Clone the Promoter of Vvcyp86a1 Gene and Analyze Its Expression.

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Abstract: The salt-induced combined promoter XM438-1 of VvCYP86A1 gene was designed, synthesized and sequence analyzed, and grape DNA was successfully extracted. After cloning the promoter of VvCYP86A1, an overexpression vector with GUS was constructed, and the verification was successful. After the transformation and identification, the results showed that salt stress could induce the gene expression driven by the promoter.

1. Introduction

Promoters can activate RNA polymerase, RNA polymerase can be accurately transcribed after combining with template DNA, but different promoters have different characteristics, which also determines the specific effect of RNA polymerase in gene expression. Therefore, an important factor in gene expression is the promoter[1].

The above is consistent with the current research results. Su et al. (2021) used enhanced green fluoresces protein (EGFP) as the reporter gene and used 5'-deletion technology to analyze the functional region of the locust Magas1 gene promoter (PMagas1). The results showed that its functional area is crucial to the activity of PMagas1. PMagas1 was used to drive the subtilisin-like protease gene Pr1A (PMagas1-Pr1A) to construct a strain of acridinium. The bioassay results showed that the virulence of the PMagas1-PR1A strain was significantly higher than that of the wild-type strain[2]. Shen et al. replaced the TEF1 promoter with a weaker promoter ($P_{Dog2p300}$) derived from the potential promoter region of 2-deoxyglucose-6-phosphate phosphatase gene for driving the antibiotic-resistant gene expression. Importantly, the $P_{Dog2p300}$ has even lower activity under carbon sources (glycerol and methanol) used for the AOX1 promoter–based production of recombinant proteins compared with glucose that is usually used for the selection process[3]. In order to study the tissue-specific expression characteristics and transcriptional regulation of the LBP gene of Meishan pigs, CAO et al. detected the expression of the LBP gene promoter in different tissues of the 35-day-old Meishan weaned piglets, and revealed the different expression and expression of LBP in Meishan pig tissues. Promoter methylation patterns reveal the potential role of DNA methylation in regulating the expression of LBP, laying a foundation for further research on the expression and function of pig LBP genes[4].

GUS gene is suitable for the expression analysis of Arabidopsis thaliana. There are many methods for determination of GUS gene in transgenic plants. Histochemical staining is the most commonly used method by researchers. The catalytic substrate is X-Gluc[5]. Almost all plant cells do not have endogenous GUS activity. The expression of GUS gene can generate hydrolase β-glucuronidase, which can decompose X-Gluc into blue substance[6-10]. In order to further verify the effect of salt stress on the expression of *VvCYP86A1* gene, we used histochemical staining method to analyze the expression of GUS gene driven by the promoter of *VvCYP86A1* gene in Arabidopsis.

1.1 Experimental Method

1.1.1 Design and Synthesis of Promoter Primers

Find the sequenced *VvCYP86A1* gene of grape varieties, design nested PCR primers with the help of NCBI website and grape genome, and name the promoter of *VvCYP86A1* gene XM438-1.

1.1.2 Dna Extraction

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The CTAB method is used to extract grape DNA:

(1) Pour liquid nitrogen into a plastic foam incubator, and use the upper young leaves of Seedless Cressen grapes to obtain materials. Put the material in a centrifuge tube with small steel balls, and then put it in an incubator. After quick freezing in liquid nitrogen, grind it with a biological tissue grinder. Depending on the degree of crushing of the material in the centrifuge tube, you can decide whether to repeat the operation. Add 800 μL of preheated The 2×CTAB solution.

(2) The ground material is water-bathed in a 65 ℃ water bath for 30 minutes, gently inverting and mixing from time to time.

(3) Put it in a centrifuge and centrifuge at 13,500 rpm for 5 minutes.

(4) Carefully transfer the supernatant of step (3) to a centrifuge tube that has added 450 μL of chloroform, gently shake it up and down, and place it on the test tube rack, and let it stand for 15 minutes.

(5) After the centrifuge tube is stratified obviously, put it in the centrifuge. Set the centrifuge program to 13500 rpm and the time is 5 minutes. Depending on the degree of supernatant in the centrifuge tube, you can decide whether to repeat the operation.

(6) Centrifuge at 13500 rpm for 5 minutes, you can extend the time of centrifugation. Wait until the supernatant is completely free of impurities, and then mix the supernatant with isopropanol. Isopropanol is doubled in volume and pre-cooled in advance. After mixing, put it in the refrigerator at -20 ℃ for two hours.

(7) The centrifuge program is set to 13500 rpm and the time is 10 minutes. Discard the supernatant, use a pipette to absorb 70% alcohol, gently clean the precipitate, and repeat the cleaning once.

(8) Put the precipitate in a 37 ℃ oven to blow dry.

(9) Add an appropriate amount of sterile water, and store the DNA in a refrigerator at -20 ℃ to prevent DNA degradation.

1.1.3 Cloning and Amplification of Vvcyp86a1 Promoter

Using grape genomic DNA as a template, using the above-designed primers and Phanta Super-Fidelity DNA Polymerase (P501-d1) kit, PCR amplification was performed on the VvCYP86A1 gene promoter sequence to obtain the entire sequence of the VvCYP86A1 gene promoter XM438-1.

Using 2xTaq Master Mix kit, the PCR results were detected by 1.0% agarose gel electrophoresis.

1.1.4 The Connection between the Promoter of Vvcyp86a1 and the Cloning Vector

After recovering the above products, mix it with the cloning vector and let it stand at room temperature (20 ℃) for 5 minutes. Then, put the centrifuge tube on ice and set the temperature in the gene amplification instrument: 20 ℃, 20 min to control the temperature.

1.1.5 Heat Shock Transformation of Recombinant Vector

Heat shock the recombinant vector at 42 ℃ for 45 seconds ,and conversion method reference Yang(2020) [11].

1.1.6 Pcr Verification of Escherichia Coli Colonies

Perform PCR verification on the above-cultured E. coli, using 2×Taq Master Mix (Dye Plus), as shown in Table 1.

Table 1 Colony Pcr Reaction System(20Ml).

Ingredient	Volume
2×Taq Master Mix(Dye Plus)	10μL
Forward	1μL
Reverse	1μL
DNA	1μL
ddH2O	7μL

Use gel electrophoresis to detect the results, and mark the positive bacteria as Laguna. The shaken bacterial solution was sent for sequencing, and the plasmid was named PEASY-Blunt-XM438-1.

1.1.7 Extract Peasy-Blunt-Vvcyp86a1 Recombinant Plasmid

Use FastPure Plasmid Mini Kit (DC201) kit to extract PEASY-Blunt-*VvCYP86A1* recombinant plasmid.

1.1.8 Restriction Digestion and Recovery of Recombinant Plasmids and Expression Vectors

The extracted recombinant plasmid PEASY-Blunt-XM438-1 (not more than 1000 ng) and the expression vector 1300-GN (not more than 1000 ng) were simultaneously digested with BamH I and EcoR I. The map of the expression vector 1300-GN is shown in the figure 1. .

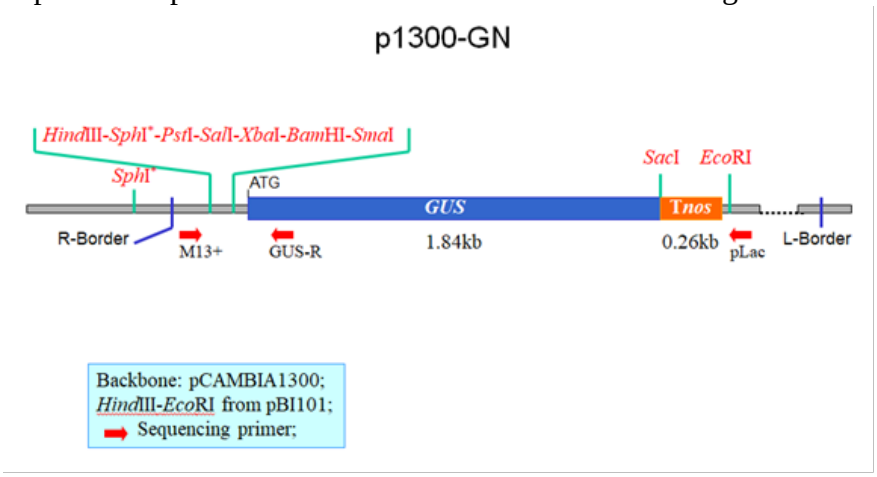

Fig.1 Structure and Restriction Site of 1300-Gn Expression Vector

The enzyme digestion reaction system is shown in the table 2.

Table 2 the Enzyme Digestion Reaction System(20 Ml)

Ingredient	XM438-1	1300-GN
BamH I	1μl	1μl
EcoR 1	1μl	1μl
10×Buffer	2μl	2μl
Plasmid	4μl	2.5μl
dd H2O	13μl	14.5μl

After adding the sample, flick the EP tube and centrifuge. In the gene thermal cycler, set the reaction program: 37 ℃, 30 min. Place it on a 1% agarose gel for electrophoresis detection. Use DC301 kit for product recovery.

1.1.9 Linkage of Promoter and Expression Vector

The connection system is as follows:

Table 3 the Connection System between Xm438-1 and Expression Vector 1300-Gn(20 Ml)

Ingredient	Volume
T4 Ligase	0.2μL
10×T4 Buffer	2μL
1300-GN	1μL
XM438-1	9μL
ddH2O	7.8μL

After adding the sample, flick the EP tube and centrifuge. In the gene thermal cycler, set the reaction program: 22 ℃, 60 min. After the reaction, the plasmid was named XM438-1-GN.

1.1.10 0. Transform Xm438-1-Gn with Agrobacterium Gv3101

Use FastPure Plasmid Mini Kit (DC201) to extract XM438-1-GN plasmid, Then use Agrobacterium competent GV3101 for transformation, the operation method is as Yang(2020) [11].

1.1.11 1. Colony Pcr Verification of Xm438-1-Gn

Use the upstream primer of the VvCYP86A1 gene and the downstream primer of the GUS gene, and randomly select the positive XM438-1-GN single colony with suitable growth status as the template, and use the 2×Taq Master Mix (Dye Plus) kit for colony PCR verification. The reaction conditions are as follows:

Table 4 Colony Pcr Reaction Conditions of Xm438-1-Gn(50 Ml)

temperature	time	Number of cycles
95℃	5min	1
95℃	20 s	30
53℃	20s	30
72℃	1min30s	30
72℃	5min	1
4℃	∞	1

1.1.12 2. Analysis of Specific Expression of Xm438-1-Gn in Arabidopsis

First, XM438-1-GN was inherited and transformed in Arabidopsis, then it performs specific expression analysis as follows:

(1) Growth of GUS stained plants

The seeds were dried for one week, and the sterilized Arabidopsis seeds were sown on 1/2MS solid medium containing Hyg (25 μg/ml) and Cef (20 μg/ml), and wild-type Arabidopsis (Col-0) was used as Contrast. After vernalization in a refrigerator at 4 ℃ for 48 hours, place in a light incubator at 23±2 ℃, 10/14 h (bright/dark) photoperiod and light intensity of 200±50 μmolm-2 s-1.

(2) GUS stained plants under NaCl stress

After the Arabidopsis overexpression line driven by the XM438-1-GN promoter and the wild-type Arabidopsis grow 2 true leaves, they are transferred to Hyg (25 μg/ml) and Cef (20 μg/ml). And 0 mM NaCl, 50 mM NaCl 1/2 MS solid medium, the culture conditions are as (1).

(3) GUS staining analysis of transgenic plants

The 13D-growing seedlings were selected for staining, and the expression of GUS gene was observed after decolorization.

GUS staining solution preparation: concentrated solution: buffer = 1:50.

The dyeing method is as follows:

(1) Seedling selection: 6 seedlings were selected and placed in a 50ml beaker.

(2) Dyeing: Put the prepared GUS staining solution into a 50ml beaker, immerse the material, seal it with a parafilm, and stain it on a shaker (25 ℃, 0 rpm) for 12 hours until the whole seedling turns blue.

(3) Decolorization: Transfer the seedlings to a beaker containing 70% ethanol and decolorize them overnight until the WT is white.

(4) Observation: Observe and take pictures under a microscope.

1.2 Results and Discussion

1.2.1 Cloning of Vvcyp86a1 Promoter

The result of electrophoresis is shown in Figure 2, and the target band of about 2208bp appears. Cut off the target strip and recycle the glue.

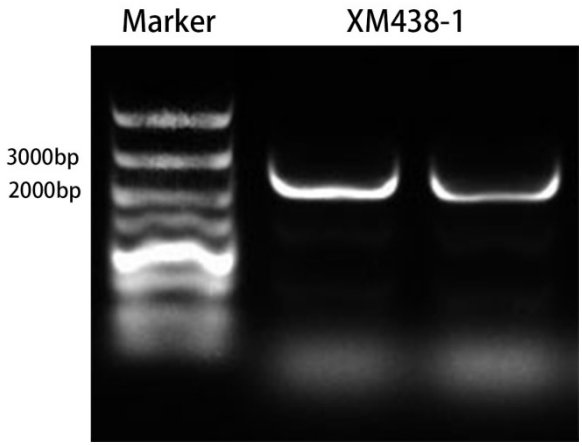

Fig.2 Vvcyp86a1 Promoter Fragment from Crimson Seedless Obtained by Pcr

1.2.2 Construction of Xm438-1 Overexpression Vector

The obtained XM438-1 fragment was connected to pEASY-Blunt Cloning Kit, and the transformed DH5α was screened and verified using 1/2MS solid medium (Kana 50ug/ml). The results indicate a band of purpose. Amplify the positive single colony, then extract the plasmid and name it PEASY-Blunt-XM438-1. We got three clear and correct purpose bands. After the digested product is recovered, the XM438-1 and the expression vector (1300-GN) are connected and transformed with T4 DNA Ligase.

1.2.3 Transform Xm438-1-Gn with Agrobacterium Gv3101

The conversion was successful. After the positive strains were expanded, the T1 generation Arabidopsis thaliana was screened for transformation. The leaves of resistant plants were randomly selected, and the DNA of T1 generation XM438-1-GN Arabidopsis seedling leaves was extracted and verified by PCR. The results of electrophoresis showed that XM438-1-GN was successfully inserted.

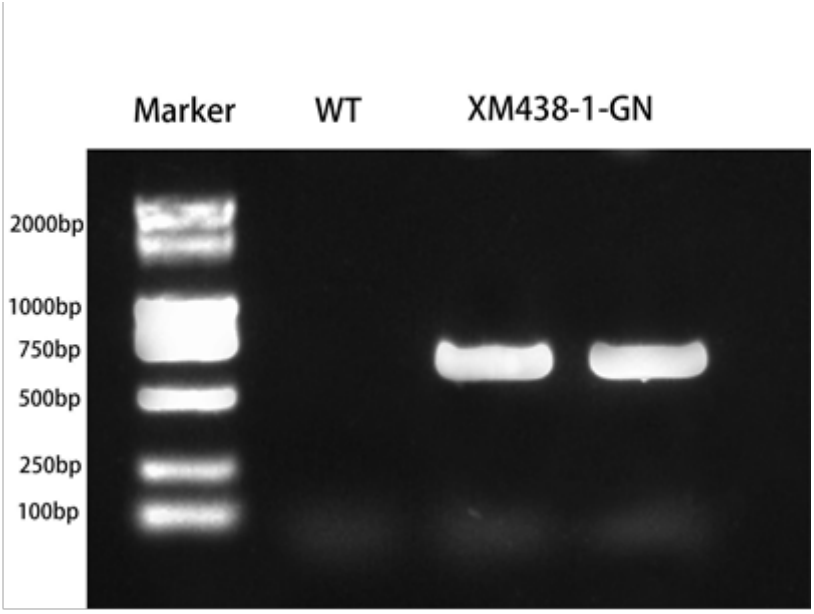

Fig.3 Pcr Identification of Transgenic Plants

1.2.4 Analysis of Expression of Vvcyp86a1 Recombinant Promoter in Arabidopsis

In this paper, the seeds obtained in 5.2.5 were cultured on 1/2 MS solid medium (containing 25 μg/ml Hyg and 20 μg/ml Cef). The seedlings grown to 13D were randomly selected and stained under the stress of 0 mM NaCl and 50 mM NaCl. After decolorization, the color difference was observed with a microscope. The photograph is shown in Figure 2.3. WT is not expressed because it

is not transferred into XM438-1-GN. Before salt treatment (such as 2.3 A) and after salt treatment (such as 2.3 B), the OE2, OE3, and OE4 (XM438-1-GN-Col-0) transferred into XM438-1-GN, the GUS gene showed varying degrees of expression. The results are shown in Figure 2.3. After salt treatment, the roots, stems, and leaves of WT did not change significantly; the GUS gene driven by XM438-1-GN was expressed to varying degrees in the roots, stems, and leaves of transgenic plants, especially It is because the roots show a tendency to gradually deepen the blue color, indicating that the degree of GUS staining is deepening, and the GUS signal is gradually increasing. XM438-1-GN was successfully transferred into Arabidopsis and can start the expression of GUS gene.

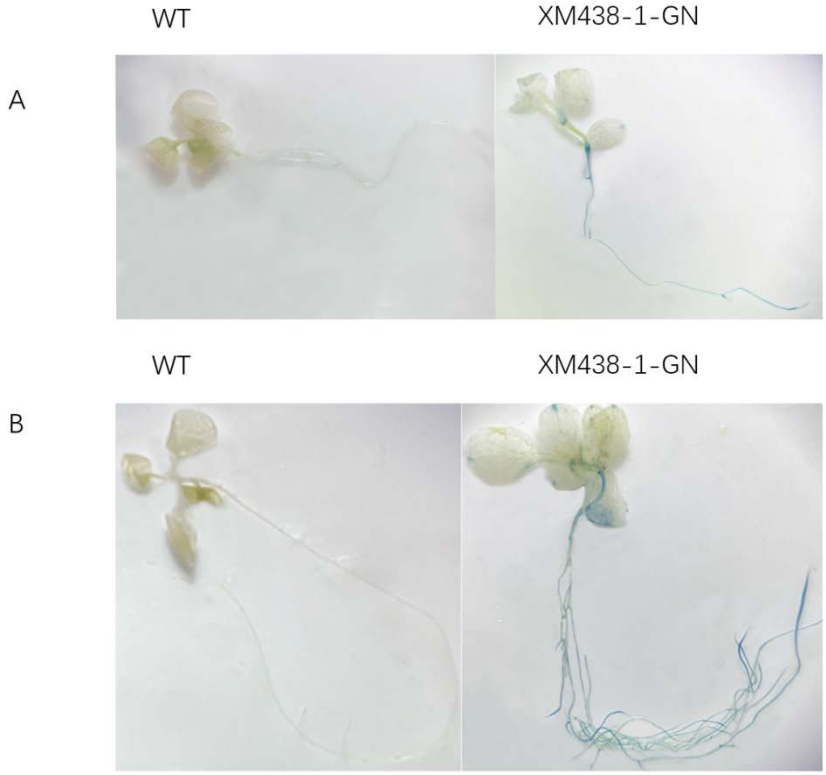

Fig.4 The Staining of Xm438-1-Gn in Arabidopsis

2. Acknowledgments

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