CHAPTER 3

MATERIALS AND METHODS

The present experiment was conducted at Biotechnology Laboratory, Saba-basha Faculty of Agriculture Alexandria University during 2012:2014. Six wheat cultivar including most of the popular varieties, collected from Wheat Research Centre at Cairo, Egypt were used for the present study. The physiological measurements (membrane thermostability, proline content and chlorophyll content), molecular (quantitative rt-PCR and sequencing) and bioinformatics analysis were obtained to achieve the main object of this study which was screening some Egyptian wheat cultivars tolerance against heat shock and snps detection based on some heat tolerance genes (hsp 101)

3.1. Plant Material and Treatments

Thirty six Egyptian wheat cultivars released from 1947 to 2011 were evaluated for their relatedness by pedigree. AS Table (1), all the cultivars were defined and categorized by their release year (RE: I = before 1960, II = 1960-1969, III = 1970-1979, IV = 1980-1989, V = 1990-1999, and VI = 2000-2011) (**Bhoja *et al*., 2011).**

Seeds of six wheat varieties Misr 1, Sids 12, Misr 2, Giza 168, Skha 93 and Gemmeiza 9 were germinated in plastic plats (18 cm diameter) filled with soil and allowed to grow with 16 hrs photoperiod, a light intensity of 200 µE/m2/s, relative humidity of 90 ± 1 (%) and at about 25^{o} C for eight days. Of the six plats of seedlings (three replications) for each cultivar three were allowed to grow in the same condition for another 48 hrs for determination of seedling measuring membrane injury (%) cell, proline content and chlorophyll content under normal condition. Another three sets of seedlings were exposed to high temperature stress at 35^{o} C for 48 hrs. The seedlings were then used also for determination of seedling measuring membrane injury (%) cell and proline content.

3.2. Physiological and biochemical Characters measurements

3.2.1 The measurement for seedlings Cell membrane thermostability:

Procedure used for measuring membrane injury to high temperature was the same as described by **Blum and Ebercon (1981)**.

Procedure

1- Flag leaf samples were collected from five randomly plants of each heat treatment and cultivar,
2- Two leaf discs (10 mm in diameter) were collected from a flag leaf and washed three times with deionized water to remove electrolytes adhering to leaf tissue, as well as electrolytes released from cut cells on the periphery of leaf discs,
3- The test tubes (25 mm x 150 mm) were also rinsed with deionized water. Then ten leaf discs from five flag leaves were placed in a test tube and a piece of cotton was put on the leaf discs inside the test tube to prevent any injury of the discs by the electrode bar during conductance measurement. There after 20 mL of deionized water was added to each tube,
4- Both control and heat treated samples were kept for 18 h in a refrigerator at 10°C after treatment period,

Table (1): Egyptian cultivars released between 1947 and 2011 and their year of release

S.N.	Code	Variety	Released Year	S.N.	Code	Variety	Released Year
1	**GZ139**	Giza139	1947	19	**GZ163**	Giza163	1987
2	**GZ144**	Giza144	1958	20	**GZ164**	Giza164	1987
3	**GZ145**	Giza145	1958	21	**GZ165**	Giza165	1991
4	**GZ147**	Giza147	1958	22	**GMZ1**	Gemmeiza1	1991
5	**GZ148**	Giza148	1959	23	**SAHL1**	Sahel1	1994
6	**GZ150**	Giza150	1960	24	**GZ167**	Giza167	1995
7	**GZ155**	Giza155	1968	25	**SIDS4**	Sids4	1995
8	**GZ156**	Giza156	1972	26	**GMZ3**	Gemmeiza3	1996
9	**SUPX**	Super X	1972	27	**GMZ5**	Gemmeiza5	1997
10	**INDS65**	Indus-65	1973	28	**GMZ7**	Gemmeiza7	1998
11	**CHB70**	Chenab70	1977	29	**GMZ9**	Gemmeiza9	1999
12	**GZ157**	Giza157	1977	30	**GZ168**	Giza168	1999
13	**GZ158**	Giza158	1980	31	**SKH93**	Sakha93	1999
14	**SKH8**	Sakha8	1978	32	**SKH94**	Sakha94	2004
15	**SKH61**	Sakha61	1980	33	**GMZ10**	Gemmeiza10	2004
16	**GZ160**	Giza160	1982	34	**SIDS12**	Sids12	2008
17	**SKH92**	Sakha92	1987	35	**MSR1**	Misr1	2010
18	**CHLEF**	Chlef	1987	36	**MSR2**	Misr2	2011

5- Conductivity readings were taken at both treatment 25°C and 35°C using an electrical conductivity meter for control (C1) and heat treated (T1) tubes,
6- The samples were then boiled for 1 h and then, a second conductivity reading of the aqueous phase (T2 and C2) was taken after the samples were cooled,
7- Leaf membrane thermal stability was estimated using equations as follows:

Relative Injury, RI (%) = 100-{[1-(T1/T2)] / [1- (C1/C2)] x100}

Where C and T refer to electrical conductivity of control and heat treated samples, and the subscript 1 and 2 refer to electric conductivity readings before and after boiling, respectively. The data were analyzed in completely randomized design and the means were separated by L.S.D. at 0.05 level.

3.2.2 The measurement for seedlings chlorophyll content

Procedure used for measuring seedlings chlorophyll content against to high temperature was the same as described by **Wettstein (1957)** as following:

Extraction solution:
85 % aqueous acetone

Procedure
1- Photosynthetic pigments contents of leaves chlorophyll a, b and total chlorophyll were determined from five disks were taken from the wheat leaves,
2- The pigment was extracted by grinding in 85 % aqueous acetone (20 ml) and a pinch of $CaCO_3$ was added to the acetone solution before grinding,
3- After filtration the volume of acetone solution was complete to 20 ml,
4- The absorbance of the extract was measured using a spectrophotometer at 663, 645 and 652 nm,
5- The total chlorophyll pigments calculated according to the equation mentioned as follows,
Chlorophyll (*a*) mg/l = 12.7 (O.D) 663 - 2.69 (O.D) 645(v\1000xw)
Chlorophyll (*b*) mg/l = 22.9 (O.D) 645- 4.68 (O.D) 663(v\1000xw)
Total Chlorophyll (mg/l) = Chlorophyll (*a*) + Chlorophyll (*b*)

3.2.3 The measurement for seedlings Proline content

Background

Overproduction of proline is a widespread response observed in plants experiencing various stresses, in particular osmotic stresses. The determination of this amino acid is therefore very useful to assess the physiological status and more generally to understand stress tolerance in plants. Here we describe a simple, fast and relatively harmless ninhydrin-based method, which is suitable for the high throughput determination of free proline content using a microplate reader. Ninhydrin (2,2-dihydroxyindane-1,3-dione, CAS number 485-47-2) is extensively used to assay amino acids. At neutral pH, it destroys each primary α-amino acid and also reacts with the released NH3 to form a deep purple chromogen referred to as Ruhemann's Purple, which has a maximum absorption at about 570 nm. At low pH, Ruthermann's purple is also yielded, but it quickly looses an amine residue, which results into colourless derivatives. The reaction with proline and other imino acids such as hydroxyproline or

pipecolic acid yields a yellow-orange product at neutral pH, as the cyclised N-group is not released. At low pH, the chromogen is red, with a peak of absorbance at 520 nm. It is important to note that ornithine and to a lesser extent lysine also yield a red chromogen at low pH, which result from one ninhydrin molecule binding two amino acids. Nevertheless, in plants under stress, levels of these amino acids are usually much lower than proline levels. In order to decrease background noise (e.g., high levels of anthocyanins that may interfere with the quantification), the chromogen is classically extracted using harmful solvents such as benzene **(Troll and Lindsey, 1955)** or toluene **(Bates *et al.*, 1973)**. We have removed this step to make the assay safer and easier, considering that interferences due to other compounds are usually low, especially in plants under osmotic stress. **CAUTION**: Ninhydrin is irritant to skin and respiratory system, thus requiring adequate precautions, in particular the wearing of gloves. This colorimetric assay is quantitative and provides reliable data about proline content. The sensitivity is of about 1 nmole and the linearity is in a range of 1-100 nmoles.

The Procedure used for measuring seedlings Proline content to high temperature was the same as described by **Bates *et al.*, (1973)** as following:

a. Extraction buffer: 3% Aqueous Sulphosalicylic Acid
b. Ninhydrin reagent: It was prepared and utilized for proline estimation within 2 h of preparation keeping it in a refrigerator in a brown bottle.

For preparing the ninhydrin reagent:

- Addition of 30 mL glacial acetic acid and 30 mL 6 M orthophosphoric acid was mixed with 1.25 g of ninhydrin,
- It was subsequently heated and stirred gently to dissolve but the temperature was not allowed to exceed 70 °C then kept cool at 4˚c until use (stored),

c. Procedure:

1- Approximately 0.5 g Leaf segments from each replication of each cultivar were taken for proline estimation and samples were crushed in mortar and pestle,
2- The material was homogenized in 10 mL 3% sulphosalicylic acid until no large segments of plant material remained,
3- Homogenate was filtered through Whatman No. 2 filter paper and washed with 3% sulphosalicylic acid and the volume was set to 25 mL. Two mL of the filtrate,
4- Each solution were reacted with 2 mL of ninhydrin reagent and 2 ml of glacial acetic acid in a test tube, boiled for 1h at 100°C in a water bath covering the tube with aluminum foil to prevent excess evaporation and then, it was cooled in an ice bath,
5- The reaction mixture was extracted with 4 mL of toluene was added to each tube using a dispensor. Each tube was then shaken vigorously for 15 to 20 s in an electrical shaker and the layer was allowed to separate for 30 min,
6- The absorbance of the layer was measured with at 520 nm using a Perkin Elmer Lambda 900 UV/VIS spectrophotometer (MC USA). Toluene was used as blank. The proline concentration was calculated using L- proline corresponding on the standard curve.

d. Proline standard curve:

Proline standard solution was prepared by dissolving 100 mg from proline in 100 ml of 3% aqueous sulfosalicylic acid. Aliquots of 10 µl (10 µg) to 50 µl (50 µg)

of the proline solution were put into test tube. Then, the total volume was increased to one ml using 3% aqueous sulfosalicylic acid. Each tube was treated as previously described. The obtained optical densities were diagramed against proline concentration.

The proline content was determined from a standard curve and calculated on a fresh weight basis as follows:
{(µg proline/ml × ml toluene)/115.5 µg/µmoles}/ (g sample/5) = µmoles proline/g of fresh plant material

3.2.4 Statistical analysis

Data in each experiment were collected in triplicates. Statistical analyses of the data were analysis of variance (ANOVA) performed using the Completely Randomized Design (CRD). Least significant difference test (LSD) was used to test for the significance of the differences among means at $P < 0.05$.

3.3 Molecular Genetics studies

3.3.1 Plant material and treatments

Seeds of six wheat varieties Misr 1, Sids 12, Misr 2, Giza 168, Skha 93 and Gemmeiza 9 were germinated in plastic plats (18 cm diameter) filled with soil and allowed to grow with 16 hrs photoperiod, a light intensity of 200 µE/m2/s, relative humidity of 90 ± 1 (%) and at about 25° C for 14 days. Six plats of seedlings (three replications) for each cultivar three were allowed to grow in the same condition for another 48 hrs as control (C). Another three sets seedlings was achieved by exposing plants to 34°C for 24 h and the temperature were shifted to 42 °C for 2 h for stress (A24+S) post-acclimation and three other sets Stressed without acclimation was achieved by exposing plants directly to 42 °C for 2 h (S). After the heat stress aerial parts were collected, frozen in liquid nitrogen and kept at -80 °C until use.

3.3.2 RNA extraction and purification

RNA isolation and purification was carried out using (NORGEN biotic corp. - Total RNA purification from plant kit).

3.3.2.1 Notes to use

- The maximum recommended input of plant is 50 mg or 5×10^6 plant cells.
- Both fresh and frozen plant samples can be used for this protocol. Samples should be flash- frozen in liquid nitrogen and transferred immediately to a-70˚C freezer for long term storage. Don't allow frozen tissue to thaw prior to grinding with the mortar and pestle in order to ensure that the integrity of the RNA is not compromised.
- It is is important to work quickly during this procedure.

3.3.2.2 Equipment required:

- Morter
- Microcentrifuge
- Adjustable pipettes suitable for accurate dispensing 10-50, 100-200 µl
- Microcentrifuge tubes and pipette tips

3.3.2.3 Procedure

1. Transfer ≤50 mg of each wheat cultivar was ground in to a mortar that contains an appropriate amount of liquid nitrogen to cover the sample. Grind the sample into a fine powder using a pestle in liquid nitrogen,
2. Allow the liquid nitrogen to evaporate, without allowing the tissue to thaw.
3. Add 600 µl of lysis solution to the tissue sample and continue to grind until the sample has homogenized,
4. Using a pipette, transfer the lysate into an RNase- free micro centrifuge tube (not provided),
5. Spin the lysate for 2 minutes to pellet any debris. Transfer the supernatant to another RNase-free micro centrifuge tube. Note the volume of the supernatant,
6. Add an equal volume of 70% ethanol that is equivalent to the lysate volume collected (100 µl of ethanol is added to every 100 µl of lysate), Then vortex to mix.

3.3.3. Total RNA purification from lysate

A. Binding RNA to column

- Assemble a column with one of the provided collection tubes
- Apply up to 600 µl of the lysate with the ethanol onto the column and centrifuge for 0ne minute,
- Discard the flow through. Reassemble the spin column with its collection tube,
- Depending on your lysate volume, Apply up to 600 µl of the lysate with the ethanol again onto the column and centrifuge for 0ne minute,
- Discard the flow through. Reassemble the spin column again with its collection tube.

B. Column Wash

- Apply 400 µl of wash solution to the column and centrifuge for 1 minute,
- Discard the flow through and reassemble the spin column with its collection tube,
- Repeat the last two steps again to wach column a second time,
- Wash column a third time by adding another 400 µl of wash solution and centrifuging for 1 minute,
- Discard the flow through and reassemble the spin column with its collection tube,
- Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

C. RNA elution

- Place the column into a fresh 1.7 ml Elution tube with the kit,
- Add 50 µl of elution solution to the column,
- Centrifuge for two minutes at 2,000 RPM, followed by one minute at 14,000 RPM then, note the volume eluted from the column. If the entire 50 µl has not been eluted, spin the column at 14,000 RPM for one additional minute.
- **Note:** for maximum RNA recovery, it is recommended that a second elution be performed into a separate micro centrifuge tube and repeat last two steps again.

D. Determining the RNA concentration

Prepare a dilution of 10 µl of RNA in 490 µl of distilled water, and another of 20 µl in 480 ll of distilled water. The absorbance at 310 nm should be zero to assure the absence of light scattering which will otherwise influence further readings. Read the absorbance at 260 nm to calculate the RNA concentration in both dilutions (1 absorbance unit at 260 nm"40 mg/ml RNA). Read the absorbance at 260 and 280 nm to calculate the ratios A_{260}/A_{280} to check the purity.

3.3.4. Database search and primer design

A search in GenBank database showed three sequences of *T. aestivum* encoding for HSP101: HSP101 (GenBank Accession No. AF083344), HSP101b and HSP101c (GenBank Accession Nos. AF097363, AF174433) (figure 1).

For qRT-PCR assay, Based on the alignment of three HSP101 isforms , two sets of specific primers were designed to amplify the primers used partial 3` coding sequence of TAHSP101c, members of hsp101/ of HSP101c were designed (Table 2).

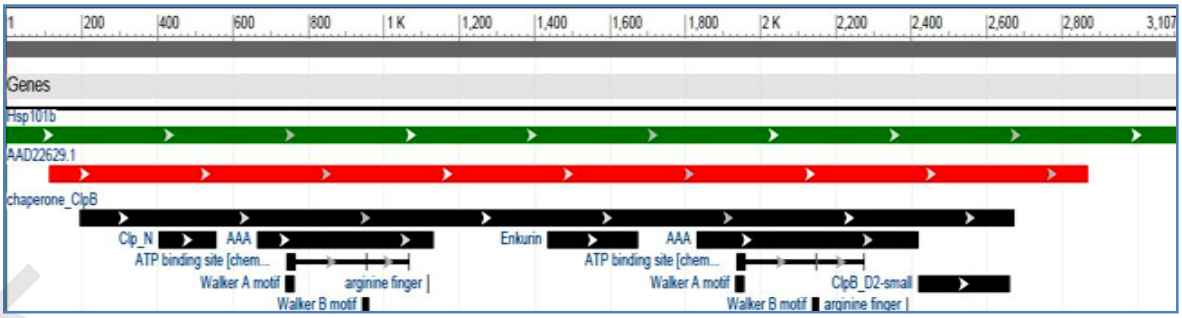
3.3.5. Real- Time PCR (RT-PCR) amplification of HSP101 gene

3.3.5.1. Principle

The Real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) has become the method of choice for the quantification of specific mRNAs. This method is fast, extremely sensitive, and accurate, requires only very small amounts of input RNA, and is relatively simple to perform.The real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) is a powerful tool for accurate quantitation of gene expression. This relative quantitation method measures changes in gene expression in test samples relative to another reference sample (calibrator), and therefore, can be used to compare RNA levels in different tissues, to monitor treatment efficacy (**Beillard E. *et al*., 2003)** .

The RQ- PCR assay utilizes a well-established three-step protocol that involves (1) a reverse transcription step that converts RNA into a complementary DNA copy (cDNA), (2) multiple rounds of amplification of the cDNA using a heat stable DNA polymerase known as *Taq* polymerase, and (3) the detection and quantification of the amplified products in real-time. (4) Real-time analysis (fluorescent measurement of total product after each cycle) of these reactions reveals a characteristic, sigmoidal curve and shows that the levels of double stranded amplicon accumulated at the end are not correlated to the number of targets present at the beginning of the reaction. In addition, exit from exponential amplification in symmetric PCR is stochastic and generates highly variable levels of double-stranded DNA among replicates. For the above reasons, symmetric PCR reactions must be analyzed in real-time to quantify the starting numbers of target molecules using measurements of the threshold cycle or CT. The CT is the cycle at which the fluorescence resulting from the accumulated product reaches a predefined threshold and is generally close to the end of exponential amplification (**Hughes T *et al*., 2006**).

i.



ii.

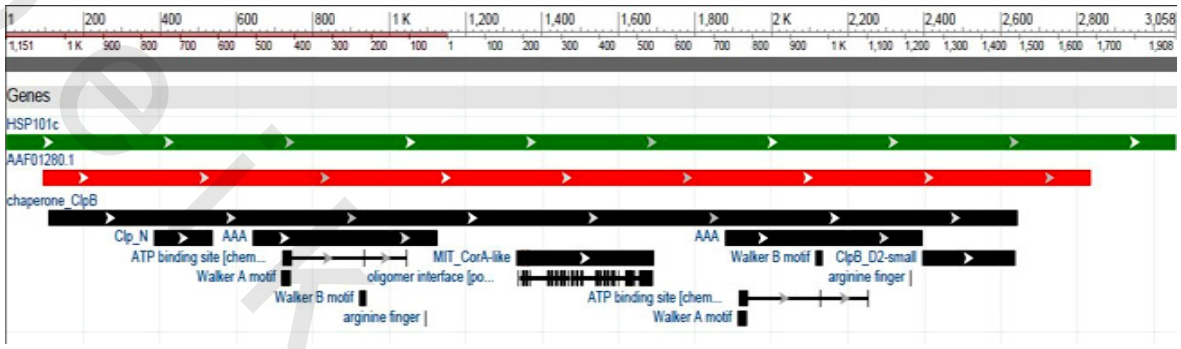


Figure (1): Graphics of *Triticum aestivum* heat shock protein 101 mRNA isoforms from GenBank database. (i) HSP101b (GenBank Accession Nos. AF097363). (ii) HSP101c (GenBank Accession Nos. AF174433).

Table (2): Primers Sequence used for the amplification TAHSP101 cDNA, Quanatitive Real-time PCR and Sequencing (Synthesized by Metabion, Germany)

Primer	Sequence	Primer case
HSP101 F1	5`-CTT CGA CGA GGT TGA GAA GG-3`	Matched
HSP101 R1	5`-CTG GAT CAG GAT GTC GGA CT-3`	
HSP101 F2	5`-CCA GCA GGT GTT CGT GGC-3`	Mismatched
HSP101 R2	5`-CTA GAG CCT TGG CGA GCT-3`	
HSP101 F3	5`- CAA GTT CAC GCA CAA GAC CA-3`	Matched
HSP101 R3	5`- CAT CGG CTT GAA CAG GTT GG-3	

β Actine were selected as internal reference gene for calculation of relative transcript levels of the genes under study. The mRNA levels of this internal reference gene were similar in the control and heat-treated samples.

3.3.5.2 Rerverse Transcrition

1. MATERIALS

1. TaqMan Reverse Transcription Reagents
2. SYBR Green PCR Master Mix (Applied Biosystems; Cat # 4309155)
3. RNase ZAP
4. PCR-tubes; 200 µl
5. RNAse and DNAse Free Water

Note: Reaction vessels MUST be DNAse and RNAse free.

2. PREPARATION

A. Decontamination

1. The workbench along with the tube racks and pipetmen should all be sprayed down with RNase ZAP before use.
2. RNases are very difficult to kill and can seriously affect the run.

B. RNA preparation

1. RNA should be thawed and stored on ice throughout the reverse transcription preparation and frozen immediately after using.
2. Repeated freeze/thaw of RNA samples causes significant degradation in RNA integrity.

3. PROCEDURE

A. Reverse Transcription (RT)

1. Thaw all reagents and store on ice.
2. Maintain an RNAse and DNAse free work environment.
3. Mix all individual reagents thoroughly and spin down.

Note: A 25-µl RT reaction efficiently converts a maximum of 4.0 µg total RNA to cDNA.

4. Master mix (for a **single 25µl reaction**):

Components	Final Concentration
4.0 µl 10X TaqMan RT Buffer	1X
2.0 µl MgCl2 (25 mM)	5.5 mM
2.0 µl dNTP Mixture	150 µM/dNTP
1.0 µl HSP101 Forward primer	33.17 nM
1.0 µl HSP101 reveries primer	46.53 nM
0.1 µl RNase Inhibitor (20 U/µl)	0.4 U/µl
0.1 µl Reverse Transcriptase (50 U/µl)	1.25 U/µl
4.0 µg Total RNA	
RNAse free Water	**to** 25 µL

Note: As with all enzymatic reactions, mix all non-enzymatic components first and then add the enzymatic components.

Note: For the reverse transcription reaction, the final volume does not have to be exact, however for sample comparison, the amount of starting material (added RNA) **MUST** be equal.

5. Combine all reagents **except RNA** and mix thoroughly and spin down.
6. Aliquot master mix into either thin wall tubes.
7. Add RNA samples to individual tubes.
8. Mix tubes and then spin down to remove any air bubbles.
9. Thermal cycling parameters:

Step	Temperature (C˚)	Time
Incubation	25˚C	10 minutes
Reverse Transcription	57˚C	30 minutes
Inactivation	95˚C	5 minutes

Note: The incubation step is necessary to maximize primer-RNA template binding.
Note: Store all cDNA samples at –15 to –25OC

3.3.5.3 Quantitative Real-time PCR amplification(for ROVALAB 2X Green dye Master Mix Kit)

1 Keep all reagents on ice during set up.

Note: It is not necessary to clean up the cDNA reactions for use in the PCR reaction.

2 Although an RNAse free environment is not necessary for the PCR of cDNA, a clean workspace is an excellent idea.

3 Mix 2X Master Mix thoroughly before use.

4. Master mix (for a **single 20 µl reaction**):

Component
10 µl 2X PCR Master Mix
1.5 µl Forward primer
1.5 µl reveries primer
RNAse free Water Up to20 µl

5. Add 18.0 µl of mix to the PCR-tubes.
6. Add 2.0 µl of cDNA to the PCR-tubes.

Note: Additional cDNA template may be added to the reaction if desired.

7. Seal PCR-tubes and mix. Spin down to remove any bubbles in the tubes.
8. Thermal Cycling Parameters:

Step	Temperature (C˚)	Time	Cycle
Initial activation	95	15 m.	1
Denaturation	94	15 sec.	45
Annealing	60	30 sec.	
Extension	72	30 sec.	

3.3.5.4. Real-time analysis

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of a co amplified internal control mRNA. During the RT-PCR assay, target Ct are compared directly to reference Ct and the results are expressed as ratios of the target-specific signal to the internal reference. This produces a corrected relative value for the target-specific mRNA product that can be compared between samples for an estimate of the relative expression of target mRNA in those samples. An evaluation of amplification efficiency is essential as this has a major influence on the accuracy of any calculated expression result. Several models have been published that correct for efficiency using various algorithms and claim to allow a more reliable estimation of the real

expression ratio. However, a crucial flaw with this approach remains in that the most common reference mRNAs are transcribed from so-called housekeeping genes whose expression is regulated and whose levels usually vary significantly with treatment or between individuals (see text below). Furthermore, if the relative levels of the housekeeping and target genes vary by orders of magnitude, the former may have entered its plateau phase by the time a Ct for the target becomes apparent. This is likely to interfere with the accurate quantiÞcation of the target mRNA **(Mackay, I. M. *et al*., 2002 ; Bustin, S. A.,2000)**.

This method involves a calculation method known as the DeltaDeltaC*T* (ΔΔC*T*) method **(Livak and Schmittgen, 2001)** and is based on a C*T* number comparison between the target gene and endogenous reference gene relative to a calibrator. The formula for this **calculation is: 2−ΔΔC*T*.**

Required calculations for this method include the following:

Calculation of the ΔC*T* for sample relative to the endogenous control and the calibrator relative to the calibrator; (2−ΔΔC*T* calculation; 2−ΔΔC*T* calculation to obtain relative quantitative value.

1) **ΔC*T*** calculations:
   ΔC*T* for test samples = average C*T* of sample − average C*T* endogenous control,
   ΔC*T* for calibrator samples = C*T* of the calibrator sample − C*T* endogenous control,
2) **ΔΔC*T*** calculation: ΔΔC*T* =ΔC*T* for test samples −ΔC*T* for calibrator samples,
3) **$2^{-\Delta\Delta CT}$** calculation > Relative quantitative value = $2^{-\Delta\Delta CT}$

3.3.6 SNP detection

Four primer pairs were designed for amplification and sequencing, all of them were designed based on the alignment of the *TaHSP101* isoforms. This region of TaHSP101c (GeneBank Acc.No. AF174433122.1) showed significant sequence identity (85% identity with 1.6% gap) with Tahsp101b (GeneBank Acc.No. AF097363) and TaHSP101 (GeneBank Acc.No. AFo83344). SNPs were automated identified using the SNPs finder software http://snpsfinder.lanl.gov **(Song, J *et al*., 2005)** and manually detection by alignment sequences by DNAMAN ® software (Lyon BioSoft, Quebec, Canada). Alignment of the deduced amino acid sequences of TdHSP101c, Gemmeiza9 HSP101 and obtained with DNAMAN® software (Lyon BioSoft, Quebec, Canada) Detection of amino acids changes by Mega 6.06 program.