Acta Physiol Plant (2017) 39:131
DOI 10.1007/s11738-017-2425-6

ORIGINAL ARTICLE

Induction of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* by abiotic and biotic stresses in *Zea mays*

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Received: 11 August 2016 / Revised: 1 May 2017 / Accepted: 6 May 2017 / Published online: 17 May 2017

Abstract Cyclotides are small plant disulfide-rich and cyclic proteins with a diverse range of biological activities. Cyclotide-like genes show key sequence features of cyclotides and are present in the Poaceae. In this study the cDNA of the nine cyclotide-like genes were cloned and sequenced using 3′RACE from *Zea mays*. The gene expression of two of these genes (*Zmcyc1* and *Zmcyc5*) were analyzed by real-time PCR in response to biotic (*Fusarium graminearum*, *Ustilago maydis* and *Rhopalosiphum maydis*) and abiotic (mechanical wounding, water deficit and salinity) stresses, as well as in response to salicylic acid and methyl jasmonate elicitors to mimic biotic stresses. All isolated genes showed significant similarity to other cyclotide-like genes and were classified in two separate clusters. Both *Zmcyc1* and *Zmcyc5* were expressed in all studied tissues with the highest expression in leaves and lowest expression in roots. Wounding, methyl jasmonate and salicylic acid significantly induced the expression of *Zmcyc1* and *Zmcyc5* genes, but the higher expression was observed for *Zmcyc1* as compared with *Zmcyc5*. Expression levels of these two genes were also induced in inoculated leaves with *F. graminearum*, *U. maydis* and also in response to insect infestation. In addition, the 1000-base-pairs (bp) upstream of the promoter of *Zmcyc1* and *Zmcyc5* genes were identified and analyzed using the PlantCARE database and consequently a large number of similar biotic and abiotic *cis*-regulatory elements were identified for these two genes.

Keywords Cyclotide-like gene · Gene expression · *Zea mays* · Real-time PCR

Communicated by Q. Wang.

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Introduction

Cyclotides are a family of bioactive small proteins from plants that contain a cyclized backbone and a knotted array of three disulfide bonds (Craik et al. 1999). They normally consist of 28–37 amino acids and are the largest family of circular proteins. The discovery of cyclotides tracs back to the time when native medicine was used in Africa in 1970. *Oldenlandia affinis* as a medicinal plant has long been used in parts of Africa by women to assist childbirth. In the early 1970s, the kalata B1 peptide was identified as the first cyclotide that is responsible for the uterotonic activity (Gran 1973). Later several other plant-derived peptides were identified with a similar circular backbone and cysteine content to kalata B1 (Daly et al. 1999).

It has been shown that cyclotides accomplish various biological activities including uterotonic activity (Gran 1973; Koehbach et al. 2013), neurotensin inhibition (Witherup et al. 1994), anti-HIV (Gustafson et al. 1994), haemolytic (Barry et al. 2003), anti-bacterial (Ovesen et al. 2011), insecticidal (Jennings et al. 2001; Gruber et al. 2007), and cytotoxic activities (Lindholm et al. 2002). Cycloviolacin VYI—a cyclotide from *Viola yedoensis* is active against influenza A H1N1 virus (Liu et al. 2014). Most of the studies have focused on animal or human antifungal and anti-bacterial activities while less works have been carried out on cyclotides effects on plant pathogens. Generally, it has been shown that cyclotides

play a role in plants against biotic/abiotic stresses. Cyclotides from Iranian *V. odorata* showed antimicrobial activity against the plant pathogenic bacteria *Xanthomonas oryzae*, *Ralstonia solanacearum*, *Ralstonia cicil*, and *Bacillus* sp. (Zarrabi et al. 2013).

Cyclotides show tissue specific expression patterns and are induced by both biotic and abiotic stresses. Variation of cyclotides in different tissues have been reported (Trabi and Craik, 2004; Seydel and Dornenburg 2006; Mylne et al. 2010; Poth et al. 2012). Semi-quantitative analysis of 20 cyclotides from *Viola uliginosa* in different tissues and in suspension cultures showed that they are differentially expressed in wild type plant tissues and suspension cultures (Slazak et al. 2015). Expression analysis of cyclotide genes from *V. baoshanensis* showed that some cyclotide genes are induced by cadmium stress and wounding (Zhang et al. 2015). This wide range of expression response of *cyclotides* in *plant* cells, is probably associated with the role of these genes in host defense against both biotic and abiotic stresses.

Most cyclotides have been isolated from the Rubiaceae and Violaceae families (Craik and Conibear 2011). However, recently cyclotides have been found in the Cucurbitaceae (Hernandez et al. 2000), Solanaceae (Poth et al. 2012), Fabaceae (Poth et al. 2011) and Poaceae (Mulvenna et al. 2006). In addition to regular cyclic cyclotides, a few acyclic variants of cyclotides have been reported. Acyclic cyclotides contain the similar cysteine arrangement and show high sequence similarity with cyclotides, but they cannot be cyclized (Ireland et al. 2006; Nguyen et al. 2011, 2012). Acyclic cyclotides have been referred as "uncyclotides". or "acyclotides" (Nguyen et al. 2011; Poth et al. 2012). Recently, nine novel linear cyclotides has been reported from *Panicum laxum* belonging to Poaceae and it has been shown that they possess a cystine knot arrangement similar to cyclotides (Nguyen et al. 2013).

Also, some cyclotide-like genes have been identified from data mining of nucleotide databases in several plants of the Poaceae (Mulvenna et al. 2006). Searching the databases using cyclotides as queries has resulted in the finding of 32 putative cyclotide-like genes, including *Zea mays* (11), *Triticum aestivum* (6), *Setaria italica* (5), *Agrostis stolonifera* (3), *Pennisetum glaucum* (1), *Sorghum bicolor* (1), *Schedonorus arundinaceus* (2), *Hordeum vulgare* (1), *Saccharum officinarum* (1), and *Oryza sativa* (1), respectively (Mulvenna et al. 2006; Nguyen et al. 2013). Cyclotide-like genes show significant similarity with other cyclotide genes. The amino acid sequence similarities include six-Cys residues, an absolutely conserved Glu residue in loop 1—a hydroxyl-bearing (Thr or Ser) residue immediately after the Glu residue in loop 1 and the last residue in loop 3 (Mulvenna et al. 2006). Deduced protein sequence of Poaceae cyclotide-like genes are classified into two broad classes. The first class shows significant sequence identity in upstream of the first Cys residue and generally has short tails after the final Cys residue. In the second class the upstream region of the Cys residue is less conserved and the tail after the last Cys residue is longer. In addition, numbers of amino acids between the first and second Cys residues are variable. These show that the Poaceae family contains a wide range of cyclotide-like genes, which have hindered their structural and functional identification.

In the present work, nine cyclotide-like genes were isolated from maize using 3′RACE-PCR. Also, the gene expression of two cyclotide-like genes including *Zmcyc*1 and *Zmcyc5* were analyzed using real-time PCR (RT-PCR). Results showed that cyclotide-like genes expression were dynamic and induced in response to fungal disease, insect attack and signaling molecule treatments which probably reflects their direct or indirect roles in plant defense systems.

Materials and methods

Plant material

Maize (*Zea mays* L. cv. SC. 704) plants were grown in 10-L pots with a 1:1:1 mix of peat: vermiculite: perlite with fertilizers in a green house with a daily cycle of 14-h light (70–80 W/m^2) at 25 °C and 10-h dark.

Stress treatment

To investigate the effect of wounding on gene expression, the leaf lamina was cut with a razor blade and harvested at different time points after wounding. Control samples were collected from healthy leaves as well. For other treatments, leaves were sprayed with 1 mM salicylic acid (SA), 0.1 mM methyl jasmonate (JA) and water as control. For drought treatment, irrigation was withheld for 96 h and three of the youngest leaves were collected from both irrigated and non-irrigated plants for gene expression analysis (Andersen et al. 2002; Boyer and McLaughlin 2007). For salinity treatments, 3-week-old plants were watered with EC = 8 deci Siemens/m (5.12 g/L NaCl) and samples were collected 48 h after treatment. The leaf samples were collected, frozen using liquid nitrogen, and were used for RNA extraction and subsequent analysis.

Fusarium graminearum inoculation

Three-week-old seedlings were subjected to pathogen inoculation using the root-dip method (Elmer and Anagnostakis 1991; Katan et al. 1994). Inoculates for root-dip assays were formulated as liquid cultures of 2% (w/v) diet

fiber at 20–28 °C with shaking and subsequently filtered through two layers of cheesecloth. Suspensions containing microconidia were prepared at the density of 10^6 cfu/ml using a hemocytometer. Roots of 3-week-old seedlings were removed from trays and carefully washed free of soil, then dipped for 3–5 min in spore suspensions for treatment(s) and in dH_2O for control plant prior to planting. Sampling was conducted at 0, 3, 6, 9 and 12 days after treatment.

Ustilago maydis inoculation

Teliospores of smut fungi were collected from a corn farm and mixed, powdered and sterilized in 5% sodium hypochlorite solution for 10 min on a shaker, washed twice with dH_2O, and then cultured on PDA medium containing 10 g/L dextrose and subsequently filtered through two layers of cheesecloth, and sporidia were collected in dH_2O (Thakur et al. 1989; Zamani et al. 2011). A mixture of sporidia with a final concentration of 10^6 cell/ml was injected into 3-week plant leaf hypodermal tissue. Sampling was conducted at 0, 3, 6, 9, and 12 days after treatment.

Aphid infection

Rhopalosiphum maydis were collected from different sections of maize plants in field. Three weeks after planting, each plant was infested with adult insects. Aphids were transferred to experimental plants with a fine paint brush (Gao et al. 2008). Samples were collected at 0, 3 and 6 days after infection. For the all treatments, leaves of 5 plants per treatment bulked as one replicate and at the end 3 replicates were used in each treatment for gene expression analysis.

RNA extraction

Total RNA was extracted from roots, stems, male and female flowers, coleoptile, coleorhiza, control leaves, and in the leaves of plants subjected to different stresses using TRIzol reagent (Invitrogen), following the manufacturer's instructions. The quality of RNA was checked by visualizing the ethidium bromide (EtBr)-stained ribosomal RNA in 1% agarose gels. The ratio of 260/280 and 230/260 nm were determined by Eppendorf BioPhotometer Plus.

cDNA synthesis

For cDNA synthesis, 5 µg of total extracted RNA were treated with RNase-free DNase (Promega) according to the manufacture's instruction to ensure no DNA contamination exist. First strand cDNA was synthesized in a 20 µl reaction system (Fermentas) containing1 µl oligo dT, 1 µl dNTP (50 mM), 8 µl total RNA (1 µg) at 65 °C for 5 min, then 2-min incubation on ice followed by addition of 0.5 µl M-MLV reverse transcriptase (Fermentas), 2 µl reaction buffer and 7.5 µl distilled water and subsequently placed at 42 °C for 60 min.

Isolation of partial sequences by 3′RACE

The sequence of putative cyclotide-like genes of *Zea mays* including CF060985, CF014141, CK369406, BM379838, CF630454, CF013901, CN070702, BI674581, BM080572, and CK368015 were aligned using CLUSTALW. A forward primer SF1, 5′ AGAGAGAGAGGAAAGCTAGC was designed based on conserved regions of maize sequences (Table 1). First strand cDNA for 3′ RACE was synthesized through reverse transcription using anchor primer (5′ GACCACGCGTATCGATGTCGACTTTTTT TTTTTTTTTTV 3′) and First strand cDNA synthesis kit (Thermo-Fisher Scientific former. Fermentas, Schwerte, Germany). The first cycle of PCR was done in a total volume of 25 µl including 8 µl H_2O, 12 µl Master Mix (CinnaGen), 2 µl First strand cDNA template, 1.5 µl SF1, and 1.5 µl PCR anchor primer, GACCACGCGTATCGATGTCGAC. PCR temperature program was 1 cycle of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 53 °C, 40 s at 72 °C, 1 cycle of 20 min at 72 °C. The 1% agarose gel was used to separate PCR product.

Molecular cloning and DNA sequencing

The expected PCR fragments were purified from the gels using a Nucleic Acid Extraction kit (Vivantis) and were ligated into the TA vector using the TA cloning kit (Fermentas). Recombinant plasmids were transformed into competent cells of *Escherichia coli* DH5a strain. Positive white clones were checked by colony PCR, and their plasmids were extracted. Sequencing of cloned fragments were done by a commercial sequencing service (Bioneer Inc. Bioneer Corporation).

Bioinformatics analysis

The sequence of putative cyclotide-like genes were blasted using BLAST program at National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov/) to their homology. Deduced protein sequence of cyclotide-like genes were aligned using Clustal-Omega. In addition to our new cyclotide-like genes, other cyclotide-like gene sequences were obtained from the GenBank database for phylogenetic analysis (Table 2). The phylogenetic tree of cyclotide-like gene was constructed using MEGA4.0.2 software based on the method of Neighbor-Joining (NJ).

Table 1 Propretise of primers that were used in this study

Primer name	Primer sequence (5′ → 3′)	Product length (bp)	Annealing temperature (°C)
Actin 1 Fw	ATGTTGCTATCCAGGCTGTTCT		61
Actin 1 Rev	TTCATTAGGTGGTCGGTGAGGT	175	61
Zm cycl Fw	GGAGAGTGGCAGCAAGAAG		60
Zm cyc1 Rev	CGAGAGAGAAAGATCAAAGACTG	275	59
Zm cyc5 Rev	GTCGTCATGGAAATTAAACAGC	275	59
ZF (forward)	AGAGAGAGAGGAAAGCTAGC		58
ZR (reverse)	CAATAAGTTGAACACCACCG		57
Oligo dT anchor primer	GACCACGCGTATCGATGTCGAC TTTTTTTTTTTTTTTV		68
PCR anchor primer	GACCACGCGTATCGATGTCGAC		66
Specific primer	AGAGAGAGAGGAAAGCTAGC		59

Theoretical isoelectric point and mass values for the protein was predicted using ExPASyProtParam tool (http://us.expasy.org/tools/protparam.html). To sequence translate to protein, cyclotide sequence was translated by online software ExPASy in each 6 ORF and then the obtained sequences were analyzed for Cys position and true cyclotide structure. For promoter sequence analysis, first the maize chromosomes were downloaded from ensemblgenome.org (ftp.ensemblgenome.org/pub/plants/release-23/fasta/zea_mays/dna/) and then aligned with cyclotide sequence by offline software stand-aloneBLAST version 2.2.26. After finding the gene position on chromosome, a 1000 bp sequence in upstream of gene was analyzed using PLACE (http://www.dna.affrc.go.jp) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

qRT-PCR analysis

Real-time PCR runs was done using Applied BioSystem thermal cycler instrument. For quantification of real-time PCR product, the threshold cycle (*C*t) of the cyclotide-like genes were normalized with *Actin1* as reference gene. The relative gene expression for each selected genes was quantified for both the control and treatment plants. For real-time PCR expression analysis three independent biological replicates and three technical replicates of each biological replicate were used. The PCR efficiency of each primer pair for target genes and reference genes was evaluated on five log serial dilutions. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for qRT-PCR. Melting curve analysis was conducted by increasing temperature from 60 to 95 °C (0.5 °C per 10 s) along with gel electrophoresis of the final product to check the specific amplification. To confirm the absence of genomic DNA negative control reactions were done using RNA as a template. For relative expression fold changes of each gene, its *C*t value was normalized to the *C*t value of the reference gene and was calculated relative to a calibrator using the Δ*C*t method as follows: $\Delta Ct = Ct(\text{target gene}) - Ct(\text{Actin 1})$ and the RGE as: $RGE = \text{POWER}(2^{-\Delta Ct})$ (Livak and Schmittgen 2001).

Statistical analysis

The expression data was analyzed using SAS version 9.1 (SAS Institute, Inc, Cary, NC, USA). One-way analysis of variance (ANOVA) with LSD post hoc tests was used to reveal significant differences among treatments.

Results

Isolation and sequence analysis of *Zmcyc* genes

Based on the alignment of 10 expressed sequence tags (ESTs) of *Z. mays,* a band of the predicted size (~500 bp) was observed after PCR amplification using forward and oligo dT anchor PCR primers. The cDNA synthesized from total RNA extracted from whole plant tissues was used as a template in PCR . The expected fragment was excised from agarose gel and cloned into the plasmid vector (TA cloning, Fermentas). Positive clones were picked and used for screening. Twenty unique clones were chosen for DNA sequencing, of which 9 clones contained the primer sites. BLAST analysis against GenBank database revealed that 9 sequences were highly similar to cyclotide-like genes with *e*-value of $5e^{-25}$ to $1e^{-155}$. Conceptual translations of the above 9 sequences revealed that they code for two classes of cyclotide-like proteins named as *Zmcyc1* to *Zmcyc9* (Fig. 1). These sequences were classified into two groups. The first group had a continuous open reading frame (ORF) and the characteristics of six cysteine motifs and was significantly similar to Poaceae cyclotide-like genes in database. *Zmcyc1*, *Zmcyc2*, and *Zmcyc3* had an identical amino

Table 2 Properties of cyclotide-like gene in Poaceae family

Accession number	Molecule weight (k dalton)	Isoelectric point	Amino acid number	Nucleotide number (bp)	Plant species	Cyclotide-like name
CF060985	10.139	7.74	64	474	*Zea mays*	*ZmCF060985*
CF014141	11.193	4.89	64	502	*Zea mays*	*ZmCF014141*
CK369406	9.295	7.75	65	505	*Zea mays*	*ZmCK369406*
BM3798383	8.95	6.73	65	526	*Zea mays*	*ZmBM37983*
CF630454	8.985	5.64	64	518	*Zea mays*	*ZmCF630454*
CF013901	9.24	5.48	66	538	*Zea mays*	*ZmCF013901*
CN070702	8.901	6.71	87	392	*Zea mays*	*Zm CN07070*
BI674581	9.52	8.85	43	496	*Zea mays*	*Zm BI674581*
BM080572	9.428	8.84	43	571	*Zea mays*	*ZmBM080572*
CK368015	8.701	7.77	43	671	*Zea mays*	*ZmCK368015*
CF061604	8.97	6.03	66	526	*Zea mays*	*ZmCF061604*
Current study	7.075	4.74	66	465	*Zea mays*	*Zmcyc 1*
Current study	7.075	4.74	66	461	*Zea mays*	*Zmcyc 2*
Current study	7.075	4.74	66	466	*Zea mays*	*Zmcyc 3*
Current study	7.075	4.74	66	322	*Zea mays*	*Zmcyc 4*
Current study	6.690	4.95	64	488	*Zea mays*	*Zmcyc 5*
Current study	14.2	8.22	93	338	*Zea mays*	*Zmcyc 6*
Current study	15.00	8.92	134	470	*Zea mays*	*Zmcyc 7*
Current study	9.53	5.78	88	413	*Zea mays*	*Zmcyc 8*
Current study	6.8	4.98	59	442	*Zea mays*	*Zmcyc 9*
KC182530	5.835	5.57	53	456	*Panicum laxum*	*PlKC182530*
KC182531	3.914	7.59	34	473	*Panicum laxum*	*PlKC182531*
KC182533	5.706	5.00	64	489	*Panicum laxum*	*PlKC182533*
KC183532	6.744	8.27	53	481	*Panicum laxum*	*PlKC182532*
CA617438	8.901	6.71	86	429	*Triticum aestivum*	*TaCA617438*
CK154330	14.419	9.61	53	907	*Triticum aestivum*	*TaCK154330*
CK154890	10.987	8.30	32	889	*Triticum aestivum*	*TaCK154890*
CA595705	10.53	8.85	59	596	*Triticum aestivum*	*TaCA595705*
BE591233	12.884	8.95	63	466	*Triticum aestivum*	*TaBE591233*
HX171398	6.491	6.06	58	485	*Triticum aestivum*	*TaHX171398*
BE125990	10.377	8.91	68	383	*Sorghum bicolor*	*SbBE125990*
CD725989	9.107	7.79	37	503	*Pennisetum glaucum*	*PgCD72598*
AL450615	11.052	6.98	53	562	*Hordeum vulgare*	*HvAL450615*
CK803164	4.092	5.92	35	323	*Sorghum arundinaceus*	*SaCK803164*
CA274667	4.538	5.30	43	842	*Sorghum officinarum*	*SoCA274667*
EX575351	6.355	8.65	59	494	*Hordeum vulgare*	*HvEX575351*
Ju116752	4.050	5.51	36	449	*Agrostis stolonifera*	*AsJu116752*

acid sequence in spite of different nucleotide sequences. All of the group I cyclotide-like genes, *Zmcyc1* and *Zmcyc5* encode an endoplasmic reticulum signal peptide with 34 amino acids and a putative precursor protein with 54 amino acids that includes a C-terminal domain with 26 amino acids that in turn contains six Cys residues at positions 62, 66,71, 78, 80, and 85 similar to known cyclotides. All the isolated sequences contain a short tail with three residues after the C-terminal Cys residue (Fig. 2).

The second group includes four sequences which showed less conservation of the six-Cys domain, and also displayed an expansion in the number of amino acids between the first and second or other Cys residues. The lengths of these sequences also varied from 60 to 140 amino acids.

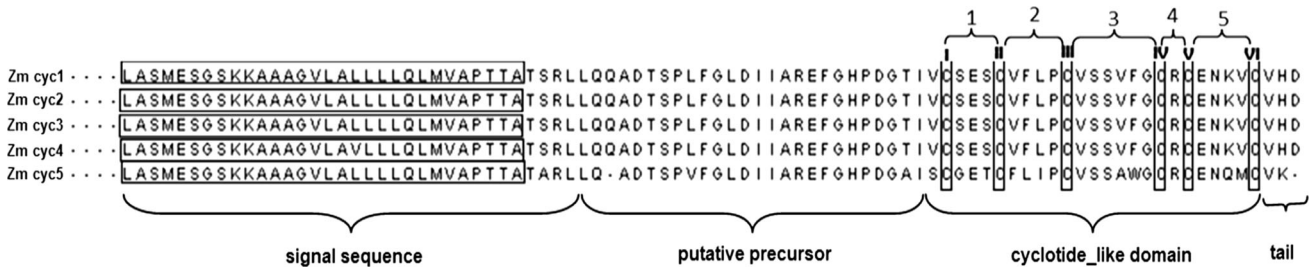

Fig. 1 Putative cyclotide-like genes identified in *Z. mays*. *Zmcyc1-Zmcyc5* shows the conserved structure of the genes in Poaceae species. Each sequence contains a signal sequence (*horizontal boxes*), a precursor region, the six-Cys domain, and a short C-terminal tail region. The numbering of the loops connecting each Cys residue is indicated at *top*

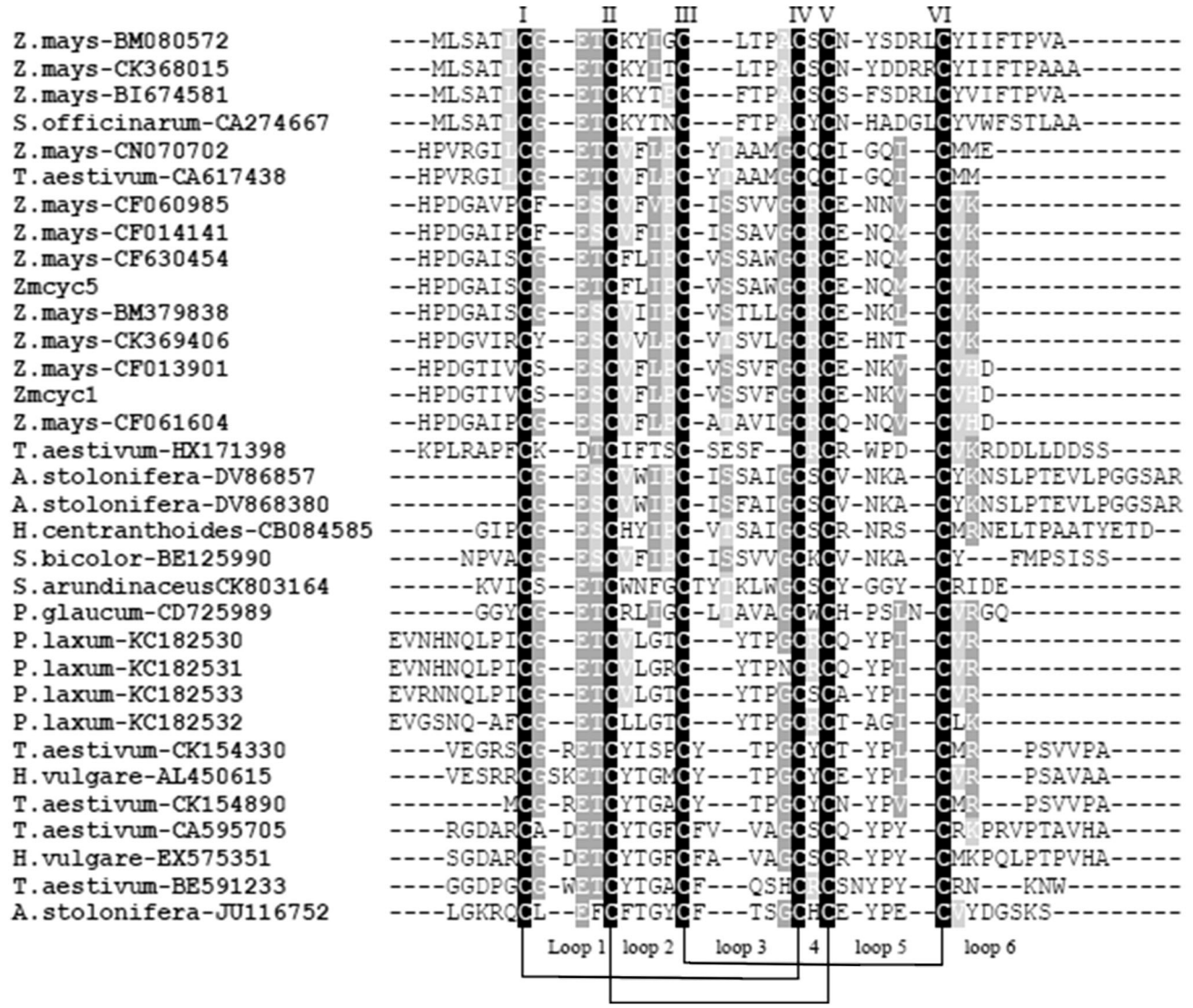

Fig. 2 The peptide sequence alignment of different cyclotide-like genes in Poaceae family. The cysteine residues and the backbone loops between them are numbered on the structure. The sequence showed variability, despite conservation of the six cysteines. The cysteine positions are shown in *black*

Phylogenetic profiling of gramineous cyclotide-like genes was carried out using MEGA software. The phylogenetic tree showed that gramineous cyclotide genes were separated into two distinct clusters, consistent with the amino acid sequence similarity analysis as shown in Fig. 3. All of the isolated cyclotides-like genes in this study along

with nine other putative cyclotide-like genes from maize were placed in one cluster. In another cluster, cyclotide-like genes from other gramineous plants were located along with four linear cyclotides from *P. laxum*. However, linear cyclotides were found in a separate sub cluster.

Database assisted sequence analysis

Based on genome draft of maize the 1000 bp upstream of both *Zmcyc1* and *Zmcyc5* genes were isolated and analyzed using PlantCARE. Comparison of the two promoter

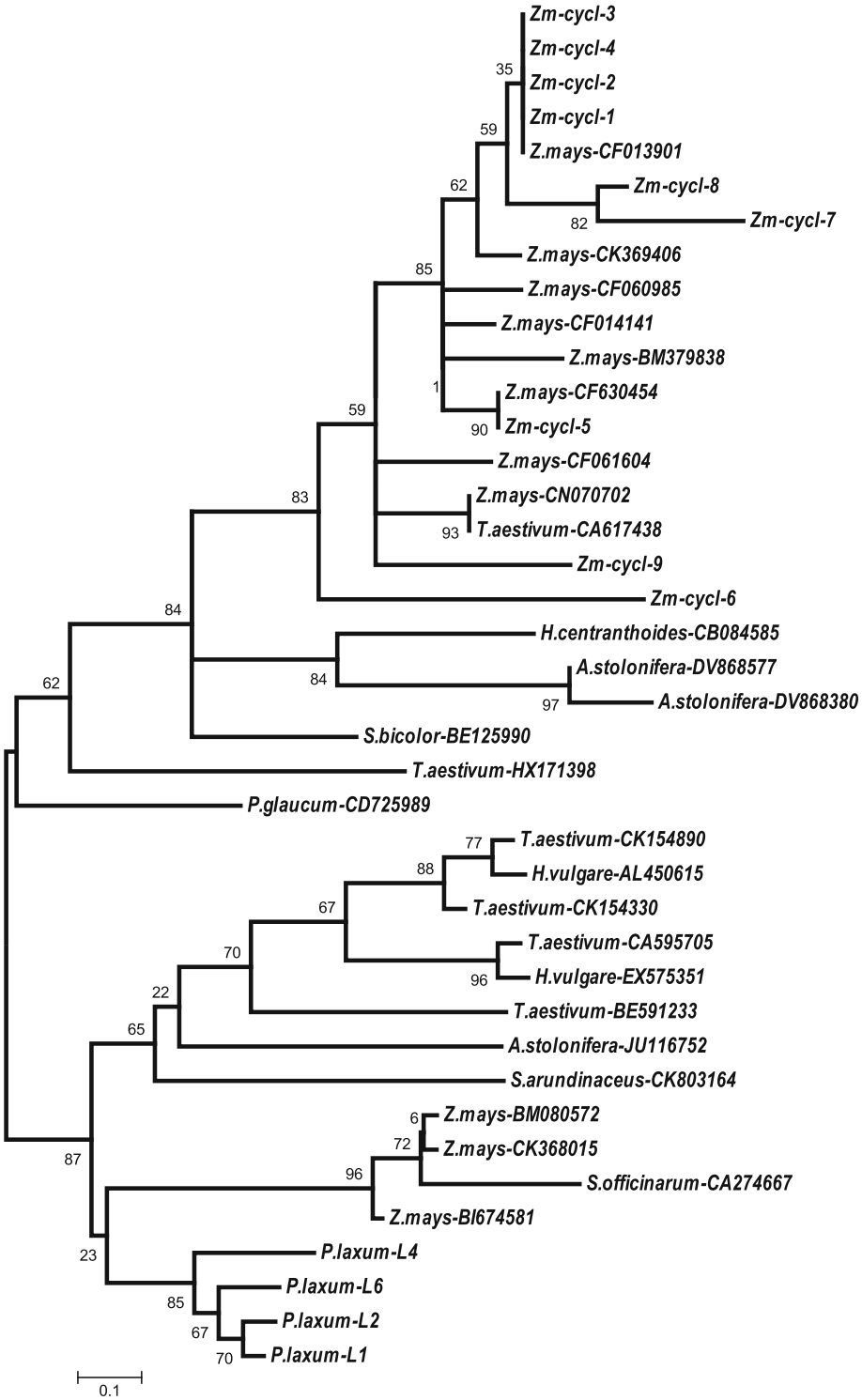

Fig. 3 Dendrogram of cyclotide-like peptide sequence in Poaceae family. Accession number for each sequence are listed on the right side of the plant name: more details are listed in Table 2. This phylogram is plotted by Criterion neighbor-joining method

sequences revealed the presence of different cis-elements at different positions. The promoter sequences of both genes were AT rich with 60.5% and 56.34% AT for *Zmcyc1* and *Zmcyc5,* respectively. The results of some of the identified general transcription and potential regulatory cis-elements for both *Zmcyc1* and *Zmcyc5* have been summarized in Tables 3 and 4.

Most of cis-elements found in both genes promoters were similar, but some different elements were also found. For both genes TATA box sequence elements required for the critical and precise transcription initiation. However, in *Zmcyc5* INRNTPSADB sequence that is called initiator and works in TATA less promoter instead of TATA box were also detected. The CAAT box sequences which are responsible for the tissue specific promoter activities were also found in both gene promoters. In both promoters calcium responsive cis-element ABRERATCAL and ACGTATERD sequence elements were identified. Conserved sequence of CBFHV was found in both promoters that is a low temperature response element. Abiotic stress related elements including LTRECOREATCOR15, MYB1AT, MYB2CONSENSUSAT, and MYBCORE were also detected in both promoters. Some important wounding and disease induced regulatory elements like, WBOXATNPR1, WBOXHVISO1, WBOXNTCHN48, WBOXNTERF3, and WRKY71OS were also found in the promoter region of both genes.

Expression of the *Zmcyc1* and *Zmcyc5* in different tissues and development stage

Quantitative real-time PCR showed significant differences in gene expression of *Zmcyc1* and *Zmcyc5* between different tissues and the relative gene expression of *Zmcyc1* was

Table 3 Putative *cis*-acting elements present in the promoter of the *Zmcyc 1*

Cis-element	Sequence	Position	Function
ABRELATERD1	ACGTG	598 (+).597 (−)	ABRE; etiolation; erd
ABRERATCAL	MACGYGB	596 (+).596 (−)	ABRE; calcium
ACGTABOX	TACGTA	907 (±)	A-box; ACGT element; Gmotif; sugar; repression; seed
ASF1MOTIFCAMV	TGACG	45 (+). 849 (−)	TGACG; root; leaf; CaMV; 35S; promoter; auxin; salicylic acid; light; as-1; TGA1a, TGA1b; CREB; ASF1; TGA6; shoot; xenobiotic; stress; SAR; SA; Disease resistance
BIHD1OS	TGTCA	579 (+).198 (−)	HD; homeodomain
CAATBOX1	CAAT	94, 114, 167 396, 479 (+)	CAAT; legA; seed
		96, 208, 243, 255, 367, 612, 622, 655, 788, 824, 927 (−)	
CBFHV	RYCGAC	715 (−)	CBF; AP2 domain; CRT/DRE; low temperature
CCAATBOX1	CCAAT	622, 824 (−)	HSE (Heat shock element); CCAAT box
CURECORECR	GTAC	393, 906, 910 (±)	Copper; oxygen; hypoxic
GT1CONSENSUS	GRWAAW	296, 433, 629, 223, 889 (−)	Promoter influences the level of SA-inducible gene expression
LTRECOREATCOR15	CCGAC	102 (+)	Low temperature; cold; LTRE; drought; ABA; cor15a; BN115; leaf; shoot; phytochrome
MYB1AT	WAACCA	386 (+)	MYB; rd22BP1; ABA; leaf; seed; stress
MYB2CONSENSUSAT	YAACKG	141 (+), 523 (−)	MYB; rd22BP1; ABA; leaf; seed; stress
		523, 932 (+)	MYB; dehydration; water; stress; flavonoid biosynthesis; leaf; shoot
	MYBCORE	CNGTTR	520, 141 (−)
TATABOXOSPAL	TATTTAA	136 (−)	TBP; TFIIB; pal; DNA binding and bending
WBOXATNPR1	TTGAC	44 (+)	NPR1; WRKY; WRKY18; disease resistance; SA; W box
WBOXHVISO1	TGACT	544, 662, 984 (−)	Sugar; SURE; patatin; WRKY; isoamylase; SUSIBA2
WBOXNTCHN48	CTGACY	544 (−)	W box; W box; WRKY; elicitor
WBOXNTERF3	TGACY	710, 720 (+) 544, 662, 984, 217 (−)	W box; ERF3; wounding
WRKY71OS	TGAC	45, 198, 710,720 (+) 218, 545, 580, 663, 850, 985 (−)	WRKY; GA; MYB; W box; TGAC; PR proteins

The 1000 bp upstream of both *Zmcyc1* and *Zmcyc5* genes were isolated in *Z. mays* genome draft and analyzed using PlantCARE

Table 4 Putative *cis*-acting elements present in the promoter of the *Zmcyc 5*

Cis-element	Sequence	Position	Function
ABRELATERD1	ACGTG	955 (+). 954 (−)	ABRE; etiolation; erd
ABRERATCAL	MACGYGB	954 (+)	ABRE; calcium
ACGTATERD1	ACGT	955 (±)	ACGT; etiolation; erd
ASF1MOTIFCAMV	TGACG	241, 307 (+)	TGACG; root; leaf; CaMV; 35S; promoter; auxin; salicylic acid; light; TGA6; shoot; xenobiotic stress; SAR; SA; Disease resistance
BIHD1OS	TGTCA	10 (−)	HD; homeodomain
CAATBOX1	CAAT	13, 206, 272, 616, 759, 973, 982 (−)	CAAT; legA; seed
CACGTGMOTIF	CACGTG	954 (±)	G box; G-box; rbcs; chs; ACGT
CBFHV	RYCGAC	561 (−)	CBF; AP2 domain; CRT/DRE; low temperature
CCAATBOX1	CCAAT	205, 758 (+), 417, 453, 982 (−)	HSE (Heat shock element); CCAAT box
DRE2COREZMRAB17	ACCGAC	561 (−)	Rab17; ABA; drought response; DBF1; DBF2
DRECRTCOREAT	RCCGAC	561 (−)	DRE/CRT; drought; high-light; cold; DREB; DREB1; DREB2; CBF
GT1CONSENSUS	GRWAAW	849, 989 (+)	GT-1; light; TATA; TFIIA; TBP; HR; SAR; TMV; leaf; shoot
INRNTPSADB	YTCANTYY	392 (+). 255, 321 (−)	Initiator; light responsive transcription; TATA less promoter; psaDb; Inr element
LTRECOREATCOR15	CCGAC	561, 919 (−)	Low temperature; cold; LTRE; drought; ABA
MYB1AT	WAACCA	528 (−)	MYB; rd22BP1; ABA; leaf; seed; stress
MYB2CONSENSUSAT	YAACKG	889 (+). 662 (−)	MYB; rd22BP1; ABA; leaf; seed; stress
MYBCORE	CNGTTR	441, 662, 1030 (+), 543, 780, 886 (−)	MYB; myb; dehydration; water; stress; flavonoid biosynthesis; leaf; shoot
MYBCOREATCYCB1	AACGG	887 (+). 137, 909 (−)	Cyc; M phase; Myb
MYBST1	GGATA	1055 (+). 211 (−)	MYB; myb; Myb
MYCATERD1	CATGTG	621 (−)	Water-stress; erd
MYCATRD22	CACATG	621 (+)	Dehydration; Water-stress; ABA; MYC; myc; leaf; shoot
ROOTMOTIFTAPOX1	ATATT	276, 971 (+) 207, 273, 970 (−)	Root; rolD
TATAPVTRNALEU	TTTATATA	495 (+). 189 (−)	TATA; tRNA; re-initiation; GapC4; Myb; TATA binding protein; TBP
WBBOXPCWRKY1	TTTGACY	581 (−)	W box; WRKY
WBOXATNPR1	TTGAC	9 (+). 582 (−)	NPR1; WRKY; WRKY18; disease resistance; SA; W box
WBOXHVISO1	TGACT	581, 1083 (−)	sugar; SURE; patatin; WRKY; isoamylase
WBOXNTERF3	TGACY	581,858,936, 1083 (−)	W box; ERF3; wounding
WRKY71OS	TGAC	10, 241, 307 (+) 582, 859, 937,1084 (−)	WRKY; GA; MYB; W box; TGAC; PR proteins

The 1000 bp upstream of both *Zmcyc1* and *Zmcyc5* genes were isolated in *Z. mays* genome draft and analyzed using PlantCARE

significantly higher than that of *Zmcyc5*. Despite different levels in gene expression of two genes the expression patterns in different tissues were similar. For both genes the highest expression was observed in leaves and stems, respectively. The gene expression of *Zmcyc1* and *Zmcyc5* in leaves were approximately eight- and three-fold higher than that observed in roots and flowers, respectively (Fig. 4a, b). The lowest gene expression was observed in roots.

Results of gene expression during leaf development showed a dynamic pattern of both genes as that the lowest level of expression for both genes were in 30-day-old leaves, but the highest level of expression for *Zmcyc1* was at the time of emergence of female flowers (70-day-old leaves) and for *Zmcyc5* at the time of emergence of male flowers (50-day-old leaves) (Fig. 4c, d). The relative expression at these stages was about 70- and 40-fold higher than that of younger leaves (30-day-old leaves).

Wounding, aphid attack

Results showed that the expression of *Zmcyc1* was elevated under mechanical wounding and within 24 h

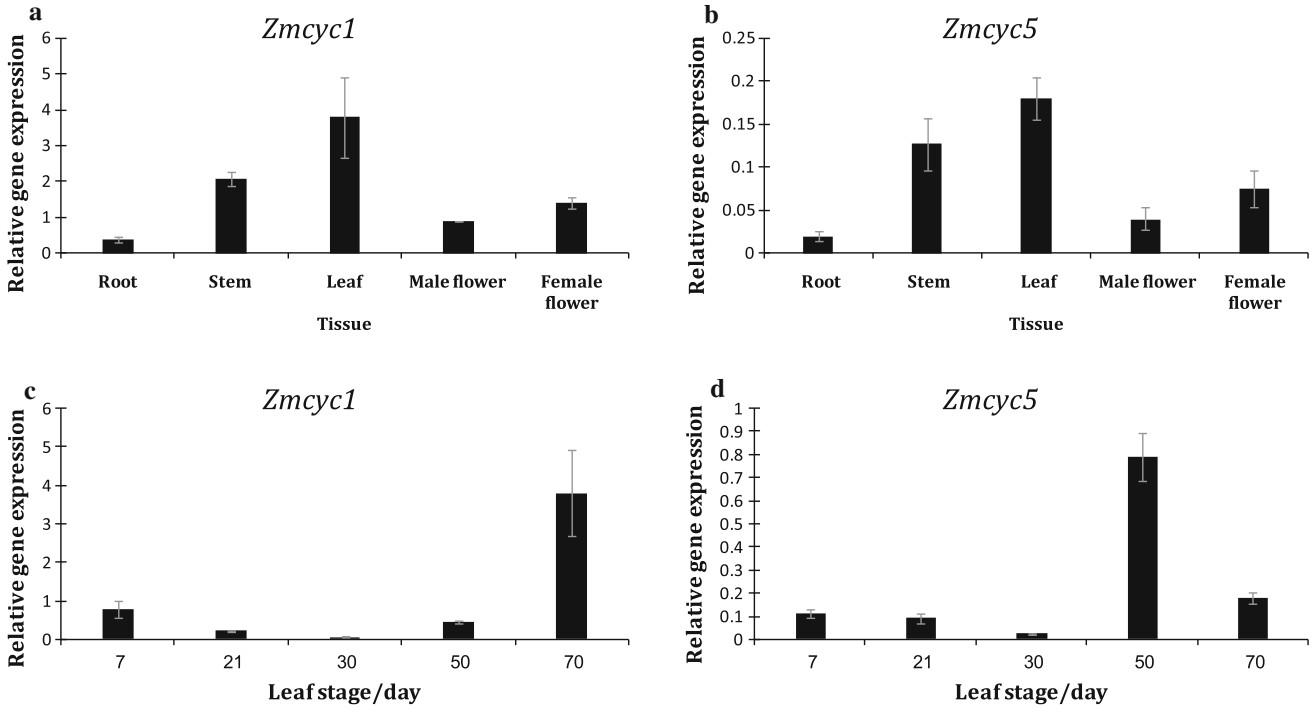

Fig. 4 Expression of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* at mRNA level **a**, **b** in different tissues (root, stem, leaf, male and female flower) and **c**, **d** in different leaf stages over 70-day period at the time of 7 day (cleoptile), 21 day (trifoliate), 30 day (the first stem node), 50 day (appearance of male flowers), and 70 day (appearance of female flowers). *Error bars* are standard deviations from three biological triplicates ($P < 0.05$)

reached highest level (about ten-fold higher than the control). Afterwards, *Zm cyc1* expression was reduced gradually, but after 72 h the gene expression level was still seven-fold higher than control plants (Fig. 5a). For *Zmcyc5* after 24 h the gene expression level was dropped quickly down to the level of control plants. Results showed that the gene expression patterns for both genes were similar and the gene expression reached the highest level 6 days after insect feeding (Fig. 5b, c).

Salicylic acid and methyl jasmonate treatments

Results showed that during the first 24 h after salicylic acid treatment the expression level of *Zmcyc1* significantly increased, then gradually decreased at 48 h and remained constant until 72 h (Fig. 6a). However, the expression level of *Zmcyc5* was reduced after 48 h and reached the control plants level.

The expression of *Zmcyc1* increased significantly 24 h after methyl jasmonate treatment, remained constant until 48 h and increased sharply 72 h after treatment (Fig. 6b). *Zmcyc5* transcript levels gradually increased and reached the highest level 48 h after treatment and decreased at 72 h. The expression levels of *Zmcyc1* were higher than *Zmcyc5* after methyl jasmonate treatment.

Ustilago mydis and *Fusarium graminearum*

Gene expression analysis of *Zmcyc1* and *Zmcyc5* under infection by *F. graminearum* showed that 3 days after inoculation, the expression levels of both genes increased significantly,

but at day 4 decreased gradually until day 12 then reached the level of control plants (Fig. 7a, d).

The gene expression level for cyclotide-like genes under infection by *Ustilago maydis* sporidium increased significantly in comparison with control plant and the gene expression coordinately increased with the progression of the disease and symptoms. Three days after infection, the gene expression of both genes increased and remained at that level until day 6 (Fig. 7b, c). The highest gene expression was observed at day 9, when gall symptoms were observed on leaves. 12 days after infection, the level of expression of both genes was decreased to the level of Day 6.

Drought and salinity

The expression levels of *Zmcyc1* and *Zmcyc5* were significantly increased in plants subjected to drought treatment. In treated plants, the expressions of *Zmcyc1* and *Zmcyc5* were almost 6 and 8 times higher than control plants at 96 h after latest irrigation, respectively (Fig. 8a). Results showed that the expression of *Zmcyc1* and for *Zmcyc5* at 48 h after

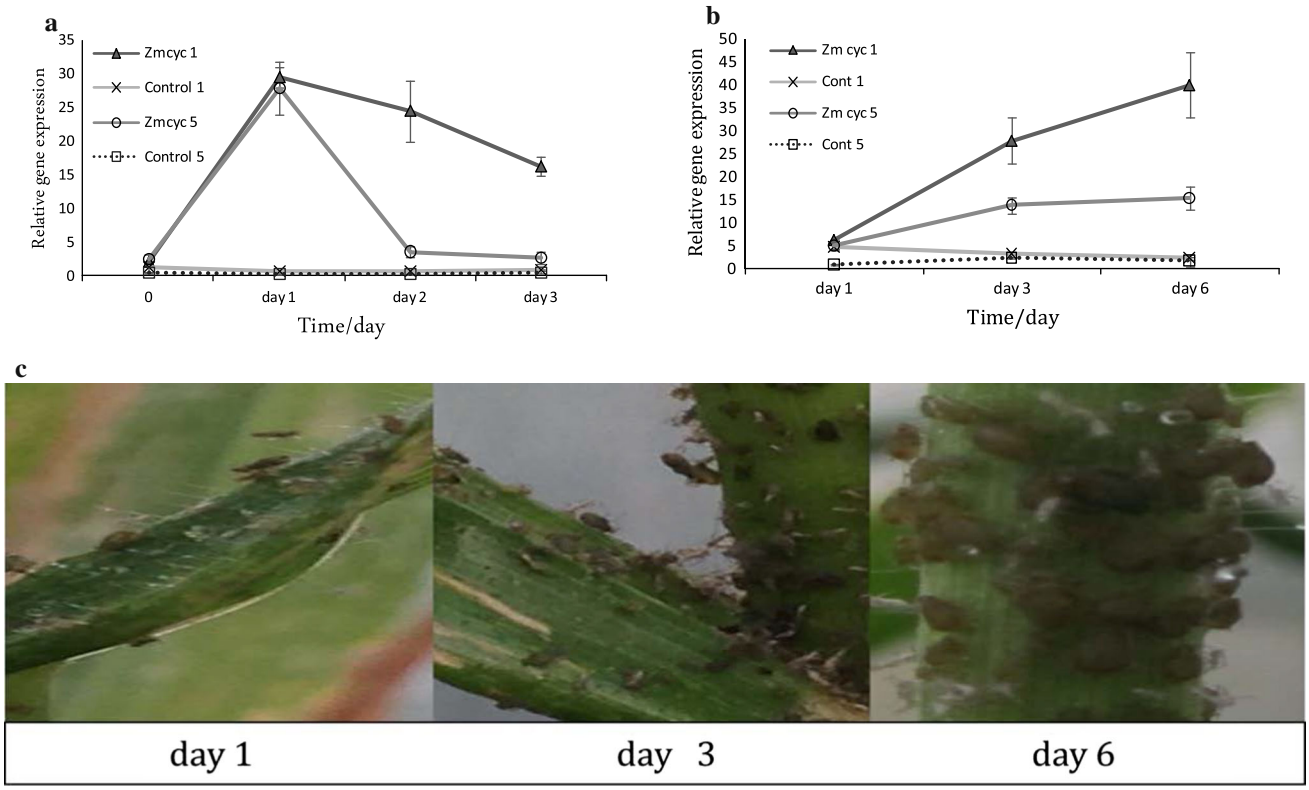

Fig. 5 Expression of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* at mRNA level under wonding on leaf **a** intact leaf in control plant at the time of 0, 24. 48 and 72 h and **b** insect attack of corn aphids inside control plant at the time of day 1, day 3 and 6. **c** Corn leaf aphid infestation on corn leaf in 6-day period. *Error bars* are standard deviations from three biological triplicates ($P < 0.05$)

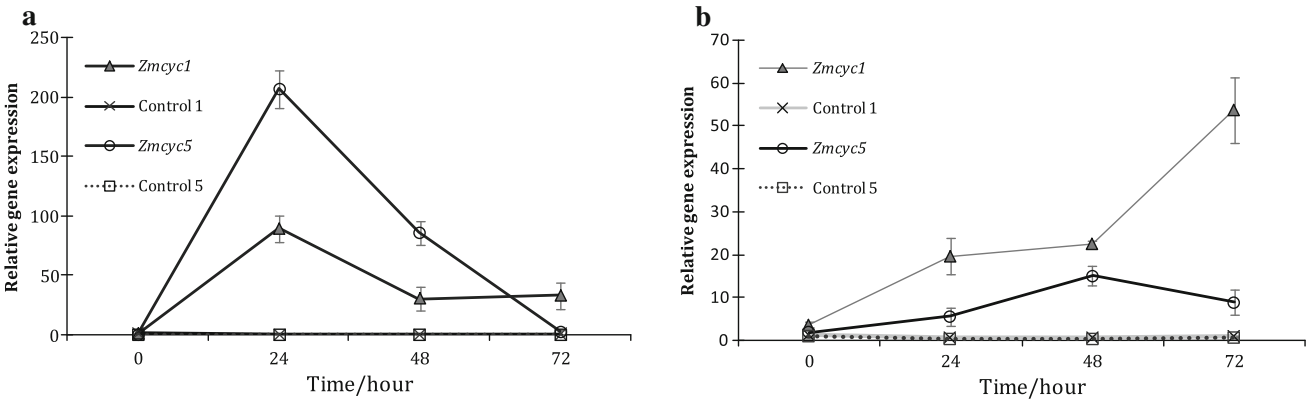

Fig. 6 Expression of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* at mRNA level under treatment with **a** 1 mM salicylic acid and **b** 0.1 mM methyl jasmonate. *Error bars* are standard deviations from three biological triplicates ($P < 0.05$)

irrigation with salt water (EC = 8) increased 5.2 fold as compared with non-irrigated condition (Fig. 8b).

Discussion

In this study, nine putative cyclotide-like genes from maize were cloned and sequenced using 3′RACE. The obtained cyclotide-like genes were classified in two groups. Five of them contained six cysteine residues and showed high similarity to the computationally identified cyclotide-like genes in gramineous plants. In Violaceae and Rubiaceae, cyclotides belong to a relatively large family. For example, more than 50 cyclotides have been identified in *Viola hederacea* (Trabi and Craik 2004). However, less putative cyclotide-like genes have been identified in gramineous plants. Most gramineous plants have less than ten and within them the majority have one or two cyclotide genes

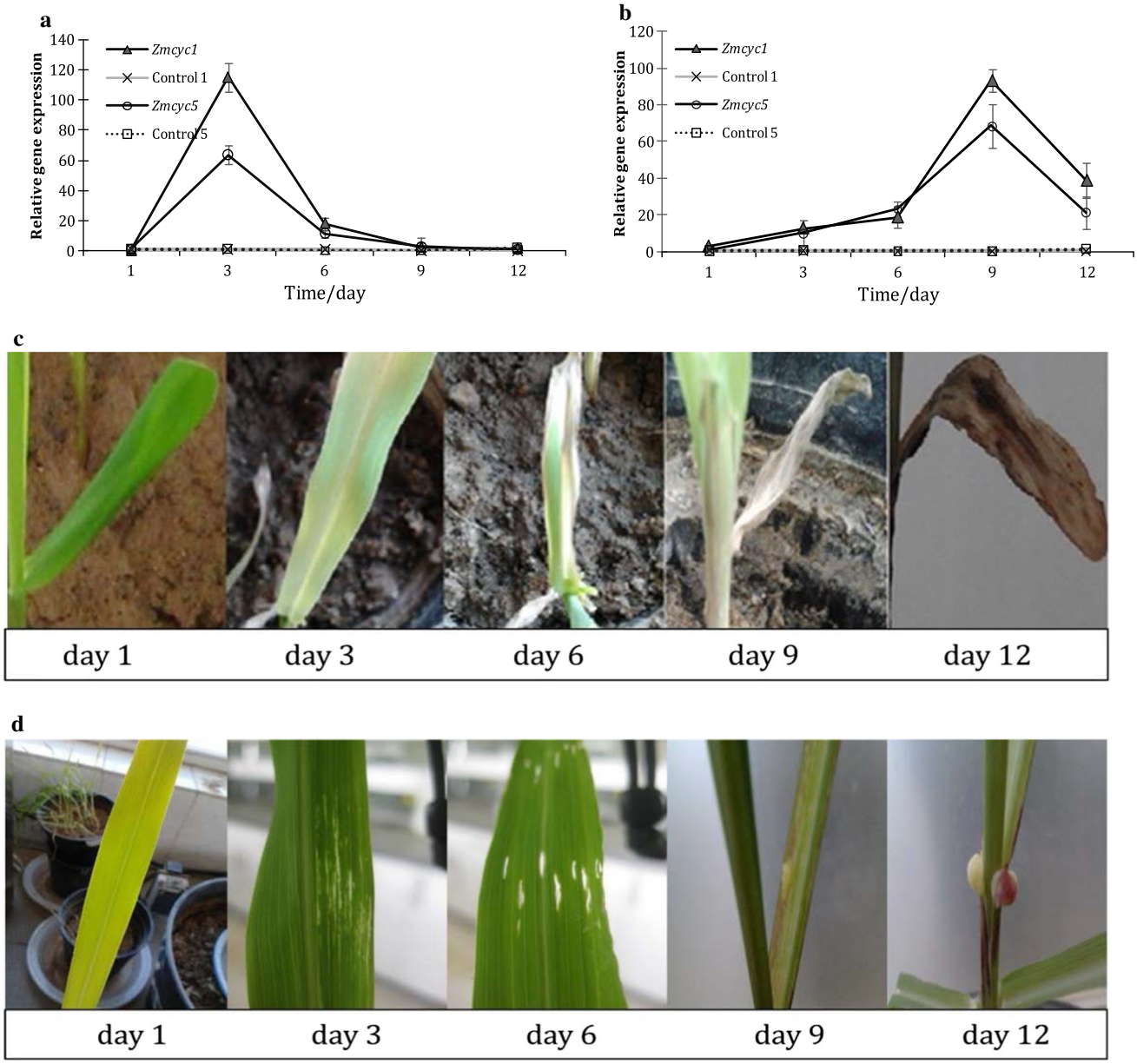

Fig. 7 Expression of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* at mRNA level under treatment with fungal **a** *F. graminarium* agent of ear rot and **b** *U. maydis* agent of smut after 1, 3, 6, 9 and 12 days. **c** *U. maydis* infection in 12-day period, symptoms appeared after 3 days and gall after 9 days. **d** *F. graminearium* infection in 12-day period, symptoms appeared after 3 days. *Error bars* are standard deviations from three biological triplicates ($P < 0.05$)

(Mulvenna et al. 2006). There are no comprehensive studies on cyclotide-like genes in gramineous plant and most of the studies are based on similarity searches in EST databases. In addition, a recent study showed that cyclotide-like genes in gramineous plant show variation in their structure and types. Nine novel linear cyclotides have been reported from the *Panicum laxum* of the Poaceae family (Nguyen et al. 2013). Linear cyclotides have been reported in two species including *A. stolonifera* and *S. italica*, from the Poaceae by searching nucleotide databases. Linear cyclotides possesses a cysteine knot arrangement similar to cyclotides, hence it is possible to identify more cyclic or acyclic cyclotide genes from gramineous plants in the future.

Four of the cloned putative cyclotide-like genes from maize were completely different in the number of cysteine residues, their positions and also in the length of the putative peptides. They showed significant similarity to cyclotide-like genes and clustered in a phylogenetic tree with cyclotide-like genes from maize and wheat. Current functions of these genes still remains poorly understood. They may come from a new class of cyclotide-like genes

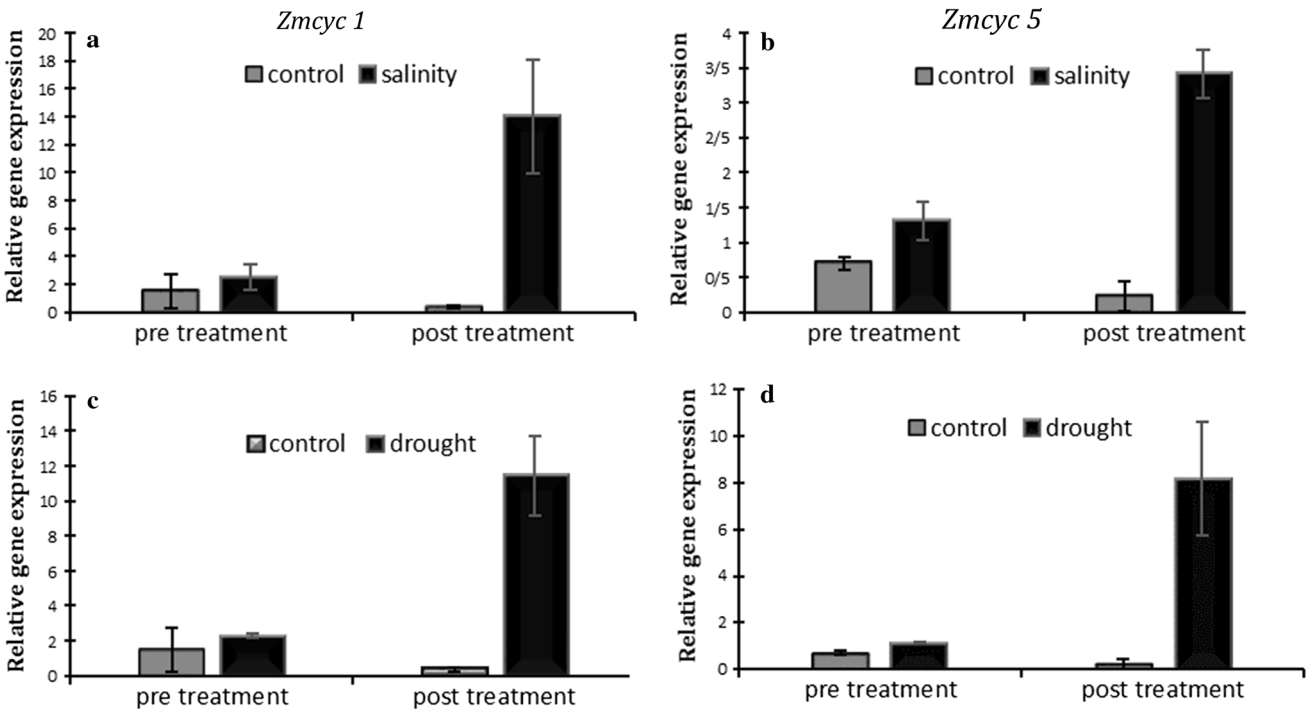

Fig. 8 Expression of two cyclotide-like genes *Zmcyc1* and *Zmcyc5* at mRNA level under salinity and drought condition. **a** *Zmcyc1* under control and salinity, **b** *Zmcyc5* under control and salinity, **c** *Zmcyc1* under control and drought, **d** *Zmcyc5* under control and drought. For drought treatment, irrigation was withheld for 96 h and three of the youngest leaves were collected for both irrigated and non-irrigated plants for expression analysis. For salinity treatments 3-week-old plants were watered with EC = 8 dSiemens/m (5.12 g NaCl per liter) and samples were collected at 48 h after treatment. *Error bars* are standard deviations from three biological triplicates ($P < 0.05$)

that have not yet been studied. It is also possible that they are a new class of small peptides similar to cyclotide genes or maybe pseudogenes.

In the present work, the gene expression patterns of two cyclotide-like genes including *Zmcyc1* and *Zmcyc5* were studied under different conditions and treatments, both biotic and abiotic. Both *Zmcyc1* and *Zmcyc5* genes were expressed in all mature plant tissues, but a higher expression levels were observed in leaves and stems. The highest expression of both genes was detected in mature leaves. In *Viola hederacea* different cyclotide genes are expressed in different tissues, e.g., a root specific cyclotide has been identified (Trabi and Craik 2004). Gene expression analysis of a cyclotide-like gene *Bcl 1* in barley has shown that *Bcl 1* gene expression in the coleoptile and in the first leaf is higher than other tissues (Mulvenna et al. 2006). *Rcl* is a cyclotide-like gene in rice expressed in all tissues except flowers and its highest expression is observed in roots (Mulvenna et al. 2006). The expression of *Barley Bcl1* in the coleoptile over 7 days shows that its expression gradually increased until day 5. *Zmcyc1* and *Zmcyc5* genes showed a similar expression pattern in different tissues and, in different developmental stages of leaves. Analysis of cis-elements in *Zmcyc1* and *Zmcyc5* promoters showed that they contain similar cis-elements, consistent with their similar expression patterns.

Methyl jasmonate and salicylic acid act as global signals for defense genes. Cross-talk between methyl jasmonate and salicylic acid pathways appears to be very common and important in the regulation of defense gene expression. There is no report on the expression of cyclotide-like genes in response to signaling molecules such as JA, ethylene and SA. Many reports show that SA and JA regulate many pathogenesis-related (PR) genes. To our knowledge there are no reports on expression analysis of cyclotides following signaling molecule treatments. Cyclotides show antimicrobial activities against a wide range of pathogens, hence they might be responding to signaling molecules such as SA and JA.

There are many reports of antifungal activity of cyclotides (Jennings et al. 2001; Tam et al. 1999). It has been reported that infection of maize plants with the smut fungus *Ustilago maydis* upregulates the expression of many genes including a cyclotide-like named *Umi11* that is significantly induced, especially in gall development stage (Basse 2005). In agreement with the latter study, both *Zmcyc1* and *Zmcyc5* expression significantly increased in 9 days after inoculation which coincided with the time of gall symptoms emergence. Similar results were observed for both genes after *F. graminearum* infection in maize. Also integrated analysis of proteomics and transcriptomics data

showed that the resistance to Fusarium Head Blight (FHB) is mediated by regular and coordinated expression of signal molecules, e.g., salicylic acid, jasmonic acid, ethylene, and ROS inside secondary metabolites (Ding et al. 2011). It has been reported that PR proteins have a role in resistance to FHB in wheat (Gottwald et al. 2012; Li and Yen 2008; Xiao et al. 2013). Consistent with this, it is possible that *Zmcyc1* and *Zmcyc5* encoding PR proteins could also play defense roles in the maize defense systems against fungi.

To examine whether *Zmcyc1* and *Zmcyc5* are dynamically regulated by abiotic stresses, maize plants subjected to drought and salinity and their expression were examined over 48 h and results showed that their expression increased after treatments. Already few reports suggest that cyclotides are induced by abiotic factors. In *Oldenlandia affinis*, a cyclotide kalata B5 is only detected during winter (Plan et al. 2010). Also, cyclotide peptide profile between two plants of the same species of *Viola hederacea* is different at diverse locations (Trabi and Craik 2004). Furthermore, the levels of some cyclotides are significantly different in Swedish violets during the warm month of July relative to April and September (Trabi and Craik 2004). In addition, six cyclotides from *Viola baoshanensis* show cadmium-dependent up-regulation (Zhang et al. 2009). These authors have speculated that cyclotide proteins in *V. baoshanensis* possibly play a role as a reactive oxygen species scavenger. Therefore, the up-regulation of these cyclotide-like genes in abiotic stress may be related to a similar function.

Taking together, cyclotide like genes in this study showed various response to a range of biotic and abiotic stresses in maize. Therefore, it is possible that they spatially and developmentally regulated and their expression is triggered by different stimuli. Further analysis and experimentation would need to be performed to determine the detail function of these genes in each stress and whether or not they provide enhanced plant defense.

Author contribution statement BB conceived and designed the experiments; HS performed the experiments; HS, and MM analyzed the data; and BB and HS wrote the paper.

Acknowledgements This project was supported by the University of Kurdistan, Faculty of Agriculture.

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