C. R. Biologies 337 (2014) 302–310

Contents lists available at ScienceDirect

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Molecular biology and genetics/Biologie et génétique moléculaires

Isolation and functional characterization of DNA damage repair protein (DRT) from *Lepidium latifolium* L.

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ARTICLE INFO

Article history:
Received 23 January 2014
Accepted after revision 15 March 2014
Available online 26 April 2014

Keywords:
Cold stress proteins
Arabidopsis
Lepidium
Plastocyanin
Real-time polymerase chain reaction

ABSTRACT

We have isolated and *in silico* characterized a cold regulated plastocyanin encoding gene from *Lepidium latifolium* L designated as *LlaDRT*. Its cDNA sequence (JN214346) consists of a 504 bp ORF, 48 and 205 bp of 5′ and 3′ UTR regions, respectively encoding a protein of 17.07 KDa and pI 4.95. *In silico* and phylogenetic analysis of *LlaDRT* suggested that the protein has features of a typical plastocyanin family member and of a nearest relative of the predominant isoform of *Arabidopsis* (PETE2) plastocyanin. Validation of stress response of *LlaDRT* by qPCR under different abiotic stress regulators viz salicylic acid, jasmonic acid, calcium chloride, ethylene and abscisic acid revealed its possible regulation and crosstalk amongst different pathways.

1. Introduction

Studies involving abiotic stress tolerance in plants have identified that the expression of many genes vary in response to various abiotic stresses *viz.* temperature stress (both low and high), drought, salinity, oxidative stress, etc. [1], which resulted in reduced crop productivity [2,3]. Studies carried out for genome wide mRNA abundance have validated that the expression of around 5–30% of the genes are controlled by various abiotic stresses [4,5]. Plants have attained different strategies (mainly adaptive) at morphological, physiochemical, biochemical and cellular levels, which help them survive environmental extremes. Upregulated genes have always been given more importance, and extensive research on members of DREBs, MYB, bZIP proteins, and zinc finger families represents their importance in response to various environmental stresses. However, only a handful of literature [6] is available on genes, which are downregulated and research on all these genes are still lacking behind for elucidating their possible roles in the regulation of plants responses to stress.

Reports on genes encoding chlorophyll *a*/*b*-binding proteins, plastocyanin, and PSI subunit proteins have revealed their downregulation in response to cold stress [7–9]. It has been established that the plants acquire increased tolerance to photo-inhibition [10,11] particularly, owing to the shifting of photosynthetic carbon metabolism [12]. Plastocyanin, a type-I copper-containing a protein is found in cyanobacteria, algae, and plant chloroplasts. It is considered as one of the most abundant proteins in thylakoids [13,14] and plays a role in both linear and cyclic photosynthetic electron transport [15]. Plastocyanin, synthesized in the cytosol, acquires its mature form and Cu^{2+} co-factor [16] in the thylakoid lumen [17]. Light-stimulated responses are causative

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http://dx.doi.org/10.1016/j.crvi.2014.03.006
1631-0691/

agents for the increase of plastocyanin mRNA levels and have been reported to play an important role in *Arabidopsis*, *Hordeum*, and *Nicotiana* [18,19]. *Arabidopsis, Nicotiana, Oryza* and other higher plants have been observed to possess/express two isoforms of plastocyanin [20–23]. PETE2 (DRT112) protein has been preferentially observed to be more abundant than PETE1 and their independent regulation for performing different roles in copper homeostasis as well as electron transport is known [24,25]. Studies on double mutant generated for *Arabidopsis* plastocyanin genes revealed their failure for growth due to an inhibition in light-driven electron transport [26]. Overexpression of the *Spinacia* plastocyanin promoter in *Nicotiana* has revealed a differential regulation of plastocyanin expression by oxidized and repressed plastoquinone pool [27].

Lepdium latifolium L. ecotype Ladakh shows remarkable ability to withstand harsh environmental stress conditions [28] at different developmental stages, making it an excellent candidate for the exploration of genes related to abiotic stresses. Hence, *Lepidium* can serve as a source of genes and regulatory elements for abiotic stress tolerance through translational genomics approach and may also further aid in providing a deeper insight into the interaction of genes and networks, clarifying the interaction of genes and regulatory networks under adverse conditions [29]. Here, we describe the isolation, full-length cloning and expression pattern of DNA damage repair/toleration protein from *Lepidium latifolium* L. in response to various abiotic stresses.

2. Materials and methods

2.1. Plant material

Seeds of *L. latifolium* L. (Ladakh ecotype) were procured from Leh, India (11,500 ft asl). The collected seeds were germinated and seedlings were grown at $25 \pm 2\,^{\circ}C$, 150 $\mu mol\, m^{-2}\, s^{-1}$ light intensity, 75% relative humidity and a 16:8 h photoperiod [30].

2.2. Rapid amplification of cDNA ends (RACE) and intron mapping

Total RNA was isolated from *L. latifolium* plants (three-week seedlings) by RNeasy mini kit (Qiagen, Germany) using the manufacturer's protocol from unstressed (control) and stressed leaves (4 °C for 24 h) of *L. latifolium*, quantified by spectrophotometry (Biorad, USA) and stored at −80 °C until further use. Based on EST sequence (FG618360), gene specific primers using Primer3 [31] were designed for RACE, which was carried out using GeneRacer™ kit (Invitrogen, USA) following the manufacturer's protocol. The amplified fragments were cloned in pGEM-T Easy vector (Promega, USA) and sequenced by M13 universal primers. The sequenced 5′ and 3′ RACE fragments were aligned together for removal of overlapping sequences and generation of full-length *LlaDRT* from *Lepidium*. Subsequently, extreme forward and reverse primers were designed from the ends of the full-length *DRT* gene sequence. All the primers designed for the study of *LlaDRT* are listed in Table 1.

2.3. Multiple alignments and bioinformatics analysis

The full-length *LlaDRT* (JN214346) cDNA and the deduced amino acid sequence were compared against a non-redundant database of NCBI. Multiple alignments of the retrieved full-length amino acid sequences from the database were generated using ClustalW [32] and a bootstrapped phylogenetic tree [33] was obtained following the neighbour-joining method [34] using MEGA5 software [35]. The conserved motifs in sequences encoded by DRT cDNAs were detected by analysing protein sequences by MEME and BLOCKS software [36]. Conserved domains in DRT encoding sequences were analysed using

Table 1
Description of the oligonucleotides used in this study.

S. No	Name	Sequence (5′–3′)	Length (mer)
1	Primers for 3′ RACE		
	LlaDRT F1	CGAGGTTGCCTTGACTGAGC	20
	LlaDRT F2	GCCACATCAGGGTGCTGGTA	19
	GeneRacer™ 3′ outer	GCTGTCAACGATACGCTACGT AACG	25
	GeneRacer™ 3′ nested	CGCTACGTAACGGCATGACAG TG	23
2	Primers for 5′ RACE		
	LlaDRT R1	GCCATACAACAATCACGGTCCA	22
	LlaDRT R2	CCTCTAATTGAGGCCGAGAC	20
	LlaDRT R3	TACCAGCACCCTGATGTGGC	20
	GeneRacer™ 5′ outer	CGACTGGAGCACGAG GACACT GA	23
	GeneRacer™ 5′ nested	GGACACTGACATGGA CTGAAG GAGTA	26
	GeneRacer™ RNA oligo sequence	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA	44
	Oligo dT primer	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA	60
3	Primers for full-length cloning and intron mapping		
	LlaDRT F	ATGGCCTCAGTAACCTCAGCC	21
	LlaDRT R	TTAGTTAACGGTGAGTTTACCG	22
4	Primers for qPCR		
	26SrRNA F	CACAATGATAGGAAGAGCCGAC	22
	26SrRNA R	CAAGGGAACGGGCTTGGCAGAAT	23
	LlaDRT F1	CGAGGTTGCCTTGACTGAGC	20
	LlaDRT R1	GCCATACAACAATCACGGTCCA	22

the online utilities PROSITE (http://web.expasy.org/docs/swiss-prot_guideline.html) and SMART (http://smart.embl-heidelberg.de/).

2.4. Comparative quantification of transcript accumulation by qPCR

To investigate the expression patterns of *LlaDRT* in different concentrations of abiotic stress treatments (salicylic acid, absciscic acid, jasmonic acid, calcium chloride, and ethylene) at a given point of time, total RNA was isolated from *Lepidium* plants by RNeasy mini kit (Qiagen, Germany), following the manufacturer's instructions. The treatments of *Lepidium* seedlings (three weeks) were carried out following the procedure in [37]. cDNA was synthesized from purified (RNase free DNase treatment for genomic DNA contamination removal) RNA and quantified using a spectrophotometer. Equal quantities of synthesized cDNA were subjected to quantitative PCR (Mx3005P Stratagene/Agilent, USA) with SYBR green mastermix (Qiagen, Germany) and gene specific primers for *LlaDRT* in the reaction set-up. A housekeeping gene (26S rRNA) was used as the reference gene for the normalization/calibration reaction, which was amplified separately. The PCR conditions followed for the reaction set-up were initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The qPCR reactions were performed thrice to assess the reproducibility. C_t values obtained were analysed using statistical program Cropstat version 7.2 (IRRI, Philippines).

3. Results

3.1. Rapid amplification of cDNA ends (RACE)

The first round of 5′ RACE was carried out using the designed primers, which yielded an amplified product of 683 bp using Generacer™ 5′ outer primer and *LlaDRT* R1. Subsequent nested PCR with diluted template (1:50) of the above reaction with Generacer™ 5′ nested primer and *LlaDRT*R2 followed by Generacer™ 5′ nested primer and *LlaDRT*R3 resulted into the amplification of 597 bp and 526 bp, respectively (Fig. 1). In a similar fashion, 3′ RACE was carried out, first with Generacer™ 3′ outer primer and *LlaDRT* F1, followed by nested amplification using the primer set Generacer™ 3′ nested primer and *LlaDRT* F2 yielded amplified products of 296 bp and 251 bp, respectively (Fig. 2). The resulting clones obtained from RACE were cloned, sequenced and aligned to obtain full-length *LlaDRT* (JN214346) and reconfirmed by PCR amplification using *LlaDRT* F and *LlaDRT* R primers designed after the ORF prediction.

3.2. Intron mapping of LlaDRT in L. latifolium L. Genome

cDNA and genomic DNA were amplified using *LlaDRT*F and *LlaDRT*R primers to find out the presence of introns, which revealed a similar product size of 504 bp (Fig. 3), which was confirmed by sequencing. This proves that *LlaDRT* from *L. latifolium* L. genome is devoid of introns.

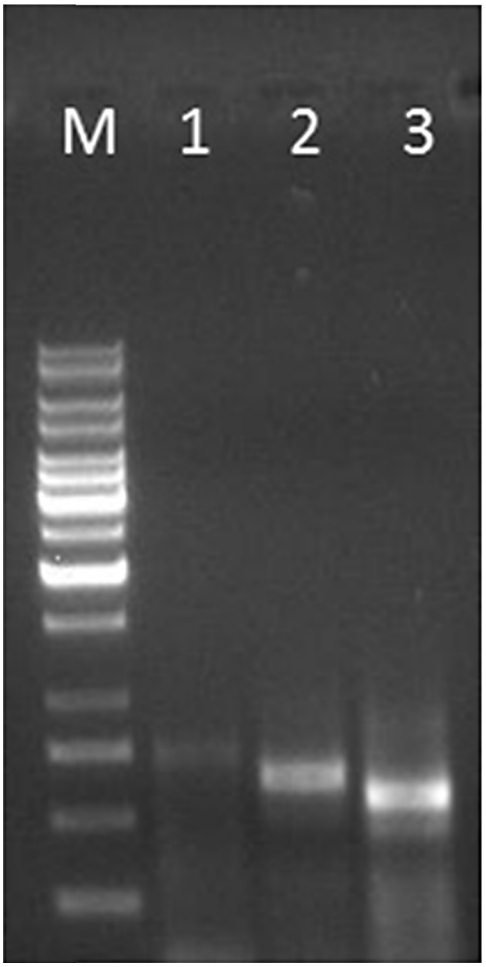

Fig. 1. The amplicons obtained after 5′ RACE in *Lepidium latifolium* L. (M – 1 Kb DNA ladder (Fermentas, Lithuania); L1 – product amplified (683 bp) using *LlaDRT* R1 and GeneRacer™ 5′ outer; L2 – product amplified (597 bp) using *LlaDRT* R2 and GeneRacer™ 3′ nested primer; L3 – product amplified (526 bp) using *LlaDRT* R2 and GeneRacer™ 3′ nested primer).

3.3. Bioinformatics predictions for isolated LlaDRT

Retrieval of amino acids sequence using BLAST analyses and subsequent alignment by ClustalW revealed significant homology of *LlaDRT* with *Arabidopsis* (86%) and over 60% with most of the other similar protein sequences from dicots and monocots (Table 2). The bootstrapped phylogenetic tree constructed confirmed the closeness of *LlaDRT* with *Arabidopsis*, which was likely, since both are members of the Brassicacae family (Fig. 4). The multilevel consensus sequences in related sequences of *LlaDRT* were detected by MEME and BLOCKS (Table 3, Fig. 5), which indicates a 130-aa long conserved motif stretch with a match of *P*-value < 0.0001 and *e*-value < 10, suggesting a high conservation of the plastocyanin gene amongst plant species. Domain analysis elucidated a conserved copper-containing domain (TIGR02656) from 70–149 (cd length of 99) with an *e*-value of 5.48e–45. The members of this family contain blue copper protein associated/related closely with pseudoazurin, halocyanin, amicyanin, etc., located in the thylakoid lumen [26]

and are considered as performing electron transport to photosystem I in cyanobacteria and chloroplasts. Another multidomain PETE (COG3794) was also detected from 73–167 (cd length of 128), having an e-value of 4.56e-16, which signifies the putative role that *LlaDRT* may play regarding energy production and conversion. The domain and other signatures sequences are probably retained by *L. latifolium* L. during the course of evolution, surpassing other selective constraints and thus, enabling plants to retain the properties associated with it. However, during the evolutionary processes, divergence from other similar sequences has also been observed by phylogenetic analysis, and it is possible that despite several similarities and conserved nature, it may have additional roles to play.

3.4. *Quantitative expression analysis of* LlaDRT *in response to various abiotic stresses*

Temporal gene expression studies of *LlaDRT* in response to various abiotic stresses were carried out by qPCR as described in the Materials and methods section. Documented studies on the induction of salicylic acid by abiotic stress in mutants and transgenic plants are available [38], and the expression level of the transcript when analysed by qPCR revealed the downregulation of *LlaDRT* by 0.67-fold and 0.73-fold when exposed to 10 μM and 50 μM salicylic acid stress, respectively compared to the control treatment (Fig. 6a), which may contribute to severe damage in abiotic stress. Considering that salicylic acid does not operate alone but moves in tandem with other phytohormones, like jasmonic acid and ethylene [39,40], we analysed the expression of *LlaDRT* under jasmonic acid and ethylene treatments. Jasmonic acid-related genes are reported to regulate defence mechanisms during pathogenesis and wounding [41]. Reports are available for jasmonic acid application for reduction of injury caused by cold stress in fruits [42]. Subsiding the salicylic acid operation with jasmonic acid, we also thought that plants might be responding to low temperature stress in a similar way to that they respond to pathogenic response/wounding stresses. qRT–PCR analysis of *Lepidium* seedlings submitted to 10 μM and 50 μM jasmonic acid foliar sprays showed significant transcript decreases (0.43-fold and 0.20-fold, respectively) compared to that of the control treatment set (Fig. 6b), reflecting its possible role in the freeze tolerance of plants, which may be further examined either by reverse genetics or metabolomics. Transcript accumulation was observed as 0.88-fold and 1.03-fold

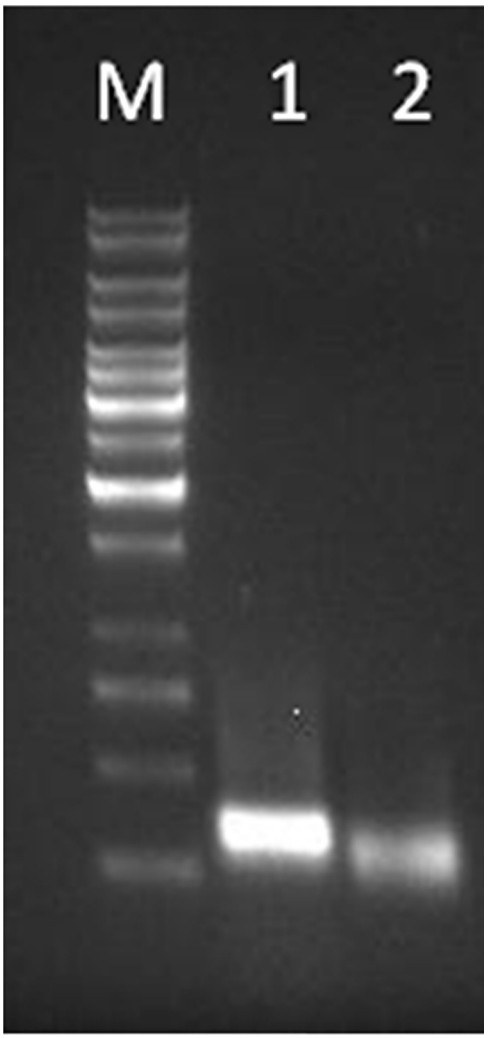

Fig. 2. The amplicons obtained after 3′ RACE in *Lepidium latifolium* L. (M – 1 Kb DNA ladder (Fermentas, Lithuania); L1 – product amplified (296 bp) using *LlaDRT* F1 GeneRacer™ 3′ outer; L2 – product amplified (251 bp) using *LlaDRT* F2 and GeneRacer™ 3′ nested primer).

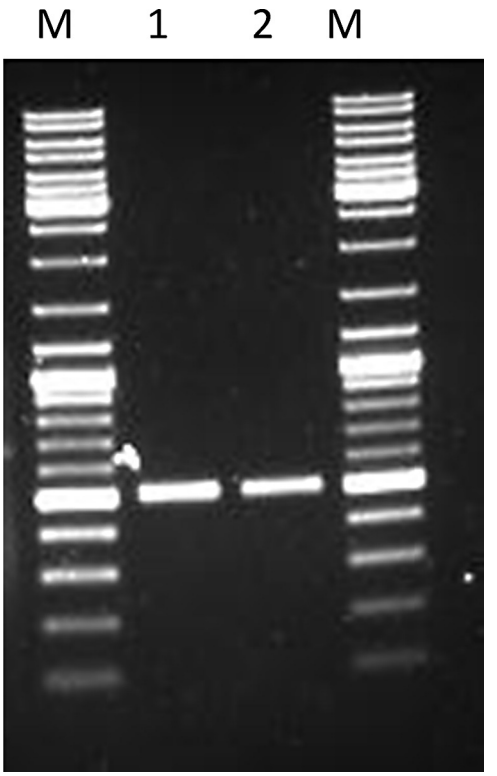

Fig. 3. Full-length amplification of *LlaDRT* from *Lepidium latifolium* L. (M – 1 Kb DNA ladder mix (Fermentas, Lithuania); L1 – product amplified (504 bp) using *LlaDRT* F *LlaDRT* R using genomic DNA as a template; L2 – product amplified (504 bp) using *LlaDRT* F and *LlaDRT* R using cDNA as a template).

Table 2
Similarities in protein sequences of *LlaDRT* with reported sequences from other plants.

S. No	Plant	Gene name	Accession no	Length (aa)	% Similarity with *LlaDRT*
1	*Arabidopsis lyrata*	DNA damage repair/toleration protein 112	XP_002893099	167	86
2	*Arabidopsis thaliana*	Plastocyanin major isoform	AEE29963	167	86
3	*Arabidopsis thaliana*	DRT112	AAA32787	167	83
4	*Ricinus communis*	Plastocyanin A, chloroplast precursor	XP_002510603	168	68
5	*Pisum sativum*	Plastocyanin	P16002	168	67
6	*Silene latifolia*	Plastocyanin, chloroplastic	P07030	165	67
7	*Nicotiana benthamiana*	Chloroplast plastocyanin precursor	ACV32157	169	69
8	*Cucumis sativus*	Plastocyanin, chloroplastic like	XP_004136366	166	70
9	*Lotus japonicus*	Unknown	AFK36672	167	67
10	*Populus trichocarpa*	Predicted protein, plastocyanin	XP_002307754	168	62
11	*Medicago trunculata*	Plastocyanin	XP_003603843	167	66
12	*Solanum tuberosum*	Plastocyanin precursor	AFW90573	170	66
13	*Solanum commersonii*	Plastocyanin precursor	CAJ19272	171	62
14	*Plantago major*	Plastocyanin	CAJ34813	160	61
15	*Zea mays*	Plastocyanin	NP_001147504	155	63
16	*Hordeum vulgare*	Plastocyanin precursor	CAA68696	155	53
17	*Oryza sativa*	Plastocyanin precurssor	AAB63590	154	57

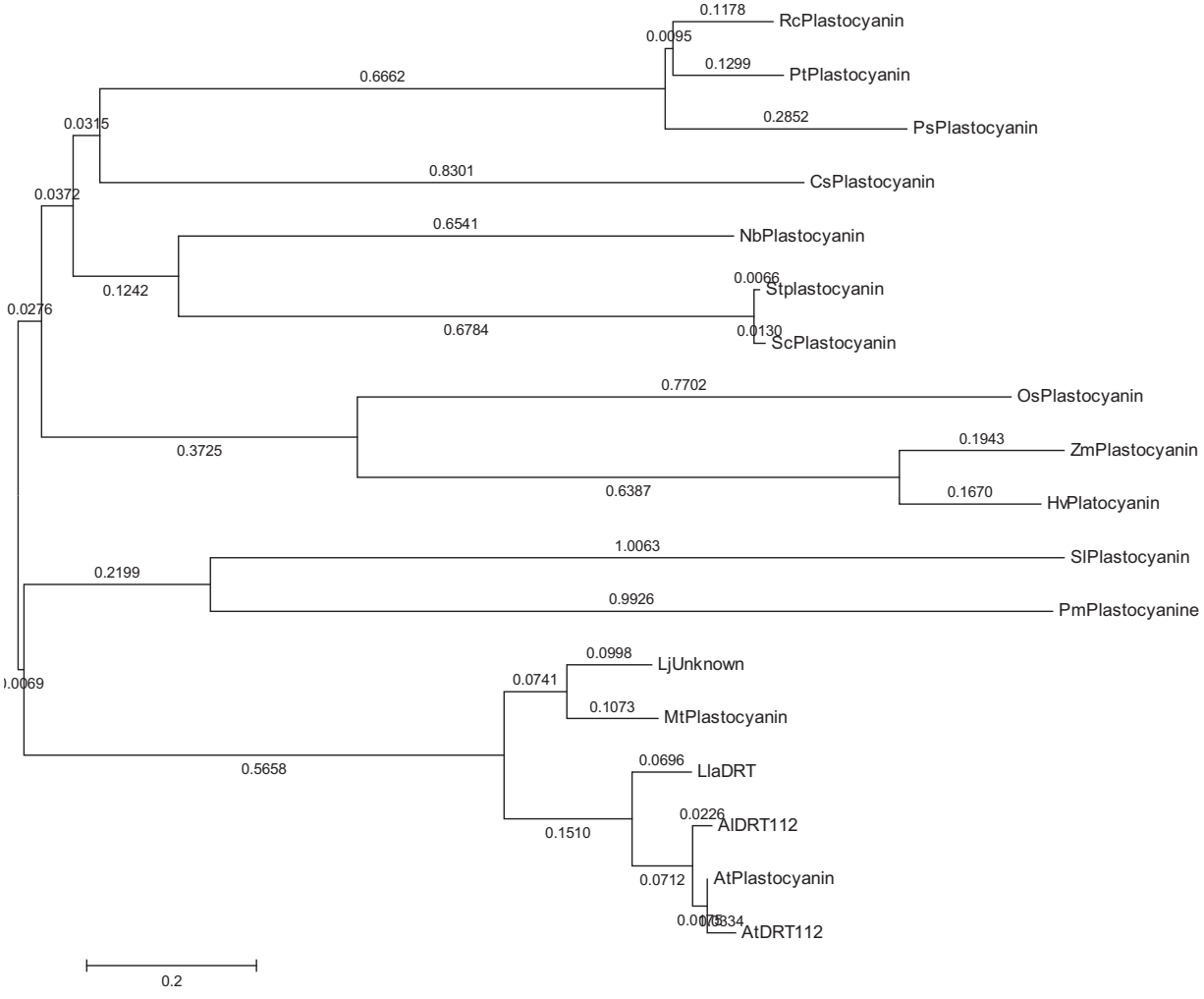

Fig. 4. Phylogenetic relationship of *LlaDRT* in 15 different taxa deduced on the basis of protein sequence similarities.

Table 3
Multilevel consensus sequences for the MEME defined motifs among different *DRT* gene sequences. The motif match has a *P*-value < 0.0001 and the sequences used have an *e*-value < 10.

Motif	Width	Multilevel consensus sequence
1	130	PRLCIKASLKDFGVAVVATAASIMLASNAMAIEVLLGGDDGGLAFIPNDFSIAKGEKITFKNNAGFPHNVVFDEDEIPSGVDVSKISMDEQDLL-NAPGETYEVTLTEKGTYSFYCAPHQGAGMVGKVTVN
2	21	MATVTSAAVAIPSFTGLKAGR
3	64	LLAGGAMAQDVLLGANDGVLVFEPNDFTVKAGETITFKNNAGFPHNVVFDEDECPSGVDWSKIS
4	29	QEEYLNAPGETYSVTLTVPGTYGFYCEPH
5	14	IKSSATVRIQTAAV
6	11	QGAGMVGKVTV
7	11	RRRRWVVRASL
8	14	KVSAIAKIPTSNSQ
9	8	CSSSRVST
10	6	PKWWIR

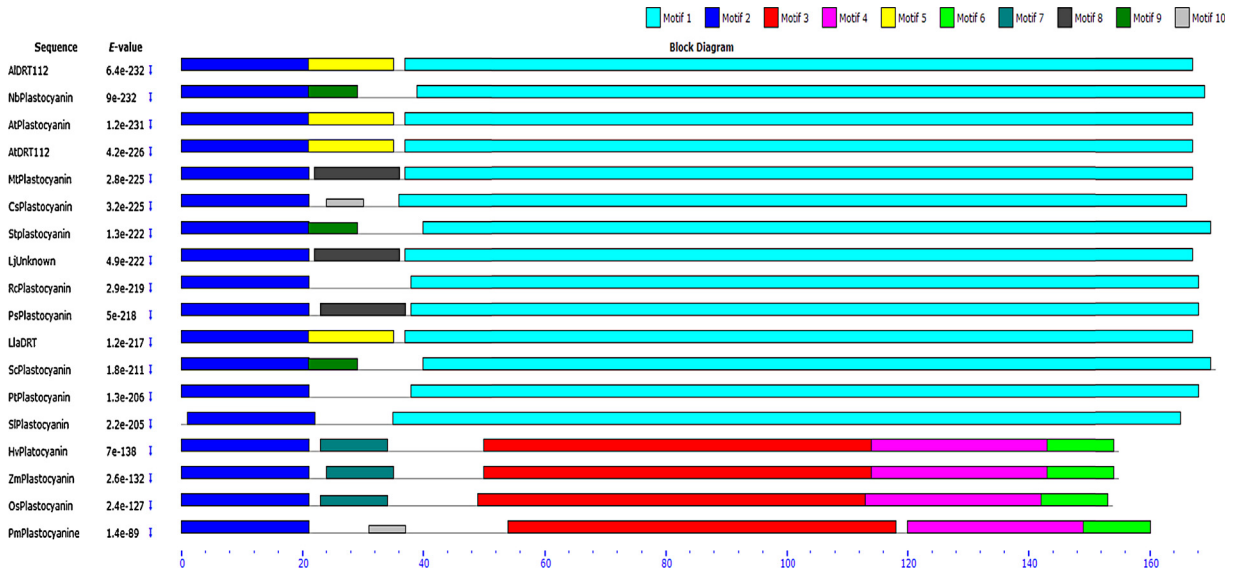

Fig. 5. (Colour online.) Motif identified using MEME and BLOCKS.

change by 10 μM and 50 μM ethylene foliar spray (Fig. 6c), respectively, suggesting that the ethylene responsive genes are not affecting *LlaDRT* and are independent of ethylene responsive pathways for its regulation. ABA is involved in the regulation of diverse plant processes and mediates expression of various stress responsive genes [43,44]. However, studies on *Arabidopsis* have shown that ABA responsive genes are induced by drought, but seem to be independent of ABA for the cold effect [45]. Seedlings exposed to ABA foliar sprays of 10 μM and 50 μM, when analysed with qPCR, showed significant downregulation of the LlaDRT transcript falling to 0.08-fold and 0.11-fold respectively, in comparison to the Mock/control treatment (Fig. 6d) qPCR carried out on *Lepidium* seedlings sprayed with 1 mM and 5 mM of calcium chloride, which showed a significant increase of the transcript with a 5 mM foliar spray. The fold change recorded for 1 mM and 5 mM sprays were 1.17 and 5.57 in comparison with the control (Fig. 6e), suggesting that the secondary messenger plays a role in *LlaDRT* regulation.

4. Discussion

DNA damage repair/toleration protein (DRT112), a copper ion binding/electron carrier, is one of the two reported *Arabidopsis* plastocyanin genes (*PETE*1 and *PETE*2), and is thought to be a predominant form, expressed around ten times stronger than *PETE*1. *PETE*2 (*DRT*) is expected to be post-transcriptionally regulated via copper homeostasis and thought to participate in the electron transfer between P700 and the cytochrome b6f complex in PSI. Physiological, biochemical and molecular changes [10,46] triggered by abiotic responses result from stable and long-term adaptations, explaining complex but necessary developmental responses [46]. Under the influence of low temperatures, inhibition of various metabolic processes, including photosynthetic carbon dioxide assimilation, occurs, which in turn creates imbalance and photo-oxidative damage [47]. Thus, energy imbalance is created; as a consequence of that, plants are forced to downregulate the photosynthetic genes for reduction in the absorbed light energy [48] and its recovery

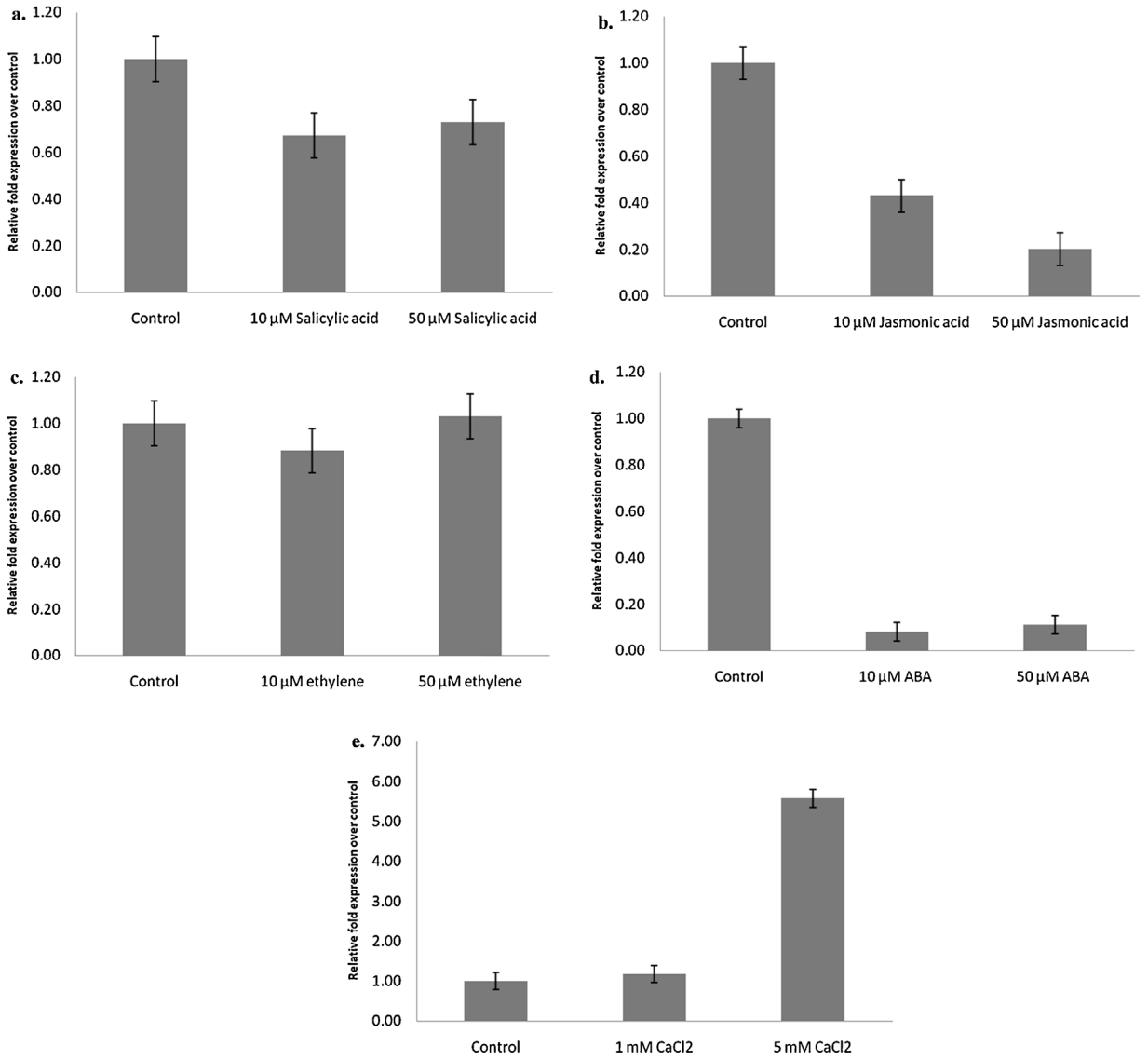

Fig. 6. Relative quantitative expression of *LlaDRT* in response to different abiotic stress regulators, *viz.* salicylic acid, jasmonic acid, ethylene, absciscic acid and calcium chloride. *Lepidium latifolium* seedlings exposed to (a) 10 μM and 50 μM of salicylic acid, (b) 10 μM and 50 μM of jasmonic acid, (c) 10 μM and 50 μM of ethylene, (d) 10 μM and 50 μM of absciscic acid, (e) 1 mM and 5 mM of calcium chloride. The treatment of stress regulators was given for 8 h and 26srRNA was used as an internal control. The error bars in each figure represent SE.

occurs by re-programming of the associated carbon metabolism [12,46]. This study highlights stress specific changes in the transcript level by application of different stress regulators. The increase in level of transcripts may be correlated with carbohydrate-mediated repression of photosynthesis when plants are shifted to a colder environment [48,49]. The fact that the same kind of results was not observed for all the studied abiotic stress treatments reflects that these stresses have different impacts on photosynthesis, both in shorter and longer durations. This indicates that if the studied gene is used for the generation of transgenic or breeding programs, it may provide some interesting regulations that had not yet been unfolded so far. This study also makes a major point in potential values of studying plastocyanin-related genes; the results obtained in the frame of our study may provide useful points for investigating the molecular mechanism of photosynthetic genes regulation during abiotic stress.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgement

Vimlendu Bhushan Sinha is thankful to the Defence Research Development Organization (DRDO), India, for financial support. The authors also thank the Defence

Institute of High altitude Research (DIHAR), Leh, India for their cooperation in collection of the plant material.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2014.03.006.

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