Seed-specific expression of the class 2 *Phytoglobin* (*Pgb2*) increases seed oil in *Brassica napus* L.

by

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DEDICATION

Dedicated to my mother Saifura Begum and my mother-in-law Halima Begum, whose prayers save me in every difficult time,

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TABLE OF CONTENTS

Contents **Page no.**

ACKNOWLEDGEMENTS……..…….ii

DEDICATION……..…….iii

LIST OF FIGURES……...vi

LIST OF ABBREVIATIONS……..viii

FOREWARD…….ix

1. LITERATURE REVIEW…….1
   1.1 Canola, an economically important crop……1
   1.2 Canola: oil quality and nutritional value…….4
   1.3 Seed development and embryogenesis……6
   1.4 Fatty acid (FA) synthesis and oil accumulation……...8
   1.5 Seed oil manipulation in canola……11
   1.6 Transcription factors regulating seed oil accumulation…….13
   1.7 Phytoglobins: overview of their function……..17

2. ABSTRACT……...21

3. INTRODUCTION…….22

4. MATERIALS AND METHODS……..…….25

   4.1 Generation of transgenic *B. napus* plants with seed specific expression of *BnPgb2*……25
   4.2 Plant growth conditions, collection of seeds for expression analyses and agronomic characteristics……...…25
   4.3 Histological localization of starch, proteins and oil bodies……...…27
   4.4 Gene expression analyses……..27
   4.5 Determination of seed protein level, oil content and fatty acid (FA) composition……28
   4.6 Statistical analyses…….28

TABLE OF CONTENTS (cont.)

Contents **Page no.**

5. RESULTS……..……..…..29

5.1 *BnPgb2* expression in seeds of natural germplasm ……..29

5.2 Changes in seed oil level and fatty acid (FA) composition by *BnPgb2*……...…….33

5.3 Time course analyses of *BnPgb2* expression and storage deposition in developing seeds…….38

5.4 *BnPgb2* regulation of transcription factors and enzymes of sucrose metabolism and fatty acid (FA) synthesis……..41

6. DISCUSSIONS…….…..……45

7. CONCLUSIONS…….…….….50

8. FUTURE DIRECTIONS…….…..….51

9. REFERENCES……..53

10. SUPPLEMENTARY TABLES……..…..72

LIST OF FIGURES

No.	Title	Page
1	Top 30 rapeseed-producing nations in 2019	3
2	Developmental stages of Arabidopsis seeds	7
3	Overview of seed fatty acid synthesis	9
4	Overview of the regulatory network and interaction of key transcription factors controlling seed development and oil synthesis	16
5	Simplified Pgb/NO cycle	18
6	Sequence of the Cruciferin 1 promoter used to drive the expression of *BnPgb2*	26
7	Gel confirming the presence of the insert in genomic DNA of the transformed Cru:BnPgb2 lines	26
8	Total oil and expression of *BnPgb2* in *B. napus* seeds of high (HO) or low (LO) oil genotypes.	30
9	Morphology of the developing (10-18 DAP) embryos in the three high (HO) or low (LO) oil genotypes	31
10	Localization of protein bodies in the developing (14-35 DAP) embryos of the three high (HO) or low (LO) oil genotypes	32
11	Localization of oil bodies in the developing (21-35 DAP) embryos of the three high (HO) or low (LO) oil genotypes	34
12	Expression of *BnPgb2* and quantification of storage products in seeds of the *BnPgb2* over-expressing Cru:BnPgb2 lines (1, 2, 3, 6, and 10)	35
13	Fatty acid composition measured during the 2021 growing season in dry seeds of WT and Cru:BnPgb2 plants.	36

LIST OF FIGURES (Cont.)

No.	Title	Page
14	Measurements of oil, proteins, and FA composition in WT plants and plants over-expressing *BnPgb2* [Cru:BnPgb2]. Measurements were performed during the 2022 growing season	37
15	Time course expression of *BnPgb2* and localization of storage products in developing WT seeds and seeds of the selected Cru:BnPgb2 lines 1 and 6	39
16	Morphology of developing (10-22 DAP) embryos in the WT line and the selected Cru:BnPgb2 lines 1 and 6	40
17	Expression levels of the transcription factors LEAFY COTYLEDONS1 (*LEC1)*, *FUSCA3* (*FUS3)* and *WRINKLED* (*WRI1*), and enzymes related to oil synthesis in developing seeds (14-35 DAP) of the WT line and the selected Cru:BnPgb2 lines 1 and 6	43

LIST OF ABBREVIATION

ACCA- Acetyl-CoA carboxylase

ACP- acyl carrier protein

ADP- Adenosine diphosphate

ATP- Adenosine triphosphate

DAG- Diacylglycerol

DAP- Day after pollination

FA- Fatty acid

GSL- Glucosinolates

HO- High oil

KAS- Ketoacyl-ACP synthase

LO- Low oil

NO- Nitric oxide

Pgb- Phytoglobin

TAG- Triacylglycerol

WT- Wild type

FORWARD

This thesis follows the paper style outlined by the Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. The manuscript follows the style recommended by the Journal of Plant Physiology. This thesis is presented as a single manuscript, containing an abstract, introduction, materials and methods, results, discussion, and conclusion. A literature review precedes the manuscript, while supplementary figures are positioned after the body of the manuscript.

1. LITERATURE REVIEW

1.1 Canola, an economically important crop

Canola (*Brassica napus* L.), the most widely cultivated mustard in the world, is a member of the *Brassicaceae* family (also known as the Cruciferae), which also comprises 338 genera and 3709 species (Warwick et al., 2006), with about 39 species in the *Brassica* genus. *Brassica napus* is one of the most important species in this family and the second-most widely produced oil crop in the world after soybean (Raboanatahiry et al., 2021). Due to its very low percentage of saturated fatty acids (FA) and a balance of omega-6 and omega-3 FA, canola oil's value in international markets has increased considerably over the past several years (Cardoza and Stewart, 2007). The amount of protein and glucosinolates in the canola seed is one indicator of its quality (Elahi et al., 2016). Canola meal has been suggested as an alternative source of human food (Uppstrom, 1995) in addition to its usage as cattle feed (Uruakpa and Arntfield, 2005).

Around 7500 years ago, turnip rape (*B. rapa*) and cabbage (*B. oleraceae*), through a spontaneous hybridization, gave rise to rapeseed (Chalhoub et al., 2014). Now, rapeseed is widely produced around the world. Rapeseed was first cultivated in India around 4000 BC and later spread to China and Japan around 2000 BC. It was naturally introduced to Europe and New Zealand, where wild forms of ancestors were also identified (Snowdon et al., 2006; Shahzadi et al., 2015). In the sixteenth century, rapeseed was commercially grown in the Netherlands as a lubricant for steam engines (Gupta and Pratap, 2007). Around the world, 66 countries produce approximately 70 million tons (MT) of canola annually, including 34 countries in Europe, 15 countries in Asia, 9 countries in the Americas, 6 countries in Africa, and 2 countries in Oceania (Raboanatahiry et al., 2021). Canada (19 MT), China (13.1 MT), and India (7.7 MT) were the top canola producing

countries in 2019/2020; meanwhile the European Union produced 16.83 MT of canola (Raboanatahiry et al., 2021) (Fig. 1). The Canadian Oilseed Processors Association has trademarked the title "canola," which refers to cultivars that yield seed oil with less than 2% erucic acid and meal with less than 30 micromoles of glucosinolates per gram (Harvey and Downey 1964; Fu et al., 2015; Ton et al., 2020). Dr. Baldur Stefansson registered Tower as the world's first canola cultivar in 1974 at the University of Manitoba (Scarth and Tang, 2006), and since then canola has become an international oilseed crop (Cabak, 2013).

Canola's contribution to the Canadian economy has more than tripled in the last 10 years, according to the newest independent analysis commissioned by the Canola Council of Canada. Canadian-grown canola contributes $29.9 billion to the Canadian economy each year, including more than 207,000 Canadian jobs and $12 billion in wages (Canola Council of Canada, 2020). Canada produced a total of 18.2 million metric tons of canola in the 2022 calendar year, from which 8.77 million metric tons were crushed to generate 3.65 million metric tons of oil (Statistics Canada, 2022). The Canola Council of Canada has predicted that by 2025, the amount of vegetable oil demand would increase to 250 MT from the 150 MT in 2015. A new goal for 2025 is to raise canola output to 26 million metric tons, in order to fulfill the growing demand of the worldwide market. Over 90% of Canada's canola production and processing occur within the prairie provinces of Saskatchewan, Alberta and Manitoba (Canola Council of Canada, 2014).

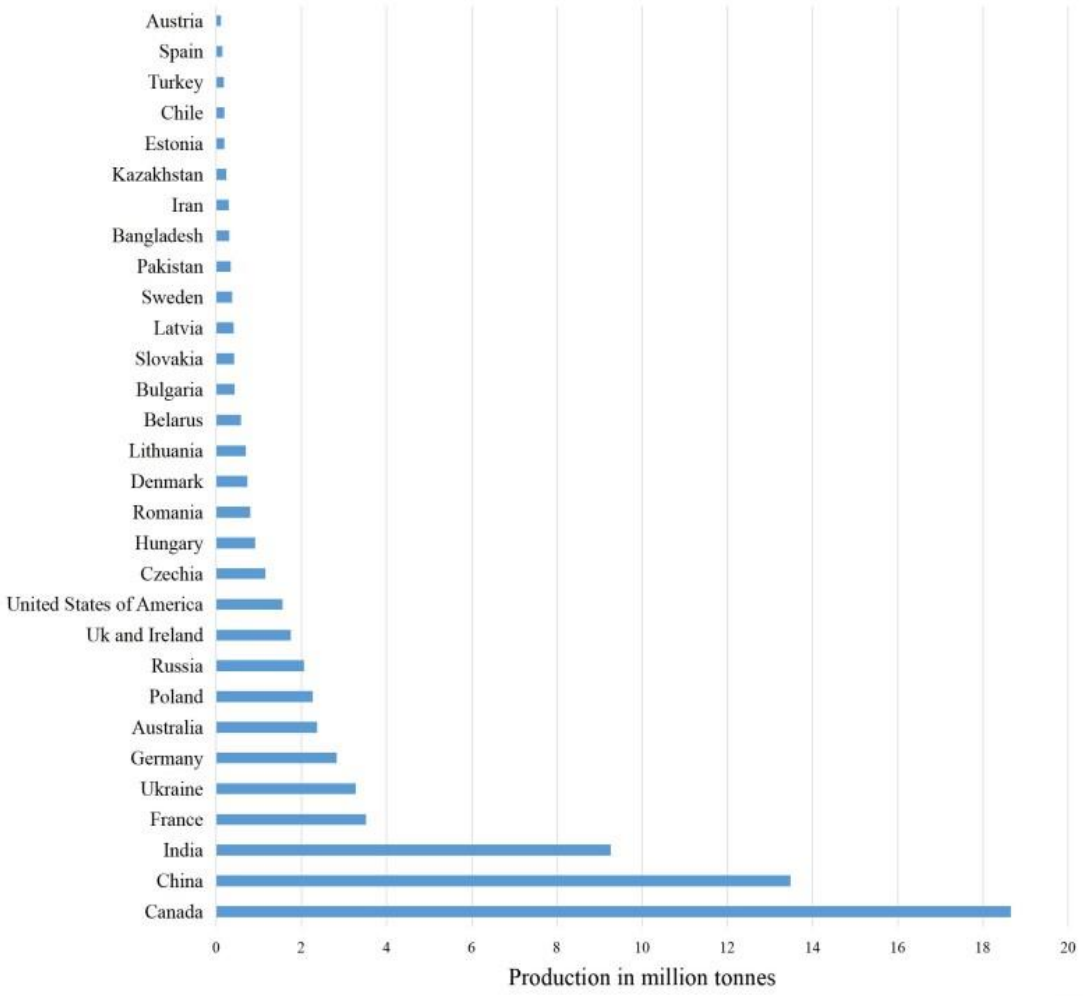

Figure 1. Top 30 canola-producing nations in 2019 (FAOSTAT, 2021; Raboanatahiry et al., 2021, permission obtained).

Canola oil is highly nutritious for human consumption and is also important for a wide range of industrial uses including cosmetics, biofuels, lubricants, and paints (Przybylski et al., 2005; Weselake et al, 2009). Canola oil's fatty acid (FA) composition and other compounds enhance its positive effects on human health, making it appropriate for use by people with a variety of conditions or even as a means of disease prevention. Canola seed oil and protein contents vary in different cultivars, as well as other components such as glucosinolates, phenols, and phytic acid

(Raboanatahiry et al., 2021). Improvements in oil content and FA composition continue to be a top priority in many breeding projects, to match the economic demand of oilseed. Therefore, it is crucial to research methods to increase seed oil content and yield per unit area of land.

1.2 Canola: oil quality and nutritional value

Plant-based oils are essential compounds for overall human health and provide benefits towards a wide range of health conditions such as stiffness, dry skin, and nervous system illnesses (Boelsma et al., 2001; Carter et al., 2013). As suggested by the Food and Agricultural Organization of the United Nations, canola meal protein contains all essential amino acids and has the potential to be consumed by humans (World Health Organization, 1985). Plant-based oils naturally include antioxidants and vitamin E, which reduce the risk of heart disease and cancer (Newkirk and Daun, 2011). Plant-based oils are not only employed in human nutrition; almost 15% of plant oil production is used in the manufacturing of chemical feed stocks, soaps, lubricants, cosmetics, and paints (Carlsson, 2009). More recently, plant-based oils have also become important as a source of biofuel to minimize the dependence on fossil fuels (Durrett et al., 2008).

The final product of plant-based oil ultimately depends on the FA profile. Canola's oil is enriched in many desirable FA, including palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (Ohlrogge and Browse, 1995; Töpfer et al., 1995). Specifically, canola oil is characterized by higher percentages of α-linolenic (C18:3), linoleic (C18:2), and oleic (C18:1), and lower levels of the undesirable erucic acid (C22:1) (Eskin and Przybylski, 2003). Improved stability of oil under high cooking temperatures for food processing is provided by low quantities of polyunsaturated fatty acids and higher amounts of monounsaturated fatty acids like oleic acid (18:1) (Abbadi and Leckband, 2011). Canola oil is considered one of the healthiest

dietary oils as it contains nearly 61% monounsaturated fat, 32% polyunsaturated fat, and only 7% saturated fat (reviewed by Elahi et al., 2016). In addition, linolenic acid (C18:3) is also considered an essential FA (Vaisey-Genser and Eskin, 1987). It has been observed that the use of linolenic acid in diets reduces the levels of low and high-density lipoproteins (LDL and HDL), which are the main contributors to several cardiovascular diseases (Vaisey-Genser and Eskin, 1987; Scarth and McVetty, 1999).

The amount glucosinolates and erucic acid are two significant factors that affect the nutritional quality of canola (Ali et al., 2009). Glucosinolates, common secondary metabolites produced by members of the Brassicaceae, are synthesized from glucose and amino acids (Sønderby et al., 2010). Being highly bioactive, they are detrimental to biological processes (Fenwick and Curtis, 1980), and at high levels can be harmful for human consumption causing thyroid, liver, and kidney disorders (Walker and Booth 2001). Canola meal contains low levels of total glucosinolates (less than 30 μmoles/g meal) (Sønderby et al., 2010).

Erucic acid, also known as cis-13-docosenoic acid, is a FA primarily found in large amounts in the seeds of Brassicaceae species and can account for more than 40% of the total seed FA content (Guan et al., 2014; European Food Safety Authority, 2016). Diets high in erucic acid have negative health effects (Beare et al. 1963). Canola oil contains less than 2% erucic acid (Food and Drug Administration, 2015). High erucic acid rapeseed cultivars are solely intended for non-food uses, to fulfill the demand of the oleochemical sector (Guan et al., 2014; European Food Safety Authority, 2016).

1.3 Seed development and embryogenesis

Events related to oil production are strictly linked to processes underlying seed development (Tian, 2020). The model plant *Arabidopsis thaliana* has been utilized as an experimental system in many seed developmental studies due to the abundance of molecular and genomic resources (Gepstein and Horwitz, 1995; Tian, 2020). Furthermore, the genetic similarities between *Arabidopsis thaliana* and *Brassica napus* have been exploited to gain knowledge on seed development and oil synthesis (Mayer et al., 1991; West and Harada, 1993; Verma et al., 2022).

The process of embryogenesis can be defined by three distinct steps: development, maturation, and dormancy (West and Harada, 1993; Baud and Lepiniec, 2009; Elahi, 2016). Development comprises morphogenesis and organogenesis, delineating the formation of the embryo (Goldberg et al., 1994; Jo et al., 2019). In higher plants, development is initiated by the double fertilization resulting in the formation of the diploid zygote (Harada, 1999; Tian, 2020). While one sperm fertilizes the egg, a second sperm nucleus fuses with the polar nuclei of the central cell forming a triploid endosperm that supports and nourishes the developing embryo (Harada, 1999; Baroux and Grossniklaus, 2019; Verma et al., 2020). In Arabidopsis, the zygote becomes polarized and gives rise to two asymmetrical daughter cells: a small apical cell and a large basal cell (Harada, 1999). Divisions of the apical cell give rise to the embryo proper, while the basal cell generates the suspensor (Kawashima and Goldberg, 2010). Through a very defined and conserved cell division and differentiation pattern, the embryo develops into a globular, heart-shaped, early cotyledonary, and late cotyledonary embryo (Nogler, 1984; Elahi, 2016). The body of fully developed embryos consists of a hypocotyl separating the root and shoot apical meristems at the opposite poles (Goldberg et al., 1994). The developmental phase overlaps with the maturation phase characterized by the initiation of storage product accumulation: starch, proteins,

and lipids (Harada, 1999; Jo et al., 2019). In Arabidopsis, maturation starts around the early heart stage of development and lasts until the seed desiccates (Fig. 2); storage products mainly accumulate in the cotyledons and embryo axis, since the endosperm is a transient structure. The type and composition of seed storage is species specific (Harada, 1999; Braybrook and Harada, 2008); Arabidopsis and Brassica seeds primarily accumulate lipids, such as triacylglycerols (TAGs), and seed storage proteins (12S globulins and 2S albumins) (Baud et al., 2002, 2008). Starch is found in very low concentrations in mature seeds. The acquisition of desiccation tolerance and dormancy marks the last phases of embryogenesis (Thomas 1993; Pelletier et al., 2017).

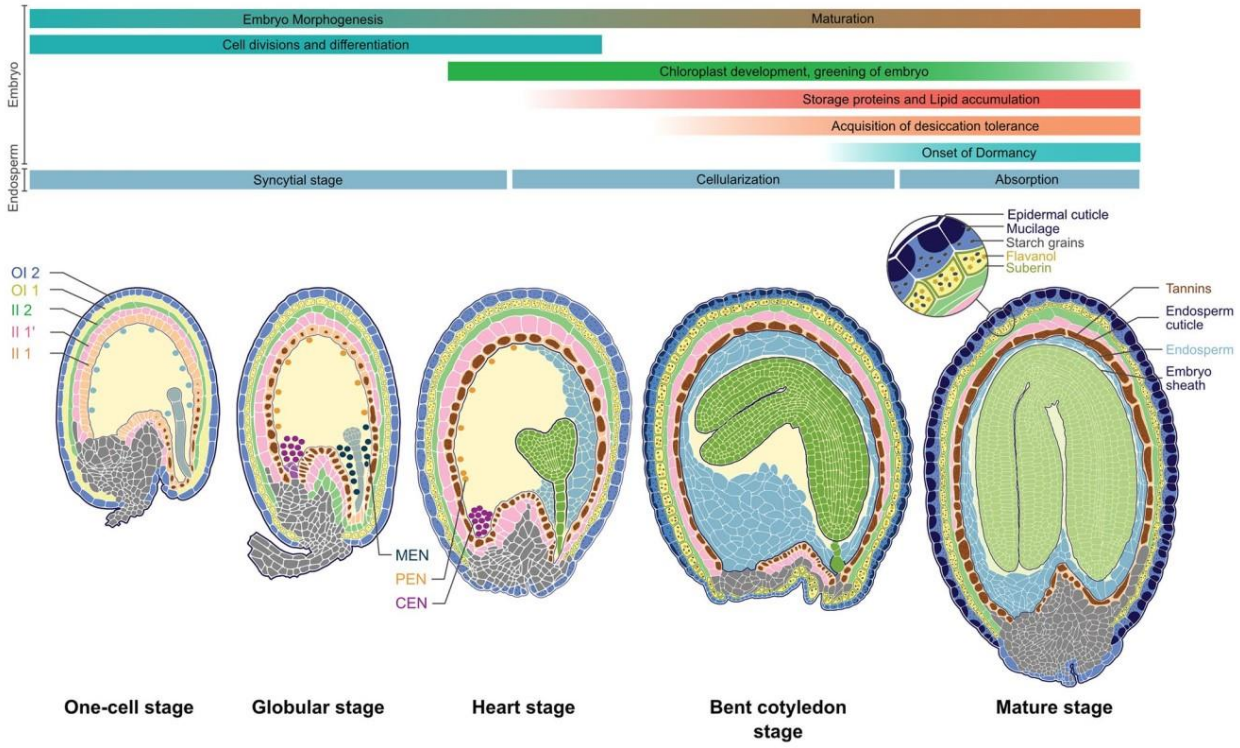

Figure 2. Developmental stages of Arabidopsis seeds (Verma et al., 2022, permission obtained).

1.4 Fatty acid (FA) synthesis and oil accumulation

Brassica seeds mainly accumulate lipids, primarily stored as triacylglycerol (TAG) which is formed by the esterification of FAs to the glycerol backbone (Murphy et al., 1989; Schwender and Ohlrogge, 2002). Oil synthesis, involving metabolic pathways housed in several compartments (Baud et al., 2002; Li-Beisson et al., 2013) (Fig. 3), can be limited by the availability of sucrose produced during photosynthesis (Baud and Lepiniec, 2009; Schwender et al., 2003). Flow of carbohydrates for FA synthesis is initiated in the glycolytic pathway where the oxidization of sucrose generates pyruvate through reactions producing ATP. Catabolic steps of hexoses in the pentose phosphate pathway also generate energy in the form of nicotinamide adenine dinucleotide phosphate (Schwender et al., 2003; Baud et al., 2007).

Synthesis of FA is initiated with the two-carbon molecule, acetyl-CoA, (Ohlrogge and Browse 1995) which is converted to malonyl-CoA by the enzyme Acetyl-CoA carboxylase (ACCase) (Konishi et al., 1996; Baud and Lepiniec, 2010; Li-Beisson et al., 2013) (Fig. 3). Through malonyl-CoA:acyl carrier protein malonyltransferase, malonyl group moieties are converted into an acyl carrier protein (ACP) that enters the FA biosynthesis pathway (Baud et al., 2008; Li-Beisson et al., 2013; Yang et al., 2022). Acetyl-CoA and malonyl-thioester undergo several condensation reactions (Li-Beisson et al., 2013), the first of which is catalyzed by 3-ketoacyl-ACP synthase (KAS III) and results in the production of 4:0-ACP. KAS I and KAS II catalyze other condensation reactions that produce 16:0-ACP and 18:0-ACP, respectively (Baud and Lepiniec, 2010; Yang et al., 2022). Two carbon molecules are added to the extended fatty acyl chain for every condensation reaction (Li-Beisson et al., 2013). Stearoyl-ACP desaturases are required for the next step in converting 18:0 to 18:1-ACP (Browse and Somerville, 1991).

Hydrolysis of 16:0-ACP and 18:1-ACP by acyl-ACP thioesterase (Fat) releases free FA into the cytoplasm (Baud et al., 2008; Li-Beisson et al., 2013).

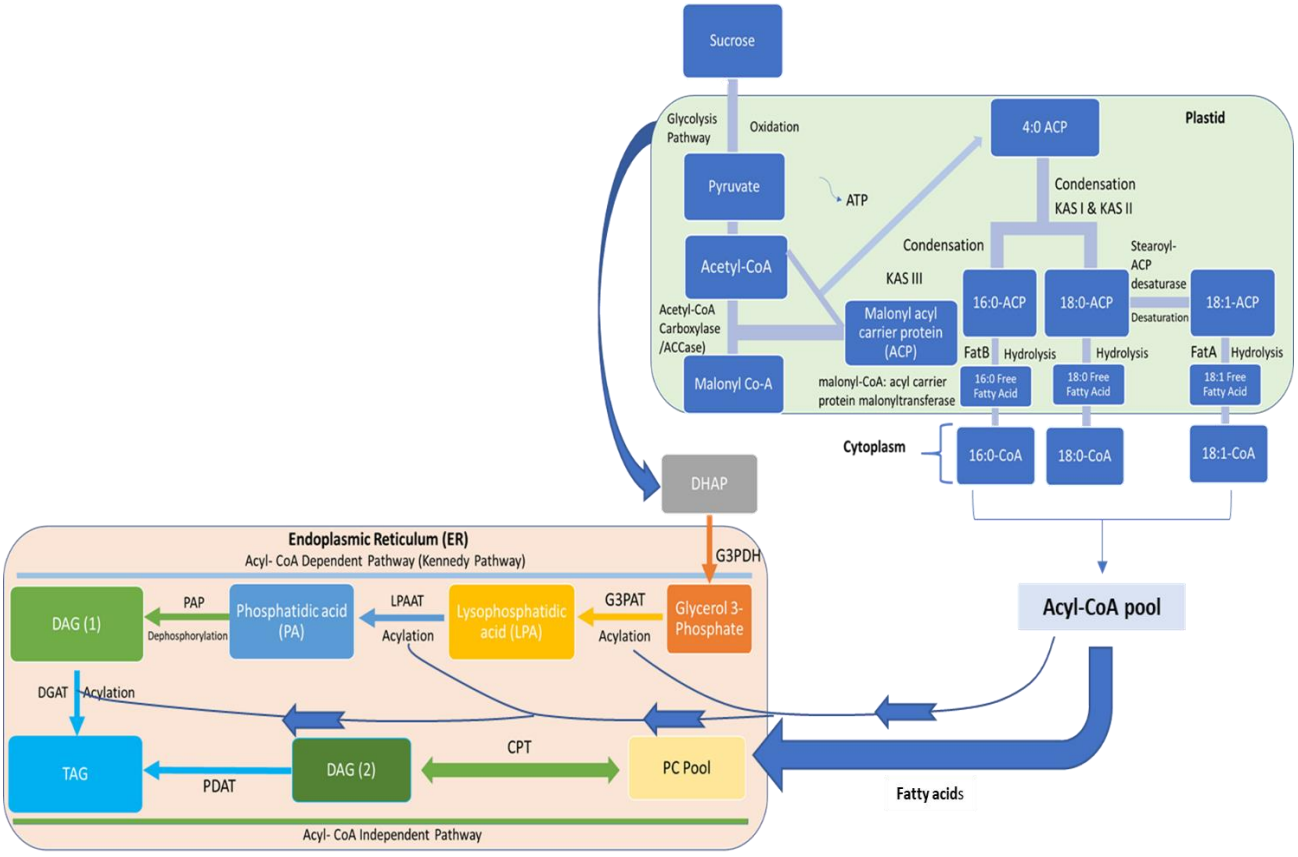

Figure 3. Overview of seed fatty acid synthesis (adapted from Elahi et al., 2016). KAS III, II and I (3-ketoacyl-ACP synthase isoform III, II and I), Acyl ACP thioesterase (i.e. FatB and FatA), CoA (Coenzyme A), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:3 (linolenic acid), DHAP (dihydroxyacetone phosphate), G3PDH (glycerol-3-phosphate dehydrogenase), G3PAT (glycerol-3-phosphate acyltransferase), LPAAT (lysophosphatidic acid acyltransferase), PAP (phosphatidic acid phosphatase), DAG (diacylglycerol), DGAT (diacylglycerol acyltransferase), CPT (cholinephosphotransferase), PDAT (phospholipid: diacylglycerol acyltransferase), TAG (triacylglycerol).

FatA and FatB are the two types of acyl-ACP's theioesterases which cleave acyl-ACP (Salas and Ohlrogge, 2002; Bonaventure et al., 2003). In general, FatA hydrolyzes 18:1-ACP while FatB hydrolyzes 16:0-ACP (Bonaventure et al., 2003; Li-Beisson et al., 2010; Bates et al., 2013). By using acyl-CoA synthethases, the free FA are converted into coenzyme A esters. These esters are then moved from the cytoplasm to the endoplasmic reticulum where they participate in elongation and desaturation reactions (Li-Beisson et al., 2010).

The elongation pathway, comprising four reactions: condensation, reduction, dehydration, and a final reduction produces long FA (Somerville et al., 2000; Li-Beisson et al., 2010). Desaturation involves the addition of a double bond in the acyl chain, which results in the reduction of a dioxygen molecule to water, producing an unsaturated FA (Shanklin and Cahoon, 1998). Two independent pathways form polyunsaturated FA in plants; one in the microsomes or ER, the other in the plastids (Sperling et al., 1993; Miklaszewska et al., 2021). The main FA synthesized in oilseeds is free 18:1 (oleic acid), while small quantities of 16:0 (palmitic acid) and 18:0 (stearic acid) are also produced (Miklaszewska et al., 2021). The major source of polyunsaturated FA in Arabidopsis seeds is linoleic (18:2) and linolenic (18:3). Oleic acid (18:1) is desaturated to form linoleic (18:2) and linolenic acids (18:3) (Ohlrogge and Browse 1995; Baud et al., 2008). Oleate desaturase (FAD2) and linoleate desaturase (FAD3) enzymes convert C18:1-PC (phosphatidylcholine) into C18:2-PC, and C18:2-PC into C18:3-PC, respectively (Baud et al., 2008; Ozseyhan et al., 2018).

Seed storage lipid TAG are stored in specialized organelles called “oil bodies,” believed to assemble within the endoplasmic reticulum (Baud et al., 2008; Elahi et al., 2016). Different routes and components involved in TAG synthesis vary based on how FA are synthesized. In the endoplasmic reticulum, TAG is formed via the Kennedy pathway (Fig. 3). In this pathway,

dihydroxyacetone phosphate (DHAP) is converted to G3P (glycerol-3-phosphate) by the enzyme G3PDH (glycerol-3-phosphate dehydrogenase) (Baud et al., 2008; Li-Beisson et al., 2013). After that, glycerol-3-phosphate acyl transferase (G3PAT) converts G3P into lysophosphatidic acid (LPA), and lysophosphatidic acid acyltransferase (LPAAT) forms phosphatidic acid (PA) by adding an acyl group to LPA (Baud et al., 2008). Then phosphatidic acid phosphatase (PAP) facilitates the dephosphorylation of PA to DAG (diacylglycerol) (Li-Beisson et al., 2013; Miklaszewska et al., 2021). The diacylglycerol acyltransferase enzyme (DGAT) catalyzes the addition of an acyl group to DAG to produce TAG (Baud et al., 2008; Li-Beisson et al., 2013). In order to incorporate DAG into the PC (phosphatidylcholine) pool, CPT (cholinephosphotransferase) is required, and FAD (fatty acid desaturase) enzymes desaturate the remaining acyl groups (Li-Beisson et al., 2013). DAG requires an acyl group which may also be provided by FA in the PC pool, through reactions requiring PDAT (phospholipid: diacylglycerol acyltransferase) to produce TAG (Fig. 3).

1.5 Seed oil manipulation in canola

Due to increasing population and sustainable energy initiatives, canola oil is in high demand for the food industry, biofuel, and various industrial uses (Nath et al., 2016; Ton et al., 2020). Research and development programs to enhance the quality of *B. napus* seeds have focused on nutritional concerns recommended by consumers and the food sector (Hannoufa et al., 2014). Based on geographical locations, rapeseed breeding programs have utilized a variety of parental lines contributing to the three ecotypes of *B. napus*: spring type (widely cultivated in Canada, Australia, and northern Europe), winter type (Asia and the remaining European territory) (Hayward, 2012), and semi-winter type (the primary rapeseed in China) (Wei et al., 2017; Ton et al., 2020). Canola seed oil has been manipulated by researchers using both conventional breeding

and transgenic methods. Conventional breeding produces canola cultivars with desirable characteristics through controlled sexual recombination, typically achieved by crossing and selfing (Sleper and Poehlman, 2006). These techniques have been used to modify the composition and content of seed oils for several commercial vegetable oils (Burton et al., 2004; Scarth and Tang, 2006). The changes in FA content are the result of both induced and natural genetic modifications (Przybylski et al., 2005; Sleper and Poehlman, 2006). A combination of conventional breeding, selection and mutations have resulted in producing regular canola and specialty canola (low linolenic and saturated FA levels, as well as cultivars having high oleic and low linolenic acid levels) (Przybylski et al., 2005; Scarth and Tang, 2006) and high erucic acid rapeseed (HEAR). However, modern and targeted genetic engineering is becoming increasingly faster and more reliable than conventional breeding (Scarth and Tang, 2006). Additionally, because of gene pool limitations, conventional breeding approaches can be difficult and time consuming (Scarth and Tang, 2006; Zhang et al., 2019).

Transgenic technology has been applied to modify important FA synthetic genes in many crop species (Tan et al., 2011; Ton et al., 2020). One of the major goals has been to improve the FA composition of seed oil by increasing the production of specific FA with higher industrial and nutritional value (Cahoon et al., 2007; Vigeolas et al., 2011). For example, the polyunsaturated α-linolenic acid is regarded as an essential FA as it has several health benefits, but it cannot be synthesized by humans (Crawford et al., 2000; Vigeolas et al., 2011). Successful attempts to elevate the level of α-linolenic acid have been reported by over-expressing FA desaturases, despite a depression in overall seed oil (Kinney et al., 2002; Anai et al., 2003). The use of genes from diverse organisms has also been employed to increase production of other polyunsaturated FA,

including long-chain omega-3 polyunsaturated FA, such as eicosapentanoic acid and docosahexaenoic acid (Napier, 2007; Napier and Graham, 2010).

Several genetic engineering methods have also been applied to alter the lipid biosynthetic enzymes (Scarth and Tang, 2006). Oils rich in caprylic, capric, and palmitic acids were produced by overexpressing acyl-ACP thioesterase (FatB) (Dehesh et al., 1996; Voelker et al., 1997). Additionally, oil with higher quantities of stearic acid was obtained by over-expressing the enzyme acyl ACP thioesterase (FatA) and suppressing the enzyme stearoyl-ACP desaturase (Töpfer et al., 1995; Scarth and Tang, 2006). Finally, oleate desaturase (FAD2) was silenced to produce oils with increased levels of oleic acid (Stoutjesdijk et al., 2000; Okuzaki et al., 2018). Although considerable progress has been achieved by genetically altering the FA composition (Dehesh et al., 1996; Sharma et al., 2008), limitations have been encountered when attempting to enhance total seed oil content (Thelen and Ohlrogge, 2002; Weselake et al., 2009). Fatty acid synthesis is a highly coordinated process involving not only FA synthesizing genes but also genes participating in carbon metabolism; thus, manipulation of a single gene might not produce the expected results (Jaworski and Cahoon, 2003; Weselake et al., 2009). An alternative approach is to alter the expression of "master" regulators of FA synthesis, including key transcription factors operating upstream of the FA biosynthetic pathway (Ohlrogge and Jaworski, 1997; Mu et al., 2008).

1.6 Transcription factors regulating seed oil accumulation

Several studies have showed that LEC1 (LEAFY COTYLEDON1), WRI1 (WRINKLED1) and FUS3 (FUSCA3) are essential transcription factors required for the synthesis of seed storage products (Boulard et al., 2017; Lepiniec et al., 2018; Jo et al., 2019). The function of these genes,

highly expressed in the developing embryo and endosperm (Le et al., 2010; Tian, 2020) will be described in detail.

LEC1 (LEAFY COTYLEDON1) belongs to the NF-YB/HAP3 subunit of the CCAAT binding factor family, and it is expressed during the entire course of embryogenesis (Lotan et al., 1998; Tian, 2020; Verma et al., 2022). NF-YB subunits include a conserved histone-like region (the central B domain), which also contains residues essential for DNA binding (Lee et al., 2003). Seeds express *LEC1* more during embryonic development and less during the maturation phase (Schmid et al., 2005, Klepikova et al., 2016). The *lec1* mutation causes impaired embryo development and maturation, resulting in abnormal suspensors and defective cotyledons (Lotan et al., 1998, Elahi et al., 2016). The cotyledon identity appears to be regulated by *LEC1*, as *lec1* homozygous seedlings display leaf-like traits in the cotyledons, including the accumulation of anthocyanins and formation of trichomes (in Arabidopsis trichomes are present only on leaves, but not on cotyledons) (West and Harada, 1993; Meinke et al., 1994; Stone et al. 2008). Expression of *LEC1* has been linked to auxin signaling, important during the formation of the embryo, and the synthesis of sugars (Casson and Lindsey, 2006). The function of LEC1 has also been documented *in vitro*, where the gene regulates the somatic-embryogenic transition (Lotan et al., 1998; Stone et al., 2001; Verma et al., 2022). In the context of seed storage products, overexpression of *LEC1* is sufficient to induce about 60% of the genes encoding enzymes in the plastidial FA biosynthesis pathway, resulting in significantly higher seed oil accumulation (Mu et al., 2008; Roscoe et al., 2015; Baud et al., 2016). In *B. napus*, the seed specific expression of *LEC1* increases the production of FA with an elevation in oleic and linolenic acids without altering other desirable agronomic traits (Tan et al., 2011). This is in contrast to *LEC1* suppression, which reduces the amount of seed oil in Arabidopsis (Meinke et al., 1994). Besides oil, *LEC1* is implicated in the

accumulation of proteins, possibly through regulation of *FUS3* and *WRI1* (Kagaya et al., 2005), as revealed by a decrease in protein content following *LEC1* suppression (Meinke et al., 1994).

The transcription factor WRI1 (WRINKLED1) encodes an APETALA2/ethylene-responsive element binding protein (Liu et al., 2010; Verma et al., 2022; Kuczynski et al., 2022). This gene's AP2 domain triggers expression of target genes by binding to their promoter region. Specifically, *WRI1* is a direct regulator of genes involved in the synthesis of FA during the late seed maturation phase (Baud et al., 2007; Kuczynski et al., 2022). In addition, *WRI1* also alters the sensitivity to abscisic acid, controls how embryos sense and respond to sugars (Cernac et al., 2006), and induces several enzymes participating in the glycolytic pathway and conversion of sugars into FA (Baud et al., 2007; Kong and Ma, 2018). While *wri1* mutant plants produce wrinkled seed coats with 80% less seed oil due to a defect in TAG biosynthesis (Focks and Benning, 1998; To et al., 2012), ectopic expression of *WRI1* increases seed oil content by 10 to 40% (Liu et al., 2010, Cernac et al., 2004).

FUS3 (FUSCA3) is another transcription factor regulating seed development, as it is necessary for the growth of embryos (Tian, 2020). *FUS3,* a member of the B3-domain family, binds to the RY element CATGCA in the promoters of various genes (Curaba et al., 2004; Santos et al., 2008). *FUS3* transcripts are primarily expressed in seeds but are also detected at low levels in tissues after germination (Luerssen et al., 1998; Tian, 2020). *FUS3* expression generally follows that of *LEC1*, especially during the middle-late stages of embryogenesis (Bäumlein et al., 1994; Roscoe et al., 2019). Among the abnormalities caused by the mutations in *FUS3* are precocious germination, desiccation intolerance, anthocyanin accumulation in developing seeds, defective hormone synthesis and altered perception of light (Meinke et al., 1994; Harada, 2001). Mutations in *fus3* in Arabidopsis and *B. napus* also depress the levels of both oil and proteins (Meinke et al.,

1994; Elahi et al., 2015). In Arabidopsis seeds overexpressing *FUS3*, the stimulation of 2S albumin and 12S cruciferin synthesis was observed along with the transcriptional induction of several FA biosynthetic enzymes such as KASI, KASII, KASIII (Wang et al., 2007). The molecular mechanisms underpinning the regulation of many target genes by *FUS3* have not been fully elucidated (Elahi et al., 2015; Roscoe et al., 2015; Verma et al., 2022).

The genetic network integrating LEC1, WRI1 and FUS3 in the synthesis of storage products, and oil in particular, is complex. Both WRI1 and FUS3 have been identified as direct targets of LEC1 although they can both alter the expression of LEC1 (Fig. 4) (Wang and Perry 2013; Pelletier et al., 2017).

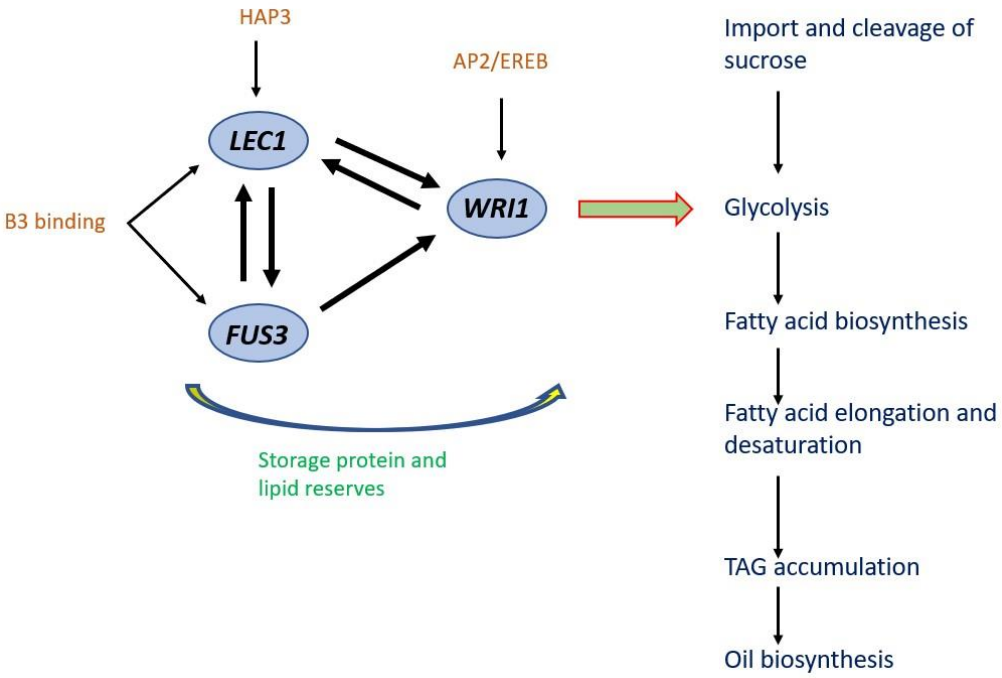

Figure 4: Overview of the regulatory network and interaction of key transcription factors controlling seed development and oil synthesis. (Adapted from North et al., 2010; Elahi et al., 2016; Verma et al., 2022).

1.7 Phytoglobins: overview of their function

Phytoglobins are heme-containing proteins related to globins identified in bacteria and eukarya (Freitas et al., 2004). They have a heme-containing group in their structure and, like animal hemoglobins, can bind oxygen (Freitas et al., 2004). The plant hemoglobin has evolved from the same ancestral globin (Riquelme and Hinrichsen, 2015). Research on plant hemoglobins started with the identification of leghemoglobin, which was the only hemoglobin-like molecule known in plants for many years. It is produced by legume plants during the symbiotic relationship with nitrogen-fixing bacteria (Appleby, 1984; Appleby, 1992). More recently, plant hemoglobins were also discovered in non-leguminous plants, and they were referred to as nonsymbiotic hemoglobins (Appleby et al., 1983). Ubiquitous in the plant kingdom, these nonsymbiotic hemoglobins with a higher oxygen affinity relative to leghemoglobin (Arredondo-Peter et al., 1998) were recently renamed “Phytoglobins” by Hill et al. (2016). Phylogenetic analyses reveal the existence of 6 clusters of plant hemoglobins (Garrocho -Villegas and Arredondo-Peter, 2008). Within these classes, the best characterized are class 1 (Pgb1) and class 2 (Pgb2). While Pgb1 is found in both monocots and dicots, Pgb2 is absent in monocots (reviewed by Hill, 2012). Both Pgbs bind oxygen, although the O_2 affinity of Pgb1 is higher than that of Pgb2. For this reason, it was suggested that Pgb1 does not act as an oxygen carrier (Hill, 2012), but rather as a scavenger of nitric oxide (NO) through the Pgb/NO cycle (Fig. 5). In this cycle oxygenation of Pgb is required for the conversion of NO to nitrate in a reaction forming Met-Pgb (Igamberdiev and Hill, 2004).

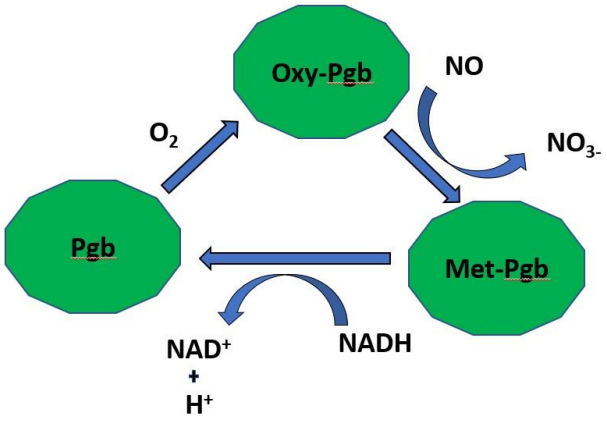

Figure 5: Simplified Pgb/NO cycle (Adapted from Igamberdiev and Hill, 2004; Dordas, 2009; Gupta et al., 2011). [Oxy-Pgb, Pgb(Fe^{2+})O_2; Met-Pgb, Pgb(Fe^{3+})].

Role of Phytoblobin 1 (Pgb1) during stress

Under non-stressed conditions, Phytoglobin 1 (Pgb1) is expressed at low levels in root tips, at the base of the cotyledons, in the undifferentiated cells of the shoot apical meristem, and in leaf hydathodes (Heckmann et al., 2006; Hebelstrup et al., 2013). Its expression increases following exposure to diverse types of stress, including water stress, oxygen deficiency, and temperature stress (Guo et al., 2003; Zhao et al. 2008; Campbell et al., 2015; Youssef et al. 2016). During low oxygen stress (hypoxia), expression of *Pgb1* exercises a protective function by enhancing plant tolerance to the stress. This was initially documented in Arabidopsis, where over-expression of *Pgb1* increased the survival rate to low oxygen (Hunt et al., 2002), and then observed in several other species including barley (*Hordeum vulgare*) (De Castro, 2022), maize (*Zea mays*) (Mira et al., 2016; Youssef et al., 2016), and soybean (*Glycine max*) (Mira et al., 2021). The Pgb1 protective role is mainly the result of its NO scavenging properties. By lowering the level of NO accumulated during the stress, Pgb1 contributes to the reduction of reactive oxygen species (ROS) and ROS-induced damage in the tissue (Mira et al., 2016). This has been well documented in hypoxic roots

where the stress causes the degradation of the root apical meristem. The production of NO and ROS within the stem cells triggers their premature differentiation, disrupting their function. These abnormalities are mitigated by the over-expression of *Pgb1* (Mira et al., 2016; 2020). In conjunction with the reduction of NO and ROS, Pgb1 preserves the root stem cells by retaining the basipetal auxin flow required for the specification of the stem cells (Mira et al., 2016; 2020). The Phytoglobin 1 gene also influences maize somatic embryogenesis, where embryos are produced *in vitro* from somatic cells (Huang et al., 2014). Suppression of Pgb1 elevates NO which triggers the death program in the embryogenic cells, reducing the number of embryos (Huang et al., 2014).

Role of Phytoglobin 2 in plant development

Relative to Pgb1, the function of Pgb2 is poorly understood. Phytoglobin 2 has a lower affinity for O_2 than Pgb1 (Hoy and Hargrove, 2008), and based on these properties, the protein might facilitate the transport of oxygen (Vigeolas et al., 2011). In support of this hypothesis is the expression pattern of *Pgb2*, which is highly abundant in metabolically active tissues requiring high levels of O_2. They include immature fruits, as well as young leaves and seeds (Hunt et al., 2001; Wang et al., 2003). Unlike Pgb1, Pgb2 is not induced by low oxygen stress, but rather by hormonal treatments, including cytokinin (Hunt et al., 2001) and cold stress (Trevaskis et al., 1997). The ability of Pgb2 to scavenge NO is debatable, although high levels of NO have been detected in tissues where *Pgb2* was down-regulated (Hebelstrup and Jensen 2008; Elhiti et al., 2013). In Arabidopsis, suppression of *Pgb2* favors the formation of somatic embryos by influencing auxin synthesis and transport (Elhiti et al., 2013). Phytoglobin 2 has also been studied in the context of seed oil. Its over-expression is sufficient to elevate the level of oil in both Arabidopsis (Vigeolas et al., 2011) and field cress (Ivarson et al., 2016). In Arabidopsis, the seed-expression of *Pgb2*

increased the total FA by about 40% and enhanced the oil nutritional value by altering the composition of FA in favor of high C18:2/C18:1 and C18:3/C18:2 ratios. These effects occurred without negative repercussions on key agronomic traits (Vigeolas et al., 2011). In the same study, the authors suggested that the Pgb2 effects were not linked to a reduction in NO (as over-expression of *Pgb1* did not have any effect on seed oil), but rather on the ability of Pgb2 to elevate the levels of ATP (possibly by favoring the movement of O_2) in the hypoxic seeds. Seeds are highly hypoxic (Vigeolas et al., 2003; Rolletschek et al., 2007) and the lack of oxygen reduces the level of ATP required to sustain the synthesis of FA (Vigeolas et al., 2011). However, as indicated above, the ability of Pgb2 to scavenge NO cannot be discounted. The reduction of NO resulting from over-expression of *Pgb2* could in fact be conducive to the synthesis of oil. This notion is based on the work of Liu et al. (2020), revealing the importance of NO levels for seed oil accumulation in Arabidopsis. Genetic and pharmacological treatments reducing seed NO increased the amount of oil, while those elevating NO repressed the synthesis of oil. These alterations in NO influenced the expression of *LEC1* and *WRI1*, but also the activity of some key enzymes involved in FA synthesis through post-translational modifications related to S-nitrosylation. Among the S-nitrosylated proteins was pyruvate kinase, involved in the last step of glycolysis, and regulating the carbon flow towards the synthesis of FA (Liu et al., 2020).

The function of *Pgb2* over-expression on seed oil has been described in Arabidopsis (Vigeolas et al., 2011), but not in oil-producing crops, like *B. napus*, where an increase in seed oil would be more desirable. The objective of this thesis is to examine the effects of the seed-specific expression of the *B. napus BnPgb2* on oil content and FA composition. The results will be presented in paper format.

Seed-specific expression of the class 2 *Phytoglobin* (*Pgb2*) increases seed oil in *Brassica napus* L.

Md Ehsanul Haq, Mohammed M. Mira, Robert W. Duncan, Robert D. Hill and Claudio Stasolla

Note: The transgenic lines were prepared by Mohammed Mira. Oil analyses and FA profile were performed by Kelsey Dickson and Debbie Witko.

Abstract

To examine the function of phytoglobin 2 (Pgb2) on seed oil level in the oil-producing crop *Brassica napus* L., we generated transgenic plants in which *BnPgb2* was over-expressed in the seeds using the cruciferin1 promoter. Over-expression of *BnPgb2* elevated the amount of oil, which showed a positive relationship with the level of *BnPgb2*, without altering the oil nutritional value, as evidenced by the lack of major changes in composition of fatty acids (FA), and key agronomic traits. Two key transcription factors, *LEAFY COTYLEDON1* (*LEC1*) and *WRINKLED1* (*WRI1*), known to promote the synthesis of fatty acids (FA) and potentiate oil accumulation, were induced in *BnPgb2* over-expressing seeds. The concomitant induction of several enzymes of sucrose metabolism, *SUCROSE SYNTHASE1* (*SUS*) *1* and *3*, *FRUCTOSE BISPHOSPHATE ALDOLASE* (*FPA*), and *PHOSPHOGLYCERATE KINASE* (*PGK*), and starch synthesis, *ADP-GLUCOSE PHOSPHORYLASE* (*AGPase*) suggests that *BnPgb2* favors sugar mobilization for FA production. The two plastid FA biosynthetic enzymes *SUBUNIT A OF ACETYL-CoA CARBOXYLASE* (*ACCA2*), and *MALONYL-CoA:ACP TRANSACYLASE* (*MCAT*) were also up-regulated by the over-expression of *BnPgb2*. The requirement of *BnPgb2* for oil deposition was further evidenced in natural germplasm by the higher levels of *BnPgb2* in seeds of high-oil genotypes relative to their low-oil counterparts.

3. INTRODUCTION

Seeds of many crop species are utilized for human consumption, as they accumulate diverse levels of storage products including carbohydrates, proteins and lipids. Among the major oil producing crops is canola (*Brassica napus* L.), being the source of one of the healthiest edible oils. The high nutritional value of canola oil is due to its characteristic fatty acid (FA) composition with low percentages of polyunsaturated and saturated FA relative to unsaturated FA, as well as low levels of erucic acid (less than 2%) and glucosinolates (less than 30 μmoles per gram) (Harvey and Downey 1964; Dupont et al., 1989; Eskin et al., 1996). Due to the high demand of vegetable oil worldwide, elevating production of oil and ameliorating its nutritional value are two important goals in breeding programs (Weselake et al., 2009).

Synthesis of seed oil involves diverse metabolic pathways housed in several cellular compartments including the cytoplasm, plastids and endoplasmic reticulum (Ohlrogge and Browse 1995; Baud et al., 2002). Multiple condensation steps of acetyl-CoA and malonyl-CoA moieties, produced by the oxidation of carbohydrates in the cytoplasm, generate 16:0-acyl carrier proteins (ACP) and 18:0-ACP in the plastids (Baud et al., 2002, Pidkowich et al., 2007). Further desaturation reactions form 18:1-ACP, which is then utilized for the production of unsaturated and long-chain FA (Sasaki and Nagano, 2004). Additional modifications occur in the endoplasmic reticulum where the Kennedy pathway produces triacylglycerol (TAG) (Baud et al., 2002).

Many genes participating in FA synthesis are direct or indirect targets of transcription factors which are also implicated in morphogenic events during embryo development (Braybrook and Harada, 2008). Among these factors are *LEAFY COTYLEDON1* (*LEC1*), *FUSCA3 (FUS3)*, and *WRINKLED1* (*WRI1*) (Meinke et al., 1994, Gazzarrini et al., 2004; Baud et al., 2007). As a member of the HAP3 subunit of the CCAAT binding factor family (Lotan et al., 1998), *LEC1* is

expressed during many stages of seed development (Parcy et al., 1994). By influencing key reactions of FA condensation, elongation, and desaturation (Mu et al., 2008), *LEC1* elevates the accumulation of seed oil (Shen et al., 2010; Tan et al., 2011). One of the direct targets of *LEC1* is *WRI1* which up-regulates several enzymes of the upper (Maeo et al., 2009) and lower (Kuczynski, 2022) glycolytic pathway. As for *LEC1*, over-expression of *WRI1* is sufficient to increase oil synthesis (Cernak and Benning 2004; Liu et al., 2010; Shen et al., 2010). Another transcription factor promoting FA synthesis is *FUS3* (Zhang et al., 2016); positively regulated by *LEC1* (Vicient et al. 2000), and an inducer of *WRI1* (Wang and Perry, 2013). Efforts to augment the production of seed oil have predominantly used breeding and genetic approaches. Varying degrees of success have been reported by altering the function of single enzymes in FA or TAG synthesis (Ruuska et al., 2002; Scarth and Tang, 2006), or over-expressing *LEC1*, *WRI1* and *FUS3* (Lotan et al., 1998, Kwong et al., 2003, Cernac and Benning, 2004, Tan et al., 2011; Wang and Perry 2013). In the majority of cases, however, the constitutive manipulation of these genes was accompanied by developmental abnormalities in plant growth (Lotan et al., 1998; Cernac and Benning, 2004; Wang et al., 2007; Mu et al., 2008; Shen et al., 2010).

Deposition of oil occurs in a hypoxic (low oxygen) environment, which is typical of developing seeds in which the level of oxygen can be as low as 2% (Rolletscheck 2005, 2007). Under hypoxic conditions, phytoglobins (Pgbs), proteins able to scavenge nitric oxide (NO), are induced (Hebelstrup et al., 2006). While mainly examined in the context of low oxygen stress (reviewed by Hill, 2012), two independent studies show the participation of Pgbs in seed oil accumulation. Over-expression of the Arabidopsis class 2 Phytoglobin (*Pgb2*), in both Arabidopsis (Vigeolas et al., 2011), and cress (Ivarson et al., 2016), elevated the amount of seed oil and, in the case of Arabidopsis, ameliorated the FA composition by enriching polyunsaturated FAs. The

unchanged amount of oil measured in Arabidopsis seeds over-expressing the class 1 *Pgb1* (able to effectively remove NO, Hebelstrup et al., 2006) prompted the authors to discount the NO scavenging role of Pgb2, in favor of a function related to the maintenance of the energy state in the hypoxic seeds (Vigeolas et al., 2011). The authors suggested that the high ATP/ADP ratio supported by *BnPgb2* expression is conducive to the synthesis of FA, an energy consuming process. While the role of *Pgb2* in promoting oil synthesis has been established in model plants, it has not been demonstrated yet in economically relevant oilseed species such as *B. napus*, where its effect would be more desirable. To address this gap, we first generated transgenic *B. napus* plants with seed-specific expression of *BnPgb2*, to circumvent yield penalties sometimes associated with the constitutive expression of *Pgbs* (Cochrane et al., 2017 and unpublished data from our lab). Then, we measured the seed oil level and FA composition and linked these parameters to transcriptomic changes of key transcription factors and enzymes participating in FA synthesis.

4. MATERIALS AND METHODS

4.1 Generation of transgenic *B. napus* plants with seed specific expression of *BnPgb2*

Brassica napus L. cv Westar plants over-expressing *Phytoglobin 2 (BnPgb2,* BnaA05g28340D) under the control of the seed specific cruciferin (Cru)1 promoter (BnaA01g08350D) were generated by using the procedure described in Clough and Bent (1998). The complete *BnPgb2* open reading frame was cloned downstream of a functional segment of the Cru1 promoter (Fig. 6). Amplification was conducted using Phusion™ High-Fidelity DNA Polymerase with primers containing *BamH1* and *ECOR1* sites (Supplementary table 1). The amplicon was inserted into pENTR1A Dual Selection Vector (Thermo Fisher), and DNA sequencing (Macrogen) confirmed the inserted sequences. The construct was then cloned into the final destination vector PBGWFS7 using the Gateway® LR Clonase® II Enzyme mix (Thermo Fisher Scientific). Plants were transformed using the floral-dip method, exactly as reported by Clough and Bent (1998).

Microspores of PCR-positive plants were utilized to generate doubled haploid (DH) plants exactly as reported in Fletcher et al. (1998). Positive DH plants were then selected and grown to maturity for seed production. From the subsequent generation several positive Cru:BnPgb2 independent lines (line 1, 2, 3, 6, and 10) were selected for the current study (Fig. 7).

4.2 Plant growth conditions, collection of seeds for expression analyses and agronomic characteristics

Plants were grown in 15 cm pots in the greenhouse at the University of Manitoba with an average temperature of 25°C and a 17 h of photoperiod. Initially, the seedlings were fertilized with NPK (10:52:10) to encourage root development and watered every second day until the

BnCRU1 - BnaA01g08350D

ACGTAGGACCACGTAACAACGAGAGATTATAAATTCGGATGATGAACAATTAAGGATCTAAATTATACTCTCTT
TCTAATCGACCTTCCGTGGGGTAAAGTGGTCAATTACATTCTTTATGGATGAGCTTGATTGAGAATGGGTTTAT
GGGTTATGACAAGGGCATGTACAAATGTCACTGCCTCTTGACATGCAACCGAACAGTTGGCGACTCAAGTCGC
AGAAGATACAACGGACCAAACCCTCCGAGTGTCGCCGCGTCTATTAAGTGTCACCTTTTTGTCTCCTTTCCTTAA
AAATTGGTAACTCATTTTTCAAAAAAAAAGAATAAGATAGTTTTGGCTGTATCTCCTAAACTATTCGATCACAAC
GCCAGATATTTTAATACTGGATACTAGTGATGTGATTTGATTTGTTAATTGTCAAAAAGTAGATTCTCCTATCTC
GTTTTTAGTTCAATTATTATATGGTTAAATGAATTTAAGTCGATTAGAAATGATTAGTTAATCAACCAGAGTTGC
TCTATAAGTCTATACTGATAACATGAACCATTTTCTAAAAATGAGATAGATACATTTGAATTTTGTCGTGGTTTG
GAGTATGCGGAGATAGTGCTACGCGCATGAACATCATGAGACACTTGCTTCAGCTCACAGAGTGACGTGTAAA
GACCATAGACCCACGACTTCATGCAAACCCATTCCTACGTGGCACAAACCTTCATGCTCACTCCACATATATAAA
CTCCTACCAAGTCTCCCTGTTTCTTCATCCATCTATCACAAAAACACACAAACAAT

Figure 6. Sequence of the Cruciferin 1 promoter used to drive the expression of *BnPgb2*

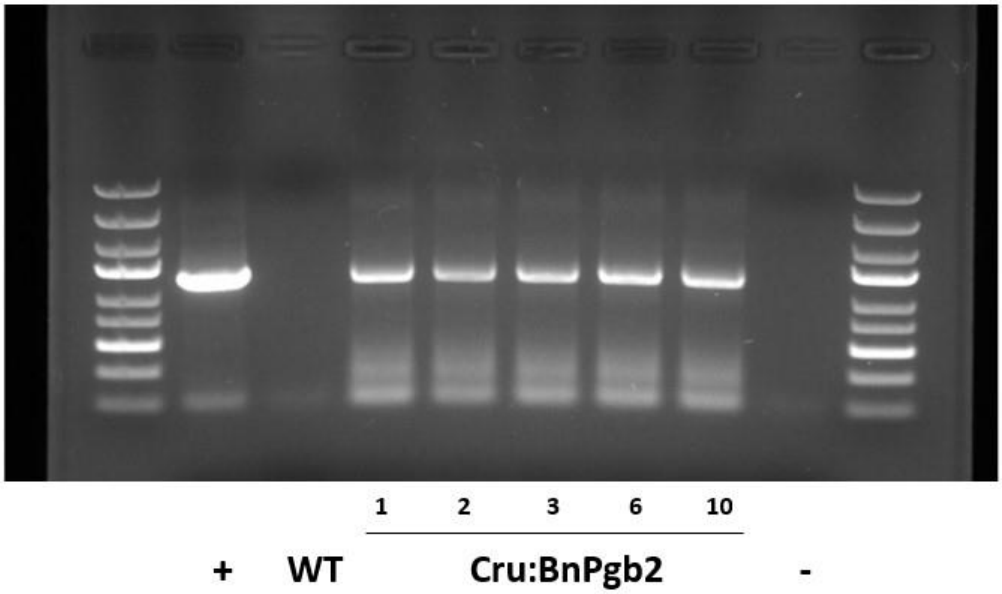

Figure 7. Gel confirming the presence of the insert in genomic DNA of the transformed Cru:BnPgb2 lines. The primers proCRU-F and BnPgb2-R amplify part of the Cruciferin 1 promoter and the *BnPgb2*. The sequences of the primers are listed in Supplementary table 1. +, positive control (plasmid); -, negative control (water).

bolting stage at which point the pots were fertilized with NPK (20:20:20) at a rate of approximately 3g per 1L of water. All necessary treatments for the control of pathogens were conducted as needed. In developing *B. napus* seeds, significant accumulation of oil starts at 28 days after pollination (DAP) and reaches its maximum level at 35 DAP (Fowler and Downey, 1970). Thus, seeds were collected at 14, 21, 28 and 35 DAP for gene expression and histological studies (Elahi et al., 2015). Fully mature seeds (35 DAP) were utilized for total oil content and FA composition analyses.

General agronomic characteristics (plant height, number siliques per plant, seeds per silique, 1000 seed weight and yield per plant) were recorded according to Elahi et al. (2015).

4.3 Histological localization of starch, proteins and oil bodies

For histological localization of storage products, developing seeds (7, 14, 21, 28, and 35 DAP) were treated exactly as reported by Mira et al. (2016). Seeds were fixed immediately in 2.5% glutaraldehyde and 1.6% paraformaldehyde in a 0.1 M phosphate buffer, pH 6.8, for 12h. After dehydration using the ethanol series, the seeds were infiltrated and subsequently embedded in paraffin wax. Semithin sections (10 μm) were dewaxed using 100% xylene followed by decreasing concentration of ethanol (100%-20%). Sections were then immediately stained and visualized. For starch localization, sections were incubated for 15 min in 0.5% periodic acid followed by 30 min in Shiff's reagent (Flütsch et al., 2018). Oil bodies were visualized by Sudan Red 7B (Brundrett et al., 1991), while proteins by Amido Black 10B (Vuosku et al., 2015).

4.4 Gene expression analyses

Extraction of RNA from developing seeds was performed using TRI Reagent (Elahi et al., 2015). The total RNA was treated with DNase I (recombinant, RNase-free, Roche) and 1μg RNA was used for cDNA synthesis. The High-Capacity cDNA Reverse Transcription Kit (Applied

Biosystems) was used for cDNA synthesis. Quantitative RT-PCR was carried out exactly as previously described by Elahi al. (2015). All primers used for gene expression studies are listed in Supplemental Table S1. The relative gene expression level, analysed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), was normalized to BnACTIN (AF111812) as a reference (Elahi et al., 2015). All PCR reactions were performed using the CFX96TM Optics Real- Time System (Bio-Rad, Hercules, CA).

4.5 Determination of seed protein level, oil content and fatty acid (FA) composition

Seed protein and oil levels were measured using near infrared reflectance (NIRSystem model XDS, FOSS, Eden Prairie, USA) spectroscopy at the University of Manitoba Oilseed Quality Laboratory (certified by the Canadian Grain Commission) following established standard methods (Daun et al. 1994). Oil and protein contents were determined based on reflectance spectra and reported as a percentage of the seed weight. Fatty acid (FA) composition analyses were conducted using gas chromatography (Varian, Walnut Creek, USA) as documented by Hougen and Bodo (1973). Experiments were duplicated over the 2021 and 2022 growing season (two greenhouse seasons).

4.6 Statistical analyses

Data were analysed by one way ANOVA using the SPSS program (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). Treatment means were compared by Duncan's test ($P<0.05$) to differentiate the significance of difference between genotypes. Unless otherwise specified, all experiments were performed in three biological replicates each consisting of a minimum of five plants.

5. RESULTS

5.1 *BnPgb2* expression in seeds of natural germplasm

To establish an initial link between *BnPgb2* expression and seed oil levels, we selected six *B. napus* genotypes from our collection at the University of Manitoba: three accumulating high oil (HO-37, 44, and 22), and three accumulating low oil (LO-27, 28 and 52). The seed oil ranged from about 46 to less than 38 (% of seed weight) (Fig. 8A). *BnPgb2* transcripts were then measured in developing seeds of the same genotypes at different days after pollination (DAP). Within the HO lines, the *BnPgb2* seed expression peaked between 14 and 28 DAP in HO-44 and especially in HO-22 (Fig. 8B). The levels of *BnPgb2* transcripts gradually increased in developing HO-37 seeds. Small fluctuations in *BnPgb2* expression were detected during development of the LO seeds, which compared to their HO counterparts accumulated fewer *BnPgb2* transcripts after 14 DAP.

To discount the possibility that these differences were due to asynchronous embryonic development, we followed the morphology of the developing embryos and the accumulation of proteins and oil, the two major storage products in *B. napus*. Embryos sampled at different DAP followed very similar developmental stages (Fig. 9). Completion of development, as denoted by the presence of an extended embryonic axis and large cotyledons, occurred at 18 DAP in all genotypes.

In *B. napus*, storage products are mainly found in the embryos, as the endosperm is consumed rapidly during development; thus, our studies focussed on cortical cells within the embryonic axis and cotyledons. Signal for protein bodies, detected in all genotypes at 14 DAP, increased from 14-21 DAP (Fig. 10). At 35 DAP, axial and cotyledonary cells accumulated a large amount of proteins, with no visible differences among the HO and LO genotypes.

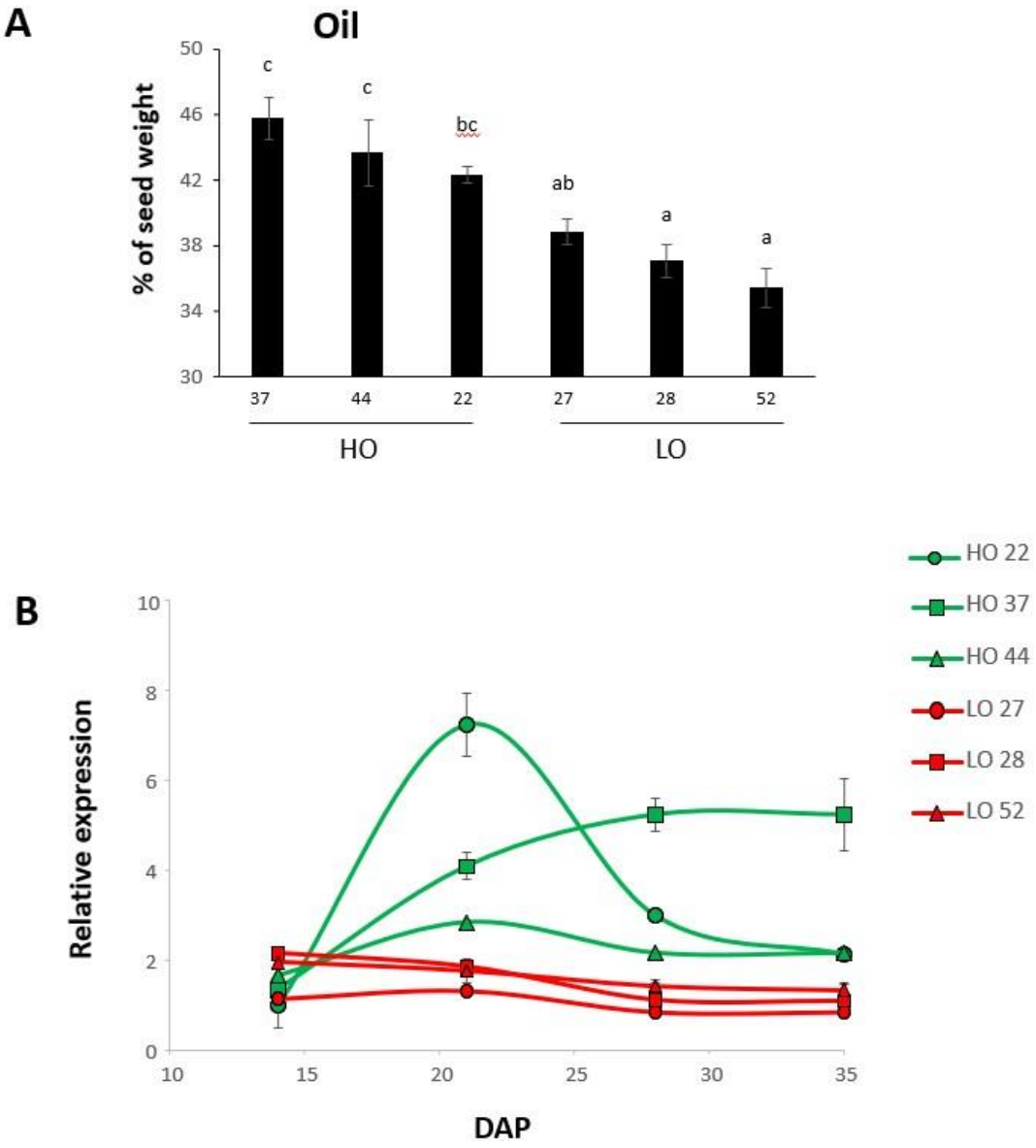

Figure 8: Total oil and expression of *BnPgb2* in *B. napus* seeds of high (HO) or low (LO) oil genotypes. **(A)** Total lipids measured in dry seeds of three HO genotypes (37, 44, and 22) and three LO genotypes (27, 28, and 52). Values are means ± SE. Letters on bars denote statistically significant differences ($p<0.05$). **(B)** Expression of *BnPgb2* in developing (14-35 DAP) seeds of the HO and LO genotypes. Values ± SE are means normalized to the value of HO 22 (at 14 DAP) set at 1.

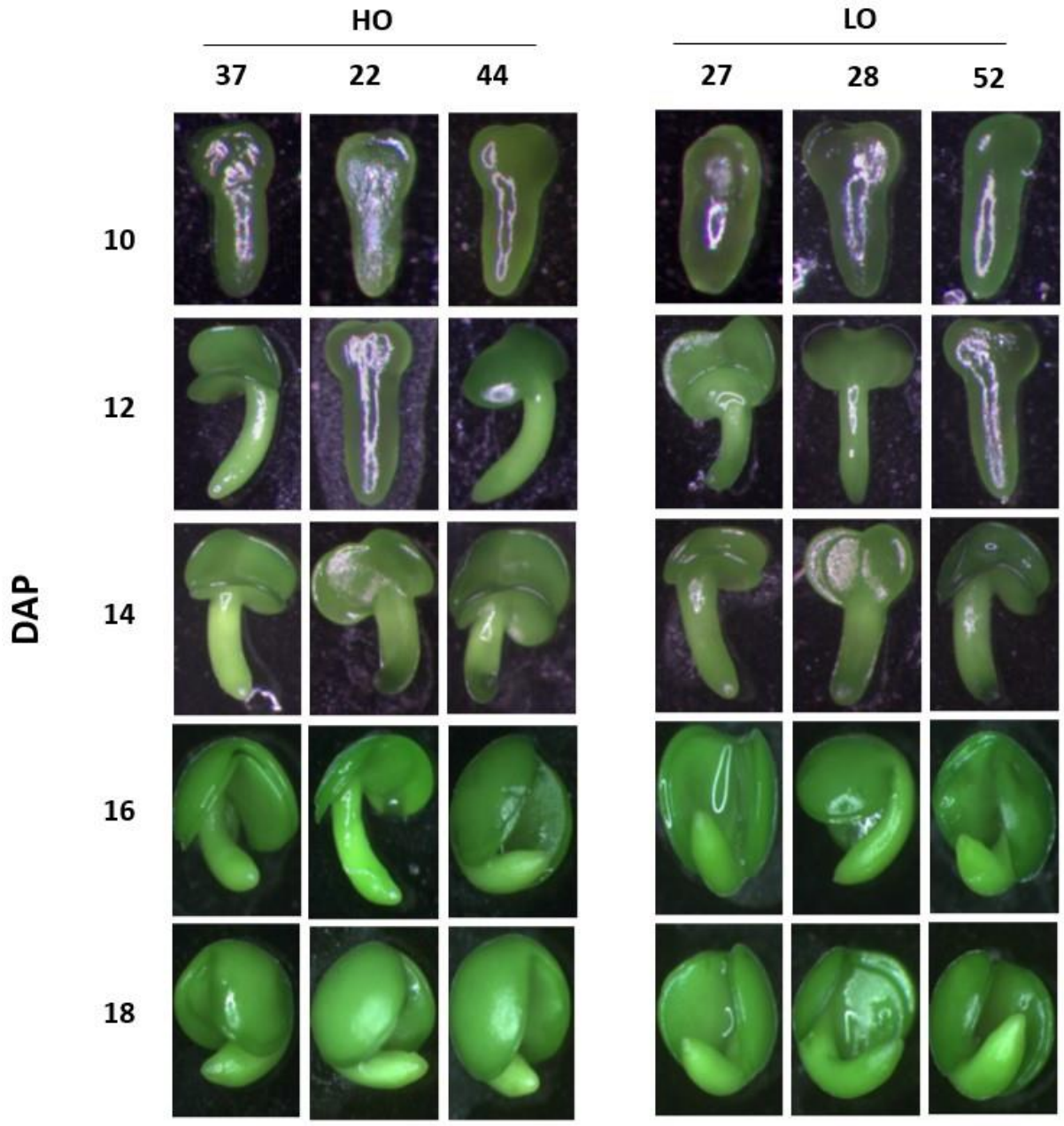

Figure 9: Morphology of the developing (10-18 DAP) embryos in the three high (HO) or low (LO) oil genotypes.

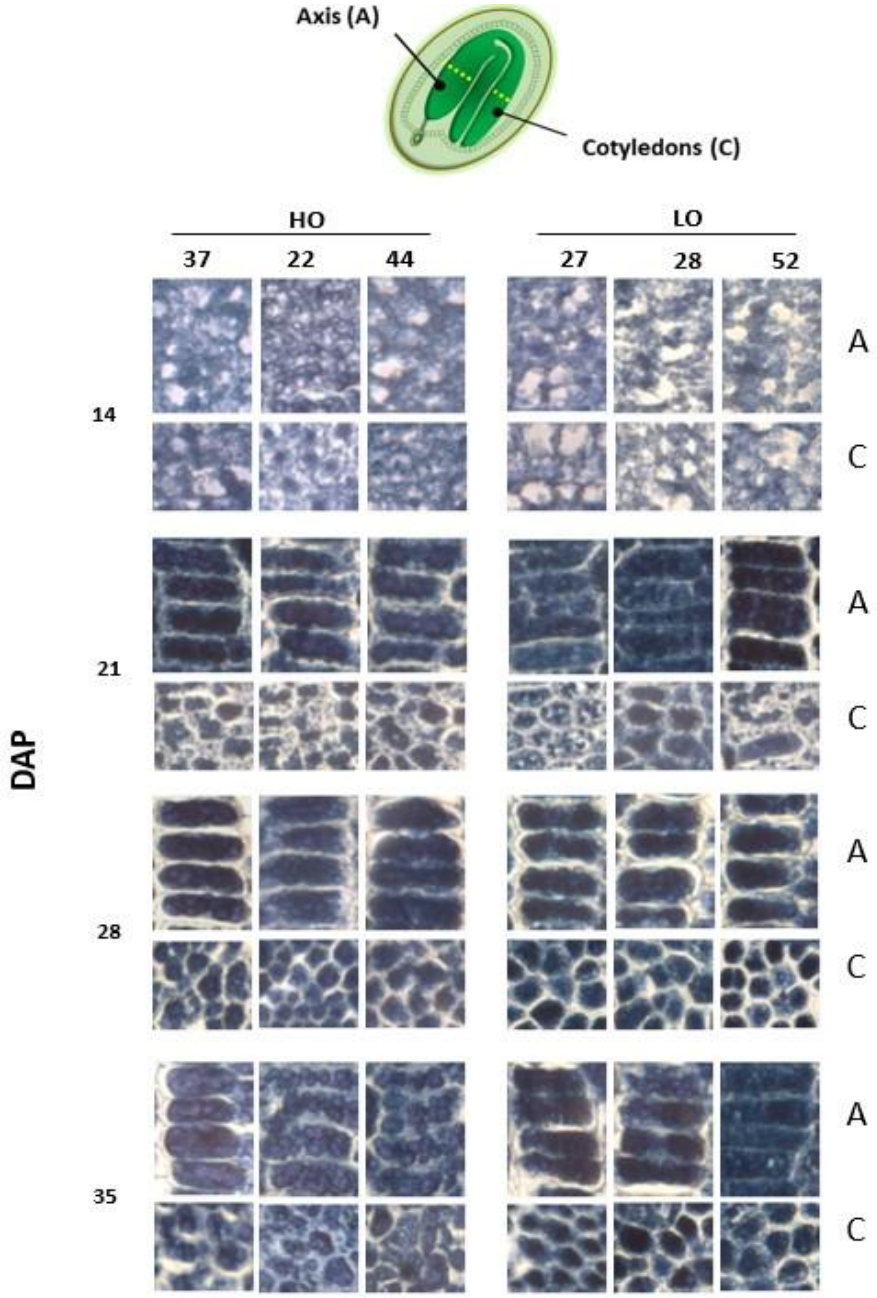

Figure 10: Localization of protein bodies in the developing (14-35 DAP) embryos of the three high (HO) or low (LO) oil genotypes. Protein bodies were visualized in cells located in the middle (yellow lines) of the embryonic axis (A) or cotyledons (C).

Relative to protein accumulation, oil accumulates later during development (Verma et al., 2022), and oil bodies were stained from 21 DAP. Oil staining increased from 21-28 DAP, especially in HO embryos (Fig. 11). Differences in staining patterns among genotypes were also apparent at 35 DAP, with a more intense signal observed in the axial cells of the HO embryos relative to LO embryos. No differences were noted in cotyledonary cells.

All together, these results indicate that the greater seed induction of *BnPgb2* after 14 DAP in HO genotypes relative to LO genotypes is not the result of asynchronous seed development.

5.2 Changes in seed oil level and fatty acid (FA) composition by *BnPgb2*

To further understand the requirement of high *BnPgb2* levels on seed oil deposition, we generated five transgenic lines [Cru:BnPgb2 (1, 2, 3, 6, and 10)] overexpressing *BnPgb2* under the Cruciferin1 (Cru1) promoter (Fig. 6). *Cruciferin1* transcripts in *B. napus* seeds are first detected between 14-20 DAP, and peak around 35 DAP before declining during the following stages (Sjödahl et al., 1993). Thus, the seed level of *BnPgb2* in the transgenic seeds was measured at 35 DAP. The relative expression of *BnPgb2* varied among the Cru:BnPgb2 transgenic lines, with the highest value recorded for line 1, and the lowest for line 10, which exhibited a value of *BnPgb2* expression comparable to the (WT) (Fig. 12A). A similar profile was also observed for the total seed oil content. With the exception of line 10, over-expression of *BnPgb2* increased the oil level in seeds of all transgenic lines, especially in line 1 (Fig. 12B). The amount of proteins measured in seeds showed an overall reverse relationship with the level of oil; seeds accumulating less oil (WT and line 10) was enriched in proteins, while seeds of line 1 accumulating more oil had the lowest levels of proteins (Fig. 12C). The composition of fatty acids (FA), a determinant factor for the nutritional quality of canola, did not show major changes following the over-expression of *BnPgb2* (Fig. 13). Some of the minor alterations in FA composition were not due to the *BnPgb2,*

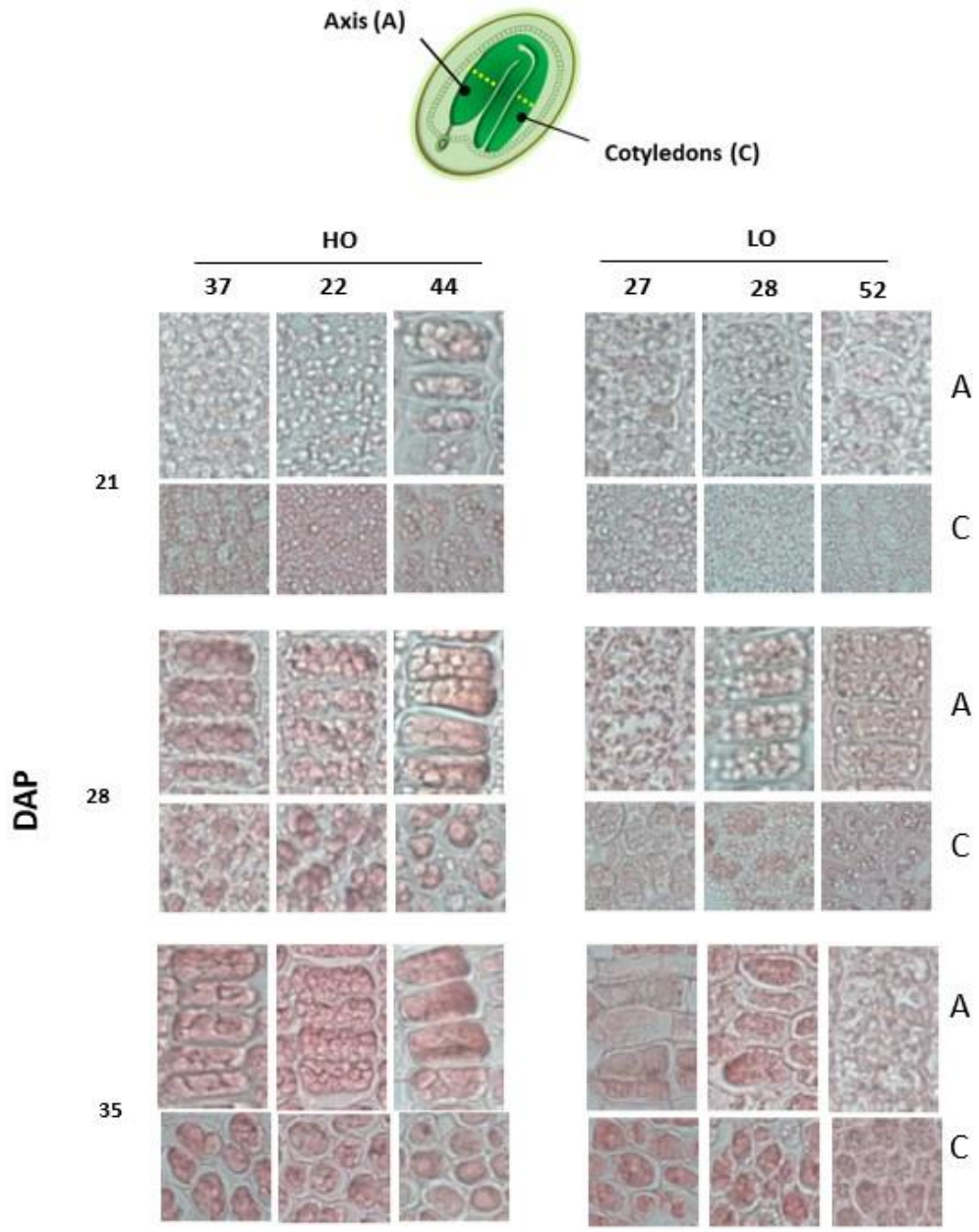

Figure 11: Localization of oil bodies in the developing (21-35 DAP) embryos of the three high (HO) or low (LO) oil genotypes. Oil bodies were visualized in cells located in the middle (yellow lines) of the embryonic axis (A) or cotyledons (C).

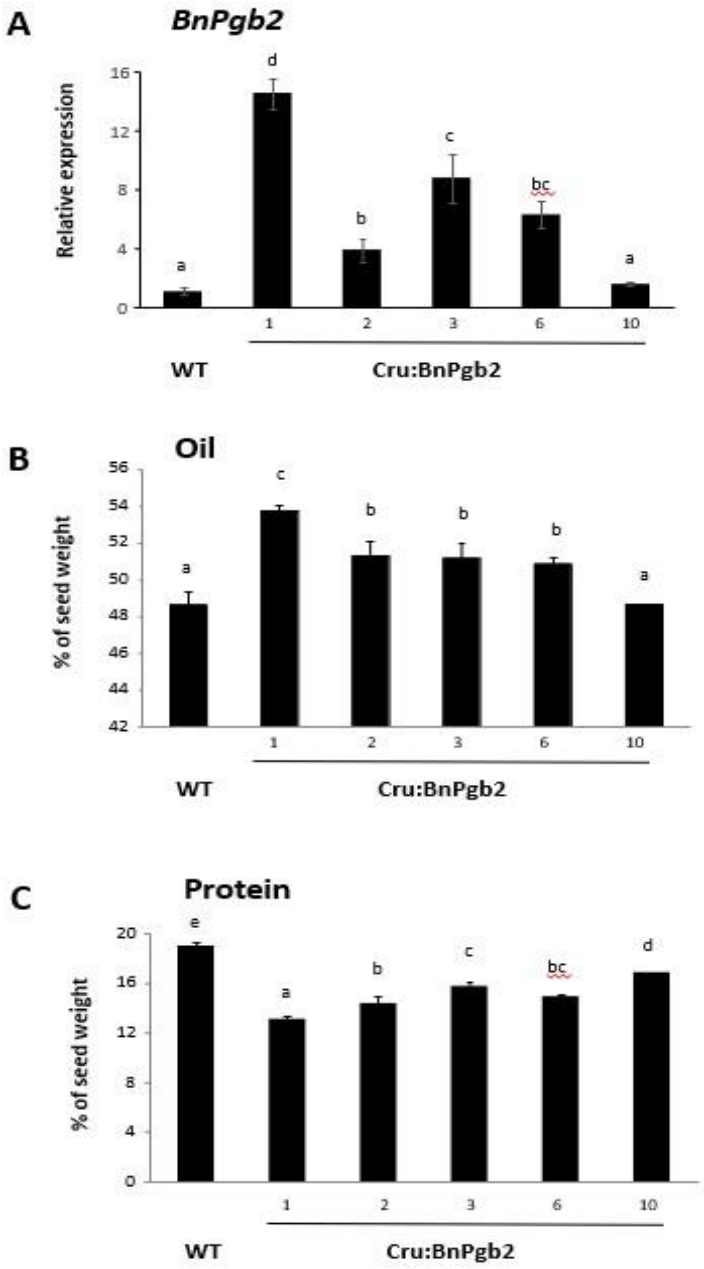

Figure 12: Expression of *BnPgb2* and quantification of storage products in seeds of the *BnPgb2* over-expressing Cru:BnPgb2 lines (1, 2, 3, 6, and 10). **(A)** Expression of *BnPgb2* at 35 DAP in WT and Cru:BnPgb2 seeds. Values, expressed as means $\pm$ SE, are normalized to the WT value set at 1. Oil **(B)** and protein **(C)** levels measured during the 2021 growing season (greenhouse) in dry seeds of the same lines used in (A). Values are means $\pm$ SE. Letters on bars denote statistically significant differences ($p<0.05$).

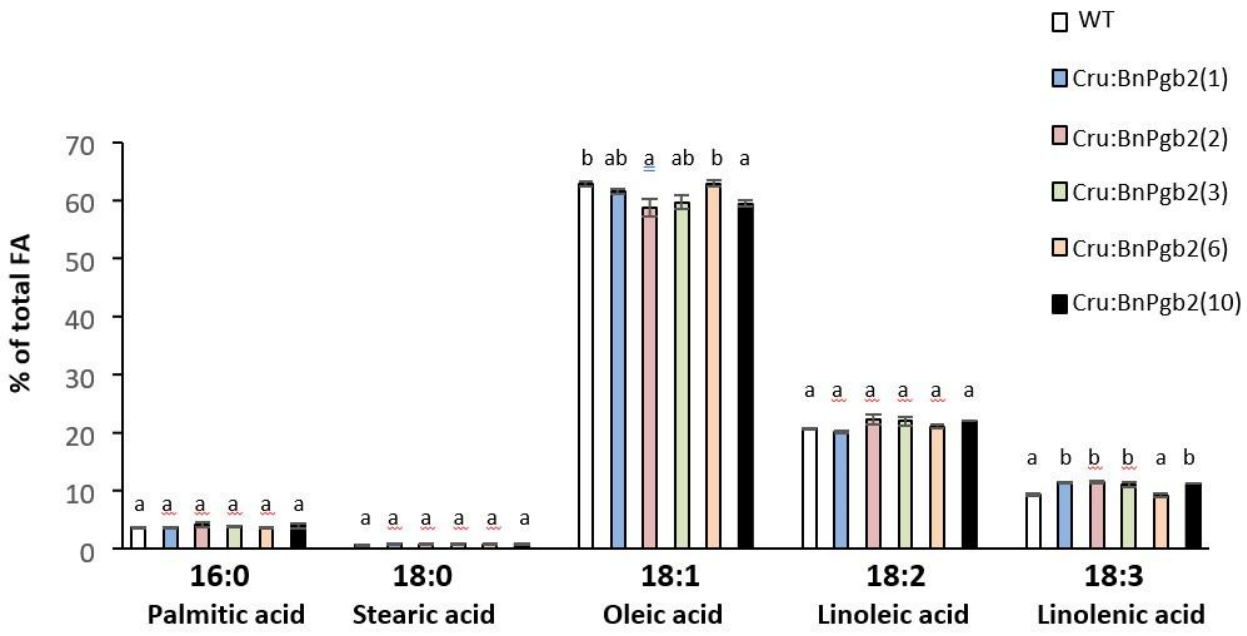

Figure 13: Fatty acid composition measured during the 2021 growing season in dry seeds of WT and Cru:BnPgb2 plants. Letters denote statistically significant differences ($P<0.05$).

as they were not consistent among all the transgenic lines.

Measurements of seed oil, proteins, and FA composition were repeated a second time during the following year (greenhouse). Except for proteins, showing no statistically significant differences among genotypes, a similar trend was observed for oil despite variations in absolute values possibly due to different environmental conditions between years (Fig. 14). Very small variation was observed for FA composition, likely not attributed to *BnPgb2* expression.

To further determine the mechanisms through which over-expression of *BnPgb2* favors the accumulation of oil, we selected the Cru:BnPgb2 lines 1 and 6 for the subsequent studies, as they exhibited the respective highest and intermediate levels of *BnPgb2* and oil (Fig. 12A and B).

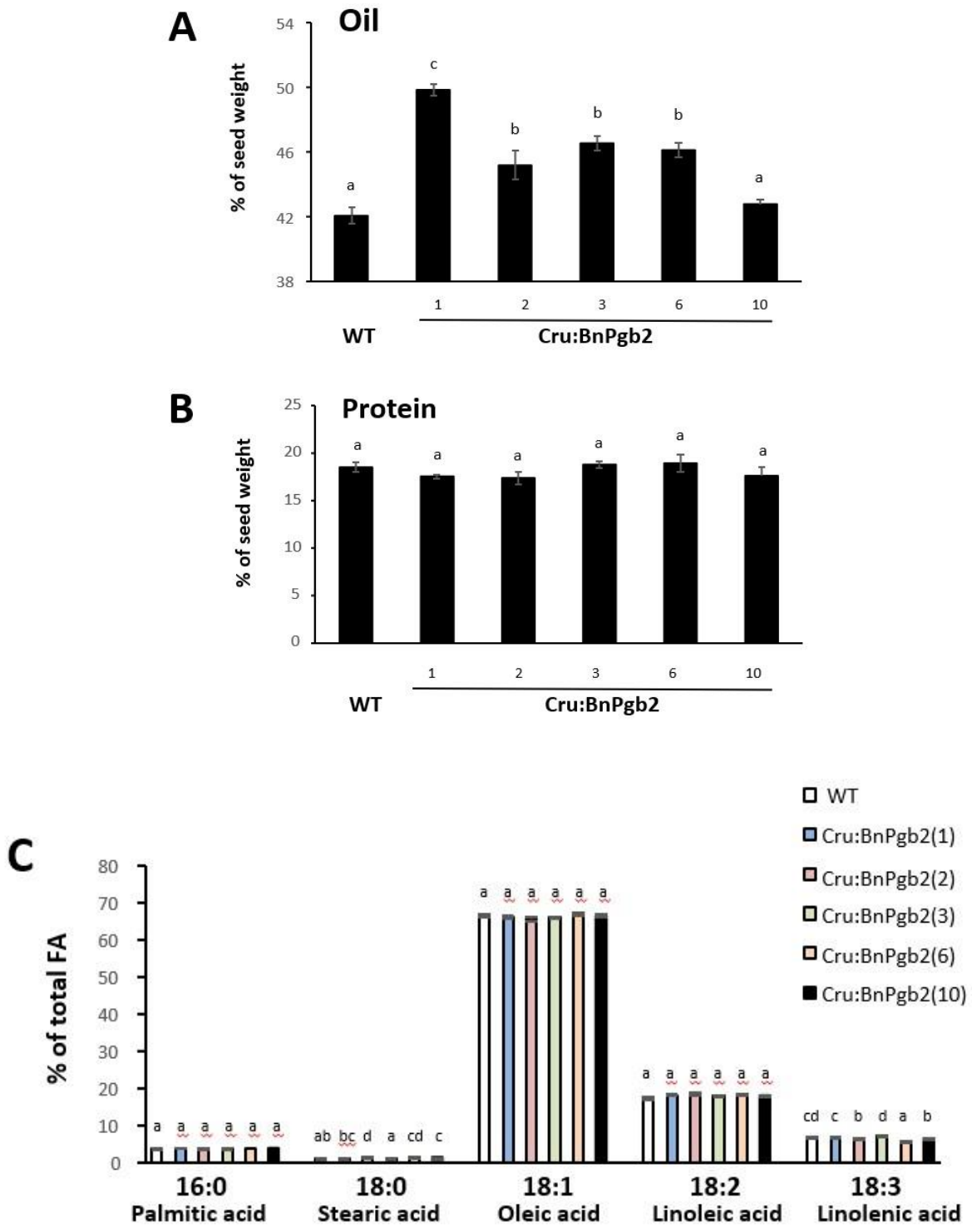

Figure 14: Measurements of oil (**A**), proteins (**B**), and FA composition (**C**) in WT plants and plants over-expressing *BnPgb2* [Cru:BnPgb2]. Measurements were performed during the 2022 growing season (greenhouse). Values are means ± SE. Letters denote statistically significant differences ($p<0.05$).

5.3 Time course analyses of *BnPgb2* expression and storage deposition in developing seeds

A time course analyses of *BnPgb2* transcript levels in developing seeds was performed during the first 35 DAP (Fig. 15A). A steady increment in *BnPgb2* expression was observed after 14 DAP in both WT and Cru:BnPgb2 lines. Relative to the WT, this increment was more pronounced in the two transformed lines, and especially in Cru:BnPgb2(1) seeds where the amount of *BnPgb2* transcripts peaked between 21 and 35 DAP. In all three genotypes the expression of *BnPgb2* reached a plateau, or declined slowly, after 28 DAP (Fig. 15A).

Starch granules were visible from 14 DAP in all three genotypes and their size increased by 21 DAP (Fig. 15B). A transient increase in starch was noted in the axial cells of Cru:BnPgb2(1) (at 21 and 28 DAP), and Cru:BnPgb2(6) (at 28 DAP) seeds. Fewer number of starch granules, smaller in size, were visible in embryonic cells at 35 DAP. A consistent and similar increase in protein bodies between 21-28 DAP, and oil bodies between 28-35 DAP, occurred in developing seeds irrespective of the genotype (Fig. 15B).

To establish if the observed alterations in total seed oil levels and starch deposition patterns in the Cru:BnPgb2 seeds were linked to differences in embryonic development, the external morphology of the embryos was examined. Despite some small variations, the three genotypes followed a very similar embryonic developmental progression, with fully developed embryos visible at 22 DAP (Fig. 16).

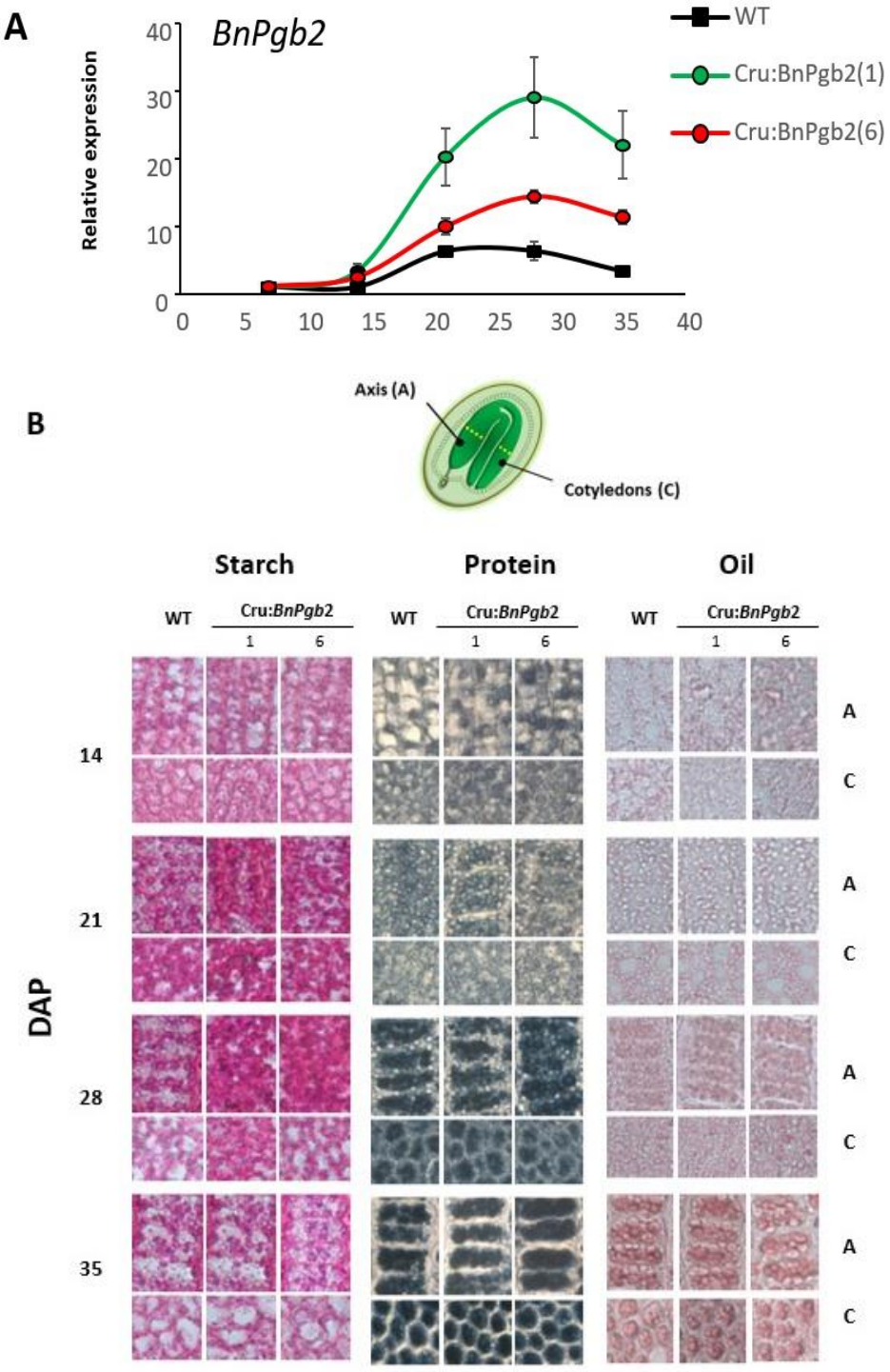

Figure 15: Time course expression of *BnPgb2* and localization of storage products in developing WT seeds and seeds of the selected Cru:BnPgb2 lines 1 and 6. **(A)** Expression levels of *BnPgb2* at 7, 14, 21, 28, and 35 DAP. Values, expressed as means ± SE are normalized to the WT value set at 1. **(B)** Staining of starch, proteins, and oil in seeds of the same lines used in (A) between 14-35

DAP. Storage products were visualized in cells located in the middle (yellow lines) of the embryonic axis (A) or cotyledons (C).

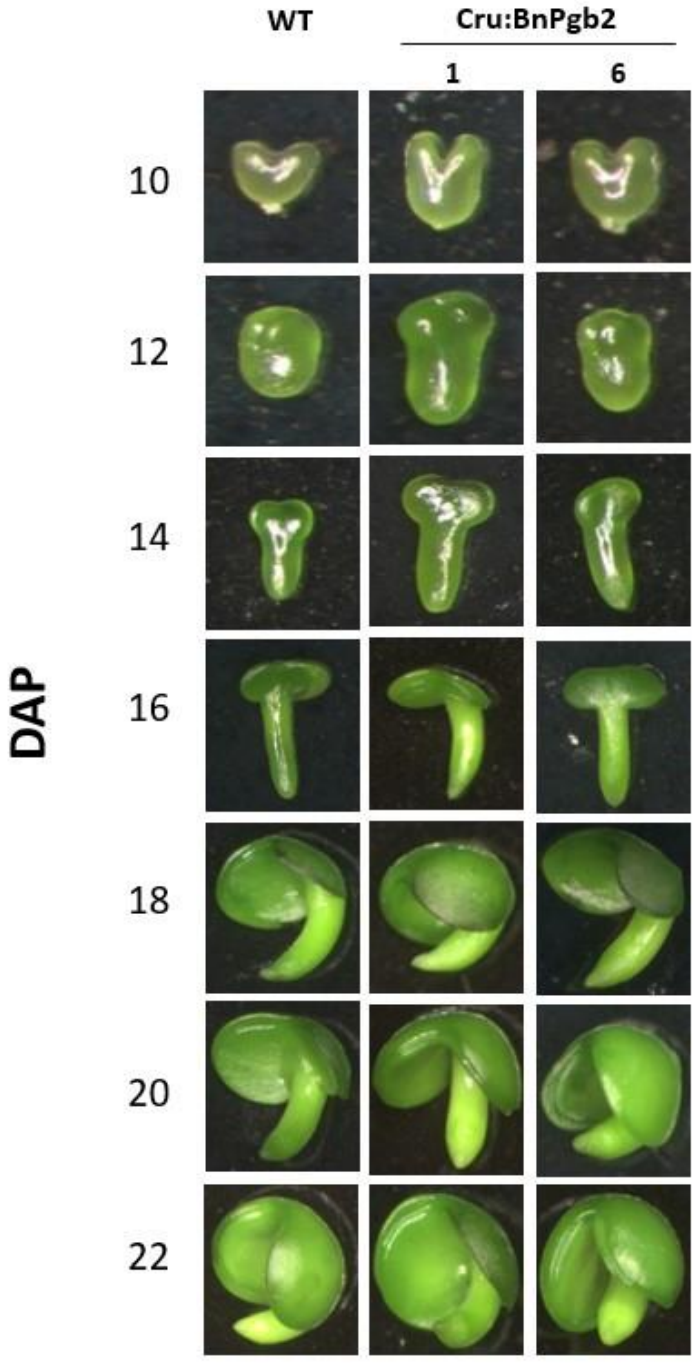

Figure 16: Morphology of developing (10-22 DAP) embryos in the WT line and the selected Cru:BnPgb2 lines, 1 and 6.

5.4 *BnPgb2* regulation of transcription factors and enzymes of sucrose metabolism and fatty acid (FA) synthesis

Synthesis of oil is tightly regulated by a set of transcription factors comprising LEAFY COTYLEDON1 (LEC1), FUSCA3 (FUS3), and WRINKLED1 (WRI1), and supported by sugars produced through photosynthesis or released from storage products (Meinke et al., 1994, Gazzarrini et al., 2004; Baud et al., 2007). In WT seeds, *LEC1* was induced after 14 DAP and its expression remained stable during the following weeks (Fig. 17A). The induction was greater in the transgenic seeds, and especially in those of the Cru:BnPgb2(1) line. No clear differences in the level of *FUS3* transcripts, which did not display marked changes during seed development, were recorded among the different genotypes. In WT seeds *WRI1* expression increased sharply after 21 DAP, and this increase was more pronounced in seeds over-expressing *BnPgb2*, especially between 21 and 35 DAP.

Among the genes participating in sugar transport and metabolism, over-expression of *BnPgb2* had no marked effects on the expression of *SUCROSE TRANSPORTER* (*SUC*) *1*, and *4* (Fig. 17B). While the former exhibited a peak between 14 and 35 DAP in all genotypes, the latter did not show great variations during seed development. In developing WT seeds, the expressions of the *SUCROSE SYNTHASE* (*SUS*) *1* and *3*, as well as the starch synthesizing enzyme *ADP-GLUCOSE PHOSPHORYLASE* (*AGPase*) did not show pronounced changes during development. The three genes were induced in Cru:BnPgb2 seeds. Among the glycolytic enzymes, no major changes were observed for *HEXOSE KINASE* (*HXK*); this was in contrast to *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*PPK*) and *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GPDH*) which were mildly induced after 14 DAP in all genotypes. Relative

to WT, over-expression of *BnPgb2* elevated the expression of *FRUCTOSE BISPHOSPHATE ALDOLASE* (*FPA*) and *PHOSPHOGLYCERATE KINASE* (*PGK*).

Transcriptional changes in FA synthesis were also monitored by measuring the expression of *SUBUNIT A OF ACETYL-CoA CARBOXYLASE* (*ACCA2*), *MALONYL-CoA:ACP TRANSACYLASE* (*MCAT*), *FA DESATURASE3* (*FAD3*), and *FA ELONGATION1* (*FAE1*). Unlike the WT, displaying minor developmental fluctuations in *ACCA2* and *MCAT*, *BnPgb2* up-regulated both genes after 14 DAP. With the exception of *FAE1* in BnCru:Pgb2(6) seeds showing a peak between 14-28DAP, no major differences in expression were measured among genotypes for *FAD3* and *FAE1* (Fig. 17B).

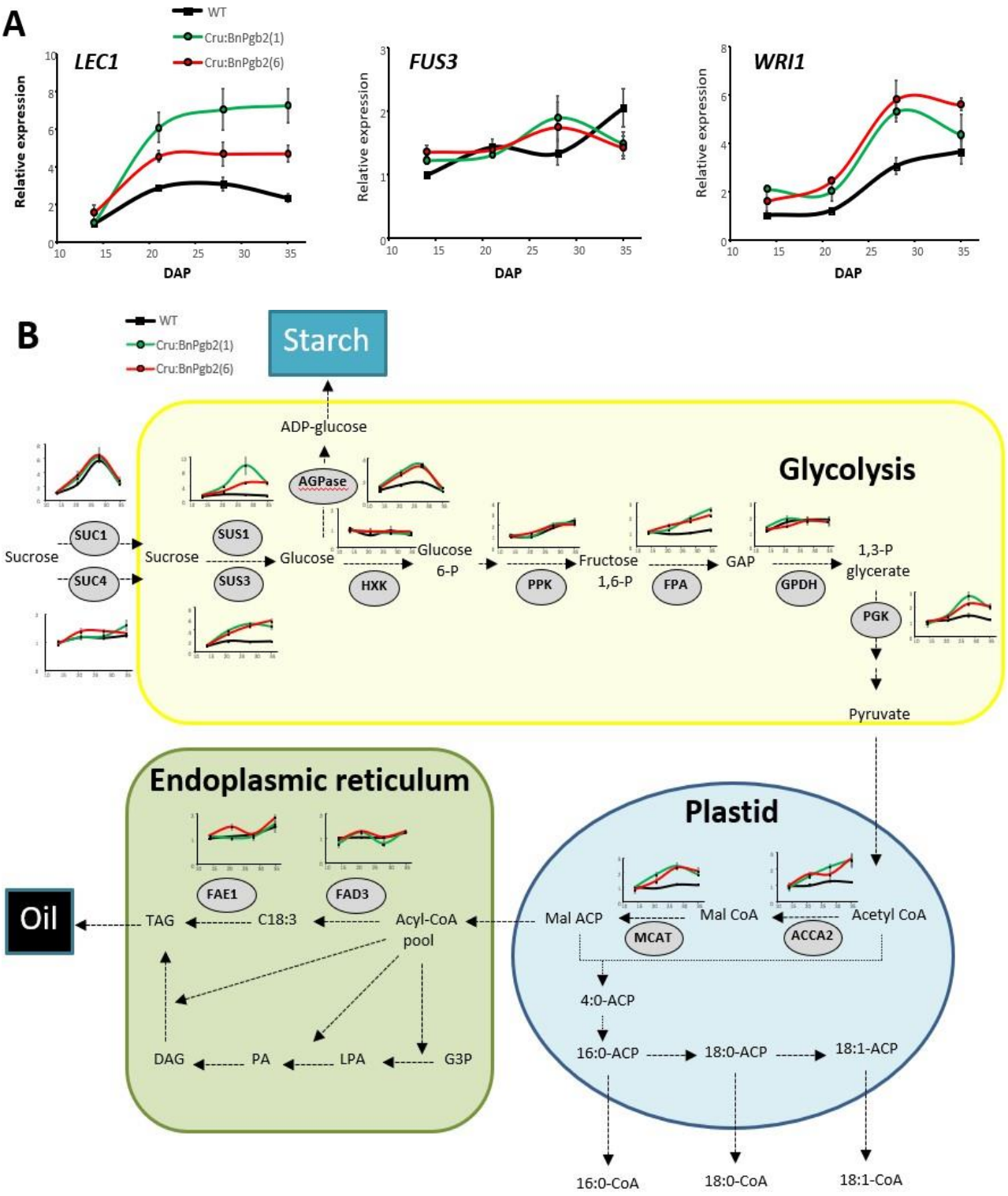

Figure 17: Expression levels of the transcription factors LEAFY COTYLEDONS1 (*LEC1)*, *FUSCA3* (*FUS3)* and *WRINKLED* (*WRI1*) **(A)**, and enzymes related to oil synthesis **(B)** in

developing seeds (14-35 DAP) of the WT line and the selected Cru:BnPgb2 lines 1 and 6. Values ± SE are means normalized to the value of WT (at 14 DAP) set at 1. Enzymes measured are *SUSCROSE TRANSPORTER* (*SUC*) *1* and *4*, *SUCROSE SYNTHASE* (*SUS*) *1* and *3*, *ADP-GLUCOSE PHOSPHORYLASE* (*AGPase*), *HEXOSE KINASE* (*HXK*), *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* (*PPK*), *FRUCTOSE* BISPHOSPHATE ALDOLASE (*FPA*), *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GPDH*), *PHOSPHOGLYCERATE KINASE* (*PGK*), *SUBUNIT A OF ACETYL-CoA CARBOXYLASE* (*ACCA2*), *MALONYL-CoA:ACP TRANSACYLASE* (*MCAT*), *FA DESATURASE3* (*FAD3*), and *FA ELONGATION1* (*FAE1*).

6. DISCUSSION

Seed oil in oilseeds crops, including *B. napus*, is mainly composed by triacylglycerols (TAG); which are the primarily source of edible oil. As such, an elevation in TAG production has become a priority in many breeding programs (Weselake et al., 2009). Genetic approaches to increase seed oil level and quality have mainly focussed on altering the expression of single enzymes involved in FA and TAG synthesis, or transcription factors operating upstream of FA synthesis, such as *FUSCA3* (Wang and Perry 2013), *LEAFY COTYLEDON1* (*LEC1*), and *WRINKLED1* (Lotan et al., 1998, Kwong et al., 2003, Cernac and Benning, 2004; Tan et al., 2011). Induction of the Arabidopsis *Pgb2* using a seed-specific promoter has also been successful in elevating oil content by about 40% (Vigeolas et al., 2011). The increment in seed oil was concomitant with an increase in linoleic and linolenic acids. Here, we provide evidence that in the major oil-producing crop *B. napus* the amount of *BnPgb2* transcripts in the seed is a determinant factor for the accumulation of oil. First, *BnPgb2* transcripts are induced preferentially in seeds of high-oil (HO) genotypes relative to their low-oil (LO) counterparts (Fig. 8), and this effect is not the result of deviations in embryonic growth progression among genotypes (Fig. 9). Second, seed oil level is increased by the over-expression of *BnPgb2*, and the pattern of oil accumulation closely reflects the level of *BnPgb2* present in the seed (Fig. 12). The increment in seed oil reported in this study, however, is inferior to that documented in *Pgb2* over-expressing Arabidopsis plants (Vigeolas et al., 2011). This discrepancy is likely the result of differences in transgene expression, possibly a consequence of the two promoters used [AtUSP in Vigeola et al. (2011) and BnCru1 in this study] to drive *Pgb2* in seeds. Unfortunately, the lack of quantitative expression data in Vigeolas et al. (2011) does not permit comparisons of *Pgb2* transcripts between the transformed Arabidopsis and *B. napus* seeds. In addition, unlike Arabidopsis, *B. napus* has undergone selection

to produce high amounts of oil, and thus due to physiological limitations, large increases in oil content are not expected.

The *Pgb2* increment in oil observed in Arabidopsis was not ascribed to the NO scavenging properties of the protein, as over-expression of *Pgb1*, which is known to scavenge NO, did not produce similar effects (Vigeolas et al., 2011). Rather the function of *Pgb2* was to mitigate the decline in energy status within hypoxic seeds. The seed environment is in fact highly hypoxic with oxygen levels ranging from 2-4% (Gibon et al., 2002; Rolletscheck 2005, 2007), and this condition depresses energy-requiring biosynthetic processes including those involved in the production of lipids and starch (Vigeolas et al., 2003; Rolletschek et al., 2005, 2007). By increasing the ATP:ADP ratio, *Pgb2* has been suggested to support these processes (Vigeolas et al., 2011). Based on the results obtained by Godee et al. (2017), the increased oil content may be the result of processes altered by the presence of BnPgb2 in the nucleus.

Transcriptome studies provide some evidence to support this thesis. There are very similar patterns between the expression of *BnPgb2* and *LEC1* (compare Fig. 15A and 17A). Both show a rapid induction in developing seeds between 14-21 DAP, and the induction of *LEC1* is greater in the *BnPgb2* enriched seeds of the Cru:BnPgb2 lines. Of interest, *LEC1* expression is regulated by histone acetylation processes (Moronczyk et al., 2022), which is well known to be affected by NO (Ageeva-Kieferle et al., 2021). Despite the evidence by Vigeolas et al. (2011), it cannot be discounted that presence of BnPgb2 in the nucleus (Godee et al., 2017) might modulate the level of NO.

Regarded as a master regulator of embryonic development (Parcy et al., 1997) LEC1 is a member of the HAP3 family of transcription factors (Kwong et al., 2003). Its over-expression is sufficient to elevate seed oil level in Arabidopsis through the simultaneous induction of about 60%

of enzymes participating in FA synthesis (Mu et al., 2008). Similar increases in seed oil following *LEC1* over-expression were also reported in maize (Shen et al., 2010), and *B. napus*, where Tan et al. (2011) measured an elevation in seed oil ranging from 2-15%. Among the direct targets of *LEC1* is the transcription factor *WRI1*, which is also preferentially induced in developing Cru:BnPgb2 seeds especially between 21 and 35 DAP (Fig. 17A). Raising seed oil when over-expressed (Cernak and Benning 2004; Liu et al., 2010; Shen et al., 2010), *WRI1* up-regulates several enzymes of the upper (Maeo et al., 2009) and lower (Kuczynski, 2022) glycolytic pathway. While still based on transcriptome data, these results suggest that the seed-specific expression of *BnPgb2* is conducive to the induction of key transcription factors responsible for the synthesis of oil and the mobilization of sugars in support of FA production.

Mobilization of sugars potentiates FA synthesis, well beyond the canonical role of providing carbon skeleton (Zhai et al., 2021). Several pieces of evidence suggest that BnPgb2 interferes with sugar metabolism of developing seeds. First, over-expression of *BnPgb2* induces the sucrose transporters *SUS1* and *SUS3* (Fig. 17B), known to reversibly regulate sucrose flow towards several pathways, including the synthesis of TAG (Zheng et al., 2011). Second, the transient accumulation of starch granules around 28 DAP, preceding the increase oil at 35 DAP (Fig. 15B), is more prominent in Cru:BnPgb2 seeds. This increase is concomitant with the induction of *ADP-Glc pyrophosphorylase* (*AGPase*) in the same seeds (Fig. 17B). The enzyme catalyses the formation of ADP-glucose which is then incorporated into starch (Kang and Rawasthorne, 1994). While originally considered as a competitive process to lipid synthesis (Bettey and Smith, 1990), transient production of starch has been shown to serve as a key source of sugar for lipid formation (Norton and Harry, 1995). The proposed positive link between starch and lipids was confirmed by the depletion (by about 40%) in oil observed in starchless Arabidopsis

mutants (Periappuram et al., 2000), and by the marked decrease in storage lipid following a reduction in AGPase activity (Vigeolas et al., 2004). Finally, from the higher induction of *fructose bisphosphate aldolase* (*FPA*) and *phosphoglycerate kinase* (*PGK*) in the Cru:BnPgb2 seeds (Fig. 17B) it cannot be excluded that BnPgb2 facilitates the carbon flux through the glycolytic pathway in support of FA synthesis.

Additional effects observed following over-expression of *BnPgb2* are the induction of *acetyl-CoA carboxylase 2* (*ACCA2*) and *Malonyl CoA-ACP Malonyltransferase* (*MCAT*). The activity of ACCA2, required for the conversion of acetyl-CoA to malonyl-CoA (Konishi and Sasaki, 1994), is considered the "determinant" step for the synthesis of FA in *B. napus* seeds (Schulte et al., 1997). While over-expression of *ACCA* in plastids increases total oil and elevates the level of oleic acid (Roesler et al., 1997), its downregulation depresses seed oil (Sellwood et al., 2000). In animals, the enzyme is inhibited by AMP-activated protein kinase present in energy depleted environment (Rubink and Winder, 1985). This raises the possibility that by maintaining a high ATP/ADP ratio, the over-expression of *BnPgb2* might promote ACCA activity in the hypoxic seeds. The second plastid enzyme induced by over-expression of *Pgb2*, *MCAT*, generates malonyl-ACP from malonyl CoA (Lessire and Stumpe, 1983). Arabidopsis seeds expressing *MCAT* under a seed specific promoter exhibited an increase in FAs of about 10%, and this was in contrast to seeds inhibiting the enzyme where the FA level decreased by a similar magnitude (Jung et al., 2019).

Besides elevating seed oil, *BnPgb2* has no consistent repercussions on other key seed development traits, where the minor changes observed are not shared among all the transgenic lines (Supplementary table 2). This observation is relevant in light of the fact that in some instances, when constitutively expressed, *Pgbs* results in growth abnormalities (Cochrane et al.,

2017). This has been observed also in transgenic *B. napus* plants constitutively expressing *BnPgb2*, with the highest levels of the transgene (our lab, unpublished data). Therefore, seed-specific induction, rather than constitutive expression, seems to be an effective strategy to ameliorate plant productivity in this species.

7. CONCLUSION

In conclusion, this work demonstrates that the seed specific expression of *BnPgb2* increases oil content in the oil-producing crop *B. napus* without major alterations in its nutritional value (fatty acid profile) and without detrimental effects on other desirable phenotypic traits. The elevated oil content was associated with the induction of the key transcription factors *LEC1* and *WRI1*, as well several enzymes participating in carbohydrate metabolism and FA synthesis. These changes, together with the transient increase in starch prior to the deposition of oil bodies, suggests that BnPgb2 might interfere with sugar metabolisms by possibly facilitating flow for carbon for production of FA. The greater induction of *BnPgb2* in developing high oil seeds, relative to low oil seeds, further supports the beneficial role of this gene in oil synthesis.

8. FUTURE DIRECTIONS

The results of our study suggest that over-expressing the *BnPgb2* gene in *Brassica napus* seeds can increase the seed oil content without negatively affecting its fatty acids profile or other key seed developmental traits. The transformed genotypes developed from this study might be replicated for multi-year and multi-location research experiments for further varietal development process. This finding has important implications for the oil-producing industry, as it can lead to increased oil yield and profitability.

Looking ahead, there are various opportunities for future research, and one potential path is to explore the possibility of over-expressing *BnPgb2* in other oil-producing crops. This would help to determine whether *BnPgb2* can have similar effects on oil content in other crops and as well as to investigate whether the same underlying mechanisms are involved.

Further research could focus on optimizing the expression of *BnPgb2* to achieve even higher oil content in *B. napus* and other oilseed crops such as soybeans or sunflowers. Knowing the possible effects of *BnPgb2* on sugar metabolism and carbon flow can provide valuable insights into the mechanisms underlying oil synthesis in seeds. The utilization of genetic methods, such as the seed-specific expression of *BnPgb2*, could help in satisfying the growing need for vegetable oils while promoting sustainable agricultural practices.

Another exciting area of research would be to investigate the potential of using *BnPgb2* as a molecular marker for crop breeding program. By selecting for higher levels of *BnPgb2* in crop seeds, it may be possible to develop new crop varieties with higher oil content and improved yield. This could involve screening germplasm collections for genotypes with high levels of *BnPgb2* and using them as parental materials for future breeding programs.

Finally, *Pgb2* is known to operate in the nucleus (Godee et al., 2017). Can seed oil be increased by targeting the gene into the nucleus? The use of Arabidopsis seeds developed by Godee et al. (2017), where dexamethasone applications translocate Pgb2 protein into the nucleus could be used as a preliminary experiment.

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10. SUPPLEMENTARY TABLES

Supplementary table 1: List of primers used in this study.

Primer name	Sequence
Primers utilized for gene expression study	
BnLEC1-F	TATCTTGCCGCAGCAACAACCAAG
BnLEC1-R	TTCACCGGTCACGAAGCTGATGTA
BnFUS3-F	TCCATCATCGTCCAGGGTTTGGAT
BnFUS3-R	AAGAAGATCGTCTCTGTCTGGGCA
BnWRI1-F	AAACCACAGAATCCCACCAGTCCT
BnWRI1-R	AGATCGTACGTATGTGCTGCTGCT
BnACCA2-F	TTGCCAGTCATAGTCCAGAGGCAT
BnACCA2-R	TCTGCTGCGATGTCCATGAAGGAT
BnAGP-F	TGCTGGAACGAGATTGTATCCGCT
BnAGP-R	ATGGCGGTTGAGAGAAGCTGAGTT
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
BnACTIN2-F	ACGAGCTACCTGACGGACAAG
BnACTIN2-R	GAGCGACGGCTGGAAGAGTA
proCRUF	ACGTAGGACCACGTAACAACGA
BnPGB2-F	GAAGCAAGAAGCTTTGGTGAAG
BnPGB2-R	TGGCTAAAGCCAAGTGATCATA
Primers utilized for Transformation and Genotyping	
BnPGB2.2-F BamH1	CGCGGATCCGCGATGGGAGAGATTGTGTTTACA
BnPGB2.2-R ECOR1	CCGGAATTCCGGTCAGGGTTTCTGTGAGTTTTC
Pro:CRU-F BamH1	CGCGGATCCGCGACGTAGGACCACGTAACAACGA

Supplementary table 2: Agronomic traits of the WT genotype and the Cru:BnPgb2 lines. Values are means ± SE. Asterisks indicate values which are statistically significant ($p<0.05$) from the WT value. Experiments were conducted using a minimum of four biological replicates each containing 5 plants.

Genotypes	Plant height (cm)	Siliques/plant	Seeds/silique	Seeds/plant (gm)	1000 grain weight(gm)
WT	43.7 ± 0.2	72.0 ± 0.9	23.8 ± 0.2	4.1 ± 0.2	3.05 ± 0.02
Cru:BnPgb2(1)	44.4 ± 0.7	73.1 ± 1.2	23.2 ± 0.1	3.9 ± 0.2	3.10 ± 0.09
Cru:BnPgb2(2)	43.8 ± 0.7	73.1 ± 1.8	22.7 ± 0.4	3.5 ± 0.2	3.02 ± 0.04
Cru:BnPgb2(3)	43.3 ± 0.5	70.5 ± 0.7	22.7 + 0.4	3.9 ± 0.2	3.08 ± 0.05
Cru:BnPgb2(6)	45.2 ± 0.5*****	74.3 ± 0.5	22.9 ± 0.2	3.6 ± 0.2	3.01 ± 0.04
Cru:BnPgb2(10)	44.4 ± 0.2	77.1 + 1.1*****	22.3 ± 0.7	3.5 ± 0.2	3.00 ± 0.03

Experimental pictures



Growing of DH plants



Sample collection and marking



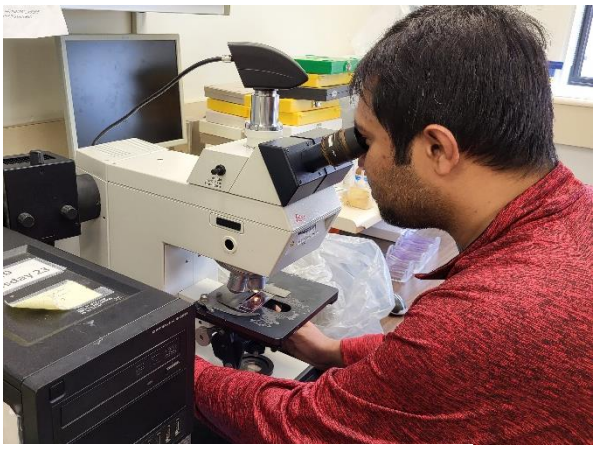
Growing plants in green house



Paraffin waxing of samples



Dewaxing using xylene



Microscopic work