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Article

Physiological and Molecular Screening of High Temperature Tolerance in Okra [*Abelmoschus esculentus* (L.) Moench]

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Abstract: Okra is a vegetable crop adapted to summer temperatures, but heat stress has been shown to reduce its growth and productivity. We measured physiological traits of 104 okra genotypes in response to high temperature, augmented by the molecular characterization of selected genotypes to identify parents for crossing. Genotypes were exposed to a short heat shock (45 °C, 4 h) in a controlled environment, followed by the assessment of chlorophyll fluorescence (F_v/F_m , F_v'/F_m') and stomatal conductance (g_s). DNA was isolated from all genotypes using a modified CTAB method with additional PVP and RNase, and the amplification of 8 polymorphic SSR markers was used to generate a dendrogram. This preliminary screening identified 33 polymorphic genotypes with less than 50% genetic similarity and contrasting F_v'/F_m' and g_s responses. More detailed physiological measurements (F_v/F_m , F_v'/F_m' , g_s , photosynthesis (A), efficiency of the open reaction centre (Φ_{PSII}), and electrolyte leakage (EL)) were conducted after exposure to 45 °C for 6 h and compared to the control (30 °C). EL did not significantly increase in the heat treatment; in contrast, there were significant genotype and treatment effects observed for fluorescence (F_v/F_m , F_v'/F_m') and photosynthetic parameters (A , Φ_{PSII} , g_s). In conclusion, cell membranes in okra remained unaffected after short periods of heat stress, whereas the ranking of differences of measured physiological traits (Δ) between control and heat-treated plants ($\Delta F_v'/F_m'$, $\Delta F_v/F_m$, ΔA , $\Delta \Phi_{PSII}$, Δg_s) was indicative of genotype sensitivity to heat.

Keywords: chlorophyll fluorescence; electrolyte leakage; heat stress; photosynthesis; SSR markers; stomatal conductance



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1. Introduction

Heat stress can significantly impact plant growth and development and result in a subsequent reduction in crop yield [1]. Different plant species or cultivars need various temperatures to optimize their productivity [2,3]; however, exposure to temperatures above the optimum (either short- or long-term) can result in the inhibition of growth. Okra (*Abelmoschus esculentus*), an important and nutritional vegetable in Africa and Asia [4], is a summer crop well adapted to elevated temperatures and has been classified as heat tolerant with optimal growth and productivity at 34 °C [5]. Nevertheless, high temperature can impair okra development and productivity [5–10]. Even a few minutes exposure to temperatures above optimal growth temperatures (i.e., heat shock) can disrupt physiological processes [1,11–13], and photosynthesis is among the first processes to be inhibited under high temperatures [14,15]. Photosynthetic reduction can result from increases in photo- and mitochondrial respiration and the inactivation of photosystem II (PSII) or Rubisco activase [16]. Heat shock between 2 min and 2 h has been shown to reduce photosynthesis, chlorophyll fluorescence, and stomatal conductance [17–20]. Chlorophyll fluorescence is

especially useful for assessing the activity of PSII and the electron transport chain in intact leaves in response to stresses [15,21–24] and can be measured as the ratio of variable to maximum fluorescence in the dark (F_v/F_m) or the light (F_v'/F_m') [21]. F_v/F_m has been used extensively to measure plant responses to stress because it is easily assessed [25]. For example, F_v/F_m was reduced as a result of increased F_o and decreased F_m under high temperatures [18,26,27]. Similarly, F_v/F_m in okra was reduced by 10.5% under water stress [28]. However, this ratio requires dark acclimation for at least 30 min to be effective [25]. Fluorescence parameters in light-adapted leaves, including F_v'/F_m' and the efficiency of open reaction centres (Φ_{PSII}), might be better suited for field measurements, as they do not need extended dark acclimation or equilibration time required for gas exchanges. Φ_{PSII} denotes the proportion of PSII reaction centres that are photochemically active, and this proportion reduces under stress [29,30]. The reductions in F_v'/F_m' and Φ_{PSII} under high temperatures indicate both structural and functional damages to PSII [14,26], which can lead to reduction in photosynthetic rate (A) and subsequent plant growth.

Photosynthesis is further reduced when plants close their stomata to prevent water loss under high temperatures, resulting in limited CO_2 availability to the Calvin cycle [18,26,31,32]. Furthermore, heat can have a detrimental effect on cell membranes, specifically the thylakoid membranes, where the photosynthetic apparatus is located. Under high temperatures, the phospholipid bilayer of a cell membrane becomes unstable and disrupts the lipid–protein interaction, leading to an increase in membrane permeability, the loss of electrolytes, and disturbance to PSII activity and ATP generation [33,34]. However, if water availability is high, plants may increase their stomatal conductance (g_s) to increase evaporative cooling and lower leaf temperature to alleviate stress, reducing the limitation to internal CO_2 concentration [14,18].

To ensure that the best varieties adapted to environmental stresses can be developed through breeding, genetic diversity is crucial. Okra is characterized by large variations in morphology [35], but little is known of its genetic diversity. Diversity has been assessed using morphological markers [36–39]; however, molecular markers provide better estimates of genetic diversity, as they are not influenced by the environment [39,40]. Although studies of okra genetic diversity are limited, some evidence has been published using Simple Sequence Repeats (SSR) [41–43], Random Amplification of Polymorphic DNA (RAPD) [39,44–46], and Amplified Fragment Length Polymorphisms (AFLP) [47].

This study aimed to identify heat-tolerant okra genotypes characterized by high genetic diversity. Physiological measurements such as fluorescence, gas exchange, and electrolyte leakage were used to screen a large pool of genotypes in a controlled environment after exposure to a heat shock in comparison to an untreated control. Thirty-five previously published SSR markers from *A. esculentus* and *Medicago truncatula* (Table A1) were used to amplify genomic DNA to identify genetic variation among the 104 okra genotypes and to identify the best SSR markers for okra screening.

2. Materials and Methods

2.1. Plant Material

One-hundred-and-four okra genotypes from different countries of origin were obtained from the Vegetable Research Institute (VRI) and the World Vegetable Centre, previously known as the Asian Vegetable Research and Development Centre (AVRDC) (Table S1). Seeds were sown in pots filled with a potting mix consisting of nine parts composted pine bark and one part washed river sand and supplemented with all trace elements at 0.4 kg m^{-3} , 1 kg m^{-3} gypsum, 1 kg m^{-3} superphosphate, 0.25 kg m^{-3} KNO_3 (13% N), 0.25 kg m^{-3} nitroform (38% N), and 1.5 kg m^{-3} magrilime. Plants were kept in a glasshouse with natural light for 4 weeks at the Plant Breeding Institute (PBI), the University of Sydney, New South Wales (NSW), Australia, with day/night temperatures of $30 \text{ }^\circ\text{C}/25 \text{ }^\circ\text{C}$. As the different lists contained different information—the AVRDC lists contained vegetable introduction (VI) numbers specific to this organization, and not all genotypes had a variety

name—genotypes were labelled by their list number and genotype number (e.g., the first genotype in Table S1 was labelled L2-1).

2.2. DNA Isolation, Quantification and Qualification

Young and tender fresh leaves (approximately 100 mg) were collected for DNA isolation 3 weeks after sowing. Samples were collected in Eppendorf tubes, frozen immediately in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$. The samples were then freeze-dried using a Kinetics Thermal System (Model: FD-1-54D, UK) for 24 h and then ground immediately with a ball mill (Retsch Mixer Mill MM 400, Retsch GmbH, Haan, Germany). A cetyl trimethylammonium bromide (CTAB) extraction method [48] was modified to extract okra DNA. The ground leaf samples were transferred to Eppendorf tubes and 1 mL of $2\times$ CTAB extraction buffer containing 2% *w/v* polyvinylpyrrolidone (PVP), and 2-mercaptoethanol (2%) was added. The solution was mixed well and incubated in a $65\text{ }^{\circ}\text{C}$ water bath for 30 min before the addition of 250 μL of cold phenol and 250 μL of cold chloroform/isoamyl alcohol to each tube. Tubes were mixed by inversion until a thick emulsion formed, centrifuged at 13,000 rpm for 30 min, and supernatants transferred to a new sterile Eppendorf tube. One volume (equivalent to supernatants volume) of cold chloroform/isoamyl alcohol was added to each tube, mixed by inversion, centrifuged at 13,000 rpm for 15 min, and the top phase was subsequently transferred to new sterile Eppendorf tubes. To precipitate DNA, 0.1 volume (equivalent to top phase volume) of 3M sodium acetate (pH 5.2) and 1 volume (equivalent to top phase volume) of cold isopropanol were added, and the tubes were stored at $-20\text{ }^{\circ}\text{C}$ overnight. The next day, tubes were centrifuged at 13,000 rpm for 30 min, the supernatant decanted, and 1 mL of cold ethanol (70%) added, mixed, and centrifuged at 13,000 rpm for 20 min. Again, the supernatant was decanted and pellets air dried before re-suspension in 200 μL of double deionised water and stored at $4\text{ }^{\circ}\text{C}$ overnight. The next day, 1 μL of RNase (100 mg mL^{-1}) was added to each tube before incubation at $37\text{ }^{\circ}\text{C}$ overnight.

DNA quantification was performed using 1 μL of material on a Nanodrop, ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The purity of DNA was assessed using the absorbance ratios of A260/A280 for protein contaminants and A260/A230 for organic contaminants, as nucleic acids absorb at 260 nm, proteins at 280 nm, and organic compounds at 230 nm. Ideally, purified DNA should have a A260/A280 ratio between 1.65 and 1.8 and a A260/A230 ratio between 1.5 and 1.8 [49]. DNA quality was assessed using a 2% agarose gel (3 g agarose in 150 mL of Tris/Borate/EDTA ($1\times$ TBE) buffer, stained with 3 μL of gel red and run in an electrophoresis system at 120 volts for 30 min. The DNA bands were analysed against the standard lambda DNA and bands were visualized under UV light (ChemiDocTM MP Imaging System, Image LabTM Software, Version 5.1). The stock DNA was then diluted with double deionised water (ddH₂O) to a final working concentration of $25\text{ ng }\mu\text{L}^{-1}$ in 200 μL .

2.3. SSR Primers and Polymerase-Chain Reaction (PCR)

Nineteen SSR primers from *A. esculentus* [42] (Table A1, Nos. 1–19) and sixteen SSR primers from *Medicago truncatula* [41] (Table A1, Nos. 20–35) were ordered from Sigma-Aldrich and used to amplify okra genomic DNA (total of 35 primers). The stock primers were diluted to a final working solution of $10\text{ ng }\mu\text{L}^{-1}$ in 200 μL ddH₂O.

PCR amplifications were conducted in a final volume of 15 μL , containing 5 μL of $25\text{ ng }\mu\text{L}^{-1}$ DNA, 4.05 μL of ddH₂O, and 5.95 μL of master mix ($10\times$ Buffer, Bioline Cat No. BIO-21040). PCR was performed using two different programs for Medicago and okra SSR markers. For Medicago SSR markers, an initial incubation of 1 min at $94\text{ }^{\circ}\text{C}$ was followed by 35 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 1 min, annealing at $45\text{ }^{\circ}\text{C}$ for 1 min, and elongation at $72\text{ }^{\circ}\text{C}$ for 2 min. A final extension of $72\text{ }^{\circ}\text{C}$ for 10 min was followed by an incubation at $4\text{ }^{\circ}\text{C}$ [41]. For okra SSR markers, the program described by Schafleitner et al. [42] was modified to an initial incubation at $95\text{ }^{\circ}\text{C}$ for 10 min, followed by 35 cycles of

denaturation at 95 °C for 30 s, annealing at 53 °C for 45 s, and elongation at 72 °C for 45 s. A final extension of 7 min at 72 °C was followed by incubation at 4 °C.

PCR products were separated using 3.5% agarose gel (7 g of agarose, 200 mL of 1× TBE buffer and 4 µL of gel red) electrophoresis. An aliquot of 1.5 µL of loading dye was added to 4 µL of PCR products and loaded on the gel against 2 µL of HyperLadder™ IV (BIOLINE) and run at 100 volts for 3 h. Bands were visualized under UV light (ChemiDoc™ MP Imaging System, Image Lab™ Software, v5.1).

2.4. Photosynthetic Parameters

F_v/F_m , F_v'/F_m' , Φ_{PSII} , A , and g_s were measured on the most recent, top mature leaf in both the control and heat treatments using a Licor 6400XT fitted with a fluorescence light source (LI-COR, Lincoln, NE, USA). Reference CO_2 was set to 400 µmol mol⁻¹, photosynthetically active radiation (PAR) to 1300 µmol m⁻² s⁻¹, and flow rate to 300 mL min⁻¹. Fluorescence parameters were assessed in the light (F_v'/F_m') and again in the dark (F_v/F_m) after dark adaptation of all plants for 1 h at 30 °C. Due to the large number of genotypes (104) in the initial screening (4 h heat shock), stomatal conductance was measured using a porometer (SC-1, Decagon Devices, Pullman, WA, USA), which was calibrated in Auto Mode, following the manufacturer's calibration procedure.

2.5. Electrolyte Leakage

Electrolyte leakage was measured on the same leaf, on which photosynthetic measurements were taken using the conductivity method described by Sullivan [50], Lafuente et al. [51], and Camejo et al. [14]. Eight 7 mm diameter leaf disks were cut using a cork borer and were placed in 50 mL Greiner centrifuge tubes (Sigma Aldrich, Castle Hill, Australia) with 20 mL of double deionized water and maintained on a shaker at 80 rpm for 20 h at room temperature. The conductivity of the solution was read with a conductivity meter (Edge, Hanna Instruments Inc. HI11310 single ceramic, double junction, and refillable pH electrode with temperature sensor, UK) before the samples were autoclaved and after autoclaving for 15 min at 121 °C to burst the cells. Electrolyte leakage was expressed as the ratio of the conductivities in percentage:

$$\% \text{ electrolyte} = (T1/T2) \times 100,$$

where T1 and T2 are the conductivities before and after autoclaving the tissues, respectively.

2.6. Initial Screening of 104 Genotypes: Four-Hour Heat Shock (45 °C)

Due to the large number of genotypes (104), one plant per genotype was considered for the initial screen. After 4 weeks growth in the glass house, plants were moved into a controlled environment facility (CEF) with a 12 h day–night cycle set at 30 °C during the day and 22 °C at night, and 70% RH for a week (high-pressure sodium lamps, 300 mmol m⁻² s⁻¹), and plants were watered regularly to avoid moisture stress. The physiological parameters F_v/F_m , F_v'/F_m' (using a LI-COR 6400) and g_s (using a porometer) were measured on the plants prior to the heat shock being applied (control). The day after the control measurements, plants were subjected to a heat shock of 45 °C for 4 h, and the chamber was cooled to 30 °C for 1 h before measuring the same physiological responses, as in the control and at a similar time of the day.

2.7. Advanced Screening of 33 Genotypes: Six-Hour Heat Shock (45 °C)

A selection of thirty-three okra genotypes from the initial screening were sown and grown in the glass house. In total, 4 weeks after sowing, the plants were moved to the CEF and arranged in a completely randomized design under the same conditions as the initial screen (3 replicates per genotype). As the fluorescence measurement did not show clear differences between control and heat treatments in the initial screening, the duration of the heat shock was extended to 6 h at 45 °C. F_v/F_m , F_v'/F_m' , A , Φ_{PSII} , and g_s using a LI-COR

6400, and EL using a conductivity meter, were measured before the heat shock (at 30 °C, control) and after heat shock (at 30 °C, one hour after termination of the heat shock).

2.8. Statistical Data Analysis

Allele frequencies for each microsatellite locus were used to calculate the polymorphic information content (PIC) manually, using the following equation, where P_i is the frequency for the i th allele, and l is the total number of alleles [52]. Markers with a PIC value greater than 0.5 were highly informative [53].

$$\text{PIC} = 1 - \sum_{i=1}^l (P_i^2) \quad (1)$$

The images obtained from agarose gels were scored manually by indicating presence (1) or absence (0) of a specific allele. The “1” or “0” data were used to generate a dendrogram using unweighted pair-group method with arithmetic means (UPGMA) [54] clustering in NTSYS-pc v2 [55]. Then, “1” and “0” data were converted to “A” and “T”, respectively, to generate a dendrogram in MEGA v6 (Molecular Evolutionary Genetics Analysis) to compare with the results from NTSYS-pc v2 [56]. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 rep), were shown above the branches on the generated dendrogram [57]. The evolutionary distances were computed using the Maximum Composite Likelihood [58].

Physiological data (photosynthetic parameters, EL) were subjected to a one- and two-sample t -test with the level of significance set at $p < 0.05$ and a general analysis of variance (ANOVA) with the level of significance set at $p < 0.05$, and a comparison of means was performed using Fisher’s unprotected LSD, using GenStat 17th Edition software (VSN International Ltd., London, UK).

3. Results

3.1. DNA Extraction and Dendrogram Generation Using SSR Primers

The average observed A260/A280 among the 104 genotypes was 1.75, of which 63.46% of the isolates produced values between 1.8 and 1.9, 23.08% had values between 1.6 and 1.7, and only 13.46% were between 1.2 and 1.5 (Table S2). The average A260/A230 was 1.2, where 38.5% of the isolates produced values between 1.5 and 2, and the remainder was less than 1.5.

Of the thirty-five SSR markers (Table A1) used to amplify genomic DNA of the 104 okra genotypes, only eight markers from *A. esculentus* (Table 1) amplified all genomic DNA under PCR at the annealing temperature of 53 °C. The eight primers amplified a total of 29 alleles among 104 isolates at an average of 3.6 alleles per primer, ranging from 7 alleles (primer 13) to 2 alleles (primers 9 and 12). The scored band size ranged from 109 to 308 base pairs. The data were used to generate a UPGMA-based phylogenetic dendrogram for clustering and similarity analysis across the isolates using both MEGA v6 and NTSYS-pc v2 software, and similar results were obtained. The dendrogram generated from the bootstrap analysis using the MEGA v6 software (Figure 1) was chosen to assess genetic diversity among genotypes with favourable physiological traits.

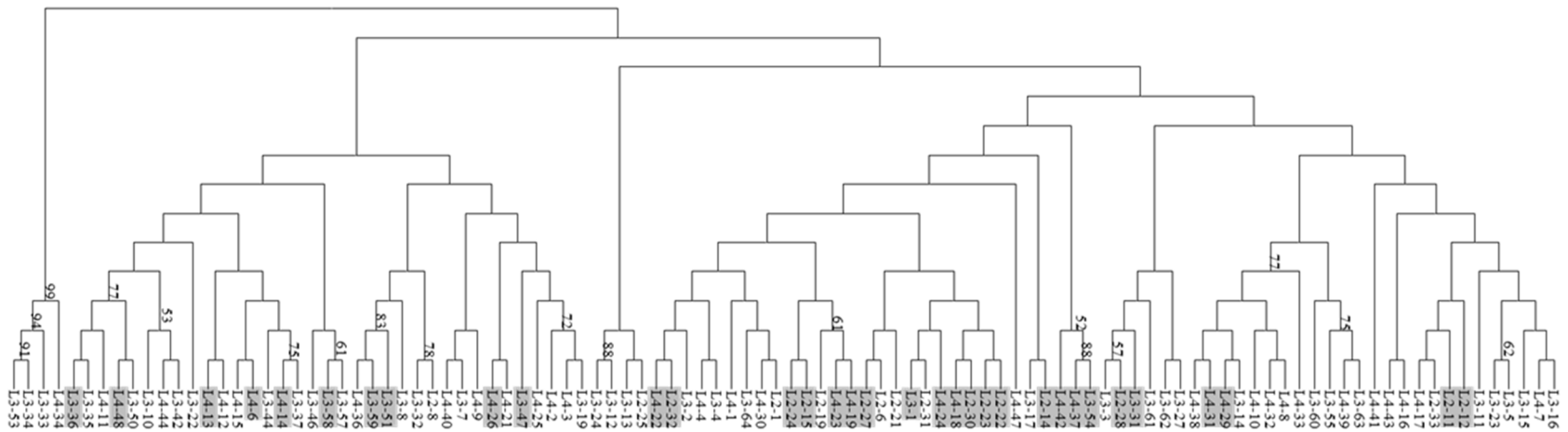


Figure 1. A phylogenetic analysis of 104 okra isolates based on 29 alleles produced by eight SSR markers. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 iterations) are shown above the branches, and values below 50 were omitted. Highlighted genotypes were selected for the in-depth physiological screening after 6 h heat shock.

Table 1. Eight polymorphic SSR markers used to amplify 104 genomic DNA at an annealing temperature of 53 °C, including information on repeated motifs, forward (F) and reverse (R) primer sequences, allele sizes (bp), number of alleles per locus, and polymorphic information content (PIC).

Primer No.	Name	Repeat Motif	Primer Sequence (5'-3')	Allele Size Range (bp)	No. of Alleles	PIC
3	AVRDC-Okra9	(AAT)12	F: ACCTTGAACACCAGGTACAG R: TTGCTCTTATGAAGCAGTGA	150–250	4	0.56
6	AVRDC-Okra28	(ATT)8	F: CCTCTTCATCCATCTTTTCA R: GGAAGATGCTGTGAAGGTAG	200–300	3	0.53
9	AVRDC-Okra54	(GAA)10	F: CGAAAAGGAAACTCAACAAC R: TGAACCTTATTTTCCTCGTG	100–170	2	0.49
11	AVRDC-Okra57	(GAA)9- (GAG)7	F: CGAGGAGACCATGGAAGAAG R: ATGAGGAGGACGAGCAAGAA	170–310	4	0.43
12	AVRDC-Okra63	(TCT)12	F: GTGTTTGAAAGGGACTGTGT R: CTTTCATCAAAACCATGCAG	200–300	2	0.27
13	AVRDC-Okra64	(TCT)22	F: AAGGAGGAGAAAGAGAAGGA R: ATTTACTTGAGCAGCAGCAG	100–300	7	0.71
18	AVRDC-Okra86	(AGC)8	F: ATGCAAACAAGCTAGTGGAT R: ATTCTCTTCAGGGTTTCCTC	250–400	4	0.65
19	AVRDC-Okra89	(AGC)8	F: TTTGAGTTCCTTCGTCCACT R: GTATTGGACATGGCGTTAT	140–200	3	0.59

3.2. Initial Physiological Screening of 104 Genotypes after Four-Hour Heat Shock (45 °C)

The rapid physiological measures of F_v/F_m , F_v'/F_m' , and g_s by porometry were used due to the large number of genotypes assessed (Table S3). Significant differences were observed between control and heat treatments for both F_v'/F_m' and g_s ($p < 0.001$) but not F_v/F_m . Under high temperatures, F_v'/F_m' increased relative to the control in 18.3% of genotypes, decreased in 52.9%, and remained similar in 28.8%. g_s varied strongly among genotypes in each treatment, and 71.2% of genotypes had lower g_s in heat treatment compared to the control, whereas the remainder had higher g_s .

Thirty-three genotypes were selected based on low genetic similarity (less than 50%), as well as contrasting responses in chlorophyll fluorescence and stomatal conductance (highlighted in Figure 1). These genotypes fell within five groups based on different responses to heat stress:

1. 45.5% with low F_v'/F_m' , low g_s ;
2. 12.1% with low F_v'/F_m' , high g_s ;
3. 6.1% with high F_v'/F_m' , high g_s ;
4. 21.2% with similar F_v'/F_m' , low g_s ;
5. 15.1% with similar F_v'/F_m' , high g_s .

Of the selected genotypes, L3-54 and L4-37 from Cambodia showed 88% genetic similarities; however, their responses to high temperatures differed, as g_s and F_v'/F_m' were lower in genotype L3-54 compared to genotype L4-37. These two genotypes had 52% similarities with the genotype L4-42 (from the USA); L4-42 showed a low g_s , such as genotype L3-54, and an F_v'/F_m' equivalent to genotype L4-37. Genotypes L3-59 from Australia and L3-51 from the Philippines were 83% similar, and, despite both having lower g_s values under high temperatures, their F_v'/F_m' responses differed.

3.3. In-Depth Physiological Screening of 33 Genotypes after Six-Hour Heat Shock (45 °C)

In this more comprehensive experiment, heat shock was extended to 6 h to elicit stronger responses in gas exchange (A , g_s), fluorescence (F_v/F_m , F_v'/F_m' , Φ_{PSII}), and EL. EL was measured for all 33 genotypes, but no photosynthetic and fluorescence data were recorded on L2-32, L3-1, L3-58, and L3-59.

The Genotype \times Treatment interaction was significant for all traits (Table 2), and genotype and treatment effects were significant for all measured parameters, except for

the treatment effect on EL ($p = 0.585$, Figure 2). Most genotypes showed lower F_v/F_m and F_v'/F_m' values after high temperature exposure, with larger differences for F_v'/F_m' (Figure 3). Some genotypes displayed higher F_v'/F_m' values after heat treatment (e.g., L4-6, L4-24, and L4-26). Generally, the ranking between the difference in fluorescence parameters in the dark and in the light ($\Delta F_v/F_m$ and $\Delta F_v'/F_m'$) was maintained, with some exceptions (e.g., L4-26).

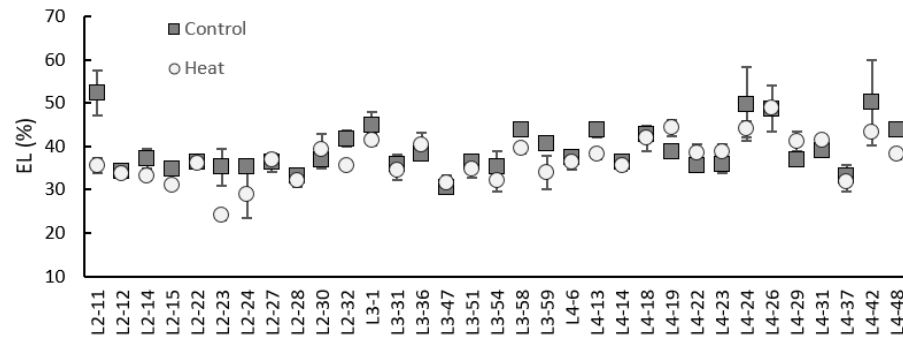


Figure 2. Electrolyte leakage (%) of 33 genotypes sorted by list number in control (grey square) and heat treatments (6 h, 45 °C, light circle). Error bars depict standard error ($n = 3$).

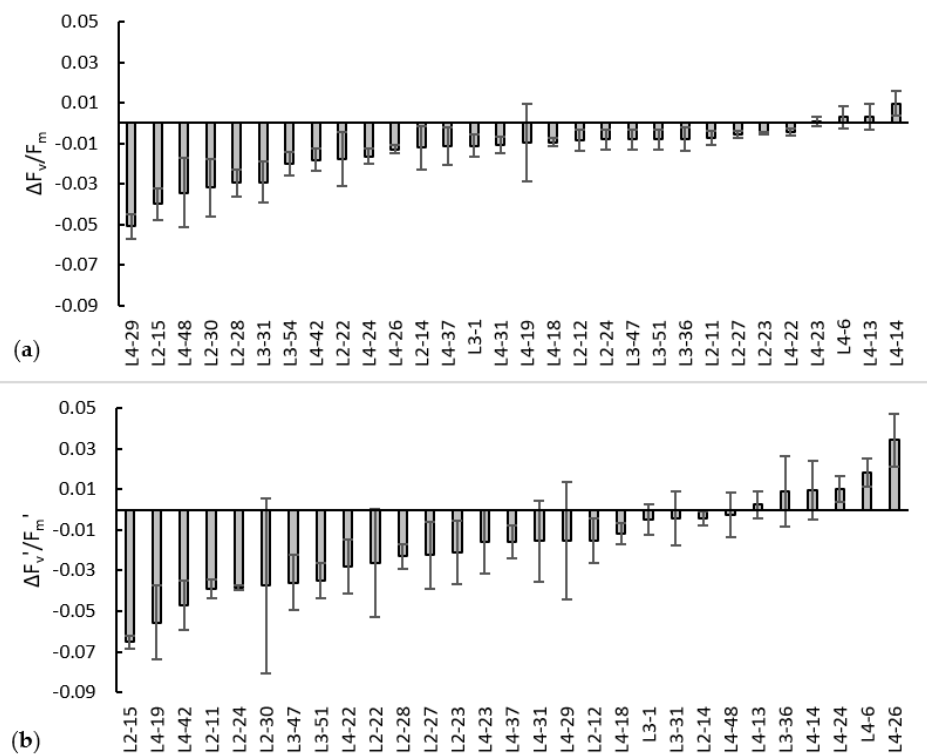


Figure 3. Average difference in chlorophyll fluorescence (Δ) of 29 genotypes ($n = 3$) calculated by subtracting measurements in control conditions from measurements after heat shock (6 h, 45 °C) (a) difference in chlorophyll fluorescence in the dark ($\Delta F_v/F_m$), (b) difference in chlorophyll fluorescence in the light ($\Delta F_v'/F_m'$). Negative values represent lower measurements in the heat treatment compared to the control. Error bars depict standard error. Photosynthesis measurement in the heat was missing for L3-54, hence not depicted in panel (b).

Twelve genotypes maintained or increased g_s values in response to heat, which generally resulted in increased A values (Figure 4). Six genotypes (L2-14, L2-27, L2-28, L3-31, L4-14, and L4-48) maintained g_s values after heat exposure but decreased A values ($p = 0.02$).

Table 2. ANOVA results (F and *p*-values) for chlorophyll fluorescence (in the dark F_v/F_m and in the light F_v'/F_m'), photosynthesis rate (*A*), efficiency of the open reaction centre (Φ_{PSII}), stomatal conductance (g_s), and electrolyte leakage (EL) of 29 genotypes (except for EL, which were 33 genotypes) in the control and heat (6 h, 45 °C).

		F_v/F_m	F_v'/F_m'	<i>A</i>	Φ_{PSII}	g_s	EL
Genotype	F	2.14	6.23	6.41	3.09	9.94	4.34
	<i>p</i> -value	0.003	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment	F	61.27	27.68	11.28	16.4	29.61	0.3
	<i>p</i> -value	<0.001	<0.001	0.001	<0.001	<0.001	0.585
Genotype × Treatment	F	2.27	1.8	4.88	2.99	5.29	1.62
	<i>p</i> -value	0.001	0.017	<0.001	<0.001	<0.001	0.031

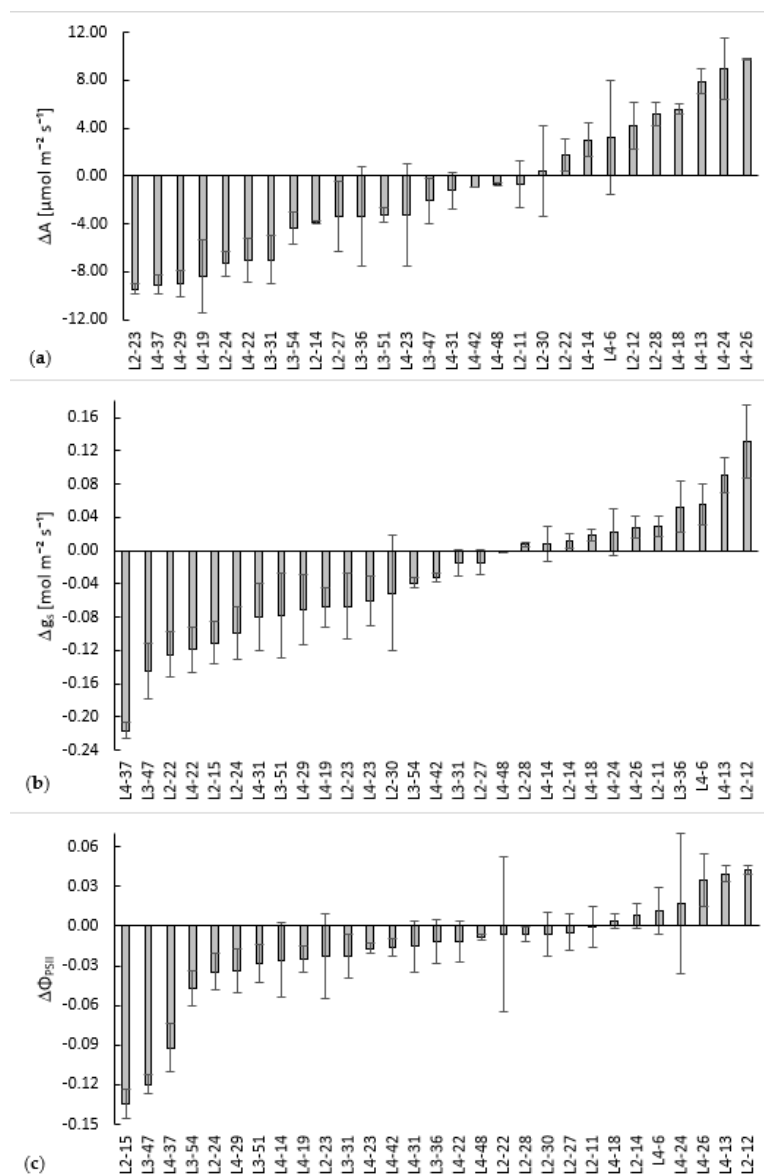


Figure 4. Average difference of physiological traits (Δ) in 29 genotypes ($n = 3$), calculated by subtracting measurements under control conditions from measurements after heat treatments (6 h, 45 °C ($n = 3$)). (a) difference in photosynthesis rate (ΔA), (b) difference in stomatal conductance (Δg_s), and (c) difference in efficiency of the open reaction centre ($\Delta \Phi_{PSII}$). Negative values represent lower measurements in the heat treatment compared to the control. Error bars depict standard error. Photosynthesis measurement in the heat was missing for L2-15, hence not depicted in panel (a).

The ranking of differences (Δ) in A , g_s , and Φ_{PSII} values was overall similar, but differences were more gradual in the A values compared to the g_s and Φ_{PSII} values. For example, genotype L4-47 showed strongest reduction in the g_s but had a similar A to L-23 and L4-29. Poorly performing genotypes reduced, whereas best-performing genotypes increased over all parameters (A , g_s , and Φ_{PSII}). Genotype L4-26 showed moderate increases in g_s , but additional increases in Φ_{PSII} resulted in overall increased ranking for A in the heat.

Poorly performing genotypes in the fluorescence measurements (both $\Delta F_v/F_m$ and $\Delta F_v'/F_m'$) also ranked low for A , g_s , and Φ_{PSII} (e.g., L2-15). However, there were some exceptions, such as genotype L4-48, which performed poorly for $\Delta F_v/F_m$ but mostly maintained $\Delta F_v'/F_m'$, A , g_s , and Φ_{PSII} .

4. Discussion

High temperature stress is a major limitation to agricultural production and food security and is expected to become more frequent with climate change [59–61]. Yield is heavily dependent on photosynthesis, but the photosynthetic pathway is sensitive to high temperature and can be affected by alteration of the PSII function and closure of stomata, resulting in CO_2 limitation and disruption of cellular function from damaged thylakoid membranes [26,33,34]. In this study, we identified heat-tolerant accessions, which can be used to breed more heat-tolerant okra by screening progeny from crosses among diverse parents for the physiological traits outlined in this paper.

4.1. Modified DNA Extraction Protocol, SSR Markers and Polymorphism in Okra

We extracted good quality DNA (confirmed by the absorbance ratio at A260/A280 nm) from okra using a modified CTAB method with the addition of PVP and RNase. Okra leaves contain polysaccharides and secondary metabolites such as polyphenols that interfere with the DNA isolation process. The glue-like texture of polysaccharides initially resulted in poor DNA quality and had a negative effect on PCR amplification through the inhibition of *Taq* polymerase activity [62]. To prevent polysaccharide contamination during DNA isolation, some studies used yellow and etiolated fresh leaves of 10–14 day old okra plants grown under dark conditions [43,63] or an additional wash with 1 M NaCl [39]. In contrast, we were able to extract high quality genomic DNA of a sufficient quantity from young, fresh, green leaf tissue without the need for additional washing, which increased the effectiveness of molecular genetic analyses using PCR-based markers.

The 16 *Medicago* SSR markers successfully used for okra by Sawadogo et al. [41] did not amplify the genomic DNA in the current study. In contrast, the 19 SSR markers specific to okra provided by Schafleitner et al. [42] amplified all genotypes, although only eight showed polymorphisms. In a previous study on Turkish okra [64], nine of the SSR markers from Schafleitner et al. [42] were used, five of which were common to the current study. Among the five common markers, the highest PIC value was observed for marker 13 (AVRDC-Okra64) in both the current study and that of Yildiz et al. [64], and had the third highest PIC in the study by Schafleitner et al. [42]. Thus, this SSR marker can be considered informative and suitable for okra genotyping. However, marker 12 (AVRDC-Okra63) had the lowest PIC value in the current study (0.27) but was greater than 0.6 in other studies, suggesting that this marker was polymorphic in only a few genotypes.

The dendrogram of 104 okra genotypes indicated that considerable genetic diversity existed among okra accessions. Interestingly, genotypes did not group by geographical origin, indicating that materials have been introduced to different regions overtime, thus blurring geographical differences. There were variations in physiological responses to high temperatures observed among some genotypes in the same grouping that were more than 50% similar. These varied responses highlighted the necessity of combining phenotyping with an understanding of genetic diversity. Clearly, crossing among heat-tolerant materials in different diversity groups would provide a higher probability of identifying higher levels of heat tolerance.

4.2. Physiological Response of Okra to Heat Shock

Heat shock has been effective in initiating physiological responses in plants, even if only imposed for short periods of time. For example, a two-hour heat shock at 45 °C decreased chlorophyll fluorescence in tomato and cucumber, indicating damage to PSII, despite an increase in g_s and the associated cooling [14,20]. PSII in okra was not strongly affected by a 4 h heat shock at 45 °C, as F_v/F_m showed no significant reduction. In contrast, crops such as cotton and tomato were characterised by lower F_v/F_m values when exposed to high temperatures, despite being warm-season crops [14,65,66]. Although fluorescence in light-adapted okra plants (i.e., F_v'/F_m') was significantly reduced in most genotypes, it was reduced only by a small amount and even increased in some genotypes compared to the control, which suggested that okra is overall tolerant to elevated temperatures. However, increasing the length of the heat shock to six hours did reduce F_v/F_m and F_v'/F_m' values more effectively in the 33 selected okra genotypes, indicating a reduction in the functionality of the PSII reaction centre [12].

Some okra genotypes showed higher F_v'/F_m' values under high temperatures compared to the control, which coincided with higher stomatal conductance and photosynthesis under stress. The opening of the stomata increased latent heat loss, which was particularly important for plants with large leaves experiencing greater thermal stresses compared to plants with narrow leaves [67]. Alternatively, these results may have been the result of the poor performance of the control rather than the tolerance of these genotypes to heat: F_v'/F_m' values in their control measurements were lower than the average of all genotypes (0.51), and their g_s and A values were nearly half the value of the average of all genotypes (0.11 mol m⁻² s⁻¹ and 12.84 μmol m⁻² s⁻¹, respectively). Although plants were regularly irrigated, the closure of the stomata and the reduction in A accompanied by a decrease in the F_v'/F_m' in the control may have been a result of transient water stress [68]. Hence, the provision of ample water to both control and heat-stressed plants (during the application of the stress) is of central importance if physiological parameters such as fluorescence and gas exchange are used for screening of heat tolerance.

Three different g_s responses to heat were observed among the okra genotypes (higher, lower, and similar to the control). As measurements were completed 1 h after the end of the heat shock, these likely reflected the g_s responses during the application of the shock. Nevertheless, this response may have been influenced by a quick (and often overlooked) recovery response [69]. For example, cotton showed a 67% increase in stomatal conductance after heat shock but returned to the control level after a 24 h recovery [70]. As noted above, genotypes with open stomata (i.e., higher g_s) were able to reduce leaf temperature when exposed to high temperature [17,71,72] and may have been able to survive even extreme heat waves [73]. Several okra genotypes had higher A values in the heat treatments, indicating that photosynthesis was mostly limited by g_s values rather than damage to PSII (i.e., reduced F_v'/F_m') under heat, corroborated by an increase in Φ_{PSII} in the heat. Although electron transport through PSII was interrupted by high temperature, and photon energy could not be used for photochemistry if the PSII was damaged [29,74], electrons can be contributed to photosystem I (PSI) by cyclic electron transportation so that a reduction in A was prevented [75]. Genotypes that displayed lower g_s values under high temperatures restricted CO₂ assimilations, resulting in lower A values, a trait characteristic of heat-sensitive genotypes [26,34]. Notably, genotypes that exhibited similar g_s in the control and heat treatments may have possessed heat tolerance, as was found in tomato [14].

Although maximum quantum yield and damage to PSII were best measured in dark-adapted leaves (F_v/F_m) [21], the ranking of the change in the physiological responses (Δ) was similar across several traits for many genotypes. Some of these traits could be measured quickly (e.g., g_s by porometry or F_v/F_m , F_v'/F_m' and Φ_{PSII} by chlorophyll fluorescence), and the instruments are highly portable, which may be an advantage in field-based screening of heat tolerance, particularly if large numbers of accessions have to be assessed. To increase confidence in these measurements, replication as well as ample water supply are recommended. Moreover, the comparison of several traits may allow a

window into different physiological adaptations to heat. For example, some genotypes displayed similar or increased g_s values but reduced A values with constant F_v'/F_m' values, suggesting a different mechanism of inhibition to PSII, possibly a reduction in Rubisco activase activity [76]. It may also allow the identification of genotypes with traits suited for specific environments. For example, high stomatal conductance may be beneficial in environments without water limitation to enable reduction in heat load on leaves [73]. In contrast, open stomata may be a disadvantage in drier environments, and genotypes maintaining constant g_s and A values may be better adapted.

The use of EL to evaluate heat damage to cell membranes has successfully been applied to crops such as cowpeas, wheat, holly, turf grass, and cotton [65]. In cotton, EL increased by 19–52% following a 3 h heat shock (45 °C) [77], 9.72–24.58% after 4 h, and 10.52–28.91% after an 8 h heat shock (40 °C) [78]. Despite heat effects on fluorescence, stomatal conductance, and photosynthesis, a six-hour heat shock only had a significant effect on electrolyte leakage of eight okra genotypes, of which only one genotype showed increased leakage, confirming the general heat tolerance of okra [79]. Hence, EL may not be an efficient screening technique for assessing short-term heat damage in okra. However, longer exposure times to high temperatures may influence EL, and this should be determined either in controlled environment or under field conditions.

5. Conclusions

DNA isolated from young, fresh, and green okra leaf tissue using the modified CTAB method with additional PVP and RNase was effective in producing high quality genomic DNA of sufficient quantity for molecular analysis. SSR markers showed that significant genetic variations exist among okra genotypes. These markers could be used in conjunction with phenotypic screening to identify okra genotypes for genetic improvement and production. One previously published SSR marker was particularly effective in discriminating materials and is recommended for okra screening.

Photosynthetic traits (F_v'/F_m' , Φ_{PSII} , A , and g_s) were good indicators of the physiological response of okra genotypes to heat shock (6 h or more at 45 °C), and the ranking of differences of measured traits (Δ) between control and heat-treated plants could be used to assess genotype sensitivity to heat. In contrast, electrolyte leakage was unable to detect damage from short-term heat exposure and may not be an effective screening tool in okra. Further studies of the physiological responses of the selected genotypes will be needed to confirm if observations under short-term heat stress equally apply to extended heat stress, particularly under field conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9060722/s1>, Table S1: List of 104 okra genotypes (names and origin); Table S2: Purity of DNA extracted from 104 okra genotypes using modified CTAB method; Table S3: Stomatal conductance and fluorescence of 104 okra genotypes after 4 h heat shock.

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Appendix A

Table A1. Thirty-five SSR primers used to amplify DNA of 104 okra genotypes, including forward (F) and reverse (R) with their repeated motif and primer sequences from *A. esculentus* (No. 1–19) and *Medicago truncatula* (No. 20–35).

No	Name (Marker)	ID	Repeated Motif	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')
1	AVRDC-Okra1	5200	(AAG)13	F:ATGGAGTGATTTTGTGGAG	R:GACCCGAACACTCACGTTACTA
2	AVRDC-Okra8	128713	(AAG)8	F:TGCTGTGGAAGGTTTTACT	R:ATGACGAAAGTGGTGAAAAG
3	AVRDC-Okra9	89235	(AAT)12	F:ACCTTGAACACCAGGTACAG	R:TTGCTCTTATGAAGCAGTGA
4	AVRDC-Okra17	8461	(AGA)7	F:ACGAGAGTGAAGTGGAACTG	R:CTCCTCTTTCCTTTTCCAT
5	AVRDC-Okra21	43380	(AGA)9	F:TCATGTCTTCCACTCAACA	R:CCAAACAAAATATGCCTCTC
6	AVRDC-Okra28	151529	(ATT)8	F:CCTCTTCATCCAICTTTTCA	R:GGAAGATGCTGTGAAGGTAG
7	AVRDC-Okra39	51708	(AG)16	F:TGAGGTGATGATGTGAGAGA	R:TTGTAGATGAGGTTTGAACG
8	AVRDC-Okra52	126731	(CAT)8-(TCA)9	F:AACACATCCTCATCCTCATC	R:ACCGGAAGCTATTTACATGA
9	AVRDC-Okra54	87311	(GAA)10	F:CGAAAAGGAAACTCAACAAC	R:TGAACCTTATTTTCTCGTG
10	AVRDC-Okra56	21030	(GAA)44	F:GGCAACTTCGTAATTTCTA	R:TGAGTAAAAGTGGGGTCTGT
11	AVRDC-Okra57	151995	(GAA)9-(GAG)7	F:CGAGGAGACCATGGAAGAAG	R:ATGAGGAGGACGAGCAAGAA
12	AVRDC-Okra63	34632	(TCT)12	F:GTGTTTGAAAGGGACTGTGT	R:CTTCATCAAAACCATGCAG
13	AVRDC-Okra64	5886	(TCT)22	F:AAGGAGGAGAAAGAGAAGGA	R:ATTTACTTGAGCAGCAGCAG
14	AVRDC-Okra66	20291	(TTC)12-(TTC)13	F:CACCAGAATTTCCCTTTTG	R:ACTGTGTTTGGCTTATGCT
15	AVRDC-Okra70	89044	(TC)11	F:GTAGCTGAACCCTTTGCTTA	R:CTATCATGGCGGATTCCTTA
16	AVRDC-Okra77	152270	(GAAATA)4-(GAAACA)7	F:CTGTTTGTTCGTCGTAATCA	R:AAAGTTTCTTCTTTCCACC
17	AVRDC-Okra78	122488	(TAT)11-(TATTGT)4-(TATCGT)4	F:CTCCGACAATTCAAGAAAAG	R:CACCCAATCAAGCTATGTTA
18	AVRDC-Okra86	461	(AGC)8	F:ATGCAAAACAAGCTAGTGGAT	R:ATTCTCTTCAGGGTTTCTCTC
19	AVRDC-Okra89	129459	(AGC)8	F:TTTGAGTCTTTCGTCCTACT	R:GTATTGGACATGGCGTTAT
20	3		(AAC)5	F:TGGTGACGACATACAAGAAAAGA	R:CCCGGTGGTTTAGGAAGTTT
21	7		(AAC)6	F:ACCACTTCTCCATCCATCCA	R:AGCTTGCTGCATGAGTGCT
22	8		(AAC)5-(AAC)6	F:CAAAGGCACTTCATCAGCAA	R:GTGAGCGTCAATGTTGGATG
23	20		(AAG)5	F:TGAAGGTCAAATTGCCAAGA	R:TCCTGTTTTTGAAGGTCACG
24	27		(AAG)6	F:CGATCGGAACGAGGACTTTA	R:CCCCGTTTTTCTTCTCTCCT
25	35		(AAG)8	F:GAAGAAGAAAAAGAGATAGATC TGTGG	R:GGCAGGAACAGATCCTTGAA
26	55		(AAG)6	F:CAGTTCGGAAGAGGACAAA	R:ATCCCAAACCAGTTCTTCA
27	62		(AT)10	F:TCCGCCCATAGTCTTTGAC	R:TGAAAGGGCTTAGAGGGTTTT
28	74		(AT)16	F:GGTGGAAAGGAACAACCTCTGG	R:CCGGCATGATTAAGACACAC
29	82		(TC)11	F:CACTTTCACACTCAAACCA	R:GAGAGGATTTCCGGTGATGT
30	95		(TCC)6	F:AAAGGTGTTGGGTTTTGTGG	R:AGGAAGGAGAGGGACGAAAAG
31	96		(TCC)6	F:CCAGTGGCAGCTACGGTACTA	R:GAGACGGAGGAGAAGTTGCTT
32	103		(TG)5	F:TGGGTTGTCCTTCTTTTGG	R:GGGTGCAGAAGTTGACCA
33	107		(AC)5	F:CAAACCAATTTCTCCATTGTG	R:TACGTAGCCCCCTTGCTCATT
34	135		(AG)10	F:GCTGACTGGACGGATCTGAG	R:CCAAAGCATAAGCATTCACTCA
35	136		(AT)5	F:TTTGTGTCGAGAGATGCACA	R:CTTGAAACTTCAACGGCATT

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