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CLONING AND CHROMOSOME LOCALIZATION OF THE LINKED SCAR MARKER (P1-P2$_{762}$) GENE IN EARLY WALNUT *(DODE)*

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Key words: Walnuts, Early-related genes, SCAR marker, Chromosomal localization

Abstract

The objectives of this paper were (1) to clone the closely linked SCAR markers (P1-P2$_{762}$) gene of the Xinjiang early walnut, and (2) *in situ* hybridization analysis of the location of the gene on the chromosome. Shoot tips of Xinjiang early walnut were used (1) to clone full-length cDNA sequences of linked markers SCAR (P1-P2$_{762}$) gene by RT-PCR and RACE and (2) to conduct chromosome sectioning. *In situ* hybridization occurred when the BCIP/NBT color liquid was diluted with 0.01 M TBS at 1 : 20. The full length gene is 1401 bp, with 320 bp containing a maximum of ORF, encoding 106 amino acids. Nucleic acid homology to the gene shows that the homology with the walnut microsatellite (EF408815.1) sequence was 84%, other. The precocious Walnut SCAR marker (P1-P2$_{762}$) gene is closely linked with the precocious, but does not directly control the fruit in it's early stages of development.

Introduction

There are many types of early Xinjiang walnuts. The trees usually begin to bear 2 - 3 years after planting. The early walnut has high heritability (Liu *et al.* 2008, Zhang *et al.* 2003). They are used not only for research and to improve the variety of walnut species, but also to control the quality of the walnuts. This helps to elucidate the mechanisms of perennial fruit trees and flower buds in their various stages of transformation (Zhang *et al.* 2005). Walnut biotechnology research has started in the late 1960s and been continuously expanding (McGranahan *et al.* 1988, Eluch *et al*. 1998, Dandekar *et al.* 1998, McGranahan *et al.* 1990). Molecular markers analysis allows the exhibition of precocious secondary flowering and fruit spike sequence, the presence of rare traits. This accounts for wild forest natural walnuts with consistent performances (Wu *et al.* 2000). Xi Kesheng (1987) found that the precocious walnut is considered as an example of stable inheritance. The dominant trait has more than two pairs of alleles that influence the phenotype of fruit (Cheng and Yang 1987). Fan *et al.* (2001) discovered that traits of the Yunnan and Xinjiang walnut shell, walnut species can be found through F_1 genetic analysis. The main traits are complex polygenic control and quantitative inheritance (Fan *et al.* 2001). Sun and Wang (2002) found that early solid walnuts have a strong heritability. Zhang *et al.* (2001) have seen that many early walnut varieties are hybrids with complex genetic bases. These varieties do not show the growth patterns or the characteristics of larger fruits, but they possess high heritability with a breeding rate between 18.2 and 62.2% (Zhang *et al.* 2001). Due to their complex genetic characteristics and limited resources studies on walnuts are not detailed enough. There is little or no information regarding walnuts on the molecular level. The focus of most previous studies were usually concentrated to walnut cultivation or physiology.

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In this research, an alternative was made to investigate the cloning of genes using the correlation techniques of molecular biology of Xinjiang walnut. This paper has been pre-screened for laboratory-related SCAR marker (P1-$P2_{762}$), verifying the genes according to the partial sequence information obtained. Primers were designed using technology to obtain cDNA RACE SCAR marker (P1-$P2_{762}$) from the complete sequence of the gene. Bioinformatics analysis of their conduct was then performed. We aimed at performing preliminary research chromosomal location and gene prediction in early solid Xinjiang walnuts.

Materials and Methods

The walnut was grown in the standard experimental land of Shihezi University. There were many resources used for our study. The leaves used for RNA extraction were frozen in liquid nitrogen immediately and stored at –70°C for future use. The restriction enzyme BamHI, PstI, M-MLV, 5'/3'-Full RACE Kit and PMD19-T vector were purchased from TaKaRa (Dalian,China), and Taq DNA polymerase was the product of Dongsheng (Guangdong, China). All chemicals were of analytical reagent grade.

Primers used to amplify the full-length sequence of the pre-obtained amplified SCAR marker (P1-$P2_{762}$) gene, primers are needed. Our primers were designed and synthesized by Shanghai Sangon's Biologieal Engineering Technology (Shanghai, China). The sequences of all primers used in this experience are shown in Table 1.

Table 1. Sequence of primers used to amplify genes.

Primer name	Primer sequence(5'-3')
3' - RACE Adaptor	DT region containing TaRaKa unique design and Adaptor Primer section
T_{3-1} (3'-RACE Outer primer)	TACCGTCGTTCCACTAGTGATTT
T_{3-2} (3'-RACE Inner primer)	CGCGGATCCTCCACTAGTGATTTCACTATAGG
T_{5-1} (5'-RACE Outer primer)	CATGGCTACATGCTGACAGCCTA
T_{5-2} (5'-RACE Inner primer)	CGCGGATCCACAGCCTACTGCTGATCAGTCGATG
P1	ACTGGGACTCCAATTGTATC
P2	ACTGGGACTCTCAACTAT
P3 (P1-$P2_{762}$-3'Outer primer)	CTCCCTGCCATCTACTCTC
P4 (P1-$P2_{762}$-3'Inner primer)	TTGCCATCATACCTCTCCAT
P5 (P1-$P2_{762}$-5'Outer primer)	CTGTATGTTGGTTGCATTCAGTCTC
P6 (P1-$P2_{762}$-5'Inner primer)	CAAAAGAACCAGTCAATGATACAAT

The total amount of RNA in the Xinjiang precocious walnut leaves was extracted (Ma 2004). This RNA functioned as a reversed template, with the first strand of cDNA synthesis product serving as a template for PCR amplification. Upstream primers P1 and downstream primer P2 were used to amplify the target gene of middle segment P1-$P2_{762}$ intermediate sequence. The annealing temperature was 56°C. Kit primer T_{3-1} and specific primers P3 that designed according to the known partial sequence were used for the first PCR reaction. Then we used the first PCR production as template to conduct nested PCR for 3 'end sequence amplification of P1-$P2_{762}$ gene. PCR reactions were listed in Table 2. 5'-RACE was conducted using 5'-Full RACE Kit (Takara, Japan). The product was recycled by agars gel recovery kits and connected PMD19-T vector, and then transformed into DH5α competent cells. The recombinants were screeninged by IPTG

inducing and X-ray gal substrate color reaction and microbial PCR. The correctly identified preliminary results were sequenced by Beijing Genomics Technology Co. DNAMAN software, NCBI Blast online tool and other related software for structural analysis and multiple alignments.

Table 2. The condition of PCR amplification.

Amplified fragment	Upstream primer	Downstream primer	Annealing temperature(°C)
Intermediate sequence	P1	P2	56
P1-$P2_{762}$-3' sequence	P3 P4	T3-1 T3-2	58
P1-$P2_{762}$-5' sequence	T5-1 T5-2	P5 P6	57

Using the shoot tips of Xinjiang precocious walnuts as materials, we produced chromosome slices synthesized with PCR probes. The PCR reaction program had the following conditions: 95°C 4 min 94°C denaturation 45s, 58°C / 55°C annealing 45s, 72°C extension 90s, 72°C extension 7 min, 32 cycles, and 4°C insulation. It was then diluted with 0.01M TBS BCIP / NBT color liquid (1 : 20) using *in situ* hybridization.

Results and Discussion

The 800 bp amplification products were preliminarily judged as the purposed fragment (Fig. 1 B). The 3' end and 5' end sequence were obtained by 3' RACE and 5' RACE PCR reaction. The fragments were 500 and 600 bp, respectively (Fig. 1 A, C). The three fragments were assembled into a 1401 bp length of cDNA sequence including an open reading frame from 816-1136 bp in total, 106 amino acids were encoded. The molecular weight and isoelectric point were 12.109 KDa and –7.07, respectively. Both the genes and the amino acid sequences are shown in Fig. 2. The BioEdit was performed analysis using the encoded amino acid sequences. Of the 106 encoded amino acids, 13 were that are strongly acidic (Fig. 2 D, E), 31 strongly basic (Fig. 2 K, H, R), 48 hydrophobic (Fig. 2 A, I, L, F, W, V, P, M) and 39 neutral hydrophilic (Fig. 2 G, S, T, C, Y, N, Q). These types of amino acids were accounted for 12.25, 10.37, 36.79, and 45.28%, respectively of the total amount.

The BLAST analysis results showed that the cDNA sequence of early solid walnut SCAR marker (P1-$P2_{762}$) had 84% homology with walnut microsatellite gene sequence (EF408815.1), while less homologous with other genes.

The results of protein homology and nucleic acid homology alignment were more consistent, however have not yet been found the presence of protein at a highly homologous rate. Only the higher rate of a homologous protein with an unknown function was observed. BioEdit software has analyzed the primary structure of the protein. Figure 3 showed that the ratios of amino acid (water soluble) chains were encoded by few hydrophobic amino acids (about 10 at the N terminal). This is due to the presence of a small amount of hydrophobic intermediate amino acids at the interior part and the C-terminal hydrophilic amino acids mainly of on the surface of molecule. The predicted amino acid sequence revealed the secondary structure (Fig. 4). It was mainly composed of α- helix, extended strand and random coil structure. Three α- helix of 22 amino acid residues were distributed throughout the protein sequences.

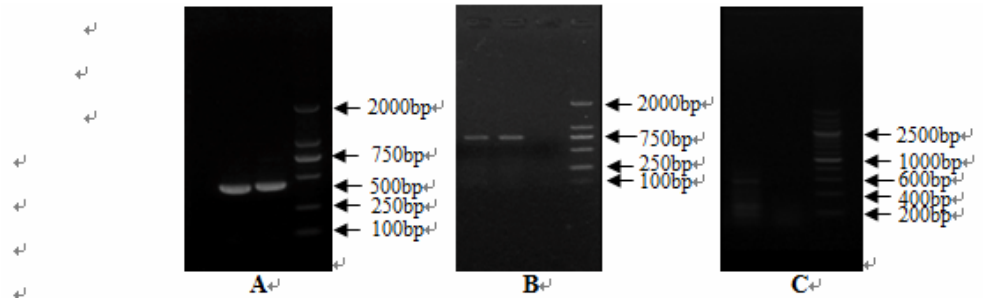

Fig. 1. The PCR result of P1-P2$_{762}$ partial sequence A. 3'-RACE; B. Partial sequence; C. 5'-RACE.

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GAAAATACGGAAATTAGCAACCTGCTCTTGATACCATGTCAAATTTGTGAAAATGGTCGA
ATGCTTGGCCTTGAGAATTCCGTATATTATATAATCTTTACAAAGCAATTCTATACCTGG
AAATAAATAATGTATACGCCTAATTCTAGCCCTATACATGGAAGGTATAATCTGTCAAAT
AATAACTAGAGAAATAAGGAAAGAGTTGTATAAGGAAAGAATAATGAACAATATGAAAAC
AACGTTGTCCATTGATAGATACATCATATGATAAGGATAATGGTAGGTAGATTACTTATA
CAAAAAAAGATAAGGGTAGGTGGTGTATGAGATTTCACATTGATTGGGAAGGAGAAGTTC
TTGCTCTTTATAAAGTTCCAATGGGGCTCCAATTGTATCATTGACTGGTTCTTTTGGAGT
ATAAGTCATGTGGTTTGGGTCTTCCATTATGGCGTTACAACTTACCTTAATTTTGTTGTT
GAGACTGAATGCAACCAACATACAGAATGGTAAAATCCTAAAAGGAAACATAGCATTAAT
TACTTAATGCGACATTTGCAAAGACCCAAGCTTCTGATTTTGAAACCAATGACCATCATC
ATCCTATATGTCAACCATTGATATAGAATTGTCAAGTTGAGAGGCAGGTGGGGTTTTAGT
TCAACTTCATTTTTAACTATGTTCGTGCACTGATGCTTCTATTACCTATTGGTTTTATTT
TGTTTTTGGGAAAGTTATGGGAAATCCTTTGATATATCGCTGGCCCTCTTTGAATACTCA
GCTTCCCTGAAGTATGAAACTATGTGGATGACGTAATGACAACAATATCTACCTCCCTGC
                                  M  T  T  I  S  T  S  L
CATCTACTCTCACTCTCAGGAGGGCTGCACACGCCTCGGTTTATGACAAGAACGAGGAGG
P  S  T  L  T  L  R  R  A  A  H  A  S  V  Y  D  K  N  E  E
ACCACACTCCGAGCGTTGTCCCGGACCATGTAATTCAGCCCCAGTCTGAAACCTACTGGG
D  H  T  P  S  V  V  P  D  H  V  I  Q  P  Q  S  E  T  Y  W
CTCCACAAACCGACCCTCGGGTATTTGGTCCCCCAGCTGAGCAAAACTCAGCTGCAGCCA
A  P  Q  T  D  P  R  V  F  G  P  P  A  E  Q  N  S  A  A  A
GTGAGAGTGGCCTCCCCTCTTCGACCGTGGATGGTGGTGAGGGGTCTGTGCTTGAACAGA
S  E  S  G  L  P  S  S  T  V  D  G  G  E  G  S  V  L  E  Q
AGGCCTGGTTTCGCCATACCAACGTTGAGGACTCGGAGAAGCCTCATACCCTCTGACCAC
K  A  W  F  R  H  T  N  V  E  D  S  E  K  P  H  T  L  *
CACAACAAACACAATAAGGTCAAGCTGGAACCGCAATATATAAGTAACTAAAATAAGTAT
AGGCTTGGCGTATGTATGCGGCTGGCTTATGTAGTAGTATTTTGGTGAGACAAATGACAC
TGGCTTTTATGTTCATGGTATAGCAGAAAATGTATTTCATTATGGAACTCGCATATCCTG
TTAATATGTCTGTATGGTTGTGCTTTGTGTTTGCACACAAGTTATTTGCTATAAAAGGCT
TGGTTATGTTAAAAAAAAAAA
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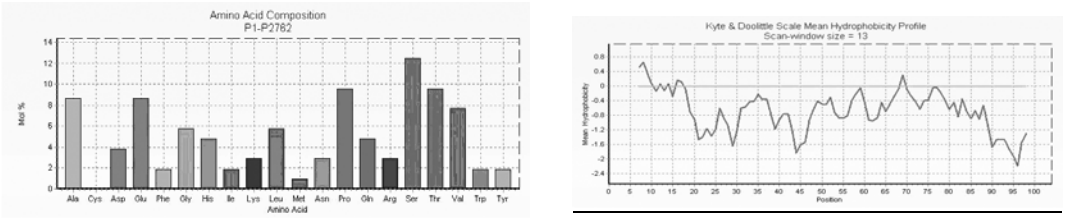
Fig. 2. The nucleotide and amino acid sequence of P1-P2$_{762}$ related to precocious character of walnut in Xinjiang.



A B

Fig. 3. Predicted hydropathy plot and composition of deduced amino acid of P1-P2$_{762}$ A. Composition of amino acid; B. Hydropathy plot.

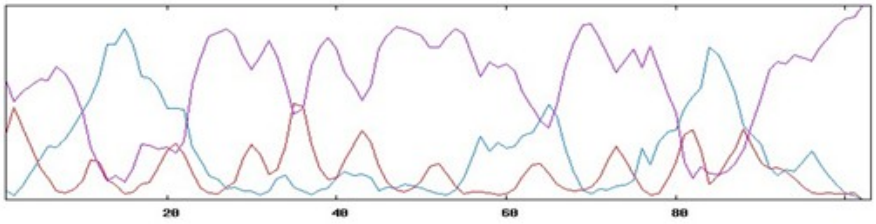


Fig. 4. Predicted secondary structure of deduced amino acid of P1-P2$_{762}$ (The helix, strand and coil were indicated, respectively with blue, red and pink lines).

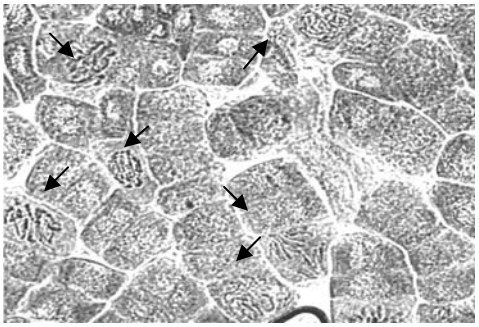


Fig. 5-1. Acid hydrolysis method to obtain chromosome wall sections.

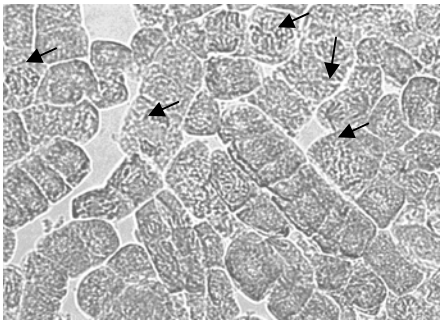


Fig. 5-2. Hypotonic method to obtain chromosome wall sections.

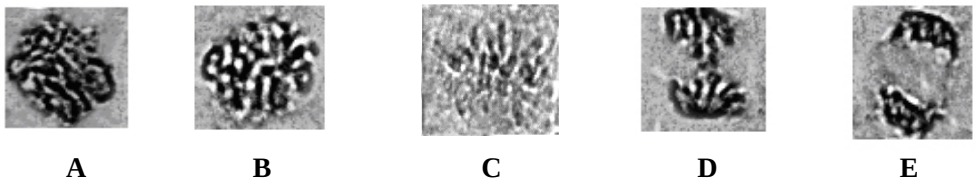


Fig. 5-3. Slices of chromosomes during division:
A. Prophase; B. Pro-metaphase; C. Metaphase; D. Anaphase; E. Telophase.

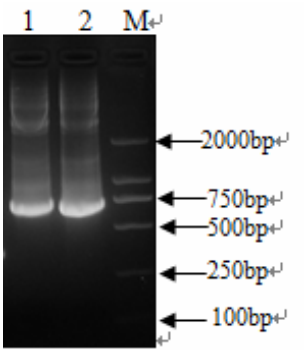


Fig. 6. The result of P1-P2$_{762}$ probes marked with Dig 1. P1-P2$_{762}$ probe;
2. P1-P2$_{762}$ Comparison; M=Marker (GM335).

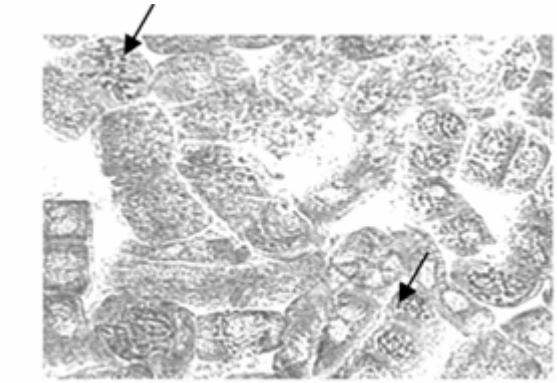
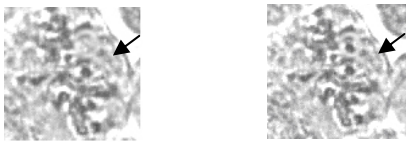


Fig. 7-1. The GISH result of P1-P2$_{762.}$



A B

Fig. 7-2. Comparing the GISH result of P1-P2$_{762}$
A. Before hybridization; B. After hybridization.

The chromosome sectioning was obtained through the wall of low permeability method and the wall of acid solution method (Fig. 5-1, 5-2, 5-3). Using the PCR method, two walnut samples with gene probe sizes 762 bp and 377 bp were used to meet the requirements of *in situ* hybridization tests. The probe results were obtained and displayed in Fig. 6. Samples of root tip and shoot tip of Xinjiang precocious walnut were used for *in situ* hybridization. Digoxigenin-labeled P1-P2$_{762}$ was used as probe. The section of chromosome with precipitation signals was found (Fig. 7-1), revealing the preliminary results of *in situ* hybridization of these two genes. Fig. 7-2 showed a comparison of the results before and after hybridization.

In this study, the gene P1-P2$_{762}$ was obtained by RAPD and AFLP markers tags related precocious screened between early and late Walnut real gene pool. Using the RT-PCR and RACE method, we successfully synthesized full-length cDNA sequences of P1-P2$_{762}$ gene. The largest open reading frame (open reading flame ORF) was analyzed by DNAMAN software. It encoded 106 amino acids. The molecular weight was 12.109 KDa, isoelectric point was –7.07. Protein kinases are large families of enzymes that take part in eukaryotic cell-mediated responses to external stimuli, regulating cellular metabolism, growth, proliferation and differentiation. This allows the gene to be studied as a breakthrough.

In situ hybridization technique (Zhang 2005) has been played an important role in gene location, genetic map rendering and more research domains. Zhang (2005) successfully located on chromosome 9 in tea tree such as coffee synthetase gene by using the technologies. Rayburn and Gill located in the wheat chromosomes with biotin labeled rye repeated sequences of DNA probe. (Rayburn *et al.* 1985). Hanson and Gomez successfully copied different chromosomes in cotton and grain sorghum by BAC - FISH and YAC - FISH technology (Hanson RE *et al.* 1995).

The wall of hypotonic and acid hydrolysis method were both used for *in situ* hybridization. The latter could obtain a little more chromosome sectioning phase. The reason might be duo to the inaccurate time of enzyme reaction that caused a certain degree of damage on chromosome. During acid hydrolysis method care should be taken to remove hydrochloric acid and clean the preparation before dyeing.

The sizes of were 762 bp and 377 bp in order to meet the requirements for *in situ* hybridization experiments. In this study, only a preliminary hybridization signals was found, however, complete knowledge about the localization of these genes on walnut chromosomes is still limited. Therefore, further study is needed to demonstrate the precise positioning of these genes on walnut chromosomes.

Acknowledgements

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