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Research paper

Depressed expression of *FAE1* and *FAD2* genes modifies fatty acid profiles and storage compounds accumulation in *Brassica napus* seeds☆

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ABSTRACT

In plants, the enzymes fatty acid dehydrogenase 2 (FAD2) and fatty acid elongase 1 (FAE1) have been shown in previous studies to play important roles in the *de novo* biosynthesis of fatty acids. However, the effects of depressed expression of *FAD2* and *FAE1* on seed storage compounds accumulation remains to be elucidated.

In this study, we produced RNA interfering transgenic rapeseeds lines, *BnFAD2-Ri*, *BnFAE1-Ri* and *BnFAD2/BnFAE1-Ri*, which exhibited depressed expression of the *BnFAD2* and *BnFAE1* genes under the control of seed-specific *napin A* promoter. These transgenic rapeseeds showed normal growth and development as compared with the wild type (CY2). Depressed expression of *BnFAD2* and *BnFAE1* genes modified fatty acid profiles, leading to increased oleic acid and decreased erucic acid contents in transgenic seeds. Consistent with these results, the ratios of C18:1/C18:2 and C18:1/C18:3 in C18 unsaturated fatty acids were greatly increased due to increased oleic acid content in transgenic seeds. Moreover, depressed expression of *BnFAD2* and *BnFAE1* genes resulted in slightly decreased oil contents and increased protein contents in transgenic seeds. Our results demonstrated that depressed expression of *BnFAD2* and *BnFAE1* greatly improves seed nutritional quality by modulating the fatty acid metabolism and storage products accumulation and that *BnFAD2* and *BnFAE1* are reliable targets for genetic improvement of rapeseed in seed nutritional quality.

1. Introduction

Rapeseed (*Brassica napus*) is one of the most important oil crops worldwide, being the world's third largest source of oilseed after palm and soybean (http://usda.mannlib.cornell.edu/usda/ers/89002/Table 47.xls). A major end use of oil derived from crushing harvested seeds of high nutritional quality is in foodstuffs [1,2]. Additionally, rapeseed oil is an important source of biodiesel fuels and industrial raw materials [3–5]. To satisfy the increased demand for oilseed production, genetic improvement of rapeseed should be performed to increase oil content and to enhance the quality of the oil for a variety of end uses'.

The nutritional quality of oilseed is mainly determined by fatty acid (FA) balance and protein content [6]. Fatty acid profiles, typically, include palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), eicosenoic (C20:1) and erucic acid (C22:1) [7]. Among these fatty acids, erucic acid (C22:1) is an undesired FA for edible oil [8,9], and , is therefore a key determinant of edible oil quality. Another criterion of oilseed oil quality is the ratio of C18 unsaturated FAs [6]. Increased oleic acid (C18:1) levels and reduced polyunsaturated FAs (linoleic, C18:2; linolenic, C18:3) levels can provide higher oil stability and produced less undesirable products at high temperatures [10,11]. Lastly, the protein content is a major contributor to the meal energy value for feed, and higher protein levels can improve the nutritional quality of the oilseed [6].

Vegetable oil is accumulated during seed development, and forms the major carbon and energy reserves for germination and seedling growth. Seed oil is predominantly composed of triacylglycerols (TAGs), which are essential for human nutrition and valuable feedstocks for the chemical industry [12]. TAGs are usually synthesized from glycerol-3-P and FAs [13–15]. In plasmids, FAs are *de novo* synthesized mainly from acetyl-CoA. The whole process is catalyzed by a series of enzymes. Acetyl-CoA carboxylase (ACCase) catalyzes the formation of malonyl-

Abbreviations: FA, Fatty acid; FAD2, fatty acid dehydrogenase 2; FAE1, fatty acid elongase 1; Ri, RNA interference; WT, wild type

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CoA from acetyl-CoA, initiating the reaction of FAs synthesis. Then, fatty acid synthase (FAS) catalyzes the transfer of a malonyl moiety to an acyl carrier protein (ACP) by adding two carbons to the growing chain, causing the formation of C16:0- and C18:0-ACP. The resulting C16:0- and C18:0 acyl-ACP are then transferred into the cytoplasm. There, FA dehydrogenase (FAD) catalyzes desaturation reactions to form unsaturated FA, and FA elongase (FAE) catalyzes elongation reactions to form long-chain FAs. Finally TAG is synthesized from glycerol-3-P and varieties of acyl chains in the endoplasmic reticulum [13–16].

Some regulatory genes have been found to be involved in the regulation of fatty acid metabolism [17–21]. Transgenic plants expressing these regulators showed altered FA synthesis [12,22–25]. Besides regulatory genes, some key FA synthetic genes were selected for genetic manipulation as most enzymes of the FA biosynthesis pathway have been characterized and genes encoding them have been cloned from various species [26–28].

FAD2, encoding fatty acid desaturase2, catalyzes the first committed step of the biosynthesis of polyunsaturated fatty acids from oleic acid to linoleic acid [13,29]. As a key enzyme controlling the proportion of polyunsaturated fatty acids in seed oil, *FAD2* has been genetically depressed to increase the proportion of oleic acid in rapeseed and cotton [30–32]. *FAE1,* encoding fatty acid elongase1, catalyzes the initial condensation step in the elongation pathway of very long chain fatty acid biosynthesis, and is thus a key gene in erucic acid biosynthesis [13,29]. Depressed expression of *FAE1* can reduce the erucic acid content to extremely low levels in seed oil [31,33–35]. In addition, previous studies have shown that over expression of *FAE1* largely increased erucic acid levels in the seed oil [5,36,37]. Whilst erucic acid is an undesired FA in edible oil, it is an important renewable raw material for the oleochemical industry. Despite these advances, the effects of depressed expression of *FAD2* and *FAE1* on storage compound accumulation in seeds need to be elucidated.

To investigate the effects of depressed expression of *FAD2* and *FAE1* on storage compound accumulation, we constructed RNA-interfering vectors of *BnFAD2* and *BnFAE1* driven by seed-specific napA promoter and subsequently produced *BnFAD2-Ri* and *BnFAE1-Ri* transgenic plants. We demonstrated that *BnFAD2-Ri* and *BnFAE1-Ri* transgenic plants showed normal morphological phenotypes. To further define the role of *BnFAD2* and *BnFAE1*, we further produced double-gene interfering transgenic rapeseeds (*BnFAD2/BnFAE1-Ri*) depressed in the expression of the *BnFAD2* and *BnFAE1* genes. We then carried out detailed molecular, biochemical and physiological analyses of these transgenic plants.

2. Materials and methods

2.1. Plant material and growth conditions

Plants of oilseed rape (*Brassica napus* 'CY2') and all related transgenic plants were grown under field conditions. In Hangzhou, Zhejiang Province, seeds were sown usually in late September or early October and harvested around late May. The developing seeds of different rapeseed genotypes were collected from the field-grown plants, immediately frozen in liquid nitrogen, and then stored at −80 °C until the use.

2.2. Plasmid construction

To generate seed-specific RNA-interfering constructs, the *napin A* promoter region was amplified from CY2 (wild type) genomic DNA and cloned into pFGC5941, resulting in a pFGCNapinA construct [33]. *BnFAE1* RNA-interfering vector has been constructed in our previous study [33]. The *BnFAD2* fragment was cloned into pFGCNapinA construct as follows: a 508 bp fragment of *BnFAD2* was amplified using primers *BnFAD2* F1 and *BnFAD2* R1 (Supplemental Table S1), and cloned in both orientations (BamHI/XbaI or NcoI/SalI sites) in pFGCNapinA, separated by the chalcone synthase (CHSA) intron of petunia hybrid to form a hairpin structure. The resulting RNAi construct was verified by extensive restriction digestion and DNA sequencing analysis.

2.3. Generation of transgenic plants

The resulting construct was transformed into *Agrobacterium tumefaciens* strain EHA105, which was then used for the transformation. Then the *BnFAD2* RNA-interfering construct was transformed into CY2 plants by hypocotyl segment transformation method as previously described [28,38].

Transgenic plants were transferred to soil for further growth. DNA was extracted from selected plants for PCR detection of the transgene. The primers used for transgenic plant detection were as described elsewhere [31].

2.4. Quantitative real-time PCR analysis

Total RNA and cDNA were prepared from the developing seeds of wild type and transgenic plants as described previously [33]. For the analysis of *BnFAD2* and *BnFAE1* expression, quantitative real-time PCR was used to quantify the levels of mRNA encoding *BnFAD2* and *BnFAE1* genes. *BnACTIN* was used as an internal control [25]. The primers used for the expression analysis of *BnFAD2* and *BnFAE1* genes are listed in Supplemental Table S1.

2.5. Cross between BnFAD2-Ri and BnFAE1-Ri transgenic plants

To create double-gene RNA-interfering transgenic plants, representative *BnFAD2-Ri* and *BnFAE1-Ri* transgenic lines were selected by qRT-PCR analysis of *BnFAD2* or *BnFAE1* expression, respectively. Then a cross between *BnFAD2-Ri* and *BnFAE1-Ri* transgenic lines was made to generate *BnFAD2/BnFAE1-Ri* transgenic plants. Among all possible genotypes of progeny seedlings in the F2 population, *BnFAD2/BnFAE1-Ri* transgenic lines were determined by qRT-PCR analysis of *BnFAD2* and *BnFAE1* expression.

2.6. Analysis of lipids and proteins

The seed oil content was analyzed by the Soxhlet extraction method as described previously [25]. Briefly, 2 g of seeds of each genotype were dried overnight at 80 °C and then cooled to room temperature in a desiccator. The dried seeds were ground to a fine powder and then transferred into a preweighted bag and sealed. The sample was dried for 6–8 h until reaching constant weight. After cooling to room temperature in a desiccator, the bagged sample was transferred into a Soxhelt tube and extracted with petroleum ether (boiling point under 50 °C) for 24 h. After the extraction, the bagged sample was dried in a hood to evaporate remaining petroleum ether, cooled in a desiccator, and then weighed. The oil content of the desiccated seeds was expressed as a percentage of the total seed dry weight.

Analysis of the fatty acid levels was performed by GC–MS as described previously [33]. The protein content was measured by the Kjeldahl method as described [25].

3. Results

3.1. Depressed expression of BnFAD2 and BnFAE1 in seeds does not affect the phenotype of transgenic plants

Multiple independent *BnFAE1-Ri* transgenic lines were generated [33]. To get *BnFAD2-Ri* transgenic plants, the *BnFAD2* RNA-interfering vector was constructed and transformed into CY2, a cultivar with high oil and high erucic acid content. Multiple independent *BnFAD2-Ri* transgenic lines were obtained by phosphinothricin resistance selection