Improvement of erucic acid level in *Brassica napus* L. Yudal

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Abstract

The improvement of erucic acid content in high erucic acid rape (HEAR) varieties is one of the important research parts, as increasing of their value as an industrial lubricant. Yudal is possible to be used to high eucic acid rapeseed variety in Korea. KCS (3-ketoacyl-CoA synthase) had been proposed as a candidate gene for explaining erucic acid level in rapeseed. To make the Yudal having the erucic acid level more than usually, we have found many fatty acid elongases with database search in *Brssica napus*, and then divided into two groups by DNA sequence homology. One of these groups were contained the FAE genes of *B. napus* HEAR Hero and *B. olearcea* high erucic acid ME103 variety as well as the partial FAE1-1 and 1-2 genes of Yudal. On basis of their DNA sequence, FAE1 cDNA about 1.5kb (YFAE1) has been able to be obtained from Yudal leaves by PCR. This gene have the 2 transmembrane regions predicted by the TMHMM2 program, and is expressed in all organ by RT-PCR, especially high in silique before harvest, and in the petal and calyx among flower organs. Green fluorescent protein (GFP) constructs under Arabidopsis SEN1, or BCB promoter was transformed transiently to rapeseed protoplast to choose the promoter working in rapeseed well besides 35S promoter. These SEN1 and BCB promoters were showed the similar GFP level to 35S promoter. So, we are transforming Yudal with the YFAE1 over-expressing construct under BCB promoter to make the improvement of erucic acid level

Key words: Brassica napus Yudal, erucic acid, FAE1, overexpression, transformation

Introduction

High erucic acid *Brassica napus* cuitivars (HEAR) have regained interest for industrial purposes because erucic acid and its derivatives are important renewable materials utilized in the manufacture of plastic films and in the lubricant and emollient industries. So many researchers have studied about the change of fatty acid composition and contents in HEAR as well as canola. Recently , progress in elucidating the organization and control of very-long-chain fatty acids (VLCFs) biosynthetic pathway has been achieved by cloning genes from different plants encoding β-ketoacyl-CoA synthase (KCS)(James *et al*., 1995; Lassner *et al*., 1996; Clemens and Kunst, 1997; Barret *et al*., 1998; Fourmann *et al*., 1998; Han *et al*., 2001; Hann *et al*., 1998; Millar *et al*., 1999; Todd *et al*., 1999; Venkateswari *et al*., 1999; Pruitt *et al*., 2000). In case of *Arabidopsis FAE1* gene, this gene expression under the control of the seed-specific napin promoter in *Brassica napus* germplasm was reported to induce the changes in VLCFA and erucic acid content in the seed oil of transgenic lines (Katavic *et al*., 2000). Winter type, Yudal, had been used to develop the RAPD and RFLP makers to localize genes controlling oligogenic traits involved in plant development and oilseed quality because of high erucic acid level, about 50%. To make the Yudal having the erucic acid level more than usually, we isolated the FAE1 gene from Yudal by sequence alignment of known clones and PCR, then has been transforming by the YFAE1 overexpression binary vector.

Material and Methods

Plant material : *Brassica napus* L. cultivars were used for the experiments, namly the HEAR cultivars Yudal. To cloning of the FAE1 gene and study of this gene expression, the organs except silique were sampled during flowering, and sillique just before harvest in green house. Protoplasts were extracted from the 4 weeks grown Yudal leaves under long day condition. The hypocotyls segment of Yudal was used for the transformation of FAE1 gene after 3-4 days growth in MS media containing 1% sucrose under the continuous light.

Cloning of YFAE1 gene : The FAE1 mRNA sequences from *Brassica napus* HEAR or LEAR varities, *Brassica oleacea* HEAR varities were reported as well as the 2 ESTs from Yudal. These FAE1 mRNA were divided to 2 groups through the sequence alignment, and then the sequence of these groups was compared to two ESTs sequence from Yudal. The oligonucleotide were designed by these sequence alignment, and then cDNA clone obtained by RT-PCR was cloned to the 326GPF vector and the modified binary vector pCAMBIA 3301 to examine the YFAE1 gene expression and transformation .

GFP gene expression : The YFAE1 gene was cloned in 326GPF vector under the control of BCB, or SEN1 promoter showing constitutive GFP expression in *Arabidopsis* as well as 35S promoter. To analysing the transient gene expression of GFP reporter gene constructs, the plasmid was transferred into the protoplasts isolated from Yudal leaves using polyethyleneglycol (PEG).

Plant transformation : The modified binary vector pCAMBIA 3301 containing BCB promoter, YFAE1 cDNA and selection marker BAR gene was transformed to *Agrobacterium tumefaciens* LBA4404 and cocultivated with light-grown

Brassica napus Yudal hypocotyl segments.

Results

Cloning of YFAE1 gene

As previously mentioned, the sequences of FAE1 genes were reported from many *Brassica* family plants including *Arabidopsis, Brassica napus, and Brassica olearcea*. 11 FAE1 genes among them and Yudal FAE1 ESTs, FAE1-1 and FAE1-2 were selected from database, and then divided into two groups (A and B group) by sequence homology. The FAE1 homolog, accession number AF009563, was showed the highest homology with Yudal 2 ESTs among them, and revealed to belong to B group . On the basis of these B group genes sequence alignment, oligonucleotides with some nucleotide modification were designed to get the cDNA clone by RT-PCR. Fig.1 shows the predicted amino acid sequence of the protein encoded by Yudal FAE1 (YFAE1) cDNA. Analysis with TMHMM2 program suggested that there are 2 transmembrane region in the protein. And the amino acid sequence among this FAE1 cDNA and ESTs is revealed that this cDNA is identical to EST FAE1-1.

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          1                                                                                          90
FAE1-1    ------------------------------------------------------------------------------------------
FAE1-2    ------------------------------------------------------------------------------------------
YFAE1     MTSINVKLLYHYVITNLFNLCFFPLTAIVAGKAYRLTIDDLHHLYYSYLQHNLITIAPLFAFTVFGSVLYIATRPKPVYLVEYSCYLPPT
          91                                                                                        180
FAE1-1    ------------------------------------------------------------------------------------------
FAE1-2    ------------------------------------------------------------------------------------------
YFAE1     HCRSSISKVMDIFYQVRKADPSRNGTCDDSSWLDFLRKIQERSGLGDETHGPEGLLQVPPRKTFAAAREETEQVIIGALENLFKNTNVNP
          181                                                                                       270
FAE1-1    ------------------------------------------------------------------------------------------
FAE1-2    ------------------------------------------------------------------------------------------
YFAE1     KDIGILVVNSSMFNPTPSLSAMVVNTFKLRSNVRSFNLGGMGCSAGVIAIDLAKDLLHVHKNTYALVVSTENITYNIYAGDNRSMMVSNC
          271                                                                                       360
FAE1-1    ----------------------------------------FRCVQQGDDENGKTGVSLSKDITDVAGRTVKKNIATLGPLILPLSEKLLFF
FAE1-2    ----------------------------------------FRCVQQGDDENGKTGVSLSKDITDVAGRTVKKNIATLGPLILPLSEKLLFF
YFAE1     LFRVGGAAILLSNKPGDRRRSKYELVHTVRTHTGADDKSFRCVQQGDDENGKTGVSLSKDITDVAGRTVKKNIATLGPLILPLSEKLLFF
          361                                                                                       450
FAE1-1    VTFMGKKLFKDKIKHYYVPDFKLAIDHFCIHAGGRAVIDVLEKNLALAPIDVEASRSTLHRFGNTSSSSIWYELAYIEAKGRMKKGNKVW
FAE1-2    VTFMGKKLFKDKIKHYYVPDFKLAIDHFCIHAGGKAVIDVLEKNLGLAPIDVEASRSTLHRFGNTSSSSIWYELAYIEAKGRMKKGNKVW
YFAE1     VTFMGKKLFKDKIKHYYVPDFKLAIDHFCIHAGGRAVIDVLEKNLALAPIDVEASRSTLHRFGNTSSSSIWYELAYIEAKGRMKKGNKVW
          451                                                 506
FAE1-1    QIALGSGFKCNSAVWVALNNVKASTNS--------------------------
FAE1-2    QIALGSGFKCNSAVWVALNNVKASTNS--------------------------
YFAE1     QIALGSGFKCNSAVWVALNNVKASTNSPWEHCIDRYPVKIDSDSGKSETRAQNGRS
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Fig.1 Amino acid sequence of the Yudal FAE1 and Alignment with 2 ESTs, FAE1-1 and FAE1-2

The amino acid sequence of the Yudal FAE1 cDNA was deduced from cDNA nucleotide sequence. Square box over the sequence show the transmembrane regions predicted by the TMHMM2 program. The sequence was aligned with Yudal FAE1 ESTs (AF054497 and AF054487), and amino acids in shadow box except one amino acid are identical to these genes.

Expression of YFAE1 mRNAs in Yudal.

To examine the tissue-specific expression of YFAE1 mRNA, we isolated RNA from root, leaf, stem, young flower, flower organ during flowering, young silque, and mature silique just before seed harvest, and analyzed the transcripts by RT-PCR (Fig.2). The result showed that this gene was expressed in all organ, and the level of this gene transcript in young silique was increased compared to that in young flower.

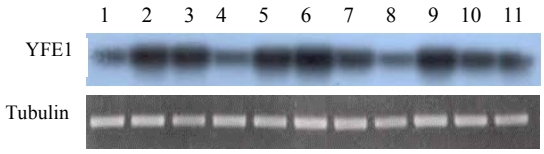


Fig.2 RT-PCR analysis of the YFAE1 transcripts in Yudal.

Total RNA (2 μg) was isolated from each *Brassica napus* Yudal organ, root (1), leaf (2), stem (3), young flower before flowering (4), calyx (5), pistil (6), stigma (7), pollen (8), young silique (9), seed coat (10), and seed (11) just before seed harvest, and probed with ^{32}P-labeled YFAE1 cDNA (1.5kb). The tubulin transcripts were shown to demonstrate equal loading of RNA in each lane.

Transient gene expression

In *Arabidopsis*, the GFP expression under the control of BCB promoter was increased more or less in compared to under the control of 35S, but in protoplast treated in high concentration of IAA solution the GFP expression under the control of SEN1 promoter was shown to be similar level to that of 35S promoter by western blot analysis using the GFP antibody (personal communication). We examined whether the FAE1 gene expression under the control of BCB and SEN1 promoter from Arabidopsis was shown to be similar level in Yudal mesophyll protoplast cells as mentioned above (Fig.3). When the protoplast was transformed with vector replaced the 35S promoter with BCB or SEN1 promoter, it seemed to be increased the number of cell showing GFP expression by BCB promoter more than by 35S and SEN1 promoter. Using the vector having the YFAE1 cDNA with the BCB or SEN1 promoter, the GFP expression level and the number of cell showing GFP expression seemed to be similar in both promoter, but higher than in 35S promoter. The BCB gene expression was reported to have been regulated by some factor including plant hormone and stress like wounding. To transform the Yudal variety by FAE1 overexpression vector, pCAMBIA 3301 vector was modified by the elimination of 35S and GUS gene region, and insertion of BCB promoter and YFAE1 cDNA.

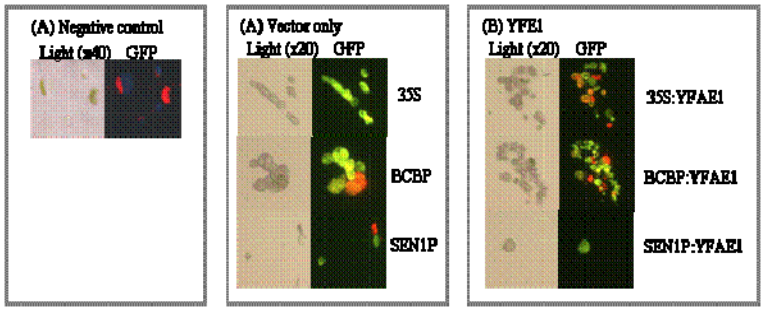
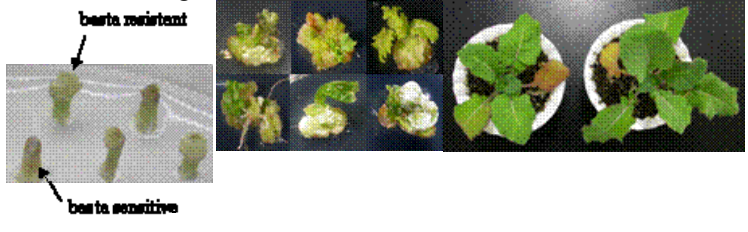


Fig.3 Transient GFP expression of YFAE1 under the control of BCB and SEN1 promoter.

Protoplast cell were studied 36 to 48 h after PEG transformation. To left for each set of panels was the transmitted light image, while the panel at the right was represented the GFP fluorescence image on the same protoplasts.

Transformation Yudal with FAE1 overexpression vector

In rapeseed, the transformation has been performed using stem internodes, stem segments, cotyledonary petioles, hypocotyl segments, microspores, and protoplast. In this experiment, hypocotyls segment (0.5 cm) from 3 or 4 day continuous light grown seedling was pretreated in callus induction condition for 2 days, and then co-cultivated with *Agrobacterium* LBA4404 containing the FAE1 overexpression vector for 2 days. The basta tolerant hypocotyls segments were selected in the callus induction media with phosphinothricin (PPT) and cefotaxime for 4 - 6 weeks. During the process of selection, the transformed hypcotyls continued to grow to produce calli. Shoots were usually regenerated within 4 or 6 weeks on the MS medium containing $AgNO_3$, cefotaxime, IAA and Zeatin. The embryonic callus induction percentage after *Agrobacterium* infection and selection was 7.9%, 32 among 405 hypocotyl explants, and 14 regeneration plants was produced these calli.



(A) Basta selection (B) Shoot induction (C) Regenerated plants

Fig. 4 The organogenesis process in *Brassica napus* Yudal.

(A) The hypocotyl segments were placed on medium containing PPT for 4 - 6 weeks. (B) Shoots were induced from transgenic calli. (C) The transformants were growing in the pots.

Discussion

High erucic acid *Brassica napus* cuitivars (HEAR) have been one of the important crops in world, because erucic acid and its derivatives are important raw materials utilized in the manufacture of plastic films, in the lubricant and emollient industries, and cosmetic industries and so on. Yudal in Korea is one of the HEAR cultivars, developed by breeding method in 1969, but recently its importance has been increased in the field of cosmetic industries. The progress in elucidating the organization and control of very-long-chain fatty acids (VLCFs) biosynthetic pathway has been achieved by cloning genes from different plants encoding β-ketoacyl-CoA synthase (KCS), and the change of FAE1 gene expression is well known to associated with the changes in VLCFA and erucic acid content in the seed oil of transgenic lines. So, this study was started to make the Yudal having the erucic acid level more than usually by change the FAE1 gene expression level.

The two genes homologous in Arabidopsis FAE1 was reported to be co-segregated with the two loci governing erucic acid content in *Brassica napus* (Fourmann *et al*., 1998). FAE1-1, and 1-2, 2 ESTs about 500bp from Yudal were belonged to the group organized by 8 genes among already known 11 FAE1 genes. Yudal FAE1 was obtained by RT-PCR using degenerated oilgonucleotides deduced from the sequence alignment of 8 FAE1 gene homologous to Yudal 2 ESTs. As the results of sequencing, YFAE1 cDNA was identical to FAE1-1, an amino acid different to FAE1-2 EST. The expression of YFAE1 by RT-PCR analysis was detected in all organ examined although not distinguish between the expression of 2 FAE1 gene, and the FAE1 gene expression level was increased in flowering flower and young silique in contrast of young flower.

At present, the cultivar Yudal have been transforming using the YFAE1 overexpression vector under the control of BCB promoter. This promoter was well expressed the GFP gene in rapeseed mesophyll protoplast transferred the plasmid containing this promoter only or with YFAE1 cDNA using PEG as known in *Arabidopsis*. In transformation, the Yudal regeneration condition was different from previously reported media composition in other *Brassica napus* including HEAR cultivar. The embryonic callus induction was more or less low in Yudal, but the regeneration efficiency was not lower than in any other the rapeseed cultivars. In future, it was selected the transformant showing the higher level erucic acid than untransformant by lipid analysis.

References

Barret, P., Delourme, R., Renard, M., Domergue, F., Lessier, R., Delseny, M and Roscoe, Y.J. (1998) A rapeseed FAE1 gene is linked to the E1 locus associated with variation in the content of erucic acid. Theor. Appl. Genet. 96, 177-186.

Clemens, S. And Kunst, L. (1997) Isolation of a Brassica napus cDNA encoding 3-ketoacyl-CoA synthase, a condensing enzyme involved in the biosynthesis of very long chain fatty acids in seed (PFR97-125). Plant Physiol. 115, 313-314

Fourmann, M., Barret, P., Renard, M., Pelletier, G., Delourme, R. And Brunel, L. (1998) The two genes homologous to Arabidopsis FAE1 co-segregate with the two loci governing erucic acid content in Brassica napus. Theor. Appl., 96, 852-858.

Han , J., Luhs, W., Sonntag, K., Zahringer, U., Borchardt, D.S., Wolter, F.P. Heinz, E., and Frentzen, M. (2001) Functional characterization of β-ketoacyl-CoA synthase genes from Brassica napus L. Plant Mol. Biol. 46, 229-239.

Hann, X., Luhs, W., Sonntag, K., Borchardt, D.S., Frentzen, M. And Wolter, F.P. (1998) A Brassica napus cDNA restores the dificiency of canola fatty acid elongation at a high level. In : J. Sanchex, E. Cerda-Olmedo and E. Matinex-Forces (Eds) Advances in Plant Lipid Research, Universidad de Sevilla, Sevilla, pp. 665-667.

James, D.W. Lim, E., Keller, J., Plooy, I. And Dooner, H.K. (1995) Directed tagging of the Arabidopsis fatty acid elongation 1 (FAE1) gene with the maize transposion acitvator. Plant Cell 7, 309-319.

Lassner, M.W., Lardizabal, K., and Metx, J.G. (1996) A fofoba β-jetiactk-CoA sybthase cDNA complements the canola fatty acid elongation mutation in transgeic plants. Plant Cell 8, 282-292.

Pruitt, R.E., Vielle-Calzada, J.P. Ploense, S.E., Grosseniklaus, U. and Lolle, S.J., (2000) FIDDLEHEAD, a gene required to supress epidermal cell interatctions in Arabidopsis, encodes a putative lipid biosynthetic enzyme. Proc. Natl., Acad. Sci. USA 97, 1311-1316.

Todd, J., Post-Beittenmiller, D. And Jaworski, J.G. (1999) KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in Arabidospsis thaliana. Plant J. 17, 119-130.

Venkateswari, J., Kanrar, S., Kirti, P.B., Malathi, V.G. and Chopra, V.L. (1999) Moleuclar cloning and characterization of FATTY ACID ELONGATION1 (BjFAE1) gene of Brassica juncea. J. Plant Biochem. Biotechnol. 8, 53-33.