The Effects of Altered Expression of Meristem Genes in *Brassica napus*

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of requirements for the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

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ABSTRACT

Yang, Cunchun. M.Sc., The University of Manitoba, August, 2012.
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The meristem genes *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1* (*CLV1*) and *ZWILLE* (*ZLL*) are essential for the formation and maintenance of the shoot apical meristem (SAM). As an extension of that work, this thesis examines the function of these genes during seed oil accumulation and microspore-derived embryogenesis. Using a transgenic approach it is demonstrated that only the over-expression of *BnSTM* increases the percentage of total seed oil and a reduction of glucosinolate (GLS) levels achieved by transcriptional regualtion. The over-expression of *BnSTM* also affected *in vitro* embryogenesis by increasing the number and quality of microspore-derived embryos (MDEs) in contrast to the MDEs down-regulating *BnSTM*. The MDEs with ectopic expression of *BnSTM* were found to regulate embryonic SAM by altering cytokinin synthesis, catabolism, perception and signaling. Taken together, these findings provide evidence for a novel function of *BnSTM* in promoting desirable changes in seed oil and GLS levels and enhancing *in vitro* embryogenesis.

ACKNOWLEDGEMENTS

I would like to thank both my supervisor, Dr. Stasolla, and my former co-supervisor, Dr. Tahir, for their guidance and support. I also like to thank Dr. Elhiti, who shared his knowledge on plant transformation and embryogenesis, and Mr. Durnin, who has assisted me in many of the experiments. Other members of the lab including Kevin Baron and Shuanglong Huang are also acknowledged for their advices and support.

A special thank goes to Dr. Renault and Dr. Ayele for providing suggestions and directions to my research. Finally, I would like to thank all people in the Department of Plant Science for their friendship and encouragements.

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LIST OF ABBREVIATIONS

ABI3, ABSCISIC ACID INSENSITIVE3

ACCA2, subunit A of ACETYL-CoA CARBOXYLASE

ACCase, acetyl-CoA carboxylase

ACP, acyl carrier protein

ACR4, ARABIDOPSIS CRINKLY4

ADP, adenosine diphosphate

AGP, ADP-GLUCOSE PHOSPHORLAYSE

AHK, ARABIDOPSIS HISTIDINE KINASE

AHP, ARABIDOPSIS HISTIDINE PHOSPHOTRANMITTER

ALE1, ABNORMAL LEAF SHAPE1

ARR, ARABIDOPSIS RESPONSE REGULATOR

At, *Arabidopsis thaliana*

AtML1, ARABIDOPSIS THALIANA MERISTEM LAYER1

ATP, adenosine triphosphate

ATR1, ALTERED TRYPTOPHAN REGULATION1

B5 medium, Gamborg B5 medium

BDL, BODENLOS

Bn, *Brassica napus*

cDNA, complementary deoxyribonucleic acid

CKXs, CYTOKININ OXIDASE

CLV, CLAVATA

CoA, Coenzyme A

CRE1, CYTOKININ RESPONSE1

CUC, CUP-SHAPED COTYLEDON

CZ, central zone

DAP, day after pollination

DAG, diacylglycerol

DAGAT, diacylglycerol acyltransferases

DHAP, dihydroxyacetonephosphate

DNA, deoxyribonucleic acid

EIF2C, elongation initiation factor 2c

FA, fatty acid

FAD, FA oleate desaturase

FAD2, FA oleate desaturase

FAD3, ω-3 FA DESATURASE

FAE1, FA ELONGATION1

FAS, FA synthase

FK, FACKEL

FPA, FRUCTOSE BISOPHOSPHATE ALDOLASE

FUS3, FUSCA3

G3P, glycerol-3-phosphates

G3PAT, glycerol-3-phosphate acyltransferase

G3PDH, glycerol-3-phosphate acyltransferase

GAs, Gibberellins

GK, GURKE

GLS, glucosinolates

GPDH, GLYCERALDEHYDES-3-PHOSPHATE DEHYDROGENASE

HAP3, Heme-Activated Proteins 3 subunit

HBT, HOBBIT

HXK, HEXOSE KINASE

IAA, indole acetic acid

IBA, indole butyric acid

IPT, isopentenyl transferase

KAS, ketoacyl-ACP synthase

KCS, 3-ketoacyl-CoA synthase

KNAT, KNOTTED1-like IN ARABIDOPSIS THALIANA

KNOX, KNOTTED1-LIKE HOMEOBOX

L1L, LEAFY COTYLEDON1-like

LEC1, LEAFY COTYLEDON1

LEC2, LEAFY COTYLEDON2

LOG, LONELY GUY

LPA, lysophosphatidic acid

LPAAT, lysophosphatidic acid acyltransferase

LPCAT, lysophosphatidylcholine acyltransferase

MAM, methylthioalkylamalate synthase

MCAT, MALONYL-CoA:ACP TRANSCYLASE

MDE, microspore-derived embryo

MP, MONOPTEROS

MS medium, Murashige-Skoog medium

MYB28, MYB-CONTAINING TRANSCRIPTION FACTOR28

NIT2, NITRILASE2

OLS, OLEOSINS

PA, phosphatidic acid

PAP, phosphatidic acid phosphohydrolase

PC, phosphatidylcholine

PCR, polymerase chain reaction

PDAT, phospholipid: diacylglycerol acyltransferase

PDF2, PROTODERMAL FACTOR2

PGK, PHOSPHOGLYCERATE KINASE

PINs, auxin efflux carrier proteins

PLL1, POLTERGEIST LIKE1

PLT, PLETHORA

POL, POLTERGEIST

PPK, PYROPHOSPHATE-DEPENDENT PHSOPHOFRUCTOSE

PZ, peripheral zone

RAM, root apical meristem

RNA, ribonucleic acid

RT, reverse transcription

RZ, rib zone

SAM, shoot apical meristem

SCR, SCARECROW

SHR, SHORTROOT

ST5a, SULFOTRANSFERASE5a

STM, SHOOT MERISTEMLESS

SUC, SUCROSE TRANSPORTER

SUR, SUPERROOT

SUS, SUCROSE SYNTHEASE

TAG, triacylglycerols

VLCFA, very long chain fatty acids

WOL, WOODEN LEG

WOX, WUSCHEL-related HOMEOBOX

WRI1, WRINKLED1

WT, wild type

WUS, WUSCHEL

ZLL, ZWILLE

1.0. INTRODUCTION

Embryogenesis is an important event during the plant life cycle which is initiated with the formation of the zygote, the product of the fusion between the male (sperm) and female (egg) gametes (reviewed by Goldberg et al., 1994). Through a coordinated pattern of cell division and differentiation, the embryo body is elaborated and at maturity it consists of an embryonic axis separating the shoot and root meristems, and one or more cotyledons (reviewed by Park and Harada, 2008). Embryogenesis is not just an *in vivo* prerogative, as it can be induced *in vitro* by culturing cells, tissues, or organs, under controlled culture conditions (reviewed by Mordhorst et al., 1997). In *Brassica napus*, *in vitro* embryogenesis can be achieved via androgenesis through the differentiation of isolated microspores into microspore-derived embryos (MDEs). This process is very effective in that a large number of synchronized MDEs can be obtained in a relatively short period of time. Furthermore, the sequence of developmental changes occurring in MDEs is similar to that observed during *in vivo* embryogenesis (Yeung et al., 1996).

Two important events observed during both *in vivo* and *in vitro* embryogenesis are the formation of the shoot apical meristem (SAM) and the deposition of storage products (Goldberg et al., 1994). The SAM is the uppermost meristem of the plant and is responsible for the formation of the above-ground organs. Genetic studies have shown that formation of the SAM during embryogenesis and its post-embryonic maintenance are governed by a complex molecular network involving several genes, including *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1* (*CLV1*), *ZWILLE* (*ZLL*), and *WUSCHEL* (*WUS*) (reviewed by Barton, 2010). *SHOOT MERISTEMLESS* encodes a homeodomain transcription regulator of the *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) family (reviewed

by Scofield and Murray, 2006). The role of *STM* is to maintain the stem cells of the SAM in an undifferentiated state by interacting with several other factors (Kanrar et al., 2006; Rutjens et al., 2009) and maintaining a high cytokinin environment (Jasinski et al., 2005; Yanai et al., 2005). Independent studies showed that elevated *STM* expression induces the expression of *ISOPENTENYL TRANSFERASE7* (*IPT7*), a cytokinin biosynthetic enzyme (Jasinski et al., 2005; Yanai et al., 2005). High levels of cytokinin within the SAM are crucial for conferring an undifferentiated state since depletion of this growth regulator results in the premature differentiation of meristematic cells (reviewed by Shani et al., 2006). Proper expression of *STM* within the SAM is ensured by ZWILLE (ZLL), a member of the EIF2C (elongation initiation factor 2c)/ARGONAUTE class of proteins involved in RNA silencing (Lynn et al., 1999; Liu et al., 2009). Suppression of *ZLL* results in the miss-expression of *STM* leading to structural and physiological abnormalities of the SAM (Moussian et al., 1998).

Another key regulator of the SAM is WUSCHEL (WUS), a homeodomain transcription factor from the *WUSCHEL-LIKE HOMEOBOX* (*WOX*) gene family (reviewed by Dodsworth, 2009). Expression of *WUS*, which is triggered by the *STM*-increase in cytokinin level, occurs very early during embryogenesis and is localized in a cluster of sub-apical cells within the SAM (Laux et al., 1996). The *WUS*-expressing domain, referred to as the "organizing center" of the SAM, is required for specifying stem cell fate in the apical cells of the meristem, by interacting with the CLAVATA (CLV) signaling pathway (Barton, 2010). The CLV signaling pathway includes CLV1, a receptor kinase which restricts the number of stem cells within the SAM in an antagonistic fashion to STM (Clark et al., 1996) and WUS (Schoof et al., 2000). The

signal cascade triggered by CLV1 also requires two additional CLV members: CLV3, a small peptide (Miwa et al., 2009) and CLV2, a receptor kinase (Jun et al., 2008). More detailed information on the genetic interaction among *STM*, *ZLL*, *WUS*, and *CLV1*, responsible for the formation of the SAM and its functionality, is presented in the literature review (section 2.2.6.).

Another important event occurring during embryogenesis is represented by the accumulation of storage products during the late developmental phases. This process, a prerequisite for successful germination (reviewed by Bewley, 1997; Pritchard et al., 2002), is well documented in economically important species, such as *Brassica napus* (canola). Canola is a Canadian developed crop grown for production of seed oil utilized not only for food, but also for basic components in a number of industrial applications (reviewed by Scarth and Tang, 2006). Oil in canola seeds is stored in specific oil bodies, oleosomes, which form during the late stages of embryo development (reviewed by Huang, 1992; Tzen and Huang, 1992). The metabolic pathways leading to the biosynthesis of fatty acids (FA) and triacylglycerols (TAG) require several proteins, most of which have been characterized. Production of FA is initiated with the condensation of acetyl-CoA and malonyl-CoA, the latter produced by the carboxylation of acetyl-CoA, which forms butyryl-ACP. Further elongation of the FA molecule is achieved by additional reactions with malonyl-ACP molecules (reviewed by Ohlrogge and Browse, 1995). Conjugations of FA with glycerol-3-phosphate (G3P) results in the formation of different TAG which are then stored in oil bodies (Baud et al., 2008). Synthesis of FA is controlled by a regulatory network of transcription factors, including *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *WRINKLED1* (*WRI1*) and

FUSCA3 (*FUS3*). While LEC1 regulates several metabolic processes such as glycolysis, FA synthesis and lipid accumulation (Mu et al., 2008), LEC2 is mainly involved in FA accumulation possibly through the action of *WRI1* (Baud et al., 2008). *WRINKLED1* has been shown to regulate some enzymes participating in glycolysis, FA synthesis and an acyl carrier protein (ACP1) (Maeo et al., 2009). Synthesis of FA is also regulated by FUS3, although the main function of this transcription factor is to induce seed storage proteins (Tiedemann et al., 2008). Genetic manipulation of these genes has been widely and successfully employed in a variety of species to alter oil composition and quantity (Wang et al., 2007; Maeo et al., 2009).

Oil quality in canola is determined by a multitude of factors, one being the level of glucosinolates (GLS), a class of organic compounds containing sulphur and nitrogen which are derived from glucose and amino acids (Sonderby et al., 2010). Due to their toxicity for both humans and animals at high doses (Office of The Gene Technology Regulator, 2002), low GLS content is highly desirable in canola seeds. Biosynthesis of GLS is regulated by two major classes of genes: cytochrome P450 genes such as *CYP79B2*, which is responsible for the core structure of the GLS molecule (reviewed by Halkier and Gershenzon, 2006), and *METHYLTHIOALKYLMALATE SYNTHASE* genes (*MAM*s) involved in the elongation steps of GLSs (reviewed by Benderoth et al., 2009). Over the past years many efforts have been directed towards reducing the levels of GLS in Brassica seeds through breeding programs and genetic manipulations of key biosynthetic genes.

1.1 Research objectives

Formation of the SAM is a key event during embryogenesis which is controlled by a complex genetic interaction involving *STM*, *ZLL* and *CLV1*. Recent work conducted in Dr. Stasolla's lab has showed that besides their involvement in SAM formation, these genes might also participate in other developmental processes (Elhiti et al., 2010). As a continuation of that study, this thesis examines the effects of altered expression of the *Brassica napus* (*Bn*) *STM*, *ZLL*, and *CLV1* on the accumulation of storage product deposition, i.e. oil, in Brassica seeds (Chapter 1), and on the ability to form MDEs in culture (Chapter 2).

Chapter 1 examines the effects of *BnSTM*, *BnZLL* and *BnCLV1* on total seed oil and shows that over-expression of *BnSTM* enhances total oil content without altering the nutritional value of the oil. Furthermore, evidence is presented indicating that the increased oil content in *BnSTM*-over-expressing lines is due to high levels of cytokinin which increase sink strength and favor carbon flow towards the synthesis of FA. Over-expression of *BnSTM* also reduces the levels of seed glucosinolates (GLS), undesirable metabolites compromising the quality of the oil.

Chapter 2 examines the effects of *BnSTM*, *BnZLL* and *BnCLV1* on the quality of the MDEs produced in culture. Over-expression of *BnSTM* enhances the quality of the MDEs and increases their ability to germinate and regenerate viable plants. These effects are discussed in relation to cytokinin synthesis, perception and signaling, which are investigated at a transcriptional level.

2.0 LITERATURE REVIEW

2.1. Plant embryogenesis: an overview

Embryogenesis in higher plants is initiated with the fusion of the sperm cell with the egg, resulting in the generation of the zygote. In flowering plants, this process occurs through the "double fertilization" event in which one sperm fertilizes the egg, producing the zygote, while a second sperm combines with two polar nuclei of the central cell of the megagametophyte (Goldberg et al., 1994). This large cell will develop into a triploid endosperm, which provides nutrients for the growing embryo. The overall embryogenetic process can be divided into two distinct phases. A developmental phase in which the body plan of the embryo is established through a precise cell division pattern of the zygote, and a maturation phase in which the embryo accumulates storage products, including starch, proteins and lipids, and becomes tolerant to desiccation. Elaboration of the embryo body during the developmental phase is guaranteed by positional cues establishing vertical (apical-basal) and horizontal (radial) axes which allow the proper differentiation of cells, tissues and organs. Experimental perturbations of these cues compromise the proper formation of the embryo. A fully developed embryo consists of shoot and root apical meristems located at the opposite extremities of the embryonic axis and one or more cotyledons originating from the apical pole. Studies illustrating the pattern of embryo formation in plants have been mainly conducted in the model species *Arabidopsis thaliana*, due to the simple structure of the embryo and above all to the available genetic resources which facilitate the development of molecular models regulating embryo growth.

2.1.1. Establishment of the embryo body in *Arabidopsis thaliana*

The development of Arabidopsis embryos is accompanied by distinct cell division patterns which can be identified from the very early stages. The process is initiated with an asymmetric division of the zygote resulting in two morphologically different cells. A small apical cell which is the progenitor of the embryo proper and a basal cell which is the precursor of the suspensor (Figure 2.1), a transient organ that plays structural and physiological roles in embryo development (Park and Harada, 2008). The uppermost cell of the suspensor, the hypophysis, will give rise to the root quiescent center and the initials of the central root cap (Park and Harada, 2008). Through one transverse and two longitudinal divisions, the apical cell produces the 8-cell (octant)-stage proembryo (reviewed by Jürgens, 2001). A periclinal division further shapes the octant-stage proembryo into an upper and a lower section, each composed of 4 cells. While the upper section will give rise to the shoot apical meristem (SAM) and cotyledons, the lower section will originate the central domain of the embryo comprising the remainder (shoulder region) of the cotyledons, the hypocotyl, and the upper section of root apical meristem (RAM). Upon further development, the 16-cell-stage embryo is produced and is characterized by the formation of the protoderm, the outer layer of cells progenitor of the epidermis (Jürgens, 2001; reviewed by Lau et al., 2010). A defined cell division pattern is apparent at the globular-stage of development with the protodermal cells dividing only anticlinally (with the plane of the new cell walls perpendicular to the surface of the embryo proper) while the inner cells divide longitudinally. It is at this stage that the first molecular markers of the shoot apical meristem (SAM) become expressed (reviewed by Lenhard and Laux, 1999), as discussed in the next section.

Inception of the cotyledons demarks the heart-stage of embryo development (Park and Harada, 2008). It is at this stage that the RAM is established through the formation of the initials originating around the quiescent cells. At this developmental phase the SAM becomes morphologically recognizable by the two emerging cotyledons.

Further embryonic phases include the torpedo stage in which cells elongate and differentiate giving rise to the complete body of the embryo which is composed of a SAM, cotyledons, embryonic axis and a RAM (Park and Harada, 2008). Although morphologically complete, torpedo embryos are not physiologically ready to germinate. In order to germinate they need to undergo a desiccation period which terminates the developmental program and triggers the post-embryonic growth (Goldberg et al., 1994). All the distinct structural events accompanying the development of the embryos are underpinned by complex molecular and genetic mechanisms, some of which have been elucidated.

2.1.2. Molecular mechanisms controlling embryo patterning

Early embryo patterning is governed by the *WUSCHEL-related HOMEOBOX* (*WOX*) genes (Figure 2.2) (Haecker et al., 2003). Microdissection studies revealed that the egg cell and the zygote express both *WOX2* and *WOX8* transcription factors, which are distributed asymmetrically. Physical separation of *WOX2* and *WOX8* mRNAs occurs with the first division of the zygote. Haecker et al. (2003) suggested that this separation is sufficient to confer different cell fate and identity to the apical cells (progenitor of the embryo proper) and the basal cells (progenitor of the RAM and suspensor).

Reinforcement of this fate is achieved through the expression of other *WOX* members, including *WOX9,* which co-localizes with *WOX8* within the basal cell. The 8-cell-stage proembryo is divided into three distinct *WOX*-expressing domains. The embryo proper expresses *WOX2*, the hypophysis *WOX8/9*, while the suspensor region is enriched with *WOX8* mRNAs. Additional *WOX*-expressing domains emerge during further embryonic development, with *WOX9* demarking the basal cells of the embryo proper, and *WUS* localizing in the sub-apical cells which will form the SAM (Haecker et al., 2003; Dodsworth, 2009). The expression pattern of *WUS* during embryogenesis has been well documented as it exemplifies mechanisms through which macromolecules segregate within dividing cells to confer specific developmental cues. In globular stage-embryos, *WUS* expression encompasses all the sub-apical cells of the embryo proper. During the heart-stage of development cell divisions within the apical pole of the embryo proper form three distinct layers (L1, L2, and L3) and *WUS* expression is lost in L1 and L2, while it is retained in L3. Retention of *WUS* mRNAs in L3 is needed for the specification of the organizing center, a domain required for the proper formation and maintenance of the SAM discussed in the next section. Another *WOX* gene expressed during the middle phase of embryogenesis is *WOX5,* which encodes a functionally equivalent transcription factor to WUS, but is involved in the specification of the embryonic root (Lau et al., 2010). Like *WUS, WOX5* is initially expressed in the inner cells of the upper tier of the embryo at the dermatogen-stage. During the globular-stage of development, its expression becomes restricted to the hypophysis and sub-sequentially to the quiescent cells of the RAM (Lau et al., 2010). The requirement of similar *WOX* genes for the establishment of the RAM and SAM indicates the presences of common mechanisms

governing meristem function in plants. Although the expression pattern of the *WOX* genes appears to be regulated by cell lineage (in which the fate of the daughter cell is inherited by the mother cell), recent studies showed that *WOX* expression is regulated by positional information cues (Lau et al., 2010). These cues, possibly established within the embryonic environment by the presence of the maternal tissue, confer positional information to the cells and induce a unique gene expression pattern. These studies add another layer of complexity to our understanding on how the *WOX* gene expression pattern is established.

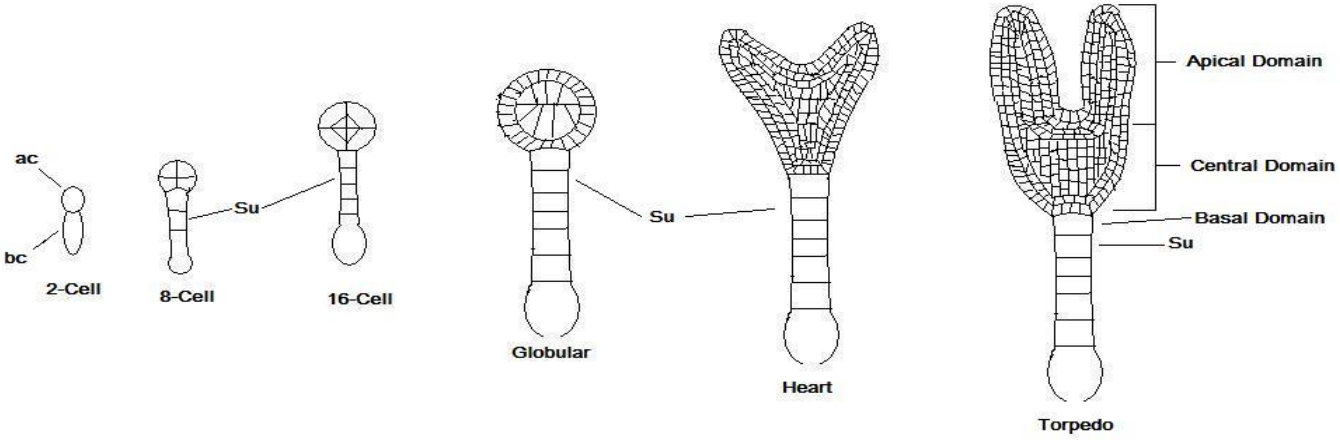


Figure 2.1. Development of the Arabidopsis embryo. Stages of development are shown, ac: apical cell; bc: basal cell; Su: suspensor. The figure is adapted from Jürgens (2001); Altamura et al. (2007), and Park and Harada (2008).

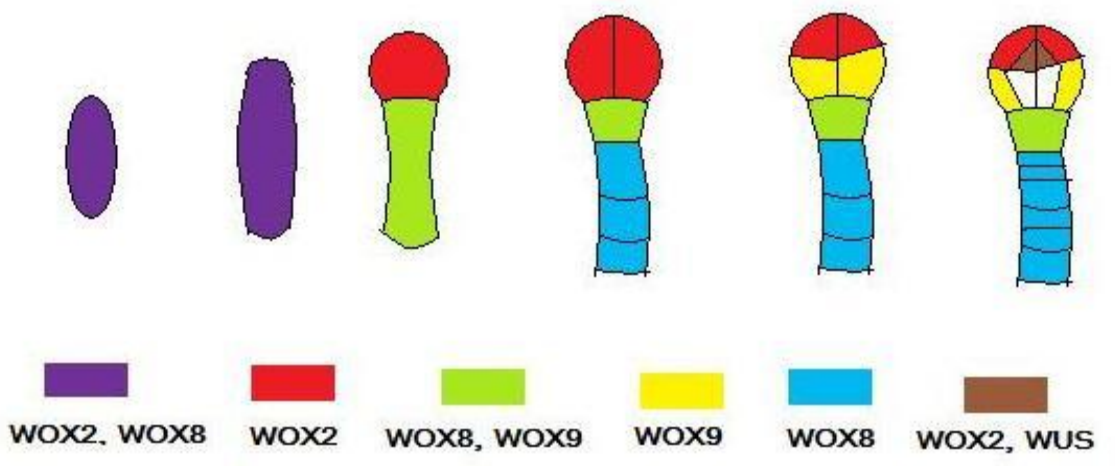


Figure 2.2. Expression pattern of *WOX* genes during early Arabidopsis embryogenesis. The figure is adapted from Haecker et al. (2003) and Lau et al. (2010).

Besides *WOX*s, several other genes have been identified as regulators of embryonic development. Consideration will be given to regulators of the protoderm, as well as regulators of the apical, central, and basal domain. Protoderm development relies on the expression of *ARABIDOPSIS THALIANA MERISTEM LAYER1* (*AtML1*), a homeodomain transcription factor also described in relation to the establishment of both apical-basal and radial patterns during plant embryogenesis (Lu et al., 1996). This gene is initially expressed in the apical cells following the division of the zygote and later it becomes restricted to the protodermal layer (Park and Harada, 2008). The function of *AtML1* appears redundant to that of *PROTODERMAL FACTOR2* (*PDF2*), which is also expressed in the protoderm and seems to be required for proper formation of the epidermis (Abe et al., 2003). Other regulators of protoderm identity include *ABNORMAL LEAF SHAPE1* (*ALE1*), a gene encoding a substilisin-like protease (Lau et al., 2010), and three receptor-like kinases, *ALE1, ALE2* and *ARABIDOPSIS CRINKLY4* (*ACR4*), which

function in the same genetic pathway (Park and Harada, 2008). ALE1, ALE2 and ACR4 regulate the expression pattern of protodermal cells and also control the expression pattern of *AtML1* (Lau et al., 2010).

Establishment of the apical domain requires many genes including *GURKE* (*GK*), playing a key role in the formation and emergence of the cotyledons (Park and Harada, 2008), and *TOPLESS*, which encodes a transcriptional co-repressor believed to inhibit the expression of genes specifying the basal development (Long et al., 2006). Within the apical domain, development of the SAM is governed by a complex genetic network involving *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA*s (*CLV*s), and several *CUP-SHAPED COTYLEDON*s (*CUC*s). While the expression of *STM*, *WUS*, and *CLV* is restricted within the cells of the SAM, *CUC1*, *2*, and *3*, which encode NAC transcription factors, are expressed at the peripheral domains of the SAM where they demark the boundaries between the meristematic cells of the SAM and the cotyledons (Park and Harada, 2008).

A representative gene defining the central domain of the embryo, i.e. the embryonic axis, is *FACKEL* (*FK*), which encodes a sterol C-14 reductase involved in sterol biosynthesis. A putative function of *FK* is to act as a signaling molecule establishing positional cues along the shoot-root axis. Another important regulator of the central domain is *MONOPTEROS* (*MP*) which is involved in auxin signaling (Hardtke and Berleth, 1998) and is required for the execution of proper cell division patterning of the apical-basal axis of the embryo.

A key event occurring in the basal domain of the embryo during the middle phases of embryogenesis is the formation of the RAM, responsible for the development of

below-ground organs during post-embryonic growth (Jürgens, 2001). In Arabidopsis the structure of the RAM is well defined and characterized by the presence of centrally located quiescent cells which maintain the surrounding initials in an undifferentiated state (Jürgens, 2001). The establishment and maintenance of the quiescent cells is regulated by the *PLETHORA* (*PLT*) genes which encode APETALA2-domain transcription factors participating in auxin signaling (Aida et al., 2004). Recent studies show that the *PLT*-regulation of quiescent cell identity also requires the expression of *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*), two transcription factors involved in the formation of the endodermal layer (Sabatini et al., 2003). The Arabidopsis root is composed of defined layers of cells converging to the RAM and characterized by precise cell division patterns modulated by *HOBBIT* (*HBT*) (Scheres et al., 1995, Willemsen et al., 1998). *HOBBIT* encodes a homolog of the CDC27 subunit of the anaphase-promoting complex, usually required for cell cycle progression and responsible for cell division and cell type specification (Willemsen et al., 1998; Blilou et al., 2002). Unlike the SAM, information is limited on the molecular mechanisms controlling RAM formation, a possible result of the sub-apical location of the RAM (due to the presence of the columella) which makes molecular studies difficult to perform.

2.1.3. Hormones involved in embryogenesis

Plant hormone synthesis, perception and signaling play crucial roles during different developmental phases of embryogenesis by modulating a variety of responses controlling patterning processes (Goldberg et al., 1994; Paiva and de Oliveira, 1995;

Hays et al., 2002; Belmonte et al., 2006; Park and Harada, 2008). Among the five classical plant growth regulators, auxin and cytokinin are the most characterized especially for their respective contribution to the apical-basal development of the early embryo and the formation of the SAM (Su et al., 2011).

The term auxin refers to a group of molecules which are chemically related and include the two well characterized members: indole acetic acid (IAA) and indole butyric acid (IBA). Auxin affects embryogenesis from its inception, as amount and localization of this plant growth regulator influence the initial asymmetric division of the zygote producing an apical and a basal cell (Lau et al., 2010). Upon further development, fluctuations in auxin maxima contribute to the precise cell and tissue patterning of the embryo. In Arabidopsis early globular embryos, auxin tends to flow upward, from the suspensor cells towards the apical region of the embryo proper. A change in auxin direction occurs prior to the formation of heart-stage embryos with the flow directed towards the developing root (Friml et al., 2003; Park and Harada, 2008). These auxin movements during embryogenesis act as positional cues for the establishment of the apical-basal pattern of development. Pharmacological approaches using auxin transport inhibitors and mutation of auxin-regulated genes compromise the proper development of the embryos by affecting tissue patterning along the embryonic axis (Friml et al., 2003). Movement of auxin along the embryo body is ensured by the temporal and spatial regulation of four auxin efflux transporters, members of the PIN family: PIN1, 3, 4, and 7 (Friml et al., 2003; Park and Harada, 2008). Friml et al. (2003) showed that *PIN7* and *PIN1* are the first *PIN*s expressed in Arabidopsis embryos (two-cell-stage). Notably, PIN7 is responsible for the initial upward flow of auxin observed in early globular

embryos through its localization along the apical membranes of the suspensor cells. This characteristic polarization pattern of PIN7 contrasts that of PIN1, which is localized in a non-polar fashion within all cells of the embryo proper. The change in auxin flow direction (basipetal) in heart-stage embryos is ensured by a switch of PIN7 to the basal membrane of the suspensor cells, the preferential distribution of PIN1 at the basal end of the provascular cells, and the accumulation of PIN4 on the hypophysis. It is also at this stage that *PIN3* expression is first detected and contributes to the basipetal movement of auxin required for the formation of the RAM (Friml et al., 2003).

The PIN-mediated distribution of auxin within the embryo body is needed to modulate the expression of genes influencing tissue patterning. For example, the expression of *WOX9* (Figure 2.2) is mediated by *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*), the former encoding the auxin responsive factor ARF5, while the latter the Aux-IAA protein (IAA12). The MP/BDL signaling controls the division of the hypophysis and is required for the establishment of the apical, central and basal domains of the embryos. Independent evidence also suggests that auxin is required for establishing and maintaining the embryonic RAM by modulating *PLETHORA* (*PLT*) genes, which specify the quiescent cells (Park and Harada, 2008). *PLT*s are induced by auxin treatments and this regulation operates through a feed-back mechanism in which *PLT*s reinforce the basipetal flow of auxin by promoting the accumulation of PIN proteins. This regulatory loop ensures the formation of the quiescent cells which in turn are responsible for the proper positioning and function of the RAM initials (Park and Harada, 2008).

While auxins are required during the early phases of embryogenesis, cytokinins play a central role in the middle and late developmental stages of embryo development.

Cytokinins, a group of signaling molecules well known for their promotive role during cell division (Kyozuka, 2007), can be divided into two broad categories: adenine-type which include kinetin and zeatin, and phenylurea-type such as diphenylurea and thidiazuron (Campbell et al., 2008). Most cytokinins synthesized in plants are adenine-type (Campbell et al., 2008). In Arabidopsis, at least three genes encode cytokinin receptors: *AHK4* [also known as *CYTOKININ RESPONSE 1* (*CRE1*) or *WOODEN LEG* (*WOL*)], *AHK2*, and *AHK3* (Yamada et al., 2001). During the middle phases of Arabidopsis embryogenesis (after the heart-stage), one of these receptors, WOL, has been implicated in the regulation of the asymmetric cell division of the procambial cells (Mähönen et al., 2000).

Over the past few years several components of the cytokinin pathway have been identified and they include *ARABIDOPSIS HISTIDINE PHOSPHOTRANSMITTER*s (*AHP*s) and *ARABIDOPSIS RESPONSE REGULATOR*s (*ARR*s). Of the 30 *ARR* genes identified in Arabidopsis some (Type-A) are characterized by a receiver domain and a short C-terminal extension, while others (Type-B) have a longer C-terminal extension and domains acting as transcription regulators (D'Agostino et al., 2000; Hwang and Sheen, 2001). Genetic work demonstrated that Type-B ARRs are transcription activators of cytokinin-induced genes, whereas Type-A ARRs are feed-back repressors of the cytokinin pathway (Hwang and Sheen, 2001). Several components of the cytokinin signaling are involved in the formation of the SAM during embryogenesis (Shani et al., 2006; Kyozuka, 2007). Notably, WUS, a key regulator of the embryonic and post-embryonic SAM, increases cytokinin response by repressing several Type-A *ARR*s including *ARR5* and *7* (D'Agostino et al., 2000; Hwang and Sheen, 2001). Biochemical

studies revealed that the WUS-regulated repression of *ARR7* occurs through a direct binding to the *ARR7* promoter (Shani et al., 2006). Independent studies confirm that an increase in cytokinin level and signaling coincide with the formation of the embryonic SAM, and these observations are also supported by *in vitro* studies showing that applications of cytokinin encourage the formation of *de novo* shoots in a variety of systems (Zhao et al., 2002).

Another class of growth regulators involved during embryogenesis are gibberellins (GAs), tetracyclic diterpenoid compounds synthesized from geranylgeranyl diphosphate (reviewed by Hedden and Kamiya, 1997) and converted into biologically active forms in the endoplasmic reticulum and cytosol (Campbell and Reece, 2002). Besides their participation in several plant developmental processes, including seed germination, leaf expansion, stem elongation, and flowering (Ogawa et al., 2003), GAs accumulate in the suspensor of heart-shaped embryos, where they facilitate cell expansion and elongation (Hays, 1996). During the formation of the embryonic SAM, GAs are excluded from the central apical pole, where the meristematic cells develop (Dodsworth, 2009). Rather, GAs tend to accumulate in the peripheral region of the SAM where they modulate cell differentiation processes (Dodsworth, 2009).

Although not directly involved in the developmental phase of embryogenesis, abscisic acid (ABA) is the growth regulator associated to the maturation of the embryo (Ramesar-Fortner and Yeung, 2006). Known as the "stress hormone" for its participation in biotic and abiotic stress responses, ABA accumulates slowly during embryo development reaching a peak in the middle of the maturation phase, before declining slowly (Rock and Quatrano, 1995). High ABA levels favor the accumulation of seed

storage products and enhance desiccation tolerance (reviewed by Johri and Mitra, 2001). Precocious germination is often observed in mutants with reduced ABA levels or impaired ABA response (reviewed by Paiva and de Oliveira, 1995). *In vitro* studies suggest that ABA might also be involved in developmental and tissue patterning processes as its exogenous applications improve the structure of the SAM and the overall quality of the embryos produced in culture (Ramesar-Fortner and Yeung, 2006). No information is currently available on the participation of ABA in the establishment of the SAM *in vivo*.

The fifth "classical" plant growth regulator, ethylene, does not appear to be directly involved in embryo development, as denoted by the paucity of information available in literature. This gaseous hormone, however, has been extensively studied during *in vitro* embryogenesis, where its undesired accumulation during the late developmental stages compromises the structural integrity of the SAM. High levels of ethylene cause the disruption of the meristematic cells and their precocious differentiation (Belmonte et al., 2006).

Collectively these studies emphasize the key role played by hormones, especially auxin and cytokinin, during plant embryogenesis, and suggest that embryo development is often regulated by the interaction and cross-talking of two or more hormones. Through this process proper cell and tissue patterning are executed, and this allows the formation of functional embryos. Two key events occurring during embryogenesis, the formation of the SAM and the deposition of storage products, will be discussed in detail.

2.2. Formation of the shoot apical meristem (SAM)

Higher plants have defined groups of undifferentiated cells that enable indeterminate growth. These groups of cells are called meristems. The shoot apical meristem (SAM) is the uppermost meristem of a plant, which is responsible for the organogenesis and histogenesis of the shoot, and the formation of the above-ground organs including leaves and flowers (reviewed by Lenhard and Laux, 1999). The SAM is formed during the globular-stage of embryogenesis and is located between two cotyledons (Lenhard and Laux, 1999).

The structural organization of the SAM varies among species and its architecture changes during development and growth conditions (Steeves and Sussex, 1989). In angiosperms, there are two models explaining the structure of the SAM: the tunica-corpus model (Figure 2.3) and the cytophysiological zonation model (Figure 2.4) (reviewed by Fletcher, 2002). According to the first model, the SAM is divided into three distinct layers: L1, L2, and L3. The first two layers (L1 and L2) are referred to as tunica and are characterized by anticlinal cell division patterns adding cells to each respective layer. Cells of L3 compose the underlying corpus region and divide in both anticlinal and periclinal orientations. Cell lineage analysis suggests that each layer contributes to specific tissues and organs. Divisions in L1 form the epidermis of the shoot, leaves and flowers; L2 contributes to ground tissue and germ cells (Fletcher, 2002); L3 is responsible for the formation of the vascular tissue and the internal tissues of leaves and flowers (reviewed by Kerstetter and Hake, 1997; Fletcher, 2002).

The cytophysiological zonation model divides the SAM into three distinct zones: the central zone (CZ), the peripheral zone (PZ) and the rib zone (RZ) (Taiz and Zeiger, 2010) (Figure 2.4). Cells within each zone are unique in staining characteristics, degree

of vacuolation, and division pattern. Cells of the CZ, often referred to as stem cells, are highly vacuolated and divide infrequently contributing cells to the other zones. The rate of division of the stem cells is crucial for ensuring the maintenance of the CZ; stem cells are produced at the same rate in which differentiating cells are incorporated into organ primordia. Through this "cellular homeostasis" the size of the CZ and the number of stem cells do not fluctuate over time. Differentiating cells from the CZ are pushed towards the PZ and RZ, where they mature into specific cell types (Figure 2.4).

The tunica-corpus model and the cytophysiological zonation model can be over-imposed in a single conceptual model to illustrate the architecture of the SAM (Figure 2.5). It must be mentioned, however, that the pronounced variations in the structure of the SAM observed among species make it often difficult to apply these models.

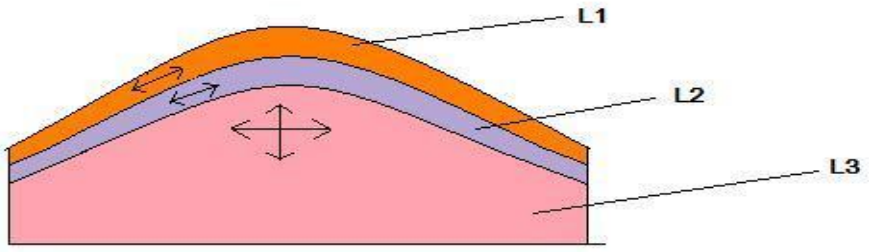


Figure 2.3. The structure of the shoot apical meristem (SAM) according to the tunica-corpus model. The SAM is divided into three layers (L1, L2 and L3). Arrows indicate the direction of cell divisions within each layer. Adapted from Fletcher (2002).

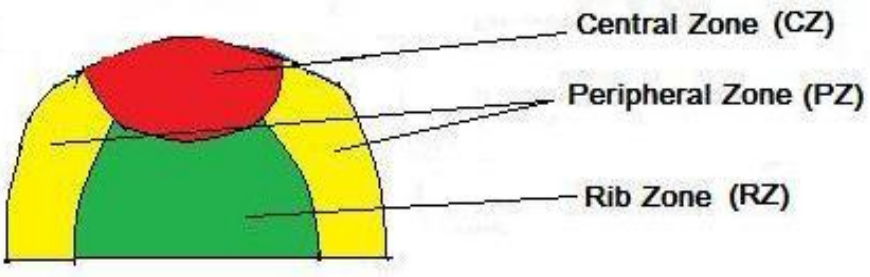


Figure 2.4. Physiological zonation of the SAM. Adapted from Fletcher (2002).

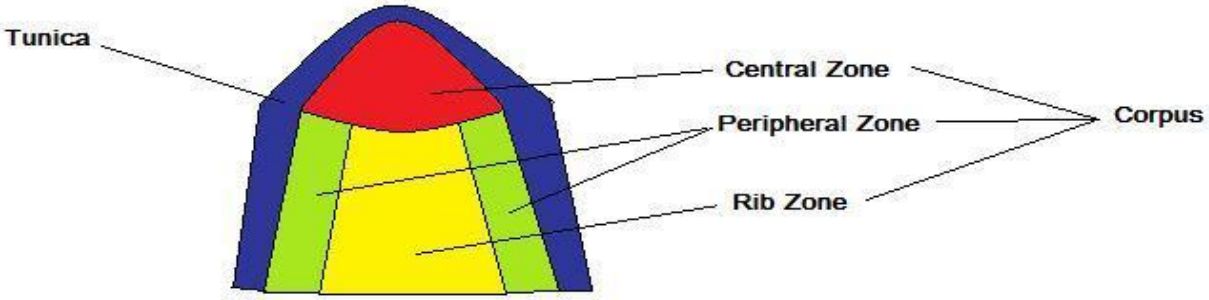


Figure 2.5. Over-imposition of the tunica-corpus model and the cytophysiological zonation model. The blue section represents the tunica and domains subtending the tunica (red - CZ, green - PZ and yellow - RZ), are included in the corpus.

2.2.1. Genetic regulation of the SAM

The precise structural characteristics of the SAM are the result of an elaborate gene network which has been described in many reviews (Fletcher and Meyerowitz, 2000; Tucker and Laux, 2007; Dodsworth, 2009). Through their integrated expression, meristem-regulating genes ensure the proper layering and zonation patterning, and most importantly, the proper balance between stem cell renewal and differentiation within the SAM. Suggestive of this refined regulation are many genetic studies showing that

mutation or miss-expression of the meristem genes affect the architecture of the SAM, and in some instances preclude normal growth and development (Fletcher and Meyerowitz, 2000; Dodsworth, 2009). Key genes involved in the development and maintenance of the SAM include *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA1* (*CLV1*) and *ZWILLE* (*ZLL*).

2.2.2. *SHOOT MERISTEMLESS* (*STM*)

SHOOT MERISTEMLESS (*STM*) belongs to the class 1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) gene family, and encodes a homeodomain transcription regulator expressed in the undifferentiated stem cells of the SAM (Scofield and Murray, 2006). Conserved domains of *STM* include the HOMEODOMAIN, required for DNA binding, the ELK domain which encodes a nuclear localization signal, and the KNOX1 and KNOX2 domains involved in transcriptional repression of target genes and in dimer formation and transactivation respectively (Scofield and Murray, 2006). Through the suppression of differentiation and the maintenance of indeterminate cell fate, STM controls homeostasis, the rate of cell divisions and differentiation, in the SAM. While *STM* over-expression increases cell proliferation and results in the generation of ectopic meristems from vegetative tissues (Brand et al., 2001; Gallois et al., 2002; Lenhard et al., 2002), down-regulation of *STM* perturbs the functionality of the SAM. Strong *stm* loss-of-function alleles produce terminated SAMs while weak *stm* alleles fail to maintain a stable SAM during post-embryonic growth as a result of the incorporation of meristematic cells into organ primordia (Barton and Poethig, 1993; Clark et al., 1996;

Endrizzi et al., 1996). Similar perturbations were also observed if the expression of *STM* homologs were altered in other species (Vollbrecht et al., 2000; Hay and Tsiantis, 2006). Several studies also showed that *STM* is sufficient to initiate the *de novo* formation of shoots when ectopically expressed in vegetative tissues (Gallois et al., 2002; Lenhard et al., 2002). Other members (*KNAT1, 2*, and *6*) of the class 1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) gene family, which include *STM*, are also involved in organ specification and plant architecture through their interaction with BELL proteins (Hamant and Pautot, 2010). The Arabidopsis STM is able to interact with several BELL proteins including ARABIDOPSIS THALIANA HOMEOBOX1 and PENNYWISE (Rutjens et al., 2009) through complexes affecting both meristem function during vegetative development (Kanrar et al., 2006; Rutjens et al., 2009) and floral patterning during reproductive development (Bhatt et al., 2004; Kanrar et al., 2006). The Arabidopsis STM is initially detected in a few cells of the globular-stage embryo (reviewed by Sharma and Fletcher, 2002). Upon further development the expression of this gene expands within the SAM and includes all the meristematic cells located between the two cotyledons, excluding the incipient organ primordia. Such expression pattern is also retained during post-embryonic growth despite small variations especially during the transition from vegetative to floral development (Long and Barton, 1998). Within the inflorescence meristem the expression of *STM* initially encompasses the whole apical region and then becomes restricted to the middle domain of the developing flower, in conjunction with the formation of the outer floral organs (Scofield and Murray, 2006).

A possible mechanism through which STM maintains the stem cells of the SAM in an undifferentiated state involves a regulation in the levels of gibberellins (GAs) and

cytokinins. Gibberellins favor cell differentiation and leaf formation; therefore, the level of GAs in the SAM must be maintained low. Several studies showed that *KNOX* genes, including *STM*, suppress GAs level by repressing GA20-oxidase, a GA biosynthetic enzyme (Sakamoto et al., 2001), as well as inducing the expression of GA2-oxidase, a GA degrading enzyme (reviewed by Shani et al., 2006). Through this STM-mediated regulation GAs maxima are excluded from the central zone of the SAM harbouring the stem cells. This concept is further supported by genetic studies documenting a reduction in phenotypic abnormalities seen in *STM* over-expressing plants upon exogenous applications of GAs (Ueguchi-Tanaka et al., 1998). As indicated in the previous section, the SAM is a site of cytokinin action (Müller and Sheen, 2008), which is a requirement for the specification of stem cell identity (Kyozuka, 2007). While cytokinin biosynthetic inhibitors reduce the number of stem cells and the size of the meristem, application of cytokinin increases the size of the SAM and the number of undifferentiated stem cells (Higuchi et al., 2004; Werner et al., 2008). *KNOTTED HOMEOBOX* (*KNOX*) genes, including *STM*, provide a high cytokinin environment by inducing the expression of *ISOPENTENYL TRANSFERASE7* (*IPT7*), which encodes an important enzyme involved in cytokinin synthesis (Jasinski et al., 2005; Yanai et al., 2005). Increasing levels of endogenous cytokinin through exogenous applications or ectopic *IPT7* expression have been shown to rescue weak *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005). The *STM* regulation of cytokinin synthesis is further complicated by the fact that cytokinin is also needed for the expression of *KNOX* genes. While cytokinin accumulation increases the expression of *KNOX* in Arabidopsis (Rupp et al., 1999), *LONELY GUY*, a cytokinin related enzyme, is needed for the induction of *KNOX* transcripts in *Oryza sativ*a

(Kurakawa et al., 2007). A positive feedback mechanism between *KNOX* genes and cytokinin is therefore apparent in the shoot meristem, whereby cytokinin induces *KNOX* expression and *KNOX* genes promote cytokinin biosynthesis. Recent studies suggest that the role of cytokinin in preserving stem cell fate within the SAM occurs through the induction of *WUSCHEL* (*WUS*) (Gordon et al., 2009), another key regulator of meristem function.

2.2.3. *WUSCHEL* (*WUS*)

WUSCHEL (*WUS*) encodes a homeodomain transcription factor from the *WUSCHEL-LIKE HOMEOBOX* (*WOX*) gene family (Dodsworth, 2009), and is expressed in the apical pole of early embryos (16-cell-stage), in approximately 10 cells (Dodsworth, 2009). Expression of *WUS* occurs throughout the vegetative SAM but terminates during the vegetative-reproductive transition of the apical meristem (Laux et al., 1996; Lenhard et al., 2001). Lenhard et al. (2001) showed that proper *WUS* expression is required for the induction of *AGAMOUS* (*AG*), one of the crucial factors initiating flower formation. The precise temporal expression of *WUS* within the embryonic SAM is ensured by high levels of cytokinins. Specifically, a higher cytokinin-auxin ratio triggers *WUS* expression (Cheng et al., 2010).

Localization of *WUS* coincides with a domain referred to as the "organizing center", which is located just below the stem cells (Dodsworth, 2009). A depletion of the organizing center, followed by stunted leaf growth and ultimately SAM abortion is often observed in *wus* mutant lines. This is in contrast to over-expression studies showing

increased meristem size, mainly due to the enlargement of the CZ, in plants with ectopic *WUS* expression (Yadav et al., 2010). Several molecular and genetic studies have revealed the involvement of *WUS* in the specification of the stem cells of the SAM through feed-back mechanisms with the CLAVATA family (discussed in the next sections). The nature of this interaction ensures proper maintenance of the SAM and cellular homeostasis, i.e. equal rate of cell division and differentiation within the meristem (Schoof et al., 2000).

2.2.4. *CLAVATA1* (*CLV1*)

CLAVATA1 (*CLV1*) encodes a receptor kinase containing 21 tandem leucine-rich repeat domains with a single pass transmembrane domain (Clark et al., 1997). This gene is crucial for the regulation of the SAM since it participates in the CLV signaling pathway which includes CLV2 and CLV3. In this pathway, the binding of CLV1 to CLV2 forms a CLV1/CLV2 receptor complex located in the sub-apical layers (L2 and L3) of the meristem. This complex binds to the ligand CLV3, which is a small protein containing the CLV3/ESR-related domain produced by the apical cells (L1), which are the stem cells of the SAM. The binding of CLV3 to the CLV1/CLV2 complex triggers a complex signal transduction pathway involving the MAPK cascade, and leading to the transcriptional repression of *WUS* (Betsuyaku et al., 2011). The downstream components of the CLV-signaling have been investigated and include *POLTERGEIST* (*POL*) and *POLTERGEIST LIKE 1* (*PLL1*) (Song et al., 2006). Both genes contribute to the specification of stem cells within the SAM. While the over-expression of *POL* or *PLL1*

represses stem cell differentiation (Song et al., 2006), the suppression of both genes increases the size of the SAM, mimicking the *wus* phenotype (Song et al., 2006; Miwa et al., 2009). From these observations it is suggested that *POL* and *PLL1* are negatively regulated by the CLV signaling and are redundant to *WUS*. No information is currently available on the down-stream components of POL and PLL1.

Mutant analyses have elucidated the function of *CLV1* during meristem formation and maintenance. Enlarged vegetative and reproductive SAMs characterized by a large pool of undifferentiated cells are observed in *clv1* mutants, suggesting that the function of this gene is to promote cell differentiation and/or repress proliferation of cells in the CZ (Betsuyaku et al., 2011). These phenotypes are also produced by *clv2* and *clv3* mutants, thus reinforcing the notion that the three *CLV* genes operate in the same signaling pathway.

2.2.5. *ZWILLE* (*ZLL*)

ZWILLE (*ZLL*; also known as *PINHEAD* and *ARGONAUTE10*) encodes a member of the EIF2C (elongation initiation factor 2c)/ARGONAUTE class of proteins involved in RNA silencing (Lynn et al., 1999; Liu et al., 2009). *ZWILLE* plays an essential role in the establishment of the central-peripheral zonation of the SAM in concert with *WUS* and *CLV*.

The expression of *ZLL* is initiated at the heart-stage of embryo development within the provascular tissue. Upon further growth of the embryo, *ZLL* becomes localized in the SAM (Moussian et al., 2003). Arabidopsis plants with suppressed *ZLL* expression

have terminally differentiated cells, and produce organs instead of stem cells during embryogenesis (Lynn et al., 1999). During post-embryonic growth, these plants form adventitious meristems, thus suggesting that the function of *ZLL* is required for the establishment of the primary embryonic SAM, but not for post-embryonic SAM formation. The mechanisms through which *ZLL* exercises its function during meristem development are mostly unknown. A proposed function of *ZLL* is to ensure the proper localization pattern of *STM* within the SAM, as miss-expression of *STM* is observed in *zll* mutant embryos (Moussian et al., 1998).

Genetic studies have revealed redundant functions between *ZLL* and other members of the *ARGONAUTE* (*AGO*) family, including *AGO1*, which is active in miRNA and siRNA pathways essential for multiple developmental events (Mallory et al., 2009). The expression pattern of *AGO1* is similar to that of *ZLL* and, as observed for *ZLL*, *AGO1* is also required for the proper expression of *STM*. Collectively these results indicate that *ZLL* is an important regulator of SAM function and that this regulation might require the expression of other *AGO* members.

2.2.6. Proposed genetic model for the regulation of the SAM

The precise expression domains of *STM*, *WUS*, *CLV1*, and *ZLL* (Figure 2.6) clearly suggest that these genes operate in concert to maintain a functional SAM during embryonic and post-embryonic development. Expression of *STM* throughout the meristem is required to maintain the meristematic cells in an undifferentiated state possibly by inducing the expression of *WUS* through increases in cytokinin levels (Figure

2.6). The expression of *WUS* coincides with the organizing center (orange domain in Figure 2.6) composed of undifferentiated cells. WUSCHEL induces the expression of *CLV3* in the apical layer (L1); *CLV3* specifies stem cells and encourages cell proliferation leading to the expansion of the SAM. Induction of CLV3 activates the CLV signaling pathway (through binding of CLV3 to the CLV1/CLV2 complex, pink domain in Figure 2.7), which results in repression of *WUS*. Through this repression, *CLV3* expression is also reduced. The WUS/CLV feedback loop is crucial for ensuring the proper functionality of the SAM and the proposed model has been verified by mutant analyses revealing the antagonistic role of *WUS* and *CLV*. The role of *ZLL* within this model is poorly understood, although evidence suggests that ZLL influences the expression pattern of *STM* (Endrizzi et al., 1996) and possibly *WUS* (Laux et al., 1996) (Figure 2.7).

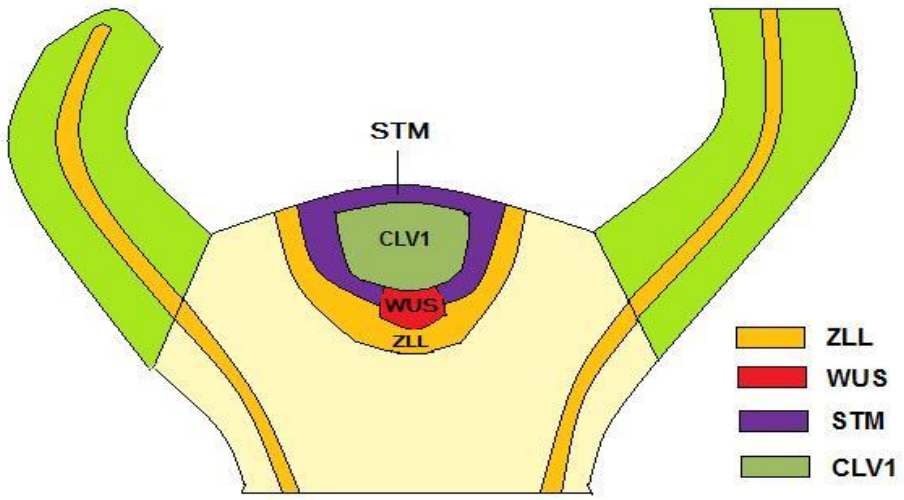


Figure 2.6. Expression domains of *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*), *CLAVATA1* (*CLV1*) and *ZWILLE* (*ZLL*) in the SAM. Adapted from Moussian et al. (1998), Tucker et al. (2008), and Dodsworth (2009).

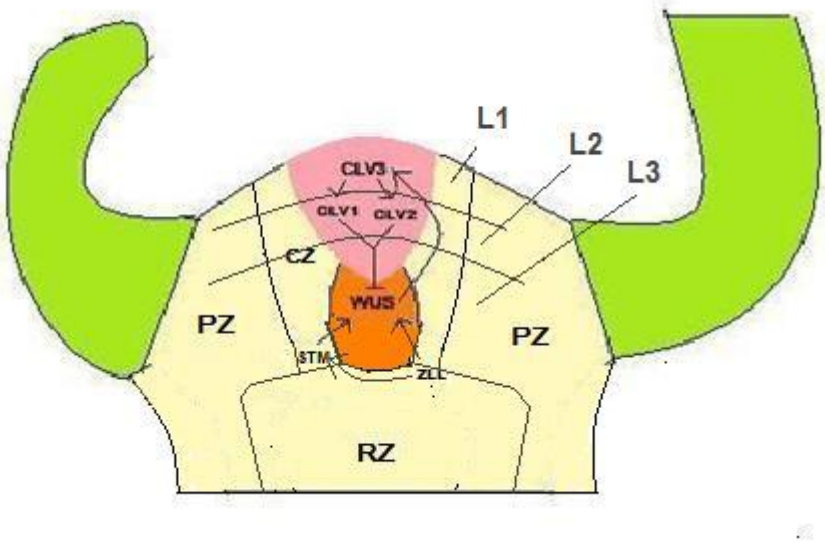


Figure 2.7. Molecular regulation of the Arabidopsis shoot apical meristem (SAM). *WUSCHEL* (*WUS*), which is expressed in the organizing center (orange domain), confers stem cell identity by inducing the expression of *CLV3*. Induction of CLV3 activates the CLAVATA signaling (pink domain) requiring CLV1, 2, and 3 and resulting in the suppression of *WUS*. Proper expression of *WUS* within the organizing center is ensured by *STM* and to a lesser extent by *ZLL*, which affects *STM* localization. (PZ: peripheral zone, CZ: central zone, RZ: rib zone) The figure is adapted from Moussian et al. (2003) and Dodsworth (2009).

2.3. Storage product accumulation

Accumulation of storage products is another important key event of embryogenesis, which is initiated during the late phases of embryo development and is promoted by increasing levels of ABA. Preferential accumulation of lipids, proteins, and/or sugar is species dependent. Canola (*Brassica napus*), has become increasingly prominent worldwide. Canola is a Canadian-developed crop extensively used for food oil but also with the potential to be employed for biofuel production (reviewed by Scarth and Tang, 2006). The original canola cultivar Tower was registered by Keith Downey and Baldur R. Stefansson from the University of Manitoba.

2.3.1. Characteristics of Canola oil

Canola oil is characterized by low levels of saturated FA (reviewed by Scarth and McVetty, 1999), high levels of monounsaturated FAs and intermediate levels of polyunsaturated FA (Vaisey-Genser and Eskin, 1987). Scientific evidence suggests that consumption of about 19 grams of canola oil daily may reduce the risk of coronary heart disease due to the presence of unsaturated fatty acids (Cassáus, 2009). A typical FA profile of canola oil consists of 61% oleic (C18:1), 21% linoleic (C18:2), 11% alpha-linolenic (C18:3) and minute levels of erucic acid (C22:1) (Canola Council of Canada, 2012). Over the years, conventional breeding and genetic engineering have been utilized to develop more suitable canola cultivars for both food and industrial uses.

Two key factors determining the nutritional quality of food oil are the levels of erucic acid and glucosinalates (GLS) (CODEX, 1999). High level (above 2% of total fatty acids) of erucic acid is harmful for human consumption, as also revealed by the abnormally high accumulation of fat in heart muscle of rats fed with elevated levels of erucic acid (Vaisey-Genser and Eskin, 1987; Office of The Gene Technology Regulator, 2002). The reduced erucic acid content of canola oil is therefore extremely desirable and several cultivars with these characteristics have been introduced over the years. Canola cultivars with high erucic acid levels which pose health concerns (Charlton et al., 1975), are usually used for the production of lubricants for industrial purposes (Scarth and Tang, 2006). In *Brassica napus*, two loci with additive genetic interaction have been identified as important for erucic acid biosynthesis. The loci encode β-ketoacyl-CoA synthase (KCS, also known as FAE) which is involved in fatty acid elongation (Scarth and Tang,

2006). Besides having reduced levels of erucic acid, canola oil is also characterized by low (below 30 μmoles/g toasted oil free meal) glucosinolate (GLS) content. Glucosinolates are a class of organic compounds containing sulphur and nitrogen, which are derived from glucose and amino acids (Sonderby et al., 2010). Produced by the plant to cope with biotic stresses, GLS are extremely toxic to human, if ingested at high doses (Office of The Gene Technology Regulator, 2002).

Besides the low levels of erucic acid and GLS, canola oil is enriched in desirable fatty acids such as linolenic, linoleic, and oleic acids. Linolenic acid (C18:3) is recognized as an essential fatty acid, being an important constituent of lipids in nerve cells (Vaisey-Genser and Eskin, 1987). Consumption of this fatty acid has been shown to reduce the levels of high and low density lipoproteins (HDL and LDL) which cause several cardiovascular diseases (Scarth and McVetty, 1999). Linoleic acid (C18:2) is also considered as an essential fatty acid since it cannot be produced by humans and animals, and is essential for the synthesis of membranes and metabolically active substances such as prostaglandin (Vaisey-Genser and Eskin, 1987). Oleic acid (C18:1) has similar effects to linolenic acid in reducing the LDL-cholesterol level (Vaisey-Genser and Eskin, 1987).

Like other oil species, accumulation of oil in canola occurs in oil bodies (reviewed by Huang, 1992), which consist of neutral lipids surrounded by a half-unit membrane of polar lipids or phospholipids, coated with abundant oleosin and some minor proteins of higher molecular mass, such as caleosins and steroleosins (Huang, 1992; Tzen and Huang, 1992). Formation of oil bodies in *Brassica napus* seeds is initiated in heart-stage embryos. Work conducted by He and Wu (2009) suggests that production of lipids in oil

bodies is driven by sucrose synthesized in the embryo. The authors showed that heart-stage embryos express genes required to execute photosynthetic reactions.

2.3.2. Fatty acid biosynthesis

In plants, the two basic precursors of FA are acetyl- and malonyl-CoA (CoA: coenzyme A) (Ohlrogge and Browse, 1995; Baud et al., 2008). As shown in Figure 2.8, malonyl-CoA is produced from the carboxylation of acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase (ACCase). This enzyme is considered as the major control point of FA biosynthetic pathway (Baud et al., 2008). Acetyl-CoA and malonyl-CoA are converted to acetyl-ACP and malonyl-ACP, respectively, by the enzymes acetyl CoA-ACP transacylase and malonyl CoA-ACP transacylase (Ohlrogge and Browse, 1995). Subsequently, malonyl-ACP undergoes a series of condensation reactions with acetyl-ACP to form 3-ketobutyryl-ACP; during these reactions one CO_2 is released (Ohlrogge and Browse, 1995). 3-Ketobutyryl-ACP is then reduced by NADPH + H^+ to 3-hydroxybutyryl-ACP in a reaction catalyzed by 3-ketoacyl-ACP reductase. The 3-Hydroxybutyryl-ACP is further converted by 3-hydroxylacyl-ACP dehydrase into trans-Δ^2-butyenoyl-ACP, which is then reduced again by NADPH + H^+ into a 4-carbon compound, butyryl-ACP. Condensation of butyryl-ACP with malonyl-ACP produces 3-ketoacyl-ACP, which represents the first product of the FA chain synthesis. Further condensation reactions of 3-ketoacyl-ACP (or other acyl-ACPs) with additional malonyl-ACP molecules elongate the fatty acids (Ohlrogge and Browse, 1995).

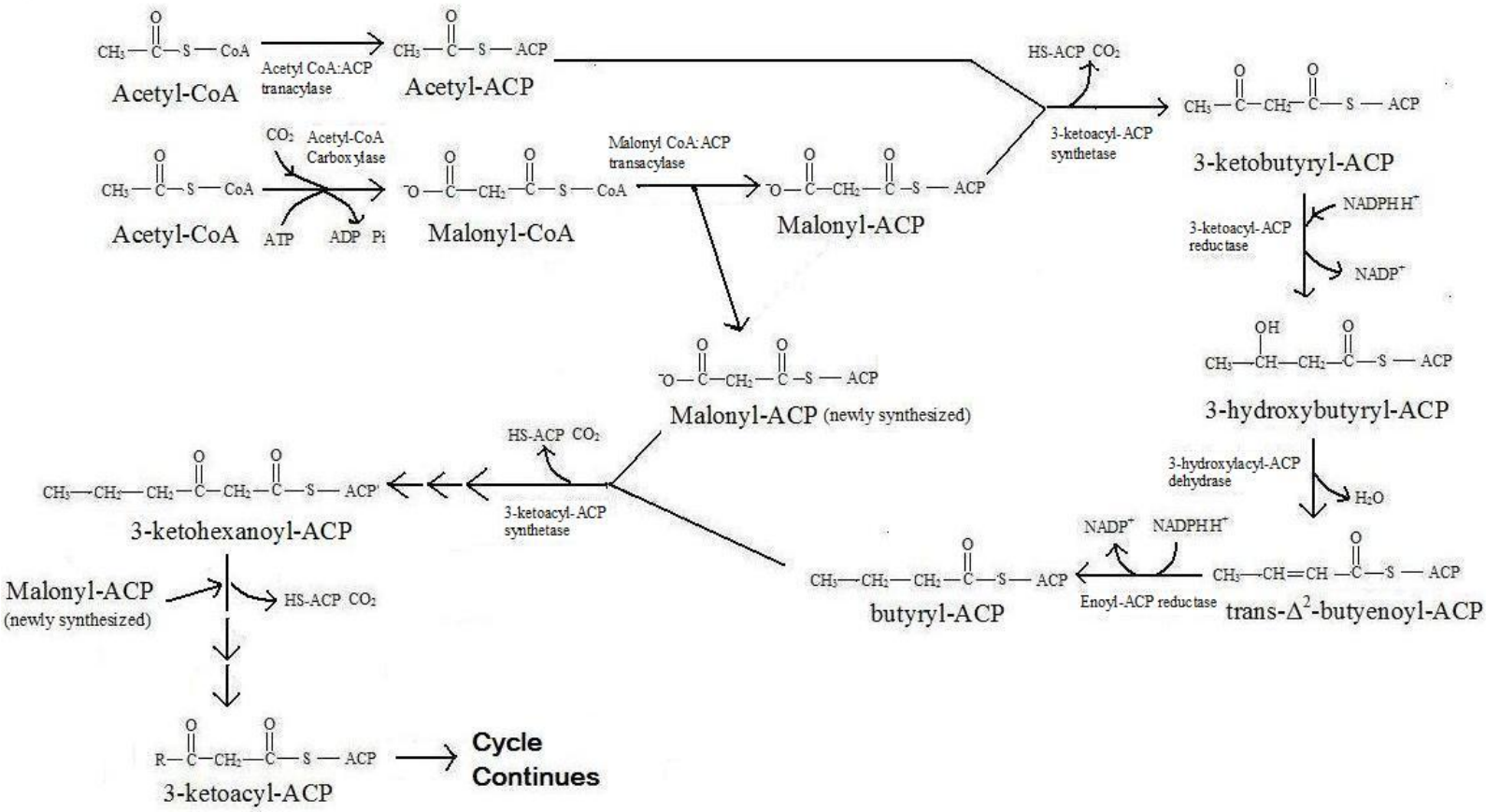


Figure 2.8. Simplified biochemical pathway leading to the synthesis of fatty acids. The figure is adapted from Ohlrogge and Browse (1995).

The pathway shown in Figure 2.8 is solely responsible for the synthesis of saturated FA. Production of unsaturated FA relies on the activity of specific enzymes, FATTY ACID DESATURASEs (FADs), which catalyse the desaturation of FA at specific positions along the FA chain. Examples of these enzymes include Δ^{12} FA OLEATE DESATURASE (FAD2), responsible for the desaturation of oleic acid (18:1) to linoleic acid (18:2) (Baud et al., 2008) and ω-3 FA DESATURASE (FAD3) which catalyzes the conversion of linoleic acid (18:2) to linolenic acid (18:3) (Tan et al., 2011).

The FA (acyl-ACPs) produced through the elongation and desaturation reactions are then esterified with glycerol to produce triacylglycerols (TAG) (Ohlrogge and Browse, 1995), which represent the major component of oil accumulated in the oil bodies of many plant species, including Brassica (Baud et al., 2008). Triacylglycerols are produced in the Kennedy pathway (Figure 2.9). In this pathway the acyl ACPs are first converted to acyl-CoAs by acyl-CoA:PC acyltransferase, and then added to glycerol-3-phosphate (G3P) through three distinct assembly steps. The first acyl-CoA assembly is catalyzed by glycerol-3-phosphate acyltransferase (G3PAT) to form lysophosphatidic acid (LPA), the second is catalyzed by lysophosphatidic acid acyltransferases (LPAAT) to produce phosphatidic acid (PA), and the last step involves an initial production of diacylglyerol (DAG) through the release of a phosphate group from the glycerol backbone catalysed by phosphatidic acid phosphohydrolase (PAP), followed by the addition of a third acyl-CoA catalyzed by diacylglycerol acyltransferases (DAGAT) (Baud et al., 2008).

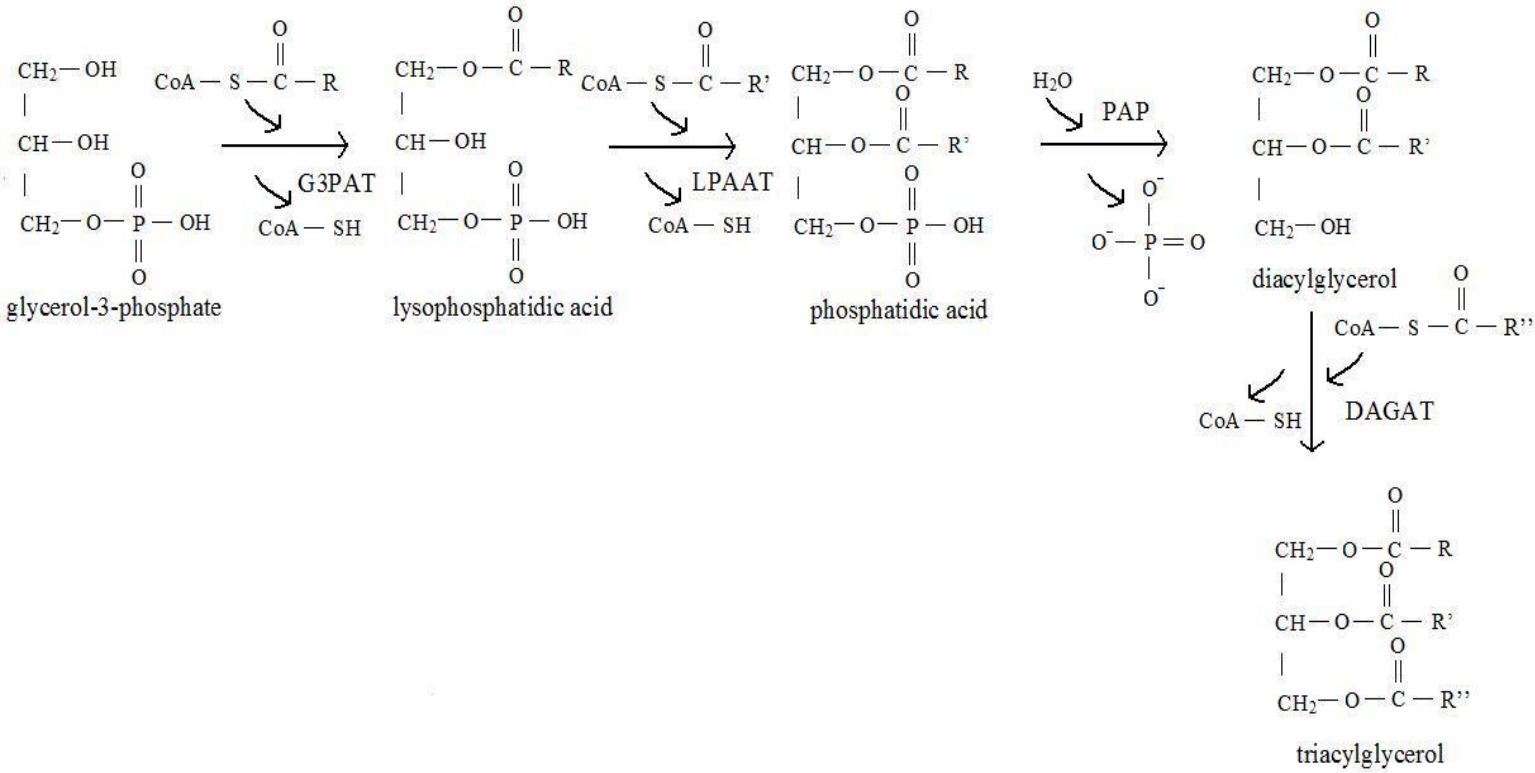


Figure 2.9. Biosynthesis of triacylglycerol (TAG) in the Kennedy pathway. Abbreviations: G3PAT: glycerol-3-phosphate acyltransferase; LPAAT: lysophosphatidic acid acyltransferases; PAP: phosphohydrolase; DAGAT: diacylglycerol acyltransferase. The figure is adapted from Baud et al. (2008).

2.3.3. Transcription factors regulating FA synthesis and oil accumulation

Fatty acids biosynthesis and oil accumulation in seed are regulated by a complex genetic network including the key transcription factors *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *FUSCA3* (*FUS3*) and *WRINKLED1* (*WRI1*). Molecular

and genetic studies have revealed that these factors are expressed during the middle-late phases of embryogenesis and they trigger synthesis of storage products through activation of down-stream components.

LEAFY COTYLEDON1 (*LEC1*) encodes a transcription factor homolog, the CCAAT box-binding factor HAP3 subunit (Lotan et al., 1998), which is implicated in a variety of embryogenic events ranging from cellular differentiation, tissue patterning, morphogenesis, and storage product deposition (Lotan et al., 1998). Over-expression of *LEC1* increases the expression levels of FA biosynthetic genes participating in key reactions of condensation, chain elongation, and desaturation (Mu et al., 2008). Genes involved in glycolysis and lipid accumulation are also up-regulated in *LEC1* over-expressing plants (Mu et al., 2008). These effects are retained across species, thus suggesting the conserved nature of LEC1 (Edwards et al., 1998; Xie et al., 2008). Like *LEC1,* over-expression of *LEC2* increases very long chain FA and TAG in leaves (Santos-Mendoza et al., 2005). *LEAFY COTYLEDON2* over-expressors are also characterized by higher levels of several oleosin genes, storage proteins, and a sucrose synthase. These broad changes in gene expression pattern evoked by LEC2 suggest a diversified function of this gene during embryo maturation. Baud et al. (2009) showed that the effects of LEC2 on FA accumulation are mediated by WRI1, which belongs to a family of transcription factors encoding an APETALA2 (AP2)-type transcription factors with two AP2 DNA-binding domains (Cernac and Benning, 2004). A common feature of members of this family is the AP2 domain, which binds to the AW-box in the promoter region of target genes and induces their activation. Several genes characterized by the presence of the AW-box and putative targets of WRI1 encode enzymes of FA

biosynthesis, including acetyl-CoA carboxylase (BCCP2), glycolysis, and an acyl carrier protein (ACP1) (Maeo et al., 2009). Genetic studies showed that *wri1* mutant plants fail to efficiently convert sucrose into precursors of TAGs, due to a reduced activity of glycolytic enzymes (Cernac and Benning, 2004). Over-expression of *Brassica napus* (*Bn*) *WRI1* in Arabidopsis increased seed oil content by 10%-40% and this effect was reproduced by over-expressing the *Arabidopsis thaliana* (*At*) *WRI1* (Cernac and Benning, 2004). These changes were due to the ability of WRI1 to induce the transcription of glycolytic enzymes and enlarging the sugar pool to be used for FA biosynthesis (Cernac and Benning, 2004; Liu et al., 2009).

Another key transcription factor regulating FA biosynthesis is FUSCA3 (FUS3), a member of the B3 transcription factor family (Tiedemann et al., 2008). While suppression of *FUS3* results in decreased protein and lipid content (Meinke et al., 1994; reviewed by Harada, 2001), the ectopic expression of *FUS3* in Arabidopsis increases the transcript levels of several FA biosynthetic genes, and several types of seed storage proteins (12S cruciferin and 2S albumin). The molecular mechanisms through which FUS3 induces these changes are not characterized.

Seed storage accumulation is also regulated by *ABSCISIC ACID INSENSITIVE3 (ABI3)*, which encodes a member of the B3 transcription factor family (Suzuki et al., 2007). Indirect evidence suggests that *ABI3* might be the target of LEC1 and LEC2 and operates in concert with *FUS3* in seed storage protein accumulation. A proposed model showing the relationship of the transcription factors discussed above is presented in Figure 2.10.

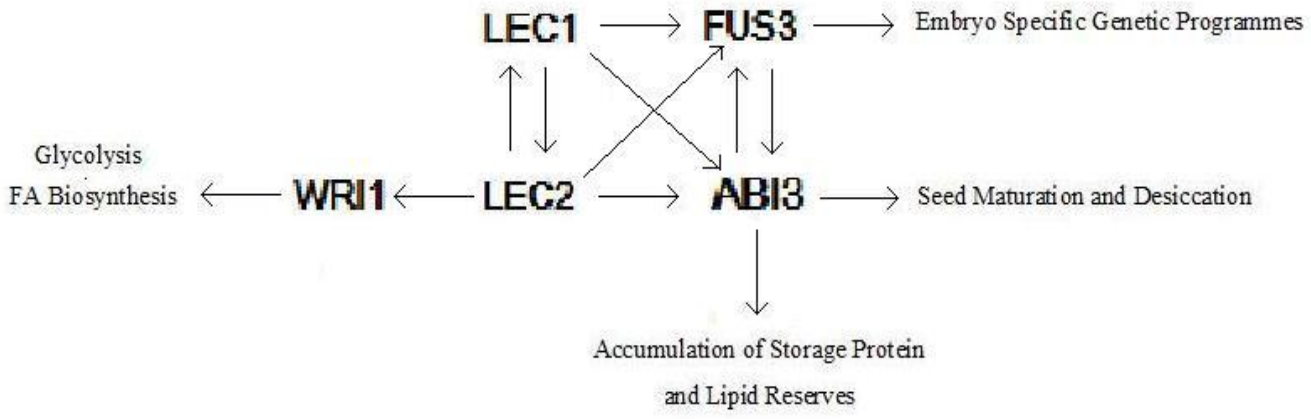


Figure 2.10. Proposed regulatory interaction between the transcription factors LEAFY COTYLEDON1 and 2 (LEC1 and LEC2), WRINKLED1 (WRI1), FUSCA3 (FUS3) and ABSCISIC ACID INSENSITIVE3 (ABI3). This model is adapted from Baud et al. (2008), Santos-Mendoza et al. (2008), and North et al. (2010).

2.3.4. Nutritional value of seed oil: glucosinolate (GLS) levels

One important determinant of oil nutritional value is represented by the glucosinolate (GLS) levels in the seed. Glucosinolates are a class of organic compounds containing sulphur and nitrogen which are derived from glucose and amino acids (Sonderby et al., 2010). Several studies have shown that GLSs play a prominent role in resistance to insects and pathogens (Sonderby et al., 2010) and innate immune response (Bednarek et al., 2009). Due to their toxicity for both humans and animals (Office of The Gene Technology Regulator, 2002), low levels of GLS are highly desirable and this is why over the past few years a lot of effort has been directed towards reducing GLS levels in the seeds (Feng et al., 2012).

Among several classes of GLS present in plants, two are predominant: aliphatic, and indole GLSs. While aliphatic GLSs are derived from methionine, indole GLSs are derived from tryptophan (Schonhof et al., 2004). A simplified GLS biosynthetic pathway is represented in Figure 2.11. Synthesis of aliphatic GLS is initiated with the conversion of methionine to alkyl aldoximes, catalyzed by the enzyme METHYLTHIOALKYLMALATE SYNTHASE1 (MAM1). This enzyme is a key regulator of GLS synthesis since silencing of *MAM1* results in a severe reduction of GLS levels (Liu et al., 2011). Alkyl aldoximes are further converted to S-alkyl thiohydroximates. The S-alkyl thiohydroximate pool also includes precursors of the indole GLS derived from tryptophan. Tryptophan-derived molecules are incorporated in the S-alkyl thiohydroximate pool through two reactions catalyzed by cytochrome P450s CYP79B2 and CYP83B1. Studies on *CYP79B2* revealed that this gene is wound-inducible and expressed in almost every plant tissue (leaves, stem, flowers and root). Over-expression of *CYP79B2* increased the level of indole GLSs (Mikkelsen et al., 2000). A key regulator of GLS synthesis is CYP83B1, since it regulates the level of indole-3-acetaldoxime committed for IAA and GLS production. High levels of IAA and reduced levels of GLS are observed in plants with suppressed levels of *CYP83B1* (Bak et al., 2001). S-alkyl aldoximes are converted to thio-hydroximates by the enzyme SUPERROOT1 (SUR1). Initial studies on SUR1 were conducted in relation to IAA production, since its mutation increased IAA synthesis and resulted in phenotypic abnormalities that are characteristic to high auxin environment (Boerjan et al., 1995). It was only later that the function of this gene was recognized in relation to GLS synthesis

(Mikkelsen et al., 2004). The last step of GLS synthesis from thiohydroximates requires the enzyme SULPHOTRANSFERASE5a (ST5a) (Piotrowski et al., 2004) (Figure 2.11).

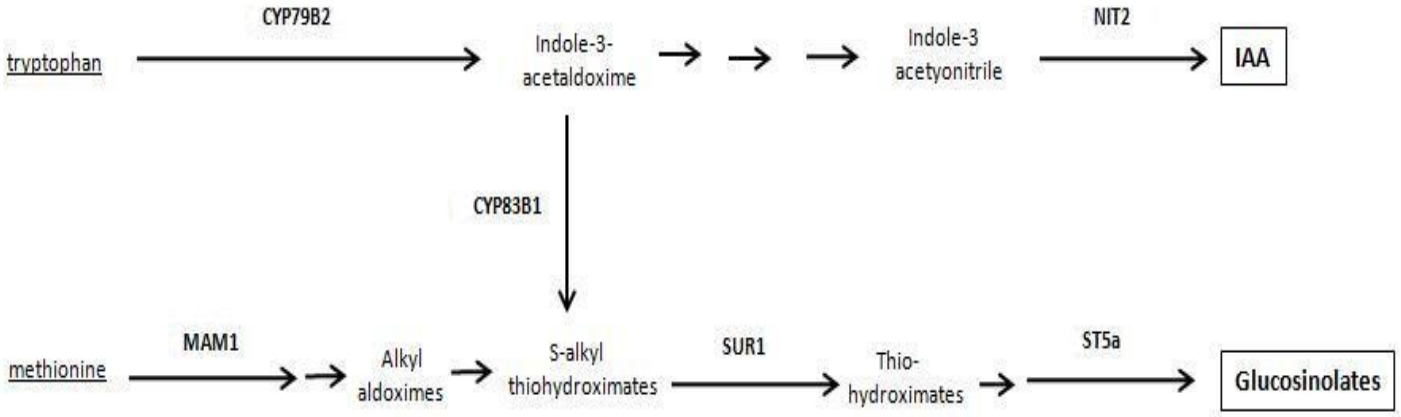


Figure 2.11 Glucosinolate biosynthetic pathway. *MAM1, METHYLTHIOALKYLMALATE SYNTHASE1; CYP79B2, CYTOCHROME P450 CYP79B2; CYP83B1, CYTOCHROME P450 CYP83B1; SUR1, SUPERROOT1*; *ST5a, SULFOTRANSFERASE5a;* and *NIT2, NITRILASE2.* The figure is adapted from Elhiti et al., 2012.

2.4. *In vitro* embryogenesis

Formation of embryos is not only an *in vivo* characteristic, since embryos can be induced in culture from different cell types. Today, formation of asexual (without fertilization) embryos can be achieved in culture through two processes: somatic embryogenesis and gametophytic embryogenesis. Somatic embryogenesis exploits the potential of somatic cells (any cell other than gametes) to embark an embryonic developmental pathway if cultured under appropriate conditions (Bhojwani and Razdan, 1996). Gamethophytic embryogenesis consists in the formation of haploid embryos using male or female gametophytes. In tissue culture practices, male gametophytic

embryogenesis is highly preferred due to the easiness in which cultured microspores and pollen grains can produce embryos (Raghavan, 2000). Both somatic and gametophytic embryogenesis have been used extensively to investigate physiological and molecular events associated to development and maturation of the embryos (Mordhorst et al., 2002). This is because all the events observed during *in vivo* embryogenesis, including the formation of the SAM and the accumulation of storage products (oil, protein, and starch), also occur during *in vitro* embryo development (Yeung et al., 1996). An important advantage of using *in vitro* embryogenesis is represented by the ability to produce a large number of embryos in a short period of time (Yeung et al., 1996). This is crucial when studying early embryogeny, since *in vivo* produced embryos are embedded in the maternal tissue and often impossible to dissect.

Some studies in this thesis will use the *Brassica napus* male gametophytic embryogenesis system, which has been well characterized (Malik et al., 2007; Stasolla et al., 2008). In this system, the initiation of the embryogenic process requires a re-differentiation step in which the genetic program of the microspores is directed towards the formation of embryos (microspore-derived embryos or MDEs). Stress treatments, including elevated temperature (Keller and Armstrong, 1979), treatments with ethanol (Pechan and Keller, 1989) and gamma irradiation (Pechan and Keller, 1989), and low temperature (Sunderland et al., 1974; Kasha et al., 1995) promote the re-differentiation step, which is manifested by changes in cytoskeletal components and a symmetric division of the microspore (Telmer et al., 1992). As observed during *in vivo* embryogenesis, the two daughter cells generated by the symmetric division of the microspore have different fates, with the apical cell forming the embryo proper through

anticlinal and periclinal divisions, and the basal cell generating the suspensor. Yeung et al. (1996) showed that development of Brassica MDEs follows the same sequence of events characteristic to seed embryos; including the formation of SAM and the accumulation of storage products.

2.5. Objectives of this study

Formation of the SAM, a key event during embryogenesis is controlled by a complex genetic interaction involving *STM, ZLL,* and *CLV1*. As shown in Figure 2.6, distinct expression and localization patterns of these genes ensure proper development of the SAM during embryogenesis and its maintenance and functionality during post-embryonic growth. To date, little information is available on the function of these genes in other developmental processes, and this is surprising, given the fact that these genes fulfill their function through changes in hormone signaling and response (D'Agostino et al., 2000; Shani et al., 2006; Cheng et al., 2010). Work by Elhiti et al. (2010) identified the *Brassica napus* (*Bn*) *STM, CLV1,* and *ZLL,* and showed that their altered expression affects Arabidopsis somatic embryogenesis. As an extension of that work, this thesis examines the effects of altered expression of these genes during *Brassica napus* embryogenesis with emphasis on storage product accumulation, mainly oil (Chapter 1) and MDE number and quality (Chapter 2).

Chapter 1. This chapter describes the characterization of *Brassica napus* lines with altered expression of *BnSTM, BnZLL,* and *BnCLV1* (generated by Dr. Mohamed

Elhiti) and the effect of the transgenes on seed oil levels. This study shows that over-expression of *BnSTM* enhances oil content and improves the nutritional value of the seeds by lowering the total GLS levels. A cytokinin-mediated model, through which *BnSTM* alters sink strength facilitating carbon flux into the seed to be used for oil production, is also presented.

Chapter 2. This chapter investigates the effects of altered expression of *BnSTM*, *BnZLL*, and *BnCLV1* on number and germination frequency of Brassica microspore-derived embryos (MDEs). It is shown that over-expression of *BnSTM* enhances both processes while its down-regulation represses the number of MDEs produced in culture and their ability to germinate. Transcriptional analysis reveals changes in cytokinin metabolism which are induced by *BnSTM* and might be implicated in the effects observed. Altered expression of *BnZLL* and *BnCLV1*, does not affect development and germination of Brassica MDEs.

CHAPTER 1: SEED OIL ANALYSIS IN BRASSICA NAPUS WITH ALTERED EXPRESSION OF *SHOOT MERISTEMLESS* (*STM*), *ZWILLE* (*ZLL*) AND *CLAVATA1* (*CLV1*)

3.0. ABSTRACT

Transgenic *Brassica napus* (*Bn*) plants with altered expression levels of *SHOOT MERISTEMLESS* (*BnSTM*), *CLAVATA1* (*BnCLV1*) and *ZWILLE* (*BnZLL*) were characterized and used to measure seed oil content. The plants with over-expression of *BnSTM* was increased in seed oil level without affecting protein and sucrose levels. These changes were accompanied by the induction of several transcription factors promoting fatty acid (FA) synthesis and key enzymes involved in sucrose metabolism, glycolysis, and FA biosynthesis. These distinctive expression patterns support the view of an increased carbon flux to the FA biosynthetic pathway in developing transformed seeds. The over-expression of *BnSTM* also resulted in a desirable reduction of seed glucosinolate (GLS) levels, ascribed to a transcriptional repression of key enzymes participating in the GLS biosynthetic pathway. Taken together, these findings provide evidence for a novel function of *BnSTM* in promoting desirable changes in seed oil and GLS levels when over-expressed in *Brassica napus* plants.

3.1. INTRODUCTION

A key event during embryogenesis in plants is the formation of the shoot apical meristem (SAM), which in Arabidopsis is controlled by several genes such as *SHOOT MERISTEMLESS* (*STM*), *ZWILLE* (*ZLL*), and *CLAVATA1* (*CLV1*). *SHOOT MERISTEMLESS* (*STM*) is a homeobox gene which encodes a member of the class-1 KNOX HOMEODOMAIN-containing proteins (Long et al., 1996; Janosevic and Budimir, 2006). The class-1 KNOX subfamily also includes KNAT1, 2, and 6 which through their interactions with BELL1-like HOMEODOMAIN proteins control developmental pathways targeting organ specification and plant architecture (Hamant and Pautot, 2010). The physical interaction between class-1 KNOX members with BELL proteins has been documented in several systems (Bellaoui et al., 2001). In Arabidopsis, STM has been shown to interact with several BELL proteins including ARABIDOPSIS THALIANA HOMEOBOX1 and PENNYWISE (Rutjens et al., 2009) through complexes affecting both meristem function during vegetative development (Kanrar et al., 2006; Rutjens et al., 2009) and floral patterning during reproductive development (Bhatt et al., 2004; Kanrar et al., 2006). Unique features of the class-1 KNOX proteins are the two domains KNOX1 and KNOX2, which are respectively involved in transcriptional repression of target genes and in dimer formation and transactivation (Sakamoto et al., 2001), the ELK domain which encodes a nuclear localization signal, and the HOMEODOMAIN required for DNA binding (Scofield and Murray, 2006). In Arabidopsis the expression of *STM* is initially detected in a few cells of the globular-stage embryo (Sharma and Fletcher, 2002). Upon further development the expression of this gene expands within the shoot apical meristem and includes all the meristematic cells

located between the two cotyledons, but not the incipient organ primordial (Sharma and Fletcher, 2002). This expression pattern is retained during post-embryonic growth despite small variations especially during the transition from vegetative to floral development (Long and Barton, 1998). Within the inflorescence meristem the expression of *STM* initially encompasses the whole apical region and then becomes restricted to the middle domain of the developing flower, in conjunction with the formation of the outer floral organs (Scofield and Murray, 2006). Perturbation of *STM* expression affects the function of the shoot meristem. Strong *stm* loss-of-function causes a failure to initiate the embryonic shoot meristem as true leaves are not observed in these mutants. Plants with mild lesions (*stm-2* and *6*) are able to form embryonic shoot meristems, but fail to maintain their function during germination due to the incorporation of the meristematic cells into organ primordia (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). Overall these genetic studies suggest that *STM* is needed for the formation and maintenance of the shoot meristem by suppressing differentiation and maintaining an undetermined cell fate within the apical pole.

Another important gene in the regulation of the SAM is *CLAVATA1* (*CLV1*), which encodes a leucine-rich repeat receptor kinase protein (Clark et al., 1997). The function of *CLV1* is antagonistic to that of *STM*. Clark et al. (1996) demonstrated a strong genetic interaction between *STM* and *CLV1* by showing that mutations at the *clv1* locus are able to suppress the *stm-1* and *stm-2* phenotypes. Therefore it was suggested that while *STM* is required for promoting meristem formation and maintenance, *CLAVATA1* represses meristematic proliferation (Clark et al., 1996; Barton, 2010). The function of CLV1 is exercised within a CLAVATA signaling pathway which also includes CLV2, a receptor

kinase which forms a CLV1/CLV2 complex located in the sub-apical cells of the SAM and CLV3, a small ligand released by the apical cells of the SAM (Ogawa et al., 2008). The binding of CLV3 to the CLV1/CLV2 complex initiates a signal transduction pathway leading to the transcriptional repression of target genes specifying stem cell fate (Betsuyaku et al., 2011). Genetic studies confirm this model and show that mutations ing *CLV1* result in the formation of enlarged SAMs characterized by a large pool of undifferentiated cells (Betsuyaku et al., 2011).

Besides *STM* and *CLV1*, a third regulator of SAM formation is *ZWILLE* (*ZLL*). *ZWILLE* encodes a member of the EIF2C (elongation initiation factor 2c)/ARGONAUTE class of proteins participating in RNA silencing mechanisms (Lynn et al., 1999; Liu et al., 2009). Expression of *ZLL* is initiated at the heart-stage of embryo development and coincides with the provascular tissue. Upon further growth of the embryo, *ZLL* becomes localized in the SAM (Moussian et al., 2003). Arabidopsis plants with suppressed *ZLL* expression have terminally differentiated cells, and produce organs instead of stem cells during embryogenesis (Lynn et al., 1999). During post-embryonic growth these plants form adventitious meristems, thus suggesting that the function of *ZLL* is required for the establishment of the primary embryonic SAM, but not for post-embryonic SAM formation. The mechanisms through which *ZLL* exercises its function during meristem development are mostly unknown. A proposed function of *ZLL* is to ensure the proper localization pattern of *STM* within the SAM, as miss-expression of *STM* is observed in *zll* mutant embryos (Moussian et al., 1998).

Besides regulating meristem architecture *in vivo*, altered levels of *STM*, *CLV1*, and *ZLL* have also been found to affect other processes, including *in vitro* organogenesis

(Gallois et al., 2002) and embryogenetic cell formation during somatic organogenesis (Elhiti and Stasolla, 2012). To further investigate novel roles of these genes in plant development, available *Brassica napus* (*Bn*) plants with altered expression of *BnSTM, BnCLV1*, and *BnZLL* were utilized to measure seed oil. While changes in *BnCLV1* and *BnZLL* expression did not influence oil levels in the seeds, the ectopic expression of *BnSTM* increased seed oil content and altered fatty acid (FA) composition without affecting protein and sucrose levels. Furthermore the expression of key enzymes regulating sucrose metabolism, glycolysis, and FA biosynthesis were up-regulated in developing seeds over-expressing *BnSTM*. These distinctive transcriptional changes suggest an increased carbon flux to the FA biosynthetic pathway in transformed seeds. The over-expression of *BnSTM* also resulted in a desirable reduction of seed glucosinolate (GLS) levels due to a transcriptional repression of key enzymes participating in GLS biosynthesis, and possibly to the differential utilization of shared intermediates between the GLS and IAA biosynthetic pathways. Taken together these findings demonstrate that *BnSTM* is a potential target to enhance seed quality in crop species.

3.2. MATERIALS AND METHODS

3.2.1. Generation of transgenic *Brassica napus* plants

Generation of transgenic plants was conducted previously in the lab using sense, antisense or RNA intereference (RNAi) mediated transformation. The following lines were available:

1) *BnSTM* sense (S) over-expressing lines: S17, S38, and S101

2) *BnSTM* antisense (A) lines: A1 and A5

3) *BnZLL* sense (S) over-expressing lines: S4, S5, and S7

4) *BnZLL* RNA intereference (RNAi) lines: RNAi1, and RNAi2

5) *BnCLV1* RNA intereference (RNAi) lines: RNAi2, RNAi3, and RNAi8.

No lines over-expressing *BnCLV1* were available.

Transgenes were transfected into the plant cells by *Agrobacterium tumefaciens* with the plasmids containing 35S promoter (cauliflower mosaic virus).

The transgenic plants were germinated in the mixed soil with Peat moss, sandy soil, black soil, Perilte and Vermiculate (6:1:1:1:1) and grown in the greenhouse with 16h/8h day/night (light intensity: 323 μmole/S m^2), the temperature was controlled by the weather outside.

3.2.2. RNA extraction

Plant tissue (0.1 g) was ground in a 1.5 ml microcentrifuge tube with 1 ml of TRI reagent (Sigma-Aldrich, Missouri, USA) and incubated for 5 min at room temperature. Chloroform (0.2 ml) was added, vortexed and incubated for 15 min. The solution then was centrifuged at 12,000 rpm (g) for 15 min at 4^{o}C. The RNA-containing aqueous phase was transferred into a 1.5 ml tube containing 0.5 ml of 2-propanol, incubated for 10 min at room temperature, and centrifuged at 12,000 rpm for 10 min at 4^{o}C. The pellet

was washed with 1 ml of 75% ethanol, centrifuged at 7500 rpm for 5 min, the ethanol was discarded and the pellet was fully dried, and re-dissolved in 20 µl DEPC treated water. DNA traces were removed from the RNA using the DNase I (Roche, USA), according to manufactory instructions. Quantity and quality of the RNA were checked using a Nanodrop spectrophotometer and by agarose gel electrophoresis.

3.2.3. cDNA synthesis

Synthesis of cDNA was performed using the Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Fermantas, Canada). DNase treated total RNA (1 pg to 5 µg) was mixed with 4 µl of 5×Reaction Mix, 2 µl of Maxima® Enzyme Mix and nuclease-free water up to 20 µl. The solution was incubated for 10 min at 25^{o}C followed by 30 min at 55^{o}C. The reaction was terminated by heating at 85^{o}C for 5 min.

3.2.4. Gene expression analysis by quantitative qRT-PCR

Analysis of gene expression in developing [7, 14, 21, and 28 days after pollination (DAP)] *Brassica napus* seeds was determined by quantitative qRT-PCR (Elhiti et al., 2010) using previously determined primer sequences (Prystenski, 2010; Tan et al., 2011). Expression studies were conducted for genes involved in sucrose transport and metabolism, glycolysis, FA/oil and GLS synthesis, and representative transcription factors regulating oil and GLS synthesis. All Primers used are compiled in Appendix 5.

The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen, (2001) using *BnACTIN* (AF111812) as a reference.

3.2.5. Analysis of lipids, proteins, and glucosinolates

Total oil, protein and glucosinolate (GLS) levels were determined using near infrared reflectance spectroscopy (Tkachuk, 1981) from the dry seeds. Analysis of FA profiles was conducted using gas chromatography with the procedure documented by Hougen and Bodo (1973) and DeClercq (2005). Analysis of GLS profile was conducted by HPLC as described in Liu et al. (2011).

3.2.6. Sucrose determination and ATP/ADP measurements

Sucrose was extracted by grinding 50 mg fresh weight of Brassica seeds (21 DAP) in 1 ml of 70% ethanol. The samples were vortexed for 5 min and subsequentially washed first with 5 ml of absolute ethanol and then with 1.5 ml of 95% ethanol. The supernatant was collected by centrifugation and dried at 100^{o}C. After the addition of 1 ml of water, 2 ml of chloroform and 1 ml of 100 mM acetate buffer (pH 4.5), the samples were centrifuged (at 2500 rpm for 10-15 min) and the sucrose containing upper phase collected. To collect residual sucrose in the sample, an additional phase separation was performed by adding 1 ml of water and 1 ml of 100 mM acetate buffer (pH 4.5), the samples were centrifuged and the upper phase containing residual sucrose was collected.

Sucrose measurements were performed spectrophotometrically using the Sucrose/D-Glucose Megazyme Kit (Megazyme International Ireland Ltd., Wicklow, Ireland).

Extraction of adenine nucleotides was performed as described by Quebedeaux (1981). ATP determination was carried out by using the ATP bioluminescence assay kit HSII (Roche, USA). ADP was converted to ATP using pyruvate kinase (Quebedeaux, 1981).

3.2.7. Statistical analysis

Unless specified, all experiments were performed using at least three biological replicates and the Duncan-Waller Post-Hoc test for multiple variance (Zar, 1999) was applied to compare differences among samples.

3.2.8. Cytokinin treatment

A solution containing benyladenine (50 mg/L), NaOH (any amount when the benyladenine was dissolved) and Tween-20 (0.5 mL/L) was used to immerse developing WT siliques at 2, 6, 12, 18, and 20 DAP. Another group of WT siliques were immersed in the solution without benyladenine for control. Seeds were harvested at day 21 for RNA extraction.

3.3. RESULTS

3.3.1. Characterization of transgenic *Brassica napus* plants with altered expression of *BnSTM, BnCLV1,* and *BnZLL*

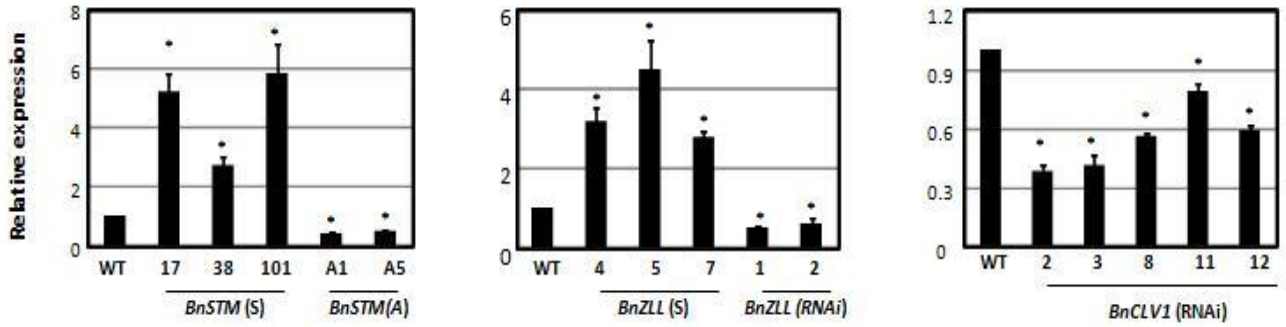
Previous work conducted by other lab members established the presence of the transgene in the genomic DNA of all the lines utilized in this study. To complete the characterization of these lines and verify the altered expression of the transgenes, quantitative RT-PCR studies were conducted in flowers of each line (Figure 3.1A). These studies confirmed that all the plants utilized for the experiments of the thesis are indeed transformed and exhibit the expected behavior (up-regulation in the S lines and down-regulation in A or RNAi lines) of the transgenes.

No phenotypic differences were observed in overall plant morphology between wild type (WT, transformed with the empty construct) plants and plants with altered expression of *BnCLV1* or *BnZLL*. However, plants transformed with *BnSTM* had definite altered phenotypes. Plants down-regulating *BnSTM* (the A lines) showed some premature shoot termination at the seedling stage (Figure 3.1B). This terminal shoot phenotype was similar to that produced by weak *stm* loss-of-function alleles in Arabidopsis (Barton and Poethig, 1993). Ectopic expression of *BnSTM* resulted in a characteristic leaf-lobing phenotype, which is a distinct feature of *KNOX1* over-expressors (Scofield and Murray, 2006). The degree of leaf lobing varied among lines (Figures 3.1B2-4) and was most severe in S101 plants which had stunted growth (Figure 3.1B4). Silique number was slightly increased in plants with a mild induction of *BnSTM* (lines S17 and S38), while a reduction in the number of siliques per plant and number of seeds per silique was observed in S101 plants, characterized by the highest expression of *BnSTM* (Figure 3.2). No differences in 1000-seed weight were measured between WT and *BnSTM* over-expressing plants.

3.3.2. Alterations in seed oil content in the transformed lines

To investigate the effects of altered expression of *BnSTM, BnCLV1* and *BnZLL* on storage oil accumulation, fully mature dry seeds were analyzed by near-infrared reflectance spectroscopy (NIR). No differences in the levels of seed oil were measured in lines with altered expression of *BnCLV1* and *BnZLL* (data not shown). The total seed oil content increased slightly in all the *BnSTM* over-expressors and this increment was more pronounced in lines S17 and S101 (Figure 3.3). The down-regulation of *BnSTM* did not alter the seed oil content (Figure 3.3). Based on these results further experiments were conducted only on lines over-expressing (S) or down-regulating (A) *BnSTM*. No differences in the levels of protein content were observed on lines over-expressing (S) or down-regulating (A) *BnSTM* (Figure 3.3).

(A)



(B)

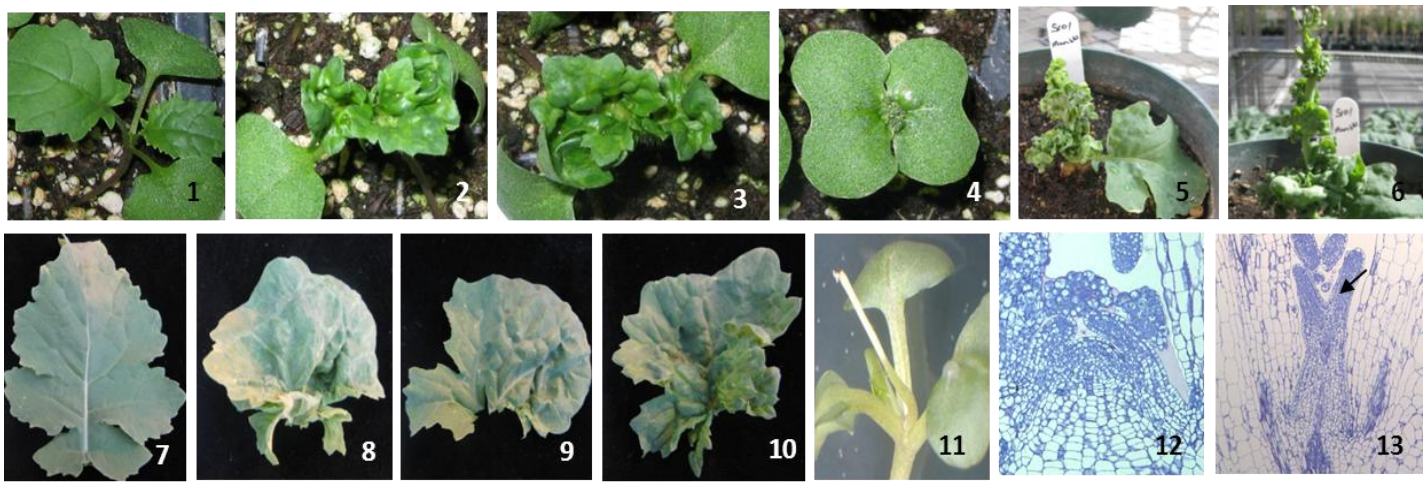


Figure 3.1. Identification and characterization of *Brassica napus* lines over-expressing (S) or down-regulating (A) *BnSTM*. **(A)** Expression level of the transgene by quantitative real time qRT-PCR in flowers which were collected as soon as petals unfolded. Values ± SE are means of at least three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators. **(B)** Comparison of seedling morphology in WT (1) and lines over-expressing *BnSTM*: S17 (2), S38 (3), and S101 (4). Morphological defects in the strong *BnSTM* over-expressor S101 line during vegetative (5) and reproductive (6) development. Compared to WT (7), lines over-expressing *BnSTM* showed lobed leaves to different degrees of severity (8-10). Several instances of meristem termination were observed in lines down-regulating *BnSTM* (11). Histological analyses in young seedlings revealed marked structural differences between a normal shoot meristem of WT plants (12) and a terminated shoot meristem (arrow) of *BnSTM* down-regulators (13).

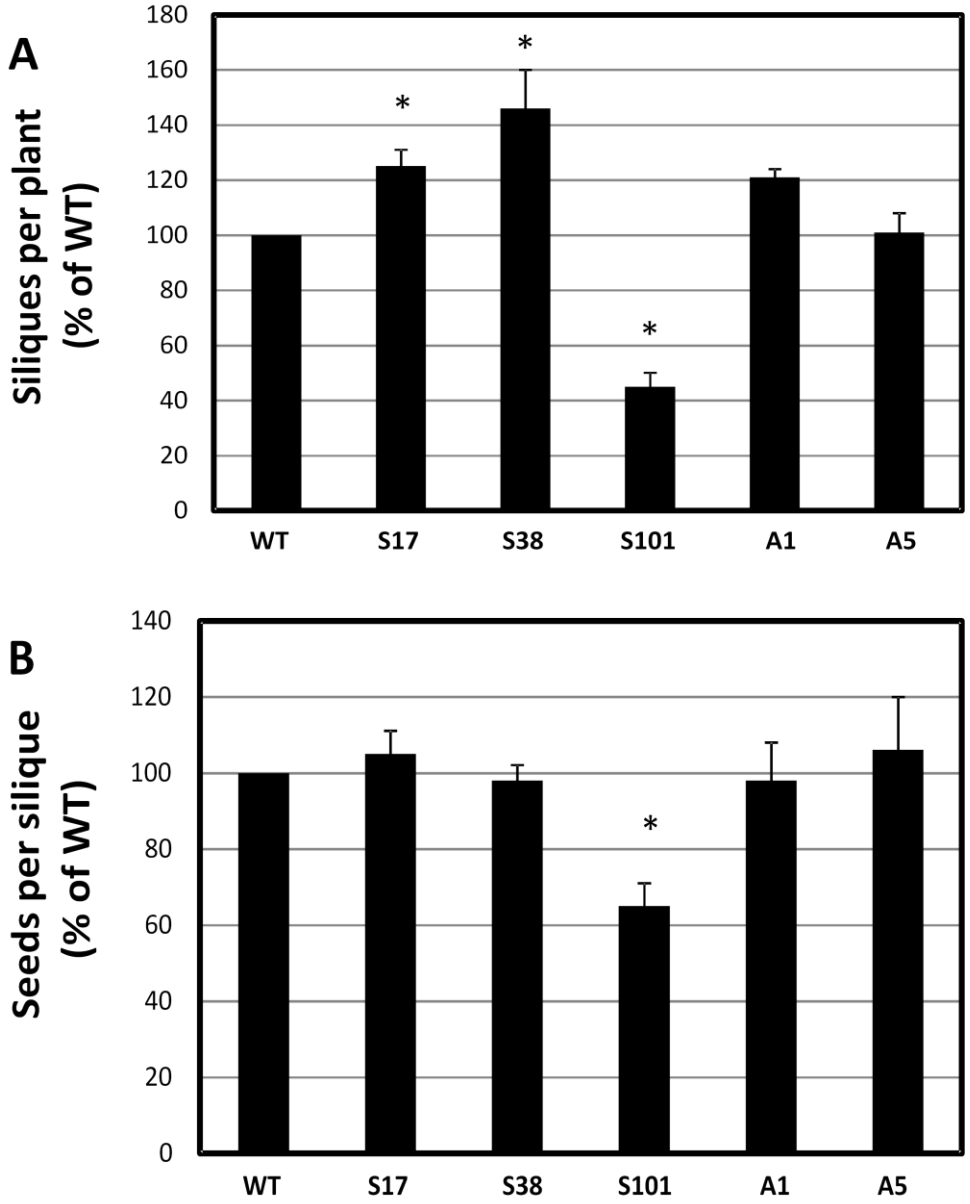


Figure 3.2. Number of siliques per plant **(A)** and seeds per silique **(B)** produced at maturity by *Brassica napus* lines over-expressing (S) or down-regulating (A) *BnSTM*. Values $\pm$ SE are means of at least three biological replicates. * indicates statistically significant differences ($p<0.05$) from the WT.

3.3.3. Fatty acid (FA composition) of seed oil

Analysis of fatty acid (FA) composition by gas chromatography (GC) showed a decrease in percentage of C18:1 oleic acid in seeds of all *BnSTM* over-expressors (Table 3.1). These changes were accompanied by a slight increase in the polyunsaturated C18:2 (ω-6) linoleic and C18:3(ω-6) linolenic acids, which reflected alterations in fatty acid desaturation. To further evaluate the nutritional value of seed oil, we estimated the ratio of these FA species in the transgenic lines. Both C18:2 (ω-6) linoleic and C18:3(ω-6) linolenic acids are highly desirable as they are important for health, and cannot be synthesized by humans. The C18:1/C18:2 and C18:1/C18:3 ratios were slightly lowered in lines with the ectopic expression of *BnSTM*, especially in S101 line, while the C18:2/C18:3 ratio remained unaltered (Table 3.2). No differences in FA ratio were observed in seeds down-regulating *BnSTM*. The nutritional quality of oilseeds is determined by the proper balance between FA, affecting the properties of the oil, and proteins, contributing to the characteristics of the meal (Nesi et al., 2008). Despite the negative correlation between oil and protein content observed in previous studies (Hao et al., 2004), the expression of *BnSTM* did not alter protein level in Brassica seeds (Figure 3.3).

Table 3.1. Percentage fatty acid composition in dry *Brassica napus* seeds. Values ± SE are means of 3 biological replicates. * Indicates statistically significant differences compared to WT values ($p<0.05$). S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

	WT	S17	S38	S101	A1	A5
C 16:0	3.73±0.07	4.03±0.05	3.94±0.06	3.95±0.05	3.67±0.03	3.82±0.01
C 16:1	0.22±0.01	0.24±0.01	0.23±0.01	0.21±0.00	0.21±0.01	0.20±0.01
C 18:0	3.20±0.19	3.28±0.05	3.05±0.04	2.85±0.04	2.31±0.85	1.92±0.01*
C 18:1	69.63±0.22	64.78±0.16*	64.92±0.25*	63.90±0.12*	68.29±0.17	69.14±0.12
C 18:2	14.01±0.03	16.74±0.12	17.02±0.23*	17.18±0.22*	15.07±0.12	15.74±0.33
C 18:3	5.48±0.03	6.33±0.33	6.32±0.08*	7.12±0.08*	6.21±0.06	5.27±0.08
C 20:0	1.07±0.04	1.07±0.07	1.03±0.07	0.87±0.09	0.84±0.14	0.73±0.05
C 20:1	1.46±0.01	1.41±0.00	1.41±0.02	1.41±0.00	1.47±0.01	1.43 ±0.01
C 22:0	0.51±0.01	0.49±0.01	0.49±0.01	0.45±0.00	0.44±0.01	0.41 ±0.01
C 24:0	0.37±0.02	0.39±0.01	0.37±0.01	0.29±0.06	0.27±0.03	0.33 ±0.01
C 24:1	0.16±0.01	0.17±0.01	0.17±0.01	0.16±0.00	0.19±0.02	0.14 ±0.00

Table 3.2. Fatty acid ratio as an estimation of nutritional value in dry *Brassica napus* seeds. The ratios were calculated using the values of Table 3.1. * Indicates statistically significant differences compared to WT values ($p<0.05$). S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

	WT	S17	S38	S101	A1	A5
18:1/18:2	4.97	3.86	3.82	3.72*	4.53	4.39
18:2/18:3	2.55	2.64	2.69	2.41	2.43	2.98

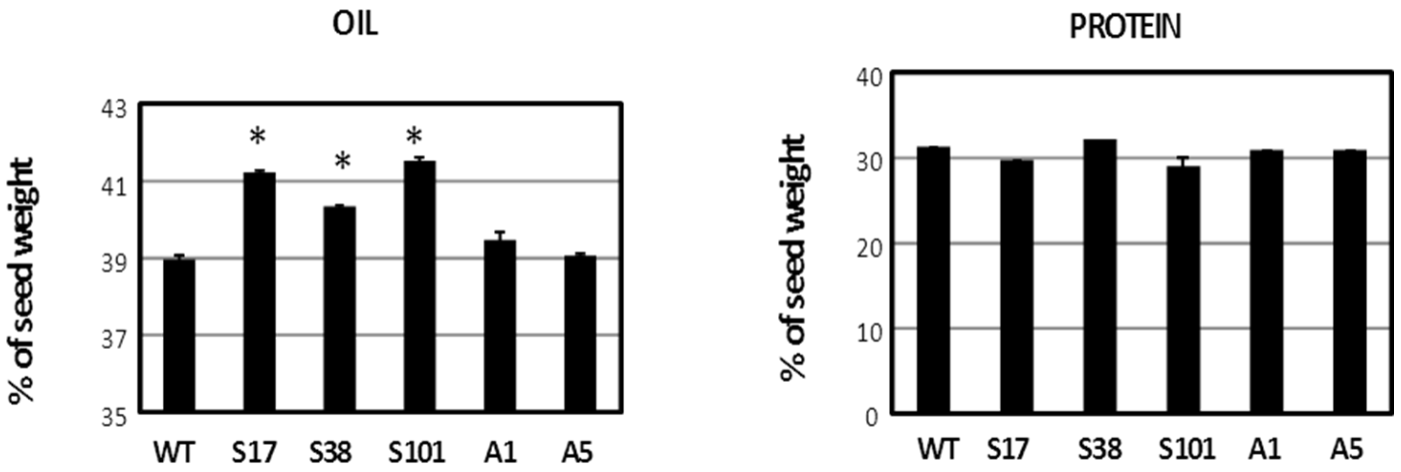


Figure 3.3. Measurements of oil and protein content in dry seeds of *Brassica napus* using near infrared reflectance spectroscopy. Values expressed as % of seed weight (at 0% humidity) $\pm$ SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

3.3.4. Increased levels of oil-related transcription factors and FA biosynthetic enzymes in developing seeds over-expressing *BnSTM*

To further investigate the mechanisms responsible for the changes in oil content evoked by the ectopic expression of *BnSTM*, the transcript level of several genes encoding transcription factors involved in oil biosynthesis were examined. Both *LEAFY COTYLEDON1* (*LEC1*) and *WRINKLED1* (*WRI1*) were induced in seeds of line S101 at 7 DAP and in all the *BnSTM* over-expressing seeds at 28 DAP (Figure 3.4). Experimental up-regulation of either gene is correlated with increased total seed oil content (Mu et al., 2008; Shen et al., 2010; Tan et al., 2011). Genetic studies have shown that *WRI1* is a target of LEC2 (Baud et al., 2007), which was induced in lines S17 and S101 at 7 DAP and in all *BnSTM* over-expressors from 21 DAP. Marginal changes were observed for the expression of *FUSCA3* (*FUS3*), another target of *LEC2* (Stone et al., 2008) except at 7 DAP and 21 DAP when the expression of this gene increased markedly in lines S101 and S17 respectively. No consistent patterns in the expressions of the transcription factors were observed in seeds with reduced levels of *BnSTM* (Figure 3.4).

In seeds, biosynthesis of FA is orchestrated by several genes and the expression of representative ones was examined. These included genes involved in desaturation (*FAD3*, ω-3 *FA DESATURASE*), elongation (*FAE1, FA ELONGATION1*), and condensation (*MCAT, MALONYL-CoA:ACP TRANSACYLASE* and *ACCA2*, subunit A of *ACETYL-CoA CARBOXYLASE*). Compared to wild type, a consistent and substantial induction of all genes was observed in the *BnSTM* over-expressing lines from 14 DAP (Figure 3.5). The most pronounced increase in expression was measured for *BnFAD3*, which catalyzes the conversion of C18:2 to C18:3. A 20 (or higher) fold change in induction of this gene occurred at 28 DAP in all *BnSTM* over-expressing lines. Down-regulation of *BnSTM* in

the A5 line repressed *BnFAD3* and *BnMCAT* at 7 DAP and 14 DAP respectively, while it induced *BnFAE1* at 7 DAP (Figure 3.5).

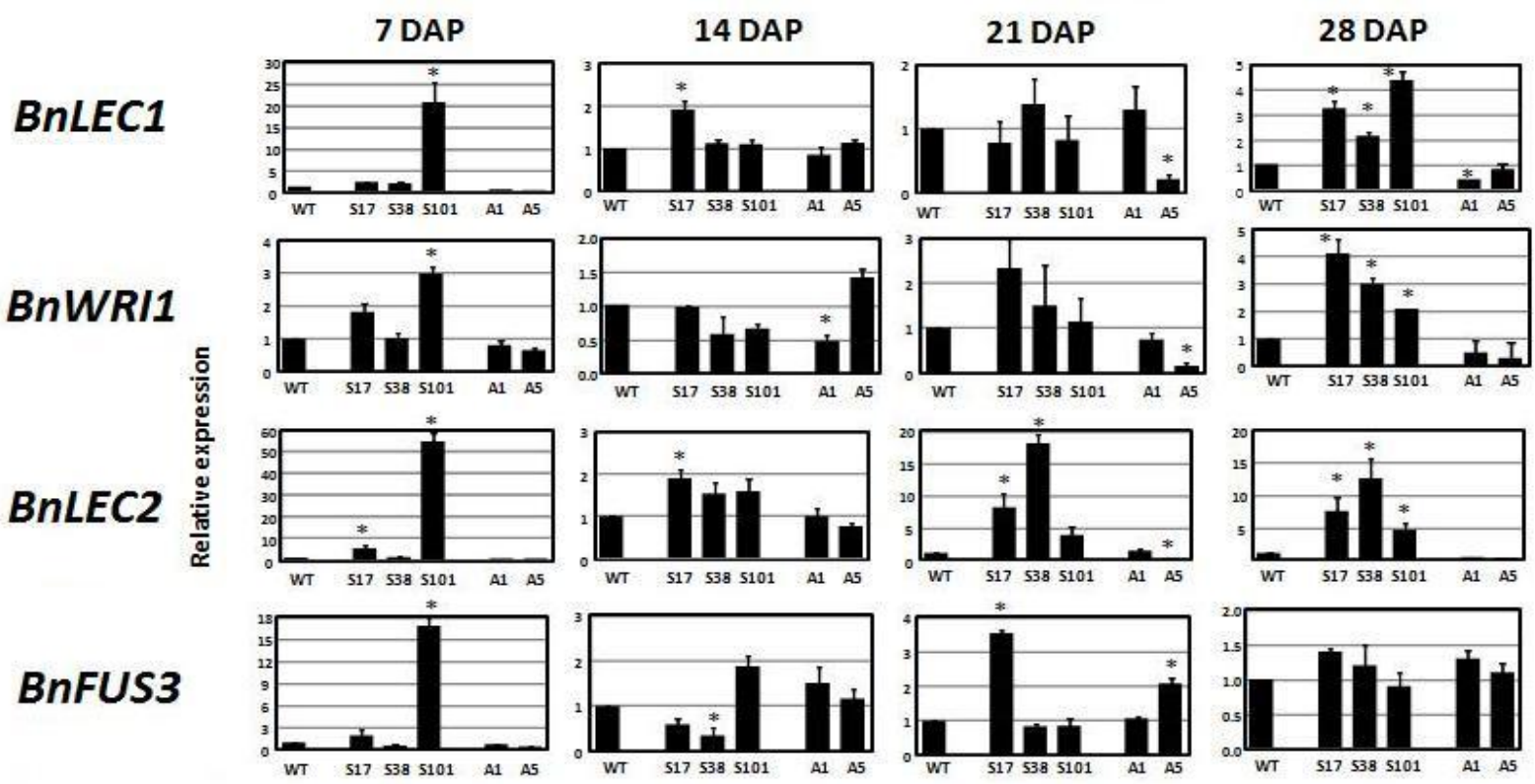


Figure 3.4. Expression levels by qRT-PCR of the transcription factors: *LEC1*, *LEAFY COTYLEDON1*; *LEC2*, *LEAFY COTYLEDON2*; *WRI1*, *WRINKLED1*; and *FUS3*, *FUSCA3;* involved in oil synthesis. Expression levels were measured in developing seeds at different days after pollination (DAP). Values $\pm$ SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

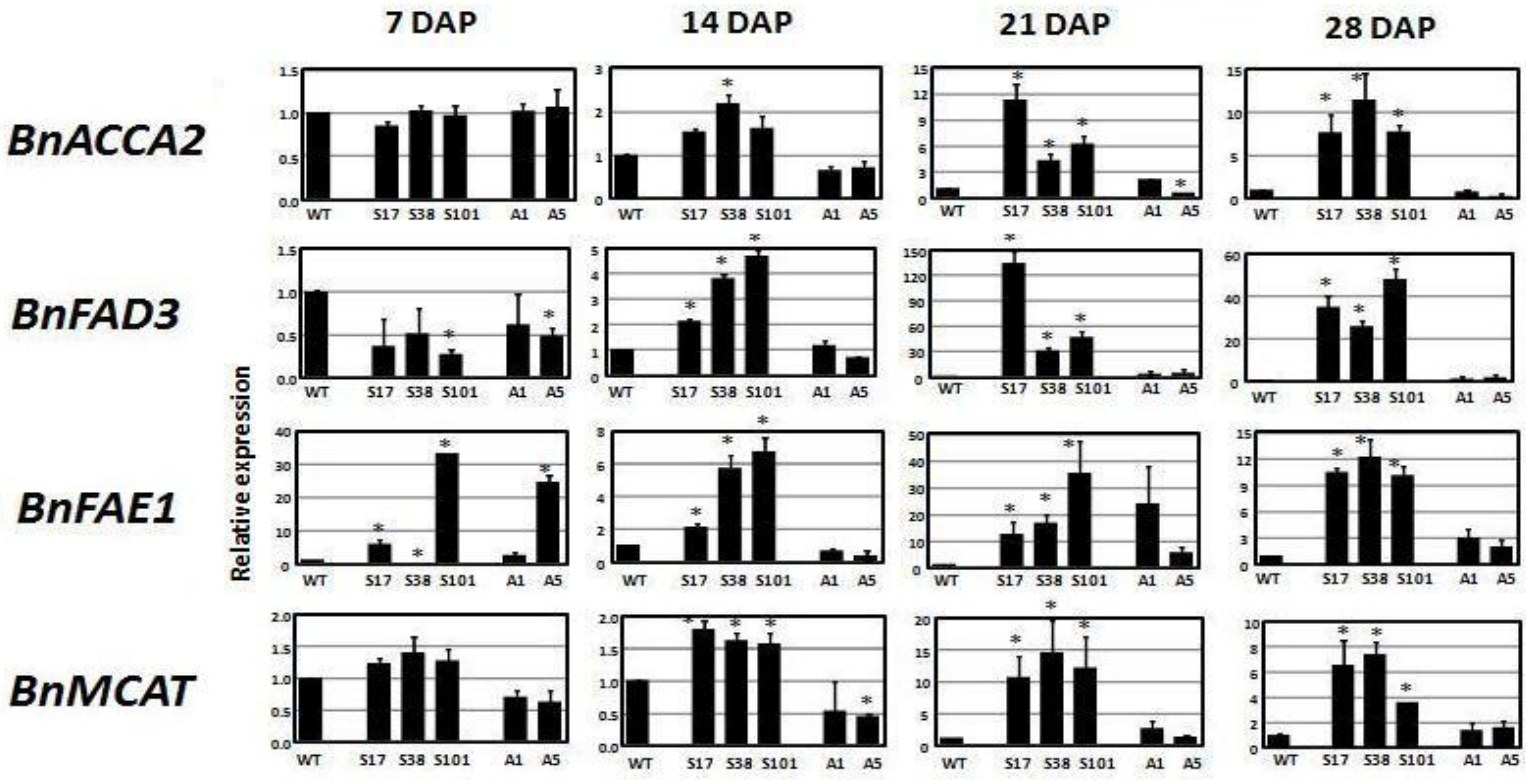


Figure 3.5. Expression level of key genes of the FA biosynthetic pathway in developing seeds of *Brassica napus* at different days after pollination (DAP). *ACCA2*, subunit A of *ACETYL-CoA CARBOXYLASE*; *FAD3*, ω-3 *FA DESATURASE*; *FAE1*, *FA ELONGATION1*; and *MCAT*, *MALONYL-CoA:ACP TRANSACYLASE*. Values ± SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

3.3.5. Ectopic expression of *BnSTM* induces genes involved in sucrose assimilation and glycolysis

The alterations in oil level and fatty acid composition in seeds over-expressing *BnSTM* prompted us to examine the expression of genes participating in sucrose metabolism and glycolysis. In developing seeds the major carbon source for oil synthesis is sucrose derived from photosynthesis, while the energy source (ATP) is generated from both photosynthetic and respiratory processes (Rawsthorne, 2002; Hills, 2004). Once imported into the seeds sucrose is hydrolysed into fructose and UDP-glucose and these hexoses are oxidized through the glycolytic pathway to acetyl-CoA, a precursor of FA synthesis (Schwender et al., 2003). Transcriptional analysis of sucrose transport and metabolism revealed a general induction of *SUCROSE TRANSPORTER1* (*BnSUC1*) and *4* (*BnSUC4*), *SUCROSE SYNTHASE1* (*BnSUS1*) and *3* (*BnSUS3*), and *ADP-GLUCOSE PHOSPHORYLASE* (*BnAGP*) in seeds over-expressing *BnSTM* from 14 DAP (Figure 3.6). This induction was more pronounced for *BnSUC1* and *BnSUC4* (note change of scale bars between 14 and 21 DAP), suggesting an active intake of sucrose in the developing seeds with high *BnSTM* levels. However, at 21 DAP which demarks the initiation of oil accumulation in rapeseed (Fowler and Downey, 1970), the induction of *BnSTM* did not elevate seed sucrose content, but did increase the energy status (ATP/ADP ratio) (Figure 3.7). In *BnSTM* antisense lines a repression of *BnSUS1* and *BnSUS3* was observed at 14 DAP, whereas the expression of *SUC1* increased in line A5 at 7 DAP and in line A1 at 14 DAP (Figure 3.6). No changes in sucrose levels and ATP/ADP ratio occurred in seeds of these line (Figure 3.7).

To more accurately analyse whether the unaltered seed sucrose content (despite the increased expression of sucrose transport and biosynthetic genes) in lines ectopically

expressing *BnSTM* was the result of higher rate of oxidation, we measured the transcript abundance of several glycolytic genes. Compared to wild type seeds the expression level of *BnFPA* (*FRUCTOSE BISPHOSPHATE ALDOLASE*), *BnPGK* (*PHOSPHOGLYCERATE KINASE*), and *BnPPK* (*PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE*) increased in *BnSTM* over-expressing seeds from 14 DAP and remained high during the following days (Figure 3.8). In these seeds, *BnGPDH* (*GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*) was induced at 28 DAP, whereas the expression of *BnHXK* (*HEXOSE KINASE*) was generally similar to wild type levels throughout seed development. Reduction in *BnSTM* expression had no profound consequences on the expression of the glycolytic enzymes except at 14 DAP when *BnFPA* and *BnHXK* were down-regulated in lines A1 and A5, and *BnPGK* was repressed in line A5 (Figure 3.8).

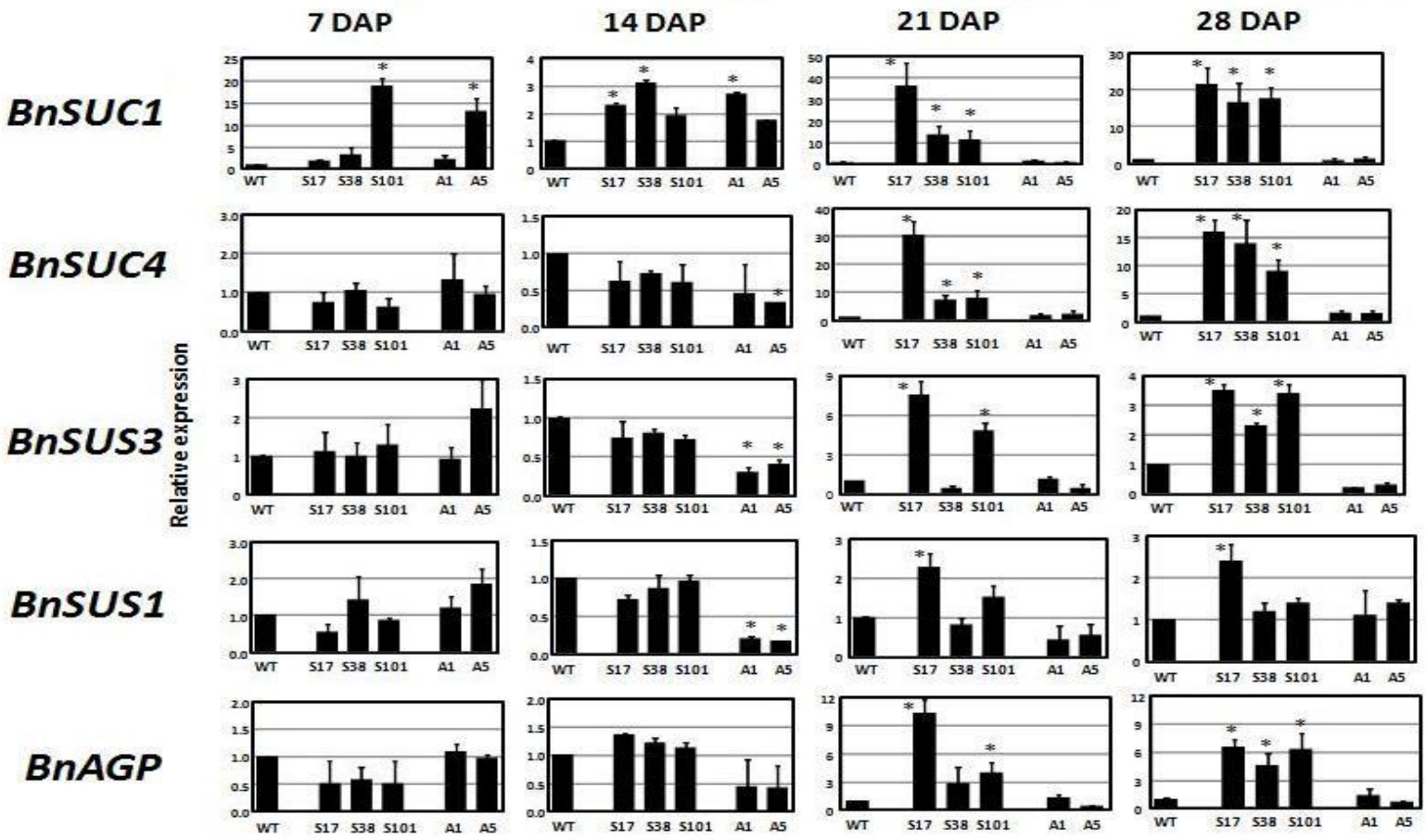


Figure 3.6. Expression levels of genes participating in sucrose transport and metabolism in developing seeds of *Brassica napus* at different days after pollination (DAP). *SUC1, SUCROSE TRANSPORTER1*; *SUC4, SUCROSE TRANSPORTER4; SUS1, SUCROSE SYNTHASE1; SUS3, SUCROSE SYNTHASE3; AGP,* and *ADP-GLUCOSE PHOSPHORYLASE*. Values $\pm$ SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

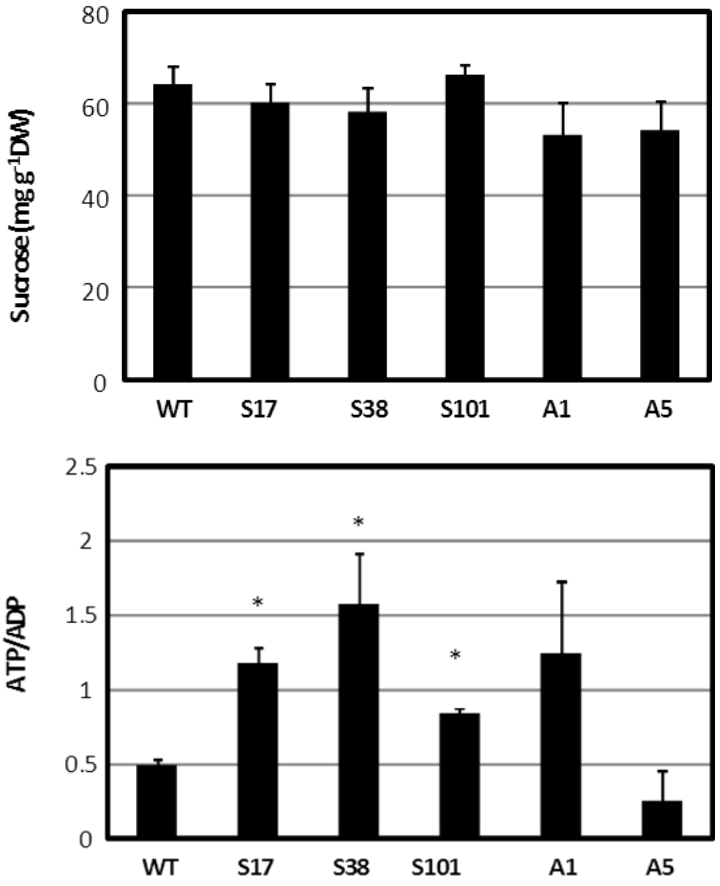


Figure 3.7. Measurements of sucrose content and energy status (ATP/ADP ratio) in *Brassica napus* seeds at 21 DAP. Values ± SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

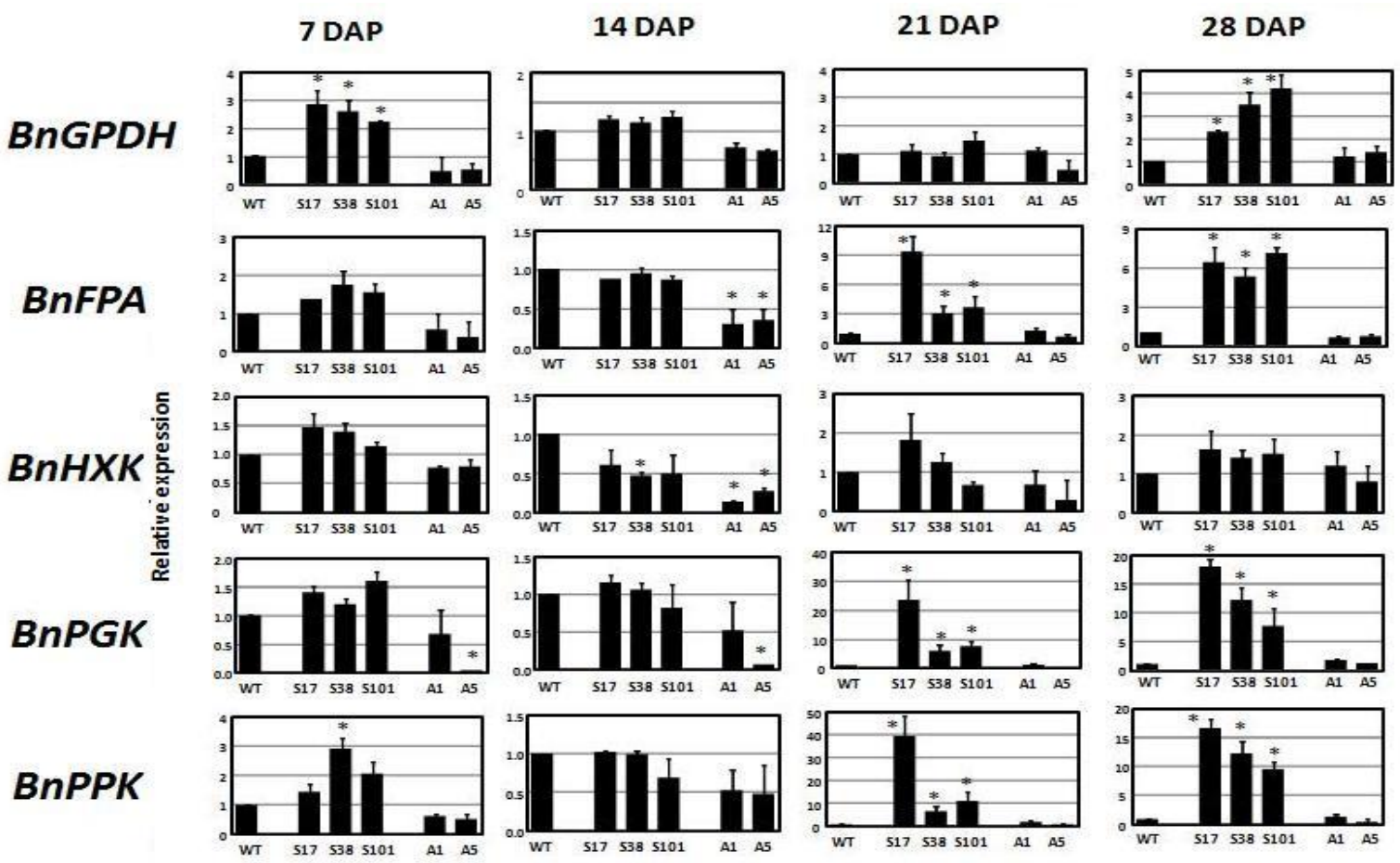


Figure 3.8. Expression levels of key genes of the glycolytic pathway in developing seeds of *Brassica napus* at different days after pollination (DAP). *FPA, FRUCTOSE BISPHOSPHATE ALDOLASE; PGK, PHOSPHOGLYCERATE KINASE*; *PPK, PYROPHOSPHATASE-DEPENDENT PHOSPHOFRUCTOKINASE; GPDH, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE;* and *HXK, HEXOSE KINASE*. Values $\pm$ SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

3.3.6. Over-expression of *BnSTM* affects the size and number of oilbodies

To investigate if the increase in oil content observed in seeds over-expressing *BnSTM* correlated to changes in the size of oilbodies, the ultracellular features of two seed compartments, the radicle/axis and cotyledons were analyzed at 21 DAP (Figure 3.9). Compared to WT, cells of *BnSTM* over-expressing seeds had an elevated number of oilbodies which appeared smaller. Stabilization of oilbodies is controlled by oleosins which prevent coalescence of the lipid particles during seed maturation, thus maintaining oilbodies as single small units (Cummins et al., 1993). These electron microscopy studies were performed by Dr. Belmonte.

Expression studies of five representative *Brassica OLEOSIN S* genes (Jolivet et al., 2009) revealed a *BnSTM* induction of *OLEOSIN S-1* and *S-2* (Figure 3.9).

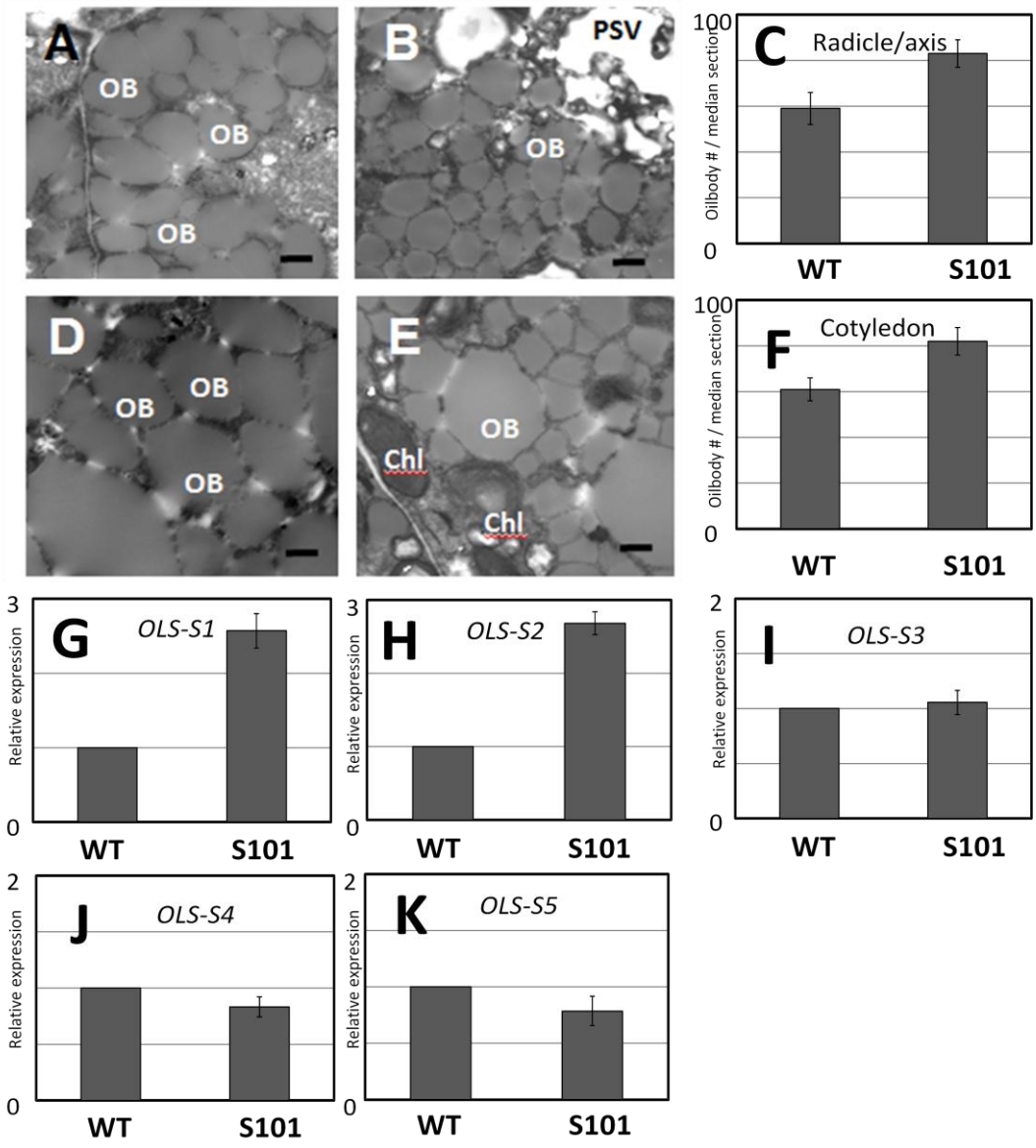


Figure 3.9. Ultrastructural examination of oilbodies and *OLEOSIN* (*OLS*) transcript levels in seeds collected at 21 DAP from the WT and the *BnSTM* over-expressing S101 line. **(A)** Oilbodies (OB) in the radicle/axis of WT seeds. **(B)** Oilbodies in the radicle/axis of S101 seeds. PSV, protein storage vacuole. All scale bars = 500nm **(C)** Number of oilbodies counted in a median section of the radicle/axis. Values ± SE are means of 10 median sections of 10 seeds. **(D)** Oilbodies in the cotyledons of WT seeds. **(E)** Oilbodies in the cotyledons of S101 seeds. Chl, chloroplast. **(F)** Number of oilbodies counted in a median section of the cotyledons. Values ± SE are means of 10 median sections of 10 seeds. **(G-K)** Expression levels of five representative *OLEOSIN*s. Values ± SE are means of three biological replicates. Values were normalized to the value of WT set at 1.

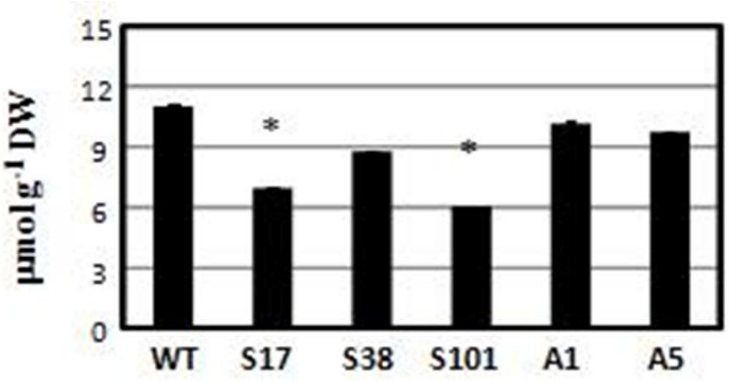
3.3.7. Over-expression of *BnSTM* reduces the level of glucosinolates in seeds

Glucosinolates (GLS), secondary metabolites found in all Brassicaceae (Fahey et al., 2001), accumulate in seeds with undesirable effects due to their anti-nutritional and goitrogenic properties. A reduction of GLS in Brassica seeds is an ongoing goal for plant breeders and geneticists (Feng et al., 2011). Total GLS level (performed by Mr. Durnin), measured at 21 DAP was unaltered in seeds down-regulating *BnSTM*, but decreased in seeds over-expressing *BnSTM*, especially in line S101 (Figure 3.10A).

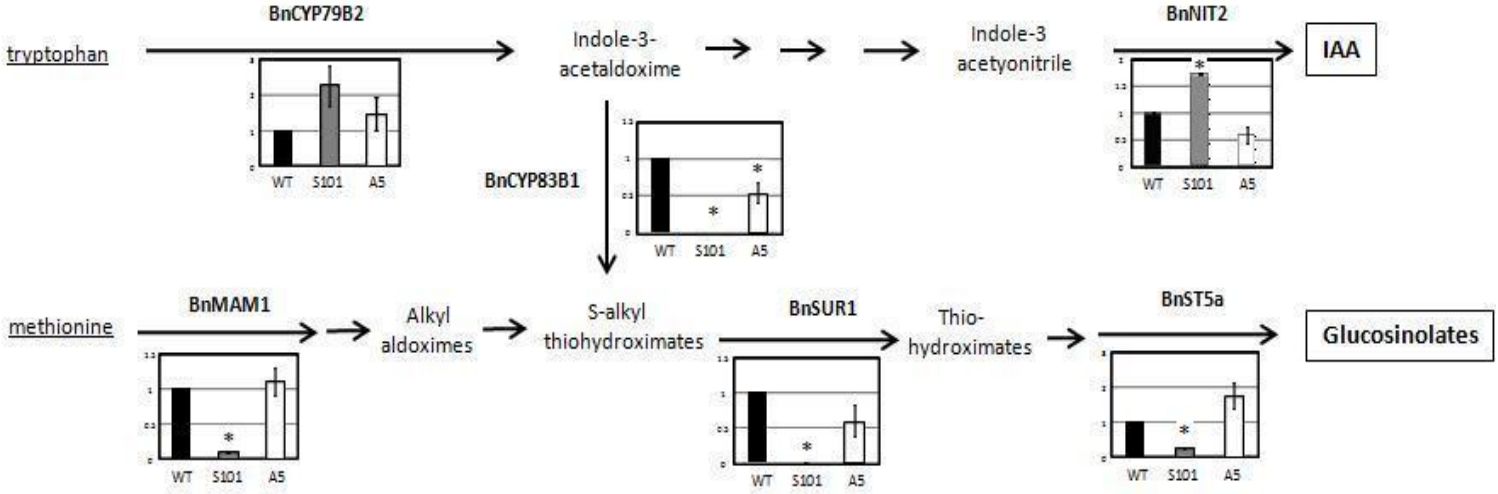
The *BnSTM* sense line S101 and antisense line A5 were selected to examine the expression level of several GLS biosynthetic genes. These included *METHYLTHIOALKYLMALATE SYNTHASE 1* (*MAM1*), involved in the condensation of deaminated methionine with acetyl-CoA; *CYTOCHROME P450 CYP79B2* (*CYP79B2*), responsible for the synthesis of indole-3-acetaldoxime from tryptophan; *CYTOCHROME P450 CYP83B1* (*CYP83B1*), producing S-alkyl thiohydroximates from indole-3-acetaldoxime, *SUPERROOT1* (*SUR1*) participating in the conversion of S-alkyl thiohydroximates to thiohydroximates; and *SULFOTRANSFERASE5a* (*ST5a*) using desulfoglucosinolates as a substrate in the last steps of GLS synthesis (Figure 3.10B). With the exception of *BnCYP79B2*, all the GLS biosynthetic genes were down-regulated in the S101 line. The reduced GLS levels in *BnSTM* over-expressing seeds were due to the diversion of indole-3-acetaldoxime (a precursor for GLS synthesis) into IAA production, the level of *NITRILASE2* (*NIT2*) involved in IAA production (Normanly et al., 1997) were measured and transcripts of this gene were elevated in the *BnSTM* over-expressing line (Figure 3.10B).

Additional analyses were conducted on upstream events of GLS synthesis by assessing the expression levels of two *MYB* transcription factors: *MYB-CONTAINING TRANSCRIPTION FACTOR28* (*MYB28*) and *ALTERED TRYPTOPHAN REGULATION1* (*ATR1*) which control GLS homeostasis by activating several GLS enzymes (Bender and Fink, 1998; Sonderby et al., 2010). Over-expression of *BnSTM* repressed both *MYB28* and *ATR1* (Figure 3.10C).

(A)


µmol g-1 DW
15
12
9
6
3
0
WT
S17
S38
S101
A1
A5

(B)


tryptophan
BnCYP79B2
Indole-3-acetaldoxime
BnCYP83B1
Indole-3 acetyonitrile
BnNIT2
IAA
methionine
BnMAM1
Alkyl aldoximes
S-alkyl thiohydroximates
BnSUR1
Thio-hydroximates
BnST5a
Glucosinolates
WT
S101
A5

(C)

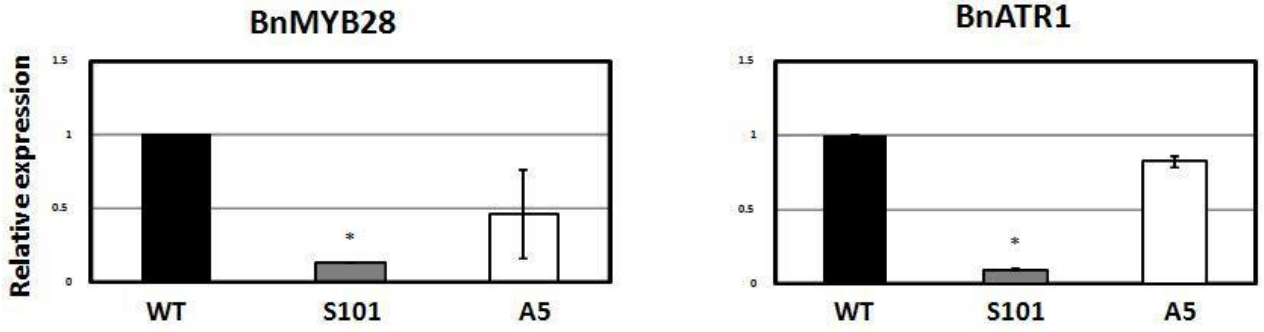

BnMYB28
BnATR1
Relative expression
1.5
1
0.5
0
WT
S101
A5

Figure 3.10. Analysis of glucosinolates (GLS) in seeds of *Brassica napus*. **(A)** Total GLS level in dry seeds of *Brassica napus* using near infrared reflectance spectroscopy. Values ± SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT. **(B)** Transcript level of genes involved in GLS biosynthesis measured at 21 DAP. *MAM1, METHYLTHIOALKYLMALATE SYNTHASE1; CYP79B2, CYTOCHROME P450 CYP79B2; CYP83B1, CYTOCHROME P450 CYP83B1; SUR1, SUPERROOT1*; *ST5a, SULFOTRANSFERASE5a; NIT2, NITRILASE2.* **(C)** Transcript levels of key transcription factors regulating GLS synthesis measured at 21 DAP. *MYB28, MYB-CONTAINING TRANSCRIPTION FACTOR28*; *ATR1, ALTERED TRYPTOPHAN REGULATION1*. Values ± SE are means of three biological replicates; * indicate statistically significant differences ($p<0.05$) from the WT value set at 1. S, *BnSTM* over-expressors; A, *BnSTM* down-regulators.

3.4. DISCUSSION

3.4.1. Over-expression of *BnSTM* affects sucrose metabolism and oil synthesis in developing seeds

Seeds of many species accumulate lipids mainly in the form of triacylglycerols (TAG), esters of glycerol and FA, as the major source of energy required for germination and post-embryonic growth. In plants, FA synthesis takes place in transcriptionally regulated steps (Millar et al., 2000) and requires the coordinated interaction of many genes, some of which have been characterized (Hobbs et al., 2004). Due to the economical relevance of seed oil for its use as nutritional feedstock and industrial applications, increasing interest has emerged to elevate the level and quality of seed oil through modifications using genetic engineering techniques. Previous attempts to enhance seed oil quality have mainly concentrated on manipulations of transcription factors involved in sugar and oil metabolism (Mu et al., 2008; Shen et al., 2010; Pouvreau et al., 2011; Tan et al., 2011), enzymes directly involved in FA synthesis (reviewed by Thelen and Ohlrogge, 2002; reviewed by Hills, 2004) or glycolysis

(Vigeolas et al., 2007), assembly of TAG (Zuo et al., 1997; Zheng et al., 2008), and in one instance a protein non integral to these processes, hemoglobin-2 (Vigeolas et al., 2011). The current work reveals that STM, a well-studied component of the embryonic developmental pathway never before reported to be in the accumulation of storage products, can be used as a target to alter seed oil production. When ectopically expressed in Brassica, this gene elevates seed lipid content (Figure 3.3), possibly by increasing the carbon flux available for the FA biosynthetic pathway and the expression of transcription factors and enzymes regulating glycolysis and FA synthesis (Figure 3.4-6 and 8). These changes were accompanied by a desirable reduction in GLS levels (Figure 3.10), without having detrimental effects on protein level (Figure 3.3) and oil nutritional value (Table 3.2). Furthermore when expressed at moderate levels, *BnSTM* has the potential to increase the number of siliques per plant (Figure 3.2). The phenotypic abnormalities observed in line S101, i.e. pronounced malformations in leaf morphology, stunted growth, and reduced number of siliques, correlate with the high expression levels of the transgene (Figure 3.1), which might not be tolerable for normal growth.

The function of *STM* during plant development is to regulate SAM formation during embryogenesis and its maintenance during post-embryonic growth by orchestrating a precise gene-expression regime which elevates the biosynthesis of cytokinin (reviewed by Veit, 2009). High levels of this hormone are needed to maintain shoot meristem homeostasis (Yanai et al., 2005). Genetic evidence further showed that both *IPT7*, which encodes an early enzyme of the cytokinin biosynthetic pathway, and the *ARABIDOPSIS RESPONSE REGULATOR5* (*ARR5*) a component of cytokinin signaling, are responsive to inducible *STM* activity (Jasinski et al., 2005). The elevated

levels of *BnIPT7* measured in Brassica seeds over-expressing *BnSTM* (Figure 3.11) agree with these studies and suggest a potential role of cytokinin in the observed phenotypes.

Cytokinin has been shown to control several parameters regulating source/sink strength within the tissue, and its accumulation establishes local metabolic sinks causing the mobilization of nutrients, including sucrose (Kuiper, 1993). Sink tissue of cytokinin-deficient plants shows a drastic reduction in soluble sugar (Werner et al., 2008), whereas increased carbon flow and storage deposition products occur in cytokinin treated tissues (Mostafa et al., 2005; Ayad and El-Din, 2011). Furthermore, local sink strength enhancement was also observed in specific regions characterized by high expression of the cytokinin biosynthetic enzyme *IPT7* (Guivarc'h et al., 2002). Therefore, if these observations hold true in Brassica, it can be suggested that elevated *BnSTM* expression reinforces seed sink strength through an increase in cytokinin synthesis resulting in the induction of genes regulating sucrose transport (*SUC1, 4*), cleavage (*SUS1, 3*), and metabolism (*AGP*) (Figure 3.6). The increased expression of these four genes in WT Brassica seeds treated with cytokinin (Figure 3.12) reinforces this notion. The *BnSTM*-induction of *AGP* (Figure 3.6), converting Glc1P to ADPGlc is a good indicator of enhanced storage product synthesis. High levels of *AGP* encourage the accumulation of starch and lipids in developing seeds, while low *AGP* expression inhibits both processes (Periappuram et al., 2000; Vigeolas et al., 2004).

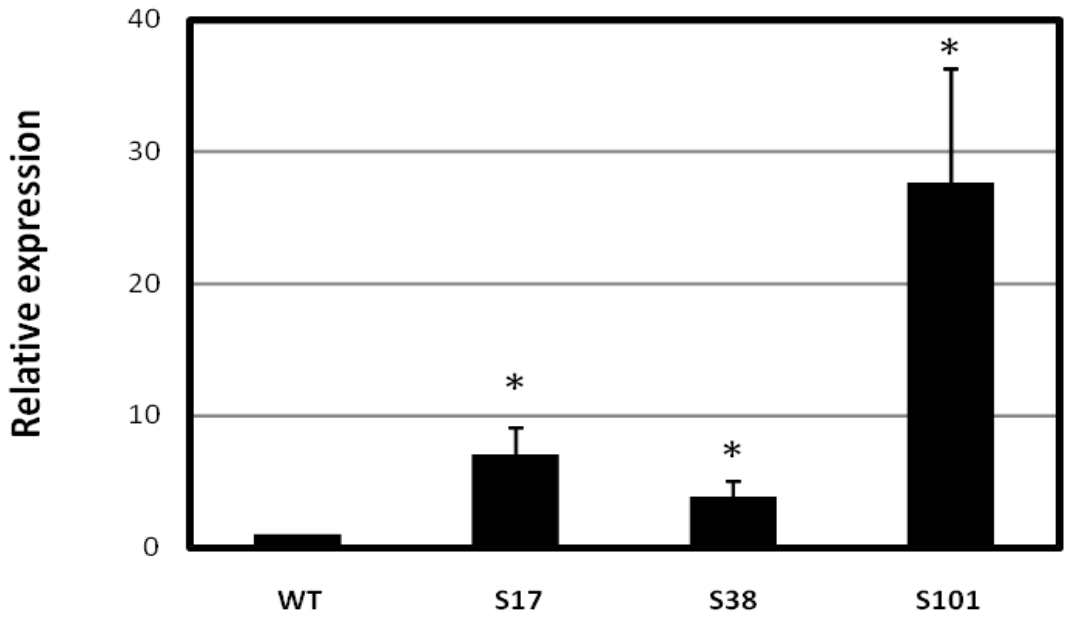


Figure 3.11. Relative expression level of *Brassica napus ISOPENTENYL TRANSFERASE 7* (*BnIPT7*) in Brassica seeds over-expressing *BnSTM* at 21 DAP. Values ± SE are means of three biological replicates. * indicates statistically significant differences ($p<0.05$) from the WT value set at 1.

Taken together these results are indicative of an elevated local sink strength and carbon mobilization in *BnSTM* over-expressing seeds, facilitated by a high energy status (ATP/ADP ratio, Figure 3.7), which is a limiting step for the import of sucrose (van Dongen et al., 2004). It must be noted, however, that the proposed increase in carbon flux in *BnSTM* over-expressing seeds does not elevate sucrose level (Figure 3.7). This might be the result of a rapid oxidation rate of sucrose through the glycolytic pathway, which is transcriptionally activated by the transgene (Figure 3.8). While it is unclear whether cytokinin also controls the glycolytic process, Suzuki et al. (1994) observed an induction of *Zea mays* phosphoenol-pyruvate carboxylase gene in response to zeatin. A fast oxidation of sucorse through the glycolytic pathway in seeds of lines with elevated *BnSTM* levels is required for enlarging the pool of FA biosynthetic precursors. This agrees with previous studies documenting a positive correlation between glycolytic activity and FA synthesis (Tan et al., 2011).

Synthesis of FA is physiologically related to seed development as the two processes share a common set of master regulators which include *LEC1*, *LEC2*, and *FUS3* (Gazzarrini et al., 2004; Mu et al., 2008; Stone et al., 2008). Both *LEC1* and *2*, HAP3 transcription factors which positively regulate oil synthesis (Mu et al., 2008; Stone et al., 2008; Tan et al., 2011), are induced by *BnSTM* (Figure 3.4), possibly through their responsiveness to cytokinin. Analysis of publically available microarray data (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) shows an up-regulation of the two genes following zeatin treatments, and our cytokinin experiments agree with this observation (Figure 3.12). Over-expression of *LEC1* in Arabidopsis encouraged the accumulation of FA through a global induction of many FA biosynthetic and

carbohydrate metabolic genes (Mu et al., 2008). By using a seed-specific expression system, Tan et al. (2011) replicated these results in *Brassica napus* and showed that elevated levels of *BnLEC1*increased the expression of *BnFAD3* and two genes involved in condensation reactions, *BnMCAT* and *BnACCA2*, which are an early control point of the FA pathway (Slabas and Fawcett, 1992; Baud et al., 2003). These three genes are also induced by *BnSTM*.

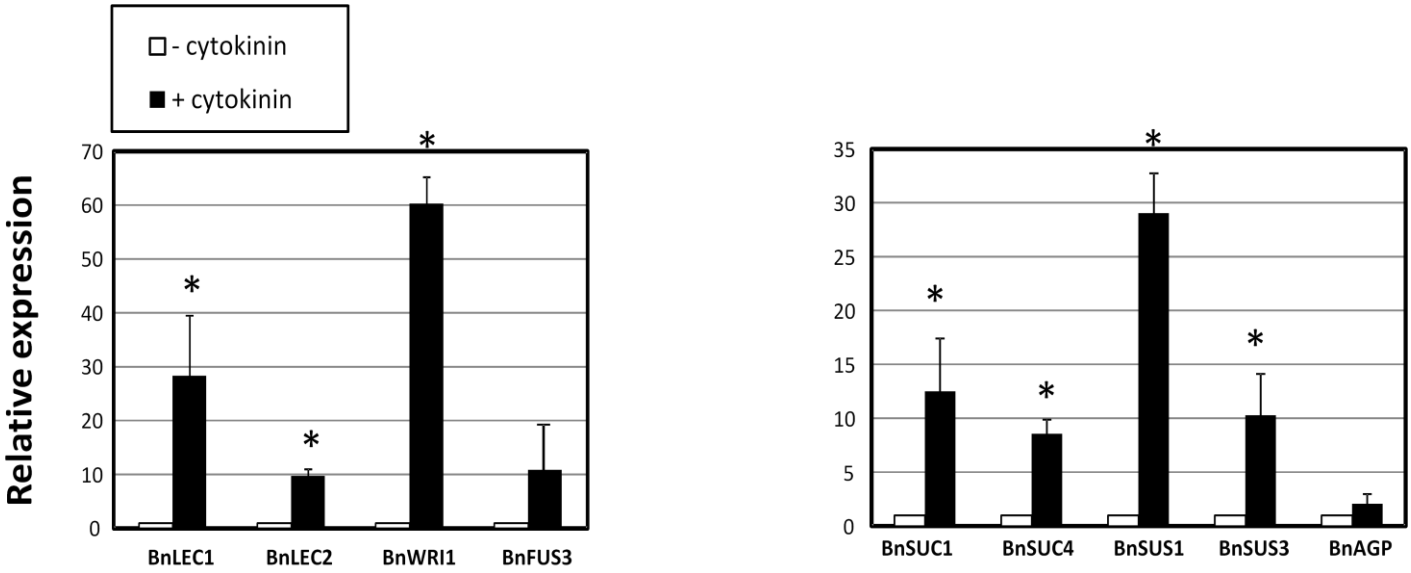


Figure 3.12. Effect of cytokinin applications on expression of genes encoding TFs related to oil synthesis (see Figure 3.4) and components of sucrose transport and metabolism (see Figure 3.6). Quantitative qRT-PCR analyses were conducted on WT seeds 21 DAP. Values $\pm$ SE are means of three biological replicates * indicates statistically significant differences ($p<0.05$) from the control (- cytokinin) value set at 1.

Another key regulator of oil synthesis is *BnLEC2* which is highly induced in *BnSTM* over-expressing seeds at 21 DAP (Figure 3.4). As for LEC1, the Arabidopsis LEC2 is also able to stimulate the accumulation of lipids in both vegetative and reproductive tissues, through the activation of oleosins and sucrose synthase genes (Santos-Mendoza et al., 2005; Stone et al., 2008). A target of LEC2 is *WRI1* (Baud et al., 2007), a transcription factor encoding an APETALA2/ethylene-responsive element binding protein (Liu et al., 2009), which is induced in seeds of *BnSTM* over-expressing lines at 28 DAP. *WRI1* is important for providing a carbon source for the FA biosynthetic pathway through the activation of glycolytic enzymes (Baud et al., 2007). Increased seed oil content as a result of *WRI1* over-expression was documented in a variety of species, including Arabidopsis (Liu et al., 2009), and maize (Shen et al., 2010; Pouvreau et al., 2011).

Ultrastructural analyses reveal a reduced size of oilbodies in two cellular compartments of seeds over-expressing *BnSTM* (Figure 3.9). This observation is highly consistent with previous work documenting a negative correlation between oil content and oilbody size (Hu et al., 2009). The small oilbody phenotype we observed in seeds with elevated levels of *BnSTM* can be ascribed to the increased expression levels of two *OLEOSIN*s, which have been implicated in maintaining the oilbodies as small units by preventing lipid coalescence during seed maturation (Ross et al., 1993).

Collectively, all these findings suggest that the over-expression of *BnSTM* enhances oil synthesis by increasing carbon flux in the developing seeds and activating regulatory components of the glycolytic and FA biosynthetic pathways. A simplified diagram summarizing these findings is shown in Figure 3.13.

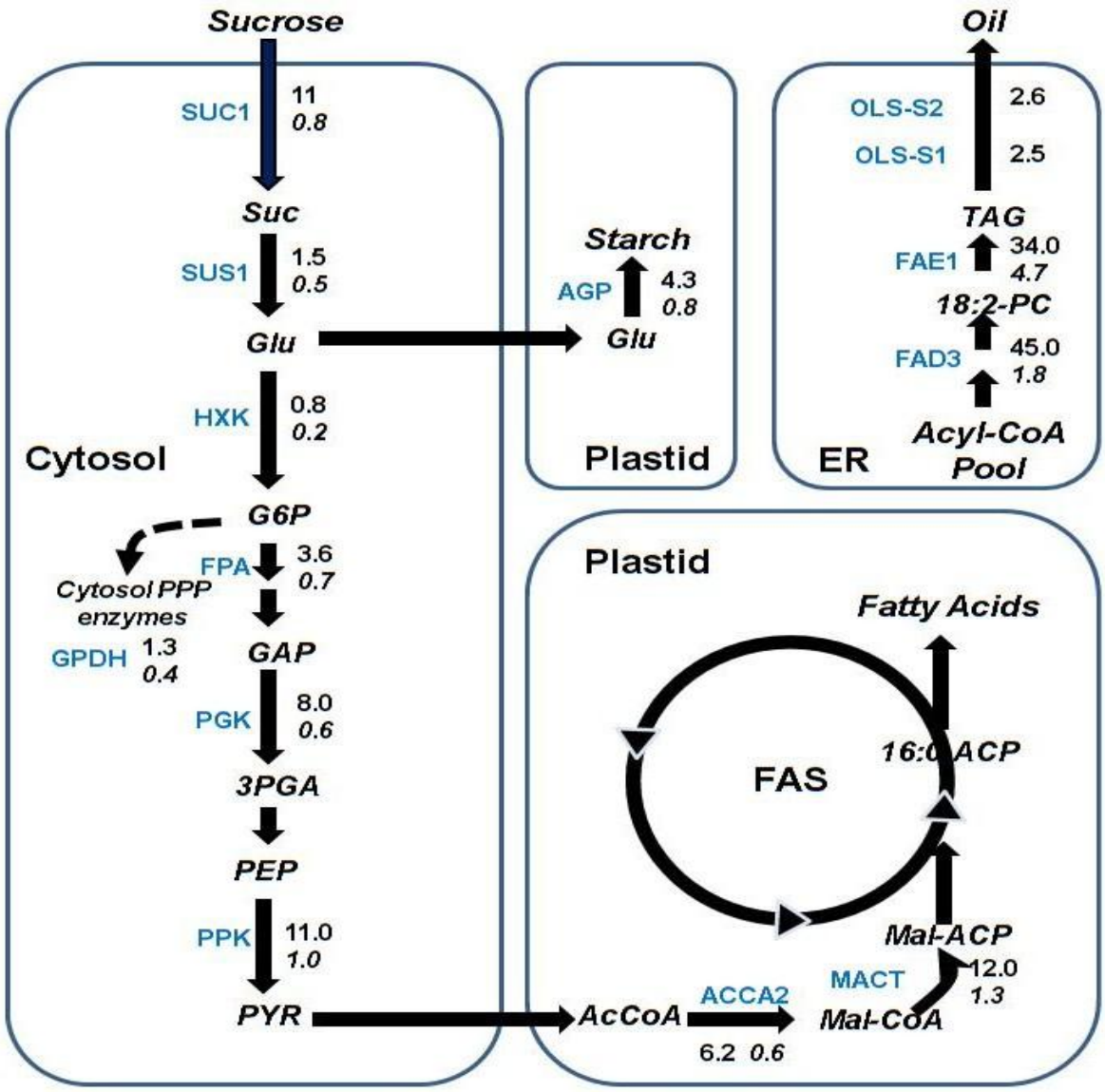


Figure 3.13. Simplified diagram showing the effects of *BnSTM* on regulatory pathways leading to FA synthesis (FAS) and oilbody formation. Numbers for each enzyme indicate fold change differences, relative to WT value set at 1, of seeds collected at 21 DAP from the *BnSTM* over-expressing line S101 (normal) or the *BnSTM* down-regulating line A5 (italic). Expression levels were derived from Figure 3.5, 6, 8 and 9. See Figure legends 3.5, 6, 8 and 9 for descriptions of enzyme abbreviations. ER, endoplasmic reticulum.

3.4.2. Over-expression of *BnSTM* reduces seed glucosinolate (GLS) levels

Glucosinolates (GLS) are sulfur-containing secondary metabolites with a prominent role in resistance to insects and pathogens (Sonderby et al., 2010) and innate immune response (Bednarek et al., 2009). Depending on their amino acid precursors, GLS can be divided into three distinct groups: indole, aliphatic, and benzenic. Many key elements of the GLS biosynthetic pathway have been identified (Feng et al., 2011). Commonly found in Brassicacea, GLS tend to accumulate in developing seeds with negative attributes for seed meal quality due to their anti-nutritional and goitrogenic properties (reviewed by Fenwick and Curtis, 1980), making reduction in seed GLS levels highly desirable. Ectopic-expression of *BnSTM* represses the accumulation of GLS and alters their relative composition (Figure 3.10 and Appendix 1). The transcript level of an early key enzyme involved in the chain elongation cycle of aliphatic GLS biosynthesis, *MAM1*, is down-regulated in seeds of lines ectopically expressing *BnSTM*. The enzyme encoded by *MAM1* is a methylthioalkylmalate synthase, which controls the side chain elongation from the precursor methionine to several side-chain aliphatic GLS. Silencing of *MAM1* causes a reduction of C4 and C5 side-chain aliphatic GLS (Liu et al., 2011). Other key downstream genes repressed by *BnSTM* are *SUR1*, and *ST5a*. The former gene encodes a C-S lyase protein, which if mutated represses GLS levels and confers a characteristics "superroot" phenotype due to IAA overproduction (reviewed by Nafisi et al., 2006), whereas ST5a is responsible for the 3'phosphoadenosine 5'-phosphosulfate-dependent sulfation of desulfoglucosinolates (Piotrowski et al., 2004).

Through repression of several biosynthetic genes, *BnSTM* might reduce GLS by diverting indole-3-acetaldoxime into IAA. Indole-3-acetaldoxime is a common substrate

for GLS and IAA (Figure 3.10B) and it is therefore an important branching point between primary and secondary metabolism in plants (reviewed by Grubb and Abel, 2006). Flux of this intermediate for GLS or IAA biosynthesis is modulated by *CYP83B1*, which converts indole-3-acetaldoxime to S-alkyl thiohydroximates. The low expression of this gene (below detectable levels) in seeds over-expressing *BnSTM* might block post-aldoxime reactions and divert indole-3-acetaldoxime into IAA synthesis. This notion, supported by the induction of the IAA biosynthetic gene *NIT2* in seeds with elevated levels of *BnSTM* (Figure 3.10B), agrees with genetic studies documenting a reduction of GLS level and an increase of free IAA in *cyp83b1* mutant plants (Bak and Feyereisen, 2001).

3.5. CONCLUSIONS

Taken together, our data provide evidence for a novel function of *BnSTM* in promoting seed oil production and desirable alterations of FAs and GLS levels when ectopically expressed in *Brassica napus* plants. While changes in oil level are most likely attributable to the *BnSTM*-reinforcement of sink strength (possibly mediated by cytokinin) when expressed in novel domains, the reduced GLS content correlates to a differential utilization of common precursors for GLS and IAA synthesis. Along with these conclusions, our findings demonstrate that *BnSTM* can be used as a target for genetic improvement of oilseed species. Future studies would be required to (1) further increase the oil content by employing a seed-specific expression system in order to maximize the expression of *BnSTM* in sink tissues without negative repercussions on

overall plant growth and (2) lower GLS content in those oil species which tend to accumulate high levels of both aliphatic and indole GLS in their seeds.

CHAPTER 2: THE EFFECTS OF MERISTEM GENES *SHOOT MERISTEMLESS* (*STM*), *ZWILLE* (*ZLL*) AND *CLAVATA1* (*CLV1*) ON *IN VITRO* EMBRYOGENESIS IN *BRASSICA NAPUS*

4.0. ABSTRACT

In vitro gametophytic embryogenesis is a process whereby immature microspores are directed to develop into microspore-derived embryos (MDEs). Microspore-derived embryo production in *Brassica napus* (*Bn*) is highly affected by the expression level of *BnSHOOT MERISTEMLESS* (*BnSTM*). Ectopic expression of *BnSTM* results in a significant increase in MDE number and quality, while the lines down-regulating *BnSTM* displayed the opposite effects. The STM-regulation of the shoot apical meristem (SAM) during *in vivo* development is mediated by cytokinin metabolism. The ectopic expression of *BnSTM* altered the gene expression in cytokinin synthesis, catabolism and perception. Cytokinin signaling was also affected by the altered expression of *BnSTM*. Several genes encoding Type-A regulators, including *BnRR5* and *BnRR7* were induced in *BnSTM* down-regulating MDEs. Overall these results show that the effect of altered *BnSTM* expression on embryo number and quality might be due to profound changes in cytokinin synthesis, catabolism, perception and signaling.

4.1. INTRODUCTION

In higher plants the sporophytic generation is initiated by the fusion of the sperm cell with the egg, resulting in the formation of a zygote (reviewed by Willemsen and Scheres, 2004). The subsequent events, referred to as embryogenesis, comprise defined morphological and cellular changes undergone by the zygote which lead to the generation of a fully mature embryo characterized by shoot and root poles, an embryonic axis, and cotyledons (reviewed by West and Harada, 1993). A simplified view of the embryogenic process comprises two distinct phases: a developmental phase in which the body plan is elaborated through precise cell division patterns, and a maturation phase delineated by the accumulation of storage products and the acquisition of desiccation tolerance. A key event during embryo development is the establishment of a fully functional shoot apical meristem (SAM) which is responsible for the reiterative formation of above-ground lateral organs during post-embryonic growth. In Arabidopsis formation and maintenance of the SAM are under the control of a complex genetic network involving several key players including *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1*, (*CLV1*), and *ZWILLE* (*ZLL*) (Clark et al., 1996; Moussian et al., 2003; Tucker and Laux, 2007; Dodsworth, 2009). *STM* is a homeobox gene encoding a member of the class-1 KNOX homeodomain-containing proteins (Long et al., 1996; Janosevic and Budimir, 2006). Members of this family are characterized by unique domains: the KNOX1 and KNOX2 domains, which are respectively involved in transcriptional repression of target genes and in dimer formation and transactivation (Sakamoto et al., 2001), the ELK domain which encodes a nuclear localization signal, and the HOMEODOMAIN required for DNA binding (Scofield and Murray, 2006). The expression of *STM* during embryogenesis,

which is initially detected in a few apical cells of the globular embryos, marks the meristematic cells of the SAM located between the cotyledons (Sharma and Fletcher, 2002). The requirement of this gene for the formation of the embryonic SAM and its subsequent post-embryonic maintenance was demonstrated by genetic studies in which the expression of *STM* was experimentally perturbed (Barton and Poethig, 1993; Endrizzi et al., 1996). While severe *stm* loss-of-function mutants resulted in the inability to initiate the embryonic meristem as true leaves, mild *stm* mutants formed an embryonic SAM, but failed to maintain it post-embryonically due to the incorporation of meristematic cells into organ primordia (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). These phenotypic aberrations were also reported in null alleles of *STM* orthologs from other species (Vollbrecht et al., 2000), thereby suggesting a conserved role of this gene. The increased cell proliferation and the generation of ectopic shoots observed in *STM* over-expressors (Brand et al., 2001; Gallois et al., 2002; Lenhard et al., 2002) are indicative that this gene regulates SAM homeostasis by suppressing differentiation and maintaining an undetermined cell fate within the apical pole. These functions are exercised in concert with other SAM-regulators, including CLAVATA1, a leucine-rich repeat receptor kinase protein (Clark et al., 1997), which promotes cell differentiation of the meristematic cells through an elaborate signaling model involving other CLV members (Dodsworth, 2009). A mutation in *CLV1* disrupts the balance between cell division and differentiation within the SAM resulting in enlarged meristems (Schoof et al., 2000). Therefore, in contrast to *STM* which maintains stem cells in an undifferentiated state by preventing meristematic cells from adopting an organ-specific cell fate, the role of CLV1 is to limit the expansion of the undifferentiated stem cell

population in the SAM, and promote cell differentiation. Another key player of SAM formation is *ZLL*, a member of the *ARGONAUTE* family, which like *STM* is required for the formation of the primary embryonic SAM, but, unlike *STM*, is not needed for post-embryonic meristem function (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999). A proper balance between *STM*, *CLV1* and *ZLL* ensures normal formation and maintenance of the SAM (Clark et al., 1996; Moussian et al., 1998; Dodsworth, 2009).

Embryogenesis is not an *in vivo* prerogative as it can be induced *in vitro* via somatic or gametophytic embryogenesis. Both systems are widely employed due to their effectiveness in providing a large number of embryos, thus representing an attractive model to examine the physiological and molecular events underpinning the vegetative-embryogenic transition. While somatic embryogenesis involves the formation of embryos from somatic cells, gametophytic embryogenesis generates haploid embryos from cells of male or female gametophytes. This latter system is more desirable to capture genetic variations through the recovery of diploid homozygous embryos (Yao et al., 1997). Overall the most effective system of gametophytic embryogenesis is androgenesis which uses microspores as the starting material to produce microspore-derived embryos (MDEs). Among the species amenable to androgenesis, *Brassica napus* is one of the preferred systems due to the large number of synchronized MDEs produced, and the ability to initiate the embryogenic pathway without hormone requirements and an intervening callus phase. Moreover Brassica shares many morphological and genetic similarities with Arabidopsis, which facilitate molecular analyses (Yeung et al., 1996; reviewed by Yeung, 2002). Studies on structural, physiological, biochemical, and

molecular events occurring during the induction of *Brassica napus* microspores and the subsequent phases of MDE development are available (Joosen et al., 2007; Malik et al., 2007; Stasolla et al., 2008).

Cytokinins are a class of hormones playing a central role in the plant life cycle, being involved in many developmental processes such as seed germination, cotyledon expansion, differentiation of vascular tissue, and SAM formation (reviewed by Schmülling, 2004). As indicated by many studies, the SAM is characterized by high levels of cytokinin required for the specification of stem cell identity (Kyozuka, 2007). While cytokinin biosynthetic inhibitors reduce the number of stem cells and the size of the meristem, application of cytokinin increases the size of the SAM and the number of undifferentiated stem cells (Higuchi et al., 2004; Werner et al., 2008). The high cytokinin environment within the SAM is ensured by the proper expression of *STM*, *CLV1*, and *ZLL*. Independent studies suggest that *STM* induces the expression of *ISOPENTENYL TRANSFERASE7* (*IPT7*), an important enzyme involved in cytokinin synthesis (Jasinski et al., 2005; Yanai et al., 2005). Increasing levels of endogenous cytokinin through exogenous applications or ectopic *IPT7* expression have been shown to rescue weak *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005). The *STM* regulation of cytokinin synthesis is further complicated by the fact that cytokinin is also needed for the expression of *KNOX* genes, which include *STM*. While cytokinin accumulation increases the expression of *KNOX* in Arabidopsis (Rupp et al., 1999), *LONELY GUY*, encoding a cytokinin related enzyme, is needed for the induction of *KNOX* transcripts in rice (Kurakawa et al., 2007). A positive feedback mechanism between *KNOX* genes and cytokinin is therefore apparent in the shoot meristem, whereby cytokinin induces *KNOX*

expression and *KNOX* genes promote cytokinin biosynthesis. Besides being required for SAM formation, cytokinins also play a key role during embryogenesis, especially during cell division and tissue differentiation (Kyozuka, 2007). Several tissue culture systems require exogenous applications of cytokinins.

In Arabidopsis, the components of cytokinin signaling have been identified and they include receptor ARABIDOPSIS HISTIDINE KINASEs (AHKs), ARABIDOPSIS HISTIDINE PHOSPHOTRANSMITTERs (AHPs) and ARABIDOPSIS RESPONSE REGULATORs (ARRs). Of the 30 *ARR* genes identified in Arabidopsis some (Type-A) are characterized by a receiver domain and a short C-terminal extension, while others (Type-B) have a longer C-terminal extension and domains acting as transcription regulators (D'Agostino et al., 2000; Hwang and Sheen, 2001). Genetic work demonstrated that Type B ARRs are transcription activators of cytokinin-induced genes, whereas Type-A ARRs are feed-back repressors of the cytokinin pathway (Hwang and Sheen, 2001). Several components of the cytokinin signaling are involved in the formation of the SAM during embryogenesis (Shani et al., 2006; Kyozuka, 2007). Cytokinins are catabolised by oxidative cleavage of the nitrogen-side chain (reviewed by Mok and Mok, 2001). Enzymes responsible for this reaction are the cytokinin oxidase/dehydrogenase enzymes (CKXs), with seven members present in Arabidopsis (Mok and Mok, 2001; Schmülling, 2004).

Over-expression of several Arabidopsis *CKX*s repressed shoot meristem activity (Werner et al., 2001; Werner et al., 2003), and increased root meristem size and activity (Werner et al., 2001). Bartrina et al. (2011) showed that *CKX3* is expressed in *WUS* expressing domains, i.e. meristematic cells of vegetative and floral meristems, and a

mutation of this gene causes an increase in cytokinin levels. A similar involvement of *CKX*s during meristematic activity was documented in orchid, where the induction of *CKX1* coincided with a repression of *KNAT1* and *STM* (Yang et al. 2003). These results clearly suggest the relevance of *CKX*s in cytokinin catabolism and control of the SAM.

The objective of this work is to evaluate the effects of altered expression of *BnSTM, BnCLV1,* as well as *BnZLL* during *Brassica napus* microspore-derived embryogenesis and to investigate how changes in embryo number and conversion frequency (the ability of the MDEs to produce viable shoot and root systems at germination) correlate to transcriptional changes of cytokinin signaling.

4.2. MATERIALS AND METHODS

4.2.1. Induction of *Brassica napus* microspore-derived embryos (MDEs)

Microspore-derived embryogenesis in *Brassica napus* cv Topas (DH4079) was induced as described by Belmonte et al. (2006). Brassica plants were grown in the greenhouse (described in section 3.2.1). Plants with flower buds were then transferred in a growth cabinet set at 12^{o}C day/ 7^{o}C night with a 12 h photoperiod. Flower buds (2-3mm) in length were harvested, sterilized in 10% bleach and ground in a mortar in half strength B5-13 medium (Appendix 2). The homogenate was centrifuged at 700 rpm (g) at 4^{o}C, and the microspore-containing pellet was re-suspended in NLN-13 medium (Appendix 3). The microspores were then further diluted in NLN-13 medium to a concentration of 10,000 microspores/ml, cultured at 32^{o}C incubator for 3 days (heat shock treatment), and subsequently incubated at 22^{o}C on a shaker set at 50 rpm. The

number of MDEs was counted after 22 days (21 day embryo), when the embryos were fully developed and characterized by fully expanded cotyledons (Belmonte et al., 2006).

4.2.2. Determination of microspore-derived embryo (MDE) quality

Embryo quality was estimated by the ability of the MDEs to convert and produce a viable shoot and root systems at germination. Fully-developed, late cotyledonary (24 day) embryos cultured from section 4.2.1 were germinated on ½ concentration Murashige & Skoog (MS) medium (Appendix 4). After 10 days germination, embryos with shoot and roots were counted.

4.2.3. RNA extraction, purification, and cDNA synthesis

The procedures for RNA extraction, purification, and DNA synthesis are described in the Material and Methods of Chapter 1.

4.2.4. Gene expression analysis by quantitative qRT-PCR of cytokinin genes

Analysis of gene expression in developing [5, 14, and 21 days] *Brassica napus* microspores was determined by quantitative qRT-PCR (Elhiti et al., 2010). Expression studies were conducted for genes involved in cytokinin biosynthesis and response. All Primers used are compiled in Appendix 6. The relative level of gene expression was

analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) using *BnACTIN* (AF111812) as a reference.

4.3. RESULTS

4.3.1. Ectopic expression of *BnSTM, BnCLV1,* and *BnZLL* on microspore-derived embryo (MDE) number and quality

Compared to WT, the number of late cotyledonary-stage (day 21) MDEs produced by lines over-expressing *BnSTM* increased, especially in line S101 (Figure 4.1). This was in contrast to lines down-regulating *BnSTM*, which exhibited the lowest number of MDEs. Altered expression of *BnCLV1*, and *BnZLL* did not have any significant effect on microspore-derived embryogenesis.

The frequency of conversion (ability to form viable shoots and roots at germination), was used as an estimation of embryo quality (Belmonte et al., 2006). Over-expression of *BnSTM* increased the conversion frequency of the MDEs, while a down-regulation of *BnSTM* repressed the ability of the embryos to convert into viable plants (Figure 4.2). Conversion frequency was not affected by the altered expression of *BnCLV1*, and *BnZLL*.

Among the meristem genes, only *BnSTM* was able to affect embryo number and quality (Figure 4.1 and 4.2). Based on this observation, transcription studies on cytokinin genes were only conducted on plants over-expressing or down-regulating *BnSTM*. Specifically, the over-expressing line S101 (which will be referred to as S line) and the down-regulating line A5 (which will be referred to A line) were used, as they exhibited the most pronounced differences from WT plants.

4.3.2. Altered expression of *BnSTM* affects the transcription of genes involved in cytokinin synthesis, perception, and catabolism.

Compared to WT, the expression level of *BnIPT7* (*ISOPENTENYL TRANSFERASE7*), an important enzyme involved in cytokinin synthesis (Jasinski et al., 2005; Yanai et al., 2005), increased in the S line over-expressing *BnSTM*. The increase was more pronounced at day 21, when the expression of *BnIPT7* in the S line was about 20 times higher than the WT. Repression of *BnSTM* in the A line resulted in a small increase in *BnIPT7* expression which was not statistically different from WT (Figure 4.3).

At day 14 in culture the expression level of the *Brassica napus* (*Bn*) gene homologous to *ARABIDOPSIS HISTIDINE KINASE* (*BnHK4*), encoding a well characterized cytokinin receptor (To et al., 2007), was higher in the S line, compared to WT (Figure 4.4). The expression of this gene slightly decreased in the A line at day 21.

Cytokinin catabolism is mediated by the *CYTOKININ OXIDASE/DEHYDROGENASE* genes (*CKX*s). A well characterized member of this family is *CKX3* which appears to modulate cytokinin level in the meristem (Bartrina et al., 2011). At both day 5 and 14 the expression level of the *Brassica napus* homolog *BnCKX3* was repressed in both S and A lines, whereas a sharp increase in *BnCKX3* transcript levels was observed at day 21 in culture in the A line (Figure 4.5).

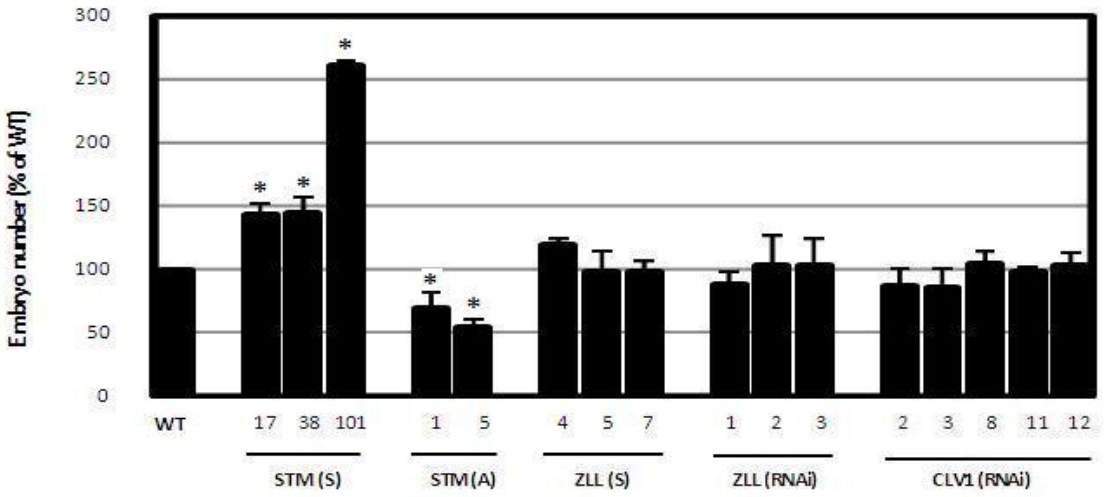


Figure 4.1. Production of microspore-derived embryos (MDEs) from *Brassica napus* plants with altered expression of *BnSTM*, *BnZLL* and *BnCLV1*. Over-expressing lines (S) and down-regulating lines (A, antisense or "RNAi" RNA interference) were utilized to generate MDEs. For each experiment, 100,000 microspores were plated and embryo number was counted at day 21. Values ±SE (*n*=3) are expressed as a percentage of the WT. * indicates values that are statistically different (P<0.05) from the WT value (set at 1) at the respective day in culture.

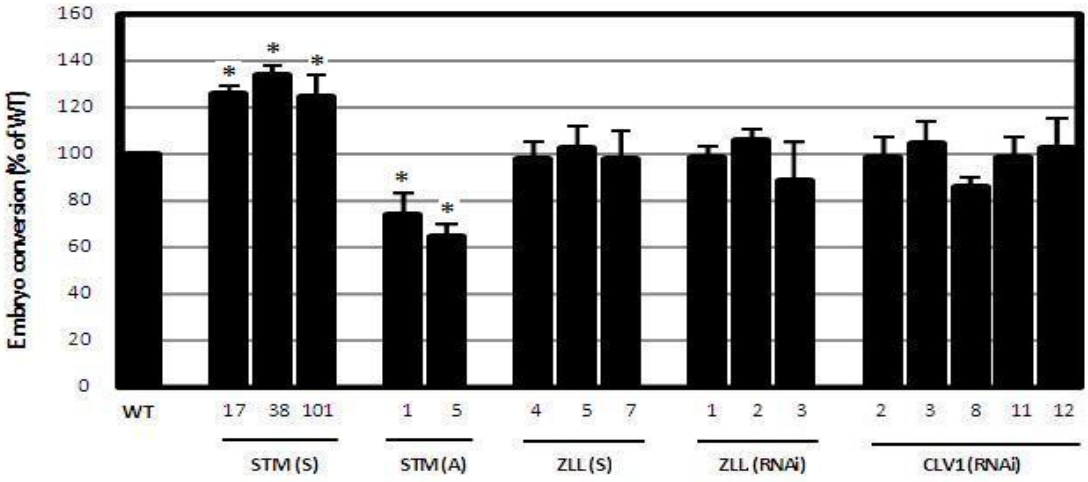


Figure 4.2. Frequency of conversion (ability to form viable shoots and roots at germination) of microspore-derived embryos (MDEs) with altered expression of *BnSTM*, *BnZLL* and *BnCLV1*. Over-expressing lines (S) and down-regulating lines (A, antisense or "RNAi" RNA interference) were utilized to generate MDEs. Values ±SE (*n*=3) are expressed as a percentage of the WT. * indicates values that are statistically different (P<0.05) from the WT value (set at 1) at the respective day in culture.

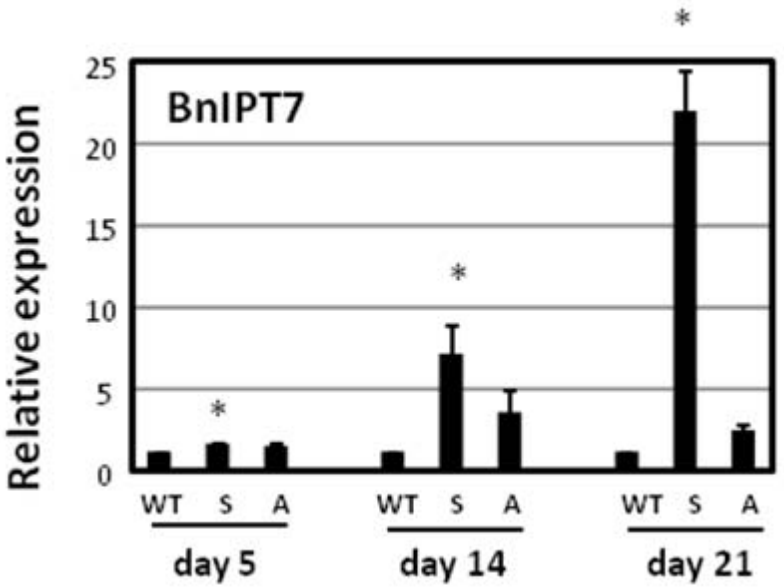


Figure 4.3. Expression of the Brassica cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE7* (*IPT7*) during microspore-derived embryogenesis in the WT line and lines over-expressing (S) or down-regulating (A) *BnSTM*. Values ± SE are means of three independent biological replicates. * indicates values that are statistically different ($P<0.05$) from the WT value (set at 1) at the respective day in culture.

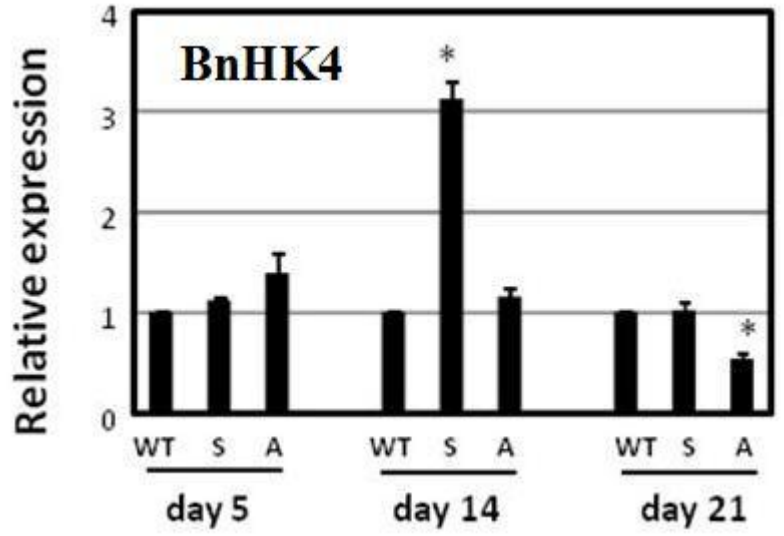


Figure 4.4. Expression of the cytokinin biosynthetic gene *Brassica napus HISTIDINE KINASE* (*BnHK4*) during microspore-derived embryogenesis in the WT line and lines over-expressing (S) or down-regulating (A) *BnSTM*. Values ± SE are means of three independent biological replicates. * indicates values that are statistically different ($P<0.05$) from the WT value (set at 1) at the respective day in culture.

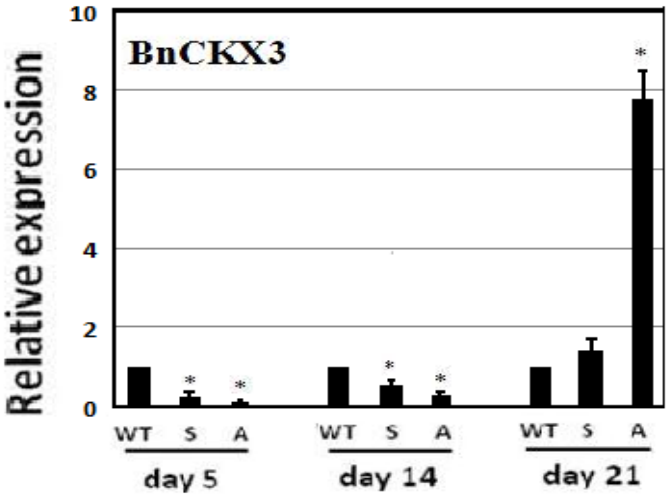


Figure 4.5. Expression of the *Brassica napus CYTOKININ OXIDASE* (*BnCKX3*) during microspore-derived embryogenesis in the WT line and lines over-expressing (S) or down-regulating (A) *BnSTM*. Values ± SE are means of three independent biological replicates. * indicates values that are statistically different ($P<0.05$) from the WT value (set at 1) at the respective day in culture.

4.3.3. Altered expression of *BnSTM* affects the transcription of genes involved in cytokinin signaling

Relay of the cytokinin signaling requires the activation of Type-B *Brassica napus RESPONSE REGULATOR*s (*BnRR*s), which induce transcription of a second group of regulators known as Type-A ARRs. Besides regulating downstream responses, Type-A ARRs act as feed-back repressors of the initial transduction pathway (Hwang and Sheen, 2001). Among the Type-A *BnRRs*, *BnRR4* was induced in early cotyledonary MDEs (day 14) of both S and A lines, whereas *BnRR5* and *7* were repressed at day 14 and 21 (Figure 4.6). The over-expression of *BnSTM* increased the transcript abundance of *BnRR15* and *16* at day 21 in culture. No major differences in expression were measured for Type-B *BnRR*s (Figure 4.7). In both transformed lines (S and A) *BnRR1*, *2*, and *10* were marginally repressed at day 21, while the transcript levels of *BnRR12* declined at day 14.

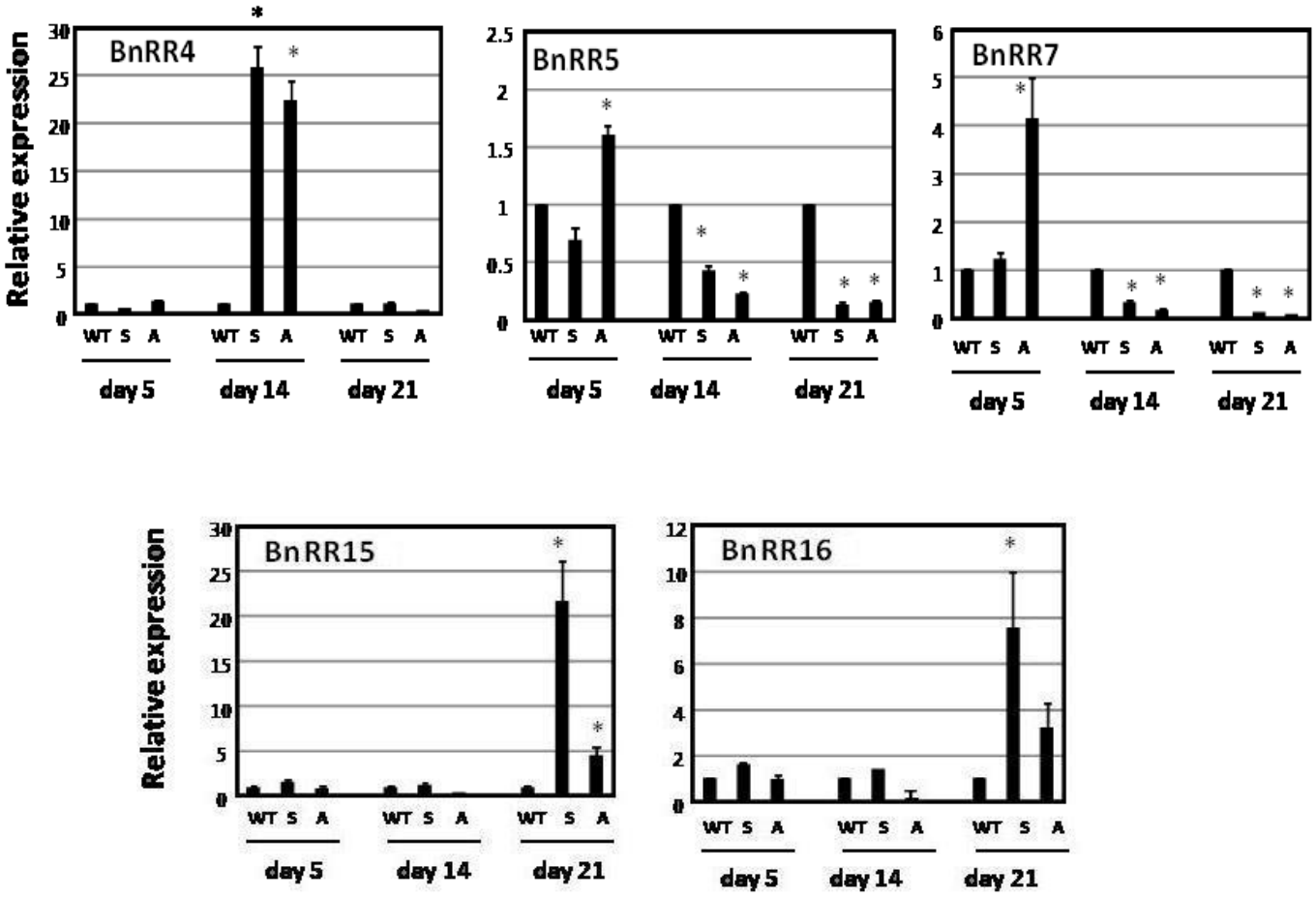


Figure 4.6. Expression of Type-A *Brassica napus RESPONSE REGULATORS* (*BnRRs*) during microspore-derived embryogenesis in the WT line and lines over-expressing (S) or down-regulating (A) *BnSTM*. Values ± SE are means of three independent biological replicates. * indicates values that are statistically different ($P<0.05$) from the WT value (set at 1) at the respective day in culture.

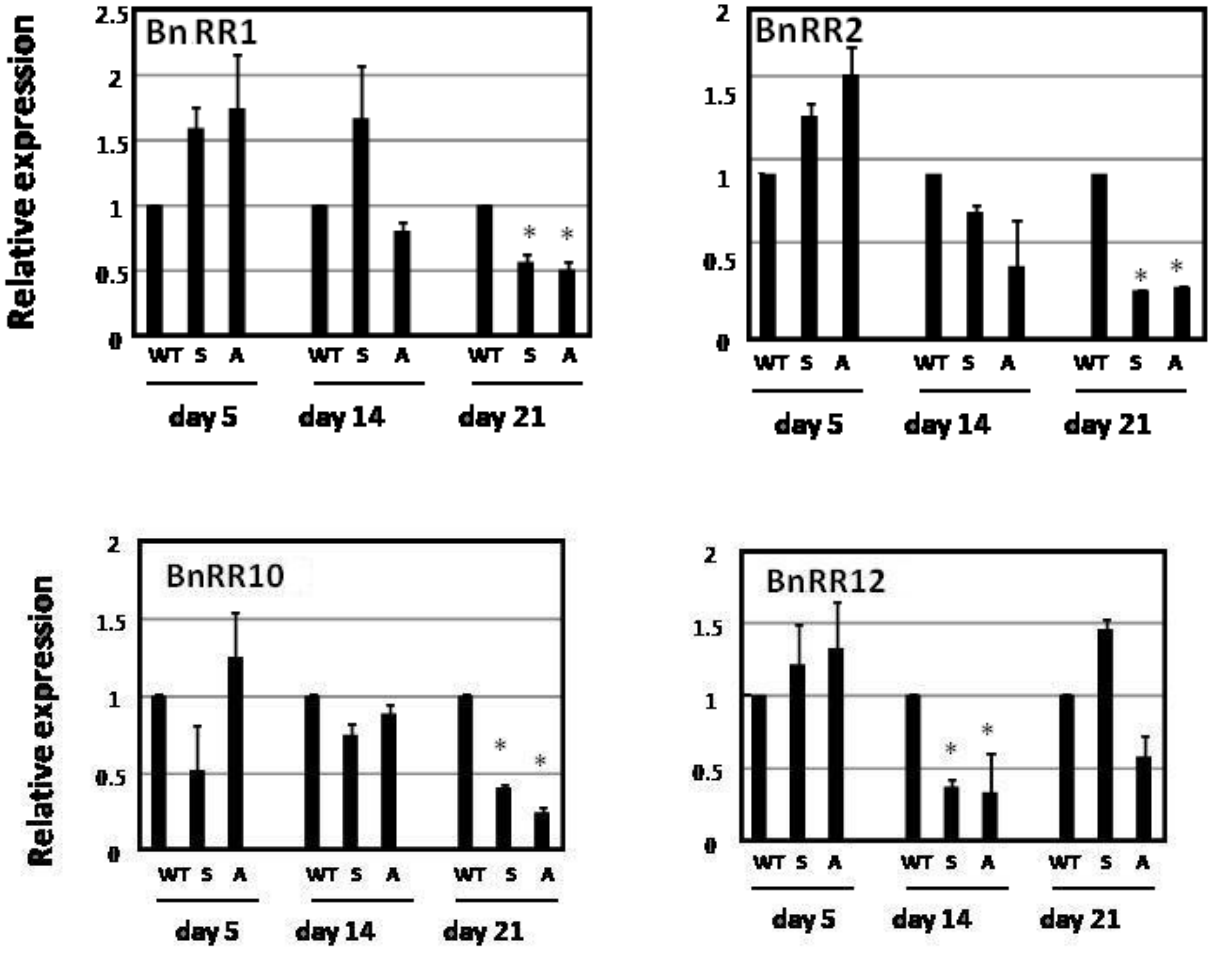


Figure 4.7. Expression of Type-B *Brassica napus RESPONSE REGULATORS* (*BnRRs*) during microspore-derived embryogenesis in the WT line and lines over-expressing (S) or down-regulating (A) *BnSTM*. Values ± SE are means of three independent biological replicates. * indicates values that are statistically different ($P<0.05$) from the WT value (set at 1) at the respective day in culture.

4.4. DISCUSSION

4.4.1. Ectopic expression of *BnSTM* on microspore-derived embryo (MDE) number and quality

In vitro embryogenesis represents a valuable model system for investigating the physiological and molecular events accompanying embryo development (Raghavan, 2000; Mordhorst et al., 2002). Through this system a large number of embryos can be obtained in a short period of time. Furthermore, embryos produced in culture are not embedded in the maternal tissue and can be harvested easily. These advantages, together with the observation that *in vitro* embryos have a similar morphology to seed embryos (Yeung et al., 1996), have been exploited to advance our knowledge on embryogenesis (Joosen et al., 2007; Malik et al., 2007; Stasolla et al., 2008).

Gametophytic embryogenesis in *Brassica napus* allows the formation embryos from immature microspores (microspore-derived embryos, MDEs). In this system, the initiation of embryogenic process requires a re-differentiation step in which the genetic program of the microspore is directed towards the formation of embryos (MDEs). Stress treatments, including elevated temperatures (Keller and Armstrong, 1979), treatments with ethanol (Pechan and Keller, 1989) and gamma irradiations (Pechan and Keller, 1989), and low temperature (Sunderland et al., 1974; Kasha et al., 1995) promote the re-differentiation step, which is manifested by changes in cytoskeletal components and a symmetric division of the microspore (Telmer et al., 1992). Fully developed MDEs are obtained at the end of the embryogenic process. Compared to seed embryos, MDEs show a reduced frequency of conversion (Belmonte et al., 2006; Stasolla et al., 2008), thus limiting the use of microspore-derived embryogenesis in breeding program (Stasolla et al.,

2008). While manipulations of the culture condition and medium composition have been successfully employed to enhance Brassica MDE conversion (Joosen et al., 2007; Malik et al., 2007; Stasolla et al., 2008), no attempts have been directed towards experimental alterations of gene expression using a transgenic approach.

A key event during embryogenesis is the formation of the SAM, a process regulated by the expression of genes including *STM*, *CLV1* and *ZLL* (Clark et al., 1996; Moussian et al., 1998; Moussian et al., 2003; Dodsworth, 2009). While some studies have investigated the role of meristem genes during embryogenesis, little information is available on how the altered expression of these three genes affects *in vitro* embryogenesis. Results from this chapter clearly show that changes in the level of *BnSTM* affect microspore-derived embryogenesis. While over-expression of *BnSTM* enhances the number and quality of MDEs, down-regulation of *BnSTM* reduces both number and conversion frequency (Figures 4.1 and 4.2). This is in contrast to *BnCLV1* and *BnZLL* which have no apparent effects on microscopore-derived embryogenesis. *SHOOT MERISTEMLESS* (*STM*) belongs to the class 1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) gene family, and encodes a homeodomain transcription regulator expressed in the undifferentiated stem cells of the SAM (Scofield and Murray, 2006). *STM* acts independently (Lenhard et al., 2002) or cooperates with other genes such as *WUS* (Clark et al., 1996; Endrizzi et al., 1996; Moussian et al., 2003; Gordon et al., 2009), and play an essential role in the formation of SAM (Lenhard et al., 2002) and maintenance of the undifferentiated stem cells (Dodsworth, 2009). While it is unclear how *BnSTM* regulates embryo number, the effect of this gene on embryo conversion might be due to its ability to alter the structure and quality of the SAM. In Arabidopsis over-expression of *STM*

favors SAM formation by increasing the number of undifferentiated cells within the meristem (Gallois et al., 2002; Lenhard et al., 2002; Dodsworth, 2009); this is in contrast to *stm* mutants plants in which meristem formation is precluded (Barton and Poethig, 1993). Based on these observations it is suggested that the increased conversion frequency in *BnSTM* over-expressing MDEs is the result of enhanced meristems which are easily able to reactivate at germination and produce viable organs and vigorous plants. In the same line, the low conversion frequency of MDEs with suppressed *BnSTM* expression is the result of poor meristems which fail to reactivate once the embryos are transferred on germination medium. This suggestion is consistent with previous studies proposing that conversion frequency during *in vitro* embryogenesis is directly related to the quality of the meristems (Kong and Yeung, 1992). Structural studies will reveal if the morphology of the SAMs is altered by the altered expression of *BnSTM*.

4.4.2. Ectopic expression of *BnSTM* affects cytokinin synthesis, catabolism, perception and signaling

The function of *STM* in the SAM is exercised through the creation of cytokinin maxima which help in maintaining the undifferentiated state of the meristematic cells. Cytokinin production in Arabidopsis is regulated by the activity of *ISOPENTENYL TRANSFERASE*s (*IPT*s) (Kyozuka, 2007) which catalyse the first and rate-limiting steps of cytokinin synthesis (Kyozuka, 2007), and the Arabidopsis STM induces the expression of *ISOPENTENYL TRANSFERASE7* (*IPT7*) leading to increasing levels of cytokinin (Jasinski et al., 2005; Yanai et al., 2005). Ectopic expression of *IPT7* can rescue weak *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005), thus confirming that the *STM* effect

is mediated by cytokinin. Cytokinin is also needed for the expression of *STM*. While cytokinin accumulation increases the expression of *STM* in Arabidopsis (Rupp et al., 1999), *LONELY GUY* (*LOG*), encoding a cytokinin related enzyme, is needed for the induction of *KNOX* transcripts in rice (Kurakawa et al., 2007). A positive feedback mechanism between *KNOX* genes including *STM*, and cytokinin is therefore apparent in the shoot meristem, whereby cytokinin induces *KNOX* expression and *KNOX* genes promote cytokinin biosynthesis. The observation that over-expression of *BnSTM* increases *BnIPT7* (Figure 4.3) is consistent with previous work and suggests that MDEs of the S line might have higher levels of cytokinin which would enhance the function of the SAM and their conversion at germination. High levels of endogenous cytokinin might also increase the number of MDEs produced, since this growth regulator is often exogenously added in protocols for *in vitro* embryogenesis (Zhao et al., 2002; Elhiti et al., 2010).

Cytokinin perception in Arabidopsis is regulated by three receptor kinases transducing cytokinin signals across the plasma membrane. They include *ARABIDOPSIS HISTIDINE KINASE2* (*AHK2*), *AHK3* and *AHK4* [also known as *CYTOKININ RESPONSE1* (*CRE1*) or *WOODEN LEG* (*WOL*)] (Yamada et al., 2001). In *Brassica napus* only the sequence of the homologous gene to *AHK4* (*BnHK4*) is currently available. The expression of *BnHK4* was up-regulated at day 14 in the S line over-expressing *BnSTM*, while it was down-regulated at day 21 in the A line (Figure 4.4). Work in Arabidopsis has shown that *AHK4* is up-regulated in a high cytokinin environment (Che et al., 2002) and contributes to cell division and differentiation. Both

processes are critical during microspore-derived embryogenesis, especially at day 14, corresponding to intense growth and development of the embryos (Belmonte et al., 2006).

Cytokinins are catabolised by the oxidative cleavage of the nitrogen-side chain, by cytokinin oxidases/dehydrogenases (CKXs) (Mok and Mok, 2001; Schmülling, 2004). Over-expression of *CKX*s results in many cytokinin-defective phenotypes, such as the deficiency in shoot growth and promotion in root growth (Werner et al., 2003). Yang et al. (2003) showed that over-expression of an orchid *CKX* (*DSCKX1*) repressed *KNAT1*, *STM* and *CycD3* in *Arabidopsis*, and suggested the negative feedback between *CKX* and *STM*. These observations are consistent with the hypothesis that *CKX*s play a negative role in meristem maintenance. The sharp increase of *BnCKX3* in the *BnSTM* down-regulating line at day 21 (Figure 4.5) is suggestive of an active cytokinin catabolic activity which might cause a depletion of cellular cytokinin in the SAM. Reduced cytokinin levels might compromise the function of the meristem and its ability to regenerate viable organs at germination, thus contributing to the low conversion frequency of the A line.

In Arabidopsis the perception of cytokinin is transduced by ARABIDOPSIS RESPONSE REGULATORs (ARRs), a group of DNA-binding transcription factors implicated in developmental events (D'Agostino et al., 2000; Hwang and Sheen, 2001; Müller and Sheen, 2008). Of the 30 *ARR*s genes identified in Arabidopsis some (Type-A) are characterized by a receiver domain and a short C-terminal extension, while others (Type-B) have a longer C-terminal extension and domains acting as transcription regulators (D'Agostino et al., 2000; Hwang and Sheen, 2001). Type-B ARRs are early transcription activators of cytokinin-induced genes, while Type-A ARRs regulate

downstream responses (Hwang and Sheen, 2001; Kakimoto, 2003). Evidence has revealed that over-expression of Type-B *ARR*s induces shoot formation *in vitro* without exogenous cytokinin (D'Agostino et al., 2000; Hwang and Sheen, 2001), while the up-regulation of Type-A *RR*s play negative roles in shoot formation (Hirose et al., 2007).

The similar regulation of Type-B *BnRR*s in MDEs produced by the S and A lines relative to WT (Figure 4.7), suggests that the early cytokinin response is affected equally by the over-expression or down-regulation of *BnSTM*. This is in contrast to the late cytokinin response which is modulated by Type-A BnRRs. The up-regulation of *BnRR5* and *7*, the best characterized Type-A regulators, in globular embryos of the A line (Figure 4.6) reflects a reduced sensitivity to cytokinin which might be the cause of the poor embryogenic capability of this line. The repressive impact of both ARR5 and 7 on various groups of cytokinin-regulated genes and responses has been documented (Hare and Staden, 1997; Lee et al., 2007). Notably, over-expression of *ARR7* reduces cytokinin sensitivity and affects basic developmental processes including cell proliferation and expansion (Lee et al., 2007). The induction of *BnRR15* and *16* in late cotyledonary embryos over-expressing *BnSTM* is difficult to interpret, but is supportive of dissimilar cytokinin responses in the transgenic lines. Taken together, these results indicate that differences in embryo number and quality in lines expressing different levels of *BnSTM* correlate to important changes in cytokinin synthesis, perception, catabolism and response.

4.5. CONCLUSIONS

Our data provide evidence that among the meristem genes tested, only the altered expression of *BnSTM* affects the number and quality of *Brassica napus* MDEs. These effects are associated to transcriptional changes of cytokinin synthesis, catabolism, perception and response. The over-expression of *BnSTM* up-regulates *BnIPT7*, encoding a rate-limiting enzyme in cytokinin synthesis, and *BnHK4*, encoding a cytokinin receptor. Transcriptional alterations in cytokinin response, mediated by *BnRR*s are also evoked by *BnSTM* and might contribute to the enhanced embryogenic competence induced by its over-expression. Overall these results suggest that *BnSTM* can be used as a target to improve embryogenic systems. Future studies would be required to analyze the effects of altered expression of meristem genes on other hormones such as auxin and ABA.

CHAPTER 3: SUMMARY AND CONCLUSIONS

Embryogenesis is an important event during the plant life cycle, initiated with the formation of the zygote. Through a coordinated pattern of cell division and differentiation, the embryo body is elaborated and at maturity it consists of an embryonic axis separating the shoot and root meristems, and one or more cotyledons (Park and Harada, 2008). Embryogenesis can also be induced *in vitro* via somatic embryogenesis, or gametophytic embryogenesis consisting in the formation of haploid embryos from immature pollen grains (microspores). An important event during both *in vivo* and *in vitro* embryogenesis is the formation of SAM at the apical pole of the embryos. The function of the SAM, which harbors stem cells, is to give rise to all above ground-organs upon germination. In Arabidopsis, the formation and maintenance of the SAM is regulated by an intricate genetic network involving several key players including *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1* (*CLV1*), and *ZWILLE* (*ZLL*) (Clark et al., 1996; Moussian et al., 2003; Tucker and Laux, 2007; Dodsworth, 2009). *STM* is a homeobox gene encoding a member of the class-1 KNOX homeodomain-containing proteins (Long et al., 1996; Scofield and Murray, 2006; Dodsworth, 2009). The function of this gene is to regulate SAM homeostasis by suppressing differentiation and maintaining an undetermined cell fate within the apical pole. This is demonstrated by genetic studies revealing the inability of severe *stm* loss-of-function mutants to form a functional embryonic SAM, while mild *stm* mutants form an embryonic SAM, but fail to maintain it post-embryonically due to the incorporation of meristematic cells into organ primordia (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996). The function of *STM* is also very conserved among species (Long et al., 1996; Yu et al., 2000; Chatterjee et al., 2011).

The function of *STM* is exercised in concert with other SAM-regulators, including CLAVATA1, a leucine-rich repeat receptor kinase protein (Clark et al., 1997), which promotes cell differentiation of the meristematic cells through a signalling model requiring other CLV members (Dodsworth, 2009). A mutation in *CLV1* disrupts the balance between cell division and differentiation within the SAM, which results in enlarged meristems (Schoof et al., 2000). Therefore, in contrast to *STM* which maintains stem cells in an undifferentiated state by preventing meristematic cells from adopting an organ-specific cell fate, the role of *CLV1* is to limit the expansion of the undifferentiated stem cell population in the SAM and promote differentiation. Another key player of SAM formation is *ZLL*, a member of the *ARGONAUTE* family, which like *STM* is required for the formation of the primary embryonic SAM, but, unlike *STM*, is not needed for post-embryonic meristem function (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999). A proper balance between *STM*, *CLV1* and *ZLL* ensures normal formation and maintenance of the SAM (Clark et al., 1996; Moussian et al., 1998; Dodsworth, 2009).

While the role of these genes in relation to SAM formation has been under scrutiny, no much information is available on their functions in other developmental processes. Towards this end, the objectives of this thesis are to analyze the effects of altered expression the *Brassica napus* (*Bn*) *STM*, *BnCLV1*, and *BnZLL* on seed storage deposition, mainly oil, and gametophytic embryogenesis. The commercial value of *Brassica napus* as on oil crop and its available protocols for *in vitro* embryogenesis makes this species a suitable model system for these studies.

The effects of the meristem genes on seed oil synthesis were reported in Chapter 1. While altered expression of *BnCLV1* and *BnZLL* did not alter oil level, over-expression of *BnSTM* increased seed oil level without affecting protein and sucrose level. These changes were accompanied by the induction of several transcription factors promoting fatty acid (FA) synthesis: *Brassica napus LEAFY COTYLEDON1* (*BnLEC1*), and *2* (*BnLEC2*), *FUSCA3* (*BnFUS3*) and *WRINKLED1* (*BnWRI1*). Key representative genes regulating sucrose metabolism, glycolysis, and fatty acid (FA) biosynthesis were induced in developing seeds ectopically expressing *BnSTM*. These distinctive expression patterns support the view of an increased carbon flux to the FA biosynthetic pathway in developing transformed seeds. The over-expression of *BnSTM* also resulted in a desirable reduction of seed glucosinolate (GLS) levels ascribed to a transcriptional repression of key enzymes participating in the GLS biosynthetic pathway, and possibly to the differential utilization of common precursors for GLS and IAA synthesis. No changes in oil and GLS levels were observed in lines down-regulating *BnSTM*. Overall these results provide evidence for a novel function of *BnSTM* in promoting desirable changes in seed oil and GLS levels when over-expressed in *Brassica napus* plants, and demonstrate that this gene can be used as a target for genetic improvement of oilseed species. Specifically, this study shows that a possible function of *BnSTM* is to alter sink strength by increasing cytokinin synthesis in developing seeds, thus favoring the flow of carbon towards the synthesis of FAs.

The effects of *BnSTM*, *BnCLV1* and *BnZLL* on microspore-derived embryogenesis were analyzed in Chapter 2. While the over-expression of *BnSTM* increased the number and the quality of the microspore-derived embryos (MDEs) by enhancing their ability to

regenerate viable plants, down-regulation of *BnSTM* decreased the number of MDEs produced and their conversion frequency. No effects on embryogenesis were observed in lines with altered expression of *BnCLV1* or *BnZLL*. The *BnSTM*-induced changes in embryogenetic performance were correlated to transcriptional changes of genes involved in the biosynthesis, catabolism, perception and signalling of cytokinin. The ectopic expression of *BnSTM* increased the expression of the cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE7* (*BnIPT7*) at day 14 and 21 in culture and that of the cytokinin receptor *HISTIDINE KINASE4* (*BnHK4*) at day 14. This was in contrast to the line down-regulating *BnSTM* where the expression of (*BnHK4*) declined at day 21. An increase in the transcript levels of *Brassica napus CYTOKININ OXIDASE* (*BnCKX3*), a gene involved in cytokinin catabolism was observed at day 21 in the same line. Cytokinin signalling was also affected by the altered expression of *BnSTM*. Several genes encoding Type-A regulators, including *BnRR5* and *7*, were induced in *BnSTM* down-regulating MDEs. Overall these results show that the effect of altered *BnSTM* expression on embryo number and quality might be due to profound changes in cytokinin synthesis, catabolism, perception and signaling.

In conclusion, these studies clearly show that besides governing the formation and maintenance of the SAM, the over-expression of *BnSTM* in novel domains, or its down-regulation, affects fundamental events of plant development, such as seed oil accumulation and *in vitro* embryogenesis. Besides contributing to fundamental knowledge, these results have valuable practical applications as genetic manipulation of *BnSTM* might have the potential to enhance oil production in other desirable crop species and to encourage *in vitro* embryogenesis in those species not amenable to be propagated

in culture. In future, studies would be embarked on (1) further improvement of oil content in quantity and qualiy and (2) analysis of altered expression of meristem genes on the regulation of other hormones.

APPENDIXES

Appendix 1. Percentage composition of several glucosinolates (GLS) in dry seeds of *Brassica* lines over-expressing *BnSTM* (S). * Indicates statistically significant differences compared to WT values ($p<0.05$). A, aliphatic GLS; I, indole GLS.

Common name	Systematic name	Type	WT	S17	S38	S101
Progoitrin	2-Hydroxy 3-Butenyl	A	7.0±0.1	5.2±0.2*	3.9±0.5*	1.6±0.1*
Sinigrin	2-Propenyl	A	0.4±0.0	0.4±0.0	0.4±0.0	0.5±0.0
Glucoalyssin	5-Methylsulfinylpentyl	A	1.5±0.1	1.3±0.1	1.1±0.1	0.7±0.1
Gluconapin	3-Butenyl	A	3.5±0.1	2.9±0.1	2.1±0.2*	1.3±0.1*
Glucoerucin	4-Methylthiobutyl	A	0.4±0.1	0.4±0.0	0.5±0.1	0.7±0.1
Glucobrassinicanapin	4-Pentenyl	A	5.2±0.3	5.0±0.3	4.2±0.2	3.4±0.1*
Glucobrassicin	3-Indolylmethyl	I	2.9±0.2	2.2±0.1	1.7±0.0	1.0±0.1
Gluconasturtiin	Phenylethyl	I	3.0±0.1	2.5±0.0	2.3±0.1	2.4±0.1
4-Methoxyglucobrassicin	4-Methoxy-Indolyl-3- Methyl	I	0.4±0.0	0.4±0.0	0.4±0.0	0.2±0.0
Neoglucobrassicin	1-Methoxy-3-Indolylmethyl	I	1.3±0.0	1.1±0.1	1.3±0.1	1.8±0.1
4-Hydroxyglucobrassicin	4-Hydroxy- Indolyl-3-Methyl	I	69.3±0.5	74.3±0.4*	77.1±0.5*	81.2±1.0*

Appendix 2. Recipe of the ½ - concentration-strength B5-13 induction medium used for microspore extraction.

Vitamin Stock (1L)

Chemical	Amount in 1 L (mg/1L)
Thiamin·HCl	1000
Nicotinic Acid	100
Pyridoxine HCl	100

Micronutrient Stock (1 L)

Chemical	Amount in 1 L (mg/1L)
KI	75
H_3BO_3	300
$MnSO_4 \cdot 4H_2O$	1000
$ZnSO_4 \cdot 7H_2O$	200
$Na_2MoO_4 \cdot 2H_2O$	25
$CuSO_4 \cdot 5H_2O$	2.5
$CoCl_2 \cdot 6H_2O$	2.5

Half-strength B5-13 Medium

Chemical	Amount in 1L
KNO_3	1500 mg
$(NH_4)_2SO_4$	75 mg
$MgSO_4 \cdot 7H_2O$	250 mg
$CaCl_2 \cdot 2H_2O$	75 mg
$NaH_2PO_4 \cdot H_2O$	75 mg
Sequestrene	14 mg
Micronutrient Stock 100×	5 mL
Vitamin Stock 1000×	0.5 mL
m-Inositol	50 mg
Sucrose (Grade I)	130 g

Appendix 3. Recipe of the NLN-13 culture medium used for microspore-derived embryo development.

NLN-13 Medium (1L)

Stock No. (Strength)	Component	Ingredient	Amount	Total volume (ml)	Stored aliquots (ml)	Final conc. mg/l
1 (20×)	**Macro-elements**	KNO_3	2.5 g	1000	50	125
		$Ca(NO_3)_2{\cdot}4H_2O$	10 g			500
		$MgSO_4{\cdot}7H_2O$	2.5 g			125
		KH_2PO_4	2.5 g			125
2 (200×)	**Micro-elements**	$MnSO_4{\cdot}4H_2O$	1250 mg	250	5	25
		$ZnSO_4{\cdot}7H_2O$	500 mg			10
		H_3BO_3	500 mg			10
		$Na_2MoO_4{\cdot}2H_2O$	12.5 mg			0.25
		$CuSO_4{\cdot}5H_2O$	1.25 mg			0.025
		$CoCl_2{\cdot}6H_2O$	1.25 mg			0.025
3 (200×)	**Iron source**	NaFe(III)EDTA	2 g	250	5	40
4 (1000×)	**Organic components I**	Nicotinic acid	500 mg	100	1	5
		Thiamine HCl	50 mg			0.5
		Pyridoxine HCl	50 mg			0.5
		Folic acid	50 mg			0.5
		Biotin	5 mg			0.05
		Glycin	200 mg			2
5 (50×)	**Organic component II**	L-Serine	2.5 g	500	20	100
		L-Glutamine	20 g			800
		Glutathion	0.75 g			30
		Myo-inositiol	2.5 g			100
	Carbon	Sucrose (Grade I)				130,000

Appendix 4. Recipe of half-Murashige & Skoog (MS) medium used for germinating microspore-derived embryo.

Recipe of MS medium with vitamins (For 1 L of medium)

Chemical	Concentration (mg/L)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot H_2O$	8.6
KI	0.83
$NaMoO_4 \cdot 2\ H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
myo-Inositol	100.0
Nicotinic acid	0.5
Thiamine•HCl	0.1
Pyridoxine•HCl	0.5
Glycine	2.0
Sucrose	30000
Agar	8000

For 1 L of MS medium

Chemical	Amount (g)
Murashige & Skoog (MS) Medium + Vitamins (plantMedia)	2.2
Sucrose (Grade II)	20
Agar	6

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

GENOTYPING	
BnSTM-F	ATGGAAAGTGGTTCCAACAGCACTT
BnSTM-R	TCAAAGCATGGTCGAGGAGATGTGA
BnACTIN-F	TAAAGTATCCGATTGAGCATGGTAT
BnACTIN-R	CGTAGGCAAGCTTCTCTTTAATGTC
35S-F	TGGACCCCCACCCACGAG
qRT-PCR	
BnLEC1-F	TATCTTGCCGCAGCAACAACCAAG
BnLEC1-R	TTCACCGGTCACGAAGCTGATGTA
BnLEC2-F	ACAAGAATCGCTCGCACTTCTCCA
BnLEC2-R	AAGCATCCGATGAGTGAAGAGGCT
BnWRI1-F	ATAGAGTACACGAGGCGCAAACGCA
BnWRI1-R	TCACAGGGAATGGGAAGACACCTT
BnFUS3-F	TCCATCATCGTCCAGGGTTTGGAT
BnFUS3-R	AAGAAGATCGTCTCTGTCTGGGCA
BnACCA2-F	TTGCCAGTCATAGTCCAGAGGCAT
BnACCA2-R	TCTGCTGCGATGTCCATGAAGGA
BnFAD3-F	TATAAGGGCGGCCATTCCTAAGCA
BnFAD3-R	AGATAGCCCAGAACAGGGTTCCTT
BnFAE1-F	TCTCCGCGATGGTCGTTAACACTT
BnFAE1-R	TCCTTGGACAAACTCACTCCGGTT
BnMCAT-F	TCAAGCAGTAGGGATGGGCAAAGA
BnMCAT-R	ACGAACACGGAGCAATTCAACAGC
BnSUC1-F	ACCAATCGTCGGTTACCACAGTGA
BnSUC1-R	ATATCCGCGGCGTATCCGATCAAA
BrSUC4-F	ACAAGGATTCAGAAGCCCATCCCT
BrSUC4-R	ATCTCCTTCCACCATGCCTTCACA
BrSUS3-F	TTAAGCAGCTTCTCGGCAACCTCA
BrSUS3-R	CGAACAAGCCGGATGTCAACCTTT
BrSUS1-F	TCTCCGTGTGCCTTTCAGAACAGA
BrSUS1-R	AGAGGCAACGAGGTTTCCATCACT
BnAGP-F	TGCTGGAACGAGATTGTATCCGCT
BnAGP-R	ATGGCGGTTGAGAGAAGCTGAGTT
BnGPDH-F	ACGGAAAGTTGACCGGAATGTCCT
BnGPDH-R	ACAACATCGTCCTCGGTGTAACCA
BnFPA-F	TGAGTTGATCGCTAACGCCGCATA
BnFPA-R	TTCTCGACGTTGATGCTGGCAAGA
BnHXK-F	ATCAGCTGCAGGGATCTACGGAAT

BnHXK-R	ACTCGCTGAACTGAGTGAGTGCT
BnPGK-F	AGGCACAAGGTCTGTCTGTTGGAT
BnPGK-R	AGCGAACTTGTCAGCAACCACAAC
BnPPK-F	AGGTGCGCCGTTTAAGAAATTCGC
BnPPK-R	AGAATGAAAGGAAGGCCGGCTACT
BnCYP79B2-F	ACTTCACCGTCGGGTAAAGATGCT
BnCYP79B2-R	ACACGTGTTGAGCTGATGGAGTCT
BnMAM1-F	AGACCATTGCCAAGACTGTAGGGA
BnMAM1-R	AACCGCCTCGATGTCTCTATGGTT
BnSUR1-F	TGGCTTCACCAGAAGAGAGCTGAA
BnSUR1-R	ACAGTAATGCCTCGTGACAACCG
BnST5a-F	ATGGCTGCTCGTATCGATGGGTTA
BnST5a-R	CCTCTGTTCCGCACCAAACAACAA
BnATR1-F	TGGCCTTATGGAGGAGTTAGAGGA
BnATR1-R	ATCAGTTACGTCAACCTCAGGCGA
BnMYB28-F	AAGCAATACTCCCGGTCAAGCTCA
BnMYB28-R	ACTGGTGTCCCATCTTTGCTGGTA
BrIPT7-F	TTGACTCCGAAGCTGGCAACCTAA
BrIPT7-R	ATGAGTTTGATCCACCGGCTACGA
BnCYP83B1-F	AAAGATGGACGTCATGACCGGACT
BnCYP83B1-R	CATCACGCCTGATCAAATGTGCGT

Appendix 6. Primer sequence (5'-3') used for qRT-PCR studies.

BnACTIN-F	TAAAGTATCCGATTGAGCATGGTAT
BnACTIN-R	CGTAGGCAAGCTTCTCTTTAAGTC
BnIPT7-F	TTGACTCCGAAGCTGGCAACCTAA
BnIPT7-R	ATGAGTTTGATCCACCGGCTACGA
BnHK4-F	ATGCCGTTTATGCAAGCGGATAGC
BnHK4-R	CACGAAACTTATCTGACCGCGCAT
BnRR1-F	AACAGAGCGGAGATGGCATTGTCT
BnRR1-R	AAGACCGACGTGTTCAAGGAGCTT
BnRR2-F	TAAATCCGGGTCAGGGAAGAGGA
BnRR2-R	ATCAGACAAGTTGGGTCATCGTCG
BnRR4-F	TAATCCGGGACTCCTCATCTTCTG
BnRR4-R	TCGCCGCTACTTTCTCCATTAGAC
BnRR5-F	ACTCTATGCCTGGGATGACTGGAT
BnRR5-R	CGAGGCAAGATGTTCTCTGAAGAC
BnRR7-F	GTGGATCGTAAAGTCATCGAGAGG
BnRR7-R	GCTCCTGTGTTTCCATCTAAGCCA
BnRR10-F	CCCATTTCGAGATCAACAAGCGTC
BnRR10-R	GTTCCATTCCAAGGCGTCTGGTTT
BnRR12-F	ACCATGTTACAACGACGAACCAGG
BnRR12-R	ACCATCCATGTCAGGCATGTCAAC
BnRR15-F	TGTCGTCTGAGAACATACAACCTCG
BnRR15-R	GCCGCTTCACATCTGCTAATTTCACC
BnRR16-F	GGCCGTGGATGATAATCTTATTGACCG
BnRR16-R	CCAATGCTCTAATCGCATTCTCTGC
BrCKX3-F	TTTCTACGCGGCGTTAGGAGGTTT
BrCKX3-R	TTCCAAGAAATGTAAACCGCCCGC

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