J. Phytol. Res. **26**(1&2) : 59-63, 2013

AMPLIFICATION OF AN ORTHOLOG OF A VACUOLAR ANION TRANSPORTER GENE (AtCLC-c) IN *LEPIDIUM SATIVUM* L.

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The study reports amplification of *Arabidopsis thaliana* vacuolar anion transporter, *AtCLC-c* (chloride channel C) gene ortholog in *Lepidium sativum* through designing and validation of gene specific primers. The designing of specific primer for a gene of an unsequenced organism, *L. sativum* was achieved using a comparative genomic approach utilizing the sequence data of a close relative, in this case, *A. thaliana*. The primers have been designed through an *in silico* approach using Primer3 software tool. Its specificity has been checked further by various bioinformatics tools like Primer Blast and MFE primer, and finally through amplicon sequencing. The designed primers (both forward and reverse) were successfully used to amplify the cDNA derived from mRNA from *L. sativum*.

Keywords: Amplicon; Brassicaceae; Cross species gene amplification; Denaturing gel elecrophoresis; RT-PCR.

Introduction

High salinity, drought and extremes of temperature are few of the most well known abiotic stresses that result in loss of productivity and even mortality in plants. A plant's capability to tolerate these stresses depends on its state of evolution in general, its surrounding environmental conditions and the capability of underlying molecular mechanisms to effectively handle extremes. Understanding the molecular basis of abiotic stress tolerance is critical for designing strategies to produce stress tolerant plants in future. Of all the abiotic stress salinity is the most challenging. Over 800 million hectares of land is affected by salinity of the soil globally[1]. High salinity adversely affects plant growth and development by disrupting ion homeostasis, resulting in membrane dysfunction, attenuation of metabolic activity and secondary effects that inhibit growth and induce cell death[2].

To be able to understand gene functions at high salt levels, the most important requirement is that the plant should be able to survive high levels of salinity. A usual glycophyte like *Arabidopsis thaliana* can only be grown to a certain high salinity levels, beyond which it struggles to survive. Hence, a halophyte has to be the subject of such a study. In the present investigation, the authors, carried out a survey of the Sambar salt lake (Rajasthan, India) peripheral region and selected *Lepidium sativum L.* a moderate halophyte to be used as a candidate for studying gene expression levels of some key genes known to be triggered during a plants response to salinity. *L. sativum* was selected for the investigation as it belongs to the family Brassicaceae, and hence a relative of *Arabidopsis,* making it ideal for comparative genomics approach. Moreover, *L. Sativum* is classified as a moderately salt tolerant plant[3]. In this approach it was envisaged to use the available genome sequence data of *Arabidopsis thaliana,* to design primer for *L. sativum,* and carry out semi-quantitative gene expression study on it.

In the present paper we report amplification of *A. thaliana* vacuolar anion transporter, *AtCLCc* (chloride channel C) gene ortholog in *L. sativum* through designing gene specific primers using *Arabidopsis thaliana* genomic information available at TAIR. *CLC-c* is a chloride channel protein coding gene located on chromosome 5 of *A. thaliana* having a locus tag AT5G49890.

L. sativum mRNA extraction and purification was perfected. cDNA synthesis was carried out followed by amplification of *CLC-c* gene using the custom designed primers through RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). The amplicon was sequenced for confirmation of gene sequence. It was observed that as expected a 477bp bands was obtained upon gel electrophoresis of the PCR product confirming that the designed primer using *Arabidopsis* gene At *CLC-c* did pick up the intended gene in *L. sativum*.

Material and Methods

(i) Primer designing: Nucleotide sequence of *CLC-c* gene

Table 1. PCR programme showing temperature and cycle details.

Step	Temperature	Time	Number of cycle
Initial denaturation	94 °C	3min	1
Denaturation	94 °C	30 sec	35
Annealing	55 °C	30 sec	
Elongation	72 °C	1 min	
Final Elongation	72 °C	5min	1

Table 2. Properties of the selected primer pairs for *A. thaliana.*

Primer	CLC-c Primer Sequences (5' - 3')	RNA sequence position	Size (b)	GC%	Tm	δTm	Product size (bp)	mRNA region	PPC (%) of amplicon
F	AGCCGTACA TGCGCAA TTTGGT	1979 - 2000	22	50	64.6	0.4	488	1979 (22/22)	93.3%
R	AGGCGGTC TCCCTGGTG TTT	2466 to 2447	20	60	64.2			2466 (20/20)	

is available for *A. thaliana.* This sequence was retrieved with its accession number from The *Arabidopsis thaliana* Information Resource (TAIR) and used for *in silico* primer designing. For a successful PCR amplification, primer designing is the most critical step. Several characteristics determine the sequence of primer such as the length of the product, its melting temperature and ultimately the yield. If a designed primer is incompetent it results in little or no product due to nonspecific amplification or it may form primer-dimer which may become competitive enough to interrupt the product formation.

The sequences of the primers used for PCR amplification have a major effect on the specificity and sensitivity of the reaction. When designing primer for *CLC-c* gene, the following guidelines were followed:

1. Ideal primer size was kept between 18 to 24 bases;
2. The last 6-10 bases (towards 3' end of primer) were kept unique;
3. The last 6 bases were kept with 50% GC ratio;
4. Overall GC % was kept between 35% - 55%;
5. Tm range for the primer was kept between 55-65°C;
6. The mean Tm difference (δTm) between the forward and reverse primer was kept within 5°C;
(The less the better. 0 differences was considered ideal ;)
7. The product size was kept between 250-600bp;

The primers were designed for *A. thaliana CLC-c* gene using an *in silico* approach and followed by testing their efficiency and ability to amplify the *CLC-c* gene of cross species *Lepidium sativum* under wet-lab condition. Primer3[4], a java based tool, was employed for designing the primers for *AtCLC-c* gene (http://frodo.wi.mit.edu/cgibin/primer3/primer3.cgi). Further specificity was checked by Primer Blast, (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). It uses BLAST and global alignment algorithm to screen primers against user-selected database in order to avoid primer pairs that can cause non-specific amplifications. MFE Primer was also used for cross checking[5] (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0). The primers were sent for synthesis (Xceleris Genomics, India).

(ii) RNA extraction, purification and quantification: Total RNA of *Lepidium sativum* was isolated from the leaf tissue using TRI reagent (Sigma-Aldrich India), according to the manufacturer's protocol. To eliminate residual genomic DNA, 1 µg of total RNA was treated with RNase free DNase1 (Sigma-Aldrich, India) according to the manufacturer's instructions. The total RNA was quantified using a nanospectrophotometer (DeNovix DS-11, USA).

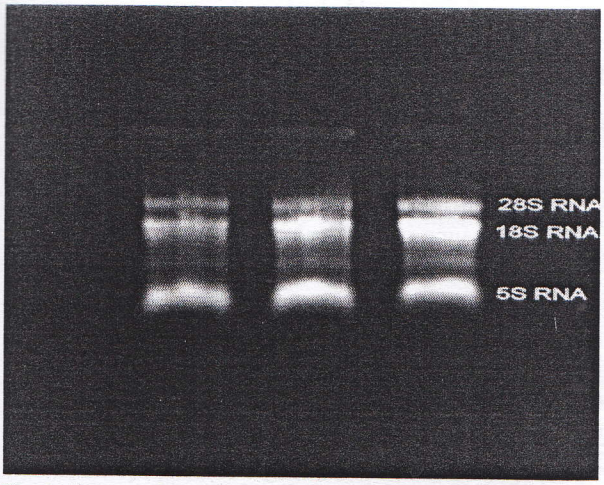

Fig.1. Denaturing RNA gel electrophoresis gel image of extracted RNA from leaf of *Lepidium sativum* showing 3 rRNA bands.

The yield was determined by measuring the absorbance at A260 and A280. The level of RNA purity was determined by the A_{260}/A_{280} absorbance ratio. Only RNA samples having A_{260}/A_{280} ratio range between 1.8-2.0 were selected for further.

(iii) Formaldehyde denaturing agarose gel electrophoresis: The overall quality of RNA is assessed by electrophoresis (Tarsons, India) on a denaturing agarose gel. This will also give some information about RNA yield. A denaturing gel system is used because, RNA can form different secondary structure due to intramolecular base pairing, and this affects its mobility in an electrical field according to its size. Hence denaturing gel electrophoresis was used by loading the RNA sample on 1% (w/v) denatured agarose gel for further check the integrity of RNA. Formaldehyde was used to keep the RNA denatured. After electrophoresis, the RNA was visualized by gel documentation system (AlphaImager EP, Alpha Innotech, USA)

(iv) cDNA Synthesis- cDNA was synthesized from 1µg of total RNA by using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, India) according to the kit instructions.

(v) Polymerase Chain Reaction: The PCR-amplification reactions were performed in a 25µl mixture containing 1 µl of cDNA, 1X PCR buffer (includes 20 mM $MgCl_2$), 0.2mM of each of the dNTPs (10mM each; Fermentas India), 0.5µM of each primer and 1.0 U of Taq polymerase (Dream Taq DNA Polymerase, Fermentas India). PCR was performed in thermal cycler (Quantas Biotech, USA and Aglient Technologies, USA) with a heated lid.

(vi) Gel Electrophoresis: Amplified products visualizing

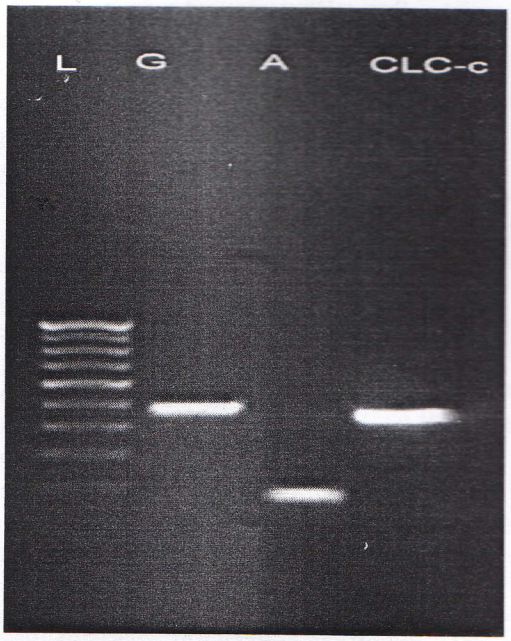

Fig.2. Amplified gene fragments, where L denotes the ladder of 100bp, G for *GAPDH* (496bp), A for *Actin* (196bp) gene and *CLC-c* (477bp) denotes the chloride channel-c gene of *L. sativum.*

the *CLC-c* amplicon were separated on 1.2% agarose gel (Sigma-Aldrich, India) in 1X TAE buffer, pre-stained with ethidium bromide (0.3µg/ml) and electrophoresis was carried out at 100V for 1 hour in 1X TAE buffer. The gels were visualized and photographed using the gel documentation system.

Reproducibility of these selected primers was evaluated using three independent PCR reactions. Negative control PCR reaction mixture contained all required PCR components except template cDNA. *GAPDH* and *Actin* gene primers were used as positive control.

Results and Discussion

This study attempts to design a new set of primers that are specific for *A. thaliana* but can be used for PCR gene amplification of *L. sativum.*

The accession numbers AT5G49890 of the *CLC-c* gene of *A. thaliana* were retrieved from TAIR database. The cDNA sequence of *CLC-c* gene in FASTA format was subjected in Primer3 program for designing of primer. Primer3 program displayed a list of several oligonucleotide sequences capable of amplifying the gene sequence of interest but only specific pairs were selected consisting of a forward and a reverse primer. Table 2 shows the designed selected primers and their properties. Primers were found to target at the position of template RNA shown in Table

2. The product length was 488bp.

Primer Blast was used as an *in silico* confirmation test for primer specificity. The designed primers were specific to *CLC-c* gene of *A. thaliana*. This was validated in cross species of *L. sativum* through wet-lab condition. RNA of *L. sativum* was extracted and its integrity was checked by denaturing formaldehyde gel electrophoresis. Three clear bands of 28S rRNA, 16S rRNA and 5S rRNA was observed (Fig.1).

This RNA was used for cDNA synthesis and RT-PCR using the finalised primer pair. The primers were found to amplify the fragments of *CLC-c* gene of *L. sativum* and generated a band of 488bp. For its final confirmation PCR reaction samples were out-sourced for sequencing (Xceleris Genomic Services India). After analysis of amplicon sequence of *L. sativum* it was found almost similar to *A. thaliana* amplicon sequence. The remaining positive control *GAPDH* gene primer and *Actin* gene primer band were located at an approximation of 496bp and 196bp as expected (Fig 2). For the identification of a gene of interest primers from *A. thaliana* were found to ideal on all parameters of being a specific and accurate to amplify *L. sativum CLC-c* gene successfully.

Salinity is one of the major factors that adversely affects quality and productivity of the crop[6-10]. Here we aimed at amplifying *CLC-c* gene of *L. sativum* using primers designed using *A. thaliana* genome sequence data. In other words, we successfully amplified an ortholog of *A. thaliana CLC-c* gene. Most of the molecular genetics study on salt tolerance has been carried out on *A. thaliana*. However, it is a salt-sensitive species and can only provide limited information about mechanisms of salt tolerance[2,11-16]. Halophytes likely are potential genetic resources both for "superior" alleles of previously identified genes and novel loci that are mechanistically involved in ionic homeostasis and salt tolerance[17]. The study of the salt tolerance mechanisms of halophytic plants has emerged as an important area because these species are well-adapted to and can overcome soil salinity more efficiently than glycophytic plants. Hence, for the understanding of the mechanism of salt tolerance ideally the study should be done on halophytes. Studies world over have been carried out on halophytes such as *Thellungiella halophila*[18] and *Puccinellia tenuiflora*, *Suaeda maritime*[19,20], *Salicornia brachiata*[21], *Mesembryanthemum crystallinum*[22], *Helianthus annuus*[23] *Mimulus guttatus*[24]. Here we selected *L. sativum*, a moderate halophyte which belongs to the family brassicaceae and hence a close relatives of *Arabidopsis* for the gene expression studies. Here we report for the first time the PCR amplification of *CLC-c* gene ortholog in an unsequenced halophyte *L. sativum*.

Specific primers are the key ingredients for selective amplification of a part of the genome using RT-PCR approach. For determining the relative presence or absence of a gene of interest in the genome it can be used as a probe. Primer designing using *in silico* approach is an important area in bioinformatics. It has manifold significance in molecular biology experiments. The *in silico* primer designing approach for the exploring gene expression study has opened up the pathway of discovering new insights. *In silico* analysis of genomic data is of greatest importance now a day. For the fast annotation of genomic data primer designing is a helpful approach. Furthermore, hypothesis put forward by *in silico* analysis can be validated by wet-lab experiments.

Acknowledgement

The authors are grateful to Indian Council of Forest Research and Education (ICFRE) for the financial support.

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