A novel mutation for low erucic acid found in *Brassica napus cv.* Zhongshuang No.9: gene cloning and molecular characterization

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Abstract

The *FAE1* genes isolated from Zhongshuang 9 both harbored a deletion of 4 nucleotides between T1366 and G1369, leading to a frameshift mutation and the premature stop of translation after 466 amino acid residues. This was evidenced and supported by heterologous expression of *FAE1* genes in yeast and immunoblot analysis. A RT-PCR and Western blot experiments with the developing seeds of Zhongshuang 9 indicated that the frameshift *FAE1* genes could be transcribed normally but failed to translate proteins to form a functional complex. It is considered that Zhongshuang 9 is the second source of LEA gene following the first finding of the LEA feed rape variety LIHO in 1960s.

Key words: Oilseed rape, low erucic acid mutation, *Fatty acid elongase 1* gene, RT-PCR,Yeast expression

Introduction

The first variant with low erucic acid content (LEA) was found in 1960s from a feed rape cultivar called LIHO[1]. The first LEA oilseed-rape cultivar ORO was bred by using the LEA LIHO as parental material [1, 2]. Since then almost all of the LEA rapeseed cultivars have been developed by traditional crossing method with the LEA gene source from ORO[3]. No other mutant for LEA content in rapeseed (*Brassica napus*) has been reported.

LEA trait of rapeseed of ORO origin was attributed to a point mutation of fatty acid elongase 1 (FAE1) gene from cytosine to thymine at the base site 845, causing substitution of a single amino acid residue from serine to phenylalanine at position 282 of the coded protein [4, 5]. In this paper we report another LEA mutation, which showed different characteristics of FAE1 genes from ORO and its derivatives. This was evidenced and supported by a transcriptional analysis, heterologous expression of *FAE1* genes in yeast and immunoblot analysis.

Materials and methods

FAE1 genes were isolated from LEA rapeseed Zhongshuang 9, LEA rapeseed Zhongshuang 6 and HEA rapeseed Zhongyou 821.

Genomic DNA was isolated from leaves of *B. napus* according to the protocol described by Saghai-Maroof et al. The FAE1 genes were amplified from the genomic DNA with KOD Plus kit (Toyobo, Osaka, Japan) using the primers FAE1F (5′ ATCGGATCCATGACGTC CGTTAACGTAAAGCTCCTT 3′) and FAE1R (5′ ATCGAATTCTTAGGACCGACCGTTTTGGACA 3′). PCR products were purified with QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The FAE1 genes from different sources were subcloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA, USA) through BamHI/ EcoRI restriction enzyme sites.

The sequenced *FAE1* genes were subcloned from pZErO-2 into the pYES2 NT C vector (Invitrogen, Carlsbad, CA, USA). The pYES2 harbored different FAE1 isoforms were transformed separately into the yeast stain InvSc1 (Invitrogen, Carlsbad, CA, USA) using a lithium acetate procedure according to the manufacturer's instruction.

Transformed yeast cells were cultured in liquid SC-ura supplemented with 2% (w/v) glucose and shaked overnight at 28 °C, then these cultures were shaked at 20 °C continuously to an OD_{600} of 1.5. Each yeast cells leavening was divided into two parts equally for immunoblot and lipids analysis.

Yeast homogenates were prepared by the method of Tillman and Bell [6]. The supernatant was collected by brief centrifugation to remove the unbroken cells and cells fragments. The microsomal membrane pellet was recovered by ultracentrifugation at 100,000 g for 60min [5]. And the pellet was suspended angain and purified with His·Bind resin (Novagen, Darmstadt, Germany) as described [7].

The purified proteins were separated on a 10% SDS-PAGE gel [8]. For Western blot analysis, proteins were transferred to nitrocellulose membrane by electrophoretic transfer with mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. Western blot analysis was performed according to the standard protocols [9]. And the protein bands were detected using the anti HisG antibody (Invitrogen, Carlsbad, CA, USA) followed by alkaline phosphatase-conjugated goat anti-mouse IgG (SABC, Luoyang, China) and color development.

Yeast cell fatty acid methyl esters (FAMEs) were prepared by the method of Katavic et al [5,6]. GC analysis was performed on a gas chromatogram (Agilent 5890N) fitted with a 30m FFAP column, ID 0.25 mm narrowbore, film thickness 0.5 μm.

The developing seeds of *B. napus* cv. Zhongyou 821, Zhongshuang 6 and Zhongshang 9 were collected at 30 DAF. The microsome was prepared according to the method described by Puyaubert et al., 2001 [10]. The microsomal proteins were separated by SDS-PAGE. And with the help of the rabbit-anti-FAE1 polyclonal antibody, the FAE1 proteins were detected by Western blot as described above.

Results

FAE1 putative genes were isolated from the HEA Zhongyou 821, LEA Zhongshuang 9 and Zhongshuang 6. The size of the products generated from PCR amplifications of genomic DNA was about 1.5 kilo base pairs (kb). Each genotype produced two isoforms of *FAE1* gene according to their sequence characteristics, corresponding separately to A-genome and C-genome [11]. Sequences of the *FAE1* isoforms from Zhongshuang 9 were delivered to Genbank with accession number AY888037 and AY888044.

After alignment analysis of the isoforms with those data from Genbank, 61 SNPs were detected in the coding sequences and 25 mutations in the deduced amino acid sequences. It was shown that Zhongshuang 9 harbored a deletion of 4 base pairs (TCAG) between T1366 and G1369 in its *FAE1* genes both in A and C genome(Fig. 1). This deletion led to a frameshift mutation and a truncated protein with 466 amino acid residues in contrast to 506 amino acid residues of HAER Zhongyou 821 and LEA rapeseed Zhongshuang 6. At position 282 of putative animo-acid sequence, a serine (Ser) residue was found in HAER Zhongyou 821 and a phenylalanine (Phe) was in LEA rapeseed Zhongshuang 6 in agreement with the characteristics reported with HAER and LEA rapeseed cultivars, respectively [4]. In Zhongshuang 9, however, a Ser residue was found at the position 282 in deduced amino acid sequence instead of Phe in the C-genome *FAE1*, which was against the results reported so far in other LEA rapeseed varieties[4].

Fig. 1. Multiple sequence alignment of the *FAE1* genes from different species.

The fused *FAE1* from *B. napus* cv. Zhongshuang 9 migrated faster than those from cv. Zhongyou 821 and cv. Zhongshuang 6, indicating that the fused *FAE1* genes from *B. napus* cv. Zhongshuang 9 encoded smaller proteins (Fig. 2).

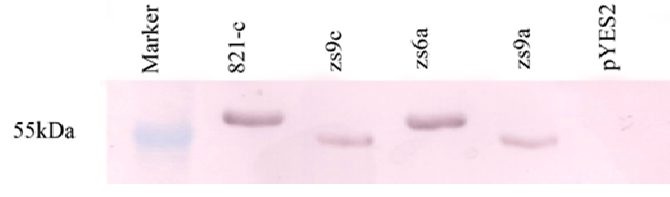

Fig. 2. Immunodetection of his-tag fused FAE1 protein expressed in yeast cells.

Discussion

Zhongshuang 9 is one of the most popular LEA rapeseed cultivars in China, which was developed by multiparental crossing and by microspore culture. Two *FAE1* genes isolated from this cultivar both showed 4 base pairs deletion between T1366 and G1369, leading to a frameshift mutation and the premature stop of translation after 466 amino acid residues (Fig.1). This was evidenced and supported by heterologous expression of *FAE1* genes in yeast and immunoblot analysis (Fig. 2).

Zhongshuang 9 differs in *FAE1* genes from lines of ORO origin either in characterization of DNA sequence or in their encoded proteins. Heterologous expression of *FAE1* mutations in yeast cells and immunoblot analysis confirmed that *FAE1* genes could be expressed and the FAE1 proteins encoded by the *FAE1* gene with the deletion were relatively smaller in

molecule weight in agreement with the result of sequence analysis. Yeast cells which normally don't produce erucic acid formed erucic acid with the functional *FAE1* gene introduced from HEA Zhongyou 821, in agreement with the findings of other researchers [4, 5, 7] but didn't form erucic acid with the *FAE1* gene either from LEA Zhongshuang9 or from Zhuangshuang 6. This result confirmed that LEA trait is really dependent on *FAE1* gene and the *FAE1* gene with either the point mutation or the four-base pair deletion or both lose its function.

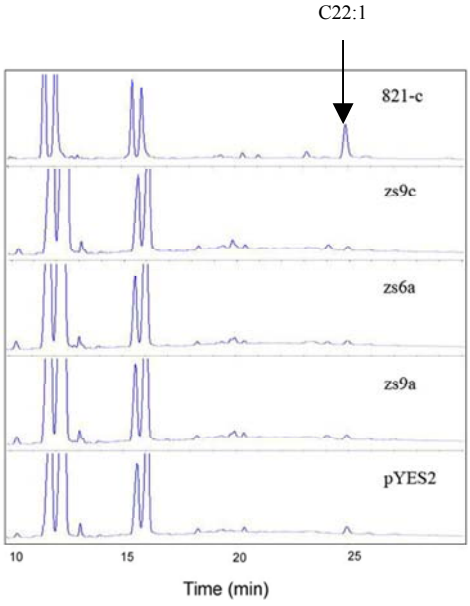

Fig. 3. GC chromatogram of FAMES showing fatty acid profiles from yeast cells transformed with his-tag fused FAE1 genes.

In conclusion, we isolated two mutated *FAE1* genes from the LEA rapeseed cultivar Zhongshuang 9. One of the isolated genes might be derived from the deletion of *FAE1* gene from C-genome of HEA Zhongyou 821 and another from A-genome of LEA parent Zhongshuang 4 and intergenomic exchange within the *FAE1* gene with the deleted *FAE1* gene from HEA Zhongyou 821. The mutated genes could transcribe normally and encode shortened proteins which are inactive and unstable in plants. The C-genome *FAE1* gene in Zhongshuang 9 was thought to be resulted independently from the origin of LEA ORO, and therefore may serve for a novel source in LEA rapeseed breeding. Due to double defects, the genes in Zhongshuang 9 should be lower in possibility of reverse mutation to HEA trait than ORO's LEA *FAE1* genes. This aspect is beneficial in keeping LEA quality of a LEA cultivar with long generations.

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