Construction of Yeast One-hybrid Bait Reporter Vector for Screening the Binding Proteins of Cassava *MeCWINV1* Promoter

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Abstract. Cellwall invertase (CWIN) hydrolyzes sucrose into glucose and fructose irreversibly, playing key roles in carbohydrate partitioning and plant defence. *MeCWINV1* is one of the CWINs in cassava, which contains several light-responsive elements and stress-responsive elements in promoter region. To analyze the regulatory function of *MeCWINV1* in the cassava starch accumulation and stress defense response, a 865 bp *MeCWINV1* promoter fragment was cloned and inserted into yeast one-hybrid bait vector to construct p*CW1*-AbAi vector, then was transformed to Y1HGold yeast strains to screen the binding proteins. It might provide a framework for further investigation on the regulation mechanism of *MeCWINV1* gene in cassava.

1 Introduction

Soluble sugars, mostly hexoses and sucrose, which not only provide energy and carbon resources, but also act as signalling molecules in plant defence. Invertase (INV) hydrolyzes sucrose into glucose and fructose, thereby playing crucial roles in primary metabolism, plant development and defence response [1]. In plant cells, invertases are found located in the cell wall, vacuole and cytosol, which are named as CWIN, VIN and CIN, respectively. CWIN and VIN are acid invertases with optimum pH 4.5-5.5, while CIN is neutral/alkaline invertases [2]. CWINs serve as key metabolic enzymes in the carbohydrate supply of sink tissues and the regulation of source-sink transitions. Meanwhile CWINs are regulated by sugar-, hormone-, stress-related signals, and generate sugar-based signals via the modulation of sucrose/hexose ratios. Hence, several signals converge at the site of CWIN regulation. CWIN acts at an integration point of hormonal, sugar, defence and diurnal/circadian regulation [3].

Cassava has six CWIN genes *MeCWINV1~6*, these genes show different spatial and temporal patterns of expression during the cassava development. *MeCWINV1* and *MeCWINV3* had higher activity than other *MeCWINVs* in cassava leaves and tubers, which suggested *MeCWINV1* and *3* were the key CWINs for catalyzing sucrose in the apoplastic space of cassava source and sink organs [4]. In previous research we found that the *MeCWINV1* gene promoter contains several light-responsive

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elements and stress-responsive elements, which might be associated to the invertase gene function of cassava starch accumulation and stress defense [5].

In this study we cloned a 865 bp *MeCWINV1* promoter fragment and constructed the yeast one-hybrid bait vector p*CW1*-AbAi vector, and further transformed to Y1HGold yeast strains then determined the minimal inhibitory concentration of Aureobasidin A for Y1HGold[*CW1*/AbAi] strains. It can be used to screen the binding proteins of *MeCWINV1* gene promoter and provide a framework to further investigate the regulation mechanism and signal pathways of *MeCWINV1* gene.

2 Materials and Methods

2.1 Materials and reagents

pAbAi Y1H bait vector, p53-AbAi vector, Y1HGold yeast strains and Aureobasidin A were purchased from Clontech Inc., DH5α/E.coli strains and p*CW1*-19T plasmid were obtained from our laboratory.

2.2 Cloning and synthesizing p*CW1*-AbAi plasmid

The forward primer with *Kpn* I site (5'-GGGGTACCATGCGACTTGAAACTT-3') and the reverse primer with *Sal* I site (5'-ACGCGTCGACCTCTATTTTCCCTCT-3') was designed according to the known *MeCWINV1* gene promoter sequences in the NCBI GenBank database (KC465190). The *MeCWINV1* promoter region was amplificated from the p*CW1*-19T plasmid by PCR using the primers. The PCR product and pAbAi Y1H bait vector were digested with *Kpn* I and *Sal* I, then ligated at 16℃ overnight with T4 DNA ligase. The promoter was inserted into the pAbAi vector to generate a recombinant plasmid p*CW1*-AbAi, then was identified by PCR, restriction analysis, and sequencing.

2.3 Generating the bait-reporter yeast strains

Linearize 1 μg of p*CW1*-AbAi plasmid and p53-AbAi plasmid (positive control) by *BstB*I restriction enzyme. Transformed Y1HGold yeast strains with 1 μg of each of the linear plasmids using the protocol for the Yeastmaker Yeast Transformation System 2. Diluted each transformation reaction 1/10, 1/100, and 1/1000, then plated 100 μl from each dilution on SD/-Ura agar medium. After 3 days picked 5 colonies and analyzed by colony PCR using the Matchmaker Insert Check PCR Mix 1 (Clontech, Cat. No.630496). Used untransformed Y1HGold colonies as negative controls. Pick one colony for each confirmed bait, and one from the p53-AbAi control, and streak them onto SD/-Ura agar medium. After 3 days at 30℃, store at 4℃ for up to 1 month. For long term storage, growed an overnight culture in YPDA broth, collect the cells by centrifugation, and resuspend them in 1 ml of freezing medium. Quick freezed, and stored at –70℃.

2.4 Testing the bait strain for AbA^r expression

Picked a large healthy colony from the p*CW1*-AbAi bait strains and p53-AbAi control strains, resuspended each colony in 0.9% NaCl and adjusted the OD600 to ~0.002 (for approximately 2000 cells per 100 μl). Plated 100 μl on each of the following media: SD/-Ura, SD/-Ura with AbA (50 ng/ml), SD/-Ura with AbA (60 ng/ml), SD/-Ura with AbA (80 ng/ml), SD/-Ura with AbA (100 ng/ml), SD/-Ura with AbA (150 ng/ml). Allowed colonies grow at 30℃ for 2–3 days, counted the number of colonies on each above media.

3 Results and Discussion

3.1 Constructon and identification of p*CW1*-AbAi bait reporter vector

A 865 bp *MeCWINV1* promoter fragment was isolated from the p*CW1*-19T plasmid (Fig.1a) and inserted into the pAbAi vector to construct the yeast one-hybrid bait vector p*CW1*-AbAi. The recombinant plasmid p*CW1*-AbAi was digested by *Kpn* Ⅰ and *Sal* Ⅰ, approximate 865 bp fragments were obtained, which was consistent with *MeCWINV1* promoters in length (Fig.1b). Sequencing results showed that we obtained the recombinant p*CW1*-AbAi vector successfully.

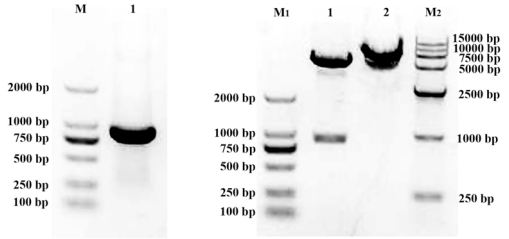

Figure 1. Clone and identification of p*CW1*-AbAi bait reporter vector
(a) The cloning of *MeCWINV1* promoter. M: 2 kb DNA ladder marker; 1: PCR amplification product of *MeCWINV1* promoter. (b) Restriction digestion of p*CW1*-AbAi vector. M1: 2 kb DNA ladder marker; M2: 15 kb DNA ladder marker; 1: p*CW1*-AbAi plasmid digested with *Kpn* Ⅰ/*Sal* Ⅰ; 2: p*CW1*-AbAi plasmid.

3.2 Generating and identifying the bait-reporter yeast strains Y1HGold[*CW1*/AbAi]

The *BstB*I linearized p*CW1*-AbAi plasmid, p53-AbAi positive control plasmid were transformed into Y1HGold yeast strains and selected for transformants on SD/-Ura media. The plasmids confirmed to integrate correctly by using a colony PCR analysis. The primers in the Matchmaker Insert Check PCR Mix 1 were located in the AbA^r gene and in the Y1Hgold genome, downstream of the *URA3* locus. The positive control Y1HGold[p53-AbAi] amplified a region of ~1.4 kb (Fig.2, line 4), each bait strain Y1HGold[*CW1*/AbAi] amplified a ~2 kb band containing 1.35 kb + insert size (865 bp) that encompassed the bait sequence and confirmed the presence of the integrated plasmid (Fig.2, line 1-3). Thus the bait-reporter strain Y1HGold[*CW1*/AbAi] is generated by homologous integration into Y1Hgold. The integrated plasmids are very stable, overnight broth media culturing without *URA3* selection will not result in loss of the integrant.

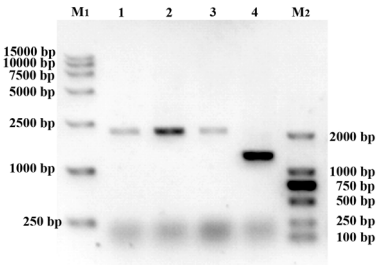

Figure 2. Confirming p*CW1*-AbAi integration by colony PCR
M1:15 kb DNA ladder marker; M2: 2 kb DNA ladder marker; 1~3: PCR product of bait plasmid p*CW1*-AbAi transformed to Y1HGold yeast genome; 4: Positive control p53-AbAi.

3.3 Determining the Minimal Inhibitory Concentration of Aureobasidin A for Y1HGold[*CW1*/AbAi] strains

Successful use of any yeast one-hybrid system depends upon no/low recognition of target sequence by endogenous yeast transcription factors. For this reason, it is critical to test the construct for AbA^r expression before screening the library. The basal expression of the bait reporter stain in the absence of prey can vary, depended on the bait sequences cloned into pAbAi vector. If 1000 ng/ml Aureobasidin A (AbA) does not suppress AbA^r in the absence of prey, the bait DNA sequence is likely being recognized by endogenous yeast transcription factors and therefore the sequence cannot be used for a yeast one hybrid screen. As recorded in the protocol, the positive control Y1HGold[p53-AbAi] has a minimal inhibitory concentration of 100 ng/ml AbA (Fig. 3). Our bait strain Y1HGold[*CW1*/AbAi] plated on SD/-Ura media with 60~80 ng/ml AbA had less clones compared to the control and no clones were appear when the concentration of AbA above100 ng/ml. Therefore, the minimal inhibitory concentration of AbA for our bait strain Y1HGold[*CW1*/AbAi] is 100 ng/ml (Fig. 3). For the follow-up library screening, we can use the minimal concentration of AbA, or a concentration that is slightly higher (by 50~100 ng/ml) to suppress the growth of the bait strain completely.

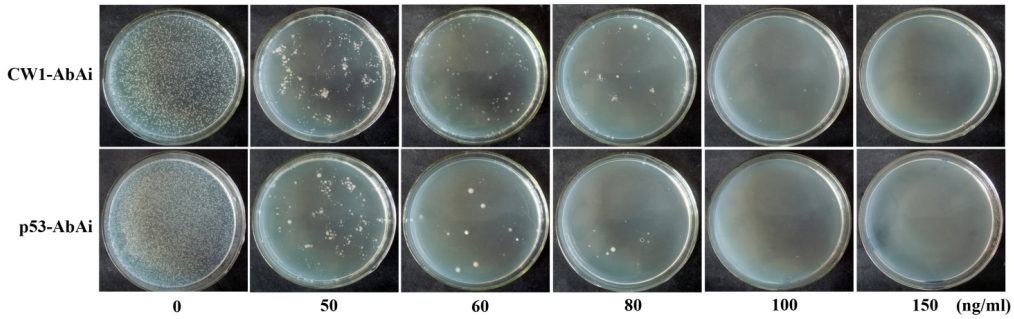

Figure 3. Testing the Y1HGold[*CW1*/AbAi] strain for AbA^r expression on SD/-Leu/AbA (0~150ng/ml) medium

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