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Research article

Knockdown of *GhIQD31* and *GhIQD32* increases drought and salt stress sensitivity in *Gossypium hirsutum*

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ABSTRACT

Drought, salinity and cold stresses have a major impact on cotton production, thus identification and utilization of plant genes vital for plant improvement Whole-genome identification and functional characterizations of the IQ67-domain (IQD) protein family was carried out in which 148, 77, and 79 *IQD* genes were identified in *Gossypium hirsutum*, *G. raimondii*, and *G. arboreum*. The entire IQD proteins had varied physiochemical properties, however; their grand hydropathy values were negative, which demonstrated that the proteins were hydrophilic, a property common among the proteins encoded by various stresses responsive genes, such as the late embryogenesis abundant (LEA) proteins. The IQD proteins were predicted to be majorly sublocalized in the nucleus; moreover, various *cis*-regulatory elements with higher role in enhancing abiotic stress tolerance were detected. RNA-seq and RT-qPCR analysis revealed two key genes, *Gh_D06G0014* and *Gh_A09G1608* with significantly higher upregulation across the various tissues under drought, salt and cold stress. Knockdown of the two genes negatively affected the ability of *G. hirsutum* to tolerate the effects of the three stress factors, being all the antioxidant assayed were significantly low concentrations compared to the oxidizing enzymes in VIGS plants under stress, furthermore, morphological and physiological traits were all negatively affected in VIGS plants. Expression levels of *GhLEA2*, *GhCDK_F4*, *GPCR (TOM1)* and *Gh_A05G2067* (TH), the stress responsive genes were all downregulated in the VIGS plants, but significantly upregulated in WT and positively controlled plants. The results demonstrated that the *IQD* genes could be responsible for enhancing drought, salt and cold stress tolerance in cotton.

1. Introduction

Environmental changes is a reality, and plants are constantly exposed to extreme environmental conditions which has negatively affected their performance (Tewari et al., 2018). But being the plants are sessile organisms, they have developed a number of survival strategies, one of which is the activation of series of signal transduction pathways which aids in coordinating and controlling the metabolic and physiological response mechanisms needed for plants adaptation (Virlouvet et al., 2018). Several plant signal pathways have so far been identified to have significant role in promoting plants response to various environmental stresses, for instance, the calcium ions (Ca^{2+}) is an important cytosolic second messenger, which plays a prominent role in many essential biological processes including plants response to abiotic stress factors (Huang et al., 2012). Moreover, when plants are exposed to abiotic and biotic stress factors, they initiates intracellular calcium transients, which decodes the calcium signals, thereby transforming the signals into cellular responses aimed at improving their performance under stress conditions (Knight and Knight, 2001).

The low solubility of calcium phosphate salts, provide a chemical

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evolution of the calcium ions as a universal second messenger within the plant cells (Al-Shaikhaly et al., 1980). The cellular energy metabolism can be destroyed by high concentrations of calcium ions (Henke et al., 2012). Thus the cellular metabolic processes are maintained by adjustments in the cytosolic calcium ions concentration (Meyer et al., 2007). The cytosolic ions concentration is adjusted through exporting calcium ions to other cellular parts, creating a steep concentration gradient thereby resulting into rapid Ca^{2+} transients in response to extracellular stimuli (Sathyanarayanan and Poovaiah, 2004). To date, several types of Ca^{2+} sensors have been identified in plants which have the Ca^{2+} binding helix-loop-helix fold known as the "EF-hand motif" (Gaburjakova and Gaburjakova, 2017). The Ca^{2+} sensors are classified into four types based on the EF-hand motifs namely, the calcineurin B-like (CBL) proteins, possessing three EF-hand motifs; calmodulin (CAM), containing four EF-hand motifs; Ca^{2+} dependent protein kinases (CDPKs), containing four EF-hand motifs and a Ca2+-dependent Ser/Thr protein kinase domain, and Ca^{2+} sensors lacking EF-hand motifs (Reddy and Day, 2001). Among the four groups of the Ca^{2+} binding proteins, calmodulin (CaM) has attracted wider studies, CaM is small, highly stable and heat resistant (Sharma and Parameswaran, 2018). The CBL proteins are the structural bases for Ca^{2+} binding, and do interact with the Serine/threonine protein kinases, thus often referred to as the CBL-interacting protein kinases (CIPKs), which are designated as CBL-interacting protein kinases (CIPKs) (Sun et al., 2015).

The CIPKs do substitute for the CBLs which lack enzymatic activity of its own, in order to receive and transmit the Ca^{2+} signals, while the CaMs and the CBL sensor proteins, also lack catalytic activity, and thus mainly referred to as calcium ion signal relayers (Yalovsky et al., 2017). Due to their duality, the CDPK proteins functions as the catalytic effectors and have significant roles in stress signaling pathways (Reddy and Day, 2001). The CaM protein sequences harbors three specific motifs which defines the role of the CaM, name the IQ motif which mediates the calcium ion independent CaM retention, 1-(5)-10 and 1-(8)-14 motifs which functions in the calcium ion dependent CaM retention and do differ with the arangement in their basic amino acid and bulky hydrophobic residues (Abel et al., 2005a). The IQ motif have so far been identified in five protein families in *Arabidopsis thaliana* which has further elucidated the putative role of IQ motif in CAM-regulated processes (McLean et al., 2013). In plants, the IQD proteins contain a specific domain of 67 highly conserved amino acid residues, thus coded IQ67 domain (Shashikanth et al., 2008), moreover, IQD1 of the IQ67 domain has been found to be integral and sufficient for CaM interaction in vitro (Bürstenbinder et al., 2013).

The plant-specific IQ67-domain (IQD) protein family have been found posses some unique characteristics; they have accurate spacing of the IQ motif, often separated by 11–15 amino acid residues; IQ motif overlap with three copies of 1-(8)-14 and four copies of 1-(5)-10 motifs and lastly the IQD proteins have a highly conserved exon-intron interuption, which occurs at 16 and 17 codons via a phase zero intron (Reddy and Day, 2001). The inherent properties enables the IQD proteins to have a protective role on various membraneous structures due to their ability to fold into amphiphilic helix structure (Parker and Stezowski, 1996). Moreover, *IQD1* gene, has been found to target microtubules, and interacts with kinesin light chain-related protein-1(KLCR1) and CaM/CaM-like proteins, indicating that the protein encoded by *IQD1* gene, could be functioning as a scaffold protein (Abel et al., 2013). Furthermore, three sets of *IQD* genes from *A. thaliana*, *IQD11*, *IQD14* and *IQD16* genes have been found to have a regulatory role in plants growth and development through a mechanism linked to CaM-dependent Ca^{2+} signaling pathway (Bürstenbinder et al., 2017). Recent investigation has led to the discovery of *GSE5* gene, which encodes a plasma membrane-associated protein with IQ domains, and found to interacts with the rice calmodulin protein, OsCaM1-1, with a higher role in improving rice grain quality (Duan et al., 2017). Furthermore, two Arabidopsis *IQD* genes, *AtIQD1* and *AtIQD22,* have been found to stimulate glucosinolate accumulation and acts as negative regulator of the plants response to gibberellin hormone, respectively (Hepler et al., 2002).

Cotton being an important agronomic crop, its production has continued to decline due to drought and salt stresses (Abdelraheem et al., 2019). Moreover, improvement of the crop to adapt to ever-changing environmental condition has paused a great challenge due to the narrow genetic base of cultivated cotton germplasm (Magwanga et al., 2018d). And thus the utilization of novel genes may help to break the bottle neck. So far, the IQD proteins have been identified and characterized in a number plant such as; *Brachypodium distachyon* (Abel et al., 2013), *Arabidopsis thaliana* and *Oryza sativa* (Abel et al., 2005b), among others, with all elucidating their functional role in enhancing the plants performance under abiotic stress condition, but no work has ever been done in any of the cotton genomes. In this research work, we undertook to carryout genome wide identification of the proteins encoded by the *IQD* genes in cotton, analysed their phylogenetic relationship, gene structure, chromosome distribution and their expression analysis under drought and salt stress conditions. We further characterized two of the highly expressed upland cotton, *G. hirsutum* *IQD* genes and evaluated their putative role in cotton through virus induced gene silencing (VIGS), in which various physiological, morphological and biochemical parameters were evaluated on the VIGs-plants, positive control and the wild types under salt and drought stress conditions.

2. Materials and methods

2.1. Identification of IQD family genes in the cotton

The IQ conserved domain PF00612 was used as the query probe for the identifications of the cotton proteins encoded by the *IQD* genes. The IQD proteins for *G. hirsutum* and *G. arboreum* were obtained from the cotton research institute website (http://mascotton.njau.edu.cn/), *G. raimondii* (D_5), *Glycine max*; *Theobroma cacao* and *Oryza sativa* were downloaded from phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), while for *Arabidopsis thaliana* the IQD proteins were downloaded from TAIR (http://www.arabidopsis.org/). The HMM profiles of the IQ functional domain PF00612 was retrieved from the Pfam database (http://pfam.xfam.org/) and quarried for the identification of the putative IQD proteins with the best domain e-value cutoffs of $< 1 \times 10^{-4}$. The NCBI database (http://www.ncbi.nlm.nih.gov/) and SMART tool (**Error! Hyperlink reference not valid.**) were employed in order to validate the HMM and BLAST search for all the IQD proteins obtained (Ludwig-Müller, 2011). The MUSCLE program was used to perform the alignment of the full-length IQD protein sequences with the default parameters (Edgar, 2004). The maximum-likelihood method was used to construct the phylogenetic relationship, MEGA7 was employed to calculate Bootstrap values with 1000 replications, with complete deletion (Tamura et al., 2011). Moreover, physicochemical parameters including the molecular weight (MW) and isoelectric point (*pI*) of each gene product were calculated using compute the *pI*/Mw tool from ExPASy (http://www.expasy.org/tools/) and the parameter (resolution) was set to average (Gasteiger et al., 2005).

2.2. Analysis of the putative promoter regions, chromosome location of the IQD gene and subcellular localization predictions of the proteins encoded by the IQD genes in cotton

The 2000-bp upstream sequences of the transcriptional start site of the *GhIQDs* were chosen to identify the *cis*-regulatory elements in the putative promoter regions. The PLACE website (**Error! Hyperlink reference not valid.**) used in identifying the putative *cis*–regulatory elements among the promoter sequences (Higo et al., 1999). Chromosomal position information were used for mapping the IQDs by use of mapchart (Voorrips, 2002). In addition, we carried out the sub cellular localization prediction of all the IQD proteins by use of an online tool

WoLF PSORT (https://wolfpsort.hgc.jp/) (Horton et al., 2006).

2.3. Plant materials

The *Gossypium hirsutum* race marie-galante 85 (M85) seeds were obtained from Institute of cotton research of Chinese Academy of Agricultural Sciences, (ICR-CAAS), located in Anyang, Henan Province, China. It is one of the accessions developed from *G. hirsutum* and it has relatively tolerant to drought and salt stresses. The seeds were pretreated and germinated in absorbent papers, upon germination, the seedlings were then transferred to a hydroponic set up with Hoagland nutrient solution (Hoagland and Arnon, 1950), in climate controlled greenhouse with 16 h/8 h light-dark and temperature at 28 °C day/25 °C night (Zhang et al., 2017). Drought and salt stress were initiated at three leaf stage, by supplementing the nutrient solution with 300 mM sodium chloride and 17% of PEG-6000, for salt and drought treatment, respectively. The leaf, stem and root tissues were collected for RNA extractions at 0 h, 3 h, 12 h, 24 h and 48 h after stress exposure.

2.4. RNA isolation and RT-qPCR analysis

Total RNA was isolated from each tissue by use of RNAprep Pure Plant kit, obtained from Tiangen, Beijing, China. Upon RNA extraction, the quality and concentration of the RNA was evaluated through Agarose gel electrophoresis. The RNA was then reverse transcribed into cDNA using the Prime Script RT reagent Kit (TaKaRa). The *IQD* gene specific primers were synthesized by use of primer 5 (Table S2) were used for RT-qPCR analysis with *GhActin* as the reference gene. The reactions were performed using a Step one plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR parameters were programmed as follows; 94 °C for 30 s, 40 cycles at 94 °C for 10 s, and 60 °C for 30 s, and then a melting curve for 61 cycles at 65 °C for 10 s was generated to check the specificity of the amplification. The relative expression level was calculated as $2^{-\Delta\Delta CT}$ [$\Delta C_T = C_{T,\ Target} - C_{T,\ CYP2}$. $\Delta\Delta CT = \Delta C_{T,\ treatment} - \Delta C_{T,\ CK}$ (0 h)]. The relative expression level [$2^{-\Delta\Delta CT,\ CK\ (0\,h)}$] in the untreated plants (without treatment) was normalized to 1 as described previously (Schmittgen and Livak, 2008).

2.5. Preparation of inocula, inoculation of plants, and abiotic stress treament for VIGS and wild type plants

Virus Induced Gene Silencing (VIGS) system applied in order to investigate the functional characterization of the two highly upregulated *IQD* genes, *IQD31* and *IQD32* gene expression in *G. hirsutum,* accession number. The 477 bp and 477 bp fragment of the gene *IQD31* and *IQD32*, respectively were transformed with specific primers Forward sequence: GTGAGTAAGGTTACCGAATTCGGCTTCCGCG AAATGCTTAC; Reverse sequence: CGTGAGCTCGGTACCGGATCCCTCATCATCAGCAAGCCGAC and *IQD31* forward sequence GTGAGTAAGGTTACCGAATTCGATCTGAACGGCATCGGGT and reverse sequence CGTGAGCTCGGTACCGGATCCTAGCCTCATTCGTGGCAGC inserted into the vector plasmid tobacco rattle virus (pTRV) via *ecoRI* and *BamHI* to develop a 35S promoter-driven pTRV2: *IQD31* and pTRV2: *IQD31*. The recombinant vector was transformed into the promoter cells of *A. tumefaciens* LBA4404 by adopting a freeze-thaw method (Corbin et al., 2017). A Phytoene desaturase (PDS) was used as positive control. The pTRV: 00 and the wild types were applied as negative control. The plants were grown up to three leaf stages, when abiotic stress was initiated; drought and salt stresses were imposed by supplementing the nutrient solutions with 17% PEG-6000 and 300 mM of NaCl solutions, respectively. Samples were collected for physiological and biochemical analysis at 0 h, 3 h, 6 h, 9 h and 12 h of post stress treatment.

2.6. Prolling of stress responsive genes on WT and VIGS plants under drought and salt stress conditions

In order to evaluate the effectiveness of the VIGS process, the two novel genes, *GhIQD31* and *GhIQD32* were profiled on three tissues, leaf, root and stem, this was to determine the target organ for future validation. Furthermore, four stress responsive genes, *GhLEA* forward primer “CGAACATCCATCCCTCCAAC” and reverse sequence “ATCATCAAGAAAACCGACCC” (Magwanga et al., 2018c), *GhCDKF4* forward sequence “TTATGTTCCGCCCTCTCTTCGTC” and reverse sequence “TGTTCCCATGTTCCTTTCACCC C” (Magwanga et al., 2018a), *GhGPCR* forward sequence “TGCGAAAGCTTTTTCATCATTGG” and reverse sequence “ACTTGTAGACGGGGCTGGTA” (Lu et al., 2018), and finally *GhTH* (GT-2) forward sequence “ATCCAATCTTTTCTCCACT” and reverse sequence “TCTTGTTTCTCAATCACCT” (Magwanga et al., 2019) with *GhActin* as the internal control.

2.7. Evaluation of physiological and biochemical parameters on the VIG-Plants and the wild type under abiotic stress conditions

We evaluated a number of physiological parameters such as chlorophyll content, cell membrane stability (CMS), excised leaf water loss (ELWL) and relative leaf water content (RLWC). Similar parameters have been extensively used in screening of various plants for abiotic stress tolerance (Barkla and Pantoja, 2010). CMS was evaluated as described by Blum and Ebercon (1981), ELWL was done as described by Clarke and McCaig (1982), while RLWL was measured as per the formula, RLWC = [[FW-DW]/[SW-DW]]*100 (Barrs and Weatherley, 1962). Furthermore, the activities of various antioxidant and oxidant enzymes were assayed. Among the antioxidant enzymes evaluated were peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD). All the antioxidant enzymes, POD, CAT, and SOD were evaluated using guaiacol colorimetric, potassium permanganate titration and NBT-illumination method, respectively (Ma et al., 2018). Whereas the oxidant enzymes hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were determined as described by Lu et al. (2018). All measurements were carried on in three biological replicates and data analysed by SPSS software.

3. Results

3.1. Identification of the cotton IQD proteins

Using the protein functional domain PF00612, a total of 304 proteins encoded by the *IQD* genes were identified in the three sequenced cotton genomes, with 148, 77 and 79 proteins in *Gossypium hirsutum* (AD), *Gossypium raimondii* (D) and *Gossypium arboreum* (A), respectively. The proportions of the proteins encoded by the *IQD* genes in the two diploid cotton species, *G. raimondii* and *G. arboreum* were higher that the number of IQD proteins in *G. hirsutum*, even though AD emerged due to whole genome duplications between A and D genomes. The low number could be attributed to either gene loss or chromosome rearrangement as previously observed in the evolution pattern of other cotton functional gene families such as the LEAs (Magwanga et al., 2018d). Moreover, in the evaluation of the physiochemical properties of the proteins encoded by the *IQD* genes, the protein lengths for the *G. hirsutum* IQD proteins ranged from 53 aa to 2144 aa, molecular weights ranged from 5.83 kDa to 245.579 kDa, a charge ranged from −24.5 to 191, the isoelectric point (*pI*) ranged from 4.845 to 12.823 and finally the grand average of hydropathy (GRAVY) ranged from −1.047 to −0.128. In relation to the two diploid cotton species, the *G. raimondii* and *G. arboreum* IQD proteins physiochemical properties showed minimal variations, in protein lengths, molecular weights and the charge, while *pI* and GRAVY values were same. The protein length ranged from 133 aa to 1668 aa, and 138 aa to 2, 143 aa in *G. raimondii* and *G. arboreum*, respectively. Their molecular weights ranged from

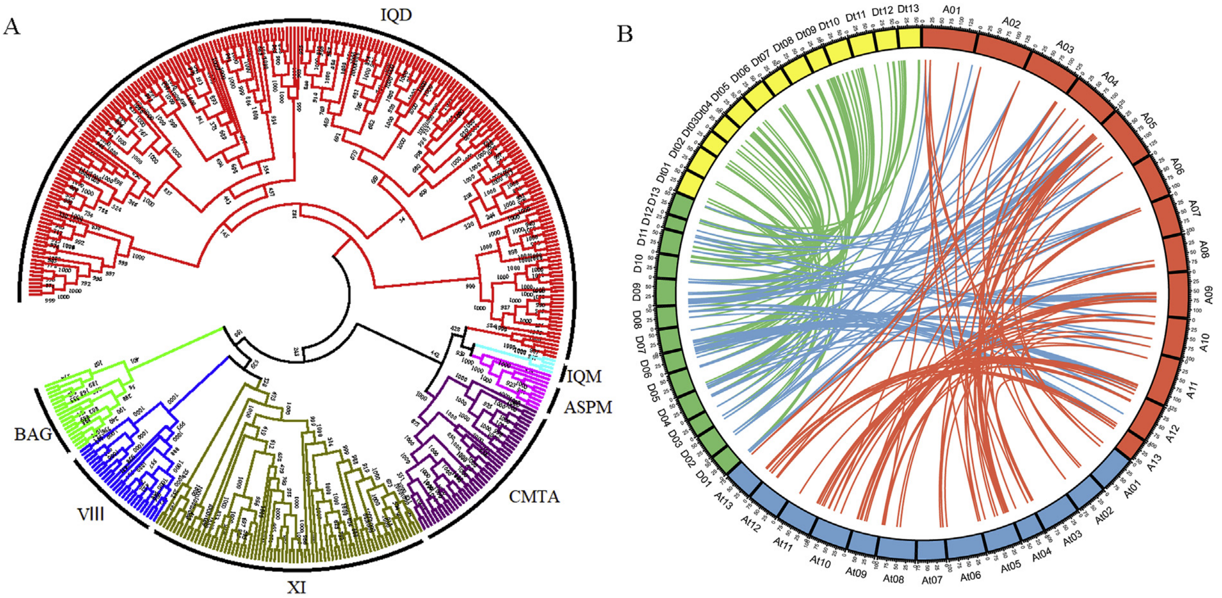

Fig. 1. Phylogeny and syntenic analysis of the IQD protein in cotton. (A). Phylogenetic tree analysis of cotton IQD proteins and other plants. (B). Synteny block formation between the three cotton species chromosomes. D: Chromosomes of *G. raimondii*, A: Chromosomes of *G. arboreum*; At and Dt: chromosomes of A and D subgenome of the tetraploid cotton, *G. hirsutum*.

15.392 to a maximum value of 245.563 kDa, with a charge range of −22 to 193.5 (Table S1). Across all the three cotton species, their GRAVY were negative, the lower value of (GRAVY) in all proteins may be a signal for the possibility of better interaction with water like a protein of hydrophilic nature.

3.2. Phylogenetic and collinearity analysis of the IQD proteins in cotton

To determine the phylogenetic relationship of the IQD proteins, a rooted phylogenetic tree was constructed from the alignments of the full IQD protein sequences, using MEGA6.0, by employing the Neighjoining (NJ) method with minimal evolution and maximum parsimony. The tree constructed grouped the cotton IQD proteins together with other plants IQD proteins into five clades with clade one being the largest. Various ortholog and paralog gene pairs were formed between the various proteins encoded by the cotton *IQD* genes. The highest proportions of the IQD proportions were detected in clade 4, with 61, 33 and 32 in *G. hirsutum*, *G. arboreum* and *G. raimondii*, respectively (Fig. 1A). Moreover, we analysed the synteny block formation between the three cotton species, we used MCScanX (Wang et al., 2012) to recognize the collinear gene pairs while circos software (Zhang et al., 2013) was used for display the result. The there was good synteny block formation between A vs D; A vs At, and finally between D vs Dt subgenome (Fig. 1B).

3.3. Chromosome distribution and subcellular localization prediction of the protein encoded by the IQD genes in cotton

The *IQD* genes were distributed in all the chromosomes of A, D and AD cotton genomes. In relation to the two diploid cotton species, A and D genomes, the gene distribution pattern was same, in *G. arboreum*, the highest gene loci were observed on chromosome, $A_2$04, $A_2$09, $A_2$11, and $A_2$13, with 9, 12, 8, and 8 genes, respectively (Fig. 2A), while in *G. raimondii*, chromosome $D_5$05, $D_5$08, $D_5$09, and $D_5$13 harbored the highest gene loci with 12, 10, 9, and 9 genes, respectively, while chromosome $D_5$02 had the least number of genes (Fig. 2B). In AD genome, the highest gene loci were observed on chromosome A_h05 and its homolog D_h05 with 12 and 11genes, respectively, while the lowest gene loci were detected on chromosomes A_h01, A_h04, and D_h01 with two genes each. The rest of the chromosome harbored a minimum of 3 to a maximum of 9 genes (Fig. 2C). Moreover, we sought to determine the subcellular localization of the proteins encoded by the *IQD* genes, through online software, Wolf sport, nine (9) different subcellular structures were found to contain the IQD proteins, however, in the two diploid cotton species, only eight (8) structures were predicted. In *G. hirsutum*, the highest proportions of the IQD proteins were embedded within the nucleus (nucl) with 96, then chloroplast (chlo) with 18 IQD proteins, cytoplasm (cyto) with 11, the mitochondrion (mito) and plasma membrane (plas) with six each, extracellular structures (extr) with five, the lowest proportions were detected in endoplasmic reticulum (E.R), peroxisome (pero) and vacuole (vacu) with 3, 2 and 1 IQD proteins, respectively. In *G. arboreum*, nucleus, chloroplast, and cytoplasm, and the mitochondrion harbored the highest proportions of the IQD proteins with 51, 11, 5 and 5, respectively, while the plasma membrane (3), peroxisomes (2) and extracellular structures (2) contained the least number of the IQD proteins. Similarly, in *G. raimondii,* nucleus (48), chloroplast (14), and cytoplasm (5) harbored the highest proportions of the IQD proteins, while extracellular structures (3), mitochondrion (3), plasma membrane (2), and peroxisome (1) had the lowest number of the IQD proteins.

3.4. Gene structure and cis-regulatory element analysis of the IQD proteins in cotton

In the analysis of the gene structures, the majority of the *IQD* gene structures were disrupted by introns. The highest level of disruption of the *IQD* gene structures were 44, 42 and 40 introns in various genes of *G. hirsutum*, *G. raimondii* and *G. arboreum*, respectively. Some of the genes which were highly interrupted by introns were; *Gh_A09G1792, Gh_D09G1914, Gh_A13G1707, Gh_D13G2056, Gh_D05G0329, Gh_D11G1387, Gh_A05G0287, Gh_A11G2268, Gh_D11G2576, Gh_D08G2294, Gh_A05G0243, Gh_D08G2063, Gh_A08G1706, Gh_D05G0388, Gh_A10G2250, Gh_A08G1928, Gh_A07G0128, Gh_D10G0537, Gh_A11G1240, Gh_A10G1061, Gh_D09G0526, Gh_A09G0530, Gh_D10G1457* with introns ranging from 32 to 44 introns, accounting for over 16% of all the *IQD* genes in upland cotton. However, six of the upland cotton *IQD* genes were intronless, *Gh_A03G1325* (A_h03), *Gh_A03G1327* (A_h03), *Gh_D02G1764* (D_h02), *Gh_D10G2510* (D_h10), *Gh_Sca115103G01* (Scaffold) and *Gh_Sca231642G01* (Scaffold). The rest of the *IQD* genes were

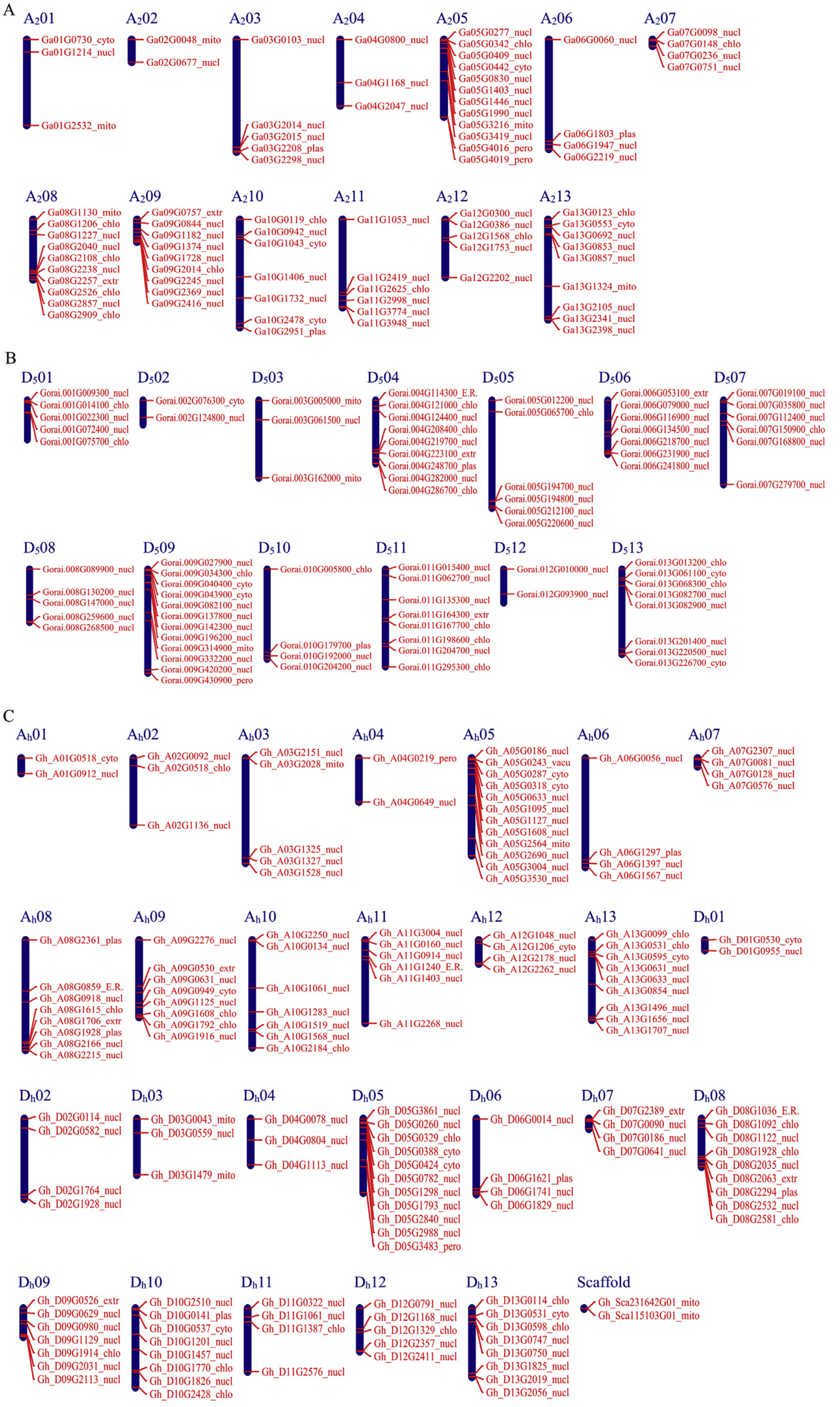

Fig. 2. *IQD* genes mapping of the various cotton chromosomes. (A). Chromosomes of *G. arboreum*, (B). Chromosomes of *G. raimondii* (C). Chromosomes of *G. hirsutum*.

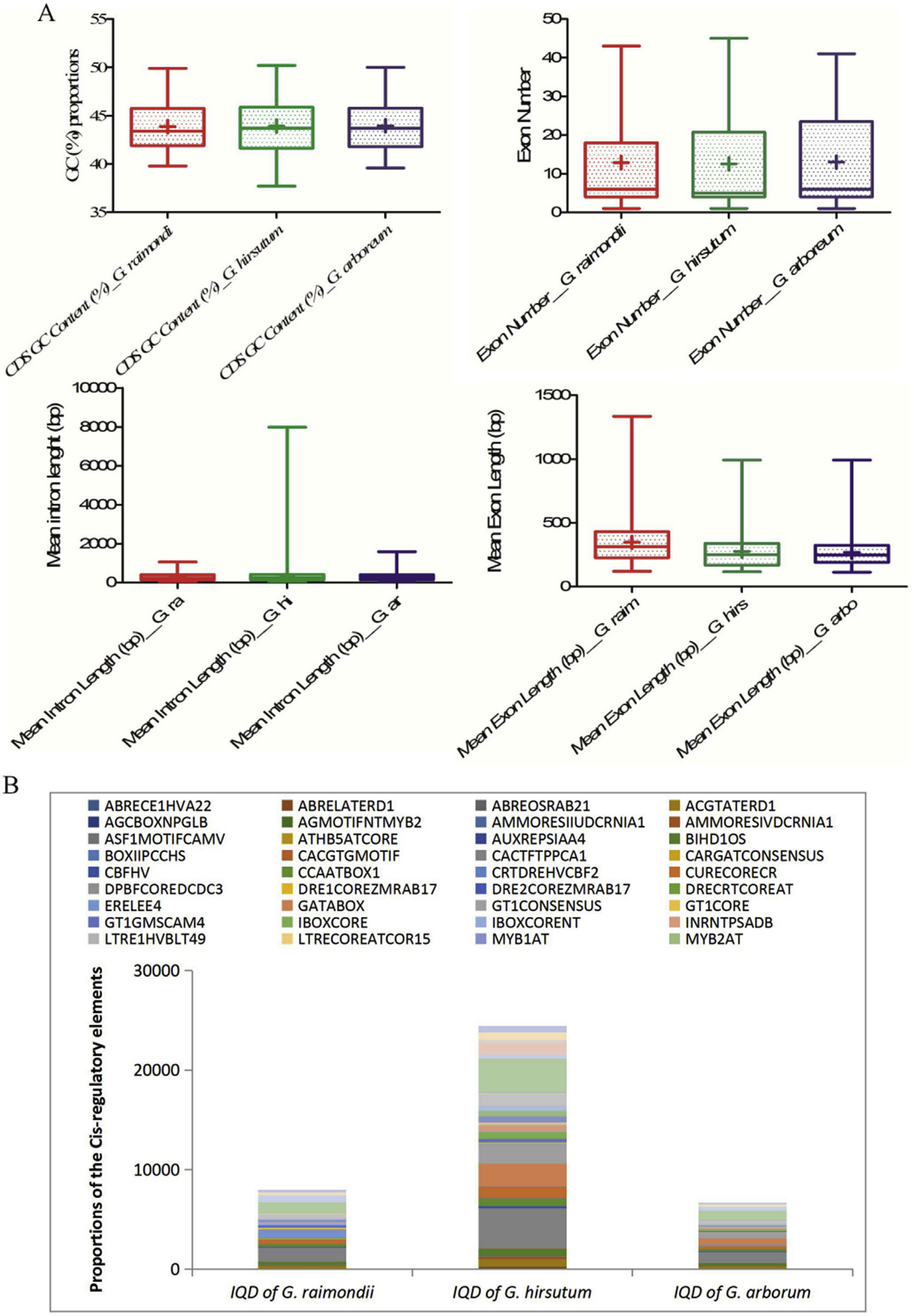

Fig. 3. Physiochemical properties and *cis*-regulatory element analysis of the proteins encoded by the cotton *IQD* genes. (A). GC content, exon number, mean intron and exon length. (B). *Cis*-regulatory elements obtained for the various proteins encoded by the *IQD* genes in the *G. raimondii*, *G. hirsutum* and *G. arboreum*.

interrupted by one to a maximum of 27 introns. Similarly, the genes obtained from the two diploid cotton species, A and D genomes, also exhibited same intron-exon distribution, with 11 in each being interrupted with over 30 introns, accounting for 13.9% and 14.3% of all the genes in *G. arboreum* and *G. raimondii*, respectively. Though interesting to note that, in each of the *IQD* genes, two were intronless in either of the cotton species and were located in the same chromosomes and their proteins being embedded within the same subcellular structure, the nucleus, *Ga03G2014* ($A_2$03) and *Ga03G2015* ($A_2$03) in *G. arboreum*, while *Gorai.005G194700* ($D_5$05) and *Gorai.005G194800* ($D_5$05) were intronless in *G. raimondii*. Moreover, the coding sequence guanine-cytosine (CDS GC) content (%) in all the three cotton *IQD* genes, varied, in which GC content in *G. hirsutum* ranged from 37.7% to 50.2%, *G. raimondii* GC content ranged from 39.8% to 49.9%, while the GC content for the *G. arboreum* ranged from 39.6% to 50% (Fig. 3A).

In the analysis of the *cis*-regulatory elements, a number of stress related *cis*-regulatory elements were observed, across the three cotton species, ACGTATERD1 (ACGTG), BIHD1OS (TGTCA), CACTFTPPCA1 (YACT), CURECORECR (GTAC), ERELEE4 (AWTTCAAA), GATABOX (GATA), MYCCONSENSU SAT (CANNTG), ACGTATERD1 (ACGT), MYB1AT (WAACCA), and GT1CONSENSUS (GRWAAW) the most dominant regulatory elements with diverse roles in enhancing plants response to various abiotic stresses such as dehydration, cold and salinity (Fig. 3B).

3.5. RNA sequence analysis and RT-qPCR validation of the upland cotton IQD genes under drought, cold and salt stress conditions

RNA sequencing data were obtained from the cotton functional genome database (https://cottonfgd.org/analyze/), transformed into log 2, all the upland cotton *IQD* genes were classified into three groups based on their level of expression. Groups 1, with 54 genes were significantly downregulated. Group 2 containing 48 genes were highly upregulated under drought and salt stress conditions, while group 3 members with 46 *IQD* genes showed differential expression, though the majority of the *IQD* genes were either downregulated or not expressed (Fig. S1). Due to the huge number of genes, 31 top ranked genes in terms of their upregulation as per the RNA sequence data were selected for RT-qPCR validation, on upland cotton tissues exposed to drought, salt and cold stress conditions. Across the three stress forms, drought, salt, and cold stresses, the expression pattern of the selected 31 *IQD* genes were grouped into two, under drought and salt stress conditions, group 1 with 15 genes were significantly upregulated, while group 2 with 16 genes exhibited differential expression with the majority of the genes being downregulated at various time points in leaf and the root tissues (Fig. 4A). Finally, under cold stress, only leaf tissues were analysed, similarly group 1 with 17 genes were significantly upregulated, while group 2 with 14 genes showed differential expression with a higher proportion being downregulated except at 12 h of cold stress exposure (Fig. 4B).

3.6. Virus induced gene silencing (VIGS) and evaluation of phyio-morphological traits under drought and salt stress conditions

Two highly upregulated genes across the three forms of stress as determined through RT-qPCR analysis, *Gh_D05G0014* and *Gh_A09G1608*, the plants which were infused with PDS showed an albino trait after 8 days, and time progressed, the entire leaf and tender regions of the shoot tip were over 95% chlorotic/bleached (Fig. 5A), this demonstrated that the vector used was effective and the knockdown of the two genes was successfully done. At three leaf stage, the VIGS plants, positively controlled (TRV:00 infused), and the wild types were exposed to drought and salt stress, by supplementing the Hoagland solution with 17% PEG-6000 and 300 mM of NaCl for drought and salt stress, respectively. Biochemical and physiological parameters were evaluated at 0 h, 3 h, 6 h, 9 h, and 12 h of stress exposure, while morphological data was determined at 0 h and 12 h which was the maximum time of stress exposure to the plants. The physiological traits measured were plant height (PH), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), dry shoot biomass (DRB) and dry root biomass (DRB). Moreover, excised leaf water loss (ELWL), saturated leaf weight (SLW), cell membrane stability (CMS) and chlorophyll content (SPAD values) were the physiological traits measured. In all the traits measured, there was a significant difference between the VIGS plants to their wild types and or positively controlled plants, the VIGS plants exhibited significant reduction in all the traits, while showing higher ion leakage (Fig. 5B), which demonstrated that the VIGS plants suffered extensive oxidative injury due to drought and salt stresses. Phenotypically, knockdown of the two *IQD* genes significantly lowered the ability of the plants to tolerate the effects of drought and salt stress as evident by drooping and wilting of the VIGS plants under drought and salt stress conditions (Fig. 5B).

3.7. Evaluation of expression levels of the knocked down IQD genes and four known stress responsive genes in the VIGS and wild type plants under drought and salt stress conditions

In order to understand the effects of knockdown of the two *IQD* genes in the plants under drought and salt stress conditions. The two

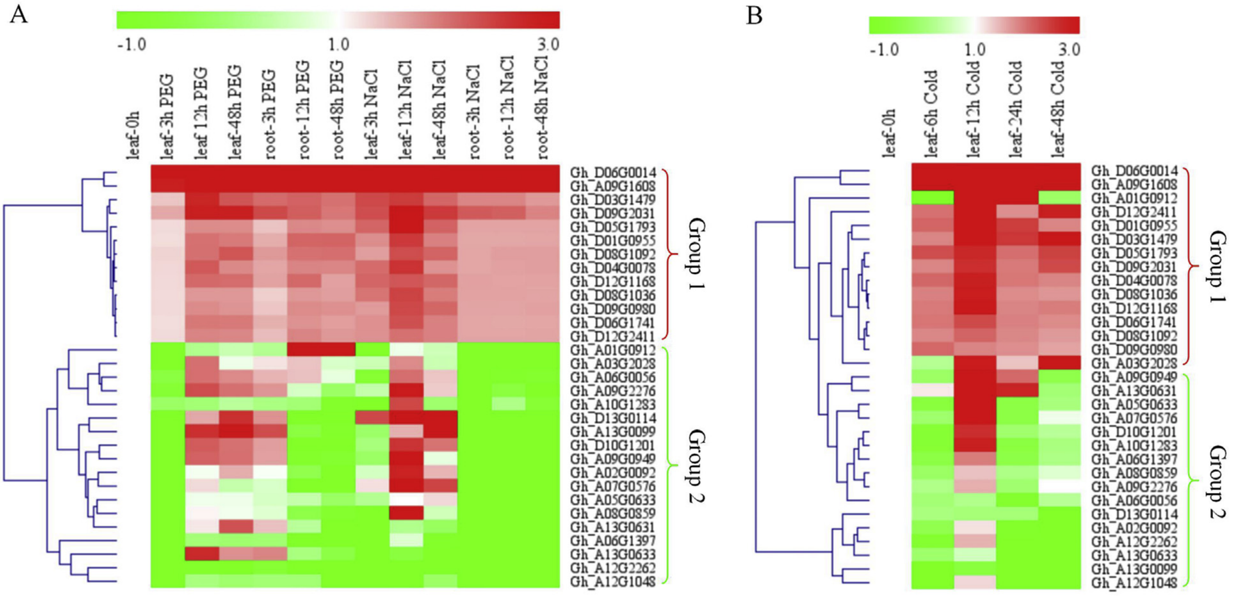

Fig. 4. RT-qPCR analysis of the cotton *IQD* genes under drought, salt and cold stress conditions. PEG: drought stress imposed by using 17% of PEG-6000, NaCl: salt stress imposed by adding 250 mM of sodium chloride solution. Cold stress imposed by keeping the plants under 4 °C for 0 h, 6 h, 12 h, 24 h and 48 h. (A). Heat map of the *IQD* genes expression under drought and salt stress conditions; (B). Heat map of the *IQD* genes expression under cold stress condition. The samples were collected at 0 h, 3 h, 12 h, 24 h and 48 h for leaf and root tissues. Red: upregulated genes; green: downregulated; Black: none expressed genes.

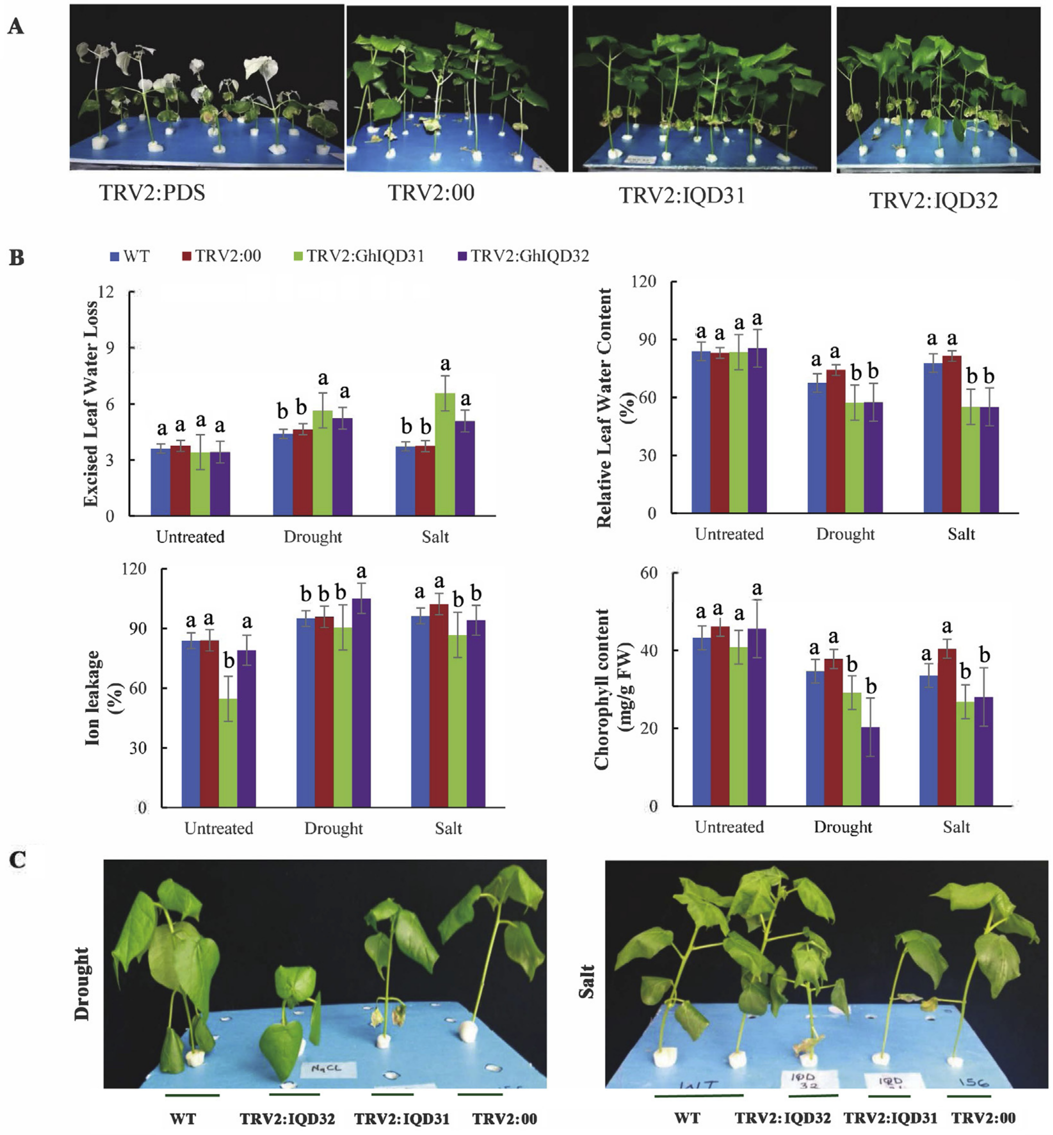

Fig. 5. Physiological trait measurements in *Gh_D05G0014 (IQD31)* and *Gh_A09G1608 (IQD32)* VIGS cotton plants under salt and drought stress conditions. (A) Representative images of VIGS (TRV: *IQDs*, silenced; TRV: 00, control) and the wild type (WT) plants. (B). Quantitative determination of excised leaf water loss (ELWL), relative water content (RLWC), cell membrane stability (CMS) as a measure of ion leakage concentration and chlorophyll content in leaves of the WT, control and *IQDs*-silenced plants (C). Representative images of VIGS (TRV: *IQDs*, silenced; TRV: 00, control) and the wild type (WT) plants after 8 days of drought and salt stress exposure. In (B), each experiment was repeated three times. Error bars of the physiological trait measurements represent the standard deviation of three biological replicates. Different letters indicate significant differences between wild-type and *IQDs*-VIGS plants (two-tailed; $p < 0.01$). CK: untreated, Salt: 300 mM NaCl treatment and Drought: 17% PEG-6000 treatment.

genes were profiled on leaf, stem and root tissues of wild type and the VIGS plants under normal conditions. The two *IQD* genes showed significant upregulation on the leaf tissues of the WT plants compared to either the stem or the root, however, on the VIGS plants, the genes were significantly downregulated across all the tissues but of significant was the leaf tissue (Fig. 6A). The downregulation of the *IQD* genes on the leaf tissues showed that the leaf play an important role, moreover, downregulation of the photosynthetic metabolism occurs due to alteration in leaf biochemistry which occurs in response to lowered carbon substrate under prolonged stresses (Chaves and Oliveira, 2004). We further evaluated known stress responsive genes such as the *GhLEA2* (Magwanga et al., 2018c), *Gh_D12G2017 (CDKF4)* (Magwanga et al., 2018a), *Gh_A07G0747 (GPCR)* (Lu et al., 2018) and a transcription factor, trihelix, *Gh_A05G2067* (Magwanga et al., 2019). The above have been intensively investigated and overexpressed in Arabidopsis and found to plat and important role in enhancing plants tolerance levels to drought and salt stress factors. All the four abiotic stress responsive genes were downregulated in the *IQD* knocked plants were significantly upregulated in the wild and positively controlled plants under drought and salt stress conditions (Fig. 6B). The downregulation of the stress responsive genes further confirmed that the knocked plants ability to tolerate the effects of drought and salt stress was significantly compromised.

3.8. Oxidant and antioxidant enzyme assayed on the VIGS and the wild types under drought and salt stress conditions

The biochemical traits evaluated were peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) as the antioxidant while hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were the oxidizing enzymes assayed on the leaf tissues of the VIGS plants and the wild types under drought and salt stress conditions. The VIGS plants

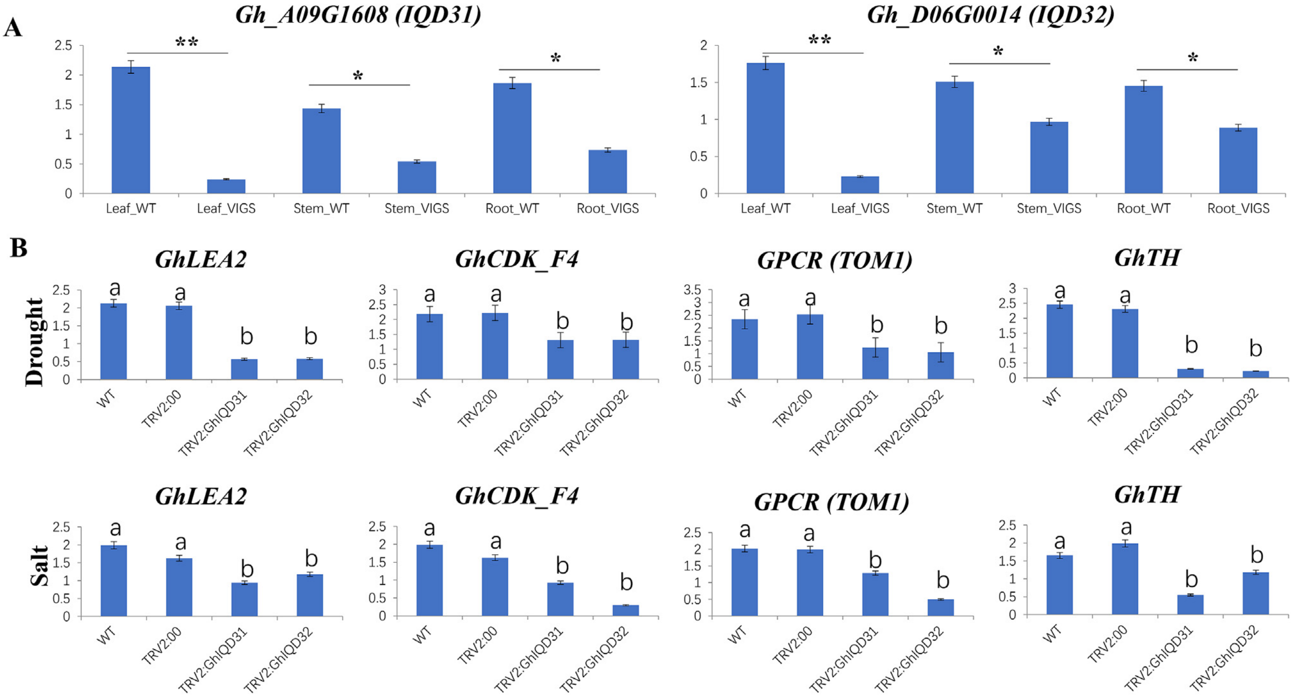

Fig. 6. RT-qPCR analysis of the change in the expression levels of abiotic stress responsive and the knockdown genes in cotton plants treated with virus-induced gene silencing (VIGS). (A). RT-qPCR expression profiling of the *Gh_A09G1608 (IQD31)* and *Gh_D06G0014 (IQD32)* in leaf, stem and root tissues of upland cotton under normal condition. (B). Profiling of the stress responsive genes ion the WT, positively controlled and VIGS plants under drought and salt stress. Bar indicates standard error (SE). Different letters indicate significant differences between wild type and VIGS-plants (ANOVA; $p < 0.05$). WT: Wild type, TRV2:GhIQD31 and GhIQD32 are the VIGS plants, TRV2:00 the positively controlled plants. Untreated: under normal condition, Drought stress imposed by adding 17% of PEG-6000, Salt stress imposed by adding 300 mM of NaCl solution.

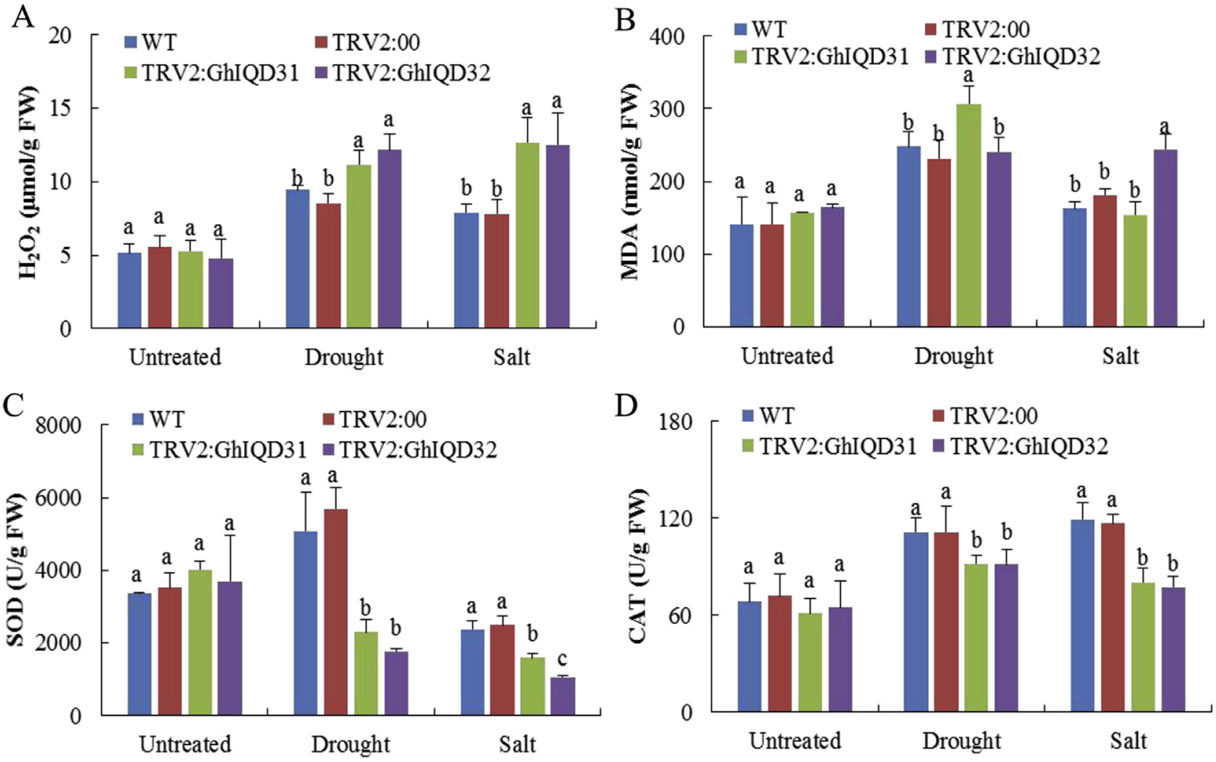

Fig. 7. Biochemical analysis of the VIGS and wild types under drought and salt stress conditions. In each experiment was repeated three times. Bar indicates standard error (SE). Different letters indicate significant differences between wild type and VIGS-plants (ANOVA; $p < 0.05$). WT: Wild type, TRV2:GhIQD31 and GhIQD32 are the VIGS plants, TRV2:00 the positively controlled plants. Untreated: under normal condition, Drought stress imposed by adding 17% of PEG-6000, Salt stress imposed by adding 300 mM of NaCl solution.

showed a significant reduction in the concentration levels of the antioxidant and twice fold increase of the oxidant enzyme concentration (Fig. 7A–D). The PODs are the first line of defense by the plants against the damage caused by the excessed reactive oxygen species, which is produced whenever the equilibrium state of ROS release and oxidation is altered (Li et al., 2019), the significant reduction of the various antioxidant and increased levels of the oxidant demonstrated that the knockdown of the two *IQD* genes in cotton compromised their ability to withstand the drought and salt stress effects.

4. Discussion

4.1. Identification of the proteins encoded by the IQD genes in cotton

Cotton is an economic important crop with huge contributing to both developing and the developed countries, but its production has undergone significant reduction to effects of various abiotic stress factors, more so drought, salt and cold stresses (Saranga et al., 2009). Efforts have been made by cotton breeders in developing a more resilient and highly tolerant cotton germplasm through conventional technique but with little progress, this is due to the narrow genetic base of the existing cotton germplasm due to prolonged and intensive selection and inbreeding (Sethi et al., 2017). Therefore, the adoption of molecular approach will help in the development of diverse cotton genotypes, being significant alleles can be identified in their wild progenitors and used in the improvement of elite cotton cultivars (Magwanga et al., 2018b). In this work, genomewide identification, characterization and functional analysis of the proteins encoded by the cotton *IQD* genes was done, in which 308 IQD protein domains were, identified in the three cotton species, with 148, 77 and 79 IQD proteins in *G. hirsutum*, *G. raimondii* and *G. arboreum*, respectively. The cotton IQD proteins had relatively high isoelectric points with an average of 9.540, 9.497, and 9.548, in *G. hirsutum*, *G. raimondii,* and *G. arboreum*, respectively. The average values were in agreement to previous findings for the IQD gene family in *Brachypodium distachyon* (10.3) (Filiz et al., 2013), Chinese cabbage (10.05) (Yuan et al., 2019) and *Arabidopsis thaliana* (10.3) (Abel et al., 2005b). The high proportions of the IQD proteins demonstrated their significant role in plants, moreover, the GRAVY values of all the IQD proteins were negative, and indication that the proteins were hydrophilic, a property common among the proteins encoded by the various stress responsive genes such as the LEA proteins (Magwanga et al., 2018d), Cyclin dependent kinases (CDKs) (Magwanga et al., 2018a). Hydrophilicity, is a vital property, and the hydrophilic proteins are significant in enhancing membrane protein stability and osmotic adjustment, has been found for the dehydrins, a member of the LEA proteins (Xie et al., 2012). Moreover, the dehydrin proteins which are hydrophilic in nature, could be playing a cryoprotective role in macromolecular stabilization by binding water molecules to their hydrophilic surfaces, in turn prevents further denaturation of cellular proteins (Gao and Lan, 2016).

4.2. Phylogenetic analysis and subcellular localization determination of the proteins encoded by the IQD genes in cotton

Phylogenetic tree analysis provides information on the evolution pattern of the various plants transcription or gene families. In the analysis of the IQD proteins together with other plants, being several plants IQD proteins have been characterized, such as Maize (Cai et al., 2016), rice and arabidopsis (Abel et al., 2005b), *Populus trichocarpa* (Ma et al., 2014), soybean (Feng et al., 2015), Chinese cabbage (Yuan et al., 2019) among other plants. In the analysis of phylogenetic tree, the cotton IQD proteins were grouped into four, which were in agreement to previous findings obtained in the analysis of the IQD proteins in various plants. Furthermore, all the chromosomes for the diploid and tetraploid cotton genomes harbored the *IQD* genes. The diverse distribution and ortholog gene pair formation demonstrated that the proteins encoded by the *IQD* genes have a significant role within the plant. In the evaluation of the subcellular localization of the proteins encoded by the *IQD* genes showed that the nucleus was the major subcellular structure which harbored the highest number of the proteins encoded by the *IQD* genes, with 91 (61.49%), 48 (62.34%), and 48 (60.76%) of all the IQD proteins in *G. hirsutum*, *G. raimondii*, and *G. arboreum*, respectively. The results were in agreement to findings obtained for the analysis of the subcellular localization prediction of the Arabidopsis IQD proteins, in which a half of all the Arabidopsis IQD proteins were localized within the cell nucleus (Abel et al., 2005b). Moreover, several basic clusters in IQD proteins that conform to the simian vacuolating virus 40-type (SV40-type), the mating-type alpha-2 (MATα2), and bipartite type of nuclear localization signals (Abel and Theologis, 1995), and by the nuclear localization of an IQD1-GFP fusion protein [37]. During oxidative stress, the release of reactive oxygen species (ROS) in various subcellular structures such as the nucleus, mitochondria, chloroplast and cytoplasm, causes oxidative injury and cell death (Langebartels et al., 2002), thus the high proportions of the proteins encoded by the *IQD* genes, demonstrate the regulatory role of reducing the deleterious effects of the ROS and thus protects the cell from oxidative injuries.

4.3. Gene expression and putative role of the proteins encoded by the IQD genes

Gene expression is a tightly regulated process which enhances the adaptation of the cells to their constantly changing environment (Link et al., 2016). It acts as both an on/off switch to control when proteins are made and also a volume control that increases or decreases the amount of proteins made. In the analysis of the RNA sequence and RT-qPCR validation of some the selected genes, the *IQD* genes were found to be inducted by both droughts, salt and cold stress factors, moreover, some of the genes such as *Gh_D06G0014* and *Gh_A09G1608* were highly upregulated in various tissues. However, some of the genes exhibited differential expression. The variation in gene expression pattern of the *IQD* genes demonstrated that the regulatory sequences that respond to a particular stress factors could have undergone modification along the evolution process of the gene family. The results obtained mirrored what has been observed among the *IQD* genes expression in other plants, for instance, the expression levels of *PeIQD* family genes under drought and MeJA treatments, 14 out of 29 genes showed the highest transcript levels in response to drought (PEG) treatment (Chen et al., 2017).

4.4. Knockdown of IQD genes increases sensitivity of upland cotton to drought and salt stresses

The possible role of the cotton *IQD* genes in relation to drought, salt and cold stress, *Gh_D06G0014* and *Gh_A09G1608* were knock down in upland cotton, the VIGS-plants were exposed to drought, salt and cold stress conditions. evaluation of the plants showed that the VIGS plants ability to tolerate the effects of various abiotic stress factors were highly affected, the concentration levels of the various antioxidant assayed were significantly reduced in the VIGS-plants compared to the wild types, moreover, morpho-physiological traits revealed that the VIGS plants suffered more compared to the wild types under drought, salt and cold stress. When plants are exposed to either abiotic and or biotic stress conditions, the equilibrium between production and elimination of the reactive oxygen species (ROS) shifts leading to excessive production, which causes massive and deleterious effects on the plant cells, and eventually plant death (Corpas et al., 2015). The first line of defense by plants against the effects of ROS is the mobilization of various antioxidant enzymes such as POD, SOD, CAT among others, being the primary sites of ROS production sites in the plant cell are the chloroplasts, mitochondria, and the peroxisomes (Bi et al., 2009). But the chloroplast and the mitochondria also functions in the maintenance of a delicate balance between energy linked functions and control of the ROS production, while peroxisomes, is the significant site important site of reactive oxygen species such as hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$), however, vital antioxidants enzymes such as catalase, as well as hydrogen peroxide producing flavin oxidases are produced by the peroxisomes (del Rio, 2006). The reduction of both morphological, physiological and biochemical traits, demonstrated that the cotton *IQD* genes play a significant role in enhancing abiotic stress tolerance, moreover, the knockdown of *SUN24* a type of *IQD* gene in tomato, significantly caused delay in seed germination but its overexpression promoted seed germination under abscisic acid (ABA) treatment, furthermore, gene expression analysis revealed that *SUN24* negatively regulated expression *Solanum lycopersicum ABA-insensitive 15* (Sl*ABI5)* and *Solanum lycopersicum ABA-insensitive 3* (Sl*ABI3*) and in germinating seeds, key ABA signaling genes (Bi et al., 2018).

5. Conclusions

In this research work, genome-wide identification, characterization and functional analysis of the cotton *IQD* genes were performed. A hundred and forty eight (148), 77 and 79 proteins encoded by the *IQD* genes were identified in *G. hirsutum*, *G. raimondii* and *G. arboreum*, respectively. The sum total of the IQD proteins in the two diploid cotton was higher than the number of IQD proteins obtained from the AD genome, despite AD emerged through whole genome duplication (WGD), the reduction could have been due to either gene loss or chromosome rearrangement. In the analysis of the various physiochemical properties of the IQD proteins, all had negative GRAVY values, which demonstrated that the cotton IQD proteins were hydrophilic, a property shared among the proteins encoded by the stress responsive genes such as the dehydrin, a member of the *LEA* gene family (Magwanga et al., 2018d). RNA sequence data and RT-qPCR analysis revealed two key genes, *Gh_D06G0014* and *Gh_A09G1608,* which were knocked down in *G. hirsutum* race Marie-galante 85 (M85), the VIGS plant's ability to tolerate the effects of drought, salt and cold stresses was significantly reduced, due to high level of oxidant enzymes and two fold reduction in antioxidant enzymes. Moreover, all the physiological and morphological traits showed the negative effects of drought, salt and drought stresses on the VIGS plants compared to the wild types. The results demonstrated that the knockdown of the two *IQD* genes significantly lowered the ability of the VIGS plants to tolerate effects of the stress factors.

Availability of data

All files supporting the findings have been submitted together with the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Y.X, R.O.M., J.N.K, F.L., and K.W conceived and designed the study. R.O.M, J.N.K, and YX performed the experiments. X.C., Y.X, L.P, F.L, K.W and Y.H supervised experiments and contributed to drafting the manuscript. All authors contributed to the analysis and interpretation of the data. Y.X, R.O.M, and J.N.K wrote and prepared the final version of the manuscript. All authors approved the final manuscript.

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Abbreviations

CMS	Cell membrane stability
ELWL	Excised leaf water loss
CAT	Catalase
POD	linear dichroism
SOD	superoxide dismutase
POD	peroxidase
MDA	malondialdehyde
PDS	Phytoene desaturase
RLWC	relative leaf water content
VIGS	Virus induced gene silencing
ROS	Reactive oxygen species

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.027.

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