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In silico primer designing of WRKY transcription factors in cumin (*Cuminum cyminum* L.) for wilt resistance from available transcriptome database

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Abstract
The yield of cumin (*Cuminum cyminum* L.) is affected by lack of superior varieties, scientific crop production technology and vulnerability to various biotic and abiotic stresses during its life cycle. Defence mechanism allows plant to tackle various stresses. Transcription factors (TFs) are at the center of the complex system controlling cellular growth, differentiation, genetic response to the environment, organism development, evolution and response to various biotic and abiotic stresses. Out of total six primers, four primers namely *EF1-α, UBQ_10, ACT-11 and Tip41* showed amplification of the endogenous genes in GC-4 and GC-2 varieties c-DNA template were selected and utilized for validation of endogenous genes through qRT-PCR. Total 33 primer pairs of WRKY transcription factors related genes were screened with DNA of both varieties and 10 primer pairs of WRKY transcription factors related genes were selected and screened with c-DNA sample. After WRKY transcription factors related primers screening with 24 hours shoot tissue c-DNA sample of GC-4 variety, WRKY14_2, WRKY25_1, WRKY27_2 and WRKY58_2 primers were selected for gene expression study through real time PCR.

Keywords: Cumin, transcription factors, WRKY

Introduction
Cumin (*Cuminum cyminum* L.) with chromosome number 2n=2x=14 is a small annual herbaceous plant that is a member of the aromatic plant family, Apiaceae. It has significant demand as a spice, all over the globe, especially in the places where spicy food is preferred. Its seeds have been commonly used for culinary and flavouring purposes due to strong and aromatic flavour and also in ethanomedical therapy since ancient times in various countries. The yield of cumin is affected by lack of superior varieties, scientific crop production technology and vulnerability to diseases like wilt, blight and powdery mildew incited by *Fusarium oxysporum* f. sp. *cumini*, *Alternaria burnsii* and *Erysiphe polygoni*, respectively. In these diseases, wilt is most common, results in yield losses up to 35% in cumin. *Fusarium oxysporum* is a causative agent of wilt disease in a wide range of economically important crops. To cope with variable biotic and abiotic stress, organisms have evolved a great capacity to extensively reprogram the transcriptome in a highly dynamic and temporal manner through an integrated network of transcription factors. Transcription factors (TFs) are at the center of the complex system controlling cellular growth, differentiation, genetic response to the environment, organismal development, evolution and response to various biotic and abiotic stresses. The amount and diversity of transcription factors directly correlates with complexity of organism (Mendoza *et al.* 2013) [6]. Currently 320370 transcription factors have been identified from 165 species, presenting a more comprehensive genomic transcription factors repertoires of green plants. (Jin *et al.* 2017) [5].

WRKY transcription factors are one of the largest families of transcriptional regulators in plants and form integral parts of signalling webs that modulate many plant processes. It is defined by presence of conserved WRKY amino acid sequence at N-terminus of the protein and zinc finger structure at C-terminus (Ruston *et al.* 2010) [7]. Phylogenetic analysis revealed that the WRKY transcriptional factor family has expanded to great extent in higher plants. WRKY proteins can activate or repress transcription and rich in potential transcriptional activation and repression domains. They play roles in regulation of seed development, seed dormancy, seed germination and senescence. They play roles in several different plant processes such as transcriptional reprogramming associated with plant immune response (Biotic stress) and regulation of many abiotic stresses. Regulation of gene expression is one of the most complex activities in the cell because it involves integration of signal transduction pathways, movement of protein between different cellular compartments, alteration of chromosome structure, RNA synthesis and processing.

To understand plant growth and development, a detailed knowledge of mechanism at transcription is required. The present study "*In silico* primer designing of WRKY transcription factors from available transcriptome database" was carried out to have insights into various reactions underlining the successful establishment of resistance to wilt in the cumin and helps in the identification of WRKY transcription factors related genes in cumin for wilt resistance.

Materials and Methods

The present investigation was carried out at Department of Agricultural Biotechnology, Anand Agricultural University, Anand during 2016-2018. Two varieties of cumin were used for the present study (Table 1). The seeds of cumin varieties were procured from Seed Spices Research Station, Sardar Krushinagar Dantiwada Agricultural University, Jagudan (Dist.: Mehsana), Gujarat (Fig 1).

Table 1: List of cumin varieties used in present study

No.	Cumin varieties	Characteristics
1	GC – 2	Wilt susceptible
2	GC – 4	Wilt resistant

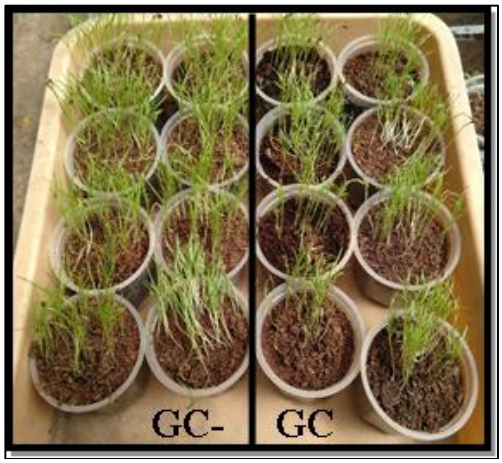

Fig. 1: Image of cumin resistant variety GC-4 and susceptible variety GC-2.

Genomic DNA Extraction from Cumin var. GC-2 and GC-4

DNA was extracted from shoots tissue using Cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1990).

Qualitative and quantitative assessment of Genomic DNA

To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA, spectrophotometry was performed. One microliter of DNA sample was loaded on to the well of Nano drop instrument. The concentration of DNA and absorbance at 260 nm and 280 nm were measured, and the data were analyzed using software Nano Drop (V.3.3.0).

Total RNA isolation from cumin seedlings var. GC-2 and GC-4

A protocol of phenol chloroform extraction method (Ghawana *et al*. 2011) [4] for RNA isolation was performed with the following requirements represented in table 3.4 and 3.5. Cumin seedlings were grown and infected with fungal inoculums (1 x 10^5 spores/ml) of *F. oxysporum* f.sp. *cumini*, roots and shoots were collected post infection of at 24, 72 and 120 hours stage.

Qualitative and quantitative assessment of total RNA

To estimate the quantity and quality (in terms of protein and DNA contamination) of isolated RNA, spectrophotometry was performed and the data was analyzed using software Nano Drop (V.3.3.0).

C-DNA Synthesis

The first strand of c-DNA was synthesized using iScript™ c-DNA synthesis kit of Bio-Rad Company for both the varieties from total RNA. c-DNA synthesis was carried out in duplicate for every RNA samples that passed quality controls**.**

In silico primer designing

Primer designing is crucial step for real-time PCR gene expression study. Primers were designed with Primer Blast (NCBI) and oligo dt software tools. The primes were also screen for hairpins, dimmer formation and target specificity. The selection of primers were based on WRKY related genes of differentially expressed transcripts/genes in defense related pathways using Primer Blast (NCBI) online software and their validation were carried out using quantitative Real Time PCR. The primers were designed according to the parameters given in the table 2 (The rest of the parameters were used as default). The IDT oligo Analyzer 3.1, an online tool was used to check the quality parameters to avoid the formation of secondary structures such as hairpin, self-dimer, heterodimer etc. IDT oligo Analyzer 3.1 analysis, less than 3 mismatches and ΔG value -9 was allowed. The best primers were selected after analyzing using IDT oligo Analyzer 3.1 and synthesized from Eurofins Genomics India Pvt. Ltd., Bangalore, Karnataka, India.

Table 2: Parameters used for primers designing for Real time PCR

S. No.	Parameters	Range	Optimum
1	Melting temperature (°C)	58-62	60
2	Primer length (nucleotides)	18-22	20
3	Amplicon size (bp)	100-200	150
4	5' complementary	1-8	5
5	3' complementary	0-3	0
6	GC content (%)	45-55	50

Primers related to WRKY transcription factor from cumin were designed by following procedure.

1. The first step is identification of WRKY transcription factor included selection of WRKY protein related sequence from Plant TFBD V.4.0.
2. The prediction of WRKY transcription factors were done through sequence similarity search in transcriptome assembly using tB LASTN. Transcriptome data of cumin were available from previous study.
3. Only non-redundant sequence were selected and translated to protein. Protein sequences were annotated by identifying conserved domain search. Only the sequence containing conserved domain is utilized for in silico primer designing.
4. The primers were designed from differentially expressed transcripts/genes (in fasta format) in GC-4 variety in defense related pathways using Primer Blast (NCBI) nline software and analyzed in Oligo Analyzer 3.1 IDT-Integrated DNA Technologies online software.
5. Validation of primers were carried out using quantitative Real Time PCR.

Screening of endogenous genes and WRKY transcription factors related primers

Using normal PCR, the specificity of primers was checked in terms of annealing temperature, primer dimer and product size using DNA and c-DNA as a template (Table 3)

Table 3: Screening PCR conditions

S. No.	Step	Temperature (ºC)	Duration
1	Initial Denaturation	94	5 minutes
2	Denaturation	94	1 minutes
3	Annealing	60	45 seconds
4	Extension	72	1 minutes
5	Final extension	72	7 minutes
6	Hold	4	∞

Electrophoresis

The PCR products were run on 1.5% (1.5 gm agarose in 100 ml 1X TBE) agarose gel containing 3 µl of ethidium bromide (10 mg/ml). PCR product of 6 µl was mixed with 1 µl of 6x gel loading dye and loaded onto the well. The 50 bp standard DNA ladder (1 µl) was also run along with the samples. The gel was run at 90 V current (constant) to separate the amplified bands approximately for 2 hours. The separated bands were visualized under U.V. trans-illuminator (Biometra, Germany) and photograph was taken using gel documentation system (Bio-Rad, California).

Results and Discussion

The experimental materials, cumin seeds were procured from the Seed Spices Research Station, Sardar Krushinagar Dantiwada Agricultural University, Jagudan (Dist.: Mehsana), Gujarat. The experiments were carried out at Department of Agricultural Biotechnology, Anand Agricultural University, Anand during 2016-2018. The results and its interpretations are discussed as below:

Qualitative and Quantitative Analysis of isolated DNA from Cumin Varieties

DNA was isolated for screening of WRKY transcription factors related primers through PCR. The quantity of DNA extracted was 1860.5 for GC-2 and 2111.1 ng/µl for GC-4 variety. $A_{260/A280}$ ratio of DNA extracted from cumin was 2.03 for GC-2 variety and 2.00 for GC-4 variety. (Fig 2)

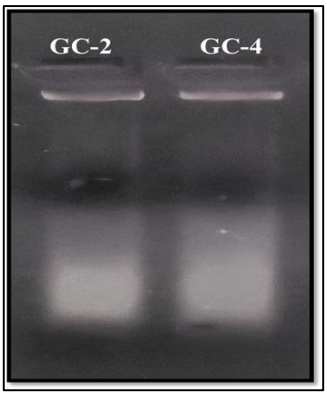

Fig 2: DNA sample of shoot tissue of cumin variety GC-2 and GC-4 on 0.8%

RNA isolation

The most important consideration in generating useful data with real time RT-PCR is the quality of the isolated RNA (Farrell, 1908). Different protocols are available for RNA isolation from various tissues. A protocol of phenol chloroform extraction for RNA isolation was used in the present experiment. All steps of RNA isolation were carried out in RNase free environment and different equipments were used for isolation. Treatment with chloroform given to remove RNase contamination. Highly purified RNA was also degraded spontaneously in solution. So, RNA was stabilized by the addition of RNase inhibitors (*e.g*., RNasin, diethylpyrocarbonate) and stored at -80°C (Fig 3).

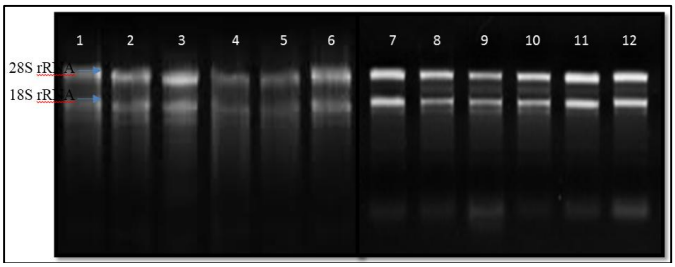

Fig. 3: RNA sample of 24, 72 and 120 hours stage in root and shoot tissue of GC-2 and GC-4 variety of cumin on 1.5% agarose gel.

GC-2: 1: (24 hours root); 2: (72 hours root); 3: (120 hours root); 7: (24 hours shoot); 8: (72 hours shoot); 9: (120 hours shoot)
GC-4: 4: (24 hours root); 5: (72 hours root); 6: (120 hours root); 10: (24 hours shoot); 11: (72 hours shoot); 12: (120 hours shoot)

Qualitative and Quantitative Analysis of Total RNA from Cumin Varieties

In present investigation, the quality, quantity and integrity of total RNA extracted from cumin was checked through nano drop spectrophotometer. The quantity of total RNA extracted was in the range of 216.59 – 2716.12 ng/µl. 24, 72 and 120 days shoot sample of resistant variety (GC-4) and susceptible variety (GC-2) had good amount of RNA (1500 ng/µl) and root sample of resistant (GC-4) and susceptible (GC-2) varieties had low quantity of RNA (300 ng/µl)

Pure preparation of RNA have an $A_{260/A280}$ ratio of between 2.0-2.2 and $A_{260/A230}$ ratio between 2.0-2.3. The quality of total RNA extracted ($A_{260/280}$ and $A_{260/230}$ ratio) ranged from 1.99-2.25 and 1.97- 2.14 respectively as shown in table 4, which is in agreement with the normal purity standards of RNA as described by Chomczynski and Mackey, (1995) [1]. Purity, quality and quantity of isolated RNA were satisfactory for c-DNA synthesis.

Table 4: Quantitative and qualitative analysis of total RNA samples of cumin

Sample Name	Stage	RNA (ng/µl)	A260/280	A260/230
GC-4 (shoot)	24 hours	2193.5	2.09	2.11
	72 hours	2716.0	2.02	2.14
	120 hours	1007.21	2.15	2.2
GC-4 (root)	24 hours	216.59	2.09	1.99
	72 hours	352.57	2.01	1.98
	120 hours	216.59	1.99	2.00
GC-2 (shoot)	24 hours	2307.50	2.12	2.05
	72 hours	1179.53	2.18	1.97
	120 hours	1046.23	2.25	2.13
GC-2 (root)	24 hours	368.20	2.02	2.07
	72 hours	267.28	1.99	2.11
	120 hours	772.46	2.04	1.97

C-DNA Synthesis: c-DNA concentration is not estimated through nano drop spectrophotometer due to leftover dNTPs, leftover RNA which fluctuate the values of nano drop. For optimization of the c-DNA concentration usually same amount of RNA was taken from all the samples for c-DNA synthesis from 2 µg of total RNA of all the samples through

iScript c-DNA synthesis kit (Bio Rad). Oligo-(dT) primers and reverse transcriptase enzyme are key component to obtain c-DNA. The synthesized c-DNA was used for primer screening study through qRT-PCR and qRT-PCR for endogenous genes and WRKY transcription factors related genes expression study.

Screening of endogenous genes and WRKY transcription factors related primers

Six endogenous gene specific primers namely Tubulin ß2, ACT-11, Tip41, UBQ_10, PP2A and EF1-α were used for screening through PCR. Template c-DNA of 24, 72 and 120 hours stage of shoot tissue of GC-4 and GC-2 varieties were utilized for screening of endogenous genes. PCR reaction was set at specific annealing temperature (T_A) of individual primer. Total 40 cycles were repeated in PCR. Gel electrophoresis was carried out for screening.

Out of total six primers, four primers showed amplification of the endogenous genes in GC-4 and GC-2 varieties c-DNA template of 24, 72 and 120 hours shoot tissue. ACT-11, UBQ_10, EF1-a and Tip41 generated single amplicon size, as expected size, as shown by the presence of single band in the samples through 1.5% gel electrophoresis as shown in fig. 4, 5, 6 and 7. Tubulin ß2 and PP2A did not amplified in all the samples and hence not selected for further downstream application.

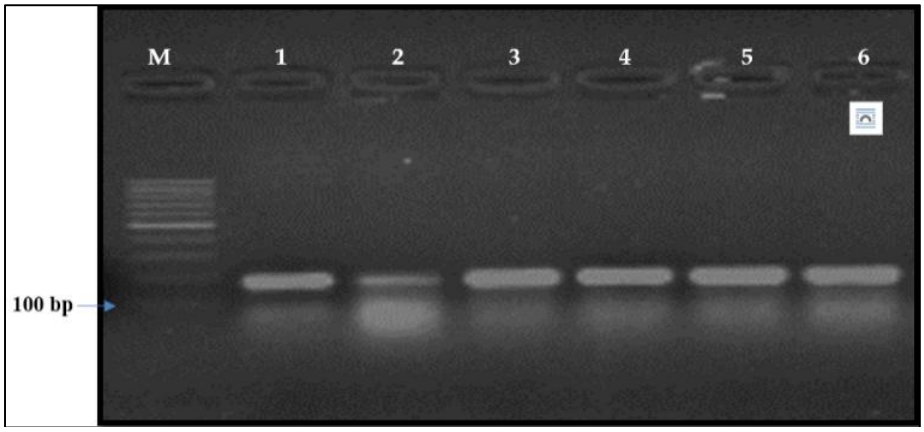

Fig 4: Banding pattern of *ACT-11* at 24, 72 and 120 hours stage having shoot tissue of GC-2 and GC-4 cumin varieties on 1.5% agarose gel

GC-2: 1: 24 hours shoot; 2: 72 hours shoot; 3: 120 hours shoot
GC-4: 4: 24 hours shoot; 5: 72 hours shoot; 6: 120 hours shoot

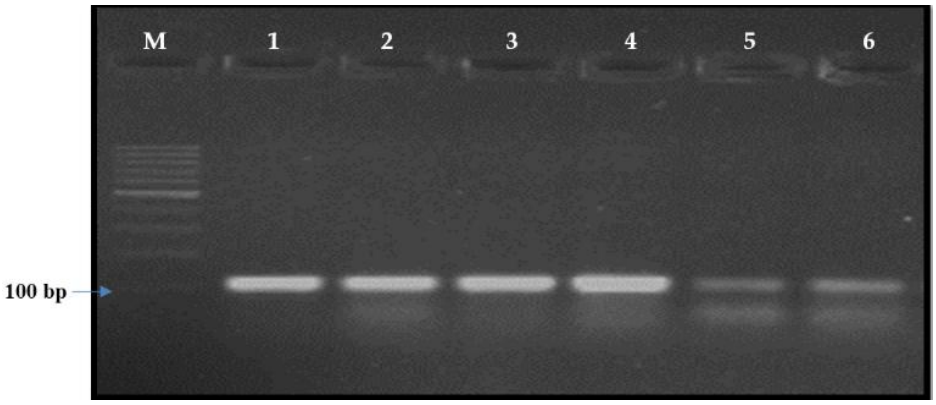

Fig 5: Banding pattern of *UBQ_10* at 24, 72 and 120 hours stage having shoot tissue of GC-2 and GC-4 cumin varieties on 1.5% agarose gel

GC-2: 1: 24 hours shoot; 2: 72 hours shoot; 3: 120 hours shoot
GC-4: 4: 24 hours shoot; 5: 72 hours shoot; 6: 120 hours shoot

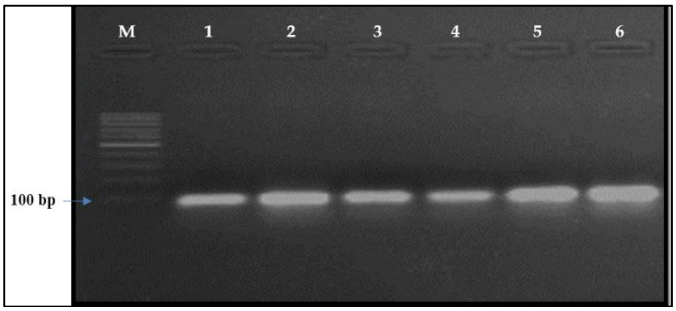

Fig. 6: Banding pattern of *EF1-α* at 24, 72 and 120 hours stage having shoot tissue of GC-2 and GC-4 cumin varieties on 1.5% agarose gel

GC-2: 1: 24 hours shoot; 2: 72 hours shoot; 3: 120 hours shoot
GC-4: 4: 24 hours shoot; 5: 72 hours shoot; 6: 120 hours shoot

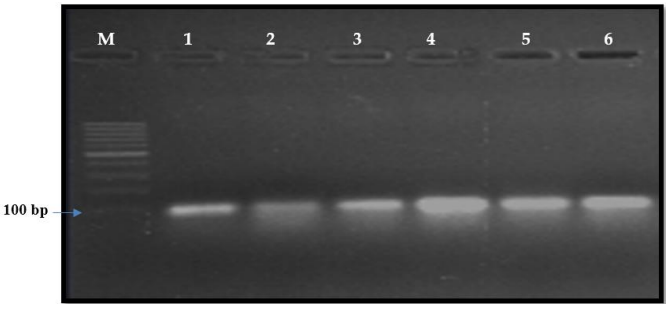

Fig. 7: Banding pattern of *Tip41* at 24, 72 and 120 hours stage having shoot tissue of GC-2 and GC-4 cumin varieties on 1.5% agarose gel

GC-2: 1: 24 hours shoot; 2: 72 hours shoot; 3: 120 hours shoot
GC-4: 4: 24 hours shoot; 5: 72 hours shoot; 6: 120 hours shoot

Table 5: List of endogenous primers

S. No.	Primer name	Primers sequences (5'- 3')		Amplicon Size (bp)
1	*ACT-11*	F	GTGGTTGCTCCACCAGAGAG	99
		R	CTCGGCCTTTGCTATCCACA	
2	*UBQ_10*	F	TCTCCGACTCCGTGGTGGTATG	108
		R	CTGCCGTCCTCCAACTGCTTAC	
3	*EF1-α*	F	TCAAGGATCTCAAGCGTGGTTATGT	115
		R	CAGCAATGTGGCAAGTGTGACAAT	
4	*Tip41*	F	GGAGGACTGTGAGGAACGAATTGAT	106
		R	ACGCAAGAGAAGGAACCAACAACT	

Total 33 primer pairs of WRKY transcription factors related genes were screened with DNA of GC-2 and GC-4 variety as shown in fig.8, 9. After screening with DNA, 10 primer pairs of WRKY transcription factors related genes were selected and screened with c-DNA sample of 24 hours stage shoot tissue of GC-4 variety through PCR at 59.5°C, 60.0°C and 60.4°C temperature as shown in fig. 4.6 and 4.7. Total 40 cycles were repeated in PCR. Gel electrophoresis was carried out for screening. Primer pairs that showed primer dimmer formation were rejected for downstream application.

After WRKY transcription factors related primers screening with 24 hours shoot tissue c-DNA sample of GC-4 variety, WRKY14_2, WRKY25_1, WRKY27_2 and WRKY58_2 primers were selected for gene expression study through real time PCR.

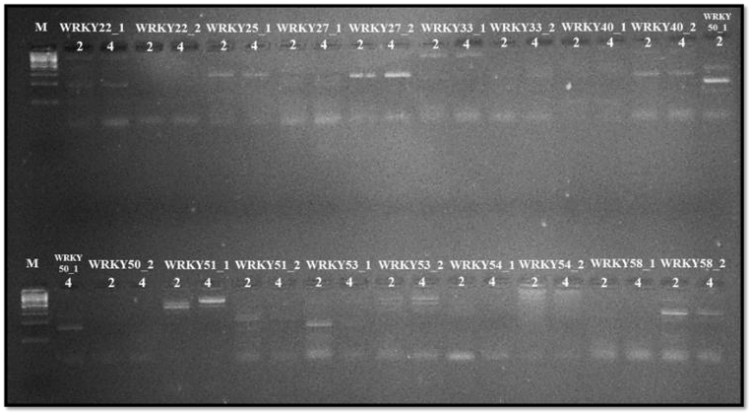

Fig 8: Screening of WRKY transcription factors related primers with DNA of shoot tissue of GC-2 and GC-4 variety of cumin on 1.5% agarose gel. 2: GC-2**;** 4: GC-4

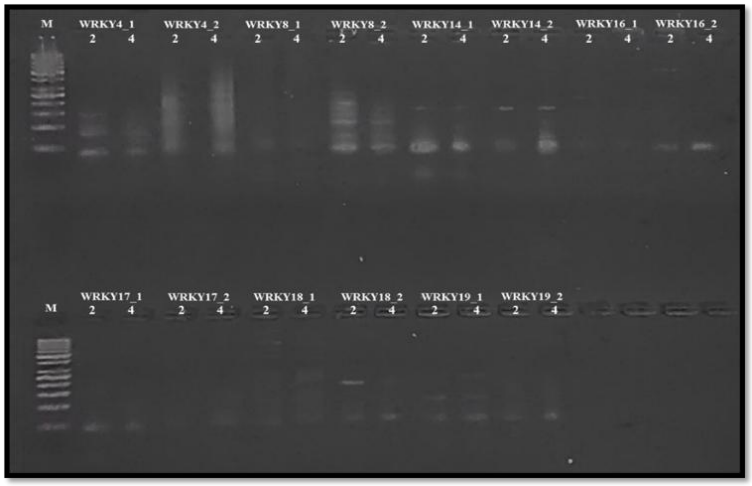

Fig 9: Screening of WRKY transcription factors related primers with DNA of shoot tissue of GC-2 and GC-4 variety of cumin on 1.5% agarose gel. 2: GC-2; 4: GC-4;

Conclusions

The present investigation entitled "*In silico* primer designing of WRKY transcription factors in cumin (*Cuminum cyminum* L.) for wilt resistance from available transcriptome database." was carried out by DNA isolation, RNA isolation, c-DNA synthesis, *in silico* primer designing, endogenous gene validation and target genes expression study trough qRT-PCR. The experimental material comprised of two cumin varieties, GC-4 (resistant to wilt) and GC-2 (susceptible to wilt). The study on DNA isolation revealed that the quantity of DNA was 1860.5 ng/µl in GC-2 variety and 2111.1 ng/µl in GC-4 variety. The study on RNA isolation revealed that the concentration of RNA was in the range of 367.54-3000.2 ng/µl with the purity level within the range of 1.97 to 2.25. Out of six of endogenous primers, four primers which produced a single intact band in both the varieties were selected. The selected primers of *EF1-α, UBQ_10, ACT-11 and Tip41* were utilized for validation of endogenous genes

through qRT-PCR. Total 33 primer pairs of WRKY transcription factor related genes were designed using primer BLAST. Four primer pairs of WRKY transcription factors related genes, WRKY14_2, WRKY25_1, WRKY27_2 and WRKY58_2 were identified and were used to study gene expression in cumin for wilt resistance.

Appendix 1: List of designed primers for cumin WRKY transcription factors

S. No.	Primer Name	Primer Sequence (5'-3')	Product size
1	WRKY4_1F	CCGTACCGCCTGGTTTAAGT	92
2	WRKY4_1R	TTGGCATTGGCTTGAACTGC	
3	WRKY4_2F	GCGTTGGATTCAGTCCTGGT	92
4	WRKY4_2R	TCCAGCGAGAAGCTGAGAGA	
5	WRKY8_1F	GTTGTCGGTGATGGTTGTGC	137
6	WRKY8_1R	CCGGATCTTGCCGGAATCTT	
7	WRKY8_2F	GTTCGCGATGGAGGAGAAGA	137
8	WRKY8_2R	CAACGATAGCCGTCTTCGAG	
9	WRKY14_1F	CCACTCCTCCTCCTACCCTC	102
10	WRKY14_1R	CTCCGGTCTGTATGGAGCAA	
11	WRKY14_2F	CCAAACCCTCAACCGCAAAC	102
12	WRKY14_2R	GAGTGGTGGAGGAAGGCAAG	
13	WRKY16_1F	GAGTCGTGACAGTCAGCAGG	108
14	WRKY16_1R	CCCACAAGTGTGTTCCTCCA	
15	WRKY16_2F	GCAGTCCTCTGGTTGTGGAG	108
16	WRKY16_2R	CTCGACAAAGGCGAAACACC	
17	WRKY17_1F	TCCTCTCTAACCGTCCCGAA	130
18	WRKY17_1R	AACCGGACCACGTCTAAACC	
19	WRKY17_2F	AAAGTGACAACTCCGGCTCC	130
20	WRKY17_2R	CCGAGCTCTTGGTTTTAGCG	
21	WRKY18_1F	GAGAACAGCTCCAGCAACGA	148
22	WRKY18_1R	CCGATGTTTCAGTAGGCACG	
23	WRKY18_2F	TCGACTGTCTACGTGCCTAC	148
24	WRKY18_2R	CTGGACAAGACGGTGCAAAC	
25	WRKY19_1F	GCACAAAAGGAGCGAAAGGG	82
26	WRKY19_1R	CACCTGTTGTTACCCCTCCC	
27	WRKY19_2F	GGTCTTTCGGGACTCTGCAT	82
28	WRKY19_2R	TGCTCCCTTTCGCTCCTTTT	
29	WRKY22_1F	AGCGTCACTAGCAAACCCAG	118
30	WRKY22_1R	TTCTGTCCGTACTTTCGCCA	
31	WRKY22_2F	CACTTACACGGCGGAGCATA	118
32	WRKY22_2R	AGCAATAGTGGTCGTCGGAG	
33	WRKY25_1F	GTTCAAGACGGCTCAACCAC	110
34	WRKY25_1R	GAGGAGCTGAGAAGCAGAGG	
35	WRKY27_1F	ACAACAATCCCAACCTCCCC	117
36	WRKY27_1R	TACGCCAAGCCCACAAATCA	
37	WRKY27_2F	CAACAATCCCAACCTCCCCT	117
38	WRKY27_2R	ACCGTATTTACGCCAAGCCC	
39	WRKY33_1F	GTGAGAGAGCCGAGAATCGT	149
40	WRKY33_1R	CAACCGATGGTTGTGCACTT	
41	WRKY33_2F	AGAGCCGAGAATCGTAGTGC	149
42	WRKY33_2R	TCCTCACTGGACAACCGATG	
43	WRKY40_1F	TCAAATGTGCTTGTGCTCCA	94
44	WRKY40_1R	CACGGACTGATCCTCCACAC	
45	WRKY40_2F	TGCTCCAAGCTGTTCTGTCAA	94
46	WRKY40_2R	TGGATGGTTGTGTTCACCCT	
47	WRKY50_1F	CCACTGCTTCTGCCGACAA	79
48	WRKY50_1R	ACCGTGTCTTGAACGCAACT	
49	WRKY50_2F	ACTGCTTCTGCCGACAACC	79
50	WRKY50_2R	CCTCGGACCGTGTCTTGAAC	
51	WRKY51_1F	GACGGGTCATCGAGTTGCAT	150
52	WRKY51_1R	TTCACCGAGCAACCTTCACT	
53	WRKY51_2F	CGGGTCATCGAGTTGCATTTA	150
54	WRKY51_2R	TCTTCACCGAGCAACCTTCAC	
55	WRKY53_1F	CCTCACCGAGCGTACAACTT	96
56	WRKY53_1R	TCCGTTTATCGATGCCGGAG	
57	WRKY53_2F	TGTAGTCCCGGTGGCAAATC	96
58	WRKY53_2R	GAACCTCCTCCATCGGCAAA	
59	WRKY54_1F	TGAGACCCGTCTTGTTTCCG	105
60	WRKY54_1R	CGGCGACGGGATCAAAAGAA	
61	WRKY54_2F	AACGCAAGGATGCAAAGCAA	105
62	WRKY54_2R	TGGTCATTGGCAGTGCATGT	
63	WRKY58_1F	CGGTGGAGATGGGCTTAGTC	112
64	WRKY58_1R	AACATGGGCGAGGTGAGAAG	
65	WRKY58_2F	CGGTCATCGGATGGACAGAT	112
66	WRKY58_2R	CACCACCAACCTCAGTGGAA	

Appendix II: List of Endogenous genes primers for cumin

S No.	Name	Primer name	Primers sequences (5'- 3')		Amplicon Size (bp)
1	Tubulin beta chain 2	*Tub ß*	F	TGTCTGGTGTGACTTGCTGT	143
			R	GAAGACGGGGGAATGGGATG	
2	Actin-11	*ACT 11*	F	GTGGTTGCTCCACCAGAGAG	99
			R	CTCGGCCTTTGCTATCCACA	
3	TIP41-like family protein	*TIP41*	F	GGAGGACTGTGAGGAACGAATTGAT	106
			R	ACGCAAGAGAAGGAACCAACAACT	
4	Polyubiquitin 10 gene	*UBQ_10*	F	TCTCCGACTCCGTGGTGGTATG	108
			R	CTGCCGTCCTCCAACTGCTTAC	
5	Protein phosphatase 2A	*PP2A*	F	GTGTATCAATGTACCACCAGCAACT	147
			R	GCTCACCAAGGAACATGACTTCTT	
6	Elongation factor 1-α gene	*EF1 α*	F	TCAAGGATCTCAAGCGTGGTTATGT	115
			R	CAGCAATGTGGCAAGTGTGACAAT	

References

1. Chomczynski P, Mackey K. Modification of TRI reagent procedure for isolation of RNA from polysaccharide and polyglycon rich sources. Biotechniques. 1995; 19(6):942-950.
2. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue! Focus. 1990; 12:13-15.
3. Farrell JR. RNA Mehtodologies, 2nd ed., Academic Press, San Diego, CA, 1998.
4. Ghawana S, Paul A, Kumar H, Kumar A, Singh H, Bhardwaj PK, Kumar S. An RNA isolation system for plant tissues rich in secondary metabolites! BMC research notes. 2011; 4(1):85.
5. Jin JP, Tian F, Yang DC, Meng YQ, Kong L, Luo JC, Gao G. Plant TFDB 4.0, toward a central hub for transcription factors and regulatory interactions in plants! Nucleic Acids Research. 2017; 45:1040-1045.
6. Mendoza AD, Sebe-Pedros A, Sestak MS, Matejcic M, Torruella G, Domazet-loso T, Ruis-trillo I. Transcription factor evolution in eukaryotes and the assembly of regulatory tool kit in multicellular lineages! Proceedings of the National Academy of Sciences. 2013; 110(50):4858-4866.
7. Ruston PJ, Somssich IE, Ringler P, Shen QJ. *WKRY* transcription factors! Trends in Plant Science, 2010; 15(5):247-258.