

# A Gene Encoding Cold-Circadian Rhythm-RNA Binding-Like Protein (*CCR*-Like) from Upland Cotton (*Gossypium hirsutum* L.) Confers Tolerance to Abiotic Stresses in Transgenic Tobacco

Gurusamy Dhandapani · Mogilicherla Kanakachari · Kethireddy Venkata Padmalatha · Mullapudi Lakshmi Venkata Phanindra · Vivek Kumar Singh · Sanagala Raghavendrarao · Narayanasamy Jayabalan · Azhagiyanavalan Lakshmi Prabha · Polumetla Ananda Kumar

Published online: 4 May 2014  
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**Abstract** Water-deficit is the major abiotic stress factor that limits the yield and quality of cotton produced around the world. We observed earlier that a *CCL* (*CCR*-like; cold-circadian rhythm-RNA binding like) gene has been differentially expressed during boll development in cotton under drought stress in the field. Isolation and functional characterization of *GhCCL* from upland cotton (*Gossypium hirsutum* L. cv. Bikaneri Narma) was carried out in the present study. We studied the *GhCCL* gene structure and organization and demonstrated for the first time that *GhCCL* may be involved in abiotic stress tolerance response in plants. RT-PCR analysis indicated that *GhCCL* is differentially regulated in cotton seedlings by abiotic stresses such as salt, mannitol, cold, heat, dehydration, wounding and jasmonic acid, salicylic acid, and hydrogen peroxide. In silico and subcellular localization analysis suggested that *GhCCL* is localized in the chloroplasts.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11105-014-0729-x) contains supplementary material, which is available to authorized users.

G. Dhandapani · M. Kanakachari · K. V. Padmalatha · M. L. V. Phanindra · V. K. Singh · S. Raghavendrarao · P. A. Kumar (✉)  
National Research Centre on Plant Biotechnology, LBS Building, Pusa Campus, New Delhi 110012, India  
e-mail: polumetla@hotmail.com

G. Dhandapani · M. Kanakachari · N. Jayabalan · A. Lakshmi Prabha  
Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

**Present Address:**  
P. A. Kumar  
Institute of Biotechnology, Acharya NG Ranga Agricultural University, Rajendranagar, Hyderabad 500030, Andhra Pradesh, India

Constitutive expression of *GhCCL* in tobacco (*Nicotiana tabacum* var. Petit Havana) conferred tolerance to water-deficit stress and salt stresses during seed germination on amended MS media. The transgenic plants showed better growth performance and increased fresh weight under long-term stress. The transgenic plants grown in the glass house tolerated water-deficit stress (by withholding water) and recovered upon rewatering (45 days) whereas WT plants did not survive. Characterization of the *GhCCL* promoter sequence by in silico analysis showed various light, stress, tissue specific, and hormone responsive cis-elements. The present study suggested that *GhCCL* positively regulates the response to abiotic stresses, especially water-deficit stress in transgenic plants and that the overexpression of *GhCCL* may enhance the stability of mRNA thereby conferring tolerance to abiotic stresses.

**Keywords** Cold-circadian rhythm-RNA binding · *Gossypium hirsutum* · Water-deficit stress · Transgenic tobacco

## Introduction

Cotton is the major source of natural fibers used in the textile industry and is cultivated across the globe (Smith and Cothren 1999). Among the four cultivated species, *Gossypium arboreum* and *G. herbaceum* are diploids with A genome and *G. hirsutum* and *G. barbadense* are allotetraploid species with AD genome. *G. hirsutum* represents more than 95 % of the cultivated cotton worldwide (National Cotton Council, 2012, <http://www.cotton.org/econ/cropinfo/index.cfm>). Cotton fiber development includes four distinct, but overlapping stages: fiber cell initiation, elongation/primary

cell wall (PCW) synthesis, secondary cell wall (SCW) biosynthesis, and maturation (Basra and Malik 1984; Ruan and Chourey 1998). The fiber cell initiation usually occurs from 2–3 days before anthesis to 2–3 days post anthesis (dpa) and fiber cell elongation occurs up to 20 dpa. However, fast elongation of fiber cell occurs between 5 and 15 dpa. Secondary cell wall synthesis starts at about 20 dpa and continues up to 45 dpa. During this period, large amount of cellulose (>90 %) deposition takes place, and the fiber cell wall becomes thick. In the final maturation stage (45–50 dpa), fibers undergo dehydration and produce mature cotton lint (Wilkins and Arpat 2005; Arpat et al. 2004; Ji et al. 2003). The quality of fiber, which is based on its final length and strength, is determined mainly during the elongation and secondary cell wall deposition stage (Li et al. 2007; Deng et al. 2012).

Abiotic stresses remain the greatest constraint to crop production. Worldwide, it has been estimated that approximately 70 % of yield reduction is the direct result of abiotic stresses (Acquaah 2007). It reduces plant productivity by inhibiting growth and photosynthesis (Taiz and Zeiger 1998). Adaptation to drought stress is undoubtedly one of the most complex biological processes. It involves numerous changes including reduced growth, transcriptional activation, inactivation of specific genes, transient increases in abscisic acid (ABA) levels, accumulation of compatible solutes and protective enzymes, increased levels of antioxidants, and suppression of energy-consuming pathways.

Various abiotic stresses severely impair growth and yield of cotton plants. Severe drought stress will slow cotton plant development and cause small bolls and squares to shed. Establishment and pre-bloom irrigations affect total yield, but water deprivation following bloom and into boll development also affects lint quality. Cotton is known to contain intrinsic mechanisms that allow it to partially withstand water-deficit and heat stresses by its deep and extensive root system and adaptive osmoregulation mechanisms (Ackerson and Hebert 1981; Grimes and Yamada 1982). However, as water-deficit stress progressively continues, the development and reproduction of cotton are severely affected, along with significant reduction in quality and yield of cotton fiber (Cothren 1999; Bradow and Davidonis 2010). Therefore, to achieve maximal cotton yield, it is essential to understand proper management strategies between water demand, irrigation, and plant responses that are seasonally variable (Martin et al. 2007).

High-throughput screening techniques such as microarray analysis have been used for efficient profiling of abiotic stress-responsive genes including drought stress in model and agronomically important crop species (Ji et al. 2003; Kawaguchi et al. 2004; Zhou et al. 2007; Aprile et al. 2009; Cohen et al. 2010; Gong et al. 2010; Padmalatha et al. 2012). Comparative transcriptomic analysis of gene expression profiles in cotton

leaf and root tissues under well-watered and water-deficit conditions (Payton et al. 2011), boll developmental stages under field drought stress condition (Padmalatha et al. 2012), and root and leaf samples from drought-resistant cultivar grown under water-deficit stressed and well-watered field conditions (Park et al. 2012) were studied extensively, to discover tissue specific and stress-responsive changes in gene expression under water-deficit stress condition in cotton. Recently, Zhu et al. (2013) identified genes involved in diverse stresses including ABA, cold, drought, salinity, and alkalinity of cotton seedling by comparative microarray analysis, and they suggested that there was crosstalk of responsive genes and pathways to multiple abiotic or even biotic stresses in cotton.

So far, a limited number of genes/transcripts which are involved in drought tolerance were isolated from cotton and functionally characterized either in heterologous systems like tobacco or in cotton to validate their role in imparting drought tolerance. *G. hirsutum* 64-amino acid type 3 metallothionein protein that was isolated through complementary DNA (cDNA) library screening showed accumulated higher levels of mRNA under salinity, drought, low temperature, heavy metal ions, ABA, ethylene, and reactive oxygen species (ROS) in cotton seedlings and overexpression of cDNA in transgenic tobacco plants conferred increased abiotic stress tolerance compared to wild-type (WT) plant (Xue et al. 2009). Transgenic cotton plants expressed with tobacco *osmotin* showed a slower rate of wilting during drought and fast recovery upon rewatering in glass house conditions; relative water content and proline levels were higher and showed reduced H<sub>2</sub>O<sub>2</sub> levels, lipid peroxidation, and electrolyte leakage as a result of which, fiber yield of transgenic plants did not suffer as much as that of their non-transgenic counterparts under drought conditions (Parkhi et al. 2009). *GHSP26* cDNA was isolated from *G. arboreum* and overexpressed in *G. hirsutum*, and the transgenic plants showed an enhanced drought tolerance (Maqbool et al. 2010). Transgenic cotton was developed by overexpression of *Annexin* gene from *Brassica juncea* which showed abiotic stress tolerance and improved fiber development and quality (Divya et al. 2010). Overexpression of Arabidopsis vacuolar H<sup>+</sup>-pyrophosphatase (*AVP1*) gene in cotton plants showed more vigorous growth, displayed significantly improved tolerance to both drought and salt stress and 20 % higher fiber yield than that of WT plants in the field under dry land conditions. They concluded that *AVP1* may improve crop performance under drought and saline conditions in areas where water and salinity are limiting factors for agricultural production (Pasapula et al. 2011; Zhang et al. 2011a). Constitutive overexpression of *G. hirsutum* mitogen-activated protein kinase (*GhMPK2*) in *Nicotiana tabacum* conferred reduced sensitivity to ABA during both seed germination and vegetative growth and decreased rate of water loss and exhibited enhanced drought

and salt tolerance which strongly suggests that *GhMPK2* positively regulates salt and drought tolerance in transgenic plants (Zhang et al. 2011b). *GhMKK1* is one of the mitogen-activated protein kinases, and it is a crucial regulator of the response to environmental stresses, overexpression in *N. benthamiana* enhances the tolerance to salt and drought stresses (Lu et al. 2013). Overexpression of Arabidopsis *LOS5* cDNA in cotton plants showed 20, 25, and 13 % of more proline content, endogenous ABA level after 3 days water withholding and increase fresh weight under 8 weeks water-deficit condition, respectively, in transgenic plants compared with WT plants (Yue et al. 2012). Overexpression of *isopentenyl transferase* gene (IPT) from *Agrobacterium tumefaciens* driven by senescence-associated receptor-like kinase gene (SARK) promoter in *G. hirsutum* cv. Coker 312 showed increased drought tolerance under water deficit condition (Kuppu et al. 2013). *Calcium neurin B-like interacting protein kinase* (CIPK6) gene was isolated from cotton somatic embryogenesis and overexpressed in transgenic Arabidopsis were significantly enhances the salt, drought, and ABA stress tolerance and indicating that *GhCIPK6* acts as a positive regulator in response to salt and drought stress tolerance (He et al. 2013). Transgenic Arabidopsis with constitute overexpression of *G. barbadense* receptor-like kinase gene exhibited a reduced rate of water loss in leaves in vitro, along with improved salinity and drought tolerance and increased sensitivity to ABA compared with wild-type Arabidopsis (Zhao et al. 2013). Transgenic tobacco with constitute expression of *G. barbadense* mitogen-activated protein kinase 3 (*GbMPK3*) was conferred with enhanced drought tolerance, reduced water loss during drought treatment, and improved plant height and survival rates after rewatering, and they suggested that *GbMPK3* may positively regulate drought tolerance through enhanced ROS scavenging ability (Long et al. 2013).

In the previous study, we reported the gene expression profiles under drought stress during the fiber developmental stages (0, 5, 10, and 20 dpa) in the field-grown cotton plants using Affymetrix cotton GeneChip Genome arrays ([http://www.affymetrix.com/estore/browse/products.jsp?productId=131430#1\\_1](http://www.affymetrix.com/estore/browse/products.jsp?productId=131430#1_1)) and identified a number of differentially expressed transcripts with  $P$  value  $\leq 0.01$  and  $\log_2$  fold change  $\geq 2$  at various stages analyzed. Based on the transcriptome study, we observed that one of the transcripts (Unigene id: Ghi.10692) encoding *CCL* (*CCR*-like) gene was upregulated by 2.5, 239.0, 4.9, and 2.3 folds at 0, 5, 10, and 20 dpa, respectively, as compared to the respective control samples (Padmalatha et al. 2012). The high level upregulation of *GhCCL* transcript was observed during fiber elongation stage (5 dpa).

In this study, we have chosen the gene *GhCCL* for molecular characterization and functional analysis in transgenic tobacco. In silico analysis of the *GhCCL* upstream sequence was carried out to find the various cis-regulatory elements.

Ectopic expression of *GhCCL* in tobacco resulted in water-deficit stress tolerance upon withholding water for 45 days and the transgenic plants made fast recovery upon rewatering. *GhCCL* transgenic tobacco plants showed similar phenotype as that of WT plants. Further, we observed early germination and faster growth rate in the transgenic plants compared to those in WT plants. This is the first report that demonstrates the involvement of cotton *CCL* gene in abiotic stress tolerance. We suggest that the ectopic expression of *GhCCL* gene in transgenic crops will confer tolerance to an array of abiotic stresses.

## Materials and Methods

### Plant Materials, Growth Conditions, and Stress Treatments

Cotton (*G. hirsutum* L. cv. Bikaneri Narma) seedlings were raised in a culture room at 25 °C with 16 hrs light: 8 h dark cycles. Seven-day-old cotton seedlings were used for different stress treatments. Seedlings were dipped in 0.5× MS (Murashige and Skoog 1962) medium containing 15 % polyethylene glycol (PEG)-6000, 200 mM each sodium chloride (NaCl), and mannitol. For methyl jasmonate (MeJA), salicylic acid (SA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and ABA treatments, leaves were sprayed with 10 mM each of MeJA, SA, H<sub>2</sub>O<sub>2</sub>, and ABA. To investigate the effect of temperature stress, the seedlings were placed in a growth chamber at either high temperature (42 °C) or low temperature (4 °C). For wounding experiments, the seedlings were punched by forceps, and dehydration-stress seedlings were removed from the bottles, and the roots were washed with water then placed on tissue paper under culture room conditions. The samples were collected from all the treatments after 1 and 12 h, frozen in liquid nitrogen, and stored at -70 °C for later use.

For expression profiling of the genes, cotton samples at different boll developmental stages under drought stress conditions were collected with their respective controls. Drought induction under field conditions and related parameters were described previously (Padmalatha et al. 2012). *Arabidopsis thaliana* ecotype Columbia seeds were sown in pots filled with a coconut pulp/cocofeed, and pots were kept in culture room at 22 °C and 16: 8 h light sources for 2 weeks, and then healthy leaf samples were collected and frozen in liquid nitrogen and stored at -70 °C for later use.

Tobacco (*Nicotiana tabacum* L. var. Petit Havana) plants have been maintained by subculturing the healthy greenish stem portion in MS medium without any phytohormones in tissue culture room at 25–27 °C temperature with a light intensity of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with 16 h light: 8 h dark cycles. One-month-old healthy tobacco leaves were used for plant transformation studies. In transgenic glass house, tobacco plants were grown at 25–27 °C temperature with 16 h light:

8 h dark cycle at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  light and 70–80 % relative humidity (RH).

#### Total RNA and RT-PCR analysis

Total RNA was isolated from cotton bolls collected from control and drought-induced plots at different fiber development stages and also from cotton seedlings subjected to various stresses using Spectrum plant total RNA extraction kit (Sigma, USA). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was employed to investigate the expression profiles of *GhCCL*. Of total RNA,  $1.0 \mu\text{g}$  was converted to single stranded cDNA using by AffinityScript QPCR cDNA Synthesis Kit (Stratagene, USA). The resulting cDNA was used as a template to perform the RT-PCR analysis using gene specific primer. The PCR conditions were as follows:  $94^\circ\text{C}$  for 1 min, initial denaturation; 30 cycles of  $94^\circ\text{C}$  for 40 s,  $60^\circ\text{C}$  for 40 s, and  $72^\circ\text{C}$  for 60 s, and a final extension at  $72^\circ\text{C}$  for 10 min. The RT-PCR for the house-keeping *Ubiquitin1* (accession number EU604080) (Li et al. 2009) gene from cotton was performed under the same conditions for normalization of the samples.

#### The Full-Length cDNA Cloning of *GhCCL* and Construction of Phylogenetic Tree

Total RNA was extracted from 5 dpa cotton bolls subjected to drought using the method discussed above. The full-length *GhCCL* cDNA was amplified by rapid amplification of cDNA ends-PCR (5' RACE kit, Invitrogen, USA) and reverse transcription-PCR (RT-PCR). The amplified cDNA was cloned in pGEM T-Easy vector and sequenced. The full-length cDNA sequence and coding sequence (CDS) were identified by the FGENESH analysis of softberry software ([www.softberry.com/](http://www.softberry.com/)) and the sequence has been submitted to the GenBank. The primer sequences used in this study are listed in Table 1. Phylogenetic tree was constructed using protein sequence of GhCCL and those of the closely related plant species by using MEGA software version 5.20 (Tamura et al. 2011).

#### Amplification of the Genomic and Upstream Sequence of *GhCCL*

Genomic DNA was extracted from the cotton leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Porebski et al. 1997). The full-length DNA sequence was amplified by PCR with the primers 5'-CGGGATCCATGCA GTCAGCAGCAGCAAC-3' (*Bam*HI site underlined) and 5'-CGAGCTCCTAATACTCCCTTTGATAC-3' (*Sac*I site underlined) designed for full-length *GhCCL* cDNA sequence. The promoter region was amplified with the genome walking

method as per the manufacturer's instructions (Genome walking kit, Clontech, USA) using primary PCR with gene specific primer (*GhCCLGSP2*) and genome walker adapter primer1 (GWAP1) and for nested PCR with gene specific primer (*GhCCLGSP3*) and genome walker nested adaptor primer2 (GWAP2). Genome walking product was cloned in pGEMT-Easy vector and sequenced. The sequences obtained from the different libraries were aligned by Lasergene software to find out the upstream promoter sequence. The sequence has been submitted to the GenBank. The program PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) was used to analyze the cis-elements in the promoter sequences.

#### Identification of *GhCCL* Copy Number in Cotton (*G. hirsutum* L. cv. Bikaneri Narma)

Twenty micrograms of genomic DNA was digested with *Bam*HI, *Hind*III, and *Eco*RV (New England Biolabs, USA). The electrophoresis of nucleic acids was performed as described (Sambrook and Russell 2001; Southern 1975), blotted overnight onto a Hybond-N<sup>+</sup> membrane (Amersham Biosciences, UK), and fixed at 22 J/cm for 2 min using Stratalinker UV crosslinker (Stratagene, USA). Immobilized nucleic acids were hybridized with  $\alpha$  [<sup>32</sup>P]-dCTP labeled 623-bp *GhCCL* genomic fragment using mega prime DNA labeling system (Amersham Biosciences, UK). Subsequent to the hybridization and stringent washes the membrane was exposed to the X-ray film with an intensifying cassette in dark and was placed at  $-70^\circ\text{C}$  for a week. The exposed X-ray film was developed to visualize the results.

#### Subcellular Localization of *GhCCL-GFP* Fusion Proteins and Agroinfiltration Study

The full-length *GhCCL* coding region was amplified without stop codon using forward primer 5'-GGAAGATCTGATGCA GTCAGCAGCAGCAAC-3' (*Bg*III site underlined) and reverse primer 5'-CGGACTAGTATACTCCCTTTGATAC CTCTCG-3' (*Spe*I site underlined). The amplified fragment was restricted and ligated to the binary vector *pCAMBIA1302-mGFP*, which generated a C-terminal fusion with the modified green fluorescence protein (mGFP) gene driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. The binary vector was mobilized to *A. tumefaciens* strain LBA4404 by freeze-thaw method (Hofgen and Willmitzer 1988). Tobacco leaves were infiltrated with induced *Agrobacterium* culture prepared with 50 mM MES buffer (pH 5.2), 100 mM MgCl<sub>2</sub>, and 100  $\mu\text{M}$  acetosyringone (Gelvin 2006) by a syringe. The infiltrated plants were kept in the culture room for 4 days. The leaf portions were observed under Leica TCS SP6 confocal microscope (Germany) using bright light, GFP channels and



**Table 1** List of primers used for this study

Primer name	Sequence (5'3')	Description
<i>GhCCLGSP1</i>	CTCTGTTGTGGTCCTC	5' RACE reverse primer, cDNA synthesis
<i>GhCCLGSP2</i>	CAACCATTGAAGGGTCGTAGGTGGTG	5' RACE reverse primer, primary and genome walking
<i>GhCCLGSP3</i>	GGAATAAGGGGTGGTGCAATGCTGAG	5' RACE reverse primer, nested and genome walking
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGII GGGIIG	5' RACE, abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	5' RACE, abridged universal amplification primer
GWAP-1	GTAATACGACTCACTATAGGGC	Primary adaptor, forward primer for promoter
GWAP-2	ACTATAGGGCACGCGTGGT	Nested adaptor, forward primer for promoter
<i>GhCCL-F</i>	<u>cgggatcc</u> ATGCAGTCAGCAGCAGCAAC	Full-length CDS amplification
<i>GhCCL-R</i>	<u>cgagctc</u> CTAATACTACTCCCCTTTGATAC	
<i>GhCCL GFP-F</i>	<u>ggaagatctg</u> ATGCAGTCAGCAGCAGCAAC	GFP fusion construct
<i>GhCCL GFP-R</i>	<u>cggactagt</u> ATACTACTCCCCTTTGATACTCTCG	
<i>GhCCL</i> qRT-F	ACAGCAACAGCAACAGCAACTCTC	Expression profile analysis
<i>GhCCL</i> qRT-R	AACCATTGAAGGGTCGTAGGTGGT	
<i>Atrd29A-F</i>	<u>cccaagctt</u> GCCATAGATGCAATTC	Amplification of <i>rd29A</i> gene specific promoter
<i>Atrd29A-R</i>	<u>gctctaga</u> TTTCCAAAGATTTTTTTC	from <i>Arabidopsis thaliana</i>
<i>GhUBI1-F</i>	ACACGATCGACAACGTTAAGGCCGA	<i>Ubiquitin1</i> normalizer gene in cotton
<i>GhUBI1-R</i>	ACGAAGGACAAGATGGAGCGTTGA	
<i>NPTII-F</i>	CCGGAATTCATGATTGAACAA	Analysis of transgenic tobacco lines for Kanamycin resistance gene
<i>NPTII-R</i>	CCCAAGCTTCAGAAGAAGCTC	

Restriction site of *Bam*HI, *Sac*I and *Bg*III, *Spe*I for *GhCCL* CDS and GFP fusion, respectively, and *Hind*III, *Xba*I for *rd29A* promoter was underlined

merged to localize the expressed *35S-GhCCL::mGFP* fusion and *35S-mGFP* proteins.

#### Construction of Plant Transformation Vector and Tobacco Transformation

Full-length coding sequence of *GhCCL* cDNA was isolated from drought-induced 5 dpa cotton bolls of *G. hirsutum* L. (cv. Bikaneri Narma) with the primers of 5'-CGGGA TCCATGCAGTCAGCAGCAGCAAC-3' (*Bam*HI site underlined) and 5'-CGAGCTCCTAATACTACTCCCCTTTG ATAC-3' (*Sac*I site underlined), and the amplified gene fragment was inserted in the corresponding sites of pBI121 binary vector (Clontech Laboratories, USA) by replacing the GUS reporter gene under the control of constitutive CaMV35S promoter. The derived vector was named as *pBI121-35S::GhCCL*. Another binary vector was constructed with *Arabidopsis thaliana rd29A* gene specific promoter (Shinozaki and Shinozaki 1994) which was amplified by PCR with primer pair 5'-CCCAAGCTTGCCATAGATGCAATTC-3' (*Hind*III site underlined) and 5'-GCTCTAGATTTCCAAAGATTTTTTTC-3' (*Xba*I site underlined) from the genomic DNA isolated from of *A. thaliana* ecotype Columbia using modified CTAB method (Porebski et al. 1997) and ligated to replace CaMV35S promoter in *pBI121-35S::GhCCL* binary vector. The derived vector was named *pBI121-rd29A::GhCCL*. These binary vectors were

individually mobilized into *A. tumefaciens* strain LBA4404 by freeze-thaw method (Hofgen and Willmitzer 1988). *Agrobacterium*-mediated transformation was carried out with co-cultivation of leaf discs obtained from in vitro grown tobacco (*Nicotiana tabacum* L. var. Petit Havana) plants, for 15 min with *Agrobacterium* strain LBA4404 harboring the recombinant binary vectors. Transformed leaf discs were selected on MS agar supplemented with 6-benzylaminopurine (2.5 mg l<sup>-1</sup>), naphthalene acetic acid (0.1 mg l<sup>-1</sup>), cefotaxime (250 mg l<sup>-1</sup>), and kanamycin (200 mg l<sup>-1</sup>). Regenerated shoots were rooted on phytohormone-free MS basal medium containing cefotaxime (250 mg l<sup>-1</sup>) and kanamycin (200 mg l<sup>-1</sup>) (Sunilkumar et al. 1999). The profusely rooted plantlets were further subcultured and maintained in tissue culture condition for further molecular and other stress-related studies. The clones were transferred to soil and grown in a glasshouse. The WT and the transgenic plants were studied under similar conditions for comparative molecular and physiological analyses.

#### Molecular Analysis of Transgenic Plants

PCR, Southern, and Northern Blot Analysis

Five lines of *35S::GhCCL* (named as A1, A2, A3, A5, and A6) and 9 lines of *rd29A::GhCCL* (named as B1, B3, B6, B11,

B15, B17, B18, B19, and B20) of transgenic and WT-rooted plantlets which were maintained in the glass house were selected for the isolation of the genomic DNA from leaf samples by following the method of Rogers and Bendish (1994). PCR analysis of the transgenic plants ( $T_0$ ) was performed using *NPTII* gene specific primers (forward primer 5'-CCGGAATTCATGATTGAACAA-3' and reverse primer 5'-CCCAAGCTTCAGAAGAAGCTC-3') and *GhCCL* gene specific primers (forward primer 5'-CGGGATCCATGCAGTCA GCAGCAGCAAC-3' (*Bam*HI site underlined) and 5'-CGAGCTCCTAATACTCCCTTTGATAC-3') (*Sac*I site underlined). PCR analysis was also carried out with selected  $T_1$  transgenic plants (A1, A2, A3, A5, A6, B1, B3, B6, B18, and B20) to amplify the specific 700-bp and 432-bp amplification fragments of *NPTII* and *GhCCL* genes, respectively. The binary vectors *pBII21-35S::GhCCL* and *pBII21-rd29A::GhCCL* were used as positive controls, and non-transformed plants (WT) and water control (W) were used as negative and environmental controls, respectively. PCR amplification was carried out in a thermal cycler (Eppendorf, Germany) programmed with an initial denaturation of DNA at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, an annealing step at 60 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1.0 % agarose gel.

Southern blot analysis was performed to confirm the stable integration and copy number of the *NPTII* gene. A 10- $\mu$ g of the genomic DNA from WT tobacco and  $T_1$  transgenic plants along with *pBII21-35S::GhCCL* binary plasmid was digested with *Bam*HI, which restricts T-DNA at single site. The digested genomic DNA was resolved on 0.8 % agarose gel and blotted on a nitrocellulose membrane (Hybond-N<sup>+</sup>, Amersham Pharmacia, UK). The blot was hybridized at 65 °C with  $\alpha$  [<sup>32</sup>P]-dCTP labeled 700-bp *NPTII* fragment using mega prime DNA labeling system (Amersham Biosciences, UK). The processing of the membrane was carried out as described before. Total RNA was isolated from  $T_1$  transgenic and WT tobacco plants using TRIzol reagent (Invitrogen Life technologies) as per manufacturer's instructions. Fifteen micrograms of total RNA was resolved on a 1.2 % formaldehyde denaturing gel and transferred to the nitrocellulose membrane (Hybond-N<sup>+</sup>, Amersham Biosciences, UK) using diethylpyrocarbonate (DEPC) treated 20 $\times$  SSC. Pre-hybridization and hybridization were performed with ULTRAhyb hybridization buffer (Ambion, USA) according to the manufacturer's instructions. The blot was hybridized at 42 °C with  $\alpha$  [<sup>32</sup>P]dCTP labeled 432-bp *GhCCL* fragment labeled with mega prime DNA labeling system (Amersham Biosciences, UK). The hybridized membrane was washed at 42 °C, and the membrane was exposed to X-ray film with an intensifying cassette under dark conditions. The cassette was placed at -70 °C

for a day, and the exposed X-ray film was developed to visualize the results.

### Analysis of Transgenic Tobacco Plants for Abiotic Stress Tolerance

#### Leaf Disc Assay

Leaf discs (1.0 cm<sup>2</sup>) were excised from healthy and fully expanded tobacco leaves of 4–6-week-old WT and  $T_0$  transgenic plants (A3, A6, B6, and B20) using a cork borer. Leaf discs were floated on 20 ml solutions of 10 % PEG, 200 mM each of mannitol for dehydration stress and NaCl for salinity stress. The experiments were carried out in three replicates. The plates were placed in the culture room for 72 h, and subsequently, the number of greenish and yellowish leaf disc were scored and plotted.

#### Seed Germination Assay Under Stress

For germination studies, WT and  $T_1$  transgenic tobacco seeds were surface sterilized and germinated on 90-mm-Petri dishes containing half strength MS agar medium supplemented with 10 % infused PEG-6000, 200 mM each mannitol or NaCl. Germination assays were carried out in three replicates, and the germination rate was scored after 20 days. Tolerance to PEG-6000, mannitol and NaCl stresses was judged based on the ability of the seeds to germinate and grow on the amended media. The number of germinated seeds was expressed as a percentage of the total number of seeds sown, and the performance of germination was compared to that of WT seeds.

#### Long-Term Growth Performance of Transgenic Plants Under Stress

Long-term stress effect was evaluated by subculturing the stem portion of WT and transgenic plants in glass bottles containing MS agar media supplemented with 10 % PEG, 200 mM each of mannitol and NaCl. The growth performance was observed after 45 days. The fresh weights were measured and plotted.

#### Water-Deficit Stress Tolerance of Transgenic Tobacco

WT and  $T_1$  transgenic tobacco plants were transferred from the culture conditions to soil in the glass house for water-deficit stress experiment. Water was withheld for 45 days at the time of flowering. The relative water content (Weatherley 1950, 1951) and total chlorophyll (Arnon 1949) were analyzed at 0 and 15 days after withholding water. Plants were allowed to recover for 20 days post 45 days of water withholding to observe the recovery in WT and transgenic plants.

## Statistical Analyses

Each experiment was replicated three times, and the values were expressed as means $\pm$ SE. All mean comparisons were done using paired *t* test for independent samples. The measurements for determining different treatments or times were analyzed by one-way analysis of variance (ANOVA).

## Results

### Isolation, Molecular Cloning, and Structural Organization of *GhCCL*

In the present study, we analyzed the possible role of *CCL* gene in water-deficit stress tolerance. In Affymetrix GeneChip, the probe for the transcript representing *CCL* gene was obtained from the cotton EST (GenBank Acc. No ES816870.1) which is 605 nucleotide in length with poly-A tail at 3' end. In order to isolate the complete gene sequence, the 5' RACE PCR technique was followed using degenerate primers to sequence 5' region. The RACE PCR product was cloned in pGEMT-Easy vector and sequenced. The sequence obtained was 671-bp in length with a 48-bp of 5' untranslated region (UTR) and 173-bp of 3' UTR. Further, coding region of the gene was identified by FGENESH of softberry online software using aligned sequence. The cDNA contained a 432-bp open reading frame (ORF) encoding a protein of 144 amino acids with a calculated molecular weight of about 15.43-kDa and an isoelectric point of 4.72. The full-length cDNA sequence of *GhCCL* was submitted to NCBI GenBank (accession number KF553636). The coding region (ORF of 432 bp) was amplified using specific primers having restriction sites and cloned in pGEMT-Easy vector and sequenced. Further, the ORF of *GhCCL* was cloned in a binary vector pBI121 with two different promoters viz., the constitutive Cauliflower Mosaic Virus 35S promoter and stress specific *Arabidopsis thaliana* rd29A promoter. The recombinant binary vectors were named *pBI121-35S::GhCCL* and *pBI121-rd29A::GhCCL*, respectively (Fig. S1) and mobilized to *Agrobacterium* strain LBA4404 by freeze-thaw method for plant transformation.

### Identification of Introns and Copy Number Determination of *GhCCL* Gene

The primers used to amplify the CDS were employed to isolate the genomic fragment of *GhCCL* from cotton (*G. hirsutum* L. cv. Bikaneri Narma) DNA and the genomic fragment was cloned in pGEMT-Easy vector and sequenced. The comparison of the full-length cDNA sequence with the corresponding genomic DNA sequence revealed that the coding region of the *GhCCL* is interrupted by two introns

(Fig. 1a). The 844-bp long sequence comprises of 5'UTR (1–48 bp), Exon 1 (49–239 bp), Intron 1 (240–334 bp), Exon 2 (335–494 bp), Intron 2 (495–595 bp), Exon 3 (596–671 bp), and 3'UTR region (672–844 bp).

To characterize the *GhCCL* in the genome, we performed Southern blotting, which could be used to obtain preliminary information on organization of the corresponding genomic sequence and the number of gene copies in cotton genome. Genomic DNA of *G. hirsutum* L. cv. Bikaneri Narma was digested with three different restriction enzymes and blotted to Hybond-N<sup>+</sup> membrane (Amersham Biosciences, UK). The blot was hybridized with  $\alpha$  [<sup>32</sup>P] dCTP-labeled 623-bp *GhCCL* fragment under high stringency conditions. The restriction enzyme *Hind*III has single site in the probe region, yielded two bands, whereas *Bam*HI and *Eco*RV having no restriction sites resulted in a single band, indicating that *GhCCL* is probably present as a single copy within the *G. hirsutum* genome (Fig. 1b).

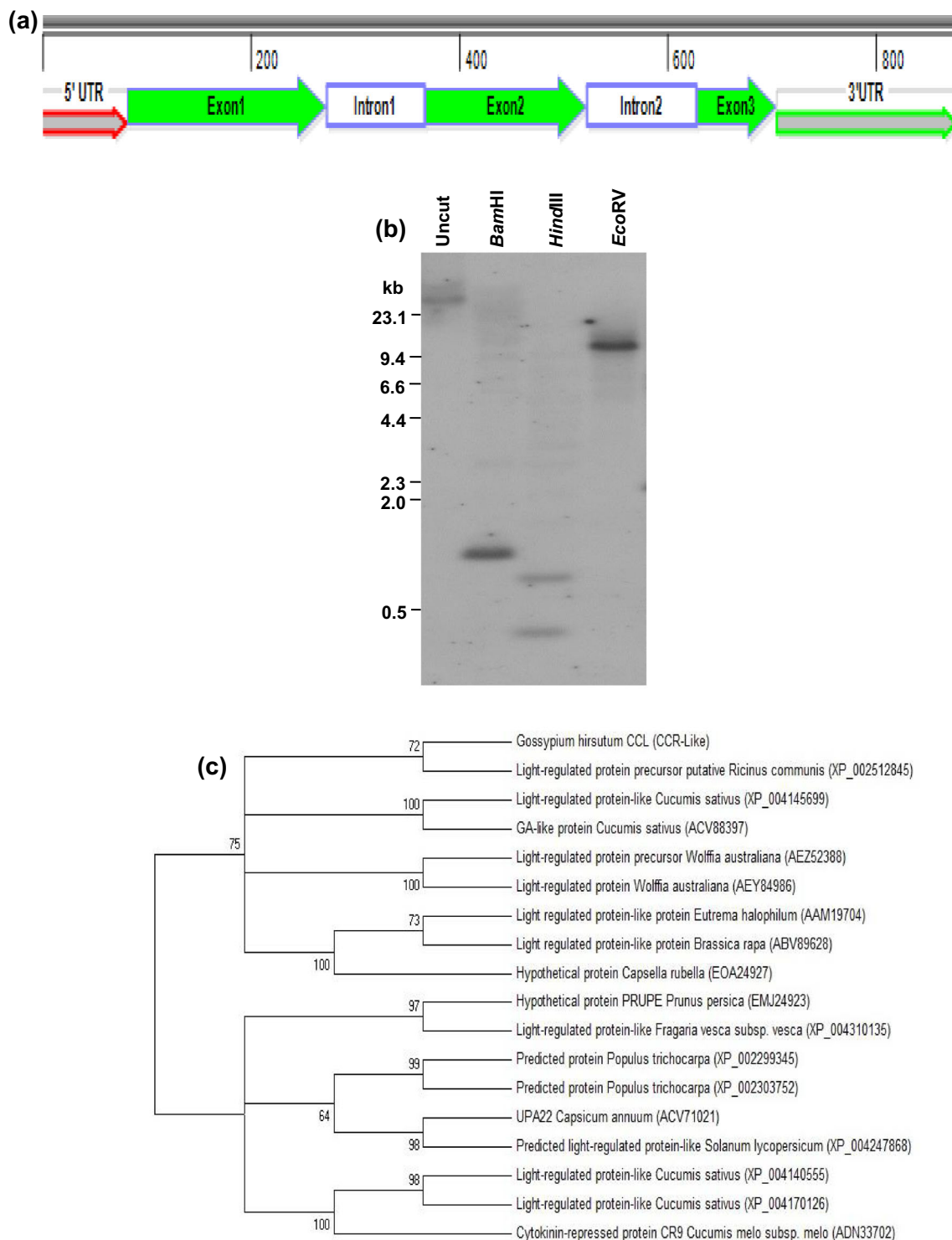
### Sequence Analysis of *GhCCL*

The sequence of *GhCCL* was aligned with *Arabidopsis CCL* sequence using BLAST algorithm to identify the homology between these sequences. We observed 69 and 48 % identity in nucleotide and protein sequences, respectively. Nucleotide composition was analyzed using BioEdit program. The percentages of A, C, G, and T nucleotides are 27.08, 28.24, 23.61, and 21.06, respectively with 51.85 % of GC content in *GhCCL* nucleotide sequence whereas the protein sequence has a high content of cysteine (28.24 %) followed by alanine (27.08), glycine (23.61), and threonine (21.06). Further, putative conserved domain called *lir1* (light, induced rice 1) superfamily (Fig. S2) was identified by NCBI-Conserved Domain Database using protein sequence of GhCCL.

BLASTP program was employed to identify the homology of the GhCCL protein sequence with that of other plant species. The alignment results showed maximum identity of 78 % with *Ricinus communis* putative light regulated protein precursor followed by 76 % with *Bruguiera gymnorrhiza* CCR protein, 75 % with *Lotus japonicus* unknown protein, 72 % with light regulated protein of *Vitis vinifera* and *Eutrema halophilum*. Phylogenetic tree showed that most of the sequences are aligned with light-regulated protein sequences of different plant species within two major clusters (Fig. 1c)

### Expression Profiles of *GhCCL* in Cotton Leaves and Boll Developmental Stages

Expression profiles of *GhCCL* gene were also studied in leaves and bolls of cotton at different stages (–2 days pre-anthesis to 0, 2, 3, 4, 5, 6, 7, 8, 10, and 20 days post anthesis) under control and drought induced in field conditions. Expression of the gene was observed in leaves and up to



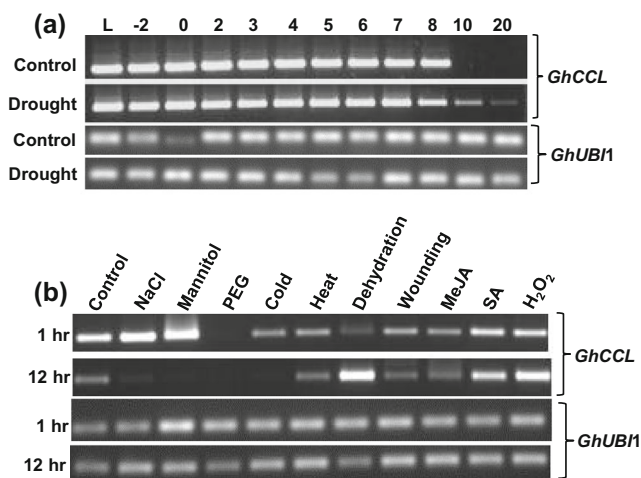
**Fig. 1** Organization of *GhCCL* gene. **a** Schematic representation of the *GhCCL* and exon-intron organization. **b** Detection of copy number of *GhCCL* by southern blot analysis. Genomic DNA (20  $\mu$ g) from cotton (cv. Bikaneri Narma) leaves was digested with *Bam*HI, *Hind*III, and *Eco*RV respectively, separated on a 0.8 % agarose gel and hybridized with  $\alpha$  [ $^{32}$ P] dCTP-labeled 623-bp of *GhCCL* as probe. Molecular marker in kb is indicated on the left. Note that *Bam*HI and *Eco*RV have

no restriction sites and *Hind*III have single restriction site within the probe sequence. **c** The phylogenetic tree showing the relationship between *Gossypium hirsutum* CCL and other plant proteins. Phylogram was generated based on protein sequences using the neighbor joining algorithm of MEGA software, version 5.05. Numbers above or below branches indicate bootstrap values (>50 %) from 1,000 replicates. The plant name is followed by the protein ID

8 dpa boll samples in control and drought-induced samples. Surprisingly, the expression of the gene was observed in 10

and 20 dpa of drought-induced bolls whereas there was no expression in respective control samples (Fig. 2a). The





**Fig. 2** Expression profiles of *GhCCL* in stage-specific control and drought-induced cotton tissues and seedlings subjected to various stresses. **a** Expression profile of *GhCCL* in field-grown cotton during different boll developmental stages under drought stress and control conditions. L leaf, -2 days pre anthesis, 0–20 days post anthesis, and lower panel indicates the expression of cotton *GhUBI1* internal gene, respectively. **b** Expression profile of *GhCCL* in response to different stresses and phytohormone treatments in cotton seedlings. One-week-old cotton seedlings were subjected to 200 mM NaCl, 200 mM mannitol, 15 % PEG-6000, cold (4 °C), heat (42 °C), dehydration, wounding, 10 mM methyl jasmonic acid, 10 mM salicylic acid, and 10 mM hydrogen peroxide for 1 and 12 h. Above panel and lower panel indicate the expression of *GhCCL* gene and cotton *GhUBI1* internal gene, respectively

samples used for RT-PCR and earlier microarray experiments were collected from two different seasons. In earlier microarray experiments (Padmalatha et al. 2012), maximum fold changes were observed in 5 dpa cotton bolls, but the RT-PCR study showed differential expression of *GhCCL* only in 10 and 20 dpa cotton bolls. This observation suggests that the circadian clock and environmental conditions may affect the expression levels in a stage-specific manner in plants. It also indicates that *GhCCL* gene may play an important role during drought stress at fiber elongation stage of cotton boll development.

#### Expression Profiles of *GhCCL* in Response to Diverse Abiotic Stresses in Cotton Seedlings

To determine the expression pattern of the *GhCCL* gene under various abiotic stress conditions, the seven-day-old cotton seedlings were exposed to PEG, mannitol, NaCl, cold, heat, dehydration, and wounding treatments for 1- and 12-hour periods to evaluate the effect of abiotic stresses. Total RNA isolated from treated cotton seedlings was used for semiquantitative RT-PCR. In mannitol, NaCl- and cold-treated seedlings, transcripts of *GhCCL* were observed within 1 h, and the expression level declined at 12 h. In the case of seedlings exposed to heat and wounding, transcripts of *GhCCL* accumulated within 1 h, and their expression is even seen at 12 h. In dehydration- and H<sub>2</sub>O<sub>2</sub>-treated seedlings, low level of

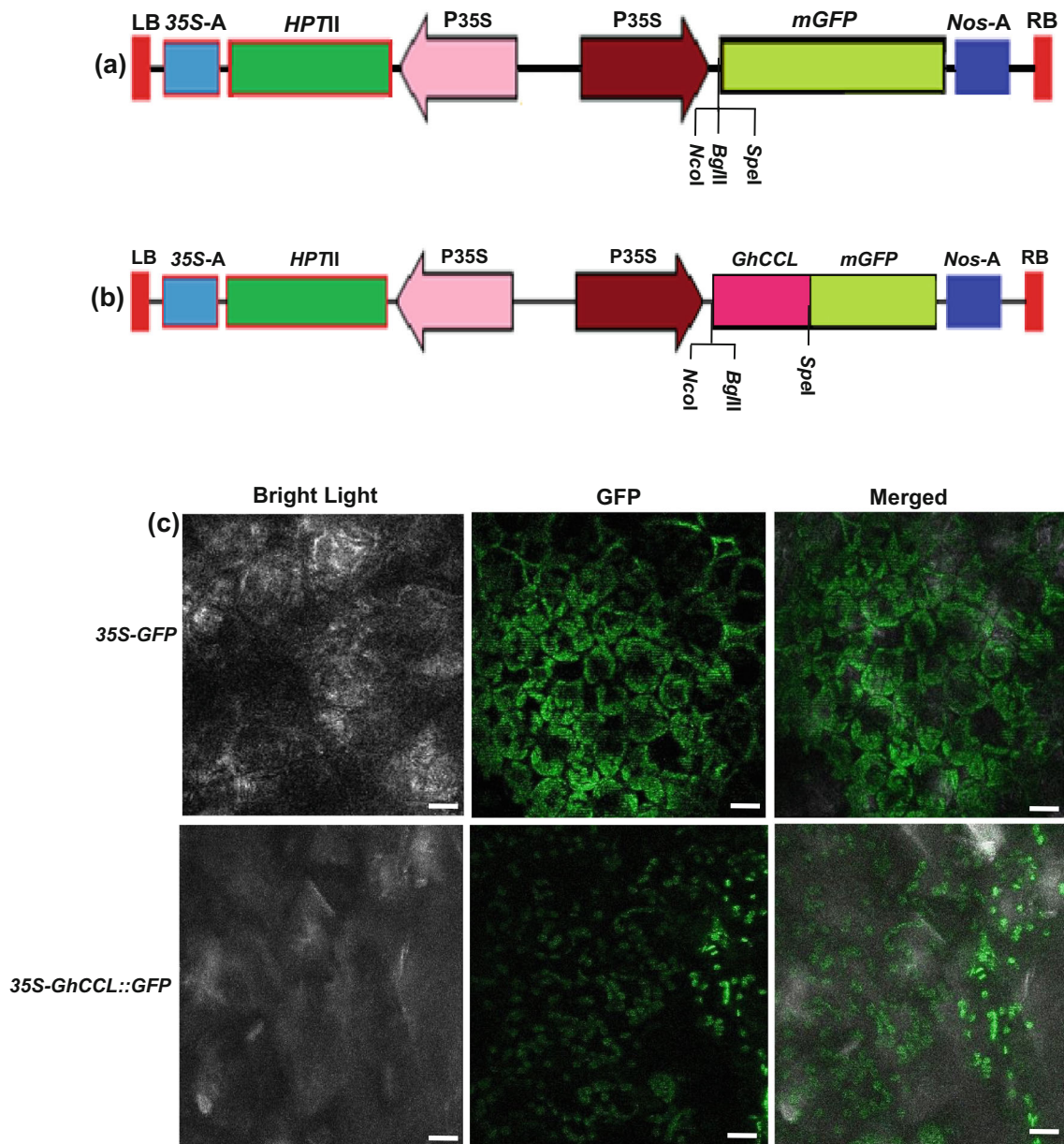
expression of transcripts was observed within 1 h, and high level of its expression was observed at 12 h. There was very low or no expression of the gene upon treatment with PEG for minimum and maximum periods tested in this study. We also observed the expression of the *GhCCL* when the cotton seedlings were treated with MeJA and SA, which are plant signal molecules that may accumulate upon pathogen infection and are involved in plant defense-signaling pathways (Agrawal et al. 2001). The expression of the gene was detected in both MeJA- and SA-treated seedlings (1 and 12 h periods) (Fig. 2b).

#### In Silico Prediction and Subcellular Localization of *GhCCL*

*GhCCL* is localized in chloroplasts as predicted by in silico analysis of *GhCCL* protein sequence using ProtComp v. 9.0 ([www.softberry.com/](http://www.softberry.com/)) and ChloroP 1.1 (<http://www.cbs.dtu.dk/services/ChloroP/>) programs (Table S1). To validate this prediction, mGFP was fused in frame to the C-terminal region of *GhCCL* under the control of CaMV 35S promoter. The binary vectors 35S-mGFP (Fig. 3a) and 35S-*GhCCL::mGFP* (Fig. 3b) were used for transient expression studies in tobacco leaves by agroinfiltration method. The injected leaf portion was visualized under TCS SP6 confocal microscope (Leica, Germany) after 4 days. As shown in Fig. 3c, the 35S-mGFP construct showed homogeneous green fluorescence throughout the cells, whereas in 35S-*GhCCL::mGFP*, green fluorescence of GFP was localized in the chloroplasts, confirming the chloroplast localization.

#### *GhCCL* Promoter Analysis

To explore the mechanism underlying *GhCCL* expression patterns, a 1,028-bp 5' flanking region of *GhCCL* was isolated by using Genome Walker kit (Clontech, USA) following the manufacturer's instructions. The genomic DNA sequence was submitted to the NCBI GenBank (accession number KF547867). Cis-acting regulatory elements were predicted using PlantCARE online tool (Lescot et al. 2002). A number of potential regulatory motifs corresponding to several known cis-acting elements related to light, stress and hormone responsive, and tissue specific expression elements were predicted (Table 2). Light-responsive elements such as G-box, I-box, as-2-box, rbcS-CMA7a among other related motifs which are involved in light response were identified. Interestingly, MRE-motif with a MYB binding site was also identified within the promoter region. Cis-regulatory elements responsive to various stresses such as elicitor (EIRE), heat stress (HSE), low temperature (LTR), and MYB binding site involved in drought inducibility were also identified. The motifs CGTCA, TGACG, and P-box elements were predicted to involve in MeJA and Gibberellic acid (GA) response,



**Fig. 3** Subcellular localization of the GhCCL protein in tobacco leaf tissue. **a, b** T-DNA regions of pCAMBIA1302 binary vector carrying *35S-mGFP* construct and *35S-GhCCL::mGFP* binary fusion construct, respectively. **c** Transient expression of *35S-mGFP* and *35S-*

*GhCCL::mGFP* in tobacco leaf tissue by Agroinfiltration method. The tissues were analyzed by Leica TCS SP6 confocal microscope after 4 days of agroinfiltration (scale bars in the lower right corners represent 20  $\mu$ m)

respectively. Cis-elements involved in tissue specific expression such as vacuole, meristem, endosperm, and shoot were predicted by in silico analysis of the *GhCCL* promoter sequence. Interestingly, the circadian element which is involved in the endogenous mechanism that allows the plant species to time their physiological changes to predict day/night cycles was also predicted. Taken together, the upstream sequence analysis showed that *GhCCL* gene may be under the control of a light, biotic, and abiotic stress-responsive promoter which is also expressed in a tissue specific manner in plants.

### Abiotic Stress Tolerance of Transgenic Tobacco

#### Overexpression of *GhCCL* in Transgenic Plants Shows no Negative Effect on Plant Growth

To evaluate the effect of over expression of *GhCCL* in tobacco, the *GhCCL* cDNA was fused to Cauliflower Mosaic Virus 35S promoter and *Arabidopsis thaliana* rd29A promoter and transferred to tobacco by *Agrobacterium*-mediated transformation. After selection on kanamycin-containing medium, the putative transgenic tobacco plants were further analyzed by

**Table 2** Putative *cis*-elements in the *GhCCL* promoter

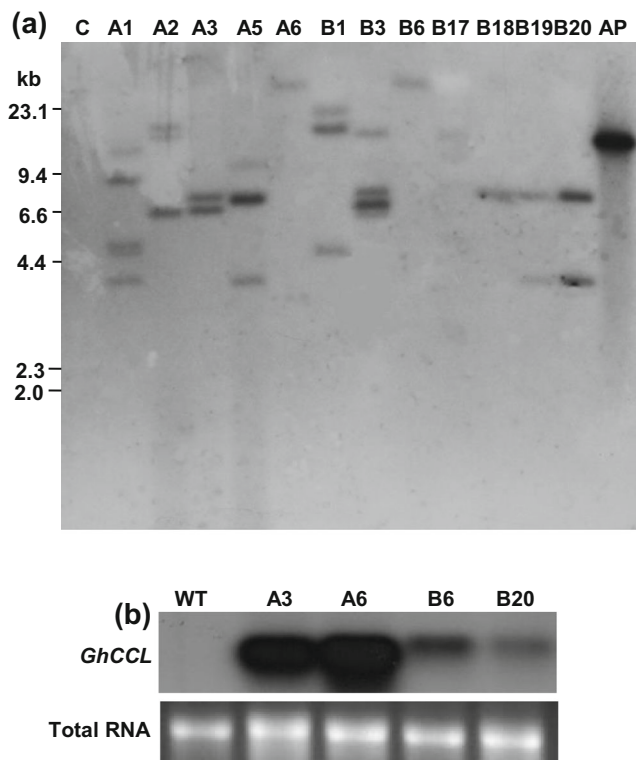
Type of motif	Sequences	Position	Strand	Function	References
Light responsive element					
ATCT-motif	CCTAAATCTGACT	778	–	Part of a conserved DNA module involved in light responsiveness	Kumar et al. (2009), Roy et al. (2012)
CATT-motif	CTTACGG	474	–	Part of a light-responsive element	Astorga and Estrella (1998)
CG-motif	GGGGTAC	579	–	Part of a light-responsive element	Staiger et al. (1989)
G-box	CACACG/CGACTC/CACGTC/CAGCAC	195, 528, 708, 711	+/-	Cis-acting regulatory element involved in light responsiveness	Zhao et al. (2012), Rose et al. (1999)
GAG-motif	GTAGAGA	961	–	Part of a light-responsive element	Karimzadeh et al. (2013)
I-box	GGAATAGA	883	–	Part of a light-responsive element	Zhao et al. (2012), Rose et al. (1999)
MRE-motif	AACCTAA	488	+	MYB binding site involved in light responsiveness	Whiteclaw et al. (1997)
Sp1	CCACCCCTTC	935	+	Light-responsive element	Aoki et al. (1995)
rbcS-CMA7a	CCTTATCGGC	275	–	Part of a light-responsive element	Chattopadhyay et al. (1998), Thanh et al. (2012)
as-2-box	GTAATAAATAG	450	–	Involved in light responsiveness	Lam et al. (1989), Wang et al. (2013)
Stress-responsive element					
EIRE	TTCGACC	608	+	Elicitor-responsive elements	Fukuda (1997)
HSE	AAAAAATTTA	1	+	Cis-acting element involved in heat stress responsiveness	Gu et al. (2013)
LTR	CCGAAA	735	+	Cis-acting element involved in low temperature responsiveness	Dunn et al. (1998), Brown et al. (2001)
MBS	CAACTG	701	+	MYB binding site involved in drought inducibility	Dabrowska et al. (2012), Karimzadeh et al. (2013)
TC-rich repeats	ATTGTCTAAC	10	+	Cis-acting element involved in defense and stress responsiveness	Fang et al. (2013), Karimzadeh et al. (2013), Nejad et al. (2013)
ARE	TTTTGGT	754	–	Cis-acting regulatory element essential for the anaerobic induction	Olive et al. (1990)
Tissue-specific expression					
AC-1	TCCAAACCCCTTCTCTATAAAATTC	929	+	Vacuole-specific expression	Gomez-Maldonado et al. (2004)
CAT-box	TCACCG	897	–	Cis-acting regulatory element related to meristem expression	Zhang et al. (2013)
Skn-1-motif	TACTGA	624, 742	–	Cis-acting regulatory element required for endosperm expression	Karimzadeh et al. (2013), Nejad et al. (2013)
as-2-box	GTAATAAATAG	450	–	Involved in shoot-specific expression	Wang et al. (2013), Lam et al. (1989)
Circadian	CAATTATAT/CAACACAAT/TTTATAGAAAC	515, 822, 904	+/-/-	Cis-acting regulatory element involved in circadian control	Piechulla et al. (1998), Zhao et al. (2012), Nejad et al. (2013)



PCR in  $T_0$  and  $T_1$  plants (Fig. S3a, b, c). Southern blot analysis of  $T_1$  transgenic plants demonstrated that the transgene was stably integrated into the genome of transgenic tobacco lines (Fig. 4a) with each carrying one or multiple copies of the transgene. RNA gel-blot analysis of the transgenic plants grown under normal and dehydration stress conditions confirmed that the transgene is expressed constitutively and in a stress-inducible manner in CaMV35S and *rd29A* promoter transgenic plants, respectively, whereas no expression was observed in the WT plants, as expected (Fig. 4b). No growth inhibition or phenotypic alterations were observed in transgenic plants compared to the WT. The transgenic tobacco plants developed normally and did not exhibit any growth retardation. Seed setting was also normal.

#### Leaf Disc and Germination Assay for Dehydration, Osmotic, and Salt Stresses

The WT and transgenic plants were exposed to dehydration, osmotic, and salt stresses, and their response was examined by



**Fig. 4** Southern and Northern blot analysis of transgenic tobacco plants. **a** Southern blot analysis of *GhCCL* expressing transgenic  $T_1$  tobacco plants showing the copy number of *NPTII*. Genomic DNA of transgenic events was digested with *Bam*HI that has no restriction site in the probe and single cutter in the T-DNA region. The blot was hybridized with  $\alpha$  [ $^{32}$ P] dCTP-labeled with *NPTII* probe. Molecular weight marker in kb indicated on the left. **b** RNA gel-blot analysis of wild-type and *GhCCL* expressing  $T_1$  tobacco plants. The ethidium bromide-stained gel demonstrates equivalent RNA quantities loaded in each lane (A3 and A6 are 35S::*GhCCL*, B6 and B20 are *rd29A*::*GhCCL* independent transgenic lines)

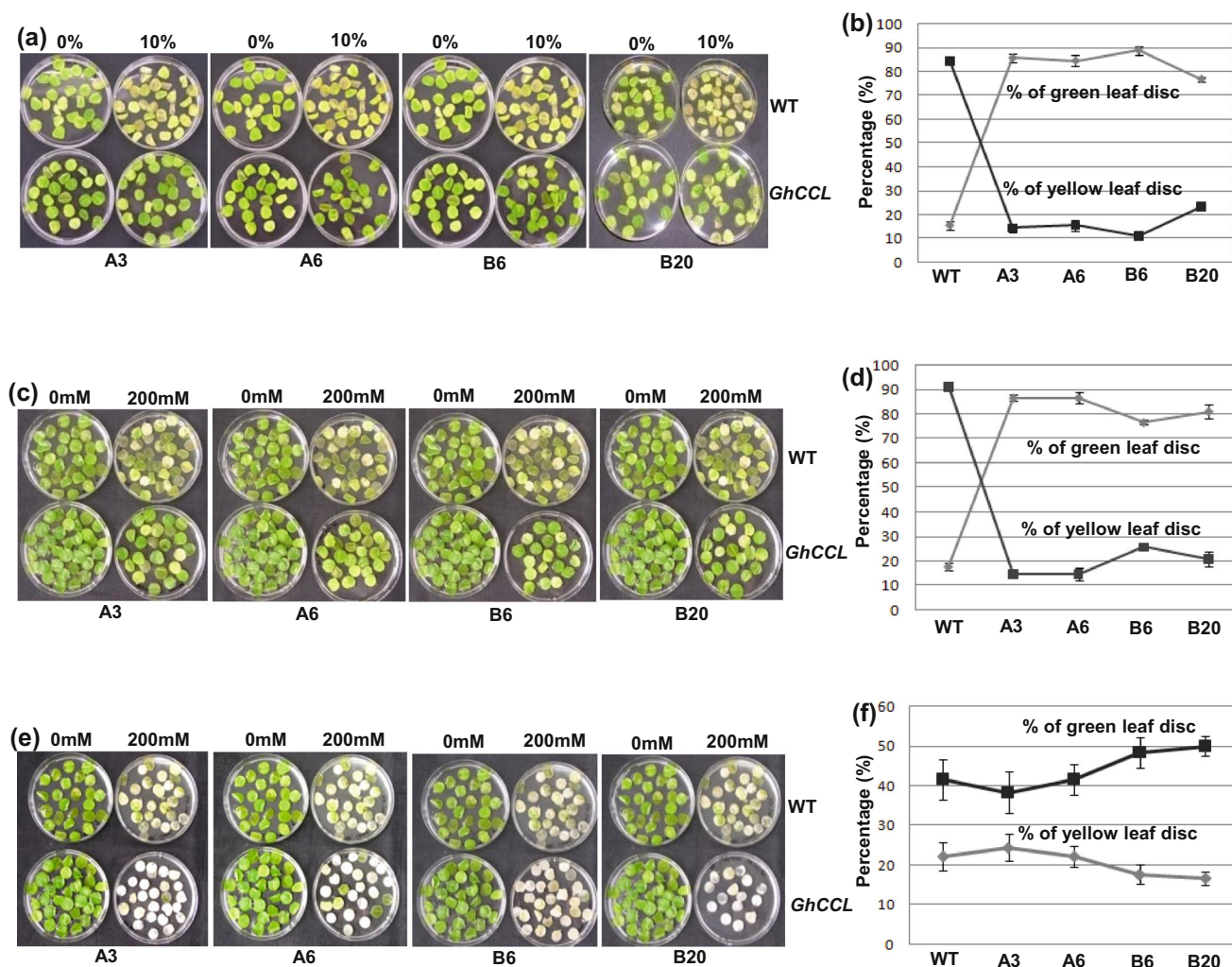
leaf-disc senescence assay under PEG-6000, mannitol and salt treatments, respectively. The leaf discs excised from mature leaves of WT and  $T_0$  transgenics of 35S::*GhCCL* (A3, A6) and *rd29A*::*GhCCL* (B6, B20) were used for the stress analysis. The maximum percentage of green leaf discs was observed in line B6 (88 %) followed by A3 (85 %) and A6 (84 %) after 72 h of treatment with 10 % PEG solution. The lines A3 and A6 showed 86.6 % of green leaf discs followed by B20 (81.1 %) and B6 (76.6 %) after 72 h of treatments with 200 mM mannitol solution. During the 72 h period of incubation in PEG-6000 and mannitol solution, the leaf discs from WT plants bleached drastically compared to the transgenic leaf discs (Fig. 5a–c). Interestingly, in NaCl-induced salinity stress samples, the WT tobacco plant performed better than the different transgenic lines tested (Fig. 5e). Leaf discs treated with distilled water devoid of PEG-6000, mannitol, and salt appeared green in both WT and the transgenics, and the percentage of green and bleached yellow leaf discs was calculated (Table S2) by ANOVA test and plotted (Fig. 5b–f).

To test the ability of seeds to germinate under dehydration, osmotic, and salt stresses, seeds from WT and transgenics of 35S::*GhCCL* (A3, A6) and *rd29A*::*GhCCL* (B6, B20) were surface sterilized and placed on 0.5 $\times$  MS agar medium supplemented with 10 % PEG-6000, 200 mM mannitol and 200 mM NaCl. The germination rate was observed (Fig. 6), and the percentage of germination was calculated (Table S3) after 20 days of germination. Higher percentage of germination was observed in the case of transgenic seeds (A3, A6, B6, and B20) as compared to the WT grown in MS media supplemented with PEG, mannitol whereas no significant differences were observed between transgenic seedlings and WT plants grown in MS agar plates without any stress. Further, seedlings of transgenic lines showed faster growth rate and produced thick green leaves compared to seedlings of WT which showed stunted growth and developed pale green leaves in the later stages. However, under NaCl-induced salinity stress, the germination rate of transgenic plants showed varied results.

Higher percentage of germination in MS media (10 % PEG-6000) was observed in A3 (97.3 %) and A6 (95 %) lines of 35S::*GhCCL* followed by B6 (82.6 %) and B20 (49.6 %) lines of *rd29A*::*GhCCL* whereas only 41.3 % germination was observed in WT tobacco seeds (Fig. 6a, b). Similarly, higher percentage of germination in MS media supplemented with 200 mM mannitol was observed in A3 (97.3 %) and A6 (86.3 %) lines of 35S::*GhCCL* followed by B6 (67.6 %) and B20 (66.6 %) lines of *rd29A*::*GhCCL* whereas only 33.6 % germination was observed in WT plants (Fig. 6c, d).

For salt tolerance analysis, surface-sterilized seeds of WT and transgenics were germinated on MS agar supplemented with 200 mM NaCl. The percentage of germination was





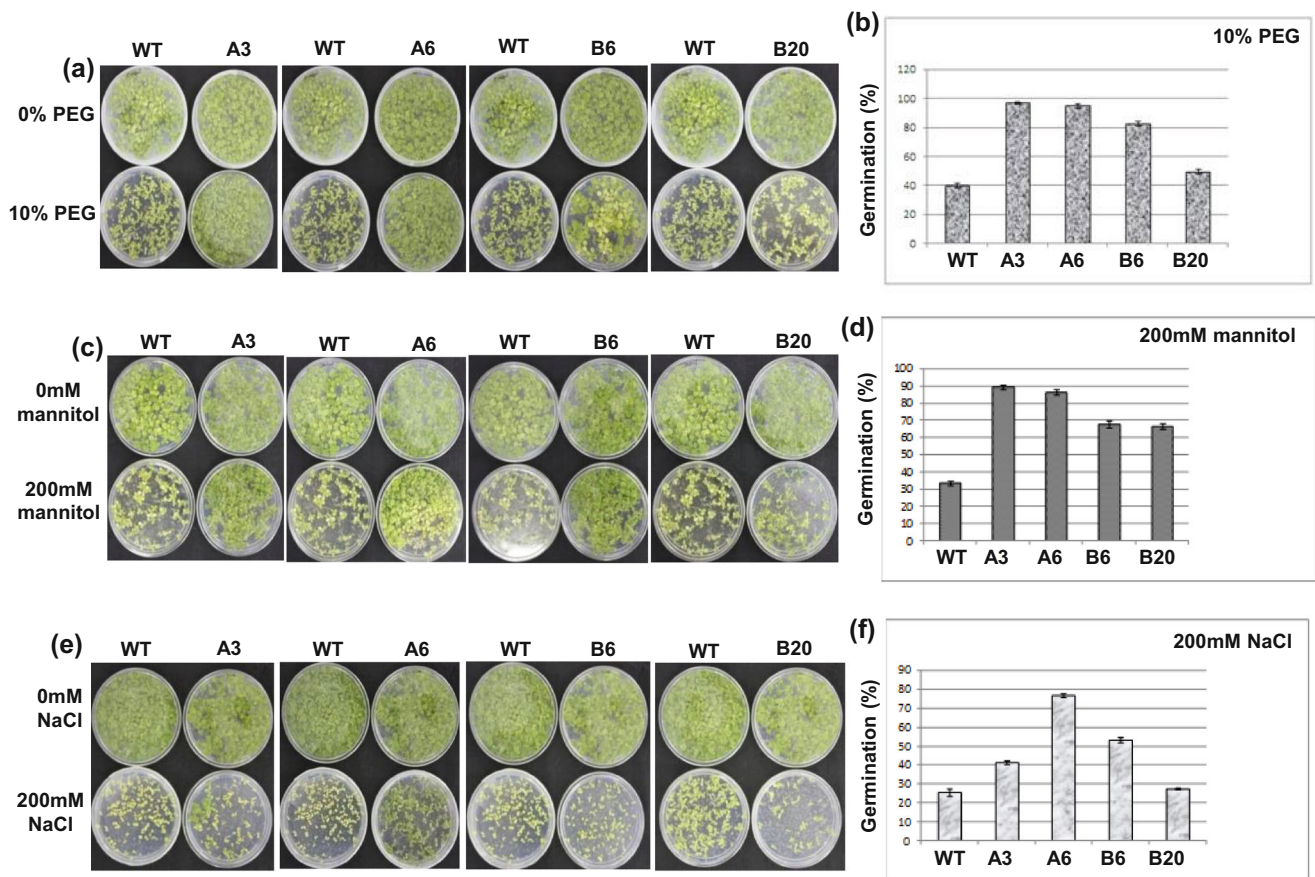
**Fig. 5** Leaf disc senescence assay of WT and  $T_0$  Transgenic plants. Photograph and bars showing the phenotypic differences and percentage response of the effect of **a, b** 10 % PEG-6000 **c, d** 200 mM mannitol and **e, f** 200 mM NaCl on the leaf-discs floated on distilled water from WT and  $T_0$  transgenic tobacco plants and leaf discs were observed after 72 h,

above and below panel as wild-type, *GhCCL* transgenic tobacco lines, respectively. Each bar value represents the mean $\pm$ of triplicates (ANOVA test), and means are significantly different from control at  $P < 0.05$  according to Dunnett's test at each treatment

observed and calculated after 20 days of sowing (Fig. 6e, f). Higher germination percentage was observed in A6 of *35S::GhCCL* (76.6 %) followed by B6 of *rd29A::GhCCL* (53.3 %), A3 of *35S::GhCCL* (41.3 %) and B20 of *rd29A::GhCCL* (27.6 %), whereas 25.6 % of germination was observed in WT plants. However, in the case of transgenic lines, we observed poor growth rate of seedlings grown under salt stress (200 mM NaCl) as compared to that of the seedlings grown under dehydration and osmotic stresses. Transgenic lines showed better tolerance to salt stress (200 mM NaCl) in seed germination assay as compared to leaf disc senescence assay.

In another set of experiment, stem portions of the WT and transgenic plants (A3, A6, B6, and B20) were subcultured in MS media supplemented with 10 % infused PEG-6000, 200 mM each of mannitol and NaCl for 45 days. Transgenic plants grew normally and initiated early root formation

whereas the WT plants showed stunted growth, delayed root formation, and pale green leaves (Fig. 7a–e). Percentage of fresh weight was calculated in the WT and transgenic plants after 45 days of exposure to the stress media (Table S4). In PEG-6000, higher fresh weight was observed in B6 (67.8 %) of *rd29A::GhCCL* followed by A6 (65.9 %) of *35S::GhCCL*, B20 (62.8 %) of *rd29A::GhCCL* and A3 (57.4 %) of *35S::GhCCL*, whereas only 48.5 % of fresh weight was observed in WT plants (Fig. 7b). In 200 mM mannitol, higher fresh weight was observed in A6 (64.4 %) of *35S::GhCCL* followed by B6 (63 %) of *rd29A::GhCCL*, B20 (59.1 %) of *rd29A::GhCCL* and A6 (51.1 %) of *35S::GhCCL*, but in WT plants, only 31.7 % accumulation of fresh weight was observed (Fig. 7d). Similarly, in 200 mM NaCl, higher fresh weight was observed in B20 (71.1 %) of *rd29A::GhCCL* followed by A3 (68.5 %) of *35S::GhCCL*, B6 (57.9 %) of *rd29A::GhCCL* and A6 (49.5) of *35S::GhCCL* whereas only



**Fig. 6** Performance and percentage (%) of WT and  $T_1$  transgenic seed germination on amended media. Sterilized seeds of WT and transgenics were germinated in petriplates having **a, b** 10 % PEG-6000 **c, d** 200 mM mannitol and **e, f** 200 mM NaCl on MS agar medium and observed

growth performance and calculated percentage of germination after 20 days. Each bar value represents the mean $\pm$ of triplicates (ANOVA test), and means are significantly different from control at  $P < 0.05$  according to Dunnett's test at each treatment

40.4 % fresh weight accumulated in WT plants (Fig. 7f). Further prolonged growth under stress resulted in stunting and bleaching of the WT plants.

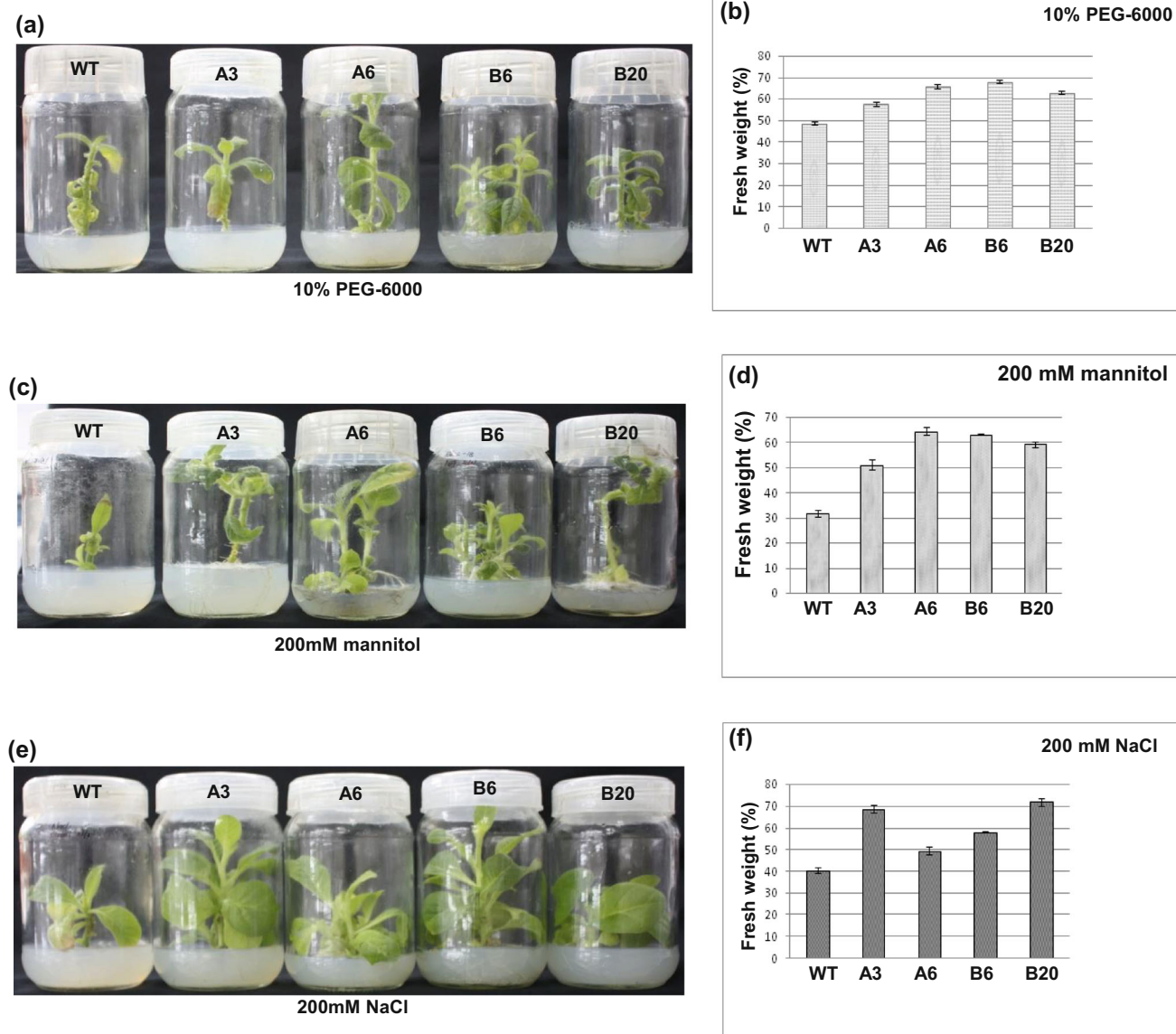
#### Stress Tolerance Study Under Glasshouse Conditions

The WT and  $T_1$  transgenic lines (A3, A6, B3, and B20) were maintained in a glass house and water-deficit stress was imposed by withholding the water at the flowering stage (Fig. 8a) as described in "Material and methods". Severe leaf wilting was observed in the case of WT plants but not in the case of transgenic plants even after 30 days of withholding the water (Fig. 8b). The stress was prolonged further to another 15–20 days, and we observed complete wilting of WT plants after 45 days whereas transgenic plants retained few green leaves, and even the stems remained green (Fig. 8c). We also tested the recovery capacity of the plants by providing water after water-deficit stress treatment for 45 days. Transgenic plants recovered within 20 days of providing the water and produced new branches and leaves. Flowering was also observed in the

later stage whereas WT plants did not show any recovery as they wilted completely after the stress treatment (Fig. 8d).

Relative water content (RWC) and chlorophyll content were estimated in WT and transgenic plants under water-deficit stress conditions at 0 and 15 days after withholding water. The percentage of RWC (Table S5) was in the range of 50.1 to 55.2 % in transgenic plants and 49.7 % in WT at 0 days of water withholding and the percentage of RWC in transgenics almost remained the same as at 0 dpa, but in WT, it declined from 49.7 to 43.1 % after 15 days of withholding water (Fig. 8e). Similarly, total chlorophyll content (Table S6) was in the range of 3.38 to 3.79 mg/g fw in transgenics and 3.41 mg/g fw in WT at 0 days of water withholding, and the total chlorophyll content was estimated in the range of 3.34 to 3.7 mg/g fw in the transgenics and 3.04 mg/g fw in WT after 15 days of withholding water (Fig. 8f). We observed the total chlorophyll content declined drastically in WT whereas in the transgenics, slight reduction was observed after 15 days of water-deficit stress. These results clearly suggested that the overexpression of *GhCCL* can impart tolerance to water-deficit stress in transgenic tobacco.





**Fig. 7** Long-term growth performance and fresh weight of WT and transgenic tobacco plants under stress medium. Stem portion of WT and transgenic tobacco were subcultured to bottles having **a, b** 10 % PEG-6000 **c, d** 200 mM mannitol and **e, f** 200 mM NaCl on MS agar medium

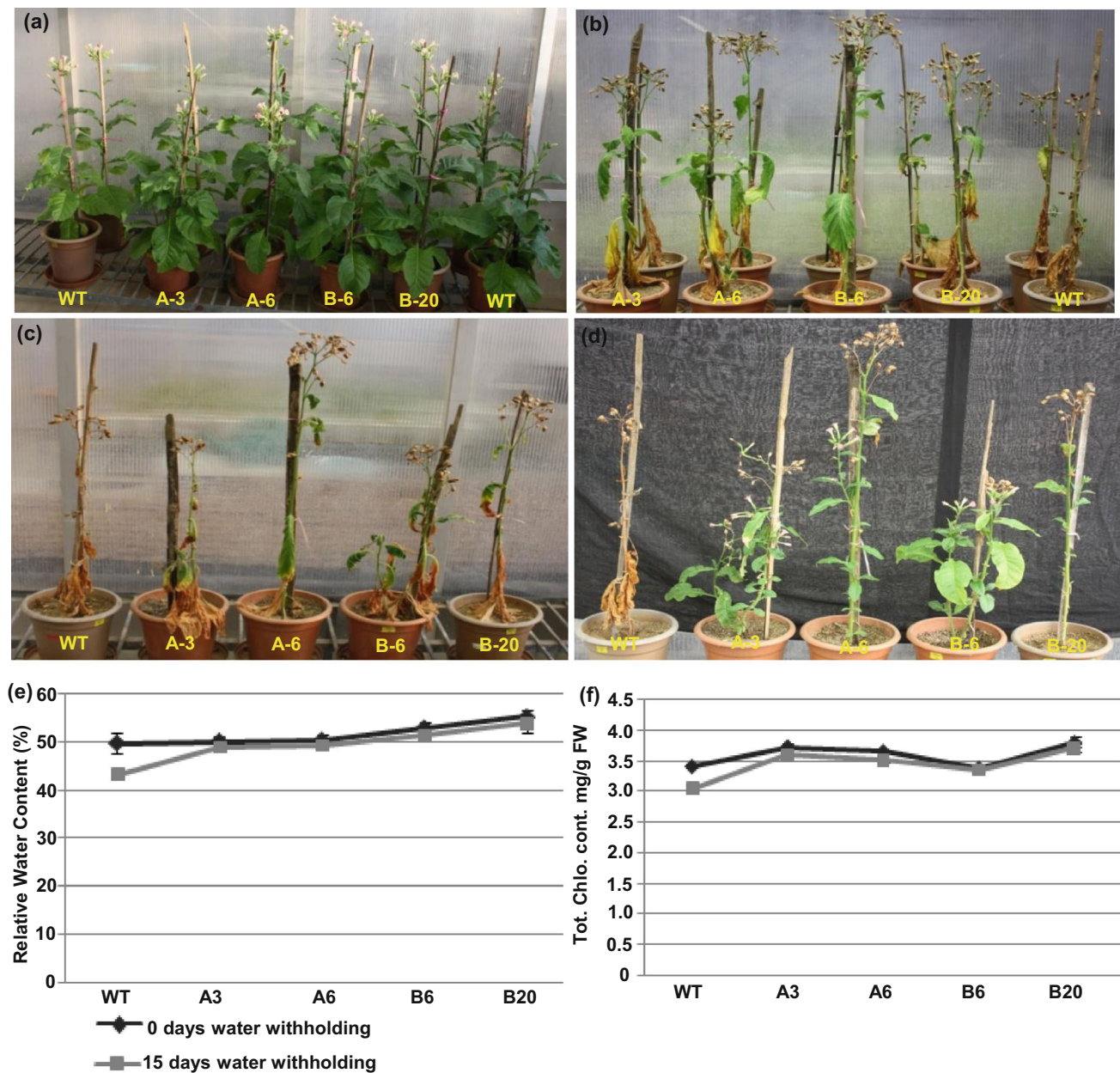
and growth phenotypes were observed after 40 days. Each *bar value* represents the mean $\pm$ of triplicates (ANOVA test), and means are significantly different from control at  $P<0.05$  according to Dunnett's test at each treatment

## Discussion

Cotton is the world's primary fiber crop and is a major agricultural commodity in over 30 countries. Like many other global commodities, sustainable cotton production is challenged by restricted natural resources. In response to the anticipated increase of agricultural water demand and constraints in water availability, research efforts in developing drought tolerant crops that can efficiently use water are being extensively carried out. Water-deficit stress is one of the most important factors that affect plant growth and development (Boyer 1982). Worldwide crop losses due to drought have multi-billion dollar impact on the economies (Mittler 2006;

Mittler and Blumwald 2010). Severe drought-induced yield reduction has been reported in crops like maize, barley, wheat, rice, and cotton (Wahid et al. 2007; Frederick et al. 2001; Pettigrew 2004). Although cotton is considered relatively drought-tolerant crop, water deficit reduces the lint quality and yield (Antony and Kutty 1975; Guinn and Mauney 1984a, b). Reduction in lint yield in cotton is due to reduced boll production because of fewer flowers and greater boll abortion when drought intensity is greater.

Drought stress impacts a network of plant gene expression mechanisms that lead to the reprogramming of a variety of physiological and metabolic processes in accordance with the stress response. Early studies primarily used model plant



**Fig. 8** Evaluation of water-deficit stress tolerance, relative water content, and total chlorophyll content analysis in WT and transgenic plants under glass house conditions. **a** WT and transgenic plants at flowering stage and 0 days of withholding water. **b** After 30 days of withholding water. **c** After 45 days of withholding water. **d** Plants recovered after 20 days of

rewatering. **e** Percentage of relative water content at 0 days and after 15 days of withholding water. **f** Total chlorophyll content at 0 days and after 15 days of withholding water. Each *bar value* represents the mean $\pm$  of triplicates (ANOVA test), and means are significantly different from control at  $P<0.05$  according to Dunnett's test at each treatment

species to identify a wide spectrum of genes that are involved in different levels of metabolism, signal transduction, osmotic regulation, stress response, and gene regulation (Seki et al. 2002; Rabbani et al. 2003; Umezawa et al. 2006). Compared to the conventional breeding methods, the genetic transformation technology has become an efficient way to accelerate the process of improving agronomic traits and economic characteristics of crops by incorporating exogenous genes encoding the desired traits (Zhang et al. 2011c).

Based on the transcriptome analysis of cotton during fiber development stages under drought-stress condition, we selected a transcript representing *CCL* gene which is highly expressed at early fiber elongation stage (5 dpa) for molecular characterization and functional analysis in transgenic tobacco. In the present study, we developed transgenic tobacco plants by overexpressing the coding region of *GhCCL* gene under constitutive (CaMV35S) and stress-specific (rd29A) promoters. The full-length gene and its 5' upstream gene



sequence were isolated using 5' RACE and genome walking PCR, respectively. The full-length gene is 844 bp in length with the coding region of 432 bp. Amplification of *GhCCL* gene from genomic DNA shows the presence of two introns. Further, subcellular localization studies showed that GhCCL is localized in chloroplasts. The gene was annotated earlier in TAIR database (<http://arabidopsis.org/servlets/TairObject?accession=locus:2090837>). *GhCCL* gene is present as single copy in the cotton (*G. hirsutum* L.) genome as identified by Southern hybridization analysis. We also observed that the *GhCCL* gene expression is modulated when cotton seedlings were exposed to various biotic and abiotic stresses. Analysis of 5' upstream sequence showed the presence of a number of potential regulatory motifs involved in regulation of light (G-box, I-box, as-2-box, rbcS-CMA7a), stress (EIRE, HSE, LTR, and MYB), hormone responses (Methyl Jasmonic acid and Gibberellic acid) and tissue specific expression elements. Analysis of the promoter region shows that the biotic and abiotic stresses can alter the expression of *GhCCL* gene. Analyzed sequence showed conserved domain for light regulation protein *lir1* family members. Reimann and Dudler (1993) demonstrated that the expression of *lir1* mRNA is controlled by light and circadian clock. The mRNA accumulates in the light, reaching maximum and minimum steady-state levels at the end of the light and dark period, which regulates growth and development and resets the circadian clock (Harmer et al. 2000). It is also reported that differential expression of many genes such as *CCL* is upregulated under low temperature stress in *Arabidopsis* (An et al. 2012). In support of this, our studies also proved that the *GhCCL* gene and its promoter region contain light regulatory, low temperature, and circadian clock responsive elements.

Cotton is most sensitive to cold at the beginning of the day and to high temperatures in the evening (Rikin et al. 1993). It has been suggested that this circadian-controlled gating of the timing of sensitivity to extreme temperatures might be a way for the plant to distinguish between changes in temperatures during the course of the day and seasonal changes in temperature. Microarray experiments have shown that around 70 % of the known clock-controlled genes may also be regulated by cold, salt, or drought stresses and regulate the mRNA levels of some pathogen-related genes in *Arabidopsis* as indication of the importance of the circadian system in regulating stress response (Kreps et al. 2002; Yakir et al. 2007b). An important adaptive feature of the circadian clock is its ability to be entrained by environmental signals such as changes in light or temperature. In general, RNA stability has an important role in the regulation of gene expression in eukaryotic cells (Ross 1995; Meyer et al. 2004). Several putative entrainment points have been described for the *Arabidopsis* oscillator. One such entrainment point is the activation of CCA1 and LHY transcription by light (Wang and Tobin 1998; Kim et al. 2003). Another well-characterized effect that light has on the

*Arabidopsis* oscillator is the regulation of proteasomal degradation of TOC1 and APRR5 proteins that is mediated by ZEITLUPE (ZTL), an F-box motif and Kelch domain protein (Mas et al. 2003; Kiba et al. 2007). ZTL itself is stabilized by a blue light-mediated interaction with GI (Kim et al. 2007). Finally, the translation rate of LHY may be light regulated; light causes an increase in the translation of LHY in transgenic plants expressing LHY from a constitutive promoter (Kim et al. 2003). Concluded that, CCA1 transcript stability and its degradation rate is regulated by light and is critical to ensure the circadian oscillator is accurately entrained by environmental changes (Yakir et al. 2007a).

The coding region of *GhCCL* was cloned in a binary vector under constitutive CaMV35S (*35S::GhCCL*) and stress-responsive rd29A promoter (*rd29A::GhCCL*) and overexpressed in transgenic tobacco. The leaf discs of the transgenic plants showed better response under 10 % PEG and 200 mM mannitol stress. However, the results showed that the transgenic lines were more prone to salinity stress.

Transgenic tobacco plants (lines # A3, A6, B6, and B20) grown in MS media supplemented with 10 % PEG, 200 mM each of mannitol and NaCl showed better growth compared to that of WT plants which suggested that high level expression of the gene might confer stress tolerance. Similarly, pot-grown transgenic and WT tobacco plants were subjected to water-deficit stress at the flowering stage. Transgenic plants could produce a few green leaves, and their stems remained green even after 45 days of withholding water whereas the WT plants completely dried. The transgenic plants recovered quickly upon rewatering and flowered normally, but the WT plants did not exhibit any signs of recovery demonstrating that the overexpression of *GhCCL* gene improved the plant growth, development, and tolerance to water-deficit stress.

Molecular mechanisms and other biological processes by which *CCL* gene is involved in stress tolerance and/or adaptation mechanism need further investigations. An earlier study reported that the CCL protein was probably involved in various physiological functions related to the adaptation of the plants to water deficit and/or saline conditions and has a vital role in the salt adaptive physiological processes in the halophyte *Suaeda maritima* exposed to NaCl treatment (Sahu and Shaw 2009). *CCL* gene encodes a highly unstable mRNA, the stability being regulated by the circadian clock. The transcript of this gene is significantly more stable in the morning than in the afternoon (Lidder et al. 2005). A high accumulation of the transcripts of the gene was observed when the plants were harvested at the evening time. It appeared that the salt treatment either had increased the stability of the *CCL* transcripts or had enhanced the expression of the gene in the plant (Sahu and Shaw 2009). Interestingly, several genes of the circadian clock were demonstrated to be involved in abiotic stress responses, and an intimate relationship between the circadian clock and ABA regulation was established (Sanchez et al.

2011). Since ABA-induced responses play a major role in conferring water-induced stress whether *CCL* gene also acts through ABA dependent pathway has to be looked into. However, the proteins of circadian clock like TOC1 and PRR5 are localized to the nucleus (Wang et al. 2010) in contrast to *CCL* protein which is found to be localized in the chloroplast. Also, *CCL* gene in Arabidopsis is under the control of circadian clock through selective mRNA degradation (Lidder et al. 2005). Whether *CCL* gene functions as a signal from or to the chloroplast in regulating its functions is not clear at this stage. Nevertheless, the steady-state levels of chlorophyll that were evident from our studies indicate that water-deficit stress-induced degradation of chlorophyll was absent in *CCL* overexpressed lines indicating that *CCL* transcripts can confer stability to the chloroplasts during abiotic stress conditions.

In conclusion, our results suggested that *GhCCL* is differentially expressed during boll developmental stages under drought stress in the field-grown cotton plants. *GhCCL* gene is also upregulated in cotton seedlings treated with different abiotic stresses. Complete molecular analysis such as gene organization, copy number, phylogenetic relationships, subcellular localization, and in silico analysis of the promoter sequence were carried out. Overexpression of the *GhCCL* gene positively regulated water-deficit stress and conferred abiotic stress tolerance to the transgenic plants which is the first demonstration of its kind. The gene is expressed better under the control of constitutive CaMV35S promoter as compared to the stress-responsive rd29A promoter. The ectopic expression of *GhCCL* in cotton would provide useful insights that would enhance our understanding of its function, regulation, and involvement in fiber quality under drought stress. Molecular mechanisms involved in the abiotic stress tolerance and the genome wide changes that are affected by the overexpression of *GhCCL* in cotton need further investigation.

**Acknowledgments** This work was supported by funds from the Indian Council of Agricultural Research (ICAR) under the National Agricultural Innovation Project (NAIP/C4/C10103). Funds from the Department of Biotechnology (DBT), Government of India are gratefully acknowledged. We thank Dr. I.S. Katageri, University of Agricultural Sciences, Dharwad for providing the drought-induced cotton boll samples used in the study and Dr. Ajay Jain, National Research Centre on Plant Biotechnology, New Delhi for providing the binary vector pCAMBIA1302. We also thank Dr. Shelly Praveen and Mr. Vipin Kumar, Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi for providing confocal microscope facility and technical help for subcellular localization study.

## References

Ackerson RC, Hebert RR (1981) Osmoregulation in cotton in response to water stress: I. Alterations in photosynthesis, leaf conductance, translocation and ultrastructure. *Plant Physiol* 67:484–488

- Acquaah G (2007) Principles of plant genetics and breeding. Blackwell, Oxford, UK
- Agrawal GK, Jwa NS, Rakwal R, Agrawal VP (2001) Signalling molecules and blast pathogen attack activates rice *OsPRIa* and *OsPRIb* genes: a model illustrating components participating during defense/stress response. *Plant Physiol Biochem* 39:1095–1103
- An D, Yang J, Zhang P (2012) Transcriptome profiling of low temperature treated cassava apical shoots showed dynamic responses of tropical plant to cold stress. *BMC Genomics* 13:64
- Antony AK, Kutty KE (1975) Effects of soil and climatic conditions on yield and fibre properties of improved strains of upland cotton. *Ind J Agri Sci* 45:199–203
- Aoki H, Tanaka K, Ida S (1995) The genomic organization of the gene encoding a nitrate-inducible ferredoxin-NADP<sup>+</sup> oxidoreductase from rice roots. *Biochim Biophys Acta* 1229:389–392
- Aprile A, Mastrangelo AM, De Leonardis AM, Galiba G, Roncaglia E, Ferrari F, De Bellis L, Turchi L, Giuliano G, Cattivelli L (2009) Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. *BMC Genomics* 10:279
- Amon DI (1949) Copper enzymes in intact chloroplast. Polyphenoxidase in *Beta vulgaris*. *Plant Physiol* 24:1–15
- Arpat AB, Waugh M, Sullivan JP, Gonzales M, Frisch D, Main D, Wood T, Leslie A, Wing RA, Wilkins TA (2004) Functional genomics of cell elongation in developing cotton fibers. *Plant Mol Biol* 54:911–929
- Astorga GA, Estrella LH (1998) Evolution of light-regulated plant promoters. *Annu Rev Plant Physiol Plant Mol Biol* 49:525–555
- Basra AS, Malik CP (1984) Development of the cotton fibre. *Int Rev of Cytol* 87:65–113
- Boyer JS (1982) Plant productivity and environment. *Science* 218:443–448
- Bradow JM, Davidonis GH (2010) Effects of environment on fiber quality. In: Stewart JM, Oosterhuis DM, Heitholt JJ, Mauney JR (eds) *Physiology of cotton*. Springer, Heidelberg, pp 229–245
- Brown APC, Dunn MA, Goddard NJ, Hughes MA (2001) Identification of a novel low-temperature-response element in the promoter of the barley (*Hordeum vulgare* L.) gene *bht101.1*. *Planta* 213:770–780
- Chattopadhyay S, Hong Ang L, Puente P, Deng XW, Wei N (1998) Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *The Plant Cell* 10:673–683
- Cohen D, Bogeat-Triboulot MB, Tisserant E, Balzergue S, Martin-Magniette ML, Lelandais G, Ningre N, Renou JP, Tamby JP, Le Thiec D, Hummel I (2010) Comparative transcriptomics of drought responses in Populus: a meta-analysis of genome-wide expression profiling in mature leaves and root apices across two genotypes. *BMC Genomics* 11:630
- Cothren J (1999) Physiology of the cotton plant. In: Smith CW, Cothren J (eds) *Cotton: origin, history, technology and production*. Wiley, New York, pp 207–268
- Dabrowska G, Adamska AM, Goc A (2012) Plant metallothioneins: putative functions identified by promoter analysis *in silico*. *Acta Biologica Cracoviensia series Botanica* 54:109–120
- Deng F, Tu L, Tan J, Li Y, Nie Y, Zhang X (2012) *GbPDF1* is involved in cotton fiber initiation via the core cis-element HDZIP2ATATHB2. *Plant Physiol* 158:890–904
- Divya K, Jami SK, Kirti PB (2010) Constitutive expression of mustard annexin, *AnnBj1* enhances abiotic stress tolerance and fiber quality in cotton under stress. *Plant Mol Biol* 73:293–308
- Dunn MA, White AJ, Vural S, Hughes MA (1998) Identification of promoter elements in a low-temperature-responsive gene (*bht4.9*) from barley (*Hordeum vulgare* L.). *Plant Mol Biol* 38:551–564
- Fang W, Zhang Y, Zhou L, Wang W, Li X (2013) Isolation and characterization of Histone1 gene and its promoter from tea plant (*Camellia sinensis*). *Mol Biol Rep* 40:3641–3648

- Frederick JR, Camp CR, Bauer PJ (2001) Drought- stress effects on branch and main stem seed yield and yield components of determinate soybean. *Crop Sci* 41:759–763
- Fukuda Y (1997) Interaction of tobacco nuclear protein with an elicitor-responsive element in the promoter of a basic class I chitinase gene. *Plant Mol Biol* 34:81–87
- Gelvin SB (2006) *Agrobacterium* virulence gene induction. *Methods Mol Biol* 343:77–84
- Gomez-Maldonado J, Canovas FM, Avila C (2004) Molecular analysis of the 5'-upstream region of a gibberellin-inducible cytosolic glutamine synthetase gene (*GS1b*) expressed in pine vascular tissue. *Planta* 218:1036–1045
- Gong P, Zhang J, Li H, Yang C, Zhang C, Zhang X, Khurram Z, Zhang Y, Wang T, Fei Z, Ye Z (2010) Transcriptional profiles of drought-responsive genes in modulating transcription signal transduction, and biochemical pathways in tomato. *J Exp Bot* 61:3563–3575
- Grimes DW, Yamada H (1982) Relation of cotton growth and yield to minimum leaf water potential. *Crop Sci* 22:134–139
- Gu L, Han Z, Zhang L, Downie B, Zhao T (2013) Functional analysis of the 5' regulatory region of the maize GALACTINOL SYNTHASE2 gene. *Plant Sci* 213:38–45
- Guinn G, Mauney JR (1984a) Fruiting of cotton. I. Effects of moisture status on flowering. *Agro J* 76:90–94
- Guinn G, Mauney JR (1984b) Fruiting of cotton. II. Effects of plant moisture status and active boll load on boll retention. *Agro J* 76:94–98
- Harmer SL, Hogenesch JB, Straume M, Chang H, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290:2110–2113
- He L, Yang X, Wang L, Zhu L, Zhou T, Deng J, Zhang X (2013) Molecular cloning and functional characterization of a novel cotton CBL-interacting protein kinase gene (*GhCIPK6*) reveals its involvement in multiple abiotic stress tolerance in transgenic plants. *Biochem Biophys Res Commun* 30:1–9
- Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucl Acids Res* 16:9877
- Ji SJ, Lu YC, Feng JX, Wei G, Li J, Shi YH, Fu Q, Liu D, Luo JC, Zhu YX (2003) Isolation and analyses of genes preferentially expressed during early cotton fiber development by subtractive PCR and cDNA array. *Nucl Acids Res* 31:2534–2543
- Karimzadeh M, Nematzadeh G, Hashemi SH (2013) Study of 5'UTR's cis-elements region of some plants P5CS gene. *Intl J Agri Crop Sci* 6:623–626
- Kawaguchi R, Girke T, Bray EA, Serres BJ (2004) Differential mRNA translation contributes to gene regulation under non stress and dehydration stress conditions in *Arabidopsis thaliana*. *Plant J* 38:823–839
- Kiba T, Henriques R, Sakakibara H, Chua NH (2007) Targeted degradation of PSEUDO-RESPONSEREGULATOR5 by a SCFZTL complex regulates clock function and photomorphogenesis in *Arabidopsis thaliana*. *Plant Cell* 19:2516–2530
- Kim JY, Song HR, Taylor BL, Carre IA (2003) Light-regulated translation mediates gated induction of the Arabidopsis clock protein LHY. *EMBO J* 22:935–944
- Kim WY, Fujiwara S, Suh SS, Kim J, Kim Y, Han L, David K, Putterill J, Nam HG, Somers DE (2007) ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. *Nature* 449:356–360
- Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic and cold stress. *Plant Physiol* 130:2129–2141
- Kumar GM, Mamidala P, Podile AR (2009) Regulation of polygalacturonase-inhibitory proteins in plants is highly dependent on stress and light responsive elements. *Plant Omics J* 2:238–249
- Kuppu S, Mishra N, Hu R, Sun L, Zhu X, Shen G, Blumwald E, Payton P, Zhang H (2013) Water-deficit inducible expression of a cytokinin biosynthetic gene IPT improves drought tolerance in cotton. *PLoS One* 8(5):e64190
- Lam E, Chua NH (1989) ASF-2: A factor that binds to the cauliflower mosaic virus 35 s promoter and a conserved GATA motif in cab promoters. *The Plant Cell* 1:1147–1156
- Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S (2002) Plant CARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucl Acids Res* 30:325–327
- Li HB, Qin YM, Pang Y, Song WQ, Mei WQ, Zhu YX (2007) A cotton ascorbate peroxidase is involved in hydrogen peroxide homeostasis during fibre cell development. *New Phytol* 175:462–471
- Li DD, Wu YJ, Ruan XM, Li B, Zhu L, Wang H, Li XB (2009) Expressions of three cotton genes encoding the PIP proteins are regulated in root development and in response to stresses. *Plant Cell Rep* 28:291–300
- Lidder P, Gutierrez RA, Salome PA, McClung CR, Green PJ (2005) Circadian control of messenger RNA stability. Association with a sequence-specific messenger RNA decay pathway. *Plant Physiol* 138:2374–2385
- Long L, Gao W, Xu L, Liu M, Luo X, He X, Yang X, Zhang X, Zhu L (2013) *GbMPK3*, a mitogen-activated protein kinase from cotton, enhances drought and oxidative stress tolerance in tobacco. *Plant Cell, Tissue Organ Cult.* doi:10.1007/s11240-013-0392-1
- Lu W, Chu X, Li Y, Wang C, Guo X (2013) Cotton *GhMCK1* induces the tolerance of salt and drought stress and mediates defence responses to pathogen infection in transgenic *Nicotiana benthamiana*. *PLoS One* 8(7):e68503
- Maqbool A, Abbas W, Rao AQ, Irfan M, Zahur M, Bakhsh A, Riazuddin S, Husnain T (2010) *Gossypium arboreum GHSP26* enhances drought tolerance in *Gossypium hirsutum*. *Am Inst Chem Eng Biotech Prog* 26:21–25
- Martin EC, Stephens W, Wiedenfeld R, Bittenbender HC, Beasley JPI, Moore JM, Neibling H and CGallian JJ (2007) Sugars, oil & fiber crops. In *Irrigation of agricultural crops*. 2nd edition. Edited by Lascano RJ and Sojka RE: Madison, Wisconsin: ASA-CSSA-SSSA30:279-335
- Mas P, KimWY SDE, Kay SA (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. *Nature* 426:567–570
- Meyer S, Temme C, Wahle E (2004) Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol Biol* 39:197–216
- Mittler R (2006) Abiotic stress, the field environment and stress combination. *Trends Plant Sci* 11:15–19
- Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: challenges and perspectives. *Annu Rev Plant Biol* 61:443–462
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nejad ES, Askari H, Hamzelou S, Gholami M (2013) Regulation of core cell cycle genes by cis-regulatory elements in *Arabidopsis thaliana*. *Plant Knowl J* 2:69–75
- Olive MR, Walker JC, Singh K, Dennis ES, Peacock WJ (1990) Functional properties of the anaerobic responsive element of the maize *Adh1* gene. *Plant Mol Biol* 15:593–604
- Padmalatha KV, Dhandapani G, Kanakachari M, Kumar S, Dass A, Patil DP, Rajamani V, Kumar K, Pathak R, Rawat B, Leelavathi S, Reddy PS, Jain N, Powar KN, Hiremath V, Katageri IS, Reddy MK, Solanke AU, Reddy VS, Kumar PA (2012) Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defense responsive genes. *Plant Mol Biol* 78:223–246
- Park W, Scheffler BE, Bauer PJ, Campbell BT (2012) Genome-wide identification of differentially expressed genes under water deficit



- stress in upland cotton (*Gossypium hirsutum* L.). *BMC Plant Biol* 12:90
- Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. *Mol Breeding* 23:625–639
- Pasapula V, Shen G, Kuppu S, Valencia JP, Mendoza M, Hou P, Chen J, Qiu X, Zhu L, Zhang X, Auld D, Blumwald E, Zhang H, Gaxiola R, Payton P (2011) Expression of an Arabidopsis vacuolar H<sup>+</sup>-pyrophosphatase gene (AVP1) in cotton improves drought- and salt tolerance and increases fibre yield in the field conditions. *Plant Biotechnol J* 9:88–99
- Payton P, Kottapalli KR, Kebede H, Mahan JR, Wright RJ, Allen RD (2011) Examining the drought stress transcriptome in cotton leaf and root tissue. *Biotech Lett* 33:821–828
- Pettigrew WT (2004) Physiological consequences of moisture deficit stress in cotton. *Crop Sci* 44:1265–1272
- Piechulla B, Merforth N, Rudolph B (1998) Identification of tomato *Lhc* promoter regions necessary for circadian expression. *Plant Mol Biol* 38:655–662
- Porebski S, Bailey G, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol Biol Rep* 15:8–15
- Rabbani AM, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Shinozaki KY (2003) Monitoring expression profiles of rice genes under cold, drought and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol* 133:1755–1767
- Reimann C, Dudler R (1993) Circadian rhythmicity in the expression of a novel light-regulated rice gene. *Plant Mol Biol* 22:165–170
- Rikin A, Dillwith JW, Bergman DK (1993) Correlation between the circadian rhythm of resistance to extreme temperatures and changes in fatty acid composition in cotton seedlings. *Plant Physiol* 101:31–36
- Rogers SO, Bendish AJ (1994) Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin SB, Schilperoort RA (eds) *Plant Mol Biol Manu.* Kluwer Academic Publishers, London
- Rose A, Meier I, Wienand U (1999) The tomato I-box binding factor LeMYB1 is a member of a novel class of Myb-like proteins. *The Plant J* 20:641–652
- Ross J (1995) mRNA stability in mammalian cells. *Microbiol Rev* 59:423–450
- Roy S, Choudhury SR, Singh SK, Das KP (2012) Functional analysis of light-regulated promoter region of *AtPolA* gene. *Planta* 235:411–432
- Ruan YL, Chourey PS (1998) A fibreless seed mutation in cotton is associated with lack of fibre cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. *Plant Physiol* 118:399–406
- Sahu BB, Shaw BP (2009) Isolation, identification and expression analysis of salt-induced genes in *Suaeda maritima*, a natural halophyte, using PCR-based suppression subtractive hybridization. *BMC Plant Biol* 9:69
- Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, New York
- Sanchez A, Shin J, Davis SJ (2011) Abiotic stress and the plant circadian clock. *Plant Signal Behav* 6:223–231
- Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enjul A, Sakurai T, Satou M, Akiyama K, Taji T, Shinozaki KY, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J* 31:279–292
- Shinozaki KY, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* 6:251–264
- Smith CW, Cothren JT (1999) *Cotton: origin, history, technology and production.* Wiley, New York
- Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517
- Staiger D, Kaulen H, Schell J (1989) A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an evolutionarily conserved nuclear protein. *Proc Natl Acad Sci U S A* 86:6930–6934
- Sunilkumar G, Vijayachandra K, Veluthambi K (1999) Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. *Plant Sci* 141:51–58
- Taiz L and Zeiger E (1998) *Stress physiology.* In: *Plant physiology*, 2nd edn. Sunderland, MA: Sinauer Associates Inc, 725-757
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thanh T, Chi VTQ, Omar H, Abdullah MP, Napis S (2012) Sequence Analysis and Potentials of the Native RbcS Promoter in the Development of an Alternative Eukaryotic Expression System Using Green Microalga *Ankistrodesmus convolutus*. *Int J Mol Sci* 13:2676–2691
- Umezawa T, Fujita M, Fujita Y, Shinozaki KY, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. *Curr Opin Biotechnol* 17:113–122
- Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. *Environ Exp Bot* 61:199–223
- Wang ZY, Tobin EM (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207–1217
- Wang L, Fujiwara S, Somers DE (2010) PRR5 regulates phosphorylation, nuclear import and subnuclear localization of TOC1 in the Arabidopsis circadian clock. *EMBO J* 29:1903–1915
- Wang K, Zhang X, Zhao Y, Chen F, Xia G (2013) Structure, variation and expression analysis of glutenin gene promoters from *Triticum aestivum* cultivar Chinese Spring shows the distal region of promoter 1Bx7 is key regulatory sequence. *Gene* 527:484–490
- Weatherley PE (1950) Studies in the water relations of the cotton plant I. The field measurements of water deficits in leaves. *New Phytol* 49:81–97
- Weatherley PE (1951) Studies in the water relations of the cotton plant II. Diurnal and seasonal fluctuations and environmental factors. *New Phytol* 50:36–51
- Whitelaw CA, Le Huquet JA, Thurman DA, Tomsett AB (1997) The isolation and characterization of the type II metallothionein-like genes from tomato (*Lycopersicon esculentum* L.). *Plant Mol Biol* 33:503–511
- Wilkins TA, Arpat AB (2005) The cotton fiber transcriptome. *Physiol Plant* 124:295–300
- Xue T, Li X, Zhu W, Wu C, Yang G, Zheng C (2009) Cotton metallothionein *GhMT3a*, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *J Exp Bot* 60:339–349
- Yakir E, Hilman D, Harir Y, Green RM (2007a) Regulation of output from the plant circadian clock. *FEBS J* 274:335–345
- Yakir E, Hilman D, Hassidim M, Green RM (2007b) CIRCADIAN CLOCK ASSOCIATED1 transcript stability and the entrainment of the circadian clock in Arabidopsis. *Plant Physiol* 145:925–932
- Yue Y, Zhang M, Zhang J, Tian X, Duan L, Li Z (2012) Overexpression of the *AtLOS5* gene increased abscisic acid level and drought tolerance in transgenic cotton. *J Exp Bot* 63:3741–3748
- Zhang H, Shen G, Kuppu S, Gaxiola R, Payton P (2011a) Creating drought- and salt-tolerant cotton by overexpressing a vacuolar pyrophosphatase gene. *Plant Signal Behav* 6:861–863



- Zhang L, Xi D, Li S, Gao Z, Zhao S, Shi J, Wu C, Guo X (2011b) A cotton group C MAP kinase gene, *GhMPK2*, positively regulates salt and drought tolerance in tobacco. *Plant Mol Biol* 77:17–31
- Zhang X, Zhen JB, Li ZH, Kang DM, Yang YM, Kong J, Hua JP (2011c) Expression profile of early responsive genes under salt stress in upland cotton (*Gossypium hirsutum* L.). *Plant Mol Biol Rep* 29: 626–637
- Zhang LF, Li WF, Han SY, Yang WH, Qi LW (2013) cDNA cloning, genomic organization and expression analysis during somatic embryogenesis of the translationally controlled tumor protein (TCTP) gene from Japanese larch (*Larix leptolepis*). *Gene* 529:150–158
- Zhao W, Wang S, Li X, Huang H, Sui X, Zhang Z (2012) Molecular cloning and characterization of the light-regulation and circadian-rhythm of the VDE gene promoter from *Zingiber officinale*. *Plant Cell Rep* 31:1381–1392
- Zhao J, Gao Y, Zhang Z, Chen T, Guo W, Zhang T (2013) A receptor-like kinase gene (*GbRLK*) from *Gossypium barbadense* enhances salinity and drought-stress tolerance in Arabidopsis. *BMC Plant Biol* 13:110
- Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong L, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high salinity stresses in shoot, flag leaf and panicle. *Plant Mol Biol* 63:591–608
- Zhu YN, Shi DQ, Ruan MB, Zhang LL, Meng ZH, Liu J, Yang WC (2013) Transcriptome analysis reveals crosstalk of responsive genes to multiple abiotic stresses in cotton (*Gossypium hirsutum* L.). *PLoS One* 8(11):e80218