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M.Sc. Thesis

ASCERTAINING THE PCR CONDITIONS AND DIEL EXPRESSION PROFILE OF DROUGHT RELATED GENES USING GENOMIC DNA AND cDNA FROM COWPEA (*Vigna unguiculata L. Walp.)* LEAVES

MURAT ATUN

Directed by

PROF. DR. MARCOS EGEA GUTIÉRREZ-CORTINES

PROF. DR. JULIA ROSL WEISS

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TABLE OF CONTENTS

LIST OF FİGURES

LIST OF TABLES

KEYWORDS

Cowpea (*Vigna unguiculata L. Walp*.), Drought Stress, Gene Expression, RT-qPCR, CPRD22, CPRD65, DREB2A, *Circadian Rhythm*, Diel Changes Gene Expression

SUMMARY

In this project, drought stress related genes of cowpea leaves have been targeted to contribute growth potential of the plant in hot conditions. To this end, ''ELITE (IT97K-499-35 breeding line)'' have been grown in growth chamber conditions with 3 variants of cowpea ('Pinkel', 'Fradel' and 'Vg166') in order to validate the growth scale. Leaf samples have been collected from ''ELITE'' plant in different time intervals (6h, 12h, 18h and 24h=0) through the day. Also 12 h L/D conditions have been provided for further analysis. Later on, primer design and gene expression analysis were conducted. REST have been used for the data analysis and the identification of reliability. It has been shown that DREB2A and CPRD65 are clearly drought stress related genes and but CPRD22 has not shown significantly differences.

RESUMEN

En este proyecto, los genes relacionados con estrés por sequía de las hojas de caupí han sido destinados a aportar potencial de crecimiento de la planta en condiciones de calor. Con este fin, ' ' Elite (IT97K-499-35 línea de reproducción) ' ' se han cultivado en condiciones de la cámara de crecimiento con 3 variantes de caupí (' Pinkel ', ' Fradel ' y ' Vg166 ') con el fin de validar la escala de crecimiento. Se han recolectado muestras de hojas de la planta ' ' Elite ' ' en diferentes intervalos de tiempo (6H, 12h, 18h y 24h = 0) durante el día. También se han proporcionado 12 h L/D condiciones para el análisis posterior. Posteriormente se realizaron análisis de primer diseño y expresión génica. El resto se ha utilizado para el análisis de datos y la identificación de la fiabilidad. Se ha demostrado que DREB2A y CPRD65 son claramente genes relacionados con el estrés de la sequía y pero CPRD22 no muestra diferencias significativas.

OBJECTIVES

The primary objective of this study was to optimize the PCR conditions for three drought stress related genes such as DREB2, CPRD22 and CPRD65 in cowpea leaves. The genes were identified based on publically available cowpea sequence information and their homology to Arabidopsis.

Moreover, it is aimed to quantify, whether a diel change in expression of these stress related genes exist under normal conditions. This knowledge is relevant for the coordination of sampling time in further experiments, as i.e. the analyse of the expression profile under drought stress conditions.

1. INTRODUCTION

1.1. Cowpea (*Vigna unguiculata L. Walp.)*

The scientists in the World search for suitable plants to be cultivated for arid and semi-arid soils. Cowpea (*Vigna unguiculata L. Walp.)* is an important plant of the legume family who has been cultivated under semi-drought conditions in some parts of the World (Carvalho, Lino-Neto, Rosa, & Carnide, 2017; Hall, 2012; Sadhukhan, Kobayashi, et al., 2014; Sadhukhan, Panda, & Sahoo, 2014; Weiss et al., 2018; Zegaoui et al., 2017a). It is one of the most important grains in the sub-Saharan Africa (Boukar, Fatokun, Huynh, Roberts, & Close, 2016) and has probably been used as a crop plant since Neolithic times (Hall, 2012). Cowpea is the most economically important local African legume crop both for human and animal consumption. Particularly in Nigeria and Niger, cowpea cultivation accounts for 66 percent of world cowpea production. Other major producers in North and West Africa are Burkina Faso, Ghana, Senegal and Mali (Agbicodo, Fatokun, Muranaka, Visser, & Linden Van Der, 2009; Boukar et al., 2016; Langyintuo et al., 2003; Singh, Chambliss, & Sharma, 1997). Currently, cowpea is a legume largely adapted and grown around the World (Butler, 1999). The average yield is relatively low (between 200 and 300 kg/ha) (Taylor et al., 2010). The cowpea grain contains 23% protein and 57% carbohydrate, and the leaves contain 27–34% of proteins(Horn, Ghebrehiwot, & Shimelis, 2016; Yao et al., 2016). This ratio shows that cowpea a very important nutritional substance in human diet as well as livestock forage. (Langyintuo et al., 2003; Yao et al., 2016) .

Cowpea is a diploid (2n = 22) and an annual plant of the grain legume family (Carvalho et al., 2017; Singh et al., 1997) and its approximate genome size is 620 Mb (X. Chen, Laudeman, Rushton, Spraggins, & Timko, 2007; Muñoz-Amatriaín et al., 2017). Hundreds of cowpea genotypes are cultivated worldwide or can be found in seed Banks (Egbadzor et al., 2014).

The name of cowpea most likely comes from the fact that the United States considered the plant an important source of chaff for cows in the south-eastern United States and in other parts of the world (Timko, Ehlers, & Roberts, 2007). Cowpea in the first instance evolved in Africa and Madagascar and wild cowpea is only found in Africa and Madagascar (Timko et al., 2007).

Cowpea has a well-developed taproot (Hall, 2012) and a compound leaf with three leaflets attached to leaf stalk (Padem, 2005; Peksen, Peksen, May, & May, 2017; Pottorff, Ehlers, Fatokun, Roberts, & Close, 2012). A coloured circle indicates the place where the seeds of cowpea connect to the fruit (Kan & Kültürleri, n.d.; Yilmaz, Atak, & Erayman, 2008). The seeds are also commonly named black eyed-pea, cowpea, southern pea, niebe or lubia depending on the geographical location (Yao et al., 2016).

The increase in the production of agricultural products is inadequate to feed the growing World population. Nutritional problems have been brought to the agenda in recent years. An additional problem consists in growing plants under increasing drought conditions, which made researchers begin to seek for drought tolerant crops and varieties (Leão et al., 2016), including the investigation of drought resistant genes (Chi et al., 2016; Padem, 2005; Properties, 2008; Thiam & Champion, 2013).

Cowpea has a top tolerance to heat and dry conditions that makes it an ideal crop for dry land environments and also, to study the molecular mechanisms of drought tolerance (Sadhukhan, Kobayashi, et al., 2014). The optimum temperature for cowpea growth is 30 °C, and reference temperature for germination is 8,5 °C and for leaf growth 20 °C (Hall, 2012). making it just available as a summer crop for most of the World. It grows optimally in regions with a yearly rainfall of between 400–700 millimeters. The optimum soils are sandy and it has better tolerance for poor and acid soils than most other crops (Hall, 2012).

1.2. Genes Known to Be Related to the Resistance to Drought Stress

Plants respond to environmental stresses by modification of physiological and developmental processes. Drought is one of the strongest environmental stresses that plants meet and it affects nearly all plant functions, including photosynthesis, growth, and development (S Iuchi, Yamaguchi-Shinozaki, Urao, Terao, & Shinozaki, 1996). When cowpea replies to drought stress, stomata close quickly and leaf transpiration is decreased (Agbicodo et al., 2009; Zegaoui et al., 2017). ABA (abscisic acid) levels in the plant greatly increase in response to water stress, resulting in the closure of stomata thereby reducing the level of water loss through transpiration from leaves and activating stress response genes (Carvalho et al., 2017; S Iuchi et al., 1996). Also, ABA has been proved to be the first hormone involved in the physiological, cellular and molecular responses of plants to osmotic and desiccation stress (Nelson, Salamini, & Bartels, 1994). When cowpea plants were dehydrated for 10 h, endogenous ABA increased more than 100 times in leaves (S Iuchi et al., 1996; Zegaoui et al., 2017a). The reaction is reversible: once water becomes available again, the level of ABA drops and stomata re-open. Increasing the plant's sensitivity to ABA has therefore been a very important target for improving drought tolerance (Hall, 2004; S Iuchi et al., 1996; Zegaoui et al., 2017b).

Stress and ABA also induce the expression of stress-related transcription factors, such as DREB2A (Agarwal, Agarwal, Nair, Sopory, & Reddy, 2007; H. Chen, Liu, Wang, Wang, & Cheng, 2016; Sadhukhan, Panda, et al., 2014; Zegaoui et al., 2017a), CPRD22(S Iuchi et al., 1996) and CPRD65(S Iuchi, Kobayashi, Yamaguchi-Shinozaki, & Shinozaki, 2000; S Iuchi et al., 1996)..These drought-inducible genes have been analyzed broadly in the model plant *Arabidopsis thaliana* (S Iuchi et al., 1996; Shinozaki & Yamaguchi-Shinozaki, 1996).

1.2.1. CPRD22

The CPRD clones represent ten different genes collectively called CPRD (cowpea responsive to dehydration) (Agbicodo et al., 2009; S Iuchi et al., 1996). The CPRD22 protein is 1129 base pairs in length and the locus code is '' D83972 ''in the *Vigna unguiculata L. Walp* plant genome (S Iuchi et al., 1996). The effects of abiotic stresses on the expression of the CPRD22 has been analysed and it was found that this gene was strongly induced by high-salinity and water stress but not by cold or heat stress (S Iuchi et al., 1996).

The sequence analysis of CPRD22 cDNAs revealed that they encoded accepted proteins that was associated late embryogenesis abundant proteins (Group 2 LEA), respectively. LEA (late embryogenesis abundant) proteins findable in both plants and animals are related with tolerance to water stress resulting from desiccation or osmotic stresses associated with low temperature. (GOYAL, WALTON, & TUNNACLIFFE, 2005; Skriver, 1990).

1.2.2. CPRD65 (VuNCED1)

The CPRD65 protein is 2432 base pairs in length and the locus code is ''AB030293'' in the *Vigna unguiculata L. Walp* plant genome(S Iuchi et al., 2000). A total of 10 cDNAs were cloned from dehydrated drought tolerant cowpea plants (*Vigna unguiculata*) by differential screening, and they were collectively called CPRD (cowpea responsive to dehydration). CPRD65 (VuNCED1) encodes a 9-cis-epoxycarotenoid dioxygenase that catalyzes the key step in ABA biosynthesis (S Iuchi et al., 2000; Schwartz, Tan, Gage, Zeevaart, & McCarty, 1997; B. C. Tan, Schwartz, Zeevaart, & McCarty, 1997). The expression of the CPRD65 responds strongly to drought stress in stems and leaves but slightly in roots (S Iuchi et al., 2000; Satoshi Iuchi, Kobayashi, Yamaguchi-shinozaki, & Shinozaki, 2000). The expression of

CPRD65 gene was also elevated under high salt condition (Suo, Zhao, David, Chen, & Dai, 2017) but not by heat or cold stress (Satoshi Iuchi et al., 2000).

1.2.3. DREB2A

VuDREB2A protein was identified in the *Vigna unguiculata L. Walp* plant genome under the locus code ‘’JN629045’’ and the length is 1505 base pairs (Sadhukhan, Kobayashi, et al., 2014). There are two different type of DREBs: DREB1 and DREB2 that were originally isolated in *Arabidopsis thaliana* (Liu et al., 1998). The DREB transcription factors belong to the APETELA2/ethylene responsive element binding protein (AP2/EREBP) family and are induced under abiotic stress conditions (Kizis, Lumbreras, & Pagès, 2001; Junya Mizoi, Shinozaki, & Yamaguchi-Shinozaki, 2012). A gene similar to VuDREB2A has been isolated and characterized from cowpea, encoding a protein of 377 amino acids exhibiting characteristics of reported DREB2-type proteins. In cowpea, VuDREB2A transcript accumulation was highly induced by desiccation, as well as heat and salt stress, but only slightly by exogenous abscisic acid (ABA) treatment (Sadhukhan, Kobayashi, et al., 2014). The cis-element of DREB2A is the dehydration-responsive element (DRE)(H. Chen et al., 2016). DREB (Dehydration-Responsive Element-Binding) is the most common cis-element involved in response to drought stress (H. Chen et al., 2016; Sadhukhan, Panda, et al., 2014; H. Tan et al., 2016).

Among the DREB gene family, the A-2 subgroup including DREB2A is more particularly involved in the response to drought, desiccation, salinity and heat stress in *Arabidopsis thaliana* and *Vigna unguiculata L. Walp* (Liu et al., 1998; Sadhukhan, Kobayashi, et al., 2014; Sakuma, Maruyama, Osakabe, Quin, feng, Seki, motoaki, Shinozaki, & Yamaguchi-Shinozaki, 2006).

All the drought related genes mentioned above were reported to be up-regulated under water stressed conditions. Overexpression of one of the cowpea genes up-regulated by water stress, *VuNCED1*(CPRD65), a key gene involved in ABA biosynthesis encoding 9-cis-epoxycarotenoid dioxygenase, developed drought tolerance in *Arabidopsis thaliana* (S Iuchi et al., 2001; Sadhukhan, Kobayashi, et al., 2014).

Many DREB (dehydration-responsive element-binding) genes have been identified and characterized in different plants under the different abiotic stresses, such as cowpea (*Vigna unguiculata L.*)(Sadhukhan, Kobayashi, et al., 2014), *Arabidopsis* (Sakuma et al., 2002), rice (*Oryza sativa L.*)(Cui et al., 2011), maize (*Zea mays L.*)(F. Qin et al., 2007) and soybean (*Glycine max L.*)(M. Chen et al., 2007; J. Mizoi et al., 2013). The abiotic stress factors affecting DREB include drought (H. Chen et al., 2016; Liu et al., 1998; Nakashima et al., 2000; Q. Qin et al., 2007; Sadhukhan, Kobayashi, et al., 2014; Sakuma et al., 2006), heat(Schramm et al., 2008), high-salinity(J. Chen, Xia, & Yin, 2009; Cong, Zheng, Zhang, & Chai, 2008; Dubouzet et al., 2003; Huang, Jin, & Liu, 2008; Nakashima et al., 2000) and low temperatures(Gutha & Reddy, 2008; Li et al., 2005; Q. Qin et al., 2007), respectively.

1.3. *Circadian Rhythm* and Genes with Diel Changes in Expression

The plants or other living organisms use environmental signals for the regulation of rhythms. Light and darkness are environmental cues that regulate the behaviour during day and night. As a result of that *circadian* clock, many phenotypical feature of plants such as leaf, organ and stomatal movements, growth and signalling, are regulated (McClung, 2006; Mizoguchi, 2005; Webb, 2003; Weiss et al., 2018). It creates an endogenous timing system that synchronizes the physiology and behaviour of many organisms, as i.e. the flowering in *Arabidopsis thaliana* (Mizoguchi, 2005). This system is known as *circadian* clock. In Latin, "*circa*" means about, "*diem*" means one day, thus describing rhythms that last roughly one day. Physiological, developmental, biochemical and transcriptional activities related to *Circadian Rhythms* have been reported

(Webb, 2003). The *Circadian clock* in plants “tells” them what season it is or when to flower for the best chance of attracting pollinators (McClung, 2006; Webb, 2003). The circadian rhythms may influence many agriculturally relevant traits such as harvest or drought stress and understanding the circadian control over these parameters may help to prevent loss of productivity (McClung, 2006; Webb, 2003).

2. MATERIAL AND METHODS

The experimental process is as shown below in the figure1. The appropriate kits used, plant samples, pipettes, tips, thermocyclers, real-time qPCR machine and other basic laboratory equipment and tools were provided by the Institute of Plant Biotechnology, Cartagena Technical University, Cartagena, Murcia/Spain.

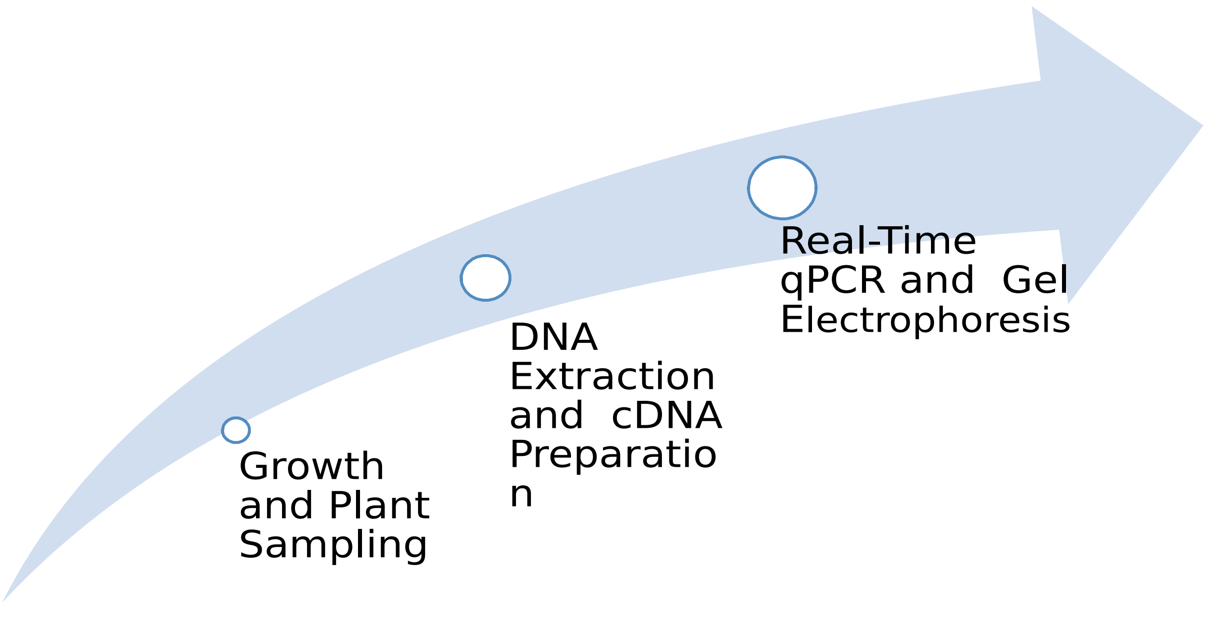

Figure 1:Process of Experiment

2.1. Plant Material & Sampling

Cowpea seeds were sown at mid-January in small bowls (2L pots) and placed in a growth chamber at 25ºC ±2 º C and 12hours light/12 hours dark period.

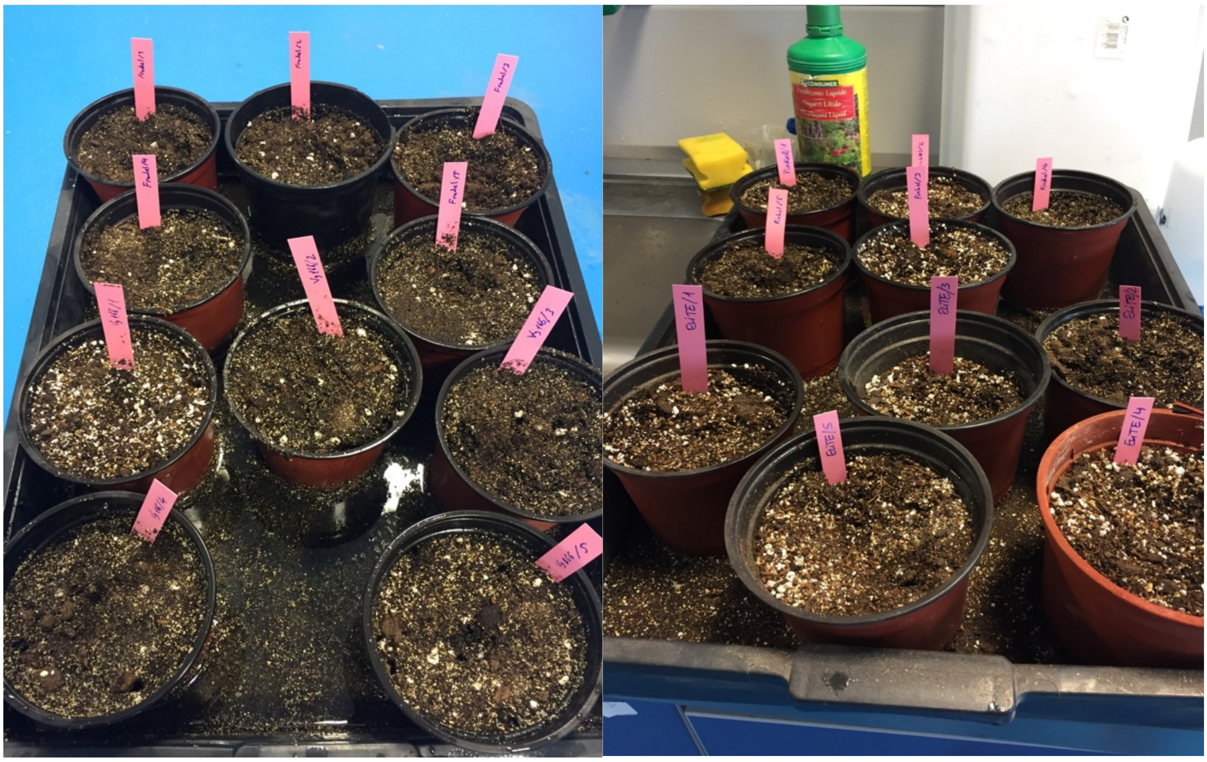

Figure 2:The sowing of seeds

Seeds belonged to the cowpea genotypes ‘’Elite’’, ‘’Vg166’’, ‘’Fradel” and “Pinkel’’. Four days after sowing in commercial substrate, 100% of germination was observed for ‘Elite’ and ‘Pinkel’ seeds, while only 75% and 0% of ‘Vg166’ and ‘Fradel’ seeds had germinated, respectively. Plants of ‘’Elite (IT97K-499-35 breeding line)’’ were used for further experiments.

Figure 3:Growing plants under the greenhouse condition (25°C)

When plants had an achieved a height of 10 cm (about 14 days after) achieved, leaf samples were collected for the extraction of genomic DNA.

For the diel expression analysis, cowpea plants of ’Elite” (IT97K-499-35 breeding line) were transferred to ‘‘Tomas Ferro’’ Experimental Agro-Food Station, Technical University of Cartagena located in southeast of Murcia region, Campo de Cartagena, Spain, where they developed under field conditions with average temperatures of 29.1 °C during sampling time. Leaf material was sampled in 6-hour intervals at 12:45pm (T6), 6:45pm (T12), 00:45am (T18) and 6:45am (T24=T0=dawn). Time of sunset was at 9:31 pm. Leaves were harvested when the first pods matured.

2.2. DNA and RNA Extraction and cDNA Preparation

2.2.1. Genomic DNA and RNA extraction

Genomic DNA (gDNA) and Total RNA was extracted using NucleoSpin® Plant II kit (Macherey-Nagel) according to manufacturer's instructions by using 100 mg of cowpea leaves, homogenized using liquid nitrogen. gDNA was stored at -20ºC. Total RNA was extracted from four leafs from three different plants at each sampling time and stored at -80ºC.

2.2.2. cDNA Preparation

Total RNA from plant samples was used for reverse-transcription in order to prepare the templates for gene expression analysis by RT-QPCR.

cDNA was prepared using the kit 'Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit' according to the manufacturer's instructions after adjusting RNA concentrations to 100 ng/µl. cDNA samples were stored at -80˚C.

2.3. Real Time Quantative PCR and Gel Electrophoresis

2.3.1. Conventional and RT-QPCR

For conventional PCR, we used 2 µl of gDNA adjusted to a concentration of 100ng/ µl. For RT-PCR we used 2 µl of cDNA. PCR was performed in both cases using the Premix Ex Taq™ kits (Takara, ThermoFisher Scientific) following the manufacturer's instructions. The kit contains all PCR components, including the fluorescent compound “SybrGreen”, Taq polymerase and dNTPs and only requires the addition of primers specific for each PCR.

PCR conditions were set as following;

1 cycle of 95˚C for 3 minutes, 40 cycles of 95˚C for 3 second, 57˚C for 15 second, and 72˚C for 15 second. Following amplification, dissociation curves were generated during 1 cycle of 95˚C for 1 minute and increasing the temperature from 55 to 95˚C in time intervals of 30 seconds. RT-PCR was performed using the equipment “Agilent Technologies Stratagene Mx3000P” with two technical replicates per leaf simple.

The primers applied for the amplification of partial sequences of the genes CPRD22, CPRD65 and DREB2 from cDNAs are represented in Table 1. Primers were designed using the software PCRefficiency (http://srvgen.upct.es/efficiency.html) and purchased from Invitrogen by Thermo Fisher Scientific.

Table 1: Primers Sequences(Forward/Reverse) and their authors

Gene Name	Primer Sequences(Forward/Reverse)	Length (bp) F / R
CPRD22 **AUTHORS**	CAAAAGTTCTTAAAATCACAACCTAT / TCCATTGGCAGAACATCTATGCCT Iuchi, S.,	26 / 24
CPRD65 **AUTHORS**	TTCCACCAGGACAACACAAA / GGATGTGGATGTGGATGTTG Iuchi, S.	20 / 20
DREB2A **AUTHORS**	TGAGACCATTGCAAAGTGGA / TGGCTCTAGCAGCTTCATCA Sadhukhan, A., Mishra, S., Panda, S.K. and Sahoo, L.	20 / 20

EF1A (reference gene)	GCCTGGTATGGTGGTGACTT / GCGAACTTCACTGCAATCTG	20 / 20

The reference gene EF1A was used in all experiments to obtain a relative expression rate between different samples. This gene was previously shown to be most appropriate for RT-PCR for diel expression analysis(Weiss et al., 2018).

The relative expression rates of housekeeping gene EF1A (Elongation Factor) was used in further statistical analysis. The normalized expression (NE) was calculated using the following formula: ''NE=2∧$^{(-\Delta Ct)}$, where ΔCt= Ct experimental – Ct normalizer (n)''(Marcolino-Gomes et al., 2014). Statistical analysis of differences in gene expression were calculated with the REST program(Pfaffl, Horgan, & Dempfle, 2002).

2.3.2. Gel Electrophoresis

The approximate length of the DNA fragments of each PCR amplicon was determined by running PCR products on an agarose gel alongside with a 1kb DNA ladder. The DNA ladder was prepared following the manufacturer's instructions (Thermo Scientific). For gel electrophoresis, 1 g of agarose was dissolved in 100 mL of buffer 1x TAE buffer in order to obtain a 1% agarose. followed by heating in a microwave for 2-3 min until the agarose was fully dissolved. After cooling down to about 50˚C, we added ethidium bromide (EtBr) which interlaces with the DNA and allows to visualize the DNA under ultraviolet (UV) light. Loading dye buffer was added to each DNA samples (approximate 10 µl). After sample loading, the gel was run at 90 volts for 35 minutes (mA 400) (Fig. 5).

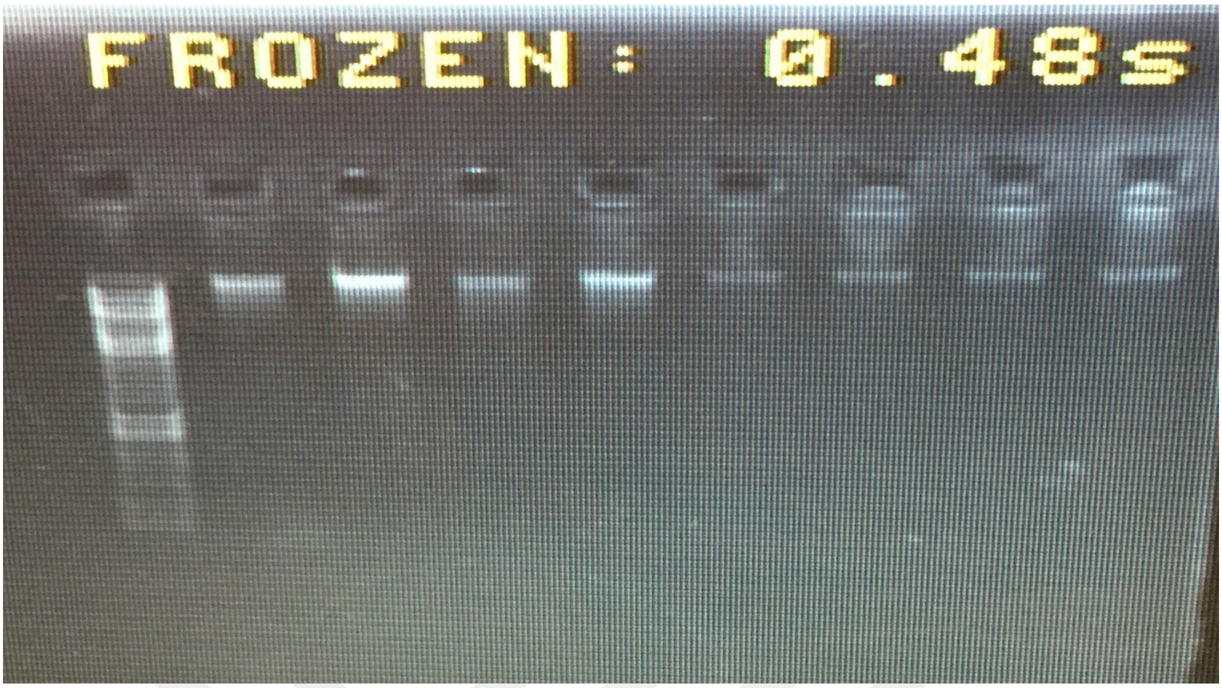

Figure 4: Genomic DNA from cowpea leaves after Gel Electrophoresis

3. RESULT

3.1. Ascertainment of PCR Conditions

Successful RT-PCR requires a prior establishment of appropriate PCR conditions for amplification. In a first step, we designed a PCR protocol adapted to the Premix Ex Taq™ kit and adjusted to the T_m of the primers. As shown in Figure 6, using gDNA, the *VuCPRD22* amplified a product of 1000bp, while *VuCPRD6*5 and *VuDREB2A* amplified a smaller product of 250bp. The intensity and quality of the DNA signal on the agarose gel indicates that PCR conditions were appropriate for the amplification of these drought related genes. This was confirmed by dissociation curve analysis. The amplicons derived from two independent gDNA extractions also showed a distinct and characteristic melting peak for the three genes.

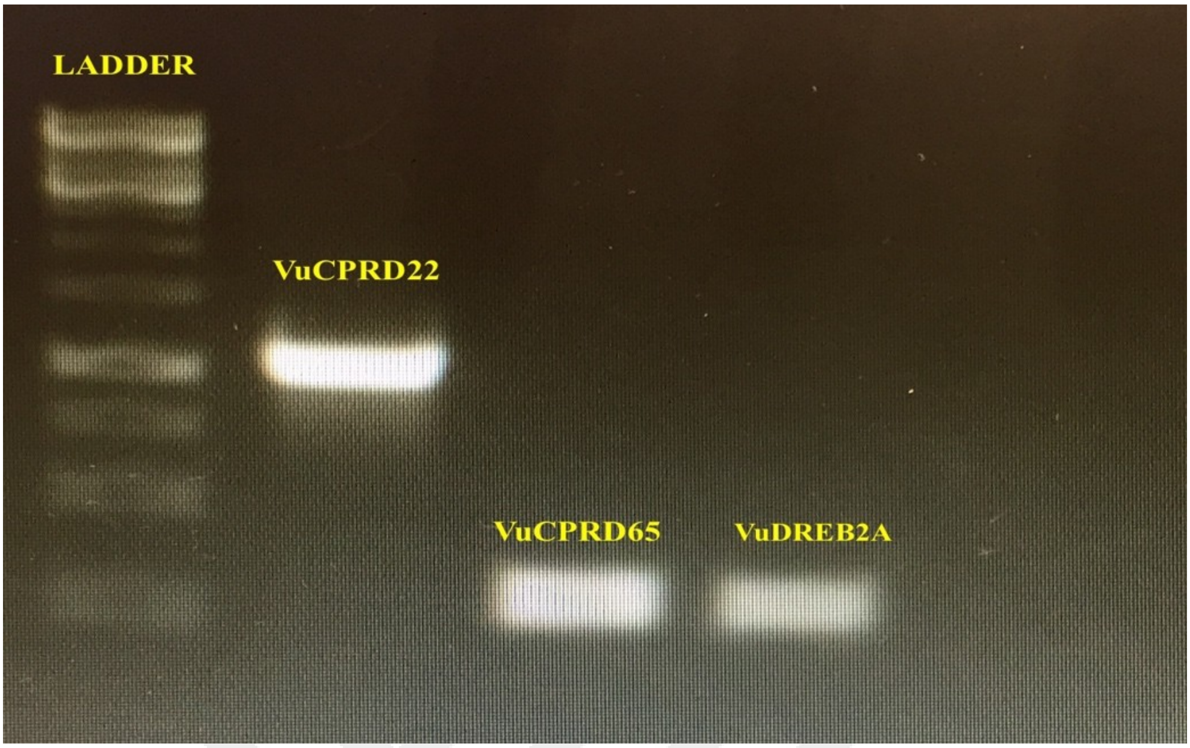

Figure 5: The primers on the gel electrophoresis after the genomic DNA expression from cowpea leaves

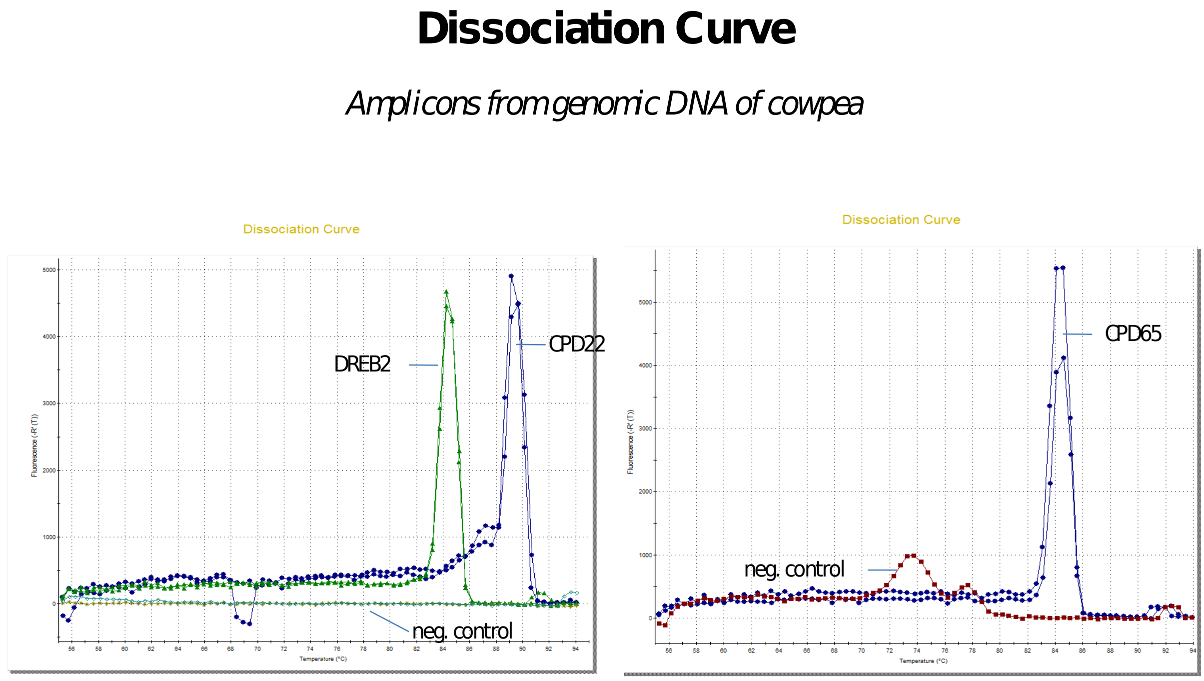

Figure 6: Amplicons from genomic DNA of cowpea leaves

3.2 Diel Expression Pattern of Drought Related Genes in Leaves

Following the establishment of successful PCR conditions for the three drought related genes, we continued to assess whether the drought related genes show a diel expression profile under control conditions in cowpea (*Vigna unguiculata L. Walp*.). Samples were therefore taken in 6h intervals during 24 hours, followed by RT-PCR.

Figures 8 and 9 shows the dissociations curves of samples from time pointe T0-T24 as well as negative controls. While *VuCPRD6*5 and *VuDREB2A* showed a Tm value identical to the amplicons derived from *gDNA, the Tm value for VuCPRD22* was inferior to amplicons derived from gDNA, which might be explained by sequence differences due to intron splicing. However, amplicon sizes as indicated by gel electrophoresis *(Fig.*

10) were identical to those produced by PCR from gDNA, indicating minor changes during RNA maturation.

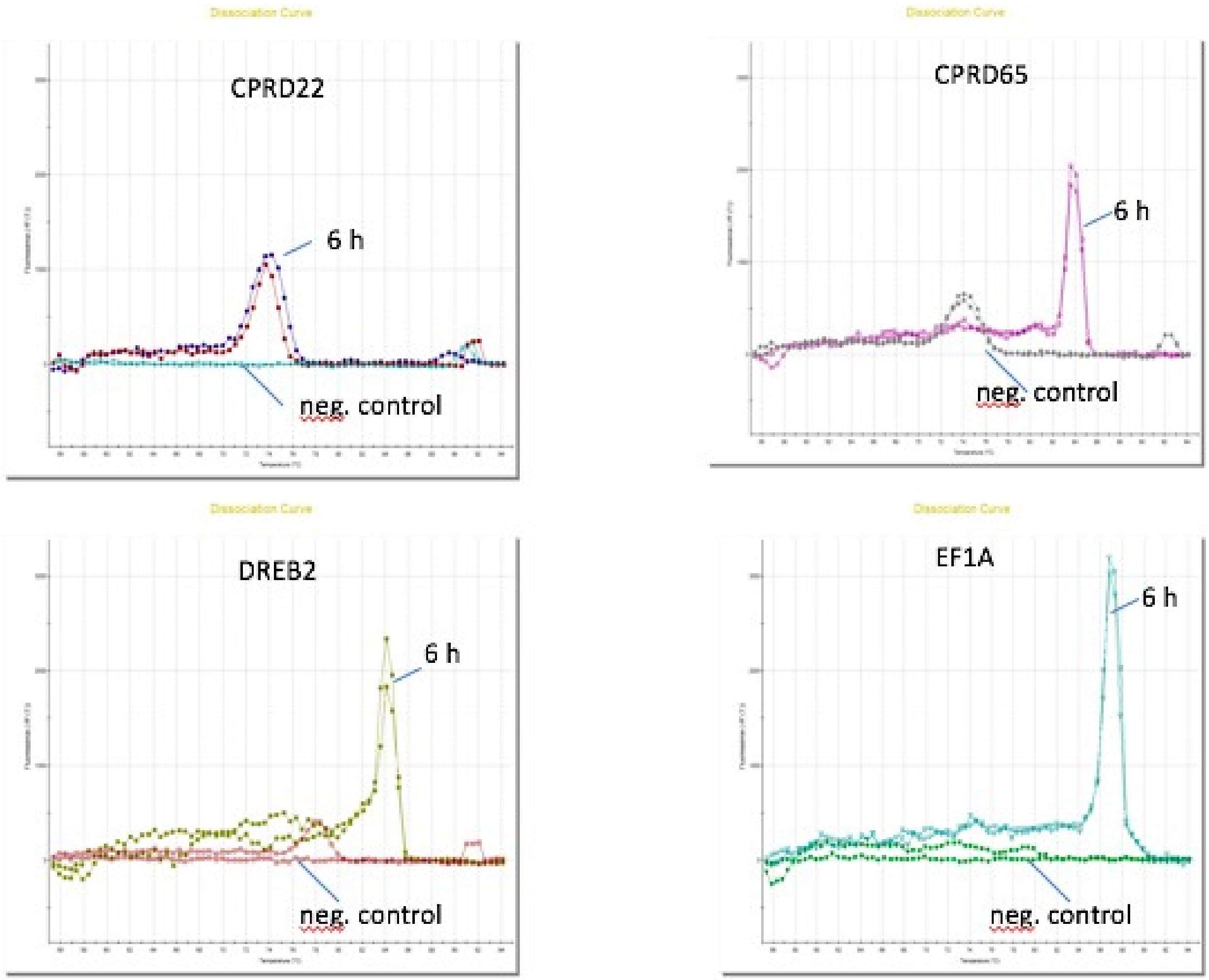

Figure 7: Genes CPRD22, CPRD65, DREB2 amplified from cDNA of leaves harvested at T6 under field condition and the corresponding negative control PCR (T0 = sun rise 6:45 am)

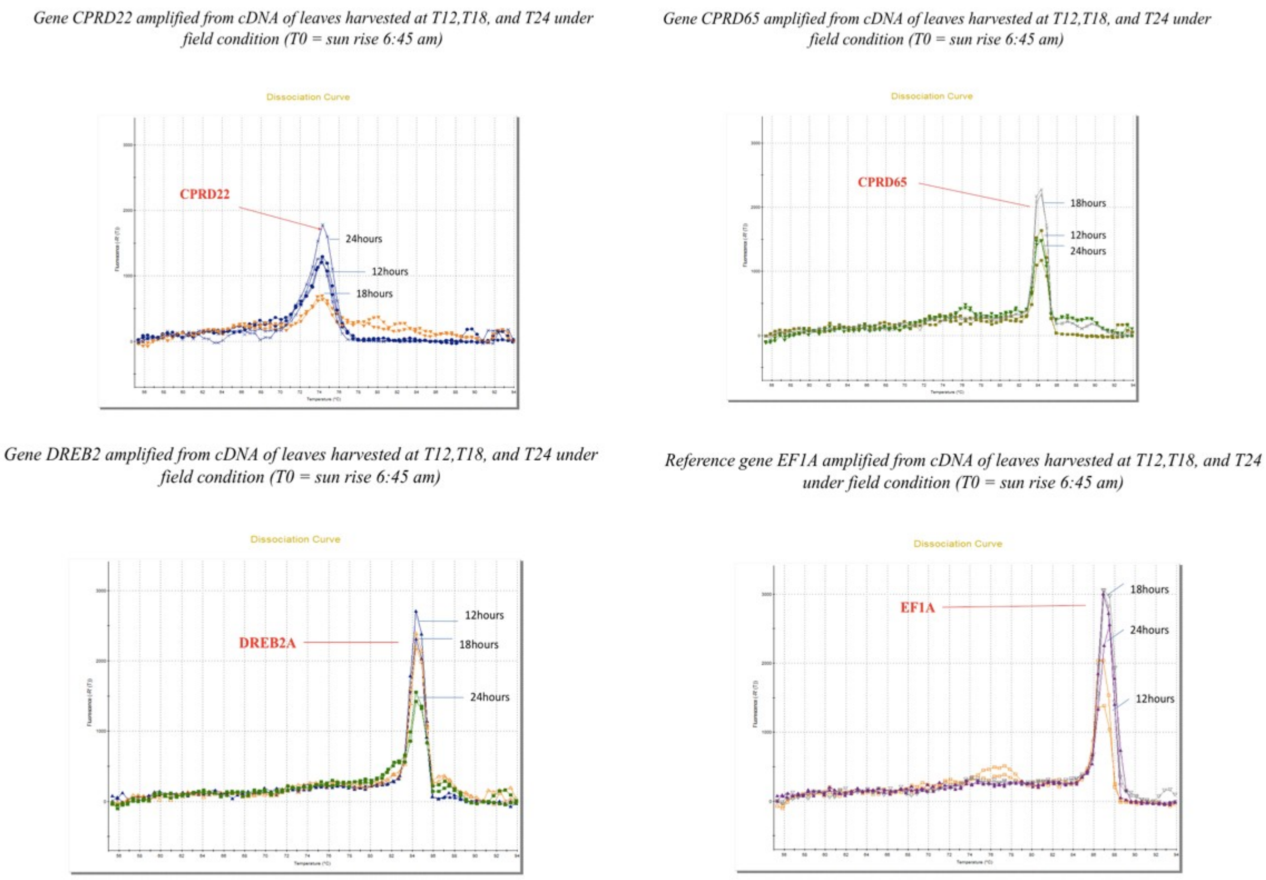

Figure 8: Genes CPRD22, CPRD65, DREB2 and EF1A amplified from cDNA of leaves harvested at T12, T18 and T24 under field condition (T0= sun rise 6;45 am)

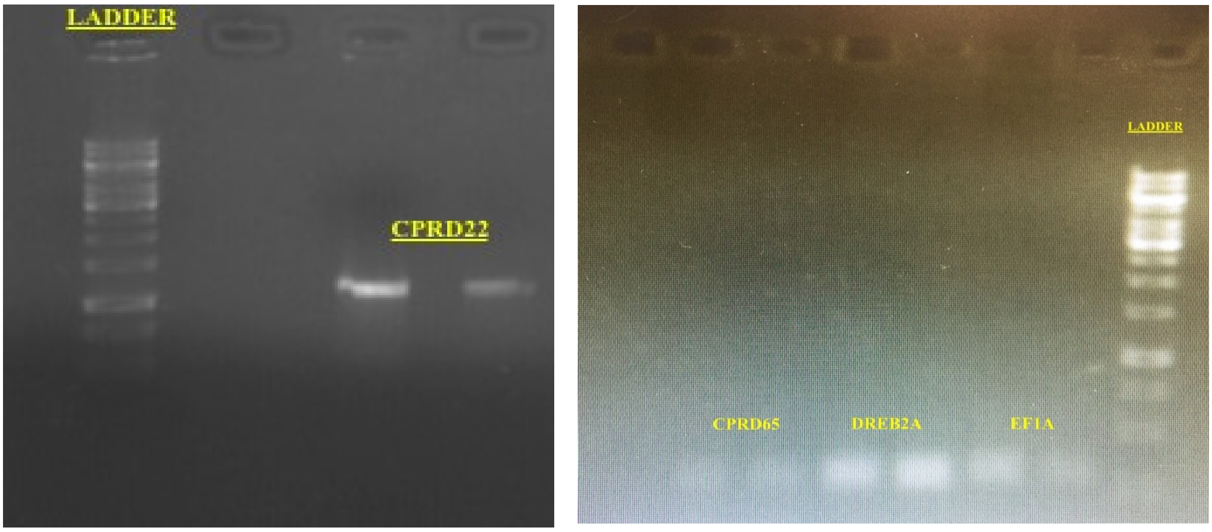

Figure 9: After the controlled condition that the Expressed Genes Shown on the Gel Electrophoresis

As indicated by RT-PCR results and dissociation curve analysis (Fig. 8-9, Table 2) all three drought related genes were expressed at each time point during the day.

However, Ct values (Table2) as well as the normalized expression values (Table 3 and 4) varied markedly during the day. As indicated in figure 11, Gene DREB2A showed the highest expression at 6h and 12h after sunrise, followed by CPRD65 and CPRD22. At 18h (00:45 am), only CPRD65 showed a marked expression and at 24hours (=T0, dawn), the expression of all three genes had dropped to minimal values.

Table 2: CT value and Efficiency of the Genes CPRD22, CPRD65, DREB2 and the Reference Gene EF1A After RT-qPCR with Leaf cDNA from Cowpea

Table 2. CT value and Efficiency of the Genes CPRD22, CPRD65, DREB2 and the Reference Gene EF1A After qPCR with Leaf cDNA from Cowpea									
		CPRD22		CPRD65		DREB2		EF1A	
Hours After Sunrise	Sample ID	Ct Value	PCR Efficiency	Ct Value	PCR Efficiency	Ct Value	PCR Efficiency	Ct Value	PCR Efficiency
6 hours (T6)	31	34,61	1,45	30,03	1,47	29,77	1,35	27,17	1,39
	31	35,35	1,57	28,27	1,63	27,06	1,57	27,86	1,46
	32	34,56	1,48	30,12	1,5	32,51	1,38	30,05	1,48
	32	34,35	1,46	29,28	1,56	28,08	1,7	28,39	1,41
	33	35,21	1,45	33,82	1,42			33,31	1,41
	33	35,21	1,45	34,94	1,41			30,83	1,33
	34	34,03	1,44	24,09	1,37	24,47	1,31	17,8	1,4
	34	34,62	1,146	23,85	1,33	24,43	1,37	17,76	1,38
12 hours (T12)	35	29,4	1,56	23,51	1,32	22,42	1,29	22,33	1,38
	35	30,12	1,66	24,18	1,32	22,2	1,32	22,33	1,38

	36	30,62	1,8	22,56	1,49	19,14	1,31	18,86	1,33	
	36	30,96	1,68	22,78	1,52	18,97	1,31	19,13	1,39	
	37	31,15	2,52	18,42	1,49	16,4	1,39	17	1,48	
	37	29,74	2,21	18,63	1,51	16,48	1,4	16,96	1,48	
	38	31,14	1,47	30,94	1,36	34,49	1,46	30,1	1,4	
	38	31,04	1,48	30,6	1,44	34,61	1,45	30,84	1,43	
18 hours (T18)	39	29,98	1,56	15,7	1,38	20,49	1,33	15,25	1,46	
	39	29,41	2,27	14,96	1,33	20,55	1,33	15,34	1,46	
	40	28,59	2,44	24,46	2,03	27,26	1,43	20,25	1,39	
	40	30,13	1,73	24,32	1,94	27,57	1,45	20,37	1,41	
	41	31,15	1,87	20,47	1,37	24,16	1,35	19	1,42	
	41	33,44	1,47	21,83	1,36	23,78	1,38	19,01	1,44	
	42	31,53	1,66	18,1	1,32	24,26	1,33	18,06	1,41	
	42	31,43	1,79	18,1	1,31	24,19	1,32	17,95	1,41	
24 hours (T24=0)	43	31,95	1,46	30,6	1,41					
	43	34,46	1,47	30,44	1,39					
	44	33,22	1,48	22,88	1,65	21,49	1,36	14,31	1,51	
	44	33,22	1,48	22,69	1,58	21,62	1,39	14,66	1,74	
	45	30,44	1,78	25,93	1,79	28,22	1,41	20,95	1,39	
	45	30,44	1,78	26,09	1,86	28,16	1,37	21,05	1,39	
	46	33,9	1,46	26,68	1,67	26,07	1,37	18,48	1,41	
	46	33,91	1,4	27,37	1,69	25,4	1,43	18,58	1,44	

Table 3:Normalized Expression Values of CPRD22, CPRD65 and DREB2, Amplified from Cowpea Leaves

Table 3. Normalized Expression Values of CPRD22, CPRD65 and DREB2, Amplified from Cowpea Leaves							
		CPRD22		CPRD65		DREB2	
Hours after sunrise		Delta CT	Normalized expression	Delta CT	Normalized expression	Delta CT	Normalized expression
6 hours (T6)	31	7,44	0,00576000	2,86	0,13774	2,6	0,16494
	31	7,49	0,00556000	0,41	0,75262	-0,8	1,7411
	32	4,51	0,04389000	0,07	0,95264	2,46	0,18175
	32	5,96	0,01606000	0,89	0,53961	-0,31	1,23971
	33	1,9	0,26794000	0,51	0,70222		
	33	4,38	0,04803000	4,11	0,05791		
	34	16,23	0,00001000	6,29	0,01278	6,67	0,00982
	34	16,86	0,00001000	6,09	0,01468	6,67	0,00982
Average			0,04841000		0,39628		0,55786
Deviation			0,09071000		0,3826		0,74317
12 hours (T12)	35	7,07	0,00744000	1,18	0,44135	0,09	0,93952
	35	7,79	0,00452000	1,85	0,27739	-0,13	1,09429
	36	11,76	0,00029000	3,7	0,07695	0,28	0,82359
	36	11,83	0,00027000	3,65	0,07966	-0,16	1,11729
	37	14,15	0,00006000	1,42	0,37371	-0,6	1,51572
	37	12,78	0,00014000	1,67	0,31425	-0,48	1,39474
	38	1,04	0,48633000	0,84	0,55864	4,39	0,0477
	38	0,2	0,87055000	-0,24	1,18099	3,77	0,0733
Average			0,17120000		0,41287		0,87577
Deviation			0,32950000		0,3295		0,55051

18 hours (T18)	39	14,73	0,00004000	0,45	0,73204	5,24	0,02646
	39	14,07	0,00006000	-0,38	1,30134	5,21	0,02702
	40	8,34	0,00309000	4,21	0,05403	7,01	0,00776
	40	9,76	0,00115000	3,95	0,0647	7,2	0,0068
	41	12,15	0,00022000	1,47	0,36098	5,16	0,02797
	41	5,59	0,02076000	1,56	0,33915	5,15	0,02816
	42	13,47	0,00009000	0,04	0,97265	6,2	0,0136
	42	13,48	0,00009000	0,15	0,90125	6,24	0,01323
Average			0,00318750		0,5907675		0,018875
Deviation			0,00717771		0,454823772		0,009425225
24 hours (T24=0)	43						
	43						
	44	18,91	0,00000203	8,57	0,00263	7,18	0,0069
	44	18,56	0,00000259	8,03	0,00383	6,96	0,00803
	45	9,49	0,00139000	4,98	0,03169	7,27	0,00648
	45	9,39	0,00149000	5,04	0,0304	7,11	0,00724
	46	15,42	0,00002000	8,2	0,0034	7,59	0,00519
	46	15,33	0,00002000	8,79	0,00226	6,82	0,00885
Average			0,00097744		0,01285833		0,00760500
Deviation			0,00073857		0,01448320		0,00126635

Table 4: Deviations of Normalized Expressions (first part is average and second part is deviations)

	CPRD22	CPRD65	DREB2
6hours	0,04841±0,09071000	0,39628±0,3826	0,55786±0,74317
12hours	0,1712±0,3295	0,41287±0,3295	0,87577±0,55051
18 hours	0,0031875±0,00717771	0,5907675±0,45482377	0,018875±0,00942522
24 hours	0,00097744±0,00073857	0,01285833±0,0144832	0,007605±0,00126635

Fig 11: Diel expressions of drought related genes (T0=06:45 am, sunrise). Values correspond to the normalized expression (NE), calculated using the formula: ‘’NE=2∧$^{(-\Delta Ct)}$, where ΔCt=Ct experimental – Ct normalizer. “2”

We used the REST program (Pflaffl et al. 2002) in order to check whether there were any significant differences between gene expression during the day (Table 5).

As indicated by the probability “P” values, gene CPRD22 did not change significantly during the day, while gene expression of gene CPRD65 was significantly lower at 24 hours (=T0, dawn) compared to 12 and 18 hours. Gene DREB2A varied significantly between all-time points except for 6 hours compared to 12 hours and with borderline significance between 6 hours and 18 hours.

Table 5: Significances of Differences in Expression Levels of drought genes under the light/dark condition

Table 5: Significances of Differences in Expression Levels (REST 2008 v2.07)

Gene	Relative expression	Error	p-	

			value	
CPRD22				
6h versus 12 h	1,622	0,14-16,126	0,597	
6h versus 18 h	0,383	0,06-4,136	0,224	
6h versus 24 h	0,322	0,016-8,014	0,334	
12h versus 18h	0,219	0,017-12,14	0,065	
12h versus 24h	0,238	0,011-6,260	0,221	
18h versus 24h	0,355	0,021-10,317	0,386	
CPRD65				
6h versus 12 h	1,567	0,752-4,970	0,19	
6h versus 18 h	2,252	0,721-8,683	0,162	
6h versus 24 h	0,468	0,095-1,747	0,171	
12h versus 18h	1,232	0,436-2,343	0,502	
12h versus 24h	0,238	0,066-1,718	0,013	*
18h versus 24h	0,147	0,028-0,600	0,009	**
DREB2A				
6h versus 12 h	2,519	0,778-9,930	0,064	
6h versus 18 h	0,348	0,116-1,610	0,06	
6h versus 24 h	0,193	0,054-0,855	0,001	***
12h versus 18h	0,155	0,111-0,200	0,000	***
12h versus 24h	0,096	0,078-0,115	0,000	***
18h versus 24h	0,631	0,486-1,037	0,013	*

- Indicate significant differences in expression among time points with*=p≤0,05; **=p≤0,01; ***=p≤0,005

4. DISCUSSION

The aim of this study was to establish successful PCR conditions for three drought related genes CPRD22, CPRD65 and DREB2A from Cowpea (*Vigna* *unguiculata L. Walp.*) as well as to investigate, whether the expression of these genes shows diel changes, which might indicate a circadian rhythmicity. We explored gene expression under controlled conditions, that means well-watered field conditions, in intervals of 6,12,18 and 24 hours after sunrise.

As shown in our work, each gene has a different pattern, but only the expression of CPRD65 and DREB2A showed significant diel changes. The expression of CPRD22 was relatively low compared to CPRD65 and DREB2A and did not seem to be under circadian regulation.

DREB2A (Dehydration-responsive element-binding protein 2A) is responsible from the activation of the b-glucuronidase reporter gene by binding to the DRE sequence(Sadhukhan, Kobayashi, et al., 2014). On the other hand, CPRD65 (Neoxanthin cleavage enzyme) is having a molecular function of oxidoreductase activity(S Iuchi et al., 2000). The CPRD22 gene has been analysed and it was found that this gene was strongly induced by high-salinity and water stress(S Iuchi et al., 1996).

These drought stress related genes are so important for future studies and make plants resistant to drought stress which were the grown plants such as cowpea at the arid and semi-arid. Also, cowpea is a model plant in legume family for drought stress that why we used these genes; these genes have been obtained from cowpea plants and we have reached the idea of using these genes based on their responses in stress conditions. Especially these three genes such as *VunDREB2A*, *VunCPRD65* and *VunCPRD22* are so suitable genes for cowpea plants.

This information prepares the groundwork for future studies, as it clearly demonstrates the importance of sampling time for comparative expression analysis. Our findings are in agreement with previous works that report on clock controls over the expression of a large fraction of abiotic stress-responsive genes. Upon abiotic stress conditions, clock gene expression and therefore their oscillation, may be altered, which then affects the downstream stress-response pathways (Grundy, Stoker, & Carré, 2015).

In case of all genes analysed here, expression increased after dawn. This is different from Arabidopsis, where the majority of drought-inducible genes peaked around dawn(Wilkins, Bräutigam, & Campbell, 2010). Nevertheless, it was stated that higher expression levels during the day may be explained with the time of high water deficit experience due to prolonged opening of stomata for photosynthetic gas Exchange (Grundy et al., 2015).

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