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PCR technique and CT values to detection the flowering gene ZmCCT9 in maize inbreds and single hybrids

Hadi Hussein Al-Baidhani[1] and Banan Hassan Hadi[2]*
[1]Directorate of planning, Ministry of Agriculture, and [2]College of Agricultural Engineering, University of Baghdad, Iraq.

***Correspondence:**
b.h.hadi@coagri.uobaghdad.edu.iq
ORCID:
https://orcid.org/0000-0003-3995-4365
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ABSTRACT

The current study aimed to molecularly detect and determine the gene expression of the flowering gene in maize inbreds and their individual hybrids using RT-PCR technique and CT values. The correlation between flowering time and the expression of the flowering gene also studied through phenotypic data related to flowering times. RNA extracted from the seeds of the studied samples using standardized methods. The extracted RNA then converted to cDNA using the reverse transcriptase enzyme. The results indicated that inbred number 11 (ZM74) gave the highest CT cycle value of 23.63 cycles (indicating a low initial amount of mRNA, which means low gene expression). The single hybrid F1 (3×15) ZAI17WR× Zi17WZ gave the lowest CT value of 21.50 cycles (indicating a large initial amount of mRNA for the flowering gene in the hybrid, which means that the gene expression for flowering gene is high). The reason for this finding attributed to the potential differences in the gene expression of the flowering gene among different inbreds and their single hybrids. These differences caused by genetic variations in the flowering gene itself, regulatory elements such as transposons that control the gene's expression, or influences from other genetic sites. The results can also help in developing inbred lines and single hybrids with higher efficiency in flowering and grain yield by understanding the molecular mechanisms controlling the flowering process in maize.

Keywords: Maize, Genotypes, RT-PCR, Inbred lines, Single hybrids.

Introduction

The maize crop is a key food and fodder grain. It ranks third in economic importance, following wheat and rice; second globally in cultivated area after wheat; and first in production. It grown extensively around the world. The maize crop has a monoecism flowering system, and flowering begins when temperatures increase. For example, in Iraq, in the spring season, flowering time occurs in May. While in the autumn fall season, flowering occurs from August to September. Flowering is the stage where vegetative growth stops and the plants begin their reproductive growth activities. It is one of the growth factors most affected by light intensity, temperature, soil moisture, and its interaction with the crop's genetic structure (Anderson et al., 2004)

The relationship between early Silking and delayed physiological maturity (the length of time between Silking and physiological maturity) ultimately results in the longest period for grain filling, which represents a crucial and important stage in the life of the maize crop. As it is responsible for producing long ears with full and healthy grains, thus increasing the yield (Al Hadi et al., 2013 The relationship during the same period shows a

positive correlation with air relative humidity throughout the growing season. There is a negative correlation between grain yield and maximum temperatures during flowering and fertilization in the spring and autumn seasons. Flowering is a complex genetic trait controlled by several pairs of genes, interacting with environmental factors during the growing season (Aziz, 2008). The importance of the duration of Silking and its synchronization with pollen shedding in maize, referred to as synchrony, lies in its effect on increasing the rate of pollination and fertilization and consequently its impact on the number of grains formed in the ear (El-Sahookie, 2009). Through the information obtained about understanding the nature of genetic actions and determining their interactions in the transfer and inheritance of flowering traits in different flowering varieties, more than 462 gene loci have identified in quantitative traits (QTL) in maize. Some pairs of flowering genes may have other functions on quantitative traits such as plant height and total leaf number (Buckler et al., 2009), which significantly contribute to phenotypic variation and whose effect is of the additive gene action type. Xie et al. (2010) indicated that approximately sixteen QTL sites are controlling seven traits of tasseling and Silking in maize, with four QTL sites responsible for the duration between tasseling and Silking, known as the Anthesis Silking Interval (ASI).

Recently, the urgent need has emerged to identify the gene responsible for the flowering process, *ZmCCT*9Gene, which contains regulatory elements, Harbinger-like transposable elements, which reduce its gene expression under long light days. Thereby helping maize to flower across a wide range of environmental conditions, particularly temperature and photoperiod factors (Huang et al., 2017). Numerous studies have indicated that cis-acting variations typically increase the expression of the ZmCCT9 gene to promote early flowering under long-day conditions (Hung et al., 2012; Salvi et al., 2007). This is Because of natural and artificial selection and hybridization, maize varieties have developed for different thermal ranges around the world, where early and late flowering varieties have found.

DNA markers, which rely on the organism's DNA, have a significant advantage over phenotypic markers in that they are not affected by surrounding environmental conditions and instead depend on the organism's genetic material, DNA. It is used to detect the genetic material (DNA) carried by the varieties as well as to identify the genetic variations among them. Among these techniques is the RT-PCR technique, which facilitates the processes of hybridization and selection for plant breeders in the creation and development of new hybrids and varieties of maize with high grain yield and good quality traits.

It is considered the best technology scientifically for estimating the gene expression of many genes from a small number of DNA samples due to its speed, high efficiency, and low cost. The reaction output measured instantly in each cycle, and the number of mRNA copies was calculated. The working principle of the RT-PCR technology is similar to the working principle of PCR. The only difference is that instead of examining the resulting bands using ultraviolet (UV) light in traditional PCR technology. Which indicates the occurrence of the reaction and the evaluation of DNA through the Gel electrophoresis of DNA samples by the appearance of the band with the required molecular weight containing the intended studied sequence (Al-Faraji et al., 2014).

The principle of RT-PCR technology does not require the electrophoresis of the reaction products. A device contains a sensor that detects the increased fluorescence of certain dyes present in the reaction mixture. The increase in fluorescence of the dyes is a clear indication of DNA amplification and the occurrence of the repetitive reaction during the Cycle of Threshold (CT). The process monitored in real-time using a camera or fluorescent dyes such as SYBR Green Dye. The RT-PCR products are analyzed using non-sequence-specific fluorescent dyes, with Sybr Green Dye, being the most commonly used due to its low cost and ease of use. It binds to all PCR reaction products, causing fluorescence upon binding. Its function is to penetrate the base pairs (it enters the double strand of the target DNA (Kumar et al., 2009; Al-Hadeithi, 2012).

The current study aims to detect and confirm the molecular presence of the flowering gene across maize single hybrids and inbreds with different flowering times using DNA-level molecular markers. This achieved through RT-PCR technique to assess the expression levels of the *ZmCCT9 gene* flowering gene and analyze CT values to determine genetic expression levels, highlighting differences between parental inbreds and single hybrids. The findings used to link gene activity with

flowering time and genetic variation. To construct genetic maps for developing the genetic structure and expanding the crop's genetic base. Additionally, these results utilized to identify the best varieties for breeding programs with desirable genetic compositions (varieties).

Materials and Methods

The molecular experiment: The molecular experiment was conducted at the laboratories of Al-Musayyib Bridge Company for Molecular Analysis, Baghdad, Al-Jadiriyah, Iraq, in 2022, to study the nan- appearance or appearance flowering gene among five inbreds bearing codes (Zi17WZ, ZA17WR, ZM19, ZM74 and ZM49W3E) and their single hybrids bearing codes (Zi17WZ x ZA17WR, ZM19×ZM49W3E, ZM49W3E x ZM74 and ZM74 × ZM19) from different sources. With different flowering times as shown in Table (1).

Table (1): Numbers, symbols, and origin of the maize pure inbreds and single hybrids used in the study: inbreds and single hybrids numbers, code and origin

Inbreds and single hybrids number	Inbreds and single hybrids code	Inbreds and single hybrids origin
Inbred line no.3	Late Zi17WZ	Locally extracted (dr. Zaid I small Abd)
Inbred line no.15	Late ZA17WR	Locally extracted (dr. Zaid I small Abd)
Inbred line no.7	Early ZM19	Locally extracted (dr. Medhat Majid Al-Sahuki)
Inbred line no.8	Early ZM49W3E	Locally extracted (dr. Medhat Majid Al-Sahuki)
Inbred line no11	Late ZM74	Locally extracted (dr. Medhat Majid Al-Sahuki)
15 × 3 Hybrid	ZA17WR * Zi17WZ	Locally extracted (dr. Hadi AL-Baidhani)
7 ×8 Hybrid	ZM19 *ZM49W3E	Locally extracted (dr. Hadi AL-Baidhani)
11 ×8 Hybrid	ZM74 * ZM49W3E	Locally extracted (dr. Hadi AL-Baidhani)
7 ×11 Hybrid	ZM19 * ZM74	Locally extracted (dr. Hadi AL-Baidhani)

The total DNA of each inbred and single hybrid extracted using Cetyltr methyl ammonium Bromide (CTAB) as mentioned by Weigand et al. (1993). In this method, it included obtaining 200 micrograms per two grams of each mature grain. The quantity and quality of the extracted DNA and performing the appropriate dilution, the mature grains taken after the harvesting process (Maniatis etal,.2001). The ears placed in a cooling box to maintain the quality of the ears (grains) until they transported to the laboratory for DNA extraction. The given range of DNA purity values (1.7-2.6) suggests the measurement is likely the **A260/A280 ratio**, which indicates varying levels of purity, from acceptable to potential contamination with RNA or other compounds. As shown in table (2).

Table (2): Concentrations of the extracted DNA purity degree for the strains and their resulting F1 hybrids

Sample ID	Abs 260	Abs 280	260/280	Con /ng/ul sample ty
Inbred no.3	1.89	0.916	2.06	94.5 ds DNA
Inbred no.15	2.254	1.192	1.89	112.7 ds DNA
Inbred no.7	0.952	0.516	1.84	40.7 ds DNA
Inbred no.8	0.998	0.558	1.79	49.1 ds DNA
Inbred no11	1.904	1.109	1.87	50.2 ds DNA
15 × 3 Hybrid	1.254	0.635	1.97	144.4 ds DNA
7 ×8 Hybrid	1.101	0.572	1.92	73.5 ds DNA
11 ×8 Hybrid	0.708	0.412	1.72	133.9 ds DNA
7 ×11 Hybrid	0.444	0.223	1.99	221.1 ds DN

The forward and reverse primers used to detect the gene expression of flowering gene using RT-PCR technique: The forward and reverse primers for the flowering gene in this study were designed using the sequences provided by (Huang et al., 2017) from the Korean company (Korea, Bioneer) and are listed in Table (3).

Table (3): The names list of two primers used and their sequences to identify the flowering gene using RT-PCR technique

Primer name	5!~ -3!~ primer sequence	Annealing temperature	Product size
forward primer ZmCCT9 gene responsible for flowering	CTACGAGAAGCAGATTCGCTAC (22nb)	58°C	191bp
forward primer ZmCCT9 gene responsible for flowering	TGACCGACATAGGTGTTTGC (20 nb)	58°C	191 bp

Molecular detection of the flowering gene: The purpose of molecular detection of flowering gene ZmCCT9 gene nan- appearance and appearance in studied samples RT-PCR and CT values applied.

First: detection using Real Time PCR technique: A laboratory technique that represents the advanced form of traditional PCR primarily relies on the PCR reaction. Which in turn depends on amplifying the DNA molecules of the studied sample after converting them to RNA through Reverse Transcription. The principle of RT-PCR technique is similar to the principle of PCR, with the only difference being that: instead of examining the resulting bands using ultraviolet (UV) light, the process monitored in real-time using a camera or any Sybr green dye detector (Van Goilder et al., 2008).

Steps of real-time polymerase chain reaction (RT-PCR) in brief:

1- cDNA used as a template in the RT- PCR reaction, which involves using specialized primers for the flowering gene. In addition to a reference gene such as the (ZmAct gene) to standardize the results.

2- The reaction mixture contains a fluorescent dye or probes that emit a fluorescent signal proportional to the amount of amplified nucleic acid in each cycle.

3- The device monitors the fluorescent signal in real-time.

RT-PCR technique reaction: The reaction was conducted using the One-step RT-PCR system, following the steps as per the protocol provided by the South Korean company Bioneer, as outlined below:

1. Add 8 microliters of GoTaq® Probe RT-PCR Master Mix Add 8 microliters of GoTaq® Probe RT-PCR Master Mix.
2. Add 1 microliter of the forward primer for the flowering gene ZmCCT9. Add 1 microliter of the forward primer for the flowering gene ZmCCT9.
3. Add 1 microliter of the reverse primer for the flowering gene. Add 1 microliter of the reverse primer for the flowering gene.
4. Add 4 microliters of the extracted DNA from the studied samples. Add 4 microliters of DNA extracted from the studied samples.
5. Add 2.5 microliters of distilled water. Add 2.5 microliters of nuclease-free water.
6. Add 0.5 microliters of reverse transcriptase. Adding 0.5 microliters of reverse transcriptase
7. Addition of RNA Sample of 3 microliters Adding 3 microliters of RNA sample
8. All the additions mentioned above are added to an Eppendorf tube containing Sybr Green dye (origin: Korea, Bioneer) as shown in Table (9). All the additions above added to an Eppendorf tube containing Sybr Green dye (originating from Korea, Bioneer) as shown in Table (4).
9. The total volume of the reaction has become 20 microliters.
10. Then, the components collectively added to the RT-PCR reaction tubes.
11. The tubes placed on the Vortex mixer.
12. run the reaction and collect the CT values for the flowering gene

cDNA synthesis and RT-PCR reaction: The method of synthesizing complementary DNA (cDNA) as DNA template to extracted RNA samples was used with the One-Step RT-PCR System provided by Bioneer, Korea-Bioneer), Korea. This system used to convert the samples after RNA extraction to cDNA, and then the latter used in the RT-PCR reaction as template. Shown in Table (4).

Table (4): Represents the reaction conditions of the RT-PCR technique

No.	steps	temperature	time	Cycle no.
1	Reverse transcription	42°C	10 Min	1
2	Enzyme activation	95°C	5 Min	1
3	Denaturation	95°C	15 sec	40
4	Annealing	57°C	15 sec	
5	Extension	72°C	30-60Sec	
6	Final extension	72°C	5 Min	1

Reference gene: called a housekeeping gene (ZmAct gene) with stable expression levels should be amplified to normalize the expression data.

Second Data analysis using CT values for studied samples:

- The RT-PCR reaction will produce CT values for each studied sample. The CT value is the cycle number at which the fluorescence signal crosses defined threshold, with lower CT values referring higher initial amount of mRNA suggests early flowering time.
- The CT values use to determine the relative expression of ZmCCT9gene in each hybrid and single hybrid, by comparing them to the CT value of housekeeping gene and control studied samples as shown in Table (4).
- Interpretation: lower relative expression of flowering gene suggests late flowering time, while higher relative expression (indicated by lower value) suggests early flowering time. The absence of CT value or CT=0 indicates the gene is unexpressed or not present in studied sample (inbred single hybrid) By comparing, the CT values between inbreds and single hybrids to determinate their difference in flowering gene expression ZmCCT9 gene and predict flowering time for studied samples.
- The difference between studied samples (inbred versus hybrid) are compared to calculate the relative change in expression (ΔΔCT)
- The result (in the form of "fold change") expresses the extent of increase or decrease in gene expression for target gene. Flowering time in single hybrids compared to parental inbreds or another studied reference.

Using these two methodologies, it is possible to determinate the hybrids and inbreds that exhibit higher or lower expression of the target gene ZmCCT9 gene, which assists understanding the molecular mechanisms controlling the flowering time process in maize and selecting the best genetic combinations for improvement purposes.

Results and Discussion

The gene expression of the flowering gene ZmCCT9 gene for selected inbreds and single F1 hybrids of maize using the RT-PCR technique. The proposed primers were effective in showing the results in light of our observation that the data provided by the technology for CT values. To verify the accuracy of the data provided by the technology. we must check the samples that yielded positive results (positive samples) which excelled in giving a lower cycle threshold value, as well as showing a curve (in a phased manner) resembling the letter S in English. The flowering gene amplified using the RT-PCR technique to study gene expression as well as, the effect of backcrossing on the expression of the flowering trait in the selected inbreds and single hybrids. It is clear that there is a direct and inverse relationship between the CT values and the flowering gene expression

The results of the relative gene expression analysis and CT threshold values obtained from the RT-PCR reaction, the forward and reverse primers with the flowering gene ZmCCT9. DNA samples of the late-flowering inbreds (Zi17WZ inbred and ZA17WR inbred and single hybrid F1 (Zi17WZ × ZA17WR)) for the maize crop: The results of the relative gene expression analysis and the CT threshold values resulting from the RT-PCR interaction of the forward and reverse primers with the flowering gene ZMCCT9. DNA samples of the late-flowering inbreds (Zi17WZ and ZA17WR) and single hybrid F1 (Zi17WZ × ZA17WR)). From the results of Table (4) and as shown in Figure (1), in light of comparing the cycle threshold (CT) values of the late×late hybrid F1 (Zi17WZ × ZA17WR), the threshold value is 21.50 cycles. The curve at the bottom) and the late-flowering strain Zi17WZ reached a (CT) value of 21.58 cycles (the middle curve), while the second late-flowering strain ZA17WR reached 21.72 (the top curve with the highest (CT) value).

The single hybrid bearing code ZA17WR (15) × Zi17WZ (3) = 21.50 cycles, the lowest curve indicating the highest gene expression. Inbred bearing code (Zi17WZ (3) = 21.58 Middle curve cycle, an intermediate state of gene expression for the gene inbred bearing code ZA17WR (15) = 21.72 upper curve cycle, first curve value, lowest gene expression of the gene.

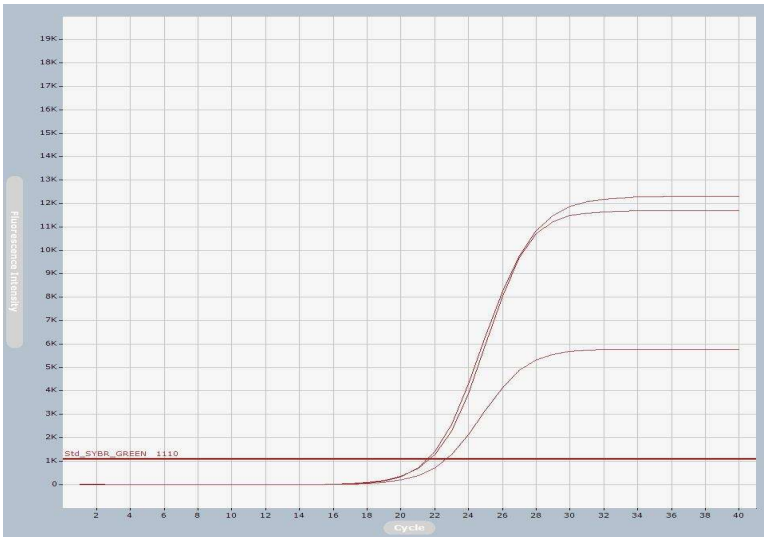

Figure (1): Analysis of RT-PCR technique results for DNA samples amplified using PCR technique with forward and reverse primers along with the flowering gene. The two late-flowering inbreds, Zi17WZ and ZA17WR, and single hybrid F1 (Zi17WZ × ZA17WR), shows differences in CT values for the selected inbreds and single hybrids.

Table (4): The data obtained from the RT-PCR reaction for the forward and reverse primers for the CT cycle threshold values to calculate the gene expression of the flowering gene.

Well	Sample name	Probe name	Type	Flu. Dye	Quencher	CT	Concentration	CT Threshold	Start Baseline	End Baseline	Gene type
A1	Inbred 3	Std _Syber _Green	Sample	Syber _Green	None	21.58	0.00E+00	1.110	Auto	Auto	ZMCCT9
A2	Inbred 15	Std _Syber _Green	Sample	Syber _Green	None	21.72	0.00E+00	1.110	Auto	Auto	ZMCCT9
A3	Hybrid 3*15	Std _Syber _Green	Sample	Syber _Green	None	21.50	0.00E+00	1.110	Auto	Auto	ZMCCT9
A4	Inbred 7	Std _Syber _Green	Sample	Syber _Green	None	22.99	0.00E+00	1.110	Auto	Auto	ZMCCT9
A5	Inbred 8	Std _Syber _Green	Sample	Syber _Green	None	22.32	0.00E+00	1.110	Auto	Auto	ZMCCT9
A6	Hybrid 7*8	Std _Syber _Green	Sample	Syber _Green	None	22.69	0.00E+00	1.110	Auto	Auto	ZMCCT9
A7	Inbred 11	Std _Syber _Green	Sample	Syber _Green	None	23.65	0.00E+00	1.110	Auto	Auto	ZMCCT9
A8	Hybrid 7*11	Std _Syber _Green	Sample	Syber _Green	None	23.05	0.00E+00	1.110	Auto	Auto	ZMCCT9
A9	Hybrid 8*11	Std _Syber _Green	Sample	Syber _Green	None	Undetermined	-	1.110	Auto	Auto	ZMCCT9

Results of the relative gene expression analysis and CT threshold values resulting from the RT-PCR interaction of the forward and reverse primers with the flowering gene. DNA samples of the two early flowering inbreds: (ZM19 and ZM49W3E) and single hybrid F1 (ZM19× ZM49W3E)) for maize crop: The results of the relative gene expression analysis and CT threshold values resulting from the RT-PCR interaction of the forward and reverse primers with the flowering gene ZMCCT9. DNA samples of the early flowering inbreds (ZM19 inbred and ZM49W3E inbred and single hybrid F1 (ZM19×ZM49W3E)). The results from Table (4) and as shown in Figure (2), in light of the comparison of the cycle threshold (CT) values for the inbred with the lowest value ZM49W3E, which reached 22.32 cycles: (the lowest curve at the bottom). The early × early hybrid F1 (ZM19×ZM49W3E) with a threshold value of 22.69 cycles (the middle curve). The early flowering inbred ZM19 reached the highest (CT) value of 22.99 cycles (the first curve). The cycle threshold values were close, indicating no difference in the relative gene expression of the flowering gene. Inbred bearing code ZM49W3E (8) = 22.32 cycles, the lowest curve at the bottom, the highest expression of the gene. Single hybrid bearing code (8) ZM49W3E× (7) ZM19 = 22.69 Middle curve cycle, an intermediate state of gene expression for the gene inbred bearing code ZM19 (7) = 22.99, the upper curve cycle, the first curve value, the lowest gene expression of the gene.

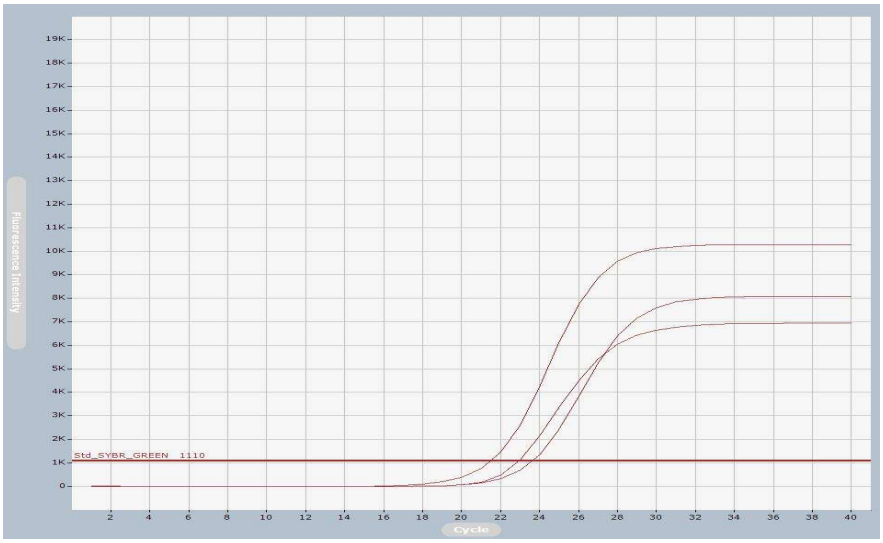

Figure (2). Analysis of the RT-PCR technique results for DNA samples amplified using PCR technology with forward and reverse primers and the flowering gene. The two early flowering inbreds, inbred ZM49W3E and inbred ZM19, and single hybrid F1 (ZM19×ZM49W3E), shows differences in CT values for the selected inbreds and single hybrids.

Results of the relative gene expression analysis and CT threshold values resulting from the RT-PCR interaction of the forward and reverse primers with the flowering gene. DNA samples of the late flowering inbred Zm74 and the single hybrid late×early F1 (ZM74×ZM19) and single hybrid early×late F1 (ZM49W3E×ZM74) for maize crop: The results of the relative gene expression analysis and the CT threshold values resulting from the RT-PCR interaction of the forward and reverse primers with the flowering gene ZMCCT9gene. The DNA samples of the late flowering inbred (ZM74) and the single hybrid late×early F1 (ZM74×ZM19) and the single hybrid early×late F1 (ZM49W3E×ZM74) are shown in Table (4) and as illustrated in Figure (3). In light of comparing the cycle threshold (CT) values, the hybrid with the lowest value F1 (ZM74×ZM19) reached 23.05 cycles (the lower curve). While the late flowering inbred ZM74 reached the highest (CT) value of 23.65 cycles (the first curve). The single hybrid early × late F1 (ZM49W3E×ZM74) had a (CT) value of Undetermined. The reason is that no mRNA came to convert it to cDNA, and thus the PCR reaction did not start, resulting in no CT value. Therefore, it considered a fingerprint for this hybrid to research in the future.

The single hybrid bearing code ZM19 (7) × (11) ZM74 = 23.05 cycle of the curve, the lower the cycle, the higher the gene expression. Inbred bearing code ZM74 (11) = 23.65, the first curve cycle with the

highest value, the least expression of the gene. Single hybrid bearing code (11) ZM74× (8) ZM49W3E = Undetermined, the curve is not present, CT value is not present.

The variation in flowering gene expression in subsequent generations inherited across generations. On the other hand, it has been interpreted based on changes in the chromatin structure, which in turn affects materials present within the genome, enabling them to exhibit several significant and impactful changes in phenotypic and quantitative traits, such as variations in the rates of traits related to yield strength and growth rate (Chen, 2013). Additionally, some researchers have suggested that the phenomenon of epigenetics, associated with DNA methylation and siRNA, plays a crucial role in revealing differences in gene activity and morphological forms related to hybrid traits, which in turn are linked to hybrid vigor and hybrid yield (Greaves et al., 2012).

Further studies conduct further studies to determine the relationship between the expression of the ZmCCT9 gene and phonological and agricultural characteristics of maize. Application of molecular marker associated with ZmCCT9 gene in breeding programs to improve selection efficiency. The study will contribute to the advancement of scientific research in the field of plant molecular biology and its applications in crop improvement.

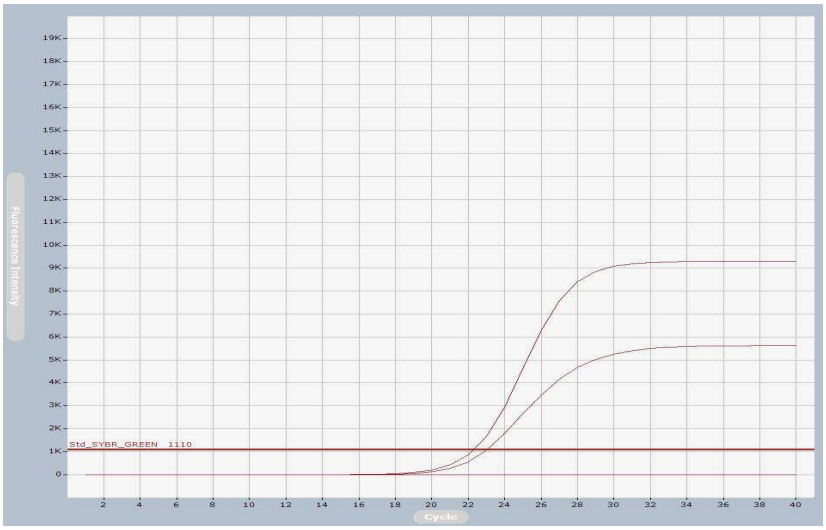

Figure (3). Analysis of RT-PCR technique results for DNA samples amplified using PCR with forward and reverse primers along with the flowering gene. The late flowering inbred (ZM74) and hybrid F1 (ZM74×ZM19) and hybrid F1 (ZM49W3E×ZM74) shows differences in CT values for the selected strains and their resulting hybrids.

Conclusion

There is a significant increase in the cycle threshold (CT) value for the 7ZM19 inbred, which recorded a CT value of 22.94 cycles compared to the single hybrid 7×11 F1 (ZM74×ZM19), which recorded a CT value of 19.10 cycles (the lowest CT value). The increase in the CT value explains the decrease in gene expression in the inbred. Due to the presence of other genes associated with the flowering gene ZmCCT9 gene, which affected the reduction in its gene expression level, or because the inbred with the high CT possesses transcription factors that reduce the mRNA copies in the inbred, and thus its gene expression. On the other hand, regarding the single hybrid early × late F1 (11×8) (ZM49W3E×(ZM74), this indicates that no mRNA was available to convert to cDNA, and thus let to the PCR reaction did not start, resulting in no CT value. Therefore, it considered a fingerprint for this hybrid to investigate in the future.

This study will contribute to understanding the role of the ZmCCT9 gene in the flowering and plant development of maize, and can provide valuable information for improving breeding programs using molecular marker. The finding can also assist in developing maize varieties and hybrids with higher flowering and productivity efficiency. Application of molecular marker associated with ZmCCT9 gene in breeding programs to improve selection efficiency. The study will contribute to the advancement of scientific research in the field of plant molecular biology and its applications in crop improvement.

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