The potential of using the *BnLEC1* and *BnFUSCA3* genes to manipulate oil content in *Brassica napus* L.

by

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ABSTRACT

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The potential of using the *BnLEC1* and *BnFUSCA3* genes to manipulate oil content in *Brassica napus* L.

Supervisors: Dr. Claudio Stasolla and Robert W. Duncan

Due to the immense utilization in food and industry, there is enormous commercial and scientific interest to manipulate canola (*Brassica napus* L.) seed oil. Seed oil accretions are influenced by genes involved in embryo and seed development. *FUSCA3* (*FUS3*) and *LEAFY COTYLEDON1* (*LEC1*) are well-known transcription factors involved during seed and embryo development. The main objective of this project was to evaluate the role of these genes during seed storage deposition and microspore-derived embryogenesis in *B. napus*. For this purpose, six *BnLEC1* transgenic lines and three *BnFUS3* TILLING mutant lines were generated. The over expression of *BnLEC1* significantly increased the seed oil content, while the down regulation of *BnLEC1* or mutation of *BnFUS3* reduced the level of seed oil. Experimental alterations of *BnLEC1* and *BnFUS3* triggered transcriptional modifications in enzymes taking part in sucrose transport and metabolism, glycolysis, and fatty acid (FA) biosynthesis. These changes are suggestive of a greater carbon pool to FA biosynthesis in tissues over-expressing *BnLEC1*, and a reduced carbon flux available for the synthesis of FA in *BnLEC1* down regulators and *BnFUS3* tilling mutants.

While the elevated oil content induced by *BnLEC1* was not accompanied by alterations in FA composition, oil nutritional value, or glucosinolate (GLS) levels, suppression of *BnLEC1* reduced seed oil accumulation and raised levels of GLS, possibly through the transcriptional regulation of *BnST5a* (Sulphotransferase5a), the last GLS biosynthetic enzyme. *BnFUS3* tilling

mutant seeds had increased levels of linoleic acid, possibly due to the reduced expression of *ω-3 FA DESATURASE* (*FAD3*). The effects of altered expression of *BnLEC1* and *BnFUS3* were also assessed during microspore-derived embryogenesis. Substantial structural abnormalities, accompanied by changes in transcript levels of several embryo marker genes were observed in embryos in which the expression of *BnLEC1* or *BnFUS3* was altered. The changes in oil level and FA profiles observed in the transformed microspore-derived embryos followed a similar trend to that described in seeds. Collectively, these observations suggest that manipulation of *BnLEC1* and *BnFUS3* can be employed as a tool to enhance seed oil production and quality in *B. napus*.

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DEDICATION

Dedicated to my parents (Mr and Mrs Noor Elahi), whose prayers save me in every difficult time and my husband M. Sabir who helped me to achieve the difficult tasks.

FORWARD

This thesis follows the manuscript style under the guidelines issued by The Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. The manuscripts follow the style recommended by Plant Physiology and Biochemistry. This thesis is presented as three manuscripts, each containing an abstract, introduction, materials and methods, results and discussion section. For each manuscript, supplemental figures and supplementary tables are located straightway after the body of the manuscript. A general introduction and literature review leads the manuscripts and general discussion and conclusions and a list of references and abbreviations follows the manuscripts.

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1 GENERAL INTRODUCTION

Canola is a member of the family Brassicaceae (syn. Cruciferae) and is the most commonly grown mustard in the world. International marketing value of canola oil has increased considerably throughout the past few years as it is characterized by very low concentration of saturated fatty acids with a balance between the omega-6 and omega-3 fatty acids (Cardoza and Stewart, 2007).

Canola seed quality is assessed by many factors, including the protein and glucosinolates (GLS) levels (Canola Council of Canada, 2010). In addition to its use in livestock feed, canola meal also has various potential uses (Uruakpa and Arntfield, 2005; Canola Council of Canada, 2009) and has been recommended as a potential source for human food (Uppstrom, 1995). Whereas the GLS are the sulphur and nitrogen containing organic compounds (Sonderby et al., 2010) and are toxic at higher levels. Therefore, low GLS levels are highly desirable.

Canola is very important in temperate climates, such as northern Europe and Canada, contributing about 12 % of total world oil and fat production (Gunstone et al., 2007). The worldwide rapeseed oil production during 2014-2015 was 26.98 million metric tons (USDA Economics, 2015). Developed from rapeseed by Keith Downey and Baldur R. Stefansson in the early 1970's (Richard, 2007; Storgaard, 2008), canola was previously considered a speciality crop for Canada, but has now become a crop grown globally. Other countries including

Australia, the United States, and several in Europe also grow canola. However, Canada and the European Union account for most of the global production (Table.1).

Table.1.1 Rapeseed production during the season 2014/2015 in the ten leading countries.

Country	Production 1000 (MT)
European Union	24,450
Canada	16,410
China	14,772
India	6,310
Australia	3,464
Ukraine	2,200
Russia	1,464
United States	1,140
Belarus	730
Kazakhstan	241

Source: USDA Foreign Agricultural Service, Official USDA Estimates. Metric tonnes (MT).

According to L.M.C International Ltd (2013), canola production contributes $19.3 billion a year to the Canadian economy (http://www.canolacouncil.org/markets-stats/industry-

overview/economic-impact-of-the-canola-industry/). In Canada, canola is mainly grown as a food crop, but canola oil is also used for various industrial purposes such as paints, lubricants and biodiesel (Scarth and Tang, 2006; Weselake et al., 2009). In 2013, Canada achieved a 29.5% increase in canola production, setting a distinctive record of eighteen million tons production (Cabak, 2013). According to the Canola Council of Canada, worldwide vegetable oil requirement will rise from 150 million tons in 2015 to 250 million tons by 2025. Thus, to meet the increasing global market demand for canola, the Canola Council of Canada has set a new target for 2025 to increase canola production to 26 million metric tons (Granger, 2014).

Currently the focus of interest of the industry is to alter the chemical composition of the seed storage products in order to improve oil quality. In this regard, during the past few years several researchers have concentrated their efforts on comparative studies among species, and the interpretation of the data from model plants to various crops (Zou et al. 1997; Bouvier-Nave et al. 2000; Jako et al. 2001; Marillia et al. 2003; Vigeolas et al. 2007; Zheng et al. 2008; Shen et al., 2010).

In the past, various conventional genetic and plant breeding methods were used to modify canola oil for several usages (Scarth and Tang, 2006; Weselake et al., 2009). With improved and varied breeding tools available for the seed oil modifications of *B. napus*, both conventional and transgenic approaches are now being used. Both of these approaches have advantages and disadvantages. The conventional approach for the production of *Brassica* oil has the major constraint set by existing genetic resources, while limitations of the transgenic approaches include site and insertion number in the genome (Scarth and Tang, 2006). Improved knowledge of the genetic regulation of seed oil biosynthesis in *Arabidopsis*, together with current progress in plant genetics and molecular biology, has opened novel research avenues which emphasize

modification of canola oil by manipulating the genetic regulation of fatty acid biosynthesis. Upgrading seed oil content and composition could improve the economic importance of *Brassica* crop production and consequently, its continuous improvement remains a main focus for *Brassica* breeders.

The introduction of biotechnology has provided plant breeders new and more precise approaches that have the capability to reduce the time needed to develop new cultivars. For example, very low levels of saturated fatty acids, high levels of short and medium chain fatty acids, high lauric acid, high caprylic acid, high palmitic acid, high stearic acid, very high oleic acid and high erucic acid have been developed using transgenic approaches (Scarth and Tang, 2006). These transgenes all have altered expression of genes encoding enzymes involved in fatty acid biosynthesis (Scarth and Tang, 2006).

The knowledge of seed developmental stages is vital for altering the seed oil in *B. napus*. The seed develops following a double fertilization event, during which one sperm cell fertilizes the egg cell of the megagametophyte, resulting in a diploid zygote, and another sperm cell fertilizes the diploid central cell, from which the triploid endosperm originates (West and Harada, 1993; Goldberg et al., 1994). Briefly, seed development can be divided into embryo morphogenesis and maturation. The process of oil synthesis involves a number of organelles and metabolic pathways within the cell. Seed oil biosynthesis is compartmentalized into plastidal fatty acid synthesis, cytosolic pools and endoplasmic reticulum based triacylglycerol synthesis pools with most of the storage product synthesis and accumulation in *B. napus* seeds occurring during the maturation phase of seed development (Hajduch et al., 2006; Baud et al., 2008; Braybrook and Harada, 2008). As a result, the maturation phase of embryo development has received great attention when attempting to manipulate the seed oil.

In the metabolic pathway for the fatty acid synthesis, there are mainly four reactions, namely condensation, reduction, dehydration, and reduction that are catalyzed by several enzymes leading to the production of acyl chains of different lengths from acetyl-CoA and malonyl-CoA. Additional elongation of the fatty acid molecule is achieved by additional reactions using malonyl-ACP molecules (reviewed by Ohlrogge and Browse, 1995). Lastly, triacylglycerol (TAG) production is completed in the endoplasmic reticulum through the Kennedy pathway (Baud et al., 2008). Triacylglycerol accumulation depends upon the efficiency of the carbon flux through photosynthesis and carbohydrate metabolism. Although the fatty acid biosynthesis and the enzymes taking part in this route are carefully understood, various studies advocate the limitations regarding the impact of these enzymes on seed oil biosynthesis (Mu et al., 2008; Baud and Lepiniec, 2009; Weselake et al., 2009). This has initiated several studies to explore additional factors affecting seed oil biosynthesis (Mu et al., 2008; Liu et al., 2010). In *Arabidopsis* more than 600 genes have been documented to participate in acyl-lipid metabolism (Li-Beisson et al., 2010). As seed oil biosynthesis and deposition is linked to various phases of embryo and seed development, it is thought that genes involved in the regulation of embryo and seed development also affect the production and accumulation of oil in seeds (Sharma et al., 2008).

In recent years more emphasis has been directed towards the modification of fatty acid biosynthesis through the alteration of transcription factors including - *LEC1* (*LEAFY COTYLEDON1*), *LEC2* (*LEAFY COTYLEDON2*), *FUS3* (*FUSCA3*), *WRI1* (*WRINKLED1*), and *ABI3* (*ABSCISIC ACID INSENSITIVE3*), which also affect the seed maturation phase of embryo development (Meinke, 1992; Meinke et al., 1994; Parcy et al., 1994; Stone et al., 2001; Cernac and Benning, 2004; Gazzarrini et al., 2004). *LEAFY COTYLEDON1* influences various

metabolic pathways such as glycolysis and lipid accumulation (Mu et al., 2008), and also regulates the expression of *LEC2* an upstream regulator of *FUS3* and *ABI3* which affects the expression of the *WRI1* gene. *WRINKLED1* has been shown to mediate the effects of *LEC2* in fatty acid metabolism (Baud et al., 2007) and some enzymes taking part in glycolysis (Maeo et al., 2009). All these genes interact with each other and play a vital role for the embryo and seed development (Meinke, 1992; Cernac and Benning, 2004 and Gazzarrini et al., 2004). These genes have been successfully used in the genetic modification of seed oil in various species (Wang et al., 2007; Maeo et al., 2009).

The *LEC1* protein has the HAP3 subunit (CBF) of the CCAAT binding factor that enables the *LEC1* to be a precise transcriptional regulator of downstream genes having the CCAAT recognition domain (Kwong et al., 2003; Lee et al., 2003). *LEAFY COTYLEDON1* expression occurs throughout the whole course of embryogenesis (West et al., 1994). During the maturation phase of seed development, *LEC1* modulates the expressions of genes affecting the abscisic acid and gibberellins homeostasis and stimulates the storage product accumulation (Braybrook and Harada, 2008). *FUSCA3* encodes a B3 protein that accumulates mostly during the maturation phase of embryo development and performs a key role during embryogenesis (Curaba et al., 2004; Santos-Mendoza et al., 2008). During seed development, reduction in lipid and protein deposition was observed in *Arabidopsis lec1* and *fus3* mutant plants (Meinke et al., 1994; Harada, 2001).

As *Arabidopsis* and *B. napus* have genomic co-linearity (Parkin et al., 2005), based on the studies in the model plant *Arabidopsis*, it is hypothesized that these transcription factors could perform similar functions in *Brassica* species. Thus, the main premise of this thesis is to determine the impact of altered expression of *BnLEC1* and *BnFUS3* on canola seed oil. For this

purpose various *BnLEC1* transgenic and *BnFUS3* TILLING mutant lines were generated. It is hypothesized that overexpression of *BnLEC1* can lead to higher oil accumulation while down regulation of either *BnLEC1* and *BnFUS3* will result in lower oil biosynthesis both in seeds and in microspore derived embryos.

2 LITERATURE REVIEW

2.1.1 The development and economic importance of canola oil

Canola (*Brassica napus* L.) (AACC = 2n = 38) is a member of the plant family *Brassicaceae* which has 338 genera and 3709 species (Warwick et al., 2006), while the genus *Brassica* contains nearly 39 species. Due to its global use as an oilseed crop, *B. napus* (polypolyploid species) is the most important in this group and ranks second after soybean in worldwide oilseed production (Iniguez-Luy and Federico, 2011).

The archeological history for rapeseed in China goes back to 5000 BC (Yan, 1990). In the Netherlands, the commercial cultivation of rapeseed as a lubricant for steam engines started in the sixteenth century (Gupta and Pratap, 2007). Canola, trademarked by the Canadian oilseed Processors Association, represents cultivars of oilseed rape that produce seed oil with less than 2% erucic acid, while the meal has less than 30 micromoles of glucosinolates per gram (Canola Council of Canada, 2014). These specific seed oil characteristics are present in varieties of *Brassica napus* L., *B. rapa* L. and *B. juncea* (L.) Czern., that are named 'canola' from Canadian oil low acid (Canola Council of Canada, 2014). Tower was the first canola cultivar registered by Dr. Baldur Stefansson in 1974 in Canada, and the canola seed quality gained acceptance worldwide (Przybylski et al., 2005; Scarth and Tang, 2006; Canola Council of Canada, 2010). With the first cultivar registered in Canada in 1974, canola has now become a global oilseed crop. In 2013, the canola production in Canada set a record with 18 million tonnes of production (Cabak, 2013). During 2014-2015, the worldwide production of oilseed rape oil was 26.98 million metric tons (USDA, 2015). Oilseed rape oil has broad-spectrum utilization as it has excellent nutritional value for human consumption and is also valuable for industrial uses such as

cosmetics, biodiesel, lubricants, and paints (Przybylski et al., 2005; Weselake et al, 2009). In order to meet increasing global demand, the Canola Council of Canada set a target of increasing canola production to 26 million metric tons by 2025 (Granger, 2014). Increasing seed oil content and yield per unit area of land is therefore of paramount importance in providing sufficient oil for food and other applications. A one percent increase in the seed oil content of *B. napus* would constitute an increase in seed yield of approximately 2.3 - 2.5 % (Wang, 2004). Increasing seed oil content would expand the economic value of oilseed rape production and consequently, oil content and fatty acid composition improvements remain a major priority in many breeding programs.

2.1.2 Nutritional value of canola seeds

This oilseed rape type is not only a source of one of the healthiest vegetable oils for human food (Downey and Bell, 1990), but also a prospective source for the development of a wide variety of industrial products such as bioplastics and biodiesel (Wu and Muir, 2008). Canola meal protein also has potential for human consumption and is composed of all essential amino acids as recommended by the Food and Agricultural Organization of the United Nations (FAO/WHO/UNU, 1985).

Canola oil is enriched in desirable fatty acids such as, 11 % α-linolenic (C18:3), 21 % linoleic (C18:2), 61 % oleic (C18:1), and reduced amounts of erucic acid (C22:1) (Canola Council of Canada, 2012). Low levels of polyunsaturated fatty acids such as linolenic acid (18:3) and higher levels of monounsaturated fatty acids such as oleic acid (18:1) offer higher stability of oil during high cooking temperatures for food processing (Abbadi and Leckband, 2011). Among the fatty acids (FA) present in canola oil, linolenic acid (C18:3) is also considered an essential fatty acid (Vaisey-Genser and Eskin, 1987). In foods, usage of linolenic acid and oleic acid

(C18:1) has been shown to decrease the concentrations of low and high density lipoproteins (LDL and HDL) which are the root causes of various cardiovascular problems (Vaisey-Genser and Eskin, 1987; Scarth and McVetty, 1999).

Two important components that influence the nutritional superiority of canola seed are the levels of erucic acid in the oil and glucosinalates in meal (Canola Council of Canada, 2010). The low levels of erucic acid of canola oil is greatly desirable as shown by the unusual high deposition of fat in heart muscle of rats nourished with food having higher erucic acid levels (Vaisey-Genser and Eskin, 1987). Rapeseed varieties having higher erucic acid contents are however valuable for the production of lubricants for industrial uses (Scarth and Tang, 2006).

As stated above, canola meal is also categorized by having less than 30 μmoles/g meal of total glucosinolate (GLS) levels. Glucosinolates (GLSs), secondary metabolites produced by *Brassicaceae*, are a class of organic compounds having nitrogen and sulphur formed from glucose and amino acids (Sonderby et al., 2010). They are produced by plants in response to stresses and higher levels are toxic for human consumption (Wittstock and Halkier, 2002). Genes playing important roles in GSL metabolism have been linked with QTL for seed GSL content and will be useful to regulate GSL levels in the seed without altering levels in other plant parts (leaves or stems) (Hasan et al., 2008).

The occurrence of phenolic compounds in the residual seed hulls adds another level of undesired characteristics of rapeseed meal, such as bitter taste and dark color. Mostly these compounds are condensed tannins (CTs) and sinapates that are thought to be anti-nutritive for livestock. In *Brassica* spp, the concentration of phenolic compounds is nearly 30 times greater than that of soybean (Shahidi, 1992).

2.1.3 Approaches used to improve canola oil

Both conventional breeding and transgenic methods have been used to manipulate canola seed oil. Conventional breeding utilizes controlled sexual recombination, usually attained by crossing and selfing, followed by selection for desirable traits (Sleper and Poehlman, 2006). This method has been used successfully to manipulate the total seed oil content and fatty acid composition to achieve various commercial requirements of vegetable oils (Burton et al., 2004; Scarth and Tang, 2006). Alterations in fatty acid composition have been the result of both natural and induced genetic mutations (Przybylski et al., 2005; Sleper and Poehlman, 2006). Conventional breeding, selection and mutations have all been used to produce conventional canola, specialty canola (low levels of linolenic and saturated fatty acids as well as varieties having high oleic and low linolenic acids) (Topfer et al., 1995; Przybylski et al., 2005; Scarth and Tang, 2006) and high erucic acid rapeseed (HEAR). However, due to the limited diversity in canola quality germplasm (Rahman et al., 2015) it is difficult to use traditional breeding to develop oils with tailored fatty acid compositions (Scarth and Tang, 2006).

Genetic manipulation has been used extensively to modify fatty acid composition, and initial attempts were directed towards the alteration of lipid biosynthetic enzymes (Knutzon et al, 1999; Scarth and Tang, 2006). Plants over-expressing *acyl-ACP thioesterase* (*FatB*) were used to develop oils containing high levels of caprylic, capric and palmitic acids (Dehesh et al., 1996; Voelker et al., 1997). Similarly, over-expression of *acyl ACP thioesterase* (*FatA*), and suppression of *stearoyl-ACP desaturase* produced oil with higher levels of stearic acid (Topfer et al., 1995; Scarth and Tang, 2006). By silencing *oleate desaturase* (*FAD2*), oils with higher levels of oleic acid were obtained (Stoutjesdijk et al., 2000; Scarth and Tang, 2006).

Success of these transgenic approaches has been affected by various factors that include the insertion number, the location of the transgene in the genome and the stability of the desired trait (Scarth and Tang, 2006). While genetic transformation has resulted in some success in modifying fatty acid composition (Dehesh et al., 1996; Tang et al., 2004; Sharma et al., 2008), limitations are encountered during attempts to increase seed oil content (Thelen and Ohlrogge, 2002; Weselake et al., 2009) .

Gene expression variation may contribute to an increased complexity of regulatory networks after polyploidization (Liu et al., 2014). In polyploid species, the use of forward and reverse genetics approaches are challenging because each gene is present in several copies (Gilchrist et al., 2013). Thus, mutations at single loci are difficult to identify as loss of function of the targeted gene can be masked by the action of the homeologous gene(s) (Gilchrist et al., 2013). TILLING (Targeting Induced Local Lesions in Genomes) is a cost-efficient reverse genetics technique that enables direct screening for point mutations in a particular gene of interest (Till et al., 2003; Wang et al., 2012). This technique allows for the recognition of mutations in distinct homeologous genes and their introgression into the same line. The phenotype can then be investigated to characterize the roles of the mutated gene (Wang et al., 2012; Gilchrist et al., 2013). Using TILLING, a diversity of loss-of-function phenotypes can be produced, while occasionally; gain-of-function phenotypes are also formed (Gilchrist et al., 2013). In most cases, however, loss of function alleles are formed by missense mutations that do not entirely abolish gene function and result in a partial loss in activity (Gilchrist et al., 2013). Due to these advantages, TILLING has been used to produce genetic variation in several polyploidy species including *B. napus* (Slade et al 2005; Wang et al., 2008; Uauy et al., 2009; Chawade et al., 2010)

Genome-engineering technologies that accurately transform genome sequence and control gene expression patterns in a site specific fashion have significant potential in plant biotechnology. Among these technologies, TALEs (Transcription activator-like effectors) and CRISPR (the clustered regularly interspaced palindromic repeats) systems have gained attention as genome-engineering platforms in various eukaryotic species. TALEs have been used with considerable success as a DNA directing module fused to functional domains for various genomic alterations (Cong et al., 2012; Li et al., 2012; Sanjana et al., 2012). Besides TALEs, the CRISPR system is the most novel genome-related technique (Barrangou et al., 2012). Using this system, two RNA molecules can be joined as a single guide RNA molecule (gRNA), which then has the ability of regulating the Cas9 protein to a specific target (Cong et al., 2013; Mali et al., 2013). On the other hand, the simplicity of engineering is escorted by a marked high rate of off-target actions of the enzyme (Fu et al., 2013; Liu et al., 2014). However, it is also anticipated that an innovative class of DNA- or RNA-guided nucleases based on the prokaryotic argonaute proteins could be created in the future to overcome the problems of the present editing platforms (Sheng et al., 2014; Swarts et al., 2014). The continuous target for all the genome-editing technologies is to increase the accuracy of changes while reducing off target effects to modify a variety of agriculturally important traits (Mahfouz et al., 2011; Shan et al., 2014).

2.2 Seed development

Arabidopsis has been extensively used as a model plant for studying various developmental processes in plant molecular biology (Mayer et al., 1991; West and Harada, 1993; Harada, 1997; Baud et al., 2002; Brocard-Gifford et al., 2003; Laux et al., 2004; Le et al., 2010). The extensive studies on *Arabidopsis* seed development and the regulatory mechanisms governing embryogenesis have been extended to economically important species, including

Brassica species to improve productivity (Mansfield et al., 1991; Mansfield and Briarty, 1991; Harada, 1997; Baud et al., 2002; Laux et al., 2004; Le et al., 2010).

Embryogenesis in higher plants starts with the double fertilization that gives rise to the diploid zygote and triploid endosperm (Harada, 1999). Fertilization occurs within the embryo sac, the mega-gametophyte embedded in maternal tissue. In *Arabidopsis*, the zygote becomes polarised and divides into two asymmetrical daughter cells, a small apical cell and large basal cell (Harada, 1999). Through precise divisions and differentiation patterns the apical cells will produce the embryo proper which undergoes defined stages of development. At the early cotyledonary stage of development, the cotyledons emerge from the embryo and the hypocotyl region elongates. The shoot and root apical meristems are formed at the opposite poles of the embryos (Kawashima and Goldberg, 2010).

Normally the whole process of seed development can be divided into three overlapping stages: development, maturation, and dormancy (Fig. 2.1) (West and Harada, 1993; Braybrook et al., 2006; Braybrook and Harada, 2008; Santos-Mendoza et al., 2008; Baud and Lepiniec, 2009). While the first stage involving morphogenesis and organogenesis is characterized by the formation of the embryo and the development of all its organs, the maturation stage is demarked by the accumulation of storage products which depending on the species, consists of a combination of starch, proteins and/or lipids. In the final stage, the seed enters dormancy, which is characterized by a reduction in metabolism (Mansfield and Briarty, 1992).

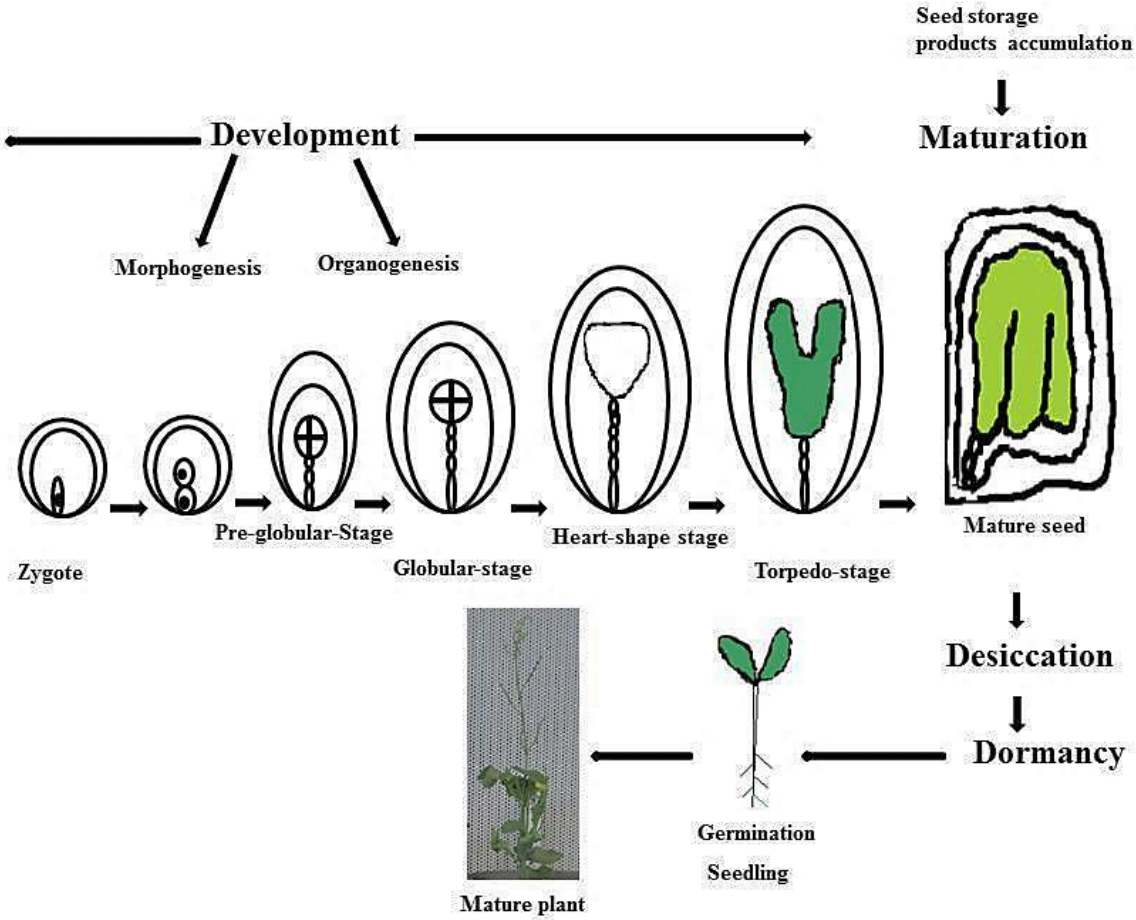

Fig. 2.1 Simple illustration of the different stages of dicot seed development as discussed by Le et al., 2010; Baud et al., 2002.

2.2.1 Early embryogenesis

During early embryogenesis the basic features of the embryo are established. This phase is characterized by precise cell division and differentiation patterns, all well described in *Arabidopsis* (Mansfield et al., 1991; West and Harada, 1993; Baud et al., 2002). This results in the formation of the embryo body consisting of a hypocotyl separating the root and shoot apical meristems, in addition to a set of cotyledons (Goldberg et al., 1994; Jurgens, 2001). Key regulators of these early processes, including many transcription factors ensuring the acquisition and establishment of polar growth have been identified (West and Harada, 1993; Harada, 2001). Many of these factors, which are characterized by precise temporal and spatial expression, are linked to auxin, the basipetal flow of which is paramount for the proper formation of the early embryo. Perturbation of auxin synthesis and/or transport results in abnormal embryos often lacking bilateral symmetry (Friml et al. 2003). Auxin also acts as a positional cue for the radial establishment of the embryos and is required for the proper formation of embryonic tissues, including the epidermis, cortex and stele (Moller and Weijers, 2009). Embryo development follows a series of developmental stages identified as globular, heart-shaped, early cotyledonary, and late cotyledonary. Numerous reviews on the topic are available (Nogler, 1984; Willemsen and Scheres, 2004)

2.2.2 Embryo maturation and accumulation of storage products

Embryo maturation is the second phase of embryogenesis which is demarked by the cessation of morphogenesis and the initiation of storage product accumulation (Harada, 1999). It is during maturation that embryonic cells (and or endosperm) accumulate macromolecules in the form of lipids, starch and/or protein which are required as a source of energy during the initial phases of germination, before the seedlings acquire the ability to photosynthesize (Harada, 1999;

Braybrook and Harada, 2008). While the composition of storage products is genetically determined and unique in different species, improper accumulation of storage reserves can be fatal for the survival of the seedling (Harada, 1999; Braybrook and Harada, 2008).

Fatty acids (FA) and lipids are the major macromolecules accumulating in *Brassica* seeds (Murphy et al., 1989; Schwender and Ohlrogge, 2002). During seed development, FA biosynthesis occurs in various organelles and is mediated by several metabolic pathways (Baud et al., 2002; Li-Beisson et al., 2010). In developing embryos, lipids are mainly stored in the form of triacylgylcerols (TAG) (Ohlrogge and Browse, 1995). Triacylgylcerols are produced from the esterification of three fatty acids to a glycerol backbone (Ohlrogge and Browse, 1995) and their production relies on the availability of sucrose produced during photosynthesis. Besides influencing the strength of the sink in various species, an active import and cleavage of sucrose is paramount for rapid production of triacylgylcerols (Schwender et al., 2003). Flow of sucrose, which is cleaved into hexoses by sucrose synthases and invertases (Schwender et al., 2003) for lipid synthesis, occurs through two distinct metabolic pathways: glycolysis and the oxidative pentose phosphate pathway (OPPP). In the glycolytic pathway, sucrose is oxidized to pyruvate through reactions producing (ATP) adenosine triphosphate (Schwender et al., 2003; Baud et al., 2007). Energy in the form of nicotinamide adenine dinucleotide phosphate is also generated during the catabolic steps of hexoses occurring in the OPPP (Schwender et al., 2003; Baud et al., 2007). The requirement of both pathways for TAG production has been documented in several species (Baud et al., 2007; Baud and Lepiniec, 2010)

2.2.2.1 Fatty acid biosynthesis

Fatty acid (FA) biosynthesis initiates in the plastids by a multi-enzyme complex termed fatty acid synthase (FAS) (Figure 2.2). Acetyl-CoA, a two carbon molecule, is the basic unit of

fatty acid synthesis (Ohlrogge and Browse, 1995). It is derived from pyruvate (Johnston et al., 1997) and converted to malonyl-CoA by the reaction catalyzed by the enzyme acetyl-CoA carboxylase (ACCase) (Konishi et al. 1996; Li-Beisson et al., 2010). Acetyl-CoA carboxylase (ACCase) is believed to be the key rate limiting step of the *de novo* fatty acid biosynthesis as it regulates the carbon flux (Baud et al., 2008). To enter the fatty acid biosynthetic route, the malonyl group moiety is converted to an acyl carrier protein (ACP) forming malonyl ACP by the activity of malonyl-CoA: acyl carrier protein malonyltransferase (Nikolau et al. 2003; Baud et al., 2008; Li-Beisson et al., 2010). Various condensation reactions can take place between acetyl-CoA and malonyl-thioester, (Li-Beisson et al., 2010). The first reaction which is catalyzed by 3-ketoacyl-ACP synthase (KAS III) results in to the production of 4:0-ACP (Baud et al., 2008). Other condensation reactions are catalyzed by KAS I and KAS II and lead to the production of 16:0-ACP and18:0-ACP, respectively (Pidkowich et al., 2007; Baud et al., 2008). Every condensation reaction is followed by two reductions and one dehydration reaction resulting in the addition of two carbon molecules to the extending fatty acyl chain (Li-Beisson et al., 2010). After this, the 18:0-ACP can be desaturated to yield 18:1-ACP, a reaction requiring stearoyl-ACP desaturase (Browse and Somerville, 1991). This characterizes the first desaturation event of a long chain acyl group that takes place in the plastid.

Due to these reactions, 16:0-ACP and 18:1-ACP are the major products which are produced by the fatty acid synthase complex. These 16:0-ACP and 18:1-ACP undergo hydrolysis which is catalyzed by the acyl-ACP thioesterase (Fat) enzyme, and release free fatty acids from the FAS machinery into the cytoplasm (Kjellberg et al. 2000; Baud et al., 2008; Li-Beisson et al., 2010). There are two main forms of acyl-ACP's theioesterases that cleave acyl-ACP: FatA and FatB. Acyl-ACP thioesterase, FatA normally hydrolyzes 18:1-ACP while 16:0-ACP is

hydrolyzed by FatB (Bonaventure et al., 2003; Li-Beisson et al., 2010). The free fatty acids formed are then converted to coenzyme A esters by acyl-CoA synthetases (Li-Beisson et al., 2010). A major number of these esters are transferred from the cytoplasm to the endoplasmic reticulum for further alterations such as elongation and desaturation (Li-Beisson et al., 2010).

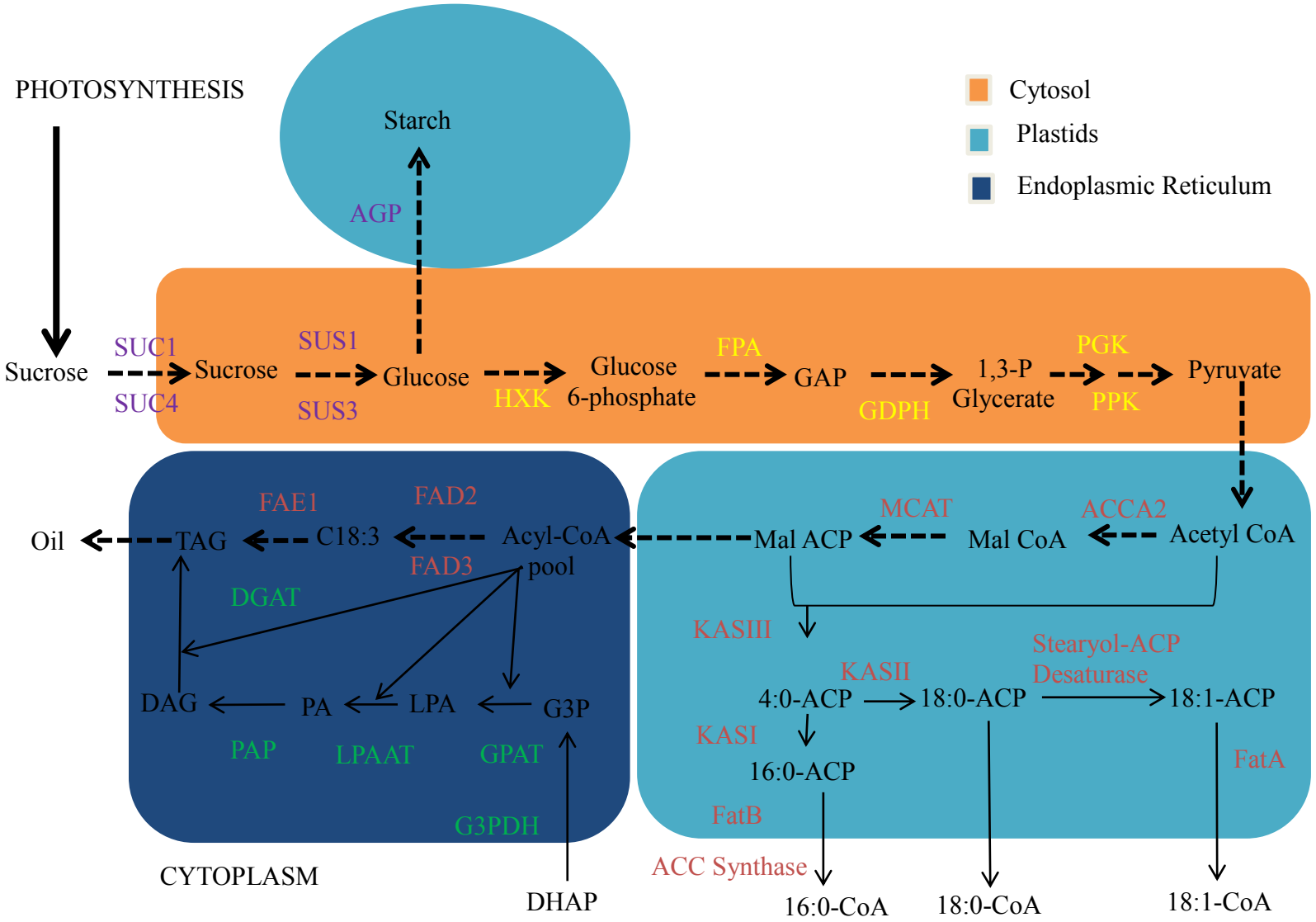
PHOTOSYNTHESIS
Starch
AGP
Cytosol
Plastids
Endoplasmic Reticulum
Sucrose
SUC1
SUC4
Sucrose
SUS1
SUS3
Glucose
HXK
Glucose
6-phosphate
FPA
GAP
GDPH
1,3-P
Glycerate
PGK
PPK
Pyruvate
Oil
TAG
FAE1
C18:3
FAD2
FAD3
Acyl-CoA
pool
DGAT
DAG
PA
LPA
G3P
PAP
LPAAT
GPAT
G3PDH
CYTOPLASM
DHAP
Mal ACP
MCAT
Mal CoA
ACCA2
Acetyl CoA
KASIII
KASII
4:0-ACP
18:0-ACP
Stearyol-ACP
Desaturase
18:1-ACP
KASI
16:0-ACP
FatA
FatB
ACC Synthase
16:0-CoA
18:0-CoA
18:1-CoA

Fig. 2.2 Simplified diagram showing the regulatory pathways leading to Fatty Acid (FA) synthesis in seeds. Enzymes involved in sucrose transport and metabolisms are highlighted in purple: SUC1 (Sucrose Transporter1), SUC4 (Sucrose Transporter4), SUS1 (Sucrose Synthase1), SUS3 (Sucrose Synthase3), AGP (ADP-Glucose Phosphorylase). Glycolytic enzymes are highlighted in yellow: HXK (Hexose Kinase), FPA (Fructose Bisphosphate Aldolase), GPDH (Glyceraldehyde-3- Phosphate Dehydrogenase), PGK (Phosphoglycerate Kinase) and PPK (Pyrophosphatase-Dependent Phosphofructokinase). Enzymes involved in FA synthesis are highlighted in red: ACCA2 (Subunit A of Acetyl-CoA Carboxylase), FAD2 (oleate desaturase) and FAD3 (linoleate desaturase), FAE1 (FA Elongation1), and MCAT (Malonyl-CoA:ACP Transacylase). 4:0-ACP (acyl carrier protein), KAS III, II and I (3-ketoacyl-ACP synthase isoform III, II and I), Acyl ACP thioesterase (i.e. FatB and FatA), ACC synthetase (Acyl-CoA synthetase), CoA (Coenzyme A). C16:0 (palmitic acid), C18:0 (Stearic acid), C18:1 (oleic acid), C18:3 (Linolenic acid), TAG: (Triacylglycerol). Enzymes used in the Kennedy Pathway are highlighted in green. DHAP (Dihydroxyacetone phosphate), (G3P) glycerol-3-phosphate, G3PDH (glycerol-3-phosphate acyltransferase), GPAT (glycerol-3-phosphate acyltransferase), LPA (lysophosphatidic acid), LPAAT (Lysophosphatidic acid acyltransferase), PA (phospatidic acid), DAG (diacylglycerol) (DAG), PAP (phosphatidic acid phosphatase). Triacylglycerols (TAGs), DGAT (diacylglycerol acyltransferase). This figure is based on the information published in Li-Beisson et al. (2010) and Elahi et al., (2015).

2.2.2.2 Fatty acid elongation

In the endoplasmic reticulum C16 and C18 fatty acids are elongated and/or desaturated to form fatty acids of different structures (Li-Beisson et al., 2010). Production of FA longer than C18 requires numerous steps taking place in the elongation pathway mediated by endoplasmic reticulum-related fatty acid elongases (Somerville et al., 2000; Li-Beisson et al., 2010).

Initially malonyl-CoA and 18:1-CoA are produced in the fatty acid elongation process, which is comprised of four reactions (Somerville et al., 2000; Baud et al., 2008). The first reaction is the condensation of acyl-CoA with malonyl-CoA that uses condensing enzymes (3-ketoacyl-CoA synthases) to produce 3-ketoacyl-CoA (Li-Beisson et al., 2010). This condensation is followed by a second reaction generating β-hydroxyacyl-CoA by using 3-ketoacyl reductase. The third reaction is the dehydration step, using 3-hydroxyacyl-CoA-dehydrase to generate enyol-CoA (Li-Beisson et al., 2010). The fourth reaction is the reduction of enoyl-CoA to acyl-CoA using the enzyme enoyl-CoA reductase. All four reactions (i.e., condensation, reduction, dehydration and final reduction) lead to the production of long fatty acids, which can be further modified in successive stages (Somerville et al. 2000; Li-Beisson et al. 2010).

2.2.2.3 Fatty acid desaturation

Desaturation, the addition of a double bond in the acyl chain, is an enzymatic reaction resulting in reduction of a dioxygen molecule to water (Shanklin and Cahoon, 1998).

Polyunsaturated fatty acids in plants are formed by two autonomous pathways with particular enzymes catalyzing the reactions, one in the microsomes or endoplasmic reticulum (ER) and the other in the plastid (Sperling et al., 1993). In Arabidopsis seeds, the most abundant unsaturated fatty acids are linoleic (18:2) and linolenic (18:3) acids (Ohlrogge and Browse, 1995; Baud et al., 2008). Both fatty acids are main membrane components and are vital for human diet (Okuley et

al., 1994). Desaturation of oleic acid (18:1) results in the formation of both linoleic (18:2) and linolenic (18:3) acids (Baud et al., 2008). The enzymes oleate desaturase (FAD2) and linoleate desaturase (FAD3) transform the C18:1-PC to C18:2-PC and C18:2-PC to C18:3-PC, respectively (Somerville et al., 2000; Baud et al., 2008). The larger majority of polyunsaturated fatty acids are produced by the activity of oleate desaturase (Okuley et al. 1994).

2.2.2.4 TAG (triacylglycerol) biosynthesis

Triacylglycerols (TAGs) are seed storage lipids that are stored in specialized organelles called oil bodies which have phospholipid monolayer and structural proteins mainly composed of oleosins (Ohlrogge and Browse, 1995; Somerville et al., 2000; Baud et al., 2008; Li-Beisson et al., 2010). The exact procedure of oil body production is unknown (Baud et al., 2008; Baud and Lepiniec, 2009). However, it is believed that TAGs assemble together within the lipid bilayer initially found within microdomains of the endoplasmic reticulum (Murphy and Vance, 1999; Baud et al., 2008). Figure 2.2 summarizes the various components and routes taking part in TAG synthesis, which differ depending on the fatty acid formed (Ohlrogge and Browse, 1995). The interaction amongst the numerous enzymes and proteins taking part in the regulation of fatty acid biosynthesis is critical for altering the level and composition of seed oil.

Triacylglycerol formation also takes place inside the endoplasmic reticulum via the Kennedy pathway (Figure 2.2) (Baud et al., 2008). In this pathway, the enzyme G3PDH (glycerol-3-phosphate acyltransferase) converts DHAP (dihydroxyacetonephosphate) to G3P (glycerol-3-phosphate) (Baud et al., 2008; Li-Beisson et al., 2010). The enzyme glycerol-3-phosphate acyl transferase (G3PAT) then transforms G3P to lysophosphatidic acid (LPA) (Baud et al., 2008), and lysophosphatidic acid acyltransferase (LPAAT) adds an acyl group to the LPA leading to the formation of PA (phosphatidic acid) (Baud et al., 2008; Li Beisson et al., 2010).

Dephosphorylation of PA to DAG (diacylglycerol) takes place with the help of PAP (phosphatidic acid phosphatase) (Weselake et al., 2009; Li-Beisson et al., 2010). For the production of TAG, an acyl group is added to the DAG, a step catalyzed by the enzyme diacylglycerol acyltransferase (DGAT) (Ohlrogge and Browse, 1995; Baud et al., 2008; Li-Beisson et al., 2010). There are many ways allowing fatty acids to move in the route leading to TAG biosynthesis (Weselake et al., 2009). Some fatty acids can also go through the desaturation process (Baud et al., 2008). DAG requires the enzyme CPT (cholinephosphotransferase) to be incorporated into the PC (phosphatidylcholine) pool and the remaining acyl groups are desaturated by FAD (fatty acid desaturase) enzymes (Li-Beisson et al., 2010). For the production of TAG, DAG needs an acyl group which may also come from fatty acids present in the PC pool, through reactions requiring PDAT (phospholipid: diacylglycerol acyltransferase). This reaction also generates a secondary substrate: (LPC) lysophosphatidylcholine (Li-Beisson et al., 2010). In the PC (phosphatidylcholine), the hydrolysis of fatty acids can also generate LPC and this reaction is catalyzed by PLA_2 (phospholipase). Facilitated by LPCAT (lysophosphatidylcholine), LPC can again enter the route of fatty acid desaturation (Weselake et al., 2009; Li-Beisson et al., 2010). Fatty acids from the acyl-CoA pool are moved into the PC pool in a reaction catalyzed by LPCAT (lysophosphatidylcholine acyltransferase) (Weselake et al., 2009; Li-Beisson et al., 2010). The fatty acid biosynthesis route is characterized and extensive research has been conducted on various genes encoding for several proteins and enzymes directly regulating fatty acid and TAG synthesis (Baud and Lepiniec, 2009). In spite of this effort, the regulatory mechanisms of this pathway are not completely understood (Thelen and Ohlrogge, 2002). Several studies have evaluated the distinct expression of genes taking part in the regulation of seed development (Mu et al., 2008; Liu et al., 2010; Le et al., 2010). Recently, interest has been

directed to manipulating transcription factors, which besides regulating embryo and seed development; influence the level of oil and the FA composition (Mu et al., 2008; Liu et al., 2010).

2.2.2.5 Glucosinolates (GLS) biosynthesis

Due to the antinutritional nature of GLS significant research has focused on decreasing GLS content in seed (Feng et al., 2012). There are various classes of GLS in plants, but the aliphatic and indole GLSs are the prominent ones and are derived from methionine and tryptophan, respectively (Schonhof et al., 2004; Elhiti et al., 2012). A simplified GLS biosynthetic pathway is represented in Figure. 2.3.

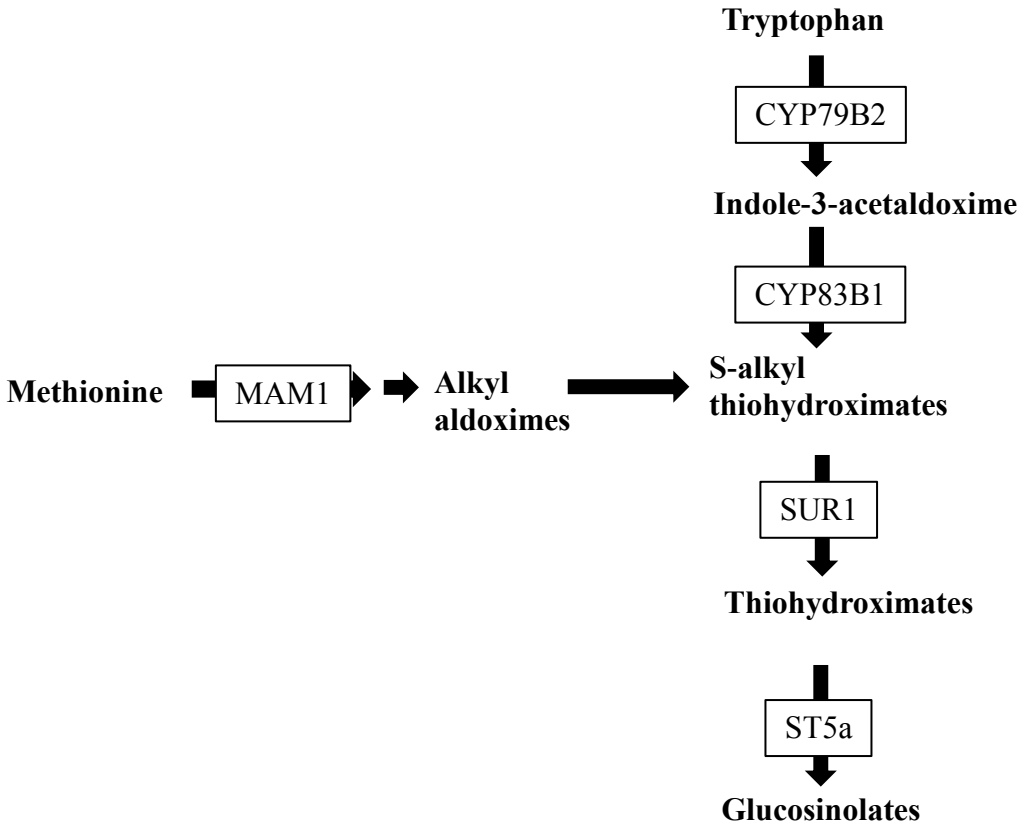

Fig. 2.3 Simplified illustration of glucosinolate (GLS) biosynthesis. MAM1 (Methylthioalkylmalate Synthase1), CYP79B2 (Cytochrome P450 CYP79B2, CYP83B1 (Cytochrome P450 CYP83B1), SUR1 (Superroot 1) and ST5a (Sulphotransferase5a). This figure is based on the information published in Elahi et al., 2016.

Initiation of aliphatic GLS biosynthesis starts with the conversion of methionine to alkyl aldoximes catalyzed by METHYLTHIOALKYLMALATE SYNTHASE1 (MAM1), which is the first key enzyme involved in the chain elongation cycle of glucosinolate biosynthesis. METHYLTHIOALKYLMALATE SYNTHASE1 (MAM1) is considered a crucial regulator of GLS biosynthesis as its down regulation significantly decreases the GLS content (Liu et al., 2011).

Then S-alkyl thiohydroximates are formed from alkyl aldoximes. The S-alkyl thiohydroximate pool is also enriched by a pathway using tryptophan as a precursor. Tryptophan-derived molecules are integrated in the S-alkyl thiohydroximate pool by two reactions which are catalyzed by cytochrome P450s CYP79B2 and CYP83B1. Studies on *CYP79B2* revealed that this gene is wound-inducible and expressed nearly in all plant tissue (root, stem, leaves and flowers) and its overexpression leads to higher levels of indole GLS (Mikkelsen et al., 2000). Cytochrome P450 CYP83B1 (CYP83B1) regulates the level of indole-3-acetaldoxime used for GLS and IAA production. Indole-3-acetaldoxime is a common substrate for GLS (Figure. 2.3), but it is also utilized in primary and secondary metabolism (Grubb &Abel, 2006). Down-regulation of *CYP83B1* reduces GLS and increases IAA (Bak et al., 2001; Elhiti et al., 2012). Then SUPERROOT1 (SUR1) converts the S-alkyl aldoximes to thio-hydroximates (discussed in Elhiti et al., 2012). Mutation of *SUR1* caused phenotypic defects that are specific to high auxin levels (Boerjan et al., 1995). This gene encodes a C-S protein whose expression influences GLS levels by producing thiohydroximates (reviewed by Nafisi et al., 2006). Lastly the thiohydroximates are converted to GLS and this last reaction is catalyzed by the enzyme ST5a (SULPHOTRANSFERASE5a) (Piotrowski et al., 2004; Elhiti et al., 2012) (Figure. 2.2). Two transcription factors, *MYB-Containing Transcription Factor28* and *Altered Tryptophan*

Regulation1 are also involved in the regulation of GLS homeostasis by activating various GLS enzymes (reviewed by Sonder by et al., 2010).

2.2.3 Regulatory network of seed development

Proper execution of the embryogenic pathways relies on the precise and often synchronized expression of several factors including transcription factors, chromatin condensation factors, mRNA permanence, as well as factors regulating DNA methylation and post-translational modification (Riechmann and Ratcliffe, 2000; Ratcliffe and Riechmann, 2002). Many studies, mainly in *Arabidopsis*, have evidenced the importance of a set of transcription factors (Riechmann and Ratcliffe, 2000; Ratcliffe and Riechmann, 2002; Spencer et al., 2007), the function of which is described below.

BABY BOOM (*BBM*)

BABY BOOM (*BBM*) is a member of the AP2/ERF family (Boutilier et al., 2002). It is a transcription factor that stimulates developmental processes linked to cell proliferation and growth, and is commonly expressed in developing seeds (Boutilier et al., 2002; Imin et al., 2007; Passarinho et al., 2008). In *Arabidopsis* and *B. napus*, overexpression of *BBM* induces somatic embryogenesis (Boutilier et al., 2002).

WUSCHEL (*WUS*) and *WUSCHEL-related HOMEOBOX* (*WOX*)

The *WUSCHEL* (*WUS*) gene has a homeobox domain and is expressed in the organizing center of the shoot meristem where it controls the stem cell identity by interacting with the *CLAVATA* (*CLV*) regulatory system (Laux et al., 1996; Mayer et al., 1998). The *CLAVATA* genes (*CLV1*, *CLV2* and *CLV3*) repress *WUS* at the transcript level (Fletcher et al., 1999; Schoof et al., 2000). Shoot meristems are self-regulatory systems in which *WUS*/*CLV* relations create a feedback loop among the stem cells and the organizing center (Schoof et al., 2000). In *Arabidopsis*, the ectopic expression of *WUS* promotes somatic embryogenesis (Zuo et al., 2002;

Gallois et al., 2002) and the expression of W*US* demarks the sites of somatic embryo formation (Su et al., 2009). *WUSCHEL* is a member of a large *WOX* family, which also contains other members required for cell fate acquisition during embryogenesis (Haecker et al., 2004). Two important members of the *WOX* family are *WOX2* and *WOX8* which are initially expressed in the zygote (Breuninger et al., 2008). After the first asymmetric division of the zygote, however, expression of *WOX2* is restricted to the apical cell, while that of *WOX8* in the basal cells (Breuninger et al., 2008). It is believed that it is this differential expression which confers the diverse "cell fate" to the cells, with the apical cell originating the embryo proper and the basal cell the suspensor (Mayer et al., 1998). Another key member of the *WOX* family is *WOX9* which plays an important role in *Arabidopsis* meristem growth and maintenance (Wu et al., 2007).

***KNOTTED –like HOMEOBOX* (*KNOX*)**

The *KNOTTED1* (*KN1*) gene was initially isolated by using transposon tagging in a maize mutant line with unusual 'knotted' leaves. Homologous genes to the maize *KN1* were later identified in *Arabidopsis* and named *KNA1, KNAT2,* and *SHOOT-MERISTEMLESS* (*STM*) (Lincoln et al., 1994). These genes were included in the *KNOX* homeodomain superfamily of transcriptional regulators (Vollbrecht et al., 1991). *KNOX* genes are diverse regulators of plant development and this multiplicity includes meristem maintenance and proper organ patterning (Hay and Tsiantis, 2010). During embryogenesis, the *Arabidopsis STM* gene is required for the formation and maintenance of the shoot apical meristem (Long et al., 1996) Overexpression of *BnSTM* significantly increases the total seed oil content (Elhiti et al., 2012). The proposed function of *STM* gene is to stimulate cytokinin biosynthesis and suppress gibberellin biosynthesis; factors required for the acquisition of "meristematic" identity (Jasinski et al., 2005).

***WRINKLED1* (*WRI1*)**

WRI1 (*WRINKLED1*) encodes an (AP2/EREB) APETALA2/ethylene-responsive element binding transcription factor (Liu et al., 2010). The AP2 domain of this gene is vital for binding to the promoter region of target genes to trigger their expression. By altering the sensitivity of the embryos to abscisic acid, *WRI1* affects the capability of the developing embryos to sense sugar (Cernac et al., 2006). *WRI1* has important roles in seed oil storage as it affects the expression of enzymes involved in glycolysis and the ability of the developing embryo to convert sucrose into the preliminary metabolites essential for fatty acid biosynthesis (Cernac and Benning, 2004; Baud et al., 2007; Santos-Mendoza et al., 2008). Studies of *wri1* mutant plants show that this gene is involved in carbohydrate metabolism and mutant seeds have wrinkled seed coats with reduced total seed oil (Focks and Benning, 1998). Additionally, ectopic expression of *WRI1* leads to the production of triacyglycerols in growing seedlings and also a 10 % to 40 % increase in seed oil content (Cernac and Benning, 2004; Liu et al., 2010).

***LEAFY COTYLEDON1* (*LEC1*)**

LEAFY COTYLEDON1 (*LEC1*) is expressed during the whole course of embryogenesis (West et al., 1994). The *LEC1* encodes the HAP3 subunit (CBF, also known as NF-Y) which has been found in the CCAAT binding factor (Lotan et al., 1998; Kwong et al., 2003; Lee et al., 2003). The HAP3 subunits consist of three domains (A, B, and C domains) with the B domain being conserved during eukaryotic cell evolution (Lee et al., 2003). The CBF enables *LEC1* to be a precise transcriptional regulator that triggers the expression of downstream genes containing the CCAAT recognition domain (Lotan et al., 1998).

During embryogenesis, *LEC1* mutation causes defects in embryo development such as abnormal suspensor and defective cotyledon morphology (Meinke et al., 1994; Lotan et al.,

1998). Mutant seedlings of *lec1* produce trichomes in the cotyledon indicating that *LEC1* regulates cotyledon identity (Lotan et al., 1998). By altering the auxin and sugar levels, *LEC1* critically affects the early stages of embryogenesis (Casson and Lindsey, 2006). In *Arabidopsis*, ectopic expression of *LEC1* produces embryo-like structures (Lotan et al., 1998; Stone et al., 2001). The ability to form somatic embryos suggests that *LEC1* regulates embryogenic competence (Lotan et al., 1998; Stone et al., 2001). By directing morphogenesis and maturation, *LEC1* stimulates a cellular environment that induces embryonic growth (Stone et al., 2001). Moreover *LEC1* overexpression results in a simultaneous over-expression of 58 % or more genes encoding enzymes having a role in the plastidal fatty acid biosynthetic pathway resulting in substantially increased seed oil accumulation (Mu et al., 2008; Elahi et al., 2016). In *Arabidopsis*, *lec1* mutants show a remarkable reduction in seed storage products (i.e., seed oil and protein) (Meinke et al., 1994), although storage proteins and oleosins were detected in the mutants suggesting that the role of *LEC1* may be partly performed by other *LEC*–like genes. It is well accepted that *LEC1* regulates the biosynthesis of seed storage protein possibly through *FUS3* and *ABI3* (Kagaya et al., 2005b). Collectively, these findings suggest that *LEC1* plays a fundamental role in storage product accumulation during seed maturation.

Besides influencing accumulation of storage products, mutations in *LEC1* lead to the premature germination (West et al., 1994) and cause intolerance to desiccation (Meinke et al., 1994). Thus, the function of this gene might be required for several aspects of embryo and seed development (Meinke et al., 1994; Lotan et al., 1998; Braybrook and Harada, 2008).

***LEC1-like* (*L1L*)**

LEC1 and *LEC1-like* proteins have distinctive roles during embryogenesis as both of these proteins have a HAP3 subunit, and a particular amino acid residue that differentiates their roles from other proteins having the HAP3 subunit (Kwong et al., 2003; Yamamoto et al., 2009). In the presence of *ABA*, both *LEC1* and *L1L* stimulate the seed storage protein Cruciferin C (CRC) by interacting with the ABA-response element (ABRE)-binding factor (bZIP67) (Yamamoto et al., 2009). Despite some functional redundancies between *LEC1*and *LIL*, their respective mutants shows different abnormalities during embryo development (Kwong et al., 2003); an observation demonstrating their distinct roles during embryogenesis (Lee et al., 2003; Yamamoto et al., 2009).

Differences in transcript abundance of *LEC1* and *L1L* are apparent during both embryonic and post-embryonic development. During embryogenesis, for example, *LEC1* transcripts appear earlier then *LIL* (Kwong et al., 2003; Lee et al., 2003; Yamamoto et al., 2009). Furthermore, differently from *LEC1*, which is expressed only during embryogenesis, expression of *LIL* occurs in the vegetative organs during post-embryonic growth (Kwong et al., 2003; Lee et al., 2003; Yamamoto et al., 2009).

***LEAFY COTYLEDON 2* (*LEC2*)**

LEC2 is a B3 domain transcription factor and is mainly expressed during seed development. The B3 domain targets the promoters of downstream genes having the RY recognition site (Stone et al., 2008; Santos-Mendoza et al., 2008). Studies overexpressing or down-regulating *LEC2* show the involvement of this gene during embryogenesis (Stone et al., 2001; Braybrook et al., 2006; Braybrook and Harada, 2008). *Leafy cotyledon2* mutant plants have various defects including elongated hypocotyl, altered anthocyanin deposition, and

trichomes on the cotyledons (Keith et al., 1994; Meinke et al., 1994; Stone et al., 2001). Like *LEC1*, over-expression of *LEC2* stimulates the expression of the genes involved in the synthesis of auxin, which in turn stimulates somatic embryogenesis (Stone et al., 2008). However, unlike *lec1*, *lec2* mutant seeds are tolerant to desiccation (Stone et al., 2008; Meinke et al., 1994). These observations suggest that the two genes might not have overlapping functions.

A key function of *LEC2* is to initiate the embryogenic program from vegetative tissue (Stone et al., 2001; Braybrook et al., 2006), and two mechanisms have been proposed for this effect. Through the first mechanism, *LEC2* activates *ABI3* and *FUS3* genes required for the acquisition of the embryogenic competence (Stone et al., 2008). Expression of *LEC2* is also required for the induction of auxin biosynthesis through the activation of *YUCCA* genes (Stone et al., 2008). The *LEC2*–mediated production of auxin is necessary for the de-differentiation of the vegetative cells and their reprogramming towards the embryogenic pathway (Stone et al., 2008). Expression of *LEC2* is also required for accumulation of seed storage products (Santos Mendoza et al., 2005). *Arabidopsis* plants ectopically expressing *LEC2* have elevated amounts of long chain fatty acids, triacylglycerol, oleosins as well as 2S and 12S proteins (Santos Mendoza et al., 2005; Stone et al., 2008). This function might be due to the ability of *LEC2* to regulate *WRI1*, an important transcription factor inducing many genes that encode enzymes involved in glycolysis and fatty acid biosynthesis (Focks and Benning, 1998; Baud et al., 2009).

***FUSCA3* (*FUS3*)**

As a member of the B3-domain family, *FUS3* identifies and binds to the RY element CATGCA (found in the promoters of several genes) and plays a very important role during seed development (Curaba et al., 2004; Santos-Mendoza et al., 2008). Mutation in *FUS3* leads to many abnormalities such as desiccation intolerance, precocious germination, anthocyanin

accumulation in developing seeds, defective hormone synthesis, perception of light and trichome formation in the cotyledons (Keith et al., 1994; Meinke et al., 1994; Harada, 2001). *Arabidopsis* and *B.napus fus3* mutants have altered accumulation of oil and proteins (Meinke et al., 1994; Harada, 2001; Elahi et al., 2015) suggesting a role of this gene in storage product accumulation (Baumlein et al., 1994). The stimulation of 2S albumin and 12S cruciferin synthesis, as well as the transcriptional induction of numerous enzymes participating in fatty acid biosynthesis such as *KAS I, KASII, KASIII, PYRUVATE DEHYDROGENASE,* and *ACETYL-COA CARBOXYLASE* was observed in *Arabidopsis* plants over-expressing *FUS3* (Wang et al., 2007). The function of *FUS3* might be related to that of *LEC2* as both suppress gibberellin biosynthesis thus, inhibiting precocious germination (Curaba et al., 2004).

***ABA INSENSITIVE3* (*ABI3*) and *ABA INSENSITIVE5* (*ABI5*)**

The *ABI3* (*ABA insensitive3*) gene is a B3 binding domain transcription factor (Vicient et al., 2000; Santos-Mendoza et al., 2008). Expressed in developing seeds, *ABI3* closely interacts with *LEC2* and *FUS3* (Vicient et al., 2000; Santos-Mendoza et al., 2008). *ABI3* mutation leads to various abnormalities during the last stages of embryogenesis resulting in the lack of dormancy and desiccation intolerance (Ooms et al., 1993; Nambara et al., 1995). During seed development, overexpression of *ABI3* enhances the expression of the genes involved in the synthesis of storage proteins (Parcy et al., 1994) in a similar fashion to that noted following the over-expression of *FUS3* (Wang et al., 2007). It is postulated that *FUS3* triggers the expression of *ABI3* (Parcy et al., 1994), an observation suggestive that the two genes participate in a similar pathway.

ABI5 is another leucine zipper transcription factor involved in seed maturation. Studies overexpressing of *ABI5* in *abi3* mutants suggest that *ABI5* acts downstream of *ABI3* (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001, 2002).

2.2.3.1. Gene network governing seed storage accumulation

From the description of some of the key genes involved in embryogenesis and seed development two general points are apparent. Firstly, genes required for embryo morphogenesis also influence the deposition of storage products, thus denoting that the two events are linked by common molecular mechanisms. Secondly, genes participating in seed storage, such as *LEC1*, *LEC2*, *ABI3*, *FUS3* and *WRI1* often interact with each other sharing common regulatory mechanisms. Some of these interactions are described in Fig. 2.4.

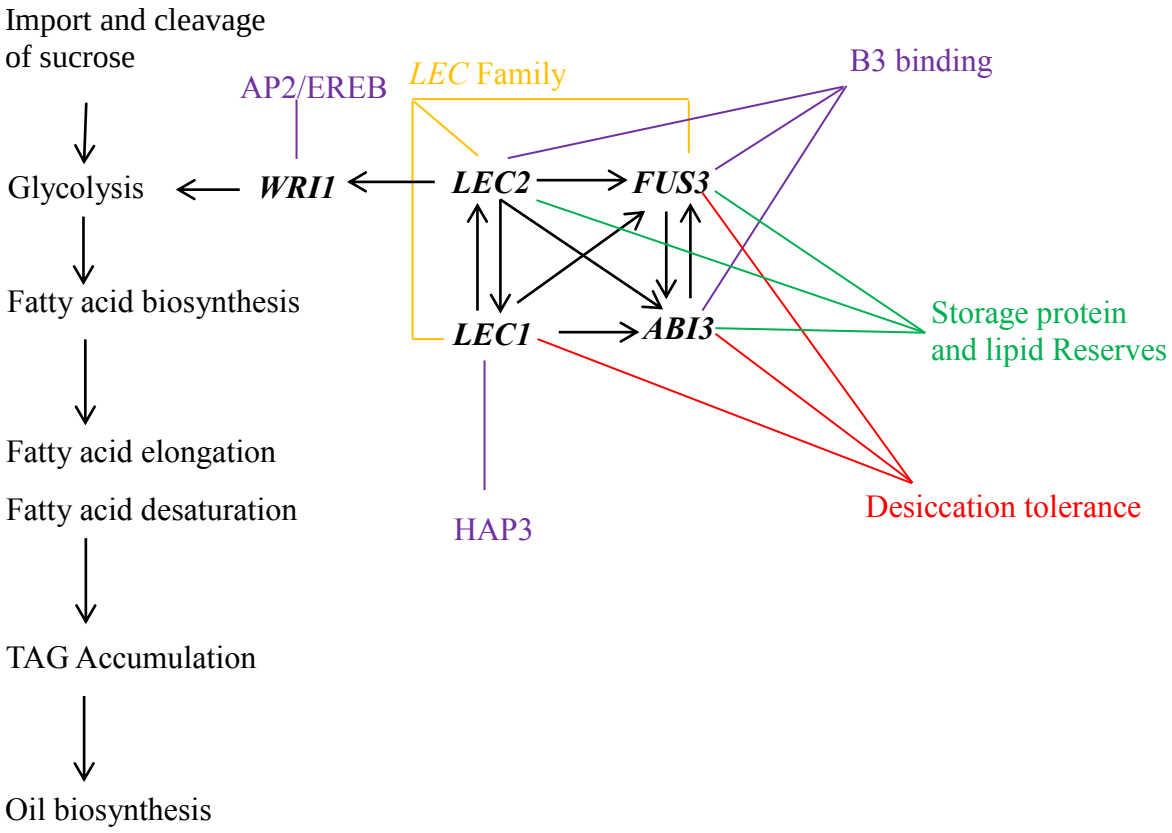

Fig. 2.4 Regulatory interaction and classification of structural characteristics of key transcription factors controlling seed development. *LEC1* and *LEC2* (*LEAFY COTYLEDON1* and *2*), *ABI3* (*ABSCISIC ACID INSENSITIVE3*), *FUS3* (*FUSCA3*) and *WRI1* (*WRINKLED1*), as discussed by Baud et al. (2008), Santos-Mendoza et al. (2008), and North et al. (2010).

2.3 *In vitro* Embryogenesis

Due to advancements of plant tissue culture techniques, *in vitro* regeneration of plants has been extensively used for various applications such as propagation and genetic modification (Hicks, 1994). *In vitro* plant regeneration can be attained through several methods, some of which producing a bipolar structure, i.e. embryos, via somatic or gametophytic embryogenesis, while others generating organs, i.e. roots or shoots, via organogenesis (Phillips, 2004). Both methods can occur directly or indirectly i.e. with or without an intervening callus phase.

As it was described in the previous sections, embryogenesis in higher plants starts with the double fertilization resulting in the formation of a single-cell zygote and the endosperm. Growth of the zygote produces fully matured embryos. Formation of embryos *in vivo* can also occur without fertilization by the formation of asexual embryos, called apomictic embryos originating from the maternal tissue, or from the unfertilized egg cell (Nogler, 1984). Apomixis is indicative of the fact that cues required for embryogenesis are not necessarily induced by the fertilization event, but rather can be generated by proper environmental conditions which are reproducible *in vitro*. The morphology of *in vitro* produced embryos is similar to that described in seed embryos, as globular, heart and cotyledonary-staged embryos are observed (Willemsen and Scheres, 2004). Due to these similarities, cultured embryos can be used as a model system to examine molecular and physiological events (Nogler, 1984) which would be difficult to examine using *in vivo* embryos, which are embedded in the maternal tissue and therefore not easily accessible. *In vitro* embryogenesis methods offer several advantages including: 1) the large number of embryos that can be obtained in a short period of time, 2) in several systems embryo production is synchronized, and embryos at similar stages of development can be harvested, 3)

the absence of the maternal tissues which allows for an easy collection of the embryos and 4) the lack of a dormancy.

Formation of embryos *in vitro* can occur via somatic embryogenesis and/or gamethophytic embryogenesis (Phillips, 2004). Somatic embryogenesis involves production of embryos from somatic cells, i.e. cells other than gametes, while gametophytic embryogenesis uses males or female gametophytes as the initial explants (Huang and Yeoman, 1995; Barinova et al., 2004; Belles-Boix et al., 2006; Malik et al., 2007; Malik et al., 2008). Choice of the technique is often species dependent and in Brassica gametophytic embryogenesis, from the male gametophyte (microspores) is the propagation method routinely used to generate embryos in culture (Bhojwani and Razdan, 1996; Raghavan, 2000; Maraschin et al., 2005).

Independent studies have used *B. napus* microspore-derived embryogenesis (MDE), for physiological and molecular studies (Malik et al., 2007; Stasolla et al., 2008). During the process, an important event is the re-differentiation step which allows the gamethophytic cells to acquire a new embryogenic fate and form embryos. This gametophytic-embryonic transition can be stimulated by various stress treatments such as high or low temperatures, (Sunderland et al., 1977; Keller and Armstrong 1979; Custers et al. 1994; Kasha et al., 1995), gamma irradiation (Pechan and Keller, 1989), sucrose deprivation (Touraev et al., 1996), colchicine treatment (Zhao et al., 1996), and alterations in the medium pH (Barinova et al., 2004). These stress treatments stimulating the re-differentiation phase cause changes in cytoskeletal organization (Telmer et al., 1992), and induce the division of the microspore (Yeung, 2002).

The two daughter cells formed from the microspore have different fates. Through anticlinal and periclinal divisions, the apical cell forms the “proembryogenic cell” that gives rise

to a cluster of cells demarking the globular phase of embryogenesis while the basal cell forms the suspensor of the embryo (Elhiti, 2010).

Approximately after fifteen days in culture the globular embryos grow into heart-shaped embryos which then develop further into fully mature embryos. The development of the *B. napus* MDEs is very similar to that observed in seed embryos (Yeung et al., 1996).

2.3.1 Genetic features of *in vitro* embryogenesis

The in vitro embryogenesis system is suitable for studying the molecular and physiological bases of plant embryogenesis (Singla et al., 2007; Malik et al., 2008; Karami and Saidi, 2010; Karami et al., 2009). As discussed before, embryogenesis in *Brassica* species requires the de-differentiation of the gametophytic cells and their redirection towards an embryonic fate. This transition has been the center of several investigations in an effort to identify underlying regulatory mechanisms.

Tsuwamoto et al. (2007) identified 136 non-redundant ESTs related to the gametophytic-embryonic transition. Out of these 136 ESTs, the authors selected 14 genes, 10 of which were defined as androgenesis-related and were suppressed in the microspores by the imposed heat-shock treatment. The remaining 4 genes were expressed soon after, during the initial stages of embryo development (Tsuwamoto et al., 2007). A more comprehensive study was performed by Malik et al. (2007) who identified 16 "embryogenesis-marker" genes, seven of which (*LEC1*, *LEC2*, *FUS3*, *ABI3*, *BBM1*, *WOX2*, *WOX9*) are transcription factors. Depending on their unique expression during microspore-derived embyrogenesis, these molecular markers were further categorized into the following three distinct groups.

Embryo-specific genes Embryo-specific genes (*FUS3*, *LEC1*, *LEC2*, *UP1*, and *UP2*) were expressed only in MDEs and in developing zygotic seeds during different developmental phases (Malik et al. 2007).

Embryo-expressed genes Besides being expressed in MDEs and in developing zygotic seeds, the embryo-expressed genes (*NAPIN*, *BBM1*, *BnFAD1*, *WOX9*, *AB13*, and *ATS1*) also showed expression in other non-seed tissues. *BBM1* for example, showed strong root expression (Malik et al. 2007).

Sporophyte-expressed genes The sporophyte genes (*LRR1*, *CP1*, *CYP78A*, *WOX2*, and *CYP81F*) were preferentially expressed in the sporophytic tissues (Malik et al. 2007).

These studies were followed by others utilizing some of these genes to assess the behavior of Brassica MDEs under specific culture conditions. Elhiti et al. (2012) used nine genes: *SERK-like kinase*; *LEAFY COTYLEDON1* and *2* (*LEC1* and *LEC2*) *UNKNOWN PROTEIN1* (*UPI1*); *BABYBOOM1* (*BBM1*), *WUSCHEL-related HOMEOBOX9* (*WOX9*), *ABSCISIC ACID3* (*ABI3*), *CYTOCHROME P78A* (*CYP78A*) and *WUSCHEL*-related *HOMEOBOX2* (*WOX2*) to evaluate proper embryonic growth. Out of these nine molecular markers, six are transcription factors (i.e., *LEC1*, *LEC2*, *ABI3*, *BBM1*, *WOX2* and *WOX9*) and were discussed above. Of the remaining three, the function of *UPI1* is unknown, although its expression is embryo-specific and can be used to differentiate embryogenic tissue from non-embryogenic tissue (Malik et al., 2007). Its preferential expression in embryogenic cells suggests that *UPI1* is somehow linked to the acquisition of embryogenic competence.

SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE (*SERK*) was initially isolated from carrot suspension cultures (Schmidt et al., 1997). During embryogenesis, its expression was not observed until the globular stage of zygotic embryogenesis (Salaj et al., 2008). Albertini et al. (2005) showed that production of apomictic embryos in *Poa pratensis L.* is linked with the *PpSERK* expression, and overexpression of *AtSERK* in *Arabidopsis* enhances the competence of somatic embryogenesis (Hecht et al., 2001). All these studies indicate that *SERK* promotes

embryogenesis both *in vivo* and *in vitro*. This idea was further substantiated in other studies linking the expression of this gene to the stress-conditions imposed in culture which are necessary for the acquisition of embryogenic competence (Feher et al., 2003). The last gene used as an embryogenesis molecular marker gene by Elhiti et al. (2012) is *CYP78A5* which was initially detected throughout floral and ovule development (Zondlo and Irish, 1999). It is thought that *CYP78A5* creates a mobile growth-promoting signal that controls organ size (Anastasiou et al., 2007). Signal produced by *CYP78A5* coordinates the floral organ growth and its symmetry (Eriksson et al., 2010). The function of this gene might also be required during embryogenesis. Embryos of the double knock-out mutant *cyp78a5 cyp78a7* are smaller in size and have unusual shoot apical meristems and cotyledons (Wang et al., 2008). Therefore, the role of this gene in fundamental developmental events in embryogenesis cannot be discounted.

Based on the information reviewed above, it appears that the alteration of the expression of transcription factors that affect the seed maturation phase of development (*LEC1*, *LEC2*, *FUS3*, *WRI1*, and *ABI3*) also may modify the FA biosynthesis in *B. napus*. This hypothesis was tested based upon the projects described below and found in the following three chapters.

Chapter 1 *BnLEC1* transgenic lines were generated using *BnLEC1* version A (GU945399) and version B (GU945398) and subsequently evaluated for seed oil level, FA profile, and transcription of genes participating in oil biosynthesis.

Chapter 2 *BnFUS3* TILLING mutant lines were produced and characterized using the same parameters listed in Chapter 1.

Chapter 3 *Brassica napus* microspore-derived embryos with altered expression of *BnLEC1* and *BnFUS3* were assessed to determine the impact of the two genes on embryo development and oil biosynthesis.

3 CHAPTER ONE: Modification of oil and glucosinolate content in canola seeds with altered expression of *Brassica napus LEAFY COTYLEDON1**

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Rob Duncan and Claudio Stasolla planed the experiments and assisted in writing the manuscript. Nosheen Elhai conducted all the experiments, contributed to some of the planning for some experiments and wrote the manuscript.

3.1 A b s t r a c t

Over the last few decades, research focusing on canola (*Brassica napus* L.) seed oil content and composition has expanded. Oil production and accumulation are influenced by genes participating in embryo and seed development. The *Arabidopsis LEAFY COTYLEDON1* (*LEC1*) is a well characterized regulator of embryo development that also enhances the expression of genes involved in fatty acid (FA) synthesis. *B. napus* lines over-expressing or down-regulating *BnLEC1* were successfully generated by *Agrobacterium* mediated transformation. The constitutive expression of *BnLEC1* in *B. napus* var. Polo, increased seed oil content by 7-16%, while the down-regulation of *BnLEC1* in *B. napus* var. Topas reduced oil content by 9 -12%. Experimental manipulation of *BnLEC1* caused transcriptional changes in enzymes participating in sucrose metabolism, glycolysis, and FA biosynthesis, suggesting an enhanced carbon flux towards FA biosynthesis in tissues over-expressing *BnLEC1*. The increase in oil content induced by *BnLEC1* was not accompanied by alterations in FA composition, oil nutritional value or glucosinolate (GLS) levels. Suppression of *BnLEC1* reduced seed oil accumulation and elevated the level of GLS possibly through the transcriptional regulation of *BnST5a* (*Sulphotransferase5a*), the last GLS biosynthetic enzyme. Collectively, these findings demonstrate that experimental alterations of *BnLEC1* expression can be used to influence oil production and quality in *B. napus*.

Keywords: *Brassica napus*, Canola, Glycolysis, Glucosinolates, *LEAFY COTYLEDON1*, Seed oil

3.2 Introduction

Canola (*Brassica napus* L.) is the member of the Brassicaceae family (syn. Cruciferae) and is the most commonly grown Brassica species in the world. During 2014-2015, the worldwide production of rapeseed oil was 26.98 million metric tons (USDA Economics, 2015).

Originally developed in Canada through breeding programs in the early 1970's (Przybylski et al., 2005; Canola Council of Canada, 2010), canola has now become a global oilseed crop. Canola seeds are high in nutritive qualities, as they contain less than 30 mmoles per gram of glucosinolates (GLS), less than 2% erucic acid (Eskin et al., 1996), high concentrations of unsaturated C: 18 fatty acids (FA), and low levels of undesirable FA (Harvey and Downey, 1964; Dupont et al., 1989). Among the storage compounds which are accumulated during seed development, oil is the major carbon and energy reserve for germination and seedling growth. The process of oil synthesis, which occurs during seed maturation, involves a number of organelles and metabolic pathways within the cell. In higher plants, biosynthesis of FA and lipids has been well described by independent studies (Ohlrogge and Browse, 1995; Beisson et al., 2003). Oil synthesis occurs in the plastids where acyl chains of varying lengths are produced from acetyl-CoA and malonyl-CoA. This initial step is the beginning of FA biosynthesis and is considered to be the major rate limiting step in the entire FA metabolic pathway, as it regulates the carbon flux in the pathway (Baud et al., 2008). Among the acetyl-CoA and malonyl-ACP moieties, numerous condensation reactions lead to the formation of 16:0-ACP (Baud et al., 2008; Pidkowich et al., 2007; Li-Beisson et al., 2010) and 18:0-ACP, which can be desaturated to produce 18:1-ACP (Ohlrogge and Browse, 1995; Baud et al., 2008) and then translocated into the cytoplasm (Ohlrogge and Jaworski, 1997; Sasaki and Nagano, 2004). In the cytoplasm, the enzyme FAD (Fatty Acid Dehydrogenase) catalyzes the formation of unsaturated FA, while FAE (Fatty Acid Elongase) successively adds two-carbon units to the developing acyl chain to form long-chain FA, which are then exported to the endoplasmic reticulum for further modifications (Baud et al., 2008; Li-Beisson et al., 2010). Finally, triacylglycerol (TAG) production is completed in the endoplasmic reticulum through the Kennedy pathway (Baud et al., 2008)

involving reactions catalyzed by lysophosphatidic acid acyltransferase and diacylglycerolacyltransferase (Baud et al., 2008; Li Beisson et al., 2010; Voelker and Kinney, 2001). TAG biosynthesis is strongly dependent on photosynthesis and carbohydrate metabolism for a source of carbon. Therefore, higher carbon flux is essential for increasing the levels of FA biosynthesis. Recently, marked improvements in oil accumulation and quality have been reported in many species following experimental manipulations in gene expression (Sharma et al., 2008). Examples include the development of transgenes characterized by low levels of saturated FA or high levels of short, medium and long chain FA, such as lauric acid, caprylic acid, palmitic acid, stearic acid, oleic acid and erucic acid (Scarth and Tang, 2006). While manipulations of a single enzyme do produce drastic phenotypic consequences, alterations of transcription factors regulating multiple downstream cascades, such as FA and TAG synthesis, might be more desirable (Scarth and Tang, 2006; Ruuska et al., 2002; Mu et al., 2008). In many crop species transcription factors participating in embryo development have been shown to regulate FA biosynthesis. Well known examples include the Arabidopsis *LEAFY COTYLEDON 1* and *2* (*AtLEC1*, and *AtLEC2*), *WRINKLED 1* (*AtWRI1*) and *FUSCA 3* (*AtFUS3*) (Lotan et al., 1998; Kwong et al., 2003; Cernac and Benning, 2004; Wang and Perry, 2013). Embryogenesis in Arabidopsis occurs in two phases; a developmental phase characterized by the growth of the embryo, followed by a maturation phase required for critical physiological changes needed for the subsequent germination step (Stone et al., 2001). Among the identified transcription factors governing embryogenesis, *LEC1*, a member of the HAP3 subunit of the CCAAT binding factor family (Lotan et al., 1998), controls embryo maturation (Parcy et al., 1994), through an unknown regulatory network involving hormone responses (Braybrook and Harada, 2008). Altered expression of *LEC1* in Arabidopsis influences the transcript levels of other factors required in FA

synthesis and other seed maturation processes. These include *WRI1*, which enhances the flow of carbon to oil (Maeo et al., 2009), *FUS3* which together with *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) is essential for the control of developmental processes during late embryogenesis (Bäumlein et al., 1994), and *LEC2*, an upstream regulator of *ABI3* and *FUS3* which also influences the expression of *WRI1*. Together, *WRI1* and *LEC2* regulate a subclass of genes taking part in glycolysis and the integration of sucrose into TAG (Cernac and Benning, 2004; Baud et al., 2007; Meinke et al., 1994; Gazzarrini et al., 2004). The use of these transcription factors for modifying seed oil content and composition is often limited by severe and undesirable pleiotropic effects observed in the transformed plants (Shen et al., 2010). In the current study, the constitutive expression of *BnLEC1* in *B. napus* significantly increases total seed oil without major changes in FA and GLS levels. This is in contrast to plants down-regulating *BnLEC1* that show a decrease in seed oil content and an undesirable accumulation of GLS. Together, these findings demonstrate that *BnLEC1* can be used to modulate oil and GLS content in canola.

3.3 Materials and methods

3.3.1 Generation of transgenic *B. napus* plants

Full-length *BnLEC1* cDNAs i.e., version A (GU945399) and version B (GU945398), which differ by 13 nucleotides (Supplementary Fig. 3.1) and 4 amino acids (Supplementary Fig. 3.2) were isolated in previous studies (Prystenski, 2011). The two cDNAs were introduced in the Gateway entry clone pDONR_221 (Invitrogen, USA) and then inserted into the pK2GW7 (for sense transformation) and pK2WG7 (for antisense transformation) vectors carrying the 35S promoter and the 35S terminator (http://www. psb.ugent.be/gateway/index.php) (Karimi et al., 2002). The constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 which was used to infect wild-type *B. napus* plants as described previously (Bhalla and Singh, 2008). In short, surfacesterilized seeds of *B. napus* varieties Polo and Topas were germinated in Magenta

GA-7 boxes (7 seeds per box) for 7 days on halfstrength MS-B5 medium supplemented with 1% sucrose. Hypocotyl explants (0.5 cm long) isolated from 7-day-old *B. napus* seedlings were placed on pre-cultured media [MS-B5 medium supplemented with 3% sucrose, 1 mg/L 2, 4-D, and 1 mg/L kinetin] for three days after which they were inoculated with *A. tumefaciens* containing pK2GW7 (for sense transformation) or pK2WG7 (for antisense transformation) for 30 min in 100 x 15 mm polysterene disposable petri dishes. Following a co-cultivation period of 3 days, the explants were plated on callus induction medium [MS-B5 supplemented with 3% sucrose, 1 mg/L 2,4-D,1 mg/L kinetin, 0.5 g/L 2-(N-morpholino) ethanesulfonic acid (MES), 5 mg/L AgNO3, 300 mg/L Timentin and 250 mg/L carbenicillin] for 2 weeks. After 2 weeks, the explants were plated on regeneration (shoot initiation) medium (MS-B5 supplemented with 3% sucrose, 0.5 g/L MES, 3 mg/L BAP, 5 mg/L AgNO3, 300 mg/L timentin, 250 mg/L carbenicillin, 1 mg/L zeatin and 50 mg/L kanamycin) for 4 weeks. De novo shoots were initially transferred on elongation medium (MS-B5 supplemented with 3% sucrose 0.5 g/L MES, 3 mg/L BAP, 300 mg/L timentin and 50 mg/L kanamycin) for three weeks and subsequently plated on rooting medium (MS-B5 supplemented with 3% sucrose, 0.5 g/L MES, 0.2 mg/L IBA, 300 mg/L Timentin and 50 mg/L kanamycin) for 4 weeks. All the cultures were incubated at 25^{o} C under a photoperiod of 16/8 h light/dark conditions using cool white fluorescent lights 60 µmol m^{-2} s^{-1} in Magenta GA-7 boxes. Rooted shoots were transplanted into soil and grown in growth chambers at 20^{o} C with 16/8 h day/night photoperiod, light intensity of 350 µmol m^{-2} s^{-1} produce fully mature plants (T0). Transformation was attempted in two varieties of *B. napus*: Polo and Topas. The variety Polo was registered by Mycogen Canada, Inc. (Dow AgroSciences) in 1994 for cultivation in western Canada. The variety Topas was developed by Svalof A.B., Sweden and was registered in 1987 for cultivation in eastern Canada. Regenerated plants over-expressing

BnLEC1 were only obtained from *B. napus* variety Polo, while only two transformants down-regulating *BnLEC1* were regenerated exclusively from the *B. napus* variety Topas. Twenty T1 plants obtained from independent transformation events were selected by PCR using the 35S forward and the respective *BnLEC1* primers [*BnLEC1*(R) for sense insertion and *BnLEC1*(F) for antisense insertion] (see primer list in Supplemental Table 3.1). Homozygous (T3) plants were obtained through controlled self-pollination and 6 independently transformed lines: 4 over-expressing *BnLEC1* (S1-4) and two down-regulating *BnLEC1* (A1-2) were used for further agronomic and molecular studies.

3.3.2 Agronomic characteristics

To study the effects of altered expression of *BnLEC1* on agronomic characteristics and on oil content and profile, the six transgenic lines selected, along with the respective controls (*B. napus* variety Polo for the *BnLEC1* over-expressors and *B. napus* variety Topas for the *BnLEC1* down-regulators) were grown in the Department of Plant Science greenhouses at the University of Manitoba in Winnipeg from April 2013 to July 2013. Plants were grown using an average temperature of 21°C and a period of 17 h of light. To study the effects of the environmental conditions on the results obtained from the greenhouse experiment, plants were also grown in growth chambers with 16/8 h day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1} and day/night temperature of 20°C /15 °C. For total oil and FA profile, at least three biological replicates (each consisting of seeds harvested from nine plants) were used. Overall, nine biological replicates for each line were used to record the data for all other agronomic characters. For measuring the number of seeds per silique, five siliques were randomly selected from each plant within each replicate.

3.3.3 Determination of glucosinolate, protein and lipid content

The seed samples were cleaned to remove plant debris prior to seed quality analysis. Oil, protein and total glucosinolate measurements were made on all individual samples using a FOSS Model Systems NIRS 6500 scanning near infrared (NIRS) spectrophotometer (FOSS, Eden Prairie, MN). The NIRS instrument was calibrated to and verified against the appropriate reference methods as described by DeClercq et al., 1997; W.Thies, 1980; Madsen, 1976; Daun and DeClercq, 1994; Tkachuk, 1981 and Kim et al. 2007. In short, approximately 5 g of seed samples were scanned after placing them in a ring cup made of metal with replaceable Quartz lens with an outer and inner diameter of 47 mm and 36mm, respectively. The reflectance analyses were calibrated using parallel analyses from an inner ceramic disc. The data were noted as log 1/*R* (the logarithm of reciprocal of reflectance) using the wavelengths of 400 to 2500 nm (Kim et al. 2007). The NIRS procedure for scanning and statistical investigation was completed with the WinISI II software (Windows version 1.04a, Foss and Infrasoft International LLC, USA).

Fatty acid composition analyses were conducted using gas chromatography as documented by Hougen and Bodo (Hougen and Bodo, 1973). All NIRS and gas chromatography analyses were conducted in the University of Manitoba seed quality lab, which is certified annually by the Canadian Grain Commission. For gas chromatography, 300 mg of seed was crushed with a test cylinder from Carver Press and placed in placed in 13 x 100 test tubes. Three ml of heptane was added to each sample. After 24 h, supernatants were transferred into clean 13 x 100 test tubes and 500 ul of 0.5 N sodium methoxide (13.5 g of sodium methylate powder in 500 ml anhydrous methanol) was added and each sample was shaken for 30 min. Then, 100 ml of acidified water (0.3% acetic acid) was added and the sample was placed at 4 °C for 1-2 hours. Finally, 500 ml of reaction mixture was pipetted into a 2 ml auto sampler (Fisher brand vial

CAT# 03-391-16) (Christie, 1989; Liu, 1994). Gas chromatography was carried out on a Varian model 3900 fitted with a CP-Wax 52 CB capillary column and a flame ionization detector. The column was 15 m x .32 mm i.d. fused silica coated with a .025 micron polyethylene glycol phase (Varian, Walnut Creek, USA). The carrier gas was Ultra High Purity (UHP) Helium with a flow rate of 2.0 ml/min. The split vent ratio was set at 100:1, with the septum purge flow set at 4 ml/min. The total hydrogen flow rate to the detector was 30 ml/min, the make-up gas (UHP Helium) flow rate was 30 ml/min, and the detector gas was supplied at a flow rate of 300 ml/min using breathing grade air. Injections were made by a Varian model CP-8400 autosampler; the injector was a Varian model CP-1177, the inlet liner was a cup splitter with 10% OV-1 on Chromsorb-WHP, operated at 250 °C. The column oven temperature was programmed from 190 °C to 240 °C, and the detector temperature was 280 °C. Peak areas were measured using the Varian Star Workstation software system. A reference standard, GLC # 421, purchased from Nu-Check Prep (Elysian, Minnesota) was used to ensure proper GC operation.

3.3.4 Gene expression analysis by quantitative (q)RT-PCR

Gene expression analyses were performed on samples grown in the greenhouse under conditions described above. In developing canola seeds, significant accumulation of oil starts at 21 DAP (days after pollination), and reaches its maximum level after 35 DAP (Fowler and Downey, 1970; Gurr et al., 1972). Therefore, the transcription studies for the genes involved in glycolysis, sucrose transport and metabolism and FA synthesis were conducted at 21 and 35 DAP (days after pollination). For the analysis of the expression levels of transcription factors taking part in oil biosynthesis i.e., *BnLEC1*, *BnLEC2*, *BnWRI1* and *BnFUS3*, seed samples were collected at 14, 21, 28 and 35 DAP. For all other expression studies, seeds were collected at 35 DAP unless otherwise stated. All gene expression analyses were conducted by quantitative (q) RT- PCR (Elhiti et al., 2010) using existing primer sequences (all primers used are listed in Supplementary

Table 3.1) (Prystenski, 2011; Elhiti et al., 2012). Total RNA was extracted using the TRIZOL reagent (Invitrogen CAT #AM9738) as described by Chomczynski and Mackey (1995) and treated with RNase-free DNase I (Promega CAT#04716728001). The concentration and quality of the extracted RNA were estimated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and by running 2 ml of RNA samples on a 1.3% agarose gel stained with 1 mg/ml of ethidium bromide. Two mg of total RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems CAT# 4368813) according to the manufacturer instructions. The concentration of the cDNA was determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Burlington, ON). Analyses of gene expression in all samples were conducted by qRTPCR (Elhiti et al., 2010). The relative level of gene expression was estimated with the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001) using *UBC21* (EV086936, ubiquitin-conjugating enzyme 21) as a reference. This gene is stably expressed during both vegetative development and seed maturation and therefore is recommended as a reference gene (Chen et al., 2010). All PCR reactions were performed using the CFX96^{TM} Optics Real- Time System (Bio Rad Hercules, CA) with an initial denaturation step at 95 ºC for 3 minutes, followed by 40 cycle of 95 ºC for 5 seconds (denaturation), and 59 ºC for 10 seconds (annealing/extension). Melting curve rose from 65 ºC to 95 ºC by increments of 0.5 ºC for 5 seconds. Expression analyses included genes involved in glycolysis: *BnFPA* (*Fructose Bisphosphate Aldolase*), *BnPGK* (*Phosphoglycerate Kinase*), *BnGPDH* (*Glyceraldehyde-3-Phosphate Dehydrogenase*), *BnHXK* (*Hexose Kinase*) and *BnPPK* (*Pyrophosphatase-Dependent Phosphofructokinase*); sucrose transport and metabolism: *BnSUC1* and *BnSUC4* (*Sucrose Transporter1* and *4*), *BnSUS1* and *BnSUS3* (*Sucrose Synthase1* and *3*), *BnAGP* (*ADP-Glucose Phosphorylase*); FA biosynthesis: *BnACCA2* (*Subunit A of Acetyl-CoA Carboxylase*, *BnFAD3*

(*ω-3 FA Desaturase*), *BnFAE1* (*FA Elongation 1*), *BnMCAT* (*Malonyl-CoA:ACP Transacylase*); GLS biosynthesis: *BnMAM1* (*Methylthioalkymalate Synthase 1*), *BnCYP79B2 (Cytochrome P450 CYP79B2*), *BnCYP83B1* (*Cytochrome P450 CYP83B1*), *BnSUR1* (*Superroot1*), *BnST5a* (*Sulphotransferase5a*); transcription factors regulating oil: *BnLEC1* (*Leafy Cotyledon1*), *BnLEC2* (*Leafy Cotyledon 2*), *BnWRI1* (*Wrinkled1*) and *BnFUS3* (*Fusca3*); and transcription factors regulating glucosinolate synthesis: *BnMYB28* (*MYB-Containing Transcription Factor28*), and *BnATR1* (*Altered Tryptophan Regulation1*).

Analyses were performed in three biological replicates.

3.3.5 Statistical analysis

The statistical analyses were performed using the SAS® 9.3 program (SAS Institute Inc., Cary, NC, USA.). The Fisher's Least Significant Difference (LSD) test was applied to observe the main effect of the mean for each transformed line and the wild type control (using at least three biological replicates for all experiments). For gene expression analysis, samples were collected from greenhouse experiments and comparison of means was performed using the SAS® 9.3 program. Fisher's LSD was used to determine if there were significant differences among the relative transcript levels between the transgenic and wild type lines.

3.4 Results

3.4.1 Genotypic and phenotypic characterization of the transformed lines

Six independently transformed *BnLEC1* lines were characterized: two (S1, S2) overexpressing version A of *BnLEC1* (GU945399), two (S3, S4) overexpressing version B of *BnLEC1* (GU945398), and two (A1, A2) down-regulating version B of *BnLEC1*. Presence of the transgene in the genomic DNA extracted from leaves was confirmed in all the lines analyzed (Supplemental Fig. 3.3A). Compared to their respective WT control (*B. napus* Polo for the four over-expressors and *B. napus* Topas for the two down-regulators), the endogenous level of

BnLEC1 RNA was significantly increased in the over-expressors and reduced in the down-regulators. This pattern was observed in both leaves and 35 DAP seeds (Supplemental Fig. 3.3B).

Agronomic characteristics revealed that plants overexpressing *BnLEC1* [both version A (S1, S2) and version B (S3, S4)] are usually taller, but produce fewer siliques per plant and seeds per silique (Supplemental Table 3.2). These plants also have a higher 1000 seed weight and a reduced overall yield per plant. With the exclusion of plant height, all of the values for the agronomic characteristics measured were lower in the downregulating *BnLEC1* lines (A1, A2) compared to the WT control (Topas) (Supplemental Table 3.2). The major difficulty in regenerating viable *BnLEC1* down-regulating plants was due to an early abortion before reaching the reproductive stage and in some instances the premature formation of inflorescences producing seedless siliques (Supplemental Fig. 3.4).

Table 3.1: Measurements of total oil content in dry seeds of *Brassica napus* using Near Infrared Reflectance (NIR) spectroscopy. Plants were grown in the Department of Plant Science greenhouse at the University of Manitoba from April 2013 to July 2013. Values are means of three biological replicates ± SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over-expressors; A1-A2, *BnLEC1* down-regulators.

Line	Total oil (% dry weight)	Percentage increase	Line	Total oil (% dry weight)	Percentage decrease
WT (Polo)	47.12±0.53	-	WT(Topas)	47.31±0.31	-
S1	53.2±1.64*	12.9	A1	42.99±0.97*	9.14
S2	54.73±0.91*	16.15	A2	41.23±0.59*	12.85
S3	50.46±0.59*	7.09			
S4	50.81±0.31*	7.83			

3.4.2 Altered *BnLEC1* expression affects seed storage accumulation

To examine the influence of altered *BnLEC1* expression on seed storage product accumulation, mature dry seeds were analyzed by NIRS. As compared to the control, total seed oil content increased by 7%-16% in the *BnLEC1* over-expressing lines (S1-4) (Table 3.1). Greater numeric values were observed in the lines S1 and S2 overexpressing the A version of *BnLEC1* (GU945399). Oil accumulation was significantly reduced in the *BnLEC1* down-regulating lines (A1, A2). Analysis of seed FA composition was performed by GC. Compared to WT seeds, the percentage of oleic acid (C18:1) was significantly higher in two (S1 and S4) *BnLEC1* over-expressing lines, but lower in the S3 line (Table 3.2). Up-regulation of *BnLEC1*

also resulted in minute (and inconsistent among lines) percentage changes in the polyunsaturated C18:2 linoleic and C18:3 linolenic acids, thus denoting possible alterations in the desaturation processes. Comparable changes in total seed oil and FA composition were also observed in plants grown in growth chambers (Supplemental Tables 3.3 and 3.4). Suppression of *BnLEC1* did not consistently alter the FA composition (Table 3.2 and Supplemental Tables 3.4 and 3.5). The nutritive superiority of oilseeds is determined by specific proportions (ratios) of various FA, affecting the quality of oil (Nesi et al., 2008). Both 18:2 linoleic and C18:3 linolenic acids cannot be synthesized by humans and are therefore highly desirable. No consistent differences in FA ratios were observed in lines overexpressing *BnLEC1* and in lines with suppressed *BnLEC1* levels (Table 3.3 and Supplemental Table 3.5). The accumulation of seed oil is often negatively correlated to protein content in the seed (Hao et al., 2004). Lines over-expressing *BnLEC1* showed an overall reduction in protein content (Fig. 3.1 and Supplemental Fig. 3.5). This reduction was more pronounced for lines S1 and S2, characterized by the highest accumulation of oil. Protein level was significantly increased in seeds suppressing *BnLEC1* (Fig. 3.1 and Supplemental Fig. 3.5).

Table 3.2: Fatty acid profile (% composition) in transgenic *Brassica napus* seeds harvested from plants grown in the Department of Plant Science greenhouse at the University of Manitoba from April 2013 to July 2013. Values are means of three biological replicates ± SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over-expressors; A1-A2, *BnLEC1* down-regulators.

	WT (Polo)	**S1**	**S2**	**S3**	**S4**	**WT(Topas)**	**A1**	**A2**
C16:0	4.47±0.17	3.62±0.10*	3.74±0.03*	4.19±0.08	4.40±0.06	4.26±0.07	4.53±0.20	4.36±0.09
C18:0	1.29±0.07	1.15±0.03	1.23±0.06	1.22±0.08	1.36±0.04	1.30±0.06	1.40±0.22	1.10±0.05
C18:1	66.94±0.18	69.30±0.7*	68.09±1.01	64.31±0.65*	69.74±0.79*	60.53±1.18	60.47±1.8	59.31±2.09
C18:2	16.74±0.11	15.41±0.50	15.21±0.84	17.93±0.59	14.43±0.52*	20.90±0.77	21.99±0.6	21.97±1.24
C18:3	7.99±0.30	7.99±0.22	9.17±0.46*	9.78±0.18*	7.49±0.34	10.42±0.26	9.5±0.42	10.32±0.81
C20:0	0.49±0.03	0.46±0.01	0.49±0.04	0.47±0.01	0.54±0.03	0.51±0.022	0.59±0.08	0.48±0.017
C20:1	1.12±0.03	1.11±0.04	1.18±0.08	1.12±0.07	1.13±0.04	1.2±0.02	1.28±0.04	1.34±0.01
C22:0	0.30±0.02	0.27±0.01	0.319±0.03	0.27±0.002	0.34±0.02	0.29±0.02	0.38±0.04	0.34±0.01

Table 3.3: Nutritive value of the transgenic *Brassica napus* seeds. The ratio of different C18 unsaturated FA is calculated according to data presented in Table 3.2. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over expressors; A1-A2, *BnLEC1* down-regulators.

	WT (Polo)	**S1**	**S2**	**S3**	**S4**	**WT(Topas)**	**A1**	**A2**
18:1/18:2	3.99±0.04	4.51±0.2	4.51±0.31	3.6±0.16	4.85±0.23*	**2.91±0.16**	2.76±0.17	2.7±0.24
18:1/18:3	8.40±0.33	8.68±0.33	7.48±0.50	6.58±0.14*	9.36±0.54	**5.82±0.25**	6.4±0.47	5.85±0.62
18:2/18:3	2.10±0.1	1.93±0.01	1.66±0.03*	1.84±0.08*	1.93±0.02	**2.0±0.03**	2.3±0.03	2.14±0.07

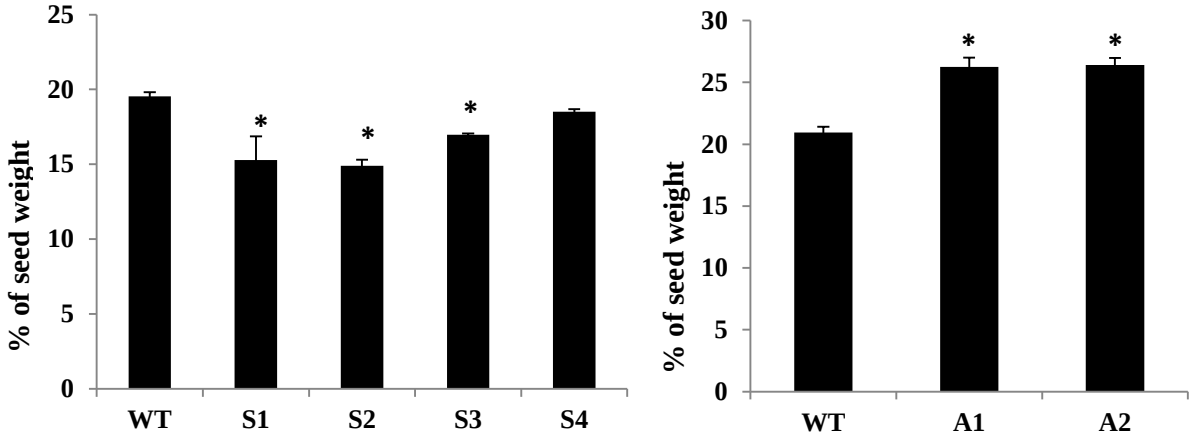


Fig.3.1 Measurements of protein content in dry seeds of *Brassica napus* using Near Infrared Reflectance Spectrophotoscopy harvested from plants grown in the Department of Plant Science greenhouse at the University of Manitoba from April 2013 to July 2013. Values, expressed as percentage of seed weight (at 0% humidity) ±SE, are means of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from theWT [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)].

3.4.3 Altered expression of *BnLEC1* affects the expression of oil related transcription factors

To examine the mechanisms responsible for the observed alterations in oil content in the transgenic lines, the transcript levels of the oil-related transcription factors *BnLEC2*, *BnWRI1*, and *BnFUS3* were measured and compared to those of *BnLEC1*. The expression of *BnLEC1* in the over-expressing lines was always higher than the WT value at any stage of seed development, especially after 21 DAP (Fig. 3.2). This profile was also observed for *BnLEC2* and *BnWRI1*, and to a lesser extent for *BnFUS3* (sees scale bar differences). In seeds down-regulating *BnLEC1*, the expressions of *BnLEC2* and *BnWRI1* were significantly repressed at 35 DAP (for *BnLEC2*), and at 21 and 35 DAP (for *BnWRI1*). Suppression of *BnFUS3* in the down-regulating lines was observed at all stages of seed development (Fig. 3.2)

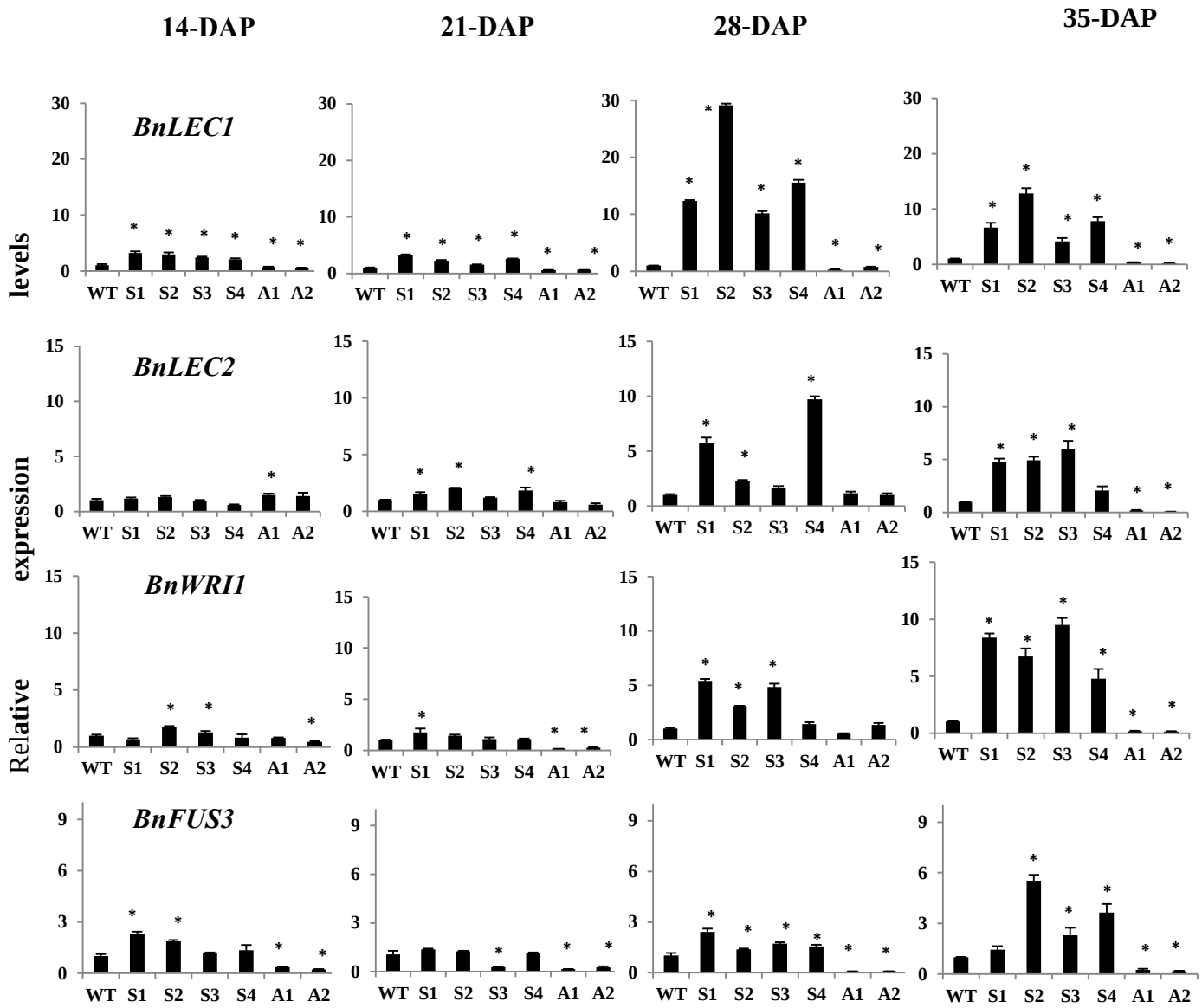
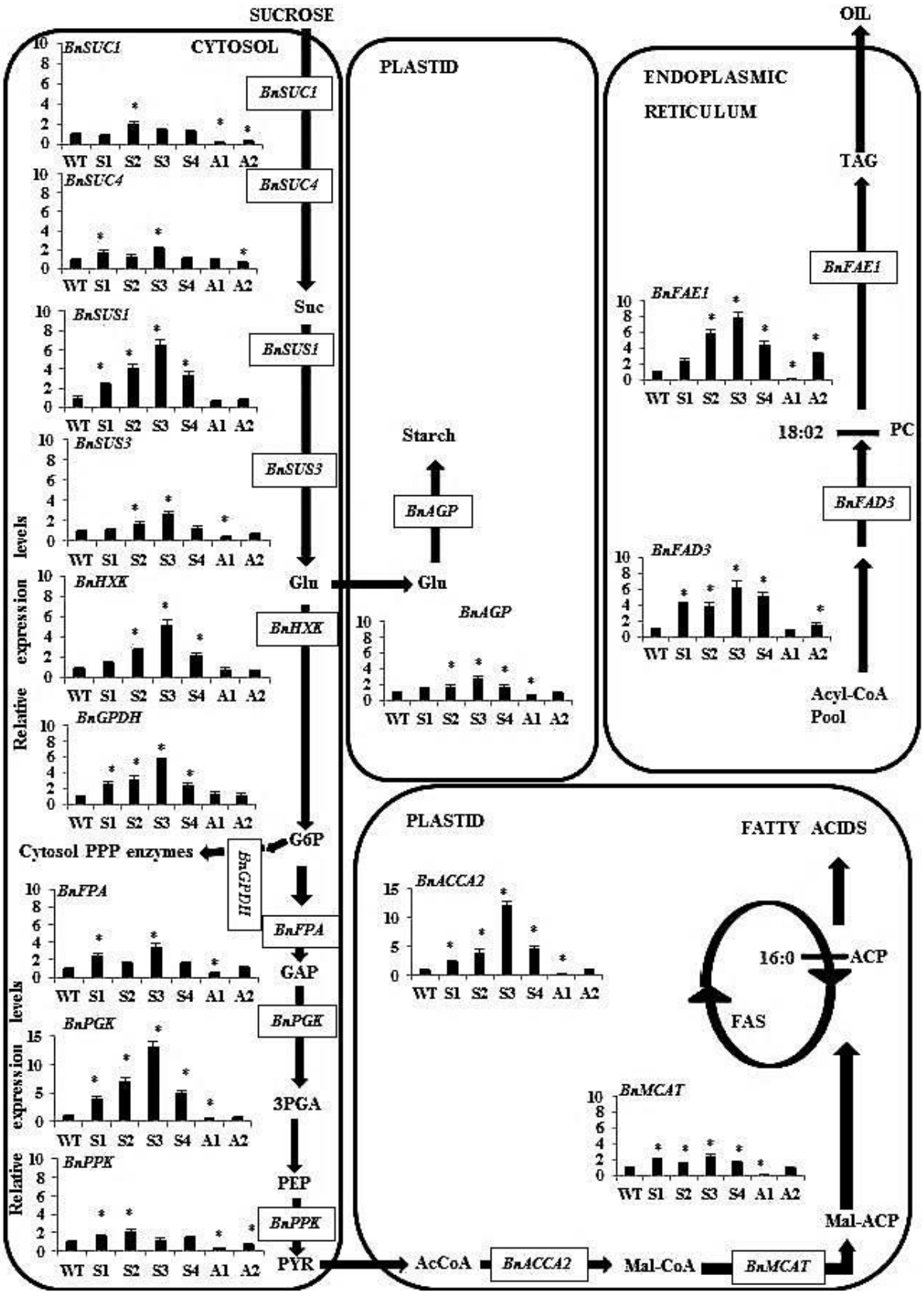

14-DAP
21-DAP
28-DAP
35-DAP
Relative expression levels
BnLEC1
BnLEC2
BnWRI1
BnFUS3
WT S1 S2 S3 S4 A1 A2

Fig. 3.2 Expression levels measured by qRT-PCR of the transcription factor (TF) involved in oil synthesis: *Brassica napus LEAFY COTYLEDON1* (*BnLEC1*); *Brassica napus LEAFY COTYLEDON2* (*BnLEC2*); *Brassica napus WRINKLED1* (*BnWRI1*) and *Brassica napus FUSCA3* (*BnFUS3*). Expression levels were measured in developing seeds overexpressing (S) or downregulating (A) *BnLEC1* at 14, 21, 28, and 35 days after pollination (DAP). Data presented are mean values ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from theWT [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2) value set at 1 at the respective DAP.

3.4.4 Altered expression of *BnLEC1* affects the transcript levels of genes involved in sucrose photo-assimilation and glycolysis

The observed variations in oil level and to a lesser extent FA composition in the transgenic seeds motivated the analysis of the transcript levels of genes encoding enzymes involved in glycolysis and sucrose metabolism. These studies were performed at 21 DAP, characterized by a significant initial accumulation of seed oil, and 35 DAP corresponding to a maximum rate of oil level (35 DAP) (Fowler and Downey, 1970; Gurr et al., 1972). The key carbon source for oil synthesis in developing seeds is sucrose derived from photosynthesis, whereas the energy (ATP) is produced mutually from respiration and photosynthesis (Hills, 2004). Once imported into the seeds, sucrose is hydrolyzed into UDP-glucose and fructose, and these hexoses are then oxidized through the glycolytic pathway into acetyl-CoA, which is a precursor of FA synthesis (Schwender et al., 2003). Transcriptional analyses of sucrose metabolism showed that over-expression of *BnLEC1* results in a general induction of *BnSUC4*, *BnSUS1*, *BnSUS3*, and *BnAGP*, suggesting an active intake of sucrose in the developing seeds (Figs. 3.3 and 3.4). This was in contrast to lines down-regulating *BnLEC1* where the expression of these genes was suppressed, especially at 35 DAP. Among the glycolytic enzymes, the transcript levels of *BnHXK*, *BnGPDH*, and *BnPGK* were induced by the over-expression of *BnLEC1* at both 21 and 35 DAP. The other two glycolytic enzymes *BnFPA* and *BnPPK* were only transcriptionally induced in a few lines (Figs. 3.3 and 3.4). This overall induction in expression of the glycolytic enzymes suggests a rapid oxidation rate in *BnLEC1* over-expressing seeds. Seeds down-regulating *BnLEC1* showed a reduced expression of several glycolytic enzymes (Figs. 3.3 and 3.4).


SUCROSE
CYTOSOL
PLASTID
ENDOPLASMIC RETICULUM
OIL
TAG
BnSUC1
BnSUC4
Suc
BnSUS1
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Glu
BnHXK
BnGPDH
G6P
Cytosol PPP enzymes
BnG6PDH
BnFPA
GAP
BnPGK
3PGA
PEP
BnPPK
PYR
Starch
BnAGP
Glu
BnFAE1
18:02
PC
BnFAD3
Acyl-CoA Pool
PLASTID
FATTY ACIDS
BnACCA2
16:0
ACP
FAS
BnMCAT
Mal-ACP
AcCoA
Mal-CoA
Relative expression levels
WT S1 S2 S3 S4 A1 A2

Fig. 3.3 Simplified illustration presenting the effects of altered *BnLEC1* expression on regulatory pathways leading to FA synthesis in seeds. Gene expression was measured in 21 DAP seeds harvested from over-expressing (S) or down-regulating (A) *BnLEC1* lines, as well as in the WT (control) line. Genes involved in sucrose transport and metabolism: *Sucrose Transporter1* (*SUC1*), *Sucrose Transporter 4* (*SUC4*), *Sucrose Synthase1* (*SUS1*), *Sucrose Synthase 3* (*SUS3*), *ADP-Glucose Phosphorylase* (*AGP*). Glycolytic genes: *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*) and *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). Genes involved in FA synthesis: *Subunit A of Acetyl-CoA Carboxylase* (*ACCA2*), *ω-3 FA Desaturase*, *FA Elongation1* (*FAE1*), and *Malonyl-CoA:ACP Transacylase* (*MCAT*). Data presented are mean values ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)] value set at 1. The diagram was adapted from Elhiti et al., 2012.

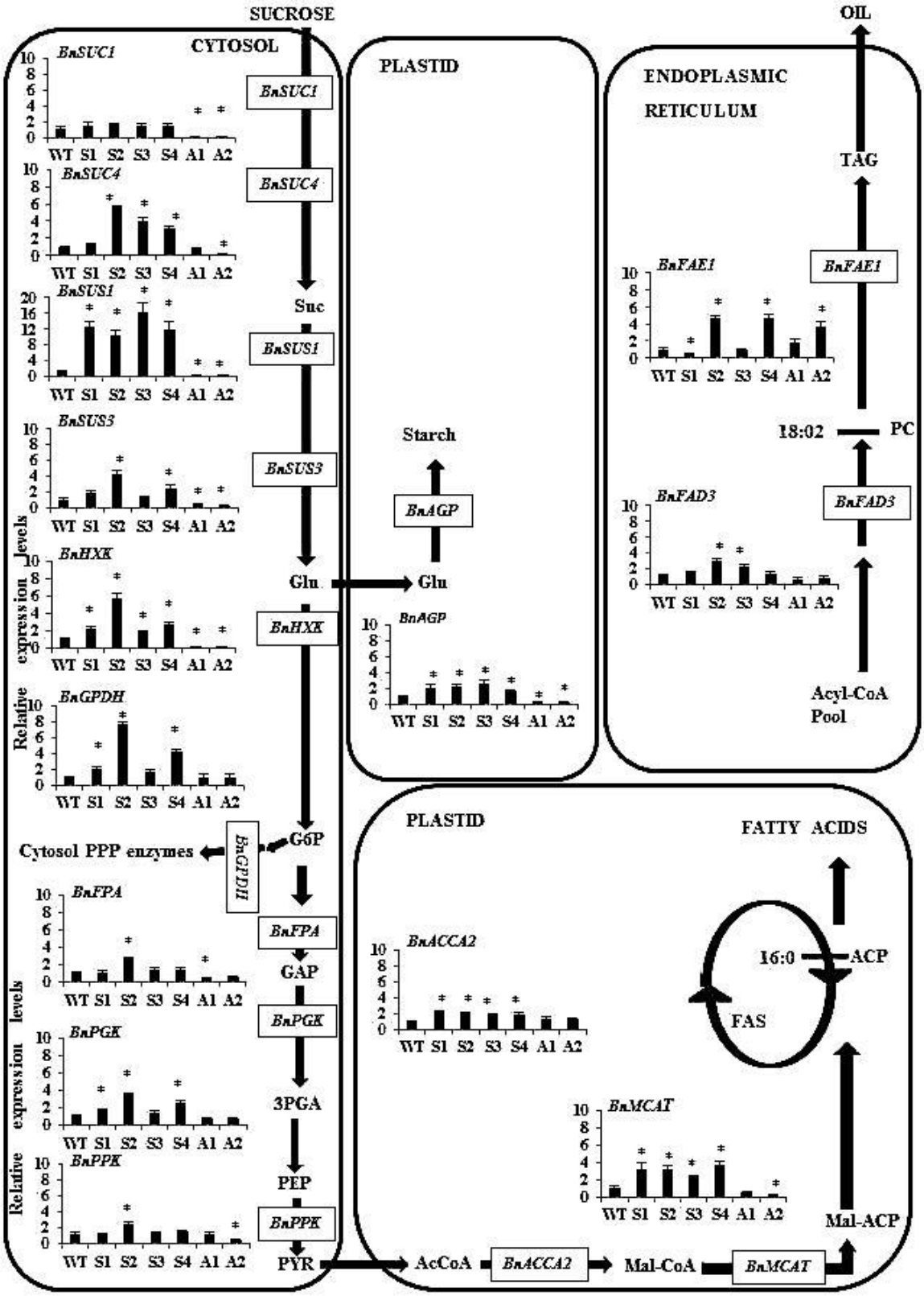
SUCROSE
CYTOSOL
BnSUC1
BnSUC4
Suc
BnSUS1
BnSUS3
Glu
BnHXK
BnGPDH
G6P
Cytosol PPP enzymes
BnFPA
GAP
BnPGK
3PGA
PEP
BnPPK
PYR
PLASTID
Starch
BnAGP
Glu
ENDOPLASMIC RETICULUM
OIL
TAG
BnFAE1
18:02
PC
BnFAD3
Acyl-CoA Pool
PLASTID
FATTY ACIDS
BnACCA2
16:0
ACP
FAS
BnMCAT
Mal-ACP
AcCoA
Mal-CoA
Relative expression levels
WT S1 S2 S3 S4 A1 A2

Fig. 3.4 Simplified illustration presenting the effects of altered *BnLEC1* expression on regulatory pathways leading to Fatty Acid (FA) synthesis in seeds. Gene expression was measured in 35 DAP seeds harvested from over-expressing (S) or down-regulating (A) *BnLEC1* lines, as well as in the WT (control) line. Genes involved in sucrose transport and metabolism: *Sucrose Transporter1* (*SUC1*), *Sucrose Transporter4* (*SUC4*), *Sucrose Synthase1* (*SUS1*), *Sucrose Synthase3* (*SUS3*), *ADP-Glucose Phosphorylase* (*AGP*). Glycolytic genes: *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*) and *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). Genes involved in FA synthesis: *Subunit A of Acetyl-CoA Carboxylase* (*ACCA2*), *ω-3 FA Desaturase* (*FAD3*), *FA Elongation1* (*FAE1*), and *Malonyl-CoA:ACP Transacylase* (*MCAT*). Data presented are mean values ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)] value set at 1. The diagram was adapted from Elhiti et al., 2012.

3.4.5 Altered expression of *BnLEC1* affects the expressions of FA biosynthetic genes

In seeds, biosynthesis of FA is coordinated by the expression of numerous genes involved in the condensation step (*MCAT* and *ACCA2*), elongation step (*FAE1*) and desaturation step (*FAD3*). Quantitative real time PCR analysis revealed that expressions of the FA synthetic genes were affected to varying degrees by the presence of the transgene (Figs. 3.3 and 3.4). Of interest, the expressions of *BnACCA2* and *BnMCAT* were induced in all the *BnLEC1* overexpressing lines, while unaltered or lower expressions of these enzymes were noted in the *BnLEC1* down-regulating lines. This trend was apparent at both 21 and 35 DAP. Conversion of C18:2 into C18:3 is catalyzed by *BnFAD3* which was induced in all the *BnLEC1* overexpressing lines and in one of the *BnLEC1* down-regulating lines (A2) at 21 DAP; while at 35 DAP it was only induced in S2 and S3 *BnLEC1* over-expressing lines. No precise expression pattern was observed for *BnFAE1*, involved in the biosynthesis of erucic acid. An increased expression of this gene was observed in the *BnLEC1* over-expressing lines S2, S3 and S4, as well as in the *BnLEC1* down-regulating line A2 (Figs. 3.3 and 3.4).

3.5.6 Suppression of *BnLEC1* increases seed glucosinolate levels

Following oil extraction in canola seeds, the remaining meal comprises numerous nutritional and anti-nutritional compounds. The main anti-nutritional compounds are GLS, sulfur containing products that occur naturally in the *Brassicaceae* (Sønderby et al., 2010). Reducing GLS levels is a major goal for plant breeders (Feng et al., 2012). Total seed GLS levels remained unaltered in the *BnLEC1* over-expressing lines (with the exception of line S3 which showed a significant decrease), while it increased significantly by more than 100% in seeds with suppressed levels of *BnLEC1* (Fig. 3.5A). A similar, but less pronounced increase in GLS content was also observed in the *BnLEC1* down-regulators grown in a controlled environment (Supplemental Fig. 3.6). The increased GLS level induced by the suppression of *BnLEC1* was

further investigated in the line A2 (characterized by a 250% increase in GLS content, Fig. 3.5A) by measuring the expression of GLS biosynthetic genes (Fig. 3.5B). These included *BnCYP79B2*, responsible for the synthesis of indole-3-acetaldoxime from tryptophan; *BnCYP83B1* producing S-alkyl thiohydroximates from indole-3-acetaldoxime; *BnMAM1*, involved in the condensation of deaminated methionine with acetyl-CoA; *BnSUR1* participating in the conversion of S-alkyl thiohydroximates to thiohydroximates; and *BnST5a* which uses desulphoglucosinolates as a substrate in the last steps of GLS synthesis. The analysis also included *BnMYC28* and *BnATR1*, two transcription factors regulating GLS homeostasis (Sønderby et al., 2010). Suppression of *BnLEC1* did not alter the expression of any of these genes, with the exception of *BnST5a*, for which the transcript levels increased by 8 times (Fig. 3.5 B and C).

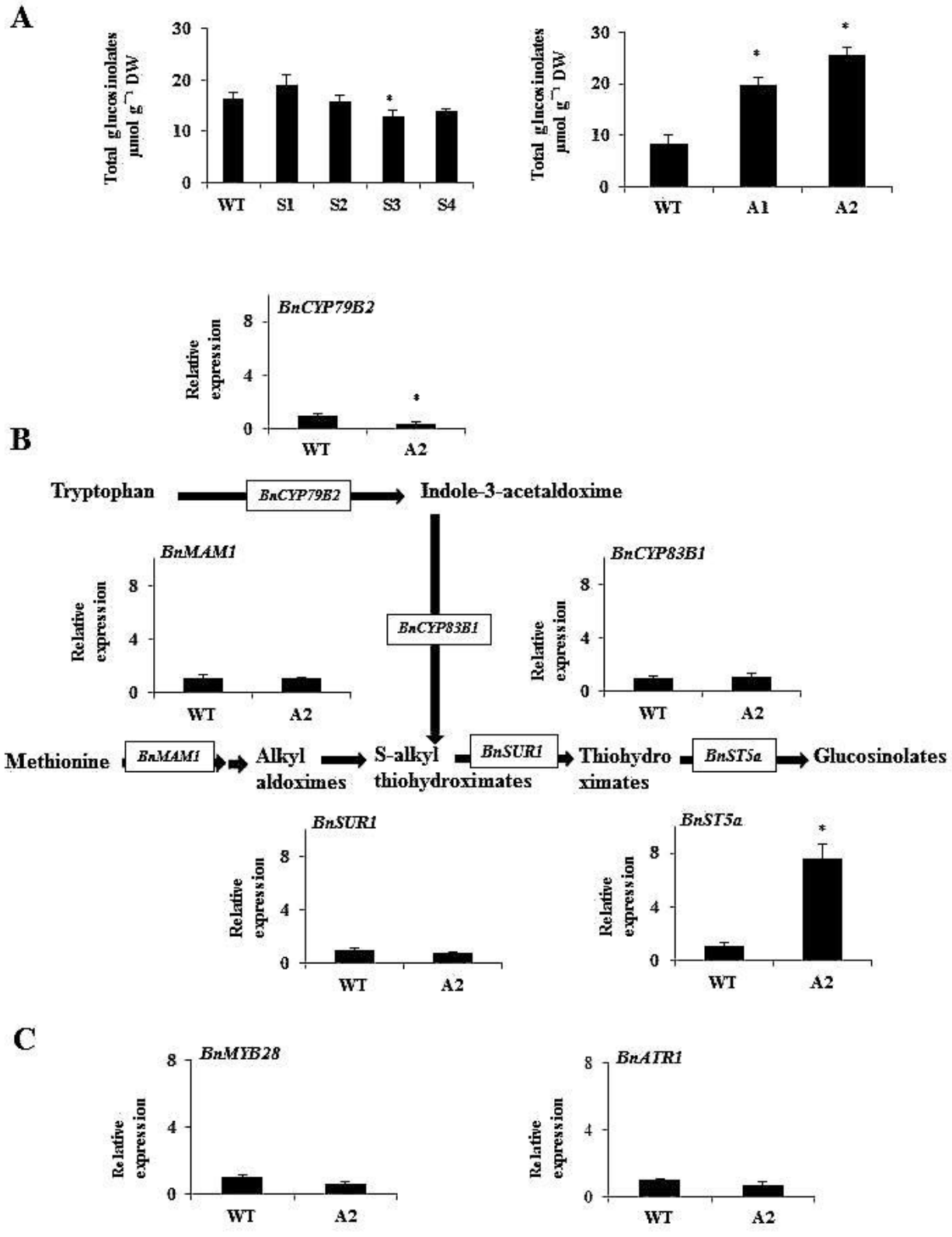
A
Total glucosinolates μmol g⁻¹ DW
30
20
10
0
WT
S1
S2
S3
S4
*
Total glucosinolates μmol g⁻¹ DW
30
20
10
0
WT
A1
A2
*
*
BnCYP79B2
Relative expression
8
4
0
WT
A2
*
B
Tryptophan
BnCYP79B2
Indole-3-acetaldoxime
BnMAM1
Relative expression
8
4
0
WT
A2
BnCYP83B1
Relative expression
8
4
0
WT
A2
BnCYP83B1
Methionine
BnMAM1
Alkyl aldoximes
S-alkyl thiohydroximates
BnSUR1
Thiohydro ximates
BnST5a
Glucosinolates
BnSUR1
Relative expression
8
4
0
WT
A2
BnST5a
Relative expression
8
4
0
WT
A2
*
C
BnMYB28
Relative expression
8
4
0
WT
A2
BnATR1
Relative expression
8
4
0
WT
A2

Fig. 3.5 (A) Total glucosinolate (GSL) levels measured by Near Infrared Reflectance Spectroscopy in dry seeds over-expressing (S) or down-regulating (A) *BnLEC1*. The respective wild type (WT) [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)] seeds were used as a control. Values are means of three biological replicates ± SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT (control) value. (B) Expression levels of key GLS biosynthetic genes. *Methylthioalkylmalate Synthase1* (*MAM1*), *Cytochrome P450 CYP79B2* (*CYP79B2*), *Cytochrome P450 CYP83B1* (*CYP83B1*), *Superroot 1* (*SUR1*) and *Sulphotransferase5a* (*ST5a*), were measured in developing seeds at 35 DAP (days after pollination). Expression levels were measured in the WT control seeds and in A2 seeds suppressing *BnLEC1*. Values are means of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT (control) value set at 1. (C) Expression levels of *MYB-Containing Transcription Factor28* (*MYB28*), and *Altered Tryptophan Regulation1* (*ATR1*) measured in developing seeds at 35 DAP (days after pollination). Expression levels were measured in the WT control seeds and in A2 seeds suppressing *BnLEC1*. Values are means of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from theWT (control) value set at 1.

3.6. Discussion

B. napus (canola) oil is considered one of the healthiest oils due to its low concentration of saturated FA and high omega-6 and omega-3 FA contents. Canola production is extensive in North America; as in 2013, only in Canada production rose to a record of 18 million tons (Cabak, 2013). Increasing seed oil content and yield per unit area of land is therefore of paramount importance to provide sufficient oil for food and other applications. Even a 1% increase in the seed oil content of *B. napus* would equate to a 2.3-2.5% increase in seed yield (Wang, 2004). The biochemical pathway involved in oil production is controlled by a complex molecular network of genes affecting the seed maturation phase. One such gene, *LEC1*, encodes a HAP3 subunit of the CCAAT-binding transcription factor which regulates the expression of several embryo- and seed-specific components (Lotan et al., 1998). Initially characterized from non-seed plants, where it played an adaptive role in protection from desiccation (Xie et al., 2008), *LEC1* has evolved as a key regulator of plant embryogenesis both *in vivo* and *in vitro* (Lotan et al., 1998; Stone et al., 2001; Xie et al., 2008; West et al., 1994). Among the roles attributed to *LEC1*, is the influence on the hormone balance required for the acquisition of totipotency and seed maturation (Braybrook and Harada, 2008). By regulating the expression of genes affecting gibberellins and ABA homeostasis, *LEC1* is also implicated in the later phases of seed maturation, especially by promoting seed storage product deposition (Braybrook and Harada, 2008). Previous studies using a seed specific promoter showed that over-expression of *ZmLEC1* in corn increases seed oil content but negatively affect seed germination and vegetative development (Shen et al., 2010). A similar effect was observed in Arabidopsis plants over-expressing *AtLEC1* under the control of an estradiol-inducible promoter, which showed improved lipid accumulation but severe seed lethality (Mu et al., 2008; Lotan et al., 1998). These severe phenotypic abnormalities, especially during the early phases of post-embryonic

development often compromise the possibility to investigate *LEC1* function. Major phenotypic abnormalities caused by the altered expression of *BnLEC1* were also observed in our study (Supplemental Table 3.2). However, contrary to previous reports (Shen et al., 2010), it was noted that constitutive overexpression of *LEC1* was not detrimental to seed germination (data not shown). Furthermore, it appears that the abnormalities in the agronomic characteristics measured in lines over-expressing *BnLEC1* might be related to the expression level of the transgene in vegetative tissue. With the exception of plant height, the mildest phenotypic deviations from WT plants were observed in the S1 line, characterized by the lowest expression level of the transgene in the leaves (8 fold greater than the WT), but still showing a 12% increase in total seed oil (Table 3.1 and Supplemental Table 3.3). It is therefore speculated that fine-tuning the expression of *BnLEC1* can be used to increase oil production without compromising agronomic performance. Over-expression of *BnLEC1* has significant effects on seed oil accumulation in *B. napus* (Table 3.1 and Supplemental Table 3.3), and this is in agreement with previous studies in Arabidopsis and maize (Mu et al., 2008; Shen et al., 2010). Of note, the highest percentage increase in seed oil was observed in the lines S1 (12.9% increase) and S2 (16.15% increase), which were transformed with version A (GU945399) of *BnLEC1*. This observation raises the possibility that the two versions (A and B), differing only by 13 nucleotides and 4 amino acids (Supplemental Figs. 3.1 and 3.2), might have different effects on seed oil accumulation. Seed oil was significantly reduced in the down-regulating lines A1 and A2 (Table 3.1 and Supplemental Table 3.3), confirming the importance of *BnLEC1* for oil production. The changes in oil content in the transgenic lines were negatively correlated to the seed protein content (Table 3.1 and Fig. 3.1), as reported in other systems (Shen et al., 2010; Hao et al., 2004). Experimental manipulations in oil content often result in alterations of the FA profile which might negatively

affect the nutritional value of the oil. Changes in FA composition were observed in both Arabidopsis and maize plants where the total seed oil level was increased by the over-expression of *LEC1* (Mu et al., 2008; Shen et al., 2010). In the current study, the constitutive expression of *BnLEC1* has no clear and consistent effects on the FA composition (Table 3.2) and the nutritional value of the oil (Table 3.3). For example, the changes in the content of palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:3) were only observed in some of the *BnLEC1* over-expressing lines, and in some cases (for oleic acid) showed contrasting results between lines. These slight variations in FA profile resulted in minute alterations in the nutritional value of the oil. The lack of any change in FA profile and oil nutritional value (Tables 3.2 and 3.3) observed in the down-regulator lines (A1 and A2) further supports the notion that *BnLEC1*, at least in *B. napus*, affects total seed oil without significant influence on FA composition. Biosynthesis of FA is physiologically connected to seed development as both processes are influenced by a common set of genes including *LEC2, WRI1* and *FUS3* (Mu et al., 2008; Cernac and Benning, 2004; Meinke et al., 1994; Gazzarrini et al., 2004; Prystenski, 2011). *BnLEC2*, highly induced in *BnLEC1* overexpressing seeds at 28 DAP and suppressed at 35 DAP in *BnLEC1* down-regulating seeds (Fig. 3.2), has been reported to enhance the accumulation of lipids in vegetative and reproductive Arabidopsis tissue (Santos Mendoza et al., 2005; Stone et al., 2008). One of the functions of *LEC2* is to induce *WRI1* (Baud et al., 2007), which show a similar expression profile as *LEC2*, with an induction in the *BnLEC1* over-expressors and suppression in the lines down-regulating *BnLEC1* during the late seed maturation phases (Fig. 3.2). *WRINKLED1* is important for providing sufficient carbon source for the FA biosynthetic pathway through the activation of glycolytic enzymes (Baud et al., 2007). Improved seed oil content as a result of *WRI1* overexpression was reported in various species, including maize

(Shen et al., 2010; Pouvreau et al., 2011) and Arabidopsis (Liu et al., 2010). Another key transcription factor, *FUS3*, encodes a B3 protein required for the transcriptional activation of genes encoding storage proteins (2S albumin and 12S cruciferin), and various enzymes involved in glycolysis and FA biosynthesis (Wang et al., 2007). The expression of *BnFUS3* also appears to be regulated by *BnLEC1* (Fig. 3.2). Collectively, these studies show a direct or indirect involvement of *BnLEC1* in the expression of seed- and oil-related transcription factors in canola seeds. The major carbon source for seed oil synthesis is sucrose derived from photosynthesis (Hills, 2004). In seeds, sucrose is rapidly converted into the hexoses fructose and UDP-glucose which are rapidly oxidized in the glycolytic pathway to acetyl-CoA, the precursor of FA (Schwender et al., 2003). The increase in transcript levels of *BnSUC4*, *BnSUS1*, *BnAGP* and to a lesser extent *BnSUS3*, in the *BnLEC1* over-expressing lines is indicative of a more active intake of sucrose into seeds (Figs. 3.3 and 3.4). In seed suppressing *BnLEC1*, this process might be compromised, as revealed by the general reduction in expression level of several genes involved in sucrose metabolism. Among the genes analyzed, *BnAGP* is the best indicator of enhanced storage product synthesis, as a high expression of this gene is required for accumulation of starch and lipids in the seed (Vigeolas et al., 2004). The overall increase in expression levels of several glycolytic enzymes, including *BnHXK*, *BnGPDH*, and *BnFPA*, observed in the *BnLEC1* lover-expressing lines (Figs. 3.3 and 3.4) is also indicative of a fast oxidation of sucrose which is possibly required for enlarging the pool of FA biosynthetic precursors, leading to increased seed oil content. This stimulation is also supported by the increased activation of *BnACCA2* and *BnMCAT* in the over-expressing lines (Figs. 3.3 and 3.4). A positive correlation between the transcriptional activation of the glycolytic pathway and enhanced oil accumulation was observed in *B. napus* plants overexpressing the transcription factor *SHOOTMERISTEMLESS* (Elhiti et al.,

2012), and in other systems (Mu et al., 2008). A reduction in *BnLEC1* expression had moderate consequences on the expression of the glycolytic and FA enzymes. However, the consistent repression of *BnHXK* observed in both A1 and A2 lines at 35 DAP suggests a reduced conversion of glucose into glucose-6-P (Fig. 3.4), which might limit the overall rate of sucrose oxidation in glycolysis. Together, these observations support the hypothesis that *BnLEC1* affects seed oil levels through the transcriptional regulation of upstream events including sucrose metabolism and glycolysis.

Glucosinolates are sulfur-containing secondary metabolites commonly found in *Brassicacea*, and often involved in stress responses (Feng et al., 2012). Due to their anti-nutritional properties and negative effects on meal quality, GLS accumulation in seeds is highly undesirable (Fenwick and Curtis, 1980). The levels of GLS do not seem to be consistently affected in all the *BnLEC1* overexpressing lines (Fig. 3.5A). Conversely, suppression of *BnLEC1* has a pronounced inductive effect on GLS levels. This effect might be due to the up-regulation of *BnST5a* regulating the 3' phosphoadenosine 5'-phosphosulphate-dependent sulphation of desulphoglucosinolates (Piotrowski et al., 2004) (Fig. 3.5B).

In conclusion, the present study provides evidence for a positive correlation between *BnLEC1* expression and seed oil accumulation. The increased level of oil induced by the ectopic expression of *BnLEC1* is most likely related to an active sucrose metabolism and glycolytic oxidation rate, and has no severe negative repercussions on FA composition, oil nutritional value and GLS content. Modulation in the expression level of the transgene in vegetative tissue is also crucial for attaining a compromise between proper plant development and desirable elevation in oil content. Oil synthesis was negatively affected by the suppression of *BnLEC1*, which also elevated GLS to undesirable levels.

3.7. Acknowledgment

This work was supported by an Agriculture, Food and Rural Development grant to CS. The technical assistance of Mr. Durnin, Mr. Kowatsch, Dave Audette and Debbie Witko is also acknowledged.

3.8 Supplemental data

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GU945399   ATGCCAATCGCAAACGTGATAAGGATCATGCGTAAAATCTTACCGCCACACGCCAAAATC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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GU945399   TCTGACGACGCAAAAGAAACGATTCAAGAATGCGTCTCCGAGTACATCAGCTTCGTGACC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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GU945399   GGTGAAGCTAACGAGCGTTGCCAACGTGAGCAACGTAAGACAATAACAGCTGAAGATATC  360
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GU945399   CTTTGGGCCATGAGCAAACTTGGGTTCGATGATTACGTTGGACCACTCAACGTGTTCATT  420
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Supplemental Fig. 3.1 Nucleotide alignment comparing two *BnLEC1* sequences: GU945399 (version A) and GU945398 (version B).

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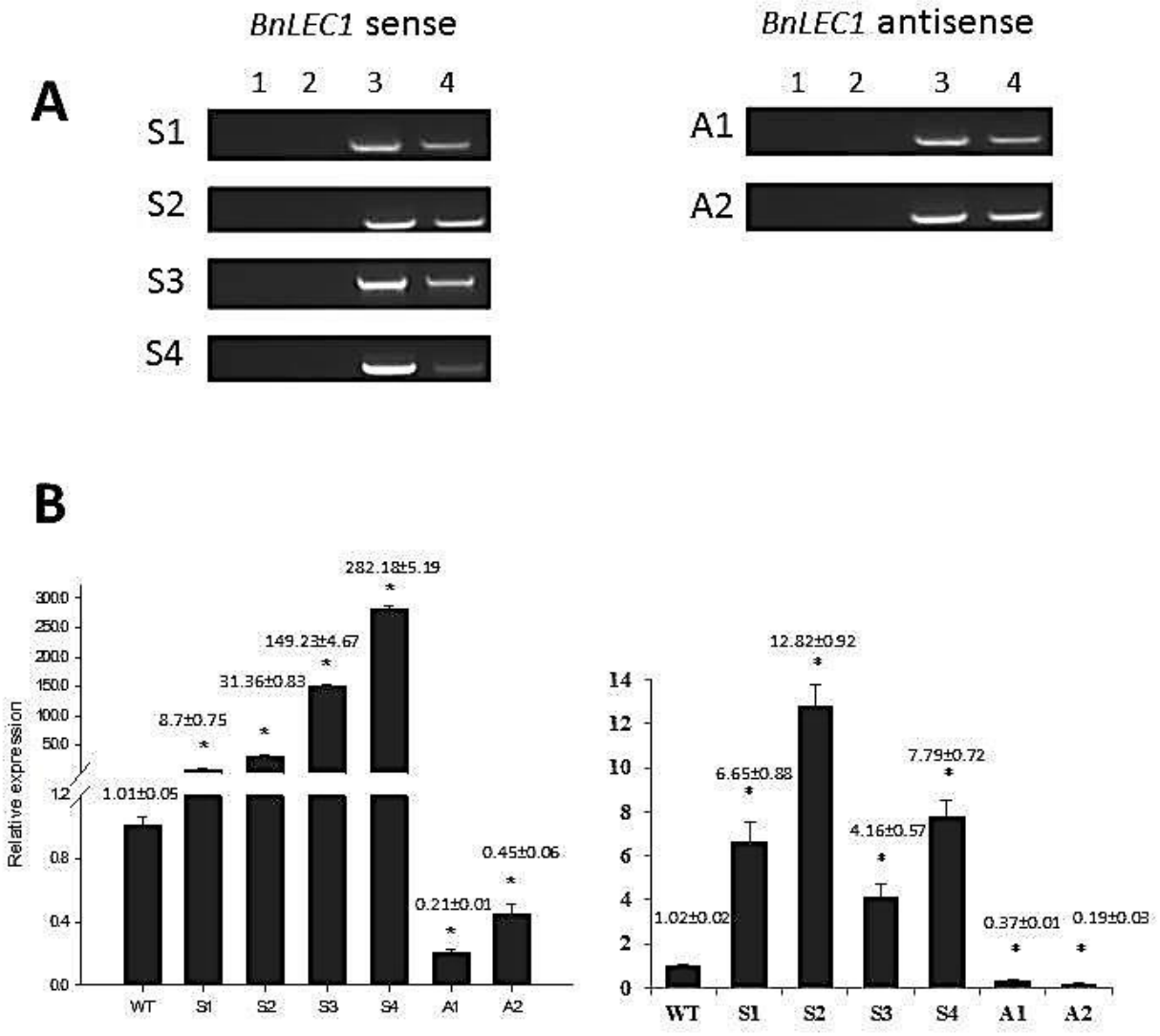
ADF81044    MERGAPLSHYQLPKSNSGLNLDQHNNSIPTMTGSIGACDDKNKTILPQQQPSMPREQDQY  60

ADF81045    MPIANVIRIMRKILPPHAKISDDAKETIQECVSEYISFVTGEANERCQREQRKTITAEDI  120
            MPIANVIRIMRKILPPHAKISDDAKETIQECVSEYISFVTGEANERCQREQRKTITAEDI
ADF81044    MPIANVIRIMRKILPPHAKISDDAKETIQECVSEYISFVTGEANERCQREQRKTITAEDI  120

ADF81045    LWAMSKLGFDDYVGPLNVFINRYREFETDRGCSLRGESSFKPVYGGSGMGFHGPPPPGSY  180
            LWAMSKLGFDDYVGPLNVFINRYREFETDRGCSLRGESSFKPVYGGSGMGFHGPPPPGSY
ADF81044    LWAMSKLGFDDYVGPLNVFINRYREFETDRGCSLRGESSFKPVYGGSGMGFHGPPPPGSY  180

ADF81045    GYGMLDQSMVMGGGRYYHNGSGQDGSVSGGGGSSSSMNGMPVYDQYGQYK  230
            GYGMLDQSMVMGGGRYYHNGSG +GSV GGGGSSSSMNGMPVYDQYGQYK
ADF81044    GYGMLDQSMVMGGGRYYHNGSGPEGSVGGGGGSSSSMNGMPVYDQYGQYK  230
```

Supplemental Fig. 3.2 Amino acid alignment comparing two *BnLEC1* sequences: GU945399 (version A) And GU945398 (version B).



Supplemental Fig. 3.3 Genotypic analysis of the transgenic lines over-expressing (S1-4) or down regulating (A1, 2) *BnLEC1*. (A) Presence of the transgene in the genomic DNA extracted from leaves using a 35S promoter forward primer and a *BnLEC1* primer (see Supplemental Table 3.1 for primer sequence). Lanes: 1, WT plant; 2, H_2O; 3, plasmid; 4, transgenic plant (B) Quantitative RT-PCR analysis of *BnLEC1* expression in leaves (left panel) and 35 DAP seeds (right panel) of transgenic *Brassica napus* lines. The expression level was normalised to the WT value (Polo for S1-4 and Topas for A1, 2) set at 1. *UBC21* (EV086936, ubiquitin-conjugating enzyme 21) was used as the internal control reference. Data presented are mean values of three experiments + standard errors. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT (control) value. All primers used are listed in Supplementary Table 3.1.

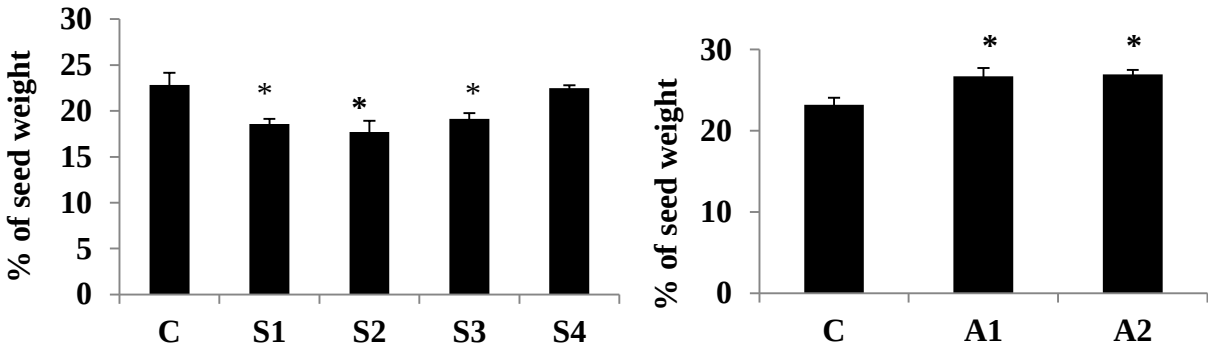
A

BnLEC1 down-regulator

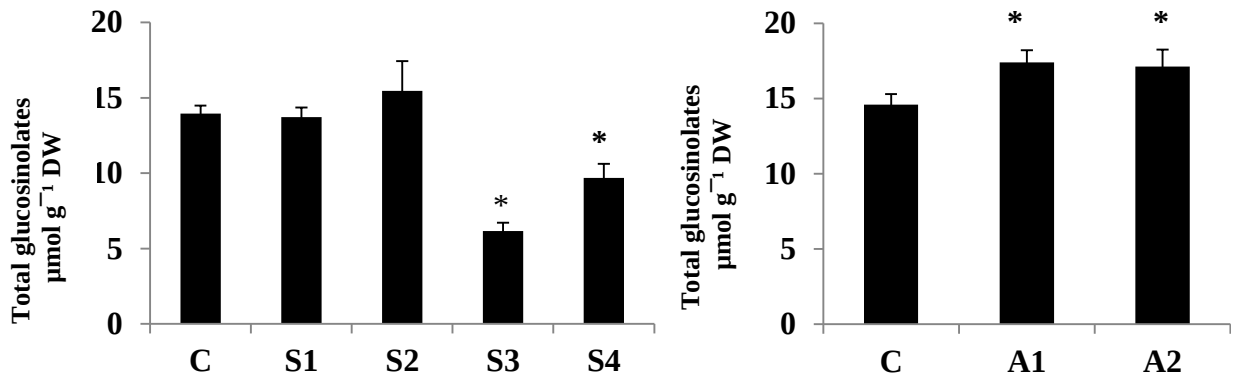
B

WT *BnLEC1* down-regulator

Supplemental Fig 3.4 Phenotypic abnormalities of the *BnLEC1* down-regulating lines (A) Precocious flowering during the regeneration of plants in tissue culture. (B) Early reproductive growth with seedless siliques.



Supplemental Fig 3.5 Measurements of protein content in dry seeds of *Brassica napus* using Near Infrared Reflectance Spectroscopy. Plants were grown in the growth chamber with 16/8 hours day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1} and day/night temperature of 20°C /15 °C. Values, expressed as percentage of seed weight (at 0% humidity) ±SE, are means of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the C (control) [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)].



Supplemental Fig 3.6 Total glucosinolate (GSL) levels measured by Near Infrared Reflectance Spectroscopy in dry seeds over-expressing (S) or down-regulating (A) *BnLEC1*. Plants were grown in the growth chamber with 16/8 hours day/night photoperiod, light intensity of 400 µmol m^{-2} s^{-1} and day/night temperature of 20°C /15 °C. The respective control (C) [Polo for the *BnLEC1* over-expressors (S1-S4) and Topas for the *BnLEC1* down-regulators (A1, A2)] seeds were used as a control. Values are means of three biological replicates + SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the C (control) value.

Supplemental Table 3.1 Primer sequence (5'-3') used for genotyping and qRT-PCR studies.

Genotyping	
35S (F)	TGGACCCCCACCCACGAG
For sense	
BnLEC1 (R)	TGGAGGTGGGCCGTGAAACC
For antisense	
BnLEC1 (R)	AAACGGCAGAGAAACAATGG
qRT-PCR	
BnLEC1-2-F	TATCTTGCCGCAGCAACAACCAAG
BnLEC1-2-R	TTCACCGGTCACGAAGCTGATGTA
BnLEC2-F	ACAAGAATCGCTCGCACTTCTCCA
BnLEC2-R	AAGCATCCGATGAGTGAAGAGGCT
FUS3-F	TCCATCATCGTCCAGGGTTTGGAT
FUS3-R	AAGAAGATCGTCTCTGTCTGGGCA
WRI1-F	ATAGAGTACAGAGGCGCAAACGCA
WRI1-R	TCACAGGGAATGGGAAGACACCTT
BnACCA2-F	TTGCCAGTCATAGTCCAGAGGCAT
BnACCA2-R	TCTGCTGCGATGTCCATGAAGGAT
BnAGP-F	TGCTGGAACGAGATTGTATCCGCT
BnAGP-R	ATGGCGGTTGAGAGAAGCTGAGTT
BnFAD3-F	TATAAGGGCGGCCATTCCTAAGCA
BnFAD3-R	AGATAGCCCAGAACAGGGTTCCTT
BnFAE1-F	TCTCCGCGATGGTCGTTAACACTT
BnFAE1-R	TCCTTGGACAAACTCACTCCGGTT
BnFPA-F	TGAGTTGATCGCTAACGCCGCATA
BnFPA-R	TTCTCGACGTTGATGCTGGCAAGA
BnGPDH-F	ACGGAAAGTTGACCGGAATGTCCT
BnGPDH-R	ACAACATCGTCCTCGGTGTAACCA
BnHXK-F	ATCAGCTGCAGGGATCTACGGAAT
BnHXK-R	ACTCGCTGAACTGAGTGTAGTGCT
BnMACT-F	TCAAGCAGTAGGGATGGGCAAAGA
BnMACT-R	ACGAACACGGAGCAATTCAACAGC

BnPGK-F	AGGCACAAGGTCTGTCTGTTGGAT
BnPGK-R	AGCGAACTTGTCAGCAACCACAAC
BnPPK-F	AGGTGCGCCGTTTAAGAAATTCGC
BnPPK-R	AGAATGAAAGGAAGGCCGGCTACT
BnSUC1-F	ACCAATCGTCGGTTACCACAGTGA
BnSUC1-R	ATATCCGCGGCGTATCCGATCAAA
BrSUC4-F	ACAAGGATTCAGAAGCCCATCCCT
BrSUC4-R	ATCTCCTTCCACCATGCCTTCACA
BrSUS1-F	TCTCCGTGTGCCTTTCAGAACAGA
BrSUS1-R	AGAGGCAACGAGGTTTCCATCACT
BrSUS3-F	TTAAGCAGCTTCTCGGCAACCTCA
BrSUS3-R	CGAACAAGCCGGATGTCAACCTTT
BnATR1-f	TGGCCTTATGGAGGAGTTAGAGGA
BnATR1-r	ATCAGTTACGTCAACCTCAGGCGA
BnMYB28-f	AAGCAATACTCCCGGTCAAGCTCA
BnMYB28-r	ACTGGTGTCCCATCTTTGCTGGTA
BrMAM1-f	AGACCATTGCCAAGACTGTAGGGA
BrMAM1-r	AACCGCCTCGATGTCTCTATGGTT
BnCYP79B2-f	ACTTCACCGTCGGGTAAAGATGCT
BnCYP79B2-r	ACACGTGTTGAGCTGATGGAGTCT
BnSUR1-f	TGGCTTCACCAGAAGAGAGCTGAA
BnSUR1-r	ACAGTAATGCCTCGTGACAAACCG
BnST5a-f	ATGGCTGCTCGTATCGATGGGTTA
BnST5a-r	CCTCTGTTCCGCACCAAACAACAA
BnCYP83B1-f	AAAGATGGACGTCATGACCGGACT
BnCYP83B1-r	CATCACGCCTGATCAAATGTGCGT
BnUBC21-F	CCTCTGCAGCCTCCTCAAGT
BnUBC21-r	CATATCTCCCCTGTCTTGAAATGC
Bn-Actin-F	TAAAGTATCCGATTGAGCATGGTAT
Bn-Actin-R	CGTAGGCAAGCTTCTCTTTAATGTC

Supplemental Table 3.2: Analysis of major agronomic characteristics of the transgenic *Brassica napus* plants (grown in greenhouse conditions from April 2013 to July 2013). Values are means + SE of nine biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the WT control [Polo for the *BnLEC1* over-expressing lines (S1-4) and Topas for the *BnLEC1* down-regulating lines (A1, A2)].

Line	Plant height(cm)	Siliques/ plant	Silique length (cm)	Seeds/ Silique	1000 seed weight (g)	Yield/plant (g)
WT (Polo)	62±1.07	72±1.6	4.94±0.08	19.2±.4	2.61±0.02	2.9±.11
S1	76.8±.38*	70±1.31	5.02±.07	19.7±.27	2.98±.02*	2.9±.15
S2	62±1.12	56±.74*	5.7± .04*	16±.23*	3.95±.03*	2.46±.06*
S3	69.8±1.18*	65±1.56*	5.16±.07	14.7±.62*	3.7±.03*	2.58±.06*
S4	76.8±.92*	49±.79*	5.02±.06	18±.33*	2.74±.02*	1.73±.07*
WT (Topas)	66.4±0.84	60.3±0.97	5.7±0.07	27±0.62	3.17±0.04	3.1±0.14
A1	68.2±0.62	40.1±0.65*	5.6±0.03	16±0.71*	2.63±0.03*	1.7±0.06*
A2	68.4±0.53	42.8±1.08*	5.3±0.13*	16.1±0.75*	2.58±0.05*	1.7±0.05*

Supplemental Table 3.3: Measurements of total oil content in dry seeds of *Brassica napus* using Near Infrared Reflectance spectroscopy (NIRS). Plants were grown in the growth chamber with 16/8 h day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1} and day/night temperature of 20 °C /15 °C. Values are means of three biological replicates + SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over-expressors; A1-A2, *BnLEC1* down-regulators.

Line	**Total oil (% dry weight)**	**Percentage of increase**	**Line**	**Total oil (% dry weight)**	**Percentage of decrease**
Control (Polo)	46.61±0.98		Control (Topas)	44.12±0.04	
S1	53.35±0.60*	14.46	A1	41.37±1.12*	6.233
S2	55.03±0.78*	18.06	A2	41.39±0.38*	6.187
S3	52.54±0.42*	12.72			
S4	49.46±0.19*	6.11			

Supplemental Table 3.4: Fatty acid profile (% composition) in transgenic *Brassica napus* seeds harvested from plants grown in the growth chamber with 16/8 h day/night photoperiod, light intensity of 400 μmol m^{-2} s^{-1} and day/night temperature of 20 °C /15 °C. Values are means of three biological replicates + SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over-expressors; A1-A2, *BnLEC1* down-regulators.

	WT (Polo)	**S1**	**S2**	**S3**	**S4**	**WT(Topas)**	**A1**	**A2**
C16:0	**4.14±0.09**	3.63±0.09*	3.66±0.02*	4.34±0.07	3.80±0.13	**4.76±0.29**	4.36±0.06	4.35±0.09
C18:0	**1.35± 0.03**	1.32±0.11	1.18±0.06	1.30±0.05	1.42±0.15	**1.83±0.25**	1.5±0.03	1.61±0.07
C18:1	**66.03±0.39**	68.61±0.62*	65.13±0.51	62.70±0.68*	67.76±0.36*	**60.61±1.02**	63.24±0.58	64.35±0.75*
C18:2	**16.92±0.22**	15.58±0.23	17.96±0.58	19.36±0.45*	16.38±0.55	**21.87±0.78**	21.32±0.33	20.63±0.45
C18:3	**8.97±0.08**	8.35±0.37	9.74±0.13	9.77±0.18	7.52±0.47*	**8.13±0.84**	6.93±0.61	6.42±0.75
C20:0	**0.46±0.01**	0.49±0.03	0.43±0.01	0.48±0.01	0.57±0.06*	**0.74±0.1**	0.56±0.03	0.58±0.04
C20:1	**1.09±0.06**	1.14±0.03	1.06±0.03	1.07±0.04	1.26±0.11	**1.35±0.03**	1.19±0.04	1.17±0.07*
C22:0	**0.25±0.003**	0.29±0.01	0.26±0.01	0.28±0.01	0.29±0.20	**0.44±0.06**	0.32±0.03	0.30±0.03*

Supplemental Table 3.5: Nutritive value of the transgenic *Brassica napus* seeds. The ratio of different C18 unsaturated FA is calculated according to data presented in Supplemental Table 3.4. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective WT (control) value. S1-S4, *BnLEC1* over-expressors; A1-A2, *BnLEC1* down-regulators.

	WT (Polo)	**S1**	**S2**	**S3**	**S4**	**WT(Topas)**	**A1**	**A2**
18:1/18:2	**3.90±0.07**	4.41±0.10*	3.64±0.14	3.24±0.11*	4.15±0.16	**2.8±0.14**	2.93±0.07	3.15±0.09
18:1/18:3	**7.36±0.11**	8.25±0.40	6.69±0.11	6.42±0.18	9.08±0.58*	**7.30±0.54**	9.29±1.05	10.65±0.83*
18:2/18:3	**1.89±0.01**	1.87±0.08	1.84±0.07	1.98±0.03	2.20±0.19*	**2.60±0.06**	3.16±0.3	3.37±0.16 *

4 CHAPTER TWO: Decreased seed oil production in *FUSCA3 Brassica napus* mutant plants

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Contributions

Drs. Duncan and Stasolla designed the experiments, helped with the interpretation of data and contributed writing the manuscript. Mrs. Elhai conducted the experiments and wrote the manuscript.

4.1 A b s t r a c t

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 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*.

Keywords:

Brassica napus; Canola; *FUSCA3*; Glycolysis; Seed oil

4.2 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; 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.

4.3 Materials and Methods

4.3.1 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 methane sulfonate) 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 4.1), 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.

4.3.2 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 cetyltrimethyl ammonium bromide (CTAB) method (Keb-Llanes et al., 2002). The DNA was suspended in 50 ml TE buffer and its concentration was determined by a Nano Drop spectrophotometer. Samples were adjusted to a final concentration of 150ng/µl H_2O. Genomic DNA samples were then amplified using primers bn36-4R and bn36-5L (Supplementary Table 4.1). 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.

4.3.3 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 Februarye-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 temperature 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.

4.3.4 Analysis of seed oil content and protein level

Total oil and protein levels were measured using near infrared reflectance (NIR System model 6500, Foss NIR Systems 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. The actual methods detail and instruments utilized are the same as written in the previous chapter (see page 48 for detail).

4.3.5 Gene expression analysis by qRT-PCR

After identification of the altered seed oil profile in the first greenhouse experiment, gene expression analyses were performed on samples grown in the second greenhouse experiment from June 2012 to September 2012. In developing *B. napus* seeds, significant accumulation of

oil starts at 28 DAP (days after pollination), and reaches its maximum level at 35 DAP (Fowler and Downey, 1970; Gurr et al., 1972). Thus, to evaluate the expression levels of transcription factors participating in oil biosynthesis i.e., *BRASSICA NAPUS LEAFY COTYLEDON1* and *2* (*BnLEC1* and *2*), *ABSCISIC ACIDINSENSITIVE 3* (*BnABI3*) and *WRINKLED1* (*BnWRI1*), seeds samples were collected at 14, 21, 28 and 35 DAP. For all other experiments, seed was collected at 35 DAP unless otherwise stated. All gene expression analyses were conducted using quantitative (q) RT-PCR (Elhiti et al., 2010) with existing primer sequences (Prystenski, 2011; Elhiti et al., 2012) listed in Supplementary Table 4.1. These analyses also included genes involved in glycolysis: *FRUSCOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *PHOSPHOGLYCERATE KINASE* (*BnPGK*), *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), *HEXOSE KINASE* (*BnHXK*) and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*); sucrose transport and metabolism: *SUCROSE TRANSPORTER 1* and *4* (*BnSUC 1* and *4*), *SUCROSE SYNTHASE 1* and *3*, (*BnSUS1* and *3*) and *ADP-GLUCOSE PHOSPHORYLASE* (*BnAGP*); FA biosynthesis: *SUBUNIT A of ACETYLCoA CARBOXYLASE* (*BnACCA2*), *ω-3 FA DESATURASE* (*BnFAD3*), *FA ELONGATION1* (*BnFAE1*), and *MALONYL-CoA:ACP TRANSACYLASE* (*BnMCAT*); and five representative *Brassica OLEOSIN* genes: *OLEOSIN S-1, S-2, S-3, S-4* and *S-5*. The relative level of gene expression was estimated with the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001) using *UBC21* (EV086936, *ubiquitin-conjugating enzyme 21*) as a reference. Analyses were performed in three biological replicates. The detail for the sample prepration, methods and instruments utilized are the same as written in the previous chapter (see pages 49 and 50 for detail).

4.3.6 Statistical analysis

The statistical analyses for agronomic traits and gene expression were performed using SAS® 9.3 (SAS Institute Inc., Cary, NC, USA.). Fisher's Least Significant Difference (LSD) test was applied to observe the main effect of the mean for each mutant line and the wild type control (using at least three biological replicates for all experiments unless otherwise specified).

4.4 Results

4.4.1 Phenotypic characterization of the *BnFUS3* TILLING mutant lines

The *B. napus FUS3* (HM370540) composed of 927 nucleotides (Supplemental Fig. 4.1), corresponding to 308 amino acids, was utilized to generate three *BnFUS3* TILLING mutant lines: M1, M2, and M3. Each mutant line was characterized by a single nucleotide substitution (Supplemental Fig. 4.2), corresponding to a respective amino acid change downstream of the B-3 DNA binding domain (Supplemental Fig. 4.3). Seed maturation in both *BnFUS3* mutant and wild type plants occurred in about 100-110 days with no visible morphological deviations in embryonic growth. A small decrease in germination frequency was observed for the mutant lines M1 and M3 (Supplemental Fig.4.4). A unique phenotypic trait in Arabidopsis *fus3* seeds is a purple coloration due to the accumulation of anthocyanins (Meinke et al., 1994). In addition to the distorted seed shape, varying degrees of reddish purple coloration were also observed in all the seeds of the three *BnFUS3* TILLING mutant lines (Supplemental Fig. 4.5), suggesting similar effects between the two species. Among the different agronomic characteristics measured, the only consistent phenotypic deviation observed in all three mutants was the reduced number of siliques per plant, resulting in decreased plant yield (Table 4.1). Similar trends were also observed during the other growing season in the greenhouse and with plants grown in the growth cabinet (Supplemental Tables 4.2 and 4.3).

Table 4.1: Analysis of major agronomic traits in the wild type (WT) and *BnFUSCA3* TILLING mutant lines (M1-3) grown in the greenhouse from June 2012 to September 2012. Values are means ± SE of ten biological replicates. Lowercase letters indicate LSD grouping used to determine significant differences (LSD: $P < 0.05$).

Line	Plant height (cm)	Siliques per plant	Seeds per silique	Silique length (cm)	1000-seed weight(g)	Yield per plant (g)
WT	70.5±1.26	37.4±1.10[a]	18.9±0.53	5.1±0.10	3.02±0.04	2.07±0.04[a]
M1	70.0±1.23	30.1±1.67[b]	18.7±0.67	5.1±0.11	2.98±0.06	1.83±0.02[b]
M2	75.5±0.86	27.0±1.14[b]	18.1±0.42	5.1±0.09	3.09±0.04	1.65±0.03[c]
M3	72.2±1.28	27.9±0.87[b]	18.6±0.54	5.14±0.13	2.84±0.04	1.62±0.03[c]

4.4.2 Mutations of *BnFUS3* affect seed storage product accumulation

To investigate the effects of *BnFUS3* on storage product accumulation, fully mature dry seeds collected from the three TILLING lines were analyzed by NIR. Compared to the wild type (WT), total seed oil content was significantly decreased in all mutant (M1-3) lines, while protein levels increased (Fig. 4.1). Similar profiles were obtained during the other growing seasons in the greenhouse and in growth cabinet; however, the decline in seed oil content relative to WT was less pronounced for *BnFUS3* mutant plants in the growth cabinet experiment (Supplemental Figs. 4.6 and 4.7). Analysis of FA composition by gas chromatography (GC) revealed minute alterations in the relative levels of palmitic (16:0) and stearic (18:0) acid content in seeds of some of the *BnFUS3* TILLING mutant lines (Table 4.2). A consistent increase in linoleic acid (18:2) was observed in all mutant lines (Table 4.2). A similar increase was also found during the other experiment in the greenhouse and with plants grown in the growth cabinet (Supplemental Tables 4.4 and 4.5).

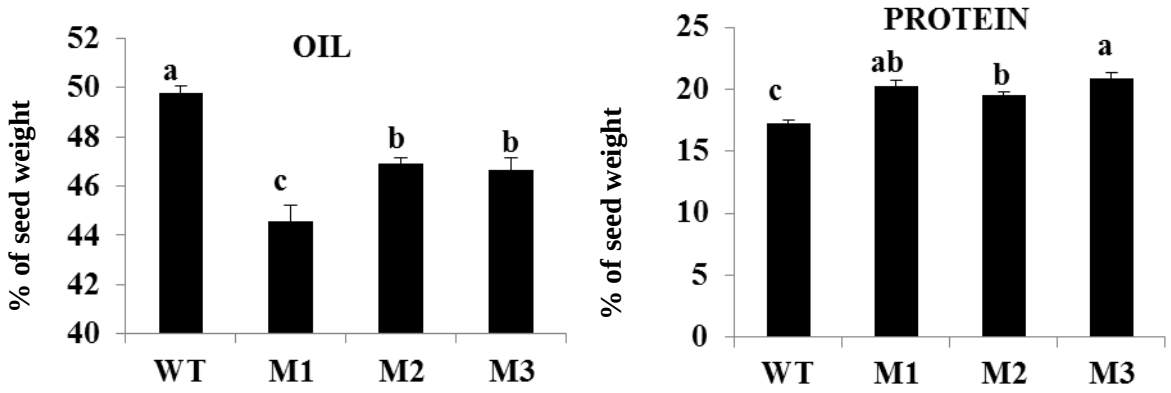

Fig. 4.1 Measurements of protein and total oil content in dry seeds of *B. napus FUS3* mutants using Near Infrared Reflectance Spectroscopy. Mature seeds were harvested from plants grown in greenhouse conditions from June 2012 to September 2012. Values, expressed as percentage of seed weight (at 0% humidity) ±SE, are means of three biological replicates. Letters indicate statistically significant differences by LSD tests at $P < 0.05$. WT, control; M1-M3, *BnFUS3* mutants.

Table 4.2: Relative fatty acid content (% dry weight) in seeds of the wild type (WT) and *BnFUSCA3* TILLING mutant lines (M1-3) grown in the greenhouse from June 2012 to September 2012. Values are means ± SE of three biological replicates. Lowercase letters indicate LSD grouping used to determine significant differences (LSD: $P < 0.05$).

Fatty Acid	WT (DH12075)	M1	M2	M3
C16:0	3.70 ± 0.03^{b}	3.82 ± 0.02^{a}	3.36 ± 0.02^{c}	3.70 ± 0.05^{b}
C16:1	0.16 ± 0.003^{ab}	0.16 ± 0.005^{a}	0.12 ± 0.004^{c}	0.15 ± 0.002^{b}
C18:0	1.51 ± 0.02^{b}	1.54 ± 0.02^{b}	1.77 ± 0.02^{a}	1.4 ± 0.02^{c}
C18:1	63.98 ± 0.22^{a}	62.92 ± 0.09^{b}	61.26 ± 0.15^{c}	63.49 ± 0.25^{ab}
C18:2	15.98 ± 0.11^{c}	17.71 ± 0.32^{b}	19.09 ± 0.26^{a}	17.32 ± 0.25^{b}
C18:3	11.46 ± 0.47^{a}	11.85 ± 0.06^{a}	11.66 ± 0.10^{a}	11.79 ± 0.15^{a}
C20:0	0.57 ± 0.01^{b}	0.63 ± 0.02^{a}	0.63 ± 0.00^{a}	0.55 ± 0.01^{b}
C20:1	1.23 ± 0.02^{b}	1.36 ± 0.01^{a}	1.32 ± 0.01^{a}	1.33 ± 0.01^{a}
C22:0	0.32 ± 0.01^{c}	0.42 ± 0.01^{a}	0.36 ± 0.00^{b}	0.33 ± 0.01^{bc}

4.4.3 Mutations of *BnFUS3* reduce the expression of genes involved in sucrose photo-assimilation and glycolysis

The main carbon source for oil biosynthesis in developing seeds is sucrose produced from photosynthesis (Rawsthorne, 2002). Sucrose is readily hydrolyzed into UDP-glucose and fructose, which are oxidized in the glycolytic pathway to Acetyl-CoA, the precursor of FA biosynthesis (Hills, 2004). The reduced level of seed oil in *BnFUS3* TILLING mutants prompted us to measure the transcript levels of genes contributing to sucrose metabolism and glycolysis. Of the five genes involved in sucrose transport and metabolism, *SUCROSE TRANSPORTER 1* (*BnSUC1*) was significantly repressed in seeds of the M2 and M3 lines, but not in those of the M1 line (Fig. 4.2). Consistent suppression among all three mutant lines was observed for *SUCROSE TRANSPORTER 4* (*BnSUC4*), *SUCROSE SYNTHASE 1* and *3* (*BnSUS1* and *3*), as well as *ADP-GLUCOSE PHOSPHORYLASE* (*BnAGP*), a key enzyme producing starch from glucose (Schwender et al., 2003; Vigeolas et al., 2004) (Fig. 4.2). Among the enzymes participating in the glycolytic pathway, the expression levels of *HEXOSE KINASE* (*BnHXK*), *FRUCTOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), and *PYROPHOSPHATASE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*) were significantly reduced in seeds of all the three *BnFUS3* TILLING mutant lines (Fig. 4.3). Relative to the WT, a repression of *PHOSPHOGLYCERATE KINASE* (*BnPGK*) was observed for the M2 line, but not for the other two mutant lines (Fig. 4.3).

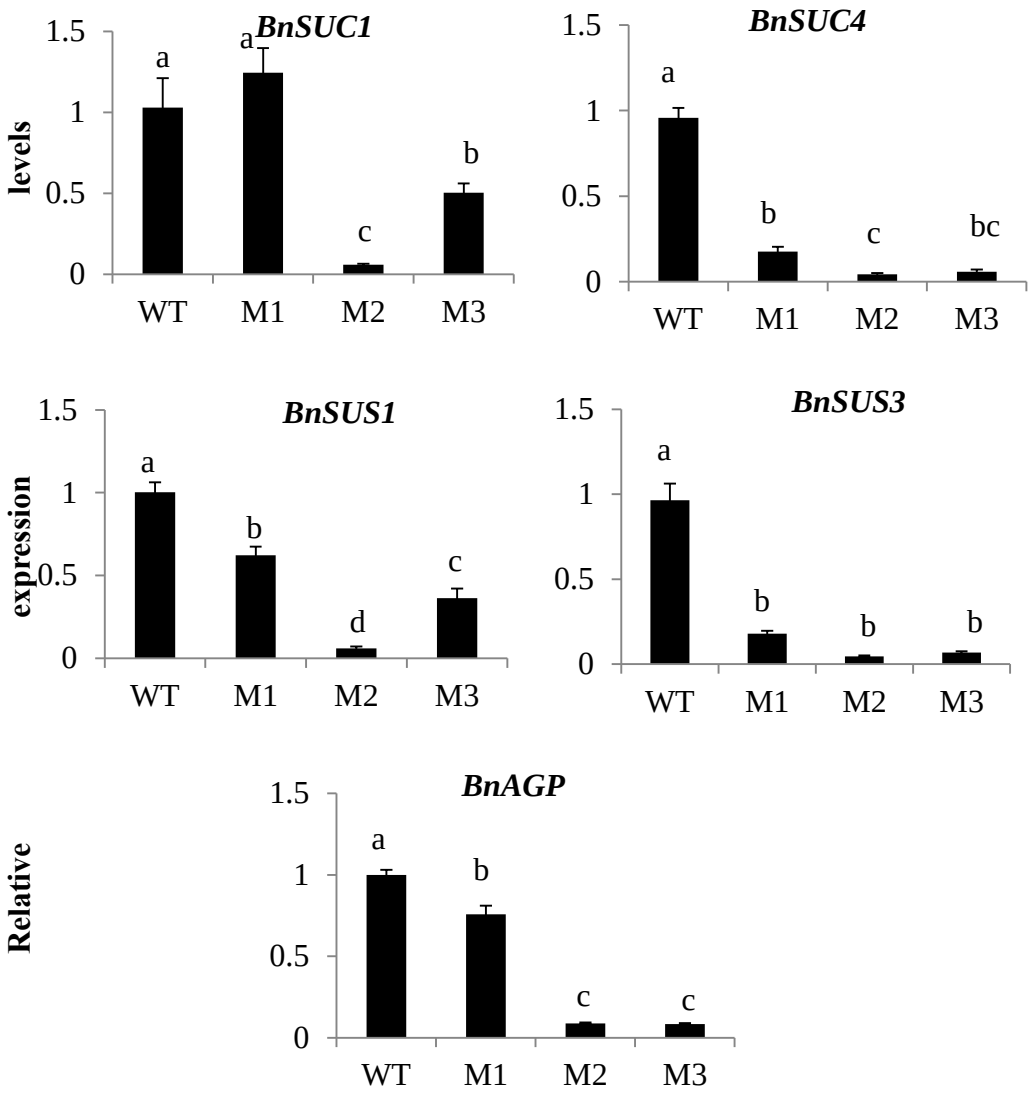

Fig. 4.2 Relative transcript levels of genes involved in sucrose transport and metabolism in dry seeds collected at 35 days after pollination (DAP) from WT and *B. napus FUS3* mutant (M1-3) plants. Transcript levels were measured for *SUCROSE TRANSPORTER 1* and *4* (*BnSUC1* and *4*), *SUCROSE SYNTHASE 1* and *3* (*BnSUS 1* and *3*), and *ADP-GLUCOSE PHOSPHORYLASE* (*BnAGP*). Values are means ± SE of at least three biological replicates and are normalized to the WT value set at 1. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.

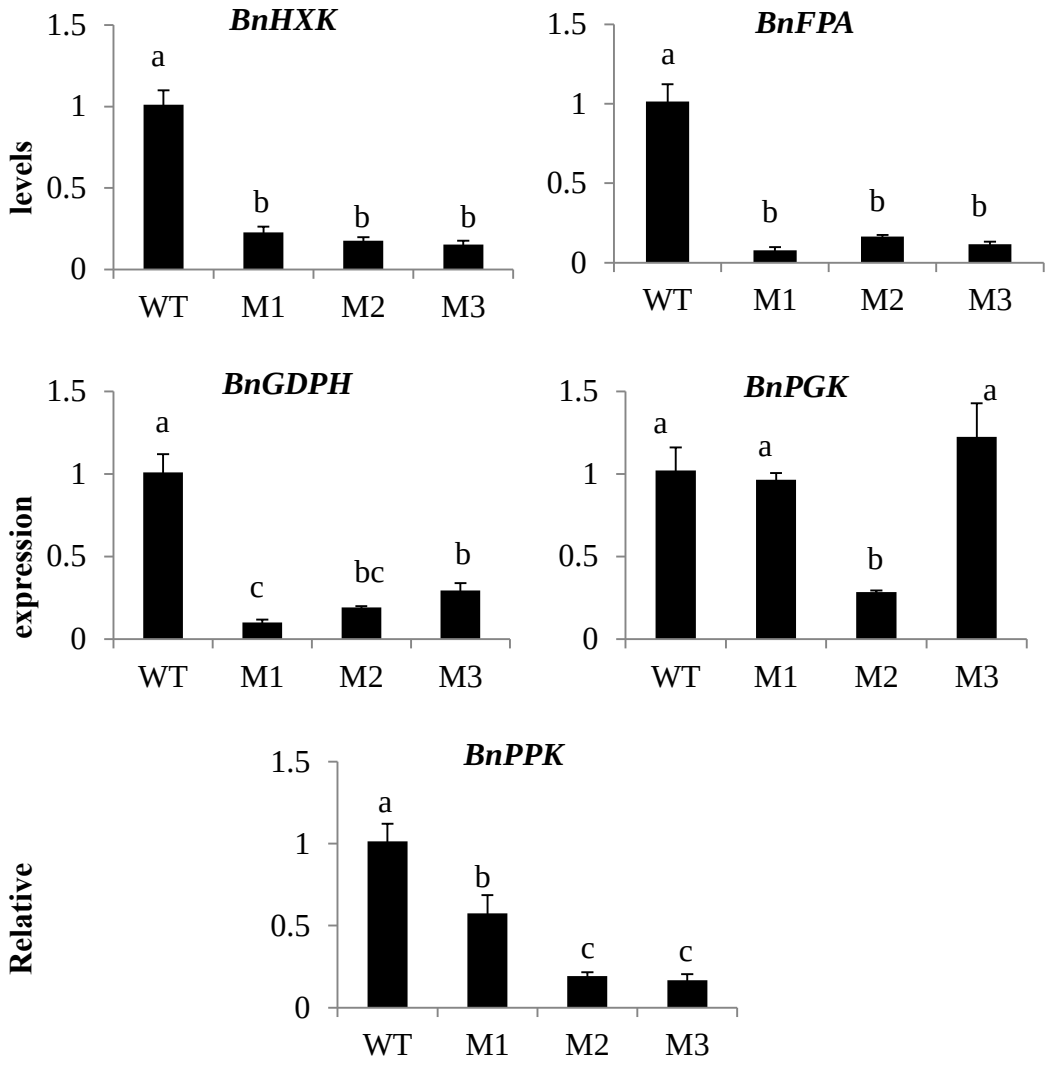

Fig. 4.3 Relative transcript levels of genes involved in glycolysis in dry seeds collected at 35 days after pollination (DAP) fromWT and *B. napus FUS3* mutant (M1-3) plants. Transcript levels were measured for *HEXOSE KINASE* (*BnHXK*), *FRUCTOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), *PHOSPHOGLYCERATE KINASE* (*BnPGK*), and *PYROPHOSPHATASE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*). Values are means ± SE of at least three biological replicates and are normalized to the WT value set at 1. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.

4.4.4 Mutations in *BnFUS3* reduce the expression of genes involved in FA synthesis and modification

Seed oil biosynthesis is regulated by the expression of transcription factors, including *LEC1* and *2*, *ABI3* and *WRI1*, the expression of which correlates positively to the final level of oil accumulated in mature seeds (Meinke et al., 1994; Cernac and Benning, 2004; Harada, 2001). Given the relevance of these transcription factors for oil production, their expression level was measured at difference phases of seed development. Relative to WT seeds, the transcript levels of *BnLEC1*, *BnLEC2*, *BnABI3* and *BnWRI1* were significantly reduced during the development of the three *BnFUSCA3* TILLING mutant lines, especially at 21 and 28 DAP for *BnLEC1* and 2, and *BnWRI1*(Fig. 4.4). A marked and consistent decrease in transcript levels in the mutant lines was also observed for *BnLEC2* and *BnABI3* at 14 DAP. No consistent patterns in the expression of the three transcription factors were observed in seeds of the mutant lines at 35 DAP (Fig. 4.4). In seeds, biosynthesis of FA is regulated by numerous genes, including those involved in condensation, *ACETYL-COA CARBOXYLASE* (*BnACCA2*) and *MALONYL-COA:ACP TRANSACYLASE* (*BnMCAT*); desaturation, *ω-3 FA DESATURASE* (*BnFAD3*); and elongation, *FA ELONGATION1* (*BnFAE1*). Of the two genes involved in FA condensation, the expression level of *BnACCA2* was suppressed in the M1 and M2 mutant lines while the expression of *BnMCAT* was suppressed only in the M2 line (Fig. 4.5). Compared to WT, all three mutant lines had a reduced transcript level for *BnFAD3* which catalyzes the conversion of C18:2 into C18:3. The expression of *BnFAE1*, involved in erucic acid biosynthesis, was only suppressed in the M1 and M2 lines (Fig. 4.5). Expression studies of five representative *Brassica OLEOSIN* genes (Jolivet et al., 2009) revealed suppression of *OLEOSIN S-5* in all *BnFUS3* mutants.

The expression level of *OLEOSIN S-2* was reduced in M1 and M2 and S-3 expression was reduced only in M1 (Supplemental Fig. 4.8).

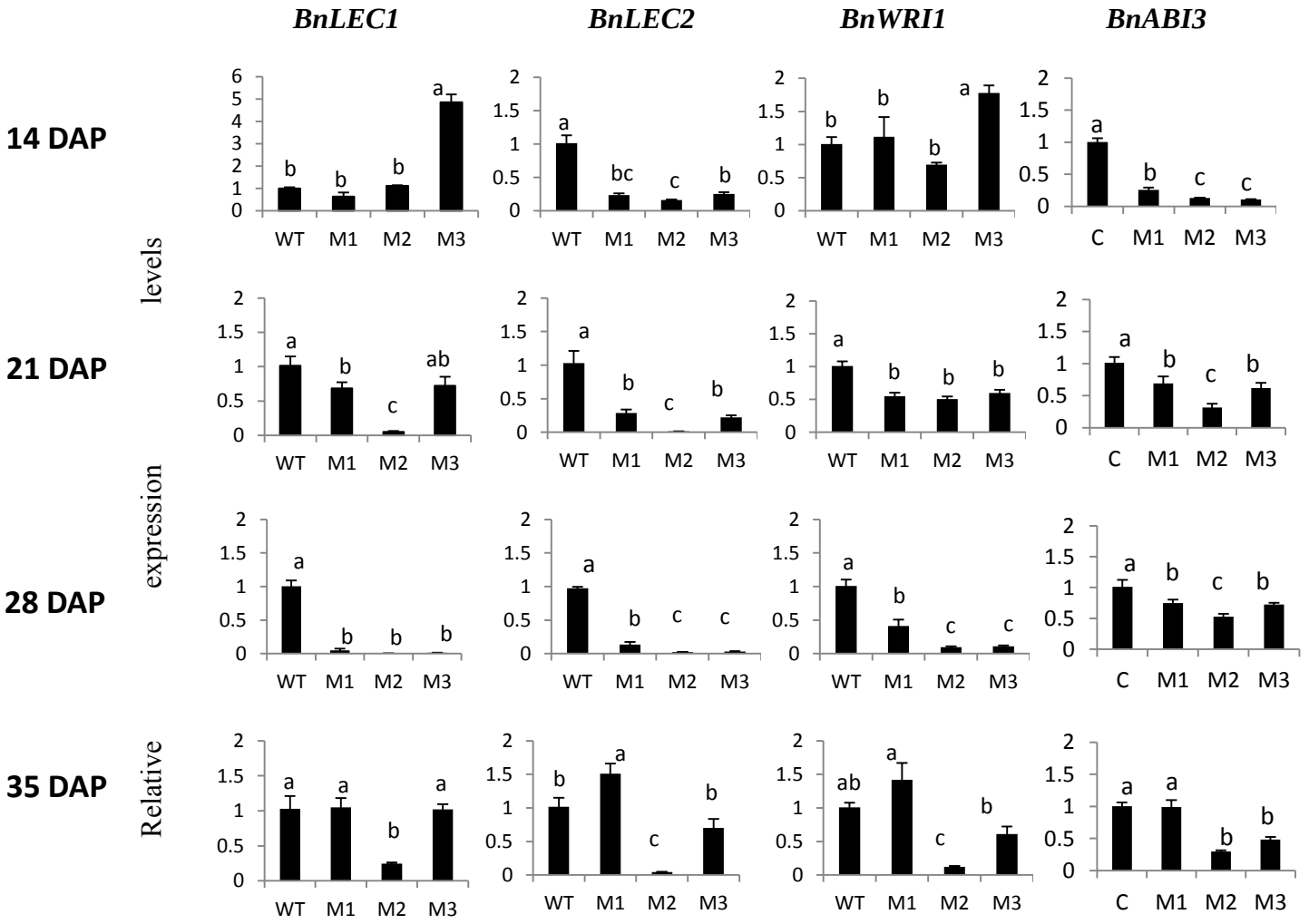

Fig. 4.4 Relative transcript levels of transcription factors participating in oil synthesis in developing seeds collected at 14, 21, 28, and 35 days after pollination (DAP) from WT and *B. napus FUS3* mutant (M1-3) plants. Transcript levels were measured for *LEAFY COTYLEDON1* and *2* (*BnLEC1* and *2*), *WRINKLED1* (*BnWRI1*) and *ABSCISIC ACID-INSENSITIVE PROTEIN3* (*BnABI3*). Values are means ± SE of at least three biological replicates and are normalized to the WT value of each specific day set at 1. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.

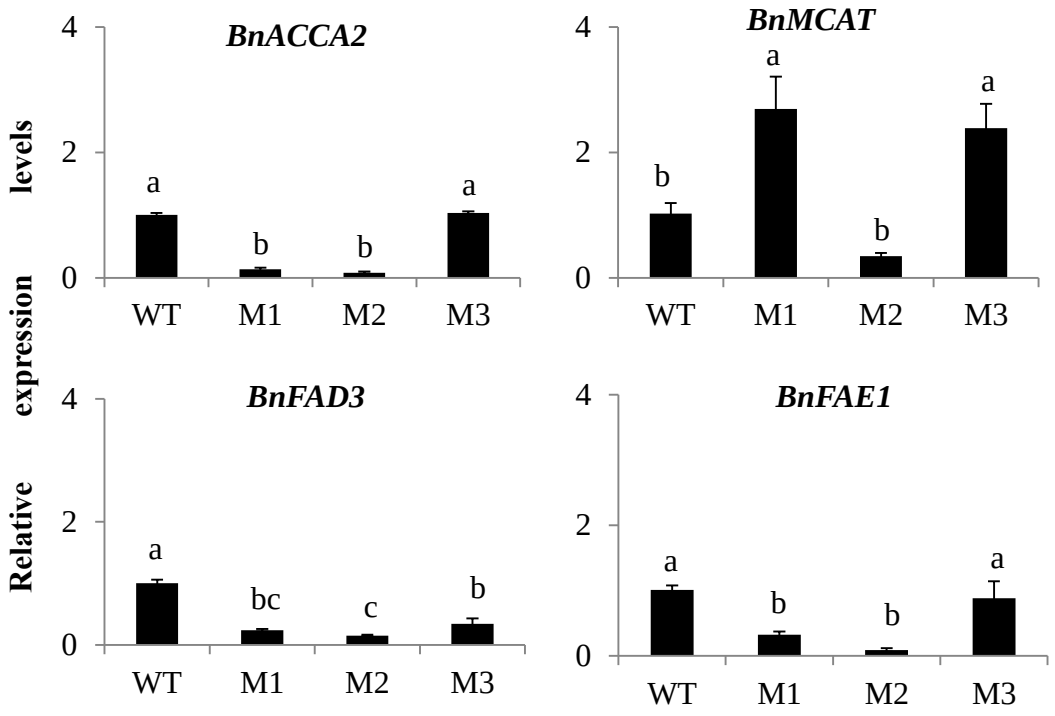

Fig. 4.5 Relative transcript levels of genes involved in fatty acid (FA) synthesis and modification in dry seeds collected at 35 days after pollination (DAP) from WT and *B. napus FUS3* mutant (M1-3) plants. Transcript levels were measured for *ACETYL-COA CARBOXYLASE* (*BnACCA2*), *MALONYL-COA:ACP TRANSACYLASE* (*BnMCAT*), *ω-3 FA DESATURASE* (*BnFAD3*), and *FA ELONGATION1* (*BnFAE1*). Values are means ± SE of at least three biological replicates and are normalized to the WT value set at 1. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.

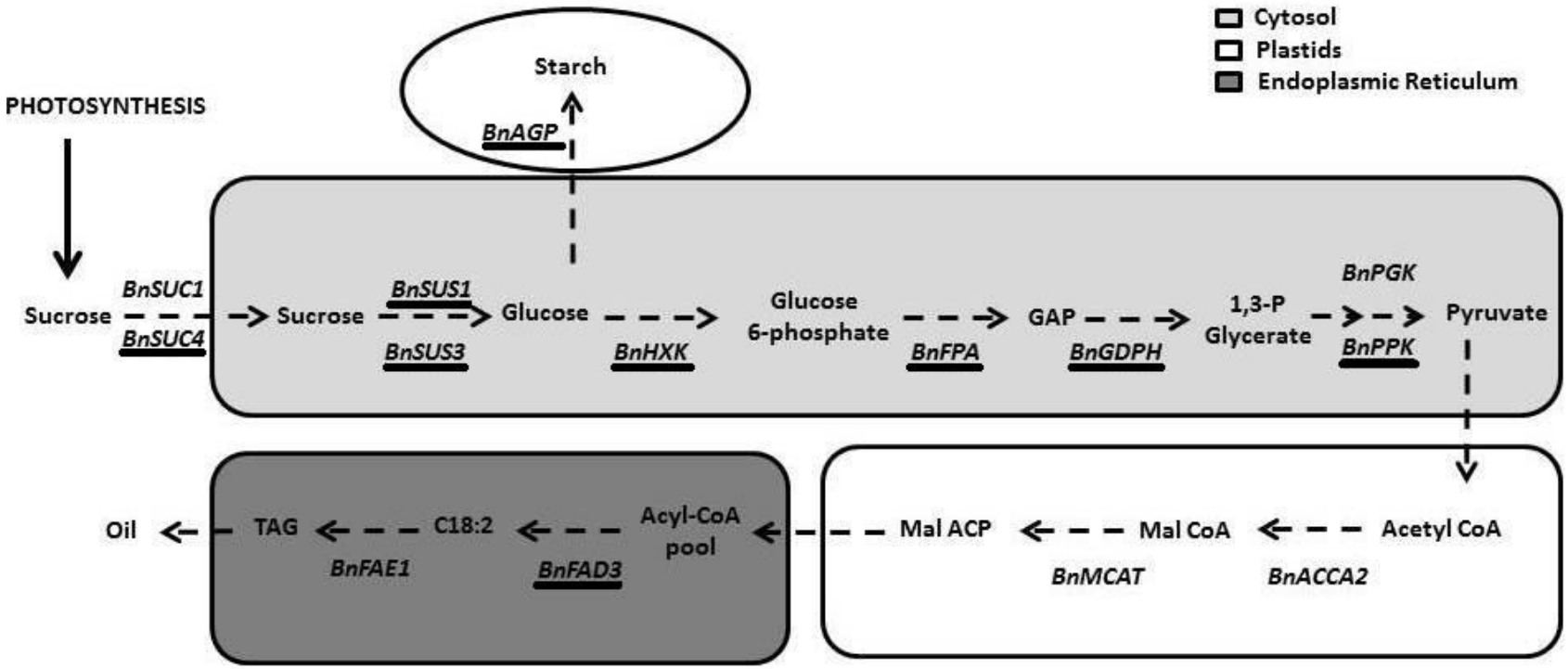

Fig. 4.6 Schematic diagram showing the carbon flow from photosynthesis, through the glycolytic pathway, to the synthesis of FA. Underlined enzymes are those transcriptionally repressed in all three *BnFUS3* mutant lines. Cellular compartments are also indicated. GAP, glyceradehyde-1, 3 bisphosphate, TAG, triacyl glycerol.

4.5 Discussion

Production of *B. napus* oil, used extensively as a food source in addition to use in industrial applications, is tightly associated to the genetic potential of selected cultivars. Biosynthesis of FA in plants occurs in several steps requiring the coordinated activity of developmentally and environmentally regulated enzymes, and the expression of several transcription factors including *LEC1*, *LEC2* and *FUS3*, which are key regulators of zygotic embryogenesis (Meinke et al., 1994; Gazzarrini et al., 2004). Loss of embryo identity, failure to accumulate storage products, and precocious initiation of germination are observed in Arabidopsis *lec1*, *lec2*, and *fus3* plants (reviewed by Harada, 2001). Like *LEC2*, *FUS3* is a member of a unique family of transcription factors characterized by a B3 domain that binds the Sph/RY (CATGCA) core element found in promoters of many seed and embryo-specific genes, and operate in conjunction with ABA responsive elements (Gazzarrini et al., 2004; Wang and Perry, 2013). Interaction between these elements is confirmed by the role played by *FUS3* in the late phases of embryo and seed development characterized by elevated levels of ABA. A putative function of *FUS3* is to regulate the temporal and spatial synthesis of ABA (Gazzarrini et al., 2004). While the requirement of *FUS3* for seed oil accumulation has been demonstrated in Arabidopsis where suppression of this gene compromises lipid accumulation (Wang et al., 2007), no information is available on the role played by *FUS3* on the economically important species *B. napus*. Furthermore, it is unclear whether the depression in oil level observed in *fus3* Arabidopsis lines (Meinke et al., 1994; Curaba et al., 2004; Vicient et al., 2000) is related to transcriptional changes in metabolic pathways upstream of FA synthesis. To address these issues, we generated *B. napus FUS3* mutants using TILLING, a technology providing a range of missense alleles of different severity for the gene of interest (Gilchrist et al., 2013). All three homozygous *BnFUS3* mutant lines obtained were characterized by single amino acid changes downstream of the B3

domain (Supplemental Fig. 4.3). These mutants developed phenotypic abnormalities characteristic of the suppression of *FUS3*. As documented in Arabidopsis *fus3* plants (Meinke et al., 1994; Harada, 2001), the *B. napus FUS3* TILLING lines produced seeds with a reddish purple coloration (Supplemental Fig. 4.5), most likely ascribed to the over-production of anthocyanins (Meinke et al., 1994). *FUSCA* genes are negative regulators of light responses, some of which rely on the biosynthesis of anthocyanins (Miséra et al., 1994). The reduction in silique number observed in our *BnFUS3* mutant lines (Table 4.1) is in line with the role played by this gene during reproductive development and its preferential expression in the siliques (Luerßen et al., 1998). The decrease in seed oil content observed in the three *B. napus* TILLING lines (Fig. 4.1) is consistent with the requirement of *FUS3* for oil synthesis (Mu et al., 2008; Stone et al., 2008). Transcriptome studies in Arabidopsis reveal that the increased expression of *FUS3* during seed development coincides with the expression of several plastidial FA biosynthetic genes (Wang et al., 2007). Using transgenic Arabidopsis plants expressing a dexamethasone (DEX)- inducible *FUS3* it was shown that transient expression of this gene is sufficient to elevate FA synthesis (Wang et al., 2007). Seeds of the *BnFUS3* lines also had higher protein content, supporting the negative correlations between oil and protein accumulation (Hao et al., 2004). The decreased oil level in the *BnFUS3* mutant lines was accompanied by a reduction in the expression of *BnLEC1*, *BnLEC2*, *BnABI3* and *BnWRI1*, especially between 21 and 28 DAP (Fig. 4.4), a phase characterized by active deposition of oil (Fowler and Downey, 1970) and by an increase in expression of *BnFUS3* in WT seeds (Supplemental Fig. 4.9). In Arabidopsis, the interaction among these genes in the regulation of embryo development and seed storage accumulation is complicated and possibly relies on feed-back regulatory mechanisms. *LEC1*, *LEC2*, *ABI3* and *WRI1* influence the production and accumulation of storage

proteins during seed development (Santos-mendoza et al., 2008; Mu et al., 2008). The suppression of the Arabidopsis *LEC1*, *2* or *WRI1* has been linked to a reduction in oil level (Meinke et al., 1994; Cernac and Benning, 2004), and *WRI1*, a transcription factor encoding an APETALA2/ ethylene responsive element-binding protein (Baud et al., 2007), is a direct target of *LEC2* and possibly *FUS3*. Expression of *WRI1* plays an important function in the regulation of glycolysis and lipid metabolism (Cernac and Benning, 2004) and its repression in the mutant lines prompted us to measure the transcript levels of genes involved in pathways upstream of oil synthesis. A tight link exists between carbon metabolism and oil production as photosynthesis provides carbon skeletons for the synthesis of FA (Rawsthorne, 2002). In *B. napus*, photoassimilation occurs actively in the tissue of the silique walls, and the sucrose produced is rapidly transported in the developing seeds by specific transporters. Within the seeds, sucrose is converted to hexosephosphates by two different routes i.e., invertase or Suc synthase (da Silva et al., 1997). The hexose-phosphates then move in the plastid through a Glc6P/Pi-translocator (da Silva et al., 1997). Within the plastid, Glc6P is processed to Glc1P by phosphoglucomutase (Periappuram et al., 2000) followed by the ATP-dependent transformation of Glc1P to ADPGlc by ADPGlc pyrophosphorylase (AGPase) following integration of the Glc moiety into the starch granule by starch synthases (da Silva et al., 1997). While variations in the expression levels of genes encoding enzymes of sucrose metabolism and glycolysis are apparent among the mutant lines, possibly as a result to the range of the missense alleles of different severity obtained by TILLING, a consistent repression was observed for many. For sucrose metabolism, the transcript levels of the sucrose transporter *BnSUC4*, *sucrose synthase BnSUS1* and *3*, and *BnAGP* were significantly reduced in the mutant *BnFUS3* seeds (Fig. 4.2). Repression of *BnAGP* is of particular interest as the expression of this gene is related to the accumulation of starch and lipids

in the seed (Vigeolas et al., 2004). The function of *BnAGP* is to form ADP glucose from Glucose 1-phosphate, which is then incorporated into starch (Vigeolas et al., 2004). A decreased level of starch and lipids occurs in seeds suppressing *AGP* (Vigeolas et al., 2004). The expression of major glycolytic enzymes: *BnHXK*, *BnFPA*, *BnGDPH*, and *BnPPK* were also consistently suppressed in seeds of the three *BnFUS3* mutant lines (Fig. 4.3). High expression levels of these genes, observed under conditions of elevated seed oil synthesis (Elhiti et al., 2012), are associated to an active glycolytic pathway producing substrates for the biosynthesis of FA (Elhiti et al., 2012). Therefore, these distinct expression patterns suggest a compromised photo assimilation capacity and decreased glycolytic activity in the three mutant lines; factors which might explain the reduced levels of oil observed in their seeds. Besides affecting the transcription of genes operating upstream of FA synthesis, suppression of *BnFUS3* results in a significant reduction in expression of *BnFAD3* which is consistent among lines (Fig. 4.5). The activity of *FAD3* is required for the production of unsaturated FA and the conversion of linoleic (18:2) to linolenic (18:3) acid (Hamada et al., 1998). Therefore its reduced expression might explain the higher percent composition of linoleic (18:2) acid observed in the *BnFUS3* mutant lines. This observation shows the potential of *BnFUS3* to influence the oil quality which may increase the economic value of plants modified to serve unique nutritional, pharmaceutical or industrial applications.

In conclusion, mutations in *BnFUS3* outside of the B3 domain, result in reduced levels of seed oil that are related to the transcriptional suppression of specific genes encoding key enzymes of pathways upstream of FA synthesis (Fig. 4.6). The selective downregulation of enzymes participating in sucrose transport and metabolism, as well as glycolysis, is an indication of a diminished carbon pool available for the synthesis of FA and ultimately oil production.

Based on these observations, it is therefore suggested that targeted manipulations of *FUS3* can be used as a tool to influence oil accumulation in the economically important species *B. napus*.

4.6 Acknowledgment

We gratefully acknowledge George Haughn and Erin Gilchrist for the TILLING work. This work was supported by an Agriculture, Food and Rural Development grant. The technical assistance of Mr. Doug Durnin and Mr. Ralph Kowatsch is also acknowledged.

4.7 Supplemental data

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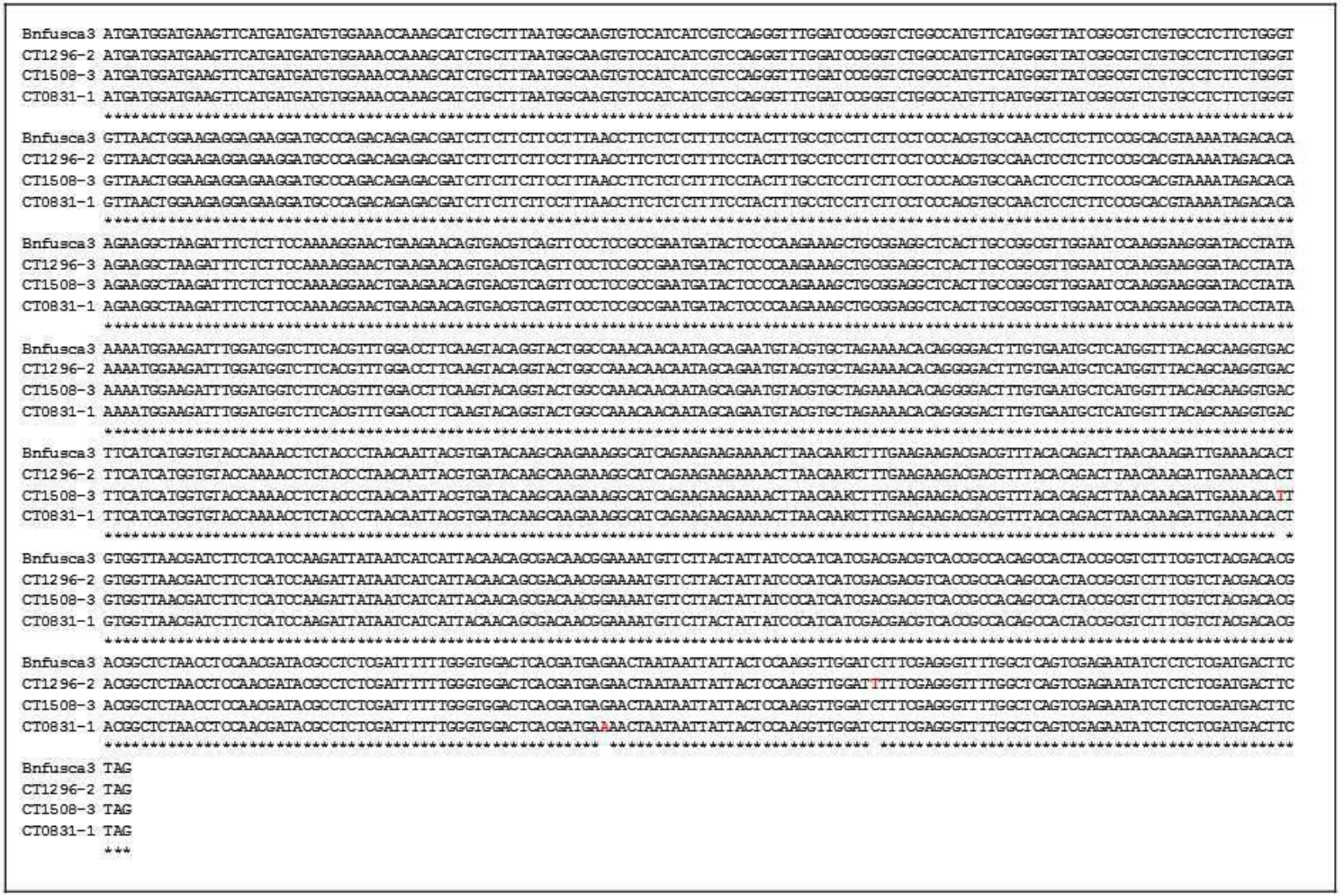
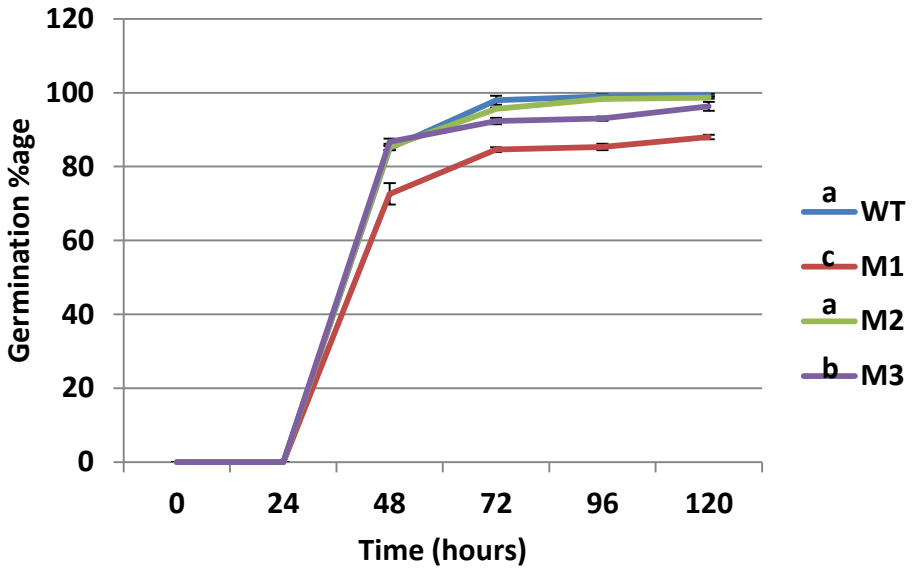
Supplemental Fig. 4.1 Nucleotide sequence of *BnFusca3* (HM370540). Untranslated regions are highlighted in yellow.

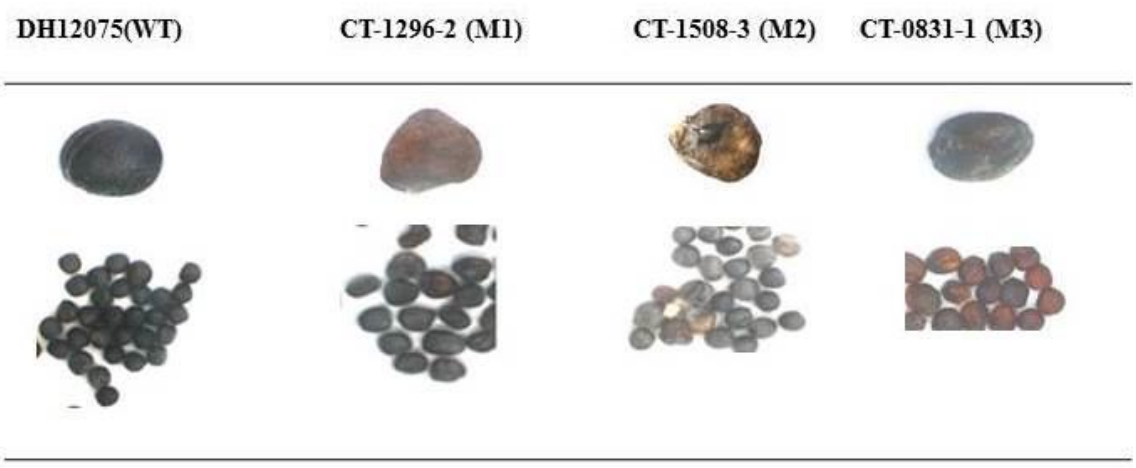


Supplemental Fig. 4.2 Nucleotide alignment comparison between *BnFusca3* and the three M1 (CT1296-2), M2 (CT1508-3), M3 (CT0831-1) mutant lines * indicate identical nucleotides. Nucleotide substitutions in the mutants are in red.

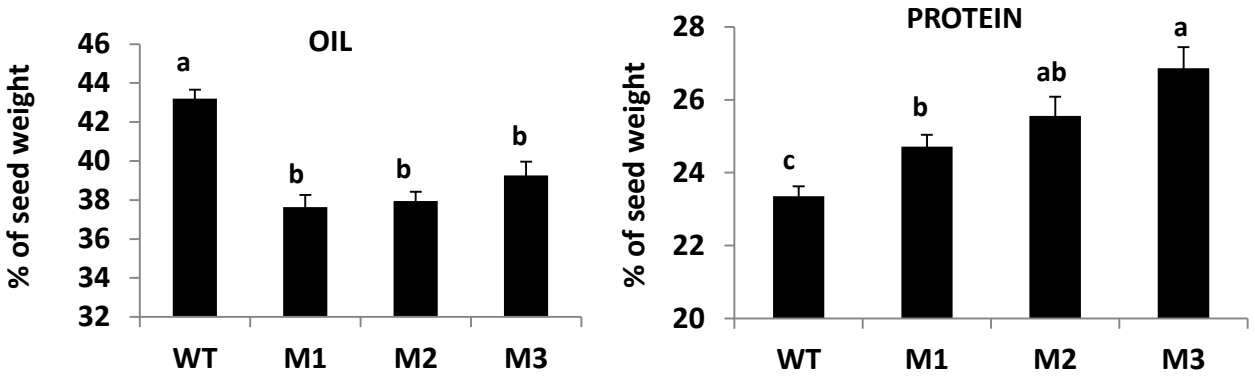
Supplemental Fig. 4.3 Amino acid comparison between *BnFusca3* and the three M1 (CT1296-2), M2 (CT1508-3), and M3 (CT0831-1) mutant lines. + indicate identical nucleotides. Amino acid substitutions in the mutants are in red. The B3 domain is highlighted in blue.



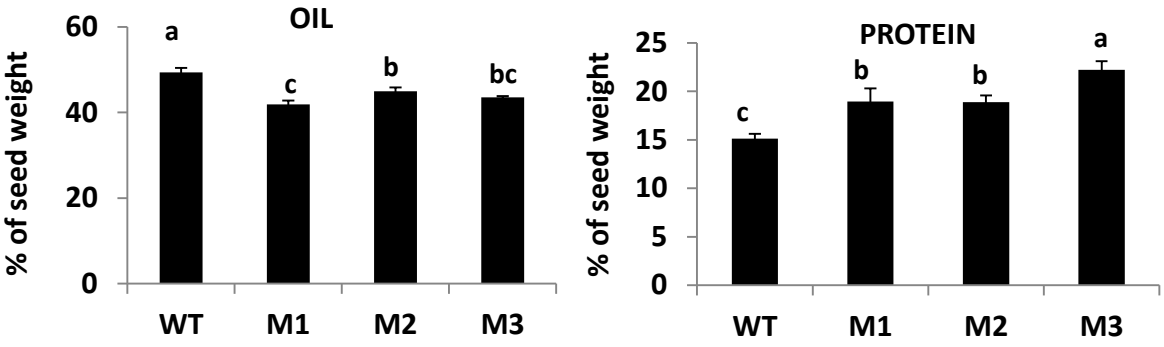
Supplemental Fig. 4.4 Germination percentage of the wild type (WT) and *BnFUSCA3* TILLING mutant lines (M1-3). WT (control) and *BnFUSCA3* mutant seeds were sown on an agar plate, and then placed in a tissue culture room (0 h in the graph). The germination percentage was examined at the time points as specified. The experiment was repeated three times ($n \geq 100$ for each sample), and the data presented are mean values of three experiments. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.



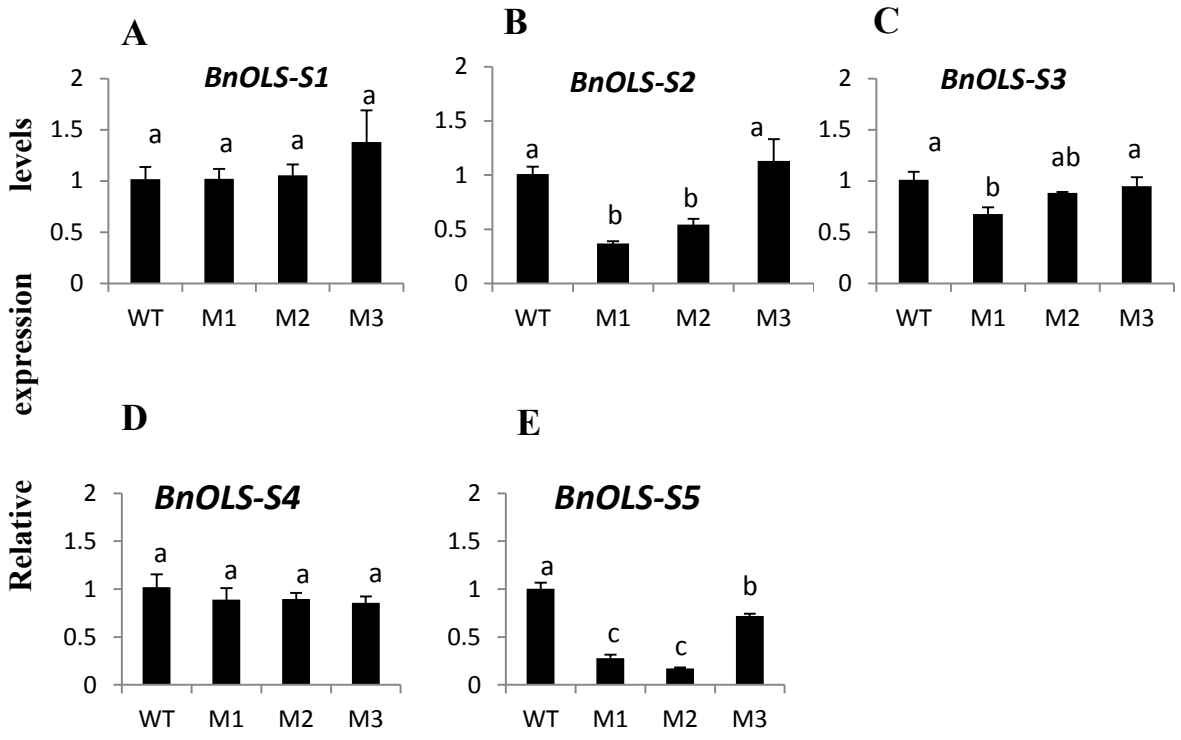
Supplemental Fig. 4.5 Seed morphology of DH12075 (WT) and *BnFUS3* TILLING mutants, M1 (CT1296), M2 (CT1508), M3 (CT0831).



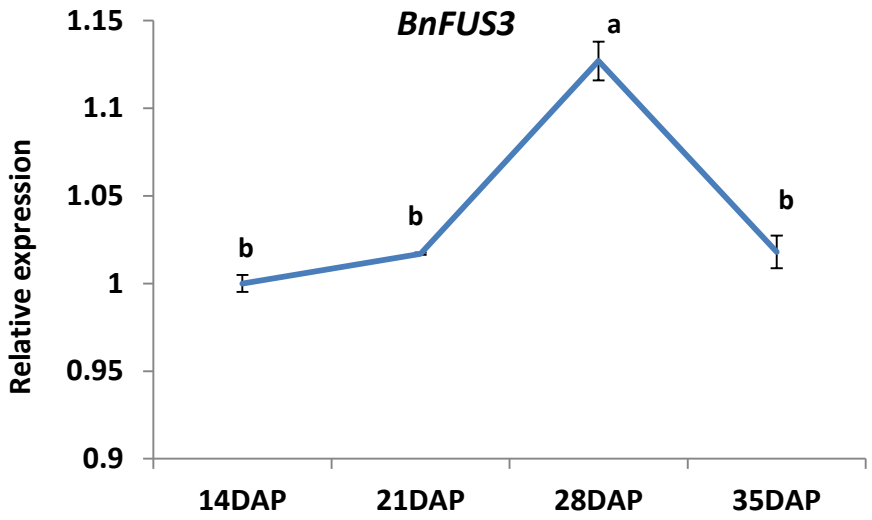
Supplemental Fig. 4.6 Measurements of protein and total oil content in dry seeds of wild type (WT) and *BnFUS3* mutant lines (M1-M3) using Near Infrared Reflectance Spectroscopy. Mature seeds harvested from plants grown under greenhouse conditions from February 2012 to May 2012. Values, expressed as percentage of seed weight (at 0% humidity) ±SE, are means of three biological replicates. Letters indicate statistically significance by LSD tests at $P < 0.05$.



Supplemental Fig. 4.7 Measurements of protein and total oil content in dry seeds of wild type (WT) and *BnFUS3* mutant lines (M1-M3) using Near Infrared Reflectance Spectroscopy. Mature seeds harvested from plants grown in growth cabinets. Values, expressed as percentage of seed weight (at 0% humidity) ±SE, are means of three biological replicates. Letters indicate statistically significance by LSD tests at $P < 0.05$.



Supplemental Fig. 4.8 Relative transcript levels of five representative *OLEOSINS* genes (*A*-*E*) in seeds at 35 days after pollination (DAP) from WT and *B. napus BnFUS3* mutant (M1-3) plants. Values are means ± SE of at least three biological replicates and are normalized to the WT value set at 1. Letters indicate statistically significant differences by LSD tests at $P < 0.05$.



Supplemental Fig. 4.9 Relative transcript levels of *BnFUS3* in developing seeds collected from WT plants at 14, 21, 28, and 35 days after pollination (DAP). Values ± SE are means of three biological replicates and are normalized to the value of 14 DAP set at 1.

Supplemental Table. 4.1: Primer sequence (5'-3') used for genotyping and qRT-PCR studies.

Genotyping

bn36-4R	CGGCTCCCATCTAGCTATGCTTGTC
bn36-2R	GTGAGTCCACCCAAAAAATCGAGAGGC
bn36-5L	GATTCTCTTTATATCTGTGTGGATCCAAGTG

qRT-PCR

BnLEC1-2-F	TATCTTGCCGCAGCAACAACCAAG
BnLEC1-2-R	TTCACCGGTCACGAAGCTGATGTA
BnLEC2-F	ACAAGAATCGCTCGCACTTCTCCA
BnLEC2-R	AAGCATCCGATGAGTGAAGAGGCT
FUS3-F	TCCATCATCGTCCAGGGTTTGGAT
FUS3-R	AAGAAGATCGTCTCTGTCTGGGCA
WRI1-F	ATAGAGTACAGAGGCGCAAACGCA
WRI1-R	TCACAGGGAATGGGAAGACACCTT
BnACCA2-F	TTGCCAGTCATAGTCCAGAGGCAT
BnACCA2-R	TCTGCTGCGATGTCCATGAAGGAT
BnAGP-F	TGCTGGAACGAGATTGTATCCGCT
BnAGP-R	ATGGCGGTTGAGAGAAGCTGAGTT
BnFAD3-F	TATAAGGGCGGCCATTCCTAAGCA
BnFAD3-R	AGATAGCCCAGAACAGGGTTCCTT
BnFAE1-F	TCTCCGCGATGGTCGTTAACACTT
BnFAE1-R	TCCTTGGACAAACTCACTCCGGTT
BnFPA-F	TGAGTTGATCGCTAACGCCGCATA
BnFPA-R	TTCTCGACGTTGATGCTGGCAAGA
BnGPDH-F	ACGGAAAGTTGACCGGAATGTCCT
BnGPDH-R	ACAACATCGTCCTCGGTGTAACCA
BnHXK-F	ATCAGCTGCAGGGATCTACGGAAT
BnHXK-R	ACTCGCTGAACTGAGTGTAGTGCT
BnMACT-F	TCAAGCAGTAGGGATGGGCAAAGA
BnMACT-R	ACGAACACGGAGCAATTCAACAGC
BnPGK-F	AGGCACAAGGTCTGTCTGTTGGAT
BnPGK-R	AGCGAACTTGTCAGCAACCACAAC
BnPPK-F	AGGTGCGCCGTTTAAGAAATTCGC
BnPPK-R	AGAATGAAAGGAAGGCCGGCTACT

BnSUC1-F	ACCAATCGTCGGTTACCACAGTGA
BnSUC1-R	ATATCCGCGGCGTATCCGATCAAA
BrSUC4-F	ACAAGGATTCAGAAGCCCATCCCT
BrSUC4-R	ATCTCCTTCCACCATGCCTTCACA
BrSUS1-F	TCTCCGTGTGCCTTTCAGAACAGA
BrSUS1-R	AGAGGCAACGAGGTTTCCATCACT
BrSUS3-F	TTAAGCAGCTTCTCGGCAACCTCA
BrSUS3-R	CGAACAAGCCGGATGTCAACCTTT
BnATR1-F	TGGCCTTATGGAGGAGTTAGAGGA
BnATR1-R	ATCAGTTACGTCAACCTCAGGCGA
BnMYB28-F	AAGCAATACTCCCGGTCAAGCTCA
BnMYB28-R	ACTGGTGTCCCATCTTTGCTGGTA
BrMAM1-F	AGACCATTGCCAAGACTGTAGGGA
BrMAM1-R	AACCGCCTCGATGTCTCTATGGTT
BnCYP79B2-F	ACTTCACCGTCGGGTAAAGATGCT
BnCYP79B2-R	ACACGTGTTGAGCTGATGGAGTCT
BnSUR1-F	TGGCTTCACCAGAAGAGAGCTGAA
BnSUR1-R	ACAGTAATGCCTCGTGACAAACCG
BnST5a-F	ATGGCTGCTCGTATCGATGGGTTA
BnST5a-R	CCTCTGTTCCGCACCAAACAACAA
BnCYP83B1-F	AAAGATGGACGTCATGACCGGACT
BnCYP83B1-R	CATCACGCCTGATCAAATGTGCGT
BnUBC21-F	CCTCTGCAGCCTCCTCAAGT
BnUBC21-R	CATATCTCCCCTGTCTTGAAATGC

oleosin S1-1F	ACAAAGGGTCATACTGCCACAGGA
oleosin S1-1R	AGAACCACAACAGGCGACTCTCTT
oleosin S2-1F	AAGAGGCGTATGGCTGATGCAGTA
oleosin S2-1R	ATGGGCCCTCCTTGTCTTACCATT
oleosin S3-1F	CTGGTGGGTTTGGCATTGCAGATA
oleosin S3-1R	TTTGGTTCCCAGCTTCATCCTTGC
oleosin S4-1F	TTGGCGGGTTCGGTGATAGGTTTA
oleosin S4-1R	TTAGACCGAACATTCCAGAGGCCA
oleosin S5-1F	TCATCATCACTGGGTTCCTTGCCT
oleosin S5-1R	TGCTTGTTGGTGTTGCTGATGGAC
BnABI3-F	TACCAACACTCCCTGACTTCCCAT
BnABI3-R	AGTTGAGGAAGAAGCAGCGGAAGA

Supplemental Table 4.2: Analysis of agronomic traits in the wild type (WT) and *BnFUSCA3* TILLING mutant lines (M1-3) grown in the greenhouse from February 2012 to May 2012. Values ± SE are means of ten biological replicates. Uppercase letters indicate LSD grouping used to determine significant differences (LSD: $P < 0.05$).

Line	Plant height (cm)	Siliques per plant	Seeds per silique	Silique length (cm)	1000- seed weight (g)	Yield per plant (g seeds\plant)
WT	72.8±1.06	45.6±1.07[a]	19.5±0.52	5.44±0.06	3.24±0.02	2±0.03[a]
M1	74.5±0.27	37.6±0.52[b]	18.7±0.52	5.48±0.06	3.22±0.07	1.66±0.05[b]
M2	74.4±0.64	33.2±.92[c]	18.6±0.45	5.28±0.05	3.034±0.05	1.63±0.08[b]
M3	73.1±0.98	28±0.79[d]	18.4±0.48	5.3±0.06	3.024±0.06	1.14±0.04[c]

Supplemental Table 4.3: Analysis of major agronomic traits in the wild type (WT) and *BnFUS3* TILLING mutant lines (M1-3) growth chamber with 16/8 hours day/night photoperiod, light intensity of 400 μmol m−2 s−1 and day/night temperature of 20°C /15 °C Values ± SE are means of ten biological replicates. Uppercase letters indicate LSD grouping used to determine significant differences (LSD: $P < 0.05$).

LINE	Plant height (cm)	Siliques per plant	Seeds per silique	Silique length(cm)	1000- seed weight(g)	Yield per plant (g seeds\plant)
WT	62.6±0.69	32.8±1.27^{a}	20.2±0.2	5.52±0.04	3.01±0.05^{a}	1.89± 0.04^{a}
M1	60.5±0.78	27.3±0.89^{b}	19.3±0.3	5.49±0.05	3.02±0.02^{a}	1.22±0.04^{c}
M2	60.8±0.81	26.9±1.03^{b}	19.2±0.61	5.44±0.02	2.88±0.04^{b}	1.729±0.06^{b}
M3	60.5±0.70	26.8±1.79^{b}	19.4±0.52	5.44±0.04	2.90±0.06^{ab}	1.29±0.07^{c}

Supplemental Table 4.4: Relative fatty acid contents (% dry weight) in seeds of the wild type (WT) and *BnFUS3* mutant (M1-3) lines. Mature seeds were harvested from plants grown under greenhouse conditions from February 2012 to May 2012. Values ± SE are means of three biological replicates. Uppercase letters indicate LSD Grouping used to determine significant differences (LSD: $P < 0.05$).

	WT	M1	M2	M3
C16:0	3.47±0.02^{a}	3.88±0.04^{a}	3.77±0.23^{a}	3.75±0.11^{a}
C16:1	0.24±0.00^{a}	0.23±0.01^{ab}	0.23±0.02^{ab}	0.19±0.01^{b}
C18:0	1.25±0.03^{c}	1.69±0.07^{a}	1.63±0.19^{ab}	1.27±0.09^{bc}
C18:1	63.82±0.23^{a}	62.79±0.19^{a}	56.89±1.45^{b}	61.87±0.07^{a}
C18:2	16.22±0.15^{c}	18.02±0.22^{b}	22.14±0.78^{a}	18.32±0.18^{b}
C18:3	12.26±0.17^{a}	10.25±0.25^{b}	12.16±0.73^{a}	12.03±0.49^{a}
C20:0	0.54±0.01^{b}	0.76±0.03^{a}	0.71±0.09^{ab}	0.57±0.05^{b}
C20:1	1.49±0.01^{ab}	1.43±0.01^{b}	1.51±0.03^{a}	1.52±0.02^{a}
C22:0	0.35±0.01^{c}	0.52±0.02^{a}	0.48±0.07^{ab}	0.36±0.03^{bc}

Supplemental Table 4.5 Relative fatty acid contents (% dry weight) in seeds of the wild type (WT) and *BnFUS3* mutant lines. Mature seeds were harvested from plants grown in growth cabinets. Values ± SE are means of three biological replicates. Uppercase letters indicate LSD grouping used to determine significant differences (LSD: $P < 0.05$).

	WT	M1	M2	M3
C16:0	3.76±0.01^{b}	3.97±0.06^{a}	3.42±0.07^{c}	3.82±0.04^{ab}
C16:1	0.18±0.01^{a}	0.19±0.01^{a}	0.19±0.02^{a}	0.18± 0.02^{a}
C18:0	1.71±0.09^{ab}	1.83±0.07^{a}	1.74±0.03^{ab}	1.57±0.07^{b}
C18:1	68.62±0.66^{a}	66.87±0.47^{ab}	65.77±0.52^{b}	66.87±0.49^{ab}
C18:2	14.00±0.41^{c}	15.24±0.38^{b}	16.73±0.49^{a}	15.45±0.33^{ab}
C18:3	8.87±0.46^{a}	8.95±0.47^{a}	9.27±0.46^{a}	9.22±0.56^{a}
C20:0	0.67±0.05^{a}	0.75±0.03^{a}	0.67±0.01^{a}	0.66±0.03^{a}
C20:1	1.21±0.05^{a}	1.22±0.06^{a}	1.33±0.02^{a}	1.33±0.02^{a}
C22:0	0.39±0.02^{b}	0.47±0.02^{a}	0.39±0.02^{b}	0.39±0.03^{b}

5 CHAPTER THREE: Effects of altered expression of *LEAFY COTYLEDON1* and *FUSCA3* on microspore-derived embryogenesis of *Brassica napus* L.

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Contributions

Drs. Duncan and Stasolla designed the experiments, helped with the interpretation of data and contributed writing the manuscript. Mrs. Elhai conducted the experiments and wrote the manuscript.

5.1 Abstract

Brassica napus (*Bn*) microspore-derived embryogenesis has become a model system to study basic aspects of plant development. Recognized transcription factors governing embryogenesis include: *FUSCA3* (*FUS3*), a member of the plant-specific B3-domain family, and *LEAFY COTYLEDON1* (*LEC1*), a member of the HAP3 subunit of the CCAAT binding factor family. The effects of altered expression of both genes were investigated during microspore-derived embryogenesis in established *B. napus* lines over-expressing or down-regulating *BnLEC1*, as well as in tilling lines where *BnFUS3* was mutated. While over-expression of *BnLEC1* decreases the yield of microspore-derived embryos (MDEs) without affecting their ability to regenerate plants, suppression of *BnLEC1* or *BnFUS3* reduced both embryo number and regeneration frequency. Embryos produced by these lines showed structural abnormalities accompanied by alterations in the expression of several embryogenesis-marker genes. Oil accumulation was also altered in the transgenic MDEs. Total oil content was increased in MDEs over-expressing *BnLEC1* and decreased in those suppressing *BnLEC1* or *BnFUS3*. Mutation of *BnFUS3* also resulted in a small but significant increase in linoleic (C18:2) acid. Together this study demonstrates the crucial role of *BnLEC1* and *BnFUS3* during *in vitro* embryogenesis.

Keywords: *Brassica napus*, *BnLEC1*, *BnFUSCA3*, embryo development, microspore-derived embryogenesis, oil content.

5.2 Introduction

Embryogenesis is one of the most important events in the life cycle of plants. The process begins with the double fertilization marking the formation of the zygote and the endosperm. During embryogenesis, the zygote divides producing characteristic embryogenic stages (globular, heart-shaped, and torpedo-shaped) that are accompanied by profound molecular, physiological, and metabolic changes (Willemsen et al., 2004). During the middle-late stages of embryogenesis, the embryos accumulate storage products and undergo desiccation prior to entering a dormant period (Harada, 1997; Bewley, 1997). Most of these events are also observed during *in vitro* embryogenesis, where embryos can be produced without fertilization. *In vitro* produced embryos proceed through a similar developmental pathway characteristic of seed embryos and are therefore utilized as a model system (Nogler, 1984)]. Studies using *in vitro* embryos have some advantages: the embryos develop in the absence of maternal tissue, can be produced in high numbers and in a synchronous fashion, and can be easily harvested. Due to the development and optimization of propagation protocols (Gatica et al., 2007; Thorpe and Stasolla, 2001), investigations on *in vitro* plant embryogenesis have grown exponentially over the past few years.

In vitro embryogenesis can be executed through different methods, with gametophytic embryogenesis being routinely used in many species. Gametophytic embryogenesis involves the utilization of microspores (or immature pollen grains) and precise culture treatments to induce embryo formation, i.e., microspore-derived embryos (MDEs, Fig. 5.1). The process uses several types of stress which repress the gametophytic pathway in favor of the embryogenic pathway (Deepak et al., 2012; Lichter, 1982; Nic-Can et al., 2015; Shariatpanahi et al., 2006; Taylor et al., 1990; Wang et al., 2007; Yang and Zhang, 2010).

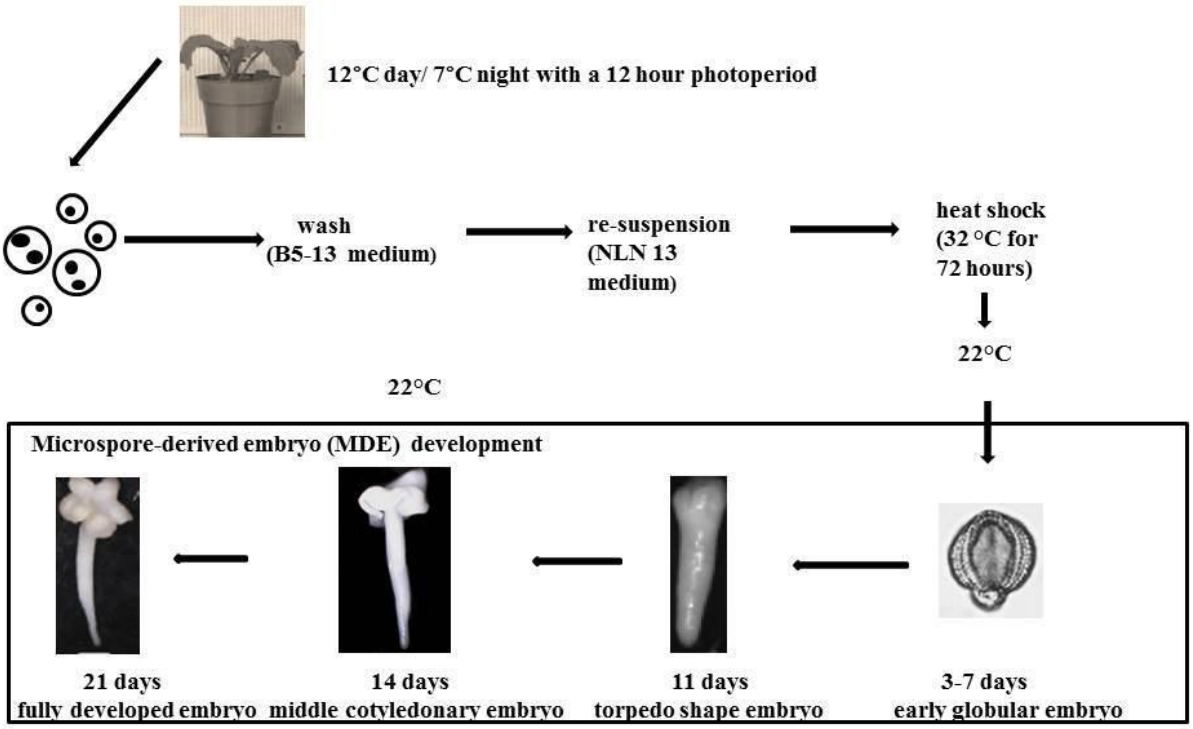


Fig. 5.1 Schematic diagram of microspore-derived embryo (MDE) development in *Brassica napus*. Plants with young buds were grown in cabinets set at 12 °C day / 7 °C night with a 12 h photoperiod. Flower buds (2-3 mm in length) were harvested, sterilized, and ground in a mortar with half strength B5-13 medium supplemented with 13 % (w/v) sucrose. The homogenate was centrifuged at 750 rpm (g) at 4 °C for 3 min and this process was repeated three times. The microspore-containing pellet was thereafter re-suspended and diluted in NLN-13 medium with 13 % sucrose (pH 5.8) to a concentration of 10,000 microspores/ml. Embryo development was triggered after an initial heat shock treatment at 32 °C for 72 h. Embryos were subsequently incubated at 22 °C on a shaker set at 80 rpm. The number below each developmental stage of MDEs shows the days in culture.

Microspore-derived embryogenesis is largely used to propagate *Brassica napus* L. (canola), an economically important species used for oil production. Oilseed rape (canola/rapeseed) oil is the third most important vegetable oil in the world (USDA, 2015). The production of canola oil relies on the genetic potential of canola cultivars to produce high seed yield and high seed oil content. The quality of canola seed oil is determined by the fatty acid (FA) composition. The process of FA biosynthesis during seed maturation is genetically controlled, and requires the synchronization of several biochemical pathways. Fatty acids and triacylglycerols (TAGs) accumulate during embryo and seed maturation (Baud et al., 2008; Braybrook and Harada, 2008), making this stage crucial when attempting to increase seed oil content. Independent studies have shown that FA biosynthesis is controlled by the expression of several transcription factors, including *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *FUSCA3* (*FUS3*), *WRINKLED1* (*WRI1*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*), which interact to regulate different phases of embryo development and seed maturation (Gazzarrini et al., 2004; Meinke et al., 1994; Stone et al., 2001). Many of these genes have critical roles during embryo development (Lotan et al., 1998). *LEC1* is expressed throughout the entire process of embryogenesis, from the initial to the late developmental stages (West et al., 1994), while *FUS3* is responsible for inducing the maturation phase (Curaba et al., 2004; Santos-Mendoza et al., 2008). The *LEC1* protein has the HAP3 subunit of the CCAAT binding factor that allows *LEC1* to be a specific transcriptional regulator of downstream genes containing the CCAAT recognition domain.

FUS3, encoding a B3 protein that accumulates mainly during seed maturation, binds to the RY element CATGCA found in the promoters of several genes (Curaba et al., 2004; Santos-Mendoza et al., 2008). Current literature indicates that ectopic expression of *LEC1* is sufficient

to induce somatic embryogenesis from vegetative tissue; thus, suggesting a role in regulation of embryogenic competence (Lotan et al., 1998; Stone et al., 2001; Braybrook and Harada 2008). *Arabidopsis* plants with a null *lec1* allele produced abnormal embryos characterized by small hypocotyls and cotyledons (Meinke et al., 1994). *Arabidopsis fus3* and *lec1* mutant plants also show a decrease in protein and lipid accumulation during seed development (Meinke et al., 1994; Harada 2001). As synthesis and storage of oil is linked to several stages of embryo and seed development, it has been suggested that the genetic regulation of embryo morphogenesis and maturation influences oil production. In our previous studies, we demonstrated that over-expression of *BnLEC1* increases seed oil accumulation in *B. napus*, while suppression of *BnLEC1* or *BnFUS3* decreases oil content (Elahi et al., 2015; Elahi et al., 2016). While these studies suggest a clear involvement of these genes during *in vivo* embryogenesis, little information is available regarding *in vitro* embryogenesis.

Assessing whether oil production and fatty acid composition during *in vitro* embryogenesis are under the control of similar genes and regulatory mechanisms operating during *in vivo* embryogenesis could have significant applications. This discovery would allow plant scientists to utilize the *in vitro* embryogenesis system as a model to study oil biosynthesis, as well as rapidly screen for desirable oil-related traits or manipulate oil production in culture prior to plant regeneration. This knowledge could save time and resources and has the potential to make significant advances in plant-based oils research. Another important factor to consider when exploiting the advantages of *in vitro* embryogenic systems is whether the introduction of the desirable trait has pleiotropic effects compromising the number of embryos produced and their quality, i.e., their ability to regenerate viable plants, as both are key parameters for propagation.

In line with these considerations, the purpose of the present study is to determine how altered expression of *BnLEC1* and *BnFUS3* influences *B. napus* microspore-derived embryogenesis, with emphasis on morphological characteristics determining embryo quantity and quality, as well as oil accumulation and fatty acid profile. The results obtained will then be compared to previous studies to assess similarities or differences between *in vivo* and *in vitro* systems.

5.3 Materials and methods

5.3.1 Plant material

Transgenic canola plants over-expressing (*B. napus* var. Polo) or down-regulating (*B. napus* var. Topas) *BnLEC1*, as well as tilling mutant lines (*B. napus* var. DH12075) suppressing *BnFUS3* were generated and characterized in previous studies (Elahi et al., 2015; Elahi et al., 2016). Two versions of *BnLEC1* were used in those studies: version A (GU945399) and version B (GU945398), which differ by 13 nucleotides (Supplementary Fig.1 in Elahi et al., 2016) and 4 amino acids (Supplementary Fig. 2 in Elahi et al., 2016). For the present work we used two lines over-expressing version A of *BnLEC1* (lines S1 and S2), two lines over-expressing version B of *BnLEC1* (lines S3 and S4), and two lines down-regulating version B (A1, and A2). Given the high similarity in nucleotide sequence of the two versions, anti-sensing version A also suppressed version B (Elahi et al., 2016).

Three *B. napus BnFUSCA3* tilling mutant lines [M1-3] with point mutations changing one amino acid downstream of the B-3 DNA binding domain of *FUS3* were generated and characterized in previous work (Elahi et al.,2015).

5.3.2 Generation of *B. napus* microspore-derived embryos (MDEs)

BnLEC1 transgenic lines and *BnFUS3* tilling mutant lines were used as the source of microspores to generate microspore-derived embryos (MDEs), following the procedure of

Belmonte *et al.* (Belmonte et al., 2006). Plants with young buds were grown in a cabinet set at 12 °C day/7 °C night with a 12 h photoperiod. Flower buds (2–3 mm in length) were harvested, sterilized in 10 % bleach and ground in a mortar in half strength B5-13 medium supplemented with 13 % (w/v) sucrose**.** The homogenate was centrifuged at 750 rpm (g) at 4 °C for 3 min. The microspore-containing pellet was thereafter re-suspended in NLN-13 medium with 13 % sucrose (pH 5.8) and further diluted in NLN-13 medium to a concentration of 10,000 microspores/ml. Embryo development was triggered after an initial heat shock treatment at 32 °C for 72 h followed by incubation at 22 °C on a shaker set at 80 rpm. The number of microspore-derived embryos was counted after 21 d in culture (Belmonte et al., 2006).

5.3.3 Determination of microspore-derived embryo quality

Embryo quality was assessed by the ability of the MDEs to regenerate viable root and shoot systems at germination. Fully mature (21 d) embryos were germinated on half concentration Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and the number of seedlings with fully developed shoots and roots were scored, as reported (Belmonte et al., 2006).

5.3.4 Microscopy

For histological examinations, fully mature embryos were fixed in 2.5 % glutaraldehyde and 1.6 % paraformaldehyde in 0.05 M phosphate buffer (pH 6.9), dehydrated with methyl cellosolve followed by three washings with absolute ethanol, and then infiltrated and embedded in Historesin (Leica, Concord, Ontario, Canada). Sections (3 µm) were stained with periodic acid-Schiff (PAS) reagent and then counterstained with toluidine blue (TBO) according to the methods of Yeung (Yeung, 1999).

5.3.5 Determination of lipid profile and fatty acid (FA) composition

All analyses were conducted in the University of Manitoba seed quality lab, which is certified annually by the Canadian Grain Commission. Total lipid contents were quantified using the modified Swedish method as described by Troëng, (Troëng, 1955). Fatty acid (FA) composition analyses were conducted using gas chromatography (Varian, Walnut Creek, USA) as documented by Hougen and Bodo (Hougen and Bodo, 1973). In short, approximately 5 g of mature *B. napus* MDEs were dried overnight at 40°C. Then, 1 g of dried MDEs was crushed and homogenized in 5ml of heptane and incubated at room temperature for 24 h. The supernatants were then poured into clean 13 x 100 test tubes and after addition of 500 µl of 0.5N sodium methoxide, the samples were shaken for 30 min. 100 µl of acidified water (0.3% acetic acid) was added to the samples which were then incubated at 4 ^{o}C for 2 h. Lastly, 500 µl of reaction mixture was poured into a 2 ml auto sampler (Fisher brand vial CAT# 03-391-16) (Christie, 1989; Liu, 1994). Gas chromatography was performed using a Varian model 3900 fitted with a CP-Wax 52 CB capillary column and a flame ionization detector. Peak areas were measured by the Varian Star Workstation software system. A reference standard, GLC # 421, bought from Nu-Check Prep (Elysian, Minnesota) was used to confirm the appropriate GC process.

5.3.6 Gene expression analysis by quantitative qRT-PCR

Analysis of gene expression in developing [7, 14, and 21 day] *B. napus* MDEs was determined by quantitative qRT-PCR (Elhiti et al., 2010). Expression studies were conducted for molecular marker genes identified in previous studies (Elhiti et al., 2012; Malik et al., 2007) and required for proper embryo formation. These genes are classified as embryo specific; *LEAFY COTYLEDON2 (LEC2*), UKNOWN *PROTEIN1* (*UPI1*); embryo expressed *BABYBOOM1* (*BBM1*), *WUSCHEL-related HOMEOBOX9* (*BnWOX9*), *ABSCISIC ACID3* (*ABI3*) and sporophyte expressed *CYTOCHROME P78A* (*CYP78A*), and *WUSCHEL-related HOMEOBOX2*

(*BnWOX2*). These analyses also included genes involved in glycolysis: *FRUCTOSE BISPHOSPHATE ALDOLASE* (*BnFPA*), *PHOSPHOGLYCERATE KINASE* (*BnPGK*), *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), *HEXOSE KINASE* (*BnHXK*) and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*BnPPK*); sucrose transport and metabolism: *SUCROSE TRANSPORTER 1* and *4* (*BnSUC 1* and *4*), *SUCROSE SYNTHASE 1* and *3*, (*BnSUS1* and *3*), *ADP-GLUCOSE PHOSPHORYLASE (BnAGP*); and FA biosynthesis: *SUBUNIT A of ACETYL-CoA CARBOXYLASE (BnACCA2), ω-3 FA DESATURASE (BnFAD3), FA ELONGATION1 (BnFAE1),* and *MALONYL-CoA:ACP TRANSACYLASE (BnMCAT)*. The relative level of gene expression was determined with the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen 2001) using *UBC21* (*EV086936, ubiquitin-conjugating enzyme 21*) as a reference. All PCR reactions were performed using the CFX96^{TM} Optics Real-Time System (Bio-Rad, Hercules, CA) with an initial denaturation step at 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 5 s (denaturation), and 59 ºC for 10 s (annealing/extension). The melting curve rose from 65 ºC to 95 ºC by increments of 0.5 ºC every 5 s. Analyses were performed on three biological replicates. All primers used are listed in Supplementary Tables 1 and 2.

5.3.7 Statistical analysis

The statistical analyses were performed using the SAS® 9.3 program (SAS Institute Inc., Cary, NC, USA.). Unless specified, all experiments were performed using at least three biological replicates and the Fisher's Least Significant Difference (LSD) test was applied to compare samples.

5.4 Results

Plants from the lines utilized have been fully characterized in previous studies (Elahi et al., 2015; Elahi et al., 2016). Of the four *BnLEC1* over-expressing lines utilized, two (S1 and S2)

over-expressed version A of *BnLEC1*, and two (S3 and S4) over-expressed version B. Expression of *BnLEC1* was induced in both vegetative and reproductive tissue of the four lines (Supplemental Fig. 3 in Elahi et al., 2016), as well as during the different phases (7, 14, and 21 day) of microspore-derived embryogenesis (Fig. 5.2). Lines with anti-sense *BnLEC1* (A1 and A2) had reduced expression of *BnLEC1* in the same tissues (Supplemental Fig. 3 in Elahi et al., 2016) and during microspore-derived embryogenesis (Fig. 5.2). The *BnFUS3* lines (M1-3) had point mutations resulting in a non-functional *BnFUS3* protein (Elahi et al., 2015).

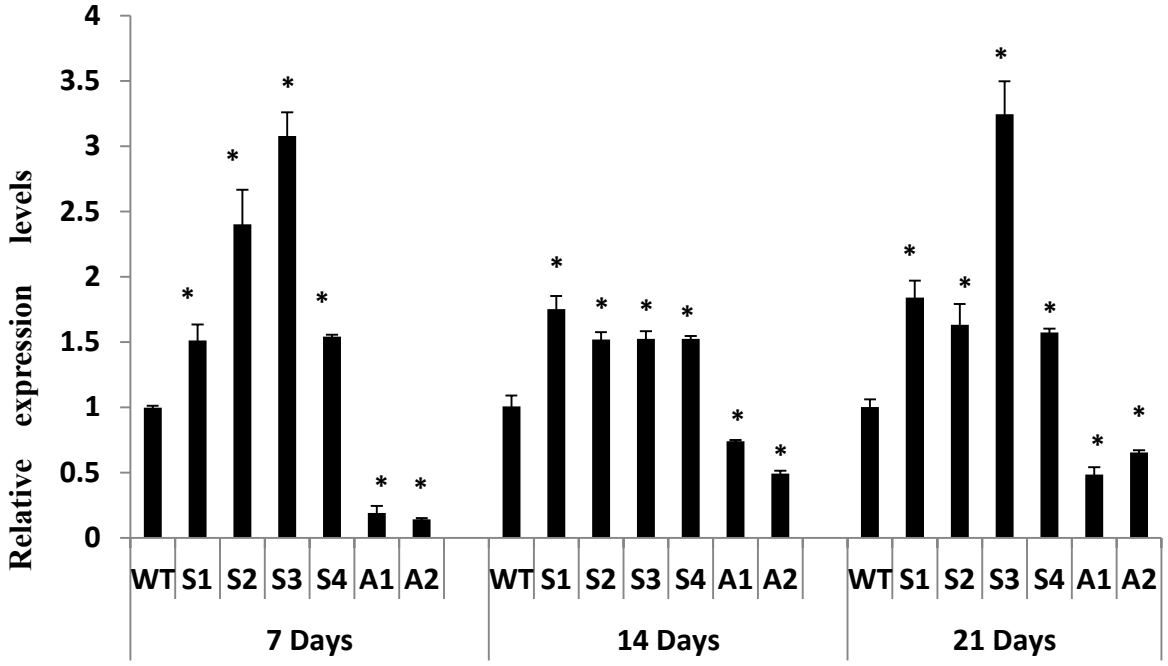


Fig. 5.2 Expression analysis by quantitative (q)RT-PCR of *LEAFY COTYLEDON1* (*LEC1*) in transgenic *BnLEC1* microspore-derived embryos at different days in culture. Data presented are mean value ± SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1-S4), *BnLEC1* down-regulators (A1-A2).

5.4.1 Effects of altered expression of *BnLEC1* and *BnFUS3* on microspore-derived embryo quantity and quality

The *BnLEC1* transgenic lines and *BnFUS3* mutant lines were utilized as the source of microspores to produce embryos in culture. Compared to the WT, the number of fully developed (day 21) MDEs produced by lines over-expressing version A (S1, S2) or version B (S3, S4) *BnLEC1*, as well as by lines down-regulating *BnLEC1* (A1, A2) were significantly repressed (Fig. 5.3A and B). With the exception of line S1, embryo formation was less than half in all *BnLEC1* transgenic lines. A similar and consistent repression in embryo number was also observed in the three lines (M1-3) where *BnFUS3* was mutated (Fig. 5.3C).

The quality of MDEs, i.e., their ability to regenerate viable plants at germination, was not affected by the over-expression of *BnLEC1* (S1-4), while it decreased in those where the expression of *BnLEC1* was suppressed (A1, A2), and in the *BnFUS3* mutant lines (M1-3) (Fig. 5.4).

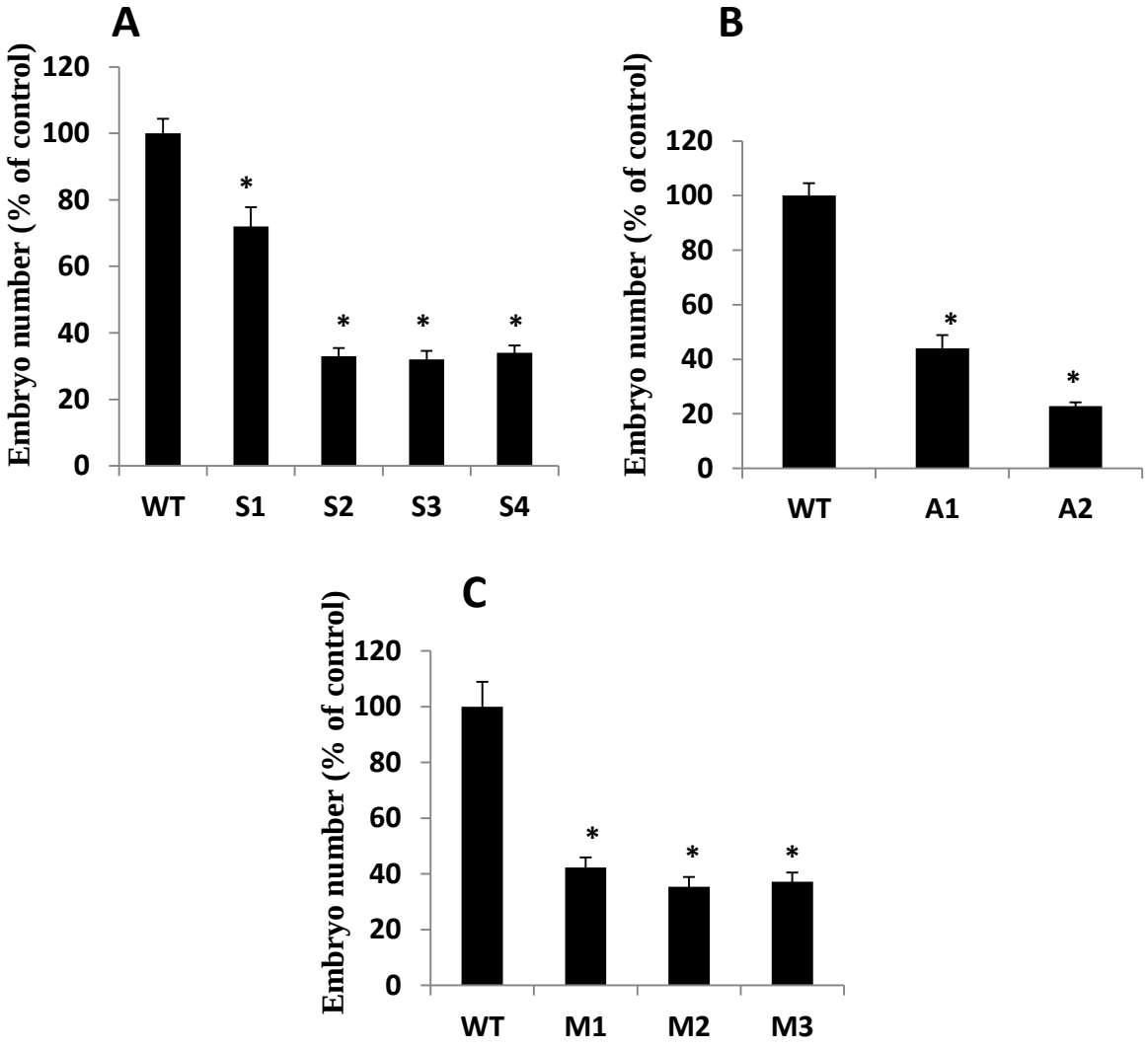


Fig. 5.3 Production of microspore-derived embryos (MDEs) from *Brassica napus* plants with altered expression of *BnLEC1* (A, B) and *BnFUS3* (C). *BnLEC1*over-expressing lines (S1-S4), *BnLEC1* down-regulating lines (A1-A2) and *BnFUS3* tilling mutant lines (M1-M3) were utilized to generate MDEs. For each experiment, 100,000 microspores were plated and embryo number was counted at 21 d. Value ±SE (*n*=5) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: P<0.05) from the respective WT (control) value (set at 100).

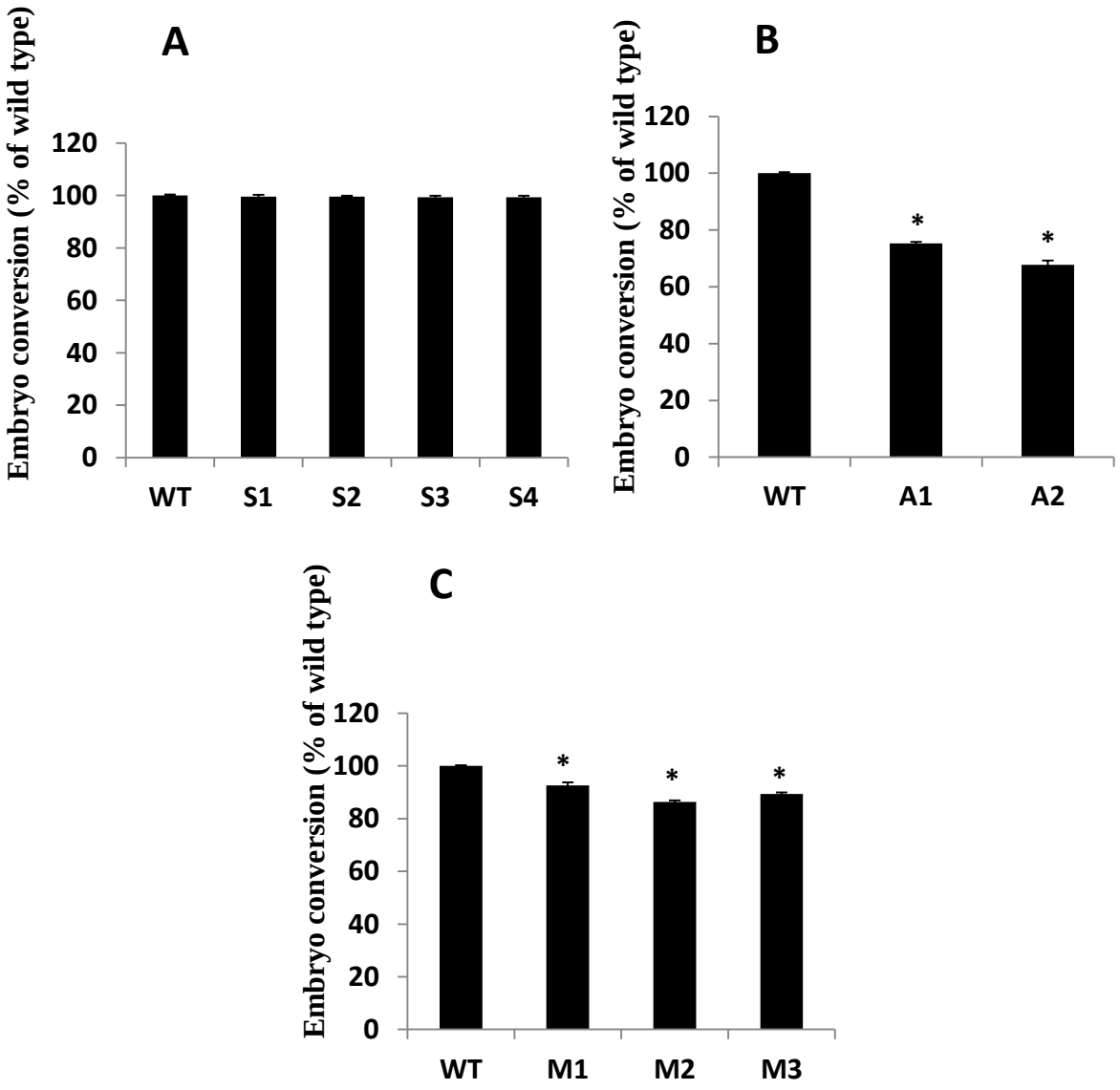


Fig. 5.4 Conversion frequency (ability to form viable shoots and roots at germination) of MDEs with altered expression of *BnLEC1* (A, B) and *BnFUS3* (C). *BnLEC1* over-expressing lines (S1-S4), *BnLEC1* down-regulating lines (A1-A2) and *BnFUS3* tilling mutant lines (M1-M3) were utilized to generate MDEs. Value ±SE (*n*=3) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: P<0.05) from the respective WT value (set at 100).

5.4.2 Effects of altered expression of *BnLEC1* and *BnFUS3* on embryo morphology

Microspore-derived embryo (MDE) morphology was assessed at day 14, corresponding to the cotyledonary stage of development and in fully developed embryos (day21). At both days identical phenotypes were observed within lines characterized by similar expression of the transgene.

After 14 d in culture, MDEs over-expressing *BnLEC1* (S1-4) had a similar morphology to their WT embryos and consisted of an elongated embryonic axis and fully expanded cotyledons (Fig. 5.5Aa-b). This was in contrast to MDEs suppressing *BnLEC1* (A1-2) where the elongation of the embryonic axis was inhibited and the proper formation of cotyledons was compromised (Fig. 5.5Ac). Embryos generated from the *BnFUS3* mutant lines (M1-3) shared similar abnormalities including embryonic axes characterized by unorganized cell proliferation (Fig.5.5Ad). The cotyledons of these embryos were also generally larger and partially fused (Fig. 5.5Ad).

The phenotypic abnormalities observed at 14 d were retained by fully developed (21 d) MDEs (Fig. 5.5Ae-h). The only exception was observed in embryos produced by the *BnLEC1* down-regulating lines (A1-2) which showed partially fused embryonic axes and abnormal cotyledons (Fig. 5.5Ag).

The most obvious morphological abnormalities were also analyzed at a structural level in relation to lack of a shoot apical meristem observed in the *BnLEC1* down-regulating embryos relative to WT embryos (Fig. 5.5Ba,b), and the presence of irregular outgrowth along the hypocotyl of *BnFUS3* tilling mutant embryos (Fig. 5.5Bc).

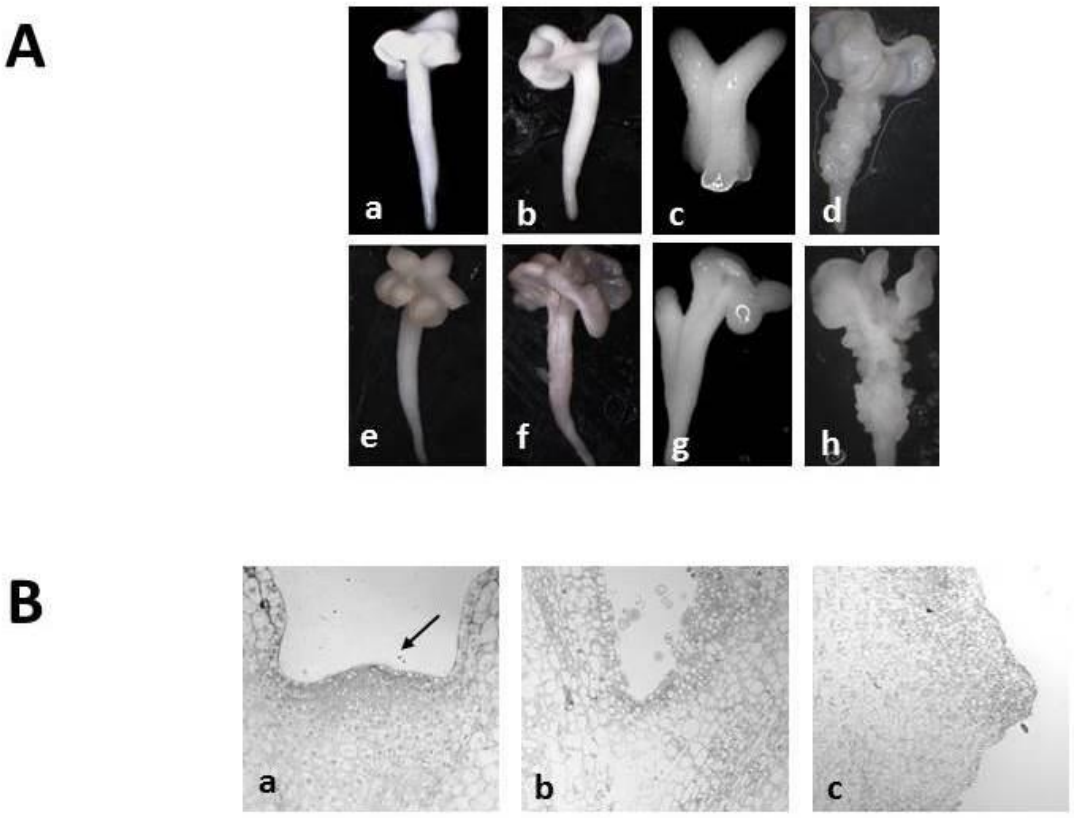


Fig. 5.5 Effects of altered expressions of *BnLEC1* and *BnFUS3* on morphology of microspore-derived embryos (MDEs). (A) Morphology of 14 and 21- day old (early and late cotyledonary stages) MDEs. Early cotyledonary stages of MDEs (a-d); (a) Wild Type, (b) S4, (c) A1, (d) M1. Late cotyledonary stages of MDEs (e-h); (e) Wild Type, (f) S4, (g) A1, (h) M1.

(B) Effects of altered expression of *BnLEC1* and *BnFUS3* on the structure of *B. napus* MDEs after 21 days in culture. (**a**) Shoot apical meristem of wild type (WT) MDEs. The meristem was dome-shaped and composed of many layers of densely cytoplasmic cells (arrow), (**b**) Poorly-developed meristem of MDEs down-regulating *BnLEC1* (line A1). (**c**) Irregular outgrowth along the epidermal layer of the hypocotyl of MDEs *BnFUS3* tilling mutant M3.

5.4.3 Expression profiles of selected molecular markers of microspore-derived embryogenesis

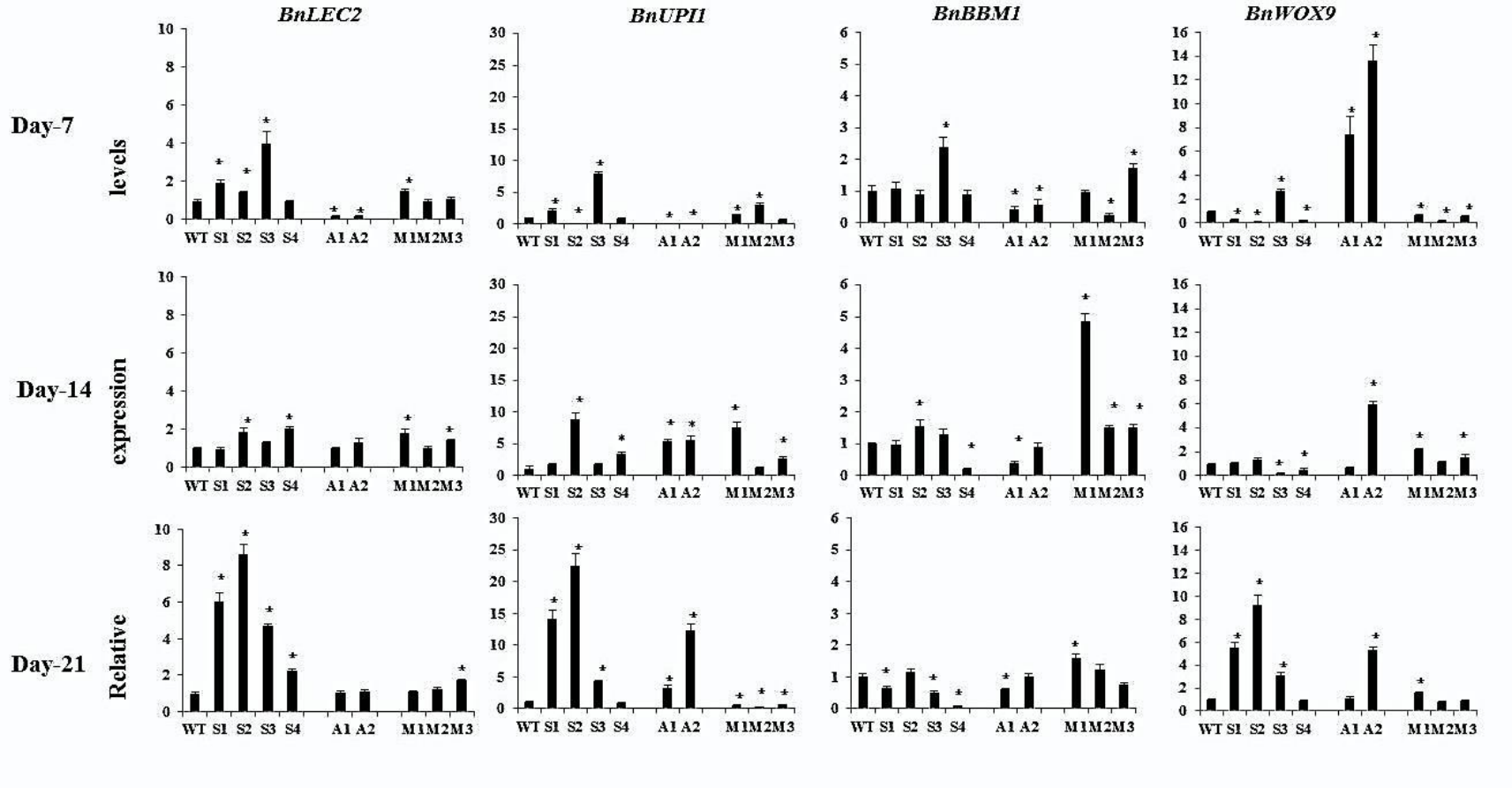
To better understand the role of altered *BnLEC1* and *BnFUS3* expression during embryogenesis and the morphological abnormalities resulting from their miss-expression, we also measured the transcript abundance of 8 molecular marker genes at different stages of MDE development (late-globular, 7 day; cotyledonary, 14 day; and fully developed, 21 day). Expression of these genes was linked to proper embryonic development (Elhiti et al., 2012; Malik et al., 2007).

An overall increase in transcript levels of *BnLEC2* and *BnAB13* was observed in late-globular (7 day) MDEs of the *BnLEC1* over-expressing lines (S1-4) (Fig. 5.6). The expression of the other genes at the same stage of development did not follow any consistent profile. At 21 d, over-expression of *BnLEC1* resulted in a significant induction of *BnLEC2* and *BnCYP78A5* in all lines (S1-S4), as well as *BnUPI1*, *BnWOX2*, and *BnWOX9* in lines S1-S3 and *BnSERK1* in lines S2-S4. A reduction in the transcript levels of *BnAB13* was observed in fully developed embryos of lines S1-S3.

In 7 day MDEs suppressing *BnLEC1* (A1, A2) an overall repression in transcript levels was measured for many of the genes analyzed including *BnCYP78A5*, *BnWOX2*, *BnLEC2*, *BnUPI4*, *BnBBM1*, and *BnABI3* (Fig. 5.6). This was in contrast to the expression of *BnWOX9*, which was highly induced in both *BnLEC1* antisense embryos. Upon further development (day 14), the transcript levels of *BnWOX2*, *BnUPI1* and *BnCYP78A5* increased in both lines. The expression of *BnSERK1* was repressed in fully developed (day 21) *BnLEC1*-suppressing MDEs and this was in contrast to *BnWOX2*, which was induced in both A1 and A2 lines.

In immature (day 7) embryos produced by the *BnFUS3* mutant lines, the only gene showing a consistent expression pattern was *BnWOX9*, which was repressed in all three (M1-3)

lines. At day 14, the expression of *BnWOX2* and *BnBBM1* was significantly induced, while that of *BnSERK1* was repressed. Fully developed (day 21) *BnFUS3* mutant MDEs were characterized by increased transcript levels of *BnABI3* and *BnWOX2* and suppression of *BnUPI1* (Fig. 5.6).


BnLEC2
BnUPI1
BnBBM1
BnWOX9
Day-7
Day-14
Day-21
Relative expression levels
WT S1 S2 S3 S4 A1 A2 M1M2M3

Continued

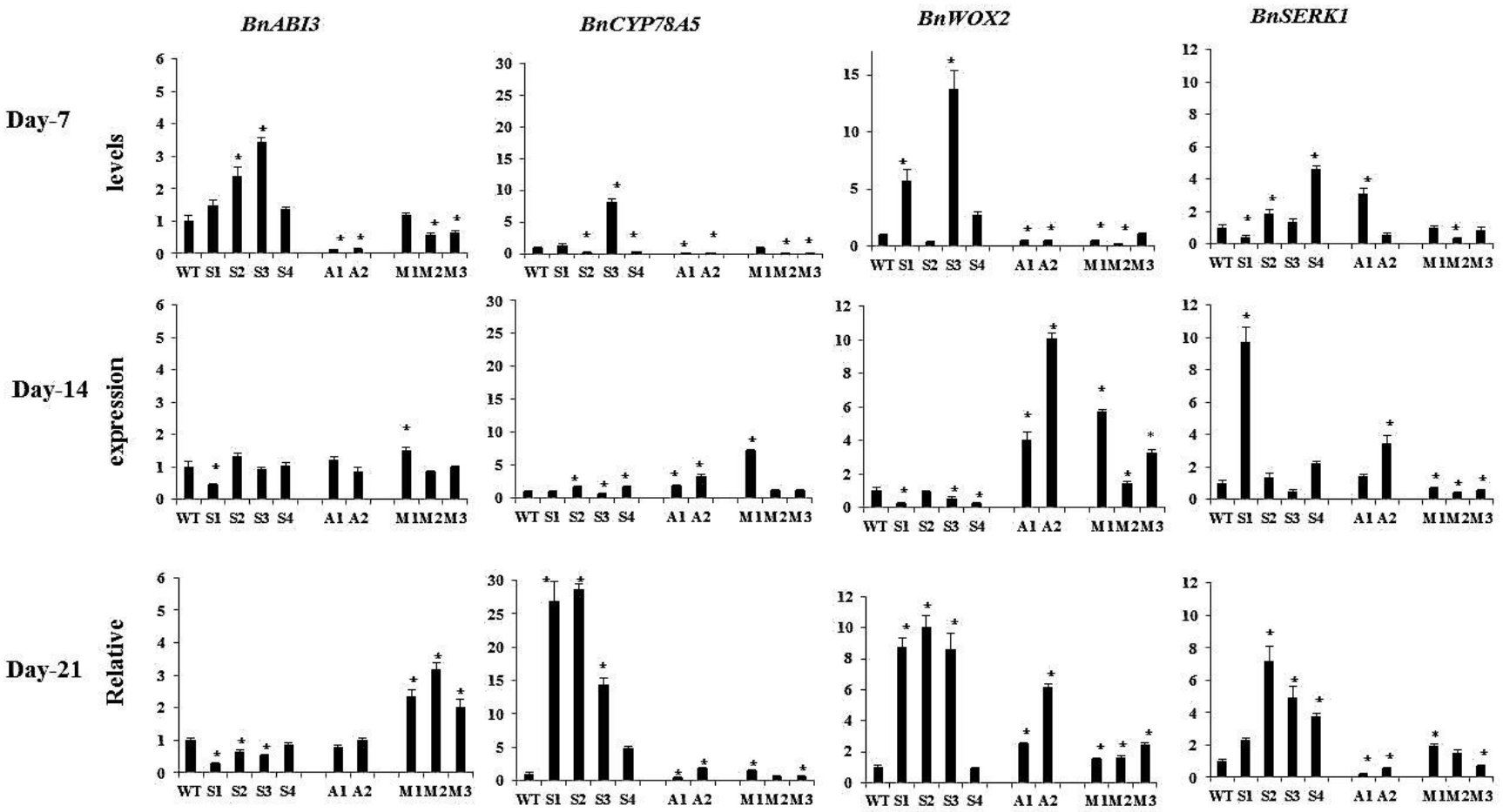

BnABI3
BnCYP78A5
BnWOX2
BnSERK1
Day-7
Day-14
Day-21
Relative expression levels
WT S1 S2 S3 S4 A1 A2 M1M2M3

Fig. 5.6 Expression analysis by quantitative (q)RT-PCR of embryogenesis-marker genes in MDEs with altered levels of *BnLEC1* and *BnFUS3*. Analyses were conducted at day 7, 14, and 21. Genes analyzed: *LEAFY COTYLEDON2* (*LEC2*), *UKNOWN PROTEIN1* (*UPI1*), *BABYBOOM1* (*BBM1*), *WUSCHEL-related HOMEOBOX9* (*BnWOX9*), *ABSCISIC ACID3* (*ABI3*), *CYTOCHROME P8A5* (*CYP78A5*), *WUSCHEL-related HOMEOBOX2* (*BnWOX2*), and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SERK1*). Data presented are mean value $\pm$ SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type (control) value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1- S4), *BnLEC1* down-regulators (A1-A2), *BnFUS3* tilling mutants (M1-M3).

5.4.4 Effects of altered expression of *BnLEC1* and *BnFUSCA3* on total lipid and fatty acid accumulation in microspore-derived embryos

The late phases of embryo development are characterized by the accumulation of storage products, which in *B. napus* consist mainly of oil. Previous studies have shown that altered expression of both *BnLEC1* and *BnFUS3* influences oil level and FA composition in seeds (Elahi et al., 2015; Elahi et al., 2016). To assess if similar changes also occur during *in vitro* embryogenesis, lipid content and FA composition were analyzed in the MDE lines.

Relative to the WT, the lipid content increased in embryos over-expressing *BnLEC1* (S1-S3), while it was reduced in those suppressing *BnLEC1* (A1) and in those where *BnFUS3* was mutated (M1, M2) (Fig. 5.7).

Analysis of FA composition revealed small alterations in the levels of stearic acid (18:0), and oleic acid (C18:1) in the M2 embryos, as well as an increase in linolenic acid (C18:3) content in S3 embryos and linoleic acid (C18:2) in M1and M2 embryos (Table 5.1).

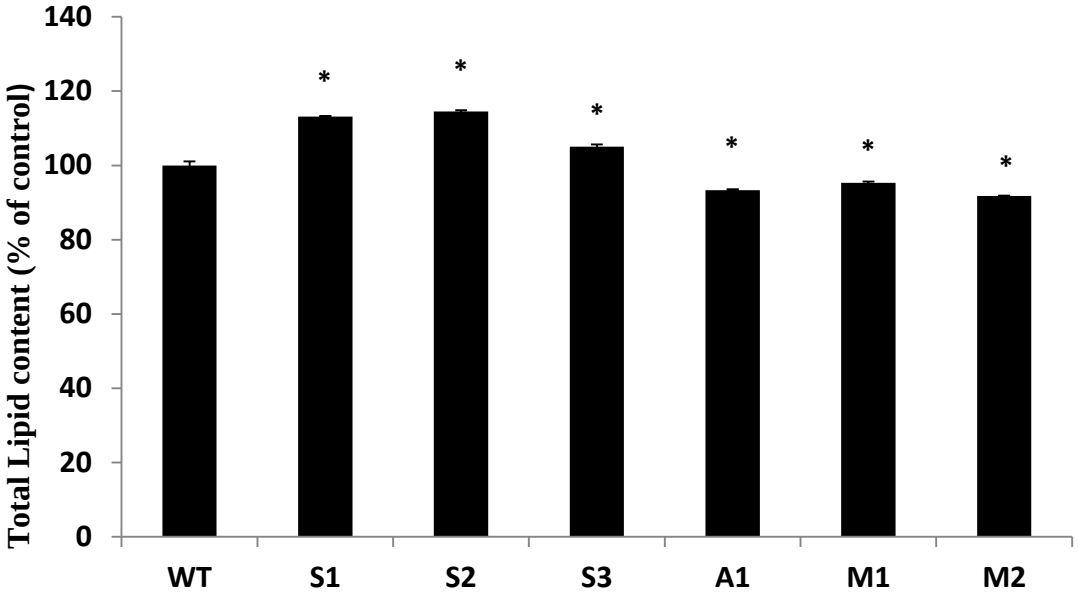


Fig. 5.7 Total lipid content in 21 day-old MDEs with altered expression of *BnLEC1* or *BnFUS3*. Value ±SE (*n*=6) is expressed as a percentage of the WT. Asterisks indicate values that are statistically different (LSD: P<0.05) from the WT value. *BnLEC1* over-expressors (S1- S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1-M3).

Table 5.1: Fatty acid profile (% composition) in transgenic *BnLEC1* and *BnFUS3* tilling mutant MDEs. Values are means of three biological replicates ± SE. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from their respective WT value. S1-S3, *BnLEC1* over-expressors; A1, *BnLEC1* down-regulators; M1-M2, *BnFUS3* tilling mutants.

	C16:0	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**
WT	5.15±0.25	4.03±0.41	68.44±1.99	13.00±1.59	5.44±0.81	1.15±0.02
S1	5.67±0.37	3.74±0.48	69.48±1.35	12.91±1.41	4.29±0.21	1.09±0.06
S2	5.35±0.25	2.91±0.68	67.08±0.95	14.59±0.23	6.95±0.06	0.94±0.12
S3	5.05±0.25	2.82±0.65	65.88±1.10	15.78±0.97	7.14±0.43*	0.93±0.11
WT	5.86±0.11	2.55±0.19	65.76±0.43	16.86±0.22	6.39±0.82	0.93±0.02
A1	5.78±0.16	2.44±0.24	65.38±0.57	16.85±0.20	6.39±0.77	0.87±0.06
WT	5.57±0.06	2.35±0.19	63.54±1.94	16.33±1.20	7.71±0.18	0.86±0.07
M1	6.01±0.27	3.21±0.49	61.16±2.62	20.10±1.05*	7.12±0.71	1.09±0.15
M2	6.61±0.49	4.09±0.53*	54.88±1.81*	22.51±0.89*	8.58±0.36	1.40±0.23

5.4.5 Transcriptional regulation of sucrose and fatty acid metabolism genes

The main carbon source for oil biosynthesis in developing seeds is sucrose produced from photosynthesis. Sucrose is readily hydrolyzed into UDP-glucose and fructose, which are oxidized in the glycolytic pathway to Acetyl-CoA, the precursor of FA biosynthesis. The changes in oil levels observed in the transformed MDEs prompted us to measure the transcript levels of genes contributing to sucrose metabolism and glycolysis. At both day 14 and 21 in culture, *BnLEC1* over-expressing MDEs had increased expression levels of several sucrose metabolic genes. These included *SUCROSE SYNTHASE1* and *3* (*BnSUS1* and *3*) at day 14 (Fig. 5.8) and *SUCROSE TRANSPORTER 4* (*BnSUC4*) and *BnSUS3* at day 21 (Fig. 5.9). Over-expression of *BnLEC1* also resulted in an increased expression of the glycolytic enzyme *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*BnGDPH*), as well as a decreased expression of the FA metabolic genes *SUBUNIT A of ACETYL-CoA CARBOXYLASE (BnACCA2)* at day 14 and *MALONYL-CoA:ACP TRANSACYLASE (BnMCAT)* at day 21 (Fig. 5.8, 5.9).

An overall suppression of several sucrose and glycolytic enzymes, including *FRUCTOSE BISPHOSPHATE ALDOLASE (BnFPA), PHOSPHOGLYCERATE KINASE (BnPGK),* and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE (BnPPK),* as well as several fatty acid metabolic enzymes such as *BnACC2,ω-3 FA DESATURASE (BnFAD3),* and *FA ELONGATION1 (BnFAE1),*were observed in MDEs with suppressed level of *BnLEC1* (Fig. 5.8, 5.9).

A similar overall repression in gene expression was also measured in the two *BnFUS3* TILLING mutant MDEs, especially at day 14 (Fig.5.8) where the majority of genes encoding enzymes of sucrose and fatty acid metabolism were transcriptionally down-regulated.

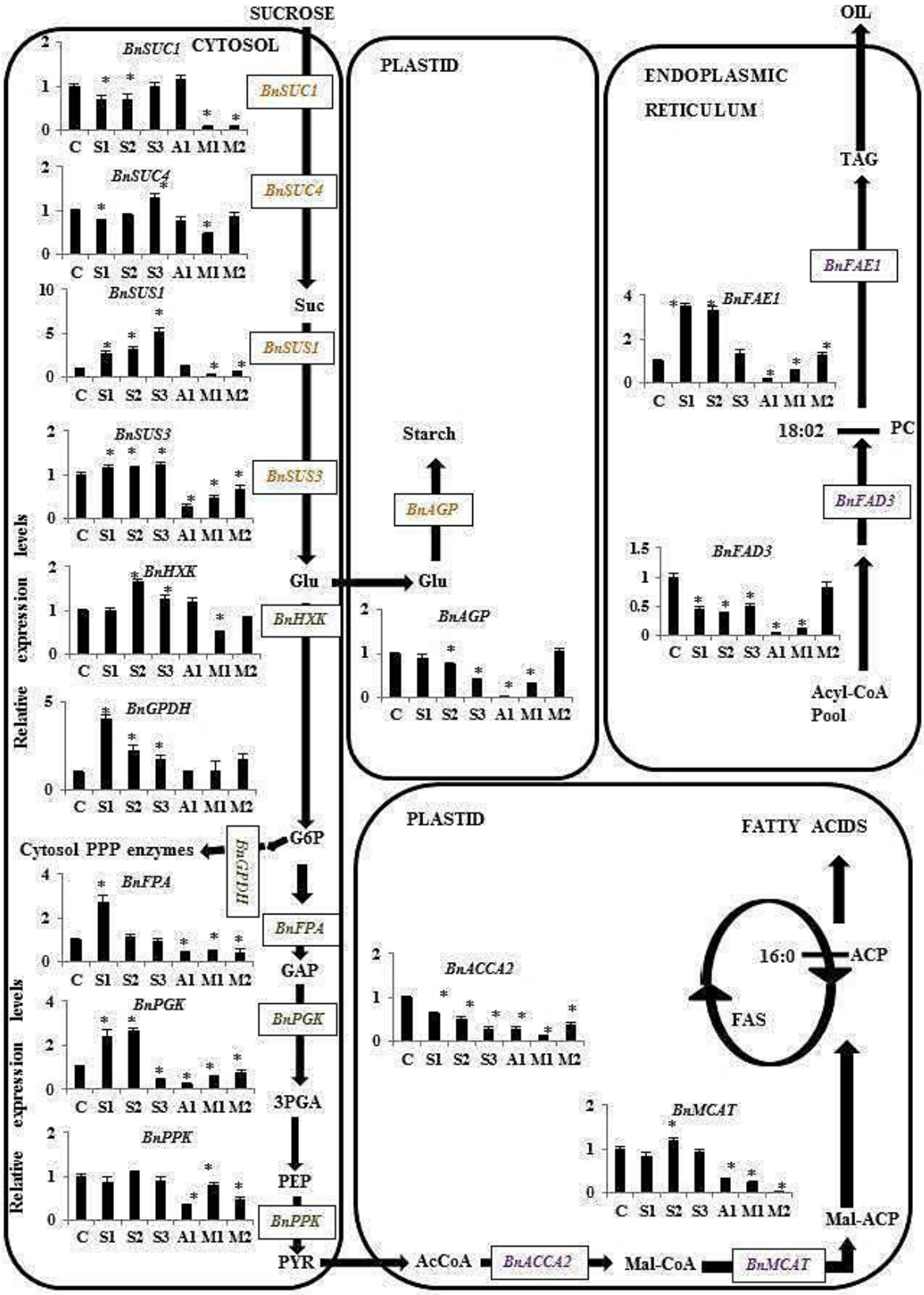

SUCROSE
CYTOSOL
PLASTID
ENDOPLASMIC RETICULUM
OIL
TAG
BnSUC1
BnSUC4
Suc
BnSUS1
BnSUS3
Glu
BnHXK
G6P
BnGPDH
Cytosol PPP enzymes
BnFPA
GAP
BnPGK
3PGA
PEP
BnPPK
PYR
Starch
BnAGP
Glu
BnFAE1
18:02
PC
BnFAD3
Acyl-CoA Pool
PLASTID
FATTY ACIDS
16:0
ACP
FAS
BnACCA2
BnMCAT
Mal-ACP
AcCoA
Mal-CoA
Relative expression levels
C S1 S2 S3 A1 M1 M2

Fig. 5.8 Expression analysis by quantitative (q) RT-PCR of genes involved in the regulatory pathways leading to FA synthesis in *BnLEC1* transgenic and *BnFUS3* tilling mutants MDEs at 14 days. *SUC1* (*Sucrose Transporter1*), *SUC4* (*Sucrose Transporter4*), *SUS1* (*Sucrose Synthase1*), *SUS3* (*Sucrose Synthase3*), *AGP* (*ADP-Glucose Phosphorylase*). *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*), *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). *ACCA2* (*Subunit A of Acetyl-CoA Carboxylase*), *FAD3* (*ω-3 FA Desaturase*), *FAE1* (*FA Elongation1*), *MCAT* (*Malonyl-CoA: ACP Transacylase*). Data presented are mean value $\pm$ SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1, S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1, M2).

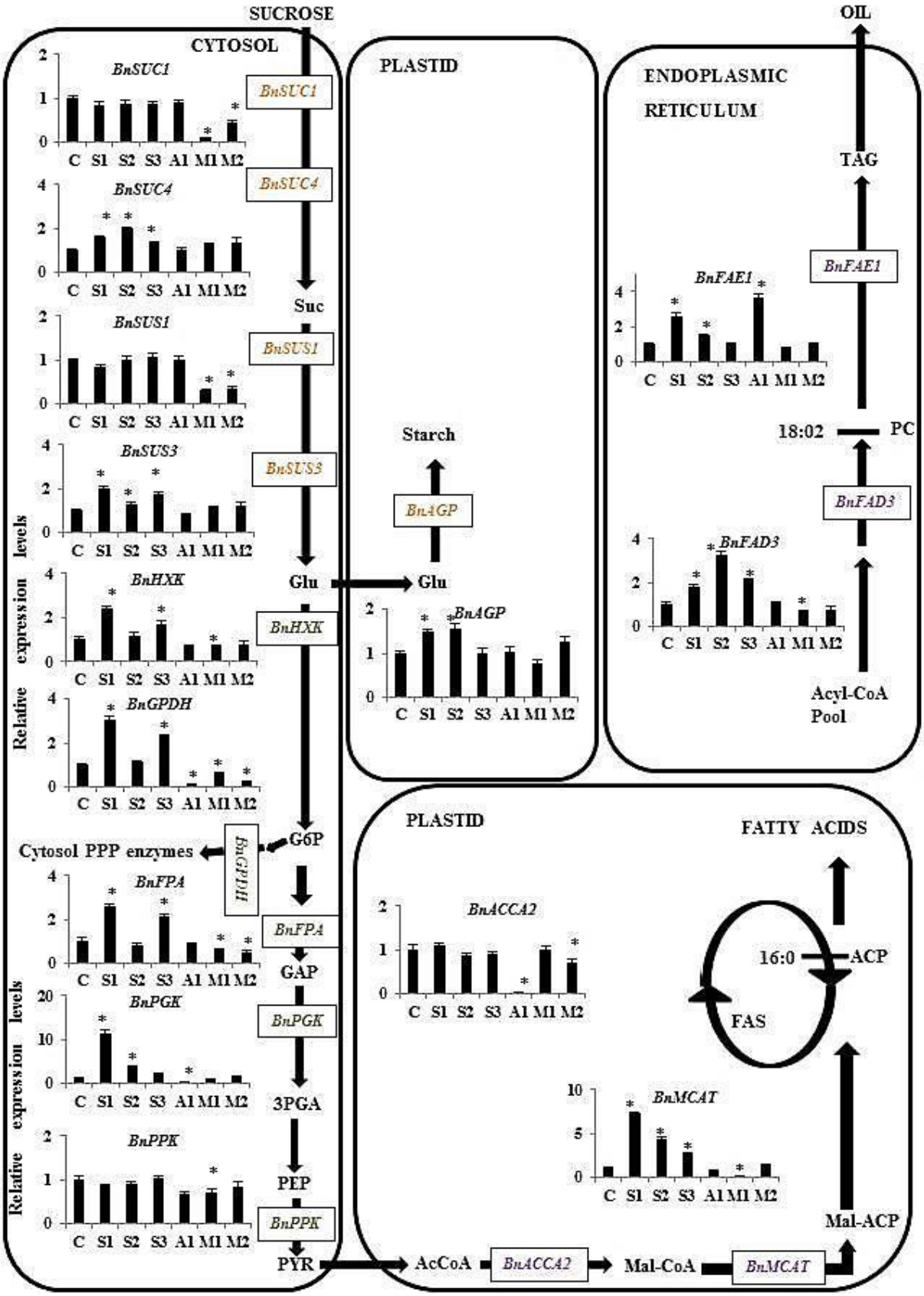

SUCROSE
CYTOSOL
PLASTID
ENDOPLASMIC RETICULUM
OIL
TAG
BnSUC1
BnSUC4
Suc
BnSUS1
BnSUS3
Glu
BnHXK
BnGPDH
G6P
Cytosol PPP enzymes
BnGPDH
BnFPA
GAP
BnPGK
3PGA
PEP
BnPPK
PYR
Starch
BnAGP
Glu
BnFAE1
18:02
PC
BnFAD3
Acyl-CoA Pool
PLASTID
FATTY ACIDS
BnACCA2
16:0
ACP
FAS
BnMCAT
Mal-ACP
AcCoA
BnACCA2
Mal-CoA
BnMCAT
Relative expression levels
C S1 S2 S3 A1 M1 M2

Fig. 5.9 Expression analysis by quantitative (q)RT-PCR of genes involved in the regulatory pathways leading to FA synthesis in *BnLEC1* transgenic and *BnFUS3* tilling mutants MDEs at 21 days. *SUC1* (*Sucrose Transporter1*), *SUC4* (*Sucrose Transporter4*), *SUS1* (*Sucrose Synthase1*), *SUS3* (*Sucrose Synthase3*), *AGP* (*ADP-Glucose Phosphorylase*). *FPA* (*Fructose Bisphosphate Aldolase*), *PGK* (*Phosphoglycerate Kinase*), *GPDH* (*Glyceraldehyde-3- Phosphate Dehydrogenase*), *HXK* (*Hexose Kinase*), *PPK* (*Pyrophosphatase-Dependent Phosphofructokinase*). *ACCA2* (*Subunit A of Acetyl-CoA Carboxylase*), *FAD3* (*ω-3 FA Desaturase*), *FAE1* (*FA Elongation1*), *MCAT* (*Malonyl-CoA: ACP Transacylase*). Data presented are mean value $\pm$ SE of three biological replicates. Asterisks indicate statistically significant differences (LSD: $P < 0.05$) from the respective wild type value (set at 1) at the respective day in culture. *BnLEC1* over-expressors (S1, S3), *BnLEC1* down-regulator (A1), *BnFUS3* tilling mutants (M1, M2).

5.5 Discussion

In vitro embryogenesis is an attractive system for studying the molecular and physiological events associated with embryo development (Mordhorst et al., 2002; Raghavan 2000). Besides producing embryos with similar morphology and developmental stages to their *in vivo* counterparts, *in vitro* embryogenic systems can generate large numbers of synchronized embryos in a short period of time (Stasolla et al., 2008). This is also true for microspore-derived embryogenesis, where the gametophytic fate of the microspores can be redirected toward an embryogenic fate through applications of stress conditions and culture treatments (Fig. 5.1).

To maximize the use of *Brassica in vitro* embryogenic systems in breeding programs, thus accelerating the selection of desirable traits such as elevated seed oil levels, it is paramount to assess if *in vitro* embryos behave in a similar fashion to their zygotic counterparts in response to gene manipulations. This is especially relevant when examining storage product deposition, a trait controlled by a complicated and partially unknown genetic network (DeLa Roche and Keller, 1977).

In *Brassica* seeds *LEC1* and *FUS3* have been identified as key regulators of oil synthesis (Elahi et al., 2015; Elahi et al., 2016). Oil content is enhanced in seeds over-expressing *BnLEC1*, while repressed in those where the expression of either *BnLEC1* or *BnFUS3* was suppressed (Elahi et al., 2015; Elahi et al., 2016). To verify if similar alterations occur *in vitro*, we measured total lipid content and FA composition during microspore-derived embryogenesis. Relative to the WT, over-expression of *BnLEC1* increased lipid content in MDEs by about 10-15%, while its down-regulation decreased oil content by 5% (Fig.5.7). These values are comparable to those observed *in vivo* (7-16% increase in *BnLEC*1 over-expressing seeds and a 9-12% decrease in *BnLEC1* down-regulating seeds) (Elahi et al., 2016). For both *in vivo* and *in vitro*, these changes were not accompanied by any major alterations in FA composition (Table 5.2 and supplementary

Table 4 in Elahi et al., 2016, and Table 5.1 in this paper), but did show major changes in the expression of genes encoding enzymes of sucrose and FA metabolism (Fig 3 and 4 in Elahi et al., 2016, and Fig. 5.8 and 5.9 in this paper). It is worth noting that both *in vivo* and *in vitro* over-expression of *BnLEC1* induce expression of sucrose metabolic genes as well as genes participating in the glycolytic pathway. This induction denotes an active utilization and oxidation of sucrose which is possibly required for enlarging the pool of FA biosynthetic precursors, leading to the observed increase in seed oil content. This process would be compromised in MDEs down-regulating *BnLEC1*, which like seeds down-regulating *BnLEC1* show a reduction in the expression of several glycolytic enzymes (Fig. 5.8, 5.9). The requirement of *BnLEC1* for oil accumulation, documented in several systems (Lotan et al., 1998; Mu et al., 2008; Shen et al., 2010), is associated with an enhancement of carbon flux toward the FA biosynthetic pathway (Elahi et al., 2016; Elhiti et al., 2012; Mu et al., 2008).

Mutation of *BnFUS3* in *B. napus* MDEs reduces oil content and increases the relative level of linoleic (C18:2) acid. A comparable decrease in oil, associated with altered sucrose metabolism and decreased glycolytic activity was also observed in *BnFUS3* mutant seeds (Elahi et al., 2015). A similar repression of the glycolytic pathway might also occur *in vitro*, as the expression of several glycolytic enzymes is repressed in *BnFUS3* TILLING mutant MDEs (Fig. 5.8, 5.9). Collectively these results show that the effects of *BnLEC1* and *BnFUS3* on oil quantity and composition, as well as on the transcription of sucrose and FA metabolic enzymes, observed in *Brassica* MDEs mimic closely those reported *in vivo*.

Embryonic yield and quality, i.e., frequency of successful regeneration, are crucial parameters of any propagation system integrated in breeding programs. It was therefore one of

the objectives of this study to evaluate if the *BnLEC1* and *BnFUS3* modulation of oil level was accompanied by changes in embryonic response in culture.

LEAFY COTYLEDON1 encodes a HAP3 subunit of the CCAAT-binding transcription factor (Lotan et al., 1998) expressed in immature *Arabidopsis* siliques, zygotic embryo and embryogenic tissue, but not in vegetative tissue (IkedaIwai et al., 2002). Elevated expression of this gene has also been measured in embryogenic carrot cells and somatic embryos (Yazawa et al., 2004), as well as immature maize somatic embryos (Zhang et al., 2002), thus suggesting its participation in embryogenesis-related pathways. During *in vitro* embryogenesis in *B. napus*, *BnLEC1* is required both for the formation of MDEs (Fig. 5.3B), as well as the ability to regenerate viable plants (Fig. 5.4B). The poor embryogenic competence observed in microspores suppressing *BnLEC1* (lines A1 and A2) suggests that the proper function of this gene is needed for the gametophytic-embryogenic transition initiating the process. This observation, supporting previous studies on the ability of *LEC1* to induce embryogenesis in vegetative tissue upon ectopic expression (Lotan et al., 1998), is also confirmed by the miss-expression of several embryogenesis marker genes in the *BnLEC1* down-regulating embryos after 7 days in culture. These include *BnABI3* which is a gene originally identified in ABA signaling (Koornneef et al., 1998) and implicated in early embryogeny (Gaj et al., 2005; Malik et al., 2007), and *BnBBM1*, which when expressed ectopically is sufficient to spontaneously form somatic embryos in *Arabidopsis*, tobacco, and *B. napus* seedlings (Boutilier et al., 2002; Srinivasan et al., 2007).

The requirement of *BnLEC1* for MDE quality (Fig. 5.4B) is a novel concept suggesting a potential function of this gene in the establishment of the embryo body, specifically the proper development of cotyledons and the elongation of the embryonic axis in immature embryos (Fig. 5.5). These phenotypic abnormalities can be partially explained by the likely function of *LEC1*

downstream of auxin responses (Gaj et al., 2005). The role of auxin in the establishment of the bilateral symmetry during embryogenesis is well established and interference with the auxin flow results in malformed and partially fused cotyledons and stunted elongation of the embryonic axis (Chandler, 2008; Tsai, 2013). These structural abnormalities, apparent in MDEs down-regulating *BnLEC1* (Fig. 5.5), compromise their regeneration and correlate to the miss-expression of several embryogenesis genes, including the auxin-regulated gene *BnWOX2*. This gene modulates the auxin effects on embryonic tissue patterning and its altered expression compromises the normal progression of embryogenesis (Haecker et al., 2004). Deviations from the normal embryogenic pathway caused by the suppression of *BnLEC1* might also be caused by the altered expression of other embryo marker genes such as *BnLEC2*, necessary for maintenance of the suspensor and specification of cotyledons (Stone et al., 2001), as well as *BnCYP78A*, and *BnUPI1*, which have been associated to the normal development of MDEs (Malik et al., 2007).

Unlike its suppression, over-expression of *BnLEC1* only affects the number of embryos produced (Fig. 5.3A), but not their morphology and quality (Fig. 5.4A). Among possible reasons for the interference with the initiation of the embryogenic pathway is the miss-expression of *BnSERK1* at day 7. This gene encodes one of the several kinase regulators of the reprogramming of embryogenesis (Hecht et al., 2001). Originally characterized in carrot, *SERK1* has been reported in many other plant species, including *Arabidopsis* and wheat where it enhances embryogenic competence *in vitro* (Hecht et al., 2001; Schmidt et al., 1997; Singla et al., 2008).

Embryo quantity and quality were greatly impaired in mutants of *BnFUS3*, a gene encoding a B3 domain-containing protein present in developing embryos from a very early stage to immediately before germination (Luerßen et al., 1998). Together with *LEC1*, *FUS3* is a key regulator of *in vivo* embryogenesis (Wang and Perry, 2013); however, the different

morphological defects caused by the suppression of the two genes suggest separate roles. Besides the repressive effect on microspore-derived embryogenesis documented in our study (Fig. 5.3C), mutations of *FUS3* also impair *Arabidopsis* somatic embryogenesis (Gaj et al., 2005), observation denoting the function of this gene are retained across different species and embryogenic systems. The abnormal behavior observed in the *BnFUS3* mutant lines is associated with profound alterations in the expression of many embryo marker genes. Of interest, are the consistent repression of *BnWOX9* at day 7 and the induction of *BnWOX2* at day 14 and 21 that are required for the establishment of polarity domains within the embryo (Haecker et al., 2004). Furthermore, the increased expression at day 21 of *BnABI3*, suggests possible alterations in ABA signaling. Abscisic acid accumulates during the middle-late stages of embryogenesis and is required for the correct completion of the embryogenic program (Suzuki et al., 2000).

Collectively these studies demonstrate that altered expression of *BnLEC1* and *BnFUS3* affects oil accumulation and composition in cultured embryos. While over-expression of *BnLEC1* favors oil accumulation, suppression of either *BnLEC1* or *BnFUS3* reduces oil level. These changes were comparable to those observed in transgenic seeds. Both embryo yield and quality were also affected by the transgenes with over-expression of *BnLEC1* reducing the number of MDEs produced, while suppression of *BnLEC1* or *BnFUS3* reducing embryo yield and regeneration frequency. Based on these results it is suggested that Brassica microspore-derived embryogenesis is a suitable *in vitro* model for oil research, in particular to further examine the role of *BnLEC1* and *BnFUS3*, although the reduction in embryo number and quality can pose limitations to its use as a propagation tool to regenerate the transformed embryos.

5.6 Acknowledgement

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5.7 Supplemental data

Supplemental Table 5.1: Primer sequence (5'-3') used for embryo specific marker genes.

BnABI3	(F)	TACCAACACTCCCTGACTTCCCAT
BnABI3	(R)	AGTTGAGGAAGAAGCAGCGGAAGA
BnBBM1	(F)	CCCGCGGCAATGACGAATAATGTT
BnBBM1	(R)	ACTGTTCACCAGCACACAACAACG
BnCYP78A5	(F)	ACATTCCTTGGAACAGCCTTGCAC
BnCYP78A5	(R)	AGCAATGGGTCTAGCCACTGTTCA
BnLEC1	(F)	TATCTTGCCGCAGCAACAACCAAG
BnLEC1	(R)	TTCACCGGTCACGAAGCTGATGTA
BnLEC2	(F)	ACAAGAATCGCTCGCACTTCTCCA
BnLEC2	(R)	AAGCATCCGATGAGTGAAGAGGCT
BnSERK1	(F)	ATGCGTTGCATACTCTGAGGGTCA
BnSERK1	(R)	ACGCTGTTCTCGTTGTTGCAAGTG
BnUPI1	(F)	GCACCATAACAACAAGGGTGCTGA
BnUPI1	(R)	CAATCCGGTTGCACTGGATCTGTT
BnWOX2	(F)	CGCAAGTCCCGTAGCAAACACAAA
BnWOX2	(R)	AAGACGATGCTGAGGAAAGAGGCT
BnWOX9	(F)	ATCACGGTTATTCTAGCGAGGGCA
BnWOX9	(R)	TCATCGCCACAATAAGTCACCGGA
BnUBC21	(F)	CCTCTGCAGCCTCCTCAAGT
BnUBC21	(R)	CATATCTCCCCTGTCTTGAAATGC

Supplemental Table 5.2: Primer sequence (5'-3') used for sucrose and fatty acid metabolism

BnACCA2-F	TTGCCAGTCATAGTCCAGAGGCAT
BnACCA2-R	TCTGCTGCGATGTCCATGAAGGAT
BnAGP-F	TGCTGGAACGAGATTGTATCCGCT
BnAGP-R	ATGGCGGTTGAGAGAAGCTGAGTT
BnFAD3-F	TATAAGGGCGGCCATTCCTAAGCA
BnFAD3-R	AGATAGCCCAGAACAGGGTTCCTT
BnFAE1-F	TCTCCGCGATGGTCGTTAACACTT
BnFAE1-R	TCCTTGGACAAACTCACTCCGGTT
BnFPA-F	TGAGTTGATCGCTAACGCCGCATA
BnFPA-R	TTCTCGACGTTGATGCTGGCAAGA
BnGPDH-F	ACGGAAAGTTGACCGGAATGTCCT
BnGPDH-R	ACAACATCGTCCTCGGTGTAACCA
BnHXK-F	ATCAGCTGCAGGGATCTACGGAAT
BnHXK-R	ACTCGCTGAACTGAGTGTAGTGCT
BnMACT-F	TCAAGCAGTAGGGATGGGCAAAGA
BnMACT-R	ACGAACACGGAGCAATTCAACAGC
BnPGK-F	AGGCACAAGGTCTGTCTGTTGGAT
BnPGK-R	AGCGAACTTGTCAGCAACCACAAC
BnPPK-F	AGGTGCGCCGTTTAAGAAATTCGC
BnPPK-R	AGAATGAAAGGAAGGCCGGCTACT
BnSUC1-F	ACCAATCGTCGGTTACCACAGTGA
BnSUC1-R	ATATCCGCGGCGTATCCGATCAAA
BrSUC4-F	ACAAGGATTCAGAAGCCCATCCCT
BrSUC4-R	ATCTCCTTCCACCATGCCTTCACA
BrSUS1-F	TCTCCGTGTGCCTTTCAGAACAGA
BrSUS1-R	AGAGGCAACGAGGTTTCCATCACT
BrSUS3-F	TTAAGCAGCTTCTCGGCAACCTCA
BrSUS3-R	CGAACAAGCCGGATGTCAACCTTT
BnATR1-F	TGGCCTTATGGAGGAGTTAGAGGA
BnATR1-R	ATCAGTTACGTCAACCTCAGGCGA
BnMYB28-F	AAGCAATACTCCCGGTCAAGCTCA
BnMYB28-R	ACTGGTGTCCCATCTTTGCTGGTA

BrMAM1-F	AGACCATTGCCAAGACTGTAGGGA
BrMAM1-R	AACCGCCTCGATGTCTCTATGGTT
BnCYP79B2-F	ACTTCACCGTCGGGTAAAGATGCT
BnCYP79B2-R	ACACGTGTTGAGCTGATGGAGTCT
BnSUR1-F	TGGCTTCACCAGAAGAGAGCTGAA
BnSUR1-R	ACAGTAATGCCTCGTGACAAACCG
BnST5a-F	ATGGCTGCTCGTATCGATGGGTTA
BnST5a-R	CCTCTGTTCCGCACCAAACAACAA
BnCYP83B1-F	AAAGATGGACGTCATGACCGGACT
BnCYP83B1-R	CATCACGCCTGATCAAATGTGCGT

6 GENERAL DISCUSSION AND CONCLUSIONS

Due to health benefits of the oil, canola oil has acquired enormous economic value, especially in Canada, where its production contributes about $19.3 billion a year to the national economy. (http://www.canolacouncil.org/markets-stats/industry-overview/economic-impact-of-the-canola-industry/). In the seed, quality of oil and protein are both important as they are valued for food and feed purposes (Pastuszewska et al., 2000; Yoshie-Stark et al., 2008).

Seed oil accumulation and biosynthesis of fatty acids (FA) are strongly linked to the genetic potential of specific cultivars. The biochemical pathways leading to the accumulation of oil in seed occur in numerous steps requiring the participation of gene networks, many of which are also implicated in embryo and seed development. Thus, understanding the molecular mechanisms governing embryogenesis is paramount for enhancing oil production.

Seed development is generally divided into two overlapping phases; the first identified as morphogenesis where the body of the embryo is established through a precise regulation of cell division and differentiation (West and Harada, 1993; Harada, 2001). This is followed by the maturation phase characterized by the accumulation of storage products which in *B. napus* mainly consist of lipids (Harada, 1999; Braybrook and Harada, 2008). Lipids are stored in the form of triacylgylcerols (TAG), the synthesis of which is dependent on the photosynthesis rate and transport of sucrose (Ohlrogge and Browse, 1995). Active photosynthesis and rapid carbon flux are key factors that improve fatty acid (FA) synthesis.

Fatty acid (FA) biosynthesis initiates in the plastids and requires the activity of a multi-enzyme complex termed, fatty acid synthase (FAS). Acetyl-CoA, derived from pyruvate (Johnston et al., 1997) is initially transferred to malonyl-CoA in a reaction catalyzed by the enzyme acetyl-CoA carboxylase (ACCase) (Konishi et al. 1996; Li-Beisson et al., 2010), which is believed to be the rate limiting step of the *de novo* FA biosynthesis (Baud et al., 2008).

Various condensation and reduction reactions are needed for the elongation of fatty acyl chains and the release of free FA into the cytoplasm. The final composition of FA, i.e., FA profile, is mediated by the combined activity of several enzymes through mechanisms still partially unknown (Baud et al., 2008; Baud and Lepiniec, 2009). It is generally accepted that polyunsaturated FA are formed in two separate compartments: the endoplasmic reticulum and plastids (Sperling et al., 1993).

As noted above, several genes participating in oil production are also involved in embryogenesis, including *LEC1*, a member of the HAP3 subunit of the CCAAT (CBF) binding factor family. Members of this family are grouped into distinct subclasses, one including non-*LEC1* genes and a second including *LEC1* type (i.e. *LEC1* and *LEC1-like*) transcription factors known to exercise crucial functions during embryo patterning (Parcy et al., 1994; Kwong et al., 2003; Lee et al., 2003; Yamamoto et al., 2009). Unique signatures of these CFB members are three domains, HAP3 (CBF-A, NF-YB), (CBF-B, or NF-YA), and HAP5 (CBF-C, NF-YC) that interact to form trimeric complexes (Sinha et al., 1995; Kim et al., 1996; Sinha et al., 1996; Maity and de Crombrugghe, 1998; Mantovani, 1999; Romier et al., 2003). The transcriptional regulation of *LEC1* is exercised on downstream genes containing the specific CCAAT motif located in their promoter region (Lotan et al., 1998). Some of these genes mediate oil synthesis and accumulation (Mu et al., 2008).

The hypothesis tested in the first chapter was whether alterations in *BnLEC1* expression influence oil synthesis and FA in *B. napus*. Various *BnLEC1* transgenic lines were developed by using the *BnLEC1* version A (GU945399) and version B (GU945398), which differ by 13 nucleotides and 4 amino acids. Seed oil content was increased in lines over-expressing *BnLEC1* while it was repressed in those where *BnLEC1* was suppressed. The highest level in oil content

was measured in the *BnLEC1* transgenic lines over-expressing version A (GU945399) of *BnLEC1*. This observation raises the probability that the two versions (A and B), might influence seed oil biosynthesis differently.

Minor phenotypic differences (from the wild type) were detected in the *BnLEC1* over-expressing line characterized by the lowest expression of the gene in leaf tissue and a 12 % increase in total seed oil. It is therefore proposed that fine-tuning the expression of *BnLEC1* can be used to increase the seed oil accumulation without affecting the agronomic characteristics of the plants. Over expression of *BnLEC1* leads to the transcriptional alterations in enzymes involved in sucrose metabolism and glycolysis, suggesting an active consumption and fast oxidation of sucrose, which is necessary for increasing the pool of FA biosynthetic precursors. Down regulation of *BnLEC1* had the opposite effect as it suppressed several sucrose biosynthetic genes and genes involved in glycolysis.

The constitutive expression of *BnLEC1* had no clear effect on the FA composition, did not influence the nutritional value of oil, and did not alter glucosinolate (GLS) contents. This was in contrast to lines suppressing *BnLEC1* which had elevated levels of GLS possibly due to the induction of sulphotransferase5a, the last GLS biosynthetic enzyme. The observation that ectopic expression of *BnLEC1* does not decrease GLS levels might indicate that a minimum threshold of GLS is required for normal seed function and plant regeneration.

Enhanced expression of *BnLEC1* also affected the transcript levels of various genes involved in FA biosynthesis and seed developmental processes. These included *BnWRI1*, known to enhance the flux of carbon towards production of seed oil (Meinke et al., 1994; Baud et al., 2007; Maeo et al., 2009) and other transcription factors containing the B3 domain such as *FUS3* and *LEC2*.

FUS3 and *LEC2* have been studied extensively in relation to embryo and seed development (Bäumlein et al., 1994; Santos-Mendoza et al., 2008; Yamamoto et al., 2009). The expression of *FUS3*, for example, influences ABA signaling, thus affecting many ABA-mediated processes, including storage product deposition occurring during late embryogenesis (Curaba et al., 2004; Gazzarrini et al., 2004; Santos-Mendoza et al., 2008; Wang and Perry, 2013). In concert with *LEC2*, *FUS3* also affects somatic embryogenesis, the ability of somatic cells to produce embryos in culture (Keith et al., 1994; Meinke, 1994).

Mutant analysis has been employed to assess the function of these transcription factors during embryogenesis. *Arabidopsis* plants where *LEC1* and *LEC2* have been mutated fail to form storage products and undergo precocious germination (reviewed by Harada, 2001; Wang et al., 2007). Consistent with this observation, mutation of *FUS3* alters protein and lipid biosynthesis, through the modulation of FA biosynthetic genes (Meinke et al., 1994; Harada, 2001). Over expression of the *Arabidopsis FUS3* induces the expression of genes encoding the two main types of seed storage proteins, 2S albumin and 12S cruciferin, as well as genes taking part in FA synthesis, including *ACETYL-COA CARBOXYLASE*, *PYRUVATE DEHYDROGENASE*, *KAS I*, *KASII*, and *KASIII* (Wang et al., 2007).

The established relationship between *LEC1* and *FUS3* during oil synthesis in *Arabidopsis* (Meinke et al., 1994; Harada, 2001; Mu et al., 2008) was the premise of the second chapter where the effects of mutations on *FUS3* on *B. napus* seed oil were assessed. Towards this end, three *BnFUSCA3* (*BnFUS3*) TILLING mutant lines characterized by single amino acid changes downstream of the B3 domain were generated. Mutation in the *BnFUS3* gene reduced the level of seed oil content and the number of siliques in mature plants. Analysis of the FA profile revealed that *fus3* seeds had increased levels of linoleic acid, possibly due to the lower

expression of *ω-3 FA DESATURASE* (*FAD3*). The reduction in oil content observed in the *BnFUS3* TILLING mutant lines was linked to the transcriptional suppression of genes encoding transcription factors stimulating FA synthesis, such as *LEC1*, *LEC2*, *ABI3* and *WRI1*, as well genes encoding key enzymes of metabolic pathways upstream of FA biosynthesis (i.e. sucrose transport and metabolism and glycolysis). Mutations outside the *BnFUS3* B3 domain might affect the downstream target genes by affecting the structure of the protein. These observations are suggestive of a reduced carbon pool available for the synthesis of FA that leads to oil biosynthesis.

In vitro embryogenesis is often used as model system to study *in vivo* embryogenesis. The process can generate a large number of somatic embryos similar in morphology to their zygotic counterparts (Raghavan, 2000; Mordhorst et al., 2002; Stasolla et al., 2008). Studies dealing with the role of *LEC1*, *LEC2* and *FUS3* during *in vivo* embryogenesis are available (Meinke et al., 1994; Lotan et al., 1998; Kwong et al., 2003; Cernac and Benning, 2004; Gazzarrini et al., 2004; Wang and Perry, 2013) and reveal specific expression patterns of these genes. While *LEC1* is expressed throughout seed development (West et al., 1994), expression of *FUS3* is restricted to the late maturation stages (Curaba et al., 2004; Santos-Mendoza et al., 2008).

As an extension of the first two research chapters, the third chapter investigates the role played by these two genes during *B. napus* microspore-derived embryogenesis. This study shows that proper expression of *BnLEC1* and *BnFUS3* is crucial for normal development of the microspore-derived embryos (MDEs) and their ability to accumulate storage products. Over-expression of *BnLEC1* reduces the yield of MDEs without affecting their quality and capability to regenerate plants. The reduced embryogenic capacity was related to the suppression of

BnSERK1, a regulator of embryogenic competence in culture (Hecht et al., 2001). Suppression of *BnLEC1* and *BnFUS3* reduced the number of MDEs, their regeneration frequency, and caused morphological defects during embryogenesis. These morphological defects were linked, at least in part, to the alteration of embryonic marker genes and to the interference of *LEC1* and *FUS3* with auxin signalling (Casson and Lindsey, 2006; Chandler, 2008; Tsai, 2013). Auxin is crucial for the establishment of embryo patterning (Chandler, 2008; Tsai, 2013). Total oil content was increased in MDEs over-expressing *BnLEC1* and decreased in those suppressing *BnLEC1* or *BnFUS3*. Mutation of *BnFUS3* also resulted in a significant increase in linoleic (C18:2) acid. These changes in oil accumulation were similar to those observed *in vivo.*

In conclusion, the present studies provide evidence for the role played by *BnLEC1* and *BnFUS3* in seed oil accumulation and during the formation of MDEs in *B. napus.* Seed oil content is increased by the over-expression of *BnLEC1* and decreased when *BnLEC1* is repressed or when *BnFUS3* is mutated. The changes in oil levels were associated with transcriptional changes of genes encoding key enzymes of sucrose metabolism and glycolysis. This observation suggests that proper expression of *BnLEC1* and *BnFUS3* is required to sustain the flow of carbon to be utilized for the synthesis of FA. The changes in oil level observed in seeds as a result of altered expression of *BnLEC1* and *BnFUS3* were also retained during microspore-derived embryogenesis, indicating a similar function of the two genes *in vivo* and *in vitro*.

Collectively, findings from this thesis support the hypothesis that targeted manipulations of *BnLEC1* and *BnFUS3* can be used as a tool to modulate the seed oil quantity and quality in the economically important species *B. napus*.

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LIST OF ABBREVIATIONS

ABA, ABSCISIC ACID

ABI3, ABSCISIC ACID INSENSITIVE3

ACCA2, SUBUNIT A OF ACETYL-CoA CARBOXYLASE

ACCase, ACETYL-CoA CARBOXYLASE

ACP, ACYL CARRIER PROTEIN

ADP, ADENOSINE DIPHOSPHATE

AGP, ADP-GLUCOSE PHOSPHORLAYSE

At, ARABIDOPSIS THALIANA

ATP, ADENOSINE TRIPHOSPHATE

ATR1, ALTERED TRYPTOPHAN REGULATION1

B5 MEDIUM, GAMBORG B5 MEDIUM

BBM1, BABY BOOM1

Bn, BRASSICA NAPUS

BPS, PHOSPHATE BUFFER

Br, BRASSICA RAPA

C16:0, PALMITIC ACID

C18:0, STEARIC ACID

C18:1, OLEIC ACID

C18:2, LINOLEIC ACID

C18:3, LINOLENIC ACID

C20:0, ARACHIDIC ACID

CBF, CCAAT BOX BINDING FACTOR

cDNA, COMPLEMENTARY DEOXYRIBONUCLEIC ACID

CJE,CELERY JUICE EXTRACT

CLV, CLAVATA

CoA, Co ENZYME A

CPT, CHOLINEPHOSPHOTRANSFERASE

CYP79B2, CYTOCHROME P450 CYP79B2

CYP83B1, CYTOCHROME P450 CYP83B1

DAG, DIACYLGLYCEROL

DAGAT, DIACYLGLYCEROL ACYLTRANSFERASES

DAP, DAY AFTER POLLINATION

DNA, DEOXYRIBONUCLEIC ACID

ER, ENDOPLASMIC RETICULUM

EST, EXPRESSED SEQUENCE TAG

FA, FATTY ACID

FAD3, ω-3 FA DESATURASE

FAE1, FA ELONGATION1

FAS, FA SYNTHASE

FAT, ACYL-ACP THIOESTERASE

FPA, FRUCTOSE BISOPHOSPHATE ALDOLASE

FUS3, FUSCA3

G3P, GLYCEROL-3-PHOSPHATES

G3PAT, GLYCEROL-3-PHOSPHATE ACYL TRANSFERASE

GAs, GIBBERELLINS

GLS, GLUCOSINOLATES

GPDH, GLYCERALDEHYDES-3-PHOSPHATE DEHYDROGENASE

HAP3, HEME- ACTIVATED PROTEINS 3 SUBUNIT

HXK, HEXOSE KINASE

IAA, INDOLE ACETIC ACID

IBA, INDOLE BUTYRIC ACID

IPT, ISOPENTENYL TRANSFERASE

KAS, KETOACYL-ACP SYNTHASE

KCS, 3-KETOACYL-COA SYNTHASE

KNOX, KNOTTED1-LIKE HOMEOBOX

L1L, LEAFY COTYLEDON1-LIKE

LB, LURIA BERTANI

LEC1, LEAFY COTYLEDON1

LEC2, LEAFY COTYLEDON2

LPA, LYSOPHOSPHATIDIC ACID

LPAAT, LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE

LPCAT, LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASE

MAM, METHYLTHIOALKYLAMALATE SYNTHASE

MCAT, MALONYL-CoA:ACP TRANSCYLASE

MDE, MICROSPORE-DERIVED EMBRYO

MS MEDIUM, MURASHIGE AND SKOOG MEDIUM

MYB28, MYB-CONTAINING TRANSCRIPTION FACTOR28

NADP+, NICOTINAMIDE ADENINE DINUCLEOTIDES PHOSPHATE OXIDIZED

NADPH, NICOTINAMIDE ADENINE DINUCLEOTIDES PHOSPHATE REDUCED

NCBI, NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION

NIR, NEAR INFRARED SPECTROSCOPY

OLS, OLEOSINS

OPPP, OXIDATIVE PENTOSE PHOSPHATE PATHWAY

PA, PHOSPHATIDIC ACID

PAP, PHOSPHATIDIC ACID PHOSPHOHYDROLASE

PAS, PERIODIC ACID-SCHIFF'S REACTION

PC, PHOSPHATIDYLCHOLINE

PCR, POLYMERASE CHAIN REACTION

PDAT, PHOSPHOLIPID: DIACYLGLYCEROL ACYLTRANSFERASE

PGK, PHOSPHOGLYCERATE KINASE

PPK, PYROPHOSPHATE-DEPENDENT PHSOPHOFRUCTOSE

QTL, QUANTITATIVE TRAIT LOCI

RNA, RIBONUCLEIC ACID

RT, REVERSE TRANSCRIPTION

SAS, STATISTICAL ANALYSIS SOFTWARE

SERK, SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE

ST5a, SULFOTRANSFERASE5a

STM, SHOOT MERISTEMLESS

SUC, SUCROSE TRANSPORTER

SUR, SUPERROOT

SUS, SUCROSE SYNTHEASE

TAG, TRIACYLGLYCEROLS

TBO, TOLUIDINE BLUE O

TFs, TRANSCRIPTION FACTORS

TIBA, 2, 4, 6-TRIIODOBENZOIC ACID

UHP HELIUM, ULTRA HIGH PURITY HELIUM

UPI1, UKNOWN PROTEIN1

WOX, WUSCHEL-RELATED HOMEOBOX

WRI1, WRINKLED1

WT, WILD TYPE

WUS, WUSCHEL