ORIGINAL ARTICLE

Construction of Sly-miR393 Over-Expression Vector and Verification of Its Target Genes in Tomato

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Abstract: To understand the function of Sly-miR393 in tomato, the precursor sequences and potential target genes of Sly-miR393 were identificated from tomato genome database by computational homology search method. The Sly-miR393 gene was amplified from the genomic DNA by PCR and cloned into plant expression vector pLP35s-100. Sly-miR393 guided-cleavage to putative target transcripts was validated u sing 5RACE RT-PCR. In this study, our results indicated that the precursor sequence of Sly-miR393 contains the complete hairpin structure. TIR1/AFB auxin receptor genes contain recognition sites with high complementarities to Sly-miR393 sequence. In tomato, Sly-miR393 directs the cleavage of SlTIR1.SlTIR1-like1 and SlAFB mRNA, then auxin receptor homologous was validated to be as target of Sly-miR393. The pLP35s-pre-SlymiR393 vector containing Sly-miR393 gene was successfully constructed, which would provide significant evidence for further study of Sly-miR393 function in auxin signaling pathway in tomato.

Keywords: Sly-miR393; Target Genes; Auxin Receptor Gene; Construction

Introduction

MicroRNA(miRNA) is a kind of endogenous small molecule non-coding RNA with a length of 21~24 bases. It specifically regulates the expression of target gene[1,2] at the post-transcriptional level by degrading or inhibiting the translation of target gene mRNA. MiRNAs most of the target genes are important transcription factors and proteins that regulate plant growth and development[3]. This, miRNA participates in the whole process of plant growth and development, including biological processes such as morphogenesis, cell differentiation, signal transduction and stress response of roots, leaves and flowers,[4,5] MiRNA. The relevant research is of great significance to clarify the complex life regulation mechanism of organisms, and is one of the frontier fields and hot spots in biological research. MiR393 is a relatively conservative miRNA family in plants. in Arabidopsis thaliana, miR393 mediated regulation target gene, TIR1.The proteins encoded by AFB1, AFB2 and AFB3 belong to F-box protein[2], which plays 45 important regulatory roles in auxin signal transduction. Recent studies show that overexpression of Arabidopsis thaliana miR393 can inhibit auxin signal transduction, thus significantly improving the plant“s resistance to bacterial infection[6] and leading to a decrease in the number of lateral roots and a decrease in auxin sensitivity in root development[7]. Xia *et al.* found that in rice, OsmiR393 downregulated TIR1/AFB and family homologous genes after overexpression, resulting in more tillers, early flowering, reduced salt tolerance and drought resistance,[8]. In addition, Sunkar et al (2004) After subjecting Arabidopsis thaliana to cold, drought, high concentration of ABA and high salt stress reactions, it was found that the expression level of miR393 was significantly up-regulated, indicating that miR393 related to Abiotic Stress Response[9] Therefore, miR393 can not only regulate auxin signal transduction pathway, but also regulate response to stress.

Tomato is one of the important model plants in plant molecular biology research. Little is known about the biological function of tomato miRNA. In particular, miR393 is an important post-transcriptional regulator of auxin signal transduction pathway, and the biological function in Solanum melongena has not been reported yet. Sly-miR393

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and target genes were isolated and identified in tomato in this study; at the same time, construct the overexpression vector plp35s-pre-slymr393, thus laying the foundation for further research on the biological function of slymr393 in tomato growth and development.

1. Materials and methods

1.1 Materials

1.1.1 Plant materials and carriers

Wild type tomato seeds (solanum lycopyrum ,cv Micro-Tom), e. coli JM109 and plant expression vector pLp100 are all stored in our laboratory.

1.1.2 Reagents

DNA extraction kit, plant total RNA extraction kit, 2×PCR Master Mix ,FastDigest restriction endonuclease, T4 DNA Ligase (purchased from Fermentas company); PrimeSTARTM HS DNA Polymerase hi-fi enzyme and 5-Full RACE kit (purchased from Takara company); Plasmid extraction kit (E.Z.N.A. Gel Extraction Kit) and gel recovery kit (E.Z.N.A. Plasmid Mini Kit I) (purchased from OMEGA company); PEASY-Blunt Clone Vector, DNA Marker plus 2K (from Beijing Quanshijin Biotechnology Co., Ltd.); Other chemical reagents are purchased from beijingsoreibao Co., Ltd. PCR.

1.2 Methods

1.2.1 Tomato genome DNA extraction

According to the DNA kit instruction, extract the DNA from tomato leaves.

1.2.2 Tomato sly-miRNA393 precursor gene prediction and cloning

In the database miRNA Registry Database (Release 17: April 2011, http://www.mirbase.org/ Index. SHTML) Downloaded to Mature Arabidopsis Ath-miR393 Sequence (miRBase Login Number: MI0001003). Based on small RNA mature sequence conservativeness, use blast tool and modify blast expected threshold (e-value) to 1e-0 in Tomato Genome Database (http://solgenomics.net/) for Alignment to Find Possible Precursor Sequence of Tomato Sly-miR393. For the predicted genome sequence completely matching Sly-miR393 mature sequence 22 nt ,select a nucleotide sequence of about 80 nt and use RNA Structure 4.6 software to analyze whether there is a typical hairpin secondary structure. if a complete neck ring structure can be formed and the mature sequence is completely located at the 5 end, the nucleotide sequence corresponding to the neck ring structure is presumed to be potential Sly-miR393 precursor (pre-SlymiR393).

Tomato genome DNA is used as a template, and specific primers are designed to carry out PCR amplification according to Sly-miR393 genome sequence containing neck ring structure. Pre-SlymiR393 primer: upstream primer: 5-CACCTCAGAGT ATTTGCGTAAGG-3; Downstream primer: 5-catgctttttgtgtgtgg-3; PCR reactant.

The line is 50L: 10L5 × PCR Buffer ,4L dNTPs (2.5mm), 1L Upstream Primer (10M), and 1L downstream primer (10 μM), 1 μL DNA (100 ng), 0.5 μL PrimeSTAR hi-fi DNA Polymerase ,32.5 μL ddH2O. The PCR reaction procedure is: 98 ℃ pre-denaturation 2 min; 98 ℃ denaturation 10 s, 55 ℃ annealing 15 s, 72 ℃ elongation 30 s, 35 cycles; Finally, 72 ℃ extension 10 min after the reaction is completed, take 5 μL PCR amplification products respectively and perform electrophoresis on agarose gel with a concentration of 1.5%. Then observe and take photos under the UV gel imaging system, recover and purify the cut glue of the target strip, and store it at -20 ℃ for later use.

1.2.3 Prediction of target genes for tomato, sly-miR393 and tomato

Sly-miR393 Sequence Pair psRNATar Website (http://bioinfo3.noble.org/psRNATarget/) Tomato in ITAG2.3cDNA Database for Blast Analysis. The target gene with highly conservative binding sequence of mature Sly-miR393 is preliminarily deduced as Sly-miR393 (maximum allowed 4 base mismatches).

1.2.4 Sly-miR393 identification of target gene shear sites

Trizol method is used to extract total RNA from tomato mixed tissues. After DNase I digestion, reverse

transcription of 5RACE RT-PCR is carried out using 2 μg RNA as template. The method refers to 5-Full RACE kit (TaKaRa) instructions. Two rounds of nested PCR were used, primers are shown in Table 1 ,the 3 end of the target gene was cleaved, the amplified product was recovered and cloned into the pEASY-Blunt vector after purification, and the site where each target gene was cleaved was analyzed by sequencing.

1.2.5 Construction of plant expression vectors

Connect the PCR product recovered in the above step 1.2.2 with the SmaI enzyme-cleaved plant expression vector pLp100-35S vector. The reaction system is: 10×T4 DNA Ligation Buffer 1 μL, PEG 4000 1 μL, T4 DNA ligase 1 μl (5u/μl), pre-SlymiR393 target fragment 7 μl, pLP100-35S vector fragment 1 μl (50 ng/μl), with a total volume of 10 μl, which is mixed evenly and connected overnight at 4 ℃ Transforming the ligation product JM109 Escherichia coli competent cells, randomly selecting 10 single colonies for PCR identification.

2 Results and analysis

2.1 Precursor sequence prediction of tomato sly-miR393

Through bioinformatics method, blast sequence alignment was carried out in tomato genome database (http://solgenomics.net/index.pl), and two genome sequences SL2.40sc04696 and SL2.40sc039022 were found to be completely matched with mature miR393 sequences respectively. The secondary structural analysis of the predicted mature miR393 flanking sequences on the corresponding genome shows that only the 80 nt sequences at both ends of the SL2.40sc04696 genome can form a complete neck ring structure and conform to the characteristics of the plant miRNA precursor. As shown in Figure 1A, the sequence indicated in bold type at the end of 5 is UCCAAAGGGAUCGCAUUGAUCC, which is the mature miR393 sequence. Therefore, we speculate that the nucleotide sequence corresponding to the neck ring structure is a potential Sly-miR393 precursor (pre-SlymiR393). The tomato genome DNA is used as a template, and specific pre-SlymiR393 front and rear primers are designed according to the two end sequences of the neck ring structure, and PCR amplification is carried out to confirm the target fragment on 1.5% agarose gel electrophoresis. Electrophoresis results are shown in Figure 1B with a single target band, DNA fragment size is consistent with expectation, which is 334 bp.

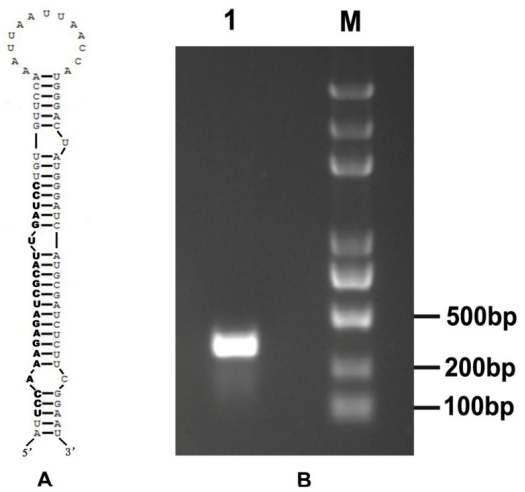

Figure 1. Hairpin structure and PCR product of tomato Sly-miR393. (A) Hairpin structure of tomato Sly-miR393; (B) PCR amplication of pre-slymiR393 target fragment 1:Sly-miR393; M: Trans2K Marker.

2.2 The predicted target gene belongs to auxin receptor F-box protein

The highly complementary sequence between plant miRNA and target mRNA is helpful for bioinformatics prediction of target gene of miRNA[10], Comparing and analyzing the Sly-miR393 sequence with the tomato ITAG2.3cDNA database in psRNATarget website (http://bioinfo3.noble.org/psRNATarget/), Solyc09g074520, Solyc02g079190 and Solyc06g008780 gene sequence and mature Sly-miR393 sequence have 3-4 base mismatches at

their binding sites, and their protein functions belong to auxin F-box protein. In the previous study, we have isolated auxin receptor, TIR1/AFBs, homologous gene in family gene, SlTIR1(GenBank login number from tomato. GQ370812) [11]. The amino acid sequence alignment of the predicted three target genes found that Solyc09g074520 is SlTIR1 ,Solyc02g079190 and Tomato.

The homology of SlTIR1 and SL TIR1 reached 55%, Solyc06g008780 and AFB gene families in Arabidopsis thaliana were 60% " Therefore, the three potential target genes of Sly-miR39 in tomato are named as: SlTIR1 ,SlTIR1-like1 ,SlAFB respectively.

2.3 Sly-miRNA393 shear identification of target genes

MiRNA negatively regulates the expression of target genes through shear degradation. In order to further verify whether Sly-miR39 in tomato has shear effect on potential target genes, modified 5RACE method is adopted to amplify 3 end shear products of three target gene mRNA. Results as shown in Figure 3A, three different target fragments were separated on agarose gel electrophoresis, recovered by gel cutting, cloned into intermediate vectors, and 8 positive clones were randomly selected for sequencing and identification. Sequencing results show that Sly-miR393 has shearing effect on targets, SlTIR1, SlTIR1-like1, SlAFB and SL AFB. Analysis of the Sly-miR393 cleavage sites of each target gene found that SlTIR1 and SlAFB were all cleaved at the CGA/UCC site, while SlTIR1-like1 was cleaved at the CGG/UGU site, but these cleavage sites were all located between the 10th and 11th bases in the mature Sly-miR393 binding sequence (Figure 2B). These results confirm that the three auxin receptor homologues in tomato, SlTIR1, SlTIR1-like1 and SlAFB, are all target genes of Sly-miR393.

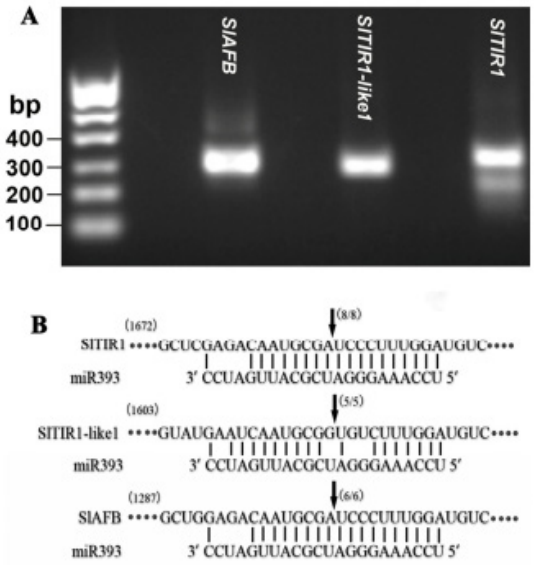

Figure 2. Validation of Sly-miR393 guided cleavage of target gene mRNA.

(A) Amplification of 3bottom sequence of cleavage products; M: DNA marker I; The electrophoretogram shows the nested PCR products representing the 3'-cleavage fragments that were cloned and sequenced for each gene

(B) Mapping of cleavage sites of SlTIR1, SlTIR1-like1 and SlAFB Cleavage sites in the targets recognition sequence are marked with arrowheads, and their frequency among sequenced clones of the same approximate size is noted above.

2.4 Sly-miRNA393 construction and identification of plant expression vectors

Colony PCR was carried out using plp35S-cx and pre-slymr 393-r as primers to identify positive clones. As shown in Figure 4. Single colonies of No.1 and No.4 can amplify the target band of 835 bp, which is consistent with the expected result, indicating that the target fragment has been successfully inserted into the plant expression vector pLP100-35S, i.e. the recombinant plasmid PL P35S-Pre-SLYMER 393 has been obtained. The recombinant plasmid which was verified to be correct initially was sequenced and the sequence was completely correct.

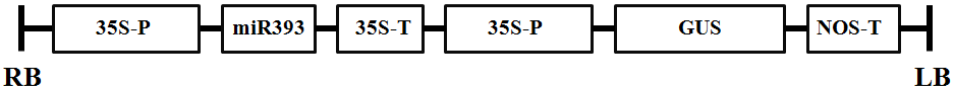

Figure 3. Construction of Sly-miR393 plant expression. vector miR393: precursor sequences of Sly-miR393; GUS: GUS gene; 35S-P: CaMV35S promoter; 35S-T: CaMV35S terminator; NOS-T: NOS terminator.

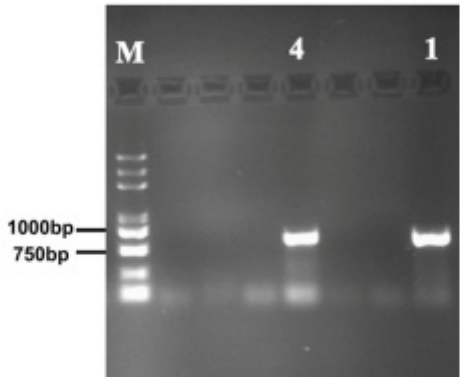

Figure 4. Detection of positive clones by PCR 1, 4: Positive clones; M: Trans2K Marker.

3. Discussion

At present, many plants miRNA have been found and isolated from plants. Based on the conservativeness and special structure of the miRNAs sequence, bioinformatics has become the most direct, fast and simple method to identify miRNAs. With the gradual improvement of tomato genome database, it will be helpful for preliminary prediction and identification of tomato miRNAs using biological information methods. Although bioinformatics methods can rapidly predict conservative miRNAs, there are still some limitations, such as incorrect prediction and incomplete information. Therefore, it is necessary to carry out experimental verification of the predicted miRNAs, miR393 between different plants is highly conservative, which facilitates the screening of target genes. Through the combination of bioinformatics prediction and the 5RACE experiment, the target genes of miR393 were identified in Arabidopsis thaliana and rice as growth hormone receptors, TIR1/AFB, gene family, respectively. In the known tomato genome sequence, through sequence alignment, we found that the Sly-miR393 target gene is also a member of the F-box protein family that codes for auxin receptor. In this study, 5RACE technology is used to prove that tomato Sly-miR393 mediates the shear degradation of target gene (SlTIR1 ,SlTIR1-like1, slafb) mrna and the shear site position conforms to the general rule that most plants miRNAs shear the target gene mostly in the middle of the binding sequence[2].

Because many plants have multiple loci of miRNA, it is difficult to find miRNA mutants with complete functional loss by positive genetic screening. although T-DNA insertion mutation mir164b (mir164b-1) is found in Arabidopsis thaliana, the phenotype is not found. The obvious defect now is that the mature miR164 is encoded by at least three loci[12]. Therefore, the function of plants miRNAs is clarified through overexpression of precursor sequences encoding miRNA or mutation of shear sites on target gene mRNA, Overexpression of the miRNA precursor gene in transgenic plants increases the accumulation level of endogenous mature miRNA but reduces the expression abundance of mRNA of the target gene. In such transgenic plants, the expression of similar target gene function loss mutation appears. At present, there are also reports of using this method to study the specific functions of miRNA in tomatoes. The transgene over-expressed Sly-miR156 shows the characteristics of increased axillary buds, smaller leaves, dwarf plants, etc. Overexpression of Sly-miR169 improving drought stress resistance and 15 in tomato. In addition, there is an obvious phenotype on the shear site on tomato through differential overexpression Sly-miR4376 and site-directed mutation target gene ACA10 :changes in flower morphology and structure and reduction in fruit yield[16]. These results all indicate that overexpressed plants miRNA can reveal their specific functions. therefore, this study has constructed Sly-miR393 plant overexpression vector plp35s-pre-slymr393, and intends to transform tomato through agrobacterium-mediated leaf disc method, aiming to obtain overexpressed transgenic plants, which is of great significance for further analysis of the role of Sly-miR393 gene in tomato growth and development.

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