UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUACÃO EM GENÉTICA

MITALLE KAREN DA SILVA MATOS

Fatores de Transcrição WRKY: Modulação da Expressão e Interações Moleculares Sob Estresse em Feijão-Caupi (*Vigna unguiculata*)

Recife
2019

MITALLE KAREN DA SILVA MATOS

Fatores de Transcrição WRKY: Modulação da Expressão e Interações Moleculares Sob Estresse em Feijão-Caupi (*Vigna unguiculata*)

Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutora em Genética.

Área de concentração: Biologia Molecular

Orientadora: Dra. Ana Maria Benko Iseppon

Coorientador: Dr. João Pacífico Bezerra Neto

Recife

2019

Catalogação na fonte
Elaine C Barroso
(CRB4 1728)

Matos, Mitalle Karen da Silva
Fatores de transcrição WRKY: modulação da expressão e interações moleculares sob estresse em feijão-caupi (*Vigna unguiculata*) / Mitalle Karen da Silva Matos – 2019.

119 f.: il., fig., tab.

Orientadora: Ana Maria Benko Iseppon
Coorientador:João Pacífico Bezerra Neto
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Genética, 2019.
Inclui referências, apêndice e anexos

1. Genética vegetal 2. Bioinformática 3. Desidratação radicular I. Iseppon, Ana Maria Benko (orient.) II. Bezerra Neto, João Pacífico (coorient.) III. Título

581.15 CDD (22.ed.) UFPE/CB – 2020-163

MITALLE KAREN DA SILVA MATOS

Fatores de Transcrição WRKY: Modulação da Expressão e Interações Moleculares Sob Estresse em Feijão-Caupi (*Vigna unguiculata*)

Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutora em Genética.

Aprovada em: 30/08/2019.

BANCA EXAMINADORA

_____

Dra. Ana Maria Benko Iseppon (Orientadora)
Universidade Federal de Pernambuco

_____

Dra. Valesca Pandolfi (Examinadora externa)
Universidade Federal de Pernambuco

_____

Dr. Lucas André Cavalcanti Brandão (Examinador interno)
Universidade Federal de Pernambuco

_____

Dr. José Ribamar Costa Ferreira Neto (Examinador externo)
Universidade Federal de Pernambuco

_____

Dra. Lidiane Lindinalva Barbosa Amorim (Examinadora externa)
Instituto Federal do Piauí

*Aos meus pais**, Paulo Jorge e Sônia Maria,***
*E ao meu esposo**, Luan Ramos,***
Dedico.

AGRADECIMENTOS

À **Deus**, fonte de sabedoria suprema, e à **Maria Santíssima**, que, guiados por seu amor incondicional, me deram proteção e forças durante toda minha caminhada.

Aos meus pais, **Paulo Jorge** e **Sônia Maria**, que sempre foram minha base, minha força, meu porto seguro e a quem devo tudo o que sou.

Ao meu amigo e meu amor **Luan Ramos**, por sua paciência, respeito e parceria, me permitindo crescer ao seu lado como ser humano. Sou muito feliz e realizada por ter você.

À professora **Ana Maria Benko Iseppon**, que me permitiu e me direcionou à conquista desse sonho. Uma das mulheres nas quais me inspiro e que tem minha admiração plena e gratidão eterna.

Ao **João Pacífico,** por toda sua paciência e carinho nos meus inúmeros momentos de desespero, me mostrando a luz da calmaria e da vitória. Você é mais que um coorientador na minha vida, você é um amigo que quero levar para sempre comigo.

À **Lidiane Amorim**, para sempre minha 'mãe científica'. A gratidão que sinto por tudo que você me ensinou nunca será expressa suficientemente. Obrigada por ter se preocupado com a minha formação desde a iniciação científica até o doutorado.

A todos da família LGBV, pelos momentos vividos, as boas e numerosas risadas e todo conhecimento compartilhado. Momentos únicos, com cada um, que levarei comigo sempre. Em particular, agradeço àqueles que me deram motivos especiais para continuar: **Flávia Czekalski, Artemisa Borges, Carolline Pires, Carlos André, Livia Maria, Vanessa Cristina, Neto Ferreira, Diogo Cavalcanti** e **Marx de Oliveira.**

À **Thialisson Cacci** e **Wilson Dias**, que me permitiram crescer como formadora ao orientá-los em seus projetos acadêmicos. A aprendizagem é mútua nessa caminhada e desejo muito sucesso aos dois.

À toda equipe gestora e docente da escola Frei Campo Mayor, que me acolheram com um carinho absurdo, facilitando minha jornada dupla. Em especial, agradeço aos meus amigos **Ivone Sales, Karla Danielle, Juliana Albertim, Ricardo Anderson, João Alfredo** e **Pablo Gonzaga**, pela honra em ter sido acolhida por vocês.

Aos membros da banca examinadora, pelas contribuições e com a minuciosa leitura para o aperfeiçoamento deste trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - **CAPES**, pela bolsa concedida durante a execução deste trabalho e pelo apoio financeiro para a geração do transcriptoma do feijão-caupi, através da Rede Intersys (Edital Biologia Computacional).

À Universidade Federal de Pernambuco (**UFPE**), por meio do Programa de Pós-Graduação em Genética (**PPGG**), pela oportunidade e suporte durante todo o período do Doutorado.

MUITO OBRIGADA!!!.

“Cada descoberta nova da ciência é uma porta nova pela qual encontro mais uma vez Deus, o autor dela”.

(Albert Einstein)

RESUMO

A seca é um dos fatores ambientais de maior impacto sobre a produtividade agrícola, podendo reduzir ou até mesmo inviabilizar o processo fotossintético. Neste cenário, o feijão-caupi [*Vigna unguiculata* (L.) Walp.] figura como uma das leguminosas mais tolerantes, porém ainda sofre com regimes de secas ocorrentes. Dentre os mecanismos de resposta vegetal, destaca-se a modulação da regulação gênica mediada pelos fatores de transcrição (*Transcription Factors* -TFs) associados a várias vias envolvidas na obtenção da tolerância da planta. Assim, buscamos identificar e caracterizar os TFs WRKY, uma das maiores famílias de TFs em plantas, no genoma do feijão-caupi e avaliar seu perfil transcricional (RNA-Seq e qPCR) sob desidratação radicular em genótipos contrastantes. Também selecionamos genes de referência adequados para as análises de expressão gênica via qPCR. Ferramentas bioinformáticas permitiram a caracterização de 92 genes *VuWRKY*, revelando a estrutura organizacional e evolutiva da família WRKY na espécie. Os dados transcricionais obtidos no banco CpGC (*Cowpea Genome Consortium*) permitiram associar 97 isoformas a 55 genes *VuWRKY*, revelando sequências promissoras para validação experimental da resposta. Para uma análise mais precisa e confiável da expressão gênica, selecionamos genes de referência para a desidratação radicular (e estresse salino), para serem utilizados na qPCR. Os resultados confirmaram o padrão de resposta dos VuWRKY, indicando um universo de 22 alvos induzidos sob desidratação radicular. Em conjunto, nossos dados fornecem potenciais candidatos para estudos biotecnológicos futuros visando à tolerância ao estresse hídrico no feijão-caupi e espécies próximas.

Palavras-chave: Bioinformática. Desidratação radicular. Genes de Referência. qPCR. Transcriptoma.

ABSTRACT

Drought is one of the environmental factors with the greatest impact on agricultural productivity, and may reduce or even render the photosynthetic process unfeasible. In this scenario, cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most tolerant legumes, but still suffers from drought regimes. Among the mechanisms of plant response, the modulation of gene regulation mediated by transcription factors (Transcription Factors -TFs), associated with several pathways involved in obtaining plant tolerance, stands out. Thus, we sought to identify and characterize WRKY TFs, one of the largest families of plant TFs, in the cowpea genome and to evaluate its transcriptional profile (RNA-Seq and qPCR) under root dehydration in contrasting genotypes. We also selected suitable reference genes for qPCR gene expression analysis. Bioinformatic tools allowed the characterization of 92 *VuWRKY* genes, revealing organizational and evolutionary structure of the WRKY family on specie. The transcriptional data obtained from CpGC database (Cowpea Genome Consortium) allowed to associate 97 isoforms with 55 *VuWRKY* genes, revealing promising sequences for experimental response validation. For a more accurate and reliable analysis of gene expression, we selected reference genes for root dehydration (and saline stress) to be used in qPCR. The results confirmed response pattern of VuWRKY, indicating a universe of 22 targets induced under root dehydration. Taken together, our data provide potential candidates for future biotechnological studies aiming at water stress tolerance in cowpea and nearby species.

Key words: Bioinformatics. Root Dehydration. Reference Genes. qPCR. Transcriptome.

LISTA DE ILUSTRAÇÕES

Revisão Bibliográfica

Artigo I

LISTA DE TABELAS

Revisão Bibliográfica

Artigo I

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ABA	*Abscisic Acid* (Ácido abscísico)
ACT	*Actin* (Actina)
AP2/ERF	APETALA 2/*Ethylene Response Factor* (Fator Responsivo ao Etileno/ APETALA 2)
AREBs	*ABA-Responsive Elements Binding Proteins* (Proteínas de Ligação ao Elemento Responsivo ao ABA)
BiFC	*Bimolecular Fluorescence Complementation* (Complementação Bimolecular da Fluorescência)
BLAST	*Basic Local Alignment Search Tool* (Ferramenta de Busca por Alinhamento Local)
BRS3T	*Salinity Sensitive Accession After Stress – bulk of three stress times* (Cultivar Sensível à Salinidade após o Estresse - Junção de Três Tempos de Estresse)
BRT0	*Sensitive Cultivar, Negative Control* (Cultivar Sensível – Controle Negativo)
bZIP	*Basic Leucine Zipper* (Zíper de Leucina Básica)
C2H2	Cisteína-2-Histidina-2
Ca	*Calcium* (Cálcio)
CABMV	*Cowpea Aphid-Borne Mosaic Virus* (Vírus do Mosaico Transmitido por Afídeos)
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
cDNA	*Complement DNA* (Dna complementar)
CGI	*Cowpea Genomics Initiative* (Iniciativa do Genoma do feijão-caupi)
CGKB	*Cowpea Genomics Knowledge Base* (Base de Conhecimentos Genômicos de Feijão-Caupi)
CHI	*Chalcone Isomerase* (Chalcona Isomerase)
CHiB	*Chitinase B* (Quitinase B)
CHS	*Chalcone Synthase Lipid Transfer Protein* (Proteína de Transferência de Lípido da Síntese de Chalcona)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico

CpGC	*Cowpea Genome Consortium* (Consórcio do Genoma do Feijão-Caupi)
CpFGC	*Cowpea Functional Genome Consortium* (Consórcio do Genoma Funcional do Feijão-Caupi)
CPSMV	*Cowpea severe mosaic vírus* (Vírus do Mosaico Severo do Caupi)
Cq	*quantification Cycle* (Ciclo de quantificação)
CRG	*Candidate Reference Genes* (Genes de Referência Candidatos)
CV	*Variance Coefficient* (Coeficiente de Variância)
DBD	*DNA Binding Domain* (Domínio de Ligação ao DNA)
DEGs	*Differentially Expressed Genes* (Genes Diferencialmente expressos)
DNA	*Deoxyribonucleic Acid* (Ácido Desoxirribonucleico)
DR	*Down-Regulation* (Regulação Negativa - Repressão)
E3	*Ubiquitin Ligase* (Ubiquitina Ligase)
EF1-α	*Elongation Factor 1-alfa* (Fator deElongação 1-alfa)
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EST	*Expressed Sequence Tag* (Etiqueta de Sequência Expressa)
FBOX	*F-box protein* (Proteína F-box)
FC	*Fold-Change*
FDR	*False Discovery Rate* (Taxa de falsa descoberta)
GAPC	*Glyceraldehyde-3-phosphate dehydrogenase C-subunit*
HT-SuperSAGE	*High-Throughput Super Serial Analysis of Genome Expression* (Super Análise Serial Da Expressão Gênica De Alto Rendimento)
LTP	*Lipid Transfer Protein* (Proteína de Transferência Lipídica)
MEME	*Multiple Em For Motif Elicitation*
MIQE	*Minimum Information for publication of Quantitative real-time PCR Experiments* (Informação Minima para a Publicação de Experimentos de qPCR)
MYB	*Myeloblastosis* (Mieloblastose)
MYC	*Myelocytomatosis* (Mielocitomastose)

NAC NAM/ATAF1/CUC2

NCBI *National Center for Biotechnology Information* (Centro Nacional para Informação Biotecnológica)

NLS *Nuclear Localization Signal* (Sinal de Localização Nuclear)

ns not significant at $p<0.05$ (não significativo a $p<0,05$)

PCR *Polymerase Chain Reaction* (Reação em cadeia da polimerase)

PlanTFDB *Plant Transcription Factor Database* (Banco de Dados de Fatores de Transcrição de Plantas)

PO Pingo de Ouro

POT0 *Tolerant Cultivar, Negative Control* (Cultivar Tolerante – Controle Negativo)

POT1-6 *Root dehydration-Tolerant Cultivar After Stress – bulk of six stress times* (Desidratação Radicular – Cultivar Tolerante após o Estresse – Junção dos Seis Tempos de Estresse)

PTS3T *Salinity Tolerant Cultivar After Stress – bulk of three stress times* (Cultivar Tolerante à Salinidade após o Estresse - Junção de Três Tempos de Estresse)

PTT0 *Tolerant Cultivar, Negative Control* (Cultivar Tolerante – Controle Negativo)

qPCR *quantitative Real-Time PCR* (PCR quantitativa em Tempo Real)

R^2 *Coefficient of Determination* (Coeficiente de Determinação)

REST *Relative expression software tool* (Ferramenta de Software de Expressão Relativa)

RNA *Ribonucleic Acid* (Ácido ribonucleico)

RNA-Seq *RNA Sequencing* (Sequenciamento de RNA)

ROS *Reactive Oxygen Species* (Espécies Reativas de Oxigênio)

RSEM *RNA-Seq by Expectation-Maximization*

SAGE *Serial Analysis of Gene Expression* (Análise Serial da Expressão Gênica)

SD *Standard Deviation* (Desvio Padrão)

SI Santo Inácio

SIT0 *Sensitive Cultivar, Negative Control* (Cultivar Sensível – Controle Negativo)

SIT1-6 *Root Dehydration-Sensitive Cultivar After Stress – bulk six stress times* (Desidratação Radicular – Cultivar Sensível após o Estresse – Junção dos Seis Tempos de Estresse)

TFs *Transcription Factors* (Fatores de Transcrição)

Tm *Melting Temperature* (Temperatura de Melting)

UBQ10 *Polyubiquitin 10* (Poliubiquitina 10)

UE21D *Ubiquitin-conjugating enzyme E2 variant 1D*

UNK *Unknown* (Desconhecido)

UR *Up-Regulated* (Super-Regulado)

VGE *Validation of Gene Expression* (Validação da Expressão Gênica)

WRKY Sequência de aminoácido WRKYGQK

ZF *Zinc Finger* (Dedos de Zinco)

β-TUB *Beta-Tubulin* (Beta-Tubulina)

SUMÁRIO

1 INTRODUÇÃO

O feijão-caupi [*Vigna unguiculata* (L.) Walp.], também conhecido como feijão verde, feijão de corda ou feijão macassar, pertence ao grupo das Leguminosas e figura como uma cultura agrícola de significativa importância socioeconômica, especialmente para o Norte/Nordeste do Brasil. A cultura é particularmente caracterizada pelo alto teor de proteínas, boa adaptação a solos de baixa fertilidade, temperaturas elevadas e ambientes de baixa disponibilidade hidrica. Embora seja usada principalmente como uma cultura de grãos, o feijão-caupi também é empregado como forrageira, para adubação verde e proteção do solo, uma vez que se associam simbioticamente com bactérias do gênero *Rhizobium*.

Apesar dessas características vantajosas, o feijão-caupi pode ter sua produtividade diminuída em regiões com limitações hídricas frequentes. Sua capacidade de produção em condições ambientais ótimas pode chegar a 3.000 $kg.ha^{-1}$. Entretanto, a produtividade média da cultura no Brasil, entre os anos de 2007 e 2017, foi de 381 $kg.ha^{-1}$ (Embrapa Arroz e Feijão, 2018). Em área plantada, a região Nordeste se destaca no cultivo do feijão-caupi, porém muitas vezes a cultura experimenta períodos de seca moderada, devido à baixa precipitação pluviométrica regional. Dessa forma, o desenvolvimento de variedades bem adaptadas para as condições de déficit hídrico aumentaria o rendimento da cultura, especialmente nessa região.

Vários são os atores moleculares envolvidos na percepção do estresse, transdução de sinal e respostas à seca em plantas. Em termos de respostas vegetais, os fatores de transcrição (*Transcription Factors* - TFs) destacam-se como elementos-chave dos mecanismos de sinalização e adaptação da planta por meio de interações específicas com elementos *cis*-regulatórios nos promotores de genes relacionados ao estresse. Tais proteínas atuam na orquestração molecular ativando ou reprimindo a expressão de genes-alvo e direcionando a expressão de forma sincronizada.

Inúmeras famílias de TFs são descritas e conhecidas por sua atuação como integradores centrais de vias específicas ativadas pela seca. TFs WRKY figuram como uma das maiores famílias de reguladores transcricionais em plantas, atuando em cascatas de sinalizações que modulam diversas respostas vegetais, incluindo

a seca. A família é caracterizada pela presença de pelo menos um domínio de ligação ao DNA (*DNA Binding Domain* – DBD), composto por 60 resíduos de aminoácidos com um heptapeptídeo [WRKYGQK] altamente conservado, seguido de um motivo dedo de zinco não canônico (C2H2 ou C2HC) na região C-terminal. Um número crescente de trabalhos tem investigado o potencial dessa família de TF para tolerância à seca em vegetais.

Neste sentido, diversos segmentos biotecnológicos têm concentrado seus esforços na criação de cultivares com características agronômicas desejáveis e que contribuam para minimizar as perdas na sua produção. A identificação de genes WRKY centrais nas vias de resposta à desidratação radicular em acessos tolerantes do feijão-caupi, pode apresentar-se como um recurso para modificação de caracteres complexos na cultura e em outras plantas relacionadas, além de se mostrarem como prováveis recursos tecnológicos para a próxima geração de cultivos biotecnológicos.

Para o feijão-caupi, a disponibilização recente do sequenciamento genômico de alta qualidade tem permitido que estudos funcionais progridam na espécie, auxiliando na compreensão da base genética frente às respostas adaptativas. A associação dessas informações ao genoma expresso em condições estressantes fornecerá candidatos em potencial para o melhoramento da cultura. Neste contexto, a base de dados CpGC (*Cowpea Genome Consortium*) possui significativa disponibilidade de dados transcricionais da espécie sob diferentes estresses ambientais, obtidos pela tecnologia do RNA-Seq.

A validação dos candidatos identificados *in silico,* envolvidos nas vias de tolerância/resistência do vegetal, tem sido crucial para os estudos em curso. A PCR quantitativa em tempo real (qPCR) é considerada um método valioso e confiável para quantificação de genes, mas apresenta algumas variáveis que devem ser consideradas para sua aplicação. Um dos pré-requisitos mais importantes da técnica é a escolha de genes de referência confiáveis para normalização precisa dos níveis de expressão.

Neste contexto, o presente trabalho objetivou identificar e caracterizar a família de TF WRKY no feijão-caupi em nível estrutural e funcional, utilizando os dados genômicos e transcriptômicos disponíveis para a espécie (CpGC), além de analisar sua modulação frente à desidratação radicular, validando os melhores candidatos para aplicações biotecnológicas. Ainda, objetivamos identificar genes

de referência para normalização das reações de qPCR para feijão-caupi quando submetido a diferentes estresses abióticos.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Identificar e caracterizar genes e transcritos codificantes dos TFs da família WRKY relacionados à resposta sob desidratação radicular no feijão-caupi, validando os melhores candidatos diferencialmente expressos e relacionando-os às interações existentes com outros grupos responsivos aos estresses.

1.1.2 Objetivos específicos

- Realizar anotação estrutural e funcional de TFs WRKY relacionados à desidratação radicular no banco de dados Phytozome, CpGC e em bancos de dados públicos;

- Estabelecer um perfil da expressão *in silico* dos transcritos modulados sob a condição analisada;

- Validar a expressão dos melhores candidatos a TFs diferencialmente expressos por meio de PCR em tempo real (qPCR).

- Identificar genes normalizadores para reações de qPCR no feijão-caupi submetido a estresse.

2 REFERENCIAL TEÓRICO

2.1 EFEITOS DOS FATORES AMBIENTAIS SOB O CRESCIMENTO E DESENVOLVIMENTO DAS PLANTAS

Os organismos vivos estão em constante interação com os fatores externos, tratando-se de situação particularmente verdadeira para as plantas. Uma vez que estão enraizadas no ambiente em que crescem, os vegetais ficam sujeitos às mudanças de condições provocadas pela multiplicidade de fatores ambientais (Pereira, 2016). Para compreender o processo de resposta de um organismo particular em uma determinada condição, as influências ambientais geralmente são consideradas separadamente. Tais fatores podem ser de natureza biótica ou abiótica e interferem diretamente no desenvolvimento e sobrevivência dos vegetais (Schulze et al., 2005).

Os fatores ambientais bióticos compreendem as interações diretas com outros seres vivos, as quais podem ser benéficas, como a simbiose com bactérias fixadoras do nitrogênio atmosférico, ou ainda prejudiciais, como infecções por patógenos, danos mecânicos por herbivoria ou pisoteio, entre outros (Hartley, 2001). Sob condições naturais, as plantas têm que lidar com diferentes organismos, incluindo outras plantas, que interagem a qualquer momento do seu desenvolvimento, seja competindo pelos mesmos recursos, se defendendo dos ataques ou colaborando com microrganismos para seu crescimento (Brooker, 2006; van Dam, 2008; Glazebrook e Roby, 2018). As vias de resposta para cada tipo de interação devem ser precisamente caracterizadas pela planta, diferenciando os sinais patogênicos *versus* mutualistas pelos sistemas de percepção/receptor, a fim de adaptar suas respostas (Plett e Martin, 2018).

Já os fatores abióticos, incluem parâmetros e recursos que determinam o crescimento de uma planta, como intensidade luminosa, temperatura, disponibilidade hídrica, minerais e CO_2, entre outros. Entretanto, o efeito de cada fator abiótico sobre a planta irá depender da quantidade ou intensidade deste, podendo ter uma influência benéfica, e assim auxiliar no seu desenvolvimento, ou atuar de maneira prejudicial, quando disponíveis em níveis extremos ou insuficientes (Schulze et al., 2005; Pereira, 2016). Os vegetais dificilmente

encontram quantidades e/ou intensidades ótimas de todos os recursos abióticos essenciais para o seu desenvolvimento. Além do crescimento, outros processos biológicos (como a reprodução, propagação da semente, etc.) podem ser afetados na planta como uma reação à variação na disponibilidade dos recursos abióticos presentes no meio (Schulze et al., 2005), gerando situações para as quais usamos o termo estresse (Kranner et al., 2010; Cramer et al., 2011). Assim, fatores ambientais que causam o estresse e suas reações podem servir como medida para classificar a força do estresse em uma escala de intensidade, variando de deficiência à oferta excessiva (Madlung e Comai, 2004; Schulze et al., 2005).

Considerando-se a quantidade de fatores interagindo simultaneamente, os estresses podem ser agrupados em três categorias: **(I)** estresse único, **(II)** múltiplo individual ou **(III)** múltiplo combinado. O estresse único ocorre quando apenas um fator afeta o crescimento e desenvolvimento do vegetal, enquanto o estresse múltiplo representa o impacto de dois ou mais fatores ocorrendo em diferentes períodos sem sobreposição (múltiplo individual) ou simultaneamente com algum grau de sobreposição entre eles (múltiplo combinado). Diferentes combinações de estresse podem resultar em uma gama de efeitos nas plantas, dependendo da natureza, gravidade e duração dos estresses (Pandey et al., 2017).

Entre os principais agentes bióticos causadores de estresse para as plantas, podemos citar bactérias, fungos, nematoides, vírus e insetos herbívoros. Doenças causadas pelo ataque de tais agentes são responsáveis por grandes perdas de rendimento das culturas agrícolas em nível mundial (Redondo-Gómez, 2013; Verma et al., 2013). Já os estressores abióticos incluem níveis extremos de luz (alta e baixa), radiação (UV-A e UV-B), temperatura excessivamente alta ou baixa (frio, congelamento), disponibilidade de água (seca, inundação e submersão), fatores químicos (metais pesados e pH do solo), excesso de sódio (salinidade), insuficiência ou excesso de nutrientes essenciais, poluentes gasosos (ozônio, dióxido de enxofre), fatores mecânicos e outros estressores de menor frequência (Redondo-Gómez, 2013; Pereira, 2016).

2.2 ESTRESSES BIÓTICOS E ABIÓTICOS EM VEGETAIS

Os vegetais desenvolveram ao longo do seu processo evolutivo, mecanismos de respostas que lhes permitam detectar alterações externas e

responder às variáveis ambientais estressantes, ajustando sua fisiologia e metabolismo, a fim de minimizar as perdas sofridas, ao manter recursos para o crescimento e reprodução em um ambiente de contínua mudança (Pinto et al., 2011; Velázquez et al., 2011; Atkinson e Urwin, 2012).

A primeira etapa para o estabelecimento da resposta de defesa nos vegetais é a percepção do estresse, permitindo uma aclimatação rápida e eficiente às condições estressantes do meio (Rejeb et al., 2014). Entretanto, em ambientes naturais, as plantas podem, frequentemente, ser acometidas por múltiplos estresses de qualquer natureza e, por vezes, de maneira simultânea. O efeito da interação simultânea de tais fatores podem ser antagônicos, sinérgicos ou aditivos para os vegetais, sendo a resposta a tais combinações ditada pela natureza da relação entre os fatores estressores (Atkinson et al., 2013; Rejeb et al., 2014; Pandey et al., 2015a, b; Choudhary et al., 2016; Ramu et al., 2016; Pandey et al., 2017). No caso de algumas coocorrências, as interações não acontecem apenas entre o fator estressor e a planta na interface da planta, mas também diretamente entre os fatores de estresse dentro e fora da interface da planta. A natureza de tais interações é determinante para a magnitude do impacto na resposta das culturas (Mittler, 2006; Pandey et al., 2017).

As chances de ocorrência de estresses abióticos no futuro tendem a um aumento gradual na frequência e amplitude frente às mudanças nas condições climáticas, afetando significativamente a produtividade das culturas (Ahuja et al., 2010; Mittler et al., 2012; Ramegowda e Senthil-Kumar, 2015). Em um cenário de mudanças climáticas, a seca e o estresse térmico são talvez os dois fatores mais limitantes do crescimento das culturas agrícolas e sua combinação induz muitas alterações fisiológicas, comprometendo o rendimento e a qualidade dos vegetais (Mittler, 2006; Prasad et al., 2011; Carmo-Silva et al., 2012; Vile et al., 2012; Jedmowski et al., 2015). Do mesmo modo, plantas que crescem em regiões áridas e semiáridas muitas vezes tendem a encarar efeitos mais acentuados do estresse salino quando combinados com o estresse térmico (Keles e Öncel, 2002; Wen et al., 2005; Li et al., 2011). Outras diferentes combinações de estresses abióticos que ocorrem na natureza podem ter um efeito prejudicial significativamente maior que o causado por cada estresse individual, o que já tem sido relatado por diversos autores (Tabela 1).

Tabela 1: Interações negativas ou positivas considerando-se as coocorrências de estresses ambientais em plantas (adaptado de Suzuki et al., 2014 e de Lamichhane e Venturi, 2015).

Tipo de interação	Natureza do estresse	Coocorrência de estresses	Referências
Interação negativa	Abiótico *vs.* Abiótico	Calor + alta luz	Mittler e Blumwald, 2010
		Calor + ozônio	Kasurinen et al., 2012
		Calor + salinidade	Li et al., 2011
		Calor + UV	Mittler e Blumwald, 2010
		Nutrientes + CO_2	Mittler e Blumwald, 2010
		Salinidade + nutrientes	Mittler e Blumwald, 2010
		Salinidade + ozônio	Mittler e Blumwald, 2010
		Seca + calor	Prasad et al., 2011; Vile et al., 2012
		Seca + compactação do solo	Alameda et al., 2012
		Seca + congelamento	Sales et al., 2013
		Seca + frio	Su et al., 2015
		Seca + metais pesados	Silva et al., 2012
		Seca + nutrientes	Mittler e Blumwald, 2010
		Seca + salinidade	Ahmed et al., 2013
		Seca + UV	Bandurska et al., 2013
		UV + metais pesados	Srivastava et al., 2012
	Abiótico *vs.* Biótico	Calor + patógenos	Zhu et al., 2010; Madgwick et al., 2011; Richerzhagen et al., 2011; Zhang et al., 2012; Ahanger et al., 2013; Aguilar et al., 2015.
		CO_2 + patógenos	Góría et al., 2013
		Nutrientes + patógenos	Mittler e Blumwald, 2010
		Ozônio + patógenos	Pollastrini et al., 2015
		Salinidade + patógenos	Mittler e Blumwald, 2010; Kissoudis et al., 2015, 2016
		Seca + patógenos	Choi et al., 2013; Valerio et al., 2013; Davis et al., 2014; Ochola et al., 2015
		Seca + calor + patógenos	Prasch e Sonnewald, 2013; Sharma e Pande, 2013
		Seca + salinidade + patógenos	Goudarzi et al., 2011
	Biótico *vs.* Biótico	Patógeno + patógeno	McMullen et al., 2012; Ma et al., 2013; Whitelaw-Weckert et al., 2013; Del Ponte et al., 2014; Kuzdraliński et al., 2014; Van Mölken et al., 2014; Lamichhane e Venturi 2015.
Interação Positiva	Abiótico *vs.* Abiótico	CO_2 + alta luz + salinidade	Perez-Lopez et al., 2013
		Ozônio + UV	Mittler e Blumwald, 2010
		Salinidade + calor	Rivero et al., 2013
		Salinidade + CO_2	Zaghdoud et al., 2013; Yi et al., 2015
		Seca + CO_2	Song et al., 2014
		Seca + ozônio	Iyer et al., 2013
	Abiótico *vs.* Biótico	Calor + patógenos	Ghandi et al., 2016
		Ozônio + patógenos	Mittler e Blumwald, 2010
		Salinidade + patógenos	Moldakimova et al., 2012
	Biótico *vs.* Biótico	Patógeno + herbívoro	Van Mölken et al., 2012

Além da forte influência exercida sobre os fatores abióticos, é presumível ainda que as alterações climáticas interfiram na relação planta-patógeno, uma vez

que podem aumentar a susceptibilidade das plantas aos organismos patogênicos e às pragas herbívoras, além de reduzir sua capacidade competitiva com ervas daninhas (Valerio et al., 2013; Suzuki et al., 2014; Pandey et al., 2017). Sabe-se que o aumento da temperatura facilita a propagação de patógenos (Kudela, 2009; Zhang et al., 2012; Ahanger, 2013; Sharma e Pande, 2013; Aguilar et al., 2015) e que solos em condições salinas e/ou de seca podem aumentar a severidade do ataque patogênico (Goudarzi et al., 2011; Choi et al., 2013; Sharma e Pande, 2013; Kissoudis et al., 2015; Ochola et al., 2015; Kissoudis et al., 2016). Semelhante às diferentes combinações citadas, as plantas ainda enfrentam mais de um estresse biótico simultaneamente ou sequencialmente (Tabela 1). A ocorrência de coinfecções fúngicas, virais, bacterianas e/ou ataque de herbívoros (Mbega et al., 2012; Van Mölken et al., 2012; Dung et al., 2013; Barros et al., 2014; Van Mölken et al., 2014; Cuellar et al., 2015) são comuns na natureza, podendo causar sintomas mais severos de uma ou de ambas as doenças.

A exposição simultânea aos diferentes estressores bióticos e abióticos resulta na implantação de estratégias adaptativas que são diferentes, mas por vezes compartilhadas com as respostas observadas sob estresses únicos nos vegetais (Atkinson e Urwin, 2012; Rejeb et al., 2014; Pandey et al., 2015). A capacidade das plantas em reconhecer, integrar e responder a tais variáveis é facilitada por fenômenos de tolerância cruzada, em que a exposição a um único estresse desencadeia uma maior tolerância a diversos outros fatores ambientais (Foyer et al., 2016). Desse modo, estudos sobre estresses individuais permitem identificar vias metabólicas e de sinalização que se cruzam, auxiliando também na elucidação dos mecanismos de resposta exclusivos de cada estresse, adaptados às necessidades específicas da planta (Atkinson e Urwin, 2012; Suzuki et al., 2014).

2.3 RESPOSTAS VEGETAIS A CONDIÇÕES ABIÓTICAS ADVERSAS

As respostas vegetais aos estresses abióticos são altamente complexas e envolvem mudanças nos níveis fisiológico, bioquímico e molecular, ativando um programa específico de expressão gênica referente à condição ambiental que lhes está sendo imposta (Atkinson e Urwin, 2012). Este rearranjo metabólico pode vir a influenciar sua viabilidade celular por meio da produção de ROS (*Reactive Oxygen Species*), responsáveis pela oxidação dos componentes multicelulares, como as

proteínas, lipídios e ácidos nucléicos, causando danos celulares extensos e inibição de processos fisiológicos, com consequente aumento do dano (Jaspers e Kangasjärvi, 2010; Zhang et al., 2014).

Para sobreviver a essas condições, as plantas ativam mecanismos de adaptação moleculares que repercutem em todos os níveis de organização. Em nível celular, as respostas incluem ajustes do sistema de membrana, modificações da arquitetura da parede celular, alterações no ciclo e na divisão celular (Fujita et al., 2006). Em nível genômico, as plantas ativam genes estresse-induzidos que estão envolvidos na proteção direta contra os estresses, que se classificam em dois grupos: **(I)** proteínas funcionais e estruturais, e **(II)** proteínas reguladoras (Benko-Iseppon et al., 2011). O primeiro grupo inclui genes codificadores de proteínas de membrana, enzimas para biossíntese de osmólitos, enzimas de desintoxicação e proteínas de proteção (Reis et al., 2012). O segundo grupo inclui proteínas reguladoras da transdução do sinal, como as quinases ou fosfatases, e os fatores de transcrição (Alves et al., 2013) (Figura 1).

Já o minucioso controle dos genes estresse-induzidos pode ocorrer em dois níveis da expressão gênica: (1) a nível transcricional (quantidade de mRNA produzido) e/ou (2) a nível traducional (quantidade de proteínas produzidas) (Alves et al., 2014). Para a maioria dos genes, essa regulação é realizada em nível transcricional, garantindo desta forma um menor gasto energético da célula, além de níveis adequados de proteínas. Dentre os diversos mecanismos regulatórios transcricionais, os fatores de transcrição (*Transcription Factors – TFs*) estão no topo de cascatas moleculares, o que faz com que qualquer alteração na sua atividade resulte na modulação das redes de adaptação da planta, tornando-os potenciais alvos para melhorar as respostas a estresses abióticos complexos (Tripathi et al., 2014). De forma genérica, a regulação da transcrição é o resultado dos efeitos combinados das propriedades estruturais do DNA e suas interações com os TFs.

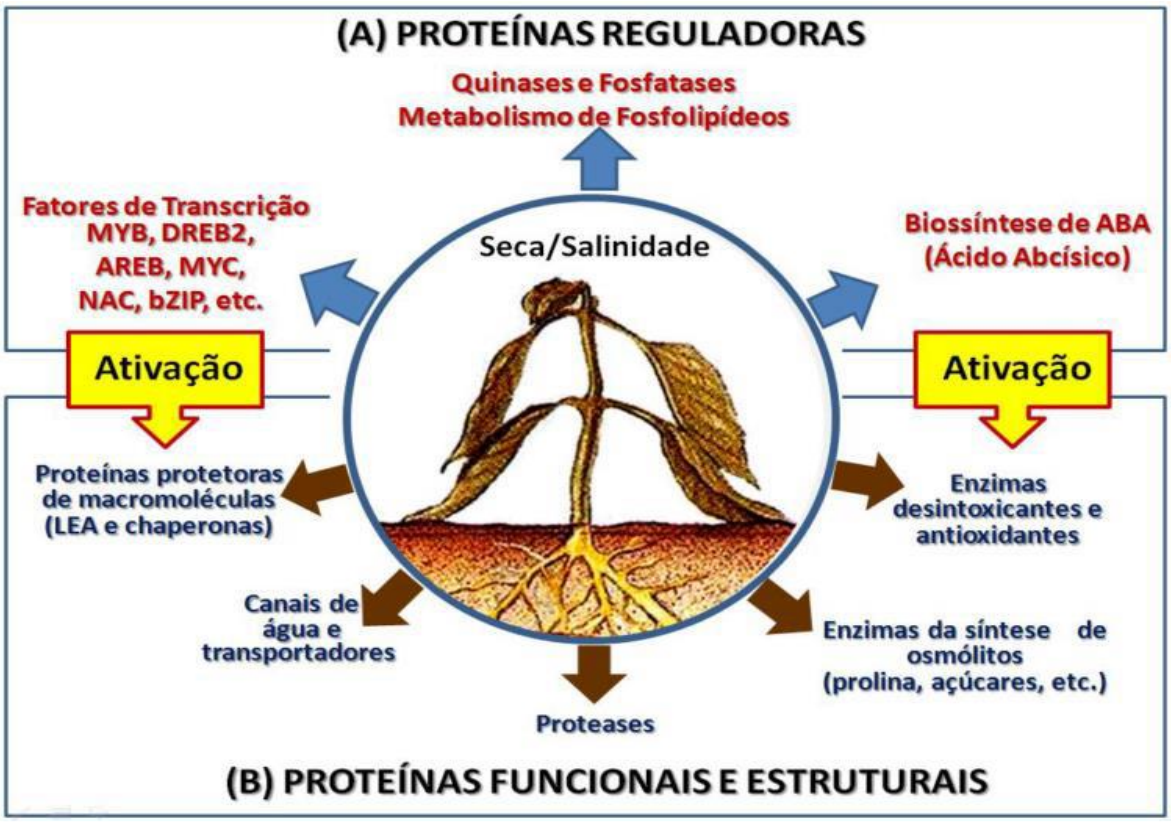


Figura 1: Principais grupos de genes envolvidos na resposta aos estresses abióticos, especialmente a seca e salinidade. As células vegetais são estimuladas frente ao estresse e enviam sinais por diferentes cascatas de sinalização (A), ativando genes que codificam proteínas envolvidas diretamente na resposta ao estresse (B). (Fonte: Benko-Iseppon et al., 2011).

2.3.1 TFs envolvidos na resposta a estresses

Os TFs estão entre os principais elementos envolvidos no processo de tolerância ao estresse, participando de cascatas de eventos moleculares alterando diretamente a expressão de genes de defesa (Amorim et al., 2017). A modulação da função dos TFs através de interações com proteínas reguladoras é um processo crucial na ativação ou repressão de caminhos de transdução de sinais (Van et al., 2009). Em *Arabidopsis*, verificou-se que estresses como seca, salinidade, injúria, metais pesados, estresse osmótico e oxidativo, induzem a expressão de TFs para modular genes específicos com regiões de ligação definidas (Figura 2). A importância desses fatores para a regulação dos genes de estresse induzidos se reflete na composição genômica dos vegetais. Em *Arabidopsis thaliana* (L.) Heynh, por exemplo, são conhecidos de 27.416 genes codificadores de proteínas, mais de 1.700 genes (6%) codificam TFs (Feller et al., 2011).

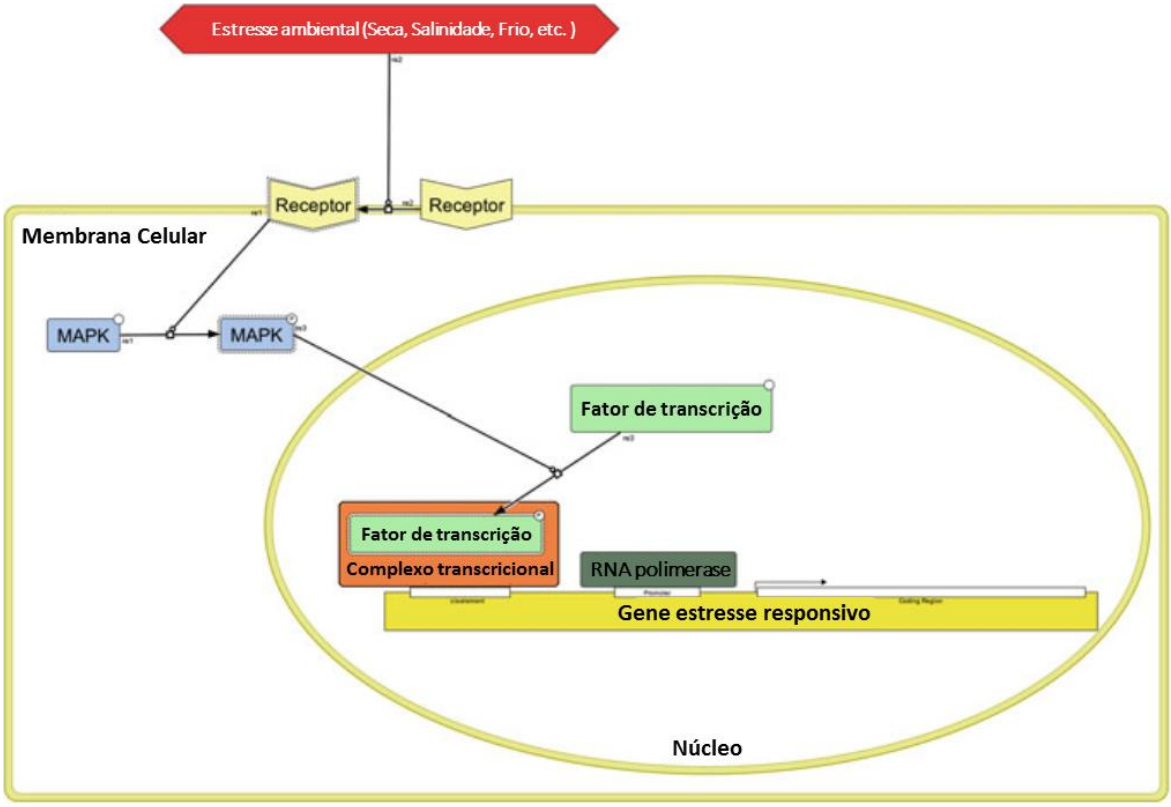


Figura 2: Modelo de ativação transcricional e regulação da expressão gênica frente ao estresse abiótico. O modelo mostra proteínas (TFs, MAPK - *Mitogen-Activated Protein Kinases*), receptores gerais, genes e complexo proteico (Fonte: Amorim et al., 2016).

Os TFs são proteínas que reconhecem motivos de DNA em regiões reguladoras ou intensificadoras de genes e, desta forma, facilitam ou inibem o acesso da RNA polimerases ao DNA. A ligação dos TFs a esses motivos é diretamente influenciada pela conservação dessas sequências, sua abundância no DNA, assim como da conservação de domínios de ligação (Amorim et al., 2017). Os TFs são compostas por, no mínimo, quatro domínios discretos: o domínio de ligação ao DNA, o sinal de localização nuclear (NLS - *Nuclear Localization Signal*), o domínio de ativação da transcrição e o local de oligomerização que, juntos, atuam como reguladores da expressão de genes-alvo por meio de transdução de sinais acionando diversas vias (Du et al., 2009).

Além disso, eles ainda interagem com a maquinaria de transcrição gênica, com as proteínas que remodelam a cromatina e até mesmo com outros TFs. Essas proteínas atuam como reguladores-chave de inúmeros processos celulares e apresentam-se como excelentes candidatos para modificar caracteres complexos em plantas cultivadas, além de mostrarem-se como prováveis recursos

tecnológicos para a próxima geração de cultivos biotecnológicos (Ambawat et al., 2013).

Os TFs são classificados em famílias distintas de acordo com suas características estruturais e a conservação dos seus domínios de ligação ao DNA, podendo ainda subdividir-se de acordo com o número e a distância das regiões conservadas. Exemplos incluem hélice-alça-hélice, dedos de zinco, hélice-volta-hélice e zíper de leucina (Liu et al., 1999; Cai et al., 2012). A modulação da função de TFs através das suas interações com outras proteínas reguladoras é um processo crucial para a ativação ou repressão de vias de transdução de sinal que são reguladas pelas interações entre proteínas com diferentes TFs (Alves et al., 2014).

Nos últimos anos, estudos têm identificado várias proteínas que interagem com TFs envolvidos na defesa vegetal. Mais de 50 famílias de TFs funcionam como integradores das complexas redes de regulação gênica ativadas por estresse (Krannich et al. 2015). Dentre as principais famílias identificadas nos organismos vegetais, merecem destaque a WRKY (contém a sequência de aminoácido WRKYGQK); AP2/ERF (APETALA 2/*Ethylene Response Factor*); bZIP (*Basic Leucine Zipper*); MYC (*Myelocytomatosis Related Proteins*); NAC (NAM/ATAF1/CUC2) e MYB (*Myeloblastosis Related Proteins*) (Alves et al., 2014). Uma fração significativa dos membros dessas famílias de TFs desempenha um papel importante no mecanismo de resposta ao estresse, o qual aumenta os seus níveis de expressão para favorecer o desenvolvimento de cultivares tolerantes.

- TFs da família WRKY

Os TFs WRKY figuram como uma das famílias de proteínas reguladoras mais abundantes do reino vegetal (Rushton et al., 2010). A família é definida pela presença de um domínio de ligação ao DNA altamente conservado, composto por 60 aminoácidos que inclui a sequência heptapeptídica [WRKYGQK] quase invariante na região N-terminal, além de um motivo dedo de zinco do tipo C_2H_2 ($Cx_{4–5}Cx_{22–23}HxH$) ou C_2HC ($Cx_7Cx_{23}HxC$), na porção C-terminal (Rushton et al., 2010).

De acordo com o número de domínios WRKY e o padrão dos motivos dedo de zinco presentes na proteína, bem como em suas relações filogenéticas, a família pode ser classificada em três grandes grupos principais (Eulgem et al., 2000).

Proteínas com dois domínios WRKY são classificadas no grupo I e têm uma maior atividade de ligação ao DNA no domínio C-terminal (Eulgem et al., 1999). Os membros dos grupos II e III apresentam apenas um domínio WRKY na sua estrutura e diferenciam-se pelas sequências de aminoácido do motivo dedo de zinco. Geralmente, proteínas dos grupos I e II compartilham o motivo dedo de zinco do tipo C_2H_2, enquanto o grupo III apresenta o padrão C_2HC. O grupo II é ainda classificados nos subgrupos IIa, IIb, IIc, IId e IIe, de acordo com na sequência primária dos aminoácidos (Eulgem et al., 2000; Rushton et al., 2010).

Yamasaki e colaboradores (2005) revelaram a estrutura tridimensional do domínio WRKY da proteína AtWRKY4 (Figura 3) de *Arabidopsis,* onde a assinatura WRKYGQK está diretamente envolvida na ligação ao DNA. O motivo reconhece e se liga ao elemento W-box com a sequência principal (C/T)TGAC(C/T) presente nas regiões promotoras dos genes-alvo (Eulgem e Somssich, 2007). Alguns genes WRKY apresentam em seus próprios promotores as sequências W-box, atuando como reguladores de seus próprios genes (Petitot et al., 2013). Alterações na sequência heptapeptídica [WRKYGQK] tem sido descritas em outras espécies (Yang et al., 2017; Wan et al., 2018; Singh et al., 2019) e podem estar relacionadas com reconhecimento de novos elementos *cis* regulatórios (Cai et al., 2008).

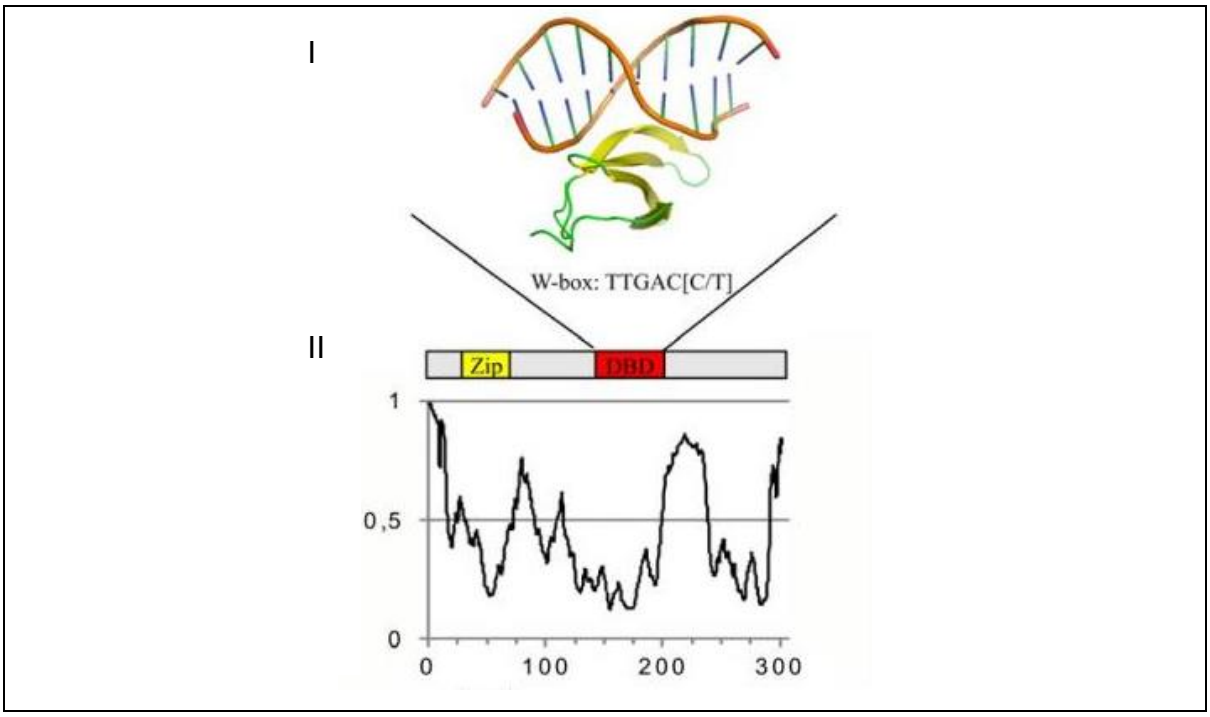


Figura 3: Estrutura por ressonância magnética nuclear (NMR - *nuclear magnetic resonance*) do domínio WRKY de *Arabidopsis* (WRKY4). **(I)** Estrutura terciária da família TF designando DBD em

complexo com DNA. (II) Estrutura primária esquemática desenhada para predições de escala e desordem para um TF selecionado. (Fonte: Lindemose et al., 2013).

TFs da família WRKY foram identificados em muitas espécies de plantas, sendo que o número de representantes varia entre espécie. Exemplos incluem *A. thaliana* (74), *Lotus japônicas* (61), *Oryza sativa* (102), *Triticum aestivum* (160), *Cicer arietinum* (78) e *Medicago truncatula* (98) (Eulgem et al., 2000; Wu et al., 2005; Okay et al., 2014; Song et al., 2014; Kumar et al., 2016). As interações dessas proteínas com outras moléculas podem desempenhar papéis na sinalização, transcrição, remodelação da cromatina e outros processos celulares importantes para a ativação da imunidade da planta (Chi et al., 2013).

2.4 TFS WRKY E A RESPOSTA À SECA EM VEGETAIS

Sob reduzida disponibilidade hídrica, o crescimento e desenvolvimento das plantas sofrem efeitos drásticos. Para minimizar tais efeitos, as plantas modificam sua arquitetura radicular, redirecionando seu crescimento para permitir sua sobrevivência (Bechtold e Field, 2018). A seca, um dos estresses mais limitantes da produtividade agrícola, ainda é responsável pela inibição da fotossíntese, o fechamento dos estômatos, alterações nos processos de respiração e assimilação de nutrientes, entre outros (Lata et al., 2011; Pinheiro e Chaves, 2011; Siddiqui et al., 2015).

Além dos efeitos citados, a deficiência hídrica estimula a produção do fitormônio ácido abscísico (ABA), um regulador central da resposta ao estresse, que confere particular tolerância à seca, induzindo a expressão de genes estresse responsivos via cascatas de sinalização (Cutler et al., 2010; Kim et al., 2015). Além dos eventos regulatórios mediados pelo fitormônio ABA, outros grupos de genes (ativados pelo déficit hídrico) conferem tolerância à planta, seja através da produção de proteínas responsivas ao estresse, seja pela regulação da expressão destas proteínas, mediada pelos TFs (Hirayama e Shinozaki, 2010; Osakabe et al., 2014).

A característica intrínseca dos TFs faz com que qualquer alteração na sua atividade resulte na modulação das redes de adaptação da planta, o que os torna potenciais alvos para melhorar as respostas a estresses abióticos complexos, como

a seca (Tripathi et al., 2014). Neste sentido, estudos acerca dos TFs que respondem ao estresse abiótico, possibilitam a elucidação de mecanismos celulares que definem a adaptação ambiental da planta (Golldack et al., 2011). O papel da família WRKY sob condições de seca, tem sido evidenciado em abordagens biotecnológicas e de transgenia para o desenvolvimento de genótipos mais tolerantes (Phukan et al., 2016; Finatto et al., 2018).

Em soja, Luo e colaboradores (2013) verificaram que a indução de *GsWRKY20* promoveu o fechamento dos estômatos mediado pelo ABA, resultando em um significativo declínio na perda da água e, consequentemente, maior tolerância à restrição hídrica. Na videira (*Vitis vinifera)* os genes *VvWRKY7*, *8* e *28* foram induzidos em resposta ao estresse hídrico pelo acúmulo de ABA, que resultou no fechamento estomático para reduzir a perda de água pela planta (Wang et al., 2014).

Em plantas de *Arabidopsis* superexpressando os genes *TaWRKY1* e *33* do trigo em condições de estresse hídrico, ambas linhagens apresentaram maior taxa de germinação quando comparadas com o tipo selvagem (He et al., 2016). Em outro estudo com *Arabidopsis* superexpressando o *GmWRKY54* da soja, também foi detectado a tolerância ao estresse hídrico (Zhou et al., 2008). Neste estudo, as plantas foram privadas de água por um período de 18 dias e, após a reidratação, apenas 30% das plantas selvagens sobreviveram, contrastando com a taxa de sobrevivência de 85% das linhagens transgênicas.

Plantas de trigo transgênicas superexpressando o TF TaWRKY2 aumentaram a tolerância à seca e o rendimento de grãos em trigo sob condições de estresse hídrico (Gao et al., 2018). Os autores identificaram uma taxa de sobrevivência maior nas linhas transgênicas do trigo, em decorrência da baixa perda de água pelas folhas, quando comparadas com as plantas selvagens. Os teores de prolina, açúcares solúveis e clorofila foram melhor protegidos dos danos oxidativos e osmóticos nas linhas transgênicas. Além disso, o aumento do rendimento de grãos refletiu os efeitos cumulativos de um maior comprimento da panícula, mais grãos por espiga e maior biomassa acima do solo, comparado as plantas selvagens.

Sob estresse hídrico, SlWRKY32 e SlWRKY74 foram significativamente induzidos no tomate (Huang et al., 2012). No arroz, foi demonstrado que a indução do OsWRKY30 em linhagens transgênicas melhorou a tolerância à seca (Shen et

al., 2012). A tolerância a múltiplos estresses, incluindo a seca, também foi observada em plantas quando um único gene WRKY é sobreexpresso (Qiu e Yu, 2009; Wang et al., 2013).

2.5 CARACTERÍSTICAS GERAIS DAS LEGUMINOSAS: CLASSIFICAÇÃO E IMPORTÂNCIA

A família Fabaceae ou Leguminosae, cujos membros são comumente conhecidos como leguminosas destaca-se como a terceira família de plantas superiores com maior riqueza de espécies, abrigando cerca de 730 gêneros e aproximadamente 19.500 espécies (Lewis et al., 2005; Yahara et al., 2013). É um dos grupos mais diversificados e abundantes da flora mundial, com distribuição cosmopolita e importante papel ecológico para quase todos os biomas do mundo, além de apresentar espécies nativas em todos os continentes, à exceção da Antártida (Schrire et al., 2005a, b). Em linhas gerais, as leguminosas apresentam uma diversidade bastante rica no que se refere à morfologia, fisiologia e ecologia de suas espécies, representando um dos grupos mais espetaculares no processo evolutivo de diversificação das plantas (LPWG, 2017). Na flora brasileira, Fabaceae apresenta-se como a família mais rica em diversidade de espécies e figura entre as cinco com maior número de indivíduos do país. São registrados para o Brasil cerca de 222 gêneros e aproximadamente 2.837 espécies de leguminosas distribuídas em todos os domínios fitogeográficos. Estima-se que o Cerrado seja o bioma que abriga maior diversidade de espécies descritas (44,48%), seguido pela Amazônia (40,46%), Mata Atlântica (35,14%), Caatinga (21,71%), Pantanal (5,81%) e Pampa (5,21%) (FDB, 2017).

A riqueza das leguminosas não se resume à sua diversidade e abundância, apresentando ainda uma grande importância ecológica e biológica, uma vez que dependem de uma ampla gama de insetos polinizadores e se relacionam com herbívoros específicos, participando de redes alimentares características (Harmon et al., 2009). O potencial econômico da família é bem acentuado, ficando atrás apenas de Poaceae, incluindo espécies exploradas pela medicina popular; na produção de óleo e resina, cortiça, lenha, carvão, vernizes, tintas e corantes; utilizadas como plantas ornamentais; pela indústria de alimentos, cosméticos e de medicamentos. Além disso, compreende espécies consideradas como modelos

vegetais (*M. truncatula*, *G. max* e *L. japonicus*), que contribuem para o desenvolvimento científico e tecnológico (Lewis et al., 2005; Saslis-Lagoudakis, et al., 2011; Souza e Souza, 2011; Yahara et al., 2013; LPWG, 2017).

Uma das principais características ecológicas das leguminosas é a capacidade que algumas espécies possuem de fixar o nitrogênio atmosférico, quando associadas simbioticamente com bactérias Gram-negativas do gênero *Rhizobium*, o que viabiliza seu uso por agricultores para o melhoramento do solo na adubação verde (Souza e Souza, 2011; Downie, 2014; LPWG, 2017). Adicionalmente, algumas espécies de leguminosas apresentam importância na alimentação animal, sendo utilizadas como forrageiras e estrume verde em regiões temperadas e tropicais (Lewis et al., 2005). Similarmente, a família tem grande destaque na dieta alimentar humana, perdendo em importância apenas para os cereais (Graham e Vance, 2003). Todavia, as leguminosas apresentam características nutricionais complementares as dos cereais, apresentando excelentes fontes de proteínas e minerais essenciais, compostos secundários de promoção à saúde, bem como de óleo vegetal processado para consumo humano, além de compreender um terço de todo o nitrogênio da proteína na dieta (Vance et al., 2000; Grusak, 2002; Graham e Vance, 2003; Gepts et al., 2005).

2.5.1 *Vigna unguiculata* L. (Walp)

O feijão-caupi [*Vigna unguiculata* L. (Walp)], popularmente conhecido como feijão-macassar, feijão verde ou feijão-de-corda, é uma leguminosa amplamente adaptada, versátil e nutritiva, destacando-se como uma das principais fontes de proteínas, vitaminas, sais minerais e fibras em várias regiões do mundo (Timko et al., 2007). Sua plasticidade fenotípica, associada à ampla variabilidade genética resulta em boa adaptação a diversos ambientes. Além disso, apresenta elevado potencial produtivo e boa capacidade de fixação de nitrogênio atmosférico, o que a torna uma cultura de elevado valor estratégico (Freire-Filho et al., 2011).

É uma leguminosa cultivada em diferentes partes do mundo, ocupando cerca de 10,4 milhões de hectares principalmente de regiões tropicais e subtropicais da América, Ásia e África. Sua produção global é estimada em 5,5 milhões de toneladas de grãos, com a Nigéria figurando como o maior produtor mundial (FAO, 2017). O Brasil, de acordo com o registrado para o ano de 2017, conta com uma

área de 1.390.198 hectares para o cultivo do feijão-caupi, com produção de 647.143 toneladas (Embrapa Arroz e Feijão, 2018) (Figura 4).

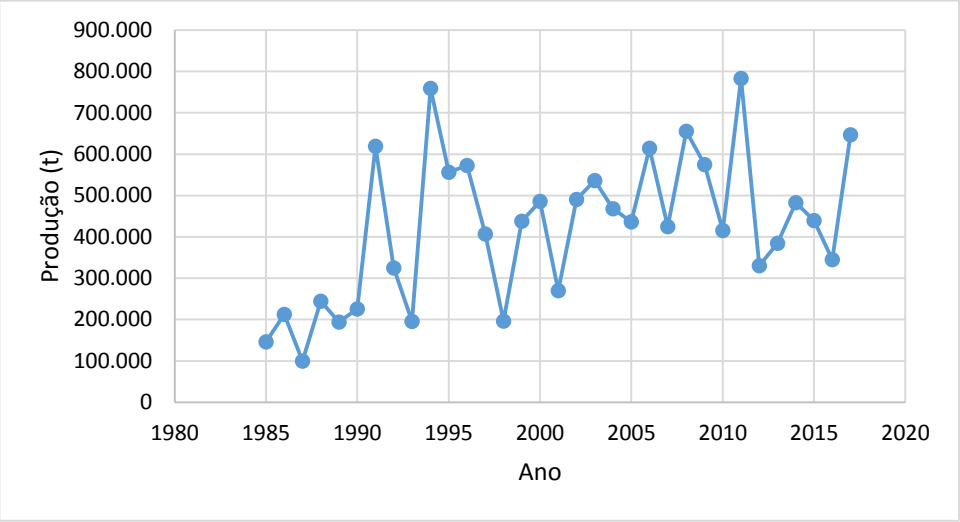


Figura 4: Produção anual de feijão-caupi no BRASIL em toneladas, entre os anos de 1985 e 2017 (Fonte: Embrapa Arroz e Feijão, 2018).

O feijão-caupi é cultivado em quase todo território brasileiro, com safra superior nas regiões Norte, Nordeste e Centro-Oeste (Embrapa Arroz e Feijão, 2018). Em especial nas regiões Norte e Nordeste do Brasil, o feijão-caupi é o principal grão que compõe a dieta da população, principalmente as de baixa renda, além de tratar-se de uma das principais fontes de emprego e renda (Lima et al., 2007; Freire-Filho et al., 2011).

A produção do feijão-caupi no Brasil, considerada há pouco tempo uma atividade exclusivamente voltada ao mercado interno, especialmente na região Nordeste, vem assumido maior importância no mercado externo. Atualmente, os principais importadores de feijão-caupi produzido no Brasil são: Índia, Egito, Paquistão, Vietnã e Indonésia, gerando uma receita de 77,5 milhões de dólares (Damasceno-Silva et al., 2016).

Visando atender às demandas internas e externas do mercado consumidor, a produção do feijão-caupi tem sofrido grandes transformações. Essa progressão tem gerado demanda por cultivares melhoradas e, consequentemente, tem impulsionado o aumento da produtividade, estabelecimento de lavoura tecnificada e produtores com novos perfis, tornando-se um agronegócio bastante promissor no país (Freire-Filho et al., 2011). O maior exemplo desta nova visão é a região Centro-Oeste, cujo cultivo em larga escala é realizado por médios e grandes produtores

que priorizam, entre outros fatores, a utilização de cultivares melhoradas, refletindo diretamente na sua produtividade.

Apesar da recente incorporação no mercado produtor de feijão-caupi (datado do ano de 2006), a região Centro-Oeste detém a maior produtividade nacional da cultura. Em contrapartida, na região Nordeste, em função de ser cultivado por pequenos produtores (agricultura familiar), o feijão-caupi ainda apresenta baixa produtividade em comparação à região Centro-Oeste, apesar de demandar uma grande área de cultivo (Embrapa Arroz e Feijão, 2018) (Figura 5).

Mesmo tendo-se em vista os avanços proporcionados pelos programas de melhoramento genético do feijão-caupi, com a obtenção de cultivares com caracteres de interesse agronômico que atendam às exigências dos pequenos, médios e grandes produtores (Damasceno-Silva, 2009; Freire-Filho et al., 2011), algumas cultivares comerciais não apresentam resistência/tolerância a muitos estresses ambientais que atingem a cultura. Assim, o feijão ainda sofre severas perdas em decorrência de diversos fatores externos. Entre estes fatores podemos destacar os de natureza abiótica, com ênfase para a seca, que se apresenta como um dos fatores mais impactantes na fisiologia do vegetal.

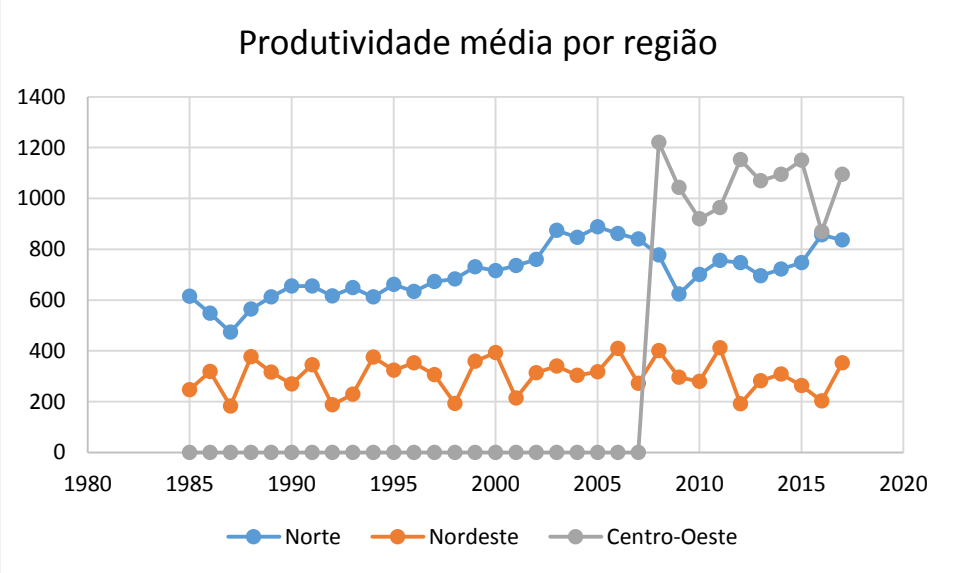


Figura 5: Dados da produtividade média da cultura do feijão-caupi nas regiões mais produtoras do Brasil. (Fonte: EMBRAPA ARROZ E FEIJÃO, 2018).

2.6 REDES DE BIOTECNOLOGIA DO FEIJÃO-CAUPI – BANCO CpGC

Redes de biotecnologia têm direcionado esforços no desenvolvimento de recursos genômicos para a cultura do feijão-caupi, o que têm viabilizado o surgimento e disponibilização de novas metodologias e informações que fornecerão subsídios aos programas de melhoramento genético vegetal dessa leguminosa. Entre os principais recursos de genômica do feijão-caupi, podemos citar: *Physical Map of cowpea* (Close et al., 2011), *HarvEST: Cowpea* (Muchero et al., 2009), *Cowpea Genespace/Genomics Knowledge Base* (CGKB) (Chen et al., 2007), *The Cowpea Genomics Initiative* (CGI) (Chen et al., 2007) e o *Cowpea consensus genetic linkage map* (Lucas et al., 2011). Mais recentemente, foi disponibilizada a montagem do genoma da variedade IT97K-499-35, com um tamanho de montagem de 519,4 Mb em 722 estruturas e 11 pseudocromossomos (Lonardi et al., 2019).

O Brasil, por sua vez, tem contribuído efetivamente na geração de bancos genômicos desta leguminosa. Com início em 2004, o Consórcio do Genoma do Feijão-Caupi (CpGC - *Cowpea Genome Consortium*), um projeto de genômica funcional, estrutural e comparativa do feijão-caupi, que tem propiciado a integração dos pesquisadores e da cultura à rede genômica internacional (Benko-Iseppon et al., 2005, 2008; Kido et al., 2011; Benko-Iseppon, 2009). Especificamente, em relação à transcriptômica, os transcritos expressos foram gerados com diferentes acessos de feijão-caupi (resistentes e susceptíveis/ tolerantes e sensíveis) sob diferentes condições de estresses bióticos [submetidos à infecção por CpSMV (*Cowpea Severe Mosaic Virus*) e CABMV (*Cowpea Aphid-borne Mosaic Virus*)] e abióticos (condições de desidratação radicular e alta salinidade) por meio de sequenciamento de ESTs (*Expressed Sequence Tags*), tags SuperSAGE (*Super Serial Analysis of Gene Expression*) e, mais recentemente, por RNA-Seq (*RNA Sequencing*).

Tais metodologias têm possibilitado a identificação de genes-candidatos potencialmente úteis para o melhoramento da cultura. Sobretudo, a tecnologia de RNA-Seq apresenta diversas vantagens sobre as tecnologias existentes, como exemplo, não se limita ao conhecimento prévio do genoma ou dos transcritos de um organismo, mostrando-se como uma técnica atrativa para espécies cuja sequência genômica não foi previamente determinada (Wang et al., 2009).

Ademais, a tecnologia promete desvendar complexidades anteriormente inacessíveis ao transcriptoma, tais como a identificação de expressão alelo-específica, isoformas de genes, identificação de mutações, novos promotores e a localização exata dos limites da transcrição (Wang et al., 2009; Oshlack et al., 2010).

Desse modo, a geração massiva de dados sobre a expressão de genes em diversas condições pode auxiliar no desenvolvimento de cultivares do feijão-caupi altamente produtivas e rentáveis, sendo a anotação desses genes um dos requisitos básicos para o entendimento da genômica funcional, ou seja, a chave para a interpretação biológica dos dados (Conesa et al., 2005; Shivashankar et al., 2006).

3 RESULTADOS

3.1 ARTIGO I

Artigo a ser submetido na Revista *Frontiers in Plant Science*

Qualis A1

Full Title: Genomic and Transcriptomic Characterization of the WRKY Transcription Factor of Cowpea and its Expression in Response to Water Deficit

Short Title: WRKY TF Family in cowpea under water stress

Mitalle Karen da Silva Matos[1], Ana Maria Benko-Iseppon[1], João Pacífico Bezerra-Neto[1], José Ribamar Feirreira-Neto[1], Yu Wang[2], Hai Liu[2], Valesca Pandolfi[1], Ederson Akio Kido[1], Lilia Willadino[3], Michael P. Timko[2], Ana Christina Brasileiro-Vidal[1*]

[1] Department of Genetics, Biosciences Centre, Universidade Federal de Pernambuco, Recife, PE, Brazil.

[2] Department of Biology, University of Virginia, Charlottesville, VA, USA.

[3] Physiology Area, Department of Biology, Universidade Federal de Pernambuco, Recife PE, Brazil.

***** Correspondence**:** brasileirovidal.ac@gmail.com

Abstract

Drought is one of the most impactful environmental stresses on agricultural productivity and cowpea (*Vigna unguiculata* L. Walp) is among the most water stress tolerant legume crops. Multiple cellular components are important in controlling plant responses to water stress, among which the WRKY family of transcription factors (TFs) has been shown to have a key role. Little information is currently available on the structure and organization of this TF family in cowpea and how its members are expressed in response to water deficit. Therefore, have used the recently released cowpea genomic assembly and transcriptomic data generated by RNA-seq analyses of cowpea plants under water deficit to identify members of the VuWRKY TF family. Ninety-two (92) complete VuWRKY genes were found in the cowpea genome that comprise three groups I (15 members), II (58 members) and III (16 members) based on their domain characteristics. Three genes having distinct domain structure were placed in unclassified group. The 92 genes are distributed across all 11 cowpea pseudochromosomes with a distribution density varying from 1 to 19.5% indicative of some gene clustering. Domain analysis of the encoded proteins identified 4 major variants of the conserved heptapeptide motif [WRKYGQK]. Analysis of cis-regulatory elements in the promoter regions of the VuWRKY genes identified 8 candidate binding motifs belonging to six TF families, most of which control abiotic stress-related gene expression. RNA-seq analysis of CpGC (*Cowpea Genome Consortium*) database identified 97 VuWRKY expressed splice variants which associated to 55 of VuWRKY genes. Of these, 83 were modulated under water deficit tolerant cultivar with expression values varying between times and isoforms analyzed. qPCR data confirmed the expression trends (up/down) and indicated a set of 22 induced genes under water stress, with *VuWRKY18, 21* and *75* exhibiting significant induction levels in the tolerant genotype. The results point out that the drought-tolerant accession exhibited an earlier stress perception, followed by a higher modulation of specific WRKYs, as compared with the susceptible genotype. The data provide a framework for the future directed modification of specific VuWRKY members to improve water stress tolerance in this import climate resilient legume in the developing world and beyond.

Keywords: abiotic stress, bioinformatics, drought, qPCR, RNA-Seq, root dehydration, validation, *Vigna unguiculata*.

Introduction

Global climate change has contributed to the rich biodiversity found in plants today by driving changes in genetic composition, phenology, growth, and species distribution through the interactive processes of species and environments (Peñuelas et al., 2013). Today, climate change is considered as one of the most serious threats to crop growth, with some individuals and organizations predicting dramatic reduction in global crop productivity due to elevated temperature and reduced water availability in coming decades (Rogelj et al., 2016; Zandalinas et al., 2018; Raza et al., 2019). Abiotic stresses, including water deficit and salinity, can lead to a loss in the average yield of some crops by more than 50%, which presents a significant challenge to the growing world population (Gill et al., 2015).

Plants respond and adapt to environmental stress by short-term responses to prevent severe damage and by long-term adaptations to acquire stress tolerance at the whole plant level (Takahashi and Shinozaki, 2019). The effects of water deficit on plants are quite variable, depending on the duration, intensity and speed at which the deficit occurs, plant developmental stage, and genotype. Usually, plants perceive dehydration stress in the roots and transmit water deficit signals to distant organs mainly using molecular signal cascades involving Ca^{+} channels and reactive oxygen species (ROS) (Christmann et al., 2013; Takahashi and Shinozaki, 2019). Changes in gene expression under stress is regulated by complex transcriptional networks that include the perception, signal transduction, gene expression and ultimately metabolic changes in the plant, thus providing stress tolerance (Mittler and Blumwald, 2010; Lata et al., 2011; Todaka et al., 2015; Amorim et al., 2017). In order to maintain growth and productivity, plants must adapt to stress conditions in molecular, physiological, and biochemical levels (Osakabe et al., 2014). At the physiological level, for example, plants can adapt to drought by reducing water loss by closing of stomata and leaf rolling, or developing adaptation mechanisms, such as developing root systems; completing life cycle in a short period of time, before a severe water deficit, or adjusting osmotic balance and turgidity (Xu et al., 2010).

At the molecular level, rapid changes in the gene expression pattern are among the primary mechanisms for the adaptation to the limiting environmental conditions. Several stresses induce a variety of genes in different plants (Hirayama and Shinozaki, 2010; Benko-Iseppon et al., 2011; Takahashi and Shinozaki, 2019). Such stress-responsive mechanisms are regulated by Transcription Factors (TFs) that can modulate a cluster of downstream target genes. Thus, TFs are excellent candidate genes for genetic manipulation of complex stress tolerance traits (Khan et al., 2018a). The response initiates when the plant recognizes the stress at the molecular level. From stress recognition, induced genes activate signal transduction pathways, which transmit information within individual cells and in the whole plant, leading to the activation of various physiological and metabolic responses (Nakashima et al., 2009; Osakabe et al., 2014).

An ever increasing number of sequencing and functional genome projects have provided valuable insights into the genes and regulatory factors associated with specific situations like abiotic/biotic stresses in plants (Langridge and Fleury 2011; Rodrigues et al., 2012; Van Verk et al., 2013), including those involved in regulation and signal transduction (Matsui et al., 2010). Transcriptional regulation of plant genes is directly controlled by networks of transcription factors, that are involved in the initial steps of gene regulation, triggering signal transduction in response to stress (Mizoi et al., 2012; Nuruzzaman et al., 2013; Tripathi et al., 2014; Amorim et al., 2016; Kimotho et al., 2019). Many TF families are plant-specific and play unique roles in stress regulation (Jiang et al., 2012). The plant-specific WRKY family is among the largest TF families, playing a significant role in several signaling pathways, including the

regulation of biotic and abiotic stresses, senescence, and germination, as well as in plant development processes (Eulgem et al., 2000; Eulgem, 2006; Rushton et al., 1995; 1996; 2010). WRKY transcription factors are classically defined by the presence of a DNA binding domain consisting of about 60 amino acids, with a signature designated as the WRKY domain with an invariant WRKYGQK sequence and an atypical zinc-finger structure at the C-terminus (motif Cx4–5Cx22–23HxH or Cx7Cx23HxC) (Eulgem et al., 2000; Bakshi and Oelmüller, 2014). The WRKY domain can bind to a DNA motif termed W-box (TGACC(A/T)) or SURE (sugar-responsive cis-element) element in target gene promoters, regulating their response under biotic and abiotic stress (Rushton et al., 1995; Sun et al., 2003; Wu et al., 2017; Yue et al., 2019). Depending on the presence of one or two domains, the amino acid sequence and the structure of its zinc finger motifs, WRKY TFs can be classified into three main Groups (I, II and III) (Eulgem et al., 2000; Yue et al., 2019). Additionally, the Group II can be subdivided into five Subgroups, based on primary amino acid sequence (IIa, IIb, IIc, IId, and IId) (Rushton et al., 2010; Yue et al., 2019).

Cowpea [*Vigna unguiculata* (L.) Walp.] is a herbaceous annual crop member of the Fabaceae family and is considered an economically and socially important legume in many parts of the developing world. Cowpea grains are high in protein (~ 25-30%), dietary fiber, vitamins, and minerals and, like other legumes, are rich in lysine but limited in its content of sulfur amino acids (Phillips et al., 2003; Singh et al., 2014). Cowpea is specifically noted for its capacity to grow under adverse environmental conditions, such as water deficit and saline soils (Ehlers and Hall, 1997; Freire-Filho et al., 1999; Singh et al., 2010; Boukar et al., 2018). As a result, it is often the primary source of dietary protein for humans and stover for animal fodder in environmentally challenged regions where subsistence farming is prevalent. It may also be used to recover degraded or poor lands through the nitrogen fixation in association with rhizobium (Bado et al. 2012; IAEA, 1998).

Once considered an orphan legume, its growth capacity under harsh conditions has made it a target for genetic improvement using both conventional and molecular assisted breeding strategies. It can also be genetically transformed thus allowing for the manipulation of endogenous genomic features or introduction of new traits (Cruz and Aragão, 2014). Cowpea is a diploid ($2n = 22$ chromosomes; Bortoleti et al., 2012), self-fertilizing plant with a relatively small genome size estimated at 620 Mbp (Arumuganathan and Earle, 1991; Boukar et al., 2018) making it ideal for genomic (Timko et al, 2008; Muñoz-Amatriaín et al., 2016) and transcriptomic (Muñoz-Amatriaín et al., 2016; Chen et al., 2017; Santos et al., 2018; Spriggs et al., 2018) studies. Recently, a high quality draft genome has been assembled and made available to the research community facilitating functional genomic studies (Lonardi et al., 2019).

The introduction of high-throughput next-generation sequencing (NGS) technologies and accompanying improvements in RNA-Seq analysis (Wang et al., 2009; Metzker, 2010; Ozsolak and Milos, 2011) and quantitative Real-Time- Polymerase Chain Reaction (qPCR), technologies has allowed for a more robust capacity to analyze transcriptomic changes in plants under physiological and aphysiological growth conditions. In this context, these functional genomic tools off the ability to explore the molecular and physiological mechanisms underlying plant abiotic stress response including response to water deficit (Gill et al., 2015; Latef and Ahmad, 2015; Moustafa and Cross, 2016).

In this present study, we identified members of the cowpea WRKY TF gene family (*VuWRKY*) using the recently published cowpea draft genome assembly (Lonardi et al., 2019)

and characterized the transcriptional response of the various VuWRKY gene family members to water deficit using a combination of RNA-Seq for root dehydration available in the CpGC (*Cowpea Genome Consortium*) database and qPCR expression profiling, to answer the following questions: (i) Do WRKY cowpea genes participate on physiological and/or adaptive processes related to water deficit? (ii) Are they early expressed? (iii) Are *VuWRKY* isoforms differentially expressed? (iv) Is there any difference between drought sensitive and tolerant cowpea expression profiles? Our data show that significant alteration occur in specific members of the family in water deficit tolerant and sensitive cultivars opening the future possibility for directed modification of this important agronomic trait through alteration of TF expression.

Materials and Methods

Plant materials, root dehydration analysis, and generation of the cowpea transcriptome.

Two cowpea accessions were used in these studies which have previously been described as being drought sensitive ('Santo Inácio' – SI) or drought tolerant ('Pingo de Ouro' – PO) (Bastos et al., 2011; Jesús-Pires et al., 2019). The seed of the two plants were germinated and seedlings transferee to a hydroponic system (Rodrigues et al., 2012) for growth. Following three weeks of growth, the plants were submitted to different times of root dehydration by the suspension of the plants such that the roots were no longer in contact with the nutrient solution in each container.

Root tissues were collected at 25 (T25), 50 (T50), 75 (T75), 100 (T100) and 150 (T150) min after the initiation of root dehydration, immediately frozen in liquid nitrogen, and stored at -80 °C until used for RNA extraction. For each experimental time, control plants (T0) were maintained in the nutrient solution and collected simultaneously to those submitted to root dehydration. Total RNA samples were obtained from the various samples using the "SV Total RNA Isolation System" kit (Promega), according to the manufacturer's recommendations. The concentration and purity of the extracted RNA were verified using a Qubit fluorimeter (Invitrogen®) and the integrity of the material was confirmed by visual examination following electrophoresis on 1% (w/v) agarose gels. Aliquots of the RNA extracted from the tolerant accession (PO) at T0, T25 and T150 were converted to cDNA and sequenced using the HiSeq Illumina 2500 platform by the paired-end method. To obtain a more robust transcriptome reads from experiments with cowpea severe mosaic virus (CPSMV) and cowpea aphid borne mosaic virus (CABMV) were added to the assay. Transcriptome assembly was performed by the 'de novo' approach through the Trinity software (Grabherr et al., 2011) and the transcriptome deposited in the CpGC database (Jesús-Pires et al., 2019).

In silico mining of cowpea WRKY sequences

Initially, a keyword search was done for cowpea WRKY genes available in the genome data (Lonardi et al., 2019) deposited in the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html; Goodstein et al., 2012). The returned genes had their encoded protein sequences confirmed for the presence of the WRKY domain and these sequences were used to mine the transcripts expressed in cowpea (CpGC bank). In addition, WRKY sequences from six other plant species (Supplementary Table 1), obtained from the Plant Transcription Factor Database v4.0 (Jin et al., 2017), were also used as a seed sequence to search for expressed WRKY. These searches were performed, via tBLASTn, against the

CpGC database for the screening of transcripts, using an e-value of e-5 as a cut-off. The mined sequences were submitted to ORF (*Open Reading Frame*) characterization with the aid of the Transdecoder v.5.0.2 (https://github.com/TransDecoder/TransDecoder/wiki; Haas et al., 2013) followed by conserved domain characterization using Batch CD-search/rps-BLAST (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; Marchler-Bauer et al., 2017). Genomic and transcriptomic sequences with incomplete ORFs and/or incomplete domains (as well as those that did not present integrity in the protein sequence) were excluded from the evaluation.

WRKY classification and chromosomal location

VuWRKY (*Vigna unguiculata* WRKY) genomic and expressed sequences were used to generate a Neighbor-Joining tree (1000 replicates) based on the alignment produced with Clustal W at the MEGA X package (Kumar et al., 2018). In addition, the gene sequences had their domains isolated through the program SMART (http://smart.embl.de; Letunic et al., 2015), which were used to generate a multiple alignment using the online tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) visualized by the Jalview v.2.11.0 (Waterhouse et al., 2009). After these steps, the *VuWRKY*s were classified into three main groups according to their structure and the proposed classification for higher plants (Eulgem et al., 2000; Rushton et al., 2010): Group I, with two WRKY domains and C2H2 (Cx4–5Cx22–23HxH) zinc finger motif; Group II, one WRKY domain and C2H2 (Cx4–5Cx22–23HxH), and Group III, one WRKY domain and C2–HC (Cx7Cx23HxC) motif instead of the standard C2H2. Group II was further subdivided in IIa, IIb, IIc, IId, and IIe based on the primary amino acid sequence, according to Rushton et al. (2010).

Using the data available in the Phytozome database and in the LIS (*Legume Information System* - https://legumeinfo.org/) database, the distribution, relative position, and abundance of WRKY sequences on the 11 cowpea pseudochromosome molecules were determined and this information used to generate the schematic representation (virtual karyotype) shown in Figure 5.

Analysis of cis-regulatory elements in the VuWRKY gene promoters

VuWRKY promoter regions, available up to 1.0 kb 5' upstream of the predicted start site of the coding region in each gene, was identified in the cowpea genome (Phytozome database) and analyzed using e MEME v5.0.5 software (http://meme-suite.org/index.html; Bailey et al., 2009). The program parameters were adjusted as described: (i) motif distribution: any number of repetitions (anr); (ii) maximum number of motifs to be found: 10. Only motifs with an *e-value* $\leq$ 1e-05 were considered. The identified motifs were submitted to TomTom v5.0.5 (http://meme-suite.org/tools/tomtom; Gupta et al., 2007) for annotation of putative cis-regulatory elements using the JASPAR (Khan et al., 2018b) database. Only candidate cis-regulatory elements associated with a target motif having highly significant p-values were considered.

Gene structural predictions and analysis of conserved protein motifs

VuWRKY gene structure was analyzed to determine the number and location of exons and introns, with the aid of the GSDS web server v2.0 (http://gsds.cbi.pku.edu.cn/; Hu et al. 2015) and compared with the sequences available from the expressed transcripts (CpGC database). Conserved motifs in the encoded proteins were identified by MEME (v.5.0.5). The defined

parameters were adjusted for: motif distribution, 0 or 1 per sequence; minimum width of the subjects, 6; maximum frame width, 60; minimum of sites by reason, 15; maximum number of motifs to be found, 50. Only motifs with an e-value ≤ 1e-10 were considered.

Ortholog groups

To identify orthologs of the cowpea *VuWRKY* genes responsive to abiotic stress tolerance in other model plants, peptide sequences of related WRKY members were downloaded from public annotated databases [*Phaseolus vulgaris* (90 sequences; http://www.phytozome.net/), *Glycine max* (199 sequences; http://www.phytozome.net/), *Arabidopsis thaliana* (71 sequences; http://www.arabidopsis.org/) and *Oryza sativa* (75 sequences; http://rice.plantbiology.msu.edu/)]. Protein sequences of WRKY genes from the above mentioned five species, including cowpea, were clustered into ortholog groups with OrthoMCL 1.4 (http://orthomcl.org/orthomcl/).

RNA-Seq expression analysis of WRKY

The gene expression level was calculated using RSEM based on the number of reads mapped against the assembled transcriptome (Li and Dewey, 2011). For the identification of differentially expressed genes (DEGs) between the control and stressed libraries, the edgeR tool (Robinson et al., 2010), implemented in the Bioconductor package (Huber et al., 2015) was used. Genes were considered differentially expressed when their transcript presented fold-change (log_2FC) values higher than 1 [$log_2FC > 1$ (up-regulated)] or lower than -1 [$log_2FC < -1$ (Down-regulated)] and with significance value (FDR - False Discovery Rate), below 0.05 (adjusted p-value <0.05). The log_2FC values of each transcript were used as input data for the Cluster 3.0 (v.1.52) software (Eisen et al., 1998) to perform the hierarchical clustering of the transcripts and the results were visualized using JAVA TreeView (Saldanha, 2004).

Primer design and quantitative real-time PCR (qPCR)

To confirm the differential expression revealed in analysis of the RNA-Seq data, a subset of the dehydration responsive genes, and with known orthologs in other plant species previously reported to be involved in water stress response (Supplementary Table 2), had their expression profiles validated by qPCR. We first used ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) to identify all possible ORF and the start and end of the longest ORF in the 26 candidate cowpea transcripts selected. Two sets of primers were designed for each transcript using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The designed primers and their respective amplicons are described in the Supplementary Table 2.

The gene specific primers were tested for their ability to amplify their selected target using RNA prepared from the root tissues of the two cowpea accessions (PO and SI) at the T0 (control), T25, T50, T75 and T100 of the root dehydration experiment. qPCR was carried out using the iQ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA) on an iCycler real-time PCR detection system (Bio-Rad, Hercules, USA), following the manufacturer's instructions. The fold changes were calculated according to Schmittgen and Livak (2008). Relative expression was normalized to by a cowpea *Ubiquitin* gene (CpGC database) obtained via tBLASTn using a 150 bp *Arabidopsis thaliana Ubiquitin* sequence (NM_001084884; GI: 240255763) as probe. The ubiquitin gene presents stable expression in cowpea under salt and water stresses, as revealed in previous experiments conducted by our group (Amorim et al., 2018). For each time

point, three biological replicates, with three technical replicates each, were analyzed. The expression values of biological replicate 2 from SI cultivar at 0 min were used for calibration.

Data were submitted to analysis of variance (ANOVA) and evaluated by *t*-Test at ($p < 0.05$ or $p < 0.01$), using the Assistat Statistical Program (http://www.assistat.com/) in an entirely randomized design, with five treatments and three replicates per treatment. The experimental unit corresponded to the average of three technical replicates in each biological replicate. Graphs were constructed using excel program, in which the relative expression value (REV) at each time point represents the average of three technical replicates of the three biological replicates. Comparisons were made between genotypes (significance level at the right side of the graphs), between times (at the bottom of the graphs, the right side of the abscissa), and for interaction genotype and time (on the left side of the graphs between both lines). Genotypes followed by the same letter were statistically similar, while the ones followed by different letters showed significant differences. On the other hand, times followed by the same uppercase letter were statistically similar, while those followed by different letters showed significant differences. For each time, the relative differential expression was also calculated by dividing the average expression value of PO by that of SI. Figure 1 shows a workflow with the major steps performed in the present work.

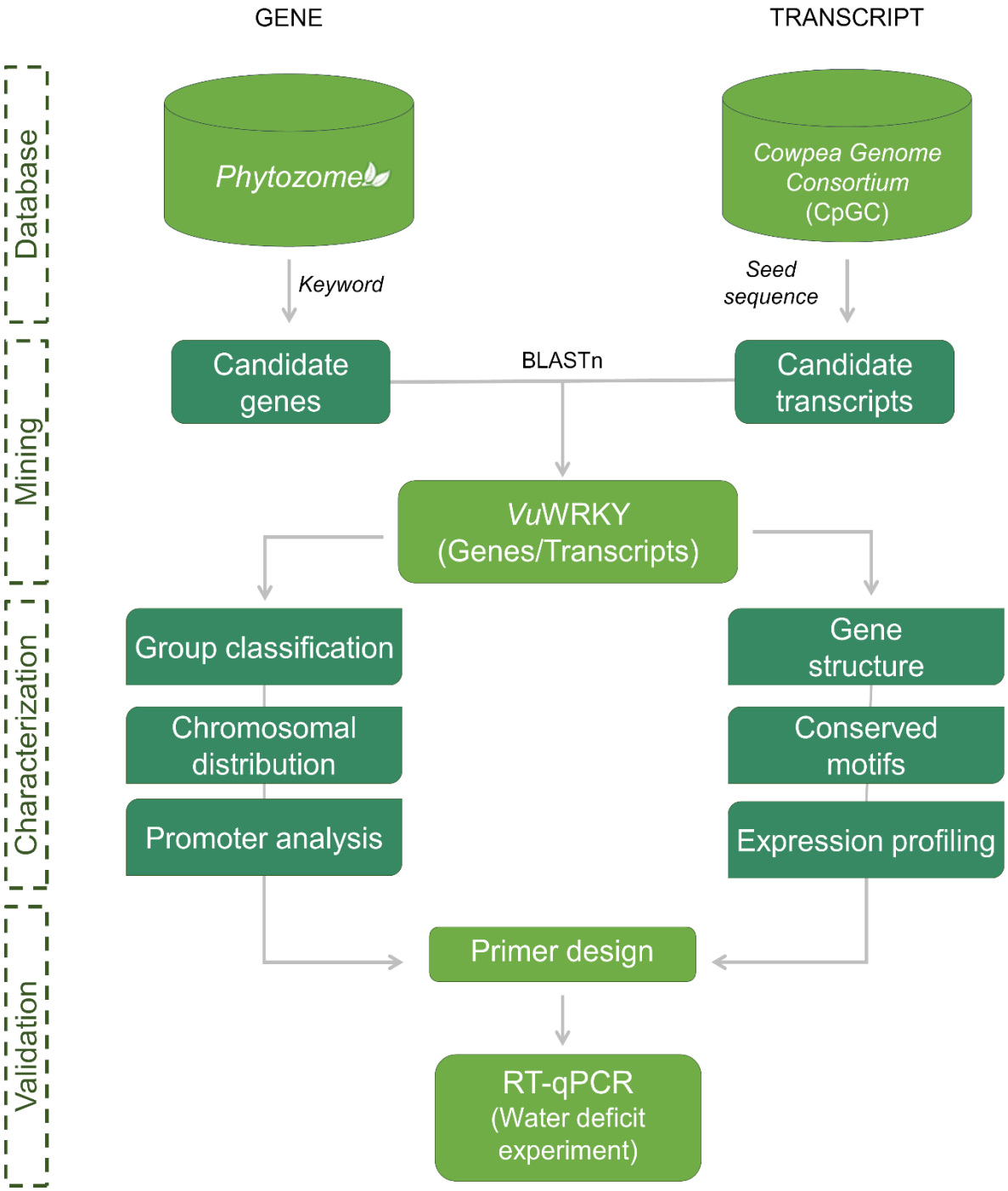


Figure 1. Workflow showing the major steps performed in the present work. Cowpea WRKY (*VuWRKY*) sequences were identified in the genome data deposited in the Phytozome database, using WRKY keyword. Afterwards, they were classified in groups and subgroups according to their structure and proposed classification for higher plants. *VuWRKY* genes were mapped in pseudochromosomes and their cis-regulatory elements in promoter regions were identified for each gene. In parallel, expressed RNA-Seq transcripts from Cowpea Genome Consortium (CpGC) bank were mined using seed sequence to search for WRKY sequences of other plant species. The correspondence between gene (Phytozome) and expressed (CpGC) sequences was performed by a local BLASTn. The expressed VuWRKY transcripts were characterized for the distribution of introns and exons of each isoform and conserved motif pattern in the protein sequence. A heatmap was generated based on log_2FC values of each modulated transcript, revealing the transcriptional profile of WRKY family under water deficit. A set of transcripts was selected based on *in silico* expression and/or gene orthology to known abiotic stress responsive sequences, followed by biological validation by qPCR.

Results

Genomic Analysis

Identification and characterization of *VuWRKY* TF gene family

Little information is currently available on the structure and organization of the WRKY TF gene family in cowpea (designated here as VuWRKY). Therefore, we key word searched the annotated genes of the recently released cowpea genomic assembly (Lonardi et al., 2019) using the term "WRKY" and recovered 93 non-redundant sequences corresponding to putative WRKY domain containing proteins. The identified genes were analyzed for the presence of the complete WRKY domain as the first basic curation criterion (Figure 1). As a result, one sequence (Vigun08g124900.1) was removed because it lacked the conserved WRKYGQK motif, while the remaining 92 candidates presented the complete heptapeptide sequence.

The sequences were then classified into one of three groups based on the number of WRKY domains present and type of atypical zinc finger binding motif they contained (as described in the materials and methods section) and phylogenetic analysis was then carried to sort the genes into groups and subgroups based on their structural similarity (Figure 2).

Of the 92 genes identified, 15 presented features characteristic of group I, 58 were placed in group II, and represented by 58 sequences, and 16 sequences were placed in group III. The members of group II could be further subdivided into subgroup IIa (6 members), subgroup IIb (15 members), subgroup IIc (20 members), subgroup IId (8 members) and subgroup IIe (9 members). Three genes (*VuWRKY90, VuWRKY91, VuWRKY92*) were difficult to classify based on their domain structure and phylogenetic position. All three genes encode proteins that cluster with members of groups I and III, but they do not present the intrinsic characteristics of the groups (presence of two WRKY domains and the C2-HC motif, respectively). We therefore designated these as "unclassified".

Datamining of expressed VuWRKY transcripts in CpGC database returned a total of 356 candidate sequences, of which 198 had the complete ORFs and 119 had complete WRKY domains. For subsequent analyses, transcripts with incomplete or truncated ORFs and those missing critical domains at the N or C-terminal regions were disregarded. In the end, a total of 97 VuWRKY transcripts were identified CpGC database that were then used in subsequent analyses. To determine the correspondence between the 97 VuWRKY and the 92 VuWRKY genes identified in the genomic assembly and a pairwise BLASTn was performed. As shown in Table 1, the 97 expressed transcripts were derived from 55 VuWRKY genes.

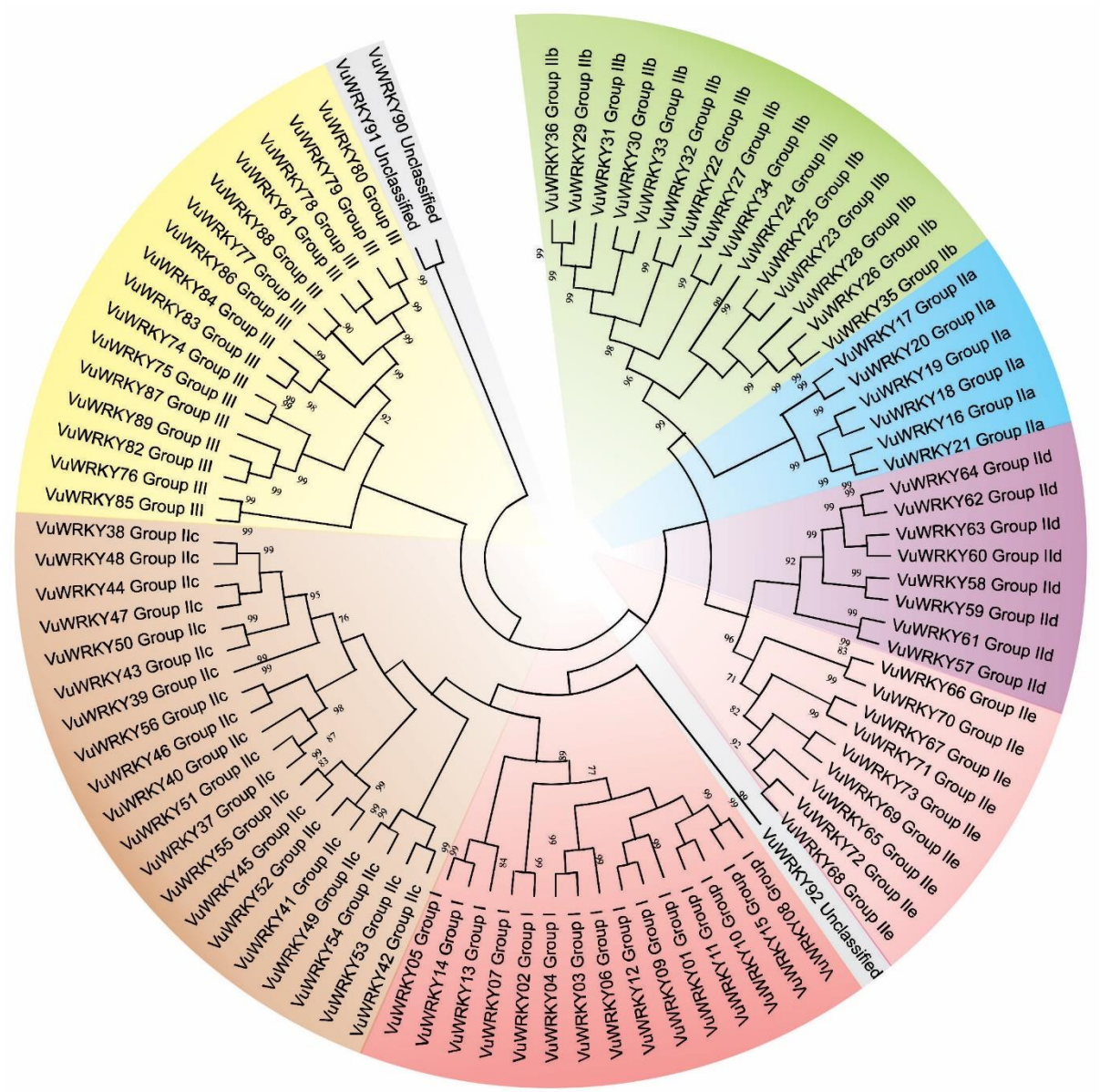


Figure 2. Neighbor-Joining (NJ) tree of 92 cowpea WRKY (VuWRKY) representatives in the cowpea genome. Bootstrap values (≥ 70) regard 1000 replicates. Groups and subgroups are distinguished by different colors (Groups: I-red; IIa-blue; IIb-green; IIc-brown; IId-purple; IIe-pink; III-yellow and unclassified-grey).

Based on their phylogenetic classification and the order/position of the genes in the pseudochromosomes within each group/subgroup (described below), the genes identified here were designated VuWRKY1 to VuWRKY92. The expressed transcripts and their encoded proteins isoforms were named based upon the gene from which they arise (e.g., VuWRKY 1-92) and given the suffix 'i' (Isoform) followed by the number associated with the name of the transcript in the CpGC databank (e.g., VuWRKY01_i2, VuWRKY01_i3, etc., see Table 1).

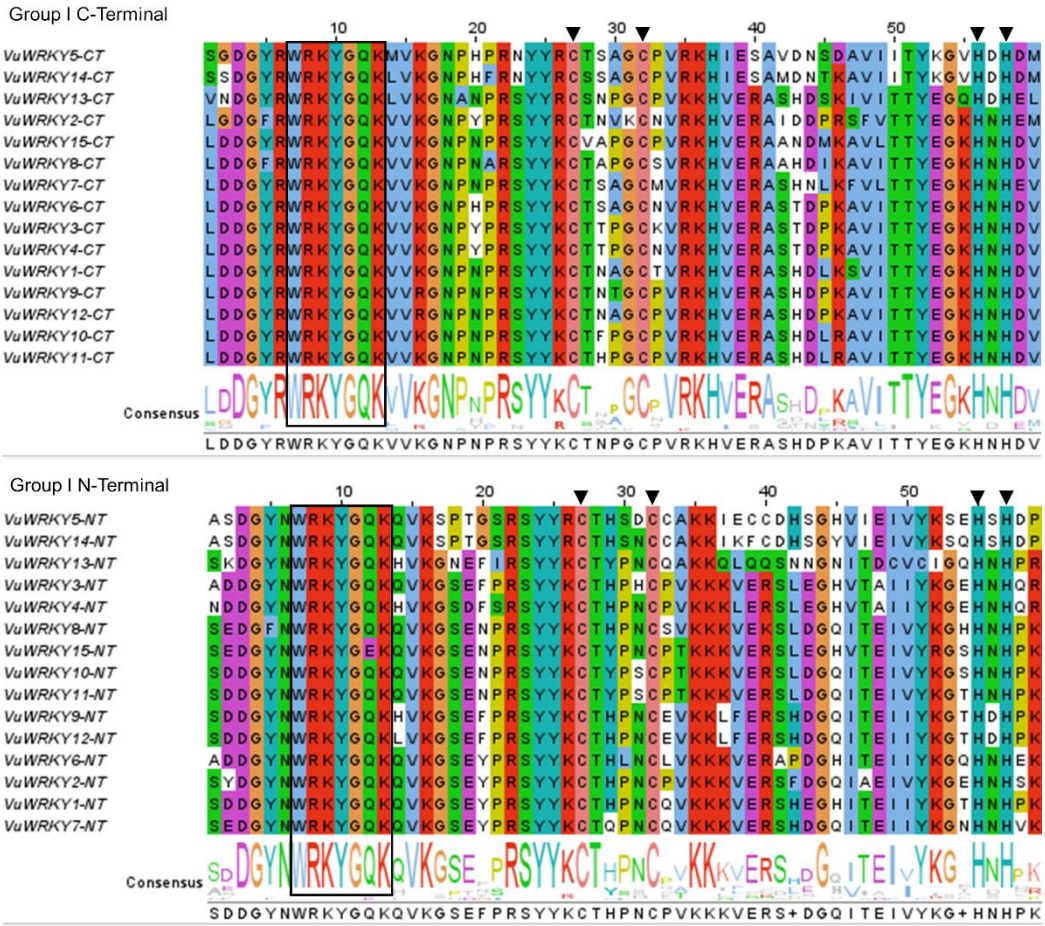
Table 1: Cowpea WRKY genes identified in the present work, with their respective Phytozome database ID, the WRKY group to which they belong (according to Eulgem et al., 2000; Rushton et al., 2010), the isoforms ID expressed in the Cowpea Genome Consortium (CpGC) database and their identification in the present work.

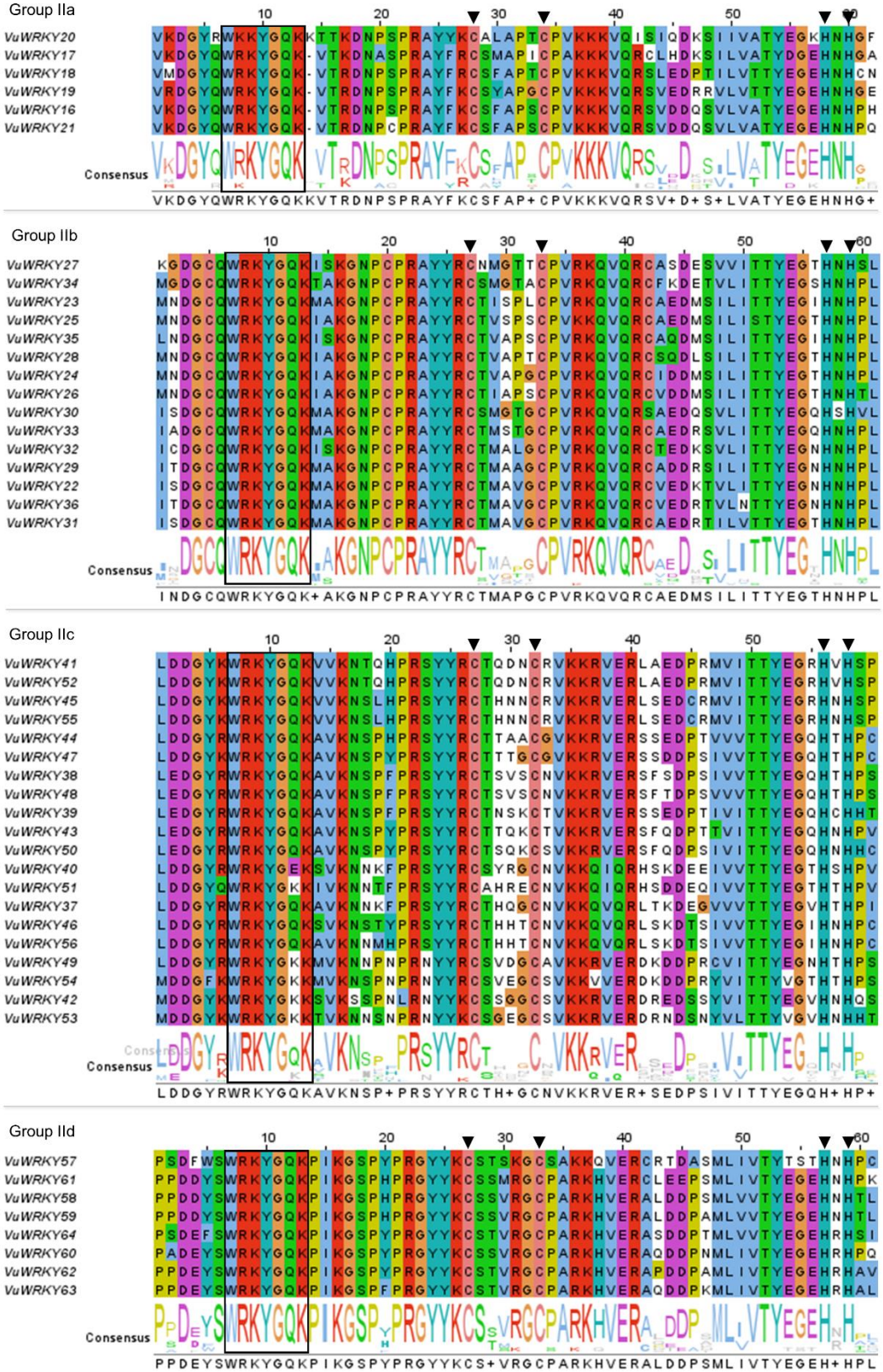
Cowpea WRKY gene Present Paper	ID Cowpea WRKY gene (Phytozome)	WRKY Group (Fig.1)	ID WRKY Transcripts (CpGC)	Cowpea WRKY Transcripts Present Paper
*Vu*WRKY01	Vigun01g006200	Group I	TR127859_c2_g2_i2.p1	*Vu*WRKY01_i2
			TR127859_c2_g2_i3.p1	*Vu*WRKY01_i3
			TR127859_c2_g2_i4.p1	*Vu*WRKY01_i4
			TR127859_c2_g2_i5.p1	*Vu*WRKY01_i5
			TR127859_c2_g2_i6.p1	*Vu*WRKY01_i6
			TR127859_c2_g2_i7.p1	*Vu*WRKY01_i7
			TR127859_c2_g2_i9.p1	*Vu*WRKY01_i9
			TR127859_c2_g2_i10.p1	*Vu*WRKY01_i10
*Vu*WRKY02	Vigun01g156000	Group I	TR4682_c0_g1_i1.p1	*Vu*WRKY02_i1
			TR4682_c0_g1_i3.p1	*Vu*WRKY02_i3
*Vu*WRKY03	Vigun02g055200	Group I	TR7693_c0_g1_i3.p1	*Vu*WRKY03_i3
*Vu*WRKY04	Vigun03g204300	Group I	TR16623_c0_g2_i1.p1	*Vu*WRKY04_i1
*Vu*WRKY05	Vigun03g371400	Group I	–	–
*Vu*WRKY06	Vigun05g038000	Group I	TR7106_c0_g1_i2.p1	*Vu*WRKY06_i2
			TR7106_c0_g1_i5.p1	*Vu*WRKY06_i5
*Vu*WRKY07	Vigun05g055800	Group I	TR117052_c0_g2_i1.p1	*Vu*WRKY07_i1
*Vu*WRKY08	Vigun05g092400	Group I	TR113959_c1_g1_i3.p1	*Vu*WRKY08_i3
			TR113959_c1_g1_i4.p1	*Vu*WRKY08_i4
			TR113959_c1_g1_i5.p1	*Vu*WRKY08_i5
*Vu*WRKY09	Vigun06g060000	Group I	–	–
*Vu*WRKY10	Vigun06g082700	Group I	–	–
*Vu*WRKY11	Vigun08g130700	Group I	TR127859_c2_g1_i9.p1	*Vu*WRKY11_i9
			TR127859_c2_g1_i11.p1	*Vu*WRKY11_i11
*Vu*WRKY12	Vigun08g206800	Group I	TR61138_c3_g1_i1.p1	*Vu*WRKY12_i1
			TR61138_c3_g1_i9.p1	*Vu*WRKY12_i9
			TR61138_c3_g1_i14.p1	*Vu*WRKY12_i14
*Vu*WRKY13	Vigun08g217500	Group I	TR121718_c0_g1_i4.p1	*Vu*WRKY13_i4
*Vu*WRKY14	Vigun09g067500	Group I	TR39449_c0_g1_i2.p1	*Vu*WRKY14_i2
*Vu*WRKY15	Vigun10g092300	Group I	TR93324_c0_g1_i3.p1	*Vu*WRKY15_i3
			TR93324_c0_g1_i4.p1	*Vu*WRKY15_i4
			TR93324_c0_g1_i5.p1	*Vu*WRKY15_i5
			TR93324_c0_g1_i6.p1	*Vu*WRKY15_i6
*Vu*WRKY16	Vigun05g298900	Group IIa	TR162834_c3_g3_i4.p1	*Vu*WRKY16_i4
			TR162834_c3_g3_i13.p1	*Vu*WRKY16_i13
*Vu*WRKY17	Vigun08g045300	Group IIa	TR72981_c0_g1_i2.p1	*Vu*WRKY17_i2
			TR72981_c0_g1_i4.p1	*Vu*WRKY17_i4
*Vu*WRKY18	Vigun08g045400	Group IIa	–	–
*Vu*WRKY19	Vigun09g215300	Group IIa	TR13411_c0_g1_i3.p2	*Vu*WRKY19_i3
			TR13411_c0_g1_i5.p1	*Vu*WRKY19_i5
			TR13411_c0_g1_i6.p1	*Vu*WRKY19_i6
			TR13411_c0_g1_i7.p1	*Vu*WRKY19_i7
*Vu*WRKY20	Vigun09g215400	Group IIa	–	–
*Vu*WRKY21	Vigun10g195700	Group IIa	–	–
*Vu*WRKY22	Vigun01g202800	Group IIb	–	–
*Vu*WRKY23	Vigun02g063800	Group IIb	–	–
*Vu*WRKY24	Vigun03g138300	Group IIb	TR52139_c1_g3_i3.p1	*Vu*WRKY24_i3
			TR52139_c1_g3_i4.p1	*Vu*WRKY24_i4
*Vu*WRKY25	Vigun03g212000	Group IIb	–	–
*Vu*WRKY26	Vigun03g333800	Group IIb	TR57532_c2_g3_i3.p1	*Vu*WRKY26_i3
			TR57532_c2_g3_i4.p1	*Vu*WRKY26_i4
*Vu*WRKY27	Vigun03g405100	Group IIb	TR14570_c0_g1_i4.p1	*Vu*WRKY27_i4
			TR14570_c0_g1_i5.p1	*Vu*WRKY27_i5
*Vu*WRKY28	Vigun05g044300	Group IIb	TR57532_c2_g1_i1.p1	*Vu*WRKY28_i1
			TR57532_c2_g1_i2.p1	*Vu*WRKY28_i2
			TR57532_c2_g1_i3.p1	*Vu*WRKY28_i3
			TR57532_c2_g1_i4.p1	*Vu*WRKY28_i4
			TR57532_c2_g1_i5.p1	*Vu*WRKY28_i5
			TR57532_c2_g1_i6.p1	*Vu*WRKY28_i6
*Vu*WRKY29	Vigun05g228000	Group IIb	–	–
*Vu*WRKY30	Vigun06g053700	Group IIb	TR134661_c0_g1_i1.p1	*Vu*WRKY30_i1
*Vu*WRKY31	Vigun06g159600	Group IIb	TR156309_c2_g1_i3.p1	*Vu*WRKY31_i1
*Vu*WRKY32	Vigun07g131500	Group IIb	–	–
*Vu*WRKY33	Vigun08g201800	Group IIb	TR103680_c0_g1_i1.p1	*Vu*WRKY33_i1
			TR139074_c0_g1_i5.p1	*Vu*WRKY33_i5
			TR139074_c0_g1_i7.p1	*Vu*WRKY33_i7

Cowpea WRKY gene Present Paper	ID Cowpea WRKY gene (Phytozome)	WRKY Group (Fig.1)	ID WRKY Transcripts (CpGC)	Cowpea WRKY Transcripts Present Paper
*Vu*WRKY34	Vigun09g044800	Group IIb	TR156309_c1_g1_i1.p1	*Vu*WRKY34_i1
*Vu*WRKY35	Vigun09g078100	Group IIb	–	–
*Vu*WRKY36	Vigun11g119100	Group IIb	–	–
*Vu*WRKY37	Vigun01g071800	Group IIc	–	–
*Vu*WRKY38	Vigun01g198600	Group IIc	TR130502_c4_g2_i2.p1	*Vu*WRKY38_i2
*Vu*WRKY39	Vigun02g051100	Group IIc	TR128374_c1_g3_i4.p1	*Vu*WRKY39_i4
*Vu*WRKY40	Vigun03g048500	Group IIc	–	–
*Vu*WRKY41	Vigun03g068900	Group IIc	TR157317_c0_g1_i2.p1	*Vu*WRKY41_i2
*Vu*WRKY42	Vigun03g069800	Group IIc	–	–
*Vu*WRKY43	Vigun03g144800	Group IIc	–	–
*Vu*WRKY44	Vigun03g412200	Group IIc	–	–
*Vu*WRKY45	Vigun05g049400	Group IIc	TR50247_c0_g1_i2.p1	*Vu*WRKY45_i2
*Vu*WRKY46	Vigun05g060300	Group IIc	–	–
*Vu*WRKY47	Vigun06g131100	Group IIc	–	–
*Vu*WRKY48	Vigun07g127100	Group IIc	TR130502_c4_g2_i4.p1	*Vu*WRKY48_i4
*Vu*WRKY49	Vigun08g042500	Group IIc	–	–
*Vu*WRKY50	Vigun08g192900	Group IIc	TR6508_c0_g1_i1.p1 TR6508_c0_g1_i2.p1	*Vu*WRKY50_i1 *Vu*WRKY50_i2
*Vu*WRKY51	Vigun09g104500	Group IIc	–	–
*Vu*WRKY52	Vigun09g128100	Group IIc	–	–
*Vu*WRKY53	Vigun09g128400	Group IIc	TR132186_c0_g1_i2.p1	*Vu*WRKY53_i2
*Vu*WRKY54	Vigun09g222700	Group IIc	TR119633_c1_g1_i1.p1 TR119633_c1_g1_i2.p1	*Vu*WRKY54_i1 *Vu*WRKY54_i2
*Vu*WRKY55	Vigun10g014400	Group IIc	TR89641_c0_g1_i1.p1	*Vu*WRKY55_i1
*Vu*WRKY56	Vigun10g138200	Group IIc	–	–
*Vu*WRKY57	Vigun01g197800	Group IId	TR109555_c0_g1_i2.p1	*Vu*WRKY57_i2
*Vu*WRKY58	Vigun02g146400	Group IId	TR115618_c0_g2_i4.p1	*Vu*WRKY58_i4
*Vu*WRKY59	Vigun03g280900	Group IId	TR121788_c1_g3_i1.p1 TR121788_c1_g3_i3.p1	*Vu*WRKY59_i1 *Vu*WRKY59_i3
*Vu*WRKY60	Vigun03g389500	Group IId	TR123540_c0_g1_i2.p3 TR123540_c0_g1_i3.p1	*Vu*WRKY60_i2 *Vu*WRKY60_i3
*Vu*WRKY61	Vigun07g103700	Group IId	–	–
*Vu*WRKY62	Vigun08g065100	Group IId	–	–
*Vu*WRKY63	Vigun09g031900	Group IId	–	–
*Vu*WRKY64	Vigun09g200200	Group IId	TR17124_c0_g1_i2.p1	*Vu*WRKY64_i2
*Vu*WRKY65	Vigun03g040900	Group IIe	TR131891_c0_g1_i1.p1	*Vu*WRKY65_i1
*Vu*WRKY66	Vigun03g096500	Group IIe	TR156951_c0_g1_i1.p1 TR156951_c0_g1_i4.p1	*Vu*WRKY66_i1 *Vu*WRKY66_i4
*Vu*WRKY67	Vigun03g138800	Group IIe	TR116592_c1_g3_i1.p1	*Vu*WRKY67_i1
*Vu*WRKY68	Vigun04g145700	Group IIe	–	–
*Vu*WRKY69	Vigun05g060600	Group IIe	–	–
*Vu*WRKY70	Vigun05g207000	Group IIe	TR58821_c0_g1_i1.p1 TR58821_c0_g1_i2.p1	*Vu*WRKY70_i1 *Vu*WRKY70_i2
*Vu*WRKY71	Vigun06g134600	Group IIe	TR116592_c1_g3_i2.p1	*Vu*WRKY71_i2
*Vu*WRKY72	Vigun07g221500	Group IIe	–	–
*Vu*WRKY73	Vigun10g134800	Group IIe	–	–
*Vu*WRKY74	Vigun02g181700	Group III	–	–
*Vu*WRKY75	Vigun03g037200	Group III	TR124875_c0_g1_i2.p1	*Vu*WRKY75_i2
*Vu*WRKY76	Vigun03g090000	Group III	–	–
*Vu*WRKY77	Vigun05g085500	Group III	TR93511_c1_g1_i1.p1	*Vu*WRKY77_i1
*Vu*WRKY78	Vigun05g182100	Group III	TR113964_c3_g1_i2.p1	*Vu*WRKY78_i2
*Vu*WRKY79	Vigun05g182200	Group III	–	–
*Vu*WRKY80	Vigun05g182300	Group III	TR146008_c0_g1_i1.p1	*Vu*WRKY80_i1
*Vu*WRKY81	Vigun05g182400	Group III	–	–
*Vu*WRKY82	Vigun06g122600	Group III	TR2910_c0_g1_i2.p1 TR2910_c0_g1_i4.p1	*Vu*WRKY82_i2 *Vu*WRKY82_i4
*Vu*WRKY83	Vigun08g112200	Group III	TR70733_c0_g2_i1.p1 TR70733_c0_g2_i2.p1	*Vu*WRKY83_i1 *Vu*WRKY83_i2
*Vu*WRKY84	Vigun08g112300	Group III	TR70733_c0_g1_i1.p1	*Vu*WRKY84_i1
*Vu*WRKY85	Vigun09g133700	Group III	TR34712_c0_g1_i2.p1	*Vu*WRKY85_i2
*Vu*WRKY86	Vigun09g133800	Group III	TR58167_c0_g1_i1.p1	*Vu*WRKY86_i1
*Vu*WRKY87	Vigun09g153200	Group III	TR102341_c0_g1_i1.p1	*Vu*WRKY87_g1_i1
*Vu*WRKY86	Vigun09g133800	Group III	TR102341_c0_g2_i1.p1	*Vu*WRKY87_g2_i1
*Vu*WRKY88	Vigun10g002700	Group III	TR79364_c0_g1_i2.p1	*Vu*WRKY88_i2
*Vu*WRKY89	Vigun10g144200	Group III	TR103577_c1_g1_i9.p1	*Vu*WRKY89_i9
*Vu*WRKY90	Vigun08g130300	Unknow	TR20888_c0_g1_i1.p1 TR20888_c0_g1_i2.p1	*Vu*WRKY90_i1 *Vu*WRKY90_i2
*Vu*WRKY91	Vigun05g118000	Unknow	–	–
*Vu*WRKY92	Vigun07g162700	Unknow	–	–

Analysis of conserved protein domain structure in the *VuWRKY* gene family members

Using the predicted proteins encoded by the various *VuWRKY* genes, multiple sequence alignments and analysis of conserved domains using MEME software analysis revealed the general features of the various family members. As noted previously all of the genes encoded proteins with defined by the presence of a DNA binding domain consisting of about 60 amino acids, with a conserved WRKYGQK motif at the N-terminal region and a zinc finger motif at the C-terminal region of the protein (Figure 3). In general, the sequence of the 60 amino acid DNA binding domain was less variable among members of subgroup IIb, with 63.9% conservation than members of group III with only 30.1% conservation. Most of the identified *VuWRKY* gene sequences (90.2%) exhibited the well-conserved heptapeptide domain [WRKYGQK]. However, in 9 *VuWRKY* genes (9.8%) contained variants of this motif with at least one amino acid substitution within the predicted invariant motif: [WKKYGQK] (VuWRKY20); [WRKYGEK] (VuWRKY15, VuWRKY40); [WRKYGKK] (VuWRKY42, VuWRKY49, VuWRKY51, VuWRKY53, VuWRKY54), and [WKKYEDK] (VuWRKY90) (Figure 4). It is interesting to note that none of the gene sequences identified had modifications in the cysteine and histidine specific residues of the zinc finger motif. Outside of the conserved DNA binding domain, the sequences of the encoded VuWRKY proteins were more variable in terms of length and amino acid sequence (data not shown).




Group IIa
VuWRKY20
VuWRKY17
VuWRKY18
VuWRKY19
VuWRKY16
VuWRKY21
Consensus
VKDGYQWRKYGQKKVTRDNPSPRAYFKCSFAP+CPVKKKVQRSV+D+S+LVATYEGEHNHG+
Group IIb
VuWRKY27
VuWRKY34
VuWRKY23
VuWRKY25
VuWRKY35
VuWRKY28
VuWRKY24
VuWRKY26
VuWRKY30
VuWRKY33
VuWRKY32
VuWRKY29
VuWRKY22
VuWRKY36
VuWRKY31
Consensus
INDGCQWRKYGQK+AKGNPCPRAYYRCTMAPGCPVRKQVQRCAEDMSILITTYEGTHNHPL
Group IIc
VuWRKY41
VuWRKY52
VuWRKY45
VuWRKY55
VuWRKY44
VuWRKY47
VuWRKY38
VuWRKY48
VuWRKY39
VuWRKY43
VuWRKY50
VuWRKY40
VuWRKY51
VuWRKY37
VuWRKY46
VuWRKY56
VuWRKY49
VuWRKY54
VuWRKY42
VuWRKY53
Consensus
LDDGYRWRKYGQKAVKNSP+PRSYYRCTH+GCNVKKRVER+SEDPSIVITTYEGQH+HP+
Group IId
VuWRKY57
VuWRKY61
VuWRKY58
VuWRKY59
VuWRKY64
VuWRKY60
VuWRKY62
VuWRKY63
Consensus
PPDEYSWRKYGQKPIKGSPYPRGYYKCS+VRGCPARKHVERALDDPSMLIVTYEGEH+HPL

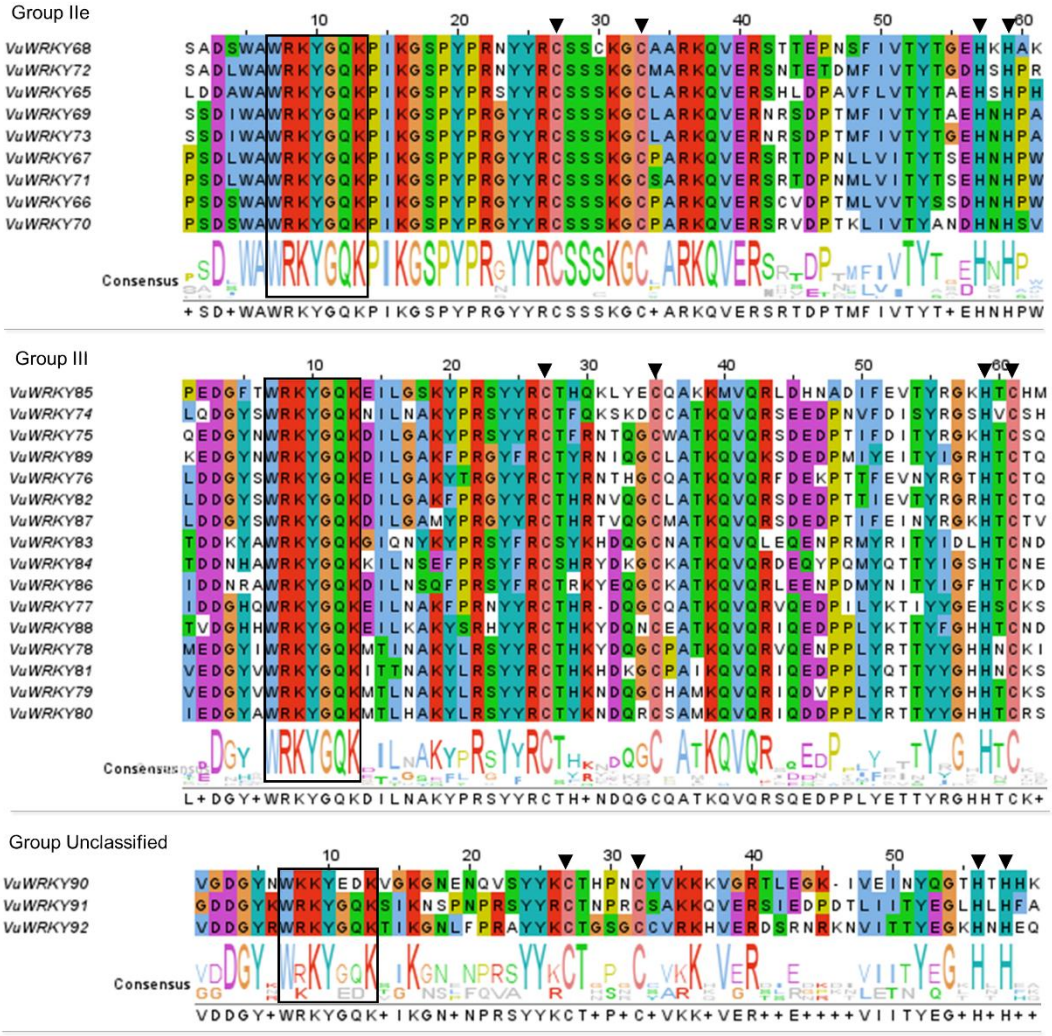


Figure 3. Alignment of cowpea WRKY protein domain regions in different groups/subgroups. WRKY domains are outlined (black boxes). Potential zinc ligands are indicated by arrowheads. For Group I of VuWRKY sequences, both WRKY domains are indicated (N- and C-terminal). Consensus sequences are shown at the bottom of each alignment.

Figure 4. Amino acid consensus sequence of the heptapeptide region of the DNA binding domain of 92 VuWRKY genes identified by the MEME tool.

Organization of the *VuWRKY* in the cowpea genome

Using tools available on the Phytozome web interface it is possible to located the 92 identified VuWRKY genes on the 11 cowpea pseudochromosomes (Figure 5). This in silico analysis showed that all 11 pseudomolecules contained at least one *VuWRKY* gene, with the distribution of genes per pseudomolecule ranging from 1 to 18 (representing 1 to 19.5 % of the total family).

The genes were concentrated in euchromatic regions. It is worth mentioning the clustering of the WRKY in specific points of the chromosomes where they appear (Figure 5).

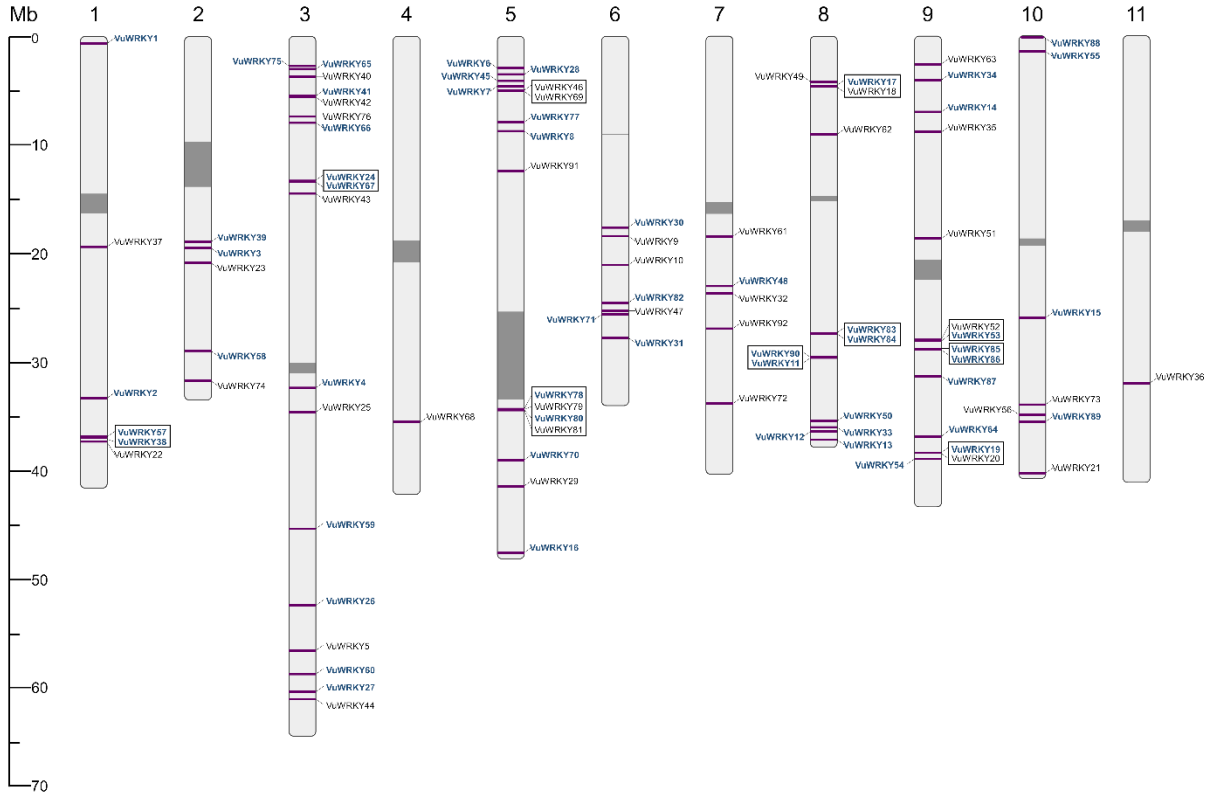


Figure 5. Schematic representation of the chromosomal locations of 92 genes WRKY genes identified in cowpea. The VuWRKY highlighted in blue correspond to the genes expressed under root dehydration (CpGC data bank). The pseudochromosomes were designed based on information available in the LIS (*Legume Information System*) database. Chromosome numbers are indicated in the upper region and the position of its centromere by a gray block, with the surrounding dark blue part including its possible variation. Black rectangles highlight possible pairs of homologous VuWRKY (Cheng et al., 2016).

Pseudomolecule 3 contained the highest number of VuWRKY genes (18) distributed among the three groups I (2 genes), II (14 genes), and III (2 genes). Pseudomolecule 5 appears contained the next largest number with 16 totl genes distributed among groups I (3 genes), II (7), III (5). It also contained the one Unclassified gene. In contrast, pseudochromosomes 4 and 11 contained only one VuWRKY gene each (subgroups IIe and IIb, respectively), representing the chromosomes with the lowest number of anchored genes (Figure 6).

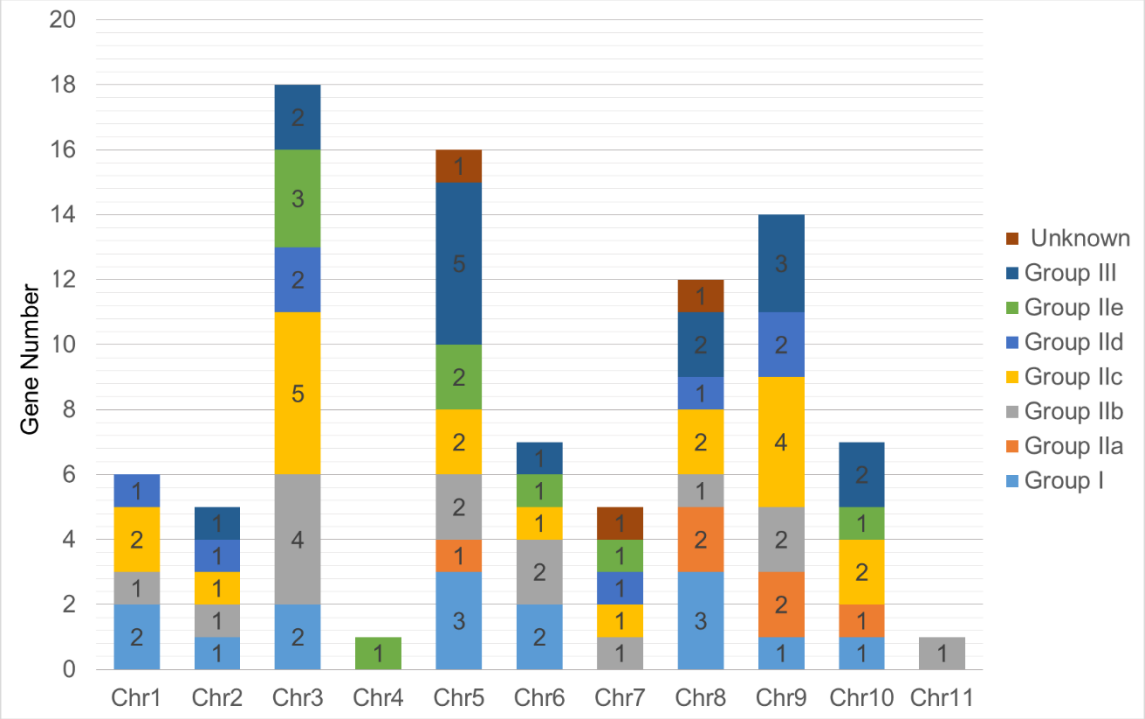


Figure 6. Distribution of 92 VuWRKY genes representatives along the 11 cowpea pseudochromosomes, also considering their classification in groups and subgroups.

Identification of cis-regulatory elements in *VuWRKY* gene promoters

The search for cis-regulatory elements in the promoter regions of the 92 VuWRKY genes returned a total of eight motifs with significant e-values and within the reliable parameters. As for the identity of the TFs, the ZF-HD families (two motifs, Figure 7A, F), BBR-BPC (Figure 7B), TCP (Figure 7C), Dof (Figure 7D), MYB (two motifs, Figure 7E, G) and CPP (Figure 7H) were associated with recognition of such target motifs.

The cis-regulatory elements associated with BB-BPC TF type (JASPAR ID MA1402.1; Figure 7B) were the most abundant, observed at 160 positions of 63 VuWRKY (Figure 8), followed by Dof TFs (JASPAR ID MA1267.1; Figure 7D) anchored at 96 positions relative to 54 VuWRKY genes (Figure 8). MYB (JASPAR ID MA1027.1; Figure 7G) and CPP (JASPAR ID MA1379.1; Figure 7H) target motifs were found in six sites of four and five VuWRKY, respectively, corresponding to the least abundant promoters among the genes analyzed. Interestingly, all identified cis-regulatory elements were located in the promoter region of at least one of the expressed VuWRKY genes under root dehydration, also confirmed by qPCR.

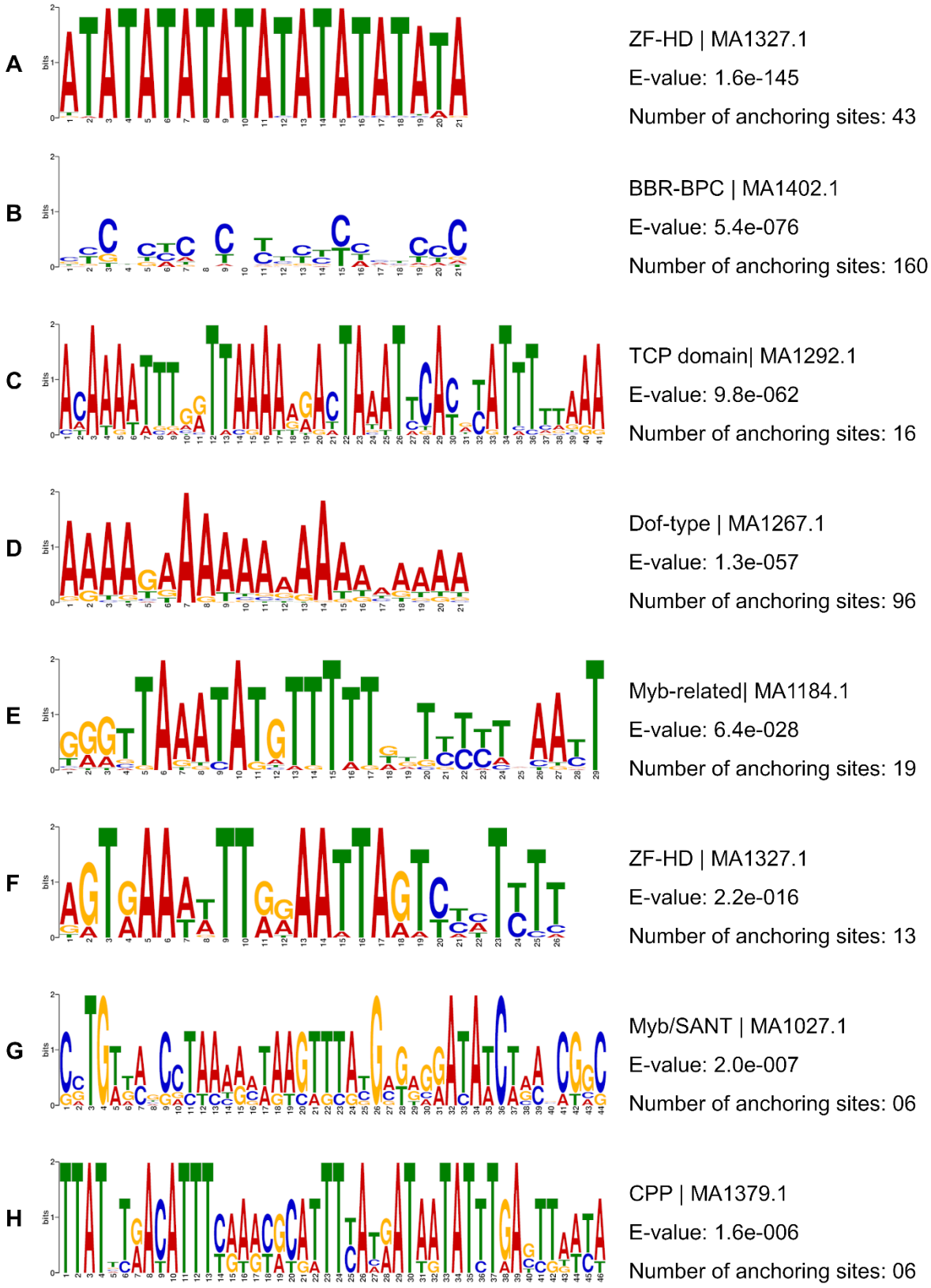


Figure 7. Predicted cis-regulatory sequences in the promoter regions of the WRKY genes of cowpea. Figures A-H show consensus sequence and below the Transcription Factor (TF) annotation associated with the indicated sequence of its JASPAR matrix (according to the Tomtom software output), as well as the e-value and number of anchored sites.

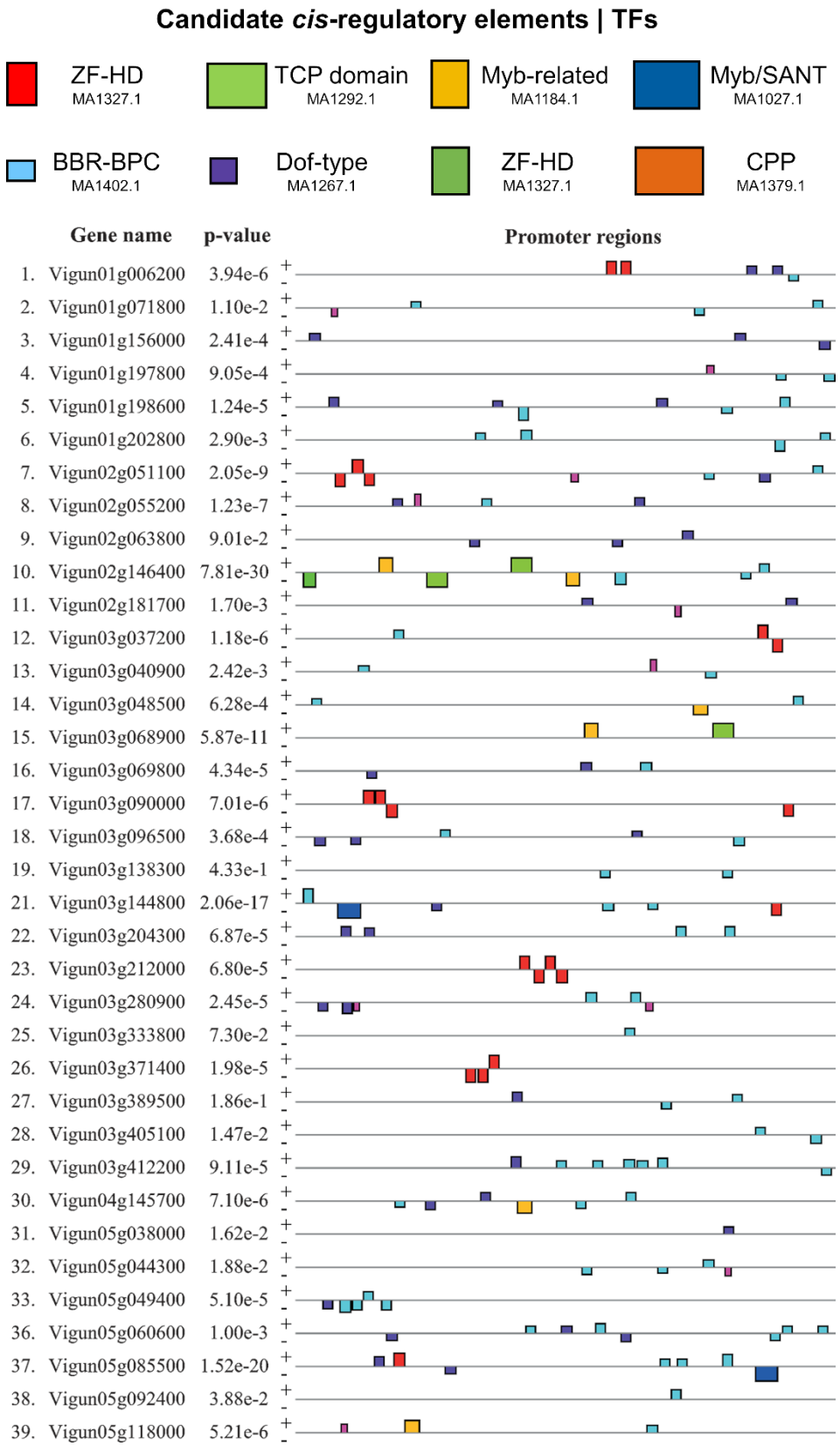


Figure 8: Distribution of predicted *cis*-regulatory elements in the promoter regions of the VuWRKY genes. Content and distribution of motifs by the promoter are represented by rectangles and colored squares. Colored boxes (section CCREs | TFs) provide information on TFs associated with the motifs identified, as well as their respective JASPAR ID matrices. The "+" and "-" signals represent *sense* and *antisense* chains of the promoter regions analyzed.

Transcriptomic Analysis

Structural variation in expressed isoforms of *VuWRKY* genes

Based on BLASTn comparisons, the 97 VuWRKY transcripts identified in the CpGC database arise from 55 members of the VuWRKY gene family by differential splicing with some genes giving rise to multiple splice variants and protein isoforms. A phenetic tree was constructed using the predicted protein coding sequence of the various isoforms (Figure 9A) and the underlying gene structure to determine the nature of the splicing variations that occurred (Figure 9B). In some cases the splice variants of a single gene gave rise to protein isoforms with significantly different protein domain compositions as defined by MEME analysis (Figure 9C).

Expressed VuWRKY transcripts exhibited some diversity in relation to the number of introns (Figure 9b), ranging from one to six, with most members (42.3%) having two introns (Supplementary Table 3). In all subgroups of the family it was possible to find transcripts with this intron pattern, with subgroup IId being the most representative, with six (85.7%) of seven members presenting two introns, followed by (sub) groups III and IIc, with 73.3% (11/15) and 72.7% (08/11) of the transcripts, respectively. Within the family groups, members of the subgroup IIb showed a higher variation in exon/intron structure, with one (VuWRKY26_i4; VuWRKY28_i2; VuWRKY28_i6) to six (VuWRKY27_i4) introns. In contrast, the expressed transcripts of subgroup IId were shown to be more conserved in terms of gene structure, with their members exhibiting a pattern of two introns, with a single exception of the transcript VuWRKY60_i2, which presented only one intron. Interestingly, three transcripts exhibit no intronic region, being one of group I (VuWRKY15_i4) and two of the 'Unclassified' (VuWRKY90_i1; VuWRKY90_i2) group (Supplementary Table 3).

A total of 38 conserved motifs varying in length from 11 to 60 amino acids were identified among the 97 VuWRKY expressed at the CpGC bank (Table 2). As expected, the DNA binding domain containing the conserved WRKYGQK heptapeptide sequence (motif 1) was present in all of the proteins. The only exception was VuWRKY80_1 which appear to be a splice variant giving rise to an isoform missing most of the N-terminal portions of the full-length protein. Almost all transcripts contained motif 2 (including VuWRKY80_1), which comprises part of the zinc finger structure of the protein (Supplementary Table 3). Motif 3 corresponds to the WRKY domain of the C-terminal region of group I proteins, is absent in only four isoform (VuWRKY13_14, VuWRKY14_12, VuWRKY15_14, and VuWRKY15_15).

Some motifs were restricted to specific VuWRKY groups. For example, motifs 05 and 21, are only found isoforms in subgroup IIb, and motifs 07 and 08 are unique to members of group I. Likewise, six motifs were shared between members of only two groups (motif 04, groups I and IIc, motif 06, groups IIa and IIb, motifs 16, 18 and 30, groups I and IIb, motif 28, groups I and IIe). Notably, most members of the same subgroup shared one or more motifs outside the WRKY domain and sometimes had the same specific location in the protein sequence (Figure 9C). Thus, it was possible to identify patterns conserved within the same VuWRKY group, as well as exon/intron patterns present in the expressed isoforms, validating the groups observed in the NJ tree (Figure 2).

Table 2: Consensus sequence of the 38 motifs identified by the MEME tool in VuWRKY transcripts expressed in the CpGC database, followed by the values of statistical validation (e-value), number of anchored sites and the size of the motif identified.

Logo	Consensus Motif	E-value	Sites	Width
1.	LDDGYRWRKYGQKVVKGNPNPRSYYRCTS	4.1e-2204	96	29
2.	PGCPVRKQVZRASEDPSILITTYEGKHN	6.7e-1520	87	28
3.	DDGYNWRKYGQKQVKGSEYPRSYYKCTHPNCQVKKKVERSHDGHITEIIYKGTHNHPKPQ	5.3e-1316	25	60
4.	GATRTVREPRVVVQTTSEVDI	5.0e-322	33	21
5.	HPLPPSATAMASTTSAAASMLLSGSSTSS	7.3e-280	18	29
6.	LVEAATKAJTTDPNFRAALAAAISSIIGS	1.7e-269	25	29
7.	HDVPAARASSHVNANASNANQ	4.7e-174	23	21
8.	YDGEGDEPESKRRKLESYNDD	1.8e-166	23	21
9.	IQSPPSTSSPHLFPTITLDLT	2.6e-143	27	21
10.	MLSNLAMSGLGHMQAKLPVMPVHPFLAQQ	5.5e-163	15	29
11.	LVKVQEENKNLLHLLADVAESYPELQIKL	6.2e-187	26	29
12.	KHENLETTSSASIGPEYCNQSTNLQTQNGGTLNDSGEAVDA	1.5e-140	15	41
13.	NPSNDYGFMMPKGEPNVEAIPEHVGGLNL	1.2e-134	16	29
14.	NVKLSGDLGLASPSPLTFFSK	1.5e-111	15	21
15.	SGTSFHRGAGRKINPTTLPQQSHPGIEVS	4.4e-145	15	29
16.	SGFNEPSETTDGSSZRTATDSEYVAQLSFSTEQAPNKREGQSNSTAGSGGFNANNANKSV	1.4e-332	17	60
17.	GIGSVNPHTDMQVDNPEHVELHNGGDGDI	6.4e-138	15	29
18.	DQRAFDDVGGGTEHSTPIEEHGDEZGBNR	2.1e-168	19	29
19.	ASKSQEAGNFILLQPPGPFGFSKNVTSQI	2.5e-109	15	29
20.	ASGVLHSHVHRPEPSQVHNGMGRLERPSL	3.5e-106	16	29
21.	TDSATQSPIKKARVSVRARSE	5.4e-105	15	21
22.	SSTFSNEEEEFEQGTQGSVSG	3.1e-098	24	21
23.	PNGSSVYQEIMSRMPLGPHM	3.7e-069	15	20
24.	RQQLAHSHGFSFGMN	9.4e-058	16	15
25.	IRPSFVNGYSFSDSFNNSRPK	1.5e-067	15	21
26.	PEKCKNNGFDDIYTSSFAFKP	1.9e-058	15	21
27.	WGNVQKGNIVGAANW	2.9e-054	15	15
28.	EENVTRSISEHSGSGRTGEFF	4.6e-045	15	21
29.	PQPTVGKFLFESEGNVRHSEL	1.3e-042	17	21
30.	GNGDSVTGGVGGAPS	9.7e-056	15	15
31.	SAANSAALDSEFGFTQFGLDNSNFKFVNP	2.3e-036	15	29
32.	NFSEFSFHSQT	2.1e-020	27	11
33.	LLADKVVSSFEKVLSLLNCGA	2.5e-014	15	21
34.	NPLFAQNLNNSLEEQQQHQQQ	7.9e-019	15	21
35.	SYKRRKTRPKWTERV	6.9e-022	16	15
36.	HFYQQCTTNQQTPSR	5.3e-015	15	15
37.	EVIEELJQGKELATKLKNLL	1.1e-013	15	20
38.	VSKIFVRTDASDTSL	8.9e-013	15	15

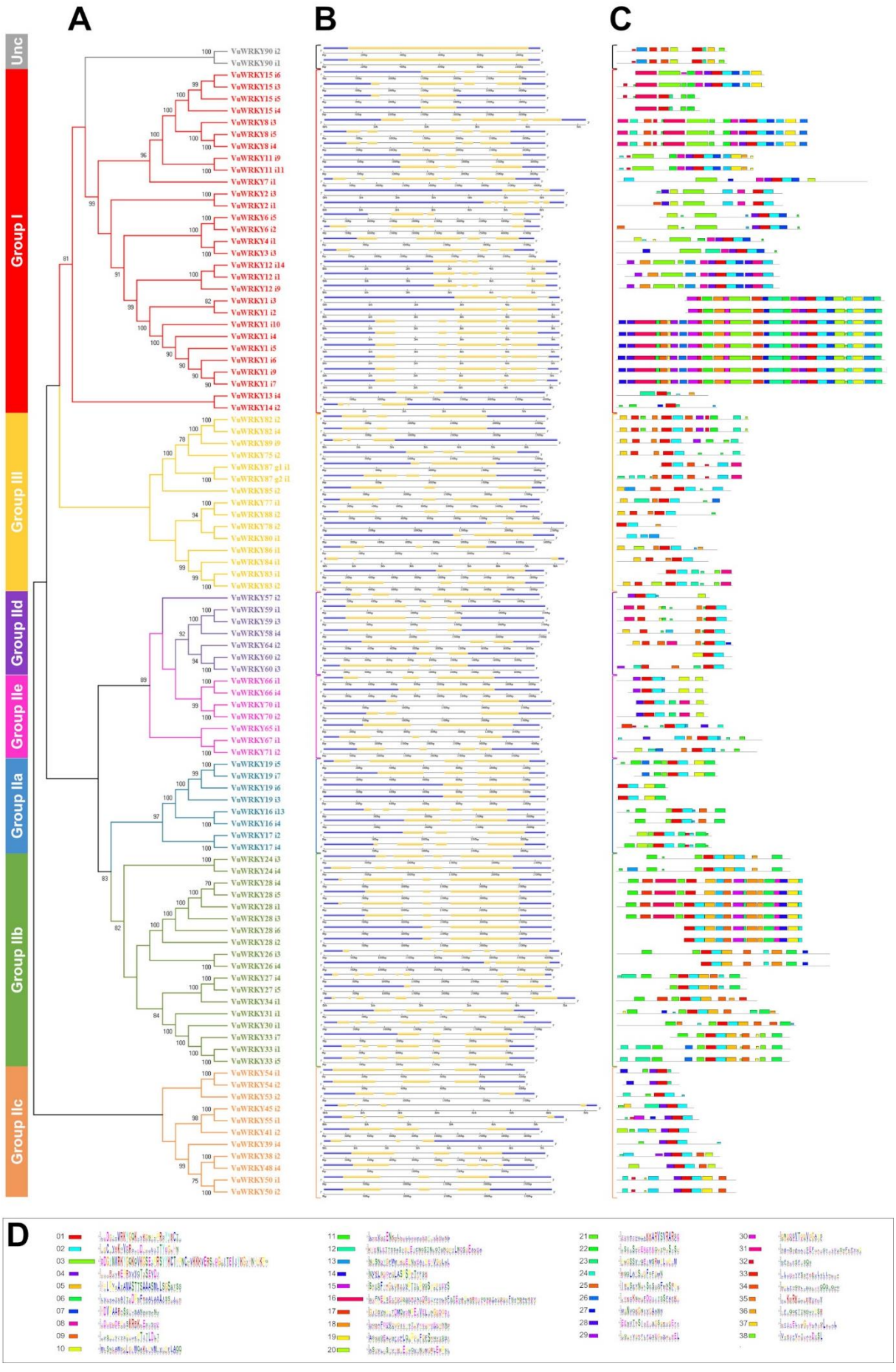

A
B
C
D
Unc
Group I
Group III
Group IId
Group IIe
Group IIa
Group IIb
Group IIc
VuWRKY90 i2
VuWRKY90 i1
VuWRKY15 i6
VuWRKY15 i3
VuWRKY15 i5
VuWRKY15 i4
VuWRKY8 i3
VuWRKY8 i5
VuWRKY8 i4
VuWRKY11 i9
VuWRKY11 i11
VuWRKY7 i1
VuWRKY2 i3
VuWRKY2 i1
VuWRKY6 i5
VuWRKY6 i2
VuWRKY4 i1
VuWRKY3 i3
VuWRKY12 i14
VuWRKY12 i1
VuWRKY12 i9
VuWRKY1 i3
VuWRKY1 i2
VuWRKY1 i10
VuWRKY1 i4
VuWRKY1 i5
VuWRKY1 i6
VuWRKY1 i9
VuWRKY1 i7
VuWRKY13 i4
VuWRKY14 i2
VuWRKY82 i2
VuWRKY82 i4
VuWRKY89 i9
VuWRKY75 i2
VuWRKY87 g1 i1
VuWRKY87 g2 i1
VuWRKY85 i2
VuWRKY77 i1
VuWRKY88 i2
VuWRKY78 i2
VuWRKY80 i1
VuWRKY86 i1
VuWRKY84 i1
VuWRKY83 i1
VuWRKY83 i2
VuWRKY57 i2
VuWRKY59 i1
VuWRKY59 i3
VuWRKY58 i4
VuWRKY64 i2
VuWRKY60 i2
VuWRKY60 i3
VuWRKY66 i1
VuWRKY66 i4
VuWRKY70 i1
VuWRKY70 i2
VuWRKY65 i1
VuWRKY67 i1
VuWRKY71 i2
VuWRKY19 i5
VuWRKY19 i7
VuWRKY19 i6
VuWRKY19 i3
VuWRKY16 i13
VuWRKY16 i4
VuWRKY17 i2
VuWRKY17 i4
VuWRKY24 i3
VuWRKY24 i4
VuWRKY28 i4
VuWRKY28 i5
VuWRKY28 i1
VuWRKY28 i3
VuWRKY28 i6
VuWRKY28 i2
VuWRKY26 i3
VuWRKY26 i4
VuWRKY27 i4
VuWRKY27 i5
VuWRKY34 i1
VuWRKY31 i1
VuWRKY30 i1
VuWRKY33 i7
VuWRKY33 i1
VuWRKY33 i5
VuWRKY54 i1
VuWRKY54 i2
VuWRKY53 i2
VuWRKY45 i2
VuWRKY55 i1
VuWRKY41 i2
VuWRKY39 i4
VuWRKY38 i2
VuWRKY48 i4
VuWRKY50 i1
VuWRKY50 i2

Figure 9. (A) Neighbor-joining tree of expressed VuWRKY identified at CpGC data bank. The groups and subgroups are delimited by the colors red (I), blue (IIa), green (IIb), orange (IIc), yellow (III) and gray (Unclassified). Gene structure of expressed VuWRKY regarding exon-intron composition is indicated in **(B)**. Gray lines and yellow boxes represent the introns and exons, respectively, of the expressed genes, while the blue regions correspond to the 5 'and 3' UTRs. **(C)** Distribution of conserved motifs identified by MEME in VuWRKY proteins. **(D)** The consensus sequence of each identified motif is represented by different boxes/colors and sizes, numbered from 1 to 38.

Expressed VuWRKY under root dehydration

To further analyze the role of the VuWRKY gene family in response to dehydration stress, we determined the transcription profiles of the family members in the roots of a drought sensitive ('Santo Inácio' – SI) and drought tolerant ('Pingo de Ouro' – PO) cultivar before and after imposition of dehydration stress. RSEM values were compared between control (T0) *vs.* stressed (T25 and T150) and 83 of 97 previously identified VuWRKY transcripts were expressed, with 3 variants expressed exclusively in the T25 samples (VuWRKY15_i5; VuWRKY28_i2; VuWRKY90_i1) and 6 in the T150 samples (VuWRKY01_i3; VuWRKY01_i9; VuWRKY02_i3; VuWRKY33_i1; VuWRKY80_i1; VuWRKY82_i2) (Figure 10). Five transcripts present in the CpCG data bank (VuWRKY01_i7; VuWRKY12_i9; VuWRKY59_i1; VuWRKY83_i1; VuWRKY83_i2) were not expressed in any of the dehydration treatment samples.

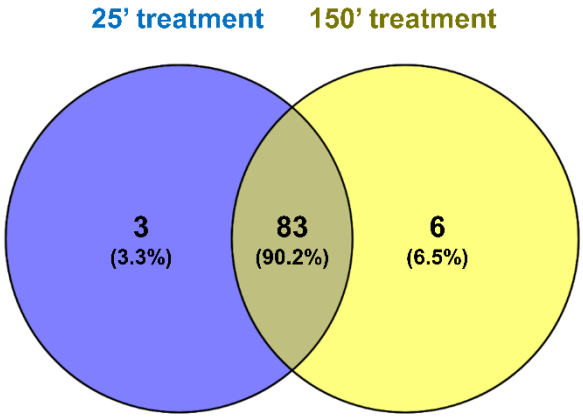


Figure 10: Venn diagram representing the number of VuWRKY transcripts expressed under root dehydration as compared with the not stressed control (T0) *vs.* t treatments with different exposition times (T25 and T150). The blue and yellow circles correspond to the genes expressed at 25 min and 150 min after stress imposition, respectively, and the intersection indicates the genes expressed in both treatments.

Based on the here defined statistical reliability criteria, 18 and 23 VuWRKY transcripts were differentially expressed at the times of 25 and 150 min after stress imposition, respectively. However, when considering such criteria to determine the intersection of the transcripts expressed in both comparisons, only eight transcripts achieved statistical confidence in terms of their differential expression (induction/repression). Considering their log_2FC, the 83 VuWRKY transcripts were grouped into four large groups (I, II, III and IV), with group IV subdivided into two subgroups (IVa and IVb) (Figure 11a, Supplementary Table 4).

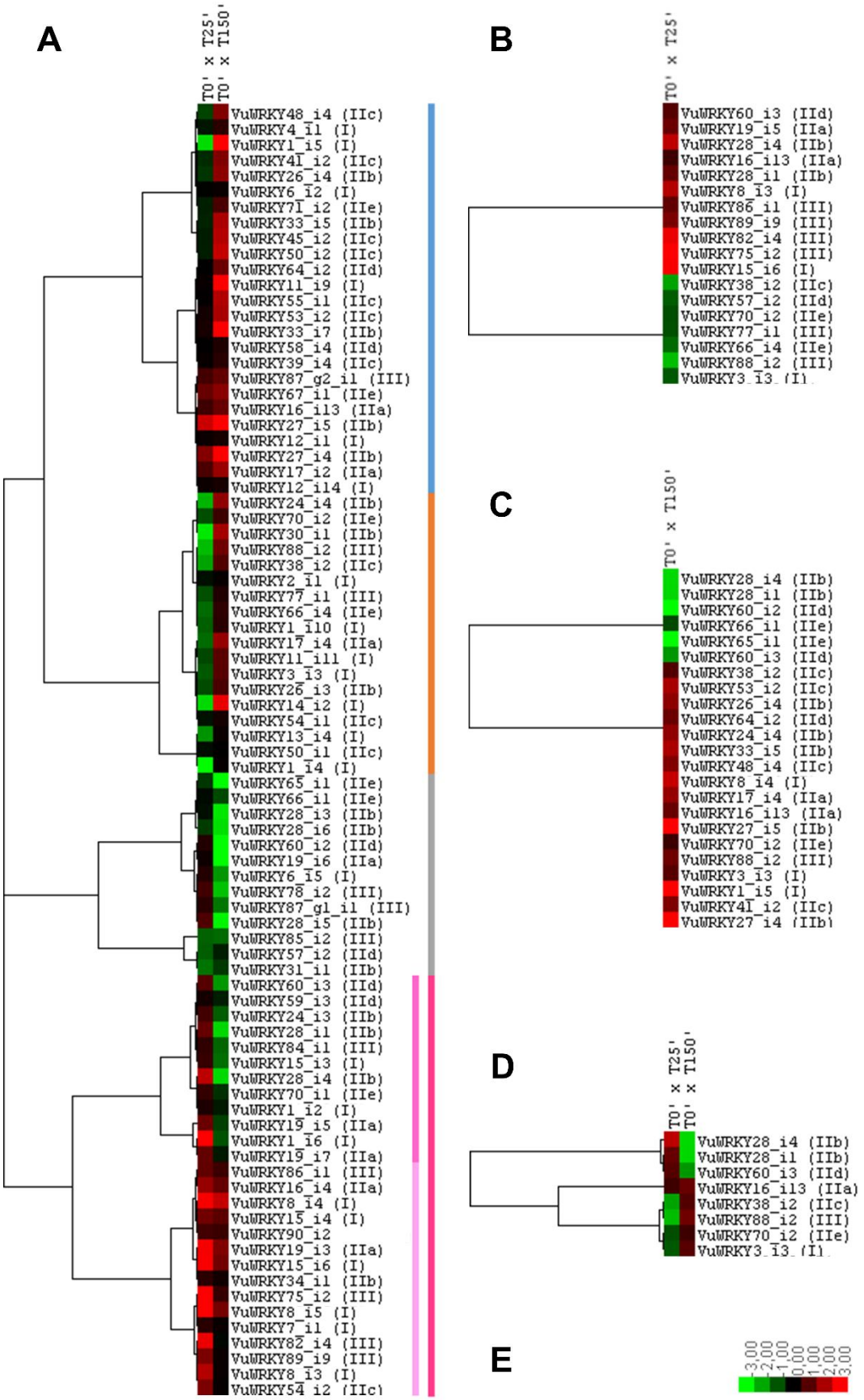


Figure 11: Gene expression profiles of WRKY TFs identified in the cowpea transcriptome under root dehydration. Expression levels are indicated by the intensity of the color, where red indicates induction and green indicates repression of the associated transcript when compared to the not stressed control in each analyzed time.

(A) *Heatmap* of the 83 VuWRKY expressed in the analyzed condition. Colored bars limit the different clusters [*cluster* 1 = blue; *cluster* 2 = orange; *cluster* 3 = gray; *cluster* 4 = pink (shades of pink delimit the subdivision of the group 4)]. When considered p-values and FDRs with 0.05 of reliability, **(B)** 18 transcripts were expressed in T25 and **(C)** 23 transcribed in T150. At the intersection of the times **(D)** eight transcripts are within the statistical reliability criteria. **(E)** Intensity scale of induction or repression of the transcript.

Cluster I comprised 25 transcripts whose expression varied at the initial time (log_2FC between -2.59 and 2.39), most of them being induced at the late (150 min) evaluated time (18 transcripts with log_2FC from 1.16 to 7.99). Cluster II included 18 repressed or constitutively expressed transcripts at T25 (log_2FC from -4.87 to -0.17), but their expression increased at T150 (log_2FC 0.02 - 2.77). Cluster III was composed of 13 repressed up to not modulated VuWRKY representatives in T25 (log_2FC -1.37 to 0.93), but in T150 most of the transcripts showed a tendency to be repressed (log_2FC between -3.56 and -0.35). 12 transcripts of subgroup IVa presented constitutive expression or induction in T25 (log_2FC: 0.22 to 4.21), decreasing or repressing their expression in T150 (log_2FC: 2.57 to -0.3), while most of the 15 transcripts of the subgroup IVb presented induction in both times or at least in one of them (T25 = log_2FC 0.49 to 5.77; T150 = log_2FC -0.08 to 2.33).

Considering transcripts with expression values within the statistical reliability criteria at T25, ten, five and three VuWRKY were induced (log_2FC: 1.02 to 3.35), repressed (-2.23 to -1.06) or were not modulated (-0.87 to 0.95), respectively (Figure 11b). Furthermore, in the final time (T150) 15 transcripts had their expression induced (log_2FC: 1.03 to 7.99), and five were repressed (-3.56 to -1.80) whereas three showed constitutive expression (-0.87 to 0.95) (Figure 11c).

The intersection with transcripts expressed in both treatments, presents a set of eight VuWRKY grouped into three clusters (A, B and C), the first one formed by three induced transcripts (log2FC: 1.02 to 2.24) in the first comparison that were later repressed (-1.8 to -2.57). The second group formed by a single transcript that increased its expression during the course of stress (T25: 0.73 and T150: 1.22). In turn, the third group was composed of four transcripts that showed induction in their expression along the evaluated times (T25: -2.23 to -0.93; T150: 0.72 to 1.31) (Figure 11d).

Validation of VuWRKY transcription revealed qPCR

Of the 83 VuWRKY expressed sequences under root dehydration, we selected 26 for further analysis. These genes also were selected because their orthologs in other plants species had been previously reported to be responsive to abiotic stress such as drought and salt (Supplementary Table 5). A set of two primers pairs were designed for each of the VuWRKY genes (Supplementary Table 2). The selected genes span the range of different VuWRKY groups and subgroups (i.e., groups I (5 sequences); IIa (5); IIb (2); IIc (3); IId (5); IIe (1) and III (5).

qPCR allowed to identify the transcriptional behavior of each of the 26 selected transcripts. Total RNA was isolated from the roots of the dehydration sensitive SI cultivar and the tolerant PO cultivar before T0 and at 25 (T25), 50 (T50), 75 (T75), and 100 (T100) min after the initiation of root dehydration and the relative abundance of the various VuWRKY transcripts determined by qRT-PCR (Figure 12).

Among the 26 investigated transcripts, three reached expression levels with log_2FC between 10 and 20 (VuWRKY11, 86 and 89), three between 25 and 50 (VuWRKY8, 16 and 82), two between 60 and 90 (VuWRKY53 and 87) and three between 150 and 300 (VuWRKY18, 21 and 75). The remaining 15 exhibited a relative expression level below 10.

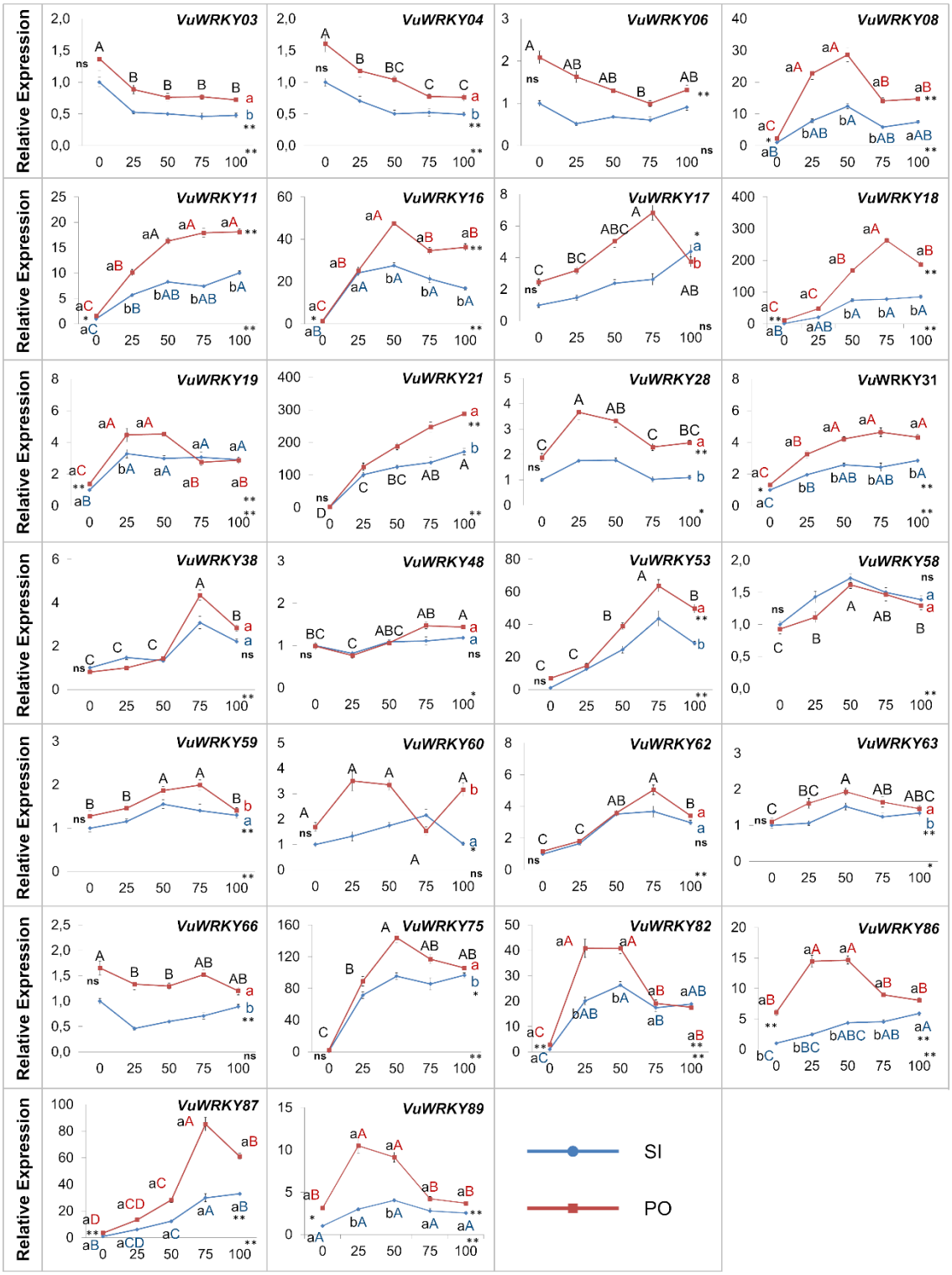


Figure 12. Water deficit responsiveness of 26 cowpea *Vu*WRKY genes. Real-time PCR was performed with cDNAs derived from five different time points (0, 25, 50, 75 and 100 min) after water deficit for both analyzed genotypes ('Santo Inácio' – sensitive/ blue; 'Pingo de Ouro' – tolerant/ red). The level of statistical significance was shown as ** (t-Test at $p < 0.01$), * (t-Test at $p < 0.05$) and ns (not significant by t-Test): **(1)** on the right side of the graph, for comparison between genotypes; **(2)** at the bottom, right side of the abscissa, for comparison between times, and **(3)** on the left side of the graph between the two lines, for interaction genotype x time. Genotypes followed by the same letter were statistically similar, while when followed by different letters showed significant differences. On the other hand, times followed by the same uppercase letter were statistically similar, while when followed by different letters showed significant differences. The graphics show the values of fold change in the comparison of relative levels of expression between genotypes for each analyzed time.

Most of the transcripts (22 out of 26) were induced compared to time 0 (zero) for both genotypes with the exception of VuWRKY3, 4, 6, and 66. For the tolerant genotype (PO), among the 22 induced transcripts, six reached their highest expression level at T25, five at T50, 10 T75 and only one at T100 in the tolerant SI genotype. In turn, in the sensitive genotype (SI - blue in the graphs; Figure 10), only two reached their highest level of expression at T25, 10 at T50, three at T75 and six at T100. Thus, in general, the transcripts of the sensitive genotype responded later and with lower expression values when compared to the tolerant.

Comparing the results of RNA-Seq techniques (only for the tolerant PO genotype) and the qPCR results in the same accession, the repressed transcripts (VuWRKY3, 4, 6 and 66) presented the same tendency in qPCR in all or at least in one of the times (Figure 11a). Regarding transcripts induced in qPCR, eight were associated with only one isoform, of which five (VuWRKY48, 53, 75, 86 and 89) were validated in terms of their expression identified by RNA-Seq in T25. The remaining induced transcripts (14) were associated with more than one isoform and may reflect the response of more than one transcript. However, eight (VuWRKY8, 11, 16, 19, 28, 60, 82, and 87) of these transcripts were validated in at least one of the times evaluated by the RNA-Seq.

Other relevant data for qPCR were the values of the relative Differential Expression between both genotypes [DEG, calculated by dividing the average expression value of PO by that of SI in each time point . It is worth mentioning that the vast majority of the analyzed genes showed DEgs higher than 1.0 (one) at time 0 (zero), indicating a higher level of relative expression the genes in the PO tolerant genotype even before applying the water deficit treatment, reaching 6.1x for VuWRKY86, 7.0x for VuWRKY53 and 11.8x for VuWRKY18. Exceptions to this rule were VuWRKY38 and VuWRKY58, with DEG before water deficit treatment smaller than 1.0 and VuWRKY48 with a value equal to 1.0. After water deficit treatment, in comparison with sensitive genotype, the tolerant genotype kept its induction in all times tested (values greater than 1), with DEg values ranging between 0.7 and 5.9x in the 22 genes up-regulated. Additionally, among the analyzed genes, 10 stood out for having a high level of expression after treatment with water deficit, with DEg values high or above average (VuWRKY8, 11, 16, 18, 21, 75, 82, 86, 87 and 89). Six of these genes showed orthology with genes from other species involved in abiotic stress tolerance pathways (see Supplementary information 2).

Discussion

Since the description of the WRKY family in plants (Ishiguro and Nakamura, 1994), many members have been described, including monocot species such as corn (*Zea mays*, 120 representatives, Zhang et al., 2017a), rice (*Oryza sativa* L. ssp. *indica*, 102 and ssp. *japonica*, 98 members, Ross et al., 2007), wheat (*Triticum aestivum* L., with 171; Ning et al., 2017), and dicots as eucalyptus (*Eucalyptus grandis*, with 79; Fan et al., 2018), cotton (*Gossypium raimondii* with 112 and *G. arboretum* with 109 members, Ding et al., 2015) and melon (*Cucumis melo* L., 56 members, Jiao et al., 2018). Within legumes, the number of WRKY encoding genes varies significantly (Song et al., 2018) with 70 representatives reported for the diploid species chickpea (*Cicer arietinum* L.; Waqas et al., 2019) and 188 for the tetraploid soybean (*Glycine max;* Yu et al., 2016). In the present work, the WRKY family was deeply studied in cowpea, uncovering 92 WRKY encoding genes in the cowpea genome recently published by Lonardi et al. (2019). This number indicates a relative conservation in the number of representatives when compared to its congener species (*Vigna angularis* with 91 VaWRKY and *V. radiata* with 85 VrWRKY; Srivastava et al., 2018).

The classification of cowpea VuWRKY proteins in three large groups (I, II and III) and five subgroups (IIa to IIe) after NJ analysis corroborated the existing infrafamilial classification previously observed for *A. thaliana*, *G. max*, *O. sativa* and *Populus trichocarpa* (Rushton et al. 2010). As observed for other legumes (Song et al., 2018; Waqas et al., 2019), in cowpea group II was the largest and most diverse, with subgroup IIc having the highest number of representatives. The evolutionary analysis of the family suggests that the diversification into groups and subgroups had a more significant expansion within the subgroup IIc, after their origin through the loss of the N-terminal domain from members of group I (Zhang and Wang, 2005; Brand et al., 2013).

It is worth noting that, proteins placed in the 'unclassified' group, VuWRKY92 is more closely related to groups I and IIc members, even do not present these groups characteristics. In an evolutionary context, this unclassified variant may indicate a diversification of WRKY members in cowpea. Besides, the independent positioning of VuWRKY90 and 91 in the NJ tree may denote the loss of the N-terminal domain, with sub-functionalization and retention of the ancestral function (Karanja et al., 2017). Therefore, the deletion of a domain seems to be the driving force in the expansion of this family into new WRKY variants in cowpea. Another hypothesis is based on the possibility that unclassified genes are remnants of sequencing errors and/or assembly, appearing as pseudogenes on cowpea genome (Jimmy and Babu, 2019). In addition, the non-induction of isoforms by the VuWRKY91 and VuWRKY92 genes in transcriptome data on CpGC bank, reinforce such evidence (Xie et al., 2018).

WRKYs bind to W-box elements (through the central motif TTGACC/T) in the promoter regions of the target genes to regulate signal transduction and metabolic pathways during stress response (Rushton et al. 2010; Phukan et al., 2016). Structural analysis of the VuWRKY domains revealed that, although most family members have the conserved heptapeptide sequence [WRKYGQK], some proteins contain variant motifs in their domain. Changes in the WRKYGQK pattern may interfere with the binding affinity of TF to DNA (Ciolkowski et al., 2008) or may, still, allow recognition of novel cis-binding sequences, other than the W-box element (Cai et al., 2008; van Verk et al., 2008; Zhou et al., 2008). The specific interaction between a WRKY-TF and its DNA target occurs due to the three-dimensional structure of the WRKY domain, which includes a positively charged β chain, responsible for binding to the cis-regulatory element in the target promoter. Brand et al. (2013) suggested that the element recognized by WRKY (W-box) is formed by an ultra-conserved 'GAC' core, preceded by a thymine and a pyrimidine at the downstream position. This central nucleus is responsible for interacting with the WRKYs, while the other residues will direct the recognition by specific TFs. Changes in any of these patterns may interfere with the precise recognition affinity and binding. In this study, four variant isoforms (two expressed from the *VuWRKY15* gene and one from each *VuWRKY53* and *54*) were induced under conditions of root dehydration, suggesting a functional activity of these proteins in cowpea under water deficit. In fact, variants of the WRKY heptapeptide motif have been reported in several species (Satapathy et al., 2014; Kumar et al., 2016; Karanja et al., 2017; Cui et al., 2018), demanding further studies to determine the binding functions and specificities of these protein variants in the plant response under stress.

To infer on the distribution, relative position and abundance of WRKY family members in the cowpea genome, an in silico mapping of the identified VuWRKY was carried out, revealing some clustered groups of tandem duplicated genes in the pseudochromosomes of cowpea, besides some disperse representatives. Serial and segmental duplications played a key role in the expansion of gene families during the evolution (Bellieny-Rabelo et al., 2013; Jiang et al., 2013), besides inducing gene neofunctionalization (Panchy et al., 2016). In fact, tandem and segmental duplications have also been considered among the driving forces

behind the expansion and diversity of the WRKY family (Zhang et al., 2005; Yin et al., 2013; Silva et al., 2017; Zhang et al., 2017b). In another Fabaceae, chickpea (*C. arietinum* L.), the evolution of WRKY genes was shown to be predominantly associated with segmental duplications. Also, in soybean duplicated and clustered WRKY genes with similar structural characteristics were detected, but with distinct gene expression patterns in the analyzed tissues (Song et al., 2018).

Our results indicate that segmental duplications are also associated with WRKY expansion in cowpea. Interestingly, three clustered VuWRKY genes duplicated in tandem (at pseudochromosome 8 e 9) and correspond to proteins with variations in the heptapeptide domain sequence (namely VuWRKY20, 53 and 90). Of these, VuWRKY90 and VuWRKY19 (a VuWRKY20 analog) were expressed under root dehydration. In contrast, the VuWRKY20 gene and the VuWRKY53 variants were not regulated against the stress analyzed, leading us to infer that (despite their common origin) there is a diversification of their biological functions.

Cis-regulatory elements present in gene promoters are important transcriptional switches involved in the expression control through TF-biding, regulating an extensive network of genes involved in various biological phenomena (Zhang et al., 2005). The specific interaction of these regulatory factors is able to induce molecular cascades, controlling the activation, suppression, and modulation of plant signaling pathways in developmental processes and in response to stress (Sheshadri et al., 2016). In this context, *VuWRKY* genes identified herein had the sequences of their predicted promoters evaluated for the presence of the cis-regulatory elements as well as the identity of the associated binding TFs. This approach allows to infer on the potential physiological and/or adaptive processes in which the WRKY TFs participate, once the functions of the regulating factors are determined. The MEME analysis allowed the identification of eight motifs, with statistical reliability, in the VuWRKY promoter regions, associating the potential cis-regulatory elements to other six TF-families.

As expected, the identified elements are known to be involved in plant development processes and in responses to environmental stresses, according to literature data. This is the case of BBR-BPC, present in the cis-regulatory region of many VuWRKY. They are plant-specific TFs and regulate gene induction or repression in association with plant development and phytohormone signaling, such as ethylene, cytokine and abscisic acid (ABA) (Monfared et al., 2011; Simonini and Kater, 2014; Mu et al., 2017; Shanks et al., 2018). ABA accumulation is known to be associated with water stress, regulating the closure of the stomata to reduce water loss through transpiration (Schroeder et al., 2001). This process was impaired in an *Arabidopsis* mutant for the *ABO3* gene encoding AtWRKY63, confirming WRKY involvement in the ABA-dependent water stress response pathway (Ren et al., 2010). In cowpea, the promoter regions of 63 WRKY genes had cis-elements recognized by BBR-BPC TF members, being 39 regulated under root dehydration, confirming the role of the WRKY TFs in response to water deficit in this legume crop.

Considering the Zinc-finger Homeobox family (ZF-HD), for instance, Rizhsky et al. (2004) observed that the expression of AtWRKY25 was dependent on the ZF protein encoded by the *AtZat12* in *Arabidopsis* mutants subjected to oxidative stress. Similarly, Dof genes may be involved in a direct interaction with other TFs, including members of the WRKY family, suggesting that they are essential in regulating plant physiological processes (Le Hir and Bellini, 2013). In the present work with water deficit stress in cowpea, at least one of the six TF families recognized the motifs in the promoter regions of the 55 regulated WRKY genes, where 97 isoforms were expressed in the CpGC data bank. Of these, 26 genes were validated for their response to water deficit via qPCR and were associated mainly with BBR-BPC and Dof TFs, besides associations with TCP, MYB-related and ZF TFs. Notably,

many cowpea WRKY genes showed more than one cis element in their promoter region, suggesting that their regulation may occur in response to different physiological conditions, or even under various environmental stresses.

In this scenario, we analyzed the architecture of isoform VuWRKY, including exon/intron characterization and position, in order to reveal isoform structural diversification. The alternative splicing is one of the molecular mechanisms that most contributes to isoform diversity, modulating the function of the protein (Hiller et al., 2005). In fact, alternative splicing was responsible for the regulation of 55 VuWRKY genes that triggered 97 isoforms of all WRKY subgroups, with the genes *VuWRKY01* and *28* figuring as the most induced variants. In addition, splicing is related to the evolutionary history of the group, since the preserved splicing sites in WRKY domains contribute, among other factors, the evolution and classification of the family (Agarwal et al., 2011).

Although intronic regions are untranslated units, they may contain elements that regulate gene expression (Koralewski and Krutovsky, 2011) and a positive correlation was identified between their size and gene expression (Ren et al., 2006; Das and Bansal, 2019). The protein variants identified here were quite diverse with splicing giving rise to isoforms with significant structural differences. In agreement with this fact, we observed that the expressed VuWRKY exhibited diversified motifs along with its composition and distribution but were conserved within the groups and subgroups of the family, further illustrating their relation in each group. Most variations in motif structure occurred outside the WRKY domain, providing binding specificity for the VuWRKY under different conditions as proposed in other studies (Phukan et al., 2016). In the present work, some identified isoforms of subgroup IId (VuWRKY58_i4, VuWRKY59_i3, VuWRKY59_i1, VuWRKY64_i2) and group III (VuWRKY83_i2, VuWRKY84_i1, VuWRKY86_i1 and VuWRKY88_i2) exhibited the motif LXXLL, reported to function as activators of biological processes (Savkur and Burris, 2004) and in plant defense (Pandey and Somssich, 2009; Ishihama and Yoshioka, 2012). Interestingly, one of the expressed isoforms of VuWRKY83 does not exhibit the motif described as a result of alternative splicing, leading us to infer that such isoforms participate in different signaling pathways. Other studies also report the presence of LXXLL motif in WRKY of subgroup IId in grape (Zhang and Feng, 2014) and of group III in common bean (Wang et al., 2016).

Rigorous control and fine-tuning of WRKY protein regulation during the responses to abiotic stresses in plants contribute to the induction of complex signaling networks (Chen et al., 2012). It is also known that these TFs are involved in the initial stages of the signaling cascade in response to adverse conditions (Franco-Zorrilla and Solano, 2016). In fact, cowpea RNA-Seq data showed that VuWRKY transcripts are strictly modulated in the tolerant access of cowpea during the first minutes of exposure to stress, including expression of variant isoforms. For instance, induction of VuWRKY01 leads to the generation of five isoforms in the tolerant genotype and with distinct log_2FC values.

The qPCR validation confirmed the regulation of this gene in the initial periods after stress imposition, as well as its modulation in the drought susceptible accession. In a similar approach in soybean under root dehydration (Ferreira-Neto et al., 2013), results of HT-SuperSAGE validated by qPCR, which showed that *GmWRKY20* (ortholog to *VuWRKY75*) was induced in the tolerant genotype at all early times analyzed (25, 50, 75 and 100 min), whereas in the susceptible genotype the induction of the gene was restricted to the initial times evaluated (25 and 50 min). In the present work, RNA-Seq and qPCR revealed that VuWRKY75 was also induced at all times tested for both genotypes. However, the induction in the tolerant genotype was significantly higher than that found for the susceptible genotype, indicating a role of this gene in stress tolerance response.

The qPCR validation confirmed in the majority of cases the same type of expression indicated by our RNA-Seq data. The high similarity of the responses between the results from different transcriptomic techniques was also observed in other reports. For example, Nuruzzaman et al. (2014) validated the transcriptional profile of differentially expressed WRKY in rice lineages with contrasting tolerance to drought based on microarray data. The expression profile revealed that most of the *OsWRKY* genes were activated in response to water stress in the tolerant lineage during the reproductive stages of plant growth. Besides, results showed a similarity of gene expression measured by RT-PCR/qPCR techniques. In another report in corn with RNA-Seq, ZmWRKYs were analyzed under leaf dehydration (Wang et al., 2018a) uncovering 14 induced genes, where *ZmWRKY106* was selected for validation in different situations. qPCR results indicated that the treatment with high-temperature plus dehydration significantly induced expression of *ZmWRKY106* in the early hours of stress, whereas ABA and high salinity treatments induced more discreet gene modulation (Wang et al., 2018a), thus confirming the trend in the positive regulation indicated by the RNA-Seq data. Under stress, differences in the expression level of regulatory proteins may lead to significant changes in the degree of tolerance to abiotic stress as a consequence of the distinct regulation of genes located downstream of TFs (Nakashima et al., 2014).

In cowpea, the results of the root dehydration assay indicate that a group of WRKY genes play a prominent role in water deficit tolerance mechanism, because of their differential expression and early response in the tolerant genotype, in relation to the initial time and to the control treatment in the susceptible genotype. The relative expression values obtained by qPCR confirmed a general later activation of *VuWRKY* genes in the drought susceptible genotype and, still, with lower induction values, as compared to the values of the tolerant accession. A similar response was observed among common bean accessions, where differences in the transcriptional regulation of seven *WRKY* genes were observed among tolerant and drought susceptible genotypes, of which only *PvWRKY33* showed a lower expression level in the tolerant genotype as compared with the susceptible one (Wu et al., 2017). Similarly, in our data, the *VuWRKY38*, *48*, *58*, and *62* were the only genes, among the validated ones, that did not follow such behavior. Their expression values were similar between the genotypes tested, with a slightly higher response in susceptible access.

Recent studies confirm the association of WRKY protein expression with drought tolerance in different plant species (Chu et al., 2015; Kiranmai et al., 2018; Wang et al., 2018b; Liu et al., 2019). For instance, Ma et al. (2019) analyzed the expression pattern of *GmWRKY16* to multiple abiotic stresses, including drought. The authors verified that the induction of *GmWRKY16* conferred higher tolerance to drought and salinity, being more efficient at rehydration and water loss, with adequate accumulations of proline, ABA and MDA (Malondialdehyde), under the analyzed stress conditions. Qiao et al. (2016) evaluated mutant *Arabidopsis* plants for *wrky1* insertion and observed that AtWRKY1 (ortholog to VuWRKY13) acts on the ABA-dependent pathway, being involved with stomatal closure. Besides, AtWRKY1 was able to bind to the W-box elements of *MYB2*, *ABCG40*, *DREB1A* and *ABI5* genes, controlling their transcripts in response to water stress or ABA treatment (Qiao et al., 2016). Wheat transgenic plants overexpressing TaWRKY2 also exhibited increased drought tolerance under conditions of water deficit (Gao et al., 2018). A higher survival rate was observed in the wheat transgenic lines, due to the lower water loss by the leaves when compared to the wild plants. Also, proline, soluble sugar, and chlorophyll content were more efficiently protected from oxidative and osmotic damage in transgenic lines. In another investigation, the overexpression of AtWRKY30 (ortholog to VuWRKY82) was evaluated under drought and heat conditions in transgenic wheat plants (El-Esawi et al., 2019) leading to a significant increase in gas exchange efficiency and relative leaf water

content, plant biomass, higher osmolyte, and chlorophyll content, besides a reduction of ROS-induced oxidative stress. qPCR revealed the induction of genes related to the tolerance (*ERF5a*, *DREB1*, *DREB3*, *WRKY19*, *TIP2*, and *AQP7*) in the transgenic line under conditions of heat and drought, compared to the normal conditions. Taken together, the results showed that the induction of AtWRKY30 increased the stress tolerance efficiency in transgenic wheat line when compared to the wild type.

More than 20 cowpea genes can be associated with ortholog genes of the analyzed species previously reported for their importance on abiotic stress tolerance. For example: *AtWRKY33* (ortholog to *VuWRKY11*), is involved in oxidative, salt and ABA stresses (Jiang and Deyholos, 2009); *AtWRKY40* (ortholog to *VuWRKY21* and *VuWRKY18*) is involved in ABA signaling (Shang et al. 2010); *GmWRKY54* (ortholog to *VuWRKY48*) participates in salt and drought tolerance (Zhou et al., 2008); *GmWRKY13* (ortholog to *VuWRKY60* and *VuWRKY64*) is involved in salt and mannitol tolerance (Zhou et al., 2008); *OsWRKY45* (ortholog to *VuWRKY75* and *VuWRKY86*) is involved in water deficit and salt tolerance (Qiu and Yu 2009), and *AtWRKY46* (ortholog to *VuWRKY87*) is involved in heat tolerance and osmotic stress (Suzuki et al. 2005).

Cowpea transcriptome data (RNA-Seq), as well as qPCR results, uncovered promising *VuWRKY* genes responsive to root dehydration, highlighting, as previously mentioned, a potential involvement of these proteins in stress tolerance, besides the differences in time and intensity comparing tolerant and susceptible genotypes. Considering our qPCR data, the genes *VuWRKY18* (group IIa), *VuWRKY21* (group IIa) and *VuWRKY75* (group III) deserve mentioning due to their significant induction under root dehydration in the tolerant genotype [262.84x (T75), 287.79x (T100), 143.98x (T50), respectively], indicating their participation in cowpea drought tolerance response, deserving complementary studies.

Concluding remarks and perspectives

Taken together, our data indicate that:

- Cowpea presents 92 complete *VuWRKY* genes in its genome, of which 55 were associated with 97 transcripts expressed under root dehydration (CpGC RNA-Seq data bank).
- VuWRKY of all known classes were identified, as well as three with distinguishing characteristics, annotated as “unclassified” WRKY members, some induced under the here studied stress condition.
- Most of the genes validated by qPCR (22 of 26) were upregulated under root dehydration conditions and, comparing RNA-Seq and qPCR data, similar expression tendencies were recognized.
- Water stress tolerance in cowpea is associated with a rapid perception of stress, followed by a more efficient and higher modulation of WRKY TFs.
- Although the susceptible accession also induced VuWRKY in the early stages of exposure to root dehydration, qPCR data showed a faster response of the tolerant genotype, which triggers its genes at more significant expression levels
- Due to their role in activating molecular signaling cascades, the regulation of VuWRKY in the cowpea drought tolerant accession seems to provide a more targeted and accelerated response when compared to susceptible accession. In this way, the plant is able to induce vital physiological processes at significant levels so as to tolerate, for a more extended period of time, the low availability of water in the substrate.

Considering that cowpea presents superior performance under abiotic stress (including drought) when compared to other grain legumes (Varshney et al., 2009) the present work is

a pioneering study showing the transcriptional profile of most induced WRKY genes under root dehydration in tolerant and susceptible genotypes using RNA-Seq and qPCR approaches. In view of the results, the candidates identified in the tolerant accession deserve functional evaluation aiming to potential applications including the generation of molecular markers for breeding purposes as well as their biotechnological use for plant transformation.

References

Agarwal, P., Reddy, M. P., and Chikara, J. (2011). WRKY: its structure, evolutionary relationship, DNA-binding selectivity, role in stress tolerance and development of plants. *Mol. Biol. Rep.* 38, 3883–3896. doi: 10.1007/s11033-010-0504-5

Amorim, L. L. B., Fonsêca-dos-Santos, R., Bezerra-Neto, J. P., Guida-Santos, M., Crovella, S., and Benko-Iseppon, A. M. (2017). Transcription factors involved in plant resistance to pathogens. *Curr. Protein Pept. Sci*. 18, 335-351. doi: 10.2174/1389203717666160619185308

Amorim, L. L. B.; Bezerra-Neto, J. P., Santos, R. F., Ferreira-Neto, J. R. C., Kido, E. A., Matos, M. et al. (2016). "Transcription Factors Involved in Plant Drought Tolerance Regulation" *In* Drought Stress Tolerance in Plants: Molecular and Genetic Perspectives, ed. Hossain, M. A., Wani, S., Bhattacharjee, S., Burritt D., and Tran, L. S. (New York, USA, Springer International Publishing), 315–358.

Amorim, L., Ferreira-Neto, J., Bezerra-Neto, J. P., Pandolfi, V., de Araújo, F. T., da Silva Matos, M. K., et al. (2018). Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR. *Plant Methods*. 14, 88. doi: 10.1186/s13007-018-0354-z

Arumuganathan, K. and Earle, E. D. (1991). Nuclear DNA content of some important plant species. *Plant Mol. Biol*. 9, 208-218. doi: 10.1007/BF02672016

Bado, B. V., Lompo, F., Bationo, A., Segda, Z., Sedogo, M. P., Cescas, M. P., et al. (2012). Nitrogen recoveries and yields improvement in cowpea sorghum and fallow sorghum rotations in West Africa Savannah. *J. Agric. Sci. Technol. B*. 2, 758-767.

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., et al. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res*. 37, W202–W208. doi:10.1093/nar/gkp335

Bakshi, M., and Oelmüller, R. (2014). WRKY transcription factors. *Plant Signal Behav*. 9, e27700. doi: 10.4161/psb.27700

Bastos, E. A., Nascimento, S. P., Silva, E. M., Freire-Filho, F. R., and Gomide, R. L. (2011). Identification of cowpea genotypes for drought tolerance. *Rev. Cienc. Agron.* 42, 100-107. doi: 10.1590/S1806-66902011000100013

Bellieny-Rabelo, D., Oliveira, A. E., and Venancio, T. M. (2013). Impact of whole-genome and tandem duplications in the expansion and functional diversification of the F-box family in legumes (*Fabaceae*). *PloS One*. 8, e55127. doi:10.1371/journal.pone.0055127

Benko-Iseppon, A. M. Soares-Cavalcanti, N. M. Belarmino, L. C. Bezerra-Neto, J. P. Amorim, L. L. B. Ferreira Neto, J. R. C. et al. (2011). Prospecção de genes de resistência à seca e à salinidade em plantas nativas e cultivadas. *In*: Volume Especial do III Simpósio de Mudanças Climáticas e Desertificação no Semiárido Brasileiro, *R Bras. Geogr. Fís*. 6, 1112-1134.

Bortoleti, K. C. A., Benko-Iseppon, A. M., Melo, N. F., and Brasileiro-Vidal, A. C. (2012). Chromatin differentiation between *Vigna radiata* (L.) R. Wilczek and *V. unguiculata* (L.) Walp. (Fabaceae). *Plant Syst. Evol*. 298, 689-693. doi: 10.1007/s00606-011-0551-y

Boukar, O., Belko, N., Chamarthi, S., Togola, A., Batieno, J., Owusu, E., et al. (2018). Cowpea (*Vigna unguiculata*): Genetics, genomics and breeding. *Plant Breed*. 00, 1– 10. doi: 10.1111/pbr.12589

Brand, L. H., Fischer, N. M., Harter, K., Kohlbacher, O., and Wanke, D. (2013). Elucidating the evolutionary conserved DNA-binding specificities of WRKY transcription factors by molecular dynamics and in vitro binding assays. *Nucleic Acids Res*. 41, 9764–9778. doi:10.1093/nar/gkt732

Cai, M., Qiu, D., Yuan, T., Ding, X., Li, H., Duan, L., et al. (2008). Identification of novel pathogen-responsive cis-elements and their binding proteins in the promoter of *OsWRKY13*, a gene regulating rice disease resistance. *Plant Cell Environ*. 31, 86-96. doi: 10.1111/j.1365-3040.2007.01739.x

Chen, H., Wang, L., Liu, X., Hu, L., Wang, S., and Cheng, X. (2017). De novo transcriptomic analysis of cowpea (*Vigna unguiculata* L. Walp.) for genic SSR marker development. *BMC Genetics*. 18, 65. doi: 10.1186/s12863-017-0531-5

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., and Yu, D. (2012). The role of WRKY transcription factors in plant abiotic stresses. *Biochim. Biophys. Acta*. 1819, 120-128. doi: 10.1016/j.bbagrm.2011.09.002

Cheng, Y., Yao, Z. P., Ruan, M. Y., Ye, Q. J., Wang, R. Q., Zhou, G. Z., et al. (2016). In silico identification and characterization of the WRKY gene superfamily in pepper (*Capsicum annuum* L.). *Genet. Mol. Res.* 15. doi: 10.4238/gmr.15038675

Christmann, A., Grill, E., and Huang, J. (2013). Hydraulic signals in long-distance signaling. *Curr. Opin. Plant Biol.* 16, 293–300. doi: 10.1016/j.pbi.2013.02.011

Chu, X., Wang, C., Chen, X., Lu, W., Li, H., Wang, X., et al. (2015). The Cotton WRKY Gene *GhWRKY41* Positively Regulates Salt and Drought Stress Tolerance in Transgenic *Nicotiana benthamiana*. *PloS One*. 10, e0143022. doi:10.1371/journal.pone.0143022

Ciolkowski, I., Wanke, D., Birkenbihl, R. P., and Somssich, I. E. (2008). Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. *Plant Mol. Biol.* 68, 81–92. doi:10.1007/s11103-008-9353-1

Cruz, A. R. R. and Aragão, F. J. L. (2014). RNAi-based enhanced resistance to Cowpea Severe Mosaic Virus and Cowpea Aphid-borne Mosaic Virus in transgenic cowpea. *Plant Pathol*. 63, 831-837. doi: 10.1111/ppa.12178

Cui, Q., Yan, X., Gao, X., Zhang, D., He, H., and Jia, G. (2018). Analysis of WRKY transcription factors and characterization of two *Botrytis cinerea*-responsive LrWRKY genes from *Lilium regale*. *Plant Physiol. Biochem.* 127, 525–536. doi:10.1016/j.plaphy.2018.04.027

Das, S., and Bansal, M. (2019). Variation of gene expression in plants is influenced by gene architecture and structural properties of promoters. *PLoS One*. 14, e0212678. doi: 10.1371/journal.pone.0212678

Ding, M., Chen, J., Jiang, Y., Lin, L., Cao, Y., Wang, M., et al. (2015). Genome-wide investigation and transcriptome analysis of the WRKY gene family in *Gossypium*. *Mol. Genet. Genomics.* 290, 151–171. doi: 10.1007/s00438-014-0904-7

Ehlers, J. D., and Hall, A. E. (1997) Cowpea (*Vigna unguiculata* L. Walp.). *Field Crop. Res.* 53, 187-204. doi: 10.1016/S0378-4290(97)00031-2

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14863–14868. doi: 10.1073/pnas.95.25.14863

El-Esawi, M. A., Al-Ghamdi, A. A., Ali, H. M., and Ahmad, M. (2019). Overexpression of *AtWRKY30* Transcription Factor Enhances Heat and Drought Stress Tolerance in Wheat (*Triticum aestivum* L.). *Genes*. 10, 163. doi:10.3390/genes10020163

Eulgem, T. (2006). Dissecting the WRKY web of plant defense regulators. *PLoS Pathog*. 2, e126. doi: 10.1371/journal.ppat.0020126

Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* 5, 199-206. doi: 10.1016/S1360-1385(00)01600-9

Fan, C., Yao, H., Qiu, Z., Ma, H., and Zeng, B. (2018). Genome-wide analysis of *Eucalyptus grandis* WRKY genes family and their expression profiling in response to hormone and abiotic stress treatment. *Gene*. 678, 38-48. doi: 10.1016/j.gene.2018.08.003

Ferreira-Neto, J. R., Pandolfi, V., Guimarães, F. C., Benko-Iseppon, A. M., Romero, C., Silva, R. L., et al. (2013). Early transcriptional response of soybean contrasting accessions to root dehydration. *PloS One*. 8, e83466. doi:10.1371/journal.pone.0083466

Franco-Zorrilla, J. M., and Solano, R. (2016). Identification of plant transcription factor target sequences. *Biochim. Biophys. Acta*. 1860, 21-30. doi: 10.1016/j.bbagrm.2016.05.001

Freire-Filho, F. R. Ribeiro, V. Q. Barreto, P. D. and Santos, C. A. F. (1999). Melhoramento genético do caupi (*Vigna unguiculata* (L.) Walp.) na região Nordeste. *In*: Recursos genéticos e melhoramento de plantas para o Nordeste Brasileiro. M. A. Queirós, C. O. Goedert, S.R.R. Ramos (Eds), Embrapa Semiárido, Petrolina, 1999.

Gao, H., Wang, Y., Xu, P., and Zhang, Z. (2018). Overexpression of a WRKY transcription factor *TaWRKY2* enhances drought stress tolerance in transgenic wheat. *Front. Plant Sci*. 9, 997. doi: 10.3389/fpls.2018.00997

Gill, S. S., Anjum, N. A., Gill, R., Mahajan, M., and Tuteja, N. (2015). Abiotic stress tolerance and sustainable agriculture: a functional genomics perspective. *In*: cidation of abiotic stress signaling in plants, Functional Genomic Perspectives. G.K. Pandey (Ed), Vol. 2, Springer, New York, 2015, pp. 439-472.

Goodstein, D. M, Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., et al. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40, 1178–1186. doi: 10.1093/nar/gkr944

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol*. 29, 644–652. doi: 10.1038/nbt.1883

Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L., and Noble, W. S. (2007). Quantifying similarity between motifs. *Genome Biol.* 8, R24. doi:10.1186/gb-2007-8-2-r24

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., et al. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc*. 8, 1494-512. doi: 10.1038/nprot.2013.084

Hiller, M., Huse, K., Platzer, M., and Backofen, R. (2005). Creation and disruption of protein features by alternative splicing -- a novel mechanism to modulate function. *Genome Biol*. 6, R58. doi:10.1186/gb-2005-6-7-r58

Hirayama, T., and Shinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J*. 61, 1041-1052. doi: 10.1111/j.1365-313X.2010.04124.x

Hu, B., Jin, J., Guo, A. Y., Zhang, H., Luo, J., and Gao, G. (2015). GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics.* 31, 1296–1297. doi:10.1093/bioinformatics/btu817

Huber, W., Carey, V. J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B. S., et al. (2015). Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 12, 115–121. doi:10.1038/nmeth.3252

IAEA, Improving yield and nitrogen fixation of grain legumes in the tropics and sub-tropics of Asia Results of a co-ordinated research program organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. International Atomic Energy Agency (IAEA). (1998) Available at http://www-pub.iaea.org/MTCD/publications/PDF/te_1027_prn.pdf (accessed 24 August 2015)

Ishiguro, S., and Nakamura, K. (1994). Characterization of a Cdna encoding a novel DNA-inding protein, SPF1, that recognizes SP8 sequences in the 5N upstream regions of genes coding for sporamin and beta-amylase from sweet potato. *Mol. Gen. Genet.* 244, 563-571.

Ishihama, N., and Yoshioka, H. (2012). Post-translational regulation of WRKY transcription factors in plant immunity. *Curr. Opin. Plant Biol.* 15, 431-437. doi: 10.1016/j.pbi.2012.02.003.

Jesús-Pires, C., Ferreira-Neto, J. R. C., Bezerra-Neto, J. P., Kido, E. A., Pandolfi, V. Wanderley-Nogueira, A. C., et al. (2019). Plant Thaumatin-like Proteins: Function, Evolution and Biotechnological Applications. *Curr. Protein Pept. Sci.* doi: 10.2174/1389203720666190318164905

Jiang, S. Y., González, J. M., and Ramachandran, S. (2013). Comparative genomic and transcriptomic analysis of tandemly and segmentally duplicated genes in rice. *PloS One*. 8, e63551. doi:10.1371/journal.pone.0063551

Jiang, Y., and Deyholos, M. K. (2009). Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. *Plant Mol. Biol.* 69, 91-105. doi: 10.1007/s11103-008-9408-3

Jiang, Y., Zeng, B., Zhao, H., Zhang, M., Xie, S., and Lai, J. (2012). Genome-wide transcription factor gene prediction and their expressional tissue-specificities in maize. *J. Integr. Plant Biol.* 54, 616– 630. doi: 10.1111/j.1744-7909.2012.01149.x

Jiao, Z., Sun, J., Wang, C., Dong, Y., Xiao, S., Gao, X., et al. (2018). Genome-wide characterization, evolutionary analysis of *WRKY* genes in Cucurbitaceae species and assessment of its roles in resisting to powdery mildew disease. *PloS One*, 13, e0199851. doi: 10.1371/journal.pone.0199851

Jimmy, J. L., and Babu, S. (2019). Variations in the Structure and Evolution of Rice *WRKY* Genes in *Indica* and *Japonica* Genotypes and their Co-expression Network in Mediating Disease Resistance. *Evol. Bioinform.* 15, 1–12. doi: 10.1177/1176934319857720

Jin, J. P., Tian, F., Yang, D. C., Meng, Y. Q., Kong, L., Luo, J. C., et al. (2017). PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res.* 45, D1040-D1045. doi: 10.1093/nar/gkw982

Karanja, B. K., Fan, L., Xu, L., Wang, Y., Zhu, X., Tang, M., et al. (2017). Genome-wide characterization of the WRKY gene family in radish (*Raphanus sativus* L.) reveals its critical functions under different abiotic stresses. *Plant Cell Rep*. 36, 1757–1773. doi:10.1007/s00299-017-2190-4

Khan, A., Fornes, O., Stigliani, A., Gheorghe, M., Castro-Mondragon, J. A., van der Lee, R., et al. (2018b). JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic Acids Res.* 46, D260–D266. doi:10.1093/nar/gkx1126

Khan, S. A., Li, M. Z., Wang, S. M., and Yin, H. J. (2018a). Revisiting the Role of Plant Transcription Factors in the Battle against Abiotic Stress. *Int. J. Mol. Sci*. 19, 1634. doi: 10.3390/ijms19061634

Kimotho, R. N., Baillo, E. H., and Zhang. Z. (2019). Transcription factors involved in abiotic stress responses in maize (*Zea mays* L.) and their roles in enhanced productivity in the post genomics era. *PeerJ Preprints* 7, e27549v1. doi: 10.7287/peerj.preprints.27549v1

Kiranmai, K., Lokanadha-Rao, G., Pandurangaiah, M., Nareshkumar, A., Amaranatha-Reddy, V., Lokesh, U. et al. (2018). A Novel WRKY Transcription Factor, *MuWRKY3* (*Macrotyloma uniflorum* Lam. Verdc.) Enhances Drought Stress Tolerance in Transgenic Groundnut (*Arachis hypogaea* L.) Plants. *Front. Plant Sci.* 9, 346. doi:10.3389/fpls.2018.00346

Koralewski, T. E., and Krutovsky, K. V. (2011). Evolution of exon-intron structure and alternative splicing. *PloS One*. 6, e18055. doi:10.1371/journal.pone.0018055

Kumar, K., Srivastava, V., Purayannur, S., Kaladhar, V. C., Cheruvu, P. J., and Verma, P. K. (2016). WRKY domain-encoding genes of a crop legume chickpea (*Cicer arietinum*): comparative analysis with *Medicago truncatula* WRKY family and characterization of group-III gene(s). *DNA Res.* 23, 225–239. doi:10.1093/dnares/dsw010

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547-1549. doi: 10.1093/molbev/msy096

Langridge, P., and Fleury, D. (2011). Making the most of 'omics' for crop breeding, *Trends Biotechnol*. 29 33-40. doi: 10.1016/j.tibtech.2010.09.006

Lata, C., Yadav, A. and Prasad, M. (2011). Role of plant transcription factors in abiotic stress tolerance. *In*: Abiotic Stress Response in Plants, ed. Shanker, A. and Venkateshwarulu, B., (INTECH Open Access Publishers), 269–296.

Latef, A. A. H. A., and Ahmad, P. (2015). Legumes and breeding under abiotic stress: An overview, in: M.M. Azooz, P. Ahmad (Eds.), Legumes under Environmental Stress: Yield, Improvement and Adaptations, John Wiley & Sons, 2015, pp. 1-20.

Le Hir, R., and Bellini, C. (2013). The plant-specific dof transcription factors family: new players involved in vascular system development and functioning in *Arabidopsis*. *Front. Plant Sci.* 4, 164. doi:10.3389/fpls.2013.00164

Letunic, I., Doerks, T., and Bork, P. (2015). SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res.* 43, D257–D260. doi: 10.1093/nar/gku949

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics.* 12, 323. doi:10.1186/1471-2105-12-323

Liu, Y., Yang, T., Lin, Z., Gu, B., Xing, C., Zhao, L., et al. (2019). A WRKY transcription factor *PbrWRKY53* from *Pyrus betulaefolia* is involved in drought tolerance and AsA accumulation. *Plant Biotechnol. J.* doi: 10.1111/pbi.13099

Lonardi, S., Muñoz-Amatriaín, M., Liang, Q., Shu, S., Wanamaker, S. I., Lo, S., et al. (2019). The genome of cowpea (*Vigna unguiculata* [L.] Walp.). *Plant J.* 98, 767-782. doi:10.1111/tpj.14349

Ma, Q., Xia, Z., Cai, Z., Li, L., Cheng, Y., Liu, J., et al. (2019). *GmWRKY16* Enhances Drought and Salt Tolerance Through an ABA-Mediated Pathway in *Arabidopsis thaliana*. *Front. Plant Sci.* 9, 1979. doi:10.3389/fpls.2018.01979

Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., et al. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* 45, D200–D203. doi:10.1093/nar/gkw1129

Matsui, A., Ishida, J., Morosawa, T., Okamoto, M., Kim, J. M., Kurihara, Y., et al. (2010). *Arabidopsis* tiling array analysis to identify the stress-responsive genes, *Method. Mol. Biol*. 639, 141-55. doi: 10.1007/978-1-60761-702-0_8.

Metzker, M. L. (2010). Sequencing technologies - the next generation, *Nat. Rev. Genet*. 11, 31-46. doi: 10.1038/nrg2626.

Mittler, R., and Blumwald, E. (2010). Genetic engineering for modern agriculture: challenges and perspectives. *Annu. Rev. Plant Biol*. 61, 443–462. doi: 10.1146/annurev-arplant-042809-112116.

Mizoi, J., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2012). AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta*. 1819, 86-96. doi: 10.1016/j.bbagrm.2011.08.004

Monfared, M. M., Simon, M. K., Meister, R. J., Roig-Villanova, I., Kooiker, M., Colombo, L., et al. (2011). Overlapping and antagonistic activities of BASIC PENTACYSTEINE genes affect a range of developmental processes in *Arabidopsis*. *Plant J.* 66, 1020–1031. doi: 10.1111/j.1365-313X.2011.04562.x

Moustafa, K., and Cross, J. M. (2016). Genetic Approaches to Study Plant Responses to Environmental Stresses: An Overview. *Biology*. 5, 20. doi:10.3390/biology5020020

Mu, Y., Zou, M., Sun, X., He, B., Xu, X., Liu, Y., et al. (2017). BASIC PENTACYSTEINE proteins repress *ABSCISIC ACID INSENSITIVE4* expression via direct recruitment of the polycomb-repressive complex 2 in *Arabidopsis* root development. *Plant Cell Physiol*. 58, 1–39. doi: 10.1093/pcp/pcx006

Muñoz-Amatriaín, M., Mirebrahim, H., Xu, P., Wanamaker, S. I., Luo, M., Alhakami, H. et al. (2016). Genome resources for climate-resilient cowpea, an essential crop for food security. *Plant J*. 89, 1042–1054. doi: 10.1111/tpj.13404.

Nakashima, K., Ito, Y., and Yamaguchi-Shinozaki, K. (2009). Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiol.* 149, 88–95. doi: 10.1104/pp.108.129791. 149 (2009) 88-95.

Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2014). The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* 5, 170. doi: 10.3389/fpls.2014.00170

Ning, P., Liu, C., Kang, J., and Lv, J. (2017). Genome-wide analysis of WRKY transcription factors in wheat (*Triticum aestivum* L.) and differential expression under water deficit condition. *PeerJ*. 5, e3232. doi: 10.7717/peerj.3232

Nuruzzaman, M., Sharoni, A. M., and Kikuchi, S. (2013). Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front. Microbiol*. 4, 1-16. doi: 10.3389/fmicb.2013.00248

Nuruzzaman, M., Sharoni, A. M., Satoh, K., Kumar, A., Leung, H., and Kikuchi, S. (2014) Comparative transcriptome profiles of the WRKY gene family under control, hormone-treated, and drought conditions in near-isogenic rice lines reveal differential, tissue specific gene activation. *J. Plant Physiol.* 171, 2–13. doi: 10.1016/j.jplph.2013.09.010

Osakabe, Y., Osakabe, K., Shinozaki, K., and Tran, L. S. (2014). Response of plants to water stress. *Front Plant Sci*. 5, 1-8. doi: 10.3389/fpls.2014.00086

Ozsolak, F. and Milos, P.M. (2011). RNA sequencing: advances, challenges and opportunities. *Nat. Rev. Genet.* 12, 87-98. doi:10.1038/nrg2934

Panchy, N., Lehti-Shiu, M., and Shiu, S. H. (2016). Evolution of Gene Duplication in Plants. *Plant Physiol.* 171, 2294–2316. doi:10.1104/pp.16.00523

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi:10.1104/pp.109.138990

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., et al. (2013). Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Glob. Chang. Biol.* 19, 2303-2338. doi: 10.1111/gcb.12143

Phillips, R. D., Mcwatters, K. H., Chinnan, M. S., Hung, Y. C., Beuchat, L. R., Sefa-Dedeh, S. et al. (2003). Utilization of cowpea for human food. *Field Crop. Res*. 82, 193-213. doi: 10.1016/S0378-4290(03)00038-8

Phukan, U. J., Jeena, G. S., and Shukla, R. K. (2016). WRKY Transcription Factors: Molecular Regulation and Stress Responses in Plants. *Front Plant Sci*. 7, 760. doi:10.3389/fpls.2016.00760

Qiao, Z., Li, C. L., and Zhang, W. (2016). WRKY1 regulates stomatal movement in drought-stressed *Arabidopsis thaliana*. *Plant Mol. Biol.* 91, 53–65. doi:10.1007/s11103-016-0441-3.

Qiu, Y. P., and Yu, D. Q. (2009). Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. *Environ Exp. Bot.* 65, 35-47. doi: 10.1016/j.envexpbot.2008.07.002

Raza, A., Razzaq, A., Mehmood, S. S., Zou, X., Zhang, X., Lv, Y., and Xu, J. (2019). Impact of Climate Change on Crops Adaptation and Strategies to Tackle Its Outcome: A Review. *Plants.* 8, 34. doi:10.3390/plants8020034

Ren, X. Y., Vorst, O., Fiers, M. W., Stiekema, W. J., and Nap, J. P. (2006). In plants, highly expressed genes are the least compact. *Trends Genet.* 22, 528-32. doi: 10.1016/j.tig.2006.08.008

Ren, X., Chen, Z., Liu, Y., Zhang, H., Zhang, M., Liu, Q., et al. (2010). *ABO3*, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in *Arabidopsis*. *Plant J.* 63, 417–429. doi: 10.1111/j.1365-313X.2010.04248.x

Rizhsky, L., Davletova, S., Liang, H., and Mittler, R. (2004). The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *J. Biol. Chem.* 279, 11736-43. doi: 10.1074/jbc.M313350200

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, 26, 139–140. doi: 10.1093/bioinformatics/btp616

Rodrigues, F. A., Marcolino, J., Carvalho, J. D. F. C., Nascimento, L. C. D., Neumaier, N., Farias, J. R. B., et al. (2012). Using subtractive libraries to prospect differentially expressed genes in soybean plants submitted to water deficit. *Genet. Mol. Biol.* 35, 304–314. doi: 10.1590/S1415-47572012000200011

Rogelj, J., den Elzen, M., Höhne, N., Fransen, T., Fekete, H., Winkler, H., et al. (2016). Paris Agreement Climate Proposals Need a Boost to Keep Warming Well below 2 °C. *Nature.* 534, 631-9. doi: 10.1038/nature18307.

Ross, C. A., Liu, Y., and Shen, Q. J. (2007). The WRKY gene family in rice (*Oryza sativa*). *J. Integr. Plant Biol.* 49, 827-842. doi: 10.1111/j.1744-7909.2007.00504.x

Rushton, P. J., MacDonald, H., Huttly, A. K., Lazarus, C. M., and Hooley, R. (1995). Members of a new family of DNA-binding proteins bind to a conserved cis-element in the promoters of α-Amy2 genes. *Plant Mol. Biol*. 29, 691-702. doi: 10.1007/BF00041160

Rushton, P. J., Somssich, I. E., Ringler, P., and Shen, Q. J. (2010). WRKY transcription factors. *Trends Plant Sci.* 15, 247–258. doi: 10.1016/j.tplants.2010.02.006

Rushton, P. J., Torres, J. T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I. E. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley *PR1* genes. *EMBO J.* 15, 5690–5700.

Saldanha, A. J. (2004). Java Treeview - extensible visualization of microarray data. *Bioinformatics.* 20, 3246-3248. doi: 10.1093/bioinformatics/bth349

Santos, J. R. P., Ndeve, A., Huynh, B., Matthews, W. C., and Roberts, P. (2018). QTL mapping and transcriptome analysis of cowpea reveals candidate genes for root-knot nematode resistance. *PLOS ONE*. 13. e0189185. doi: 10.1371/journal.pone.0189185

Satapathy, L., Singh, D., Ranjan, P., Kumar, D., Kumar, M., Prabhu, K. V., et al. (2014). Transcriptome-wide analysis of WRKY transcription factors in wheat and their leaf rust responsive expression profiling. *Mol. Genet. Genomics.* 289, 1289-1306. doi: 10.1007/s00438-014-0890-9

Savkur, R. S., and Burris, T. P. (2004). The coactivator LXXLL nuclear receptor recognition motif. *J. Pept. Res.* 63, 207–212. doi: 10.1111/j.1399-3011.2004.00126.x

Schmittgen, T. D. and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc*. 3, 1101-1108. doi: 10.1038/nprot.2008.73

Schroeder, J. I., Kwak, J. M., and Allen, G. J. (2001). Guard cell abscisic acid signaling and engineering drought hardiness in plants. *Nature*. 410, 327-330. doi: 10.1038/35066500

Shang, Y., Yan, L., Liu, Z. Q., Cao, Z., Mei, C., Xin, Q., et al. (2010). The Mg-chelatase H subunit of *Arabidopsis* antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell*, 22, 1909–1935. doi:10.1105/tpc.110.073874

Shanks, C. M., Hecker, A., Cheng, C., Brand, L., Collani, S., Schmid, M., et al. (2018). Role of *BASIC PENTACYSTEINE* transcription factors in a subset of cytokinin signaling responses. *Plant J.* 95, 458-473. doi:10.1111/tpj.13962

Sheshadri, S. A., Nishanth, M. J., and Simon, B. (2016). Stress-mediated cis-element transcription factor interactions interconnecting primary and specialized metabolism in planta. *Front. Plant. Sci*. 7, 1725. doi: 10.3389/fpls.2016.01725

Silva, E. G., Ito, T. M., and Souza, S. G. H. (2017). *In silico* genome-wide identification and phylogenetic analysis of the WRKY transcription factor family in sweet orange (*Citrus sinensis*). *Aust. J. Crop. Sci.* 11, 716. doi: 10.21475/ajcs.17.11.06.p471

Simonini, S., and Kater, M. M. (2014). Class I BASIC PENTACYSTEINE factors regulate *HOMEOBOX* genes involved in meristem size maintenance. *J. Exp. Bot.* 65, 1455–1465. doi: 10.1093/jxb/eru003

Singh, A., Dahiru, R., Musa, M., and Sani Haliru, B. (2014). Effect of Osmopriming Duration on Germination, Emergence, and Early Growth of Cowpea (*Vigna unguiculata* (L.) Walp.) in the Sudan Savanna of Nigeria. *International Journal of Agronomy*, 1–4. doi:10.1155/2014/841238

Singh, S. K., Kakani, V. G., Surabhi, G. K., and Reddy, K. R. (2010). Cowpea (*Vigna unguiculata* [L.] Walp.) genotypes response to multiple abiotic stresses, *J. Photochem. Photobiol. B.* 100, 135-46. doi: 10.1016/j.jphotobiol.2010.05.013

Song, H., Sun, W., Yang, G., and Sun, J. (2018). WRKY transcription factors in legumes. *BMC Plant Biol.* 18, 243. doi:10.1186/s12870-018-1467-2

Spriggs, A., Henderson, S. T., Hand, M. L., Johnson, S. D., Taylor, J. M., and Koltunow, A. (2018). Assembled genomic and tissue-specific transcriptomic data resources for two genetically distinct lines of Cowpea (*Vigna unguiculata* (L.) Walp) *Gates Open Res.* 2, 7. doi:10.12688/gatesopenres.12777.1

Srivastava, R., Kumar, S., Kobayashi, Y., Kusunoki, K., Tripathi, P., Kobayashi, Y., et al. (2018). Comparative genome-wide analysis of WRKY transcription factors in two Asian legume crops: Adzuki bean and Mung bean. *Sci. Rep.* 8, 16971. doi: 10.1038/s41598-018-34920-8

Sun, C., Palmqvist, S., Olsson, H., Borén, M., Ahlandsberg, S., and Jansson, C. (2003). A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. *Plant Cell*, 15, 2076–2092. doi:10.1105/tpc.014597

Suzuki, N., Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., and Mittler, R. (2005). Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. *Plant Physiol.* 139, 1313-1322. doi: 10.1104/pp.105.070110

Takahashi, F., and Shinozaki, K. (2019). Science Direct Long-Distance Signaling in Plant Stress Response. *Curr. Protein Pept. Sci*. 47, 106–11. doi: 10.1016/j.pbi.2018.10.006.

Timko, M. P., Rushton, P. J., Laudeman, T. W., Bokowiec, M. T., Chipumuro, E., Cheung, F., et al. (2008). Sequencing and analysis of the gene-rich space of cowpea. *BMC Genomics*. 9, 103. doi: 10.1186/1471-2164-9-103.

Todaka, D., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2015). Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Front. Plant Sci.* 6, 84. doi:10.3389/fpls.2015.00084

Tripathi, P., Rabara, R. C., and Rushton, P. J. (2014). A systems biology perspective on the role of WRKY transcription factors in drought responses in plants. *Planta*. 239, 255-266. doi: 10.1007/s00425-013-1985-y

Van Verk, M. C., Hickman, R., Pieterse, C. M. J., and Van Wees, S. C. M. (2013). RNA-Seq: revelation of the messengers. *Trends Plant Sci.* 18, 175–179. doi:10.1016/j.tplants.2013.02.001

Van Verk, M. C., Pappaioannou, D., Neeleman, L., Bol, J. F., and Linthorst, H. J. (2008). A Novel WRKY transcription factor is required for induction of *PR-1a* gene expression by salicylic acid and bacterial elicitors. *Plant Physiol.* 146, 1983–1995. doi:10.1104/pp.107.112789

Varshney, R. K., Close, T. J., Singh, N. K., Hoisington, D. A., and Cook, D. R. (2009). Orphan legume crops enter the genomics era! *Curr. Opin. Plant Biol.* 12, 202-210. doi: 10.1016/j.pbi.2008.12.004

Wang, C. T., Ru, J. N., Liu, Y. W., Li, M., Zhao, D., Yang, J. F., et al. (2018a). Maize WRKY Transcription Factor *ZmWRKY106* Confers Drought and Heat Tolerance in Transgenic Plants. *Int. J. Mol. Sci.* 19, 3046. doi:10.3390/ijms19103046

Wang, C. T., Ru, J. N., Liu, Y. W., Yang, J. F., Li, M., Xu, Z. S., et al. (2018b). The Maize WRKY Transcription Factor ZmWRKY40 Confers Drought Resistance in Transgenic *Arabidopsis*. *Int. J. Mol. Sci.* 19, 2580. doi:10.3390/ijms19092580

Wang, N., Xia, E. H., and Goa, L. Z. (2016). Genome-wide analysis of WRKY family of transcription factors in common bean, *Phaseolus vulgaris*: Chromosomal localization, structure, evolution and expression divergence. *Plant Gene*. 5, 22–30. doi: 10.1016/j.plgene.2015.11.003

Wang, Z. Gerstein, M. and Snyder, M. (2009). RNAseq: a revolutionary tool for transcriptomics. *Nature Rev. Genet*. 10, 57-63. doi: 10.1038/nrg2484

Waqas, M., Azhar, M. T., Rana, I. A., Azeem, F., Ali, M. A., Nawaz, M. A., et al. (2019). Genome-wide identification and expression analyses of WRKY transcription factor family members from chickpea (*Cicer arietinum* L.) reveal their role in abiotic stress-responses. *Genes Genom*. 5, 22-30. doi:10.1007/s13258-018-00780-9

Waterhouse, A. M., Procter, J. B., Martin, D. M. A, Clamp, M. and Barton, G. J. (2009). Jalview Version 2 - a multiple sequence alignment editor and analysis workbench. *Bioinformatics.* 25, 1189-1191. doi: 10.1093/bioinformatics/btp033

Wu, J., Chen, J., Wang, L., and Wang, S. (2017). Genome-wide investigation of WRKY transcription factors involved in terminal drought stress response in common bean. *Front. Plant Sci.* 8, 380. doi: 10.3389/fpls.2017.00380

Xie, T., Chen, C., Li, C., Liu, J., Liu, C., and He, Y. (2018). Genome-wide investigation of WRKY gene family in pineapple: evolution and expression profiles during development and stress. *BMC genomics*. 19, 490. doi:10.1186/s12864-018-4880-x

Xu, Z., Zhou, G., and Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signal Behav. 5*, 649–654. doi:10.4161/psb.5.6.11398

Yin, G., Xu, H., Xiao, S., Qin, Y., Li, Y., Yan, Y., et al. (2013). The large soybean (*Glycine max*) WRKY TF family expanded by segmental duplication events and subsequent divergent selection among subgroups. *BMC Plant Biol.* 13, 148. doi:10.1186/1471-2229-13-148

Yu, Y., Wang, N., Hu, R., and Xiang, F. (2016). Genome-wide identification of soybean WRKY transcription factors in response to salt stress. *SpringerPlus.* 5, 920. doi:10.1186/s40064-016-2647-x

Yue, H., Chang, X., Zhi, Y., Wang, L., Xing, G., Song, W., and Nie, X. (2019). Evolution and Identification of the WRKY Gene Family in Quinoa (*Chenopodium quinoa*). *Genes*, 10, 131. doi:10.3390/genes10020131

Zandalinas, S. I., Mittler, R., Balfagón, D., Arbona, V., Gómez-Cadenas, A. (2018). Plant adaptations to the combination of drought and high temperatures. *Physiol. Plant.* 162, 2–12. doi: 10.1111/ppl.12540.

Zhang, C., Wang, D., Yang, C., Kong, N., Shi, Z., Zhao, P., et al. (2017b). Genome-wide identification of the potato WRKY transcription factor family. *PloS One*, 12, e0181573. doi: 10.1371/journal.pone.0181573

Zhang, T., Tan D., Zhang L., Zhang X. Y., and Han, Z. (2017a). Phylogenetic analysis and drought-responsive expression profiles of the WRKY transcription factor family in maize. *Agri. Gene.* 3, 99–108. doi: 10.1016/j.aggene.2017.01.001

Zhang, W., Ruan, J., Ho, T. H., You, Y., Yu, T., and Quatrano, R. S. (2005). Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic acid-and abiotic stress-responsive genes in *Arabidopsis thaliana*. *Bioinformatics.* 21, 3074–3081. doi: 10.1093/bioinformatics/bti490

Zhang, Y., and Feng, J. C. (2014). Identification and characterization of the grape WRKY family. *Biomed. Res. Int.* 2014, 787680. doi: 10.1155/2014/787680

Zhang, Y., and Wang, L. (2005). The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. *BMC Evol. Biol.* 5, 1. doi: 10.1186/1471-2148-5-1

Zhou, Q. Y., Tian, A. G., Zou, H. F., Xie, Z. M., Lei, G., Huang, J., et al. (2008). Soybean WRKY-type transcription factor genes, *GmWRKY13*, *GmWRKY21*, and *GmWRKY54*, confer differential tolerance to

abiotic stresses in transgenic *Arabidopsis* plants. *Plant Biotechnol. J.* 6, 486-503. doi: 10.1111/j.1467-7652.2008.00336.x

Complementary material. Access the link: http://twixar.me/WSy1.

4 DISCUSSÃO GERAL

Os TFs WRKY são integrantes de uma das maiores família de reguladores transcricionais em plantas, atuando em cascatas de sinalizações que modulam diversas respostas vegetais. Desde seu primeiro relato em plantas, a família tem sido identificada em várias espécies. A conclusão recente do sequenciamento e montagem do genoma do feijão-caupi permitiu a identificação de 92 sequências genômicas codificantes de proteínas WRKY, com características e propriedades particulares da família e da espécie.

Análises estruturais e evolutivas das sequências VuWRKY permitiram o agrupamento da família nos grupos I, II e III e nos subgrupos IIa, IIb, IIc, IId e IIe, consistente com o reportado na literatura para outras plantas superiores. No entanto, três genes não puderam ser classificados nos grupos previstos, sendo alocados no grupo *'unclassified'*.

Analisados em conjunto, nossos dados sugerem que membros da família WRKY podem estar sofrendo uma diversificação gênica na espécie. Uma das evidencias se baseia na estrutura do domínio WRKY que, segundo a literatura, apresenta uma sequência heptapeptídica altamente conservada [WRKYGQK] responsável pelo reconhecimento dos motivos W-box. Nossos achados revelaram alterações nesta assinatura proteica para alguns genes com atividade sob condições de desidratação radicular.

Além disso, pôde-se verificar a presença de genes duplicados *em tandem* nos cromossomos do feijão-caupi, incluindo um do grupo '*unclassified'* (VuWRKY90), reforçando a hipótese da diversificação gênica e com manutenção da função biológica. É interessante notar que três genes WRKY duplicados *em tandem* correspondem às proteínas com variações na sequência heptapetídica do domínio, os quais foram modulados em condições de desidratação radicular.

Consistente com os relatos da literatura, os dados de RNA-Seq (banco CpGC) mostraram que os transcritos VuWRKY são rigorosamente modulados no acesso tolerante do feijão-caupi durante os primeiros minutos de exposição ao estresse, apresentando, inclusive, variações na expressão das isoformas. Para confirmação deste padrão regulatório, validamos a resposta biológica de alguns genes por qPCR. Uma das etapas primárias para realização da técnica é a escolha

de bons genes de referência, visto as inúmeras variações existentes no processo. Aqui, identificamos um grupo de genes normalizadores (*VuAct, UE21D, Ukn* e *F-BOX*) que, em combinações específicas, podem ser aplicados em reações de qPCR para normalizar os valores de expressão dos genes do feijão-caupi, quando a planta é submetida à desidratação radicular ou sob alta salinidade.

Com isso, o padrão de expressão da família WRKY foi confirmado pela técnica de qPCR em acessos contrastantes para desidratação radicular no feijão-caupi. Apesar do acesso sensível também induzir os VuWRKY nos estágios iniciais de exposição à desidratação radicular, os dados da qPCR evidenciam a eficácia na resposta do genótipo tolerante, que aciona seus genes mais rapidamente e em níveis mais significativos permitindo uma resposta mais direcionada e acelerada a desidratação radicular. Com isso, assim como verificado em outros vegetais, a planta consegue manter seus processos fisiológicos vitais em níveis significativos de modo a tolerar, por um período maior de tempo, a baixa disponibilidade de água no meio.

5 CONCLUSÕES

- A cultura do feijão-caupi possui em sua configuração genômica 92 sequências destinadas a codificar TFs WRKY, com representantes classificados nos três grupos e cinco subgrupos da família;
- Três genes *VuWRKY* não apresentaram as características estruturais definidoras de nenhum grupo, podendo compreender sequências em processo de diversificação da função biológica na espécie.
- Sob condições de desidratação radicular, o acesso tolerante do feijão-caupi aciona 55 dos 92 genes *VuWRKY* identificados no genoma de referência, modulando 97 diferentes isoformas;
- As isoformas moduladas no acesso tolerante são resultantes de *splicing* alternativo dos genes, apresentando diferentes valores de expressão.
- Variações na assinatura padrão do domínio WRKY parecem não interferir na atividade funcional de algumas proteínas, uma vez que foram induzidas sob desidratação radicular.
- A qPCR evidenciou que a resposta do genótipo tolerante ocorre de maneira mais rápida e em níveis mais significativos, comparativamente ao genótipo sensível.
- A tolerância a desidratação radicular no feijão-caupi parece estar associada a uma rápida percepção do estresse, seguida da modulação eficiente dos seus genes reguladores, como os TF WRKY.
- Em condições de desidratação radicular e estresse salino, o feijão-caupi apresenta genes que não alteram sua expressão, podendo ser utilizados como normalizadores para reações de qPCR.
- Diferentes combinações entre genes *VuAct*, *UE21D*, *Ukn* e *F-BOX*, mostraram-se como os melhores genes de referência para normalização de dados de qPCR para o feijão-caupi, quando submetido à restrição hídrica e alta salinidade.

REFERÊNCIAS

ABAD, L. R. et al. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. **Plant Science**, v. 118, p. 11–23, 1996.

ACHARYA, K. et al. Overexpression of *Camellia sinensis* thaumatin-like protein, CsTLP in potato confers enhanced resistance to *Macrophomina phaseolina* and *Phytophthora infestans* infection. **Molecular Biotechnology**, v. 54, n. 2, p. 609–622, 2013.

AGUILAR, E. et al. Effects of elevated CO_2 and temperature on pathogenicity determinants and virulence of Potato virus X/Potyvirus-associated synergism. **Molecular Plant-Microbe Interactions**, v. 28, n. 12, p. 1364-73, 2015.

AHANGER, R. A. et al. Impact of climate change on plant diseases. **International Journal of Modern Plant and Animal Sciences**, v. 1, n. 3, p. 105-115, 2013.

AHMED, I. M. et al. Genotypic differences in physiological characteristics in the tolerance to drought and salinity combined stress between Tibetan wild and cultivated barley. **Plant Physiology and Biochemistry**, v. 63, p. 49-60, 2013.

AHUJA, I. et al. Plant molecular stress responses face climate change. **Trends Plant Science**, v. 15, n. 12, p. 1-11, 2010.

ALAMEDA, D.; ANTEN, N. P. R.; VILLAR, R. Soil compaction effects on growth and root traits of tobacco depend on light, water regime and mechanical stress. **Soil Tillage Research**, v. 120, p. 121-129, 2012.

ALVES, M. S. et al. Plant bZIP Transcription Factors Responsive to Pathogens: A Review. **International Journal of Molecular Sciences**, v. 14, p. 7815-7828, 2013.

ALVES, M. S. et al. Transcription Factor Functional Protein-Protein Interactions in Plant Defense Responses. **Proteomes**, v. 2, p. 85-106, 2014.

AMBAWAT, S. et al. MYB transcription factor genes as regulators for plant responses: an overview. **Physiology and Molecular Biology of Plants**, v. 19, n. 3, p. 307-321, 2013.

AMORIM, L. L. B. et al. Transcription Factors Involved in Plant Drought Tolerance Regulation. *In*: HOSSAIN, M. A. et al. (eds) Drought Stress Tolerance in Plants, Molecular and Genetic Perspectives. **Springer**, Suíça, pp 315-358, 2016.

AMORIM, L. L. B. et al. Transcription Factors Involved in Plant Resistance to Pathogens, **Current Protein & Peptide Science**, v. 18, n. 4, p. 1-17, 2017.

ATKINSON, N. J.; LILLEY, C. J.; URWIN, P. E. Identification of genes involved in the response to simultaneous biotic and abiotic stress. **Plant Physiology**, v. 162, p. 2028-2041, 2013.

ATKINSON, N. J.; URWIN, P. E. The interaction of plant biotic and abiotic stresses: from genes to the field. **Journal of Experimental Botany**, v. 63, n. 10, p. 3523-3544, 2012.

AZANI, N. et al. A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny: The Legume Phylogeny Working Group (LPWG). **Taxon**, v. 66, n. 1, p. 44-77, 2017.

BANDURSKA, H.; NIEDZIELA, J.; CHADZINIKOLAU, T. Separate and combined responses to water deficit and UV-B radiation. **Plant Science**, v. 213, p. 98-105, 2013.

BARROS, G. G. et al. Pathogenicity of phylogenetic species in the *Fusarium graminearum* complex on soybean seedlings in Argentina. **European Journal of Plant Pathology**, v. 138, p. 215-222, 2014.

BECHTOLD, U.; FIELD, B. Molecular mechanisms controlling plant growth during abiotic stress. **Journal of Experimental Botany**, v. 69, n. 11, p. 2753-2758, 2018.

BENKO-ISEPPON A. M. Identificação e Validação de Genes Importantes para a Biotecnologia e Produtividade a partir do Transcriptoma do Feijão-Caupi (*Vigna unguiculata*). Projeto Submetido ao Edital MCT/CNPq/CT-AGRO/CT-BIOTEC Nº 42/2009. **Programa GENOPROT** - Rede Integrada de Estudos Genômicos e Proteômicos, 34 pp, 2009.

BENKO-ISEPPON, A. M. et al. Genômica funcional, estrutural e comparativa de feijão-caupi (*Vigna unguiculata*). *In*: Anais do I Encontro de Avaliação do Programa Rede Nordeste de Biotecnologia – **RENORBIO**. Brasília: Ministério da Ciência e Tecnologia, v. 1, p. 7-8, 2008.

BENKO-ISEPPON, A. M. et al. Prospecção de genes de resistência à seca e à salinidade em plantas nativas e cultivadas. **Revista Brasileira de Geografia Física**, v. 6, p. 1112-1134, 2011.

BENKO-ISEPPON, A. M. Genes associated with biotic and abiotic stresses in cowpea [*Vigna unguiculata* (L.) Walp.] and other angiosperms, pp.350-359. *In:* Nogueira, R. J. M. C. et al. (eds) Environmental Stresses: damages and benefits to plants. **UFRPE University Press**, Recife, 500 pp, 2005.

BROOKER, R. W. et al. Plant–plant interactions and environmental change. **New Phytol**, v. 171, p. 271-284, 2006.

CAI, H. TIAN, S.; DONG, H. Large Scale In Silico Identification of MYB Family Genes from Wheat Expressed Sequence Tags. **Molecular Biotechnology**, v. 52, p. 184-192, 2012.

CAI, M. et al. Identification of novel pathogen-responsive cis-elements and their binding proteins in the promoter of OsWRKY13, a gene regulating rice disease resistance. **Plant, Cell & Environment**, v. 31, p. 86-96, 2008.

CARMO-SILVA, A. E. et al. Decreased CO_2 availability and inactivation of Rubisco limit photosynthesis in cotton plants under heat and drought stress in the field. **Environmental and Experimental Botany**, v. 83, p. 1-11, 2012.

CHEN, X. et al. CGKB: an annotation knowledge base for cowpea (*Vigna unguiculata* L.) methylation filtered genomic genespace sequences. **BMC Bioinformatics**, v. 8, p. 129-132, 2007.

CHI, Y. et al. Protein–Protein Interactions in the Regulation of WRKY Transcription Factors. **Molecular Plant**, v. 6, n. 2, p. 87-300, 2013.

CHOI, H. K. et al. Water deficit modulates the response of *Vitis vinifera* to the Pierce's disease pathogen *Xylella fastidiosa*. **Molecular Plant-Microbe Interactions**, v. 6, p. 643-57, 2013.

CHOUDHARY, A.; PANDEY, P.; SENTHIL-KUMAR, M. Tailored responses to simultaneous drought stress and pathogen infection in plants. *In*: HOSSAIN, M. A. et al. (eds) Drought Stress Tolerance in Plants. Vol 1. **Springer International**, pp 427-438, 2016.

CLOSE, T. J. et al. Genome resources for cowpea genotype IT97 K-499-35. *In*: Proceedings of Plant and Animal Genome Conference XIX P028 (San Diego, CA). 2011.

CONESA, A. et al. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. **Bioinformatics**, v. 21, p. 3674-3676, 2005.

CRAMER, G. R.et al. Efeitos do estresse abiótico em plantas: uma perspectiva de biologia de sistemas. **BMC Plant Biology,** v. 11, p. 163, 2011.

CUELLAR, W. J. et al. Synergistic interactions of Begomoviruses with *Sweet potato chlorotic stunt virus* (genus *Crinivirus*) in sweetpotato (*Ipomoea batatas* L.). **Molecular plant pathology**, v. 16, p. 459-471, 2015.

CUTLER, S. R. et al. Abscisic acid: Emergence of a core signaling network. **Annual Review of Plant Biology**, v. 61, p. 651-679, 2010.

DAMASCENO-SILVA, K. J.; ROCHA, M. M.; FREIRE-FILHO, F. R. Processamento indústrial do feijão-caupi. *In*: Congresso Nacional de Feijão-Caupi. Da agricultura de subsistência ao agronegócio: anais. Belém, PA: **Embrapa Amazônia Oriental**. p. 146-157, 2009.

DAMASCENO-SILVA, K. J.; ROCHA, M. M.; MENEZES, J. J. Socioeconomia. *In:* FREIRE-FILHO, F. R. A cultura do feijão-caupi no Brasil. **Embrapa Meio-Norte**, Teresina, 6-12 pp, 2016.

DAVIS, R. F.; EARL, H. J.; TIMPER, P. Effect of simultaneous water deficit stress and *Meloidogyne incognita* infection on cotton yield and fiber quality. **Journal of Nematology**, v. 46, n. 2, p. 108-118, 2014.

DEL PONTE, E. M. et al. Regional and field-specific factors affect the composition of Fusarium head blight pathogens in subtropical no-till wheat agroecosystem of Brazil. **Phytopathology**, v. 105, p. 246-254, 2014.

DOWNIE, A. Legume nodulation. **Current Biology,** v. 24, p. R184-R190, 2014.
DU, H. et al. Biochemical and Molecular Characterization of Plant MYB Transcription Factor Family. **Biochemistry**, v. 74, n. 1, p. 1-11, 2009.

DUNG, J. K. S.; JOHNSON, D. A.; SCHROEDER, B. K. Role of co-infection by Pectobacterium and *Verticillium dahliae* in the development of early dying and aerial stem rot of Russet Burbank potato. **Plant Pathology**, v. 63, p. 299-307, 2013.

EULGEM, T. et al. Networks of WRKY transcription factors in defense signaling. **Current Opinion in Plant Biology**, v. 10, p. 366-371, 2007.

EULGEM, T. et al. The WRKY superfamily of plant transcription factors. **Trends Plant Science**, v. 5, p. 199-206, 2000.

EULGEM, T.; SOMSSICH, I. E. Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. **The EMBO Journal**, v. 18, n. 17, p. 4689–4699, 1999.

FELLER, A. et al. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. **The Plant Journal**, v. 66, p. 94-116, 2011.

FINATTO, T. et al. Can WRKY transcription factors help plants to overcome environmental challenges?. **Genetics and Molecular Biology**, v. 41, n. 3, p. 533-544, 2018.

FOYER, C. H. et al. Cross-tolerance to biotic and abiotic stresses in plants: a focus on resistance to aphid infestation. **Journal of Experimental Botany**, v. 67, n. 7, p. 2025-37, 2016.

FREIRE-FILHO, F. R. et al. Feijão-Caupi no Brasil: Produção, Melhoramento Genético, Avanços e Desafios. Teresina: **Embrapa Meio-Norte**, 2011. 84 p.

FUJITA, M. et al. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. **Current Opinion in Plant Biology**, v. 9, p. 436–442, 2006.

GAO, H. et al. Overexpression of a WRKY transcription factor *TaWRKY2* enhances drought stress tolerance in transgenic wheat. **Frontiers in Plant Science**, v. 9, p. 997, 2018.

GEPTS, P. et al. Legumes as a Model Plant Family: Genomics for Food and Feed Report of the Cross-Legume Advances through Genomics Conference. **Plant Physiology**, v. 137, p. 1228-1235, 2005.

GHANDI, A. et al. Tomato yellow leaf curl virus infection mitigates the heat stress response of plants grown at high temperatures. **Scientific Reports**, v. 6, p. 19715, 2016.

GLAZEBROOK, J.; ROBY, D. Plant biotic interactions: from conflict to collaboration. **The Plant Journal**, v. 93, p. 589–591, 2018.

GOLLDACK, D.; LÜKING, I.; YANG, O. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. **Plant Cell Reports**, v. 30, p. 1383–1391, 2011.

GÓRIA, M. M.; GHINI, R.; BETTIOL, W. Elevated atmospheric CO_2 concentration increases rice blast severity. **Tropical Plant Pathology**, v. 38, n. 3, p. 253-257, 2013.

GOUDARZI, A.; BANIHASHEMI, Z.; MAFTOUN, M. Effect of salt and water stress on root infection by *Macrophomina phaseolina* and ion composition in shoot in sorghum. **Iranian Journal of Pathology**, v. 47, n. 3, p. 69-83, 2011.

GRAHAM, P. H.; VANCE, C. P. Legumes: Importance and Constraints to Greater Use. **Plant Physiology**, v. 131, p. 872-877, 2003.

GRUSAK, M. A. Enhancing mineral content in plant food products. **Journal of the American College of Nutrition**, v. 21, p. 178-183, 2002.

HARMON, J. P.; MORAN, N. A.; IVES, A. R. Species response to environmental change: Impacts of food web interactions and evolution. **Science**, v. 323, p. 1347-1350, 2009.

HARTLEY, S. E. Plant Interactions with Biotic Factors. University of Sussex, Brighton, 10 pp, 2001.

HE G. H. et al. Drought-responsive WRKY transcription factor genes *TaWRKY1* and *TaWRKY33* from wheat confer drought and/or heat resistance in *Arabidopsis*. **BMC Plant Biology**, v. 16, p. 116, 2016.

HIRAYAMA, T.; SHINOZAKI, K. Research on plant abiotic stress responses in the post-genome era: past, present and future. **The Plant Journal,** v. 61, p. 1041-1052, 2010.

HUANG, S. X. et al. Genome-wide analysis of WRKY transcription factors in *Solanum lycopersicum*. **Molecular Genetics and Genomics,** v. 287, p. 495–513, 2012.

IYER, N. J.; TANG, Y.; MAHALINGAM, R. Physiological, biochemical and molecular responses to a combination of drought and ozone in *Medicago truncatula*. **Plant Cell Environ**, v. 36, n. 3, p. 706-20, 2013.

JASPERS, P.; KANGASJARVI J. Reactive oxygen species in abiotic stress signaling. **Physiologia Plantarum**, v. 138, p. 405-413, 2010.

JEDMOWSKI, C. et al. Impact of drought, heat, and their combination on chlorophyll fluorescence and yield of wild barley (*Hordeum spontaneum*). **Journal of botany**, v. 9, p. 10, 2015.

KASURINEN, A. et al. Interactive effects of elevated ozone and temperature on carbon allocation of silver birch (*Betula pendula*) genotypes in an open-air field exposure. **Tree Physiology**, v. 32, p. 737-751, 2012.

KELES, Y.; ONCEL, I. Response of antioxidative defence system to temperature and water stress combinations in wheat seedlings. **Plant Science**, v. 163, p. 783-790, 2002.

KIDO, E. A. et al. Identification of plant protein kinases in response to abiotic and biotic stresses using SuperSAGE. **Current Protein & Peptide Science**, v. 12, n. 7, p. 643-656, 2011.

KIM, N. et al. Functional characterization and reconstitution of ABA signaling components using transient gene expression in rice protoplasts. **Frontiers in Plant Science**, v. 6, p. 614, 2015.

KISSOUDIS, C. et al. Responses to combined abiotic and biotic stress in tomato are governed by stress intensity and resistance mechanism. **Journal of Experimental Botany**, v. 67, n. 17, p. 5119-32, 2016.

KISSOUDIS, C. et al.Combined biotic and abiotic stress resistance in tomato. **Euphytica**, v. 202, n. 2, p. 317-332, 2015.

KRANNER, I. et al. What is stress? Concepts, definitions and applications in seed science. **New Phytologist**, v. 188, p. 655–73, 2010.

KRANNICH, C. T. et al. Network Candidate Genes in Breeding for Drought Tolerant Crops. **International Journal of Molecular Sciences**, v. 16, p. 16378-400, 2015.

KUDELA, V. Potential impact of climate change on geographic distribution of plant pathogenic bacteria in central Europe. **Plant Protect Science**, v. 45, p. 27-32, 2009.

KUMAR, K. et al. WRKY domain-encoding genes of a crop legume chickpea (*Cicer arietinum*): comparative analysis with *Medicago truncatula* WRKY family and characterization of group-III gene(s). **DNA Research**, v. 23, n. 3, p. 225-239, 2016.

KUZDRALIŃSKI, A. et al. Early PCR-based detection of *Fusarium culmorum, F. graminearum, F. sporotrichioides* and *F. poae* on stem bases of winter wheat throughout Poland. **European Journal of Plant Pathology**, v. 140, p. 491-502, 2014.

LAMICHHANE, J. R.; VENTURI, V. Synergisms between microbial pathogens in plant disease complexes: a growing trend. **Frontiers in Science**, v. 6, p. 385, 2015.

LATA, C. YADAV, A.; PRASAD, M. Role of Plant Transcription Factors in Abiotic Stress Tolerance. *In*: Abiotic Stress Response in Plants. Physiological, Biochemical and Genetic Perspectives. 10 Ed. InTech, 296 pp, 2011.

LEWIS, G. P. et al. Legumes of the world. **Kew Publishing**, 592 pp, 2005.

LI, W. et al. The combined effect of salt stress and heat shock on proteome profiling in *Suaeda salsa*. **Journal of Plant Physiology**, v. 168, p. 1743-1752, 2011.

LIMA, L. C. P.; GARCIA, F. C. P.; SARTORI, A. L. B. Leguminosae nas Florestas Estacionais do Parque Estadual do Itacolomi, Minas Gerais, Brasil: Ervas, Arbustos, Subarbustos, Lianas e Trepadeiras. **Rodriguésia**, v. 58, n. 2, p. 331-358, 2007.

LINDEMOSE, S. et al. Structure, function and networks of transcription factors involved in abiotic stress responses. **International Journal of Molecular Sciences**, v. 14, n. 3, p. 5842-78, 2013.

LIU, L.; WHITE, M. J.; MACRAE, T. H. Transcription factors and their genes in higher plants: Functional domains, evolution and regulation. **European Journal of Biochemistry**, v. 262, p. 247-257, 1999.

LONARDI, S. et al. The genome of cowpea (*Vigna unguiculata* [L.] Walp.). **Plant Journal**, v. 98, p. 767-782, 2019.

LUCAS, M. R. et al. Cowpea-soybean synteny clarified through an improved genetic map. **Plant Genome**, v. 4, p. 218-224, 2011.

LUO, X. et al. Expression of wild soybean WRKY20 in *Arabidopsis* enhances drought tolerance and regulates ABA signalling. **Journal of Experimental Botany**, v. 64, p. 2155–2169, 2013.

MA, L. et al. Phylogenetic diversity of bacterial endophytes of Panax notoginseng with antagonistic characteristics towards pathogens of root-rot disease complex. **Antonie Van Leeuwenhoek, v.** 113, p. 299-312, 2013.

MADGWICK, J. et al. Impacts of climate change on wheat anthesis and fusarium ear blight in the UK. **European Journal of Plant Pathology**, v. 130, p. 117-131, 2011.

MADLUNG, A.; COMAI, L. The effect of stress on genome regulation and structure. **Annals of Botany**, v. 94, n. 4, p. 481-95, 2004.

MBEGA, E. R. et al. Five species of Xanthomonads associated with bacterial leaf spot symptoms in tomato from Tanzania. **Plant Disease**, v. 96, p. 7602, 2012.

MCMULLEN, M. P. et al. A unified effort to fight an enemy of wheat and barley: Fusarium head blight. **Plant Disease**, v. 96, p. 1712-1728, 2012.

MITTLER, R. Abiotic stress, the field environment and stress combination. **Trends in Plant Science**, v. 11, p. 15-19, 2006.

MITTLER, R.; BLUMWALD, E. Genetic engineering for modern agriculture: challenges and perspectives. **Annual Review of Plant Biology**, v. 61, p. 443-462, 2010.

MITTLER, R.; FINKA, A.; GOLOUBINOFF, P. How do plants feel the heat? **Trends in Biochemical Sciences**, v. 37, p. 118-125, 2012.

MOLDAKIMOVA, N. A. et al. Effect of salinity on viral disease spread in plants. **Journal of Stress Physiology & Biochemistry**, v. 8, n. 3, p. 17, 2012.

MUCHERO, W. et al. Mapping QTL for drought stress-induced premature senescence and maturity in cowpea [*Vigna unguiculata* (L.) Walp.]. **Journal of Stress Physiology & Biochemistry**, v. 118, p. 849-863, 2009.

OCHOLA, D. et al. Effects of water stress on the development of banana Xanthomonas wilt disease. **Plant Pathology**, v. 64, n. 3, p. 552-558, 2015.

OKAY, S.; DERELLI, E.; UNVER, T. Transcriptome-wide identification of bread wheat WRKY transcription factors in response to drought stress. **Molecular Genetics and Genomics**, v. 289, p. 765-781, 2014.

OSAKABE, Y. et al. Response of plants to water stress. **Frontiers in Plant Science**, v. 5, p. 86, 2014.

OSHLACK, A.; ROBINSON, M. D.; YOUNG, M. D. From RNA-seq reads to differential expression results. **Genome Biology**, v. 11, p. 220, 2010.

PANDEY, P. et al. Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-morphological Traits. **Frontiers in Plant Science**, v. 8, p. 537, 2017.

PANDEY, P. Impact of concurrent drought stress and pathogen infection on plants, in Combined Stresses in Plants. *In*: MAHALINGAM R (eds**). Springer International Publishing**, Australia, 203-222 pp, 2015b.

PANDEY, P.; RAMEGOWDA, V.; SENTHIL-KUMAR, M. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. **Frontiers in Plant Science**, v. 6, p. 723, 2015a.

PEREIRA, A. Plant Abiotic Stress Challenges from the Changing Environment. **Frontiers in Plant Science**, v. 7, p. 1123, 2016.

PEREZ-LOPEZ, U. et al. Lettuce production and antioxidant capacity are differentially modified by salt stress and light intensity under ambient and elevated CO_2. **Journal of Plant Physiology**, v. 170, p. 1517-1525, 2013.

PETITOT, A. S. et al. Promoter analysis of the WRKY transcription factors CaWRKY1a and CaWRKY1b homoeologous genes in coffee (*Coffea arabica*). **Plant Cell Reports**, v. 32, p. 1263-1276, 2013.

PHUKAN, U. J.; JEENA, G. S.; SHUKLA, R. K. WRKY Transcription Factors: Molecular Regulation and Stress Responses in Plants. **Frontiers in Plant Science**, v. 7, p. 760, 2016.

PINHEIRO, C.; Chaves, M. M. Photosynthesis and drought: can we make metabolic connections from available data?. **Journal of Experimental Botany**, v. 62, p. 869-882, 2011.

PINTO, M. S. T.; RIBEIRO, J. M.; OLIVEIRA, E. A. G. O estudo de genes e proteínas de defesa em plantas. **Revista Brasileira de Biociências**, v. 9, n. 2, p. 241-248, 2011.

PLETT, J. M.; MARTIN, F. M. Know your enemy, embrace your friend: using omics to understand how plants respond differently to pathogenic and mutualistic microorganisms. **The Plant Journal**, v. 93, p. 729–746, 2018.

POLLASTRINI, M. et al. Early physiological responses of *Pinus pinea* L. seedlings infected by *Heterobasidion* sp. pl. in an ozone-enriched atmospheric environment. **Tree Physiology**, v. 35, n. 3, p. 331-340, 2015.

PRASAD, P. V. V. et al. Independent and Combined Effects of High Temperature and Drought Stress During Grain Filling on Plant Yield and Chloroplast EF-Tu Expression in Spring Wheat. **Journal of Agronomy and Crop Science**, v. 197, p. 430-441, 2011.

PRASCH, C. M.; SONNEWALD, U. Simultaneous application of heat, drought, and virus to *Arabidopsis* plants reveals significant shifts in signaling networks. **Plant Physiology**, v. 162, p. 1849-1866, 2013.

QIU, Y. P. et al. Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. **Environmental and Experimental Botany,** v. 65, n. 35–47, 2009.

RAMEGOWDA, V.; SENTHIL-KUMAR, M. The interactive effects of simultaneous biotic and abiotic stresses on plants: mechanistic understanding from drought and pathogen combination. **Journal of Plant Physiology**, v. 176, p. 47-54, 2015.

RAMU, V. S. et al. Transcriptome analysis of sunflower genotypes with contrasting oxidative stress tolerance reveals individual- and combined- biotic and abiotic stress tolerance mechanisms. **PLoS ONE**, v. 11, p. e0157522, 2016.

REDONDO-GÓMEZ, S. Abiotic and Biotic Stress Tolerance in Plants. **Springer**. Universidad de Sevilla, Spain, 22 pp, 2013.

REIS, S. P.; LIMA, A. M.; SOUZA, C. R. B. Recent molecular advances on downstream plant responses to abiotic stress. **International Journal of Molecular Sciences**, v. 13, p. 8628–8647, 2012.

REJEB, I. B.; PASTOR, V.; MAUCH-MANI, B. Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. **Plants**, v. 3, n. 4, p. 458-475, 2014.

RICHERZHAGEN, D. et al. Impact of climate change on the temporal and regional occurrence of Cercospora leaf spot in Lower Saxony. **Journal of Plant Diseases and Protection**, v. 118, n. 5, p. 168-177, 2011.

RIVERO, R. M. et al. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. **Plant, Cell & Environment**, v. 37, n. 5, p. 1059-1073, 2013.

RUSHTON, P. J. et al. WRKY transcription factors. **Trends Plant Science**, v. 15, p. 247–258, 2010.

SALES, C. R. et al. Superoxide dismutase and ascorbate peroxidase improve the recovery of photosynthesis in sugarcane plants subjected to water deficit and low substrate temperature. **Plant Physiology and Biochemistry**, n. 73, p. 326-336, 2013.

SASLIS-LAGOUDAKIS, C. H. et al. The use of phylogeny to interpret cross-cultural patterns in plant use and guide medicinal plant discovery: An example from *Pterocarpus* (Leguminosae). **PloS One**, v. 6, p. e22275, 2011.

SCHRIRE, B. D.; LAVIN, M.; LEWIS, G. P. Global distribution patterns of the Leguminosae: Insights from recent phylogenies. **Biologiske Skrifter**, v. 55, p. 375–422, 2005b.

SCHRIRE, B. D.; LEWIS, G. P.; LAVI, M. Biogeography of the Leguminosae. *In*: LEWIS, G. P. et al. (eds.), Legumes of the World. Richmond, **Royal Botanic Gardens**, 54 pp, 2005a.

SCHULZE, E.; BECK, E.; MULLER-HOHENSTEIN, K. Plant Ecology. **Springer**, Berlin Heidelberg, New York, 692 pp, 2005.

SHARMA, M.; PANDE, S. Unravelling effects of temperature and soil moisture stress response on development of dry root rot [*Rhizoctonia bataticola* (Taub.)] Butler in chickpea. **American Journal of Plant Sciences**, v. 4, p. 584-589, 2013.

SHEN, H. et al. OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice. **Plant Molecular Biology,** v. 80, p. 241-253, 2012.

SHIVASHANKAR, H. N.; GASSER, R. B.; RANGANATHAN, S. A hitchhiker's guide to expressed sequence tag (EST) analysis. **Briefings in Bioinformatics**, v. 8, n. 1, p. 6-21, 2006.

SIDDIQUI, M. H. et al. Response of different genotypes of faba bean plant to drought stress. **International Journal of Molecular Sciences**, v. 16, p. 14-27, 2015.

SILVA, N. D.; CHOLEWA, E.; RYSER, P. Effects of combined drought and heavy metal stresses on xylem structure and hydraulic conductivity in red maple (*Acer rubrum* L.). **Journal of Experimental Botany**, v. 63, p. 5957-5966, 2012.

SINGH, A. et al. Understanding the Role of the WRKY Gene Family under Stress Conditions in Pigeonpea (*Cajanus Cajan* L.). **Plants (Basel)**, v. 8, n. 7, p. 214, 2019.

SONG, Y.; YU, J.; HUANG, B. Elevated CO_2-mitigation of high temperature stress associated with maintenance of positive carbon balance and carbohydrate accumulation in Kentucky Bluegrass. **PLoS One**, v. 9, n. 3, p. e89725, 2014.

SOUZA, N. M.; SOUZA, L. A. G. Levantamento do potencial de aproveitamento das leguminosas no distrito da Barreira do Andirá, Barreirinha, AM. **Enciclopédia Biosfera**, v. 7, n. 12, p. 1-23, 2011.

SRIVASTAVA, G. et al. Nickel and ultraviolet-B stresses induce differential growth and photosynthetic responses in *Pisum sativum* L. seedlings. **Biological Trace Element Research**, v. 149, p. 86-96, 2012.

SU, L. et al. A novel system for evaluating drought–cold tolerance of grapevines using chlorophyll fluorescence. **BMC Plant Biology**, v. 15, p. 82, 2015.

SUZUKI, N. et al. Abiotic and biotic stress combinations. **New Phytologist**, v. 203, p. 32-43, 2014.

TIMKO, M. P.; EHLERS, J. D.; ROBERTS, P. A. Cowpea: pulses, sugar and tuber crops. **Genome Mapp Molecular Breed Plants**, v. 3, p. 49-67, 2007.

TRIPATHI, P.; RABARA, R. C.; RUSHTON, P. J. A systems biology perspective on the role of WRKY transcription factors in drought responses in plants. **Planta**, v. 239, p. 255-266, 2014.

ÜLKER, B.; SOMSSICH, I. E. WRKY transcription factors: from DNA binding towards biological function. **Current Opinion in Plant Biology**, v. 7, p. 491-498, 2004.

VALERIO, M. et al. The role of water availability on weed–crop interactions in processing tomato for southern Italy. **Acta Agriculturae Scandinavica, Section B - Soil & Plant Science**, v. 63, n. 1, p. 62-68, 2013.

VAN DAM, N. M. How plants cope with biotic interactions. **Plant Biology**, v. 11, p. 1-5, 2008.

VAN MÖLKEN, T. et al. Consequences of combined herbivore feeding and pathogen infection for fitness of *Barbarea vulgaris* plants. **Oecologia**, v. 175, n. 2, p. 589-600, 2014.

VAN MÖLKEN, T. et al. Virus infection decreases the attractiveness of white clover plants for a non-vectoring herbivore. **Oecologia**, v. 170, n. 2, p. 433-444, 2012.

VAN, M. C. V.; GATZ, C.; LINTHORST, H. J. M. Transcriptional regulation of plant defense responses. **Advances in Botanical Research**, v. 51, p. 397-438, 2009.

VANCE, C. P.; GRAGAM, P. H.; ALLAN, D. L. Biological nitrogen fixation: Phosphorus a critic future need. *In*: PEDROSA, F. O. et al. (eds) Nitrogen fixation: from molecules to crop productivity. **Kluwer Academy Publishers**, Dordrecht, 506-514 pp, 2000.

VELÁZQUEZ, S. F.; GUERRA, R. R.; CALDERÓN, L. S. Abiotic and Biotic Stress Response Crosstalk in Plants. *In*: Shanker, A. (ed). Abiotic Stress Response in Plants. *Physiological, Biochemical and Genetic Perspectives*. InTech, Croatia, 346 pp, 2011.

VERMA, S.; NIZAM, S.; VERMA, P. K. Biotic and Abiotic Stress Signaling in Plants. **Springer**, New York, 23 pp, 2013.

VILE, D. et al. *Arabidopsis* growth under prolonged high temperature and water deficit: independent or interactive effects?. **Plant, Cell & Environment**, v. 35, p. 702-718, 2012.

WAN, Y. et al. Identification of the WRKY gene family and functional analysis of two genes in *Caragana intermedia*. **BMC Plant Biology**, v. 18, n. 1, p. 31, 2018.

WANG, C. et al. A wheat WRKY transcription fator TaWRKY10 confers tolerance to multiple abiotic stresses in transgenic tobacco. **PLoS One**, v. 8, p. e65120, 2013.

WANG, M. et al. Genome and transcriptome analysis of the grapevine (*Vitis vinifera* L.) WRKY gene family. **Horticulture Research**, v. 1, p. 14106, 2014.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-seq: a revolutionary tool for transcriptomics. **Nature Reviews Genetics**, v. 10, n. 1, p. 57–63, 2009.

WEN, X. et al. Enhanced thermotolerance of photosystemII in salt-adapted plants of the halophyte *Artemisia anethifolia*. **Planta**, v. 220, p. 486-497, 2005.

WHITELAW-WECKERT, M. A. et al. Co-infection by Botryosphaeriaceae and Ilyonectria spp. fungi during propagation causes decline of young grafted grapevines. **Plant Pathology**, v. 62, p. 1226-1237, 2013.

WU, K. L. et al. The WRKY family of transcription factors in rice and *Arabidopsis* and their origins. **DNA Research**, v. 12, n. 1, p. 9-26, 2005.

YAHARA, T. et al. Global legume diversity assessment: Concepts, key indicators and strategies. **Taxon**, v. 62, p. 249-266, 2013.

YAMASAKI, K. et al. Solution structure of an *Arabidopsis* WRKY DNA binding domain. **Plant Cell**, v. 17, p. 944-956, 2005.

YANG, Y. et al. Characterization of soybean WRKY gene family and identification of soybean WRKY genes that promote resistance to soybean cyst nematode. **Scientific Reports**, v. 7, p. 17804, 2017.

YI, C. et al. High atmospheric carbon dioxide-dependent alleviation of salt stress is linked to Respiratory burst oxidase 1 (RBOH1)-dependent H_2O_2 production in tomato (*Solanum lycopersicum*). **Journal of Experimental Botany**, v. 66, n. 22, p. 7391-7404, 2015.

ZAGHDOUD, C. et al. Elevated CO_2 alleviates negative effects of salinity on broccoli (*Brassica oleracea* L. var Italica) plants by modulating water balance through aquaporins abundance. **Environmental and Experimental Botany**, v. 95, p. 15-24, 2013.

ZHANG, J. Y. et al. Global reprogramming of transcription and metabolism in Medicago truncatula during progressive drought and after re-watering. **Plant Cell Environ**, v. 37, p. 2553–2576, 2014.

ZHANG, X. et al. Temperature-dependent survival of Turnip crinkle virus-infected *Arabidopsis* plants relies on an RNA silencing-based defense that requires dcl2, AGO2, and HEN1. **Journal of Virology**, v. 86, p. 6847-6854, 2012.

ZHOU, Q. Y. et al. Soybean WRKY-type transcription factor genes, *GmWRKY13*, *GmWRKY21*, and *GmWRKY54*, confer differential tolerance to abiotic stresses in transgenic *Arabidopsis* plants. **Plant Biotechnology Journal**, v. 6, p. 486-503, 2008.

ZHU, Y.; QIAN, W.; HUA, J. Temperature modulates plant defense responses through NB-LRR proteins. **PLoS Pathogens**, v. 6, p. e1000844, 2010.

Recursos da Internet

EMBRAPA ARROZ E FEIJÃO. Dados conjunturais da produção de feijão comum (*Phaseolus vulgaris* L.) e caupi (*Vigna unguiculata* (L.) Walp) no Brasil (1985 a 2017): área, produção e rendimento. Santo Antônio de Goiás: Embrapa Arroz e Feijão, 2018. Disponível em: <http://www.cnpaf.embrapa.br/socioeconomia/index.htm>. Acesso em: 01/08/19.

Food and Agriculture Organization – FAO FAOSTAT. Crops. Cow peas, dry. Available:http://faostat3.fao.org/home/index.html#DOWNLOAD (Acesso em: 08 ago. 2017).

FDB (2017) - Flora do Brasil 2020 em construção http://floradobrasil.jbrj. gov.br/ Acesso: Outubro 26, 2017.

APÊNDICE A

Artigo publicado na Revista *Plant Methods*

Qualis A1

Full Title: Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR

Short Title: Novel Reference Genes for Cowpea

Lidiane Lindinalva Barbosa Amorim[1,2†], José Ribamar Costa Ferreira-Neto[2†], João Pacífico Bezerra-Neto[2], Valesca Pandolfi[2], Flávia Tadeu de Araújo[2], **Mitalle Karen da Silva Matos[2]**, Mauro Guida Santos[3], Ederson Akio Kido[2] and Ana Maria Benko-Iseppon[2*]

[1] Instituto Federal de Educação, Ciência e Tecnologia do Piauí, Oeiras, Piauí, Brazil.

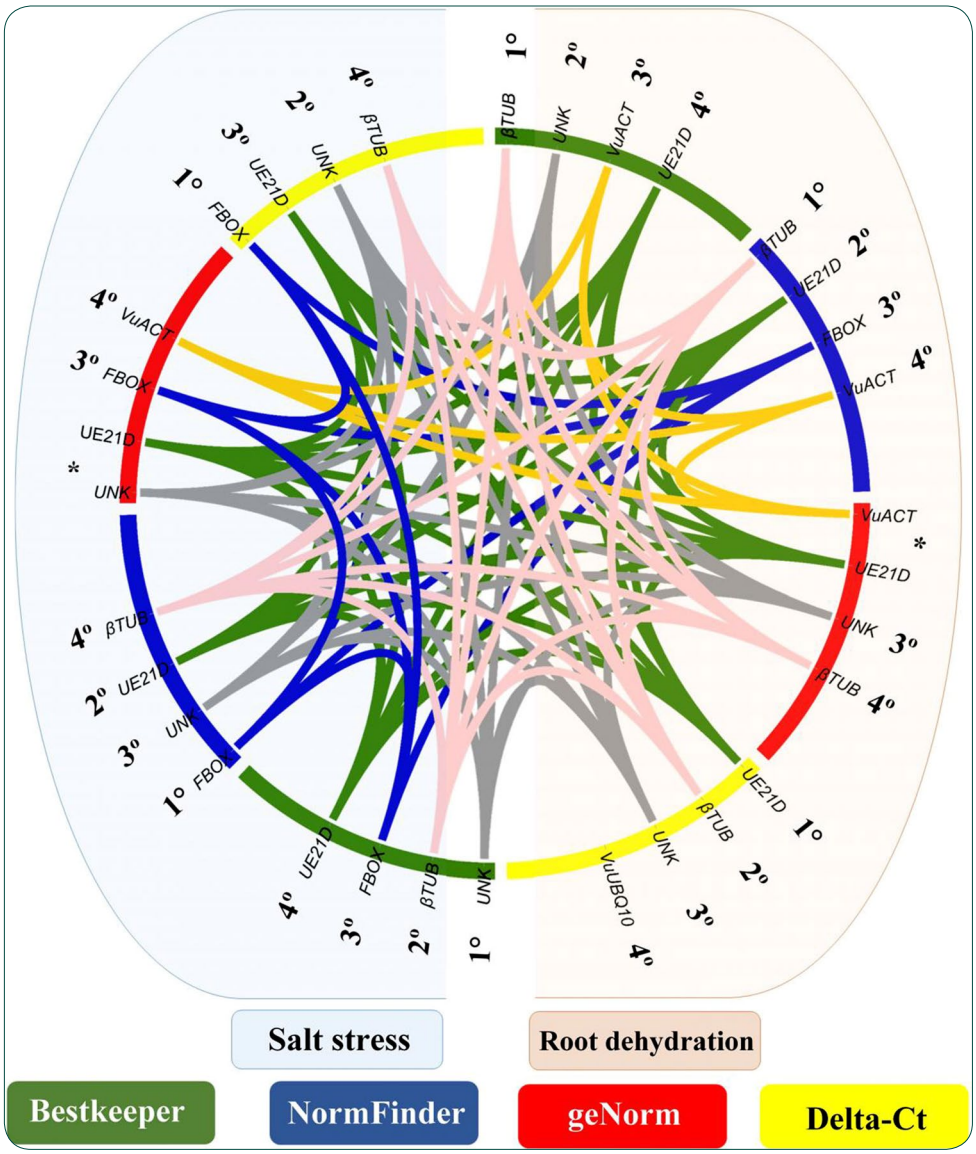
[2] Genetics Department, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.

[3] Botany Department, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.

[†] These authors contributed equally to this work.

***** Corresponding author.

E-mail: ana.benko.iseppon@pq.cnpq



Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR

Amorim *et al.*

Amorim *et al. Plant Methods (2018) 14:88*
https://doi.org/10.1186/s13007-018-0354-z

Plant Methods

RESEARCH

Open Access

Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR

Lidiane Lindinalva Barbosa Amorim[1,2†], José Ribamar Costa Ferreira-Neto[2†], João Pacífico Bezerra-Neto[2], Valesca Pandolfi[2], Flávia Tadeu de Araújo[2], Mitalle Karen da Silva Matos[2], Mauro Guida Santos[3], Ederson Akio Kido[2] and Ana Maria Benko-Iseppon[2*]

Abstract

Background: Due to cowpea ability to fix nitrogen in poor soils and relative tolerance to drought and salt stresses, efforts have been directed to identifying genes and pathways that confer stress tolerance in this species. Real-time quantitative PCR (qPCR) has been widely used as the most reliable method to measure gene expression, due to its high accuracy and specificity. In the present study, nine candidate reference genes were rigorously tested for their application in normalization of qPCR data onto roots of four distinct cowpea accessions under two abiotic stresses: root dehydration and salt (NaCl, 100 mM). In addition, the regulation of four target transcripts, under the same referred conditions was also scrutinized.

Results: geNorm, NormFinder, BestKeeper, and ΔCt method results indicated a set of three statistically validated RGs for each stress condition: (I) root dehydration (actin, ubiquitin-conjugating enzyme E2 variant 1D, and a *Phaseolus vulgaris* unknown gene—*UNK*), and (II) salt (ubiquitin-conjugating enzyme E2 variant 1D, F-box protein, and *UNK*). The expression profile of the target transcripts suggests that flavonoids are important players in the cowpea response to the abiotic stresses analyzed, since chalcone isomerase and chalcone synthase were up-regulated in the tolerant and sensitive accessions. A lipid transfer protein also participates in the cowpea tolerance mechanisms to root dehydration and salt stress. The referred transcript was up-regulated in the two tolerant accessions and presented no differential expression in the sensitive counterparts. Chitinase B, in turn, generally related to plant defense, was an important target transcript under salt stress, being up-regulated at the tolerant, and down-regulated in the sensitive accession.

Conclusions: Reference genes suitable for qPCR analyses in cowpea under root dehydration and salt stress were identified. This action will lead to a more accurate and reliable analysis of gene expression on this species. Additionally, the results obtained in this study may guide future research on gene expression in cowpea under other abiotic stress types that impose osmotic imbalance. The target genes analyzed, in turn, deserve functional evaluation due to their transcriptional regulation under stresses and biotechnological potential.

Keywords: Housekeeping genes, Root tissue, Salt stress, Drought, Phenylpropanoids, Legume

*Correspondence: ana.benko.iseppon@pq.cnpq
†Lidiane Lindinalva Barbosa Amorim and José Ribamar Costa Ferreira-Neto authors contributed equally to this work
[2] Genetics Department, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil
Full list of author information is available at the end of the article

Background

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the most important legumes cultivated by subsistence farmers, for both human and livestock consumption, mainly in the semi-arid regions of Africa [1] and Brazil [2]. In Africa, it is used for the livelihoods of millions of people in the semi-arid regions of West and Central regions [3] and is considered the most important grain legume crop in the sub-Saharan region. Regarding its potential for grain supply in dry areas (due to its wide genetic variability and good nitrogen fixation capacity), this crop minimizes the almost exclusive dependence on common bean, conferring to cowpea a strategic value in Brazil [2].

Given the cowpea great economic and socio-cultural importance, in addition to its rusticity and phenotypic plasticity on adverse soil and climatic conditions (with peculiar features of tolerance/resistance to stresses), the *Cowpea Functional Genome Consortium* (CpFGC) was created [4]. Using different methodological approaches (i.e. ESTs, HT-SuperSAGE and RNA-Seq) this effort resulted in the generation of millions of transcripts obtained from cowpea plants under different abiotic [root dehydration and salt (NaCl, 100 mM)] and biotic (infection by Cowpea Aphid-borne Mosaic Virus—CABMV and Cowpea Severe Mosaic Virus—CPSMV) stresses [4]. These data provide a good start for identifying putative genes and gene families associated with resistance/tolerance to such challenging conditions [4].

Real-time quantitative PCR (qPCR) is the most commonly used method of validation of transcriptomic studies, due to its high sensitivity and specificity [5]. Despite the advantages, its reliability depends on various factors, such as the integrity and purity of RNA, the efficacy of various reagents and enzymes used in RNA extraction, sample quantification, and reverse transcription, among others [5]. Such variables can cause quantitative and qualitative differences between the analyzed samples. Thus, the careful planning of protocols is mandatory when implementing qPCR, and the use of reference genes (RGs) as normalizers is an essential prerequisite. RGs should ideally be constitutively expressed in the studied tissue or cell type and should not be affected by the treatments performed. Additionally, uniform transcript abundance across the different groups being analyzed (e.g., across treatments) is necessary, serving as a "calibrator" to compare the different samples of the same quantitative level. This way, the use of suitable RGs ensure that the observed variation in the relative quantification of the target transcripts is due to changes in the gene expression, avoiding false positives or negatives in the transcriptome analysis.

Housekeeping genes are required for basal procedures and cellular survival, being often stably expressed and therefore used as normalizers without validating their suitability [6]. Nevertheless, increasing evidence showing that the transcription level of commonly used RGs (such as 18S ribosomal RNA, 25S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase, elongation factor 1-alpha, among others [7, 8]) can vary considerably depending on the state of development and physiological conditions, especially under stress. Therefore, various statistical methods have been developed to validate the expression stability of candidate RGs, and to allow the selection of the most suitable candidates for particular condition/tissue/species. NormFinder [9], geNorm [10] and BestKeeper [11] algorithms, besides the ΔCt method [12], are the most widely used approaches.

Unfortunately, only a limited number of studies addressing gene expression and qPCR have been reported for cowpea so far. Coetzer et al. [13] used the glyceraldehyde-3-phosphate dehydrogenase C-subunit gene as normalizer in the evaluation of target transcripts in leave tissues of cowpea plants under drought. Mellor et al. [14] used the actin gene as normalizer to evaluate *RSG3-301* (acronym of "resistance to *S. gesnerioides* race 3") transcript levels involved in the resistance response of transgenic cowpea roots (Blackeye cultivar) to the attack by the root parasitic weed, *Striga gesnerioides.* Huang et al. [15] also used the actin gene as an endogenous control to analyze target genes expression of cowpea (cultivar B301) during compatible and incompatible interactions with different races (SG3 and SG4z) of *Striga gesnerioides*. In turn, Shui et al. [16], used the small nuclear RNA U6 gene as an endogenous control to analyze target microRNAs in the leaves and roots of cowpea plants under drought treatment. The studies above have in common the fact that the respective RGs employed were not previously subjected to careful statistical analysis to determine their stability, following statistical tests, in the condition/tissue/plant analyzed. In addition, only one reference gene was used in each proposed assay, which reduces the statistical robustness of the results. According to MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines [5], which presents a series of indications on good practices in experiments involving qPCR, normalization should be carried out against multiple RGs, chosen from a variety of candidate RGs tested from independent pathways with the application of at least one algorithm [5, 10]. However, few works have focused on the selection of RGs in cowpea based on different statistical software. The first study was conducted by Da Silva et al. [17], who evaluated the expression stability of eight candidate genes in cowpea under drought stress during biological nitrogen fixation, using geNorm and NormFinder algorithms. The genes of the regulatory subunit of phosphatase 2A

protein (*VuPp2A*) and polyubiquitin 28 (*VuUbq28*) were the best normalizers suggested by both algorithms, for global analysis (nodules and leaves tissues).

The present study was undertaken to select and validate suitable RGs tested for effective normalization of cowpea qPCR data. Root tissues of four contrasting cowpea accessions (tolerant and sensitive), under two different abiotic stresses [root dehydration or salt (NaCl, 100 mM)] were analyzed through three different algorithms (NormFinder [9], geNorm [10], BestKeeper [11]), and also the ΔCt method [12], to find the most suitable RGs for each experimental condition. In addition, the transcriptional regulation of four cowpea target transcripts [chalcone isomerase (*VuCHI*), chalcone synthase (*VuCHS*), lipid transfer protein (*VuLTP*), and chitinase B (*VuCHiB*)] was scrutinized. The gene expression data obtained from a Next Generation Sequencing approach (HT-SuperSAGE) has been used to evaluate the participation of these targets in response to the studied stresses. The proposed RGs will serve to validate RNA-Seq and HT-SuperSAGE data generated by the CpFGC, as well as will benefit future studies on gene expression in cowpea and related species.

Methods

Plant material and treatments (root dehydration and salt stresses)

Two independent experimental trials were performed in the present study: one with root dehydration (Additional file 1: Fig. S1A) and the other with salt stress (Additional file 1: Fig. S1B). For root dehydration assay (Additional file 1: Fig. S1A), cowpea cultivars 'Pingo de Ouro' (PO; drought-tolerant) and 'Santo Inácio' (SI; drought-sensitive) were grown in a greenhouse at Embrapa-Soybean station (Londrina, Brazil), under hydroponic conditions (30 L plastic containers, pH 6.6, and balanced nutrient solution, as reported by Kulcheski et al. [18]). Briefly, cowpea seedlings, with the first trifoliate leaf fully developed, were submitted to root dehydration (in the dark) for 0 minutes (negative control), 25 (T1), 50 (T2), 75 (T3), 100 (T4), 125 (T5), and 150 (T6) minutes (Additional file 1: Fig. S1A) after removal of the nutrient solution from the tray. At the end of each treatment, the roots were frozen in liquid nitrogen and stored at − 80 °C until total RNA extraction.

For salt stress (Additional file 1: Fig. S1B), seeds of two contrasting cultivars, named 'Pitiúba' (PI; salt-tolerant; [19, 20]) and 'BR14-Mulato' (BR; salt-sensitive; [21]) were grown in pots with washed sand watered with 200 ml of 1/2 strength Hoagland's Solution. Seedlings (with first trifoliate leaf fully developed) were submitted to different periods of exposition to salt (NaCl added to the Hoagland's Solution to a final concentration of 100 mM). Roots were collected at 0 minutes (negative control; only with Hoagland Solution and water), 30 (T1), 60 (T2) and 90 (T3) minutes after irrigation with saline (NaCl, 100 mM) Hoagland's Solution (Additional file 1: Fig. S1B).

The treatment times mentioned in the trials were distinct because the events were independent, and these treatments took into account physiological analyzes (manuscript in preparation) indicating that the plants begin to undergo stress effects. The experimental designs were factorials (cultivars × extent of the stress) with three biological replicates (five plantlets composed each replicate).

Total RNA isolation and cDNA synthesis

Total RNA of cowpea root tissues (in all cultivars and treatments) was isolated using 'SV Total RNA Isolation System Kit' (Promega, Madison, WI) following the manufacturer's instructions. The genomic DNA (gDNA) was eliminated by RNase-free DNase I digestion during the isolation procedure. The quantity and quality of the isolated RNA were evaluated, respectively, using a NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and by electrophoresis agarose gels 1% (w/v), stained with Blue Green (LGC, São Paulo, Brazil). For each sample, the total RNA (1 µg) was reverse-transcribed into cDNA, using the 'Improm-II™ Reverse Transcriptional System' (Promega) with oligo (dT) primers following the manufacturer's recommendations. The quality of each cDNA was assessed by using standard PCR reaction with an actin primer pair [F: GGAACA TCCCGTTCTCTTGA and R: CTCTCAGGAGGAGCA ACCAC, amplicon of 708 bp; template Contig16004 (CpFGC database; Additional file 2: S1 Appendix)] that spanned intronic regions.

HT-SuperSAGE libraries, statistical analysis, and unitag-gene annotation

The HT-SuperSAGE libraries were synthesized for root dehydration and salt stresses. For root dehydration assay, two HT-SuperSAGE libraries were generated for each cultivar: POT1-6 (drought-tolerant cultivar under stress—a bulk with similar amounts of RNAs poly A^+ from samples covering the six stress times; Additional file 1: Fig. S1A), and POT0 (drought-tolerant cultivar, negative control; Additional file 1: Fig. S1A); SIT1-6 (drought-sensitive cultivar under stress—bulk of six stress times; Additional file 1: Fig. S1A), and SIT0 (drought-sensitive cultivar, negative control; Additional file 1: Fig. S1A). The salt stress included the following libraries: PTS3T (salt-tolerant cultivar Pitiúba under stress—bulk of three stress times; Additional file 1: Fig. S1B), and PTT0 (salt-tolerant cultivar, negative control;

Additional file 1: Fig. S1B); BRS3T (salt-sensitive accession BR14-Mulato under stress—bulk of three stress times), and BRT0 (salt-sensitive cultivar, negative control; Additional file 1: Fig. S1B). All HT-SuperSAGE libraries were generated at GenXPro GmbH (Frankfurt, Germany) as described by Matsumura et al. [22] and submitted to Illumina sequencing technology.

HT-SuperSAGE tags (26-bp) were analyzed to find unique and differentially expressed unitags ($p < 0.05$) based on Poisson statistics developed by Audic and Claverie [23], as implemented in DiscoverySpace (v.4.01) software [24]. The singlets (tags sequenced only once) were excluded from the evaluation. Unitags were annotated by BLASTn against nucleotide sequences from *Vigna unguiculata* available at Phytozome Database (https://phytozome.jgi.doe.gov/pz/portal.html#). The BLASTn alignments (unitag-EST) with e-value ≤ 0.001 and scores higher than 50 (i.e., with a maximum of one mismatch) were identified between the plus/plus matches. Unitags with mismatch regarding the four first bases "CATG" were not accepted to guarantee the integrity of the unitags.

Selection of the candidate RGs, target transcripts and primers design

The workflow of qPCR assay and stability analysis of the RGs is depicted in Additional file 3: Fig. S2. For the selection of the candidate RGs, a literature search in PubMed Database (https://www.ncbi.nlm.nih.gov/pubmed/) was carried out using the terms '*Phaseolus vulgaris* AND qPCR'; '*Vigna unguiculata* AND qPCR'. In addition, a data mining for candidate RGs also was performed in the CpFGC database.

Nine candidates were selected for the present study (Table 1) including:

- six (*β-TUB*: beta-tubulin; *EF1-α*: elongation factor 1-alfa; *FBOX*: F-box protein; *UE21D*: ubiquitin-conjugating enzyme E2 variant 1D; *UNK*: *Phaseolus vulgaris* unknown gene; and *ZMP*: zinc metalloproteinase) anchored in *Phaseolus vulgaris* genes;
- one (*GAPC*: glyceraldehyde-3-phosphate dehydrogenase C-subunit) anchored in soybean (*Glycine max*) gene;
- and two candidate RGs (*VuACT*: actin and *VuUBQ10*: polyubiquitin 10) anchored in *Vigna unguiculata* genes, previously designed by our group (CpFGC, Cowpea Functional Genome Consortium).

The candidate RGs obtained from in *V. unguiculata* genes were identified by BLASTn search (cutoff $< e^{-10}$) in CpFGC database, using predicted *Vigna radiata* (actin) and *Arabidopsis* (ubiquitin10) genes as queries (Additional file 2: S1 Appendix). All candidates were also selected based on their involvement in diverse plant cellular processes reducing, thus, the probability of co-regulation.

The primer pairs were designed using the online Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) with the following parameters: annealing temperature of 57–63 °C (optimal 60 °C), primer length of 18–22 bp (optimal 20 bp), GC contents of 45–55% (optimal 50%) and amplicon length of 100–200 bp (Table 1).

The target transcripts (*VuCHiB*, *VuLTP*, *VuCHI*, *VuCHS*; Table 1), whose expression was analyzed in the present work, were chosen because of their presence in both analyzed assays (root dehydration and salt stress HT-SuperSAGE libraries) and up-regulation ($FC > 5$; $p < 0.05$) in the 'Pingo de Ouro' accession, considered tolerant to the drought stress. Fold change (FC) is a measure describing how the unitag expression modulated after the stress. The FC values were based on the ratio (R) of the normalized unitag frequencies considering two libraries (treatment and control). In the case of $R < 1$, the $FC = -1/R$, being the negative FC values indicator of repressed unitags; in the case of 'zero' frequency in a library, this value was replaced by 'one.'

qPCR setup, amplification efficiency, and relative expression analysis

Although the root dehydration assay covered six exposition stress times, three of them (25, 75 and 150 min.) was chosen for the RTqPCR data validation, representing the initial, intermediate, and late stress exposition times, respectively. For salt stress, the studied points were 30, 60 and 90 min. Three biological and three technical replicates per sample were used to ensure statistical reliability. The same number was used for the not stressed controls maintained under the same condition. The qPCR reactions were performed in 96-well plates and performed on the LineGene 9660 (Bioer), using SYBR Green detection. Reactions were prepared in a total volume of 10 µL containing: 1 µL of 10 fold diluted template, 5 µL 'HotStart-IT SYBR Green qPCR Master Mix 2x' (USB), 0.05 µL of ROX, 1 µL of each primer (500 nM) and nuclease-free water to a final volume of 10 µL. The PCR program was adjusted to 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 15 s. After amplification, dissociation curves were produced (60–95 °C at a heating rate of 0.1 °C/sec and acquiring fluorescence data every 0.3 °C) to confirm the specificity of the PCR products.

The amplification efficiency ($E = 10^{(-1/\text{slope of the standard curve})}$; Additional file 3: Fig. S2) for all primer pairs was determined from a 5-point standard curve generated by

serial dilutions of cDNA (10-fold each) in technical triplicates. Slopes in the range of -3.58 to -3.10 were considered acceptable for the qPCR assay [28]. These slope values correlated to amplification efficiencies between 90% (E = 1.9) and 110% (E = 2.1; Additional file 3: Fig. S2).

The Rest2009 software package (standard mode) was used for relative expression analysis of target transcripts. REST bases its performance on pairwise comparisons (of target transcripts and reference genes) using randomization and bootstrapping techniques—Pair-wise Fixed Reallocation Randomization Test© [29, 30]. Hypothesis testing ($p < 0.05$) was used to determine whether the differences in target transcripts expression between the control and treatment conditions were significant.

Statistical analyses of candidate RGs expression stability

The expression stability of each candidate RG was evaluated by four different strategies (Additional file 3: Fig. S2).

GeNorm algorithm [10] calculates an expression stability value (M) for each candidate RG. Then, the algorithm determines the pairwise variation (V) of each candidate RG with all of the others studied. At the end of the analysis, by stepwise exclusion of the gene with the highest M-value (less stable), this tool allows for the ranking of the tested RGs according to their expression stability. The optimal number of RGs required for normalization was determined by pairwise variation V_n/V_{n+1}. According to Vandesompele et al. [10], a cut-off value of $V_{n/n+1} < 0.15$ dispense the inclusion of additional RG. Despite the possibility to achieve this prerequisite with the use of only two stable RGs, it is recommended the use of at least three reference genes (NF, $n = 3$) for calculation of a qPCR normalization factor [10].

The NormFinder [9] algorithm provides a stability value (SV) for each candidate RG. Different from geNorm, the NormFinder takes information by comparing the variation within and between user-defined sample groups, such as "Untreated/Treatment I/Treatment II, etc." The

Table 1 Candidate reference genes, target transcripts and respective primers pairs used in the present work

Gene	Anchor specie*	Cellular function	Primer sequences	Amplicon size (bp)	References
VuACT	*Vigna unguiculata* (Contig16004)	Diverse functions, ranging from cell motility to maintenance of cell shape and polarity	F: TCAGGTGTCCAGAGGTGTTGTA R: ATGGTTGTGCCTCCTGAAAGTA	151	CpFGC Database
VuUBQ10	*Vigna unguiculata* (Contig282)	Protein ubiquitination pathway	F: GTCTAAGGGGAGGAATGCAGAT R: CAAAGATCAACCTCTGCTGGTC	150	CpFGC Database
β-TUB	*Phaseolus vulgaris* (XM_007147394.1)	Internal cell architecture maintenance drives cytoplasmic streaming and others	F: CCGTTGTGGAGCCTTACAAT R: GCTTGAGGGTCCTGAAACAA	117	[25]
EF1-α	*Phaseolus vulgaris* (XM_007151727.1)	Enzymatic release of aminoacyl tRNAs to the ribosome	F: GGTCATTGGTCATGTCGACTCTG R: GCACCCAGGCATACTTGAATGACC	146	[26]
FBOX	*Phaseolus vulgaris* (XM_007131876.1)	Mediation of protein–protein interaction	F: CACCAGGATGCAAAAGTGG R: ATCCGCTTGTCCCTTGAAC	163	[27]
UE21D	*Phaseolus vulgaris* (XM_007145751.1)	Protein ubiquitination pathway; DNA repair pathway	F: AGAAAAGCCCCCAAGTGTTC R: CTGCCATCTCCTTCTTCAGC	161	[27]
UNK	*Phaseolus vulgaris* (XM_007131494.1)	Unknown function, putatively a membrane-associated protein	F: ATTCCCATCATGCAGCAAAG R: AGATCCCTCCAGGTCAATCC	192	[27]
ZMP	*Phaseolus vulgaris* (XM_007162147.1)	Metalloproteinase (i.e., protease enzyme whose catalytic mechanism involves a metal)	F: GCAACCAACCTTTCATCAGC R: AGAAATGCCTCAACCCTTTG	156	[27]
GAPC	*Glycine max* (XM_003526927.3)	Catalyzes an essential energy-yielding step in carbohydrate metabolism	F: ATCAGCCAAGGACTGGAGAG R: ACGGAATGCCATACCAGTCA	130	[13]
VuCHiB	*Vigna unguiculata* (Contig5335)	Hydrolytic enzyme that breaks down glycosidic bonds in chitin. It plays an important role not only in plant defense but also in various abiotic stresses	F: CCATCTGGTTCTGGATGACC R: CCGTTGATGATGTTCGTCAC	130	CpFGC Database
VuLTP	*Vigna unguiculata* (Contig14261)	Play important roles in biotic and abiotic stresses responses	F: TGTGATGATGGAAGCGAATG R: TGAGCAGCAATCAGAGGTTG	124	CpFGC Database
VuCHI	*Vigna unguiculata* (Contig12804)	Catalyzes the conversion of naringenin chalcone to naringenin and is strictly required for flavonoid production	F: CACATACCATTTCCCAGCAG R: TGGAAGACACTGCCCTTGAG	149	CpFGC Database
VuCHS	*Vigna unguiculata* (Contig7106)	Catalyzes the first committed step in the flavonoid biosynthetic pathway	F: GACTGCACAGACCATTGCAC R: GGATCGAAGGCTTCAGAAAG	144	CpFGC Database

Candidate reference genes (*VuACT*: actin; *VuUBQ10*: polyubiquitin 10; *β-TUB*: beta-tubulin; *EF1-α*: elongation factor 1-alfa; *FBOX:* F-box protein; *UE21D*: ubiquitin-conjugating enzyme E2 variant 1D; *UNK*: *Phaseolus vulgaris* unknown gene; *ZMP*: zinc metalloproteinase; *GAPC*: glyceraldehyde-3-phosphate dehydrogenase C-subunit). **Target transcripts** (*VuCHiB*: chitinase B; *VuLTP*: lipid transfer protein; *VuCHI*: chalcone isomerase; *VuCHS*: chalcone synthase). *Vu* (*Vigna unguiculata*). *Based on RefSeq-NCBI and Cowpea Functional Genome Consortium (CpFGC) databases

SV is given by a combined measure of intra-and inter-group-variation associated to the candidate RGs expression. The lower SV, the more stable are the expressed RGs. The fundamental principle is that a stable candidate RG should have minimal variation across experimental groups and subgroups [9].

The BestKeeper [11] algorithm determines the most stable gene from a panel of up to ten potential candidate RGs. The geometric mean of the Cq values for each sample across all potential RGs are combined together to form the BestKeeper index. Then, each individual gene is compared in a pairwise fashion by Pearson correlation coefficient to the BestKeeper index (gene with the highest coefficient of correlation with the BestKeeper index indicates the highest stability and the highest ranked gene is the most stable). Pfaffl et al. [11] suggest the use of the best three to four most stable RGs to provide adequate normalization of the results.

The ΔCt method compares the relative expression of all pairwise combination of candidate RGs within each condition to identify which pairs show less variability and hence which gene(s) has the most stable expression by calculating the average SD of the relative expression of the pair of genes (the lower the average SD, the more stable the candidate RG expression) [12].

MIQE guidelines

In the present work, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [5] was followed aiming experimental stringency and transparency, in order to increase the reliability and integrity of the data obtained (Additional file 4: Table S1).

Results

RGs and target transcripts: data mining and expression under abiotic stresses

All proposed candidate RGs present known functions/annotations (Table 1) and are involved in basal or vital cellular processes, as expected for a potential normalizer gene. The candidate RG "*UNK*" (XM_007131494.1; namely as "unknown" by Borges et al. [27]) was annotated in our work (through genetic ontology) as putative membrane protein (similar to AT3G13410; *Arabidopsis thaliana*). The initial screening of nine candidate RGs (except *ZMP* and *GAPC*) and four target transcripts (Table 1) by qPCR showed that all evaluated primer pairs were functional in all cowpea samples, amplifying a single band as indicated by the presence of a single peak in melting curves (Additional file 5: S2 Appendix). Means of Cq (quantification cycle) for each candidate RG varied from 16.58 (*VuUBQ10*) to 22.17 (*FBOX*), for root dehydration stress (Additional file 6: Table S2), and from 15.58 (*VuUBQ10*) to 21.61 (*FBOX*), for salt stress (Additional file 7: Table S3). It is noteworthy that *VuUBQ10* and *FBOX* presented, respectively, the highest and smallest transcripts average abundance in both analyzed conditions. Considering, preliminarily, a stringent Cqs SD (standard deviation) < 1 associated to CVs (coefficient of variance), all potential RGs were constitutively expressed in the treatments evaluated, with the exception of *EF1-α* (11.36 ± 1.90), *β-TUB* (6.70 ± 1.33), and *VuUBQ10* (6.64 ± 1.04), concerning samples under salt stress (Table 2). However, *β-TUB*, *VuUBQ10*, and *EF1-α* were maintained in the study, in order to corroborate its expression using the more robust strategies.

The selection of target transcripts (*VuCHI*, chalcone isomerase; *VuCHS*, chalcone synthase; *VuLTP*, lipid transfer protein and *VuCHiB*, chitinase B) (Table 3) was based on their regulation in the HT-SuperSAGE libraries (see Methods) of the Cowpea Functional Genome Consortium (CpFGC). Despite their presence in both experiments, their regulation was distinct (Table 3). All target transcripts were up-regulated (UR) in 'Pingo de Ouro' (drought-tolerant accession), whereas in 'Santo Inácio' (drought-sensitive accession) their expression was variable including UR, down-regulation (DR) or not differential expression [also denominated not significant (ns) at the level of $p \leq 0.05$] (Table 3). In turn, HT-SuperSAGE data for salt treatment indicated *VuLTP* and *VuCHS* as interesting target transcripts. While the *VuLTP* was UR in the 'Pitiúba' (salt-tolerant accession) and DR in the 'BR14-Mulato' (salt-sensitive accession); *VuCHS* was UR, in the salt-tolerant and "ns" in the salt-sensitive accession (Table 3).

Considering the functional primer pairs, all amplification efficiency values in the qPCR analysis presented acceptable values (90 to 110% [28]) and ranged between 95.68 and 106.28% (Table 4). The y-intercept values varied from 33.12 to 38.03, while linear regression coefficients (r^2) for all seven genes were ≥ 0.990 (Table 4).

Expression stability of candidates RGs based on four different statistical analyses

In the present study, the expression stability of the candidate RGs was analyzed in accessions under abiotic stress [root dehydration or salt (NaCl, 100 mM)], using four different approaches: geNorm, NormFinder, BestKeeper and ΔCt method.

According to **GeNorm** analysis, all candidate RGs tested showed reduced "M" values (Fig. 1a, b), below 1.5, which is the default limit. Considering that the RGs are not co-regulated, stepwise exclusion of the gene with the highest "M" value brings a combination of two RGs that had the most stable expressions of the tested samples. For root dehydration, the most stable

candidate RGs were *VuACT*/*UE21D*, followed by *UNK* and *β-TUB* (Fig. 1a). The two most stable RGs cannot be ranked in order because of the required use of gene ratios for gene stability measurements [10]. For salt stress treatment, after the stepwise exclusion of the gene with the highest "M" value, the four best RGs were *UNK*/*UE21D*, followed by *FBOX* and *VuACT* (Fig. 1b).

The geNorm also gives an estimate of the optimal number of RGs necessary for reliable normalization. This value is obtained from the "V" value analysis. A V-value below the established 0.15 threshold suggested by Vandesompele et al. [10] indicates that inclusion of an additional gene is not required for data normalization. Since this value was already reached after the first analysis (V2/3) for both assays (Fig. 2a, b), the inclusion of an additional candidate RG is not required. Thus, the two RGs could be used for normalization under these conditions; however, the use of the three most stable RGs for calculation of a qPCR normalization factor is recommended [10].

The best candidate reference genes according to **NormFinder** are those with the lowest stability value (Table 5), with minimal intra- and inter-group variation. Regard to root dehydration, the most stable were: *β-TUB* (0.104); *UE21D* (0.122); *FBOX* (0.138); and *VuACT* (0.141) (Table 5). For salt stress, the best candidate RGs were: *FBOX* (0.099); *UE21D* (0.116); *UNK*; and *β-TUB* (Table 5). *UNK* and *β-TUB* presented a stability value of 0.125 and assumed different positions for ranking purposes in Table 5.

The output of the NormFinder analysis revealed similar results to the geNorm. Both algorithms suggested *UNK*, *UE21D*, and *FBOX* as three of four most stable genes (Fig. 3) for salt stress; for root dehydration assay, the referred strategies listed *UE21D*, *VuACT*, and *β-TUB* among the four most stable (Fig. 3).

The **BestKeepe**r algorithm computes Pearson correlation coefficient to the BestKeeper index. The candidate reference gene with the highest Pearson coefficient of correlation with the BestKeeper index presents the highest stability. The analysis revealed *β-TUB* (0.958), *UNK* (0.925), *VuACT* (0.910), and *UE21D* (0.875; Table 6) as the four most stable RGs for root dehydration [following the results from geNorm (Fig. 3)]. Besides these, *β-TUB*, *UE21D*, and *VuACT* were also among the most stable, as indicated by NormFinder (Fig. 3).

For salt stress, the four most stable RGs were *UNK* (0.982), *β-TUB* (0.979), *FBOX* and *UE21D* (both 0.975) (Table 6). *FBOX* and *UE21D* exhibited identical "r" values (despite presenting different positions in Table 6 due to their ranking). The four most stable candidate RGs indicated by BestKeeper were the same as those indicated by NormFinder (Fig. 3); while in geNorm, *UNK* and *FBOX* and *UE21D* also figured among the four most stable (Fig. 3).

The **ΔCt method** is based on the comparison of 'pairs of genes' using a simple ΔCt approach. All pairs of candidate reference genes are compared to each other, and the genes are ranked according to the average standard deviation (SD) based on the relative expression of the pair of genes (the lower the average SD, the more stable is the candidate RG). For root dehydration, the most stable were, respectively: *UE21D* (0.60), *β-TUB* (0.62), *UNK* (0.64), and *VuUBQ10* (0.66) (Fig. 4). This set contains 75% of RGs also ranked as more stable by geNorm and BestKeeper (Fig. 3); compared to NormFinder this result was 50% (Fig. 3). For salt treatment, the most stable, respectively, were *FBOX* (0.70), *UNK* (0.71), *UE21D* (0.72), and *β-TUB* (0.76) (Fig. 4). Again, a confluence of 75% of the data was observed qualitatively in relation to the other approaches used (Fig. 3).

Considering both stresses analyzed, the gene *EF1-α* was the less stable candidate RG, as also revealed by geNorm, NormFinder and Bestkeeper (Figs. 1a, b, 4; Tables 5, 6).

Conservation of the candidates RGs stability between both abiotic stresses

Comparing the results, based on the data of the two analyzed assays and the algorithms employed, including the ΔCt method, some of the RG candidates presented as the most stable on one stress condition were also considered stable in the other stressful situation. Among the candidate RGs showing this expression stability, it is worth mentioning:

Table 2 Coefficient of variance (CV) and standard deviation (SD) based on Cqs values of the candidates to cowpea reference genes

Gene	Salt stress (NaCl, 100 mM)	Root dehydration
	CV (%) ± SD	CV (%) ± SD
UNK	4.54 ± 0.82	3.57 ± 0.73
β-TUB	6.70 ± 1.33	3.37 ± 0.67
FBOX	4.23 ± 0.91	2.97 ± 0.66
UE21D	4.54 ± 0.84	3.24 ± 0.62
VuUBQ10	6.64 ± 1.04	3.17 ± 0.53
*Vu*ACT	4.40 ± 0.90	3.28 ± 0.71
EF1-α	11.36 ± 1.90	3.66 ± 0.62

Vu (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *EF1-α* (elongation factor 1-alfa); *VuACT* (actin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *UNK* (*Phaseolus vulgaris* unknown gene); *FBOX* (F-box protein); *VuUBQ10* (polyubiquitin 10)

Table 3 Transcriptional modulation of selected cowpea targets transcripts in the accessions and treatments analyzed

Gene	Unitag name	Root dehydration				Salt stress (NaCl, 100 mM)			
		Tolerant accession (Pingo de Ouro)		Sensitive accession (Santo Inácio)		Tolerant accession (Pitiúba)		Sensitive accession (BR14-Mulato)	
		FC	Reg. (*)	FC	Reg. (*)	FC	Reg. (*)	FC	Reg. (*)
VuChiB	Cp1020	5.10	UR	− 1.09	ns	1.20	ns	4.40	UR
VuLTP	Cp1050	11.21	UR	− 1.94	DR	2.80	UR	− 7.90	DR
VuCHI	Cp1131	5.65	UR	1.15	ns	1.20	ns	9.50	UR
VuCHS	Cp2022	47.70	UR	13.70	UR	2.20	UR	1.40	ns

Reg. (gene regulation); FC [Fold change: measure describing how much a quantity changes going from an initial (control) to a final value (treatment)]; UR: (up-regulated); DR: (down-regulated); ns (not significant at the level of $p \leq 0.05$). *Vu* (*Vigna unguiculata*); *VuCHiB* (chitinase B); *VuLTP* (lipid transfer protein); *VuCHI* (chalcone isomerase); *VuCHS* (chalcone synthase)

*Gene regulation at the level of $p \leq 0.05$

Table 4 Characterization of cowpea qPCR reactions, indicating the category of the primer pairs used, amplified CRG or TT name, source (reference), efficiency (%) and sensitivity (y-intercept)

Category	Name	Reference	Slope (−)	Efficiency (%)	R^2	y-intercept
CRG	*VuACT*	CpFGC	3.18	106.28	− 0.989	35.09
CRG	*β-TUB*	[25]	3.25	103.09	− 0.993	35.34
CRG	*EF1-α*	[26]	3.38	97.63	− 0.995	33.12
CRG	*FBOX*	[27]	3.28	101.78	− 0.982	35.77
CRG	*UE21D*	[27]	3.30	100.92	− 0.995	34.63
CRG	*VuUBQ10*	CpFGC	3.40	96.84	− 0.997	38.01
CRG	*UNK*	[27]	3.35	98.84	− 0.989	35.17
TT	*VuChiB*	CpFGC	3.40	96.84	− 0.996	38.03
TT	*VuLTP*	CpFGC	3.38	97.24	− 0.995	35.56
TT	*VuCHI*	CpFGC	3.43	95.68	− 0.996	37.01
TT	*VuCHS*	CpFGC	3.31	100.50	− 0.996	33.69

CRG (Candidate Reference Gene); TT (Target Transcript); Cowpea Functional Genome Consortium (CpFGC); *Vu* (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *EF1-α* (elongation factor 1-alfa); *VuACT* (actin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *UNK* (*Phaseolus vulgaris* unknown gene); *FBOX* (F-box protein); *VuUBQ10* (polyubiquitin 10); *VuCHiB* (chitinase B); *VuLTP* (lipid transfer protein); *VuCHI* (chalcone isomerase); *VuCHS* (chalcone synthase); R^2 (Coefficient of Determination)

- *UE21D*, which is one of the four most stable during salt stress assay, considering the four analytical approaches; and one the two most stable during the root dehydration assay, considering the geNorm, NormFinder, and ΔCt strategies (Fig. 3);
- *UNK*: it was considered among the three most stable during the salt stress assay (based on the four analytical approaches), and also among the three most stable during the root dehydration assay based on the geNorm, BestKeeper and ΔCt approaches (Fig. 3).

Reference genes choice and HT-SuperSAGE data validation by qPCR

Considering that HT-SuperSAGE and qPCR are different approaches, the expression levels in both methods are not expected to be identical. So, to validate the HT-SuperSAGE data, the samples were not pooled for the qPCR analysis (as they were for HT-SuperSAGE libraries analysis; see Methods section). An agreement between both approaches was considered when, at least in one-time point, similar gene expression regulation was demonstrated in both approaches (HT-SuperSAGE and qPCR), as adopted by Ferreira Neto et al. [31].

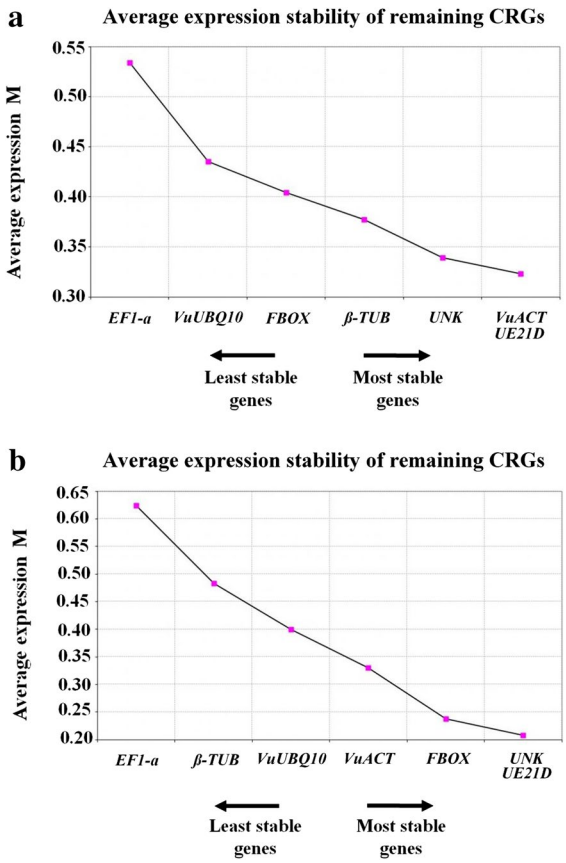


Fig. 1 geNorm analysis, indicating the average expression stability (M value) of all seven candidate reference genes in cowpea accessions under: **a** root dehydration stress; and **b** salt stress (NaCl, 100 mM). The most stably expressed genes present lower M values. CRGs (Candidate Reference Genes); *Vu* (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *EF1-α* (elongation factor 1-alfa); *VuACT* (actin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *UNK* (*Phaseolus vulgaris* unknown gene); *FBOX* (F-box protein); *VuUBQ10* (polyubiquitin 10). *The two most stable RGs of the geNorm analysis cannot be ranked in order because of the required use of gene ratios for gene-stability measurements [10]

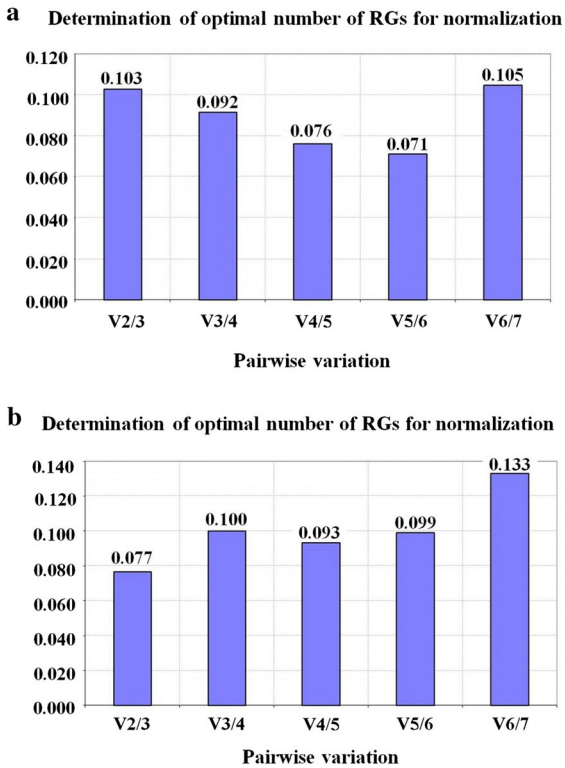


Fig. 2 geNorm output, calculated by pairwise variation analysis between normalization factors NF_n and NF_{n+1}, indicating the optimal number of reference genes (RGs) required for reliable normalization in cowpea accessions under: **a** Root dehydration stress and **b** salt stress (NaCl, 100 mM). RGs (Reference Genes)

Table 5 The NormFinder analysis of candidate reference genes (RGs) showing stability values in both experiments performed (a lower value indicates a more stable expression)

RGs acronym	Root Dehydration stress		Salt stress (NaCl, 100 mM)	
	Stability value	Rank	Stability value	Rank
β-TUB	0.104	1	0.125	4
UE21D	0.122	2	0.116	2
FBOX	0.138	3	0.099	1
VuACT	0.141	4	0.162	5
UNK	0.144	5	0.125	3
VuUBQ10	0.190	6	0.177	6
EF1-α	0.227	7	0.324	7

Vu (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *FBOX* (F-box protein); *VuACT* (Actin); *UNK* (*Phaseolus vulgaris* unknown gene); *VuUBQ10* (polyubiquitin 10) and *EF1-α* (elongation factor 1-alfa)

The most stable genes under both stresses suggested by the geNorm (which were also the most stable by the others algorithms) (Fig. 3) were used as reference genes for the expression validation of the HT-SuperSAGE data. Thus, *VuACT*, *UE21D*, and *UNK* (Fig. 1a) were the selected RGs for validation of the root dehydration assay, whereas *UNK*, *UE21D*, and *FBOX* (Fig. 1b) were chosen for salt stress assay.

Concerning the root dehydration qPCR assay, the target *VuLTP* was up-regulated in the drought-tolerant accession (Pingo de Ouro) only 150 min after the stress imposition (Table 7; Additional file 8: Table S4). In turn, in relation to the drought-sensitive accession (Santo Inácio), this target showed no differential expression (ns) during all exposition times (Table 7; Additional file 8: Table S4). Considering the salt stress assay, *VuLTP* also presented up-regulation by the salt-tolerant accession (Pitiúba) at

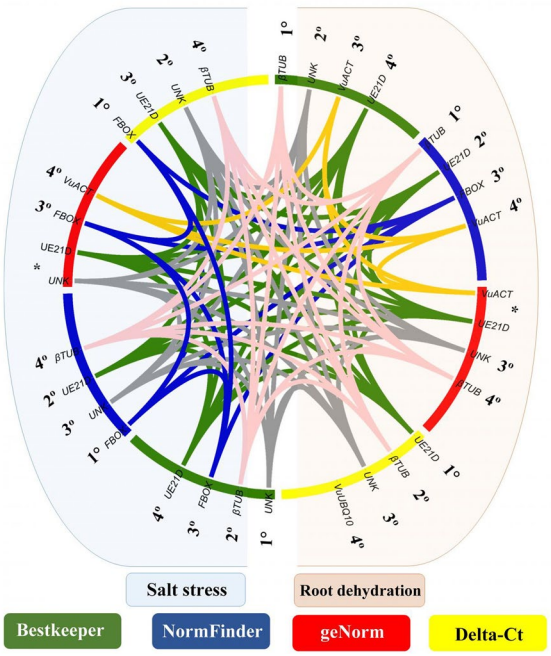


Fig. 3 Intra- and inter-assay comparisons of the four most stable cowpea candidate reference genes, considering geNorm, NormFinder, BestKeeper, and ΔCt approaches. *Vu* (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *EF1-α* (elongation factor 1-alfa); *VuACT* (actin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *UNK* (*Phaseolus vulgaris* unknown gene); *FBOX* (F-box protein); *VuUBQ10* (polyubiquitin 10); *The two most stable candidate reference genes of this algorithm cannot be ranked in order because of the required use of gene ratios for gene-stability measurements [10]

all the analyzed stress times (Table 7; Additional file 9: Table S5), opposite to the salt-sensitive accession (BR14-Mulato), which showed no differential expression in the referred time points (Table 7; Additional file 9: Table S5).

The contrasting drought-responsive accessions differed regarding the expression level of *VuCHiB*, in the course of the qPCR-tested times. Santo Inácio showed up-regulation only at the first time point (25 min). However, the expression of *VuCHiB* did not change at any time in Pingo de Ouro (Table 7; Additional file 8: Table S4). Considering the salt stress, Pitiúba showed up-regulation only at the last time (90 min), while BR14-Mulato showed down-regulation in the first two times (Table 7; Additional file 9: Table S5). *VuCHiB* was the only down-regulated transcript from the stress imposition analyzed by qPCR.

qPCR analysis indicated that the *VuCHS* gene was up-regulated in both accessions under root dehydration over all the stressful times (Table 7; Additional file 8: Table S4). For salt stress, the same transcript was up-regulated in 'Pitiúba' in 30, 60, and 90 min; while BR14-Mulato showed up-regulation in 30 and 60 min (Table 7; Additional file 9: Table S5).

The up-regulation of *VuCHI* transcript in cowpea Pingo de Ouro accession was confirmed by qPCR at 75 and 150 min. However, contrary to results from HT-SuperSAGE, qPCR data showed that the Santo Inácio accession was up-regulated in all the analyzed times after stress (Table 7; Additional file 8: Table S4). In salt stress assay, *VuCHI* was also up-regulated in both Pitiúba and BR-14 Mulato accessions, in all the stress times (Table 7; Additional file 9: Table S5).

The results of qPCR and expression libraries were validated for nine of sixteen comparisons (approximately 56%) (Table 7). In addition, qPCR data suggests *VuLTP*, *VuCHI*, and *VuCHS* (in both abiotic stresses studied) as potential targets for biotechnological approaches. This is due to two findings: (1) *VuLTP* is up-regulated in both tolerant accessions and has different regulation on both respective sensitive accessions, for both stress conditions studied; (2) the results indicate the participation (up-regulation) of the *VuCHI* and *VuCHS* in response to the abiotic stresses analyzed, even in cowpea accessions quite genetically distinct (tolerant and sensitive).

On the other hand, *VuCHiB* presents biotechnological potential regarding salt stress, only. The tolerant accession up-regulated a transcript coding the referred enzyme, while sensitive accession presented down-regulation of this target (Table 7; Additional file 9: Table S5).

Discussion

Due to the existence of potential errors during the preparation, synthesis, sequencing, and analysis of transcriptomic libraries (e.g., subtractive libraries, HT-SuperSAGE, RNA-Seq, among others), a second technique is required to validate (corroborate) the gene expression results. The currently most used technique for such purpose is quantitative real-time PCR [32], considered a gold standard validation method. Therefore, in order to ensure the reliability and precision of qPCR data, the MIQE guidelines [5] was applied for the acquisition of the results presented here (Additional file 4: Table S1). There is a lack of a systematic validation of RGs in cowpea (i.e., out of five works [13–17] addressing this theme, four omitted any information on how RGs expression stability and primer efficiency were evaluated). The present work represents a pioneering effort for a detailed analysis of candidate RGs in tolerant and sensitive cowpea cultivars in response to abiotic stresses (root dehydration and salt) rigorously tested for effective normalization of the qPCR data. These RGs may be also useful in the qPCR analysis of gene expression studies in other closely related species.

In terms of standardization and quality, all qPCR reactions (Table 4) showed amplification efficiencies between

Table 6 qPCR descriptive statistics of the candidate reference genes in cowpea under root dehydration and salt stress (NaCl, 100 mM), measured by BestKeeper software

Abiotic stress	Candidate reference gene	Rank	Pairwise correlation (PC) coefficient						Min	Max	SD	PC coefficient	*p* value
			GM	AM	min	max	SD	CV	[x-fold]	[x-fold]	[± x-fold]	[r]	
Salt stress	*UNK*	1	19.91	19.93	18.60	22.10	0.64	3.24	− 2.47	4.50	1.58	0.982	0.001
	β-TUB	2	19.81	19.85	18.00	23.40	1.00	5.03	− 3.60	12.71	2.03	0.979	0.001
	FBOX	3	21.59	21.61	20.10	24.10	0.68	3.13	− 2.85	5.84	1.62	0.975	0.001
	UE21D	4	18.40	18.42	17.00	20.70	0.60	3.24	− 2.65	4.99	1.52	0.975	0.001
	VuUBQ10	5	15.55	15.58	13.80	18.00	0.78	5.03	− 3.27	5.28	1.74	0.920	0.001
	VuACT	6	20.41	20.43	18.70	23.00	0.66	3.23	− 3.44	6.50	1.59	0.915	0.001
	*EF1-α**	7	16.58	16.68	14.40	22.80	1.36	8.16	− 4.43	70.08	2.62	0.948	0.001
Root dehydration	*β-TUB*	1	19.93	19.95	18.60	21.30	0.55	2.76	− 2.57	2.63	1.48	0.958	0.001
	UNK	2	20.35	20.37	19.30	22.20	0.60	2.95	− 2.07	3.56	1.53	0.925	0.001
	VuACT	3	21.74	21.75	20.60	23.50	0.53	2.45	− 2.28	3.56	1.46	0.910	0.001
	UE21D	4	19.04	19.05	18.07	20.40	0.51	2.65	− 1.97	2.59	1.43	0.875	0.001
	FBOX	5	22.16	22.17	21.20	23.70	0.56	2.52	− 1.96	2.96	1.48	0.870	0.001
	VuUBQ10	6	16.57	16.58	15.80	17.90	0.45	2.70	− 1.68	2.47	1.37	0.816	0.001
	EF1-α	7	16.84	16.85	15.80	18.50	0.47	2.78	− 2.03	3.11	1.39	0.475	0.019

Vu (*Vigna unguiculata*); *UNK* (*Phaseolus vulgaris* unknown gene); *β-TUB* (beta-tubulin); *FBOX* (F-box protein); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *VuUBQ10* (polyubiquitin 10); *VuACT* (actin); *EF1-α* (elongation Factor 1-alfa); GM: the geometric mean of PC; AM: the arithmetic mean of PC; Min PC and Max PC: the extreme values of PC; SD: the standard deviation of the PC; CV: the coefficient of variance expressed as a percentage of the PC level; Min [x-fold] and Max [x-fold]: the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD [± x-fold]: standard deviation of the absolute regulation coefficient; [r]: Pearson correlation coefficient

*Inconsistent data: SD > 1 [11]

90 and 110%, considered, therefore, acceptable [28]. According to Pfaffl et al. [29], uncorrected small efficiency differences between target and reference genes generate false expression ratio, resulting in over/underestimation of the 'real' initial RNA amount. The y-intercept (33.12 to 38.03) and r^2 (≥ 0.990) values (Table 4), for all seven RG candidates, revealed that the adopted setup for qPCR was also sufficient to obtain good efficiency, accuracy, and sensitivity [33].

Previous studies have reported the importance of using more than one statistical method for reference gene stability evaluation. Thus, it is expected that the comparison using different approaches might provide a more reliable set of RGs under a given experimental condition. Based on this, here we applied the four most common approaches (geNorm, NormFinder, Bestkeeper and the ΔCt method) to evaluate a set of candidate RGs for qPCR normalization of cowpea accessions after the abiotic stresses (root dehydration or salt) application. Intra-assay, for salt stress, there was a convergence of at least 75% in the genes indicated as most stable RGs, in regard to the four most stable suggested by all strategies (Fig. 3). For root dehydration, this value has been reached considering geNorm, BestKeeper, and NormFinder (Fig. 3). The variation occurred especially regarding their ranking (Fig. 3). Such discrepancies in ranking are not surprising, has been reported in previous studies [e.g., 34–36].

Considering its fundamental role in the protein biosynthesis [37], the housekeeping gene *EF1-α* has been used for normalization of qPCR data in some crop species, such as *Vigna mungo* [38], coffee [39] and potato [40], during salt stress. In the legume crop *Caragana intermedia* under osmotic, salt, cold and heat stress, the *EF1-α* gene showed to be stable using geNorm, NormFinder, and BestKeeper algorithms [41]. Also, in combination with *SAND* (SAND family protein) and *UNK2* (hypothetical protein), *EF1-α* was appropriate for normalizing gene expression data in salt-treated and in cold-treated leaves of the same species [41]. Contrarily, in our study, *EF1-α* gene was found to be one of the least stable, indicating that it is not a suitable reference gene in cowpea under root dehydration or salinity stress. Although housekeeping genes are generally indicated as good normalizers in qPCR data, these contrasting results have shown that they need to be evaluated efficiently in different species, tissues, and stress conditions.

In addition to *EF1-α,* actin and tubulin genes (both evolved in basic and essential processes in the cell) are also known as traditional RGs in plants [42]. *β-TUB* was ranked among the most stable genes considering the root dehydration samples and the applied strategies (Fig. 3), while *VuACT* was recommended using the geNorm, NormFinder, and Bestkeeper strategies (Fig. 3). Similarly, paralogous of actin (i.e., *ACT-1, 2, 4* or *11*) were ranked as the top most stable genes in cotton (for different stages

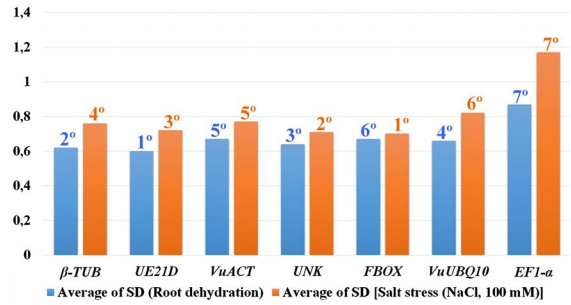


Fig. 4 Cowpea candidate reference genes evaluated by the ΔCt method and ranked according to expression stability for root dehydration and salt assays. *Vu* (*Vigna unguiculata*); *β-TUB* (beta-tubulin); *UE21D* (ubiquitin-conjugating enzyme E2 variant 1D); *VuACT* (actin); *UNK* (*Phaseolus vulgaris* unknown gene); *FBOX* (F-box protein); *VuUBQ10* (polyubiquitin 10) and *EF1-α* (elongation factor 1-alfa)

of development of flower verticils and fruit) [43], in rice under salt stress [44], and in peanut under biotic and abiotic stresses [45].

Another candidate RG whose expression stability deserves mentioning codify a *UE21D* (Ubiquitin-conjugating enzyme E2 variant 1D). *UE21D* carries out the transfer of ubiquitin to a protein substrate and figures among the main enzymes in the regulatory step for the selective protein degradation mechanism. It is a crucial regulatory step for an essential housekeeping role by removing abnormal proteins that arise through biosynthetic errors and natural proteins that acquire non-native conformations, supplying amino acids needed to produce new proteins [46]. All applied methods indicated the *UE21D* among the four most stable for both stress types (Fig. 3). Thus, this candidate RG is indicated as a major actor for the standardization of qPCR reactions in cowpea root tissue under abiotic stresses, in contrast to its reduced expression stability previously observed by Borges et al. [27] in common bean (*Phaseolus vulgaris*) leaves under fungal infection (*Colletotrichum lindemuthianum*).

The difference between gene expression stability of *UE21D* and *VuUBQ10* is worth mentioning. As previously mentioned, *UE21D* was among the four most stable gene applying the four evaluated approaches, concerning both abiotic stresses studied (Fig. 3). In turn, *VuUBQ10* (also associated with ubiquitination processes) fell outside the list of the four most stable gene, in both analyzed situations (except for root dehydration, using the ΔCt method; Fig. 3). The fact that both genes are involved in ubiquitination mechanisms could, a priori, indicate a possible co-regulation, with close results in the rankings, reducing the reliability of our results. However, it was observed in Arabidopsis that even within the polyubiquitin group (divided in *UBQ3/UBQ4* and the *UBQ10/UBQ11/UBQ14* subtypes) the RNA level of their constituents are independently modulated, within and between subtypes [47]. One major limitation in geNorm is its insensitivity to coregulated candidate RGs, therefore demanding the choice of candidates preferentially from different pathways and functional classes [10]. However, it is sometimes difficult to avoid using coregulated genes for geNorm, especially when dealing with unknown, hypothetical or poorly annotated genes [9]. In this context, it is noteworthy that the supposed molecular function of *UNK*, based on the genetic ontology, is a membrane protein (Table 1), with no functional overlap with another candidate RGs analyzed here. When coregulated genes are absent, geNorm and NormFinder usually provide almost the same general

Table 7 Comparison between the HT-SuperSAGE expression libraries and qPCR data in cowpea roots under abiotic stress treatments

Gene	Root dehydration										Salt stress (NaCl, 100 mM)									
	HT-SS assay†		qPCR assay*								HT-SS assay†		qPCR assay*							
			(Pingo de Ouro) TOL			VGE‡	(Santo Inácio) SEN			VGE‡			(Pitiúba) TOL			VGE‡	(BR14-Mulato) SEN			VGE‡
	TOL	SEN	25′	75′	150′		25′	75′	150′		TOL	SEN	30′	60′	90′		30′	60′	90′	
VuChiB	UR	ns	ns	ns	ns	No	UR	ns	ns	Yes	ns	UR	ns	ns	UR	Yes	DR	DR	ns	No
VuLTP	UR	DR	ns	ns	UR	Yes	ns	ns	ns	No	UR	DR	UR	UR	UR	Yes	ns	ns	ns	No
VuCHI	UR	UR	ns	UR	UR	Yes	UR	UR	UR	Yes	UR	ns	UR	UR	UR	Yes	UR	UR	UR	No
VuCHS	UR	ns	UR	UR	UR	Yes	UR	UR	UR	No	ns	UR	UR	UR	UR	No	UR	UR	ns	Yes

HT-SS (HT-SuperSAGE); *Vu* (*Vigna unguiculata*); *VuCHiB* (Chitinase B); *VuLTP* (Lipid transfer protein); *VuCHS* (Chalcone synthase); *VuCHI* (Chalcone isomerase)

† and * $p < 0.05$. TOL (tolerant accession); SEN (sensitive accession); UR (up-regulated); DR (down-regulated); ns (not significant at $p < 0.05$); 25′, 75′ and 150′ (minutes under root dehydration); 30′, 60′ and 90′ (minutes under salt stress), VGE (Validation of Gene Expression); ǂ between HT-SuperSAGE and qPCR data

ranking, with only minor differences in order, as observed in the present work (Fig. 3).

Another interesting result was that two candidate RGs (*UE21D* and *UNK*) considered among the most stable in root tissue under dehydration were also stable in the same tissue under salt stress (Fig. 3). It is known that plants exhibit a range number of response to environmental changes and that molecular mechanisms include signal recognition, signal transduction, and signal responses, among others [48]. Some of these mechanisms are shared among distinct stresses since plants are often exposed to a myriad of abiotic and biotic stresses under field conditions [48]. Thus, such RGs become strong candidates for application of stability tests on other abiotic stress types, with emphasis on those that have a similar physiological impact on tissues, like freezing, that imposes osmotic stress on plants, similarly to what occurs with drought and salt stresses [49].

The RG *F-BOX*, in turn, presented as one of the three most stable genes using the performed strategies and considering the salt stress applied. However, similar behavior was not evidenced (at least comprising the top four) considering the root dehydration stress samples (except by the NormFinder result; Table 5). Despite this, for the referred assay, the observed stability indices of this RG are within the acceptable standards indicated by the four approaches (stability value, NormFinder [9]; M-value, geNorm [10]; coefficient of correlation with the BestKeeper index [11]; and average SD for relative expression of 'pairs of genes'; ΔCt approach [12]). Therefore, *F-BOX* is another important RG to be considered in gene expression of cowpea addressing saline or other osmotic stress.

Regarding the target transcripts, only the expression of the *VuCHI* gene was validated in both accessions under root dehydration (Table 7; Additional file 8: Table S4). The *VuCHI* (EC 5.5.1.6) is an enzyme of the isoflavonoid pathway in plants and catalyzes the cyclization of chalcone into (2S)-naringenin. Naringenin defines a critical branch point for the synthesis of several major classes of flavonoids, including flavanones, flavonols, and anthocyanins [50, 51]. Flavonoids have a significant contribution to the response mechanisms of higher plants to a variety of abiotic stresses. Its function is mainly associated with the inhibition of cellular reactive oxygen species (ROS) production [52]. Even considering the validation index of approximately 56%, it is important to emphasize that qPCR analyses indicated that these targets have biotechnological potential.

Besides *CHI*, also the gene encoding *VuCHS* presented up-regulation in both situations, and accessions studied, differently than the HT-SuperSAGE data indicated (Table 7). *VuCHS* (EC 2.3.1.74) is another key enzyme involved in the regulation of flavonoids biosynthesis. *VuCHS* is the entry point of the flavonoid pathway and catalyzes the transformation of the 4-Coumaroyl-CoA and Malonyl-CoA to chalcone, leading phenylpropanoids pathway to flavonoids biosynthesis [50, 51]. Thus, there are indications that compounds derived from the enzymatic action of *VuCHS* and *VuCHI* actively participate in the process of tolerance to stresses that cause an osmotic imbalance (such as root dehydration and salt stress) in cowpea.

The gene codifying a *VuLTP*, in turn, presented contrasting regulation between the accessions (Table 7; Additional File 8: Table S4; Additional File 9: Table S5), considering both conditions analyzed by qPCR. *VuLTP* gene was induced in the two tolerant employed accessions, while presented no differential expression in the sensitive counterparts. The involvement of this gene in response to drought in other plant species has been reported. Guo et al. [53] found that the rice loss-of-function mutant *LTP3* was sensitive to drought stress, whereas overexpressing plants were drought tolerant. Additionally, Wang et al. [54] observed that a wheat lipid transfer protein 3 (w*LTP*3) could enhance the basal thermotolerance and oxidative stress resistance in Arabidopsis.

The only transcript analyzed whose qPCR indicated down-regulation was *VuCHiB*, in the salt-sensitive accession. With regard to the salt-tolerant accession, *VuCHiB* was up-regulated in the last time point analyzed (90 min; Table 7; Additional File 9: Table S5). Chitinases are enzymes that degrade chitin (a linear polymer of β-1, 4-N-acetylglucosamine). Chitin is the second most abundant biopolymer on the planet [55] and is found in the outer skeleton of many organisms (insects, algae, yeasts, crabs, fungi, among other [56]). Plant chitinases are classified as PR (pathogen-related) proteins that act in plant self-defense against phytopathogens and pests [57]. Some chitinases genes are up-regulated in response to abiotic stresses, such as drought in cucumber [58] and high salt, in rice [53]. This fact, now, is also reported in cowpea tolerant accessions under radicular dehydration and salt stresses. Data mining in the CpFGC datasets uncovered just over a dozen *VuCHiB* isoforms (data not shown).

Conclusion

Taken together, the here applied approaches allowed the identification of converging RGs within and between trials, considering the four different cowpea accessions analyzed. The presenting work represents the first evaluation of RGs for cowpea subjected to root dehydration or salt stress. Except for *EF1-α* (using BestKeeper algorithm for salt stress assay), all other candidates showed acceptable stability thresholds according to the four strategies. For

root dehydration stress, the candidates *VuACT*, *UE21D*, used in qPCR validation activities, were ranked among the most stable genes in the four approaches scrutinized. The *UNK* gene also applied in the qPCR analysis with the root dehydration samples ranked among the most stable RGs considering three approaches (geNorm, BestKeeper, and ΔCt method). In turn, the candidates *UNK*, *UE21D*, and *FBOX* were the most stable genes for salt stress, based on all used strategies. In summary, these findings provide useful tools for the normalization of qPCR experiments and enable accurate and reliable gene expression evaluations related to cowpea transcriptomics.

Regarding the comparative analysis of the target genes, both qPCR and HT-SuperSAGE approaches presented a 56% agreement on results. This data may reflect a random sampling deviation or real technique variation, demonstrating the importance of validation of gene expression results in transcriptomic studies. The target genes represent promising candidates for biotechnological use after these validations. Our results suggest that flavonoids (or its derivatives) are essential players in the cowpea response to the conditions analyzed. This fact was observed from the up-regulation, in all accessions and situations, of transcripts encoding *VuCHS* and *VuCHI*, key enzymes in the synthesis of those compounds. According to qPCR analyses, *VuLTP*s also participate in the tolerance processes. This gene presented contrasting regulation between the accessions in the analyzed situations. The analyzed *VuLTP* was up-regulated in the tolerant ones, and no differentially expressed in the sensitive counterparts, for both stresses. Since these proteins are involved in multiple actions, their specific involvement or function still needs to be scrutinized. *VuCHiB*, in turn, is an interesting target gene only in cowpea under salt stress, due to its differential expression in both analyzed accessions (up-regulated in the tolerant; down-regulated in the sensitive). Such a target is commonly associated with plant responses to pathogenic organisms, adding value to its biotechnological potential since this gene can act in diverse situations.

Besides, cowpea beans, when compared to other legumes of economic importance, are still a crop with limited gene expression research. The present study provides some grounds for future research on the gene expression of cowpea under root dehydration or saline stress, or similar conditions that impose osmotic imbalance. The analyzed targets, in turn, aggregate information on the molecular physiology of cowpea under unfavorable conditions, as well as revealing a potential for future use in breeding programs.

Additional files

Additional file 1. Figure S1A and S1B. Experimental design for the assays [radicular dehydration and salt (NaCl, 100 mM) presented in this work. Dashed lines represent the bulks formation to the HT-SuperSAGE libraries synthesis.

Additional file 2. Appendix S1. Sequences used in the present work.

Additional file 3. Figure S2. Schematic representation of the steps performed to analyze the candidate reference genes evaluated in the present study. **Legend**: SD (Standard Deviation); r^2 (Pearson´s Correlation Coefficient); CRGs (Candidate Reference Genes); CpFGC (Cowpea Functional Genome Consortium).

Additional file 4. Table S1. MIQE checklist for reviewers, and editors. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available.

Additional file 5. Appendix S2. Melting curve for seven candidate reference genes [(*FBOX*) F-box protein; (*VuACT*) actin; (*VuUBQ10*) polyubiquitin 10; (*eEF-1α*) eukaryotic elongation factor 1α; (*β-TUB*) beta-tubulin; (*UNK*) *Phaseolus vulgaris* unknown gene; (*UE21D*) ubiquitin-conjugating enzyme E2 variant 1D] with single peak.

Additional file 6. Table S2. Cqs obtained for the candidate reference genes analyzed for the root dehydration assay.

Additional file 7. Table S3. Cqs obtained for the candidate reference genes analyzed for the salt stress assay (NaCl, 100 mM).

Additional file 8. Table S4. Data explored by REST software to analyze the relative expression of target transcripts in cowpea under root dehydration stress.

Additional file 9. Table S5. Data explored by REST software to analyze the relative expression of target transcripts in cowpea under salt stress.

Authors' contributions

Conceived and designed the experiments: AMBI, MSG, and EAK. Planned and performed the glasshouse experiments: VP, LLBA, JRCFN, MKSM, FTA, and MSG. Performed the laboratory experiments and analyzed the data: VP, LLBA, JRCFN, JPBN, MKSM, and FTA. Contributed with reagents/materials/analysis tools: AMBI and EAK; Wrote the manuscript: JCRFN, LLBA, and AMBI. All authors read and approved the final manuscript.

Author details

[1] Instituto Federal de Educação, Ciência e Tecnologia do Piauí, Oeiras, Piauí, Brazil. [2] Genetics Department, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. [3] Botany Department, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data generated or analyzed during this study are included in this published article and its supplementary information files.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

The authors acknowledge the support of "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq, Brazil) and the "Fundação de Amparo à Pesquisa do Estado de Pernambuco" (FACEPE, Brazil). The authors also acknowledge the fellowships granted by CNPq and "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES, Brazil).

Publisher's Note

Received: 14 June 2018 Accepted: 26 September 2018
Published online: 12 October 2018

References

1. Nsa IY, Kareem KT. Additive interactions of unrelated viruses in mixed infections of cowpea (*Vigna unguiculata* L. Walp). Front. Plant Sci. 2015;6:1–13. https://doi.org/10.3389/fpls.2015.00812.
2. Freire Filho et al., 2011: Freire Filho FR, Ribeiro VQ, Rocha MM, Silva KJD, Nogueira MSR, Rodrigues EV. Feijão-caupi no Brasil: produção, melhoramento genético, avanços e desafios. 1st edition. Embrapa Meio-Norte; 2011.
3. Langyintuo AS, Lowenberg-DeBoer J. Potential regional trade implications of adopting Bt cowpea in West and Central Africa. AgBioForum. 2006;9:111–20.
4. Benko-Iseppon AM. NordEST Network. 2013; Brazilian Cowpea Genome Consortium: understanding responses against biotic and abiotic stresses. IV Simpósio Brasileiro de Genética Molecular de Plantas, Bento Gonçalves/RS. http://www.sbg.org.br/IV_SBGMP/index.html. Accessed 7 May 2018.
5. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J. The MIQE guidelines: minimum information for publication of quantitative Real-Time PCR Experiments. Clin Chem. 2009;55(4):611–22. https://doi.org/10.1373/clinchem.2008.112797.
6. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 2008;8:131. https://doi.org/10.1186/1471-2229-8-131.
7. Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, Griffiths LR. β-Actin—an unsuitable internal control for RT-PCR. Mol Cell Probes. 2001;15:307–11. https://doi.org/10.1006/mcpr.2001.0376.
8. Lee PD, Sladek R, Greenwood CMT, Lee PD, Sladek R, Greenwood CMT, et al. Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res. 2002. https://doi.org/10.1101/gr.217802.
9. Andersen C, Jensen J, Orntoft T. Normalization of real-time quantitative reverse transcription—PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64:5245. https://doi.org/10.1158/0008.
10. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002. https://doi.org/10.1186/gb-2002-3-7-research0034.
11. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26:509–15. https://doi.org/10.1023/B:BILE.0000019559.84305.47.
12. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol. 2006;7:33. https://doi.org/10.1186/1471-2199-7-33.
13. Coetzer N, Gazendam I, Oelofse D, Berger DK. SSHscreen and SSHdb, generic software for microarray based gene discovery: application to the stress response in cowpea. Plant Methods. 2010;6:10. https://doi.org/10.1186/1746-4811-6-10.
14. Mellor KE, Hoffman AM, Timko MP. Use of ex vitro composite plants to study the interaction of cowpea (*Vigna unguiculata* L.) with the root parasitic angiosperm Striga gesnerioides. Plant Methods. 2012;8:22. https://doi.org/10.1186/1746-4811-8-22.
15. Huang K, Mellor KE, Paul SN, Lawson MJ, Mackey AJ, Timko MP. Global changes in gene expression during compatible and incompatible interactions of cowpea (*Vigna unguiculata* L.) with the root parasitic angiosperm Striga gesnerioides. BMC Genom. 2012;13:402–17. https://doi.org/10.1186/1471-2164-13-402.
16. Shui XR, Chen ZW, Li JX. MicroRNA prediction and its function in regulating drought-related genes in cowpea. Plant Sci. 2013;210:25–35. https://doi.org/10.1016/j.plantsci.2013.05.002.
17. Da Silva HAP, Nardeli SM, Alves-Ferreira M, Simões-Araújo JL. Evaluation of reference genes for RT-qPCR normalization in cowpea under drought stress during biological nitrogen fixation. Crop Sci. 2015;55:1–13. https://doi.org/10.2135/cropsci2014.10.0738.
18. Kulcheski FR, de Oliveira LF, Molina LG, Almerão MP, Rodrigues FA, Marcolino J, et al. Identification of novel soybean microRNAs involved in abiotic and biotic stresses. BMC Genom. 2011;12:307. https://doi.org/10.1186/1471-2164-12-307.
19. Dantas BF, Ribeiro LDS, Aragão CA. Physiological response of cowpea seeds to salinity stress. Rev Bras Sementes. 2005;27:144–8. https://doi.org/10.1590/S0101-31222005000100018.
20. Praxedes SC, Ferreira TM. Increase of proline and aminoacids in cowpea cultivars with differential salt stress tolerance. Rev Caatinga. 2009;22:211–4.
21. Kido EA, Barbosa PK, Ferreira Neto JRC, Pandolfi V, Houllou-Kido LM, et al. Identification of plant protein kinases in response to abiotic and biotic stresses using SuperSAGE. Curr Protein Pept Sci. 2011;12(7):643–56.
22. Matsumura H, Yoshida K, Luo S, Kimura E, Fujibe T, Albertyn Z, Barrero RA, Krüger DH, Kahl G, Schroth GP, Terauchi R. High-throughput SuperSAGE for digital gene expression analysis of multiple samples using next generation sequencing. PLoS One. 2010;5(8):12010. https://doi.org/10.1371/journal.pone.0012010.
23. Audic S, Claverie J. The significance of digital gene expression profiles. Genome Res. 1997;7:986–95. https://doi.org/10.1101/gr.7.10.986.
24. Robertson N, Oveisi-Fordorei M, Zuyderduyn SD, Varhol RJ, Fjell C, Marra M, et al. DiscoverySpace: an interactive data analysis application. Genome Biol. 2007;8:R6. https://doi.org/10.1186/gb-2007-8-1-r6.
25. Eticha D, Zahn M, Bremer M, Yang Z, Rangel AF, Rao IM, et al. Transcriptomic analysis reveals differential gene expression in response to aluminium in common bean (*Phaseolus vulgaris*) genotypes. Ann Bot. 2010;105:1119–28. https://doi.org/10.1093/aob/mcq049.
26. Guerrero-Gonzalez ML, Rodriguez-Kessler M, Rodriguez-Guerra R, Gonzalez-Chavira M, Simpson J, Sanchez F, et al. Differential expression of Phaseolus vulgaris genes induced during the interaction with Rhizoctonia solani. Plant Cell Rep. 2011. https://doi.org/10.1007/s00299-011-1055-5.
27. Borges A, Tsai SM, Caldas DGG. Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. Plant Cell Rep. 2012;31:827–38. https://doi.org/10.1007/s00299-011-1204-x.
28. Biassoni R. Quantitative real-time PCR: methods and protocols. In: Biassoni R, Raso A, editors. Methods in molecular biology. New York: Springer; 2014.
29. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30:e36. https://doi.org/10.1093/nar/30.9.e36.
30. REST 2009 Software User Guide For gene expression analysis using real-time. Sample & Assay Technologies. 2009.
31. Ferreira Neto JRC, Pandolfi V, Guimaraes FCM, Benko-Iseppon AM, Romero C, Silva RLDO, et al. Early transcriptional response of soybean contrasting accessions to root dehydration. PLoS ONE. 2013;8:1–20. https://doi.org/10.1371/journal.pone.0083466.
32. Adamski MG, Gumann P, Baird AE. A method for quantitative analysis of standard and high-throughput qPCR expression data based on input sample quantity. PLoS ONE. 2014. https://doi.org/10.1371/journal.pone.0103917.
33. Scott Adams. Adams, 2006: Scott Adams, P. Data analysis and reporting. In Real-time PCR; Dorak, M.T., Ed.; Taylor & Francis Group: Abingdon, UK, 2006; pp. 39–62.
34. Cassan-Wang H, Soler M, Yu H, Camargo ELO, Carocha V, et al. Reference genes for high-throughput quantitative reverse transcription—PCR analysis of gene expression in organs and tissues of *Eucalyptus* grown in various environmental conditions. Plant Cell Physiol. 2012;53:2101–16. https://doi.org/10.1093/pcp/pcs152.
35. Liu D, Shi L, Han C, Yu J, Li D, Zhang Y. Validation of reference genes for gene expression studies in virus-infected *Nicotiana benthamiana* using quantitative Real-Time PCR. PLoS ONE. 2012. https://doi.org/10.1371/journal.pone.0046451.

36. Mariot RF, De Oliveira LA, Voorhuijzen MM, Staats M, Hutten RCB, Van Dijk JP, et al. Selection of reference genes for transcriptional analysis of edible tubers of potato (*Solanum tuberosum* L.). PLoS ONE. 2015;10:1–13. https://doi.org/10.1371/journal.pone.0120854.
37. Suhandono S, Apriyanto A, Ihsani N. Isolation and characterization of three cassava elongation factor 1 alpha (MeEF1A) promoters. PLoS ONE. 2014;9:32–4. https://doi.org/10.1371/journal.pone.0084692.
38. Kundu A, Patel A, Pal A. Defining reference genes for qPCR normalization to study biotic and abiotic stress responses in *Vigna mungo*. Plant Cell Rep. 2013;32:1647–58. https://doi.org/10.1007/s00299-013-1478-2.
39. De Carvalho K, Bespalhok Filho JC, Dos Santos TB, De Souza SGH, Vieira LGE, Pereira LFP, et al. Nitrogen starvation, salt and heat stress in coffee (*Coffea arabica* L.): Identification and validation of new genes for qPCR normalization. Mol Biotechnol. 2013;53:315–25. https://doi.org/10.1007/s12033-012-9529-4.
40. Nicot N, Hausman JF, Hoffmann L, Evers D. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot. 2005;56:2907–14. https://doi.org/10.1093/jxb/eri285.
41. Zhu J, Zhang L, Li W, Han S, Yang W, Qi L. Reference gene selection for quantitative Real-Time PCR normalization in Caragana intermedia under different abiotic stress conditions. PloS One. 2013. https://doi.org/10.1371/journal.pone.0053196.
42. Kumar V, Sharma R, Trivedi PC, Vyas GK, Khandelwal V. Traditional and novel references towards systematic normalization of qRT-PCR data in plants. Aust J Crop Sci. 2011;5:1455–68.
43. Artico S, Nardeli SM, Brilhante O, Grossi-de-Sa MF, Alves-Ferreira M. Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol. 2010;10:49. https://doi.org/10.1186/1471-2229-10-49.
44. Caldana C, Scheible W-R, Mueller-Roeber B, Ruzicic S. A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. Plant Methods. 2007;3:7. https://doi.org/10.1186/1746-4811-3-7.
45. Morgante C, Guimaraes P, Martins A, Araujo A, Leal-Bertioli S, Bertioli D, et al. Reference genes for quantitative reverse transcription-polymerase chain reaction expression studies in wild and cultivated peanut. BMC Res Notes. 2011;4:339.
46. Vierstra RD. Proteolysis in plants: mechanisms and functions. Plant Mol Biol. 1996;32:275–302. https://doi.org/10.1007/BF00039386.
47. Sun CW, Callis J. Independent modulation of *Arabidopsis thaliana* polyubiquitin mRNAs in different organs and in response to environmental changes. Plant J. 1997;11:1017–27. https://doi.org/10.1046/j.1365-313X.1997.11051017.x.
48. Pandey P, Ramegowda V, Senthil-Kumar M. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. Front Plant Sci. 2015;6:1–14. https://doi.org/10.3389/fpls.2015.00723.
49. Xiong L, Schumaker KS, Zhu JK. Cell signaling during cold, drought, and salt stress. Plant Cell. 2002;14:S165. https://doi.org/10.1105/tpc.000596.S166.
50. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. Plant Cell. 1995;7:1085–97.
51. Weisshaar B, Jenkins GI. Phenylpropanoid biosynthesis and its regulation. Curr Opin Plant Biol. 1998;1:251–7. https://doi.org/10.1016/S1369-5266(98)80113-1.
52. Di Ferdinando M, Brunetti C, Fini A, Tattini M. Flavonoids as antioxidants in plants under abiotic stresses. In: Ahmad P, Prasad MNV, editors. Abiotic stress responses in plants. New York: Springer; 2012. p. 159–79.
53. Guo XL, Bai LR, Su CQ, Shi LR, Wang DW. Molecular cloning and expression of a class III chitinase in upland rice. Genet Mol Res. 2013;3:6860–70.
54. Wang F, Zang X, Kabir MR, Liu K, Liu Z, Ni Z, et al. A wheat lipid transfer protein 3 could enhance the basal thermotolerance and oxidative stress resistance of Arabidopsis. Gene. 2014;550:18–26. https://doi.org/10.1016/j.gene.2014.08.007.
55. Shahidi. Chitin, chitosan and co-products: chemistry, production, applications and health effects. Adv Food Nutr Res. 2005;49:93–135.
56. Bhattacharya D, Nagpure A, Gupta RK. Bacterial chitinases: properties and potential. Crit Rev Biotechnol. 2007;27(1):21–8.
57. Gooday N. Aggressive and defensive roles of chitinases. EXS. 1999;87:157–69.
58. Chen. Intercellular 27 kD protein is a chitinase induced by water stress or *Pseudoperonospora cubensis* in cucumber leaves.

ANEXOS

Anexo A: Normas para submissão na revista *Plant Methods* disponível em: https://plantmethods.biomedcentral.com/submission-guidelines/preparing-your-manuscript

Anexo B: Normas para submissão na revista *Frontiers in Plant Science* disponível em: https://www.frontiersin.org/journals/plant-science#author-guidelines

Anexo C: Capítulo de livro publicado na Springer International Publishing.

Chapter 12
Transcription Factors Involved in Plant Drought Tolerance Regulation

Lidiane L. Barbosa Amorim, João Pacífico Bezerra-Neto, Rômulo da Fonseca do Santos, José Ribamar Costa Ferreira Neto, Ederson Akio Kido, Mitalle Matos and Ana Maria Benko-Iseppon

12.1 Introduction

The complex genetic basis related to drought tolerance requires a deeper understanding of molecular and physiological mechanisms, also concerning regulation of networks involved in related responses such as water deficit, osmotic, oxidative, and heat stress. The recognition of these mechanisms is fundamental for the development of adapted cultivars to each type of abiotic stress [180, 227]. When exposed to drought, plants induce a series of changes at the physiological, biochemical, and molecular levels. Such modifications may affect cell viability due to the production of reactive oxygen species (ROS) responsible for the oxidation of multicellular components, such as proteins, lipids, and nucleic acids [90, 258].

Drought is also responsible for inhibition of respiration, stomatal closure, stimulation of root growth, changes in photosynthesis, and assimilation of nutrients, among others [123, 175, 201]. In addition to the mentioned effects, the refered stress stimulates the production of abscisic acid (ABA), a central regulator of the stress response, which confers tolerance, increasing expression of many stress-responsive genes by inducing signaling cascades [37, 112].

In addition to regulatory events mediated by ABA, other groups of genes activated by drought confer tolerance to the plant, including transcription factors (TFs) that play a role as regulators and central molecular switches in the control of

L.L. Barbosa Amorim · J.P. Bezerra-Neto · R. da Fonseca do Santos · J.R.C. Ferreira Neto · E.A. Kido · M. Matos · A.M. Benko-Iseppon (✉)
Department of Genetics, Laboratory of Plant Genetics and Biotechnology, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, 50670-423 Recife, PE, Brazil
e-mail: ana.iseppon@gmail.com

L.L. Barbosa Amorim
Federal Institute of Education, Science and Technology of Piauí, Rua Projetada, s/n, CEP 64500-000 Oeiras, PI, Brazil

M.A. Hossain et al. (eds.), *Drought Stress Tolerance in Plants, Vol 2*,
DOI 10.1007/978-3-319-32423-4_12

CURRICULUM VITAE (LATTES)

Mitalle Karen da Silva Matos

Currículo Lattes

Formação acadêmica/titulação

2015 Doutorado em andamento em Genética (Conceito CAPES 4).
Universidade Federal de Pernambuco, UFPE, Brasil.
Título: *Fatores de Transcrição WRKY: Modulação da Expressão e Interações Moleculares Sob Estresse em Feijão-Caupi*
Orientador: Ana Maria Benko-Iseppon.
Coorientador: João Pacífico Bezerra Neto.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
Grande área: Ciências Biológicas

2013 - 2015
Mestrado em Genética (Conceito CAPES 4).
Universidade Federal de Pernambuco, UFPE, Brasil.
Título: *Expressão Diferencial e Diversidade de Fatores de Transcrição da Família MYB em Feijão-Caupi*, Ano de Obtenção: 2015.
Orientador: Ana Maria Benko-Iseppon.
Coorientador: Lidiane Lindinalva Barbosa Amorim.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

2015 - 2017
Graduação em Licenciatura Plena em Ciências Biológicas.
Universidade Federal Rural de Pernambuco, UFRPE, Brasil.

2009 - 2013
Graduação em Ciências Biológicas.
Universidade Federal Rural de Pernambuco, UFRPE, Brasil.
Título: Filogenia Molecular de Espécies do Gênero *Croton* Usando Marcadores ITS.
Orientador: Ana Maria Benko-Iseppon.

Formação Complementar

2017 - 2017
R para Geneticistas. (Carga horária: 8h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2017 - 2017
Banco de Dados Biológicos e Conceitos Básicos de Linux. (Carga horária: 6h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2017 - 2017
Bioinformatics with Python for Biologists. (Carga horária: 60h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2016 - 2016
Visualization of Biological Data. (Carga horária: 45h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2016 - 2016
Computational Biology for the Analysis of Biological Networks. (Carga horária: 45h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2016 - 2016
Desmistificando o Ensino da Genética. (Carga horária: 3h).
Sociedade Brasileira de Genética, SBG, Brasil.

2016 - 2016
Introduction to High Performance Computing for Bioinformatics Analysis. (Carga horária: 60h).
Sociedade Brasileira de Genética, SBG, Brasil.

2015 - 2015
Genética Forense. (Carga horária: 4h).
Universidade Federal de Pernambuco, UFPE, Brasil.

2015 - 2015
Sistema CFX e Introdução a PCR em tempo real e suas aplicações. (Carga horária: 16h).
Sociedade Brasileira de Genética, SBG, Brasil.

Atuação Profissional

Secretaria de Educação de Pernambuco, SEPE, Brasil.

Vínculo institucional
2016 - Atual
Vínculo: Enquadramento Funcional: Professora, Carga horária: 40
Outras informações
Professora do Programa de Educação Integral do Estado de Pernambuco

Projetos de pesquisa

2015 - Atual
Rede InterSys: Biologia Sistêmica no Estudo de Função Gênica em Interações Bióticas

Descrição: O projeto envolve nove instituições e 17 subprojetos. Pretende estabelecer a rede INTERSYS, voltada para a formação de pessoal e geração de conhecimento científico de alto nível envolvendo interações bióticas a partir de abordagens multidisciplinares de biologia sistêmica (ômicas, biologia celular e bioinformática) através da integração de grupos nacionais e internacionais experientes.
Situação: Em andamento; Natureza: Pesquisa.

Integrantes: Mitalle Karen da Silva Matos - Integrante / ANA BENKO - Coordenador.

Projetos de extensão

2017 - Atual
Adote um Gene
Descrição: O referido projeto de extensão envolve aulas teóricas e práticas de bioinformática com alunos do ensino médio do Colégio Militar do Recife..
Situação: Em andamento; Natureza: Extensão.

Integrantes: Mitalle Karen da Silva Matos - Integrante / A M Benko-Iseppon - Coordenador / José Ribamar Costa Ferreira-Neto - Integrante / Carolline de Jesús Pires - Integrante / João Pacífico - Integrante / Flávia Araújo - Integrante / Artemisa Borges - Integrante / Flavia Figueira Aburjaile - Integrante / Romulo da Fonseca dos Santos - Integrante.

Prêmios e títulos

2018 Menção Honrosa na área de, Sociedade Botânica do Brasil.
2018 1° Lugar - Doutorado, VIII Jornada de Pós Graduação em Genética.
2017 Menção Honrosa na área de Genética, Sociedade Botânica do Brasil.
2016 3º Lugar Geral - Doutorado, Sociedade Brasileira de Genética.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

1.
AMORIM, LIDIANE LINDINALVA BARBOSA; FERREIRA-NETO, JOSÉ RIBAMAR COSTA; Bezerra-Neto, João Pacífico; PANDOLFI, VALESCA; DE ARAÚJO, FLÁVIA TADEU; **DA SILVA MATOS, MITALLE KAREN**; SANTOS, MAURO GUIDA; Kido, Ederson Akio; Benko-Iseppon, Ana Maria. Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR. Plant Methods JCR, v. 14, p. 88, 2018.

Capítulos de livros publicados

1.
Barbosa Amorim, Lidiane L.; Bezerra-Neto, João Pacífico; da Fonseca do Santos, Rômulo; Ferreira Neto, José Ribamar Costa; Kido, Ederson Akio; **Matos, Mitalle**; Benko-Iseppon, Ana Maria. Transcription Factors Involved in Plant Drought Tolerance Regulation. Drought Stress Tolerance in Plants, Vol 2. 1ed.: Springer International Publishing, 2016, v. 2, p. 315-358.

Resumos publicados em anais de congressos

1.
Araújo, F. T.; **MATOS, M. K. S.**; Amorim, L.L.B; Pandolfi, V.; Benko-Iseppon, A. M.. Genes Candidatos para Normalização em Estudos de Expressão de *Stylosanthes scabra* Vogel (Fabaceae). In: 35ª Reunião Nordestina de Botânica, 2017, Recife. 35ª Reunião Nordestina de Botânica, 2017.
2.
Rêgo, M. S.; Silva, R. L. O.; **MATOS, M. K. S.**; ARAUJO, F. T.; Silva, J. B.; Lemos, A. B.; Araújo, A. C. C.; Oliveira, M. F.; Benko-Iseppon, A. M.. Desenvolvimento e Seleção de *Primers* para Validação de Genes Responsivos à Virose em *Vigna unguiculata* (L.) Walp. In: XXI Encontro de Genética do Nordeste, 2016, Recife. XXI Encontro de Genética do Nordeste, 2016.
3.
ARAUJO, F. T.; BEZERRA-NETO, J. P.; **MATOS, M. K. S.**; OLIVEIRA, W. D.; Amorim, T. C. V.; Amorim, L.L.B; Benko-Iseppon, A. M.. Identificação e Análise Fenética de Aquaporinas no Transcriptoma do Feijão-Caupi. In: 67º Congresso Nacional de Botânica, XXXVI ERBOT e 8ª Jornada Capixaba de Botânica, 2016, Vitória - ES. 67º
4.
OLIVEIRA, W. D.; Amorim, T. C. V.; ARAUJO, F. T.; **MATOS, M. K. S.**; BEZERRA-NETO, J. P.; Benko-Iseppon, A. M.. Identificação In Silico e Caracterização de Microssatélites no Genoma do Feijão-Caupi. In: XXI Encontro de Genética do Nordeste, 2016, Recife. XXI Encontro de Genética do Nordeste, 2016.
5.
MATOS, M. K. S.; Leal, D. R. S.; Pires, C. J.; Benko-Iseppon, A. M.. Inferência Filogenética de Fatores de Transcrição MYB em Plantas Superiores. In: XXI Encontro de Genética do Nordeste, 2016, Recife. XXI Encontro de Genética do Nordeste, 2016.
6.
Lemos, A. B.; Silva, R. L. O.; **MATOS, M. K. S.**; ARAUJO, F. T.; Oliveira, M. F.; Rêgo, M. S.; Benko-Iseppon, A. M.. Seleção de Genes Candidatos Associados à Resistência ao Vírus do Mosaico Severo em Feijão-Caupi. In: XXI Encontro de Genética do Nordeste, 2016, Recife. XXI Encontro de Genética do Nordeste, 2016.
7.
OLIVEIRA, W. D.; ARAUJO, F. T.; **MATOS, M. K. S.**; Amorim, L.L.B; Benko-Iseppon, A. M.. Marcadores Gênicos Polimórficos entre Acessos de Feijão-Caupi [*Vigna unguiculata* (L.) Walp]. In: I Encontro de Limnologia e Botânica da Paraíba, 2015, João Pessoa. I Encontro de Limnologia e Botânica da Paraíba, 2015.
8.
MATOS, M. K. S.; ARAUJO, F. T.; Amorim, L.L.B; Ferreira-Neto, J. R. C.; Benko-Iseppon, A. M.. Expressão Diferencial de Fatores de Transcrição MYB em Feijão-Caupi Submetido a Estresse Viral. In: V Jornada de Pós-Graduação em Genética, 2015, Recife. V Jornada de Pós-Graduação em Genética, 2015.
9.
OLIVEIRA, A. R. S.; **MATOS, M. K. S.**; ARAUJO, F. T.; Ribeiro, L. M. S.; Lima, S. T. S.. Base Nacional Comum Curricular: Levantamento Sobre a Difusão do Tema entre os Profissionais da Educação Básica. In: XV Jornada de Ensino, Pesquisa e Extensão da UFRPE, 2015, Recife. XV Jornada de Ensino, Pesquisa e Extensão da UFRPE, 2015.
10.
OLIVEIRA, W. D.; **MATOS, M. K. S.**; ARAUJO, F. T.; Ferreira-Neto, J. R. C.; Amorim, L.L.B; Benko-Iseppon, A. M.. Polimorfismo de Marcadores SSR's Derivados de Sequências Transcriptômicas do Feijão-Caupi. In: XV Jornada de Ensino, Pesquisa e Extensão da UFRPE, 2015, Recife. XV Jornada de Ensino, Pesquisa e Extensão da UFRPE, 2015.
11.
OLIVEIRA, W. D.; **MATOS, M. K. S.**; ARAUJO, F. T.; Amorim, L.L.B; Benko-Iseppon, A. M.. Triagem de Marcadores SSRs Polimórficos entre Genótipos de Feijão-Caupi Contrastantes Quanto à Tolerância à Seca. In: V Jornada de Pós-Graduação em Genética, 2015, Recife. V Jornada de Pós-Graduação em Genética, 2015.

Apresentações de Trabalho

1.
MATOS, M. K. S.; Bezerra-Neto, J. P.; Araújo, F. T.; Amorim, T. C. V.; BENKO-ISEPPON, A. M. Prospecção e Caracterização In Silico de Proteínas Reguladoras da Transcrição em Resposta à Desidratação Radicular no Feijão-Caupi. 2017. (Apresentação de Trabalho/Congresso).
2.
MATOS, M. K. S.; Bezerra-Neto, J. P.; Araújo, F. T.; Borges, A. N. C.; Benko-Iseppon, A. M. Identificação global in silico das famílias de fatores de transcrição expressas no transcriptoma do feijão-caupi. 2017. (Apresentação de Trabalho/Congresso).
3.

MATOS, M. K. S.; BEZERRA-NETO, J. P.; Ferreira-Neto, J. R. C.; Kido, E. A.; Benko-Iseppon, A. M.. Expressão de Fatores de Transcrição Dof (Dna-Binding With One Finger) em Genótipos Selecionados de Feijão-Caupi sob Estresses Bióticos e Abióticos. 2016. (Apresentação de Trabalho/Congresso).

4.

MATOS, M. K. S.. Fatores de Transcrição: Modulação da Expressão e Interações Moleculares sob Estresse em Fabaceae Cultivadas. 2015. (Apresentação de Trabalho/Seminário).

5.

MATOS, M. K. S.. An RNA-Seq based gene expression atlas of the common bean. 2015. (Apresentação de Trabalho/Seminário).

6.

MATOS, M. K. S.. Transcription Factor Functional Protein-Protein Interactions in Plant Defense Responses. 2015. (Apresentação de Trabalho/Seminário).

Bancas

Participação em bancas de comissões julgadoras

Outras participações

1.

Guilherme, B. C.; **MATOS, M. K. S.** VIII Mostra do Laboratório de Ensino de Ciências Biológicas (LECBio) - Biologia: Docência, Experiências e Saberes. 2017. Universidade Federal Rural de Pernambuco.

Eventos

Participação em eventos, congressos, exposições e feiras

1.

6º Seminário Internacional, 20º Seminário Nacional. 2018. (Seminário).

2.

35ª Reunião Nordestina de Botânica. Prospecção e Caracterização In Silico de Proteínas Reguladoras da Transcrição em Resposta à Desidratação Radicular no Feijão-Caupi. 2017. (Congresso).

3.

VII Jornada de Pós-Graduação em Genética. Identificação global in silico das famílias de fatores de transcrição expressas no transcriptoma do feijão-caupi. 2017. (Simpósio).

4.

VIII Ciclo de Seminários em Genética e Biotecnologia Vegetal. 2016. (Seminário).

5.

III Ciclo de Seminários em Bioinformática. Unravelling Peptidomes by in silico Mining. 2015. (Seminário).

6.

III Ciclo de Seminários em Bioinformática. De Novo Assembly an Discovery os Genes That Are Involved in Drougth Tolerance in Tibetan *Sophora moorcroftiana*. 2015. (Seminário).

7.

IV Ciclo de Seminários "Genética Vegetal". Transposon Variants and Their Effects on Gene Expression in *Arabidopsis*. 2015. (Seminário).

8.

VI Ciclo de Seminários.Global reprogramming of transcription and metabolism in *Medicago truncatula* During Progressive Drought and After Rewatering. 2015. (Seminário).

9.

VI Ciclo de Seminários "Genética Vegetal". Genome Organization and Characteristics of Soybean microRNAs. 2015. (Seminário).

10.

V Jornada de Pós-Graduação em Genética. Expressão diferencial de fatores de transcrição MYB em feijão-caupi submetido a estresse viral. 2015. (Simpósio).

Organização de eventos, congressos, exposições e feiras

1.

Benko-Iseppon, A. M.; Aburjaile, F. F.; Bezerra-Neto, J. P.; **MATOS, M. K. S.**; Pires, C. J.; Borges, A. N. C.; Araújo, F. T.; Santos, R. F.. EXPO UFPE 2017 - Projeto Adote um Gene. 2017. (Exposição).

2.

MATOS, M. K. S. Mostra do Projeto Adote um Gene. 2017. (Outro).

3.

Benko-Iseppon, A. M.; **MATOS, M. K. S.**. Adote um Gene ? viajando nos genomas com a bioinformática. 2016. (Exposição).

Orientações

Iniciação científica

Orientações e supervisões concluídas

1.
Wilson Dias de Oliveira. Desenvolvimento e Validação de Marcadores Moleculares tipo Microssatélites a Partir do Transcriptoma de *Vigna unguiculata* (L.) Walp. Início: 2017. Iniciação científica (Graduado em Licenciatura Plena em Ciências Biológicas) - Universidade Federal Rural de Pernambuco, Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco. (Orientador).

Orientações e supervisões em andamento

2.
Thialisson Cacci do Vale Amorim. Prospecção e Caracterização de Genes Induzidos em *Vigna unguiculata* sob Infecção pelo Vírus do Mosaico Severo. Início: 2017. Iniciação científica (Graduando em Ciências Biológicas) - Universidade Federal de Pernambuco, Conselho Nacional de Desenvolvimento Científico e Tecnológico. (Orientador).