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Cloning and analysis of *ZmLTP3* gene promoter in maize

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Abstract

Plant lipid transfer proteins (LTPs) play important roles in many processes in planta; however, its roles in abiotic stresses and the regulation mechanisms were seldom reported. In our previous study, the Arabidopsis overexpressing maize ZmLTP3 enhanced salt tolerance. In the present study, the 1302 bp sequence upstream of the ZmLTP3 were cloned. Analysis via Plantcare showed that the CAAT-box, TATA-box and many elements related to biotic and abiotic stresses were present in this region. The results laid a foundation for further investigation to determine the function and upstream regulation mechanisms of *ZmLTP3*.

Keywords: Maize; Lipid transfer protein; Promoter; Cloning; Functional analysis

Introduction

Plant LTPs are a class of small molecular proteins with the ability to bind and transport lipids. Because of their high affinities to several different lipids, such as coenzyme A, phospholipids and fatty acids, LTPs are often named non-specific lipid transfer proteins (nsLTPs) (Kader 1996; Edstam *et al*., 2011; Smith *et al*., 2013). They are a class of basic proteins, each with eight highly conserved cysteine residues. These eight cysteine residues form four disulphide bonds, which endows the LTPs with high resistance to high temperature and denaturation (Edstam *et al*., 2014). Based on the molecular masses, LTPs have traditionally been classified into two families, I and II type (Yeats *et al*., 2008). Recently, based on the distance of the cysteins, position of conserved intron and the occurrence of GPI-anchor, LTPs are classified into A, B, C, D, E, F, G, H, J and K type. Plant LTPs were firstly thought to participated in the synthesis of biomembrane system because of their ability of lipid transport between membranes (Kader *et al*., 1984). Subsequent finding of N-terminal hydrophobic and extracellular location indicates their newer functions. Recent researches show that LTPs may play important roles in cutin synthesis and transport, development of reproductive organs, tolerance to stresses, and promotion of the elongation of cell wall (Orellana *et al*., 2010; Yang *et al*., 2011; Fan *et al*., 2013). Among them, the roles in cutin synthesis and transport, reproductive development and stresses tolerance were more reported.

In previous study, we have cloned a member of *LTPs* family from maize named *ZmLTP3* (GenBank accession no. JX435819.1). Overexpression of *ZmLTP3* in Arabidopsis significantly enhanced salt tolerance (Zou *et al*., 2013). In NCBI database, the promoter sequences upstream of *ZmLTP3* gene were not entirely sequenced, this makes it difficult to study the regulatory mechanisms of *ZmLTP3* gene (sentence not self-explanatory). In this study, the full promoter sequence of *ZmLTP3* gene was cloned sequenced and bioinformatics analysis was conducted. This research makes it possible for the following deep research of the roles and upstream regulation mechanisms of *ZmLTP3*.

Materials and Methods

In NCBI database (https://www.ncbi.nlm.nih.gov/), the sequence upstream *ZmLTP3* gene was not sequenced thoroughly. There is a gap in the candidate promoter sequence. CTAB method was used to extract genome DNA from

maize inbred line (B73) leaves (Zou *et al*., 2006). The upstream sequence of *ZmLTP3* gene of 2881 bp (excluding gap) was downloaded from NCBI. Primers (5'-ggcgactgtgacactatcc-3', sense primer and 5'-aagtagggctatcgaaacagg-3', antisense primer) were designed outside the gap, to amplify complete promoter sequence. The PCR product was sequenced (Direct sequence or cloned and sequenced) and blasted with the already reported sequence at NCBI. The sequenced candidate promoter sequence was analyzed using Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Results and discussion

Cloning of the *ZmLTP3 gene* promoter

In NCBI database, there is a gap at -1270 bp upstream the initiation codon ATG of *ZmLTP3* gene, furthermore, there are several repeats and inverted repeats (Figure 1). To amplify complete sequence, the primers were designed far away from the gap. The sequencing result showed that there are only 1302 bps from the sense primer to ATG, but not 2492 bps (excluding gap) in NCBI database. Blast results showed that the deduced nucleotide sequence had the identity of 99% with the sequence in NCBI database, and there are no repeats and inverted repeats (Figure 2).

Analyses of the candidate promoter sequence of *ZmLTP3* gene

Many cis-acting elements were found via analyses using Plantcare including 14 TATA-boxes, and the nearest one from ATG is at the position of -149 bp, the furthest one from ATG is at the position of -1196 bp. 7 CAAT-boxes were also found in the sequence. Besides the necessary elements for promoter (TATA-box and CAAT-box), this sequence contained other important binding sites and functional elements such as the elements responsive to plant defense and stresses (TC-rich repeats), ABA (ABRE), drought (MBS), SA (TCA-element) and light (Table 1). These functional elements showed that *ZmLTP3* gene may response to various biotic and abiotic stresses, and play important roles.

Conclusion

To our knowledge, more researchers focus on the biological roles, few on the mechanisms of *LTPs* genes, especially on their upstream molecular regulatory mechanisms. Although the first cDNA encoding plant *LTP* cloned from maize, the subsequent researches about the gene family and their roles in maize were seldom. In this study, the analyses about *ZmLTP3* gene promoter sequence laid a foundation for further researches about the upstream molecular regulatory mechanisms of *ZmLTP3.* The completion of maize genome sequencing provides more convenience for maize molecular biology researches. But some segments are still not entirely sequenced because of the complexity of the maize genome structure; this brings difficulties for some related studies. For example, the promoter sequence of *ZmLTP3* gene in this study is not entirely sequenced. Based on the original sequence in NCBI, we successfully cloned the complete promoter sequence, filling up the gap in NCBI. Blast results showed that the sequenced product had 99% similarity with the related sequences at NCBI. This study provides more convenience for the subsequent work.

Analyses using Plantcare showed that besides the classic elements for promoter, this sequence contains many functional elements responsive to various abiotic and biotic stresses, indicating its important roles in abiotic and biotic stresses. This result tallied with our previous report. However, its precise role remains to be further studied.

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CATCCAGGATGTCCTCTCCATCACCACCTGTAAAAAAGTAACATAAAACACATGGTGAAACCCTATATCC
AAAAATGTGGCTAAAATGTGAAGGACGCGTTGGAGTGCGACATCTGCTAAAAAGTCAGATTACCATGCAT
GTATATATACAGTACATATTGATCGGTAGACTCCAGCATGATGTTCTGCCCTGTGTAAAATCAGTGCAAG
AACGGGCATTCTGGATGTGCAAAGTGCTGCATCCGCACGGACGGTAAGTGCTGCACCTGCTCCGAGCGCA
TTGGCAACAATAGCTCTCTCTCCCTCTCCCTCTCCTCTCAGGGTTGGAGGATGAGGAAGAGGCGACTGTGA
CACTATCCATGGAGAAGGACGCGTTGGAGTGCGACATCTGCTGCCTACCCTTCCAGTCCGAAGTTTTCATG
GCAAGTCAGATTACCATGCATGTATATATACAGTAAATATTGATCGGTATATATACAAATGTAGTTAGAC
TACTTTATCTCTTCCAATAAAAGTAGTTAAACTACTTTATCTCTAACACATGGTGAAACCCTATATCCAAA
ATGTGGCTAAAATGGAGGAAAAATAAACAAAATTTGGCAAAAGAAACATAAGCCAAACTTTCCTAGCAA
CTAATAAACAAAAAAAAATTAATACCCAAACCTATCTTTAACATATGGTGAAAGCCTAGTTTCAAAATGT
AGTTAAAATTGAGCAAAAATAAACAATAACTAACAATTGAAACATAATTAAACTAATCTTCCATACAACA
CATTCACCATTCATTAAGCAACTAAACATGAAAAAGAGAGGGAATAGACAAAAACTAACCATCTTATGC
AACATCATTTGAGAAAATAGAGAAAATTACCTATCTATACTCTTCTCTCTCACATGTGAAACCCTAGATCC
AAAATGTGGCTAAAATTGAGCAAGAATGAACAAAAATTGACAAAGAAACATAAACCAACCTTTCTTATC

CACCTTCTTCCAAGAAATGAAGACCAAAACCTCCCCCCTTATATTTTTGTGAAATCTGGACCTCCAAAATC
GCCTCCAATGGAAGCTGGCTGCGAGCATACAGTTCACTGTCGCGGGGAAGATGGGTATTTTATAACAGAC
AGTTCACTGGCGGTTGGTTTGAAAAACCGCCAGTGGAAACCTATTTCCACTGGCGGTTTTTCAAACGAAC
CGCCAGTGAACTGTCTGTTATAAAATACCCATCTTCCCCGCGACAGTGAACTGTATGCTCGCAGCCAGCTT
CCATTGGAGGCGATTTTGGAGGTCCAGATTTCACAAAAATATAAGGGGGGAGGTTTTGGTCTTCATTTCTT
GGAAGAAGGTGGATAAGAAAGGTTGGTTTATGTTTCTTTGTCAATTTTTGTTCATTCTTGCTCAATTTTAGC
CACATTTTGGATCTAGGGTTTCACATGTGAGAGAGAGAGTATAGATAGGTAATTTTCTCTATTTTCTCAAA
TGATGTTGCATAAGATGGTTAGTTTTTGTCATTCCTCTCTTTATCATGTTTAGATGCTTAATGATGGTGATG
TGTTGTATGGAA 100bp gap
TGCTTCTGATGAATCAGCTTGATTCGTTGCTTCTTGGCGAAGACAGGGCCTCGGGCGAGCCGGAAATATG
TTAGCCGCTGGAGGGGGGCCTCGGGCGAGGCGGAGATCCTCCGGGGTTGGCTGCCCTTGTCCGAGGCTAG
GCTCGGGCGAGGTGTGATCGTGTCCCTCGAATGGACCGATCCCCGACTTAATCACACCCATCAGGCCTTT
GCAGCTTTATGCTGATAGGGGTAACCAGCTGAGAATTAGGAGCCTTGAGGGTACCCCTAATTATGGTCAC
CGATAGTAGCCCCCGAGCCTCGAAGGGAGTGTTAGTACTTGATTGGAGGCTTATAATTAACTTTTTTGTCA
GGGGACCAGCCTTTCATGGTTGCGTTTTGTAAAGGTGAAAAAGAGAGGGAATAGACAAAAACTAACCAT
CTTATGCAACATCATTTGAGAAAATAGAGAAAATTACCTATCTATACTCTTCTCTCTCACATGTGAAACCC
TAGATCCAAAATGTGGCTAAAATTGAGCAAGAATGAACAAAAATTGACAAAGAAACATAAACCAACCTT
TCTTATCCACCTTCTTCCAAGAAATGAAGACCAAAACCTCCCCCCTTATATTTTTGTGAAATCTGGACCTC
CAAAATCGCCTCCAATGGAAGCTGGCTGCGAGCATACAGTTCACTGTCGCGGGGAAGATGGGTATTTTAT
AACAGACAGTTCACTGGCGGTTGGTTTGAAAAACCGCCAGTGGAAACCTATTTCCACTGGCGGTTGTGTT
AAGATAACCGCCAGTGAAAATGGGTTTCCACTGGCGGTTCCTAAATCGGGTCCACCTTGTTTTTTTTACTG
GCGCGTGATAACTGAAACCGCCAGTGATAATTTATGGGTGCCGCAGGCTTTGAGCTCTTTTCTACTAGTGA
AAGTAGGGCTATCGAAACAGGTCCACATGAGCCATGACCAAACGTTGAGAGTGCAGCTAGCACTGCTACT
CTAGCTCGCTGTTAAAAGAACTCCTACAGGCTACAGGTGGTAGTAATTCACCGGAGCGATGCATCTACCA
GCGAACCATCTTAACTCCTCCCCTGAATGCACTCACCCACCACCCGTAATAGTAACTTTCCCTCCGCTATA
TAACCCCCACTTGTGAAACCCTCGTATCCCCACAACACCAGAATCCGCGAATCACAGACGCGTCTATCTC
AGCTTGCTGCACTGCACTACCCTGCCCTGCCATCATATCGTACGTGAGCCCGGCCGAGCGAGAGCGAGGG
AGAGGCATGGCTGCTCCGAAGCTC

Fig. 1: Sequence upstream *ZmLTP3* gene in NCBI database.

The red segments are repeats; the red and blue segments are inverted repeats; the green segments are primers; atg is the initiation codon of *ZmLTP3* gene.

AGGCGACTGTGACACTATCCATGGAGAAGGACGCGTTGGAGTGCGACATCTGCTGCCTACCCTTCCAGTC
CGAAGTTTTCATGGCAAGTCAGATTACCATGCATGTATATATACAGTAAATATTGATCGGTATATATACAA
ATGTAGTTAGACTACTTTATCTCTTCCAATAAAAGTAGTTAAACTACTTTATCTCTAACACATGGTGAAAC
CCTATATCCAAAATGTGGCTAAAATGGAGGAAAAATAAACAAAATTTGGCAAAAGAAACATAAGCCAAA
CTTTCCTAGCAACTAATAAACAAAAAAAAATTAATACCCAAACCTATCTTTAACATATGGTGAAAGCCTA
GTTTCAAAATGTAGTTAAAATTGAGCAAAAATAAACAATAACTAACAATTGAAACATAATTAAACTAATC
TTCCATACAACACATTCACCATTCATTAAGCAACTAAACATGAAAAAGAGAGGGAATAGACAAAAACTA
ACCATCTTATGCAACATCATTTGAGAAAATAGAGAAAATTACCTATCTATACTCTTCTCTCTCACATGTGA
AACCCTAGATCCAAAATGTGGCTAAAATTGAGCAAGAATGAACAAAAATTGACAAAGAAACATAAACCA
ACCTTTCTTATCCACCTTCTTCCAAGAAATGAAGACCAAAACCTCCCCCCTTATATTTTTGTGAAATCTGG

ACCTCCAAAATCGCCTCCAATGGAAGCTGGCTGCGAGCATACAGTTCACTGTCGCGGGGAAGATGGGTAT
TTTATAACAGACAGTTCACTGGCGGTTGGTTTGAAAAACCGCCAGTGGAAACCTATTTCCACTGGCGGTTT
TTCAAACGAACCGCCAGTGTAATAGGGTCTTTACACTGGCGGTTCTGTTACACCAACCGCCAGTGAAAAT
AGGTTTCCACTGGCGGTTGGTTTGAAAAACCGCCAGTGGAAACCTATTTCCACTGGCGGTTGTGTTAAGAT
AACCGCCAGTGAAAATGGGTTTCCACTGGCGGTTCCTAAATCGGGTCCACCTTGTTTTTTTTACTGGCGCG
TGATAACTGAAACCGCCAGTGATAATTTATGGGTGCCGCAGGCTTTGAGCTCTTTTCTACTAGTGAAAGTA
GGGCTATCGAAACAGGTCCACATGAGCCATGACCAAACGTTGAGAGTGCAGCTAGCACTGCTACTCTAGC
TCGCTGTTAAAAGAACTCCTACAGGCTACAGGTGGTAGTAATTCACCGGAGCGATGCATCTACCAGCGAA
CCATCTTAACTCCTCCCCTGAATGCACTCACCCACCACCCGTAATAGTAACTTTCCCTCCGCTATATAACC
CCCACTTGTGAAACCCTCGTATCCCCACAACACCAGAATCCGCGAATCACAGACGCGTCTATCTCAGCTT
GCTGCACTGCACTACCCTGCCCTGCCATCATATCGTACGTGAGCCCGGCCGAGCGAGAGCGAGGGAGAGG
CATG

Fig. 2: Sequenced candidate promoter sequence of *ZmLTP3* gene.
The green segments are primers; atg is the initiation codon of *ZmLTP3* gene.

Table 1: Cis-acting elements in the candidate promoter sequence of *ZmLTP3* gene

Site name	Sequence	Function
ABRE	TACGTG	cis-acting element involved in the abscisic acid responsiveness
AE-box	AGAAACAT, AGAAACAT	part of a module for light response
ARE	TGGTTT, TGGTTT	cis-acting regulatory element essential for the anaerobic induction
ATCT-motif	AATCTAATCT	part of a conserved DNA module involved in light responsiveness
Box 4	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	TTTCAAA	light responsive element
CAAT-box	CAAAT, CCAAT, CAATT	common cis-acting element in promoter and enhancer regions
G-box	CACATGG, TACGTG, GTACGTG	light responsive element
MBS	TAACTG	MYB binding site involved in drought-inducibility
RY-element	CATGCATG	cis-acting regulatory element involved in seed-specific regulation
Sp1	CC(G/A)CCC	light responsive element
TATA-box	TATATATA, ATATAT, TATA, ATATAT, TAATA, TTTTA	core promoter element around -30 of transcription start
TC-rich repeats	ATTTTCTCCA	cis-acting element involved in defense and stress responsiveness
TCA-element	GAGAAGAATA	cis-acting element involved in salicylic acid responsiveness