Improved Method of Determination of Membrane Thermostability for Screening Heat-Tolerant and Sensitive Varieties in *Brassica*

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Abstract

We developed an improved method of determination of membrane thermostability (MT) for screening heat-tolerant and sensitive varieties in *Brassica* which is simple, rapid and precise. The use of two leaf discs 13 mm in diameter was sufficient to measure the electric conductivity for screening for temperature tolerance at the young stage. Elevated temperature at 55℃ was more suitable for distinguishing heat-tolerant varieties from heat-sensitive ones in *Brassica* within 3-4 h compared to the previous method where the sample was kept overnight at 10℃. Calculation method is very simple and accurate compared to the previous method, as it does not require a control value. Among the four varieties of cabbage (*Brassica oleracea* var. *capitata* L.) studied, the varieties Yoshin and YR Kinshun were the most tolerant followed by Sousyu, while the variety Kinkei No.201 was heat-sensitive. The heat killing time of the heat-tolerant and sensitive varieties was 150 and 120 min at the elevated temperature of 55℃ respec tively. In Chinese cabbage (*B. campestris* var. *pekinensis* L.), the varieties Kenshin and No. 11 were heat- tolerant, whereas the variety Chihiri 70 was heat-sensitive. In Chinese kale (*B. oleracea* var. *alboglabra* Bailey), the varieties Full white and Sen-yo shirobana were heat-tolerant while the variety Large leaf was heat-sensitive. In general, heat tolerance ability was higher in Chinese kale compared to cabbage and Chinese cabbage. The heat tolerance trait in the interspecific somatic and sexual hybrids between cabbage × Chinese cabbage, and between Chinese kale × Chinese cabbage was intermediate between that of the parents. The present results suggest that the improved method for the determination of MT is efficient and useful for screening and analyzing the genetic variability of heat tolerance in *Brassica*.

Additional key words: Temperature stress, EC, heat killing time

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INTRODUCTION

Membrane thermostability (MT) is one of the important methods used to evaluate tolerant and sensitive varieties in various crops such as sorghum (1, 2), soybeans (3), wheat (4), bean, potato and tomato (5). Sullivan (1) had described a procedure to estimate the electrolyte leakage from the leaf-disc induced by high or low temperature stress through electrical conductance. The amount of electrolytes leaked is a function of membrane permeability. The higher the value, the lower the tolerance and a longer heat killing time indicated a higher level of heat tolerance (6).

The production of *Brassica* crops like cabbage, Chinese cabbage, cauliflower and Chinese kale is often impaired in the tropics, when the growth temperature exceeds the critical level (7). Therefore, it is essential to develop a suitable and simple screening method in *Brassica* for ensuring the successful growth of these crops in tropical and subtropical countries. The present report describes an improved, simple and efficient method of determination of membrane thermostability (MT) for screening heat-tolerant and sensitive varieties and hybrids in *Brassica*.

MATERIALS AND METHODS

Plant materials: For screening heat-tolerant (HT) and sensitive (HS) varie ties, four varieties of cabbage (*Brassica oleracea* var. *capitata* L.) and Chinese cabbage (*B. campestris* var. *pekinensis* L.) and three varieties of Chinese kale (*B. oleracea* var. *alboglabra* Bailey) were used as follows: Yoshin (HT), Sousyu F_1 (HT), YR Kinshun F_1 and Kinkei No. 201 F_1 for cabbage; Kenshin (HT), Kensui F_1, Chihiri 70 F_1 (HS) and No. 11 F_1 for Chinese cabbage; and Sen-yo shirobana F_1, Full white and Large leaf for Chinese kale. At least one putative heat-sensitive variety was included for the present investigation.

Seeds were sown in vermiculite and one week after germination, seedlings were transplanted into pots 12 cm in size, filled with a soil:compost (3:1) mixture. Fertilizer was applied at the rate of 0.5 g mixed NPK (14-14-14), 1 g single super phosphate and 2g magnesium carbonate per kg of pot soil. The seedlings were grown in a glass house under natural day light conditions at 25/20 ± 2℃ and 30/25 ± 2℃ day/night temperatures. The leaf samples were collected from young 30-days old seedlings for membrane thermostability determination.

For the studies on genetic variability, three interspecific hybrids and their parents were used as follows: a somatic hybrid between the cabbage variety Yoshin and Chinese cabbage variety Kenshin, a sexual hybrid of the same combination (Yoshin × Kenshin) and another sexual hybrid between the Chinese kale variety Sen-yo shirobana and Chinese cabbage variety Kenshin.

Fifty-days old seedlings were used for the determination of membrane thermostability. The other conditions and tempera ture were the same as those for cabbage, Chinese cabbage and Chinese kale. All the experiments were replicated three times.

Improved method of determination of membrane thermostability (MT):

Two leaf discs were collected using a leaf-puncher 13 mm in diameter and washed 3 times with distilled water using a magnetic stirrer. The leaf samples were then placed in 25 × 150 mm test tubes and a piece of cotton was put on the leaf-disc. Thereafter, 20 ml of distilled water was added. Then, the initial conductivity reading (I) was taken with an EC meter (Model CM-115, Kyoto Electronics Co. Ltd, Japan). The test tubes were covered by aluminum foil or a stopper and placed in a thermostatically controlled water bath maintained at the constant temperature of 55℃. Electric conductivity reading (E) was taken every 30 min up to 4 h at the elevated temperature of 55℃. Subsequently, the samples were autoclaved at 121℃ for 15 min to completely kill the leaf tissues. The samples were again placed in a water bath at 55℃ to adjust the elevated temperature and after 30 min of incubation, final conductance (F) was measured. The percentage of injury induced by the elevated temperature during the time course (30 min) was calculated as follows:

$$\text{Injury (\%)} = \frac{E - I}{F} \times 100$$

where, I is the initial conductance; E is the elevated temperature conductance; F is the final conductance after autoclaving. The time required to cause 50 % injury at 55℃ was defined as the heat killing time.

RESULTS

The improved method of determination of membrane thermostability (MT) developed for the screening of heat-tolerant and sensitive varieties in *Brassica* was rapid, nondestructive and efficient (Fig. 1). Two leaf discs 13 mm in diameter were used from each plant. Application of some cotton pieces into the test tubes on the top of the sample was very effective to prevent the leaf sample from becoming damaged by the electrode bar during the conductance measurement. Addition of 20 ml distilled water was useful for the submergence of the electrode bar. Five different elevated temperatures such as 50, 55, 60, 65 and 70℃ were tested for distinguishing the varieties. Elevated temperature of 55℃ was most appropriate to distinguish varieties within the shortest period of time. When the temperature was below 55℃ it took more than 5 h to reach the end point of conductance. On the contrary, higher temperature than 55℃ gave very close results which did not enable to evaluate the temperature tolerance of the varieties. Conductance measurement at 55℃ for 3 h is suitable and enables to obtain rapid results. Final conductance reading was taken after autoclaving at 121℃ when leaf tissues were killed completely. Similar results were also observed when the samples were placed into boiling water for 30min. However, the final conductance reading was 8.41% lower, when the samples were frozen at -70℃ compared to autoclaving at 121℃. It was important to adjust the sample at the elevated temperature of 55℃ before taking the final conductance reading as the value was more accurate than at room temperature, where the value of conductance was approximately 25% lower. Finally, the calculation formula developed in the present method was convenient and accurate. Since the control value was not necessary, the procedures were simplified and less time-consuming. The major improvement in the calculation method was the subtraction of the initial value from the value induced at elevated temperature for a specified period of time.

Improved and Rapid Method of Determination of Membrane Thermostability (MT)

Take 2-leaf-discs for each treatment using a leaf puncher (13 mm Dia)

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Wash the sample 3-times with distilled water using a magnetic stirrer

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Put the sample into a test tube; Place 1 cm 2 cotton layer on the sample and add 20 ml of distilled water; Measure initial conductance (I)

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Close the test tube with a marble stopper and place it in a thermostatically controlled water bath at 55°C; Measure conductance every 30 min up to 3 h (E)

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Cover test tubes with aluminum foil and autoclave for 15 min at 121°C; Place test tubes again in a water bath at 55°C for 30 min and measure final conductance (F)

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Leaf injury is measured in percentage as follows:

$$\text{Injury (\%)} = \frac{E - I}{F} \times 100$$

where, I is the initial conductance; E is the elevated temperatureconductance; and F is the final conductance

Fig. 1. Flow diagram of the improved method of determination of membrane thermostability in *Brassica*.

Screening of heat-tolerant and sensitive varieties in *Brassica*:

The heat killing time of the heat-tolerant cabbage varieties Yoshin, YR kinshun and Sousyu was longer (150 min) than that of a heat sensitive variety Kinkei No.201 (120 min) at a growth temperature of 25/20 ± 2℃ in a glass house under natural day light conditions. Among the four varieties, Yoshin and YR Kinshun were the most heat-tolerant showing a 51% injury during 150 min at a temperature of 55℃ while the variety Sousyu showed a 56% injury under the same conditions (Table 1). However, when the same varieties were grown under 30/25 ± 2℃ temperature conditions, the heat killing time for Yoshin and YR Kinshun was 210 min, while the heat killing time of Sousyu and a heat-sensi tive variety Kinkei No.201 was 150min. The trend of heat toler ance in these varieties was similar for both growth temperatures. Difference in heat killing time caused by the growth temperature clearly indicated that the acclimation capacity of the heat-tolerant varieties was higher than that of the sensitive ones, under high temperature stress conditions. Among the four varieties, Yoshin and YR Kinshun were the most heat-tolerant, followed by Sousyu, while Kinkei No. 201 was heat-sensitive.

The heat tolerance ability in the four Chinese cabbage varieties differed significantly under 25/20 ± 2℃ and 30/25 ± 2℃ growth conditions (Table 2). The heat killing time was longer in Kenshin and No.11 (120 min) than in the others at 25/20 ± 2℃ and their percentage of killing injury was 54.6% and 57.1%, respectively. When these two varieties were grown at 30/25 ± 2℃, the heat killing time was 150 min for both varieties (Table 2), while the heat killing time was shorter for Chihiri 70 (90 min) and Kensui (90 min) which exhibited a 58.4% and 50.5% injury, respectively at 25/20 ± 2℃. When Chihiri 70 and Kensui were grown at 30/25 ± 2℃, the heat killing time varied significantly and reached values up to 120 min. The results revealed that among the four varieties of Chinese cabbage studied, the varieties Kenshin and No.11 were heat-tolerant, Kensui was intermediately tolerant, while Chihiri 70 was heat-sensitive.

Heat-killing time of Chinese kale was longer for the variety Sen-yo shirobana (180 min) and Full white (180 min) than for the variety Large leaf, in which the percentage of killing injury was 56.9% and 55.0%, respectively (Table 3), while the variety Large leaf required only 120 min for reaching 52.0% killing injury at the growth temperature of 25/20 ± 2℃. When theses varieties were grown at 30/25 ± 2℃, the heat killing time was the longest in Full white and Sen-yo shirobana (210 min). The shortest heat killing time was recorded in the variety Large leaf (120 min) under 30/25 ± 2℃ growth conditions. The MT results suggest that the varieties Full white and Sen-yo shirobana were heat-tolerant while Large leaf was heat sensitive. In general, the heat tolerance ability was higher in Chinese kale compared to cabbage and Chinese cabbage (Fig. 2).

Table 1. Screening for heat tolerance in cabbage based on the percentage of injury at the elevated temperature of 55℃.

Exposure time(min) at 55℃	Injury(%)							
	Yoshin		Sousyu		YR Kinshun		Kinkei No.201	
	25[a]℃	30[a]℃	25℃	30℃	25℃	30℃	25℃	30℃
30	14.52	12.43	24.29	13.45	18.30	16.38	15.98	15.69
60	21.71	20.45	30.24	26.28	29.27	22.02	29.81	27.75
90	32.47	26.86	40.86	39.24	38.59	27.46	43.25	40.74
120	41.01	35.50	47.88	44.87	45.60	36.81	50.96	47.61
150	51.38	42.00	56.03	50.78	51.17	42.85	60.64	54.11
180	54.49	47.50	57.15	56.41	55.95	49.20	64.43	60.75
210	61.40	54.30	65.30	63.28	63.75	55.80	68.94	66.20
240	66.58	57.52	70.64	66.58	66.77	58.82	72.62	67.40

[a] Growth temperature

Table 2. Screening for heat tolerance in Chinese cabbage based on the percentage of injury at the elevated temperature of 55℃.

Exposure time(min) at 55℃	Injury(%)							
	Kenshin		Kensui		Chihiri 70		No.11	
	25℃[a]	30℃[a]	25℃	30℃	25℃	30℃	25℃	30℃
30	20.54	9.02	27.41	19.25	43.19	30.42	21.63	13.72
60	32.53	19.18	39.82	36.27	47.93	46.59	36.96	24.51
120	54.59	38.62	61.53	54.68	69.98	61.71	57.11	46.41
150	64.56	59.94	68.69	67.01	76.69	71.58	67.30	61.80
180	68.62	63.21	77.09	75.17	84.99	78.47	70.95	68.69
210	73.85	68.85	79.58	77.73	86.29	81.30	73.26	69.91
240	74.34	70.95	80.98	79.52	88.88	85.02	76.72	73.74

[a] Growth temperature

Table 3. Screening for heat tolerance in Chinese kale based on the percentage of injury at the elevated temperature of 55℃.

Exposure time(min) at 55℃	Injury(%)					
	Sen-yo shirobana		Full white		Large leaf	
	25℃[a]	30℃[a]	25℃	30℃	25℃	30℃
30	22.15	12.73	14.10	19.10	25.09	22.72
60	29.80	18.81	26.44	24.17	41.48	37.64
90	36.17	29.84	35.64	31.31	49.71	47.66
120	45.10	34.91	42.81	36.44	51.96	50.33
150	49.28	41.61	47.41	41.15	58.25	56.26
180	56.93	45.49	55.03	42.67	62.45	61.20
210	62.38	59.35	61.22	52.37	78.58	68.86
240	65.98	60.99	63.71	54.72	81.64	71.33

[a] Growth temperature

Genetic variation for heat tolerance in the interspecific hybrids between cabbage × Chinese cabbage, and between Chinese kale × Chinese cabbage was examined. As shown in Fig. 3, the heat killing time was longer in the cabbage variety Yoshin (150 min) than in the Chinese cabbage variety Kenshin (90 min). However, the heat killing time of their interspecific somatic (120 min) and sexual hybrids (120 min) was intermediate between that of the parents (Fig. 3). Heat tolerance trait of the other interspecific hybrids between the Chinese kale variety Sen-yo shirobana and Chinese cabbage variety Kenshin was also intermediate (Fig. 4), and closer to that of their heat-tolerant parent. The heat killing time was 150 min for both Chinese kale and the hybrids. Heat killing time was only 90 min for the paternal parent of Chinese cabbage, although the injury level was different in Chinese kale (51.4%) from that of the hybrid (54.3%) for the same heat killing time (150 min).

DISCUSSION

Dexter (8) first reported the use of electrical

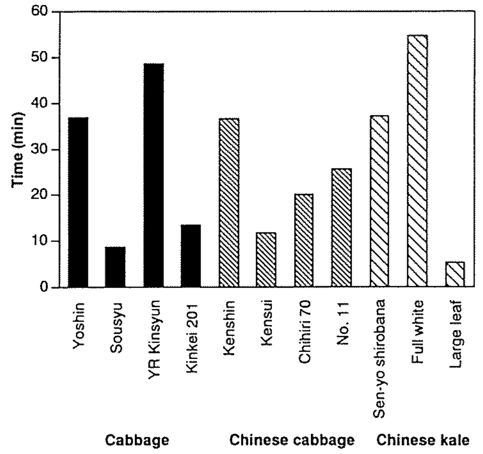

Fig. 2. Difference in time required for inducing 50 % killing injury in cabbage, Chinese cabbage and Chinese kale when the plants were grown at 25℃ and 30℃.

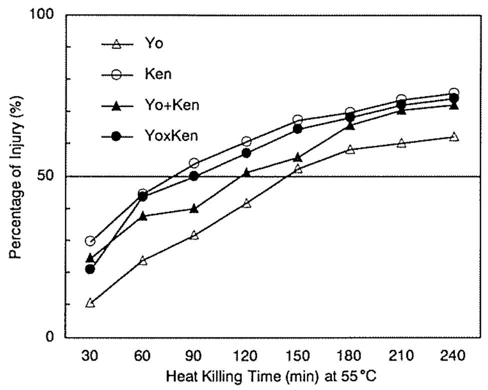

Fig.3. Heat killing time in the interspecific somatic and sexual hybrids between the cabbage variety Yoshin (Yo) and Chinese cabbage variety Kenshin (Ken) with their parents.

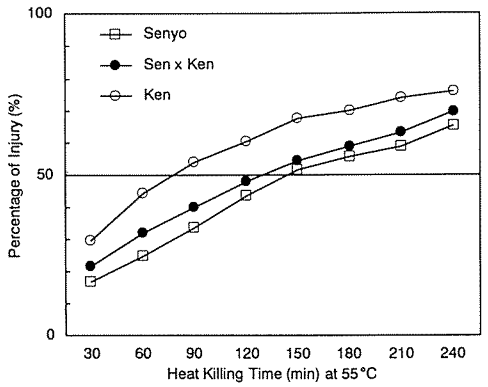

Fig. 4. Heat killing time in the interspecific hybrid between the Chinese kale variety Sen-yo shirobana (Sen) and Chinese cabbage variety Kenshin (Ken) with their parents.

conductivity to measure heat and drought tolerance. This method was modified by Sullivan and Kinbacher (9) and Sullivan (1) to evaluate the drought tolerance in sorghum. Thereafter many researchers applied this method to evaluate the heat tolerance in soybean, bean, tomato, country bean, pepper and wheat by using various elevated temperatures (3, 10, 11, 12, 13, 14, 15). In the reported method, a large number of leaf samples was used, which prevented the application in the young stage and 2 days were required to measure one set of leaf sample, because the samples were kept over night in an incubator at 10℃ after exposure to 48℃ (1). The method always required duplicate samples, one for the control at room temperature and the other for the exposure to various elevated temperatures, which is laborious. However, instead of using various elevated temperatures, Chen (5) determined the heat killing time which allowed a better differentiation between heat-tolerant and heat-sensitive varieties. The degree of heat tolerance could be expressed either by the heat killing temperature or the heat killing time, the latter being a more sensitive indicator. Heat killing time was considered to correspond to the time needed to cause 50% injury at 55℃. On the other hand, since the heat killing temperature corresponds to the temperature which causes 50% injury after treatment, it is necessary to test the various temperatures applied which is difficult and sometimes creates problems. Therefore, we developed an improved method by using the heat killing time after identifying the appropriate elevated temperature for rapid evaluation and screening between heat tolerant and sensitive varieties in *Brassica*. The present method is simple and does not require duplicate samples. Only two leaf discs 13mm in diameter are sufficient for the evaluation. In addition, a most accurate and convenient calculation formula had been developed to obtain precise results. In the method described by Chen et al. (5), the final conductance was measured at room temperature after exposure to 45 to 63℃. We observed that the final reading of conductivity at room temperature gave a value 25%

lower than the value recorded at the elevated temperature at 55℃. Therefore, in the present method, it is recommended to take the final reading of conductance at an elevated temperature.

In general, the heat killing time was longer in plants grown at 30/25 ± 2℃ day/night temperatures than in those grown at 25/20 ± 2℃ in the same variety which indicated that the plants developed their high temperature acclimation mechanism under high temperature stress (Tables 1-3). The capacity of acclimation to high temperature was higher in the heat-tolerant variety Yoshin for cabbage than in the heat-sensitive variety Kinkei No.201 (Fig. 2), because the difference in the heat killing time was longer in the heat-tolerant varieties than in the sensitive ones at 25℃ and 30℃ (Table 1). Similar results were also reported in Chinese cabbage and Chinese kale. The present results are in agreement with the report of Chen et al. (5) who observed that none of the genotypes of tomato, bean, soybean and potato differed in heat tolerance below a 30℃ acclimating temperature. The temperature at which genotypes showed the greatest difference in the heat killing time was 37.5℃ for soybean and potato, and 40℃ for tomato and bean. Generally, the heat-tolerant genotypes, after hardening at temperatures above 30℃, can survive for a long period of time. Therefore, the MT method enables to evaluate the heat tolerant varieties in *Brassica* under high temperature stress. The heat tolerance trait in the interspecific somatic and sexual hybrids between the cabbage variety Yoshin and Chinese cabbage variety Kenshin was intermediate between that of parents. Similar results have also been obtained in another interspecific hybrid between Chinese kale and Chinese cabbage for the heat tolerance characteristic, though in this hybrid the trait was closer to that of the heat- tolerant parent. In addition, Martineau et al. (16) reported that in soybean, the mean of progeny in tolerant X intolerant cross was intermediate for the tolerance characteristic and the heat-tolerance trait was close to that of the tolerant parent.

The results suggest that the improved MT method could be useful for screening heat-tolerant and sensitive varieties in cabbage, Chinese cabbage and Chinese kale at the seedling stage, and also effective to study the genetic variability of temperature tolerance in the hybrids of *Brassica*.

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アブラナ科葉菜類高温耐性品種スクリーニングのための細胞膜温度耐性測定法の改良

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摘要

アブラナ科葉菜類の高温耐性及び感受性品種スクリーニング細胞膜温度耐性（Membrane Thermostability）測定の改良を試みた結果，本改良法は簡便で迅速かつ信頼性の高い方法であることが判明したので報告する。

本改良法の電導率測定では，直径13mm葉片２枚の検体で充分なことから，幼苗期でのスクリーニングが可能である。また，従来の方法では試料を10℃で一晩静置してから測定するのに対し，本法の測定所要時間は，サンプリングから３～４時間と大幅に短縮された。加温処理温度55℃は，アブラナ科葉菜類の高温耐性及び感受性品種の識別に極めて有効な設定温度であった。また，55℃での電導率測定値は，従来の温室での測定よりも約25%程度高い値を示した。電導率の計算方法も，対照値を必要としないことを等，従来の方法に比して簡便で信頼性の高い値を得ることができた。

本改良法を用いて，アブラナ科葉菜類の高温耐性品種間差異を比較した結果，キャベツ（*Brassica oleracea* var. *capitata* L.）供試４品種のうち“金系No.201”は高温感受性であった。55℃加温処理における高温耐性品種及び感受性品種の“heat killing time”は，それぞれ150分と120分であった。ハクサイ（*B.campestris* var. *pekinenseis* L.）の品種“捲心”と“No.11”は高い高温耐性を示したが，品種“チヒリ70”は高温感受性であった。カイラン（*B. olercea* var. *alboglabra* Bailey）では，品種“Full White”と“尖葉白花”が高温耐性を示したが，“ラージリーフ”は高温感受性であった。一般的にカイランはキャベツやハクサイに比べると高い高温耐性を示した。また，キャベツとハクサイの種間体細胞雑種及び交雑種の高温耐性特性を比較すると，それぞれの親の中間的性質をそなえていた。

キーワード：湿度ストレス，電導率，ヒート・キリング・タイム