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Full Length Research Paper

Cloning and sequence analysis of the *defective in anther dehiscence1* (*DAD1*) gene fragment of Chinese kale

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To clone the *defective in anther dehiscence1* (*DAD1*) gene fragment of Chinese kale, about 700 bp product was obtained by PCR amplification using Chinese kale genomic DNA as the template and a pair of specific primers designed according to the conserved sequence of *DAD1* genes of *Arabidopsis thaliana* and *Brassica rapa*. The amplified product was ligated into the T vector and sequenced. The results show that the gene fragment was 678 bp long without introns. It shared 89% identity with the nucleotide sequence of the *DAD1* gene of *A. thaliana* and the sequence identity was as high as 97 to 99% with those of other plants belonging to the same genus as Chinese kale. The amino acid sequence deduced from the nucleotide sequence had 91% identity with that of *A. thaliana*. It was shown that the cloned fragment was a part of Chinese kale *DAD1* gene.

Key words: Chinese kale, *Brassica oleracea* var. *alboglabra*, *defective in anther dehiscence1* (*DAD1*), gene clone.

INTRODUCTION

Recent studies show that jasmonates are related to plant pollen development (Park et al., 2002; Mandaokar et al., 2003; Song et al., 2011). It was found that jasmonic acid cannot be normally synthesized in male sterile *Arabidopsis* plants, which are the mutants losing ones of key enzymes in the synthesis of jasmonic acid (McConn and Browse, 1996; Stintzi and Browse, 2000; Ishiguro et al., 2001). Ishiguro et al. (2001) got a male sterile *Arabidopsis thaliana* mutant by T-DNA insertion, showing defects in anther dehiscence. It was named *defective in anther dehiscence1* (*DAD1*) mutant. The defects could be rescued by the exogenous application of jasmonic acid or linolenic acid. Further studies showed that the *DAD1* gene tagged with T-DNA encoded a phospholipase A1, which catalyzed phospholipids into linolenic acid that was the initial step in jasmonic acid biosynthesis. Hatakeyama et al. (2003) used antisense inhibition of the nuclear gene, *BrDAD1*, in *Brassica rapa*. Three plants transformed with the antisense gene showed a defect of anther dehiscence at the flower bud opening stage and produced inviable pollen. The male sterile and flower-opening phenotypes were also rescued by the application of jasmonic acid as well as linolenic acid. Furthermore, all these characteristics could be inherited by the next generation. Chen et al. (2010) used broccoli as the material to study the same gene and obtained similar results. These results demonstrate a novel control system for hybrid seed production by the use of nuclear genes.

However, few researches have been done on other plants. Chinese kale (*Brassica oleracea* L. var. *alboglabra*) is an annual and biennial herb of the genus *Brassica* (*Cruciferae*), belonging to cole vegetable crops. In this study, primers were designed according to the conserved sequence of the known *DAD1* gene of *Arabidopsis thaliana* and *B. rapa* for amplification, followed by obtaining the corresponding region of the gene of Chinese kale and then, its sequence was analyzed, which lay the foundation for its use in creating male sterile materials of Chinese kale.

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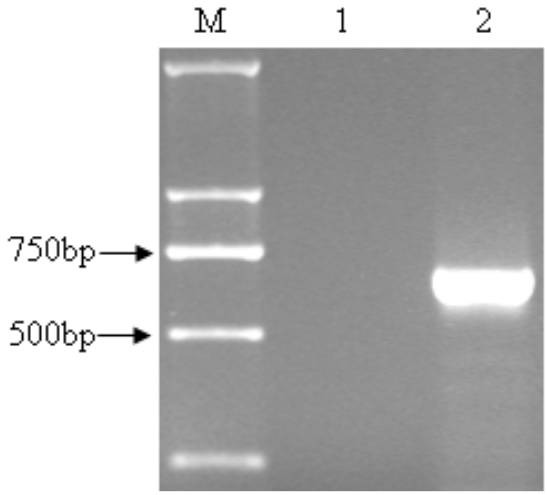

Figure 1. PCR amplification. M, DL2000 Marker; 1,CK$^-$ (No template); 2, the product amplified from genomic DNA of Chinese kale.

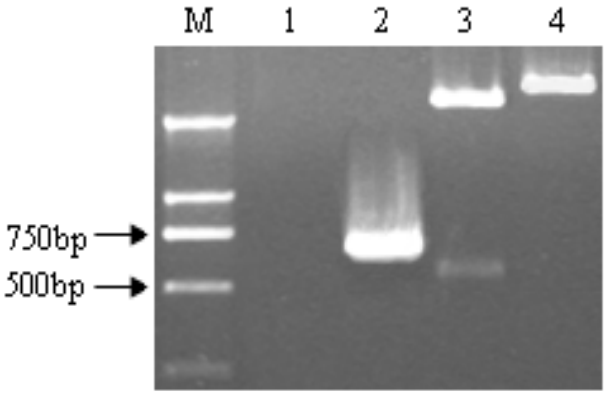

Figure 2. Identification of the recombinant by PCR and enzyme digestion. M, DL2000 Marker; 1, PCR of the plasmid from a blue colony; 2, PCR of the plasmid from a white colony; 3, enzyme digestion of the plasmid from a white colony; 4:,enzyme digestion of the plasmid from a blue colony.

MATERIALS AND METHODS

Chinese kale (*B. oleracea* L. var. *alboglabra* cv. Zhongchi in Guangdong, China) was used in DNA extraction. Vector pMD19-T (D102A; Takara, Dalian, China) and *Escherichia coli* DH5α were used to clone the gene fragment.

Primers design

A pair of specific primers was designed according to *DAD1* gene conserved sequence of *A. thaliana* (accession no. AB060156) and *B. rapa* (accession no. AB073401) deposited in the GenBank database. The upstream primer: 5'-GTCATCTCCTCCCGT GGAACC-3' and the downstream primer: 5'-GAATGGACACGT GGAGCTCAC-3' were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd.

PCR amplification

Genomic DNA was extracted from fresh leaves of Chinese kale using the procedures described by Chen and Ronald (1999). The PCR components contained: sterile distilled water (19.8 µl), 10×buffer (2.5 µl), dNTPs (10 mmol/L, 0.5 µl), upstream primer (10 µmol/L, 0.5 µl), downstream primer (10 µmol/L, 0.5 µl), template DNA (1 µl) (20 to 50 ng), TaqDNA polymerase (5 U/µL, 0.2 µl). The samples were denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and then final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis.

Cloning of the gene fragment

The purified fragment was cloned into a pMD19-T vector (Takara, Dalian, China). After transformation into DH5α competent cells, ten white colonies were randomly selected and their plasmids were extracted by the alkaline lysis method (Sambrook and Russell, 2001). The DNA inserts of the recombinant clones were amplified by PCR with the primers used earlier, and were then assayed by double digestion with *Eco*RI and *Hin*dIII. The positive clones were sequenced at Sangon Biotech (Shanghai, China) Co., Ltd.

Sequence analysis of the gene fragment

Searching of similar sequences was performed using blastn in the NCBI website (http://www.ncbi.nlm.nih.gov). A phylogenetic tree based on nucleotide sequences was constructed by DNAman software (Lynnon Biosoft, Canada).

RESULTS

PCR amplification

Using Chinese kale genomic DNA as the template and a pair of specific primers designed, about 700 bp product was obtained by PCR amplification (Figure 1), which is consistent with the expected fragment length.

Identification of the recombinant

PCR and enzyme digestion detection of the recombinant is shown in Figure 2. PCR produced the same long fragment as the expected one, while the negative control was unable to amplify any fragment. The digested fragment length was less than that of the PCR product, in which there might be *Hin*dIII or *Eco*RI site, confirmed by the following sequence analysis. It was shown that the gene fragment had been ligated into pMD19-T.

Sequence analysis of the gene fragment

The sequencing results show that the amplified fragment length was 678 bp (GenBank accession no. FJ648777). Its nucleotide sequence is shown in Figure 3. After sequence alignment by blastn analysis in the GenBank

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1    GTCATCTCCTCCCGTGGAACCGCCACGTGTTTCGAGTGGTTAGAGAATCTCCGCGCCACG
1     V  I  S  S  R  G  T  A  T  C  F  E  W  L  E  N  L  R  A  T
61   CTGACTCATCTCCCTGATGGGCCGAGTGGACCTAATCTAAACGGGTCTAACTCTGGGCCC
21    L  T  H  L  P  D  G  P  S  G  P  N  L  N  G  S  N  S  G  P
121  ATGGTCGAAAGTGGATTCTTAAGCTTGTACACATCAGGGGCCCACAGTTTGAGAGACATG
41    M  V  E  S  G  F  L  S  L  Y  T  S  G  A  H  S  L  R  D  M
181  GTAAGACAAGAGATCTCGAGACTGCTCCAGTCTTACGGCGACGAGCCGTTGAGTTTAACG
61    V  R  Q  E  I  S  R  L  L  Q  S  Y  G  D  E  P  L  S  L  T
241  ATAACGGGGCACAGTCTCGGCGCTGCGATCGCGACGTTAGCGGCGTACGATATCAAGACG
81    I  T  G  H  S  L  G  A  A  I  A  T  L  A  A  Y  D  I  K  T
301  ACGTTTAAACGTGCGCTGATGGTCACCGTTATGTCTTTCGGAGGTCCACGTGTCGGAAAC
101   T  F  K  R  A  L  M  V  T  V  M  S  F  G  G  P  R  V  G  N
361  AGATGCTTCAGGAGACTCCTTGAGAAGCAAGGCACCAAGGTGTTGAGGATCGTTAACTCC
121   R  C  F  R  R  L  L  E  K  Q  G  T  K  V  L  R  I  V  N  S
421  GACGACGTCATCACCAAAGTTCCAGGTGTCGTTTTAGATAACCGAGAGAAAGATAACGTG
141   D  D  V  I  T  K  V  P  G  V  V  L  D  N  R  E  K  D  N  V
481  AAGATGACGGCGTCAATGCCGAGCTGGATACAGAAACGAGTGGAGGAGACGCCGTGGGTT
161   K  M  T  A  S  M  P  S  W  I  Q  K  R  V  E  E  T  P  W  V
541  TACGCTGAGGTCGGGAAAGAGCTTCGGCTGAGCAGCCGTGACTCTCCGTACCTGAACGGC
181   Y  A  E  V  G  K  E  L  R  L  S  S  R  D  S  P  Y  L  N  G
601  ATCAATGTTGCCACGTGTCACGAGCTGAAGACGTATCTACATTTAGTAGATGGGTTTGTG
201   I  N  V  A  T  C  H  E  L  K  T  Y  L  H  L  V  D  G  F  V
661  AGCTCCACGTGTCCATTC
221   S  S  T  C  P  F
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Figure 3. Nucleotide sequence of the cloned fragment and the putative amino acids sequence.

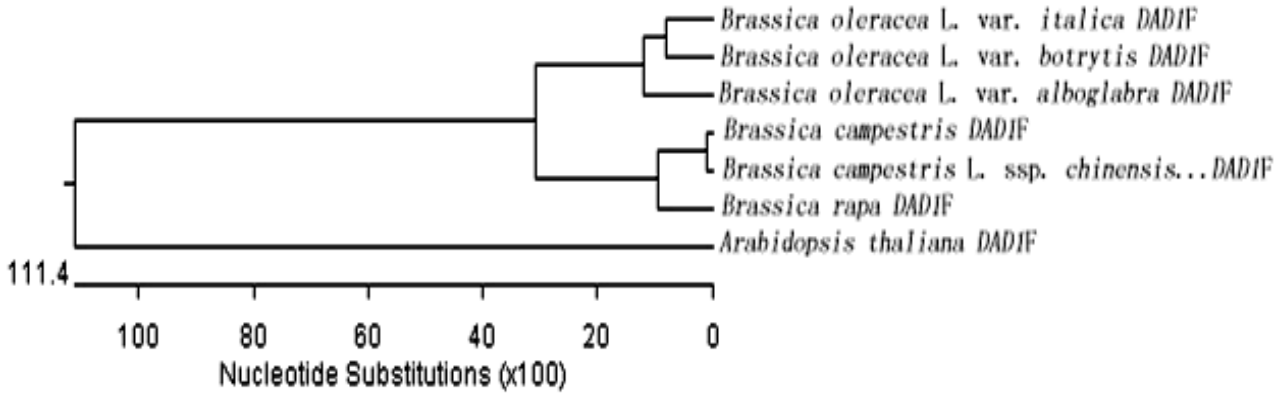

Figure 4. A phylogenetic tree for seven species based on *DAD1* gene fragment sequences.

database, it was found that it shared 89% identity with the nucleotide sequence of the *DAD1* gene of *A. thaliana* and the identity was as high as 97% with that of *B. rapa* belonging to the same genus as Chinese kale.

According to known *DAD1* gene fragment (*DAD1*F) sequences, the phylogenetic tree for seven species was constructed. As shown in Figure 4, *Brassica oleracea* L. var. *italica*, *B. oleracea* L. var. *botrytis* and Chinese kale were first clustered together, followed by *Brassica campestris*, *Brassica campestris* L. ssp. *chinensis* var. *utilis* Tsen et Lee and *B. rapa*, reflecting their genetic relationship.

The amino acid sequence deduced from the nucleotide sequence in Figure 3 shared 91% identity with that of *A. thaliana*, including the lipase active site characterized by a GHSLG motif and the catalytic triad S85, D142 and H207 (or H214). As a result of high sequence identity, they were identified as homologous genes, and the fragment obtained was a part of Chinese kale *DAD1* gene sequence.

DISCUSSION

Cloning and sequence analysis of the gene fragment

Since Ishiguro et al. (2001) first reported the *DAD1* gene, related studies have been rarely done. According to the conserved region of reported genes in *A. thaliana* and *B. rapa* to design primers, a specific gene fragment was successfully amplified from Chinese kale. Its nucleotide and putative amino acid sequence identity is high with that of *A. thaliana*. It contains no introns, as reported previously, sequence comparison between the genomic and cDNA clones revealed that the *DAD1* gene of *A. thaliana* had no introns. In addition, using the same method, we have also cloned related gene fragments from *B. oleracea* L. var. *botrytis*, *B. oleracea* L. var. *italica* and *B. campestris* L. ssp. *chinensis* var. *utilis* Tsen et Lee; the nucleotide sequence identity was as high as 97 to 99% with that of Chinese kale. The relationship revealed by the phylogenetic tree based on the nucleotide sequences is consistent with the actual genetic relationship among these species, which indicates that *DAD1* genes have relative conservatism in closely related plants and there is a co-evolution between the genes and the species with different degrees of variation. The authors conclude that they are homologous genes and the cloned fragment is a part of Chinese kale *DAD1* gene.

The use of the *DAD1* gene

DAD1 encodes a phospholipase A1 that catalyzes the initial step of jasmonic acid biosynthesis. The gene silencing can cause defects in anther dehiscence, pollen maturation and flower opening, which can be rescued by the exogenous application of jasmonic acid or linolenic acid (Ishiguro et al., 2001). It offers the possibility to create adjustable male sterile plants through genetic transformation. The cloned fragment in this study can be used to construct the antisense expression vector or RNA interference vector for genetic transformation of Chinese kale to inhibit the expression of the *DAD1* gene and develop male sterile materials.

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