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Optimization of PCR conditions for amplifying an AT-rich amino acid transporter promoter sequence with high number of tandem repeats from *Arabidopsis thaliana*

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Abstract

Objective: The aim of the present study is to optimize the PCR conditions required to amplify the promoter sequence of an amino acid transporter having an AT-rich base composition with a high number of tandem repeats.

Result: Results show that successful amplification can be achieved by performing a 2-step PCR at a lower extension temperature of 65 °C for an increased extension period of 1.5 min/kb, with MgCl₂ concentration ranging from 2.5 to 3.0 mM. The results also suggest that the DNA concentration of about 25–30 ng/μl was essential to achieve this amplification.

Keywords: AT-rich, PCR, Optimization, Tandem repeats, Extension

Introduction

PCR is one of the indispensable techniques in molecular biology for in vitro amplification of a specific segment of DNA [1]. It is highly reliable because of its sensitivity, accuracy, and speed [2, 3]. However, under specific requirements such as to amplify templates, which are AT- or GC-rich, or have a high number of tandem repeats the PCR conditions need to be optimized. Plant promoter regions are generally difficult to amplify by PCR as they are highly AT-rich and sometimes contain tandem repetitive DNA sequences [4, 5]. Tandem repeats represent two or more copies of short segments of DNA occurring repeatedly from head-to-tail within the coding and regulatory regions [6]. The problem with these templates is that they need lower annealing and extension temperatures which can result in the amplification of undesired products [7, 8]. The aim of this work is to amplify a promoter sequence (1781 bp) of an amino acid transporter

(AT2G40420) from *Arabidopsis thaliana*, which is highly AT-rich and has a high number of tandem repeats.

The in silico analysis of the promoter sequence reveals that it possesses many important *cis*-acting regulatory elements such as light-responsive, auxin-responsive, salicylic acid-responsive, and abscisic acid-responsive elements along with 16 copies of an ACGT motif [9]. Studies suggest that the *cis*-elements with ACGT core sequence responds to light, anaerobiosis, phytohormones like abscisic acid, jasmonic acid, salicylic acid and auxin [10, 11]. Furthermore, Zou et al. [12] conducted a study where they concluded that around 19.6% of the total pCREs (putative *cis*-regulatory elements) identified in the promoter regions of abiotic stress responsive genes have ACGT as a core sequence. Therefore, tapping this promoter sequence for its response to abiotic stress conditions can potentially bring forth important characteristics that can further find wide application for generation of transgenic plants with high stress tolerance. As, a suitable promoter is needed to achieve desired expression levels of a transgene [13]. In the study, the promoter sequence (AT2G40420, 1781 bp) was amplified from *Arabidopsis thaliana* genome. However, the sequence is 65.2% AT-rich and has 15.5 copies of 28 base long tandem repeat

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[14], which makes it difficult to amplify by PCR (Fig. 1). These tandem repeat sequences have a binding site for bZIP (basic leucine zipper) transcription factors (TFs). Reports suggest that tandem repeats possessing binding sites for transcription factors in the promoter regions can affect the transcriptional rate of a gene [15]. To check the effect of all these TF binding sites localized in tandem repeats on the downstream gene expression, isolation of the promoter sequence with all the copies of tandem repeats was highly desirable.

Main text

Methods

Plant material and growth conditions

Arabidopsis thaliana, ecotype Columbia (Col-0) was used in this study. *Arabidopsis* seeds were procured from LEHLE SEEDS Company (Catalog number: WT-02), Texas, USA. Seeds were vernalized at 4 °C in the dark for 3 days before sowing in 5.08 cm plastic pots filled with soilrite. Thereafter, the pots were transferred into a growth chamber (Daihan Labtech, LGC-5101, India) maintaining a 16-h light/8-h dark photoperiod, 22 °C temperature, 75% relative humidity. After 3 weeks, rosette leaves were harvested to extract the genomic DNA.

Genomic DNA isolation

Genomic DNA was isolated from the leaves of *Arabidopsis thaliana* (ecotype Col-0) using the DNeasy Plant Mini Kit (Qiagen, Cat No./ID: 69104) according to the manufacturer’s recommendations. The DNA integrity was confirmed by running 0.8% agarose gel electrophoresis at 80 V for 30 min.

Primer design

Primers were designed to amplify a 1781 bp promoter sequence of the amino acid transporter (AT2G40420)

(Table 1) using the Primer3 program [16]. Their specificity was ensured by performing primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) with the *Arabidopsis* genome. Further, the OligoAnalyzer tool supported by Integrated DNA Technologies (<https://www.idtdna.com/SciTools/SciTools.aspx>) was used to check for the presence of any secondary structure or primer–dimer formation.

PCR conditions

Each 20 µl PCR contained 2 µl of genomic DNA (~ 50 ng), 4 µl of 5X Phusion HF buffer, 0.4 µl of 10 mM dNTPs, 0.8 µl of each 10 µM forward and reverse primer, 0.2 µl of Phusion DNA polymerase (2U/µl), and varying concentrations of MgCl₂ ranging from 1.5 to 3.5 mM. All the reagents were procured from Thermo Fisher SCIENTIFIC (Catalog number: F530S, Waltham, MA, USA) and MB grade nuclease-free water from Himedia (Catalog number: ML024). A 2-step PCR was carried out using the Applied Biosystems® Veriti® 96-Well Thermal Cycler (Catalog number: 4375786, Foster City, CA, USA) with conditions as follows: Initial denaturation at 98 °C for 1.5 min; followed by 35 cycles of denaturation at 98 °C for 30 s, extension at 60/65/68/72 °C for 3 min and final extension at 60/65/68/72 °C for 7 min. PCR for each extension temperature with varying MgCl₂ concentrations were performed separately and in triplicates. PCR products were checked by electrophoresis in 1% (w/v) agarose gel, at 80 V for 30 min.

Table 1 Forward and reverse primer specifications for AT2G40420 promoter sequence

Primer	Primer sequence (5'→3')	Tm °C	GC%	Product size
AT2G40420F	CCTACTAGTTCGTGATACTG	52.05	45.00	1781 bp
AT2G40420R	CGAACGATTCCTTCATCACC	57.02	50.00	

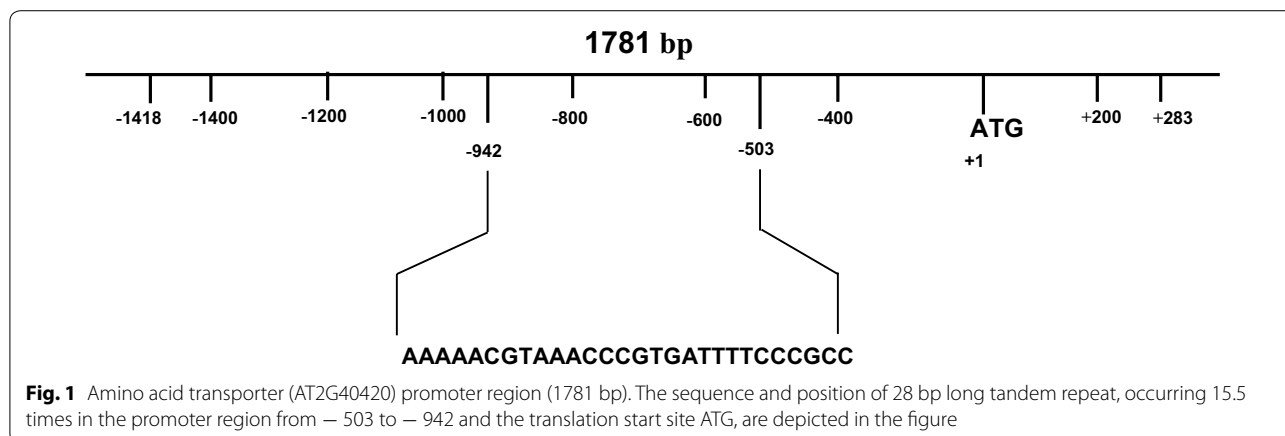


Fig. 1 Amino acid transporter (AT2G40420) promoter region (1781 bp). The sequence and position of 28 bp long tandem repeat, occurring 15.5 times in the promoter region from – 503 to – 942 and the translation start site ATG, are depicted in the figure

Amplicon sequence analyses

The QIAquick Gel Extraction Kit (Qiagen, Catalog number: 28704) was used to purify the PCR products. The purified PCR product along with the primers used for amplification, was then directed for sequencing to verify the specificity of the amplified product. The amplicon specificity was confirmed by analysing the obtained sequencing results with the reference sequence deposited in the TAIR database (<https://www.arabidopsis.org>) of the amino acid transporter promoter region [17].

Results and discussion

Concentration of magnesium ions

The magnesium ion concentration greatly influences the PCR as DNA polymerase requires Mg^{2+} ions for its proper functioning [18, 19]. Therefore, to achieve maximal PCR yield the $MgCl_2$ concentration needs to be optimized. As, a high Mg^{2+} ion concentration can hinder the reaction by preventing proper melting of template DNA and can also promote non-specific binding of primers. Even a low Mg^{2+} ion concentration can adversely affect the product yield. With this aim, varying concentrations of $MgCl_2$ such as 1.5, 2.0, 2.5, 3.0, 3.5 mM were tried. The desired amplicon yield was obtained at a 3.0 mM $MgCl_2$ concentration (Fig. 2).

Extension temperature

For successful amplification, the extension time and temperature need to be carefully optimized. Xin-Zhuan Su et al. [20] reported that to amplify an AT-rich DNA, reduced extension temperatures are needed. In the present study, a two-step PCR (denaturation and amplification) was performed at four different extension temperatures 60, 65, 68 and 72 °C with increased extension time from the usual 1 to 1.5 min/kb. Successful amplification was achieved at an extension temperature of 65 °C with 2.5 mM $MgCl_2$ yielding a faint band while an intense band was observed with 3 mM $MgCl_2$ concentration (Fig. 2). No results were obtained at other extension temperatures (60, 68, and 72 °C) at any of the five $MgCl_2$ concentrations tested (data not shown).

Conclusions

The promoter region of the amino acid transporter was difficult to amplify by PCR owing to its high AT content and a high number of tandem repeats. Successful amplification can be achieved, after optimization of $MgCl_2$ concentration and extension temperature with the DNA template of desired concentration.

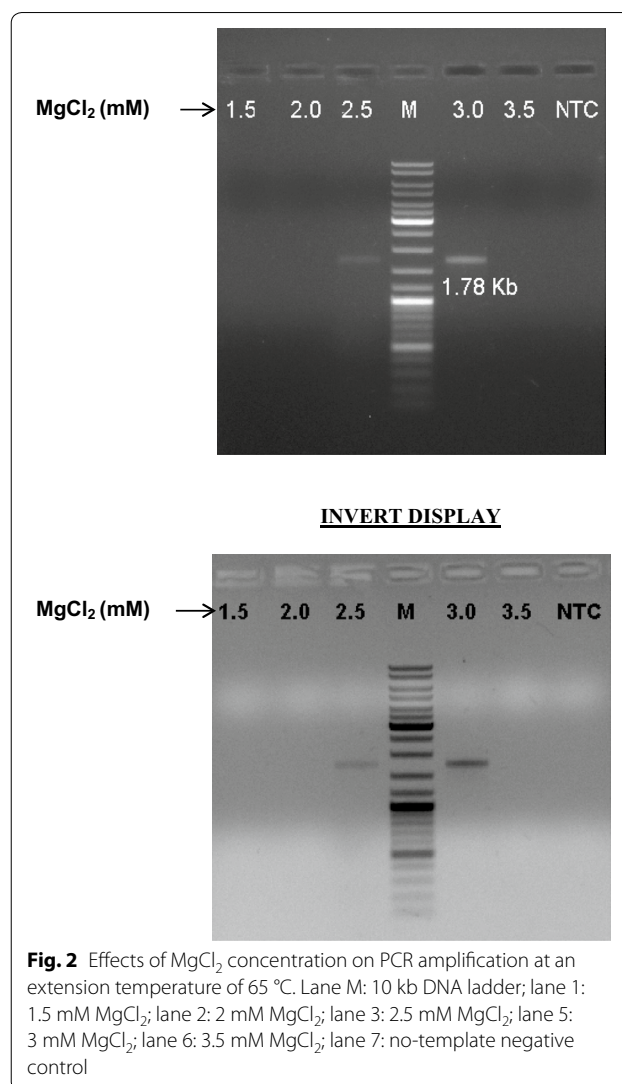


Fig. 2 Effects of $MgCl_2$ concentration on PCR amplification at an extension temperature of 65 °C. Lane M: 10 kb DNA ladder; lane 1: 1.5 mM $MgCl_2$; lane 2: 2 mM $MgCl_2$; lane 3: 2.5 mM $MgCl_2$; lane 5: 3 mM $MgCl_2$; lane 6: 3.5 mM $MgCl_2$; lane 7: no-template negative control

Limitations

- The DNA template should be pure, homogeneous and concentration should be around 50–60 ng for setting up a 20 μ l PCR reaction.
- Efficient for AT-rich DNA templates.

Abbreviations

dNTP: deoxyribonucleotide triphosphate; PCR: polymerase chain reaction; TRs: tandem repeats; TFs: transcription factors; pCREs: putative *cis*-regulatory elements.

Author's contributions

PD designed, performed the experiments and wrote the manuscript. RM and SM gave critical inputs on designing of experiments, supervised the study and corrected the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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