The Role of Stress during in Vivo and in Vitro Plant Reproductive Development: Implications for Cropping Systems and Germplasm Enhancement in Canada

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ABSTRACT

High-yielding cereal and oilseed cultivars are integral components of modern cropping systems in Canada. Climate change occurring at both regional and global scales, along with increased frequency of extreme weather events, has resulted in greater emphasis upon yield stability or safety in local breeding programs. The reproductive development of crop and model plant species is particularly sensitive to environmental stress with undesirable reductions in seed yield linked to pollen sterility and ovule abortion along with defects in embryogenesis, storage product accumulation and seed maturation. In contrast to the detrimental role environmental stress plays in reducing harvestable yields, plants also employ controlled ‘stress’ programs at various checkpoints throughout the plant lifecycle. Modern in vitro tissue culture techniques which support breeding programs also employ stress as a means of reprogramming plant development. The following review covers recent molecular and physiological studies that have improved our understanding of the mechanism(s) through which both model and crop species cope with environmental or imposed stress during in vitro and in vivo reproductive development. Through approaches such as germplasm screening or genetic engineering plant biologists can utilize the information provided to enhance the stress tolerance of species of importance to the Canadian agriculture and forestry sectors.

Keywords: abscisic acid, androgenesis, embryogenesis, reproductive development, stress

INTRODUCTION

In the last decade it has become apparent that human activities have contributed significantly to global climate change. For the Canadian agriculture sector rising mean temperatures, regional changes in annual precipitation and increased frequency of weather extremes (drought, heat, excess water, cold temperatures or frost) are specific challenges that producers, agronomists and plant breeders will encounter in coming years (Porter and Semenov 2005; Motha and Baier 2005; Schindler and Donahue 2006). Forecasted climate changes also present opportunities for introgression of early maturing warm-season crops (e.g. maize, soybean, dry beans) to regions previously unable to support such crops (Cutforth et al. 2007).

The aforementioned changes to Canadian cropping systems also occur within the context of worldwide population growth predicted to reach 9-10 billion by the year 2050 (Rothstein 2007). Issues of food scarcity are further heightened by competing demands from the biofuel sector as well as a disproportionate increase in production of primary grain and oilseeds to support dietary trends in developing countries (Naylor et al. 2005). In any given growing season Canadian cropping systems encounter resource limitations or stress conditions that prevent producers from attaining the true genetic yield potential of modern cultivars. In the age of rapid climate change, however, yield stability has emerged as a focus of modern breeding programs (Porter and Semenov 2005). The following review examines how in vivo and in vitro plant reproductive development is affected by environmental or imposed stress. Recent molecular and physiological studies conducted in both model systems and crop species have provided critical insights into aspects of carbohydrate transport and assimilation, phytohormone metabolism or signaling, heat shock protein (HSP) production and reactive oxygen species (ROS) metabolism which affect the success or failure of plant reproductive development in the face of environmental stress.

Although the following review explores fundamental molecular, physiological and developmental aspects of in vivo and in vitro plant reproduction, we emphasize areas where additional efforts may be focused or integrated into...
breeding programs to enhance the stress tolerance and yield stability of cropping systems. The reader is also directed to reviews covering related aspects of plant reproductive development (Saini and Westgate 2000; Liu et al. 2005; Barnabás et al. 2008; Hedhly et al. 2008; Donahue et al. 2009; Thakur et al. 2010).

MICROSPOROGENESIS, MICROGAMETOGENESIS AND POLLEN DEVELOPMENT

Male reproductive development in angiosperms commences with stamen primordia formation within floral organs and generation of a filament-supported anther housing specialized meiotic male cells (microspore mother cell = MMC) (Ma 2005). Maternal sporophytic tissues, including the epidermis, endothecium, middle layer and tapetum, support and nourish the MMCs. Interior to the tapetum MMCs undergo meiosis and subsequently dissociate from the tapetum wall forming the locule, an intercellular space. At this stage of development microsporogenesis is complete and microgametogenesis initiates with two rounds of meiosis to generate a tetrad of haploid microspores (Scott et al. 2004; Ma 2005) (Fig. 1). Microspores then undergo asymmetric mitotic cell division and differentiation to form pollen grain containing a vegetative cell and two sperm cells. With subsequent pollen coat formation, anther dehiscence and pollen release, viable pollen grains are capable of germinating following contact with compatible stigma. Following pollen tube germination and growth within the transmitting tract, sperm cells are delivered to the female gametophyte during double fertilization (Kandasamy et al. 1994; Berger et al. 2008; Courtois and Yamófsky 2008).

The capacity for environmental stress to reduce pollen fertility and subsequent seed sets has been known for some time (reviewed in Barnabás et al. 2008). However, recent physiological and molecular studies of drought or cold-induced pollen sterility in wheat (Dorian et al. 1996; Koonjul et al. 2005) or rice (Oliver et al. 2005, 2007), respectively, revealed commonalities in the underlying mechanism through which environmental stress triggers pollen sterility. The young microspore stage (tetrad to uninucleate) of pollen development was noted for being particularly vulnerable to applied stresses, with reduced invertase activity, impaired sucrose transport or loading, and elevated abscisic acid (ABA) contents serving as correlative markers of reduced pollen viability or abortion (Koonjul et al. 2005; Oliver et al. 2007).

At the young microspore stage of pollen development the tapetum functions at maximum capacity to support and nourish symplastically-isolated pollen by loading carbohydrates and proteins into the locules (Oliver et al. 2007). An apoplastic unloading pathway supports this process, with sucrose transporters exporting sucrose from phloem sieve cells and extracellular invertases hydrolysing transported sucrose to monomers of glucose or fructose (Roitsch and González 2004). In turn sink cells utilize high-affinity hexose transporters to enhance monomer uptake and create a localized concentration gradient, thus increasing the combined sink strength of actively growing tissues (Roitsch and González 2004).

Antisense repression and RNAi silencing of invertase genes in tobacco (Nin88) and tomato (LIN5), respectively, result in male sterility or severe defects in pollen viability (Goetz et al. 2001; Zanor et al. 2009). These independent studies provide direct evidence that pollen abortion is linked to impaired invertase activity and sucrose cleavage. In comparing pollen sterility between cold-resistant (R31) and cold-sensitive (Doongra) rice cultivars, Oliver et al. (2007) identified ABA as a regulatory signal that could phenocopy cold-induced pollen sterility and similarly modify invertase and monosaccharide transporter gene expression. Further probing of cultivar-dependent differences in pollen sterility between R31 and Doongra revealed anthers of the former maintained lower endogenous levels of ABA following exposure to cold stress. Lower endogenous ABA levels in R31 also correlated with the activity of genes involved in ABA metabolism (Oliver et al. 2007). Similar genotype-dependent alterations in ABA appear to underlie pollen sterility in reproductive structures of Brassica napus, with maximal ABA contents of GMS (genic male sterile) lines observed in mature stamens (Singh and Sawhney 1992; Shukla and Sawhney 1994). Correlative evidence of a role for ABA in pollen sterility was first described for wheat where ABA could phenocopy the reduced seed set and
altered pollen morphology of drought-stressed wheat plants (Morgan 1980). However, several lines of evidence, including localization of enzymes involved in ABA biosynthesis, reduced fertility of ABA-deficient mutants, and immuno-localization of ABA to distinct male gametophytic tissues, all lend support to a promotive role for basal ABA levels in facilitating male gametophyte development (Cheng et al. 2002; Tan et al. 2003; Peng et al. 2009). However, the signaling of the phytohormone methyl jasmonate has also been implicated in defective male gametophyte development which contributes to reduced seed sets, with a possible feedback mechanism proposed to operate between jasmonate and ABA signaling pathways (Cippolini 2007; Kim et al. 2007). In the case of rice plants, overexpression of jasmonic carboxyl methyltransferase (JMT) and exogenous methyl jasmonates treatments enhanced the ABA content of reproductive structures. In turn, ABA biosynthesis and signaling appears to serve as a prerequisite for jasmonate signaling (Adie et al. 2007). For plant biologists seeking to enhance the stress tolerance of reproductive processes and elucidate the physiological basis of natural variation for related traits, it may be advantageous to expand the complement of phytohormones assayed (e.g. ethylene, GA, auxin, brassinosteroids, etc). Developmental programs that underlie reproductive processes are particularly dynamic with respect to the recruitment of phytohormone biosynthesis and signaling.

Heat shock proteins (HSPs) also accumulate during pollen development with expression patterns appearing complex and sometimes weak in contrast to the heat shock response of vegetative tissues (Crone et al. 2001; Young et al. 2004; Volkov et al. 2005; Frank et al. 2009). From these observations pollen is proposed to carry a subset of HSPs induced by a developmental program and an additional subset responsive to heat stress (Volkov et al. 2005). It is also noteworthy that higher basal levels of HSP expression may underlie natural variation for thermotolerance amongst tomato cultivars (Frank et al. 2009). Similar profiling of HSPs has occurred for Brassica napus, although it has yet to be determined if natural variation in thermotolerance amongst Brassica cultivars can be linked to differential expression or activity of HSPs (Young et al. 2004). Microspore maturation is also associated with activation of genes involved in scavenging reactive oxygen species (ROS) (Frank et al. 2009). However, in rice anthers undergoing programmed cell death (PCD) and pollen abortion in response to drought stress, hydrogen peroxide (H$_2$O$_2$) levels rise with a concomitant down-regulation of antioxidant transcripts and depletion of ATP pools (Nguyen et al. 2009). The need for work is required in discerning which genotypic variation in stress tolerance can be attributed to the capacity of male gametophytic tissues to support or repair oxidative damage.

TdT-mediated dUTP nick-end labelling (TUNEL) assays that detect fragmentation of genomic DNA indicate PCD is developmentally controlled during in vivo male gametophyte development (Wang et al. 1999; Vincze-Barren and Wilson 2006). However, similar TUNEL assays indicate endogenous and exogenous ABA suppresses DNA fragmentation of barley microspores, leading to enhanced microspore viability during androgenesis. From these observations enhanced synthesis or impaired catabolism of ABA is proposed to function towards improving microspore viability and suppressing cell death programs which ultimately lead to pollen abortion (Wang et al. 1999). Therefore, any manipulation of pollen tube growth (Singh et al. 2001; pollen tube guidance and ovule targeting) might influence the utility of pollen-based assays in isolating thermotolerant genotypes (Liu et al. 2006; Salem et al. 2007; Singh et al. 2008). The polyamine titre of mature pollen grains was also reported to correlate with rates of pollen germination, depth of pollen tube growth, and enhanced pollen tube growth during high temperature stress (Chibi et al. 1994; Song et al. 1999). From these results it is readily apparent that pollen-based assays have proven utility in isolating thermotolerant genotypes during reproductive development. However, some authors have advocated the use of alternative assays or screens to isolate genotypes with thermotolerant vegetative tissues (Salem et al. 2007).

At the molecular level NADPH oxidase-mediated reactive oxygen species (ROS) production, in addition to reactive nitrogen species (RNS), have been implicated as signaling molecules in pollination events (Tung et al. 2005; Li et al. 2007; Vriezen et al. 2007). Stigmatic cells further present an over-representation of stress-related transcripts suggesting that genetic programs for pollen recognition overlap with stress responses to some extent (Lan et al. 2005). The synchrony of anther dehiscence with stigma receptivity during reproductive development has also been considered in the context of climate change (Hedhly et al. 2009). In general increasing temperatures accelerate male and female gametophyte development, with low temperature prolonging stigmatic receptivity and longevity of ovules.

For several crop species (e.g. soybean, cabbage, cotton) in vitro pollen-based assays have been applied towards isolating thermotolerant genotypes (Liu et al. 2006; Salem et al. 2007; Singh et al. 2008). The polyamine titre of mature pollen grains was also reported to correlate with rates of pollen germination, depth of pollen tube growth, and enhanced pollen tube growth during high temperature stress (Chibi et al. 1994; Song et al. 1999). From these results it is readily apparent that pollen-based assays have proven utility in isolating thermotolerant genotypes during reproductive development. However, some authors have advocated the use of alternative assays or screens to isolate genotypes with thermotolerant vegetative tissues (Salem et al. 2007).

At the molecular level NADPH oxidase-mediated reactive oxygen species (ROS) production, in addition to reactive nitrogen species (RNS), have been implicated as signal transduction pathways in pollen germination (Singh et al. 2001; pollen tube growth (Singh et al. 2001; pollen tube guidance and ovule targeting) (Mclnnis et al. 2006; Potocky et al. 2007; Prado et al. 2008). Genetic lesions which impair ROS/RNS generation during pollen tube growth and ovule targeting are similar to those which operate during ABA-mediated stomatal closure (Kwak et al. 2003; Bright et al. 2006). Mutant or transgenic plants altered in gibberellin (GA) metabolism or signaling also highlight GA as a regulator of pollen tube growth (Smith et al. 2004; Hu et al. 2008). GA-deficiency or overdose are similarly linked with seed sets and localized promotion of fruit development (Vivian-Smith et al. 1999; Cox and Swain 2006; Hu et al. 2008). The localization of ABA-receptor complexes and signaling intermediates to pollen grains, growing pollen tubes and developing seeds/siliques (Brocard et al. 2002; Ma et al. 2009) also invites speculation that mechanisms of ABA-GA antagonism which have been well characterized for seed development or dormancy might...
also be operative during intermittent aspects of sexual plant reproduction (Seo et al. 2006; Piskurewicz et al. 2008; Toh et al. 2008). The versatile nature of ROS/RNS as intermediates in hormonal signaling, plant development and programmed cell death also warrants consideration when aspects of reproductive development are superimposed by conditions of environmental stress.

FEMALE GAMETOPHYTE DEVELOPMENT

In both eudicot and monocot species, ovules are specialized structures that develop from the ovary wall and consist of three basic structures: the nucellus, one or two integuments and the funiculus (Reisinger and Fischer 1993). Within ovules the nucellus gives rise to a megasporeocyte and subsequently generates the embryo sac, or female gametophyte, during the process of megagametogenesis. At the completion of megagametogenesis species with a Polygonum-type embryo sac consist of one (1) egg cell, one (1) central cell, three (3) antipodal cells and two (2) synergids. Following double fertilization of the egg cell and central cell, endosperm (central cell) nuclei proliferate and facilitate transport of nutrients and signal molecules from the maternal sporophyte (Berger et al. 2008).

In Arabidopsis embryo growth initially proceeds at a very slow rate immediately following fertilization, with rapid embryo development (globular → heart → torpedo) occurring after endosperm replication has ceased. At the torpedo stage the embryo undergoes rapid cell expansion and crushes the endosperm. Although differences in seed development and anatomy are apparent amongst Arabidopsis, legumes and cereals (Chaudhury et al. 2001; Weber et al. 2005; Sabelli and Larkins 2009) the basic steps in endosperm development appeared to be conserved between eudicots and monocots, with cell divisions and differentiation in the developing embryo invariably delayed relative to endosperm.

Cell biology studies examining the movement of GFP variants in Arabidopsis thaliana have determined the outer integument serves as a symplastic extension of the funicular phloem (Stadler et al. 2005). However, the adjacent cells of outer and inner integument are symplastically isolated from one another with little or no plasmodesmatal connections. Developing embryos are also symplastically isolated from the endosperm with the suspensor serving as the conduit for delivery of assimilates, hormones or other signaling molecules (Stadler et al. 2005). Therefore apoplastic barriers reside between outer and inner integuments, between the inner integument and endosperm and between the endosperm and embryo. Similar approaches using phloem-mobile dyes indicate ovule abortion in maize is accompanied by decreased delivery of carbohydrates through a post-phloem unloading pathway (Makela et al. 2005).

In the context of environmental stress, there exist numerous stages at which the female gametophyte or developing zygote can abort or senesce. For Arabidopsis, a number of publications have described in detail the anatomical changes which accompany stress-induced abortion of Arabidopsis ovules (Sun et al. 2004, 2005; Hauser et al. 2006). TUNEL assays identified maternal nourishing tissues (integuments, chalaza) as sites of DNA fragmentation during stress-induced abortion of female gametophytes (Sun et al. 2004). In comparison, developing embryo and endosperm tissue did not undergo significant DNA fragmentation. Changes in mitochondrial membrane potential, callose deposition, ROS generation and downregulation of ROS detoxification genes were also identified as correlative markers of ovules which had committed to abort (Sun et al. 2005; Hauser et al. 2006).

Although the seed anatomy of Arabidopsis differs from that of cereals or legumes, numerous studies reinforce the concept that pre- and post-fertilization abortion of reproductive sinks is tied to decreased non-structural carbohydrate delivery (Patrick and Ollier 2001; Makela et al. 2005). Furthermore, in line with the putative role proposed for invertebrate(s) in mediating pollen viability and abortion, the abortion of female reproductive tissues has also been associated with changes in invertase gene expression and activity (Anderson et al. 2002; reviewed in Boyer and McLaughlin 2007).

Two recent and independent studies conducted in tomato have highlighted the role of invertase inhibitors as putative intermediaries coordinating ABA and invertase activities during male and female reproductive development (Jin et al. 2009; Zanor et al. 2009). Proteinaceous invertase inhibitors have previously garnered attention as regulators of sucrose transport or metabolism although their exact physiological role has remained somewhat enigmatic (Huang et al. 2007). At the transcriptional level ABA and polyethylene glycol (PEG) treatments are reported either induce or repress inhibitor transcripts (Rausch and Greiner 2004; Koh et al. 2008) and reversible protein phosphorylation serves as an intermediary in ABA-mediated regulation of invertase activity (Pan et al. 2005; Huang et al. 2007). More detailed localization and chromatography studies with a kernel-specific maize invertase inhibitor protein (INVINH1) revealed inhibitor transcripts were transiently localized to a defined region around the embryo. The authors proposed that transient and contained INVINH1 expression immediately following fertilization could coordinate hexose supply and contrasting developmental programs of the embryo and endosperm (Bate et al. 2004). An INVINH1-homolog identified in tomato was found to physically interact in vivo with LIN5, a well-characterized cell wall invertase (Jin et al. 2009). Silencing INVINH1 increased LIN5 invertase activity via release of post-translational inhibition, with increases in seed weight and size reported. In contrast, overexpressing INVINH1 in a tobacco cell wall invertase inhibitor dramatically reduced seed sets or resulted in complete infertility (Jin et al. 2009). In situ mRNA localization studies revealed LIN5 and INVINH1 transcripts co-localize to the placental vasculature. In addition to post-translational regulation, earlier studies revealed the LIN5 promoter is responsive to multiple hormones (GA, ABA, auxin) with LIN5 transcripts localizing to male and female gametophytic tissues (Godt and Roitsch 1997; Proels et al. 2003). RNAi silencing of LIN5 also induces aberrant floral and fruit phenotypes that are concordant with altered hormone biosynthesis and signaling (Zanor et al. 2009).

ZYGOTIC EMBRYOGENESIS AND SEED DEVELOPMENT

Zygotic embryogenesis is an inherently complex developmental process due in large part to the enclosure and physical interaction of three genetically discrete tissues (zygotic embryo, tripliod endosperm and maternal integuments) within the developing seed (Chaudhury et al. 2001; Berger et al. 2006). Studies of seed development are further complicated by the reciprocal interaction of individual seeds and whole fruits with maternal sporophytic tissues (Vivian-Smith et al. 1999). During early stages of seed development, maternal tissues hold a dominant position in determining the metabolic state and sugar composition of the embryo and endosperm. Throughout early and late stages of seed development carbohydrates such as glucose and sucrose serve as both nutrients and signal molecules (Wobus and Weber 1999; Borisjuk et al. 2004). By altering the temporal and spatial distribution of these precursors and metabolic events seed developmental programs including cell division and enlargement, transfer cell formation, embryo and endosperm differentiation, endoreduplication, photosynthetic activity and storage product accumulation, can be coordinated. As developing seeds progress from fertilization through to storage product accumulation a sequential transition from maternal to filial control occurs and can be correlated with defined transcriptional changes mediated in part by altered concentrations of metabolites and phytohormones (ABA or...
GA) alongside modifications to the cellular energy status (ATP) (Weber et al. 2005).

As with pollen development certain HSPs are expressed during embryogenesis and seed maturation with distinct regulatory pathways triggering expression of individual family members in response to heat stress or developmental cues (Wehmeyer et al. 1996; Kotak et al. 2007). AB13, a transcriptional activator involved in ABA signaling, has recently been implicated in a transcriptional downregulating HSP expression during seed development (Kotak et al. 2007). However, it is also intriguing that ab13 mutants were isolated in a genetic screen to detect high temperature resistant germination mutants (Tamura et al. 2006). ABA and reactive oxygen species (ROS) have been further implicated in the development of seeds (Wang et al. 2002). Nonetheless, under environmental stress ABA produced in maternal sporophytic tissues (e.g. leaves) can be transported to reproductive tissues where it accumulates within developing seeds and subsequently endosperm cell division arrests and reduplication (Mambell and Setter 1998; Setter and Flanagan 2001). In severe cases ABA accumulation triggers seed abortion and reduces seed set (Ober and Setter 1990, 1992; Setter et al. 2001). How direct a role ABA plays in triggering ovule/ovary abortion has been questioned (Asch et al. 2001; Andersen et al. 2002). Nonetheless, under environmental stress ABA produced in maternal sporophytic tissues such as the placenta which directly support endosperm and zygote development appear to demonstrate enhanced rates of ABA catabolism (Wang et al. 2002). These observations parallel those observed during pollen development under stress, with steady-state maintenance of endogenous ABA levels correlated with enhanced viability of developing seeds (Wang et al. 2002). Seed development, in turn, produces signals that stimulate fruit expansion, with endosperm-derived GA strongly implicated in this process (Hu et al. 2008; Dorey et al. 2009). In the future, the dynamic interplay between GA and ABA pathways warrants further investigation from both a developmental and agronomic standpoint. For example, whether environmental stress affects the flux of GA emanating from developing seeds and whether or not GA impinges upon ABA metabolism and signaling in surrounding maternal tissues.

Genetic approaches have further reinforced that an extensive genetic interplay exists among parental and zygotic components of seed development (Dilkes et al. 2002; Johnston et al. 2007). For example, in cdc2a mutants of Arabidopsis preferential delivery of a single sperm cell to the egg cell triggers endosperm proliferation, suggesting a positive signal emanates from the fertilized egg cell to trigger proliferation of the central cell (Nowack et al. 2006). Bayer et al. (2009) also recently discovered that embryonic patterning in Arabidopsis thaliana is mediated by male gamete-specific transcripts of the SHORT SUSPENSOR (SSP) gene. SSP transcripts produced in a parent-of-origin manner are subsequently delivered to the egg cell or central cell at which point SSP transcripts are translated to initiate asymmetric cell divisions in the developing zygote (Bayer et al. 2009). Maternal parent-of-origin effects have also been described for several genes (e.g. MEDEA (MEA), FERTILIZATION INDEPENDENT ENDOSPERM (FIS), MULTI-COPY SUPPRESOR of IRA1 (MIS1) in Arabidopsis which involve the differential or strict mono-allelic expression of maternal transcripts over paternal transcripts (reviewed in Chaudhry et al. 2001). For these particular genes parent-of-origin effects are derived through genomic imprinting which adds specific epigenetic marks to maternal (MEA1) or paternal (MEA0) alleles. In turn, these epigenetic marks result in repressive chromatin structure which selectively silences the maternal or paternal alleles. Thus far, PHERES (PHE1) has emerged as the only imprinted gene where higher levels of expression are observed from the paternal allele over that of the maternal allele. Several additional genes expressed predominantly in male or female gametophytic tissues (FERONIA, LORELEI, ANXUR1 and ANXUR2 of Arabidopsis interact to coordinate pollen tube targeting, rupture and delivery of sperm cells to the egg cell and synergid (Berger 2009). Nonetheless, in all cases of the genes listed above have been isolated in studies which sought to elucidate fundamental aspects of plant reproductive development, there is tremendous potential to explore these signaling pathways in the context of environmental stress and reproductive failure. The evolution of genomic imprinting is often hypothesized to arise from maternal and paternal conflicts over resource allocation when multiple offspring require nourishment (Köhler and Makarevich 2006). Differential dosage hypothesis also exist to explain the consequences of paternal or maternal excess in driving the growth rate and final size of developing seeds (Dilkes and Comai 2004). From an agronomic standpoint reciprocal crosses (control/stressed pollen × control/stressed pistil) represent an important starting point towards unravelling the molecular intricacies of maternal (megagametophyte) or paternal (microgametophyte) control of reproductive outcome during abiotic stress.

STRESS DURING IN VITRO MORPHOGENESIS

Cellular totipotency, proposed by Haberlandt (1902) is the inherent ability of plant cells to form organs or embryos in culture. This concept was first demonstrated by Levine (1947) who was able to obtain embryo-like structures from carrot cells. These observations were further elaborated by Steward et al. (1958) and Reinert (1958) who are accredited with an accurate description of the process. Today in vitro plant propagation represents an effective way to clone desirable species and capture genetic gain over a relatively short period of time. Over the past few years methods of propagation have prospered thanks to the optimization of culture conditions and the refinement of improved media and addenda. These efforts have been pivotal in increasing the number of species able to propagate in culture (see Thorpe and Stasolla 2001).

In vitro embryogenesis can be achieved through two distinct processes. The first is somatic embryogenesis, which is the capacity of somatic or non-sexual plant cells to form embryos by a process very similar to that occurring for zygotic (or seed) embryogenesis. This process leads to the formation of bipolar structures with a defined root-shoot axis and a closed vascular system. The morphology of somatic embryos is close to that of their zygotic counterparts and stages equivalent to globular, heart, and torpedo can be observed in culture. Embryos can also be generated in vitro through gametophytic embryogenesis, the formation of haploid embryos from cells of the male or female gametophyte. This process is very suitable for the capture of endogenous genetic variation through recovery of diploid homozygous chromosomes following artificial chromosome doubling (Yao et al. 1997). As a general rule the most efficient and routinely used system for gametophytic embryogenesis is androgenesis in which immature pollen grains, referred to as microspores, can be used as starting material for the production of microspore-derived embryos (MDEs) (Fig. 1). Initial work was conducted using anther cultures but in recent years isolated microspore cultures have emerged as the more suitable alternative as they offer the advantage that the sporophytic anther wall does not interfere with the development of the embryos. Androgenesis has been demonstrated in a large variety of species and several protocols have been optimized over the years. In recent years this process has been fully integrated in many breeding programs aimed at recovering and propagating desirable lines.

The imposition of stress in the form of osmoticum, heat, or oxidative stress has often been used in both somatic and
Microspore-derived embryogenesis during either the induction phase or to stimulate embryonic development. In this review two species will be used as model systems to describe the role of stress during morphogenesis: *Brassica napus* (canola) for androgenesis and *Picea glauca* (white spruce) for somatic embryogenesis. Both species have profound ecological and economical significance to Canada. According to the Canola Council of Canada (2010) canola-related production in Canada can be estimated as $13.8 billion. Canola is not the main source of oil for foreign exchange by the export but also has diverse domestic usage for food, animal feed, chemical feedstocks and fuel. Like canola, white spruce holds a key position in Canada where it is used in the forestry sector serving as the most economically important tree for paper and pulp production (NRCAN 2007).

**STRESS DURING BRASSICA MICROSPORE- DERIVED EMBRYOGENESIS**

Embryogenesis from immature pollen requires a redirection of the gametophytic pathway into the sporophytic pathway. Early worked show that several forms of stress imposed to donor plants, such as short days, nitrogen starvation, as well as cold treatments, and/or chemical treatments of the excised inflorescences, induced the number of microspores initiating the embryogenic pathways (see Sunderland 1982). As reviewed by Touraev et al. (2001) a better understanding on the role played by stress during the initiation of microspore-derived embryogenesis was only reached with the realization that spores isolated from non-stressed donor plans, were also responsive to stress treatments. In the absence of plant growth regulators, a heat shock of 8 h at 32°C is sufficient to induce isolated *Brassica napus* microspores to initiate the embryogenic process and the resulting embryos are able to regenerate into viable plants (Custers 1994). The duration of the heat stress was further increased to 2 d (Ferrie and Keller 1995) to increase the number of *Brassica* microspore-derived embryos produced. The embryos produced with these stress treatments are generally similar to that of their zygotic counterparts although they do not display the same initial cell division and differentiation patterns observed in vivo (Tykarska 1976, 1979), as well as lacking a well developed suspensor (Yeung et al. 1996). Zygotic-like microspore-derived embryos were obtained by Joosen et al. (2007) who used a milder heat stress (25°C), together with a narrower range of microspore developmental stages. Such embryos display an elongated suspensor and the embryo proper originates from the distal cell of the suspensor and undergoes the same ordered events occurring during embryogenesis. The ability to “reproduce” embryos with zygotic-like features in culture has made this system a model for molecular studies which besides elucidating the genetic network responsible for the induction process (Joosen et al. 2007) have shed light on the role of the suspensor during embryogenesis (Supena et al. 2008). These advantages have prompted many labs to use in vitro embryogenesis as an alternative system for studying zygotic embryogenesis.

The role of heat shock for the redirection of the gametophytic pathway into the sporophytic pathway is not restricted to *Brassica* but also documented in other species, including tobacco where it triggers embryogenesis in isolated unicellular microspores (Touraev et al. 1996), and in wheat when combined with temporary starvation (Kyo and Harada 1986). Characteristic stress-induced morphological changes have been observed during microspore-derived embryogenesis in a variety of species including *Brassica*, rice, wheat, and tobacco. Following the heat treatments the microspores swell and their nucleus moves in a central position. This is followed by the formation of cytoplasmic strands connecting the perinuclear cytoplasm with the subcortical cytoplasm through the vacuole (reviewed by Touraev et al. 2007). These structural cellular alterations, which have been associated to a state of highly mitotic activity, are needed for the reactivation of the cell cycle and the initiation of the embryogenic process. During normal pollen development microspores undergo an asymmetric cell division. The generative cell becomes arrested in the G2 phase of the cell cycle after a round of DNA replication, whereas the vegetative cell arrests in G1 phase (Zarsky et al. 1992). This is in contrast to “stressed” microspores in which the vegetative nucleus proceeds from the G1 to the S phase and the generative nucleus is blocked as S13 in the G2 phase. *Canola* is not the main source of oil for foreign exchange by the export but also has diverse domestic usage for food, animal feed, chemical feedstocks and fuel. Like canola, white spruce holds a key position in Canada where it is used in the forestry sector serving as the most economically important tree for paper and pulp production (NRCAN 2007).

Changes in gene expression as a result of stress have also been documented during the initiation of the embryogenic process. Independent studies have shown that in both *Brassica* and tobacco the inductive starvation of microspores promotes the transcription of several mRNAs (Garrido et al. 1993; Cordewener et al. 1994). In some instances some of these transcripts remain transcriptionally inactive, as also observed in other developmental systems (i.e. animal oocytes), in which cells remain in a quiescent state prior fertilization, whereas in other they are translated into proteins. Touraev et al. (1997) showed that these differences might be attributed to the fact that in some species, including *Brassica*, cell arrest is not a requirement for embryogenic induction. As a result, several genes induced at the onset of *Brassica* microspore-derived embryogenesis belong to different classes of heat shock proteins (Cordewener et al. 1994), making it difficult, if not impossible, to discriminate between stress-induced and embryogenic-induced transcription. These problems have recently been overcome by the use of refined isolation methods coupled with advanced molecular and proteomic techniques. Joosen et al. (2007) identified 135 robust markers for the transition of *Brassica* microspore-derived embryogenesis and several of these markers were co-regulated at the gene and protein expression levels. These findings are exciting as they open new avenues for unravelling the genetic network induced by stress and involved in embryo initiation.

Oxidative stress, can be defined as the “imbalance in the pro- versus anti-oxidant ratio in the cells” (Cassells and Curry 2001) resulting in high levels of ROS such as hydrogen peroxide, superoxide dismutase, and peroxyl and alkoxyl radicals. These molecules are harmful for the cellular environment as they cause several aberrations including DNA mutations, hyper- and hypomethylation, changes in chromosome numbers, and structural damages to membranes and other cellular components. Oxidative stress is inevitable in culture as tissue manipulation, sterilization, and the subsequent subculturing cause woundage, which are a known cause of oxidative burst (Yahraus et al. 1995). Chemicals used in sterilization solutions are also elicitors of oxidative stress, as well as sub-optimal light and temperature requirements (reviewed by Cassells and Curry 2001). Both plant and animal cells respond to oxidative stress through changes in metabolism of glutathione, a small peptide which which is synthesized enzymatically and not as a result of mRNA translation (Alsher 1989). This molecule can exist in a reduced form (GSH) or as an oxidized form (GSSG), with a disulfide bridge linking two molecules (reviewed by Yeung et al. 2005). Since glutathione acts as a major redox buffer within plant cells it is not surprising that cells maintain a balance between GSH and GSSG through several feedback mechanisms regulating the pro-oxidant/pseudo-oxidant ratio which is needed for the reactivation of the cell cycle and the initiation of the embryogenic process. During normal pollen development microspores undergo an asymmetric cell division. The generative cell becomes arrested in the G2 phase of the cell cycle after a round of DNA replication, whereas the vegetative cell arrests in G1 phase (Zarsky et al. 1992). This is in contrast to “stressed” microspores in which the vegetative nucleus proceeds from the G1 to the S phase and the generative nucleus is blocked as S13 in the G2 phase. *Canola* is not the main source of oil for foreign exchange by the export but also has diverse domestic usage for food, animal feed, chemical feedstocks and fuel. Like canola, white spruce holds a key position in Canada where it is used in the forestry sector serving as the most economically important tree for paper and pulp production (NRCAN 2007).

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ation and maintenance of the Arabidopsis root meristem. The ROOT MERISTEMLESS 1 (RML1) gene is needed for cell proliferation within the root tip (Cheng et al. 1995) and encodes γ-glutamylcysteine synthetase, which is a precursor of the de-novo synthesis of GSH. In the same study it was found that plants homozygous for the rml1 mutation show a reduction in root growth and have lower levels of cellular GSH. It is interesting to note that the shoot apical meristem of RML1-mutant plants remained functional, producing vegetative structures. Additional evidence on the participation of glutathione on root development comes from maize seedlings (Jiang et al. 2003). Quiescent center integrity of the root meristem is proposed to be regulated by the redox state of the ascorbate and glutathione pool (Jiang et al. 2003).

Cell proliferation and elongation are two key components of plant growth that govern morphogenesis. It appears that there is a strong correlation between glutathione levels and mitotic activity. Elevated concentrations of GSH, which result in a high GSH/GSSG ratio, are often associated with rapidly growing tissues including meristematic regions and newly expanding leaves of pea (Bielawski and Joy 1986). However, at present, it is not clear as to how glutathione regulates mitotic activity and cell cycle events (Potters et al. 2003).

The realization that alteration in the glutathione redox state (ratio between GSH and GSSG) could affect morphogenesis in vitro was first ascribed to Marre and Arrigoni (1957). Using a variety of systems they concluded that the auxin-induced changes is growth rate were the results of endogenous fluctuations of the glutathione redox state, with an oxidized environment (low GSH/GSSG ratio) inhibiting growth and a reduced environment (high GSH/GSSG ratio) promoting cell proliferation. The concept that oxidative stress, mediated by the glutathione redox state, could be use to manipulate in vitro embryogenesis was proved in several species including Brassica microspore-derived embryogenesis. The imposition of a glutathione oxidized environment (low GSH/GSSG ratio) through applications of buthionine sulfoximine (BSO), a specific GSH biosynthetic inhibitor and enhanced significantly the quality of Brassica MDEs and their ability to convert into viable and vigorous plants (Belmonte et al. 2006). The authors showed that applications of 0.1 mM BSO increased the percentage of 25 day old MDE able to convert, i.e. form a fully developed shoot and root system at germination, from 26% to more than 60%. Histological analyses revealed that compared to their control counterparts BSO-treated embryos form well organized meristems and accumulate storage products in a similar pattern to that observed in seed embryos. The imposed oxidized stress was beneficial especially for the differentiation of shoot apical meristems which displayed a zygotic-like appearance having a dome-shaped architecture and a well defined zonation pattern, i.e. visible tunica-corpus boundaries. This was in contrast to control MDEs which had meristems disrupted by the presence of intercellular spaces and exhibited signed of cellular differentiations.

Production of endogenous ABA is tightly linked to the “stress level” of the tissue as synthesis of this plant growth regulator generally increases under sub-optimal conditions. Several studies have shown that ABA increases both in vivo and in vitro as a result of osmoticum. Yeung and Brown (1982) documented that the liquid endosperm of flowering plants has more negative osmotic values than those of the embryo. This negative osmotic potential is important for a slow development of the zygotic embryo, changes were related to morphological characteristics of the embryos and their post-embryonic performance. BSO applications also activated many genes controlling meristem formation and function, including ARGONAUTE 1, and ZWILLE, SHOOT-MERISTEMLESS, and ARGONAUTE 1. The authors speculated that increased expression of these genes may contribute to the improved structural quality of the shoot poles observed in the presence of BSO (Stasolla et al. 2008). The role of BSO in the induction of a developmental pathway towards the embryo-fate, whereas the oxidative stress might be required for the subsequent stages of development characterized by histodifferentiation and tissue patterning.

Overall the understanding of how stress regulates morphogenesis, and in particular embryogenesis, is key for the Canadian breeding industry, where haploid plants derived from microspores are often used to propagate elite genotypes to be introduced in agronomic programs.

STRESS DURING WHITE SPRUCE SOMATIC EMBRYOGENESIS

White spruce somatic embryogenesis is a multi-step process which includes (1) induction and maintenance of the embryogenic tissue, and (2) embryogenesis and maturation. As reviewed by Stasolla et al. (2003) embryogenic tissue is initiated from immature zygotic embryos and maintained either on solid or liquid medium in the presence of levels of auxin and cytokinins which need to be optimized for each cell line. During both processes, i.e. induction and maintenance, tissue is constantly under stress, due to the excision of the zygotic embryos and the sub-culturing of the embryogenic tissue on a regular basis. However, the role played by stress during these phases remains elusive. It is during the developmental phases, in which filamentous embryos increase in size that the role of stress, especially water and oxidative stresses, has been investigated in detail.

Embryo development is achieved through the removal of auxin and cytokinin, addition of abscisic acid (ABA), and applications of increased osmolarity in the medium. When all these conditions are applied fully developed embryos are produced (see Stasolla et al. 2003). The role of ABA during somatic embryogenesis is mainly related to promotion of storage product accumulation and the prevention of precocious germination. The requirement of ABA during embryonic development has also been described in vivo. Studies conducted by Kong et al. (1997) revealed that ABA levels in developing white spruce seeds are low during the initial phases of development, reaching a maximum point during the middle phase of maturation, prior to declining again at later stages when seeds dry.

In order to investigate the molecular basis of the glutathione oxidized environment during micropore-derived embryogenesis, Stasolla et al. (2008) monitored the profile of 15k transcripts between control MDEs and MDEs treated with BSO. BSO applications induced major changes in transcript accumulation patterns, especially during the late phases of embryogenesis. Several key enzymes involved in ascorbate metabolism, which resulted in major fluctuations in cellular ascorbate levels were affected by BSO. These
necessary for regulating the pattern of histodifferentiation (Yeung 1995). Restriction of water uptake in vitro can be achieved through the use of either permeating osmoticum agents, such as mannitol, sucrose, and inorganic acids, or by non-permeating osmotica, such as polyethylene glycol (PEG) and dextran (reviewed by Stasolla et al. 2003). Utilization of PEG as an osmotic agent has been extensively reported during white spruce somatic embryogenesis. Importantly, deprivation of water through applications of PEG (5-10%) resulted in a three-fold increase in the maturation frequency of white spruce somatic embryos and produced somatic embryos with superior appearance to those matured with ABA alone. These embryos also exhibited an increased tolerance to drying, a nine-fold increase in the amount of storage lipid triacylglycerol with a fatty acid composition resembling that of zygotic embryos (Attree and Fowke 1993), and a three-fold higher protein content than somatic embryos matured in the absence of PEG (Misra et al. 1993). The physiological “maturity” observed in PEG-treated white spruce somatic embryos was also confirmed by the appearance of some of the major matrix and crystalloid polypeptides which were absent from somatic embryos matured in ABA and low osmoticum, but present in mature seed embryos (Misra et al. 1993). In the same study it was observed that effects of ABA on growth and development of specific organs, regulation of somatic embryo development, especially protein synthesis, appear to be additive. Crystalloid protein synthesis is first initiated by ABA alone, but sequentially regulated by PEG at a translational or post-translational level (Misra et al. 1993).

Spruce somatic embryos which are morphologically mature cannot successfully germinate and convert into viable plantlets unless they undergo a desiccation period, either through applications of non-plasmolysing osmoticum or by a partial drying treatment, which lowers water content of about 20% (reviewed by Stasolla et al. 2003). To date there are only very few studies dealing with the physiological and biochemical events occurring during the desiccation period. Changes in hormone level are most likely to occur as the embryos dry. Compared to mature embryos, partially dried white spruce somatic embryos had a lower endogenous level of ABA, as well as a reduced sensitivity to ABA (Kong and Yeung 1995). Kong (1994) suggested that these alterations in ABA metabolism are beneficial for both germination and conversion processes. The poor post-embryonic growth of white spruce somatic embryos following applications of ABA in the germination medium supports this notion. Similarly to ABA, ethylene production was significantly reduced in partially dried embryos (Kong 1994). It appears that a specific combination of hormone level treatment may be required for increasing the ability of white spruce somatic embryos to generate purine and pyrimidine nucleotides in preparation for the resumption of growth at germination. Both uridine and adenine salvage enzymes, uridine kinase (UK) and adenine phosphoribosyltransferase (APRT) were found to increase in partially dried embryos (Stasolla et al. 2001a). High activities of these enzymes will be required for the extensive salvage of both purine and pyrimidine precursors occurring at the inception of germination, before the restoration of the de novo nucleotide biosynthesis (Stasolla et al. 2001b, 2001c). The critical role played by the salvage pathway at germination is also demonstrated by the observations that inclusions of ascorbic acid in the germination medium increased the conversion frequency of white spruce somatic embryos (Stasolla and Yeung 1992). As well as the ability of the embryos to salvage purine nucleotides for nucleotide production (Stasolla et al. 2001b).

As observed during microspore-derived embryogenesis, the imposition of an oxidative stress, effected by applications of oxidized glutathione GSSG promotes proper histodifferentiation and tissue patterning in developing somatic embryos. Several studies (Belmonte et al. 2005; Belmonte and Stasolla 2007) revealed that white spruce somatic embryos cultured in a low GSH/GSSG ratio have better organized shoot apical meristems, which are able to reactivate promptly at the onset of germination. The apical pole of these embryos also exhibit a larger expression pattern of HBK1, a gene preferentially expressed in the meristematic cells and involved in proper meristem formation (Belmonte et al. 2005).

These studies are relevant to the Canadian biotechnology/forestry sector dealing with propagation of coniferous and non-native species. High levels of germination and tissue differentiation in vitro are desired to enable the reintroduction practices in the Canadian boreal forest are putting pressure on the private sector to provide suitable genotypes to be integrated in reforestation practices. This holds true especially for spruce, which is the preferred species utilized for paper and pulp production. Therefore the understanding on how stress can be applied in culture to enhance embryonic yield and quality is crucial for accelerating the propagation of elite genotypes.

CONCLUSIONS AND PERSPECTIVES

In the following review we have discussed molecular and physiological aspects of plant reproductive development, highlighting the dual role which stress plays in reducing reproductive output but also serving as a tool to reprogram embryogenesis. For example, the maturation of the somatic embryo sector maintaining or improving the productivity of cropping systems is of paramount importance and is most frequently assessed according to the quantity and quality of seed derived from reproductive processes. Abiotic stress and variable climatic conditions are both hallmarks of Canadian cropping systems: past, present and future. However, in response to forecasted climate changes and rising input costs, an increased focus has been placed upon yield stability or safety in modern breeding programs.

In an evolutionary context, reproductive processes are associated with continuation of the genetic line and may actually be at odds with agronomic goals. The maternal sporophyte exerts a dominant influence in allocating resources to reproductive processes and developing progeny. Abiotic stress or resource limitation might also trigger the maternal sporophyte to undergo developmental arrest or invest in metabolic processes which improve stress tolerance, thus delaying reproductive processes or reducing reproductive output.

While the following review has focused on molecular and cellular aspects of stress in specific reproductive structures it is important to emphasize that yield is determined by a hierarchy of yield components which operate at the tissue, organ, whole plant and population level. For example, specific communication systems exist within the reproductive processes and developing progeny. As observed, ABA is crucial in promoting growth in vitro while a high GSH/GSSG ratio is required to promote growth in vivo. It is also important to emphasize that an understanding of the molecular mechanisms which underlie stress responses in reproductive processes is essential for the development of robust stress-tolerant genotypes. Therefore, the identification of the genetic basis of stress tolerance in reproductive processes is not only critical for the development of improved genotypes but also serves as a tool to reprogram the process of propagation of elite genotypes.
enhanced crop productivity or yield stability in extreme environments. Crop growth, development and yield are affected by environmental stress in both linear and nonlinear ways depending upon severity of the imposed stress, developmental stage of the crop, and defined thresholds of growth assigned to individual species and cultivars (Porter and Semenov 2005). Moreover, in the field crop plants can be exposed to a combination of stress factors, which reinforces the importance of studying genotype × environment interactions across different management practices.

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