Functional Analysis of Two NAC Transcription Factors and Transcriptome Analysis under Drought in *Citrullus Colocynthis*

By

Zhuoyu Wang

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Approved by

Fenny Dane, Chair, Professor of Horticulture
Narendra K.Singh, Professor of Biology
Aaron Rashotte, Associate Professor of Biology
Elina Coneva, Associate Professor of Horticulture

Abstract

Citrullus colocynthis (L.) Schrad, closely related to watermelon (*Citrullus lanatus* L.), belongs to the *Cucurbitaceae* family. It can survive arid environments by maintaining its water content without wilting of the leaves or desiccation under severe stress conditions.

NAC (NAM, ATAF1,2, CUC2) transcription factors play important roles in plant growth, development, and responses to abiotic and biotic stress. Two novel NAC transcription factors (CcNAC1 and CcNAC2) were isolated from *Citrullus colocynthis*. The characterization of the two genes was studied in order to gain an understanding of their function under abiotic stress conditions. Manipulation of transcription factors represents a potential strategy for development of transgenic stress tolerant plants. Promoter regions, transgentic GUS assays etc. studies results indicated that CcNAC1 and CcNAC2 may have multiple functions to regulate the plant's defense responses to abiotic stress.

Further studies on the functional role of these genes to different qualities of light and auxin were based on the *in silico* analysis of the *CcNAC1* and *CcNAC2* promoter regions, which revealed the presence of several light-associated motifs. The impact of both light and auxin on *CcNAC1* and *CcNAC2* gene expression was examined in *C. colocynthis* leaves, and using reporter (*pCcNAC1, 2::GUS*) lines in *Arabidopsis*. Furthermore the effects of constitutive overexpression (*OE-CcNAC1, 2*) lines in *Arabidopsis* were also examined under a range of conditions to confirm reporter line linkages. White, blue, red, and far red light treatments

resulted in similar patterns of quantitative changes in *CcNAC1*and *CcNAC2* expression in both species, with the highest transcript increases associated with red light. Photomorphogenic changes in *Arabidopsis* hypocotyls were correlated with changes in gene transcript levels. In the absence of light hypocotyls of OE-CcNAC1 and OE-CCNAC2 lines were significantly longer as compared to hypocotyls of wild type seedlings. The addition of exogenous auxin (+IAA) to growth medium also resulted in changes to the hypocotyl lengths of overexpression lines and spatiotemporal reporter line changes in *Arabidopsis* seedlings. Our data suggest that *CcNAC1, 2* might be functionally important in the light signaling pathway, and appear connected to the phytohormone auxin. Different light and dark treatments resulted in quantitative and apatiotemporal changes in *CcNAC1* and *CcNAC2* expression patterns. This study points to the relationship between the auxin, light and NAC TFs.

We also used high throughput mRNA Illumina sequencing technology and bioinformatic strategies to analyze the leaf transcriptome of *C. colocynthis* under drought treatment. Leaf samples following 4 days of water deficit treatment were used for RNA extraction for library construction and Illumina sequencing. qRT-PCR analysis of drought induced genes was performed to confirm the accuracy of RNA sequencing. More than 5038 whole cDNAs were identified and 2545 genes showed significantly changes during drought as compared with Day 1. Principle component analysis showed that drought was the major factor in regulation of the transcriptome changes and many candidate drought stress related genes were detected. Our data imply that there are transcriptional changes in *C.colocynthis* under drought stress, providing the understanding of the molecular regulation mechanism of plant drought resistance.

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I Introduction and literature review

Plants have evolved several molecular mechanisms to cope with biotic and abiotic stress. Successful adaptation to stress is regulated through the activation or repression of the effects of transcription factors on specific target genes. The NAC (NAM, ATAF and CUC) transcription factors (TFs), which constitute one of the largest transcription factor family in plants, have been reported to be involved in plant development and biotic and abiotic stress regulation and thus might be promising candidates for improving a plants' stress tolerance.

Plants are exposed to various biotic and abiotic stresses in the natural environment, which are regulated by gene specific transcription factors that activate or repress the transcription apparatus by binding at target gene promoters (Fujita et al., 2006). Transcription factors are grouped into families on the basis of sequence similarities, most often in the DNA binding domain. NAM (no apical meristem), ATAF, CUC (cup-shaped cotyledon) or NAC domain proteins comprise one of the largest plant specific transcription factors, represented by ~105 genes in Arabidopsis (Jensen et al., 2010), ~151 in rice (*Oryza sativa*)(Fang et al., 2008; Nuruzzaman et al., 2010), ~163 in *Populus* (Hu et al., 2010) and ~205 in the soybean genome, ~152 members in tobacco (Tran et al., 2010). NAC transcription factors have multiple functions, some of which have been well elucidated in *Arabidopsis* and *Oryza sativa* (Tran et al., 2009). Identification of NACs involved in biotic and abiotic stress is beneficial for elucidating the

molecular mechanisms of the stress response and for determining a biotechnological approach toward plant improvement.

Structure of NACs

N-terminal regions of NACs

NAC transcription factors contain a highly conserved N-terminal DNA-binding domain and a diverse regulatory C-terminal domain (Ernst et al., 2004). The N-terminal region comprises nearly 160 amino acid residues which are divided into five main conserved sub-domains, named A to E (Ooka et al., 2003). NACs in rice can be classified into 15 types (Ooka et al., 2003). NACs with complete A-E sub-domains are typical A-E type NACs. F-O type NACs which are not classified based on conserved motifs, are called NAC-like proteins (Tran et al., 2009). The architecture of the NAC domain of 60 residues consists of a twisted β-sheet surrounded by a few helical elements (Ernst et al., 2004). It was determined from in vitro gel mobility shift assays that residues Val 111-Lys 168 in *Arabidopsis* ATNAM constitute the DNA-binding domain, which corresponds to the inside part of the helix-turn-helix structure (Ernst et al., 2004). AtNAC1, AtNAM and ANAC019 from *Arabidopsis* can bind to a fragment of the CaMV 35S promoter (Xie et al., 2000; Duval et al., 2002; Tran et al., 2004). ANAC019/055/072 bind to the core element CACG in the ERD1 (early response to dehydration 1) promoter (Tran et al., 2004). GmNAC11 and GmNAC20 from soybean bind to the core CGT(G/A) sequence, which is complementary to CACG. However, other sequences can also be bound by NACs (Hao et al., 2011).

C-terminal regions of NACs

The C-terminal regions of NAC proteins are highly divergent and confer the regulation diversities of transcriptional activation activity. For example, CarNAC3 from *Cicer arietinum*, AtNAC2 and AtNAM have C terminal trans-activation domains (Duval et al., 2002; He et al., 2005; Peng et al., 2009). The C-terminal regions of GmNAC20 from soybean and NAC1 from *Arabidopsis* have transcriptional activation ability, but full length NACs have little transcriptional activity, which indicates that NAC family proteins may have different mechanisms for transcriptional regulation (Hao et al., 2010; Xie et al., 2000).

Most NACs were found to function as transcriptional activators (Hao et al., 2010), but Calmodulin binding (CB)NAC from *Arabidopsis* and GmNAC20 from soybean are NAC-like proteins which function as transcriptional repressors (Kim et al., 2007; Hao et al., 2011). Deletion experiments detected an active repression domain with 35 amino acids, named NARD (NAC Repression Domain+ in the d subdomain of the NAC DNA binding domain (Hao et al., 2010).

Plasma membrane-bound NAC transcription factors

Membrane-bound motifs are contained in the C-terminal region of some NACs (Tran, et al., 2009). Controlled proteolytic cleavage of membrane-associated transcription factors (MTFs)

is an adaptive strategy to abrupt environmental changes, which ensures rapid transcriptional responses to incoming stimuli (Pj et al., 2008). Some NACs in *Arabidopsis* are MTFs and most of the putative NACs are up-regulated by stress conditions, suggesting that they may be involved in stress responses. Kim et al. have identified a group of membrane-bound NAC transcription factors (designated NTLs), which are released from the membranes by proteolytic cleavage, responding to stress conditions and transported into the nucleus (Kim et al., 2007). Also, some rice NACs are also membrane-associated (Kim et al., 2007). Studies with a GFP-NTM2 (for NAC with Transmembrane Motif2) fused gene showed that the full-size NTM2 protein was localized at the plasma membrane, whereas the C-terminal deleted form was predominantly in the nucleus (Park et al., 2011). Similarly, NTM1, which regulates cell division by modulating cytokinin signaling, has a transmembrane motif1 (Kim et al., 2006). A fructose specific quantitative trait locus (FSQ6) was identified via map-based cloning in *Arabidopsis* and shown to encode the NAC domain encoding protein 89 (ANAC089).The Cvi allele of FSQ6/ANAC089 was found to be active without a membrane-bound domain in the nucleus and could suppress fructose sensitivity via the ABA-signaling pathway in *Arabidopsis* (Li et al., 2011).

NACs involved in the regulation of biotic and abiotic stress

NACs and Salt Stress

High salinity is a major abiotic stress condition which results in both ionic stress and osmotic stress. Salt cress (*Thellungiella halophila*), an *Arabidopsis*-related halophyte, showed differential gene expression profiles in full-length *Arabidopsis* cDNA microarray studies. At1g01720 (ANAC002) showed significant up-regulation in *Arabidopsis* following 2h exposure to 250mM NaCl (Taji et al., 2004). The NAC transcription factor NAM2/*Arabidopsis* NAC domain-containing protein 69 (for NAC with Transmembrane Motif1) was found to mediate a salt signaling cascade during seed germination via the IAA30 gene, which functioned as a negative regulator of auxin signaling (Park et al., 2011). A salt-responsive NTL in *Arabidopsis*, NTL8, was found to mediate salt-responsive flowering via the FLOWERING LOCUS T (FT) (Kim et al., 2007). MTFs of *Arabidopsis*, NTL3 and NTL6, were dramatically up-regulated by NaCl similarly to CBF2 (C-repeat/dehydration responsive element binding factor 2) (Kim et al., 2007). RD (Responsive to Desiccation) 26 mediates both the ABA-dependent and ABA-independent pathway. Microarray analysis showed that antioxidant-, defense- and senescence-responsive genes were up-regulated in RD-26 overexpressed *Arabidopsis* plants (Fujita et al., 2004).

NACs and Temperature Stress

Plants implement multiple molecular mechanisms to cope with heat stress, which include maintenance of membrane stability, scavenging of reactive oxygen species (ROS), production of antioxidants, accumulation and adjustment of compatible solutes, induction of mitogen-activated

protein kinase (MAPK), calcium-dependent protein kinase (CDPK) cascades, chaperon signaling and transcriptional activation (Wahid et al., 2007). Acclimation of plants to temperature stress involves the simultaneous alteration of many genes since multiple characters are involved in tolerance over a wide range of temperatures (Iba, 2002). MTFs of *Arabidopsis*, NTL1 and NTL11, were induced by heat (37C) (Kim et al., 2007a), while NTL4 and NTL7 were induced by cold (4C), and NTL2 and NTL3 were broadly influenced by cold, drought, and NaCl (Kim et al., 2007b).

NACs and Drought Stress

Drought is a worldwide problem, effecting global crop production and quality. Scavenging of reactive oxygen, cell membrane stability, expression of aquaporins and stress proteins are vital mechanisms of drought tolerance (Anjum et al., 2011). Microarray analysis showed that about 40% of the upregulated genes in rice plants overexpressing OsNAC6 were responsive to dehydration, high salinity or cold stress. A large number of peroxidase genes were identified among the upregulated genes, which might be direct targets of OsNAC6 and provide protection to the cells under stress (Nakashima et al., 2007). NACs were found to not only be positive regulators, but also negative regulators. For example, an *ataf1* mutant could increase the rate of recovery after drought stress along with a stimulation of the expression of some stress-inducible marker genes, which means that ATAF1 is a negative drought response regulator in *Arabidopsis.* Also, enhanced drought tolerance contributed to the increased expression of

dehydration tolerance genes rather than physical changes during stomatal movement (Lu et al., 2007). OsNAC6 overexpression caused growth retardation, low productivity, though it improved tolerance towards blast disease, dehydration and high-salt (Nakashima et al., 2007).

NACs and Osmotic Stress

Water availability is fundamental to almost all aspects of plant physiology. Plants can only survive within a certain range of water status. Significant changes in water potential can impose osmotic stress to plants, resulting in damage to cellular activities or plant death. Drought, high salinity and freezing cause osmotic stress to plants (Xiong and Zhu, 2002). Osmotic stress can activate the transduction of a cell death signal through N-rich proteins (NRPs), therefore, endoplasmic reticulum (ER)-stress and osmotic stress are integrated into a circuit of cell death through NRP-mediated signaling. *GmNAC6* (*Glycine max* NAC6) was found to be induced by the osmotic stress inducer, PEG, and the ER-stress-inducing agents, tunicamycin (TUN) and L-azetidine-2-carboxylic acid (AZC) individually. When TUN and AZC were combined, the stress signals promoted a synergistic accumulation of GmNAC6 transcripts (Faria et al., 2011). NTL9 is implicated in osmotic stress signaling during leaf senescence and its pathway is independent of known growth hormones (abscisic acid, ethylene, salicylic acid, and jasmonic acid) signaling pathways or may act upstream of growth hormone biosynthesis and signaling (Yoon et al., 2008).

NACs and Oxidative Stress

Oxidative stress results in extensive changes in plant metabolism. During oxidative stress reactive oxygen species (ROS) and other radicals are produced, which result in damage to key biomolecules such as proteins, lipids and DNA leading to cellular dysfunction and ultimately cell death (Halliwell, 2006). Microarray analysis in *Arabidopsis* revealed that the overall metabolic response of plant cells to oxidative stress is remarkably similar to oxidative stress response of bacteria and yeast (*Saccharomyces cerevisiae*). Significantly altered functional classes of transcripts at the 2 and 6h time points during oxidative stress included NACs. RD26 - overexpressed plants showed up-regulation of the GLY (glutathione transferase) gene, which is known to be involved in the antioxidant defense system. The transactivation experiment showed that RD26 could recognize the cis-acting element of GLY (Fujita et al., 2004). Overaccumulation of iron in plant cells leads to oxidative stress. IEDF2 (for Iron Deficiency Responsive Element 2) is known to encode a member of the NAC family of TFs. Transgenic IDEF2 *Arabidopsis* plants exhibited increased accumulation of Fe under iron sufficient conditions, while lower than normal levels of iron were present in iron deficient plants, indicating an important role of IDEF2 in maintaining optimal levels of iron in tissues (Walker and Connolly, 2008).

NACs and Light Stress

Senescence of plants can be induced following dark treatment (Yoshida, 2003). As dark-induced senescence occurs, the expression of some molecular regulators (e.g. transcriptional regulators) change. Whole-genome ATH1 Genome Array studies showed that more than one quarter of NAC proteins in *Arabidopsis* leaves were upregulated under dark treatment (Lin and Wu, 2004).

High-light (HL) stress can result in damage to the photosynthetic apparatus of plants. cDNA microarray analysis in *Arabidopsis* indicated that some *NACs* are involved in photosynthetic protection (Kimura et al., 2003). For example, ANAC078 in *Arabidopsis* was shown to regulate flavonoid biosynthesis under HL stress (Morishita et al., 2009). Light includes UV-B and UV-A. Short wavelength, higher intensity UV-B could modulate ANAC13 by an yet unknown and COP1 (CONSTITUTIVE PHOTOMORPHOGENESIS 1)-independent signaling cascade. ANAC13 was also modulated by the plant hormone abscisic acid (ABA) and different abiotic stresses (Safrany et al., 2008).

NACs and Biotic Stress

Biotic stress occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial and harmful insects, weeds, and cultivated or native plants. The interactions between plants and pathogens have resulted in the evolution of many complex defense pathways (Maor and Shirasu, 2005). Generally, biotic stresses from

necrotrophic and biotrophic microbes are perceived in plants by the jasmonic acid-ethylene and salicylic acid (JA-ET-and SA)-dependent signaling pathways, respectively (Glazebrook, 2005; Jensen et al., 2008). Age-related resistance (ARR) affords increased resistance to normally virulent pathogens as some plants mature (Carviel et al., 2009). SA accumulation is required for resistance (R) gene (specific resistance receptor) –mediated responses, basal resistance responses and systemic acquired resistance (SAR) (Delaney et al., 1994;1995; Ukenes et al., 1993; Vernooij et al., 1994; Carviel et al., 2009). ANAC055 (At3g15500) and ANAC092 (At5g39610) were identified as two new ARR-associated genes via microarray analysis, which contributed to ARR. Besides this analysis, there are also some reports about the expression of ANAC055 and ANAC092 in response to NaCl and ABA treatment (He et al., 2005; Tran et al., 2004). ANAC055 is also up-regulated by methyl jasmonate (MeJA) treatment (Bu et al., 2008; He et al., 2005). NAC092 is a positive regulator of the onset of leaf senescence (Guo and Gan, 2006). In conclusion, some NACs may contribute to ARR gene regulation, which is also regulated by other factors.

GmNAC6 does not only play a role during osmotic and ER stress, but is also induced by biotic signals, which could further induce the pathogenesis-related gene1 (PR1) and result in necrotic lesions (Faria et al., 2011). ATAF1 insertion mutations were reported to enhance the penetration rate of the fungal pathogen *Blumeria graminin* f.sp.*hordei* into epidermal cells (Jensen et al., 2007). Two NAC proteins, ANAC019 and ANAC055, were found to act downstream of AtMYC2 (identified player involved in JA-signaling in *Arabidopsis*) to regulate JA-signaled defense pathway. Their double mutants showed decreased expression of MeJA-

induced VEGETATIVE STORAGE PROTEIN1 (VSP1) and LIPOXYGENASE2 (LOX2), but decreased resistance to the necrotrophic pathogen *B. cinerea* (Bu, et al., 2008). OsNAC19 (Oryza sativa NAC19) protein is involved in rice's response to infection by the blast fungus *Magnaporthe grisea* and maybe a component in the MeJA-mediated signaling pathway (Lin et al., 2007).

ATAF2 can rapidly be induced by wounding, while pathogen-induced genes are induced in overexpressed ATAF2 *Arabidopsis*. The systemic acquired resistance marker PDF1.2 (in jasmonate /ethylene signaling) and PR1 (in SA-mediated signaling pathway) are higher in knockout than in wild type *Arabidopsis*, which indicates that ATAF2 mediates the pathogen-related signaling pathway (Delessert et al., 2005).

ABA has been studied mainly in abiotic stress, especially with respect to regulation under drought stress, low temperature and salinity (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Jensen et al., 2008). But more and more evidence accumulates which indicates that ABA also has some correlation with biotic stress (Jensen et al., 2008). Global transcript profiling of ABA-responsive genes is one way to elucidate ABA signaling components (Huang et al., 2007; Rabbani et al., 2003; Jensen et al., 2008). ATAF1 is one of the identified genes (Lu et al., 2007). It has been hypothesized that ATAF1, the previously reported drought-responsive gene, negatively regulates ABA synthesis (Lu et al., 2007), and enhances the expression of JA/ET defense signaling indirectly. ATAF1 has an effect on ABA signaling, which is required for

maintenance of effective penetration resistance toward *Blumeria graminis* f.sp.*hordei* (Jensen et al., 2008).

NACs and Phytohormones

Phytohormones such as SA, JA, ET, and ABA primarily regulate the protective responses of plants to both biotic and abiotic stresses via synergistic and antagonistic actions, often referred to as signaling crosstalk (Bostock, 2005; Lorenzo and Solano, 2005; Mauch-Mani and Mauch, 2005; Fujita et al., 2006). Biotic and abiotic stress cannot be isolated from different signaling pathways, but constitute complex signaling circuits, with either antagonistic or synergistic interaction (Gupta et al., 2000; Spoel et al., 2003; Jensen et al., 2008). ABA mainly acts as a regulator of abiotic stress, and ET, SA, and JA play central roles in biotic stress signaling. But NACs are viable candidate molecules that potentially regulate both biotic and abiotic signaling, besides AtMYC2 and other transcription factors (Fujita et al., 2006). RD26, one of dehydration-responsive NAC transcription factor, is induced by JA, hydrogen peroxide (H2O2), pathogen infections, drought, high salinity and ABA treatment (Fujita et al., 2006; Fujita et al., 2004; Zimmermann et al., 2004). ATAF2 is not only pathogen-related, but also related to salt, blast disease, dehydration and wounding (Delessert et al., 2005). JA regulates plant defense responses against herbivore attack, pathogen infection and mechanical wounding. JA signaling defense response was differentially regulated by AtMYC2, either positively or negatively. ANAC019 and ANAC055 were not only associated with drought tolerance, but also regulated JA-induced

expression of defense genes downstream of AtMYC2 (Bu et al., 2008). NAC transcription factor RIM1 is as a host factor involved in multiplication of rice dwarf virus (RDV), and RIM1, are also involved in JA signaling in rice plants. Through a 26S proteasome-dependent pathway, RIM1 could be degraded by JA treatment. RIM1 interacts with MYC2, one key transcription factor in the JA pathway, to regulate the JA-responsive genes. RIM1 does not only play critical roles in JA signaling but also in other biological processes (Yoshii et al., 2010).

ABA might inhibit the SA-mediated responses in some plants. NRPs (N-rich protein) - mediated cell death response might function in the SA pathway, which acts antagonistically to suppress the ABA-mediated response. Also, because ABA is a central regulator in the plant drought regulation pathway, and GmNAC6 was regulated by NRPs and ABA, it is possible that NRP mediates GmNAC6 through a cell-death response, which could promote tolerance to dehydration (Fujita et al., 2006; Foria et al., 2011).

During seed germination, both ABA and gibberellic acid (GA) play roles. ABA can break seed dormancy, whereas GA promotes seed germination (Okamoto et al., 2006). Auxin interacts with NTM2-mediated salt stress signals during seed germination and part of the NTM-mediated salt signals is mediated by IAA30, which functions as a negative regulator of auxin signaling (Park et al., 2011).

Previous reports have indicated that ethylene response genes can influence ABA biosynthesis. Cross talk exists between ABA and ethylene (Ghassemian et al., 2000). The salt response of AtNAC2 can be induced by plant hormones ABA and NAA as well as the ethylene

precursor ACC. Salt stress induction of AtNACs requires the ethylene and auxin signaling pathways, acting downstream. Ethylene receptor EIN2 did completely block AtNAC2 induction under salt stress, whereas other upstream components in the ethylene or auxin signaling pathway could only partially inhibit the induction. Auxin resistant mutant tir1-1could suppress salt induction of AtNAC2 (He et al., 2005). Xie et al. (2000) showed that NAC1 is induced by auxin in *Arabidopsis* and He et al (2005) similarly showed that AtNAC2 was induced by multiple plant hormones (auxin, ABA and ethylene) and high salinity.

Figure 1. 1. Signaling network of NACs in biotic and abiotic stresses.

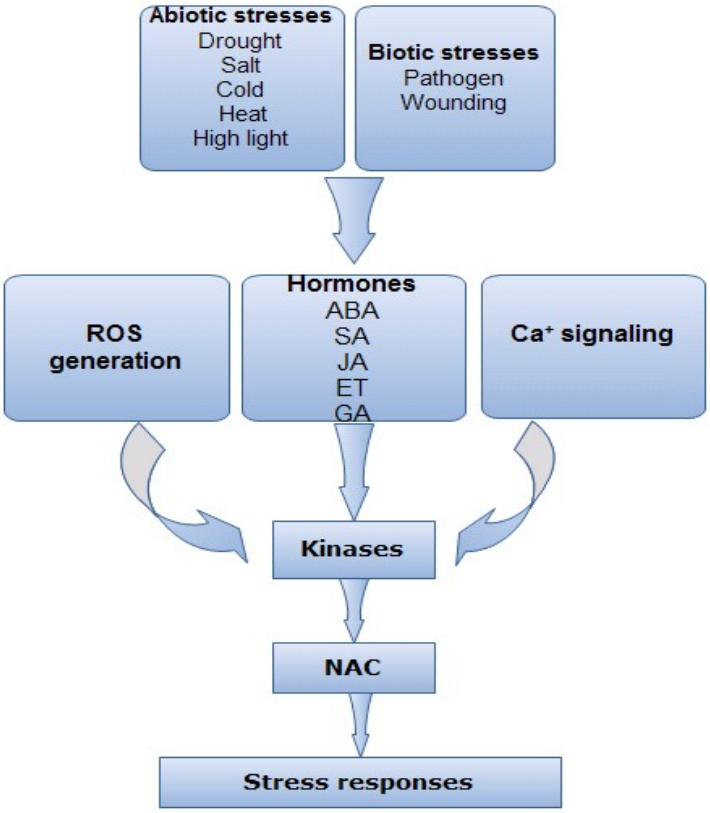


Figure1. 2.The correlation between NACs and different phytohormones.

Plant hormones are indicated in green, NACs in red, the NAC family in yellow. SA, JA, IAA, ET mainly regulate herbivory, pathogen, wound responses, while ABA regulates drought, cold, heat, salt and high light stress responses. Different phytohormones regulate different NACs, which can bind to NACRS (NAC Recognition Sequence) to respond to abiotic and biotic stresses.

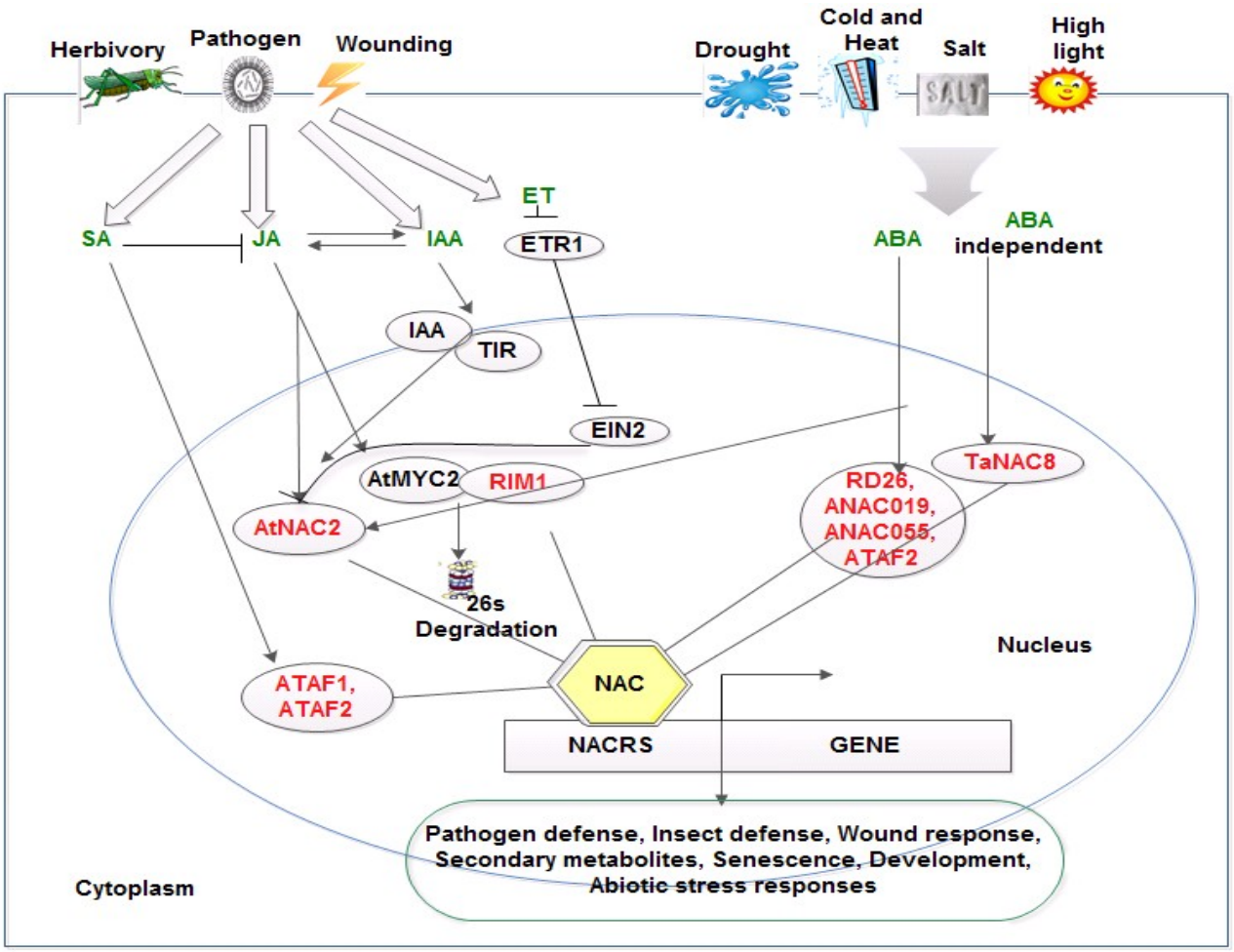
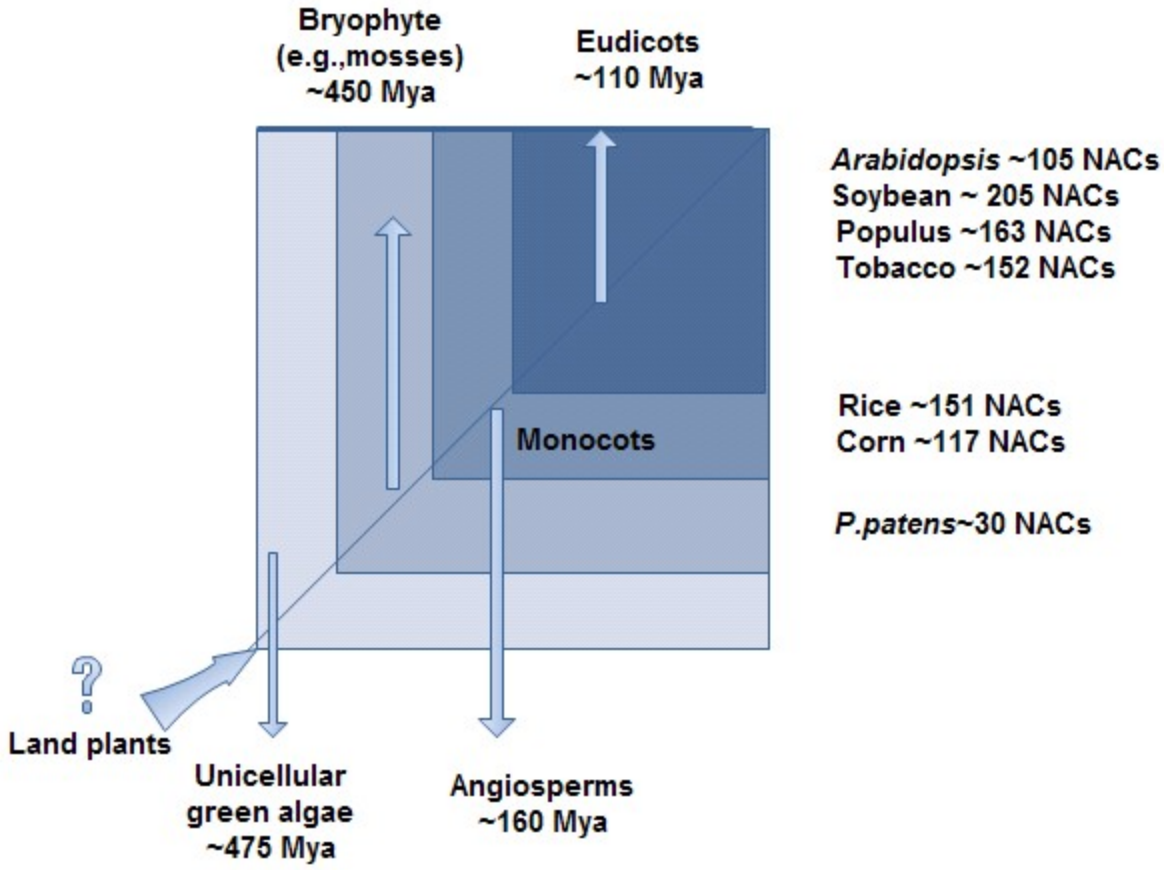


Figure1. 3. NAC gene evolution in plants.

The cladogram (not drawn to scale) represents the plant tree of life with major phylogenetic groups noted. The number of *NACs* is also indicated for each species.



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II Two NAC transcription factors from *Citrullus Colocynthis*, CcNAC1, CcNAC2, implicated in multiple stress responses

Abstract

NAC (NAM, ATAF1,2, CUC2) transcription factors play important roles in plant growth, development, and responses to abiotic and biotic stress. Two novel NAC transcription factors were isolated from *Citrullus colocynthis*, a highly drought tolerant cucurbit species: CcNAC1 and CcNAC2 each with conserved A-E NAC domains. Subcellular location of CcNAC1 and CcNAC2 investigated via transient expression of 35S::*CcNAC1*::*GFP* and 35S::*CcNAC2*::*GFP* fusion constructs in *Arabidopsis* protoplasts, revealed nuclear localization. The transactivation ability of CcNACs was examined in the GAL4 yeast assay system, and showed that only the C-terminal domain of CcNAC1 has the ability to activate reporter genes *LacZ* and *His3*. The CcNAC genes accumulated in a tissue specific manner with expression levels in male flowers of *C. colocynthis* higher than leaves, hypocotyls or roots. Genome walking was used to isolate the CcNAC1 and CcNAC2 promoter regions. A high number of stress-related sequence motifs were detected, especially in the CcNAC1 promoter. *C. colocynthis* seedlings were treated with PEG, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), H_2O_2, ethylene, gibberellic acid (GA), wounding or salt. High CcNAC1 expression levels were detected following JA application and wounding, while high CcNAC2 levels followed treatment with GA, JA, SA and wounding, indicative of differential regulation of these stress responsive transcription factors in this cucurbit species.

Keywords

Citrullus colocynthis, NAC transcription factor, CcNAC1, CcNAC2, *Arabidopsis*, stresses

Introduction

Plant responses to abiotic and biotic stresses involve changes at the transcriptome, cellular and physiological levels. The interaction between biotic and abiotic stress is orchestrated by hormone signaling pathways (Atkinson and Urwin 2012). Responses to stress require the production of important functional proteins, such as those involved in the synthesis of osmoprotectants, and regulatory proteins, kinases, and transcription factors (TFs), operating in the signal transduction pathways (Saibo et al. 2009). Several different techniques can be used to study the transcriptome during multiple stress responses. Affymetric ATH1 microarrays are commonly used to investigate universal components of the plant's response to different stress conditions (Mongkolsiriwatana et al. 2009, Swindell 2006). Quantitative trait analysis (QTL) can be used for the identification of useful regions of genomes (Ashraf 2010), and Next-Generation high-throughput sequencing (Quail et al. 2012) offers whole plant transcriptome surveys (Wang et al. 2010), but gene functional analyses are still needed to study plant development and gain an understanding of responses to biotic and abiotic stress conditions.

Transcription factors are DNA-binding proteins that activate or repress transcription of downstream genes by binding to a consensus sequence in their promoters. The NAC TF family is one of the largest TF families in plants, with more than 100 members identified in both *Arabidopsis* and rice (Wang and Dane 2013) and 80 in *Citrullus lanatus* (Guo et al. 2013). The acronym NAC originates from the NAM (no apical meristem), ATAF (*Arabidopsis* transcription activation factor 1 and 2) and CUC (cup-shaped cotyledon) genes. NAC proteins typically share a well conserved N-terminal NAC domain, which is divided into five conserved subdomains (A-E), and a diversified C-terminal transcription regulatory domain (Puranik et al. 2012). NAC transcription factors are specific to plants and associated

with many biological functions during embryonic, floral and vegetative development, and stress-related processes (Olsen et al. 2005, Atkinson and Urwin 2012). A number of NAC proteins interact with pathogens, the hormones ABA, JA and SA and exhibit interactions with both biotic and abiotic stress responses. Signaling crosstalk among phytohormones in NAC associated pathways regulate the protective responses in plants via synergistic or antagonistic actions (Tuteja and Sopory 2008). Different phytohormones, which can bind to the NAC recognition sequence, can regulate different NACs and further regulate stress-related genes. Many *NAC* genes are associated with stress, and some of the *NAC* genes have multiple functions. *OsNAC6* in rice, for example, is involved in both abiotic and biotic stresses (Nakashima et al. 2007). *CsNAC1* is induced by salt stress, cold and ABA (Oliveira et al. 2011). Overexpression of ONAC045 results in enhanced drought and salt tolerance (Zheng et al. 2009), while RD26 was induced by drought, ABA and high salinity (Fujita et al. 2004). NACs have long been associated with stress signaling, and recent discoveries suggest that they may make excellent targets for improving broad-spectrum tolerance in crops through genetic engineering (Nakashima et al. 2007, Xu et al. 2011). Although quite a few NACs have been functionally characterized primarily in model plants like *Arabidopsis*, the functions of the majority of the members of the large NAC gene family remain unknown (Hu et al. 2010).

Citrullus colocynthis (L.) Schrad, closely related to domesticated watermelon (*Citrullus lanatus* var. *lanatus*), is a non-hardy drought-tolerant perennial herbaceous species in the *Cucurbitaceae* family (Jeffrey 2008, Al-Zahrani and Al-Amer 2006). It can survive arid environments by maintaining its water content without wilting of the leaves or desiccation under severe stress conditions. *C. colocynthis* has a rich history as an important medicinal plant and as a source of valuable oil (Dane et al. 2006). Its seeds appear in several early Egyptian, Libyan and Near Eastern sites from about 4000 BC (Zohary and Hopf

2000). The species grows in sandy areas throughout northern Africa, southwestern Asia and the Mediterrranean region (Zamir et al. 1984, Burkill 1985, Jarret et al. 1997). Drought tolerance studies in *C. colocynthis* pointed to several drought-inducible genes, including a partial NAC transcript (GenBank accession number GH626169), with complex adaptive transcriptional regulation (Si et al. 2009, 2010a, 2010b). To further characterize the function of stress tolerant genes in *C. colocynthis*, two novel plant specific transcription factors, CcNAC1 (KC814686) and CcNAC2 (KC814687) and their promoter regions, were cloned using 5'RACE and the genome walker kit. Here, we report the characterization of the *CcNAC1* and *CcNAC2* genes to gain an understanding of their function under stress conditions. Manipulation of transcription factors represents a potential strategy for development of transgenic stress tolerant plants. Results indicate that CcNAC1 and CcNAC2 may have multiple functions to regulate the plant's defense responses to abiotic stress.

Materials and methods

Plant materials

Citrullus colocynthis seeds (No. 34256) from Israel with high tolerance to drought were sown in potting mix in the greenhouse with a 14 h photoperiod and temperatures ranging from 22℃ to 33℃ and ambient relative humidity and light conditions (600-720 μmol m^{-2} s^{-1}). *Arabidopsis* seeds were planted for leaf protoplast isolation. Wild-type *Arabidopsis* (*A. thaliana*, ecotype Columbia) was grown in growth chambers for 5-6 weeks, as previously described (Jensen et al. 2007).

NAC isolation and phylogenetic analysis

NAC domain proteins from more than ten different species were used as query sequences for Blastx searches of the GenBank database (http://www.ncbi.nlm.nih.gov/) for analysis of the conserved domains of NAC proteins. Primers were designed based on conserved domain sequences for cloning of the *CcNAC* genes into T-easy-vector (Promega, Madison, WI), followed by sequence analysis and re-blasting into the NCBI database. The 5'RACE cloning technique (Clontech Lab Inc, Mountain View, CA) was used to obtain full length *NAC* sequences from *C. colocynthis*. Vector sequences and low quality sequences were manually removed following sequencing. The non-redundant *C. colocynthis* sequences with the highest similarity to the query sequences were investigated as putative NAC domain genes. Evaluation of putative open reading frames (ORFs) of the identified sequences was based on (i) the sequences near the translation start site corresponding to the eukaryotic consensus sequence GCC(AG)CCATGG, (ii) the sequence length and homology to the *in vitro* translated product, (iii) the conserved eukaryotic polyadenylation signal 50-AATAAA-30 following the stop codon. The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to detect ORFs and predict amino acid sequences. Primers described in Table 1 were used for cloning of the full length *CcNAC1* and *CcNAC2* genes. Con-F and Con-R were used for obtaining the conserved regions of *CcNAC1* and *CcNAC2*; GSP1 and GSP2 for the 5' region of *CcNAC1* ; 2.1-GSP1 and 2.1-GSP2 for 5' region of CcNAC2; CcNAC1R/F and CcNAC2 F/R were used separately to clone the ORF of *CcNAC1* and ORF of *CcNAC2*.

Previously published plant NAC-like gene sequences were retrieved from the GenBank database: ATAF1 (X74555), AtNAC2 (AB049071), AtNAM (AF123311), CUC3 (AF54194), AtNAC3 (AB049070), ANAC (AY11722), ANAC019 (At1g52890), NAC1 (AF198054), CUC1 (AB049069),

CUC2 (AB002560), TIP (AF281062), NAP (At1g69490), BnNAC5-11 (AY245884), BnNAC14 (AY245886), OsNAC19 (AY596808), OsNAC5 (AB028184), OsNAC4 (AB028183), OsNAC6 (AB028185), GmNAC8 (EU661911). Multiple sequence alignment of NAC proteins was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was based on Neighbor-joining (NJ) using MEGA5 (Tamura et al. 2011) with 1000 bootstrap replications. Jones Taylor Thornton (JTT) model was used as substitution model, while Gamma distribution with invariant sites (G+I) as range substitution pattern. Amino acid sequences of NAC genes with high homology to CcNAC1 and CcNAC2 (AEF80001, XP_004161162, AFY26893, ACS94038, XP_004149802, XP_004172335, ACS94038) were aligned to investigate conserved NAC domains.

Green Fluorescent protein (GFP) conjugated plasmid construction

The cDNA was amplified with primers CcNAC1F/R and CcNAC2F/R (Table 2. 1), and the resulting PCR product was fused into the pCR8/GW/TOPO entry vector. After a sequencing check, the insert was transferred into pMDC43 via the LR reaction (Gateway^{R} Entry vector, Life Technologies). The resulting plasmids containing Pro35S: CcNAC-GFP insert were used in electroporation experiments to determine the subcellular localization of *CcNAC*.

Protoplasts of *Arabidopsis* were isolated and transformed essentially as previously described in Sheen et al. (1999) with minor modifications. The tissues for protoplasts isolation were *Arabidopsis* leaves. The leaves were collected before flowering, excised and cut into 1 mm strips and immediately placed into an enzyme solution for overnight digestion in the dark. The enzyme solution which contained 2% cellulose R10, 0.5% macerozyme R10, 0.5% driselase, 2.5% KCl, 0.2% $CaCl_2$, pH 5.7, was filter sterilized. After overnight incubation, leaf tissue was gently shaken for 30 min at 40 rpm to release protoplasts, followed by filtration through a 40 µm cell sifter to remove debris and centrifugation at 150g

to pellet the protoplasts. Protoplasts were washed twice with a washing solution (0.5 M mannitol, 4mM MES pH 5.7, and 20 mM KCl) and re-centrifuged at 150g. The protoplasts were suspended in washing solution on ice for electroporation.

Protoplasts were transformed in a manner essentially as previously described (Sheen et al. 1999, Rashotte et al. 2006). Electroporation was typically carried out with 1-2x10^5 protoplasts in 200 µl of wash solution and about 1-2 µl 400 ng/µl of plasmid DNA. Protoplasts were electroporated at 300 V in a 0.1 mm cuvette using an Eppendorf Electroporator 2510 (Hauppauge, NY). After overnight incubation in the dark, protoplasts were examined under Accu-scope 3025 phase fluorescence microscope (New York Microscope Company, Inc.). A GFP filter was used to block the chlorophyll autofluorescence and a UV filter was used to detect Hoechst 33342 fluorescence under UV light. All photographs were taken with a Qimaging Fast 1394 digital camera (Imaging).

Transcriptional activation activity

The yeast strain YPG-2 containing *His3* and *LacZ* reporter genes was used as an assay system (Stratagene, La Jolla, CA, USA). The coding sequences of *ccNAC1* and *ccNAC2* and the *ccNAC1* and *ccNAC2* N-terminal and C-terminal fragments were obtained by PCR using primers described in Table 2. 2. The PCR products were cloned into the vector containing the GAL4 DNA binding domain to obtain pBD-ccNAC1, pBD-ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C. According to the protocol of the manufacturer (Stratagene), pBD-ccNAC1, pBD-ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C and the positive control pGAL4 and the negative control

pBD vector were all transformed into the yeast YPG-2 competent cells. PCR products were inserted into the *Sal*I-*P*stI site of pDB vector containing CcNAC1/ CcNAC2-F, -N, -C, respectively.

The transformed strains were confirmed by PCR and streaked on YPAD or SD/His- plates. The transcription activation activities of each protein were evaluated according to their growth status. The underlined nucleotide bases in Table 2. 2 indicate restriction enzyme digestion sites.

Isolation of CcNAC1 and CcNAC2 promoters and in silico promoter analysis of CcNAC1 and CcNAC2 promoters

The promoters (1585 bp and 1299 bp) of CcNAC1 (KC814688) and CcNAC2 (KC814689) were obtained using the Genome walker universal kit (Clontech Cat NO.638904). Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), a database of plant cis-acting regulatory elements and a portal of tools for *in silico* analysis of promoter sequences (Lescot et al. 2002), was used to identify consensus motifs in the promoter sequences of *CcNAC1* and *CcNAC2*.

Abiotic treatments

Treatments were conducted on seedlings at the 5-6 leaf stage. Seedlings were placed in 20% PEG8000 solution (-0.5 MPa osmotic potential) or 50mM NaCl to induce drought or salt treatment or water as the control treatment. Hormone and hydrogen peroxide treatments were conducted on fourteen-day-old seedlings. Seedlings were treated with 100μM MeJA, 200μM ABA, 100μM SA, 100μm

ethephon (ET), 20mM H_2O_2, or 40µM GA. The leaves were also wounded using a hemostat (wounding). Leaves were harvested following each treatment at specific time points: 0h, 1h, 2h, 6h, 12h and 24h.

cDNA synthesis and relative quantitative (RQ) real-time RT-PCR

RNA was extracted from leaf material using the Trizol (Invitrogen Life Technologies, Grand Island, NY) method. To eliminate the remaining genomic DNA, RNA was treated with Dnase I (Ambion Life Technologies) according to the manufacturer's instruction. cDNA was synthesized using RETROscript™ (Ambion).

qRT-PCR was carried out using an Bio-Rad, iCycler Real Time PCR (Hercules, CA) system and iCycler detection system software. The *C. colocynthis*-specific actin gene (ccActin154F/R), used as the reference gene, was amplified in parallel with the target gene, allowing normalization of gene expression and providing quantification. Primers were designed based on specific regions. Primers sequences of the *CcNAC1* (Q-NAC1F/R), *CcNAC2* (Q-NAC2F/R) and Actin (ccActin154F/R) are listed in Table 2. 3. Detection of RQ real-time RT-PCR products was conducted using the SYBR ® Green PCR Master mix kit (Applied Biosystems, Life Technologies) following the manufacturer's recommendations. Quantification of the relative transcript levels was performed using the comparative C_T method. The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT= (C_{T,\text{ target gene},} - C_{T,\text{ actin}})_{\text{ treatment}} - (_{CT,\text{ Target}} - C_{T,\text{ actin}})_{\text{control}}$. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in the target sample versus the reference sample (Pfaffl 2001).

Results

Cloning and sequence analysis of the CcNAC1 and CcNAC2 genes

Earlier research on gene expression changes in response to drought in *C. colocynthis* had identified a partial NAC transcript (GH626169), which showed high expression levels in shoots following drought (PEG) treatment and in response to different hormones such as ABA, JA, and SA (Si et al. 2009, 2010a). Following primer design based on conserved regions of NAC genes, two NAC genes from *C. colocynthis* were sequenced and cloned and designated as *CcNAC1* and *CcNAC2*. *CcNAC1* encodes a 900 bp (300 amino acids) long sequence. *CcNAC2* encodes an 888 bp (296 amino acids) long sequence. Amino acid alignment of CcNAC1, CcNAC2 and other NAC proteins was used to construct a phylogenetic tree (Fig. 2. 1A). Phylogenetic analysis indicated that NAC proteins can be classified into several subgroups based on similarities to published NACs (Ooka et al. 2003). Major subgroups are shown in Fig. 2. 1. Both CcNAC1 and CcNAC2 align with proteins in the ATAF subgroup composed of ATAF1, BnNAC5-11, GmNAC8, OsNAC19, OsNAC5, OsNAC4, OsNAC6 and BnNAC14. Amino acid alignment analysis (Fig. 2. 1B) indicated that CcNAC1 and CcNAC2 show high homology to NACs with conserved A-E domains (five N-terminal subdomains). Even though the *CcNACs* are variable, especially at the C-terminal region, several conserved amino acid domains were detected.

CcNAC1 and CcNAC2 are localized to the nucleus

To identify the subcellular localization of the CcNACs, the following constructs were made: 35S::*CcNAC1*-GFP and 35S::*CcNAC2*-GFP. The constructs were used to transform *Arabidopsis* leaf protoplasts. Analysis of more than 20 protoplasts showed nuclear localization of the fusion protein of

CcNAC1 and *CcNAC2* with GFP, as illustrated in Fig. 2. 2, whereas the GFP protein was distributed ubiquitously in protoplasts transformed with vector plasmid control, PMDC43 (Fig. 2. 2A2). These results indicated that both *CcNAC1* and *CcNAC2* are nuclear proteins.

CcNAC1 –C terminal has transcription activation function

CcNAC1, CcNAC2, their N-terminal domain and C-terminal domain, and full length coding sequence were fused to the GAL4 DNA binding domain to investigate their transcription activation activity. The yeast strain YRG-2 was transformed with the fusion plasmids pBD-ccNAC1, pBD- ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C, the positive control pGAL4 and the negative control pBD. As shown in Fig. 2. 3, all transformed cells can grow well on YPAD medium, but only pBD-ccNAC1-C can grow on SD medium without histidine. The filter lift assay showed that the yeast cells that grew on the SD medium without histidine turned blue in the presence of 5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) due to the activation of another reporter gene *LacZ*. The results indicate that only the C terminal region of CcNAC1 has transcription activation activity, while the full length and N-terminal region of CcNAC1 do not have this activity. This phenomenon was also observed in GmNAC20 where the C-terminal has transcriptional activation ability (Hao et al. 2010). *CcNAC2* did not show transcriptional activation activity using yeast assay, since blue color was not observed using the X-Gal assay (data not shown).

CcNAC1 and CcNAC2 expression patterns

Expression analysis of CcNAC1 and CcNAC2 was conducted using semi-quantitative real-time PCR. Actin, used as an internal control for constitutive expression, was uniformly expressed in all organs. As shown in Fig. 2. 4, *CcNAC1* and *CcNAC2* are expressed in every tissue of *C. colocynthis.* *CcNAC1* and *CcNAC2* showed the highest level of expression in male flowers, and low expression levels were detected in fruits. Comparisons between the two genes also indicated that *CcNAC2* is highly expressed in the hypocotyl of *C. colocynthis*.

In silico identification of stress-related promoter motifs

CcNAC1 and CcNAC2 promoters were isolated using the genome walking method. A 1585 bp region upstream of the *CcNAC1* gene and a 1298 bp region upstream of the *CcNAC2* gene were cloned, which should contain most of the regulatory domains. For further analysis of stress-related motifs in the two promoters, PlantCARE was used. Table 2. 4 shows the details of stress-related motifs detected in the two promoters, with in the attachment the promoter sequences and motifs in color (Fig. 2. 5). The *CcNAC1* promoter contains the ABA response element (ABRE) (Yamaguchi-Shinozaki et al. 1989; Mundy et al. 1990; Michel et al. 1993; Giraudat et al. 1994; Barker et al. 1994), ARE motif (Manjunath and Sachs, 2005), CE-3 (coupling element 3) (Hubo et al. 1999), CGTCA motif, TC-rich motif, TGA-box and TGACG-motif, which are correlated with ABA response, anaerobic induction, JA response and auxin response. The *CcNAC2* promoter contains an ABRE motif, ARE motif, HSE (heat stress responsiveness), MBS (MYB binding site), Box-W1 and TC-rich repeats, which are correlated with ABA response, drought stress, anaerobic stress, JA response and auxin response. Promoter motifs provide evidence for the involvement of *CcNAC1* and *CcNAC2* in biotic and abiotic stresses. The two promoters contain several identical motifs, such as ABRE, ARE, TC-rich repeats, which indicate that both promoters might have similar functions. However, some key differences in the composition or distribution of putative

stress-related *cis*-acting elements (Fig. 2.5) were observed. Five ABRE motifs are in the *CcNAC1* promoter, but only two ABRE in the *CcNAC2* promoter. *CcNAC2* promoter contains three TC-rich repeats, while *CcNAC1* promoter contains only one TC-rich repeats. Both contain some special motifs, for example, *CcNAC1* contains CE3, CGTCA-motif and TGA-motif, whereas the *CcNAC2* promoter contains Box-W1, HSE and MBS motifs. These special characteristics indicate that although both promoters have similar regulatory domains, they might be regulated by different factors. It can also be deduced that the CcNAC1 and CcNAC2 transcription factors might have differential regulation.

CcNAC gene expression during stress and plant hormone treatments

NAC transcription factors are known to play important roles in plant growth and development, and can be induced by multiple biotic and abiotic stresses (Wang and Dane 2013). The complex regulatory and interaction network occurring between hormone-signaling pathways allows the plant to activate responses to different types of stimuli (Bari and Jones 2009). *CcNAC1* and *CcNAC2* gene expression was studied under different stress (drought, wounding, and salt) and phytohormone (ABA, JA, SA, GA, ET) treatments as shown in Fig. 2. 6. Results indicated that both genes, *CcNAC1* and *CcNAC2,* show similar expression patterns under hormonal and stress treatments. Both genes are induced by stress factors and hormones, even though differences in the timing of induction and level of expression were observed. Hormones can thus regulate CcNACs expression levels. For example, treatment with GA resulted in a 10 fold up-regulation of *CcNAC1* at 2h, while much higher levels of *CcNAC2,* 40 fold increases, were observed at a later time point, 24h. Similarly treatment with JA resulted in 30 fold up-regulation of *CcNAC1* at 24h, while the highest levels of *CcNAC2* (40 fold) were detected following JA

treatment at 12h. Treatment with ET resulted in higher expression levels of *CcNAC2* than *CcNAC1*, although both showed the highest level at 6h. The highest levels of *CcNAC1* were detected 10h following ABA treatment, while *CcNAC2* showed the highest levels already at 2h (40 fold). Treatment with SA resulted in 6-8 fold up-regulation of *CcNAC1* from 6h to 24h, while *CcNAC2* was expressed from 1h-12h, and reached very high levels (40 x).

Abiotic stresses do regulate *CcNAC*s expression. For example, H_2O_2 treatment did result in high expression of *CcNAC1,* more than 15 fold at 24h, while it did result in up-regulation of *CcNAC2* more than 40 fold at 40h. PEG treatment did not induce changes in expression of *CcNAC1*, but did cause high *CcNAC2* expression, 15-25 fold. The effect of salt treatment did effect the expression of *CcNAC2* more than *CcNAC1*; both showed similar patterns. Wounding resulted in high (40x) up-regulation of *CcNAC1* and *CcNAC2* after 2 h, and 24h and similar expression patterns.

Discussion

In this study, two members of the NAC gene family in *Citrullus colocynthis* were identified and NAC gene expression in different tissues and under different treatments was examined. This is the first report of molecular characterization of NAC genes in *C. colocynthis* The Cucurbitaceae is a large and diverse family containing several domesticated species such as watermelon, melon and cucumber (*Cucumis* species), squashes, pumpkins and gourds (*Cucurbita* species). *C. colocynthis* is a source of genetic improvement for drought resistance, since this species is widely distributed in the Sahara-Arabian desert areas and well adapted to drought stress (Dane et al. 2006). Tolerance to drought stress is a complex phenomenon, comprising a number of physio-biochemical processes at both the cellular and whole plant level which are activated during different stages of plant development. Molecular mechanisms involved

in different stresses have been revealed in other plant species, and transcription factors are one of the promising players in stress signaling pathways (Fujita et al. 2006).

The plant specific NAC proteins constitute a major transcription factor family implicated in many developmental processes (Puranik et al. 2012). Like most NACs, CcNAC1 and CcNAC2 contain conserved NAC domains. NAC domains are N-terminal regions of NACs that can bind both DNA and other proteins (Ernst et al. 2004). N-terminal amino acid substitutions can abolish NAC DNA binding or structural integrity (Olsen et al. 2005). CcNAC1 and CcNAC2, like most other NACs, contain conserved NAC N-terminal and variable C-terminal domains. Protoplast transformation experiments indicated that both CcNAC1 and CcNAC2 are localized in the nucleus, which is where most transcription factors function. Research has indicated that C-terminal regions of many NACs possess trans-activation activity (He et al. 2005, Peng et al. 2009). Yeast assay experiments showed that CcNAC1 has trans-activation activity, while CcNAC2 does not have that ability. Some NACs have been reported to function as transcriptional repressors. Hao et al. (2010) reported that NARD (NAC Repression domain) contributed to the transcriptional repression function of GmNAC20, with the LVFY motif essentially required for suppression.

Examination of *CcNAC1* and *CcNAC2* expression in different plant tissues pointed to similar expression patterns, although both genes were expressed mainly in male flowers and the hypocotyl. Tissue specific expression of members of the NAC gene family has also been studied in other species. For example, ATAF2, which is a pathogenesis-related gene in *Arabidopsis,* showed expression mainly in roots, leaves and mature flowers (Delessert et al. 2005). ANAC036 which caused a dwarf phenotype in *Arabidopsis thaliana,* was expressed mainly in rosette leaves (Kato et al. 2010). AtNAC2 was expressed mainly in root tissues and involved in salt stress responses and lateral root development (He et al. 2005).

ATAF1 in *Arabidopsis* showed expression in every tissue, but mainly in stems, flowers and seedlings. Its overexpression resulted in severe developmental defects in *Arabidopsis* (Kleinow et al. 2009).

Phytohormone and stress treatments induced CcNAC1 and CcNAC2 expression to different levels. Since the promoters of both genes contain the anaerobic related motif ARE, this indicates that both genes might be correlated with oxidative stress. Salt and drought stress are worldwide problems, effecting global crop production and quality. Both genes were regulated by salt and drought stress, and a drought related motif was also detected in the *CcNAC2* promoter. Elevated levels of ABA, JA and anthocyanin are metabolic signatures of oxidative stress (Steppuhn et al. 2010). While the impact of the different treatments was similar on both genes, differences in gene expression were detected. NAC TFs are candidate molecules that potentially regulate aspects of both biotic and abiotic signaling (Fujita et al. 2006). In signaling pathways, different hormones play different parts and crosstalk with each other. Earlier experiments had indicated that many NACs play a role in phytohormone pathways (Kim et al. 2008). NTL8, a membrane-bound NAC transcription factor, plays a role in GA-mediated salt signaling in *Arabidopsis* (Kim et al. 2008). Studies of gene expression in wild-type and mutant *Arabidopsis* genotypes in response to pathogens revealed interactions among SA, JA and ethylene (Maleck et al. 2000, Tao et al. 2003, Salzman et al. 2005). It has been suggested that ethylene produced during wounding can activate JA biosynthesis, and ethylene can also interact with the JA pathway to induce a number of pathogenesis-related and defense genes (Laudert and Weiler 1998, Kunkel and Brooks 2002). JA operates in a distinct defense pathway, which interacts with the SA pathway. JA is known to effectively mediate the defense of necrotrophic pathogens, while SA is effective against biotrophic fungi, bacteria and viruses (Murphy and Carr 2002). SA is thought to be antagonistic to JA, indicating that SA can block the JA induction pathway (Doares et al. 1995). Ethylene and JA are also associated with pathogen-induced wounding (Kunkel and

Brooks 2002). JA is one of the main components of the wound repair signal in plant tissues, and the formation of JA is activated by ABA, ethylene, hydrogen peroxide, UV, whereas SA and nitric oxide inhibit the synthesis of JA (Vasyukova et al. 2011). Both JA and wounding did induce CcNAC1 and CcNAC2 expression, which indicates that both genes might play a role in the signaling of pathogen resistance and wounding response. Similar to AtNAC2, CcNAC1 and CcNAC2 were also up-regulated by ethylene. SA is a major component of the systemic acquired resistance (SAR) response which refers to induced resistance to pathogens (Bostock 2005). *ANAC055, ANAC092* and *GmNAC6* genes were identified as SA signaling components (Delessert et al. 2005, Faria et al. 2011). SA similarly induced changes in *CcNAC1* and *CcNAC2* expression, especially to a large degree in *CcNAC2* expression. The fungal elicitor motif Box-W1 was detected in the *CcNAC2* promoter, which is further evidence that *CcNAC2* has a function in biotic stress responses. JAZ (JASMONATE-ZIM DOMAIN) family proteins are JA co-receptors and transcriptional repressors in JA signaling in *Arabidopsis*. Research has indicated that JAZ orchestrates the crosstalk between JA and other hormone signaling pathways such as ethylene, gibberellic acid, SA and auxin (Kazan and Manners 2012). Both CcNAC1 and CcNAC2 were regulated by GA as well. It is known that some NACs act as regulators in several phytohormone pathways. AtNAC2 is a transcription factor downstream of the ethylene and auxin signaling pathway (He et al. 2005).

In conclusion, two NAC transcription factors CcNAC1 and CcNAC2 were identified in *C. colocynthis*. Different stresses and phytohormones did induce *CcNAC1* and *CcNAC2* gene expression, which may provide clues for a better understanding of NAC gene family in this drought tolerant cucurbit species. The identification of novel transcription factors regulating abiotic stress tolerance will enable further enhancement of stress tolerance in cultivated cucurbit species.

Primer	Tm	Length	Aim	Sequence (5'-3')
Con-F	60	27	Conserved region	TTCCATCCAACGGATGAGGAGCTCCGT
Con-R	60	27	Conserved region	TCAAAACGGCTTCTGCAGGTGCATAAA
GSP1	64	29	5' region	TGGCTTCTCCTCCTCCTCCTCATTTTCAA
GSP2	65	28	3' region	AACGCCTTTGATTTTGCAGCTGGACGAT
ccNAC1 F	58	20	NAC1 CDS	ATGGCCGCCGATTTGCAGTT
ccNAC1 R	56	30	NAC1 CDS	TCAAAAAGGCTTGTGAATATACATGAACAT
2.1-GSP2	64	24	5'region	TTTTACAGAACTGGCCTTGCACGGAGAGA
2.1-GSP1	63	23	3'region	TTCGGCTTCTCGTCTTCTTCCTCTTCGTAG
ccNAC2F	56	24	NAC2 CDS	ATGACCACCGAGTTGACTCAGCTG
ccNAC2R	58	21	NAC2 CDS	TCAGAACGGCTTCGGCAGGTG

Table 2. 1. Primer sequences for CcNAC1 cDNA and CcNAC2 cDNA cloning.

Table 2. 2. Primers used for transcription activation assay of CcNAC1 and CcNAC2

Primer	Sequence (5'-3')	Product length	Tm
ccNAC1-N-F	CCGGAATCCATGGCCGCCGATTTGCAG	478bp	55C
ccNAC1-N-R	ACGCGTCGACCGCCCTTCTTGTTGTATATACGG	478bp	
ccNAC1-C-F	CCGGAATCC GTAATCGAGAAACAGCAACAGC	421bp	50C
ccNAC1-C-R	ACGCGTCGACATGACCACCGAGTTGACTCAGCTG	421bp	
ccNAC2-N-F	ACGCGTCGACATGACCACCGAGTTGACTCAGCTG	481bp	56C
ccNAC2-N-R	AAAACTGCAG CGCCTTTCTTGTTGTAAATCCGG	481bp	
ccNAC2-C-F	ACGCGTCGACGTAATCGAGAAGCGAAATCAGATAGC	408bp	55.8C
ccNAC2-C-R	AAAACTGCAG GAACGGCTTCTGCAGGTGCAT	408bp	

Table 2. 3. **Q-RT PCR primers for detection of relative expression levels of *CcNAC1, 2***

Primers	Sequence(5'-3')	Tm	Product length
ccActin 154F	CACCATCACCAGAATCCAGCACGA	59℃	140bp
ccActin 154R	GGCTCCACTCAACCCAAAGGCTAAC	59℃	140bp
Q-NAC1F	GTCAACCGAGAATGAAAGAAGAGTA	59℃	132bp
Q-NAC1R	TATACATGAACATATCCTGCAATGG	59℃	132bp
Q-NAC2F	GTGCCGGATTTACAACAAGAA	59℃	106bp
Q-NAC2R	AATCTTCGGCTTCTCGCTTC	59℃	106bp

Table 2. 4. Details of stress-related elements in CcNAC1 promoter and CcNAC2 promoter

Name of cis element	Sequence	Number of cis elements	Function	Reference
CcNAC1 Promoter				
ABRE	CCTACGTGGC/ CGCACGTGTC/ GACACGTGGC/ CACGTG/	5	ABA responsiveness	Barker et al. 1994
ARE	TGGTTT	4	Anaerobic induction	Walker et al. 1987
CE-3	GACGCGTGTC	1	ABA and VP1 (seed-specific transcription factor) responsiveness	Hubo et al. 1999
CGTCA motifs	CGTCA	3	MeJA-responsiveness	Wang, et al. 2011
TC-rich repeats	ATTTTCTCCA	1	defense and stress responsiveness	Diaz-De-Leon et al. 1993
TGA-box	TGACGTAA	1	auxin-responsive element	
TGACG-motif	TGACG	3	MeJA-responsiveness	Rouster et al. 1997
CcNAC2 Promoter				
ABRE	CACGTG/ACGTGGC	2	ABA responsiveness	Barker et al. 1994
ARE	TGGTTT	3	Anaerobic induction	Walker et al. 1987
Box-W1	TTGACC	1	fungal elicitor responsive	Shi et al. 2011

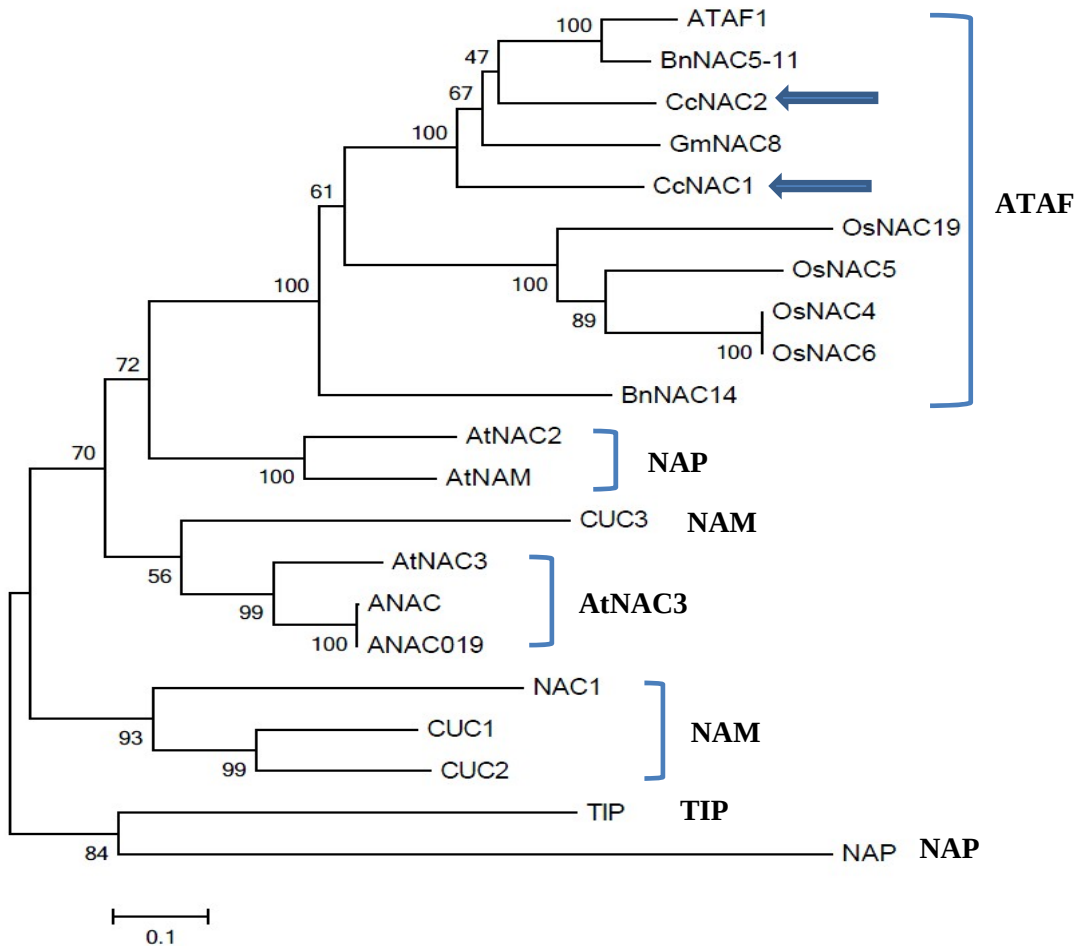
			element	
HSE	AAAAAATTTC	1	heat stress responsiveness	Schramm et al. 2006
MBS	CAACTG	1	MYB binding site involved in drought-inducibility	Mongkolsiriwatana et al. 2009
TC-rich repeats	GTTTTCTTAC/ATTTTCTTCA	3	defense and stress responsiveness	Diaz-De-Leon et al. 1993

Figure2. 1. Phylogenetic tree of CcNAC1, 2and analyses of their amino acid sequences.

(A) Phylogenetic tree of CcNAC1, CcNAC2 with other NACs proteins in plants. Numbers at the nodes of the trees represent the bootstrap vales for the node (100 replicates). CcNAC1and CcNAC2 proteins are indicated by arrows.

(B) Analyses of amino acid sequences of *Cc NAC1* and *CcNAC2*. The five sub-domains (A-E) are underlined by dashes (-). A-E domains are conservative domains of NAC transcription factors.

A



B

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CcNAC1          MAADL-QLPPGFRFHPTDDELVTHYLCRKCASQPISVPIIAEIDLYKYNP 49
 cNAC2          MTTELTQLPPGFRFHPTDEELVMHYLCRRCASQPIAVPIIAEIDLYKFDP 50
 P_004161162    MASDL-QLPPGFRFHPTDDELVTHYLCRKCASQPISVPIIAEIDLYKYNP 49
AEF80001        MTAEL-QLPPGFRFHPTDDELVMHYLCRKCASQSIAVPIIAEIDLYKYDP 49
AFY26893        MTAEL-PLPPGFRFHPTDEELVMHYLCRKCASQPIAVPIIAEIDLYKYDP 49
ACS94038        MASEL-QLPPGFRFHPTDEELVMHYLCRKCTSQPISVPIIAEIDLYKYDP 49
XP_004149802    MTTDLTQLPPGFRFHPTDEELVMHYLCRRCASQPIAVPIIAEIDLYKFDP 50
XP_004172335    MTTDLTQLPPGFRFHPTDEELVIHYLCRRCASQPIAVPIIAEIDLYKFDP 50
                *:::*  ***********:*** *****:*:**.*:***********::*
                                    A                B
CcNAC1          WDLPEKALYGEKEWHFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGR 99
CcNAC2          WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
XP_004161162    WDLPERALYGEKEWYFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGR 99
AEF80001        WELPRMALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGH 99
AFY26893        WELPGMALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGH 99
ACS94038        WDLPGMASYGEKEWYFFSPRDRKYPNGSRPNRAAGSGYWKATGADKPIGH 99
XP_004149802    WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
XP_004172335    WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
                *:**  * :*****:*****:***********:**:**********:
                                          C
CcNAC1          PKPVGIKKALVFYSGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
CcNAC2          PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
XP_004161162    PKAVGIKKALXFYSGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
AEF80001        PKAMGIKKALVFYAGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
AFY26893        PKAVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
ACS94038        PKPVGIKKALVFYAGKAPKGDKTNWIMHEYRLADVDRSIRRKNSLRLDDW 149
XP_004149802    PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
XP_004172335    PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
                **.:****** **:****:*:****************** *.**.*:****
                                    D
CcNAC1          VLCRIYNKKGVIEKQQQLQNVSINRPEMNTIGFVENEEEEEKPEILNERA 199
CcNAC2          VLCRIYNKKGVIEKRNQIA---VNR-----NTNVYEEEEDEKPKIFTSRG 192
XP_004161162    VLCRIYNKKGAIEKQNPP---EMNT-----IGFFENEEQEEKPEILNDRA 191
AEF80001        VLCRIYNKKGTTEKQQQ-G---INL-----QKMSDSEMEDKKPENLTIGG 190
AFY26893        VLCRIYNKKGTIEKQQQQI---INR------RMNSFETEEKKPEILTNGG 190
ACS94038        VLCRIYNKKGTIEKQPNSG--VINR------KTDPSEIEDKKPEILTRGS 191
XP_004149802    VLCRIYNKKGVIEKRYQMA---MNR-----NTKGLEEVEEEKPKIFISGG 192
XP_004172335    VLCRIYNKKGVIEKRYQMA---MNR-----NTKGLEEVEEEKPKIFISGG 192
                **********. **:       :*           * :::**: :   .
                  E
CcNAC1          VSGR--IPSPS----PSQ--APPVNDYMYFDPSDSIPRLHADSSCSEHVV 241
CcNAC2          GADTL--IPAV----AAA--PASVDDYIHYDTSDSIPRLHTDSSCSEHVV 234
XP_004161162    ISGR--IPSASPLQGPPS--SGVVNDYVYFDPSDSIPRLHADSSCSEHVV 237
AEF80001        ATS-AILSGPA----ATR--TAAGDDYVYFDTSDSVPRLHTDSSCSEHVV 233
AFY26893        TTPAPLLPAPP----PSAVPKAAVNDYTYFDTSDSVPRLHTDSSCSEHVV 236
ACS94038        G--L--PPHPP----PQA--TAGMRDYMYFDTSDSIPKLHTDSSCSEHVV 231
XP_004149802    GDGGG--DVLM----PSA--TASVGDFVHFETSDSIPRLHTDSSGSEQVV 234
XP_004172335    GGGGG--DVLM----PSA--TASVGDFVHFETSDSIPRLHTDSSGSEQVV 234
                           .        *: :::.***:*:**:*** **:**
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CcNAC1          SSEFT-SEVQSQPRMKEEY--SGFPYNYMDT-SLENAFCAQFPTLNQMSP 287
CcNAC2          SPEFTTGEVQSEPKWKELENAFNFQYNYIDNNSLDHEFATQFQSGNQMSP 284
XP_004161162    SSEFT-SEVQSEPRLKEEYCGLGFQYNYTDS-SLESAFCAQFPSLHQMSP 285
AEF80001        SPEFT-CEVQSEPKCW--EKTIDYHFNYMDA-TLDNGFGAQFQSSNQMSP 279
AFY26893        SPEFT-CEVQSEPKYW--EKPMDFSFNYMDA-TFDNGFGSQFQSNNQLSP 282
ACS94038        SPEFA-SEVQSEPKWNEWEKHLEFPYNYVDT-TLNSGFGSQFQSNNQMSP 279
XP_004149802    SPEFTTGEVESERKWKEMENAFSYRYNYIDDSLLDHEFATQFQNGNQMSP 284
XP_004172335    SPEFTTGEVESERKWKEMENAFSYRYNYIDDSLLDHEFATQFQNGNQMSP 284
                *.**:  **:*: :         : :** *   ::  * :** . :*:**

CcNAC1          LQDMFMYIHKPF 299
CcNAC2          LQDMFMHLQKPF 296
XP_004161162    LQDMFMY--KPF 295
AEF80001        LQDMFMYMQKPF 291
AFY26893        LQDMFMYLQKPF 294
ACS94038        LQDMFMYLPKTF 291
XP_004149802    LQDMFMYLQKPF 296
XP_004172335    LQDMFMYLQKPF 296
                ******:  *.*
```

Figure2. 2. The subcellular localization of the CcNAC1, 2 in *Arabidopsis* protoplasts.

A representative example of 35S:CcNAC1:GFP, and 35S: CcNAC2:GFP fusion in a leaf mesophyll protoplast of *Arabidopsis* are B1-B3 and C1-C3 . The control (A1, A2, A3) protoplast was transformed with vector pMDC43. Protoplasts were visualized under white light (A1, B1, C1), GFP excitation (A2, B2, C2 with filter blocking UV and other wavelengths) and UV light (A3, B3, C3).

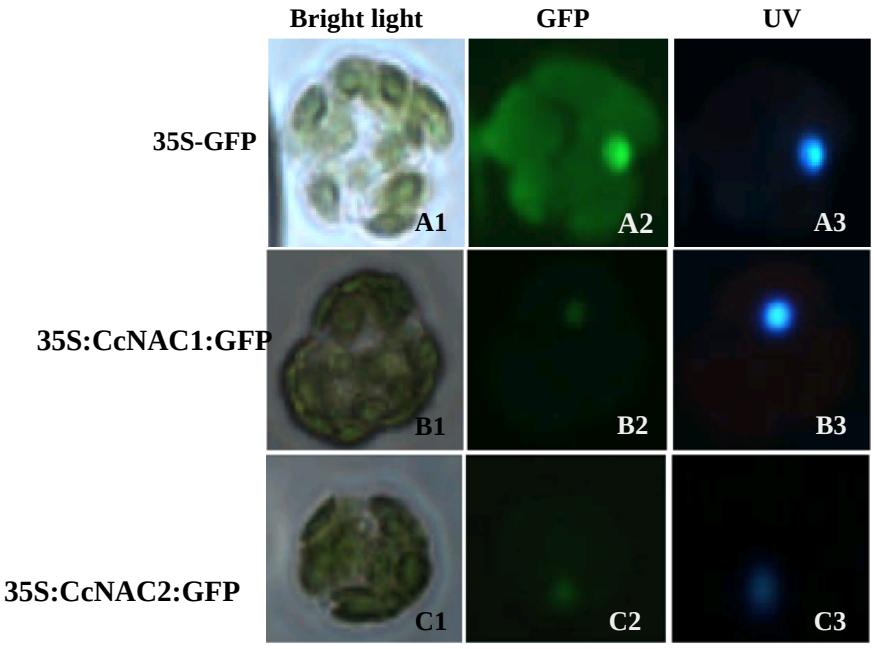


Figure2. 3.Transactivation activity of CcNAC1.

(A) Diagram of arrangement of transformants.

(B) pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F and pBD transformants were streaked on YPAD medium.

(C) pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F and pBD transformants were streaked on SD-His$^-$ medium.

(D) Filter lift assay results of pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F and pBD transformants.

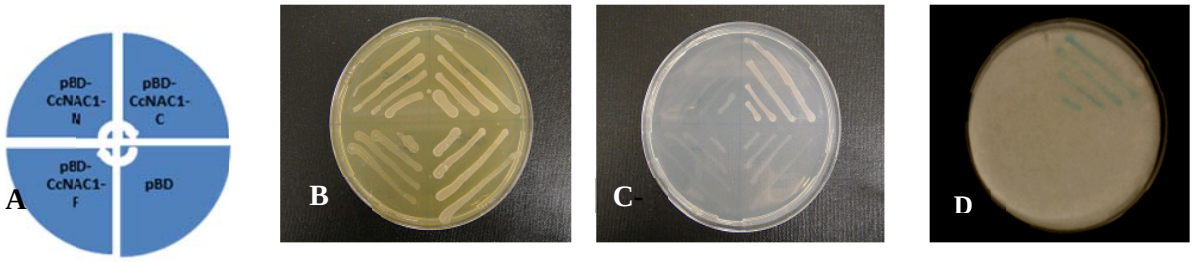


Figure2. 4. *CcNAC1, 2* relative expression in different plant tissues.

Results are relative to expression in fruits. Arrow bars show SE (n=3).CcNAC1 expression was shown as solid black, and CcNAC2 was shown as gray dashed lines.

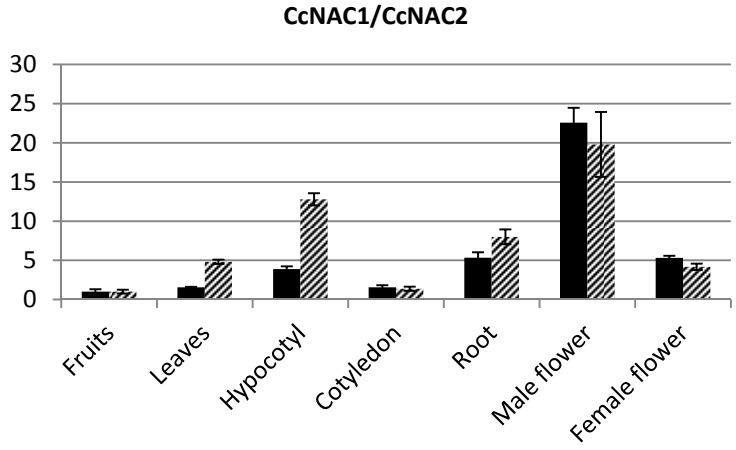


Figure2. 5. Location of stress-related *cis*-regulatory elements in *CcNAC1 , 2* promoters.

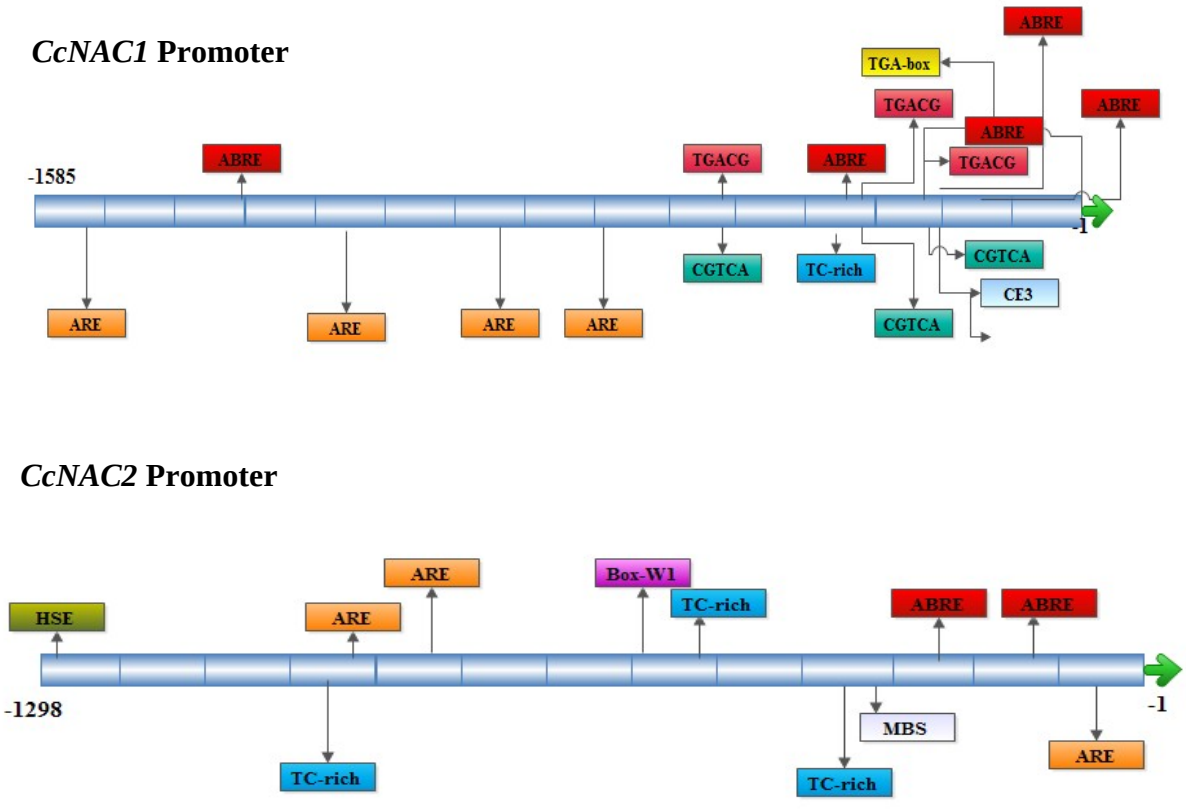
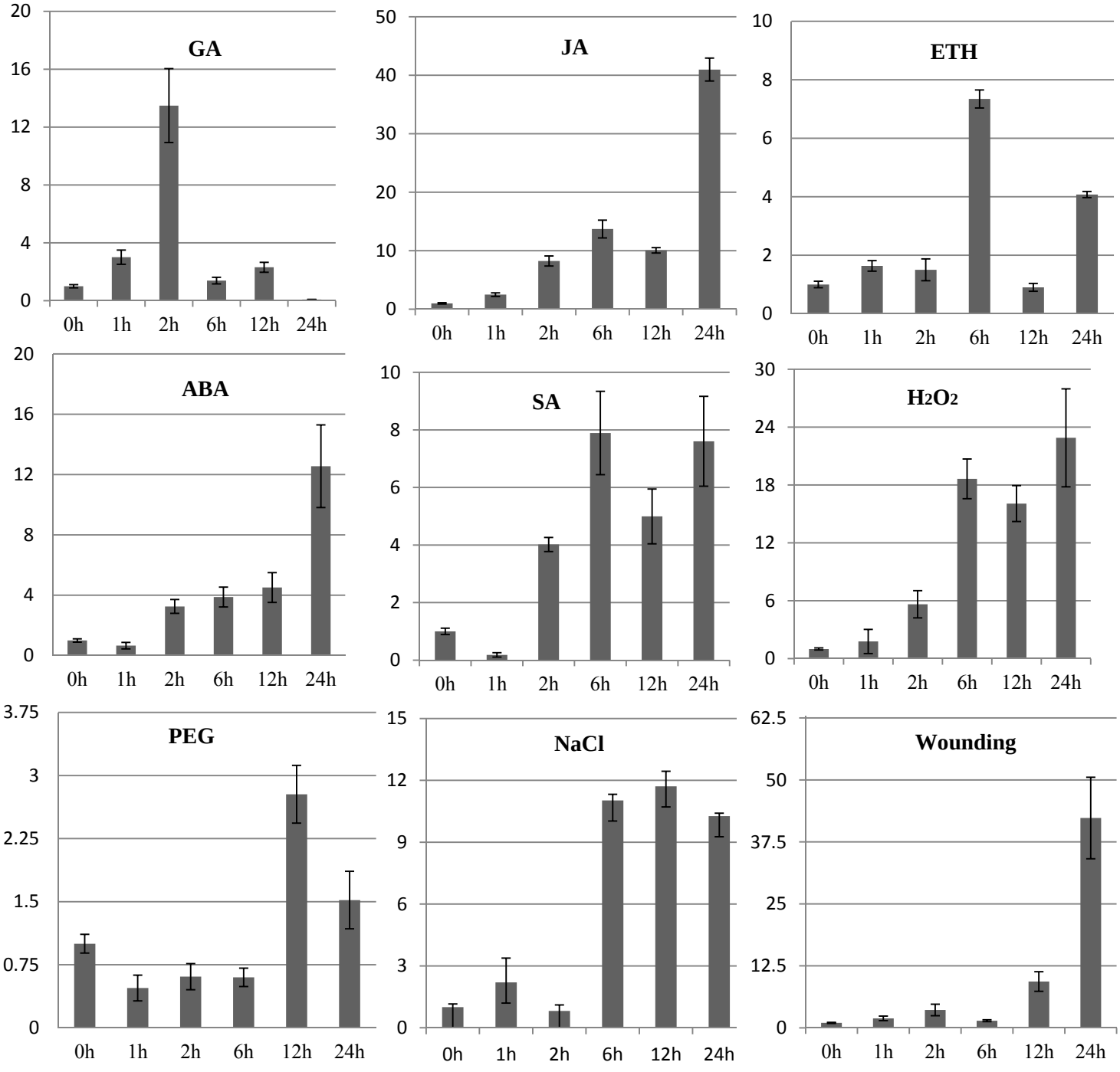
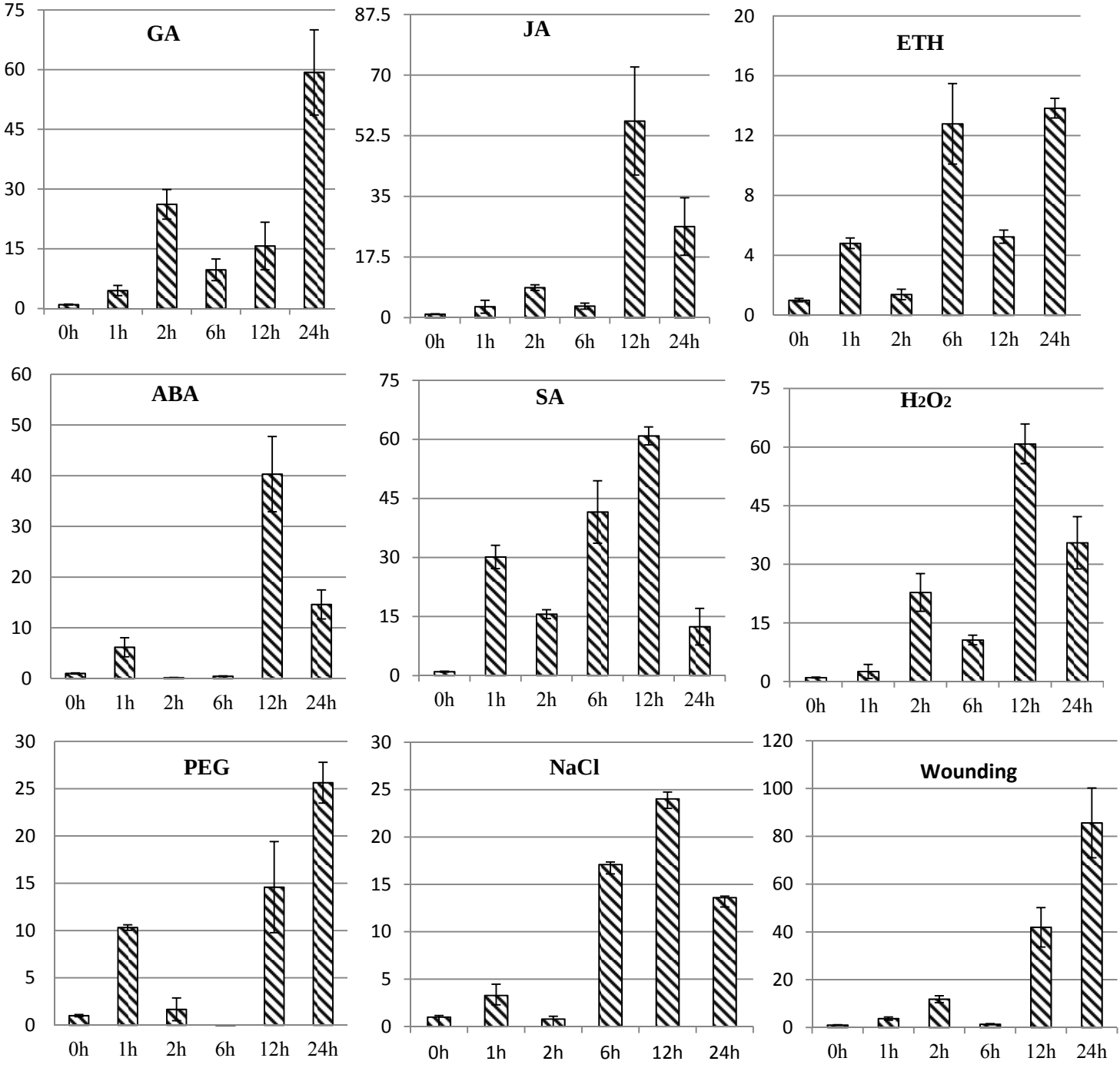


Figure2. 6. *CcNAC1* and *CcNAC2* expression profiles under different treatments.

The gray figures are expression patterns of *CcNAC1*, while the figures with slanted lines show expression patterns of *CcNAC2*. Gene expression was normalized by comparing ΔΔCT to control (0h) (n=3). Y-axis shows the expression level of *CcNAC1* and *CcNAC2*.




GA
0
15
30
45
60
75
0h 1h 2h 6h 12h 24h
JA
0
17.5
35
52.5
70
87.5
0h 1h 2h 6h 12h 24h
ETH
0
4
8
12
16
20
0h 1h 2h 6h 12h 24h
ABA
0
10
20
30
40
50
60
0h 1h 2h 6h 12h 24h
SA
0
15
30
45
60
75
0h 1h 2h 6h 12h 24h
H2O2
0
15
30
45
60
75
0h 1h 2h 6h 12h 24h
PEG
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5
10
15
20
25
30
0h 1h 2h 6h 12h 24h
NaCl
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20
25
30
0h 1h 2h 6h 12h 24h
Wounding
0
20
40
60
80
100
120
0h 1h 2h 6h 12h 24h

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III Expression and function analysis of *CcNAC1* and *CcNAC2* genes in *Citrullus Colocynthis* using promoter region analysis and grafting onto *Citrullus Lanatus* (watermelon)

Abstract

NAC transcription factors are part of the largest family of transcription factors (TF) found in a wide range of land plants with important roles in plant growth and development. The expression patterns of two NAC TFs from *Citrullus colocynthis*, *CcNAC1* and *CcNAC2,* were analyzed on different rootstocks and under different environmental conditions. Results indicated that both genes were up-regulated by drought treatment on *C. lanatus* and *C. colocynthis* rootstocks. To investigate the transcriptional regulation governed by the CcNAC gene promoters, transgenic *Arabidopsis* reporter (*pCcNAC1, 2::GUS*) lines were generated. GUS expression analysis under the control of the CcNAC gene promoters revealed that both *CcNAC1* and *CcNAC2* promoters were activated in response to dehydration stress. The *pCcNAC1, 2 ::GUS* reporter lines showed similar expression patterns at different developmental stages, in different tissues and throughout floral development. Transgenic *Arabidopsis* reporter lines under the control of different fragments of each promoter were generated. GUS assays suggested that different regions have differential responses under different regulation. From these data, we propose that the *CcNAC1* and *CcNAC2* TFs play important roles in plant drought tolerance and throughout plant development.

Keywords

NAC. *C. colocynthis*. *Arabidopsis*. Grafting. Promoter

Introduction

To cope with abiotic stress, plants have evolved adaptive molecular mechanims that result in the expression of a large number of genes that alter plant physiology depending on the specific type of environmental stress. One of the pivotal events in these responses is the perception of stress, which activates the expression of stress-response genes via signal transduction (Shinozaki and Yamaguchi-Shinozaki, 2006). The core signaling components involved in the regulation of stress-responsive genes during abiotic stress include kinases, phosphatases and transcription factors. Transcription factors play major roles in regulation and responses of plants under drought stress (Narsai et al. 2013).

NAC (NAM/ATAF/CUC) transcription factors (NAC-TFs) form a unique class of transcription factors found in a wide range of land plants. The characteristic feature of NAC TFs is the presence of a conserved NAC-domain at the N-terminus and variable C-terminal sequences (Voitsik et al. 2013). A number of NAC TFs are also important regulators under plant abiotic and biotic stresses. For example, NTL4 can induce the *Atrboh* genes, resulting in reactive oxygen species (ROS) accumulation and triggering of leaf senescence, which are direct drought stress responses (Lee et al. 2012). RD26 is induced by drought, ABA and high salinity, thus functioning as a transcription activator in ABA-inducible gene expression under abiotic stress in plants (Fujita 2004). ATAF1 in *Arabidopsis* is induced by drought and salt stresses, and ATAF-type SNAC1/2 TFs are induced by drought, high salt, low temperature, injury and abscisic acid (ABA) (Christianson et al. 2010; Hu et al. 2008*). RhNAC2* is involved in the regulation of dehydration on flower opening and senescence in rose (Dai et al. 2012). *TaNAC2* transgenic tobacco shows higher fresh weight and dry weight than non-transgenic tobacco under drought conditions, which indicates that overexpression of *TaNAC2* improves tobacco tolerance to drought treatment (Tang et al.

2012). Many *NAC* genes are altered by drought in V6 and/or R2 leaves in *Glycine max*, whichdid contribute to identifying excellent drought-responsive candidate genes (Le et al. 2012). *ZmSNAC1* of maize enhanced drought tolerance in transgenic *Arabidopsis* (Lu et al. 2012). Field experiments indicated that *OsNAC9* in rice can enhance drought resistance without reduction of grain yield (Redillas et al. 2012). As a negative stress regulator, overexpression of the *GmNAC2* gene reduces abiotic stress tolerance in tobacco (Jin et al. 2013). NAC3 from *Arachis hypogaea* acted as a positive regulator under drought stress, and is known to enhance drought tolerance in tobacco by increasing superoxide scavenging (Liu et al. 2013). Also, *SNAC1* enhanced the tolerance of transgenic wheat to drought and salt stresses in multiple generations (Saad et al. 2013).

Citrullus colocynthis (L.) Schrad, closely related to the domesticated and widely cultivated watermelon (*Citrullus lanatus* var. *lanatus*), is a non-hardy, perennial herbaceous and medicinal plant species in the *Cucurbitaceae* family. It is a drought tolerant species, which can survive arid environments by maintaining its water content under severe stress conditions, which is accomplished by extending its root system into deep ground water (Dane et al. 2007). Its rootstock has been investigated as a potential rootstock for the improvement of drought tolerance mechanism of watermelon (Si et al. 2010).

Grafting has been used extensively in horticulture to enhance the performance of certain vegetables and woody fruit trees (Wu et al. 2013; Savvas et al. 2010). Grafting is also an effective way to enhance the viability of transgenic plants, for example, in safflower (Belide et al. 2011). It has been proposed that mRNAs (eg, from root-stock) could be reverse-transcribed to produce cDNAs and be integrated into the new host (scion) genome, thus generating *bona fide* genetic changes (Liu 2006). It was also demonstrated that DNA and small RNA could be exchanged by grafting (Stegemann and Bock 2009; Bai et al. 2011; Shaharuddin et al. 2006). Posttranscriptional gene silencing (PTGS) signal did migrate

from cell-to-cell in advance of the infection front and was transported over long distances through the phloem from the donors to recipients (Voinnet et al. 2000). In addition, the grafting process between genetically divergent cells (hetero-grafting) might generate novel epigenetic marks in the scion, a portion of which might be inherited to the next generation (Wu et al. 2013). Turnbull et al. (2002) also developed a cotyledon micrografting (Cot-grafting) method, unlike other grafting method, in which a graft donor and a graft recepient are used that can be of different genotypes, which allowed the protein from the graft to be transported via its native route from leaves to the shoot apex (Yoo et al. 2013).

To reconstruct the transcriptional regulatory network in a particular plant species, we first have to identify the general and specific TFs (Ravcheev et al. 2011). Here, we analyze the expression patterns of two transcription factors, *CcNAC1* and *CcNAC2,* from a very drought tolerant cucurbit species during grafting and study their promoter expression patterns.

Materials and methods

Plant materials and growth conditions

C. colocynthis seeds (No. 34256) from Israel were sown in soil with a 14h photoperiod at temperatures ranging from 22℃ to 30℃ and ambient relative humidity. *Arabidopsis thaliana* seeds were germinated on ½ MS medium and after one week, transferred to vermiculite soil. The conditions for *Arabidopsis* were 23℃ and a 16/8h photoperiod and 60% relative humidity. The growth stages were designated as described by Boyes et al. (2001). For the water withholding treatment, 7d old *Arabidopsis* seedlings were placed at 0h, 1h, 2h time points into GUS staining buffer for histochemical assay and GUS assay.

Grafting analysis

C. colocynthis seedlings were grafted following the procedure of one cotyledon or the slant graft method (Davis et al. 2008). To facilitate rootstock and scion union, seedlings were placed in a shaded plastic tunnel with a humidifier (Fedders, Sanford, NC) to maintain 100% humidity and temperatures around 28℃ for a period of 7-10 d, followed by acclimation for 7d to the natural conditons in the greenhouse. Seedings at the 3-4 leaf stage were placed into natural conditions without watering as drought treatment. The leaf samples were collected at 0, 2, 6, 9, 13, 17, 23, 25, 26, 29, 30 and 31d and immediately immersed into liquid nitrogen for storage at -80℃.

RNA isolation and cDNA synthesis

RNA was extracted from the samples of *C. colocynthis* and grafted *C. colocynthis* leaves according to the RNeasy Plant Mini Kit (Qiagen, USA). cDNA was synthesized using ProtoScript cDNA systhesis kit (New England Biolabs, USA).

Relative quantitative (RQ) realtime RT-PCR

The first strand of cDNA was diluted 50 times before it was used in the qRT-PCR reaction. qRT-PCR was performed with the SYBR-Green chemistry in an Eppendorf Mastercyler ep realplex with gene specific primers (Table 3. 1). Each reaction contains 10 µl of SYBR-Green supermix, 1ul of forward and reverse primers (4 um), 7 ul water and 1 ul of cDNA sample. The qRT-PCR program consisted of one

cycle of 95℃ for 2 min, followed by 40 cycles of 15 sec at 95℃, 15 sec at 58℃, and 30 sec at 72℃, and melting curve to check for specificity of the program. *C. colocynthis* specific *actin* was used as the relative expression gene, with primers for actin (ccActin 154F and ccActin 154R), and for *CcNAC1* and *CcNAC2* genes (primers Q-NAC1F/R and Q-NAC2F/R) as in Table 3. 1.

Construction of plant expression vectors and plant transformation

The promoters (1585 bp and 1299 bp) of *CcNAC1* (KC814688) and *CcNAC2* (KC814689) were obtained as described by Wang et al. 2014. The different sections of the two promoters were amplified with the primers as shown in Table 3. 2 and the resulting PCR products were fused into the pCR8/GW/TOPO entry vector. After the sequencing check, the insert was transferred into pMDC162 via the LR reaction. The recombinant plasmid containing the *pNAC*::*GUS* fusion was transferred to *Agrobacterium tumefaciens* LBA4044, and the resulting strain was used to transform *Arabidopsis* wild-type by floral dip method (Cough and Bent 1998). Transformants were selected on 20 μg ml^{-1} hygromycin and grown under white light. At least ten independent plants were analyzed. Histochemical staining was conducted according to Altamura et al. (1991) on different tissues at different developmental stages.

Histochemical assay

β-glucuronidase activity was assessed by histochemical assay (Jefferson et al. 1987). Histochemical GUS analysis was done by immersing different tissues in a GUS reaction buffer. Samples were incubated for 16-18h at 37℃, followed by 70% ethanol rinse to clear the tissues. Fluorometric GUS assays were

performed on seedlings of each line. 100 mg of seedling tissues were ground in GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosin, 0.1% Triton X-100) in one 1.5 ml microfuge tube, using a drill fitted with autoclaved plastic pestles. A sample of 475 ul was taken from the clarified extract and warmed to 37℃ for 1h after the addition of 25 ul GUS assay buffer (2 mM MUG in GUS extraction buffer). The reaction was stopped by pipetting 500 ul of the reaction mixture into 1.95 ml of carbonate stop buffer (0.2 M Na_2CO_3). A DyNA Quant 200 Fluorometer (Hoefer, Inc., San Francisco, CA) was used with a 4-MU standard solution. To calibrate the instrument, 1.9 ml of carbonate stop buffer was added to 100 ml of the 4-MU standard solution (1mM). The GUS activity was calculated as fluorescent units per mg fresh weight of tissue.

Drought treatment of pCcNACs::GUS in Arabidopsis by withholding water

pCcNAC1::GUS and *pCcNAC2::GUS* seedlings were grown on ½ MS medium until they were 7-8 d old. More than five seedlings per line were used for GUS staining and separately for GUS histochemical assay.

Results

Expression profiles of CcNAC1 and CcNAC2 genes in C. colocynthis during drought

In order to investigate the *CcNAC1* and *CcNAC2* gene expression under drought treatment and the importance of the rootstock to *C. colocynthis* gene expression, *C. colocynthis* scion was grafted onto *C. lanatus* rootstock. Gene expression in *C. colocynthis* leaves was studied using *C. lanatus* as rootstock (CC/CL) or its own rootstock (CC), as graphically shown in Fig. 3. 1. Nonsignificant differences in

expression levels were observed for the *CcNAC1* and *CcNAC2* genes until 23 days after the start of water deficit treatment. After 23 days, both genes showed up-regulation, and gene expression gradually increased until the grafted (CC/CL) plants died at 26 d. Although *CcNAC1* showed an almost 3 times increase in gene expression in CC/CL 26 days after the start of the drought treatment, *CcNAC2* showed more than five times an increase at 26 d as compared to gene expression levels before 23 d. *NAC1* and *NAC2* gene expression in ungrafted *C. colocynthis* leaves was not much different up to 26 days of treatment, but showed strong up-regulation from 26 d to 31 d before plant death. The range of up-regulation was much higher for *CcNAC2* than *CcNAC1*. It is clear that rootstock did have a major effect on onset of senescence. Wilting of CC on CL rootstock occurred earlier and resulted in death at least 3 days before wilting and senescence on CC rootstock. However, water deficit stress did influence gene expression of *CcNAC1* and *CcNAC2* to a high degree before senescence of the plants. After the plants showed wilting, both genes showed gradually up-regulation until the death of the plants, both in CC/CL and CC plants. It is also clear that roots of *C. colocynthis* were more efficient for water transport function than the roots of *C. lanatus*.

Expression patterns of the CcNAC1 or CcNAC2 promoters in Arabidopsis during drought treatment

To further study the correlation of *CcNAC1* and *CcNAC2* with drought tolerance, water was withheld from 7d old *pCcNAC1::GUS* and *pCcNAC2::GUS* transgenic *Arabidopsis* plants. The results as shown in Fig. 3. 2 indicated that at 0h time point, both transgenic seedlings showed low levels of GUS expression, with *pCcNAC1::GUS* transgenics at 0h time point having little more GUS expression than *pCcNAC2::GUS* seedlings. At 1h and 3h time points, transgenic seedlings showed an increase in GUS

expression levels as compared to control (0h time point). From this experimental result, we can conclude that both *CcNAC1* and *CcNAC2* were activated during drought treatment.

GUS assays were subsequently used as shown in Fig. 3. 3. The GUS assay showed that both transgenic lines showed higher GUS levels during drought treatment. Figs. 3.2 and 3.3 indicate that after withholding water, *pCcNAC1::GUS* showed 67.1 times the level of expression at 1h treatment as compared to control, and 87.5* following 2h treatment. *pCcNAC2::GUS* had similar expression pattern as *pCcNAC1::GUS,* with increases of 52.7 times and 60.8 times expression at 1h and 2h time point after treatment. In conclusion, both *pCcNAC1::GUS* and *pCcNAC1::GUS* were up-regulated during drought treatment.

Developmental regulation of promoters of CcNAC1 and CcNAC2 during early plant development

The *pCcNAC1* and *pCcNAC2*-driven expression of GUS activity in developing *Arabidopsis* seedlings was studied (Fig. 3. 4). *pCcNAC1::GUS* and *pCcNAC2::GUS* transgenic plants were grown on ½ MS medium and subjected to histochemical staining for GUS activity following a 2-10 day treatment. In epigeal seedlings (2d-3d), both promoters were expressed mainly in the hypocotyls, while the promoter of *CcNAC1* was also expressed to a limited degree in the cotyledons. At day 4, promoters of both genes were mainly expressed in hypocotyls and again to limited degree in the cotyledons. From day 5 to day 6, some *GUS* activity was detected in the newly developed leaves of both transgenics. From day 7 to day 10, GUS expression was reduced in leaves, but increased in the petioles of both promoter transgenic plants. No expression was detected in the roots of the seedlings. Thus, the promoters of *CcNAC1* and *CcNAC2* showed similar expression patterns during seedling developmental stages.

Developmental regulation of pCcNAC1::GUS and pCcNAC2::GUS expression in Arabidopsis organs

The *pCcNAC1::GUS and pCcNAC2::GUS* expression in developing *Arabidopsis* organs is illustrated in Fig. 3. 5. GUS was expressed in micropyles of seeds of GUS transgenic lines. Also promoters of both genes were expressed mainly at both ends of the siliques, but not in seeds, even though the *CcNAC2* promoter showed low expression in the siliques. Since GUS expression at cutting edges of the stems was observed in the transgenics, this might be the result of wounding. In the cauline leaves, GUS expression was detected all across the leaves. *pCcNAC1::GUS* showed higher levels of expression on the rosette leaf than *pCcNAC2::GUS*. GUS assay on organs indicated that both promoters showed similar expression patterns in different organs.

PCcNAC1-driven and pCcNAC2-driven GUS expression during flower development of A. thaliana

PCcNAC1::GUS and *pCcNAC2::GUS* histochemical staining was conducted during different stages of flower development (Fig. 3.6) Promoters of both genes showed expression at all developmental stages in the stigmas, and petals and both expressed to a higher degree in the late stages as compared to the early stages of flower development. On the ninth day of flower developmental stages (fds9), GUS expression was detected in the stigma and some in the petals. In fds13, when the bud opened, and petals became visible, expression was observed more in the petals and stigmas. During fds15 with extension of the stigma above long anthers, the petals and stigmas showed higher expression levels. Differences with earlier stages of flower development with expression in filaments and anthers were detected. Differential expression during flower developmental stages indicated that the promoters of both *CcNAC* genes showed similar expression patterns during flower development.

Different CcNAC1, 2 promoter fragments show differential expression patterns

GUS assay was used to detect the effect of length of promoter region on *Arabidopsis* seedling expression following 7 days of growth. It can be deduced from Fig. 3.7 that different fragments showed differential expression levels. The *pCcNAC1::GUS*, Pro1.2 (1142 bp) fragment showed higher expression levels than shorter fragments of the *CcNAC1*promoter region. Section Pro1.3 (631 bp), Pro1.5 (340 bp) and Pro 1.8 (129 bp) showed expression levels 1.29 times, 1.05 times and 1.18 times as compared to Pro1.0 (1585 bp). One short section of promoter *CcNAC1* Pro1.6 (227 bp) showed the lowest expression level (0.85*). The *pCcNAC2::GUS* fragment Pro2.1 (858 bp) showed the highest expression (*2.49) as compared to other fragments. Pro2.3 (346 bp) showed more expression (1.63*) than Pro2.0 (1299 bp). The shortest promoter fragments of *CcNAC2* Pro2.4, Pro2.5 and Pro2.6 showed less expression as compared to the whole sequence of *pCcNAC2.* Differential expression indicates that different promoter regions have different regulatory responses. The different promoter fragments might contain different motifs, which responded to treatment differently, and show variable expression level under normal (*in situ*) conditions.

Discussion

NAC TFs are involved in the regulation of biotic and abiotic stresses. The signaling pathways include crosstalk between biotic and abiotic stresses and NACs play important parts in the crosstalk (Olsen et al. 2005). NACs were found to not only be positive regulators, but also negative regulators under drought treatment. For example, ATAF1 is a negative drought response regulator in *Arabidopsis*, because its mutant could increase the rate of recovery after drought stress along with stimulation of the

expression of some stress-inducible marker genes (Lu et al. 2007). *OsNAC6*, functions as a positive regulator, and improves tolerance towards blast disease, dehydration and high-salt (Nakashima et al. 2007). In earlier studies of *CcNAC1* and *CcNAC2* the up-regulation was observed during many abiotic stress treatments (Wang et al. 2014). In this study of seedling grafting and GUS expression in transgenic *Arabidopsis* it can also be concluded that CcNAC1 and CcNAC2 are drought-related transcription factors. *CcNAC1* and *CcNAC2* both were up-regulated during the later stages of drought treatment (Fig. 3.1).

Gene functions can be investigated by plant grafting, morphological studies, mutant characterization, and gene expression analysis (Simons et al. 2007). Although there are many NAC genes in plants, there might be significant variations in the way the genes appear to function in different rootstocks. These include differences in the patterns of gene expression in response to grafting (Sorefan et al. 2003; Foo et al. 2005; Snowden et al. 2005). There are about 80 NACs in watermelon (*Citrullus lanatus*) (Guo et al.2013). These two *NAC* genes from *C. colocynthis* showed up-regulation both on rootstock of *C. lanatus* and *C. colocynthis*, especially their own rootstock during drought treatment, and thus are drought stress-related genes. Further studies using next generation sequencing methods might help us discover more of their functional roles under stress conditions.

NAC transcription factors have been studied by promoter analysis to reveal transcriptional control. ANAC092 expression was studied during leaf and flower ageing and in response to salt stress (Balazadeh et al. 2010). ANAC012, which negatively regulates xylary fiber development in *A. thaliana*, was found to be preferentially localized to the (pro) cambium region of inflorescence stem and root (Ko et al. 2007). Histochemical analysis of *RD26* promoter::GUS in transgenic *Arabidopsis* showed that *RD26* expression was higher during dehydration (Fujita et al., 2004). Similarly the NTL4 transcription factors show higher expression during drought treatment (Lee et al. 2012). The promoters of *PtNAC068* and *PtNAC154* from

poplar showed more complex expression patterns in poplar than in *Arabidopsis* (Han et al. 2012). Also, NST1 and NST3 were found to regulate pod shattering in a partially redundant manner in siliques of *Arabidopsis* (Mitsuda and Ohme-Takagi 2008). Also, the spatial and temporal expression patterns of the *VND-INTERACTING2* (*VNI2*) gene are correlated with leaf aging and senescence (Yang et al. 2011).

In this study of *CcNACs* during flower developmental stages in *Arabidopsis*, it was detected that the *CcNAC1* and *CcNAC2* promoters showed higher levels of expression in mature anthers. Whether this is correlated with maturity of anthers or senescence, needs to be investigated in more detail. Since the *CcNAC1* and *CcNAC2* promoters showed very similar expression patterns in *Arabidopsis* tissues and organs, the two *NACs* might have a similar function. Because different regions of the promoters showed differential regulation, the transcriptional control factors might be different in two promoters. *NAC* gene functions are relatively conserved within subfamilies and *NAC* gene lineages have experienced extensive duplications (Zhu et al. 2012). *CcNAC1* and *CcNAC2* have much similarity in structure and functions, so we can deduce that both NACs probably were the result of *NAC* gene duplication in *C. colocynthis*.

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Table 3. 1.Q-RT PCR primers for detection of relative expression levels of *CcNAC1* and *CcNAC2*

Primers	**Sequence(5'-3')**	**Tm**	**Product length**
ccActin 154F	CACCATCACCAGAATCCAGCACGA	59℃	140bp
ccActin 154R	GGCTCCACTCAACCCAAAGGCTAAC	59℃	140bp
Q-NAC1F	GTCAACCGAGAATGAAAGAAGAGTA	59℃	132bp
Q-NAC1R	TATACATGAACATATCCTGCAATGG	59℃	132bp
Q-NAC2F	GTGCCGGATTTACAACAAGAA	59℃	106bp
Q-NAC2R	AATCTTCGGCTTCTCGCTTC	59℃	106bp

Table 3. 2.Primers for different sections of promoter of CcNAC1 (Pro1) and CCNAC2 (Pro2).

Usage	Forward primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')
section 1.0	Pro1.0F	AAAAATGTATGCATGTTGACTCAATATT	Pro1.0R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section 1.2	Pro1.2F	CATAAAGGTTCCTACGTTGCTAAAATAA	Pro1.2R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section1.3	Pro1.3F	TCCAACTCTTCACTAATCGCTTTCTT	Pro1.3R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section 1.5	Pro1.5F	TCATAAAAGCGGTCCCAAAAAGA	Pro1.5R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section 1.6	Pro1.6F	CACGCACCCCACTTTCTTTGT	Pro1.6R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section 1.8	Pro1.8F	CACGTGTCTCTTTCTTTTCCTCTATAA	Pro1.8R	GATTTCTTCTTTCCCTTTTTTCTTCTT
section 2.0	Pro2.0F	ATCAAAAAATTTAGAAGTCAAACAAGACAT	Pro2.0R	TTTTCTTCTGATATTTCTCCGGCG
section 2.1	Pro2.1F	AAGTTTTTAGTTTGGTTTCGATTTAGTTC	Pro2.1R	TTTTCTTCTGATATTTCTCCGGCG
section 2.3	Pro2.2F	GAGGCGAGTAAATGGAATCTGCAG	Pro2.2R	TTTTCTTCTGATATTTCTCCGGCG
section 2.4	Pro2.3F	CGCATGCACTTCACGTCTTAATCC	Pro2.3R	TTTTCTTCTGATATTTCTCCGGCG
section 2.5	Pro2.5F	CCCTCTCTCCCACCCCTATAAATAC	Pro2.5R	TTTTCTTCTGATATTTCTCCGGCG
section 2.6	Pro2.6F	CACAAACACCAAAAAAAAAAACCATA	Pro2.6R	TTTTCTTCTGATATTTCTCCGGCG

Figure 3. 1. Expression profiles of the *CcNAC1*, 2 in CC and CC/CL with holding water.

CC/CL: *C.colocynthis* grafted onto the *C.lanatus* var. *lanatus* rootstock. CC: *C. colocynthis*. Gene expression was normalized by comparing ΔΔCt to control (0d) (n=3).

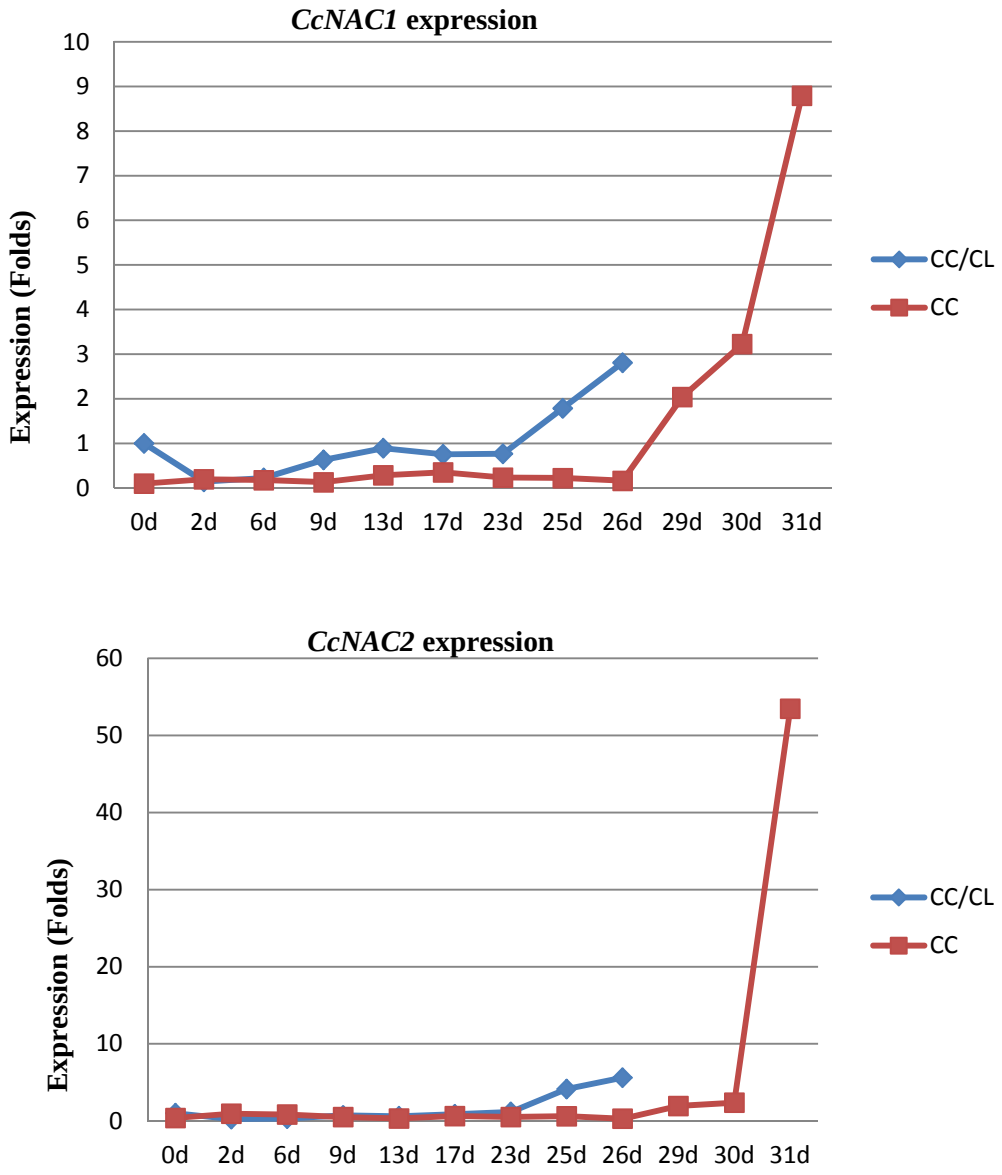


Figure 3. 2. *pCcNAC1::GUS* and *pCcNAC2::GUS* expression with holding water.

Treatment 0h, 1h, 3h indicate the number of hours following withholding of water. Bars are 500 μm in the panels.

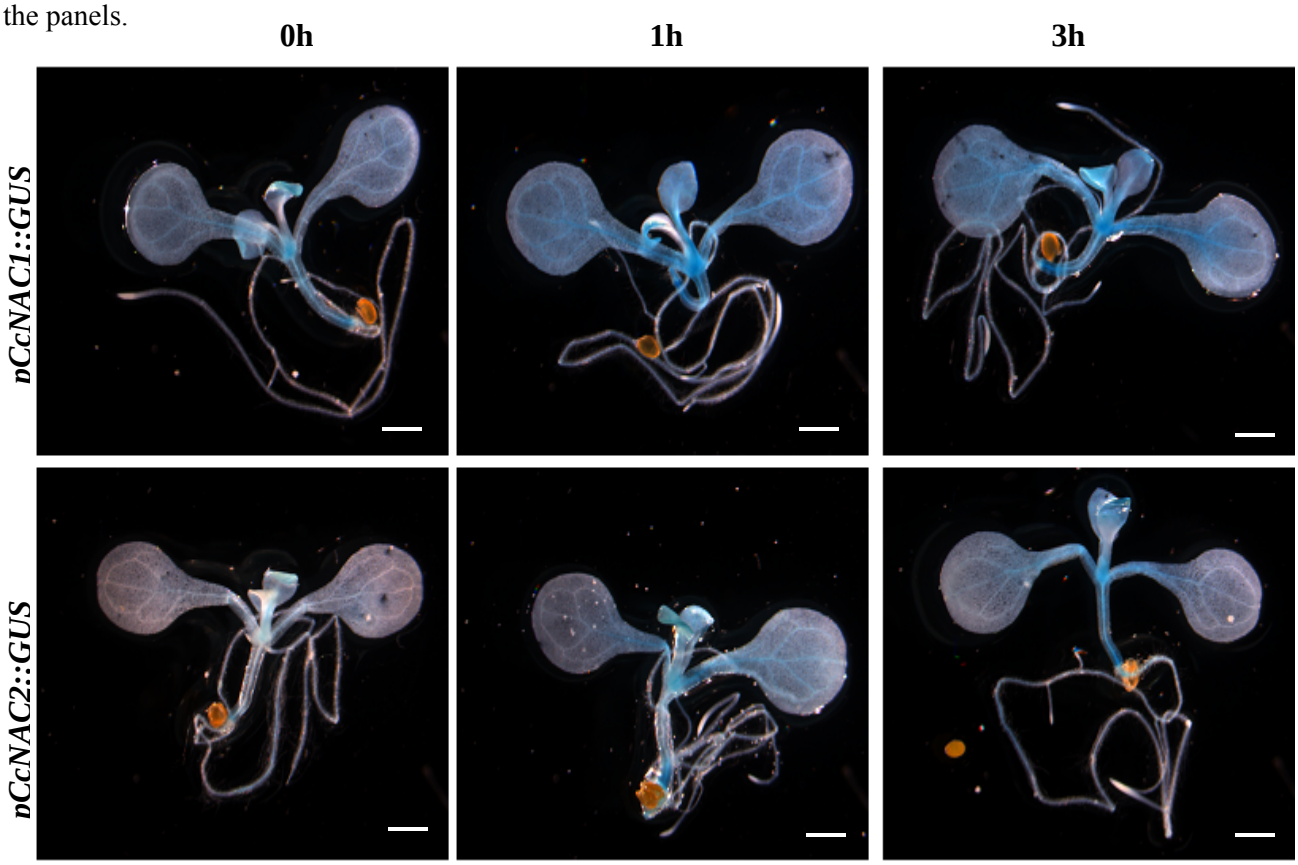


Figure 3. 3. Activity of *pCcNAC1::GUS* and *pCcNAC2::GUS* after withholding water.

0h, 1h and 2h seedlings were assayed after withholding water. N1 represents *pCcNAC1::GUS*, and N2 represents *pCcNAC2::GUS*. Fold induction is shown above each treatment, and numbers indicate up-regulation of GUS as compared to control for each transgenic line. GUS activities represent the mean values of at least 5 different lines of transgenic *Arabidopsis*.

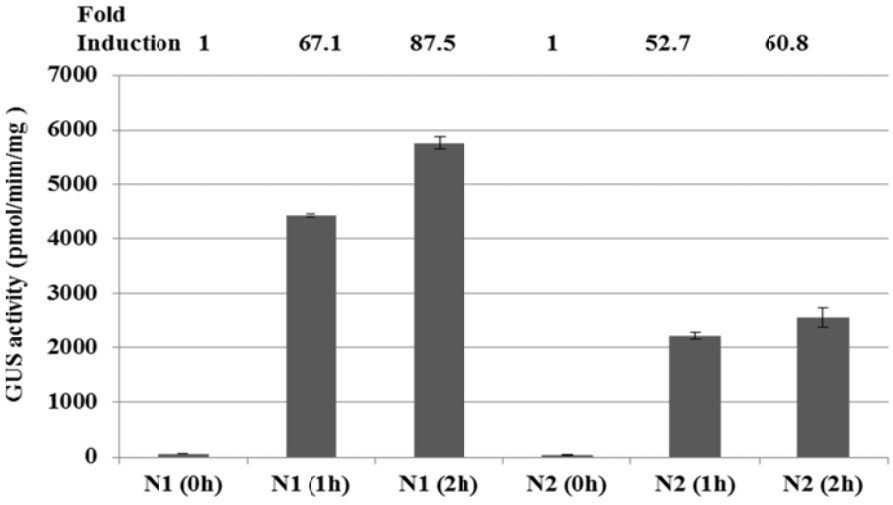
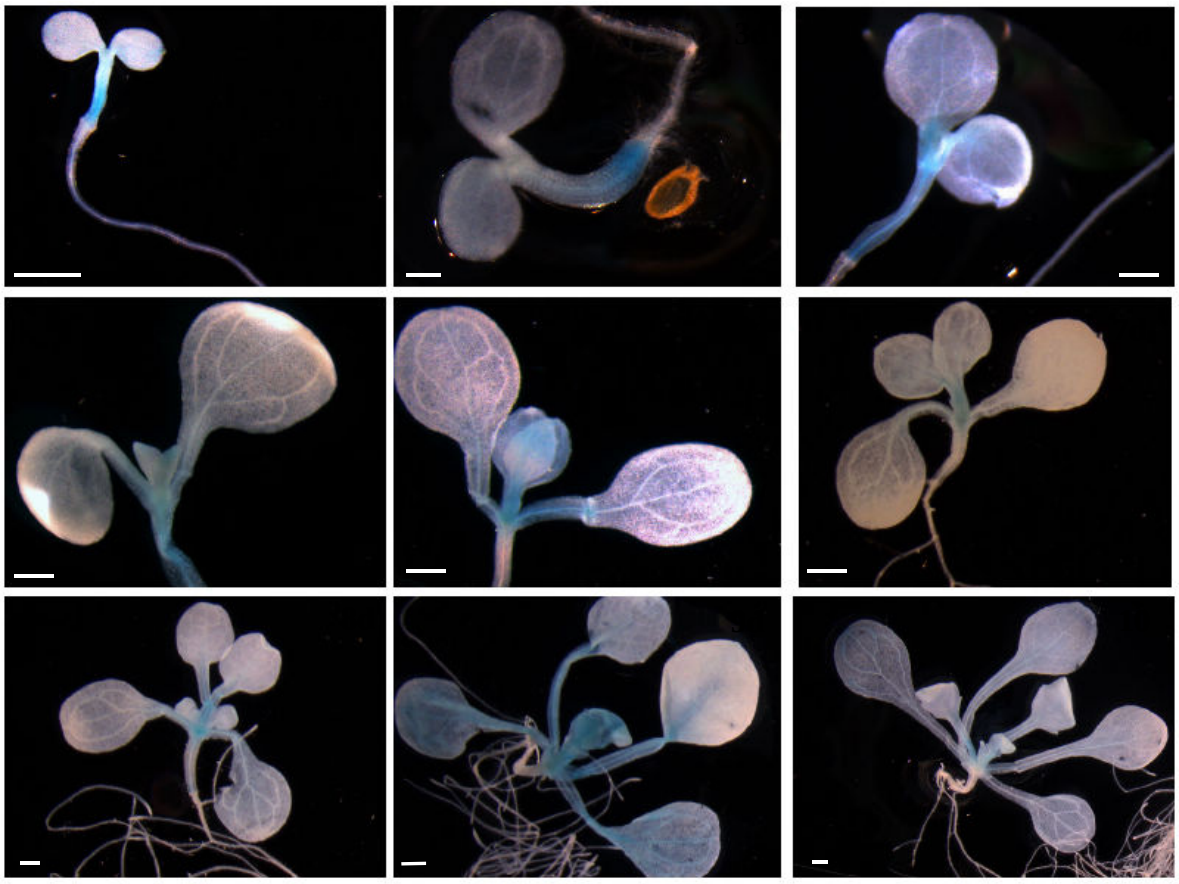


Figure 3. 4. *pCcNAC1::GUS* and *pCcNAC2::GUS* in early seedlings of *Arabidopsis*.

Histochemical assays show *pCcNAC1::GUS* and *pCcNAC2::GUS* expression patterns in from day2 (2d) to day10 (10d) seedlings under normal growth conditions. Scale bars are 500 μm.

pCcNAC1::GUS



pCcNAC2::GUS

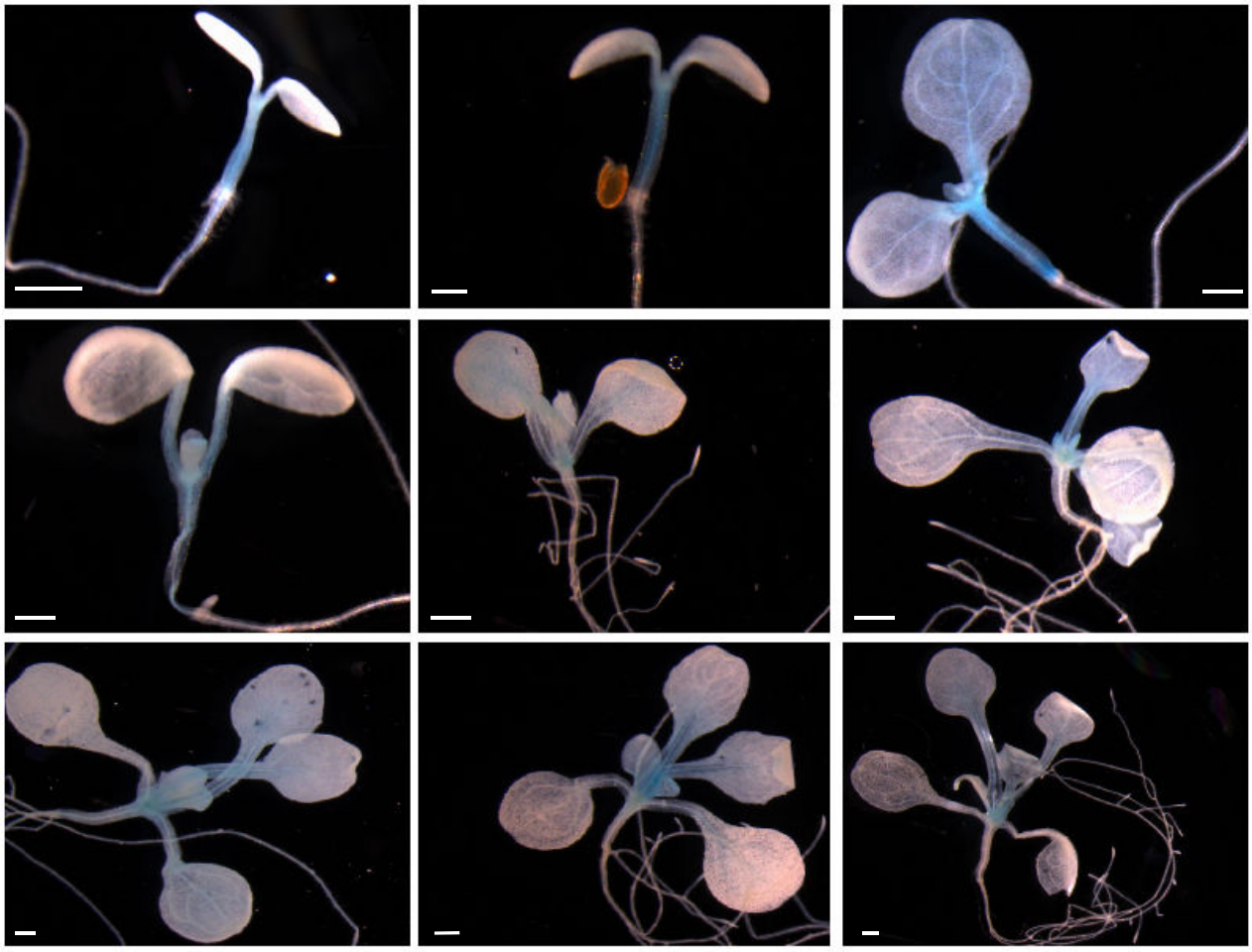


Figure 3. 5. GUS staining in *Arabidopsis* different tissues.

A1-E1 came from promoter of *CcNAC1*; A2-E2 came from promoter of *CcNAC2*. A1 and A2 are seeds. B1 and B2 are siliques. C1 and C2 are stems. D1 and D2 are cauline leaves. E1 and E2 are rosette leaves. Arrow points to GUS staining in the micropylar endosperm after testa rupture. Scale bars are 500μm.

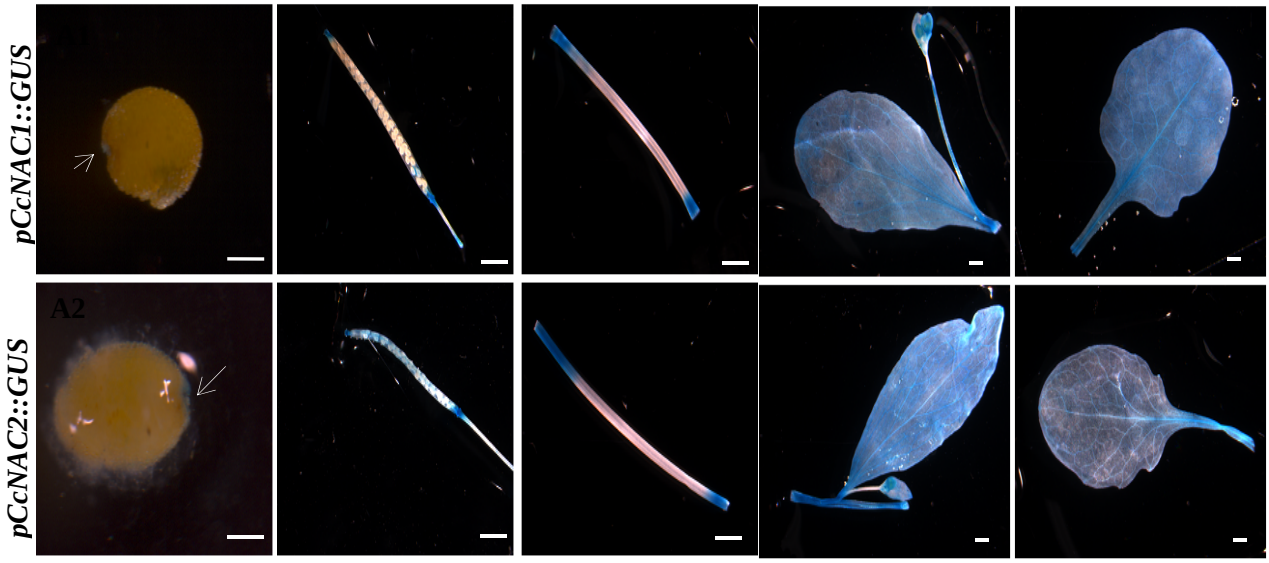


Figure 3. 6. ***pCcNAC1::GUS*** **and** ***pCcNAC2::GUS*** **expression in flowers.**

The numbers indicate flower development stages (fds). Bars are 500 μm in the panels.

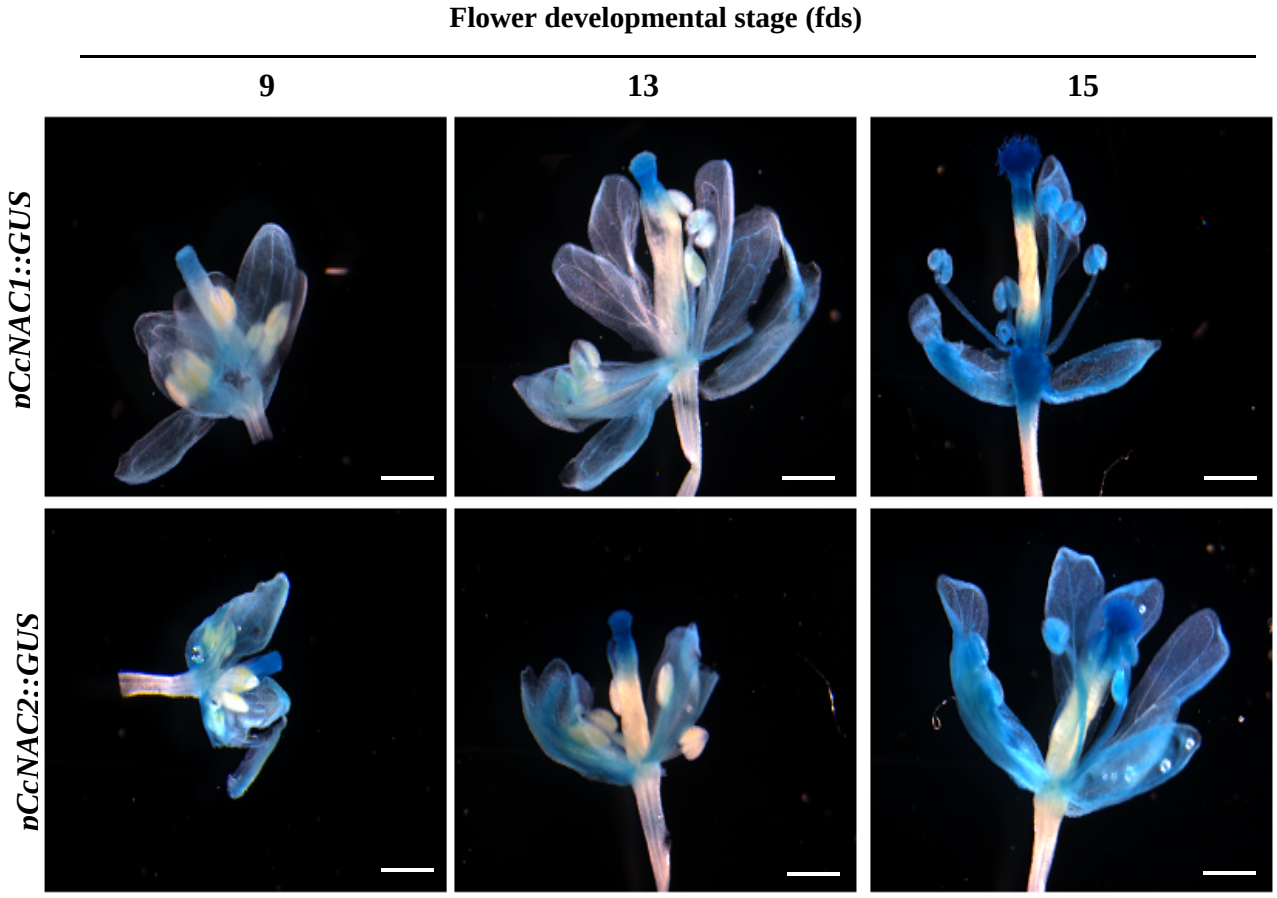
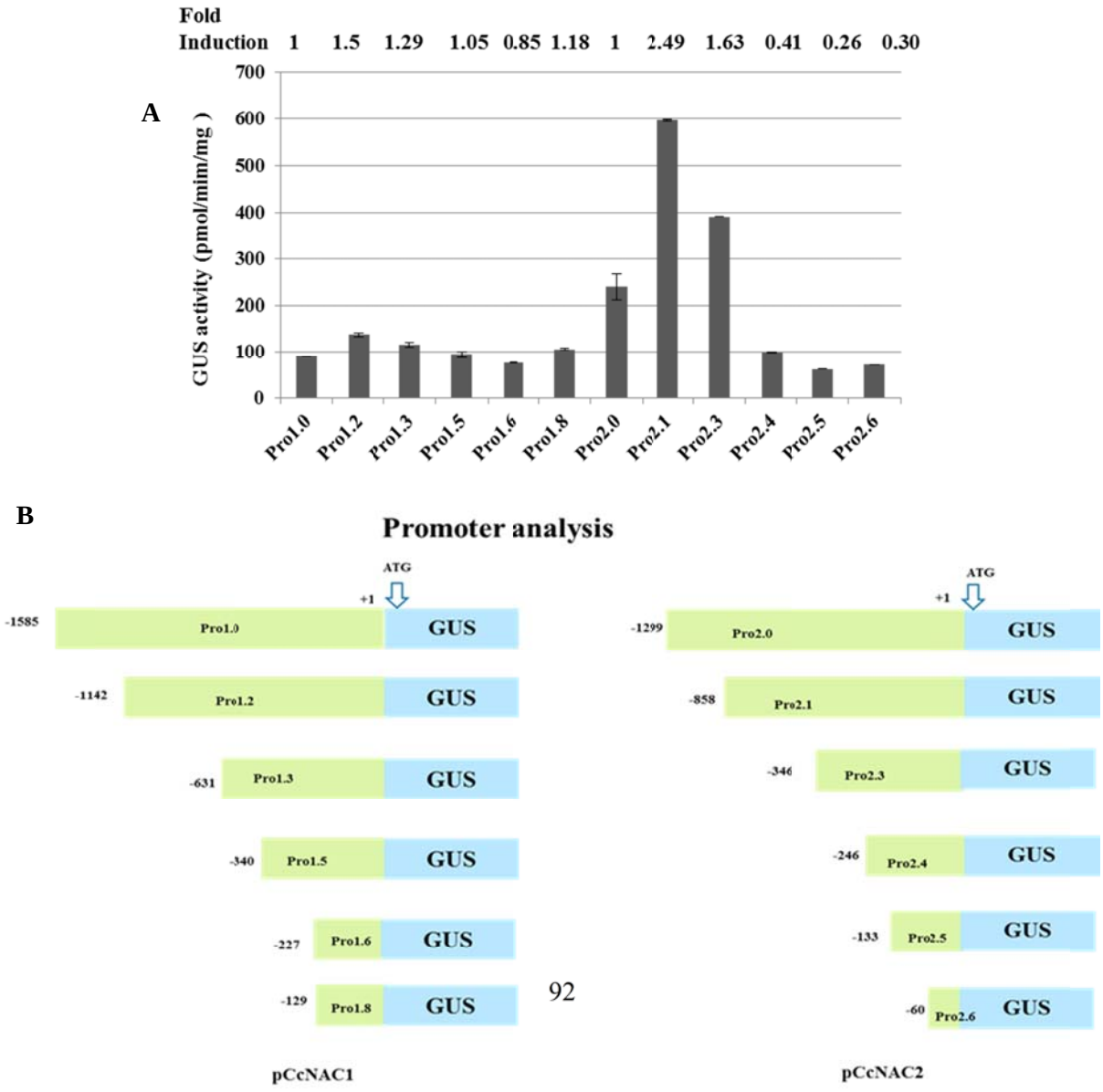


Figure 3. 7. Different promoter section activity of *pCcNAC1::GUS* and *pCcNAC2::GUS.*

(A) Different promoter section activity of *pCcNAC1::GUS* and *pCcNAC2::GUS,* with fold induction indicated on top of each promoter region. The numbers indicate the level of induction of GUS as compared to the longest promoter section Pro1.0 or Pro2.0. GUS activities represent the mean values of at least 5 different lines of transgenic *Arabidopsis*. The promoter segments correspond to promoter section vector construction shown in Fig.4 (B).

(B) Promoter section vector construction. The length of the promoter sections are shown in front of different GUS construction figure. All of the sections were linked to the GUS gene. ATG site is the gene transcription starting site.



IV *Citrullus Colocynthis* NAC trasncription factors CcNAC1 and CcNAC2 are involved in light and auxin signaling

Abstract

NACs (NAM, ATAF1, 2, CUC2) are conserved in plants, and have multiple functions in plant growth and development. Two NAC transcription factors, CcNAC1 and CcNAC2, were recently identified in the highly drought-tolerant cucurbit species, *Citrullus colocynthis*. This study further examines the functional role of these genes to different qualities of light and the associated hormone auxin based on the *in silico* analysis of the *CcNAC1* and *CcNAC2* promoter regions which revealed the presence of several light-associated motifs. The impact of both light and auxin on *CcNAC1* and *CcNAC2* gene expression was examined in *C. colocynthis* leaves, and using reporter (*pCcNAC1, 2::GUS*) lines in *Arabidopsis*. Furthermore the effects of constitutive overexpression (*OE-CcNAC1, 2*) in *Arabidopsis* were also examined under a range of conditions to confirm reporter line linkages. White, blue, red, and far red light treatments resulted in similar patterns of quantitative changes in *CcNAC1*and *CcNAC2* expression in both species, with the highest transcript increases coming with red light. Photomorphogenic changes in *Arabidopsis* hypocotyls were also correlated with changes in gene transcript levels. In the absence of light hypocotyls of OE-CcNAC1 and OE-CCNAC2 lines were significantly longer as compared to WT. The addition of exogenous auxin (+IAA) to growth medium also resulted in changes to the hypocotyl lengths of overexpression lines and spatiotemporal reporter line changes in seedlings. Our data suggest that *CcNAC1, 2* might be functionally important in the light signaling pathway, and appear connected to

the hormone auxin. This is the first study to indicate that *NAC* genes might play a role in both light and auxin signaling pathways.

Keywords

Citrullus colocynthis, NAC, transcription factors, promoters, light, auxin, CcNAC1, CcNAC2

Introduction

Plants use light signals as a source of information to adjust growth and development in response to changing environmental conditions via different classes of photoreceptors involved in light signal transduction pathways (Briggs and Huala 1999; Parks 2003; Chen et al. 2004; Shikata et al. 2012). The phytochrome family of photoreceptors are known to respond to far-red light (FR) and red light (R), the cryptochromes (cry1 and cry3) to blue and UV-A light, and the phototropins (phot1 and phot2) to UV-B and UVR8 (Bhatia et al. 2008; Rizzini et al. 2011). Phytochromes are red/far-red absorbing chromoproteins that regulate a wide range of plant development, from seed germination to flowering (Shikata et al. 2012). Blue light induces various developmental responses, including phototropic bending, cotyledon opening, photoperiodic flowering, leaf flattening, de-etiolation, stomatal opening, chloroplast movements, anthocyanin accumulation, gene expression, and the inhibition of hypocotyl elongation (Inoue et al. 2008).

Many transcription factors are involved in light regulation and some regulate hypocotyl elongation. Hypocotyls have proven to be excellent systems for studying signal interplay in the

regulation of growth and developmental responses (Vandenbussche et al. 2005). For example, UGT73C5, which catalyzes 23-O-glycosylation of the brassinosteroid brassinolide (BL) and castasterone, has been shown to regulate the hypocotyl phenotypes (Poppenberger et al. 2005). The *HY5* mutant exhibits elongated hypocotyl under various wavelengths of light, suggesting that functionally HY5 is downstream of multiple photoreceptors (Oyama et al. 1997; Koornneef et al. 1980; Chattopadhyay et al. 1998; Ang et al. 1998). SHORT HYPOCOTYL IN WHITE LIGHT1 (SHW1) is a master repressor of photomorphogenesis involved in *Arabidopsis* seedling development (Bhatia et al. 2008). The GT or trihelix family of transcription factors are known to bind GT elements in light regulated genes, and phyA is a member of the GT transcription factor family (Kaplan-Levy et al. 2011). GT-1 ortholog RML1 in rice is down regulated by light and shows a circadian expression pattern (Wang et al. 2004). Many HELIX-LOOP-HELIX (bHLH) transcription factors are involved in light signaling. For example, the basic bHLH transcription factor PRE3/bHLH135/ATBS1/TMO7 is involved in the regulation of light signaling pathway in *Arabidopsis* and its overexpression line showed less expression under red, far-red and blue light (Castelian et al. 2012). PIFs also belong to the bHLH family (Monte et al. 2007).

Light signaling is associated with many phytohormones. The HY5 transcription factor integrates light and hormone signaling pathways because HY5 promotes the expression of negative regulators of auxin signaling (Cluis et al. 2004). STF1 (SHORT HYPOCOTYL 1) in soybean (*Glycine max*) is a homolog of HY5 with a role in light and hormone signaling, including root development of short hypocotyl, accumulation of chlorophyll and root gravitropism (Song et al. 2008). Low-fluence red light has been found to increase the transport and biosynthesis of auxin (Liu et al. 2011). Light and auxin also control many aspects of plant growth and development in an overlapping manner. Two homologous ATP-binding cassette transporter proteins, AtMDR1 and AtPGP1, regulate *Arabidopsis* photomorphogenesis

and root development by mediating polar auxin transport (Lin and Wang 2005). The increase in plant stem elongation is regulated by plant hormones, mainly by gibberellins, but also the involvement of ethylene, auxins, and cytokinins has been reported (Kurepin et al. 2010). It has also been reported that the salicylic acid pathway interacts with light-induced signaling pathway in plant defense responses (Genoud et al. 2002). Cho et al. (2012) uncovered that when phyB was activated by red light, seed germination was promoted by epigenetic transcriptional activation of gibberellic acid biosynthetic enzymes via histone demethylation, connecting the light and hormone-mediated induction of seed germination in *Arabidopsis*. HY1, MYC2 and HY5 are functionally interrelated in the light and JA signaling pathways (Rajendar et al. 2012). Differential expression of the auxin primary response gene MASSUGU2/IAA19 was shown in tropic responses of *Arabidopsis* hypocotyls (Saito et al. 2007). Low light intensity-induced differential petiole growth requires blue-light, auxin signaling and polar auxin transport and is, at least in part, genetically separate from well-characterized ethylene-induced differential growth (Millenaar et al. 2009). ABCB19-mediated polar auxin transport in seedling photomorphogenesis is mediated by cryptochrome 1 and phyB (Wu et al. 2010).

Another family of transcription factors, the NACs (NAM, ATAF1, 2, CUC2), which play important roles in plant growth and responses to stress, has also been implicated in light mediated regulation of plant development. Whole-genome ATH1 Genome Array studies showed that more than one quarter of NAC proteins in *Arabidopsis* leaves were up-regulated under dark treatment (Lin and Wu 2004). cDNA microarray analysis in *Arabidopsis* indicated that some *NACs* are involved in photosynthetic protection (Kimura et al. 2003). For example, *ANAC078* in *Arabidopsis* can regulate flavonoid biosynthesis under high light stress (Morishita et al. 2009). Short wavelength, higher intensity UV-B could modulate *ANAC13* by a yet unknown and COP1 -independent signaling cascade. *ANAC13*

was also modulated by abscisic acid (ABA) and different abiotic stresses (Safrany et al. 2008). Auxin and blue light are known to regulate the phototropic response in *A. thaliana* (Sun et al. 2013). Some NACs are also implicated in the auxin response. A mutation in one of the auxin receptors (*tir1*-1) was found to suppress the salt stress induction of *AtNAC2* (He et al. 2005). It was also shown that *NAC1* was induced by auxin in *Arabidopsis* and *AtNAC2* was induced by multiple plant hormones (auxin, ABA and ethylene) and high salinity (Xie et al. 2000; He et al. 2005). Auxin associated genes such as *IAA30* have also been link to a salt signaling cascade mediated by the NAM-ATAF1/2-CUC2 transcription factor NTM2 during seed germination (Park et al. 2011).

Expression of many structural, metabolic and regulatory genes is required for light-related morphogenesis of plants. Light-inducible promoters may regulate their transcription. Numerous cis-acting elements have been identified in light-regulated genes (Terzaghi and Cashmore 1995). The ACE motif has been known to stimulate transcription of a parsley basic region/ leucine zipper (bZIP) transcription factor, a common plant regulatory factor 1 (CPRF1) in light responses (Feldbrugge et al. 1994). The AE (Activation Element) box dimmer was found to be involved in the regulation of the nuclear gene GapA, which encodes the A subunit of glyceraldehyde 3-phosphate dehydrogenase, and confers light responsiveness (Park et al. 1996). The AT1 motif has been found to exist in the promoter of a Bell1-like gene of potato, which can be light-activated (Chatterjee et al. 2007). G-boxes, elements with the core CACGTA, are found in the promoters of many genes that respond to a variety of different stimuli, especially light (Terzaghi and Cashmore 1995). GT-1 sites appear important for light regulation, and can be found in many light-regulated genes including RbcS (ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit) from many species (Donald et al. 1990; Viret et al. 1994), and in phyA from oats and rice (Kuhn et al. 1993; Dehesh et al. 1990). MRE motif sequences are MYB binding

sites known to be involved in light responsiveness (Nalbandi et al. 2012). The Sp1 motif is also known as a potential light-correlated motif (Thanh et al. 2012). CHS promoters from several species have an element known as Box1 (H-box), which is essential for light regulation (Terzaghi et al. 1995), while Box4 was a light-responsive promoter motif correlated with specific phototransduction pathway (Yamada et al. 1992; Cerardo et al. 1996). Gap boxes are necessary for GAPB gene transcription, which encodes the B subunit of chloroplast glyceradehyde-3-phosphate dehydrogenase (GADPH) of *Arabidopsis thaliana* (Chan et al. 2001). TCT in the promoter of the RbcS (small subunit of ribulose-1,5-biphosphate carboxylase) gene was found to be a light responsive element in poplar (Wang et al. 2013).

Here we report on the regulation of two NAC transcription factors from *C. colocynthis* by different light signals, which at least in part, are associated with the auxin response. This is the first report on the interaction of light, auxin and NAC transcription factors from this drought tolerant cucurbit species.

Materials and Methods

Bioinformatic analysis

The partial promoters of *CcNAC1* (KC814688) and *CcNAC2* (KC814689) (1585 bp and 1299 bp lengths, respectively) were cloned as previously described (Wang et al. 2013). Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), a database of plant cis-acting regulatory elements and a portal of tools for *in silico* analysis of promoter sequences (Lescot et al. 2002), was used to identify consensus motifs in the promoter sequences of *CcNAC1* and *CcNAC2* using standard program parameters.

GUS constructs and analysis

The promoters were amplified with the primers shown in Table 1. The resulting PCR products were fused into the pCR8/GW/TOPO Gateway entry vector, then transferred into pMDC162 via the LR reaction using manufacture's protocol. The recombinant pMDC162 plasmid containing the p*CcNAC*::*GUS* fusion was transferred to *Agrobacterium tumefaciens* LBA4044, and the resulting strain was used to transform *Arabidopsis* wild type (Col). At least ten independently transformed plants for *CcNAC1* and *CcNAC2* were identified and further analyzed using histochemical staining according to Altamura et al. (1991) on different tissues at different developmental stages as described in the text and a representative line for each is shown.

β-glucuronidase activity was assessed by histochemical assay (Jefferson et al. 1987). Histochemical GUS analysis was done by immersing different tissues in a GUS reaction buffer. Samples were incubated for 16-18 h at 37℃, followed by a 70% ethanol rinse to clear the tissues. Fluorometric GUS assays were performed on seedlings of each line using 100 mg of tissue ground in GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosin, 0.1% Triton X-100). A sample of 475 ul was taken from the clarified extract and warmed to 37℃ for 1 h after the addition of 25 ul GUS assay buffer (2 mM MUG in GUS extraction buffer). The reaction was stopped by pipetting 500 ul of the reaction mixture into 1.95 ml carbonate stop buffer (0.2 M Na_2CO_3). A DyNA Quant 200 Fluorometer (Hoefer, Inc., San Francisco, CA) was used with a 4-MU standard solution to calibrate the instrument. For calibration, 1.9 ml of carbonate stop buffer was added to 100 ml of the 4-MU standard solution (1mM). The GUS activity was calculated as fluorescent units per mg fresh weight of tissue.

Overexpression plasmid construction and transformation

Two previously cloned genes *CcNAC1* and *CcNAC2* (Wang et al. 2013) and their cDNA were amplified with primers CcNAC1F/R and CcNAC2F/R. The resulting PCR products were fused into the pCR8/GW/TOPO entry vector, followed by transfer into pMDC32 via the LR reaction (Gateway^{R} Entry vector, Life Technologies) using the manufacturer's protocol. The recombinant pMDC32 plasmids containing 2X35S: CcNAC (CDS) insert were used for transformation into *Agrobacterium tumefaciens* LBA4404. *Arabidopsis* plants were transformed via the floral dip method (Clough and Bent 1998) and selected on hygromycin-containing medium. Homozygosity of transgenic plants was determined by segregation analyses based on the presence or absence of the hygromycin selection marker, and PCR analysis of the transgenic plants.

Different light/dark/IAA treatment of C. colocynthis

Citrullus colocynthis seeds (No. 34256) from Israel with high tolerance to drought were sown in potting mix in the greenhouse with a 14 h photoperiod and temperatures ranging from 22℃ to 33℃ and ambient relative humidity and light conditions (600-720 μmol $m^{-2}sec^{-1}$). The plants were exposed for 24h to red/far-red/ blue/ dark/white light treatments when the seedlings showed 2-3 true leaves. Light intensities used in the LED chamber were 60 μmol $m^{-2}sec^{-1}$ for far-red light, 90 μmol $m^{-2}sec^{-1}$ for red light, 30 μmol $m^{-2}sec^{-1}$ for blue light treatment using the method of Folta, 2005. Following treatment, true leaves from 2-3 seedlings were immediately placed in liquid nitrogen followed by RNA extraction and qRT-PCR analysis. For IAA treatment, the plants were transferred to water plus 10 μM IAA for 24h before freezing of true leaves in liquid nitrogen followed by RNA-extraction and qRT-PCR.

Different light/dark /IAA treatment and growth measurements in Arabidopsis

White light/dark treatment: Plants were grown in petri plates on ½ MS medium (pH=6.5, 15 g/L sucrose), either under 8h light/16h dark cycle or under continuous dark conditions, as noted in the text. For determination of the effects of auxin on growth of seedlings, a final concentration of 10 µM IAA was added to the ½ MS medium.

Different light treatments: Seeds of the homozygous lines were germinated on ½ MS medium for different light treatments for a 7 day period. The light intensities used (in the LED chamber) were the same as described for treatment of *C. colocynthis* seedlings.

Growth measurements: Hypocotyl length measurements were taken on wild type and overexpression lines from digitized images using ImageJ following 7 days of growth.

cDNA synthesis and relative quantitative (RQ) real-time RT-PCR

RNA was extracted from plant material using the Trizol (Invitrogen Life Technologies, Grand Island, NY) method. To eliminate the remaining genomic DNA, RNA was treated with Dnase I (Ambion Life Technologies) according to the manufacturer's instruction. cDNA was synthesized using RETROscriptTM (Ambion). qRT-PCR was carried out using a Bio-Rad, iCycler Real Time PCR (Hercules, CA) system and iCycler detection system software. The *C. colocynthis*-specific actin gene (ccActin154F/R), used as the reference gene, was amplified in parallel with the target gene, allowing normalization of gene expression and providing quantification. Primers were designed based on specific regions of *CcNAC1* and *CcNAC2*. Primers sequences of the *CcNAC1* (Q-NAC1F/R), *CcNAC2* (Q-

NAC2F/R) and Actin (ccActin154F/R) are listed in Table 2. Detection of RQ real-time RT-PCR products was conducted using the SYBR ® Green PCR Master mix kit (Applied Biosystems, Life Technologies). Quantification of the relative transcript levels was performed using the comparative CT method. The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to 2-ΔΔCT, where ΔΔCT= (CT, target gene, -CT, actin) treatment- (CT, Target-CT, actin) control. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in the target sample versus the reference sample (Pfaffl 2001).

Results

In silico analysis of CcNAC1 and CcNAC2 promoter

Numerous cis-acting elements such as ACE, AE, AT1, BOX4, and others have repeatedly been identified in light-regulated genes (Terzaghi and Cashmore 1995). Analysis of *CcNAC1* and *CcNAC2* promoter sequences as illustrated in Fig. 1 showed the presence of several putative and specific light-regulated elements, which are not commonly found in other plant promoters. The *CcNAC1* and *CcNAC2* promoter regions both contained the ACE, ATI, and GTI motifs, G-box, MRE and Sp1 elements, while the *CcNAC2* promoter additionally contained the BOX1, BOX4, the GAP-BOX and TCT motif. This suggests that *CcNAC1* and *CcNAC2* may be light regulated.

CcNACs expression in C. colocynthis under R, FR, B light

Since *C.colocynthis* CcNAC1 and CcNAC2 promoters were correlated with light motifs, the plants were exposed for 24h red/far-red/blue/white light treatments when the seedlings showed 2-3 true leaves. Afterwards, the leaves were immediately placed in liquid nitrogen for RNA extraction and qRT-PCR

analysis of *CcNAC1, 2* expression. The *CcNAC1* and *CcNAC2* expression patterns in *C. colocynthis* leaves were very similar under different light treatments (not significantly different). Compared with their expression level under white light, *CcNAC1* and *CcNAC2* expression were up-regulated by blue light, about 2 times. Red light enhanced their expression 10 and 7 times (Fig. 2), while far-red light treatment did not result in *CcNAC* gene expression level changes.

pCcNACs::GUS expression pattern and expression level under R, FR, B light

In order to gain a better understanding of the expression patterns of *pCcNACs::GUS* under R, FR, or B light, promoter-GUS transgenic lines for each NAC were generated and analyzed using GUS staining (Fig.3). *pCcNAC1::GUS* and *pCCNAC2::GUS* both showed limited expression throughout the seedling under standard white light conditions, with the strongest expression being in the stipule regions and little expression in roots and cotyledons (Fig 3A, 3E). Under red light, GUS expression became intense and ubiquitous in transgenic seedlings of both CcNAC1 and CcNAC2, including cotyledons, hypocotyls, and roots (Fig.3C and 3G). Blue light induced expression largely in cotyledons, but similar to white light expression in hypocotyls and roots. Far red light treatment resulted in low expression levels of both *pCcNAC1::GUS* and *pCcNAC2::GUS*. The *pCcNAC1::GUS* expression was detected at low levels in the shoot apical meristem, and *pCcNACs::GUS* at low levels in shoot apical meristem, the bottom of the hypocotyl and root (Fig 3D and 3H). Its tissue expression pattern under FR was quite similar to that under white light. Results from the GUS staining analysis indicated that red light up-regulated the expression of both pCcNAC1 and pCcNAC2-GUS transgenic plants, while far red light limited the expression of both promoter-regions of the NAC genes, and blue light induced expression in cotyledons (Fig. 3B and 3F),

and the level of expression under blue light was in between that of expression levels under red light and far-red light.

To accurately quantify the effect of blue, red, and far red light on *pCcNACs::GUS* expression, fluorometric GUS assays were conducted on the transgenic lines of *pCcNACs::GUS*. As shown in Fig. 4, the *pCcNAC1::GUS* expression level under blue light (N1-B) was 8.29 times higher than that of *pCcNAC1::GUS* under white light (N1-W). The *pCcNAC2::GUS* activity level under blue light (N2-B) was 11.34 times that of *pCcNAC2::GUS* under white light (N2-W). Red light resulted in even higher expression levels: *pCcNAC1::GUS* and *pCcNAC2::GUS* both showed 141.31 (N1-R) and 194.39 (N2-R) times higher levels of expression in red light as compared to those under white light. Far red light had the opposite effect as compared to other light treatments: *pCcNAC1::GUS* and *pCcNAC2::GUS* showed 0.31 and 0.53 fold changes, thus indicating that far-red light repressed the expression of both genes. The GUS assay results of Fig. 4 correspond to the GUS staining results shown in Fig.3 of *pCcNAC1::GUS* and *pCcNAC2::GUS*.

From the GUS expression patterns (Fig.3) and level of level of *pCcNAC1::GUS* and *pCcNAC2::GUS* (Fig.4) under different light conditions, we can conclude that R, FR, and B light have different impacts on the *CcNAC1/2* expression.

CcNACs expression patterns without light

QRT-PCR experiments were conducted to investigate *CcNACs* expression levels in *C. colocynthis* in the dark. As shown in Fig.5, the expression of *CcNAC1* and *CcNAC2* in *C. colocynthis* leaves was up-regulated in the dark. *CcNAC1* expression level was more than 7.5 times higher than its expression level white light, and *CcNAC2* expression level was more than 15 times higher as compared to its level under white light.

The expression patterns of *pCcNAC1::GUS* and *pCcNAC1::GUS Arabidopsis* plants in the dark are shown in Fig 6. Both promoters were mainly expressed in hypocotyls (Fig. 6A and 6D) and shoot apical meristems (Fig. 6B and 6E). Compared with their expression pattern under white light (Fig. 3A and 3E), higher levels were observed in the hypocotyls under the dark condition (Fig.6).

Photomorphogenic response of overexpression lines of CcNAC1 and CcNAC2 in the dark

To examine the impact of light on *CcNAC1* and *CcNAC2*, the photomorphogenic responses of *Arabidopsis* transgenic overexpression lines of *CcNAC1* and *CcNAC2* were studied. Hypocotyls are known to be strongly influenced by both external and internal cues known to regulate cell elongation such as light, gravity and hormones (Vanderbussche et al. 2005). Light signals do regulate gene expression and growth via a set of photoreceptors (Bertrand et al. 2005) and many genes are known to be regulated by light (Ma et al. 2001). Since *CcNAC1/2* might be associated with light regulation, the hypocotyls of the seedlings were first investigated under dark conditions. Seedlings grown under dark conditions are etiolated and have long hypocotyls and closed cotyledons, while seedlings grown in the light have a short hypocotyl and open cotyledons. Hypocotyl elongation/etiolation in the absence of light is the result of cell elongation (Gendreau et al. 2003). Under dark conditions the hypocotyls of the OE-*CcNAC1* and OE-*CcNAC2* lines were significantly (25%-30%) longer than WT hypocotyls, as shown in Fig. 7. Fig. 7C shows the average length of the WT hypocotyls and hypocotyls from overexpression lines. Hypocotyls from overexpression lines (>17.5 mm) were longer than WT hypocotyls (~ 15 mm).

Impact of Red, Far Red, and Blue light on growth of OE-CcNAC1 and OE-CcNAC2 lines

Under standard white light conditions, both OE-*NAC1* and OE-*NAC2* lines showed longer hypocotyls (~ 2 mm) than the wild type *Arabidopsis* (~1.75 mm) as shown in Fig. 8. Red and far-red light absorbing phytochromes are part of one of the major influential signaling network in plants. Phytochromes exist in two distinct but interconvertible forms in plants, the R light-absorbing Pr form and the FR light-absorbing Pfr form. The Pfr form is considered to be the active form, but there is also evidence suggesting that the Pr form has some biological activity (Shinomura et al. 2000). PhyA mediates the very low fluence response (VLFR) and the far-red high-irradiance response (HIR); phyB mediates both HIR to red light and the R/FR reversible low-fluence response (LFR) (Shinomura et al. 1996; Yanovsky et al. 1997). Our results (Fig. 8A and B) showed that R and FR light had different impacts on the overexpression lines of *CcNAC1* and *CcNAC2*. Red light enhanced the hypocotyl growth of overexpression lines, both OE-*CcNAC1* and OE-*CcNAC2* showed >6.0 mm of hypocotyl length, compared with WT (< 5.5 mm) hypocotyl length. On the contrary, FR shortened the hypocotyl length of overexpression lines. Both of the overexpression lines showed less than 2.8 mm of hypocotyl length, while WT seedlings showed longer hypocotyls (>2.8 mm).

Currently, two classes of blue light receptors have been identified in plants: the cryptochromes and the phototropins. Cry1 and cry2 are the most important members of cryptochrome family. Cry1 controls cotyledon expansion, anthocyanin formation and flavonoid biosynthetic enzymes. In addition to cry1, cry2 also mediates blue light-mediated inhibition of hypocotyl elongation and anthocyanin formation (Christie and Briggs 2001). In this study the effect of blue light on growth of hypocotyls of overexpression lines of *CcNAC1* and *CcNAC2* (Fig. 8A and B) was investigated. Under blue light, seedlings from WT and OE lines showed significant differences in hypocotyl lengths. Overexpression lines under blue light did show longer hypocotyls (1.0 mm for N1 and 1.16 mm for N2) as compared to

WT hypocotyls (0.8 mm). White light also had more effect on the hypocotyl length of OE-CcNAC1 (~1.75 mm) and OE-CcNAC2 (~1.9 mm) as compared to WT (~1.45 mm). Thus, based on results from GUS staining and assay analysis as illustrated in Fig. 3 and Fig. 4, it can be concluded that the enhanced expression of *CcNAC1* and *CcNAC2* did induce hypocotyl elongation under blue light and red light (Fig.8). On the contrary, the lower levels of expression of the two genes (Fig. 3 and 4) did result in repression of hypocotyl elongation under far-red light (Fig. 8). And OE-*CcNAC1, 2* impacts on hypocotyl lengths were partially isolated from their promoter effects, because we used constitutive35S promoters in overexpression lines. Although the hypocotyls phenotypes of OE lines were not significantly changed compared with WT, we can still draw the conclusion that *CcNAC1* and *CcNAC2* expression levels influenced hypocotyl lengths.

Auxin effects on CcNAC1 and CcNAC2 expression levels in C. colocynthis

Several mechanisms explain the correlation between light and hypocotyl elongation. It is known that auxin promotes hypocotyl, inflorescence stem and flower organ elongation via changes in gene expression (Chae et al. 2012). MSG2/IAA19 and NPH4/ARF4 are involved in tropic hypocotyl growth, and ARF2, ARF8, and ARF9 are involved in root and hypocotyl growth and development, although the function of Aux/IAA partners in these processes is not clear. The AFB5-5 mutant of auxin receptor shows almost complete resistance to picloram-induced hypocotyl growth (Greenham et al. 2011). H^+-ATPase is known to play a central role in auxin-induced hypocotyl elongation (Takahashi et al. 2012).

Hypocotyl length is affected by both cell number and cell elongation, and cell elongation is the major factor of hypocotyl length (Zhang et al. 2013). Some embryogenesis regulators might control the capability for hypocotyl elongation (Junker et al. 2012). The *Arabidopsis* TIM50 knockout can cause

mitochondrial structural changes and a reduction in intracellular ATP levels, which in turn control cell elongation in *Arabidopsis* hypocotyls through possible signaling via AMP kinase (Kumar et al. 2012). COP1/SPA can control hypocotyl elongation in response to low R:FR via auxin biosynthesis (Rolauffs et al. 2012). Circadian bursts of hypocotyl elongation occur when plants are exposed to constant light and light intensity can strongly influence growth rate. Sucrose can trigger PIF-dependent and –independent auxin response pathway and increase hypocotyl elongation (Lilley et al. 2012). By permissive gating of light-mediated hormone-related gene transcript levels to the proper time of day, circadian clock controls the seasonal and shade-appropriate plant growth, which includes the hypocotyl elongation (Nomoto et al. 2012). Since we found one auxin-related motif TGA-box in *CcNAC1* upstream (Fig.1A) and auxin levels are known to be correlated with hypocotyl growth, we conducted experiments to investigate the effect of auxin on these two *NAC* genes. As can be observed in Fig.9, *CcNAC1* and *CcNAC2* were down-regulated by IAA treatment in *C. colocynthis* (0.4 and 0.25 times, respectively).

The effects of auxin on localization of CcNAC1 and CcNAC2 under light

Light is able to manipulate plant growth and development through links to the auxin system (Halliday and Fankhauser 2003). Auxin transport through tissues and organs is integral to its action (Wu et al. 2010). The impact of light on auxin signaling is not likely to be as simple as the down regulation of auxin sensitivity or auxin levels to suppress cell elongation, because exogenous auxin does not substantially counteract the inhibitory effects of light (Tian and Reed 2001). It is known that auxin is synthesized in young leaves of the shoot system and transported downward to the root tip through the vasculature. Since light stimuli result in a directional transport of auxin, we investigated whether auxin could regulate gene expression in plant tissues. To understand the mechanism of auxin regulation of

CcNAC1 and *CcNAC2*, the effects of IAA treatment was investigated using *CcNAC1*-promoter:GUS (p*CcNAC1::GUS*) and *CcNAC2*-promoter:GUS (p*CcNAC2::GUS*) transformants. As shown in Fig. 10, the addition of IAA changes the expression of *CcNAC1* from shoot apical meristem (SAM) (A) to the bottom of the hypocotyl (B). The expression pattern of *CcNAC2* promoter under different levels of IAA was similar, since GUS expression was mainly detected in the apical meristem on ½ MS. However, *CcNAC2* showed high expression levels at the bottom of the hypocotyl, root tissue and SAM on ½ MS + IAA (Fig. 10D).

Manipulation of the CcNAC1 and CcNAC2 expression patterns under dark by auxin in early stage of growth

To better understand the effects of auxin on *CcNAC1* and *CcNAC2* expression with or without light, very young *Arabidopsis* seedlings transformed with *pCcNAC1*::*GUS* or *pCcNAC2*::*GUS* were grown in the dark on ½ MS and ½ MS + IAA. In 2-day old *Arabidopsis* seedlings, GUS expression was detected mainly in hypocotyls and cotyledons under normal light conditions as seen in Fig. 11A and D. *pCcNAC1*::*GUS* (Fig. 11B) and *pCcNAC2::GUS* (Fig. 11E) showed low levels of expression with IAA treatment under light. This phenomenon corresponds to qRT-PCR results from Fig. 9, which showed the inhibition of IAA on expression of these two genes. However, IAA treatment did induce their expression in the roots of transgenic plants under dark treatment (Fig. 11C and F).

Reversal of OE-CcNAC1 and OE-CcNAC2 phenotypes by auxin under dark conditions

To further study the effects of auxin on *CcNAC1* and *CcNAC2,* the photomorphogenic phenotypes of overexpression lines were investigated under dark conditions in the presence of 10 uM IAA. Hypocotyls of WT and overexpression lines growing on ½ MS and ½ MS + IAA were measured following a 7 day dark treatment. The addition of IAA to ½ MS medium resulted in significantly reduced growth of the hypocotyls of the *CcNAC1* and *CcNAC2* OE lines, as shown in Fig. 12 A and 12B. The length of hypocotyls from OE lines on ½ MS + IAA was significantly shorter, indicating that the growth of WT and OE hypocotyls was inhibited, especially in the OE lines. The average length of hypocotyls of WT seedlings was more than 7mm, while hypocotyls of both OE lines were less than 6 mm, indicating significantly reduced growth as compared to WT hypocotyl growth observed in Fig. 12C. The results indicate that the hypocotyls of OE-*CcNAC1* and OE-*CcNAC2* were sensitive to IAA, because IAA inhibited hypocotyl growth significantly. This phenomenon indicated that growth of overexpression seedlings was inhibited by auxin under dark treatment.

Light regulates many developmental processes, including seed germination and seedling photomorphogenesis (Lau and Deng 2010). Light and hormones control many of the same aspects of plant development (Kraepiel and Miginiac 1997). For example, auxin and gibberellins induce cell elongation, which can be inhibited by blue, red and far-red light perceived by cryptochromes and phytochromes (Kraepiel and Miginiac 1997). Romano et al. (1995) had demonstrated that the regulation of hypocotyl elongation by auxin and light are independent, since IAA overproducing plants exhibited normal responses to light. Auxin promotes hypocotyl elongation in light-grown seedlings, and several components of light signaling have been shown to affect auxin signaling (Valdés et al. 2012).

Here, our results indicate that in the presence of auxin under dark conditions, the *CcNAC* genes were expressed mainly in the *Arabidopsis* cotyledons (Fig. 11A and 11D) and roots (Fig. 11C and F) and

showed down-regulation in very young seedlings (Fig.11B and 11E). The phenotypes of OE-*CcNAC1* and OE-*CcNAC2* in the presence of auxin under dark conditions displayed shorter hypocotyls (Fig.12) than phenotypes without auxin in the dark (Fig. 7). In conclusion, the expression of the two *CcNACs* and the hypocotyls of OE-*CcNAC1* and OE-*CcNAC2* lines were regulated by auxin and light together. *CcNAC1* and *CcNAC2* both play roles in the signaling of auxin and light pathways.

Discussion

Critical responses to development and environmental stimuli are mediated by different transcription factors. Transcription factors play important roles in light mediated regulation of plant development. MYC2 and SPA1 transcription factors regulate the Z/G-box containing promoters, and mediate photomorphogenesis in *Arabidopsis* (Gangappa et al. 2013). HY5, a bZIP transcription factor, functions downstream of multiple photoreceptors and plays roles in promoting photomorphogenesis under diverse light conditions (Ulm et al. 2004). HY5 can directly bind to the ACGT-containing elements (ACEs) of the promoters of light-responsive genes (Zhang et al. 2011). PIF4 and PIF5 (also known as PHYTOCHROME INTERACTING FACTOR3-LIKE6) are crucial for the function of the ELF (EARLY FLOWERING) 4-ELF3-LUX (LUX ARRHYTHMO) complex (evening complex), which directly regulates plant growth diurnally (Nusinow et al. 2011). PhyB-mediated, post-translational regulation allows PIF3 accumulation to peak just before dawn, at which time it accelerates hypocotyl growth, together with PIF4 and PIF5, by directly regulating the induction of growth-related genes (Soy et al. 2012). COP1 stabilizes PIF in darkness. Phytochromes are known to facilitate PIF degradation and reduce COP1 activity. COP1 and PIF together mediate phytochrome degradation (Smirnova et al. 2012). Here, we identified two new transcription factors with functions in light regulation. The mechanisms of

light regulation on gene transcription have been reported in several publications. GT-elements are regulatory DNA sequences found in the promoter region of many different plant genes, with a positive or negative light-responsive function (Zhou 1999). HY5 also mediates the crosstalk between light and hormone signaling, including ABA, gibberellins and auxins (Oyama et al. 1997; Cluis et al. 2004; Lau and Deng 2010).

Light and auxin signaling pathways are intertwined (Lin and Wang 2005). IAA has been shown to directly interact with, and to be phosphorylated by oat phyA (Colón-Carmona et al. 2000). Some IAA mutants show different light responses, which result in hypocotyl changes both in the dark and in the light (Kim et al. 1998; Nagpal et al. 2000). Light has been shown to affect auxin metabolism, auxin transport, and auxin signaling/response (Lin and Wang 2005). In this study, we conducted functional characterization of *CcNAC1* and *CcNAC2* genes based on their promoter analysis and phenotypes under light and dark treatment. The promoters of both genes have light responsive motifs, which indicated that both genes were responsive to light. The responsiveness of two kinds of light and auxin factors provided future evidence for the correlation among *NAC* genes and those two signaling pathways. Under light conditions, IAA does regulate the localization of the *CcNACs* in GUS transgenic *Arabidopsis*. In the dark, the expression pattern of those two genes is changed, both in expression level and expression localization. Both of *CcNAC1* and *CcNAC2* promoters contain many light-related motifs, although the specific light regulation mechanisms of these two transcription factors need to be investigated in more detail. But in the dark, IAA could reverse the phenotype and expression patterns of *CcNACs* compared with those in dark condition alone, which means that the two signaling pathways have to be intertwined to regulate their genes expression.

The relationship between auxin and light has been studied in many plant species. Some auxin-associated element transcripts are expressed in a phyA- and PIF1-dependent manner, including two auxin resistance genes (AXR1 and AXR4), three auxin transport proteins (PIN1, PIN2, PIN7), a transcription factor (ATMYB34), and one auxin metabolism related gene (SUR1) (Ibarra et al. 2013). It was shown in stem elongation studies of pea (*Pisum sativum* L.) that the relationship between IAA content and stem growth is correlated with red, far-red and white light, but not correlated with blue light (Sorce et al. 2008). In this study, OE-*CcNAC1* and OE-*CcNAC2* hypocotyls were also influenced by white, red, blue and far red light. *BIG* gene is required for normal auxin efflux, and its mutant *asa1* can suppress the shade avoidance in *phyA phyB* null mutants in all organs other than the hypocotyl, which indicated that BIG does not only play a role in auxin signaling, but in light pathways (Kanyuka et al. 2003). Similarly to the *BIG* gene, our *NAC* genes did play roles in both auxin and light signaling.

Red light did lead to increases in both IAA biosynthesis in the apical region (meristem, cotyledons, and hook) of *Arabidopsis* seedlings and polar auxin transport in hypocotyls, leading to unchanged free IAA levels in the apical regions and increased free IAA levels in the more basal hypocotyl regions (Liu et al. 2011). It is unknown whether IAA synthesis and transport in our overexpression lines was changed, but the addition of auxin to the growth medium definitely changed the expression patterns of *CcNAC1* and *CcNAC2*. Since *NACs* function downstream of auxin and phytochromes, the *CcNACs* can function under normal light and auxin regulation. Under different light or auxin conditions, *CcNAC*s show different expression levels and localization, thus these genes are light and auxin signaling-related. *Terminal flower2* (*tfl2*) mutant, which carries a mutation in the *A. thaliana HETEROCHROMATIN PROTEIN 1* homolog, shows defects in both hypocotyl elongation and shade avoidance response. *PhyA* and *phyB* are epistatic to *tfl2* in far-red and red light. Light-dependent and

auxin-dependent genes are misexpressed in *tfl2* plants, and some of the genes overlap. Since TFL2 could bind to IAA5 and IAA19, it was suggested that TFL2 might be involved in regulation of phytochrome-mediated light response through auxin action (Valdes et al. 2012). From our experimental results, we can conclude that both auxin and light regulate *CcNAC1, 2* gene expression and there might be some correlation among auxin, light, *CcNACs*. Different kinds of light had different impact on *CcNAC1, 2* expression. It is known that COP1 is a key negative regulator in light signaling that can ubiquitinate positive transcription factors such as HY5 for degradation in the 26S proteasome pathway under different light conditions in *Arabidopsis* (Jia et al. 2013). Since different lights had different impact on the both expression patterns and phenotypes of *CcNAC1* and *CcNAC2* genes, the mechanism of the NAC TFs in the light signaling pathway remains to be investigated.

Conclusions

In this study, two *NACs* from *C. colocynthis* were further characterized for a function in light signaling and auxin signaling. R/FR and blue light were found to play major roles in regulation of *CcNAC1* and *CcNAC2*. *NAC* genes have many functions in plant species (Olsen et al. 2005; Wang and Dane 2013). This is the first report on the relationship between phytohormone, light and *NACs*. Different light and dark treatments resulted in quantitative and spatiotemporal changes in *CcNAC1* and *CcNAC2* expression patterns. However, the mechanism of NAC *gene* expression under light and auxin are unknown. The regulation of light might be controlled by specific elements in their promoter regions, which can be studied using GUS expression of different promoter regions. Also, native promoters linked to these two genes in transgenic plants could further explain the components of light regulation and their functional mechanisms.

CcNAC1 and *CcNAC2* also contain several stress related motifs in their promoter regions (Wang et al. 2014). Thus we propose that not only stress, but also light and auxin might regulate the expression of *CcNAC1* and *CcNAC2* genes in several ways, resulting in changes in localization of gene expression and gene expression levels and phenotype. It is important to know the detailed mechanisms of the regulation of these two genes, not only for understanding the correlation among *CcNACs*, light, phytohormones and stress, but also for future discoveries of *NACs* in other species. Research is needed to understand whether the interaction of auxin with *CcNAC*s in unique to this species. Techniques such as next generation sequencing, two-yeast hybridization could be used to detect other regulatory factors in overexpression lines of *CcNAC1* and *CcNAC2*.

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Table4. 1. Primers used for amplification of promoter regions of *CcNAC1*, 2

Primer	Sequence (5'-3')	Product length	Tm
pNAC1-F	AAAAATGTATGCATGTTGACTCAATATT	1585bp	60℃
pNAC1-R	GATTTCTTCTTTCCCTTTTTTCTTCTT		
pNAC2-F	ATCAAAAAATTTAGAAGTCAAACAAGACAT	1299bp	60℃
pNAC2-R	TTTTCTTCTGATATTTCTCCGGCG		

Table4. 2.Q-RT PCR primers for detection of relative expression levels of *CcNAC1*, 2

Primers	Sequence(5'-3')	Tm	Product length
ccActin 154F	CACCATCACCAGAATCCAGCACGA	59℃	140bp
ccActin 154R	GGCTCCACTCAACCCAAAGGCTAAC	59℃	140bp
Q-NAC1F	GTCAACCGAGAATGAAAGAAGAGTA	59℃	132bp
Q-NAC1R	TATACATGAACATATCCTGCAATGG	59℃	132bp
Q-NAC2F	GTGCCGGATTTACAACAAGAA	59℃	106bp
Q-NAC2R	AATCTTCGGCTTCTCGCTTC	59℃	106bp

Figure 4. 1. Motifs of *CcNAC1* promoter (A) and *CcNAC2* promoter (B).

Elements found were identified by *in silico* analysis at the Plant CARE database. The legends with different motifs are shown at the bottom of each picture. In Fig.1A, the -307 site is auxin-responsive element TGA-box.

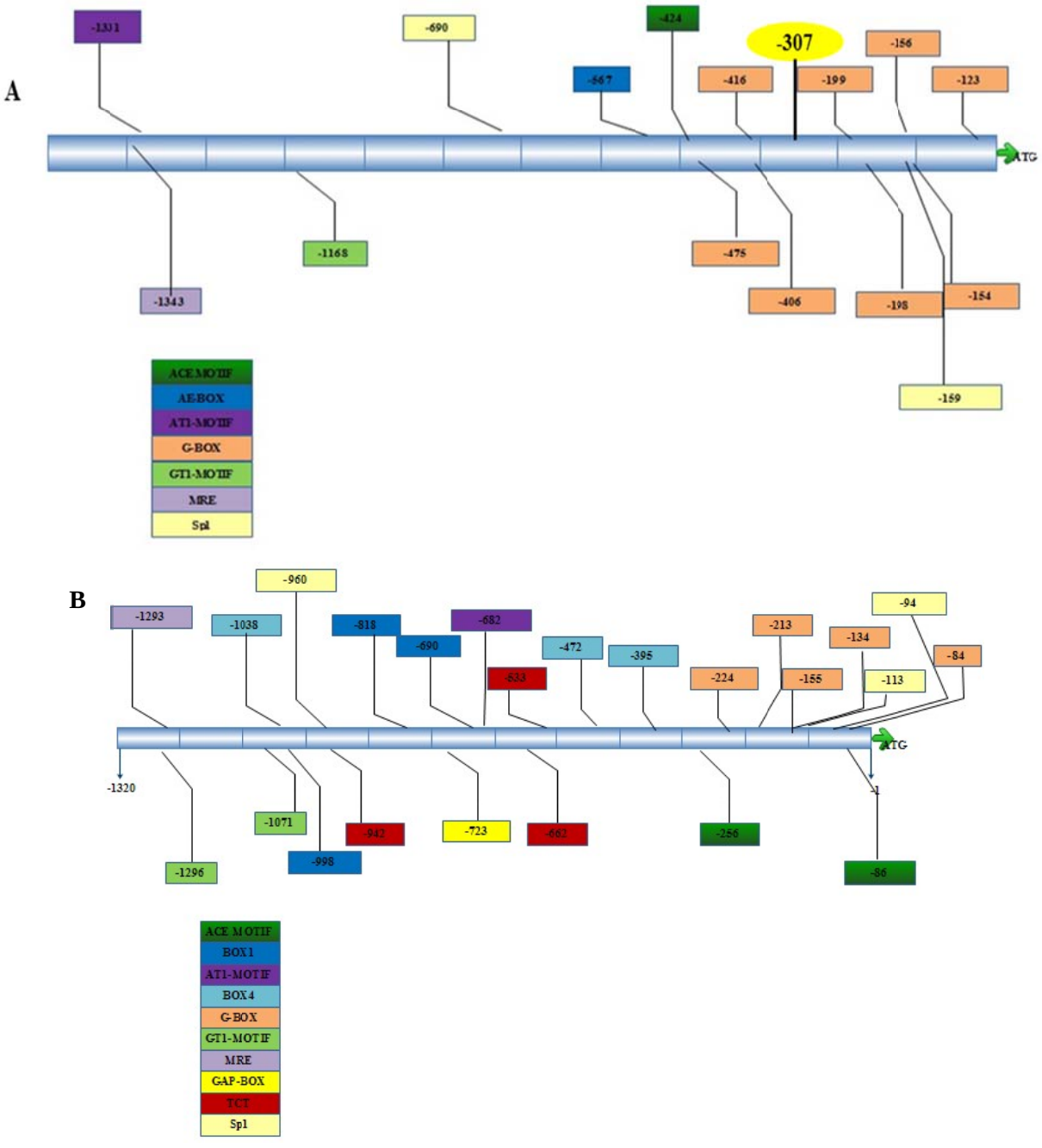


Figure 4. 2.CcNAC1, 2quantitative expression under different light in *C.colocynthis*.

The gray color indicates *CcNAC1* and black color *CcNAC2* expression. The asterisk indicates the expression is significant compared with its expression under white light.

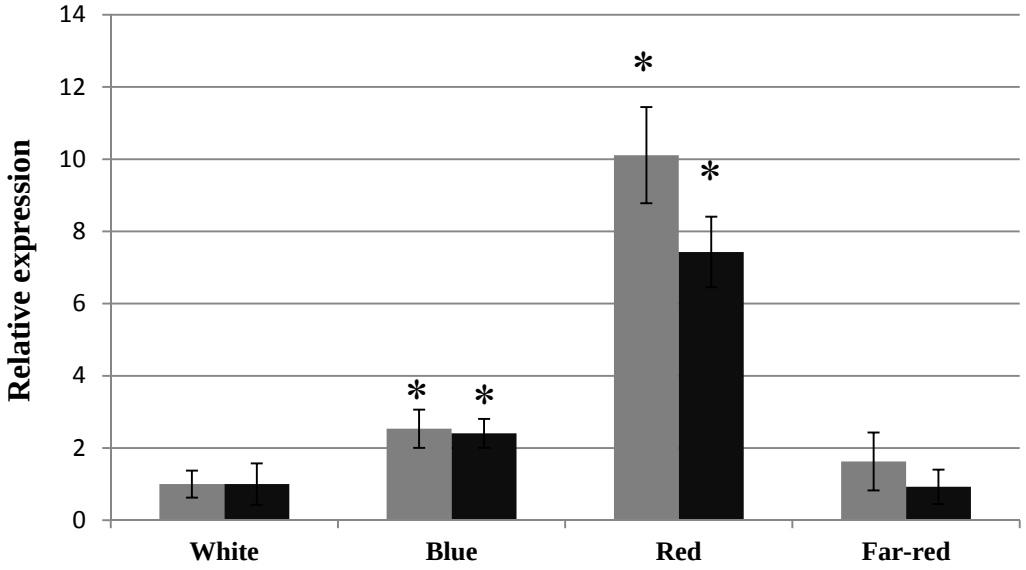


Figure 4. 3. *pCcNAC1, 2* :: *GUS* histochemical assays under different light.

A, B, C, and D show CcNAC1-promoter region in *Arabidopsis*. E, F, G and H show CcNAC2-promoter regions in *Arabidopsis*. A and E seedlings were grown under white light, B and F under blue light, C and G seedlings under red light, D and H seedlings under far-red light. The bar indicates 0.25 cm.

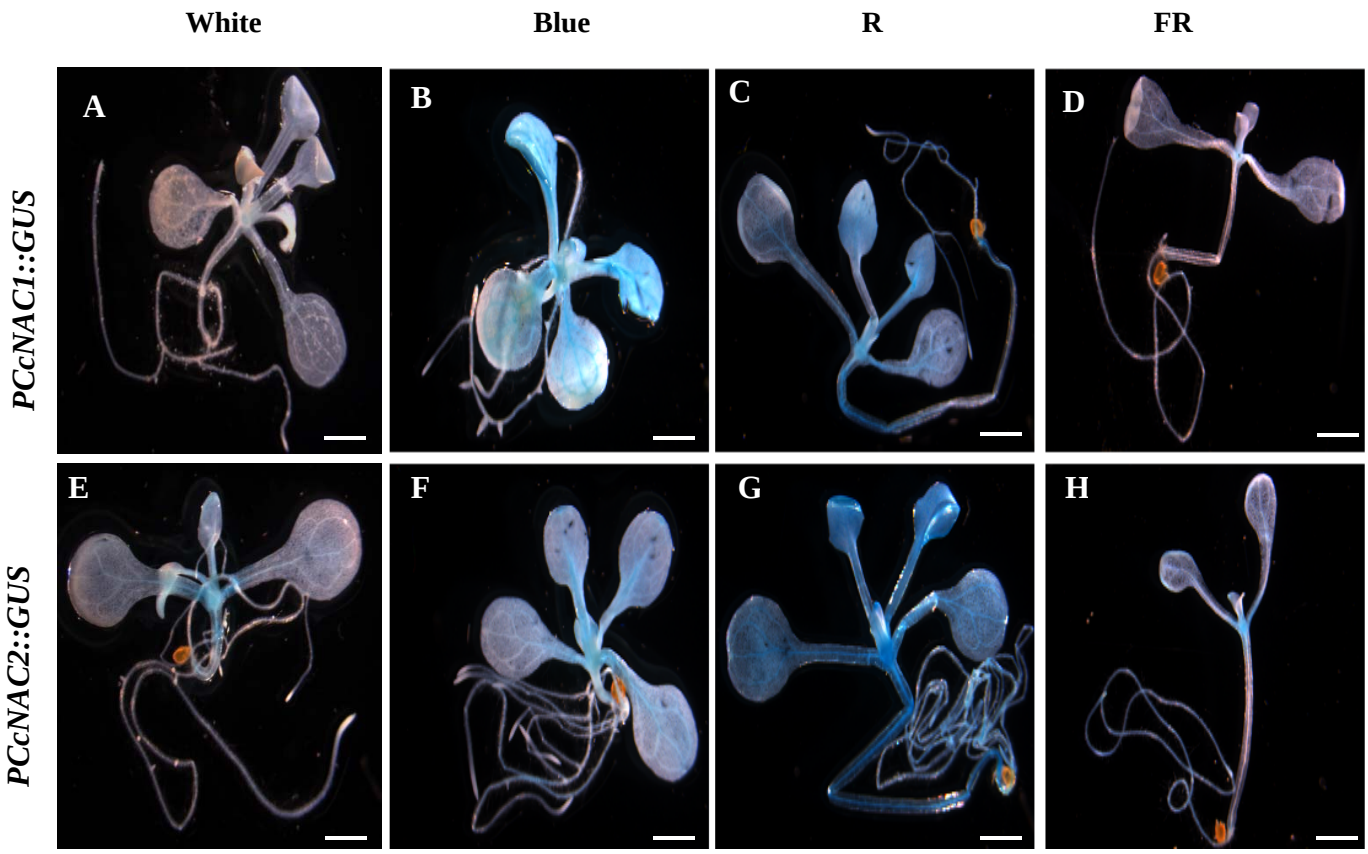


Figure 4. 4. GUS assay of *pCcNAC1::GUS* and *pCcNAC2:: GUS* under different light.

Seedlings or seeds were germinated under and treated for 7 days. N1 and N2 stand for CcNAC1 and CcNAC2 separately. N1-W and N2-W were treated under white light; N1-B and N2-B were treated under blue light; N1-R and N2-R were treated under red light; N1-FR and N2-NR were treated under far red light. Fold induction is shown in front of this figure, and number indicates the fold induction of GUS as compared to white light of each transgenic line. GUS activities represent the mean values of at least 5 different lines of transgenic *Arabidopsis*.

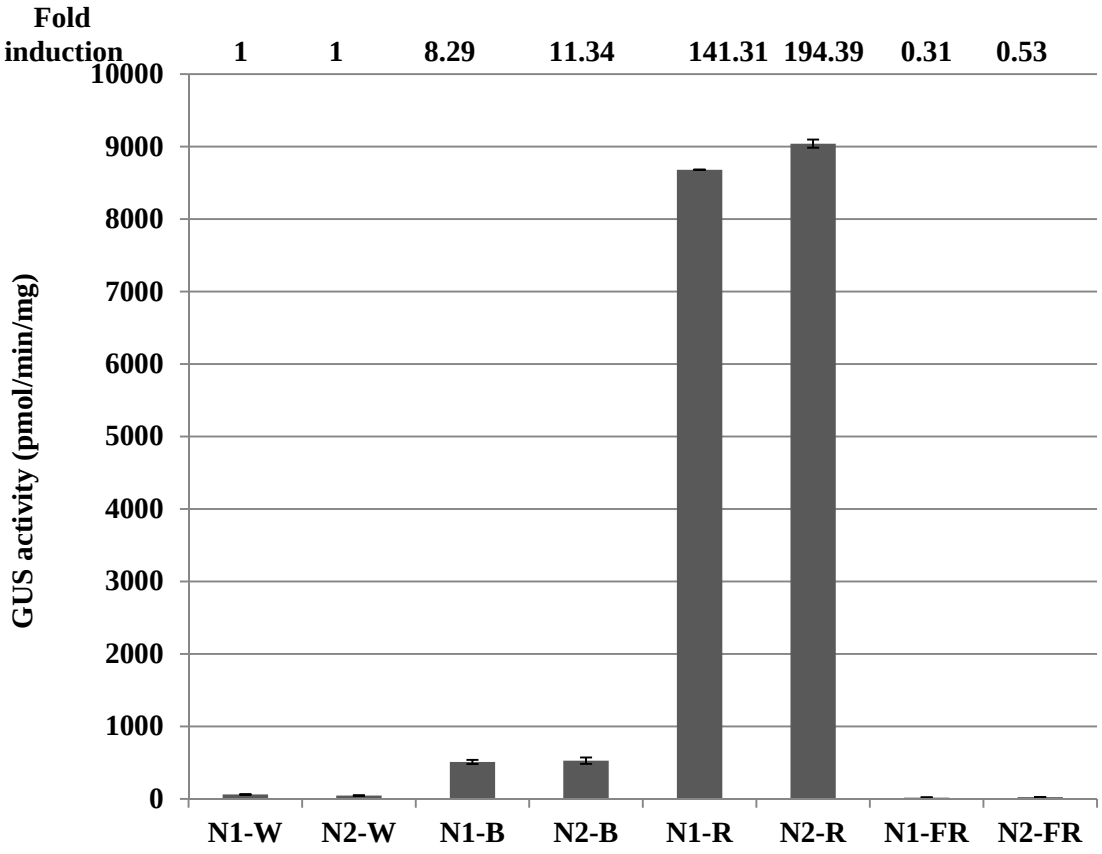


Figure 4. 5. *CcNAC1* and *CcNAC2* expression pattern in *C. colocynthis* leaves without light.

The relative *CcNAC1* (gray) and *CcNAC2* (black) expression levels were normalized to the expression levels under white light by qRT-PCR.

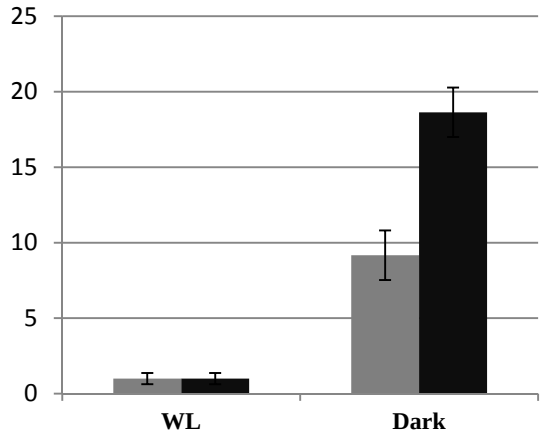


Figure 4. 6. *pCcNAC1::GUS* and *pCcNAC2::GUS* expression in *Arabidopsis* under dark.

The bar indicates 0.25cm.

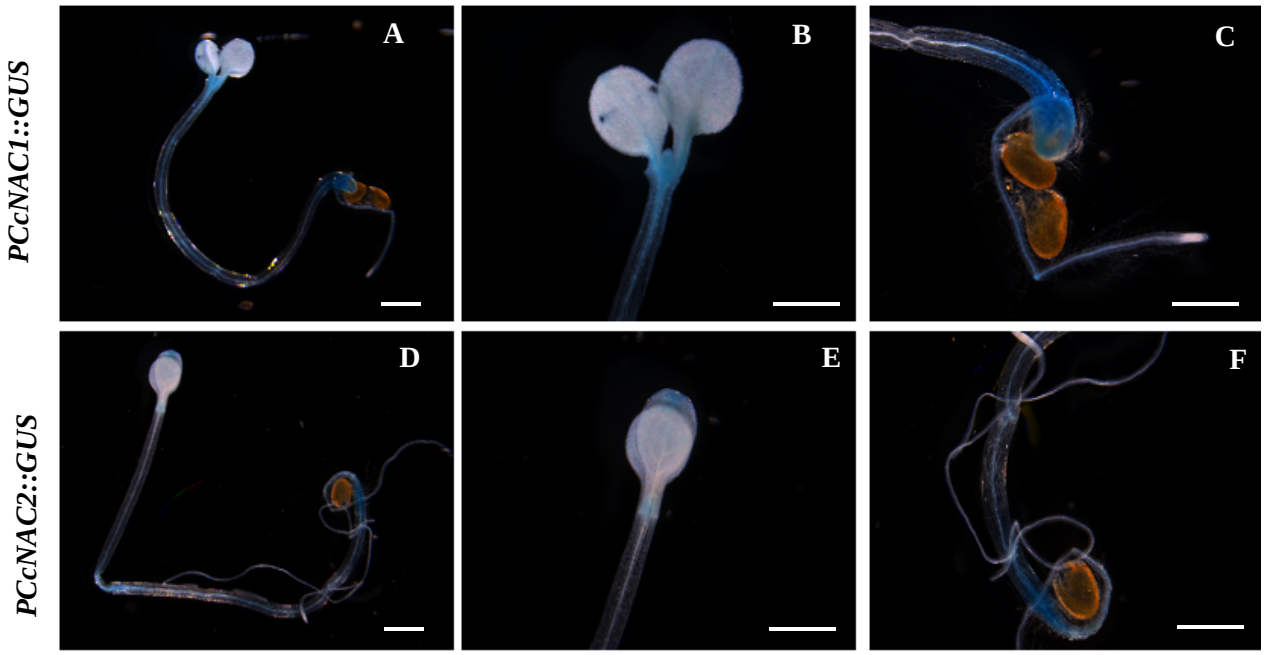


Figure 4. 7. Comparison of hypocotyl lengths of OE-CcNAC1 and OE-CcNAC2.

(A) and (B): Comparison of hypocotyl lengths of overexpression lines of *CcNAC1* or *CcNAC2* and WT *Arabidopsis* following 7d of darkness. A and B were grown on ½ MS medium. The bar indicates 5mm.

(C) Hypocotyl length measurement of WT and overexpression lines. N1-1 and N1-2 are different lines of OE-*CcNAC1*, N2-1 and N2-2 are different lines of OE-*CcNAC2*. WT = wild type *Arabidopsis*. Hypocotyl length was measured in mm. Values are means ± SD of more than 20 independent replicates.

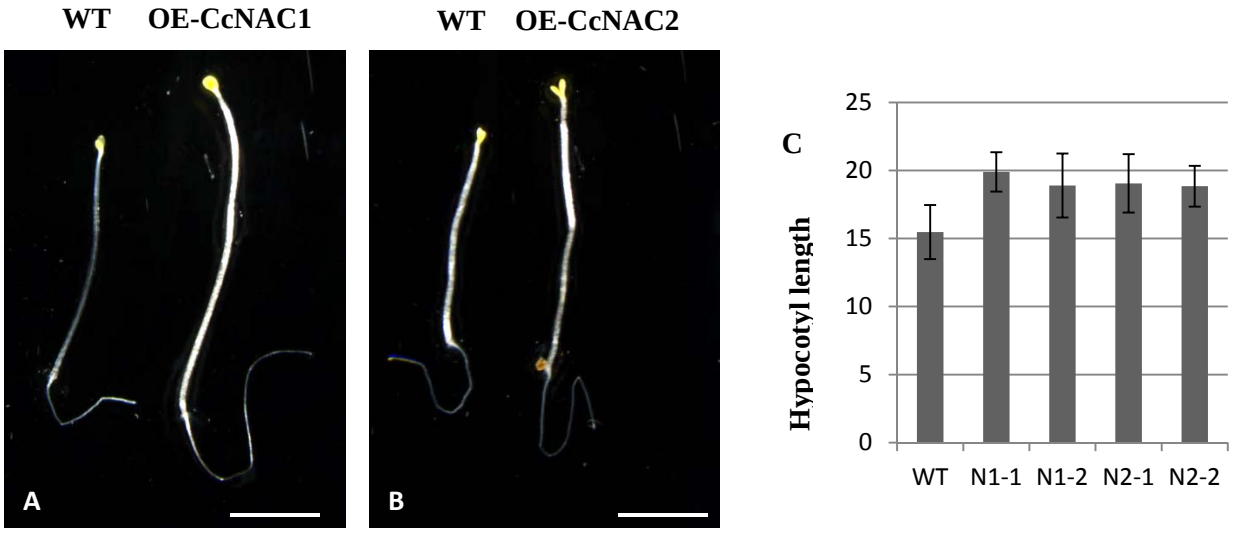


Figure 4. 8 Different light impact on hypocotyle length of OE-*CcNAC1* or *CcNAC2* .

(A) Hypocotyl comparison of overexpression lines of *CcNAC1* or *CcNAC2* with WT *Arabidopsis* under different light treatment. W, B, R, FR are instead of white light, blue light, red light and far-red light. Each treatment contains three seedlings, which are wild type, OE-N1 and OE-N2. The bar indicated 0.25mm. (B) Hypocotyl length of WT, OE-*CcNAC1* (N1) and OE-*CcNAC2* (N2) seedlings after growth under different light conditions. The light conditions include white light, blue light, red light and far-red light treatment. Seedlings were grown continuously in the indicated light treatment for 7 days. Values shown are means ± SEs of 20 seedlings.

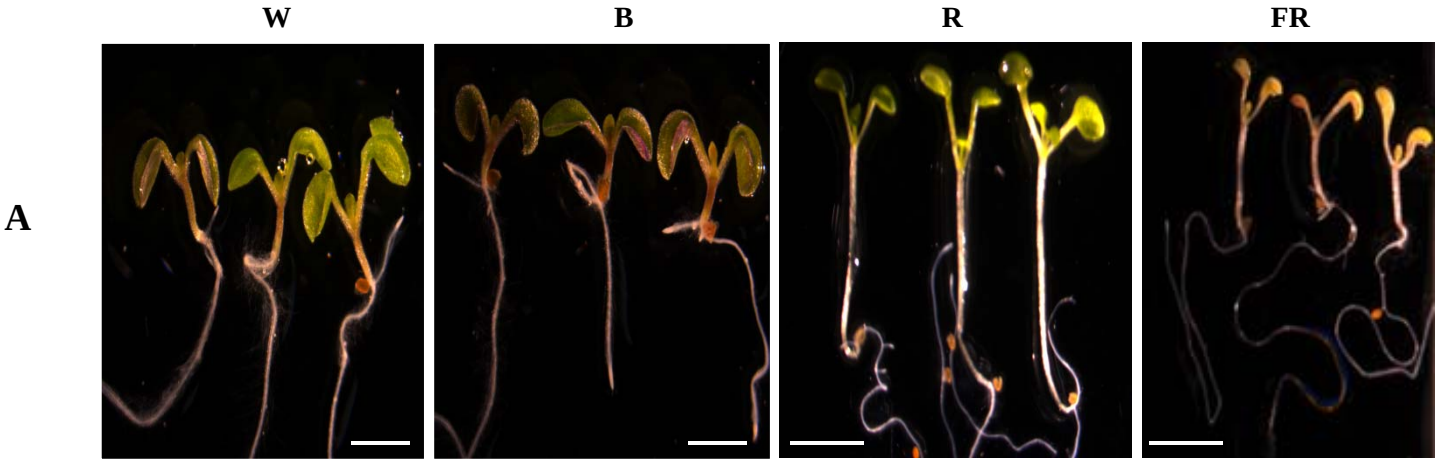


Figure 4. 9. *CcNAC1 , 2* expression pattern with and without IAA in *C. colocynthis*.

The relative *CcNAC1* (gray) and *CcNAC2* expression levels were normalized to the expression levels under white light by qRT-PCR. W/O IAA: without IAA ; W/IAA : with IAA.

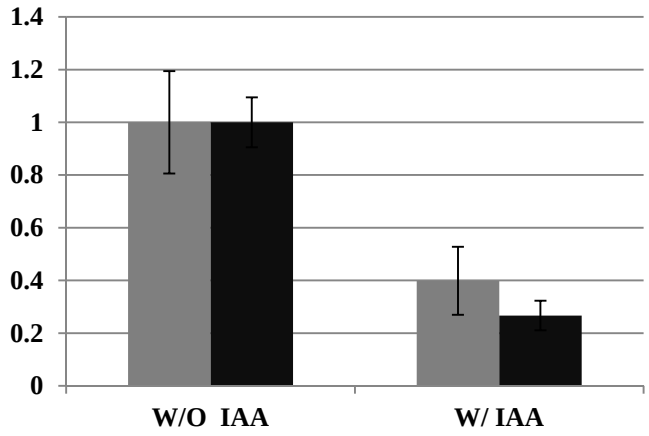


Figure 4. 10. *pCcNAC1:: GUS* and *pCcNAC2::GUS* under normal light conditions.

A, and B show *CcNAC1*-promoter transgenic *Arabidopsis*. C and D *CcNAC2*-promoter transgenic *Arabidopsis*. A and C seedlings were grown on ½ MS medium, B and D seedlings on ½ MS with 10µM IAA. The bar indicates 0.25 cm.

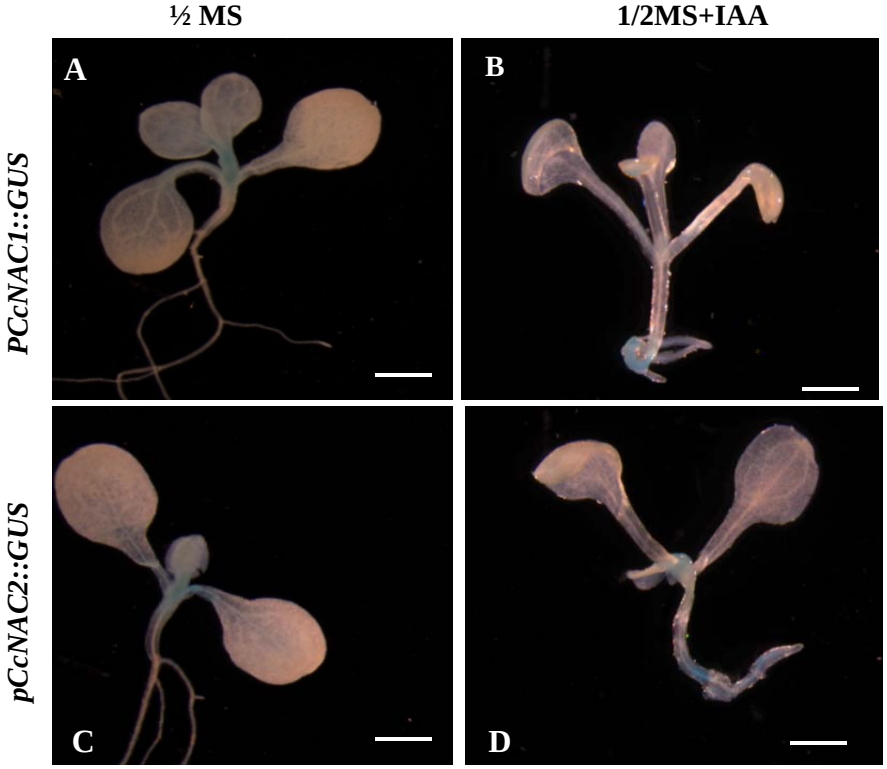


Figure 4. 11. IAA and light regualte *pCcNAC1::GUS* and *pCcNAC2::GUS* together.

A, B and C show *pCcNAC1::GUS* expression in *Arabidopsis* ; D, E and F show *pCcNAC2::GUS* expression in *Arabidopsis* .A and D are 2-days old seedlings on 1/2MS with white light; B and E are 2-days old seedlings on 1/2MS +IAA under white light; C and F are 2-days old seedlings on 1/2MS+IAA under dark. The bar indicates 1mm.

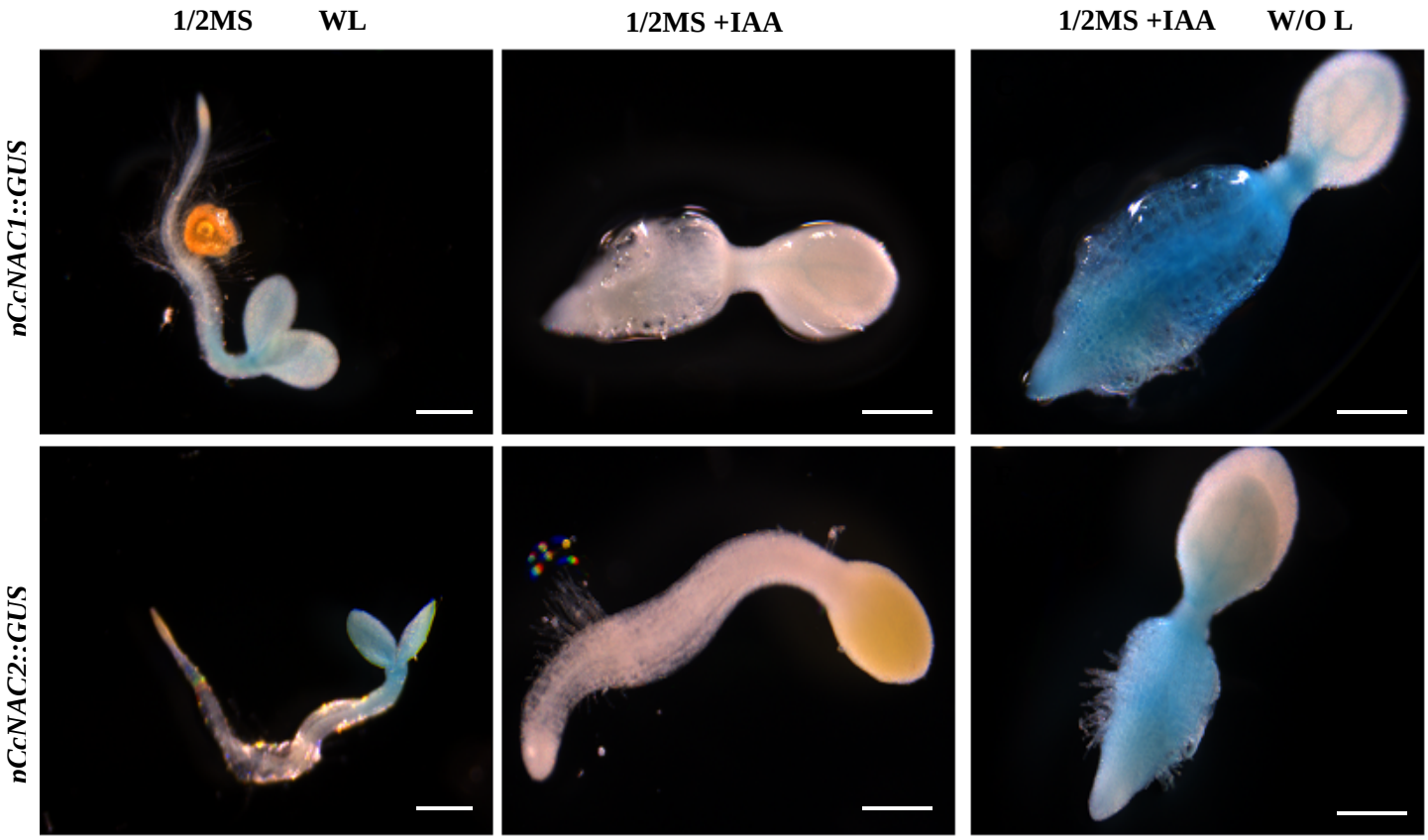
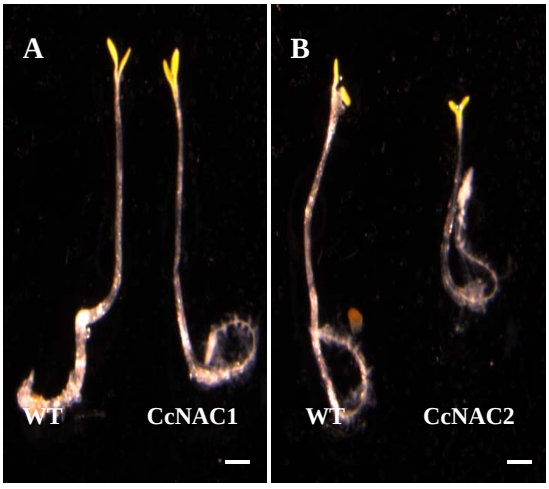
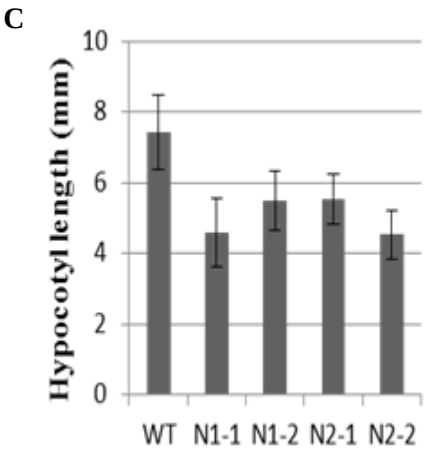


Figure 4. 12. IAA reversed impact on OE- *CcNAC1* or *CcNAC2* under 7d dark .

(A and B) Hypocotyl comparison between overexpression lines of *CcNAC1* or *CcNAC2* with WT *Arabidopsis* on ½ MS+IAA under 7 d dark . A shows the comparisons between WT and OE-*CcNAC1* seedlings, while B shows the comparisons between WT and OE-*CcNAC2* seedlings. The bar indicated 0.2mm.

(C) Hypocotyl length (in mm) of overexpression lines of *CcNAC1* and *CcNAC2* with WT 7 day-old *Arabidopsis* under dark. N1-1 and N1-2 were independent lines of OE-*CcNAC1*, N2-1 and N2-2 were independent lines of OE-*CcNAC2*. Values are the means ± SD of more than 20 independent replicates.





V Analysis of the *Citrullus colocynthis* transcriptome during water deficit stress

Abstract

Citrullus colocynthis is a very drought tolerant species, closely related to watermelon (*C. lanatus* var. *lanatus*), an economically important cucurbit crop. Drought is a threat to plant growth and development, and the discovery of drought inducible genes with various functions is of great importance. We used high throughout mRNA Illumina sequencing technology and bioinformatic strategies to analyze the *C. colocynthis* leaf transcriptome under drought treatment. Leaf samples at four different time points, day 1 (D1), day 2 (D2), day 3 (D3) and day 4 (D4), were used for RNA extraction and Illumina sequencing. qRT-PCR of several drought responsive genes was performed to confirm the accuracy of RNA sequencing. Leaf transcriptome analysis provided the first glimpse of the drought responsive transcriptome of this species. A total of 5038 full-length cDNAs were detected, with 2545 genes showing significant changes during water deficit conditions (at D2, D3 or D4) as compared to D1. Principle component analysis indicated that drought was the major contributing factor regulating transcriptome changes. Up regulation of many transcription factors, stress signaling factors, detoxification genes involved in phytohormone signaling occurred in *C. colocynthis* throughout the water deficit experiment. Similarly citrulline metabolism genes were induced under water deficit conditions. The transcriptome under water deficit thus showed transcripts, which might be the candidate genes in

the defense of drought stress, and might provide an important resource for plant research and crop genetic improvement.

Keywords

Citrullus colocynthis, drought, transcriptome

Introduction

Water is essential for plant growth in modern agriculture (Guo and Tan 2013). Drought delays the development of crops, and strongly affects morphology, as well as physiological processes like transpiration, photosynthesis, respiration and translocation of assimilates (Do et al. 2013). Drought avoidance can be achieved through morphological changes in plants, such as decreased stomatal conductance, reduced leaf area, and extensive root systems (Levitt 1980). Drought tolerance is achieved by physiological and molecular mechanisms, including osmotic adjustment, antioxidant and scavenger compounds (Bartels and Sunkar 2005). Both strategies involve the induction of specific genes and proteins, such as dehydrins (dehydration-induced proteins), key enzymes for osmolyte biosynthesis, and detoxification enzymes (Reddy et al. 2004; Shinozaki and Yamaguchi-Shinozaki 2007).

Plant species have developed diverse strategies to adapt and thrive in all kinds of climates and terrains and evolved to deal with extreme changes in the environment. These strategies are supported by rich and complex metabolic networks that enable the plant to synthesize a wide range of compounds. Plant responses to abiotic stresses involve interactions and crosstalk between many molecular pathways. High throughput screening techniques such as transcriptome sequencing have been used to study the adaptability of plants to drought (Zhao et al. 2013). This led to the discovery of many drought related genes. For example, PIP aquaporins were found to fine-tune the environment in response to declining water availability (Sŭrbanovski et al. 2013). However, few natural allelic variants have been cloned for drought related traits, so QTL, RNA sequencing and other methods are needed to improve methodology for exploring complex multivariate data (Juenger 2013; Caccamo and Grotewold 2013).

The cucurbit family is a large family with several economically important species, such as watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and several *Cucurbita* species with edible fruits (Jagadeeswaran et al. 2012). *Citrullus colocynthis* (L.) Schrad (2n=2x=22), the bitter apple, closely related to domesticated watermelon (*Citrullus lanatus* var. *lanatus*), is a very drought-tolerant perennial herbaceous species in the Cucurbitaceae family (Jeffrey 2008). It can survive arid environments by maintaining its water content under severe stress conditions. *C. colocynthis* is an important medicinal plant and a source of valuable oil (Burkill 1985; Dane et al. 2006). Its seeds were found in several early Egyptian, Libyan and Near Eastern sites from about 4000 BC (Zohary and Hopf 2000). This species grows in sandy areas throughout northern Africa, southwestern Asia and the

Mediterranean region (Zamir et al. 1984, Burkill 1985, Jarret et al. 1997). The species has been used as a model to elucidate the function of genes implicated in the stress response ultimately leading to enhancement of stress tolerance in cucurbit crops through genetic manipulation. Si et al. (2009) found dynamic gene expression changes in *C. colocynthis* root tissues by cDNA amplified fragment length polymorphism (cDNA-AFLP) technique.

Several research groups have used next generation sequencing technologies to study gene expression profiles in species of the cucurbit family. For example, Guo et al (2011) used 454 sequencing technology to study the comprehensive profile for watermelon fruit flesh tissues, while Grassi et al (2013) studied carotenoid pathway regulators in ripening watermelon fruit. The draft genome of watermelon (*C. lanatus*, 2n=2x=22, ~ 425 Mb) was analyzed by Guo et al (2013) using three different watermelon subspecies. Comparative genomic analysis provided an evolutionary scenario for the origin of the 11 watermelon chromosomes derived from a 7-chromosome paleohexaploid eudicot ancestor. The genome sequence of cucumber (*Cucumis sativus*, 2n=2x=14) has been completed, and the genome of melon (*Cucumis melo*, 2n=2x=24) is being sequenced under the Spanish Genomics Initiative (MELONOMICS) (Huang et al. 2009). Liu et al (2013) used sequencing techniques to identify conserved and novel miRNA in watermelon, while Wincker (2013) used comparative analysis of genomes between watermelon and sweet orange to detect the traits related to their domestication.

Here, high-throughput sequencing of the leaf transcriptome from *C. colocynthis* provides a glimpse at drought related genes in this uniquely drought tolerant cucurbit species, which

should facilitate the identification of valuable multiple genes, needed for complex interactions of plant species with the environment.

Results and Discussion

Transcriptome assembly results

The transcriptome of *C. colocynthis* leaves following 4 days of drought stress was assembled and assessed following paired-end (2*50bp) Illumina sequencing. The Illumina platform yielded an average of 24 million high-quality reads per sample (Table 1). All sample reads were used to construct a *de novo* assembly, and a reference assembly using the completely sequenced watermelon genome. A total of 20,581 contigs were generated (Table 2). The contigs had an average length of 1350 bp and N50 of 1870 bp. BlastX was used against SwissProt and ESTscan of translated protein sequences to detect full length cDNAs. A total of 5,038 full length cDNAs were detected in our sequencing assembly.

Principle component analysis (PCA) was implemented in the CLC workbench. The results (Fig.1) illustrate differential gene expression patterns in *C. colocynthis* seedlings following four days of withholding water. Gene expression patterns on D1 and D2 were quite similar, while results from D3 and D4 showed more differences as compared to D1 and D2, and gene expression patterns detected on D4 were very different from the other time points as a result of drought stress.

Differentially expressed genes involved in the response to drought stress in C. colocynthis

The paired-end reads were mapped to the reference genomes after filtration using the CLC genome workbench. Read counts of each unigene were converted to reads per million (RPM). The read number of each cDNA was divided by the total number of reads per day (1, 2, 3 or 4) from the data set, and multiplied by 10^6. Statistical analysis was conducted using Kal's test in the CLC workbench ($P<0.05$ and fold change ≥ 1.5). Genes showing non-significant and significant changes in read counts are shown in *Table S1*. Each sample was compared to the day 1 sample reads for analysis of their significance level of gene expression. The read results indicated that 59 genes showed significant changes at D2, D3, and D4 as compared to D1; 13 genes showed significant differential expression at D2 and D3 as compared to D1; 30 genes showed significant differential expression early, at D2, as compared to D1; 897 genes showed changes at D3 and D4 as compared to D1; 341 genes were only differentially expressed at D3; 1191 genes were regulated late, at D4 only, under drought stress. In conclusion, the *C. colocynthis* gene expression patterns showed dramatic changes with 2545 genes showing significant changes, mostly occurring late under drought conditions (D3 and D4 of withholding water).

The heat map depicted in Fig. 2 corresponded to the principal component analysis. D3 and D4 transcripts were clustered together, and D1 and D2 transcripts were clustered. Also

significant changes were seen D3 and D4 suggesting that the transcriptional response of many genes was up-regulated during drought. Strong effects were especially observed on D3 and D4.

Gene ontology (GO) classification

To functionally categorize significantly changed genes in *C. colocynthis* under drought treatment, gene ontology analysis by Blast2go was performed. *C. colocynthis* unigenes were categorized in three main GO categorizes: biological process (2672), molecular function (1368) and cellular component (1053). These GO terms were further divided into several sub-categories (Fig. 3). In the biological process category, single organism process genes accounted for more than 20% of the biological process genes. In the catalytic activity category, more than 40% of genes were associated with a molecular function. In the cellular category, more than 35% of the genes were associated with the cellular component.

Validation of Illumina expression patterns by qRT-PCR analysis

To confirm the reliability of the Illumina sequencing read analysis, 8 candidate genes were selected and their expression was compared at D4 and D1 using qRT-PCR. The expression patterns resulting from qRT-PCR showed general agreement with those from the Illumina sequencing analysis (Table 3). Discrepancies with respect to ratio of fold changes between sequencing and qRT-PCR analysis can be attributed to the essentially different algorithm and

sensitivity of the two techniques (Shi et al. 2012). In the deep-sequencing method the absolute expression rather than relative expression as in qRT-PCR analysis is used. Transcriptional qRT-PCR analyses of 16 genes during the drought treatments are shown in Fig.4.

Gene Comp5873 is homeobox-leucine zipper protein, with significant upregulation during all days of drought in *C. colocynthis* (Fig. 4). It is known that the expression of several homeobox-leucine zipper proteins is correlated to stress. Athb-12, a homeobox-leucine zipper domain protein from *Arabidopsis,* is functionally involved in salt tolerance in yeast (Shin et al. 2004). Hahb-4, a homeobox-leucine zipper gene is potentially involved in water stress in sunflower (Gago et al. 2002).

Comp862 belongs to the glutathione S-transferase (GST) family, which contains heterogeneous, multifunctional dimeric proteins. This gene is highly up-regulated (60x) during drought in *C. colocynthis*. It is thought that GSTs are involved in cellular detoxification (Park and Choung 2010). Comp1108 is a member of the NAC gene family and NACs are known to be involved in numerous biological processes, including drought stress (Shaminuzzaman and Vodkin 2013, Wang et al. 2013).

Comp10156 is a member of the GID1 (GIBBERELLIN INSENSITIVE DWARF1) family in *C. colocynthis* with high expression under drought conditions. GID1 is a soluble GA receptor in rice (Ueguchi-Tanaka et al. 2005). GA-GID1 complex interacts with DELLA proteins which are negative regulators of GA signaling pathway (Richards et al. 2001).

RD22, a known dehydration responsive gene in *Arabidopsis*, which is mediated by ABA, may have physiological and molecular significance for processes underlying memory functions of plants in response to ABA and light pulses (Yamaguchi-Shinozaki et al. 1992; Goh et al. 2003). One RD22-like protein from soybean can alleviate salinity and osmotic stress (Wang et al. 2012). RD22-like genes (Comp 6528) with significant up-regulation (>160x) under drought in *C. colocynthis* were confirmed by qRT-PCR, especially after days of withholding water.

NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE1) plays an important role in coordinating broader cellular processes in response to stress and bacterial pathogen infection (Knepper et al. 2011). The chemical ß-aminobutyric acid, which is known to induce resistance in plants, primed the expression of many genes, and NDR1/NHL10 was one of them (Chen et al. 2013). Comp7317 gene, which is a member of NDR1/NH10 showed significant changes at D2 only during the early stage of water deficit stress.

GRAS (for GA Insensitive, REPRESSOR of gal-3 (RGA), SCARECROW (SCR)) transcription factors, have a major function in plant development and environmental adaption. These TFs are particularly implicated in the modulation of plant tolerance to stressors as cold, drought, salinity by crosstalks via GA to ABA-dependent and ABA-independent pathways (Golldack et al. 2013). For example, SCL7 confers salt and drought tolerance in *Arabidopsis* (Ma et al. 2010). SCL14 is involved in the detoxification of xenobiotics and possibly endogenous harmful metabolites (Fode et al. 2008). Comp20554, which belongs to the GRAS transcription factor family, showed significant up-regulation especially on D2.

The expression profile of Comp3048, which codes for a methyltransferase, maintained high levels during drought stress, especially on D2. It was found that myo-inositol-O-methyltransferase (Imt1) responded to low temperature stress in transgenic *Arabidopsis* (Zhu et al. 2012). Trithorax-like H3K4 methyltransferase from barley is drought inducible (Papaefthimiou and Tsaftaris, 2012). The methylation of myo-inositol catalyzed by myo-inositol methyltransferase (IMT) occurs when plants are under abiotic stress. Over-expressing of IMT resulted in improved tolerance to dehydration and salt stress treatment in *Arabidopsis*. (Ahn et al. 2011).

Heat-shock proteins (HSPs) are environmentally induced proteins that enable plants to cope with heat and other environmental stresses. For example, *Trichoderma harzianum* Hsp70 transgenic *Arabidopsis* is abiotic stress tolerant (Montero-Barrientos et al. 2010). Similarly HSP22 was found to be highly upregulated in *C. colocynthis* roots during drought conditions (Si et al. 2009). Overexpression of GmHsp90S can decrease damage of abiotic stresses in *Arabidopsis* (Xu et al. 2013). Comp14675 belongs to the HSP family, and showed up-regulation at later stages from D3 to D4.

Plant cold shock proteins (CSP) are very conserved among various plant genera (Karlson and Imai 2003). The first CSP identified was WCSP1 from winter wheat, which did accumulate in response to low-temperature stress (Karlson et al. 2002). Similarly in *C. colocynthis* CSP (Comp13927) was up-regulated during later stages of water deficit.

Comp2553, one MA3 domain-containing protein gene, showed significant changes on D3 and D4 under drought conditions. Loss-of-function of ECIP1 (one MA3 domain-containing protein) resulted in enhanced ethylene response but altered salt response (Lei et al. 2011) in *Arabidopsis*.

Overexpression of plant BL-1 in *Arabidopsis* resulted in the attenuation of cell death induced by biotic stresses (pathogens) and abiotic stresses such as heat, cold, drought, salt and chemical-induced oxidative stresses (Ishikawa et al. 2011). BL-1 might function to control the level of the "pro-survival and pro-death" signals under multiple stress conditions in plants (Watanabe and Lam, 2009). For example, cucumber BAX inhibitor-1 is a conserved cell death suppressor induced by cold stress and a negative regulator of programmed cell death (PCD) (Chen et al. 2013). Comp372 encodes one BL-1 gene, which was up-regulated by drought at later stages.

Comp8117, which is a homologue of a *Populus* EST (CU233481.1), is a drought stress related gene, up-regulated at D4. Comp10586, which encodes a member of the MYB transcription factor family, is up-regulated during the late stage of drought stress. Some MYB members have been shown to regulate plant responses to biotic and abiotic stress conditions. For example, MdoMYB121 in apple confers abiotic stress tolerance in plants (Cal et al. 2013). AtMYB96 acts through the ABA signaling pathway to induce pathogen resistance by promoting salicylic acid biosynthesis, and thus regulating stomata movement, drought tolerance and disease resistance in *Arabidopsis* (Seo et al. 2009). MYB88 might function directly or indirectly, as

positive regulator of stress-responsive genes (Xie et al. 2010). TaMYB30-B from wheat did improve drought stress tolerance in transgenic *Arabidopsis* (Zhang et al. 2012).

Another family of transcription factors, the WRKY family, named for the WRKY domain of about 60 amino acids, contains a highly conserved WRKYGQK heptapeptide at its N-terminus and a zinc-finger-like motif at its C-terminus (Rushton et al. 1995, 2010; Eulgem et al. 2000). WRKY transcription factors are involved in multiple aspects of plant growth, development and stress (Niu et al. 2012). Several TaWRKY in wheat with roles in the abiotic stress response acted in an ABA-dependent manner (Zhu et al. 2013). Here, gene Comp19751encodes a WRKY gene, which showed up-regulation at D4.

MADS-box family members function in reproductive development and stress (Arora et al. 2007). For example, OsMADS25 and OsMADS27 transcripts accumulate in response to osmotic stress (Puig et al. 2013). Comp6823, which encodes for a MADS-box gene in *C. colocynthis*, is up-regulated largely at the last stage of drought (D4).

Analysis of the drought stress signaling transcriptome in C. colocynthis

Drought stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification responses pathways and growth regulation pathways (Zhu et al. 2002). Genes detected in *C. colocynthis* leaves during water deficit are listed in Table 4.

In the ion signaling pathway, calcium binding proteins are well known for their involvement in both biotic and abiotic stress response pathways (Wan et al. 2012). The calcium ion (Ca^{2+}) as a secondary messenger in plants is sensed by calmodulins (CaMs)/CaM-like protein (CMLs), the caldineurin B-like proteins (CBLs) and Ca2+-dependent protein kinases (CDPKs). CaM binds to CaM-binding proteins (CBPs), which function in different pathways under biotic and biotic stress (Ranty et al. 2006). A total of 10 Ca^{2+} binding proteins were detected in the *C. colocynthis* transcriptome.

Protein phosphorylation is a central theme in the cell's response to stress. The MAP kinase cascade in transcript levels consist of a number of protein kinases, such as two-component histidine kinase, MAPKKK, MAPKK, MAPK etc. (Morris et al. 2001). Here we detected 16 MAP kinases in the *C. colocynthis* transcriptome.

Membrane phospholipids can activate several types of phospholipases that cleave phospholipids to generate lipid messengers (eg. PA, DAG, IP3), which further regulate stress tolerance through modulation of stress-responsive gene expression (Singh et al. 2013). Several members in this pathway, such as phospholipase C (PLC), diacylglycerol (DAG) and phosphotidylinositol 4,5-bisphosphate (PIP2)-like aquaporine were detected (Table 4).

Detoxification signaling can ameliorate the damage in plants under stresses (Triantaphylidès and Havaux 2009), as noted in many other plant species. A total of 32 detoxification proteins were detected in the *C. colocynthis* transcriptome.

Molecular mechanisms regulating gene expression in response to drought stress have been studied by analyzing the functional transcription factors in ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki 2006). Several regulatory proteins in ABA-dependent or –independent pathway were detected, among which NAC, MYB/C and leucine-rich repeat proteins (LRR). These regulatory proteins can further modulate many responsive transcription factors. Several functional proteins such as heat shock protein (HSP) 70, HSP22, grpE Like Protein, RBOHD, VIRE2-Interacting protein2 (VIP2), ABA transporter-like protein, synaptobrevin-related protein, translocon outer envelope of chloroplast (Toc34-1), beta-amylase, TIP1 (TIP GROWTH DEFECTIVE 1), RD22 were detected.

Analysis of phytohormone signaling mediators in C. colocynthis

Phytohormones play important roles in regulating plant responses under biotic and abiotic stress. Elaborate phytohormone signaling networks mediate the adaptability of plants to different environmental conditions (Kohli et al. 2013). Many phytohormones such as ABA, salicylic acid (SA), jasmonic acid (JA), auxin, ethylene and gibberellic acid (GA) are being studied for their role in abiotic stress responses (Santner et al. 2009; Wolters and Jürgens 2009; Klingler et al. 2010; Lopez-Raez et al. 2010; Peleg and Blumwald 2011; Santino et al. 2013). It is known that downstream signaling proteins for auxin, GA, JA, and ABA are subjected to ubiquitin-dependent degradation (Santner et al. 2009). Putative phytohormome signaling genes detected in *C. colocynthis* during the drought response are listed in Table 5. For example, we

detected 11 GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), 4 DELLA growth inhibitors (DELLAs) and 2 F-box proteins (SLY1) and SNEEZY (SNZ), which play important roles in GA signaling pathways (Davière and Achard 2013).

The auxin response factor (ARF) family contains transcription factors that bind to auxin-responsive elements (AREs) in the promoters of primary auxin-responsive genes. Aux/IAAs are early auxin-response proteins that bind ARFs, therefore inhibiting ARE-mediated gene transcription. Aux/IAAs are involved in ubiquitin-mediated degradation, which is catalyzed by SCF E3 ubiquitin ligase. TIR1 can stimulate Aux/IAA proteolysis by binding auxin to this protein (Teale et al. 2006). All of the major auxin signaling related transcription factors found in the *C. colocynthis* transcriptome are shown in Table 5. Similar to the auxin pathway, a novel family of transcriptional regulators, the jasmonate ZIM-domain (JAZ) proteins play a target part as Aux/IAA. In addition, SOI1 plays a similar role as TIR1, while MYC and R2R3-MYB transcription factors work as ARFs (Pérez and Goossens 2013). Several JA signaling pathway related genes exist in the *C. colocynthis* transcriptome.

Ethylene signaling pathway components were ordered into a hypothetical linear pathway based on both genetic (epistasis) analysis and biochemical interactions (Bleecker 2000). Almost all of the ethylene signaling homologous members (118 ethylene response factors or ERFs) were detected in the *C. colocynthis* transcriptome. APETALA2/ethylene responsive factor (AP2/ERF) transcription factors are well-known for mediating stress responses and development in plants (Licausi et al. 2013). In the SA pathway, NPR1 (non-specific disease resistance 1) is a key

regulator in the SA-dependent defense signaling pathways (Boatwright and Pajerowska-Mukhtar 2013). Similarly WRKY and TGA play major roles as transcriptional regulators in the SA pathway. We detected 2 NPR1 related proteins, 46 WRKYs and 6 TGAs in *C. colocynthis*, which might function in the *C. colocynthis* SA pathway.

Drought triggers the production of the phytohormone ABA, which in turn causes stomatal closure and induces expression of stress-related genes (Umezawa et al. 2006). The soluble PYR/PYL/RCAR receptors function at the apex of a negative regulatory pathway to directly regulate PP2C phosphatases, which in turn directly regulate SnRK2 kinases (Cutler et al. 2010). Several of the core transcription factors in the ABA pathway are listed in Table 5.

Cellular metabolism under drought stress in C. colocynthis

Global gene expression analyses have shown substantial down-regulation of many photosynthetic genes under drought not only in *Arabidopsis* (Harb et al. 2010), but also several other species such as indica rice (Gorantla et al. 2007 ; Xu et al., 2012; Damarajua et al., 2011). Similarly, many photosystem I and II, chlorophyll a, b binding protein, and oxygen evolving enhancer protein genes, showed down-regulation during water deficit stress in *C. colocynthis* (Table 6).

Citrulline, a non-protein amino acid intermediate in the arginine biosynthetic pathway, has been found to accumulate in leaves of drought tolerant watermelon under water deficit conditions (Kawasaki et al., 2000; Kusvuran et al. 2013). Thus several factors related to the response of this model drought tolerant species to stress have been identified (Table 7). Citrulline metabolic genes (carbamoyl-phosphate synthetase, acetyl glutamate synthase, acetylornithine aminotransferase, aminoglutamate decarboxylase, acetylornithine deacetylase, and glutamate dehydrogenase) were found to be significantly up-regulated during drought.

One of the major research goals is to understand the molecular mechanisms underlying drought tolerance in plants. It is clear that drought triggers a wide variety of responses in *C. colocynthis*. Down regulation of many photosynthetic genes was observed especially at the later stages of drought. Up regulation of many transcription factors, stress signaling factors, detoxification genes, and genes involved in phytohormone signaling occurred throughout the water deficit experiment. Systematic approaches using genomic analyses should lead to the discovery of additional stress factors and provide us with a better understanding of stress tolerance mechanisms of plant species.

Materials and Methods

Plant materials and RNA extraction

C. colocynthis seedlings were grown in Sunshine Mix #8 under a 16h light /8h dark photoperiod at 26℃ day, 22℃ night temperature. Seedlings with 2-3 true leaves (2-3 week old) in 50 ml containers were exposed to drought by withholding water. True leaf samples were collected each day (day 1-day 4), flash frozen in liquid nitrogen and stored at -80C. RNA was subsequently extracted using the TRIzol method (Chomczynski & Sacchi 1987).

Preparation of cDNA library and sequencing

Illumina sequencing was performed at the HudsonAlpha Institute of Biotechnology (Hunstsville, AL) following manufacturer's instructions. RNA-Seq reads were first processed to remove rRNA sequence contamination. First strand cDNA was synthesized with reverse transcriptase and random primers using the small fragment RNAs as template. Second strand cDNA was then synthesized followed by phosphorylation by T4 DNA polymerase. The cDNA fragments were 3' adenylated and ligated to the Illumina's paired-end adapters. The enrichment of cDNA templates were conducted following fifteen cycles of PCR amplification. In total, over 20*4Mp were sequenced for mapping assembly and differential expression analysis. Raw sequence data are available for download at NCBI Sequence Read Archive under the accession (currently awaiting SRP# assignment).

Assembly

Raw sequencing data were filtered using the CLC Genome Workbench (0.05). Paired –end sequences from the four samples were used to construct the *de novo C. colocynthis* transcriptome

assembly with default parameters. Assembly reads were also assembled against the watermelon (*C. lanatus*) genome sequences, which were downloaded from the Cucurbit Database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi). Reads were filtered and assembled using CLC workbench. The parameters used were as follows: 2 points of mismatch cost, 2 points of insertion cost, 2 points of deletion cost, 0.5 as length fraction, 0.95 as similarity fraction. After the *de novo* assembly and watermelon mapping assembly, we used Trinity to assemble all the contigs with the default parameters.

Identification of full length cDNAs

Two methods were used to identify full length cDNAs. First, Blastx searching (E value:$1e^{-10}$) was used to detect the matched cDNAs in SwissProt database; second, ESTScan 2.0 was used to identify the translated sequences. Sequences with either start codon (ATG) and stop codon (TAG/TGA/TAA), or sequences with start codon (ATG) and homologue to a known protein with ≥80% similarity, were chosen as full length cDNAs.

Expression Analysis with Custom Transcriptome Reference

Pair-end sequencing reads of the four libraries were filtered using CLC Genome Workbench (0.05) before mapping to the references sequences from assembled cDNAs. First, read counts of each unigene were converted to reads per million (RPM). Secondly, statistical analysis using

Kal's test provided in CLC Genome Workbench ($p<0.05$ and fold change ≥ 1.5) was conducted. These transcripts were annotated against the reference sequences.

Gene Ontology Analysis

The functional annotation software Blast2go (http://www.blast2go.com/b2ghome) was used to conduct gene ontology analysis of *C. colocynthis* genes in this study. The databases used were Swissprot and NCBI. Blast E-value was set as $1.0e^{-3}$. The major GO analysis was determined by BLAST, mapping, and annotation. Results were presented as a bar chart showing the percent of genes belonging to each group.

qRT-PCR Analysis

For cDNA synthesis, 500ng of the total RNA for each sample (the same RNA was used for RNA-seq analysis) was used in reverse transcription with ProtoScript First Strand cDNA synthesis kit from BioLabs (NEB #E6550S). qRT-PCR was performed with SYBR-Green Supermix from Bio-Rad in an Eppendorf Mastercycler ep realplex with gene specific primers (Table 8). Each reaction contains 10µl of SYBR-Green supermix, 1µl of cDNA template, 1µl forward primer (4µm), 1µl reverse primer (4µm), 7µl ddH_2O_2. The qRT-PCR program consists of one cycle at 95℃ for 15sec, followed 40 cycles of 15sec at 95℃, 15 sec at 55℃, and 30sec at

72℃. The relative expression data was compared with actin from *C. colocynthis*. All experiments were replicated three times.

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Author Contributions

Conceived and designed the experiments: ZW, FD. Performed the experiment: ZW, HH. Analyzed the data: ZW, HH. Contributed analysis tools: LRG, SM. Contributed reagents/materials: FD. Wrote and revised the paper: ZW, FD.

Table 5. 1. Transcriptome read statistics

Time point	1d		2d		3d		4d	
	Before	After	Before	After	Before	After	Before	After
Paired	trim	trim	trim	trim	trim	trim	trim	trim
ends reads	21,194,778	20,339,776	23,566,720	22,626,577	23,435,694	22,531,826	27,388,920	26,288,785
	21,379,596	20,537,526	23,774,222	22,846,047	23,626,206	22,738,550	27,737,584	26,649,390
Average	21,287,187	20,438,651	23,670,471	22,736,312	23,530,950	22,635,188	27,563,252	26,469,088
%trimmed	96.15		96.11		96.19		96.03	

Table 5. 2. Summary details of sequences produced after assembly

	Length/Number
N50	1870bp
Average	1350bp
Min	201bp
Max	1956bp
Total contigs	20581
Full length cDNAs	5038

Table 5. 3. Validation of the RNA-Seq expression profiles of selected *C. colocynthis* genes.

Gene ID	Hit ID	Annotations	Log2FC*-RNA seq	Log2 FC-qRT-PCR
Comp14675	Cla000300	Heat shock protein	7.60	8.37
Comp13927	Cla001351	Cold shock protein	3.41	4.8
Comp2553	Cla002576	MA3 domain-containing protein	2.15	5.33
Comp372	Cla002814	Bax inhibitor-1	1.90	3.56
Comp8117	Cla012143	Drought stress related gene	2.87	4.31
Comp10586	Cla013687	MYB	4.09	5.86
Comp19751	Cla022362	WRKY	6.27	6.00
Comp6823	Cla022169	MADS	2.07	3.67

Transcripts identified as D4 significantly changed genes were compared to transcripts at D1. The table shows the log2 fold change calculated from D4 expression vs D1 expression for RNAseq and qRT-PCR analysis. FC=fold change. Gene ID is gene named in *C. colocynthis*, and Hit ID is blastx of gene with watermelon ID number.

Table 5. 4. List and number of several drought stress signaling pathway members detected in the *Citrullus colocynthis* transcriptome.

Ion Signaling	**NO.**
Calcium Binding Protein	10
Protein Kinase Pathways For Osmotic Signaling	
MAP kinase	16
Osmotic Stress-Activated Phospholipid Signaling	
PLC-like Phosphodiesterase	9
DAG	8
PIP2-like Aquaporin	5
Detoxification Signaling	
Heavy Metal Transport/Detoxification Protein	32
Transcription Regulators	
Regulatory Proteins	
MYB	131
MYC	43
NAC	42
Leucine-rich Repeat Proteins	150
Functional Proteins	
Heat Shock Protein 70	70
Heat Shock Protein 22	1
grpE Like Protein	4
RBOHD (respiratory burst oxidase)	2
VIP2(VIRE2-INTERACTING PROTEIN2)	1
ABATransporter-like Protein	100
Synaptobrevin-related protein	5
Toc34-1 (Translocon outer envelope of chloroplast)	1
Beta-amylase	9
Puruvate Kinase	11
TIP1 (TIP GROWTH DEFECTIVE 1)	1
RD22	2

Table 5. 5. Overview of *Citrullus colocynthis* genes involved in phytohormone signaling.

Function	**Ethylene Pathway Major Members**	**Number**
Ethylene-insensitive protein	EIN	5
Ethylene receptors	ETR	3
Constitutive triple response proteins	CTR	1
EIN3-like (EIL) transcription factors	EIL	1
Ethylene response factors	ERF	118
Function	**Auxin Pathway Major Members**	**Number**
Receptors/F-box proteins	TIR	1
Ubiquitin ligase component	SCF	5
Target proteins	Aux/IAA	30
Auxin response factors	ARF	30
Auxin transport protein	PIN	6
Function	**JA Pathway Major Members**	**Number**
Receptor/F-box proteins	COI1	1
Target proteins	JAZ	1
JAZ interacted proteins	NINJA	4
Activator transcription factors	R2R3-myb transcription factor	3
Activator transcription factors	MYC2,3,4	9
Function	**SA Pathway Major Members**	**Number**
Regulatory proteins	NPR1	2
SA mainly induced genes	WRKY	46
SA mainly induced genes	TGA2,3,5,6	6
Function	**ABA Pathway Major Members**	**Number**
ABA receptors	PYR/PYL/RCAR	19
PYR/PYL/RCAR interacted proteins	PP2C	3
Serine/threonine-protein kinase	SnRK2	1
SnRK2 target	ABI5	1
Function	**GA Pathway Major Members**	**Number**
GA receptors	GID1A/B/C/-like	11
E3 ubiquitin ligase	SLY1/SNZ	2
DELLA proteins	GA1, RGA, RGL1, 2 ,3	4

Table 5.6. Photosynthesis-related and chlorophyll-related gene expression profiles.

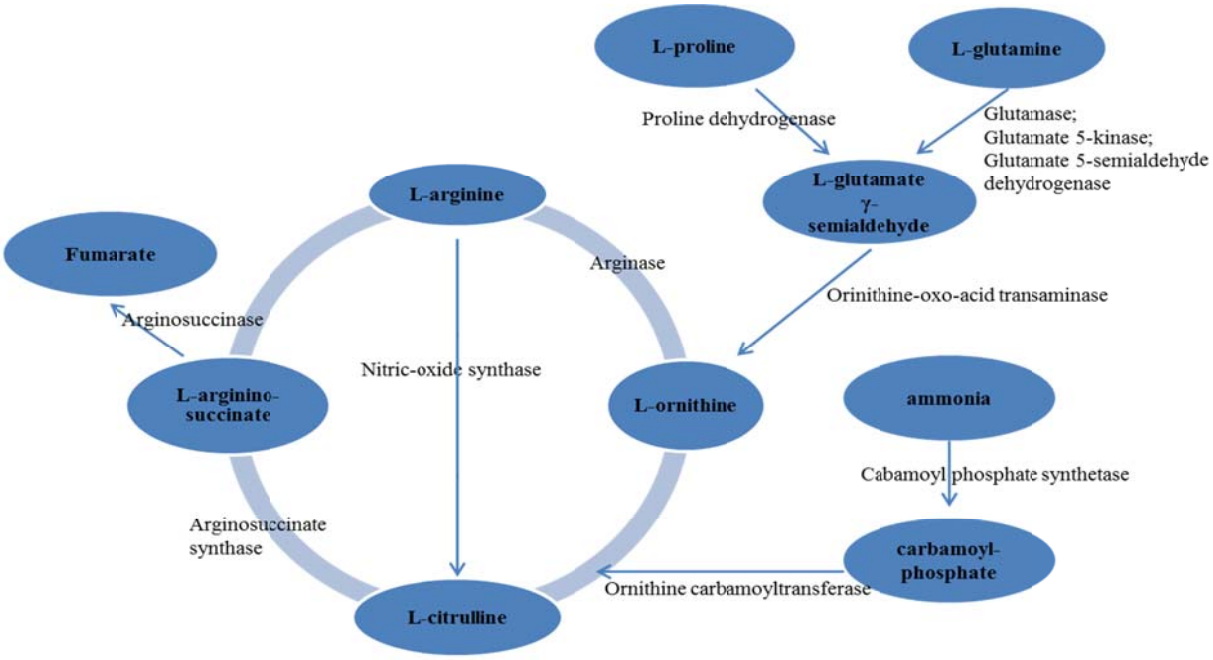
D1RPM-D4RMP are the gene expression reads per millions for each day. ↓ indicates the down-regulation of each gene on each time point.

Gene ID	Hit ID	Annotations	D1RPM	D2RPM	D3RPM	D4RPM
Comp808	Cla002545	Photosystem I psaA	1180	1066.9	513.6↓	418.8↓
Comp2604	Cla021635	Photosystem I subunit II	309	319	114↓	22.8↓
Comp9446	Cla002576	Photosystem I subunit III	470	428↓	97↓	39.1↓
Comp4444	Cla012670	Photosystem I subunit IV	274	219↓	70.4↓	8.3↓
Comp4514	Cla007871	Photosystem I subunit IV A	104.9	98.1↓	41.2↓	9.9↓
Comp2126	Cla004483	Photosystem I subunit V	292.9	281.8↓	95.6↓	23.5↓
Comp4727	Cla007940	Photosystem I subunit XI	257.2	229.5↓	61.4↓	9.6↓
Comp7482	Cla009814	Photosystem I subunit X	225.3	194.1↓	60.3↓	12.3↓
Comp5307	Cla011174	Photosystem I subunit X	199	176↓	44↓	4.6↓
Comp1999	Cla005420	Photosystem II polypeptide	225	194.1↓	60.3↓	12.3↓
Comp2011	Cla008554	Photosystem II 5 kDa protein	138.9	118.7↓	45.4↓	2.5↓
comp7744	Cla013942	Photosystem II Protein	495.8	515.8	331.4↓	116.7↓
Comp8614	Cla014815	Photosystem II reaction center W protein	310.2	265.5	117.3↓	28↓
Comp2016	Cla022723	Photosystem II core complex proteins psbY	78	76.3↓	24.7↓	7.7↓
Comp12757	Cla011748	Chlorophyll a-b binding protein 13	290.5	246.2↓	3.1↓	0.3↓
Comp6677	Cla013483	Chlorophyll a-b binding protein 3C	130.6	89.5↓	7.9↓	3.8↓

Comp1476	Cla013826	Chlorophyll a-b binding protein	671.4	583.9↓	42.9↓	8.8↓
Comp7589	Cla015680	Chlorophyll a-b binding protein 37	737.2	489.3↓	16.6↓	10.5↓
Comp2286	Cla017325	Chlorophyll a-b binding protein 3C	577.4	230.8↓	4.1↓	1↓
Comp544	Cla017983	Chlorophyll a-b binding protein 6	235.2	219.1↓	72.3↓	48.2↓
Comp3797	Cla018117	Chlorophyll a-b binding protein 6	632.9	502.3↓	154.8↓	38.3↓
Comp15099	Cla019595	Chlorophyll a-b binding protein 21	228.8	126.3↓	0.1↓	1
Comp1922	Cla001764	Chlorophyll a-b binding protein 8	609.1	470↓	241.6↓	58.6↓
Comp1584	Cla012368	Chlorophyll a-b binding protein 8	1082.2	884.8↓	111.1↓	281.1↓
Comp940	Cla009752	Chlorophyll a-b binding protein 21	652.6	514.8↓	43.3↓	3.6↓
Comp13569	Cla009753	Chlorophyll a-b binding protein 21	827.5	676.3↓	68.1↓	5.8↓
Comp4958	Cla022963	Chlorophyll a-b binding protein 7	538.2	404.7↓	110.1↓	18.8↓
Comp4043	Cla001790	Oxygen-evolving enhancer protein 1 of photosystem II	1088.6	795.9↓	334.6↓	82.8↓
Comp3077	Cla005429	Oxygen-evolving enhancer protein 2, chloroplastic	604.2	531.1↓	395.2↓	122.6↓
Comp5901	Cla019423	Oxygen-evolving enhancer protein 3	545.6	414.8↓	86.7↓	17↓

Table 5.7. Expression profiles of some citrulline metabolic genes during drought stress.

Graft shows citrulline metabolism pathway in *C.colocynthis*. D1RPM-D4RMP are the gene expression reads per millions for each day. ↑ and ↓ indicate the significant up-regulation and down-regulation of each gene on each time point.



Gene ID	Hit ID	Annotations	D1RPM	D2RPM	D3RPM	D4RPM
Comp1556	Cla006970	Carbamoyl-phosphate synthetase	64.6	70.9↑	159↑	175.8↑
Comp1556	Cla005591	Carbamoyl-phosphate synthetase	196.1	162.5	846.5↑	1542↑
Comp3843	Cla014787	Carbamoyl-phosphate synthetase	15	20.5↑	49.8↑	63.3↑
Comp11009	Cla016474	Proline dehydrogenase	2.2	3.9↑	11.6↑	31.4↑
Comp01186	Cla023055	Arinosuccinase	5.6	6.2	3.7	5.3

Comp5521	Cla020781	Orinithine carbamoyltransfrase	25.2	19.1↓	52.5↑	32.5
Comp6244	Cla015337	Acetylornithine aminotransferase	39	45.3↑	127↑	147.6↑
Comp6587	Cla008748	Glutamine amidotransferase	25.6	19.7	12.4	13.8
Comp15261	Cla017928	Glutamate 5-kinase	4.3	5.7	1.7	1.6
Comp3961	Cla019569	Orinithine-oxo-acid transaminase	19.3	30.5	28.1	17.1
Comp7707	Cla023055	Argininosuccinate lyase	5.6	6.2	3.7	5.3
Comp3468	Cla003592	Argininosuccinate lyase	22.9	26.8	35.4	25.6
Comp776_seq2	Cla002611	Arginosuccinate synthase	12.5	25.4	6	7.7
Comp776_seq1	Cla002609	Arginosuccinate synthase	18.2	18.9	2.4↓	2↓
Comp1556	Cla022915	Carbamoyl-phosphate synthetase	115.4	103.2	53.4↓	42↓

Table 5. 8. qRT-PCR primer information

Primer Name	Sequence (5' to 3')	length
Comp5873-F	TAAGCCGGAAATTGGTGATCAGTT	24
Comp5873-R	TTCTTCTATTGCTGCCTCTTCCCC	24
Comp862-F	AATGCTGCTTTGTTGCCTTCTGATC	25
Comp862-R	CCTCCTTCTTTCCTGTCTCATGCTCT	26
Comp1108-F	TCTTCGCGTATCCAAAAACAACATTA	26
Comp1108-R	CCAGCCAGACTCGCCCAATC	20
Comp10156-F	GAAGCCTCTAAGCACGTCGAAAGTT	25
Comp10156-R	ACCGAGACTACAACCGCCTTACATAC	26
Comp6528-F	ACGGAAGTCGAGAAAGATACGGATT	25
Comp6528-R	GCGTATGTTGGGTGAAATGGCA	22
Comp7317-F	TCCTCCTCACCATCCTCATCGTCT	24
Comp7317-R	GGTTGCGAGCGGAGACAGTGAG	22
Comp20554-F	CCCCCCGATTCTGCCGAC	18
Comp20554-R	ACCGCCTACTAAACTATCCATCCACTC	27
Comp3048-F	CCTTTTACCAGAGACTTTTCCCCAAT	26
Comp3048-R	CGTCCCTCTGTTCACCGGTTTC	22
Comp14675-F	TCAAACCCAGACCCCTCAAGAAAAC	25
Comp14675-R	GCGCTTGGATTGACATGCACC	21
Comp13927-F	ATCCCTTCATCCCCATTTTCCCTCT	25
Comp13927-R	CCAAAGCCGGGTATGTCGTCAAATC	25
Comp2553-F	CCCGAATTAATACGAAGCCTAGAAGA	26
Comp2553-R	CACTGATGCCATTTCTTTTTCTCTGTT	27

Comp372-F	CAGCGGGTTTATCTCACTCTTGGTT	25
Comp372-R	TTCAAAAAGAGCAGCCCCTAATAAAAT	27
Comp8117-F	AAGGAATTTGGGGATGGTTACAGAG	25
Comp8117-R	GGTTCTCTCCTTCCTTCCATTTCCA	25
Comp10586-F	AGAAAGGAACGCCATGGACTGAAGA	25
Comp10586-R	TTCTGGGCATGACTGGCTACCTGAG	25
Comp19751-F	AATGGAGGAAATATGGTCAAAAGGTG	26
Comp19751-R	ACCTTTCTCTTCACCGGACAGCTAG	25
Comp6823-F	GCTCAAGCATCAGTTCCACAAAATA	25
Comp6823-R	TGCTTCACAACGACATAACCTTCTT	25

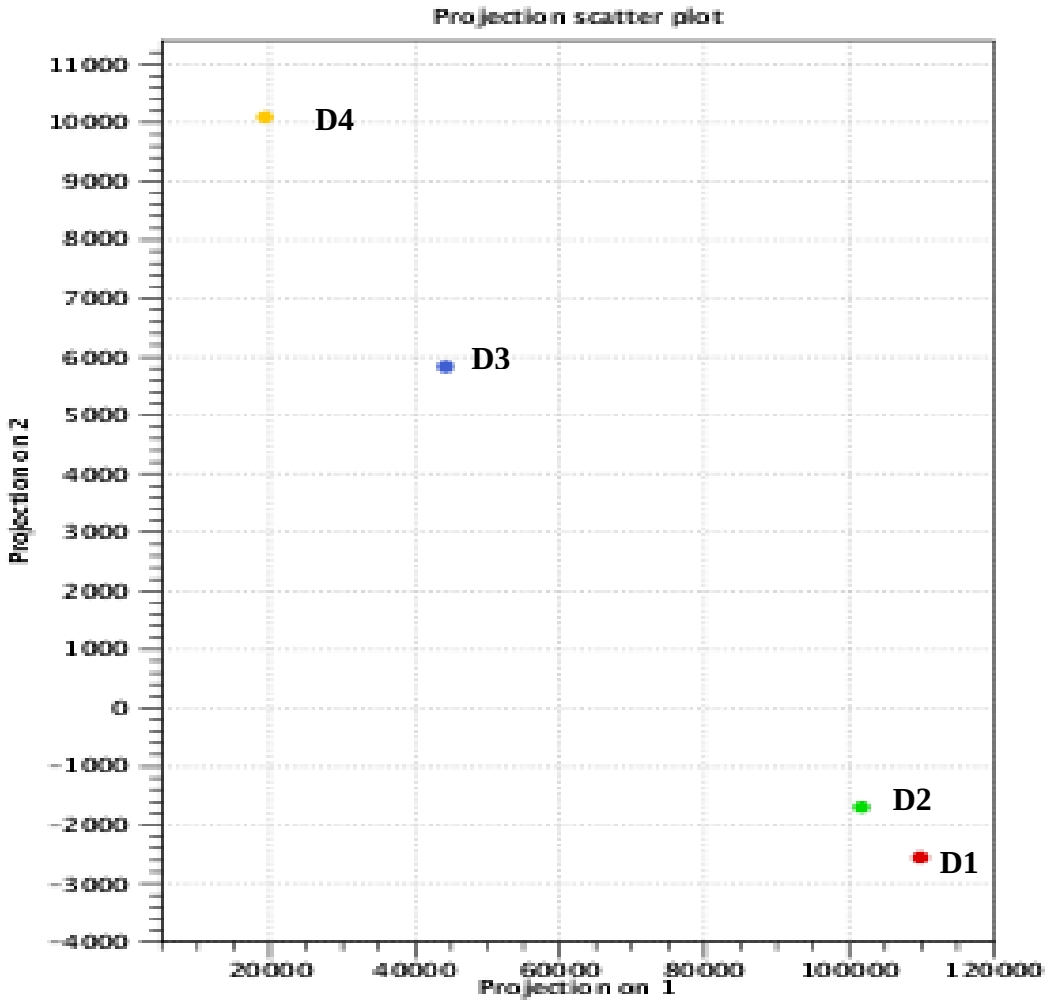


Figure 5. 1. Principal component analysis of the leaf transcriptome in four days.

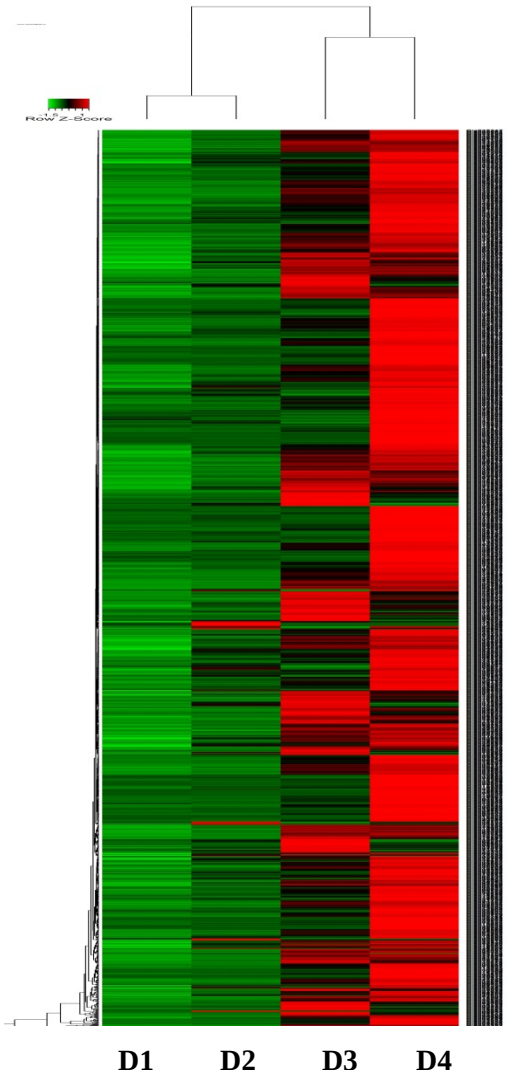


Figure 5. 2. Heat map depicting changes in transcript patterns under drought treatment. 2545 genes with clusters of transcripts across four different day (D1-D4) samples under drought stress.

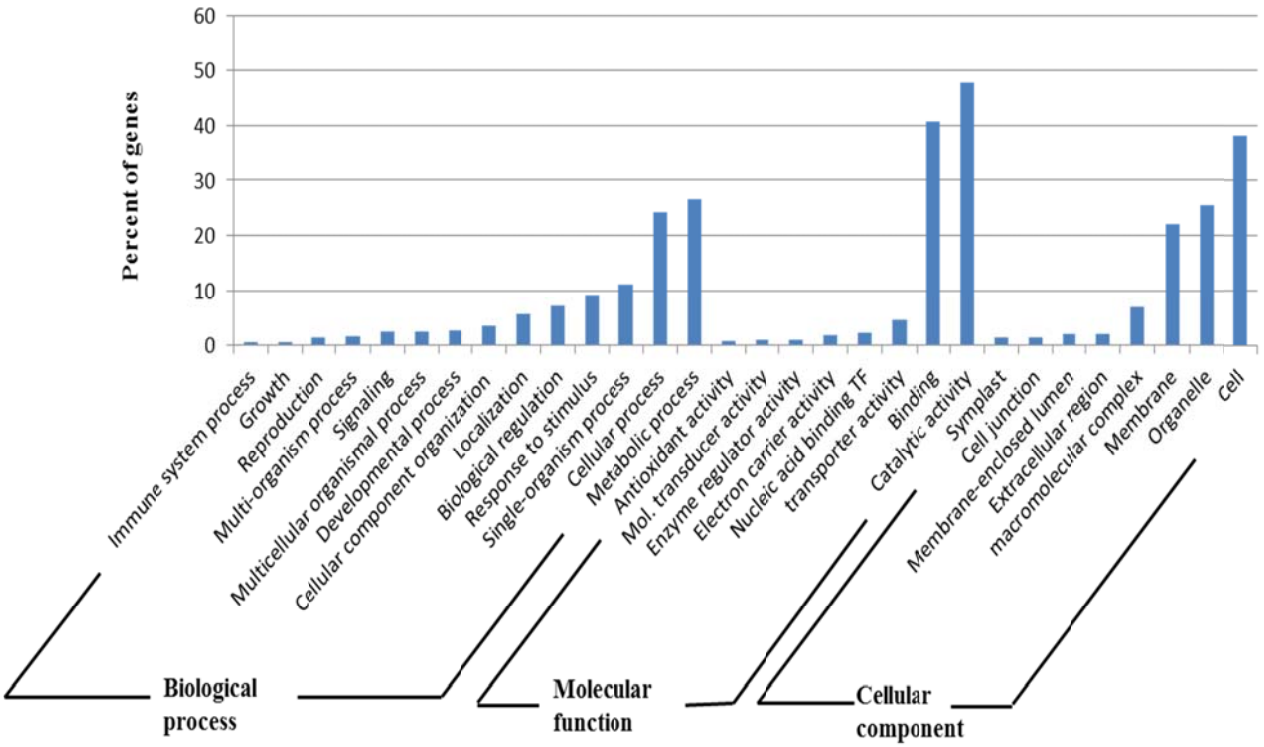


Figure 5. 3. Gene Ontology (GO) of significantly changed genes under drought stress.

Based on high-score BLASTX matches in the NR (non-redundant) plant protein database, *C. colocynthis* genes were classified into three main GO categories. The y-axis indicates the percentage of a specific category of genes in each main category.

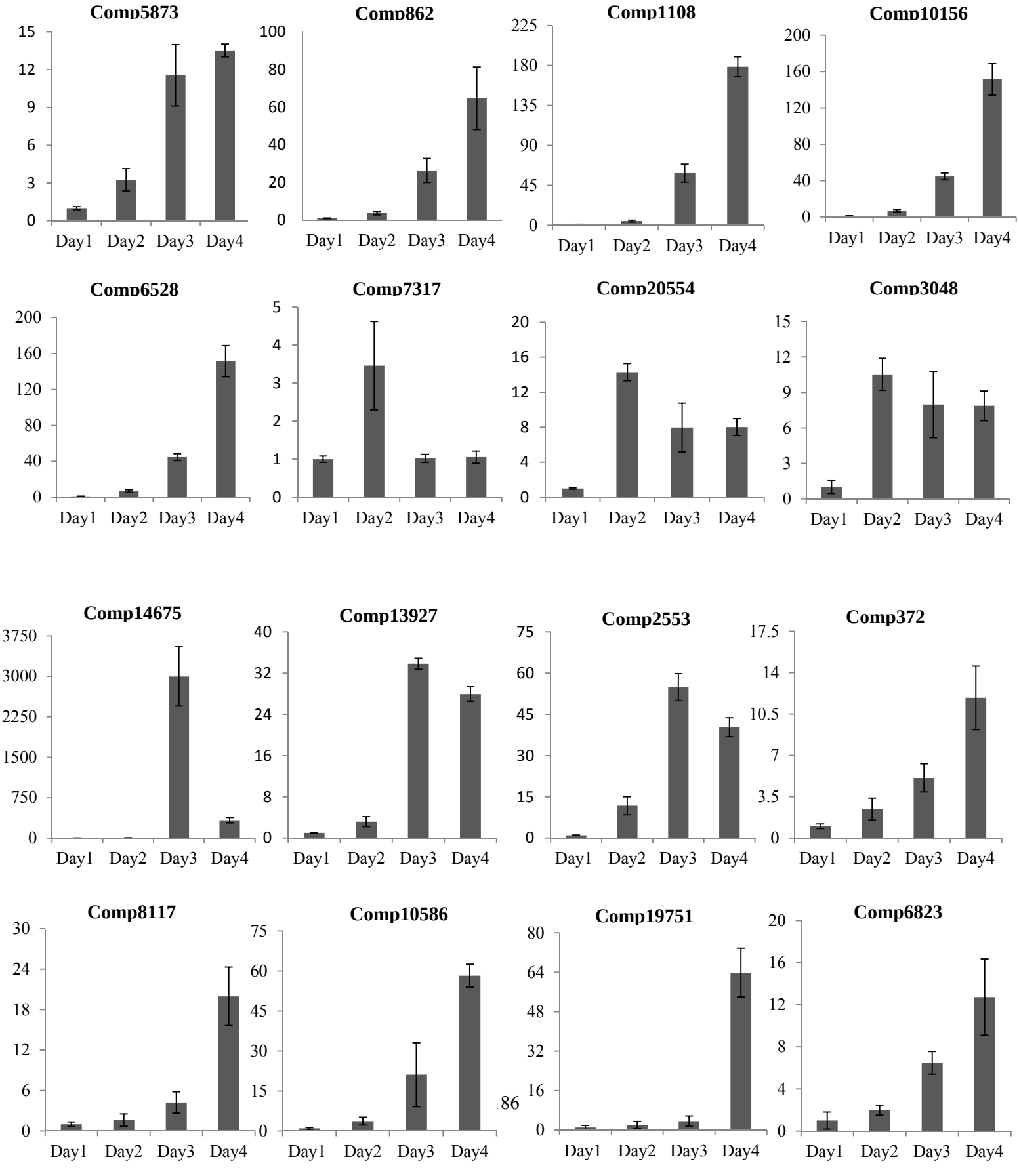

Comp5873
Comp862
Comp1108
Comp10156
Comp6528
Comp7317
Comp20554
Comp3048
Comp14675
Comp13927
Comp2553
Comp372
Comp8117
Comp10586
Comp19751
Comp6823
Day1
Day2
Day3
Day4

Figure 5. 4. qRT-PCR analysis of 16 selected genes expression under drought treatment.

The y-axis is the relative expression compared with Day 1 expression of each gene. The x-axis shows days of water-withholding time points.