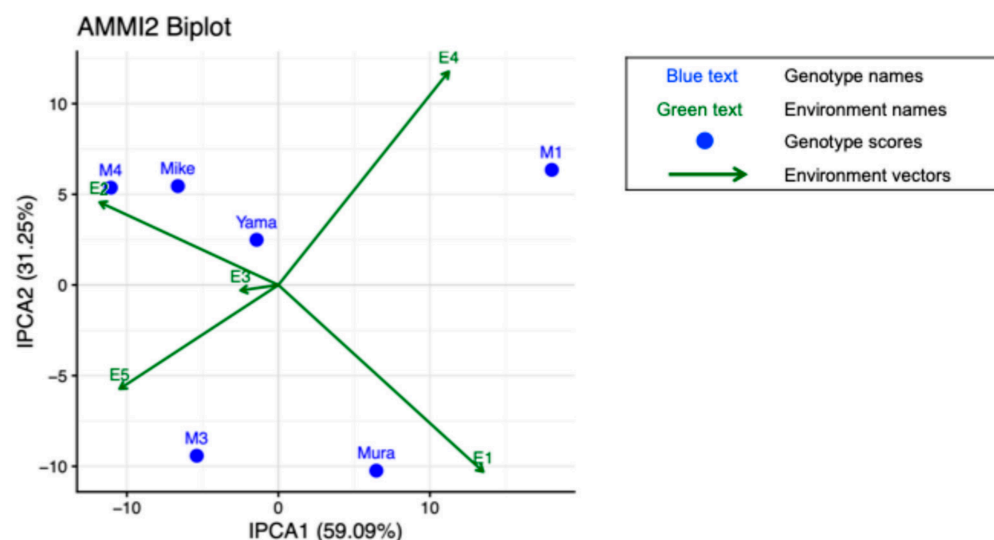


Figure 2. The AMMI biplot of cell membrane thermostability of six leaf mustard accessions across five environments. IPCA = interaction principal component axis.

The AMMI biplot was constructed using IPCA1 and IPCA2 scores as spatial coordinates for genotypes and environments [17,19]. Scores closer to zero, regardless of their positivity or negativity, indicate a smaller contribution to the $G \times E$ interaction and, consequently, greater genotype stability [19]. Consistent with this interpretation, a lower ASV indicates greater stability across the environments. In this study, ASV ranged from 3.68 to 34.71. Yama exhibited the highest stability, with the lowest ASV, followed by Miike and M3 with ASVs of 13.68 and 13.87, respectively (Table 5). In contrast, M4 and M1 showed the highest ASVs, at 21.59 and 34.71, respectively.

Table 5. AMMI stability value (ASV) for cell membrane thermostability (CMT, %).

Genotypes	IPCA1	IPCA2	ASV	Rank (ASV)	Mean CMT	Rank (Mean)	GSI
M3	−5.38	−9.42	13.87	3	44.10	1	4
Miike	−6.63	5.45	13.68	2	49.86	2	4
Mura	6.46	−10.24	15.95	4	52.45	3	7
Yama	−1.44	2.49	3.68	1	58.93	4	5
M1	18.05	6.35	34.71	6	59.58	5	11
M4	−11.06	5.37	21.59	5	60.78	6	11

IPCA = interaction principal component axis; ASV = AMMI stability value; GSI = genotype selection index.

Lower GSI values indicate a more favorable combination of mean performance and ASV [22–24]. Among the six accessions, GSI values ranged from 4 to 11 (Table 5), with M3 and Miike showing the lowest GSI values (4) while M1 and M4 showed the highest (11).

2.2.4. Genotype Main Effects Plus Genotype-by-Environment Interaction (GGE) Biplot

Given that lower CMT values indicate greater heat tolerance, CMT was transformed into reversed CMT (CMTr; $\text{CMTr} = 100 - \text{CMT}$) before GGE biplot analysis. This transformation placed higher CMTr values in the direction of greater heat tolerance, facilitating the interpretation of GEI and the which-won-where pattern.

The GGE biplot explained 89.22% of the total G + GE variation, with PC1 and PC2 accounting for 63.28% and 25.94%, respectively (Figure 3a,b). The angle between two environment vectors, expressed through its cosine, reflects the degree of association between the environments. A close relationship was observed between E1 and E4 of 2022 and among E2, E3 and E5 of 2023.

In the which-won-where GGE biplot, the polygon view delineated the patterns of genotype performance across the environments [24]. Genotypes positioned at the polygon vertices represent the most responsive genotypes, therefore showing the strongest GEI effects. These vertex genotypes define the sectors in which they exhibit the highest or lowest performance [24]. In this study, Miike, M3, Mura, M1 and M4 were positioned at the polygon vertices and collectively formed the polygon (Figure 3a).

Equality lines extending from the origin to each side of the polygon partitioned the plot into distinct sectors, each representing a potential mega-environment. The sector of M3 contained all environments except E5 and showed the highest performance. E5 was positioned on the equality line separating the M3 and Mura sectors, indicating that the relative performance of these genotypes was less distinct in this environment. A similarity was observed between M3 and Miike under the E1 condition. In contrast, the sectors defined by M1 and M4 comprised no tested environments (Figure 3a).

The mean versus stability view of the GGE biplot ranked the genotypes according to their mean CMTr values along the average environment coordination (AEC) abscissa, represented by a green line with an arrow, whereas stability was assessed based on the length of each accession's projection onto the AEC ordinate (Figure 3b). Genotypes positioned farther in the direction of the AEC arrow had higher mean CMTr, indicating greater heat tolerance. M3 was positioned at the greatest distance along the AEC axis and had a relatively short projection, thereby showing the highest and a relatively stable CMTr value. Yama also possessed a short projection; however, it was positioned on the opposite side of the AEC origin and showed a lower value CMTr.

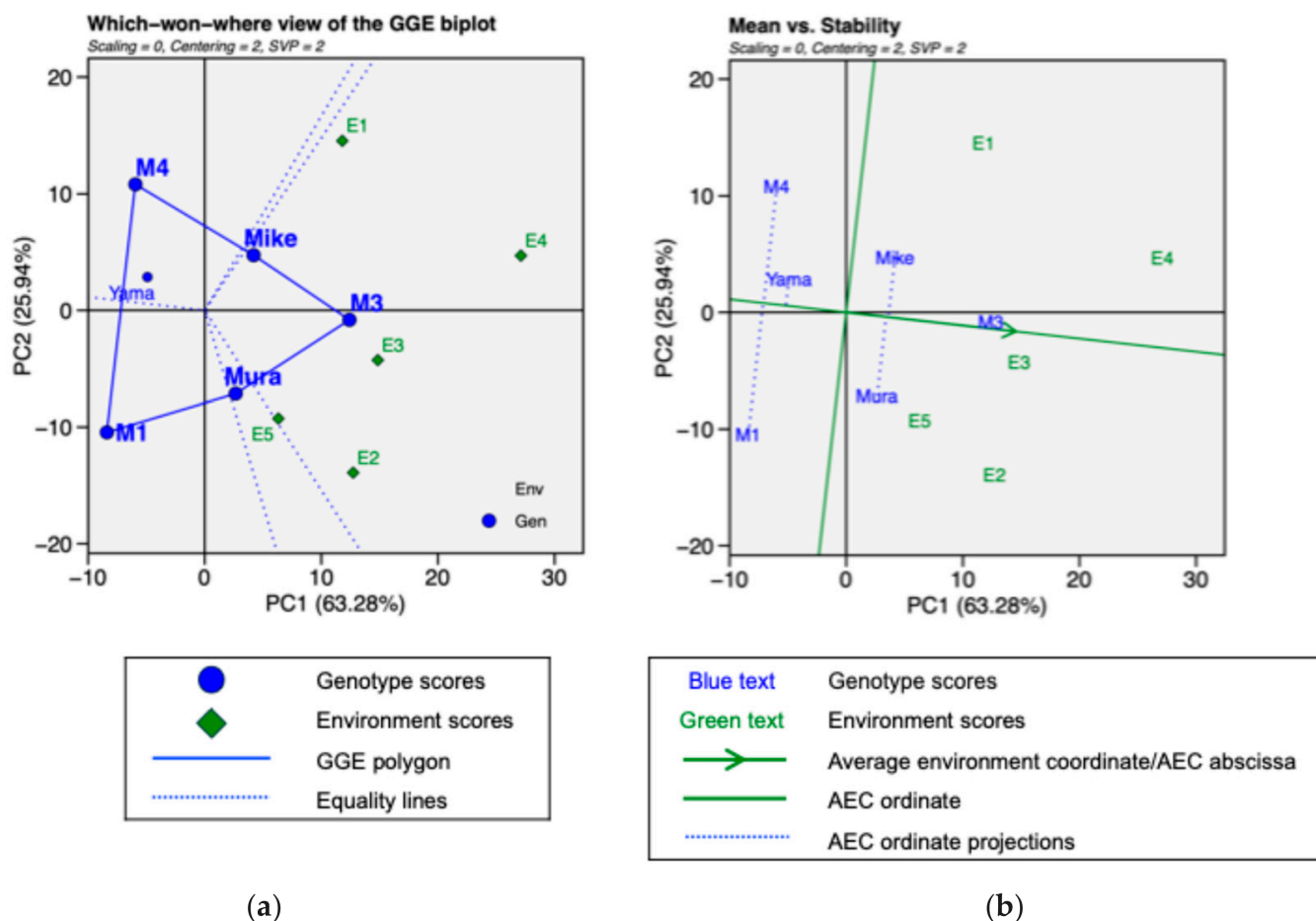


Figure 3. GGE biplot analysis of reversed cell membrane thermostability (CMTr) in six leaf mustard genotypes across five environments. (a) Which-won-where polygon view. (b) Mean performance and stability view of CMTr. Scaling = 0 refers to no scaling; Centering = 2 refers to environment-centering; and SVP = 2 refers to singular values are partitioned to the genotype scores.

2.2.5. Growth and Development Traits of Leaf Mustard Under Different Environments

To evaluate plant growth and development, plant height and number of leaves were measured five weeks after transplanting, while days to bolting were monitored throughout the growing period (Figure 4). Across the five environments, substantial variation was observed in plant growth, with mean plant height ranging from 22.41 cm in E4 to 52.20 cm in E3. A similar environmental pattern was observed for leaf production, with plants in E4 developing the fewest leaves (six leaves), whereas those in E3 produced the greatest number (14 leaves) (Figure 4a,c).

Among the genotypes, Mura exhibited the greatest plant height and produced the highest number of leaves across environments (Figure 4b,d). Bolting time also varied considerably among environments and genotypes. Plants grown in E3 bolted earliest, at 50 days after transplanting, whereas bolting was markedly delayed in E4, E5 and E2, occurring at 123, 124 and 127 days after transplanting, respectively. Among genotypes, M3 was the earliest to bolt (49 days after transplanting), whereas Miike exhibited the most delayed bolting, requiring 141 days after transplanting to bolt (Figure 4e,f).

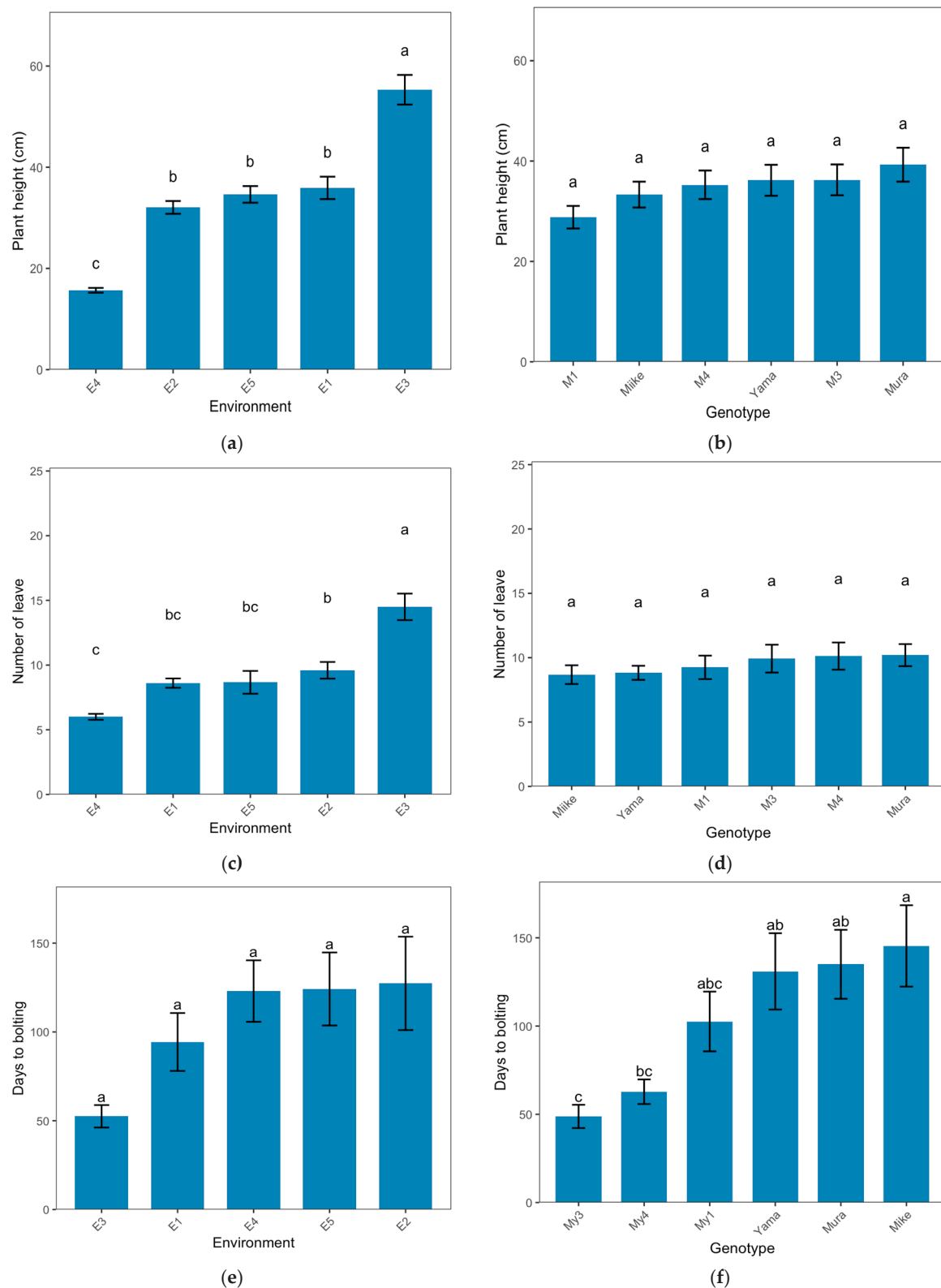


Figure 4. Bar plot of plant growth and bolting characteristics of six *B. juncea* accessions across five environments. (a) Mean seasonal plant height; (b) mean plant height by accession; (c) mean seasonal number of leaves; (d) mean number of leaves by accession; (e) mean seasonal days from transplanting to bolting; (f) mean days from transplanting to bolting by accession. Different letters of each bar are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test. Values are means of eight plants per accession.

3. Discussion

3.1. Cell Membrane Thermostability as a Reliable Indicator for Heat Tolerance in Leaf Mustard

Although the negative effects of elevated temperature on *Brassica* crops have been widely reported, effective physiological methods for evaluating heat tolerance in leafy mustard remain limited [25]. Relative injury (RI%) and cell membrane thermostability (CMT) are widely used indicators of membrane damage under heat stress and have been applied to many crop species [9,10]. Exposure to 50 °C has been successfully used in the soybean [26], cabbage [27] and leafy radish [28]. The finding from the initial experiment using sigmoidal analysis revealed significant differences in RI (%) among tested genotypes under heat treatment at 45 °C. This treatment temperature corroborates those used in the classical CMT studies [9,10].

3.2. Genotypic and Environmental Variation in CMT and Heat Acclimation of Leaf Mustard

The significant differences in CMT among the six leaf mustard genotypes indicate variation in membrane response. This suggests that CMT can be used as a physiological trait for screening heat tolerance. Notably, CMT values were generally lower under the warmer conditions, indicating greater membrane stability. This pattern may reflect heat acclimation, whereby prior exposure to higher temperatures in the field enhanced the capacity of cell membranes to withstand the subsequent heat treatment used in CMT evaluation. Such acclimation could also have contributed to the clearer differentiation among genotypes under warmer growing conditions. Similar temperature-acclimation responses have been reported in cotton and wheat [10,29,30]. Heat responses can be affected by several factors, including water status, stress intensity and duration, crop developmental stage and prior acclimation. However, Rahman found no marked difference in the extent of genetic variation for CMT between heat stress and non-stressed conditions [30].

3.3. Genotype $\times$ Environment Interaction and AMMI Analysis of Genotype Stability

The combined ANOVA showed that GEI had a great influence on CMT, indicating that under different environments the genotypes react differently. GEI is particularly important when physiological traits are used in breeding because selection based only on pooled means may overlook stable materials or flavor genotypes whose performance is more environmentally specific. Similar GEI effects have been reported for *Brassica* crops grown under different environments and stress conditions [31–34].

Conventional statistical methods, such as linear regression, ANOVA and principal component analysis (PCA), are often insufficient for evaluating complex data from multi-environment trials because of the strong effects of GEI [30]. Therefore, the AMMI model combines ANOVA and PCA in a single analytical framework to better explain GEI. AMMI analysis has been widely used in breeding programs for many crops, for example, seed oil and seed protein content in *B. napus* [35]. In addition, AMMI provides stability data such as the AMMI stability value (ASV), which quantifies the stability of genotypes for the target traits [19]. However, stability alone is insufficient for identifying promising genotypes, due to the lack of favorable performance. Therefore, mean performance and stability should be considered together, as reflected by the GSI, to provide a more informative basis for genotype selection.

3.4. Comparison of AMMI and GGE Biplot Analysis

Compared to the AMMI biplot, the GGE biplot excludes environmental effects, making it easier to interpret visually and more practical for selection [36,37] and for breeding programs [38]. The which-won-where pattern of the GGE biplot is considered effective for presenting genotype performance and GEI [39].

Based on the AMMI analysis of the ASVs, Yama was the most stable genotype, followed by Miike and M3. The GGE mean vs. stability biplot identified M3 and Yama as the most stable genotypes. Considering both CMT values and stability across environments, M3 was the most promising genotype, showing strong heat tolerance and stable performance across different conditions.

Thus, using both AMMI and GGE biplots may provide a more comprehensive evaluation of genotype performance and stability across environments, as the two methods complement each other [40].

3.5. Flowering Time Across Environments

The flowering period is an essential trait of plants that is involved in the quality and quantity of biomass. Rice and soybean are short-day plants that bolt quickly under short-day conditions [36,37], whereas leaf mustard and *Brassica* crops are controlled by the interaction between vernalization and a long photoperiod [8,41]. Long-term exposure to low temperatures induces the capacity to flower, whereas long-day conditions accelerate floral transition and bolting. The early sowing periods, May (E3) and June (E1), had the longest days, with increasing temperature, which showed the earliest flowering times. Interestingly, the August sowing period (E2), with decreasing temperature, showed later flowering times similar to those under optimal conditions (E4 and E5). Early flowering may be a heat escape mechanism that contributes to heat tolerance, helping plants avoid terminal heat stress and transition to the reproductive stage earlier, as previously reported in *B. juncea* [42].

4. Materials and Methods

4.1. Overview of the Study Design

This study consisted of two experiments. First, a preliminary experiment was conducted to determine the appropriate heat-treatment temperature for evaluating heat tolerance in leaf mustard using a cell membrane thermostability (CMT) assay. In this experiment, relative injury (RI, %) was measured across a series of temperature treatments and the temperature causing the clearest discrimination among genotypes was selected for further evaluation. Second, a multi-environment trial (MET) was conducted to evaluate the heat tolerance and stability of six leaf mustard genotypes across five sowing environments using the selected CMT condition.

4.2. Preliminary Experiment: Optimization of Heat-Treatment Temperature for CMT Evaluation

4.2.1. Plant Materials and Growth Conditions

A preliminary experiment was conducted in September 2015 at the Setagaya Campus of the Tokyo University of Agriculture, Tokyo, Japan. Ten cultivars/accessions of *B. juncea* with different geographical origins, preserved in a genebank, were used for this experiment. Seedlings were raised in a growth chamber and then transplanted to the experimental field. Plants were cultivated under a non-woven fabric covering to enhance high-temperature conditions and air temperature inside the cover was recorded during the experimental period.

Plant growth and development under high temperature were assessed once a week from 26 September to 19 October 2015, by recording plant height.

4.2.2. CMT Assay and Calculation of Relative Injury

Heat tolerance was assessed using a cell membrane thermostability assay based on electrolyte leakage. Fresh leaf disks were collected from the field using a cork borer and subjected to temperature treatments at 25, 35, 45, 55 and 65 °C for 30 min in a thermostatically controlled water bath. The 25 °C treatment was used as the control.

After heat treatment, distilled water was added to each sample, and the samples were incubated at 25 °C for 24 h before the first measurement of electrical conductivity. The electrical conductivity values of the heat-treated and control samples were recorded as T1 and C1, respectively. Subsequently, the samples were frozen at −80 °C for at least 24 h, thawed and measured again to obtain total conductivity values (T2 and C2, respectively) using a compact electrical conductivity meter (LAQUAtwin EC-33B, HORIBA Advanced Techno Co., Ltd., Kyoto, Japan). Relative injury (RI, %) was calculated using the corrected formula [9,10]:

$$RI(\%) = \left(1 - \frac{1 - \frac{T1}{T2}}{1 - \frac{C1}{C2}} \right) \times 100$$

where T and C represent the heat-treated and control samples (25 °C), respectively.

4.2.3. Determination of LT50 and Selection of Evaluation Temperature

Sigmoid response curves were generated using RI values across the temperature treatments and the temperature causing 50% membrane injury (lethal temperature at 50%—LT50) was estimated for each cultivar/accession. The relationship between LT50 and RI values at each treatment temperature was examined to identify the temperature condition that most clearly discriminated varietal differences in heat tolerance. Based on this preliminary analysis, 45 °C was selected as the evaluation temperature for the main experiment. The relationship between RI at 45 °C and plant growth under high-temperature conditions was also evaluated.

4.3. Main Experiment: Multi-Environment Evaluation of Heat Tolerance

4.3.1. Experimental Site and Environments

The main experiment was carried out in a greenhouse at the Tokyo University of Agriculture, Setagaya Campus, Tokyo, Japan (35°38'33" N, 139°37'48" E; 53 m above sea level), during the 2022–2023 and 2023–2024 growing seasons. Tokyo has a humid subtropical climate, characterized by hot and humid summers from late May to August and an average annual precipitation of approximately 1200–1800 mm.

A multi-environment trial (MET) was conducted across five sowing seasons over two years. Three sowing-trials were performed under relatively high-temperature conditions, with sowing conducted on 14 June 2022, 9 May 2023, and 22 August 2023, whereas two sowing-trials were established under relatively mild conditions, with sowing on 26 September 2022 and 22 September 2023. Daily meteorological data corresponding to each sowing period were obtained from Tutiempo Climate Data (Madrid, Spain). The experimental environments were characterized based on the vapor pressure deficit (VPD), number of days with maximum temperatures above 30 °C and heat degree days (HDD) (Table 6). Based on the combined patterns of HDD and the number of days above 30 °C, the environments were ordered from higher to lower heat exposure, with E1 representing the highest and E5 the lowest heat exposure.

Table 6. Meteorological conditions recorded across five environments.

Sowing Times	T max	T min	Days > 30 °C	RH	VPD	HDD Total	Environment
Jun 2022	37.0	15.1	39	77.7	1.13	130.1	E1
Sep 2022	29.6	7.7	0	71.5	0.72	0	E4
May 2023	33.7	10.1	11	76.3	0.85	17.3	E3
Aug 2023	34.2	12.1	25	74.2	1.03	64.2	E2
Sep 2023	26.6	5.9	0	66.1	0.82	0	E5

T max = maximum temperature (°C), T min = minimum temperature (°C), RH = relative humidity (%), VPD = vapor pressure deficit (kPa), Days >30 °C = number of days with temperature over 30 °C (days).

4.3.2. Plant Materials

Six *B. juncea* genotypes, including three Myanmar accessions and three Japanese cultivars, were used in the main experiment (Table 7). The Japanese cultivars (Yamagata seisai, Murasaki takana and Miike takana) belong to *B. juncea* var. *integrifolia* and are widely grown in the Yamagata and Fukuoka prefectures. The Myanmar accessions were collected from Shan States with prior informed consent. Detailed passport data, including collection site coordinates, elevation and accession records, are available in the NARO Genebank database (Ibaraki, Japan).

Table 7. Leaf mustard accessions and cultivars evaluated for heat tolerance across five environments.

Abbreviation	Accession No.	Collection/Variety Name	Origin	Latitude	Longitude	Altitude (m)
M1	JP256560	Monehyin	Shan, Myanmar	20°56'32" N	96°39'45" E	1199
M3	JP256550	Monehyin	Shan, Myanmar	20°38'13" N	96°33'43" E	1325
M4	JP256554	Monehyin	Shan, Myanmar	20°38'13" N	96°33'43" E	1325
Miike	-	Miike takana	Japan	-	-	-
Mura	-	Murasaki takana	Japan	-	-	-
Yama	-	Yamagata seisai	Japan	-	-	-

Accession no. = accession number of the genotypes in NARO Genebank.

4.3.3. Crop Management and Growth Trait Evaluation

Seeds were initially sown in a greenhouse and seedlings were transplanted into the greenhouse four to five weeks later at a spacing of 35 × 35 cm. Basal fertilization was provided using Akitsu Midori Ichiban cow compost (Nose Farm Co., Ltd., Nara, Japan), followed by NPK fertilizer (10:4:4) (Kumiai Fertilizer Co., Ltd., Iwate, Japan).

Plant growth and development were evaluated using eight plants per accession. Plant height (PH, cm) and number of leaves (NOL) were recorded four weeks after transplanting, whereas days to bolting (DTB) were recorded weekly throughout the experiment.

4.3.4. CMT Assay in the Multi-Environment Trial

Cell membrane thermostability (CMT) was evaluated five weeks after transplanting using three plants per accession, with three leaf samples collected from each plant. Based on the preliminary experiment, leaf disks were heat-treated at 45 °C for 30 min in a thermostatically controlled water bath. Following heat treatment, samples were incubated in distilled water at room temperature (25 °C) for 24 h, after which electrical conductivity was measured before and after freezing at −80 °C using a compact electrical conductivity meter (LAQUAtwin EC-33B). CMT was calculated as follows:

$$\text{CMT}(\%) = \frac{C_1}{C_2} \times 100$$

C1 and C2 represent electrolyte leakage measured after 45 °C and −80 °C treatment, respectively.

Because lower CMT values indicate higher heat tolerance, these values were transformed into reversed CMT (CMTr) values, with higher CMTr values representing greater heat tolerance for visualization and interpretation of the GGE biplot. Higher CMTr values therefore indicate greater membrane stability and higher heat tolerance:

$$\text{CMTr}(\%) = 100 - \text{CMT}$$

4.4. Statistical Analysis

4.4.1. Preliminary Experiment

The relative injury (RI) values calculated in Excel for each cultivar/accession across the temperature treatments were imported into SigmaPlot 12 (Systat Software, Inc., San

Jose, CA, USA). A three-parameter logistic model was fitted to the relationship between treatment temperature and the cell damage rate, and the temperature at which the cell damage rate reached 50% was determined as LT50 from the fitted regression curve using the Curve Fit function. Mean differences among cultivars/accessions at heat treatment were compared using Tukey's multiple comparison test at $p < 0.05$. The relationship between RI at 45 °C and plant growth under high-temperature conditions was also examined using Pearson's correlation analysis in Excel.

4.4.2. Main Experiment

For the main experiment, morphological and physiological data collected from the MET were analyzed using R version 4.5.2 (2025) of RStudio 2025.09.1+401 (R Foundation for Statistical Computing, Vienna, Austria). For each genotype within each environment, one plot was established, from which three plants were randomly sampled. For each sampled plant, the three leaf subsamples were averaged to obtain a plant-level CMT value. The plant-level observations were used for the combined ANOVA, with variation among plants sampled within each plot included in the residual error term as within-plot sampling variation. Mean differences in CMT values were compared using Tukey's multiple comparison test at $p < 0.05$. The effects of genotype (G), environment (E) and genotype $\times$ environment interaction (GEI) were estimated by combined analysis of variance (ANOVA). The ANOVA model was specified as:

$$Y_{ijk} = \mu + G_j + E_i + (GE)_{ij} + \varepsilon_{ijk}$$

Y_{ijk} = CMT value of the plant (k), genotype (j), in the environment (i); μ = overall mean; G_j = the main effect of genotype (6 genotypes); E_i = the main effect of environment (5 environments); $(GE)_{ij}$ = genotype $\times$ environment interaction; and ε_{ijk} = within-plot sampling variation among three sampled plants.

To evaluate genotype stability for CMT across environments, the additive main effects and multiplicative interaction (AMMI) model was applied [17,18]. The AMMI stability value (ASV) was calculated according to [19] and the genotype selection index (GSI) was obtained by integrating ASV rank with mean CMT rank using R base functions [22,23]. The AMMI biplot was visualized by using the ggplot2 package version 4.0.3. The ASV can be calculated by the following formula [19]:

$$ASV = \sqrt{\left[\frac{SSIPCA1}{SSIPCA2}(\text{IPCA1 Score})\right]^2 + (\text{IPCA2 Score})^2}$$

SSIPCA1 and SSIPCA2 = sum of squares for IPCA1 and IPCA2, respectively (the variance explained by the first and second interaction principal component analysis, respectively). IPCA1 and IPCA2 = the coordinate value of a specific genotype on the first IPCA and the second IPCA, respectively.

A genotype plus genotype-by-environment interaction (GGE) biplot was constructed to characterize genotype performance and GEI patterns across the five environments [20]. The GGE model was fitted without scaling, with environment-centered data (centering = 2) and singular value partitioning (SVP = 1) to emphasize genotype-focused interpretation. GGE biplot analyses and visualizations were performed using the metan package version 1.19.0, together with the dplyr version 1.2.1 and the ggplot2 version 4.0.3.

5. Conclusions

The present study established 45 °C as an effective temperature for discriminating heat tolerance in leaf mustard based on cell membrane thermostability (CMT). The results further suggested that prior exposure to thermal stress may induce heat acclimation, reducing

membrane injury compared with plants grown under optimal conditions. Significant effects of genotype, environment and genotype $\times$ environment interaction demonstrated that heat tolerance is determined by both genetic and environmental factors, emphasizing the importance of multi-environment evaluation.

Across the five environments, AMMI and GGE biplot analyses consistently identified M3 (JP256550) as a high-performing genotype, with particularly favorable responses in E2, E3 and E5. Its stable performance across contrasting environments indicates its potential as a parent source for developing heat-resilient leaf mustard and for subsequent QTL analyses to dissect the genetic basis of the trait. Other genotypes, such as Yama (Yamagata seisai), M4 (JP256554) and M1 (JP256560), may serve as contrasting materials for subsequent validation, with Yama being particularly valuable with greatest stability. Furthermore, the CMT-based screening method established in this study provides a rapid and cost-effectiveness approach for phenotyping and evaluating heat adaptation. Combined with multi-environment testing and molecular approaches, this method could facilitate the efficient selection and genetic improvement of leaf mustard under rising-temperature stress.

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Abbreviations

The following abbreviations are used in this manuscript:

AEC	Average environment coordination
AMMI	Additive main effects and multiplicative interaction
ASV	AMMI stability value
CMT	Cell membrane thermostability
CMTr	Reversed cell membrane thermostability
GGE	Genotype plus genotype-by-environment
GSI	Genotype selection index
IPCA	Interaction Principal Component Axis
LT50%	Lethal temperature at 50%
MET	Multi-environment trial
PCA	Principal Component Analysis
RI	Relative injury
SVP	Singular Value Partitioning

Appendix A

Table A1. Growth parameters and cellular injury under field conditions.

No.	Cultivars/Accessions	Plant Height (cm)	Group	RI (%)	Relationship Between Plant Height and RI (%)		
					26 Sep	28 Sep	05 Oct
1	Chinese mustard	35.18	1	26.6	−0.613	−0.745	−0.634
2	Okinawa karashina	36.07		28.8			
3	Yamagata seisai	39.76		21.6			
4	Murasakinaga obatakana	40.12		14.9			
5	Sendaibasyouna	44.74	2	35.9	−0.841	−0.359	−0.803
6	Unknown	43.55		74.6			
7	INT/TAINAN	46.62		46.0			
8	Yamashiona	51.33	3	26.6	−0.993 **	−0.977 **	−0.827
9	PAK-85870	51.88		40.5			
10	BGR 413	59.12		49.3			

RI (%) = relative injury; different letters indicate significant differences at $p < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test. ** refers to significant if $p < 0.01$.

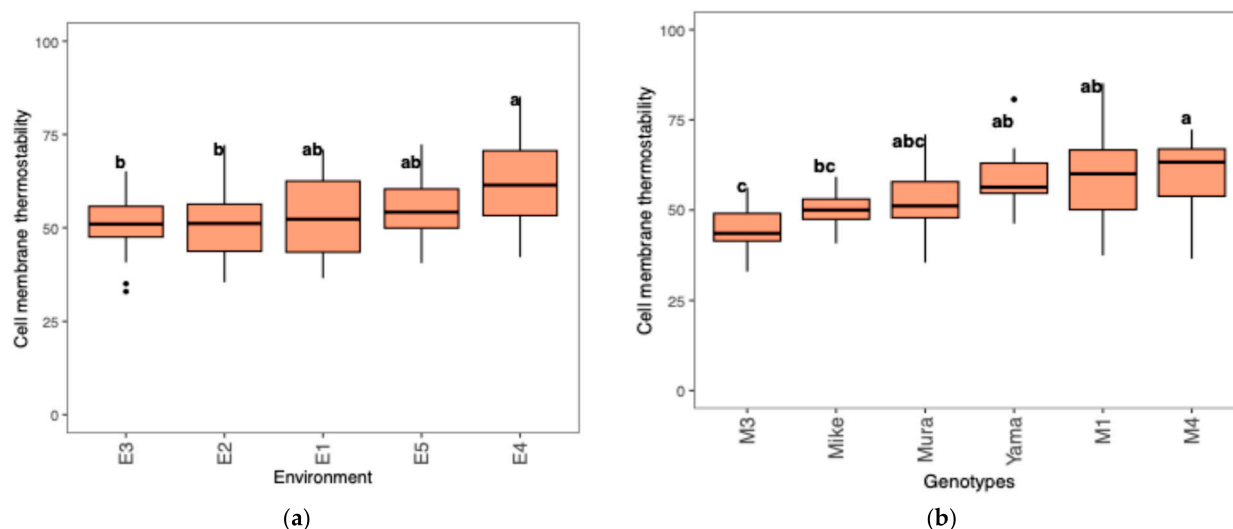


Figure A1. Boxplots of cell membrane thermostability (CMT) across environments and accessions; (a) CMT values across environments; (b) CMT values across genotypes. The boxes represent the interquartile range (IQR), lines within boxes indicate the medians, whiskers show the range within $1.5 \times \text{IQR}$, and dots indicate outliers. Different letters above the boxes indicate significant differences at $p < 0.05$ according to one-way ANOVA followed by Tukey's multiple comparison test.

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