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A Link between Early Embryogenesis Transcription Factors and *Brassica napus* Oil Production

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ABSTRACT

Oil synthesis in *Brassica napus* is composed of a plastidal fatty acid synthesis component, a cytosol pool, and an ER-based triacylglycerol component. A number of the main embryogenic transcription factors such as LEC1, LEC2, WRI1 and FUS3 appear to affect the plastidal fatty acid synthesis component during the seed development. A comparison of the relative transcript concentrations of embryogenic transcription factors between high and low oil double haploid lines shows an increase in the WRI1 and FUS3 transcripts at 21 days after pollination in high oil lines and an increase in the LEC2 transcript at 28 days after pollination in the low oil lines. This indicates that variation in the transcript levels of embryogenic transcription factors which are linked to the plastidal component of *B. napus* oil synthesis, may affect the production of total seed oil.

Keywords: FUSCA 3, LEAFY COTYLEDON 1, LEAFY COTYLEDON 2, oil biosynthesis, WRINKLED 1

INTRODUCTION

Brassica napus is an economically important oil crop ranking only behind soybean and palm oil in terms of global production. Due to its importance, the improvement of *B. napus* oil in terms of oil quantity (oil content) and quality (fatty acid profiles) is a subject of interest. Despite research efforts, the regulation of the *B. napus* fatty acid synthesis is largely unknown. However, it is known that oil synthesis is compartmentalized into plastidal fatty acid synthesis (FAS), cytosolic pools and endoplasmic reticulum (ER) based triacylglycerol (TAG) synthesis pools with most of the storage product synthesis and accumulation in *B. napus* seeds occurring during the maturation phase (14-35 days after flowering) of seed development (Hajduch *et al.* 2006). Since there is differential timing of the expression of genes involved in the FAS and the TAG metabolism, it is likely that plastidal and ER biosynthesis are regulated by different systems (Baud and Lipiniec 2009). One means of manipulating the plastidal FAS is to manipulate the main seed-specific Transcription Factors (TF) which effect embryogenesis and the maturation phase (Braybrook and Harada 2008; Baud and Lepiniec 2009). Three TF known as the LEC genes, LEAFY COTYLEDON 1 (LEC1), LEAFY COTYLEDON2 (LEC2), and FUSCA3 (FUS3), maintain the embryogenic state in *Arabidopsis* (Harada 2001; Santos-Mendoza *et al.* 2008). During the maturation phase, these genes and ABSCISIC ACID-INSENSITIVE3 (ABI3) are required for desiccation intolerance and storage reserve synthesis and accumulation (Harada 2001; Santos-Mendoza *et al.* 2008). The LEC1, a NFY-B-type or CCAAT-binding HAP3-like LEC1 upstream of other major TFs, FUS3 and LEC2 are part of the B3 TF ABI3/VP1 family, and WRINKLED (WRI1), an APETALA2–ethylene responsive element-binding protein (AP2/EREBP), are all linked to the plastidal component of FAS (Fock and Benning 1998; Lotan *et al.* 1998; Cernac and Benning 2004; Gazzarrini *et al.* 2004; Masaki *et al.* 2005; Santos-Mendoza *et al.* 2005; Cernac *et al.* 2006; Stone *et al.* 2008; Tiedmann *et al.* 2008). The importance of understanding the transcript profile of the major embryogenic TF in different *B. napus* oil phenotypes is demonstrated by the fact that an unknown B3 family and two unknown AP2/ERF family TF are upregulated in *B. napus* immature seeds 20 days post anthesis (DPA) compared to 35 DPA (Yu *et al.* 2010).

To determine the potential of embryogenic TF in the manipulation of oil biosynthesis, a link between their transcription and the developmental pathway of the *B. napus* seed is needed. Over and under-expression of these TF in Arabidopsis indicates that the LEC genes stimulate plastidal FAS (Wang *et al.* 2007; Santos-Mendoza *et al.* 2008; Liu *et al.* 2009). However, flux control experiments in *B. napus* indicate that TAG synthesis is controlled by the ER component (Weselake *et al.* 2008), despite the genetic linkage of high oil *B. napus* lines to maternal effects and increased embryo chlorophyll (Hobbs *et al.* 2004; Ruuska *et al.* 2004). A difference in transcripts levels of early embryogenic TF between homozygous lines segregating for oil content would indicate if the high oil phenotype is linked to expression levels of TF (LEC1, LEC2, WRI1, FUS3) which are connected to plastidal FAS. We hypothesize that greater transcript levels for one or all of these transcription factors is linked to increased storage oil concentrations.

MATERIALS AND METHODS

Development of double haploid high and low oil lines

A double haploid (DH) population segregating for oil content was developed using the microspores of a *B. napus* hybrid resulting from a cross between a high oil ('Polo') and low oil ('Topas') cultivar. Two high oil and two low oil DH lines were selected from this population and used for this study (**Table 1**).

The seeds of the selected DH lines were planted to grow adult plants under non-controlled greenhouse conditions of 21°C average temperature and 17-hour light (an average intensity of 500 μE). Just before flowering, the individual flowers on each plant were marked, self pollinated dated. About 10-15 siliques resulting from self-pollinated flowers were harvested at four developmental stages marked as 7, 14, 21, 28 days after pollination (DAP). The harvested siliques were placed on ice and ~10-100 mg immature

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Short Communication

Table 1 *Brassica napus* double haploid (DH) lines used for transcription factor quantification.

DH line	Pedigree	Oil content (%)
1	'Polo' x DH 'Topas'	43.5
2	'Polo' x DH 'Topas'	42.1
3	DH 'Topas' x 'Polo'	50.0
4	DH 'Polo' x 'Topas'	50.3

seeds were excised out. Jmmature seed extraction was done by slicing the silique open with a #10 surgical scalpel blade and picking out the immature seeds with a sterile needle. The immature seeds were collected in 2 mL RNase free Eppendorf tubes, placed on dry ice before their transfer at -80°C, and were ground to fine powder for RNA extraction. Total RNA was then extracted from each sample by using the Qiagen RNeasy Plant Mini Kit (Palo Alto, California) and following the manufacture's instructions. Total RNA was cleaned twice with the Qiagen RNase-free DNase Set (#79254), quantified on an Ultraspec 2100 pro UV/visible spectrophotometer, and diluted accordingly to a working concentration of 1 µg/ µL.

Relative quantification of mRNA concentrations in high and low oil lines

Using the Applied Biosystems high capacity RNA-to-cDNA kit (#4387406), 2 µg of total RNA was used for the preparation of cDNA by following the manufacture's instructions. Promega GoTaq® Hot Start Colorless PCR Master Mix (#M5133) was used to conduct semi-quantitative PCR's. The PCR reactions included 1 µL of 50% diluted cDNA as template and 10 pmol of appropriate primers (**Table 2**) per 50 µL reaction.

Using a PRC-100 programmable thermocycler (MJ Research Inc.), an initial denaturing step of 95ºC for 5 min following by a cycle of: denaturing at 95°C for 30 s; annealing at 55°C for 30 s; and extension at 72ºC for 1 min. The cycle was repeated 25, 30, 35, and 40 times depending on gene of interest, with a final extension at 72°C for 10 minutes. For each DH line at 7, 14, 21 and 28 days after pollinating, the gene of interest was amplified for 35 cycles for LEC1 and LEC2, and 30 cycles for WRI1 and FUS3. A control (ACTIN) at 30 cycles was included with each DH line. The appropriate cycle time, 5 cycles above and below that time, plus the ACTIN control were visualized in a 1% agarose 1X TAE gel by staining in 1X Invitrogen SYBR®gold (S11494) for 45 min, followed by a 30 min wash in 1X TAE. An AlphaImager® set to 302 nm, with a 500 nm filter, was used to quantify the area under the curve. The area for the gene of interest was given as a percent of the area for ACTIN.

RESULTS AND DISCUSSION

We determined the relative transcript levels of embryogenic TF in immature seeds of *B. napus* double haploid (DH) lines extracted from the siliques at four developmental times. Initial PCR experiments indicated that 35 cycles for LEC1 and LEC2, and 30 cycles for WRI1 and FUS3 were the appropriate cycle times to ensure that the gene of interest was at the end of the logarithmic phase of amplification. Duplicate samples of all reactions were done in the same experiment and the experiments were replicated over time in triplicate.

At 7 days after pollination (DAP), the immature seeds are at the globular/heart-shaped stage of pattern formation and soon to start producing chlorophyll and oil bodies (He and Wu 2009). The embryos are at the torpedo/cotyledonary stage and beginning to synthesize oil at 14 DAP (Hajdunch *et al.* 2006). Our results indicated no difference in the relative transcript concentration of LEC1 between the high and low oil lines (**Fig. 1A**), at any of the four developmental times (7, 14, 21, 28 DAP). However, the high oil lines (DH 3 and 4) had a significantly greater relative concentration of WRI1 and FUS3 mRNA at the 21 DAP period (**Fig. 1C, 1D**) or the beginning of the maturation phase. At 28 DAP when oil synthesis is the highest (Hajdunch *et al.* 2006), the high oil lines had significantly less LEC2 relative transcript (**Fig. 1B**). The increase in WRI1 and FUS3 transcription is likely linked to increases in plastidal FAS. Increases in WRI1 expression in high oil rapeseed was also found by Liu *et al*. (2009). As well, the over-expression of *B. napus* (Bn) WRI1 in an Arabidopsis background causes a high oil phenotype (Liu *et al*. 2009). The linkage between increased FUS3 transcript, decreased LEC2 transcript, and increased oil content has been noted in Arabidopsis (Wang *et al*. 2007). Since, WRI1 transcript is promoted by LEC1, LEC2, and sugar availability (Cernac and Benning 2004; Masaki *et al*. 2005; Huang *et al.* 2009), the difference in WRI1 relative transcript could also reflect an increase in the photosynthetic rate between the two lines and indicated that the greater amount of oil seen in these lines is due to an increased ability to capture available carbon from the environment. A change in WRI1 and/or FUS3 transcript is not seen in high oil lines produced transgenically increasing the ER component of oil synthesis (Sharma *et al*. 2008). They found that only LEC1 was differentially expressed between control and high oil DGAT lines and probes for WRI1, FUS3, ABI3, and ABI5 were not differentially expressed between the two, while LEC2 and GLABRA2 were not present in the microarray (Sharma *et al.* 2008). Interestingly, we found no difference in the relative LEC1 transcript between high and low oil lines.

This research indicates that a change in the transcript levels of TF which affect WRI1 and FUS3, mainly LEC1 and LEC2, may cause a change in total oil synthesis in *B. napus*, although the feedback inhibition demonstrated by Liu *et al.* (2009) would indicate that the Arabidopsis genes may be of more use for this. The isolation and change in the expression levels of BnLEC1, BnLEC2, BnWRI1, and BnFUS3 within a *B. napus* germplasm may increase oil yields, either alone or in combination with genes involved in the ER component of oil synthesis.

A measurement of the photosynthetic levels of the high and low oil lines should be done. Since, WRI1 promotes the enzymes of glycolysis, an inherent increase in WRI1 transcript could increase glycolysis and plastidal FAS which would then cause a feed-backward stimulation of photosynthesis. A communication link with plastidal FAS and glycolysis is supported by the maternal inheritance of oil content (Hobbs *et al.* 2004) and the increase of fatty acid accumulation by increased embryo photosynthesis (Ruuska *et al.* 2004). It could also be that the increase in WRI1 and FUS3 mRNA is due to a feed-forward effect caused by an inherently greater photosynthetic capability of the plant. Either way, LEC1 and LEC2 expression levels are shown to be relatively similar in high oil lines at and prior to 21 DAP, whereas WRI1 and FUS3 relative expression levels are variable. The only known factor which causes increased WRI1 transcription that has not been measured is sucrose availability.

Table 2 Primers used for semi-quantitative mRNA analysis. Primers were developed by using the GenBank Arabidopsis sequences and producing primers via the use of Primer 3 (http://frodo.wi.mit.edu/primer3/)

Gene	Forward primer	Reverse primer	Fragment size (bp)	GenBank accession number
ACTIN	taa agt atc cga ttg agc atg gtat	cgt agg caa gct tct ctt taa tgtc	450	AF111812 (M. Elhiti personal communication)
LEC1	aaa cgg ca gaga aaa caa tgg	tca ctt ata ctg acc atactg gtc	735	NM_102046
LEC2	ccc ttt ccc tct tct aac gc	cag ctc cat ttt gct tca ca	729	AY568668.1
WRI1	ccg act caa tca gag act cca	aag cag gac aac gga gaa ga	1100	AY254038.2
FUS3	gaa gga tgc cta gac aga ga	aga gga gta tcg ttg gag gt	650	AF016265.1

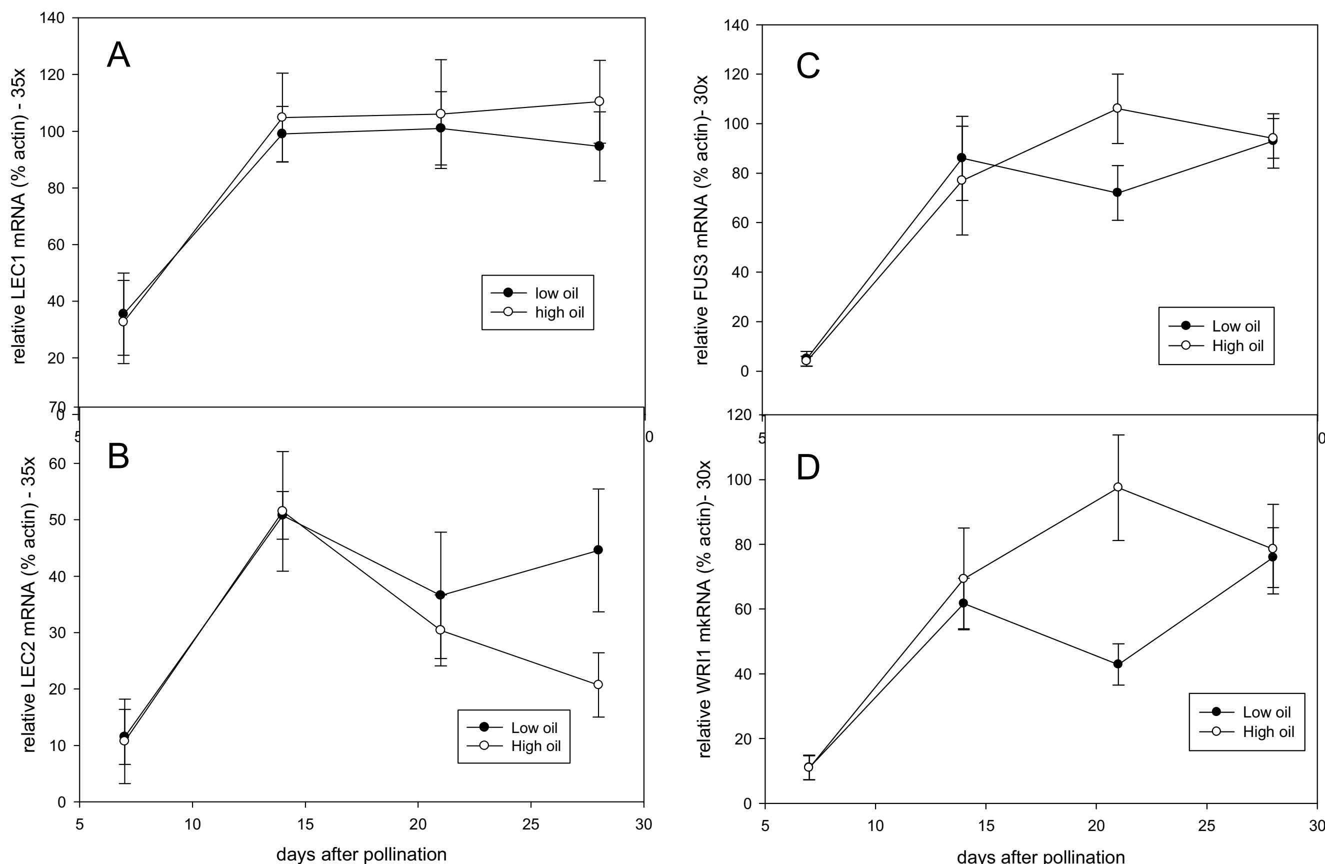

Fig. 1 The relative quantity of mRNA transcript of each gene of interest. LEC1 (**A**), LEC2 (**B**), FUS3 (**C**) and WRI1(**D**) mRNA is given as a percent of the quantity of ACTIN transcript in the total RNA population of immature embryos extracted at 7, 14, 21 and 28 days after pollination, from high oil and low oil double haploid lines (mean ± SE, n = 6 or 7).

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