Glutathione modulation of *in vitro* organized development in canola microspore derived and spruce somatic embryos

by

MARK FINDLAY BELMONTE

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MARK FINDLAY BELMONTE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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Abstract

Belmonte, Mark Findlay. PhD, The University of Manitoba, September, 2007. Glutathione modulation of *in vitro* organized development in canola microspore derived and spruce somatic embryos. Major Professor; Dr. Claudio Stasolla.

The production of good quality microspore derived embryos (MDEs) of *Brassica napus* cv. Topaz (canola) and somatic embryos of spruce including *Picea glauca* and *P. abies* (white spruce and Norway spruce, respectively) are of great importance for developmental and applied studies. One of the obstacles encountered during the culture process is the lack of organized development, especially within the shoot apical meristem (SAM). In the last few years, glutathione and its association in the ascorbate-glutathione cycle has been viewed as rational modulator of embryo development *in vivo* and *in vitro*. It is apparent that glutathione plays a fundamental role during embryogeny of both canola and spruce and that the ratio between the reduced (GSH) and oxidized (GSSG) forms of this redox compound have profound effects on development. Applications of buthionine sulfoximine (BSO), a specific glutathione biosynthesis inhibitor, to MDEs of *B. napus* shifts the glutathione redox status, i.e. GSH/(GSH+GSSG) in favour of an oxidized state and significantly enhances the quality of embryos through alterations in hormone metabolism and gene expression. Improved development is accompanied by major changes in the ascorbate-glutathione redox system. Like canola, spruce somatic embryos cultured in the presence of BSO are depleted of GSH which creates a forced shift in the glutathione redox ratio towards a more oxidized status. This shift is necessary to maintain structural integrity of the shoot apex and to prevent deterioration of the sub-apical cells. To examine whether altered embryonic development affects the glutathione redox status, the function of a class I homeobox of *knox* gene, *HBK3*, was characterized from the conifer *P. abies* (L.) Karst. *HBK3* is necessary for improved somatic embryo development in the promotion of a more stable SAM through alterations in *PgAGO* gene expression, a specific meristem marker. Cells down-regulating *HBK3* failed to develop into somatic embryos. Furthermore, glutathione metabolism in embryos down regulating *HBK3* failed to shift the GSH/GSH+GSSG ratio necessary for transdifferentiation of

somatic embryos. Overall, the findings emerging from this thesis represent a solid contribution to the improvement of canola and spruce embryos produced in culture.

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List of Symbols, Abbreviations and Nomenclature

ABA, abscisic acid

ABA-GE, glucose ester-ABA

AFRR, ascorbate free radical reductase

APX, ascorbate peroxidase

AA, ascorbic acid

AE medium, von Arnold and Eriksson medium

ATP, adenosine triphosphate

BA, N^6-benzyladenine

BSO, buthionine sulfoximine

C, control

2,4-D, 2,4-dichlorophenoxyacetic acid

d, day(s)

DHA, dehydroascorbate

DHAR, dehydroascorbate reductase

DIG, digoxigenin

DPA, dehydrophaseic acid

DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid)

F, fluridone

FW, fresh weight

GR, glutathione reductase

GSH, reduced glutathione

GSSG, oxidized glutathione

GUS, β-glucoronidase

LV medium, Litvay medium

MDE, microspore derived embryo

MS medium, Murashige and Skoog medium

N, no root or shoot development

NADPH, nicotinamide adenine dinucleotide phosphate

PA, phaseic acid

PAS, periodic acid-Schiff's reaction

PEG, polyethylene glycol

PEM, pro-embryogenic mass

PDT, partial drying treatment

PgAGO, *Picea glauca* ARGONAUTE gene

PGR, plant growth regulator (auxin and cytolinin)

PMSF, phenylmethylsulfonyl fluoride

PVP-40, polyvinylpolypyrrolidone-40

R, root growth only

RAM, root apical meristem

R+S, root and shoot development

S, shoot growth only

SAM, shoot apical meristem

SE, somatic embryo

STM, SHOOTMERISTEMLESS

TBO, Toluidine blue O

ZE, zygotic embryo

CHAPTER 1: INTRODUCTION

Recent advances in tissue culture techniques allows for large scale plantlet production via *in vitro* embryogenesis. Tissue culture technology, including somatic embryogenesis and microspore derived embryogenesis are largely based on the totipotency of plant cells. In addition to being a method for plant propagation, this technology is also suitable for the study of embryo development as a large number of embryos can be readily obtained. These embryos, produced asexually in culture, are bi-polar structures that follow similar developmental patterns to their zygotic counterparts. When combined with breeding programs and molecular techniques, somatic embryogenesis represents a cost-effective strategy to improving cropping systems. Although micropropagation of plants via *in vitro* techniques represents only a small percentage of total plant production, it is not surprising that a large number of scientists are now finding ways, both naturally and through genetic engineering, to improve many economically important species. This will hold especially true with the increase in demand for high quality genotypes with unique characteristics. Despite recent advances in plant cell tissue culture technology, the quality of embryos produced in culture can be poor as optimal tissue culture conditions for the embryogenic process have not been maximized.

The first reports of plant *in vitro* embryogenesis by Reinert (1958) were for the production of embryogenic heads from somatic cells of *Daucus carrota*. Later, Guha and Maheshwari (1964,1966) reported that microspores of the flowering plant, *Datura*, are also capable of developing into haploid plants. This discovery clearly illustrates the totipotency of microspores and their developmental potential to switch from gametophytic to sporophytic developmental programs. Successful generation of microspore derived embryos has also been reported in a number of economically important species including *Brassica* (especially canola and mustard), wheat, tobacco, barley and rice. In gymnosperms, the first report of somatic embryogenesis dates back to the late 1970s when Durzan and Chalupa (1976) generated immature somatic embryos that were unable to complete the developmental process in *Pinus banksiana* and later in *Picea glauca* (Durzan, 1980). Successful regeneration of mature viable *Picea abies*

somatic embryos as well as plantlets was achieved later in 1985 by two independent groups (Chalupa, 1985; Hakman et al., 1985). Since these initial reports in both flowering and non-flowering plants, canola microspore derived embryogenesis and spruce somatic embryogenesis have been used as model systems in the study of developmental, physiological, and molecular events occurring during embryogeny *in vitro*.

Brassica is the most economically important genus in the *Brassicaceae* family. Among the *Brassica* crops, oilseeds have the highest economic importance and are found collectively within *Brassica juncea*, *Brassica carinata*, *Brassica rapa* and *Brassica napus* (Cardoza and Stewart, 2004). *B. napus* is most often referred to as canola and is one of the most popular oilseed crops. In Canada, oilseed forms of *B. napus* are grown extensively and are suitable for human consumption because of modifications in its fatty acid composition to reduce levels of erucic acid (Downey and Craig, 1964). Millions of acres are planted in Canada each year with billions of dollars in economic value (Canola Council of Canada, Manitoba). Conifers such as spruce include *Picea glauca* (white spruce) and *Picea abies* (Norway spruce) are an important resource in the lumber and wood pulp industry for both their commercial and ornamental value (Grossnickle, 2000). Increasing exploitation of softwoods by major industries has led to growing interest in the scientific community to find ways to re-establish many of the lost stands of this valuable resource. The use of *in vitro* technologies such as microspore derived embryogenesis and somatic embryogenesis in canola and spruce respectively allows for large-scale plantlet production. The ability to manipulate the culture environment provides information concerning the regulation of embryo development. However, successful production depends on a number of factors and is often achieved through a series of steps requiring specific culture and media conditions. Optimization of the *in vitro* culture process is far from complete for canola and spruce as many of the embryos produced are of poor quality and fail to convert into viable plantlets. The failure to convert is largely dependent on un-organized development within the shoot apical meristem (SAM). Therefore, optimization of the culture conditions is imperative in achieving a good quality product for successful plant regeneration.

The SAM, formed during embryo development, is responsible for its fundamental architecture and subsequent conversion of the embryo into a plantlet, and is paramount to the continual success and viability of the plant. The low percentage of conversion observed is often a result of poor apical meristem development or defects in the meristem organization during embryogenesis *in vitro*. In zygotic embryos, the meristems, once formed, remain as "determined structures" with a fixed developmental fate (Steeves and Sussex, 1989). Determination of the SAM, is dependent on antagonistic processes between the renewal of the stem cell population and the regulation of cell differentiation (Lenhard et al., 2002). Alterations in this balance can lead to abnormal meristem formation, aberrant phenotypes and decreased somatic embryo production. Ultimately, it is the structural organization of these groups of cells that leads to the failure or the success of the somatic embryo derived plantlet. Structural deterioration of the region, via vacuolation, expansion of meristematic cells leading to the development of intercellular spaces, and a lack of storage product deposition are common properties of less functional somatic embryos (Kong and Yeung, 1994).

The intracellular redox state is described as a complex interaction in the relative concentrations of both reduced and oxidized forms of a variety of molecules (Harvey et al., 2002). Unfavourable shifts in reduction-oxidation (REDOX) states often leads to perturbed metabolic activity and unfavourable cellular conditions. Redox compounds have recently been speculated to be major players in meristem identity (Jiang et al., 2002). Because of their ubiquitous nature, compounds like ascorbic acid (AA) and glutathione (GSH) and nicotine adenine nucleotides ($NADP^+$/NADPH) are not only involved in physiological reactions, but have also been suggested to play a large role in the regulation of gene expression (Desikan et al., 2001; Tron et al., 2002; Pastori et al., 2003; Belmonte et al., 2005). Research related to these redox compounds generally focuses on AA and its antioxidant function while the role of GSH in plant growth and development is often neglected. Although AA promotes somatic embryo germination through meristemoid activation (Stasolla et al., 2006), it has little effect during embryo maturation. On the other hand, an increasing number of reports have indicated that the glutathione – glutathione disulfide (GSH-GSSG) redox pair, and ultimately the

GSH/GSSG ratio, may play an important role during embryo maturation (Earnshaw and Johnson, 1985; 1987; Jain et al., 1988; De Gara, 2003; Stasolla et al., 2003; Belmonte and Yeung, 2004; Belmonte et al., 2005; Belmonte and Stasolla, 2007).

Genetic regulation of the shoot apical meristem has been exhaustively investigated in the angiosperm *Arabidopsis* (for review see Williams and Fletcher, 2005), and to a lesser extent in gymnosperms such as spruce (Stasolla et al., 2003; Stasolla et al., 2004a; Tahir et al., 2006). Molecular markers are therefore readily available and serve as useful tools for the study of stem cell regulation in the shoot meristem. Genes associated with meristem identity include *WUSCHEL (WUS)* from *Arabidopsis* and *HOMEOBOX of KNOX (HBK)* from the *knox* (Knotted-like homeobox) family of homeobox genes from spruce in addition to *PgAGO* from the *ARGONAUTE* family. Moreover, certain *KNOX* genes have been shown to be preferentially expressed in embryos of improved quality (Belmonte et al., 2005). Mutations related to loss and gain of function mutations indicate that *knox* genes are important regulators of meristem function (Reiser et al., 2000; Barton, 2001; Belmonte et al., 2007). Although our understanding of the molecular networks involved in the establishment and continual maintenance of the SAM is well understood at the zygotic level in angiosperms, little is known about how certain genes interact during *in vitro* culturing of either microspore-derived-canola embryos or somatic embryos of spruce.

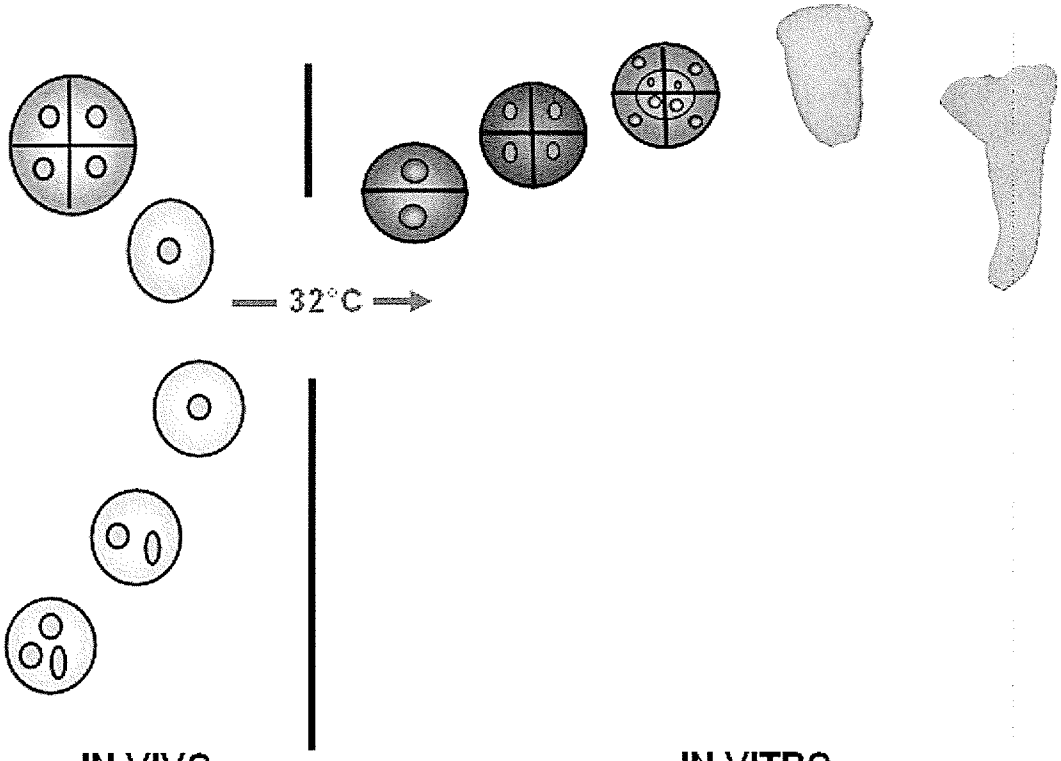
The organization and maintenance of the meristem remains as one of the fundamental questions in plant development. Although much research has been conducted on the study of genes related to meristem formation (Chan et al., 1998; Takada and Tasaka, 2002; Carles and Fletcher; 2003), the physiological and molecular mechanisms regulating meristem maintenance remain unclear, especially during embryogenesis *in vitro*. *In vitro* culturing of somatic or microspore derived embryos often leads to meristem deterioration (Kong and Yeung, 1994; Yeung et al., 1996). Recently, the GSH/GSSG redox pair, and more specifically the importance of the oxidized form, GSSG, has been shown to be paramount in maintaining meristem integrity in both roots (Sanchez-Fernandez et al., 1997; Jiang et al., 2003) and shoots (Belmonte et al., 2005). Taken together, I propose to test the hypothesis that the glutathione redox pair

plays a major role during embryogenesis. Two systems will be used in my study to test this hypothesis: 1) the canola system to examine microspore derived embryo (MDE) development and 2) the spruce system to study somatic embryo (SE) development. Both canola and spruce are model systems used to investigate angiosperm and gymnosperm embryogenesis, respectively. Based on the literature it is apparent that organized development, especially within the shoot apical meristem, is under similar mechanisms of control. Understanding these common elements will provide information for improving the quality of embryos produced *in vitro*.

The canola system: MDE development

Due to the difficulty of isolating and manipulating zygotic embryos, investigations on various aspects of embryo development have focused on using embryos derived from *in vitro* culture systems. Since the first report of isolated microspore culture of *B. napus* (Lichter, 1982), a large number of laboratories have reported the production of very high frequency embryogenic lines (Fan et al., 1987). This has led to the utilization of the microspore system in *B. napus* in developmental, histological, physiological and molecular studies. It is widely understood that an appropriate stress treatment is necessary for successful MDE development. An external stimulus often brings about changes in the developmental program which ultimately allows for plants to survive the ever-changing environment. Because of their sessile nature, the ability of a plant cell to switch its developmental fate is a totipotency characteristic of all plants and is best exemplified in MDE formation. Figure 1 outlines the process of pollen embryo development. Under normal conditions, pollen mother cells undergo meiosis which leads to the formation of haploid, uninucleate microspores. Following a round of mitosis, microspores undergo a process called microgametogenesis leading to the formation of a two-celled pollen grain with a vegetative and generative cell (Yeung et al., 1996). In general, the generative cell divides once more to give rise to two sperm cells and the development of mature pollen with three nuclei. Interestingly, in many species, through a mild heat shock, the pollen developmental program can be diverted at the late

Figure 1.1. Schematic diagram of microspore derived embryo development in canola. Normal pollen development occurs under normal conditions *in vivo*. Pollen mother cells undergo meiosis leading to the formation of a haploid uninucleate microspore (left grey). Following a round of mitosis, microgametogenesis of the microspore leads to the formation of a two celled pollen grain with a vegetative and generative cell. The generative cell then divides once more to give rise to two sperm cells leading to the development of a mature pollen grain with three nuclei. Pollen development is diverted to that of microsporogenesis in culture following a brief 32°C heat shock treatment for three days which divides the microspore symmetrically. Cells continue to divide and follow a characteristic angiosperm embryogenic pathway leading to the formation of a mature cotyledonary embryo (right).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EMBRYOS