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Research article

Decreased seed oil production in *FUSCA3 Brassica napus* mutant plants

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ABSTRACT

Canola (*Brassica napus* L.) oil is extensively utilized for human consumption and industrial applications. Among the genes regulating seed development and participating in oil accumulation is *FUSCA3* (*FUS3*), a member of the plant-specific B3-domain family of transcription factors. To evaluate the role of this gene during seed storage deposition, three *BnFUSCA3* (*BnFUS3*) TILLING mutants were generated. Mutations occurring downstream of the B3 domain reduced silique number and repressed seed oil level resulting in increased protein content in developing seeds. *BnFUS3* mutant seeds also had increased levels of linoleic acid, possibly due to the reduced expression of *ω-3 FA DESATURASE* (*FAD3*). These observed phenotypic alterations were accompanied by the decreased expression of genes encoding transcription factors stimulating fatty acid (FA) synthesis: *LEAFY COTYLEDON1* and *2* (*LEC1* and *2*) *ABSCISIC ACID-INSENSITIVE 3* (*BnABI3*) and *WRINKLED1* (*WRI1*). Additionally, expression of genes encoding enzymes involved in sucrose metabolism, glycolysis, and FA modifications were down-regulated in developing seeds of the mutant plants. Collectively, these transcriptional changes support altered sucrose metabolism and reduced glycolytic activity, diminishing the carbon pool available for the synthesis of FA and ultimately seed oil production. Based on these observations, it is suggested that targeted manipulations of *BnFUS3* can be used as a tool to influence oil accumulation in the economically important species *B. napus*.

1. Introduction

Demand for *Brassica napus* L. (canola) oil has significantly increased in the recent past due to the health benefits related to the low concentration of saturated fatty acids (FA) and a balance between omega-6 and omega-3 FA. In higher plants, the biosynthesis of FA and lipids has been well characterized by biochemical and molecular studies (Slabas and Fawcett, 1992; Beisson et al., 2003). Specifically, canola seed oil biosynthesis is composed of a plastidal FA synthesis component, a cytosolic pool and an endoplasmic reticulum-based triacylglycerol (TAG) component. Storage product deposition occurs throughout seed development reaching a peak during the later maturation phases (Baud et al., 2008; Braybrook and Harada, 2008). As a consequence, the maturation phase of seed development is of specific interest when trying to increase the quantity and quality of oil. In recent years, emphasis has been placed on controlling FA biosynthesis through a regulatory network of transcription factors: *LEAFY COTYLEDON1* and *2* (*LEC1* and *2*), *FUSCA3* (*FUS3*), *ABSCISIC ACID-INSENSITIVE 3* (*BnABI3*) and *WRINKLED1* (*WRI1*), that control overlapping aspects of embryo development and seed maturation (Meinke et al., 1994; Gazzarrini et al., 2004). These transcription factors are known regulators of diverse phases of embryo growth and maturation (Braybrook and Harada, 2008; Cernac and Benning, 2004).

FUS3, a member of the plant-specific B3-domain family of transcription factors, recognizes and binds to the RY element CATGCA found in the promoters of many genes (Curaba et al., 2004; Santos-mendoza et al., 2008). Mutation of this gene results in several phenotypes including desiccation intolerance, precocious germination, anthocyanin accumulation in developing seeds, defective hormone synthesis and perception of light (Meinke et al., 1994; Harada, 2001). Arabidopsis *FUS3* mutant plants also show altered protein and lipid accumulation (Meinke et al., 1994; Harada, 2001), implying that *FUS3* can influence FA biosynthetic genes. Enhanced expression of the Arabidopsis *FUS3* has been associated with the induction of genes encoding the two main kinds of seed storage proteins (2S albumin and 12S cruciferin), and many enzymes involved in FA biosynthesis (*KAS I*, *KASII*, *KASIII*, *PYRUVATE DEHYDROGENASE*, and *ACETYL-COA CARBOXYLASE*) (Wang et al., 2007). While transgenic studies have shown the influence of *FUS3* on FA biosynthesis, the molecular mechanisms underlying these effects are not completely characterized (Curaba et al., 2004;

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Vicient et al., 2000). For example, the activation of *FUS3* by *LEC1* is indicative of a complex gene network regulating seed development (To et al., 2006).

The majority of information relative to the function of *FUS3* derives from studies on Arabidopsis, with no information available for *B. napus*. *B. napus* is an applicable system due to its economic relevance and oil production. The lack of information is partially due to the polyploidy nature of *B. napus*, which can limit the application of forward genetic analyses. The presence of multiple copies of the same gene results in mutations at single loci not easily identified in a forward genetic screen, as the function of the mutated locus can be masked by the activity of homeologous gene(s) (Gilchrist et al., 2013). Therefore, reverse genetic approaches are appropriate for investigating the function of genes in polyploid species. Through TILLING (Targeting Induced Local Lesions in Genomes), mutations in individual homeologous genes can be identified independently and then introgressed into the same line, the phenotype of which can be analyzed to identify the function of the mutated gene (Gilchrist et al., 2013). Such mutations can result in a variety of loss-of-function phenotypes, while gain-of-function phenotypes are very rare. In the majority of the events, loss of function alleles are produced by missense mutations which do not necessarily eliminate gene function fully, but rather cause a partial loss in activity (Gilchrist et al., 2013).

In the present study, three *BnFUS3* TILLING mutant lines were generated to evaluate the requirement of a functional *FUS3* gene on FA composition, and oil and protein content. Expression studies were also conducted on genes encoding key enzymes for sucrose metabolism, glycolysis and FA biosynthesis to identify potential transcriptional regulatory mechanism underlying the observed phenotypes.

2. Materials and methods

2.1. Generation of TILLING mutants

B. napus (canola cultivar DH12075) seeds were used to generate three *BnFUS3* TILLING mutant lines, CT1296-2 (M1), CT1508-3 (M2) and CT0831-1 (M3) with the method described in http://www.botany.ubc.ca/can-till/and Gilchrist et al. (2013). In short, surface-sterilized seeds of *B. napus* were mutagenized with EMS (Ethyl methanesulfonate) and allowed to grow and produce progeny. Plants obtained from mutagenized seeds were transferred to soil and grown in a greenhouse until maturity. Seeds were used to produce second generation of plants which were grown until flowering, at which point leaves were taken from each plant for DNA extraction. Plants were allowed to grow until they had produced a minimum of 100 seeds. Upon maturity, seeds were dried and stored. Samples of DNA from leaf tissue were used as templates in polymerase chain reactions (PCR) with the primers bn36-4R and bn36-5L (Supplementary Table 1), as described by Gilchrist et al. (2013) and Colbert et al. (2001). These PCR products were then digested with Celery Juice Extract (CJE) (Till et al., 2004), which contains an endonuclease that specifically cleaves the single base pair mismatches resulting from heterozygous point mutations. The digested DNA was run on a denaturing polyacrylamide Li-cor gel in order to find DNA populations carrying a mutation in *BnFUS3*. Upon the identification of the mutation, the procedure was repeated using DNA from individuals that exhibited the mutation in the primary screen.

2.2. Planting and growth

Sixteen heterozygous seeds from each mutant line were grown in the greenhouse of the Department of Plant Science, University of Manitoba, Winnipeg, Canada in 2011. For the identification of homozygous plants, leaf tissue samples from each *BnFUS3* TILLING mutant lines were flash frozen in liquid nitrogen and stored at −80 °C. Genomic DNA was extracted from each of the sampled tissues using the cetyltrimethylammonium bromide (CTAB) method (Keb-Llanes et al., 2002). The DNA was suspended in 50 μl TE buffer and its concentration was determined by a Nano Drop spectrophotometer. Samples were adjusted to a final concentration of 150 ng/μl H_2O. Genomic DNA samples were then amplified using primers bn36-4R and bn36-5L (Supplementary Table 1). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen Gm bH, Hilden, Germany) and sequence analysis was conducted by Macrogen USA. The homozygous mutations were detected with the primer, bn36-4R for the M1 and M3 lines and bn36-2R for the M2 line. On the basis of the sequence analysis, the homozygous mutant plants were selected and grown in separate pots and covered with selfing bags to prevent cross-pollination. Nucleotide sequences were translated into amino acid sequences using the ExPASy Proteomics Server (http://expasy.org/tools/dna.html). The conserved domains and structure of the gene were identified using the NCBI (The National Center for Biotechnology Information) Search for Conserved Domains within a Protein Sequence (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The plants were grown to maturity, at which point the seeds from each individual plant derived from the independent mutant lines were collected and grown for further investigation.

2.3. Agronomic characters

To study the effects of the mutations of *BnFUS3* on plant morphology, the homozygous mutant lines (M1-3), along with control lines, were grown in 15 cm pots (two parts soil, two parts sand, and one part peat moss) twice in the greenhouse (from February–May 2012 and June–September 2012) with an average temperature of 21 °C and 25 °C, respectively, with a period of 17 h of light. Initially, the seedlings were fertilized with NPK (10:52:10) to encourage root development and watered every second day until the bolting stage at which point the pots were fertilized with NPK (20:20:20). All necessary treatments for the control of pathogens were conducted as needed. Using the same procedure, the experiment was repeated in growth cabinets with 16/8 h day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1} and day/night temperatures of 20 °C/15 °C. Overall, nine biological replicates for each mutant line were used to record the data for all agronomic characters (i.e., plant height, siliques per plant, seeds per silique, 1000 seed weight and yield per plant). For measuring the number of seeds per silique and silique length, mean values of five randomly selected siliques from each plant within each replicate were used.

The gene expression studies were conducted only in the second experiment. Thus, the data for seed quality and agronomic characteristics were not combined between experiments as the intent was to examine the seed quality and agronomic characteristics in relation to the same plants analyzed for gene expression. Seed quality and agronomic characteristics were also evaluated in the growth cabinet.

2.4. Analysis of seed oil content and protein level

Total oil and protein levels were measured using near infrared reflectance (NIRSystem model 6500, Foss NIRSystems Inc., MD, USA) spectroscopy (Tkachuk, 1981). Fatty acid (FA) composition analyses were conducted using gas chromatography (Varian, Walnut Creek, USA) as documented by Hougen and Bodo (1973). For oil and FA composition, at least three biological replicates (each replicate consisted of seeds pooled from three plants) were used.