High oleic acid rapeseed (Brassica napus L.) produced by transformation with an ihpRNA vector targeting the delta-12 desaturase gene

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Rapeseed (Brassica napus L) is one of the world's main sources of vegetable oil. Rapeseed oil has been traditionally characterized by a high polyunsaturation level, with about 20% linoleic acid and 10% linolenic acid, respectively. Oils with high linoleic acid and linolenic acid are susceptible to oxidation and deteriorate more rapidly upon exposure to air, especially at high temperatures. Oils with high oleic acid are desirable because this monosaturated fatty acid improves the nutrition and oxidative stability of the oil commodity. Moreover, high-oleic oil has many industrial applications, for example, high-oleic oil is a high-quality raw material for production of biodiesel. Consequently, Breeding new varieties with high oleic acid in seed oil has become one of the major goals for many crops (Kinney ,2001）

Increases in oleic acid content can potentially be achieved by reducing the activity of delta-12 desaturase (FAD2) which converts oleate into linoleate in the developing seed. Traditional strategy is to induce the mutants by chemical mutagenesis or radiation induction. High oleic mutants, with an oleic content ranging from 60% to 90%, have been developed in some crops. These mutants usually have defective fad2 gene (Toyoaki, 2008; Patal et al, 2004). The problem with mutants is that the fatty acid content of non seed organs is also changed, often resulting in agronomic issues (Miquel and Browse 1994). In addition, many plants have a number of fad2 genes, all of which contribute to seed linoleic content (Tang et al., 2005). This means that development of mutant lines with useful oleic acid contents often requires combining several mutant loci and provides a breeding challenge (Mikkilineni and Rocheford, 2003).

In the past few decades, the pathways, mechanisms and regulation of de novo biosynthesis of plant fatty acids have been well elucidated. Based on such knowledge, it is now possible to genetically manipulate the composition of fatty acids in some oil crops. By gene engineering, the delta-12 desaturase gene (fad2) can easily and specifically been down-regulated in the developing seeds of oil crops, led to increased oleic acid and reduced in both linoleic and linolenic specifically in seed. There have been many successful reports using transgenic strategies based on posttranscriptional gene silencing to increase oleic acid content in oil crops. (Stoutjesdijk,2000; Yin et al., 2007)

In this paper we will introduce the transgenic rapeseed line W-4 that produces seeds with increased oleic acid and decreased polyunsaturated acids.

1. Materials and methods

1.1Plant materials

The Brassica napus L. cv. westar was used in the transformation experiments to produce transgenic line. T0 plants were grown in greenhouse in Sept. 2007, T1 and T2 were grown in screen house in Sept. 2008 and 2009 respectively. Self pollination T1 and T2 seeds were harvested at maturity respectively. The non-transgenic cv. westar was used as Control.

1.2 ihpRNA vector construction and Agrobacterium -mediated transformation

An ihpRNA vector was constructed by Chen et al. (2006): a 537-bp coding region of the fad2 gene (AF243045) was amplified from genome DNA of rapeseed and cloned into the pHurrican Vector in an inverted repeated way with a spliceable fad2 intron sequence in between. The amplified napin promoter of 1147-bp (AF420598) was placed to the 5'-end of the inverted repeat cassette and a nos polyA to the 3'-end of it. Then the whole inverted repeat cassette was inserted into the multiple clone sites in the binary vector pCAMBIA2300 to form an ihpRNAi vector, pCNFIRnos.

The cotyledon with approximately 2 mm petiole of Brassica napus L.cv. westar were selected as explants and co-cultivated with Agrobacterium tumefaciens strain EHA105 harboring the construct pCNFIRnos. The transgenic plants were produced as Chen et al (2008).

1.3 Analysis of fatty acid composition

Self pollination seed obtained from T0 and T1 plants were analyzed for fatty acid composition by gas chromatography. Relative content of unsaturated fatty acid was analyzed based on the oleic acid desaturation parameter (ODP) which were calculated according to the following formula: ODP=（18：2,%+18：3,%）/（18：1,%+18：2,%+18：3,%）.

1.4 Southern blot

About 30μg of BamHI digested genomic DNA of both transgenic and non-transgenic plants was separated on a 0.8% (wt/vol) agarose gel, then blotted to nylon membrane (Hybond ™-N $^{+}$) and probed with a segment of NPTII gene labeled by DIG-dUTP through PCR using pCAMBIA2300 as template. The labeling, hybridization,

washing, development and fixing were conducted according to the kit manual. （Roche Dig nucleic acid detection kit）. Primers were 5′ -GGTGGAGAGGCTATTCGGCTA-3′ and 5′-GTAAAGCACGAGGAAGCGGTC-3′。

1.5 Real time PCR

Total RNA was extracted from the 0.5g developing seeds of 25-30 day after flowering using the RNA pure Kit (Yuanpinghao Bio). cDNA synthesis and Real-Time PCR were performed using SYBR^{R} Premix Ex Tag^{TM} II kit (TaKaRa). The relative standard curve was used to quantify the relative level of fad2 gene expression of transgenic line to the control. The cycling conditions was 30s 95°C, 40 cycles of 10 s at 95°C and 34s at 60°C, followed by the generation of a dissociation curve to check for specificity of amplification with ABI7500. Relative expression quantity was analyzed using 7500 software.2. 0.1. Primer sequences for the reference gene (actin, FJ529168.1) were 5′-CGAGGCTCCTCTTAACCCAAAGG-3′ and 5′-CACCAGAATCCAGCACAATACCG-3′ , and for fad2 gene were 5′-AAGTGTTTGTCCCCAAGA-3′ and 5′-AAGCGAAGCCG CCGTC GTA A G -3′

1.6 TAIL-PCR amplification

Thermal asymmetric interlaced PCR (TAIL-PCR) was conducted to amplify the flanking sequence to T-DNA in the transgenic line W-4 genome according to the method described by Liu et al. (1995).The nested specific

fatty acid composition (%)						
	item	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**
HO	range	3.64-5.47	3.59-2.06	75.73-84.73	1.87-6.53	2.66-3.99
(n=19)	mean±SD	4.25±0.51	2.69±0.37	81.03±2.8[a]	3.46±1.64[a]	3.37±0.53[a]
CK	range	3.94-6.32	1.97-4.11	55.28-67.43	14.65-23.42	4.78-8.55
(n=22)	mean±SD	4.65±0.62	2.4±0.45	62.1±3.67[b]	19.16±2.19[b]	7.08±0.89[b]

primers were designed based on the sequence of the vector pCNFIRnos. The primers for LB Flanking sequence were 5′ -TAG CGT TGG CTA CCC GTG ATA TTG CTG AAG AGC TT- 3′ , 5-′ TTC TAT CGC CTT CTT GAC GAG TTC TTC TGA GC -3′ , 5′-TAA AGT AGT CAC TAA TTG GAT GAC AAA GCA AAT GG- 3′ , coupled with the arbitrary degenerate primers AD1, 5′- NTC GA(G/C) T(A/T)T (G/C)G(A/T) GTT-3′, respectively, The primers for RB were flanking sequence were 5′-TAA AGT AGT CAC TAA TTG GAT GAC AAA GCA AAT GG-3′ , 5′-GAA TTG TTT CTT CCT CAA AGA AGA AGC ATT CAG CC-3′ , 5′-GAG GAC GCA GTC AGA AAG TCC AGA ATA TAA GTG GT-3′ , 5′-TCG TGA CTG GGA AAA CCC TGG CGT TAC CCAA-3′ coupled with the arbitrary degenerate primers AD2, 5-′NGT CGA (G/C)(A/T)G ANA (A/T)GA A -3′ , respectively.

2. Results and Discussion

2.1. Fatty acid composition in seeds of T1 generation

Analysis of acid composition of T1 seed showed that among the six transgenic lines, only the transgenic line W-4 was found a 9.25% increase in oleic acid content and a 9.1 percentage point decrease in ODP compare to the non-transgenic controls respectively. Further analysis of T2 seeds showed there was a significant quantitative variation for unsaturated fatty acid content among T2 population. The oleic acid level among the T2 seed of 49 progenies varied from 55.87% to 84.73% whilst that of the control varied from 55.28% to 67.43%. There are 19 individuals of the 49 progenies containing more than 75% oleic acid in the seed oil. For the 19 individuals the average content of oleic acid was 81.03±2.84%, significantly higher than that of the control. These high oleic acid (HO) individuals were also found considerable reductions in linoleic acid and linolenic acid. The oleic acid desaturation parameter (ODP) of the HO individuals was only 5.18%-12% much lower than that of the control, which had an ODP value of around 23.37%-35.92%(Table 1).

Table 1. fatty acid composition of transgenic line W-4 (HO) and CK

(data labeled with different superscript letters in the same column are significantly different at 0.01 level)

2. 2 Expression of fad2 gene in the developing seeds

We investigated the fad2 gene expression in the developing seed of T2 plants with above 80% oleic acid and took non-transgenic plants as control by real time PCR. The result showed that fad2 gene expression in young seed of transgenic line was decreased significantly compare to the control. The relative expression level of transgenic line is only about 35% that of control (figure 1). This demonstrated that the fad2 gene was effectively silenced by the expression of ihpRNA of fad2 in transgenic line and led to the decrease of delta-12 desaturase activity and high oleic acid content in seed.

Figure 1 *fad*2 gene relative expression level of transgenic rapeseed (HO) and control (CK).

2.3. Southern blot analysis

To investigate the copy of T-DNA integrated into the genome of transgenic line W-4 the southern blot was conducted. The result showed an obvious hybridization signal was detected in the four HO individual plants of W-4, but not in negative control (Figure 2). The result indicated that the T-DNA was integrated into the genome of line W-4. Based on the evidence that the T-DNA region of the binary vector (pCNFIRnos) has only one BamHI restriction sites, we hypothesized that transgenic rapeseed W-4 had only one copy of the T-DNA integrated in genome.

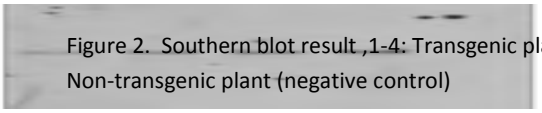

Figure 2. Southern blot result ,1-4: Transgenic plants, 5: Non-transgenic plant (negative control)

2.4 flanking sequence analysis

The specific PCR products of 470bp and 641bp corresponding to the flanking sequence to right border and the left border respectively were successfully amplified using TAIL-PCR. Sequence analysis showed that the flanking sequence to RB border includes 290bp genome sequence and 180bp vector sequence. Further sequence alignment analysis (Figure 3) showed that the 180bp was identical to the RB border of pCNFIRnos but with a 62bp deletion. The left border flanking sequence includes a 365bp genome sequence and a 276bp vector sequence. The 276bp was same with the left border of pCNFIRnos except a change of G to A. The integration of the T-DNA in the transgenic line W-4 is a vector backbone-free integration. The obtained genomic sequences were done blast on web, but did not found highly homologous sequence. We concluded that the T-DNA may integrate at non-coding site of the genome.

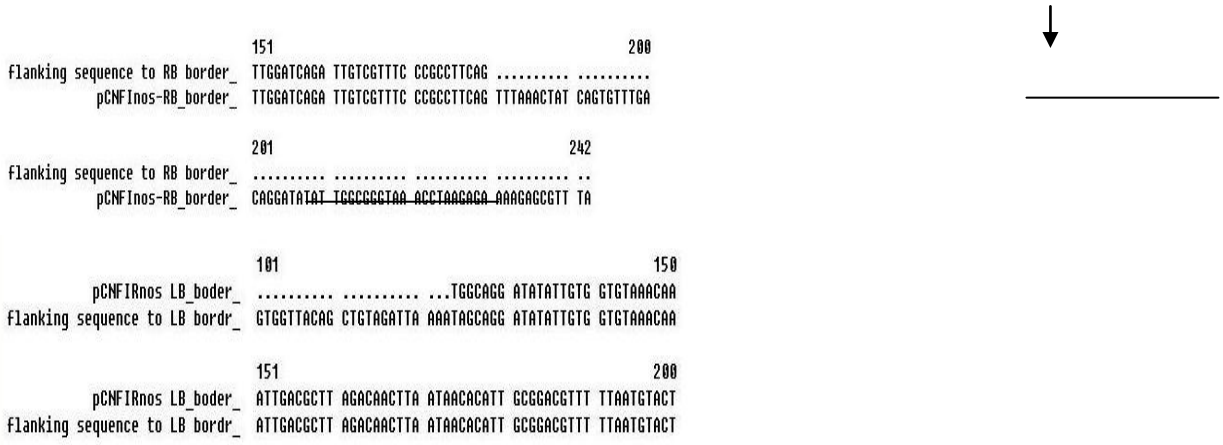

Figure 3. alignment of the partial T-DNA sequence integrated in the genome of W-4 with the right border or left boreder of pCNFIRnos respectively, the 25bp imperfect repeats indicated with line and a arrow show the G/A change.

3. Conclusions

We have successfully developed transgenic line W-4, through transferring an ihpRNA vector targeting the delta-12 desaturase gene into rapeseed by Agrobacterium-mediated method. The line W-4 has increased oleic acid up to 84%, and decreased linoleic acid and linolenic acid down to 4% and 3%, respectively. The fad2 gene expression level in developing seed of transgenic line was dropped significantly compare to the control. Southern blot analysis shows that the W-4 has one copy of T-DNA, which may integrate at a non-coding site. The integration is a vector backbone-free integration characterized with a 62bp deletion of right border and complete left border.

(11 References were omitted)