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REVIEW



Molecular confirmation of candidate Hsp70 gene associated with heat tolerance in BC₃F₂ advanced backcross lines and their phenotypic resemblance with recurrent chilli Kulai

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ABSTRACT

Domestic production of chilli in Malaysia is insufficient which accounted for 52% self-sufficiency level. Poor performance of local varieties under high temperatures above 42°C is one of the major factors affecting chilli production. This study was conducted with the objective of introgress heat-tolerant gene (Hsps) from AVPP0702 donor into Kulai using marker-assisted backcrossing. A total of 68 SSR markers, including Hsp linked markers that showed polymorphism between the parents were used to assess the generation of backcrossing; BC1F1, BC2F1, BC3F1 and BC3F2 and the average RPG percentage of the recurrent parent was found to be 81, 90, 95 and 97%, respectively. The pattern of Hsp expression in the backcross generations was similar to the donor parent (upregulated with more than 4-fold increase). Twelve improved heat tolerant chilli lines were developed. Most of the morphological and agronomical traits were recovered in the selected improved heat-tolerant genotypes from Kulai such as plant height, number of days to 50% flowering, number of fruits, fruit length and total fruit yield per plant. Improved high-yielding heat-tolerant chilli lines showed tolerance to high temperature as well as did not express any negative effect on agronomic traits in comparison with Kulai variety.

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Backcrossing; heat-tolerant lines; heat shock proteins; heat stress; marker assisted selection; simple sequence repeat markers

Introduction

Among the factors responsible for low production of chilli in Malaysia and other high-temperature areas is the unavailability of high yielding varieties suitable for cultivation under increasing high temperatures. Such conditions are important factors limiting the production of chilli (Usman et al. 2014). As domestic requirement is not fulfilled with native production, Malaysia depends on import of different food items along with chilli (MoA 2012). Chilli peppers are among the high-value important vegetable crop in the world being used as spice (Chapekar et al. 2018). Hybrid varieties have high yield potential with uniformity in maturity, tolerance to abiotic and biotic stresses, and better quality as compared to the standard open-pollinated varieties (Wilson et al. 2012).

Temperatures high enough to cause irreversible damage to plant growth and development are considered heat stress. It affects various physiological and metabolic processes in plants such as uptake of water and ions, translocation of solutes, photosynthesis and respiration, and produce inactivation of enzymes,

accumulation of unprocessed peptides, and proteolysis and is detrimental in terms of growth and productivity (Chapekar et al. 2018). The typical response to environmental stress conditions is established by the induction of a set of stress proteins that protect the organism from cellular damage. A basic response of plants to heat stress that is very common is the heat shock proteins (Hsps) expression, which is known as a mechanism in plant tolerance to heat stress (Jaswanthi et al. 2019). Incorporation of stress-responsive proteins (Hsps) for the improvement of crops plants against abiotic stress has shown considerable success (Usman et al. 2018).

Several methods were employed by researchers to study heat tolerance in plants including cell membrane stability index (CMSI), canopy temperature depression (CTD) and chlorophyll stability index (CSI) traits, which are indicative of high temperature tolerance. Gajayake et al. (2011) showed that depression of canopy temperature is a good indicator for screening plants for tolerance to high temperature and other environmental stresses such as drought. Similarly, Gajayake

et al. (2011) studied CMSI in 12 ornamental pepper genotypes indicating the possibility to screen pepper for heat tolerance. Chlorophyll stability index explains a plant's tolerance to environmental stresses (Reddy et al. 2014). As the CSI increases it showed that the negative effect of heat stress on the chlorophyll content of the plants is less. Additionally, chlorophyll stability index and cell membrane stability showed a significant positive relationship with pollen viability in peppers (Hain and Lee 2005).

The ancient breeding approach contributed immensely to the genetic manipulation of chilli germplasm decades ago (Reddy et al. 2014). Backcrossing breeding strategy is adopted to transfer targeted characters, such as heat tolerance, from an exotic line, into another elite breeding line (Lau et al. 2017). Using marker-assisted backcrossing the backcross process can often be accelerated (Lau et al. 2017). Molecular markers are effective in cultivar identification for protecting proprietary rights as well as authenticating plant cultivars. Molecular markers of necessity are assayed by chemical procedures and are of two basic types—protein and DNA markers (Ahmad et al. 2019). There are many DNA markers used which include Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphism (AFLP), and Single Nucleotide Polymorphism (SNPs) (Dhaliwal et al. 2013).

To increase production there is the need to identify genotypes having high yield potential as well as tolerance against high temperature to be suitable for lowland cultivation. Research on chilli breeding for heat tolerance had received less attention as compared to other vegetables like tomato. Recent works have indicated the role of heat shock proteins (Hsps) for the improvement of heat tolerance in plants. However, no research had been done, so far, for incorporation of heat shock proteins in chilli to improve and or develop heat-tolerant genotypes. The experience on the possibility of exploiting the hybrid vigour and heterosis in chilli has shown considerable promise. Under this context, it is imperative to identify and incorporate Hsps in chilli for the development of heat-tolerant as well as high yielding chilli variety.

Materials and methods

Plant Materials and growth condition

The advanced backcross lines population was created from the cross Kulai × AVPP002. The backcrossing scheme is presented in Figure 1. BC₃F₂ lines homozygous to AVPP0702 alleles for the Hsp gene were selected and evaluated for

Hsp gene expression, genetic background, and morphological assessment together with the parents were used in the first place to generate the advanced lines. The parental lines, AVPP0702 and chilli Kulai, used for this study were provided by AVRDC – The World Vegetable Center (AVRDC 2001) and Malaysian Agricultural Research and Development Institute (MARDI 2009).

BC₃F₂ homozygous lines having the desirable tolerance allele for the target gene and chilli Kulai were germinated on seed trays to assess their agronomic performance under normal growth conditions (29–32° C). The advanced backcrosses are sown in seed trays filled with peat moss. Emerged seedlings were transplanted four weeks after sowing into prepared polybag (25 × 25 cm). The polybags were arranged from east to the west and spaced at 75 × 150 cm. Drip system is used for both irrigation and fertilisation under normal rain-shelter conditions at Taman Pertanian Unit and Ladang 15, Faculty of Agriculture, Universiti Putra Malaysia. Morphological parameters assessed for the selected BC₃F₂ homozygous lines include plant height, stem length, stem diameter, mature leaf length, mature leaf width, days to 50% flowering, fruit yield, fruit length, and fruit weight. Morphological and physiological characters were analysed using SAS 9.4 version and *t*-test comparison was used to compare the chilli Kulai and the improved heat-tolerant chilli lines.

Molecular assessment of the BC₃F₂ improved lines

DNA extraction, polymerase chain reaction and marker analysis

Potential candidate markers (Hsp70-u2 and AGi42) associated with heat tolerance in chilli peppers (Magaji et al. 2016) were used to select advanced backcross plants possessing the gene of interest. Marker screening for polymorphism was earlier carried out between the two selected parents (Kulai and AVPP0702) using the tightly-linked (Hsp70-u2 and AGi42) markers and 250 paired SSR markers, spread across the 12-pepper chromosomes (Magaji et al. 2016). A minimum of four polymorphic SSR background markers per chromosome were used for recurrent parent genome recovery analysis. This was a follow-up experiment published by Usman et al. (2018).

Genomic DNA was extracted from fresh leaf tissues collected from five plants per parental genotype, following CTAB (cetyltrimethylammonium bromide) method (Saghai-Marouf et al. 1984) and described (Usman et al. 2018). Composition of the PCR amplification carried out was 1ul DNA template (70 ng), 1 µl each of F- and R-primer (1.0 µM), 7.4 µl 2 × Power PCR Master; mixture of DNA Polymerase, Buffer, dNTP (Bioteke) and 4.6µl

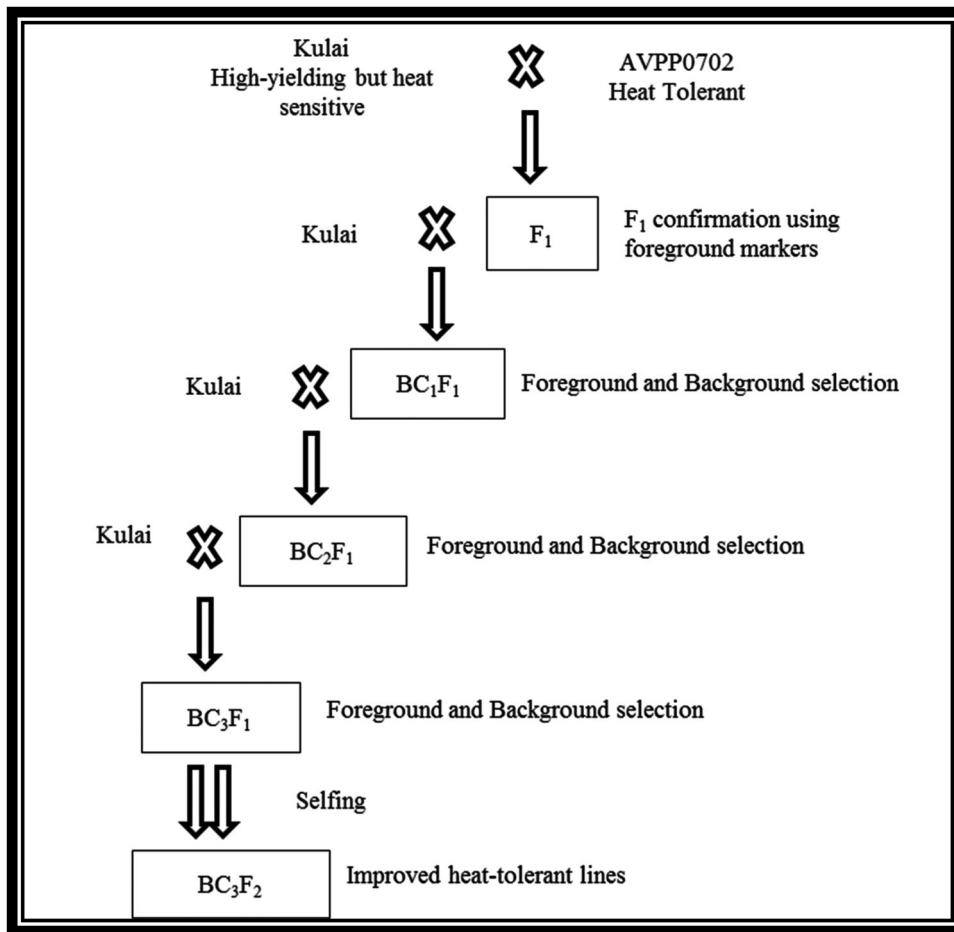


Figure 1. Schematic representation of the backcrossing procedure.

nuclease-free water amplified in BioRad thermal cycler (T100TM, Bio-Rad). A touchdown PCR programme was set at 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 60°C (−1°C per cycle) for 1 min, then 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, then 72°C for 5 min and, a final extension for 5 min at 72°C and cooled to 4°C before analysis for both target linked and recovery markers. The PCR product was run on 2.5% metaphor agarose gel at 90 volts for 60 min and visualised using the Molecular Imager® (GelDoc™ XR, Bio-Rad Laboratories Inc., USA). The banding score in the selection for the linked markers, the recipient allele is denoted as 'K', the donor allele denoted as 'A' and heterozygous allele denoted as 'H'. The χ^2 analysis for the sensitive and tolerant ratio was calculated by using the formula, chi-square = $(O-E)^2/E$, where O is the observed value, and E is the expected value. With respect to background markers for recurrent parent genome recovery analysis, the marker data were analysed using the software Graphical Genotyper (GGT 2.0) (Van Berloo 2008). The percentage of the recipient (%K), donor (%A) and heterozygous (%H) were estimated. The experiment was carried out at

the Laboratory of Molecular and Food crops, Climate-Smart Food Crop Production Laboratory, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia.

Physiological assessment

The advanced backcross population together with their parents were exposed to heat stress for 7 days in experimental plant growth chamber (GC-101C; Daeyang ETS, Hwasung-si, Kyunggi-do, South Korea) set at 40–45°C at the vegetative stage and were assessed for heat tolerance which includes cell membrane stability index (CMSI), chlorophyll stability index (CSI) and canopy temperature depression (CTD).

Cell membrane stability index (CMSI)

The procedure (Gajanayake et al. 2011) was followed for the CMSI assessment. A paired set consisting of control (C) and treatment (T) of two leaf disks, were cut from two fully expanded 3rd or 4th leaves from the top of the stem axis from each genotype. Both sets of leave

disk placed in test tubes were filled with 10 mL de-ionized water and sealed with aluminum foil to avoid evaporation. The T set of the test tubes were incubated for 20 min at 40 and 50°C in a temperature-controlled water bath (Oilbaths Model ONE 7, Memmert GmbH and Co. KG, Frankfurt, Germany); the C set of test tubes were kept at room temperature (approximately 25°C). Both sets of test tubes were then incubated at 4°C (kept in a refrigerator) for 24 h. Initial and final conductance readings of both sets (CEC 1, 2 and TEC 1, 2) was performed using a bench electrical conductivity metre (Model Starter 3000C; Ohaus Corp., Parsippany, NJ) after bringing the test tubes to room temperature (Usman et al. 2017).

$$\text{CMSI}(\%) = 1 - \frac{[1 - (\text{TEC1}/\text{TEC2})]}{[1 - (\text{CEC1}/\text{CEC2})]} \times 100$$

Measurement of canopy temperature depression (CTD)

The CTD was measured from plants between 50 and 60 days after transplanting. Four to five fully opened leaves were selected from each parent and the improved heat-tolerant chilli lines to measure the leaf temperature and their air temperature within 1200 and 1300_{HR} (cloudless, bright days) with the aid of a handheld infrared thermometer (Model OS533E-OMEGASCOPE; OMEGA Engineering, Inc., Stamford, CT). Using the following equation, CTD was calculated, where T_a denotes air temperature and canopy temperature T_c denotes:

$$\text{CTD} = T_a - T_c$$

Relative chlorophyll content (SPAD) and chlorophyll stability index (CSI)

A self-calibrating Soil and Plant Analysis Development (SPAD-502 Minolta Camera Co., Japan) chlorophyll metre was used to measure chlorophyll content. The chlorophyll content was measured from the leaf blade area at 2, 4 h, 3- and 7-days after heat treatment (40 and 50°C) in experimental plant growth chamber (GC-101C; Daeyang ETS, Hwasung-si, Kyunggi-do, South Korea). In each treatment (control and treated), chlorophyll was measured from three different plants. The chlorophyll stability index was calculated as the proportion of the amount of chlorophyll in heat treated leaf (40 and 50°C) and untreated leaf presented in percentage as follows:

$$\text{CSI}(\%) = \frac{\text{Relative chlorophyll content in heat treated sample}}{\text{Relative chlorophyll content in control sample}} \times 100$$

Statistical analysis was carried out on the physiological data using ANOVA to determine the significant differences between the parents and advanced backcross individuals under control and heat treated, and means were separated Tukey's HSD test ($p < 0.05$) using SAS 9.4.

Expression analysis of Hsp70 gene in BC₃F₂ population

Seedlings were randomly divided into two groups at four weeks after sowing, treated (35 and 45 °C) and control (30°C). For mild and severe heat stress, the plants were challenged with a gradual increase in temperature starting at 25–35°C for 2 h and then to 45°C using an experimental growth chamber (GC-101C, Daeyang^{ETS}, Korea). Heat stress treated leaves were collected and directly immersed in liquid nitrogen before further analyses (Usman et al. 2018). The extraction of total RNA was done following Trizol method (Chomczynski and Sacchi 1987) and further purification was done following Universal Plant Total RNA Extraction Kit (Spin-column) Biotek. A high-capacity cDNA synthesis kit (Biotek Corporation) was used to generate cDNA and qPCR Hsp70 primer was designed using primer3Plus software (Magaji et al. 2016). RNA isolated from these leave tissues were used for the quantitative RT-PCR analysis using Hsp70 gene-specific primers to compare the change in the expression level of the Hsp70 gene among the two parents contrasting in heat tolerance and the hybrid backcrossed improved heat-tolerant chilli lines. A CFX96 Real-Time PCR system (Bio-Rad, Mississauga, Ontario, Canada) machine was used to perform qPCR using Power 2 × SYBR real-time PCR premixture (Biotek). Three biological replicates were used per sample, along with no template negative controls. *EF1-α* was used as the internal control gene and relative gene expression was determined using the delta-delta- C_T method. The expression of Hsp gene was analysed using software provided by Bio-Rad, UK.

Results and discussion

Genotyping of BC₃F₂ population

Confirmation and selection of homozygous improved heat-tolerant chilli lines

Two linked markers (Hsp70 and AGi42) found to be polymorphic between the parental lines were used to screen the advanced backcross populations. Five confirmed F₁ plants were selected and crossed with Kulai to generate the BC₁F₁ plants. Four best plants

each from BC₁F₁ and BC₂F₁ carrying the Hsp allele and high recovery of the recurrent parent were selected for the next generation of backcross. In BC₃F₁ generation, three best selected plants were self-pollinated to produce BC₃F₂ seeds. One hundred BC₃F₂ plants were screened with the Hsp-linked SSR markers, out of which 24 of them had homozygous Hsp alleles like AVPP0702, 56 plants are carrying both alleles and 20 plants had homozygous Hsp alleles like Kulai (Figure 2). The observed ratio of 24:56:20 following Chi-square test (Table 1) was not significant to the 1:2:1 expected ratio of Mendelian segregation. In other words, the segregation analysis of the linked markers displayed a goodness of fit to the marker segregation ratio (1:2:1) expected following Mendelian principles in a single gene model. This implies that the markers are inherited together and will help to simultaneously improve the

chilli peppers for heat tolerance. Muthusamy et al. (2014) reported that linked marker segregation distortion occurred without showing consistency in maize backcross generations, irrespective of the size of the population.

Background selection

The genetic background of the advanced heat-tolerant chilli lines was analysed with 68 simple sequence repeat markers that appeared to be polymorphic, over the 1575.9 cM of the genome studied. The mean RPG recovery increased from 80.75% in BC₁F₁, 83.76% in BC₂F₁ to 94.6% in BC₃F₁ generations. After three successive generations of backcrossing and cycle of selfing, the average RPG recovery has increased to 97.90% among the advanced heat-tolerant chilli lines (Figure 3). The

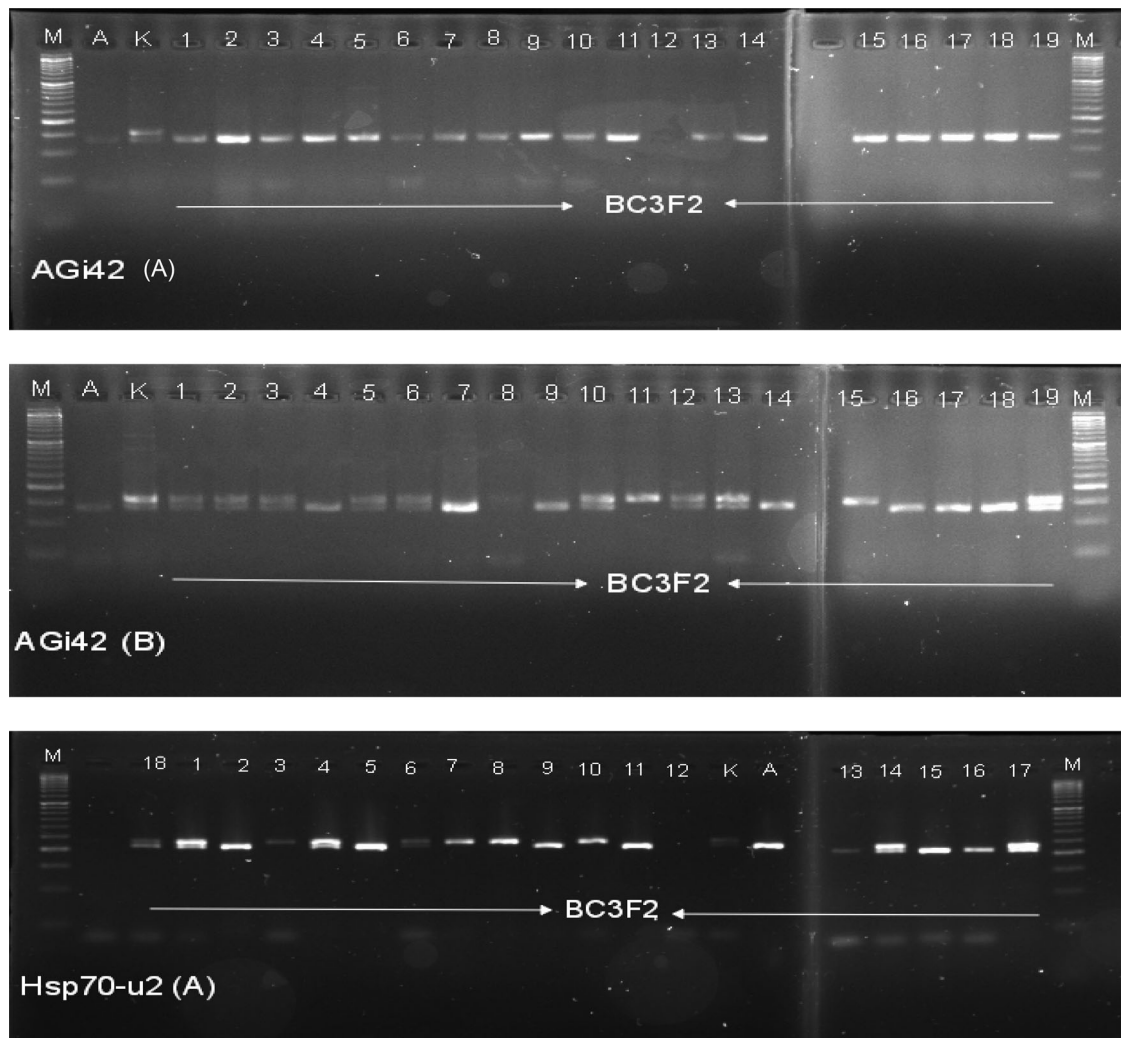


Figure 2. Improved homozygous advanced backcross lines screening using foreground makers (Hsp70-u2 and AGi42) in selected plant population P₁ (A) and P₄ (B). The banding pattern of the P₁ (A) individuals are similar, indicating that the Hsp gene was successfully introgressed. Similar banding pattern was observed for some individuals in A and B. Running on 2.5% metaphor agarose gel stained with midori green. M: 50 bp Ladder.

Table 1. Genotypic marker segregation analysis in BC₃F₂ population using chi square test of association to test the expected 1:2:1 ratio in a single gene model.

Marker	Chromosome no	Marker analysis			chi square (1:2:1)	P < 0.05
		AA	Aa	aa		
Hsp70	3	24	56	20	1.76	0.4148
AGI42	8	39	51	30	4.05	0.1320

Single dominant gene model, Tolerant (AA); Sensitive (aa) and Segregant (Aa).

RPG recovery extends between 95.9 and –99.10% among the advanced heat-tolerant chilli lines (Table 2). The plants with the highest proportion of recurrent parent genome were BC₁-P₇-P₁₀-P₁-P₇ and BC₁-P₇-P₁₀-P₄-P₁₆, with 99.10% each.

The mean RPG of the recurrent parent in advanced heat-tolerant chilli lines compared with the theory-based mean recovery of the recipient parent genome (87.5%) was higher after three successive generations of backcrossing. The result is considered likely to happen because continuous selection with polymorphic markers in BC₃ homozygous lines would result in increased high RPG recovery. On the other hand, some investigations on advanced backcross lines showed lower RPG recovery than the theory-based mean RPG recovery belonging to each backcross populations. The low RPG recovery in advanced lines is likely because of the molecular screening of genetic make-up conducted for the advanced lines but not the earlier generations; therefore, the lack of selection for the genetic make-up at marker assisted backcross breeding scheme might bring about lower than likely to happen recovery of RPG at advanced lines. The recipient genome is fully recovered on all chromosomes in the BC₃F₂ homozygous lines, the blue colour indicated the recovery of the genome after successful introgression while the red colour indicated the gene

Table 2. Background and introgressed segment analysis in selected BC₃F₂ populations.

BC ₃ F ₂ Selected lines	K (Recurrent Parent)	H	A (Donor Parent)	Total (cM)	H- Segment
BC ₁ -P ₇ -P ₁₀ -P ₁ -P ₃	98.8	0.0	1.2	1575.9	0
BC ₁ -P ₇ -P ₁₀ -P ₁ -P ₇	99.1	0.0	0.9	1575.9	0
BC ₁ -P ₇ -P ₁₀ -P ₁ -P ₈	95.9	0.0	4.1	1575.9	0
BC ₁ -P ₇ -P ₁₀ -P ₁ -P ₁₀	98.9	0.2	0.9	1575.9	1
BC ₁ -P ₇ -P ₁₀ -P ₁ -P ₁₃	98.6	0.5	0.9	1575.9	2
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₄	97.2	1.9	0.9	1575.9	1
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₇	96.0	3.1	0.9	1575.9	2
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₉	96.3	1.8	1.8	1575.9	2
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₁₄	97.8	1.3	0.9	1575.9	3
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₁₆	99.1	0.0	0.9	1575.9	0
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₁₇	98.9	0.2	0.9	1575.9	1
BC ₁ -P ₇ -P ₁₀ -P ₄ -P ₁₈	98.2	0.9	0.9	1575.9	1
Average	97.90	0.83	1.27	1575.90	1.08

H: Heterozygous, cM: centiMorgan.

introgressed (Figure 4). The recurrent parent genome recovery increases in the BC₃F₂ advanced heat-tolerant chilli lines due to the attachment of recipient parent allele from the heterozygous alleles.

In the breeding process of this study, the advanced backcross lines had a high RPG recovery percentage and agronomic performance. Successfully, in this our investigation, using MAB we incorporated the Hsp gene and reintroduced the genetic background of the recurrent parent in advanced backcross chili lines. Additionally, identifying the interesting lines with different alleles from the parents in backcrossing, reintroduction of the recipient parent genome using simple sequence repeat markers has also speed up advanced backcross chilli line development by choosing genotypes having high recipient genome recovery for developing the further backcross populations. In the BC₃F₂ generation, 12 improved heat tolerant chilli lines were confirmed and selected, which had homozygous Hsp alleles like AVPP0702 and genetic background similar to chilli Kulai.

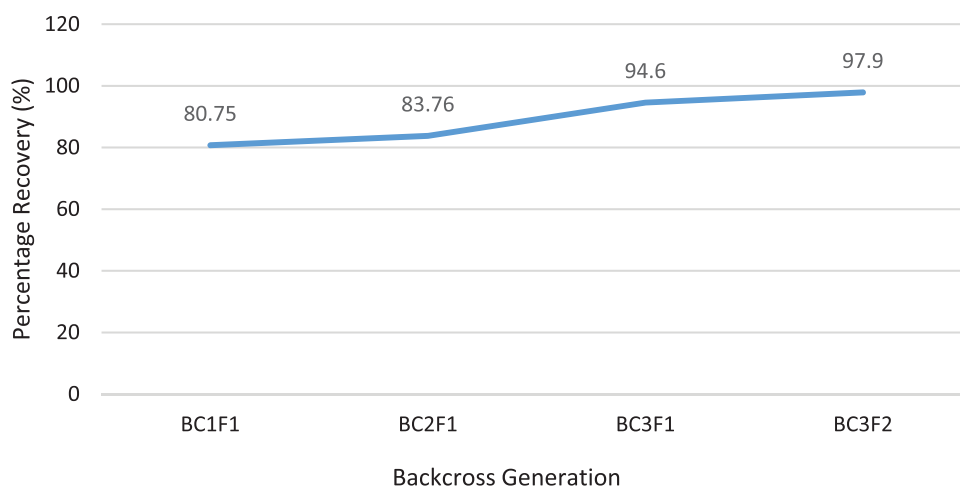


Figure 3. Recipient genome recovery increment in the backcross generations studied using the polymorphic background markers.

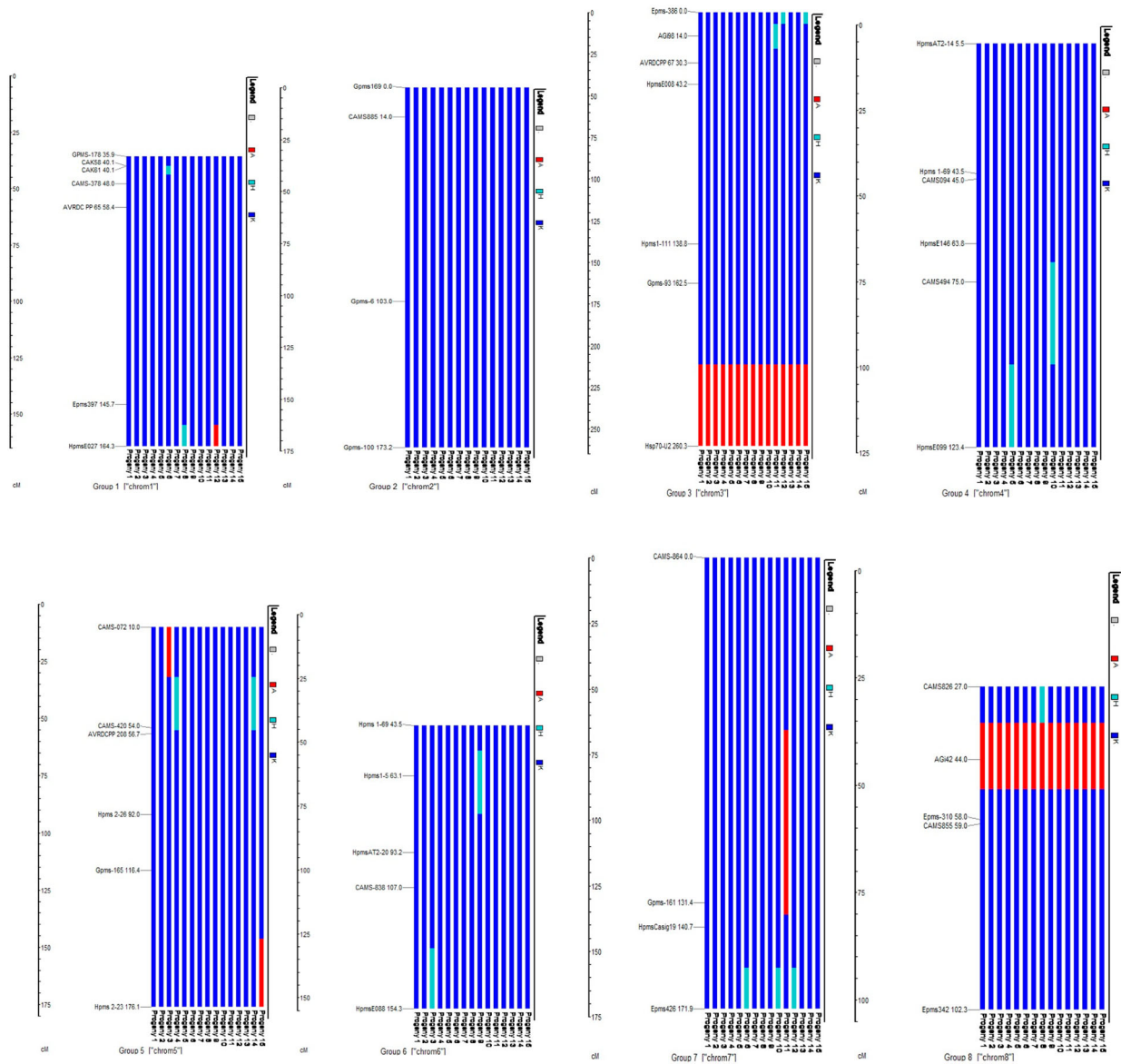


Figure 4. Graphical genotypes of the selected introgression lines in chilli Kulai genetic make-up developed in this study. A (Red): donor parent, K (Blue): recurrent parent, H (light green): heterozygous segments from GGT software 2.0v.

Phenotyping of BC_3F_2 population

Research has been conducted on chilli to evaluate the temperature tolerance of advanced backcross pepper lines using physiological parameters such as cell membrane stability index (CMSI), relative chlorophyll stability index (CSI) and canopy temperature depression (CTD). In this study, three physiological characters, CMT, CSI, and CTD were assessed to compare the parental lines and the advanced backcross chilli lines.

Cell membrane stability index (CMSI)

When tissues are subjected to high temperature, electrolyte leakages increase due to damage to the cell membrane by heat. The relative percentage damage and cell membrane

stability index (CMSI) of AVPP0702, Kulai, and the advanced backcross chilli lines were estimated at different temperature regimes. In the present investigation, a significant difference (Table 3) was observed between the parents and advanced backcross lines when exposed to high-temperature stress at 50°C. The advanced backcross chilli lines maintain a low plasma membrane damage due to high temperatures at 40 and 50°C compared to both parents (Table 4). This indicates that the advanced backcross lines resist the high-temperature stress better than the AVPP0702 (heat tolerant) donor parent. Increased electrolyte leakage indicates decreased cell membrane stability index (CMSI), which has long been used as an indirect mechanism of heat-stress tolerance in several crop plant species, including tomato (Islam et al. 2014).

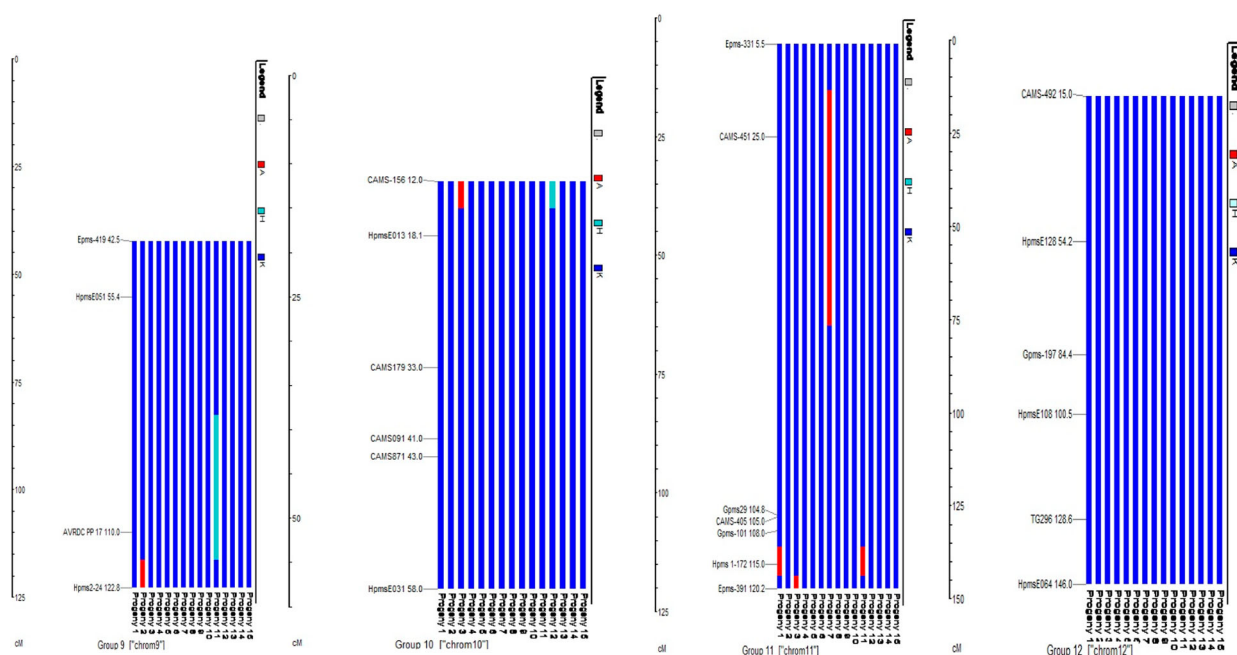


Figure 4 Continued

The integrity of the cell membrane is important because it is the first line of defence having many heat-responsive sensors that help plant to activate its defense mechanism well in advance against heat shock. The lowest CMSI was observed in the heat-sensitive variety (Kulai) with an increase in temperature to

Table 3. Analysis of variance showing the Mean Squares of percentage damage and cell membrane stability index (%) of the parental as well as the improved heat-tolerant chilli lines exposed to high temperature stress (40 and 50°C) experimental water bath.

Variables	Lines (DF = 5)	Error (DF = 12)	CV (%)
PD (30°C)	357.32**	16.37	20.88
PD (40°C)	725.97**	29.27	17.09
PD (50°C)	1552.81**	82.14	28.28
CMSI (40°C)	424.14**	20.32	5.32
CMSI (50°C)	1437.96**	111.02	12.72

Cell membrane stability index (CMSI) = $[1 - (T1/T2)] / [1 - (C1/C2)] \times 100$.
 Percentage damage (PD) = $(\text{initial conductivity} / \text{final conductivity}) \times 100$.
 DF = Degree of Freedom, ** = significant at 1% level of probability.

Table 4. Relative percentage damage (PD) of the parental as well as the improved heat-tolerant chilli lines exposed to different temperature regimes.

Lines	PD (30°C)	PD (40°C)	PD (50°C)
AVPP0702	39.17c	48.17b	51.82b
Kulai	25.00b	50.41b	68.99b
BC ₃ -1	10.43a	15.50a	16.96a
BC ₃ -3	14.65ab	36.79b	21.34a
BC ₃ -4	14.36ab	21.26a	19.09a
BC ₃ -6	12.69a	17.84a	14.10a
Sig. (<i>P</i> < 0.05)	**	**	**
SE±	1.79	2.65	3.15

SE± = standard error, ** = significant at 1% level of probability.

50°C (Table 4). Since AVPP0702 is considered as a thermotolerant chilli variety, this may be the reason for the changes in the CMSI values. In addition, the advanced backcross lines showed similar membrane stability with the thermotolerant parent indicating successful incorporation of the heat-tolerant gene into the Malaysia elite chilli Kulai variety. The mean value of CMSI of the advanced backcross lines from this investigation indicates that membrane integrity was not damaged by the high-temperature treatment of 40 and 50°C for 20 min, compared to chilli Kulai and damaged at 50°C. The unification and roles of cell membranes are sensitive to high-temperature stress. Lines having low CMSI (<30%) indicate that under high temperature there exists a lot leakage of membrane lipids and this is related to loss of physiological function. This is in conformity with the works (Gajanayake et al. 2011) who measured CMSI in 12 ornamental pepper genotypes and found in most of the genotypes to be thermotolerant with a mean of 59.50%.

Canopy temperature depression

Canopy temperature depression (CTD) defines the difference in air temperature and canopy temperature. Canopy temperature depression is a good indicator for screening plants for tolerance to high temperature and other environmental stresses such as drought (Kumar et al. 2016). From the results, significant differences exist between the parental and advanced backcross lines (Table 5) for canopy temperature depression, a

Table 5. Canopy Temperature Depression measured among the parents and improved heat-tolerant lines.

Lines	Canopy Temperature Depression °C
AVPP0702 (Donor)	-3.79 ± 0.39bc
Kulai (Recurrent)	-1.64 ± 0.59a
BC ₃ -1	-3.28 ± 0.31abc
BC ₃ -3	-1.94 ± 0.47ab
BC ₃ -4	-4.80 ± 0.33c
BC ₃ -6	-3.17 ± 0.28abc
Significance ($P > 0.05$)	**
Mean	-3.10
SE±	-3.26

SE±: standard error; **: significance at 1% level of probability, means followed by same letters are not significantly different following Tukey's (HSD) mean separation.

Table 6. SPAD chlorophyll content mean comparison among the parental lines and improved heat-tolerance chilli lines exposed to high temperature stress (40°C).

Lines	2-hours	4-hours	3-days	7-days
AVPP0702 (Donor)	41.67a	35.60ab	31.60	38.68a
Kulai (Recurrent)	26.38c	24.18c	26.35	32.50c
BC ₃ -1	31.85b	30.17ab	27.25	28.33bc
BC ₃ -3	31.85b	29.35ab	23.58	28.85bc
BC ₃ -4	34.47b	31.12b	32.20	33.23ab
BC ₃ -6	34.77b	33.72a	32.15	32.77ab
Sig. ($P > 0.05$)	**	**	ns	**
Error	5.33	2.41	35.71	6.50
CV (%)	7.18	5.33	21.80	8.27

**: significance at 1% level of probability, CV: coefficient of variation, means followed by same letters within column are not significantly different following Tukey's (HSD) mean separation.

measure of a plant's ability to lower canopy temperature through transpirational cooling. The mean CTD is -3.10, while the maximum and minimum CTD is -4.80 and -1.64 respectively (Table 5). AVPP0702 heat-tolerant parent was similar to the advanced backcross lines

indicating that the incorporation of Hsp genes was successful and increase the possible high-temperature tolerance.

Relative chlorophyll content (SPAD) and chlorophyll stability index (CSI)

Significant variation in SPAD chlorophyll content exists when the parental, as well as the advanced backcross lines, were exposed to high-temperature stress at different stages (2-, 4-hours and 7-days) except 3-days (Table 6). Results showed that the advanced backcross lines were consistently and significantly ($p < 0.05$) similar to the donor parent but statistically ($p < 0.05$) different from the recurrent (Table 6). This might be attributed to the successful introgression of the heat-tolerant (Hsp) genes from AVPP0702 into the elite chilli Kulai variety through MAB.

The CSI recorded across two parental lines and advanced backcross lines ranged from 58.61% in 'chilli Kulai at 2-hours' to 122% in 'AVPP0702 at 3-days' with a mean of 93.33% (Figure 5). Chlorophyll stability index explains a plant's tolerance to environmental stresses. As the CSI increases it showed lower amount of stress impact on chlorophyll content of the plants. Furthermore, higher CSI is a sign of plant's capability to cope under stress by maintaining more dry matter production, increasing higher rates of photosynthesis through greater stability of chloroplast membranes and higher productivity (Arjun 2019). In our investigation, the CSI of the improved heat-tolerant chilli lines appeared to be significantly ($p < 0.05$) (Table 7) similar to AVPP0702,

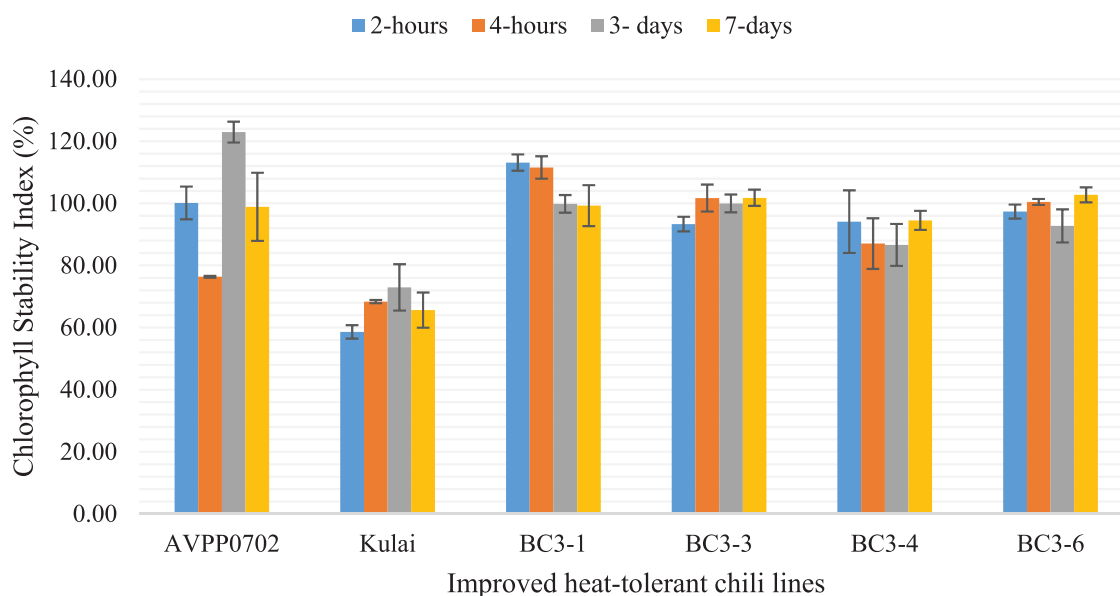
**Figure 5.** Chlorophyll stability index of the parental (AVPP0702 and Kulai) as well as the improved heat-tolerant chilli lines exposed to high temperature stress (40°C) at different exposure time in experimental plant growth chamber.

Table 7. Mean Squares of chlorophyll stability index (%) of the parental (AVPP0702 and Kulai) as well as the improved heat-tolerant chili lines exposed to high temperature stress (40°C) at different exposure time in experimental plant growth chamber.

Exposure Time	Lines (DF = 5)	Error (DF = 12)	CV (%)
2-hours	994.92**	76.04	9.40
4-hours	821.57**	49.79	7.76
3- days	831.24**	78.7	9.25
7-days	596.29**	109.38	11.15

DF: Degree of freedom, CV: coefficient of variation, **: significance at 1% level of probability.

Table 8. Pearson correlation matrix showing the relationship among chlorophyll stability index, cell membrane stability index and canopy temperature depression of the parent and improved heat-tolerant chilli lines.

Physiological Parameters	CSI	CMSI	CTD
CSI	1		
CMSI	0.95**	1	
CTD	-0.46ns	-0.55ns	1

CSI, chlorophyll stability index; CMSI, cell membrane stability index; CTD, canopy temperature depression, **: significance at 1% level of probability.

the donor parent indicating successful incorporation of the heat-tolerant genes. But were significantly ($p < 0.05$) different from Kulai, the recurrent parent (Figure 5). This showed that high-temperature stress may not affect the agronomic performance of these improved heat-tolerant chilli lines through greater stability of chloroplast membranes.

Correlation between CMSI, CTD and CSI

Phenotypic relationship between CMSI, chlorophyll stability and CTD among the parental and advanced

backcross lines would be beneficial to confirm the successful incorporation of the Hsp genes from AVPP0702 to chilli Kulai. Several studies on temperature tolerance tried to study the correlation between physiological parameters to temperature tolerance and other performances of crop plants. CSI was strongly, positively and significantly ($p < 0.05$) correlated ($R = 0.95$) to CMSI (Table 8). This implies that as the cell membrane becomes more stable under heat stress the chlorophyll becomes more stable, hence high photosynthesis efficiency. No correlation of CTD was recorded with CSI but a poor negative correlation was found with CMSI (Figure 6). Additionally, chlorophyll stability index and cell membrane stability showed a significant positive relationship with pollen viability in peppers (Gajanayake et al. 2011).

Analysis of fold difference in BC_3F_2 improved heat-tolerant chilli lines

Strong positive threshold cycle ($Cq < 29$) values were observed in AVPP0702 and advanced backcross lines indicative of abundant target nucleic acid in the samples, except for Kulai, which showed weak reaction (Cq of 38–40), indicative of a minimal amount of the target nucleic acid in the sample (Table 9). The real-time expression of Hsp70 gene in the leaf tissues under heat shock of 45°C for 2 h showed significant up-regulation (Table 9) in the heat-tolerant (AVPP0702) variety and advanced backcross lines, compared to 1.57-fold increase in heat-sensitive Kulai variety (Table 9). The gene that was up- or down-regulated by more than 4-

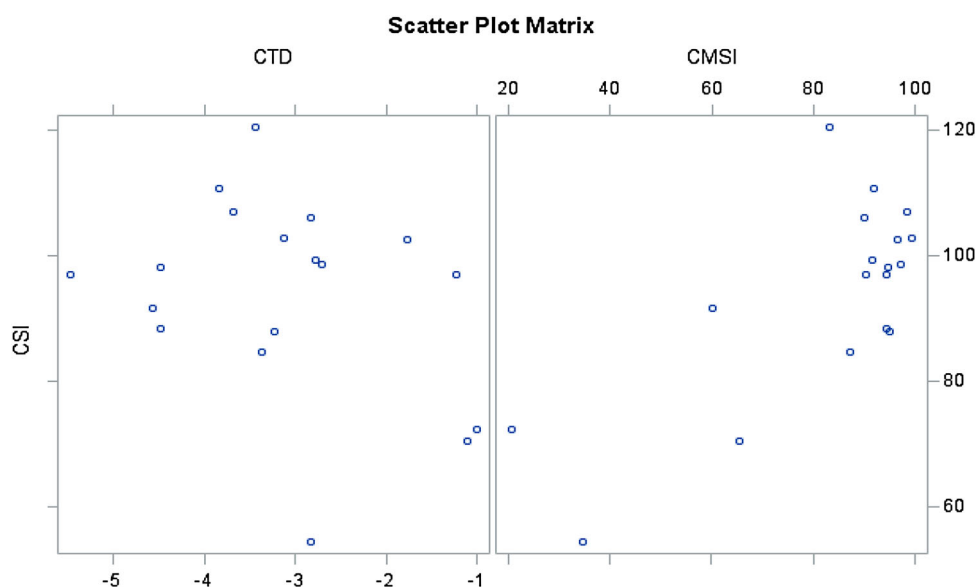


Figure 6. Correlation matrix among CSI, chlorophyll stability index; CMSI, cell membrane stability index; CTD, canopy temperature depression of the parents and improved heat-tolerant chilli lines.

Table 9. Analysis of Hsp70 gene under differential heat stress (45°C) condition among the parents and improved heat-tolerant chili lines revealed by qRT-PCR at 4-hours.

Lines	Mean Cq	Relative Normalised Expression	Regulation	Regulation Threshold	P-Value
AVPP0702 (Donor)	15.28	31.39	31.39	Up regulated	0.000
BC ₃ -1	14.56	19.76	19.76	Up regulated	0.000
BC ₃ -3	16.59	23.16	23.16	Up regulated	0.001
BC ₃ -4	17.07	16.45	16.45	Up regulated	0.129
BC ₃ -6	16.35	29.50	29.50	Up regulated	0.014
Kulai (Recurrent)	38.65	1.58	1.57	No change	0.004

Cq = Cycle threshold.

fold and with $p < 0.05$ were differentially expressed. The up-regulation of Hsp70 in AVPP0702 and the advanced backcross lines indicate that Hsp70 increases in the cell when exposed to heat stress. From the present investigations, up-regulation of the Hsp70 gene in the advanced heat-tolerant chilli lines may be attributed to the successful incorporation of the gene from the heat-tolerant donor (AVPP0702) variety. The expression of Hsp70 in thermotolerant (AVPP0702) genotype and improved chilli lines is higher than thermo-sensitive (Kulai), indicating better protection against heat stress. This indicates that upon exposure to elevated heat stress, the cellular heat shock response in the nucleus was activated. This leads to the enhanced expression of the heat stress genes, indicating that there was a better protection and for a longer time against heat shock stress. A significant positive correlation between Hsp70 gene expression and the acquisition of thermotolerance has been studied (Mishra et al. 2018), resulting in increased tolerance to high-temperature stress in plants (Zhang et al. 2017).

Morphological and physiological comparison of selected homozygous BC₃F₂ improved heat-tolerant chilli lines with Kulai (Recurrent parent) under normal growth condition

A descriptive statistic of the dependent variables is presented in Table 10. A two-sample independent t -test was conducted after checking all assumptions to

compare the morphophysiological performance of the advanced backcross lines and chilli Kulai for phenotypic resemblance from the recurrent parent genome recovery. As can be seen in Table 10, the advanced backcrossed lines and chilli Kulai distributions were sufficiently normal for conducting a t -test (i.e Skew $< |2.0|$ and Kurtosis $< |9.0|$) (Schmider et al. 2010). The homogeneity of variance determines whether to use pooled variance when calculating t . This was satisfied via Folded F 's test, $F(4) = 1.20-7.09$, $P = 0.08-0.86$ (Table 11). This indicates the equality of variances was not significant for all the dependent variables, so the pooled variance method was used to calculate t (Table 11). The improved heat-tolerant group was associated with similar growth performance in comparison with the chilli Kulai (recurrent parent) except the individual fruit length (Table 11). The independent sampled t -test was associated with a statistically non-significant effect, $t(4) = 0.08-1.94$, for all dependent variables except for individual fruit length $t(19) = -3.11$, $p = 0.01$ (Table 11). Similar observations were reported from previous research (Saha et al. 2010). Most of the advanced heat-tolerant lines are as good as the chilli Kulai with respect to the yield and yield components indicating that the agronomic performance of the introgression lines is similar with chilli Kulai and the efficiency of the selection procedure practiced (Table 11).

The improved heat-tolerant chilli lines of chilli Kulai showed no apparent yield penalty about the incorporation of Hsp-linked genes. This indicates that the

Table 10. Descriptive Statistics for the ten dependent variables of the improved heat-tolerant lines (BC₃F₂ populations) and chili Kulai (recurrent parent).

Dependent variables	N	M	SD	Skew	Kurtosis	M	SD	Skew	Kurtosis	
	BC ₃ F ₂ (Improved lines)					Kulai (Recurrent parent)				
Plant height (cm)	5	75.94	6.05	-2.06	4.36	71.86	2.77	1.80	3.30	
Stem length (cm)	5	22.30	4.94	-0.31	-0.87	17.66	2.06	-0.51	-2.24	
Stem diameter (cm)	5	6.02	0.44	-1.01	0.82	5.98	0.94	-0.91	2.32	
Leaf length (cm)	5	6.12	1.47	1.44	2.21	7.11	1.34	1.96	3.95	
Leaf width (cm)	5	3.29	0.26	0.08	-1.37	3.33	0.67	-1.33	2.23	
Days 50% flowering	5	56.54	2.25	0.19	-0.65	60.36	3.21	-1.57	2.62	
Number of fruits	5	91.60	29.41	-0.22	-1.04	69.8	15.22	-2.07	4.35	
Fruit length (cm)	5	13.28	2.02	-0.56	-0.57	18.02	2.75	-1.46	2.32	
Fruit weight (g)	5	17.45	1.11	0.59	0.70	18.96	2.96	-1.89	3.88	
Yield (g/plant)	5	862.28	57.88	0.20	1.94	869.62	108.96	-0.91	-1.60	

N, observations; M, Mean; SD, Standard Deviations; Skew, Skewness.

Table 11. Descriptive t-test and homogeneity variance test statistics for the mean improved heat-tolerant chilli lines compared with chilli Kulai (recurrent parent).

Genotype	Plant height (cm)	Stem length (cm)	Stem diameter (cm)	Leaf length (cm)	Leaf width	Days to 50% flowering
BC ₃ F ₂ (Improved lines)	75.94 ± 2.70	22.30 ± 2.21	6.02 ± 0.20	6.12 ± 0.66	3.29 ± 0.12	56.54 ± 1.01
Kulai (Recurrent Parent)	71.86 ± 1.24	17.66 ± 0.92	5.98 ± 0.42	7.11 ± 0.60	3.33 ± 0.30	60.36 ± 1.43
t-test	1.37	1.94	0.08	-1.11	-0.11	-2.18
P < 0.05	0.21	0.09	0.94	0.30	0.91	0.06
Folded F test	4.76	5.78	4.61	1.20	6.58	2.03
P < 0.05	0.16	0.12	0.17	0.86	0.10	0.51
Genotype	Number of fruits	Individual fruit length (cm)	Individual fruit weight (g)	Fruit yield (g/plant)		
BC ₃ F ₂ (Improved lines)	91.60 ± 13.16	13.28 ± 0.90b	17.45 ± 0.50	862.30 ± 25.89		
Kulai (Recurrent Parent)	69.80 ± 6.81	18.02 ± 1.23a	18.95 ± 1.32	869.60 ± 48.73		
t-test	1.47	-3.11	-1.06	-0.13		
P < 0.05	0.18	0.01	0.32	0.90		
Folded F – test	3.73	1.86	7.09	3.54		
P < 0.05	0.23	0.56	0.08	0.25		

Mean ± standard error.

production of the heat-tolerant advanced chilli developed from our study would be of great importance in heat-prone areas. The near-complete recovery of yield in the advanced backcross lines of chilli Kulai along with Hsp genes is paramount importance in this study. This could be attributed to the fact that yield and other fruit quality characters are multigenic encoded by loci that are distributed across the chilli genome. Higher recovery of the recipient genome in the advanced backcross lines of chilli Kulai was achieved through phenotype-based visual selection for morphophysiological characters throughout the consecutive generations of backcrossing, and screening of a reasonable number of backcross individuals.

This study has demonstrated the successful introgression of Hsp gene from AVPP0702 and reintroduction of Kulai genome through marker assisted backcrossing (MAB) approach. The advanced backcross lines had homozygous Hsp alleles from AVPP0702 and recovered most of the morphological and agronomical traits from Kulai. This study further confirmed that DNA markers for heat-stress tolerance (Hsp) genes could be reliable for marker-assisted breeding in chilli peppers. This finding is the first report on successful incorporation of heat shock protein genes in chilli peppers to develop advanced backcross lines. This will serve as genetic material in chili pepper breeding programmes for heat tolerance and in selecting cultivars for a niche environment. The advanced backcross lines developed could increase chilli production by enhancing and sustaining livelihoods soon and timely access and availability of food in Malaysia and other heat prone areas vis-a-vis climate change.

Disclosure statement

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