# Regulation of Metabolic Events during Embryo Development in Norway Spruce (*Picea abies* L. Karst)

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## Abstract

The overall aim of this thesis was to identify and characterize metabolic and biochemical events that are involved in regulation of embryo development in Norway spruce. Embryogenesis involves coordination of multicellular patterning events which are critical for establishment of the apical-basal plan of the plant. Due to similarities with zygotic embryogenesis, the process of somatic embryogenesis (SE) provides an excellent *in vitro* model system for investigating the regulatory mechanisms of embryo development. Recent progress in metabolomics provides new tools for innovative approaches for elucidating the metabolic pathways present in *in vitro* samples.

Gas chromatography coupled with time-of-flight mass spectrometry (GC/TOFMS) was used to identify important metabolic changes during development of somatic embryos in embryogenic cell lines of Norway spruce. The studied cell lines exhibited normal, aberrant and blocked development of embryos. The results of the metabolic analyses indicated that endogenous sucrose is beneficial for proliferation of proembryogenic masses (PEMs), early embryo differentiation and normal late embryo development. In contrast, aberrant late embryo formation was associated with elevated levels of endogenous fructose during embryo differentiation. A subsequent study found that embryogenic cultures of Norway spruce exhibited blocked development of embryos when cultured on medium containing fructose. Furthermore, the embryogenic cultures displayed elevated levels of protein fluorescence, protein carbonyl content, deoxyribonucleic acid (DNA) damage and alterations in antioxidant (glutathione) content. These results led to a hypothesis that the inhibitory effect of fructose on embryo development may be linked to the Maillard reaction.

Assessment of the biochemical effect of carbohydrates and osmoticum on embryo development revealed that, maltose and polyethylene glycol (PEG) inhibit the germination of embryos by restricting the accumulation of sucrose, raffinose family oligosaccharides (RFOs) and late embryogenesis abundant (LEA) proteins. These compounds are important for the acquisition of desiccation tolerance. Taken together, these findings show that carbohydrates play an important role during development and germination of embryos.



Keywords*:* Conifers, Norway spruce, *Picea abies*, metabolomics, carbohydrates, Maillard reaction, desiccation tolerance, somatic embryogenesis.



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To Barbara Kintu & Florence Nsubuga

*"It’s nice to be important, but it’s more important to be nice"*-Roger Federer

*"Everything will be ok..Fo shizzle!"*- Danna L. Alessandra

# Contents

# List of Publications

This thesis is based on the work described in the following papers, which are referred to by the corresponding Roman numerals in the text.

**I** **Businge E**, Brackmann K, Moritz T, Egertsdotter U (2012). Metabolite profiling reveals clear metabolic changes during somatic embryo development of Norway spruce (*Picea abies*). Tree Physiology 32(2): 232-244.

**II** **Businge E**, Bygdell J, Wingsle G, Moritz T, Egertsdotter U (2013). The effect of carbohydrates and osmoticum on storage reserve accumulation and germination of Norway spruce somatic embryos. Physiologia Plantarum 149(2): 273-285.

**III** **Businge E**, Egertsdotter U (2014). A possible biochemical basis for fructose-induced inhibition of embryo development in Norway spruce (*Picea abies*). Tree Physiology 34(6):657-69.

Papers I, II and III are reproduced with the permission of the publishers.

The contribution of Edward Businge to the papers included in this thesis was as follows:

**I** Performed the experimental work, evaluated the data and wrote the paper jointly with the co-authors.

**II** Planned and performed the experimental work, except for analysis of peptides using mass spectrometry. Evaluated the data and wrote the paper jointly with the co-authors.

**III** Planned and performed the experimental work, evaluated the data and wrote the paper jointly with the co-author.

# Abbreviations

All abbreviations are explained when they first appear in the text

# 1 Introduction

## 1.1 Why study embryo development in Norway spruce?

Norway spruce (*Picea abies* (L.) Karst) is the most economically important coniferous species in northern and central Europe (Svobodova *et al.*, 1999; Schlyter *et al.*, 2006). Norway spruce belongs to the genus *Picea*, which comprises about 35 species (Alden, 1987). The genus *Picea* is a member of the sub-division *Gymnospermae*, class *Coniferopsida*, order *Coniferae* and family *Pinaceae* (Kubitzki, 1990). According to the Swedish Forest Agency, conifers account for 81% of the total productive forest land of which 39% is Scots pine and, 42% is Norway spruce. Wood from Norway spruce accounts for 50% of the forest products (Swedish Statistical Yearbook of Forestry, 2012). With regard to propagation, Norway spruce is traditionally regenerated using seeds. However, the drawback with this method of propagation is that sporadic flowering can lead to poor seed production and lack of seeds for reforestation (Svobodova *et al.*, 1999). Furthermore, seed propagation requires long generation cycles involving extensive breeding efforts in order to obtain seeds of elite genotypes (Hogberg *et al.*, 1998; Svobodova *et al.*, 1999).

Somatic embryogenesis (SE) epitomizes a clonal propagation method with the potential for capturing the genetic gains from forest breeding programs (Hogberg *et al.*, 1998). Somatic embryogenesis is a process whereby plant growth regulators (PGRs) are used to stimulate the differentiated cells within plant explants to dedifferentiate and form somatic embryos. Subsequently, the somatic embryos are used to regenerate plants (Jimenez, 2001). The developmental stages in somatic embryogenesis are described in greater detail in chapter 1.2.3. Some of the benefits of somatic embryogenesis include; the ability to produce many somatic embryos of the same genotype in a short time (Jimenez, 2001). Additionally, embryogenic cultures of candidate elite clones can be cryopreserved while the regenerated plants are tested in the field. This

makes it possible to mass produce plants from the somatic embryos from the cryopreserved embryogenic cultures once the field trials have been completed (Martinez *et al.*, 2003).

Existing evidence suggests that somatic embryogenesis is a viable alternative for large scale propagation of important conifers such as Norway spruce (Bonga *et al.*, 2010; Nehra *et al.*, 2012). However, in order to realize the full potential of somatic embryogenesis as a propagation technique, we need to first develop a better understanding of embryo development in conifers. The more that is known about embryo development in conifers and most importantly the regulatory mechanisms of embryo development, the more precise the culture protocols can be tailored to support large scale propagation of conifers by somatic embryogenesis.

The work presented in this thesis describes an investigation into the metabolic and biochemical events during embryo development in Norway spruce using SE as a model system. We initially used GC-MS to obtain the metabolic profiles of three Norway spruce embryogenic cell lines displaying differences in somatic embryo development and plant formation capabilities (Paper I). In this study, we aimed to establish the fundamental metabolic events necessary for normal somatic embryo development in Norway spruce. Based on the findings in Paper I, we performed a biochemical evaluation of the regulatory role of reducing sugars during somatic embryo development in Norway spruce (Paper III). In particular, we investigated the underlying biochemical mechanisms of fructose-induced inhibition of embryo development. Lastly, we also investigated the biochemical effects from carbohydrates and osmoticum present during early embryo development to the subsequent accumulation of storage reserves and germination of embryos (Paper II).

## 1.2 Embryogenesis in higher plants

The model angiosperm *Arabidopsis,* is extensively used to study embryogenesis in higher plants (Zhao *et al.*, 2011). However, there are notable differences between angiosperms and gymnosperms with regard to ancestry and embryo patterning (Raghavan & Sharma, 1995; Smith *et al.*, 2010). For that reason, I will start this section by describing embryogenesis in angiosperms using *Arabidopsis* as an example. Subsequently, I will describe embryogenesis in gymnosperms for which conifers will serve as the example.

### 1.2.1 Angiosperms (*Arabidopsis*)

In *Arabidopsis*, embryogenesis commences with double fertilization which involves the fusion of two sperm cells of the male gametophyte with two cells of the female gametophyte (Fig. 1A). One sperm fertilizes the egg cell and the other sperm combines with the two polar nuclei of the large central cell of the megagametophytes to form the zygote and endosperm, respectively (Park & Harada, 2008). After fertilization, the zygote goes through an elongation phase during which it elongates by almost three times along the apical-basal axis (Lau *et al.*, 2012). Following elongation, the zygote divides asymmetrically to produce two cells, a small apical cell which develops into the embryo proper and a large basal cell which forms the hyophysis and suspensor (Yeung & Meinke, 1993; Park & Harada, 2008; Zhao *et al.*, 2011).

Embryogenesis continues with dual and single division of cells in the longitudinal and transverse planes of the apical cell resulting in an eight-celled (octant) proembryo respectively (Petricka *et al.*, 2009; Zhao *et al.*, 2011). Each of the eight cells in the proembryo divides periclinally to produce the protoderm and a dermatogen-globular stage embryo (Park & Harada, 2008). The ensuing periclinal division of cells on either side of the of the globular embryo leads to outgrowth of cotyledon lobes which later flank the shoot apical meristem (SAM) in the heart stage embryo (Boscá *et al.*, 2011). By the torpedo stage, the cotyledons, hypocotyl and root apical meristem (RAM) are clearly visible along the apical basal-axis of the embryo (Park & Harada, 2008).

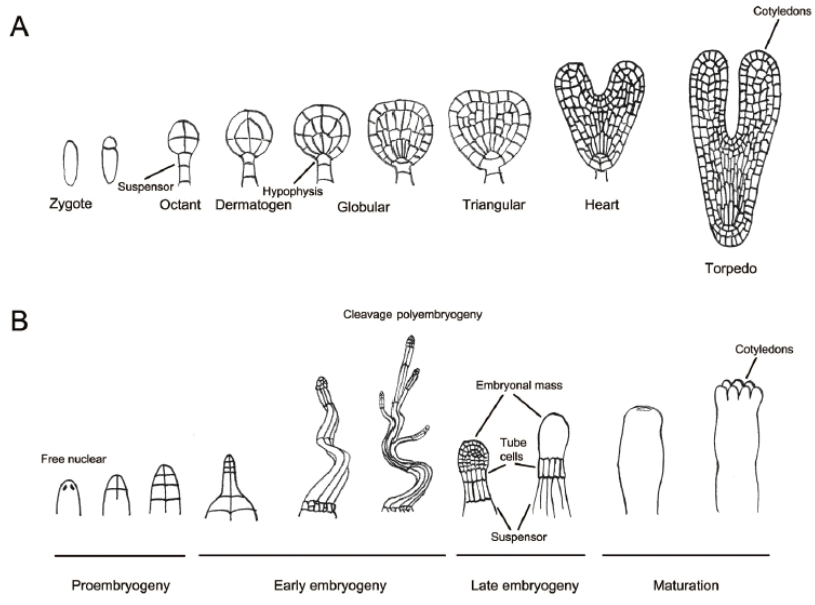


**Figure 1.** Schematic overview of embryo development in: (A) angiosperms (*Arabidopsis*) and (B) gymnosperms (conifers). Reproduced with permission from Emma Larsson, *Acta Universitatis Agriculturae Sueciae*, Doctoral Thesis No. 2011:68.

### 1.2.2 Gymnosperms (conifers)

Embryogenesis in gymnosperms, to which the conifers belong proceeds in a sequence of three stages (Singh, 1978): (i) proembryogeny – all stages before elongation of the suspensor; (ii) early embryogeny – all stages during and after elongation of the suspensor and before establishment of the root meristem; (iii) late embryogeny – establishment of the root and shoot meristem and further development of the embryo thereafter.

Proembryogeny (Fig. 1B) starts when the fertilized egg nucleus undergoes a series of divisions to produce four free nuclei. After the initial division, two tiers (the primary embryonal and upper tier) comprising of four nuclei each are formed. Localized divisions within the two tiers generate four tiers of four cells of which the lower two tiers make up the embryonal tier. The lower four cells of the embryonal tier produce the embryonal mass while the upper four cells elongate and produce the suspensor (von Arnold & Clapham, 2008).

During early embryogeny, the suspensor elongates and the cells of the embryonal tier divide to form the embryonal mass (von Arnold & Clapham, 2008). At this point, there is a marked difference between the conifers and

*Arabidopsis* with regard to the morphology of the suspensor. The suspensors in conifers are comprised of numerous non-dividing files of cells emanating from the proximal cells of the embryonal mass while the *Arabidopsis* suspensor comprises a single file of dividing cells (Fig. 1A & B) (Larsson, 2011). Also, embryo development in conifers is characterized by the occurrence of cleavage polyembryogeny, a process in which the early embryo splits into several identical embryos out of which only one dominant embryo develops to maturity while the rest of the embryos are aborted (Filonova *et al.*, 2002).

Late embryogeny (Fig. 1B) involves the specification of the root and apical meristems and establishment of the plant axis. The root apical meristem forms close to the center of the embryo while the shoot meristem originates from the distal region of the embryonal mass (von Arnold & Clapham, 2008). During maturation, the developmental program shifts from pattern formation to storage reserve accumulation in preparation for the impending germination of the embryo. Unlike the *Arabidopsis* embryo, the mature embryo in conifers contains a shoot apical meristem which is surrounded by a crown comprising of multiple cotyledons (Larsson, 2011).

### 1.2.3 Somatic embryogenesis in Norway spruce

Somatic embryogenesis requires the use of plant growth regulators (PGRs) to stimulate a sequence of developmental events that lead to formation of somatic embryos from non-zygotic cells. The developmental stages of somatic embryogenesis include: initiation of embryogenic cultures, proliferation of proembryogenic masses (PEMs), maturation and germination of somatic embryos (Pullman *et al.*, 2003; Helmersson *et al.*, 2004).

In Norway spruce, embryogenic cultures are initiated by culturing zygotic embryos on growth medium supplemented with 2,4-Dichlorophenoxyacetic acid (2, 4-D) and $N^6$-benzyladenine (BA) as the plant growth regulators. The presence of both PGRs prompts the differentiated cells in the zygotic embryos to dedifferentiate and produce proliferating PEMs. The PEMs are maintained in a proliferative state by keeping the embryogenic cultures on growth medium containing 2, 4-D and BA. The PEMs are comprised of two major cell types: the meristematic cells of the embryonal mass and the embryonal tube cells (von Arnold & Clapham, 2008). Before formation of early somatic embryos, the PEMs go through three stages of development (Filonova *et al.*, 2000a):

(i) PEM I – comprising of a small clump of closely packed meristematic cells alongside a single vacuolated cell.
(ii) PEM II – similar to PEM I but with more than one vacuolated cell.
(iii) PEM III – consisting of a large mass of meristematic cells intertwined with the vacuolated cells (Fig. 2).

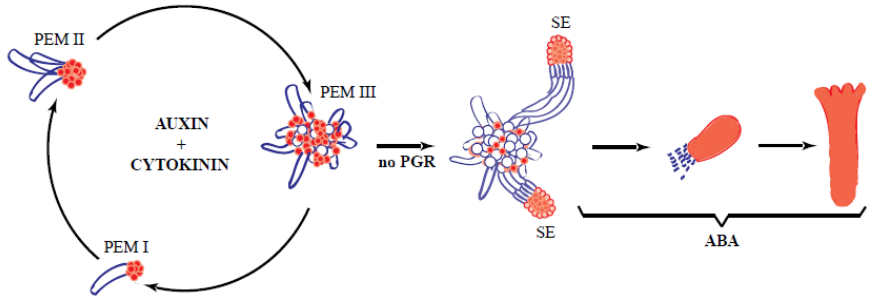


**Figure 2**. A schematic representation of the developmental pathway of somatic embryogenesis in Norway spruce (adapted from Filonova *et al.* (2000a); not drawn to scale). Proliferation of PEMs is stimulated by auxin and cytokinin. An individual PEM should pass through a series of three characteristic stages (I, II and III) to transdifferentiate to somatic embryos (SE). At stage PEM I, a cell aggregate is composed of a small compact clump of densely cytoplasmic cells adjacent to a single enlarged and vacuolated cell. Similar cell aggregates but possessing more than one vacuolated cell have been classified as PEM II. At stage PEM III, an enlarged clump of densely cytoplasmic cells appears loose rather compact; polarity is disturbed. Withdrawal of plant growth regulators (PGRs) triggers embryo formation from PEM III, whereas abscisic acid (ABA) is necessary to promote further development of somatic embryos through late embryogeny to mature forms. Shown in red are the cells of the PEMs and somatic embryos, which stain in situ blue with Evan's blue. Shown as dashed blue lines in the last but one stage of the pathway are the remnants of the degenerated suspensor in the beginning of late embryogeny. Reproduced with permission from The Company of Biologists: Filonova *et al.* (2000b), *Journal of Cell Science* 113, 4400.

Withdrawal of the PGRs stimulates the differentiation of early somatic embryos from the stage III PEMs. The early somatic embryos display a polar structure comprising of an apical embryonal mass and basal vacuolated suspensor cells (Fig. 2) (Larsson, 2011). Subsequently, the early embryos are transferred to medium containing abscisic acid (ABA) and elevated osmotic stress to undergo further development and maturation into cotyledonary somatic embryos (Filonova *et al.*, 2000b). The osmotic stress is induced by using a permeating osmoticum such as sucrose or a non-permeating osmoticum such as polyethylene glycol (PEG) (Attree & Fowke, 1993). Studies in embryos of rapeseed (*Brassica napus*) have shown that many of the ABA-induced responses are also affected by osmotic stress (Finkelstein & Crouch, 1986; Finkelstein & Somerville, 1989; Wilen *et al.*, 1990).

Abscisic acid is known to be involved in the development of embryos and the maturation of seeds in angiosperms. Abscisic acid stimulates changes in gene expression that lead to physiological and morphological changes in

developing and maturing seeds (Dunstan *et al.*, 1998). Notably, ABA has been found to inhibit precocious germination of embryos, promote synthesis of storage proteins and induce the expression of genes that encode late embryogenesis abundant (LEA) proteins which are involved in desiccation tolerance (Kermode, 1990; Dunstan *et al.*, 1998). As might be anticipated, the presence of ABA during spruce somatic embryogenesis inhibits precocious germination of embryos and promotes the deposition of storage proteins (Attree & Fowke, 1993; Dunstan *et al.*, 1998).

## 1.3 Regulation of embryo development

There are close similarities between somatic and zygotic embryogenesis in Norway spruce (Suarez *et al.*, 2004). For that reason, somatic embryogenesis is also an excellent model system for studying different aspects of embryo development in Norway spruce (Filonova *et al.*, 2000b; Egertsdotter *et al.*, 2006; Egertsdotter & Arnold, 2008; Sun *et al.*, 2010). Recent genomic and molecular studies have greatly contributed to elucidate the regulation of embryo development in angiosperms and gymnosperms. Therefore, the next section focuses on regulation of embryo development by gene expression, plant hormones and extracellular components. Since some of the aforementioned regulatory mechanisms are well described in *Arabidopsis*, I will base the discussions on the known events in *Arabidopsis* and compare them to similar events in Norway spruce and other plant species.

### 1.3.1 Embryo development regulation by gene expression

The fact that the life cycle of a plant starts with a simple zygote makes embryogenesis an amazing and important process because it's during embryogenesis that the shoot and root body pattern of the plant is established. In view of that, embryogenesis relies on well-timed changes in gene expression in order to ensure proper patterning of the embryo.

**Early embryogeny**

In gymnosperms, early embryogeny involves all stages during and after elongation of the suspensor cells and before establishment of the root meristem (Singh, 1978). During somatic embryogenesis, early embryogeny is stimulated by withdrawing the PGRs (2, 4-D and BA) from the growth medium. Recently, microarray analysis of an embryogenic cell line of Norway spruce showed that, programmed cell death (PCD)-related genes: F-actin capping protein, *CATHEPSIN B-LIKE CYSTEINE PROTEASE* and *METACASPASE 9* (*MC9*)

are up-regulated 24 hours after withdrawal of 2, 4-D and BA (Vestman *et al.*, 2011).

Programmed cell death is a process through which plants and animals eliminate unwanted cells or tissue during development (Ellis *et al.*, 1991). Metacaspases are cysteine dependent proteases found in plants, fungi and protozoa and they are involved in cell death, stress and proliferation. Additionally, metacaspases are characterized by a distinctive substrate specificity for arginine and lysine (Tsiatsiani *et al.*, 2011). In *Arabidopsis*, nine metacaspase genes (*AtMC1-AtMC9*) have been identified while a single metacaspase gene (*mcII-Pa*) has been described in Norway spruce (Suarez *et al.*, 2004; Tsiatsiani *et al.*, 2011). In *Populus*, the *AtMC9* gene has been found to be up-regulated during xylem maturation indicative of its likely involvement in PCD (Courtois-Moreau *et al.*, 2009).

Two waves of PCD have been found to occur during development of somatic embryos in Norway spruce (Filonova *et al.*, 2000b). The first wave of PCD occurs during the transition from PEMs to somatic embryos during which the PEMs are eliminated after formation of the early embryos. The second wave of PCD eliminates the terminally differentiated embryo suspensors during early embryogeny (Filonova *et al.*, 2000b). In Norway spruce, SE relies on the aforementioned waves of PCD for proper transition from PEMs to early embryos and patterning of the somatic embryos. Accordingly, embryogenic cell lines of Norway spruce which are capable of forming normal somatic embryos have been found to exhibit a high level of PCD compared to cell lines with blocked development of embryos (Smertenko *et al.*, 2003). Furthermore, silencing of the Norway spruce metacaspase gene (*mcII-Pa*) has been shown to suppress PCD, block suspensor differentiation and cause developmental arrest at the early stage of embryogenesis (Suarez *et al.*, 2004).

### Late embryogeny and maturation

During late embryogeny, the root and shoot meristems are established and the embryo continues with its development thereafter (Singh, 1978). Establishment of the shoot apical meristem (SAM) in somatic embryos of Norway spruce requires the expression of *KNOTTED1*-like homeobox (*KNOX*) genes (Larsson, 2011). Homeobox refers to a 180 bp consensus DNA sequence which is found in genes that are involved in developmental processes. Homeobox genes play a fundamental role during plant development by controlling cell specification and patterning events (Chan *et al.*, 1998). The *KNOX* genes code for transcription factors that are members of the homeobox gene family (Guillet-Claude *et al.*, 2004). Proteins of the KNOX family have a structure which comprises of six regions including a conserved KNOX domain, a highly

conserved ELK domain and a homeodomain that binds DNA (Ito *et al.*, 2002). The plant *KNOX* genes are divided into two classes: I and II based on their expression pattern and sequences (Kerstetter *et al.*, 1994). The class I *KNOX* genes are mainly expressed in meristemic tissues while the class II *KNOX* genes are expressed in all tissues but not much is known about their functions (Kerstetter *et al.*, 1994; Guillet-Claude *et al.*, 2004). In *Arabidopsis*, the class I *KNOX* gene *SHOOT MERISTEMLESS* (*STM*) is expressed within the SAM. As a result, the *stm* mutants are incapable of maintaining a functional SAM during embryogenesis (Long *et al.*, 1996; Long & Barton, 1998).

In Norway spruce, four class I *KNOX* genes: *HBK1*, *HBK2*, *HBK3* and *HBK4* have been identified (Sundås-Larsson *et al.*, 1998; Hjortswang *et al.*, 2002; Guillet-Claude *et al.*, 2004; Larsson *et al.*, 2012). Initially, it was found that the *HBK2* gene was expressed only in embryogenic cell lines which are capable of forming fully mature cotyledonary embryos (Hjortswang *et al.*, 2002). However, using quantitative real-time polymerase chain reaction (qRT-PCR), it was further discovered that the *HBK2* and *HBK4* genes are both significantly up-regulated during formation of the SAM (Larsson *et al.*, 2012). Therefore, it was suggested that up-regulation of the *HBK2* and *HBK4* genes is necessary for formation of a functional SAM in somatic embryos of Norway spruce (Larsson *et al.*, 2012).

### 1.3.2 Hormonal regulation of embryo development

On the molecular level, embryogenesis is also under the control of plant hormones just like most developmental processes in plants. Plant hormones are naturally occurring organic substances which at minute concentrations regulate growth and development. In the previous years, molecular studies have demonstrated the role played by the versatile plant hormone, auxin during embryogenesis. The question; *what is auxin and how is auxin transported from cell to cell?* is vital for highlighting the role that auxin plays during embryogenesis.

**Auxin**

Indole-3-acetic acid (IAA) is the most common of the natively occurring plant auxins. Besides its role during plant embryogenesis, auxin is involved in apical dominance, tropic responses and cell elongation. There are two pathways for auxin biosynthesis namely the tryptophan dependent and tryptophan independent pathway (Jenik & Barton, 2005; Benjamins & Scheres, 2008; Davies, 2010). Since auxin is not synthesized in all plant cells, it must be transported from the synthesis site to its point of action. Therefore, auxin is

transported into one cell and out to another cell by a set of carriers known as the auxin influx (AUX) and efflux (PIN) proteins, respectively. In *Arabidopsis*, 8 PIN proteins (PIN1-PIN8) have been identified (Jenik & Barton, 2005; Benjamins & Scheres, 2008; Zažímalová *et al.*, 2010). A key feature of auxin transport is that, because of the asymmetric intracellular localization of the PIN proteins, auxin is directionally transported through the cells which leads to the term polar auxin transport (PAT) (Jenik & Barton, 2005; Benjamins & Scheres, 2008).

Figure three shows auxin transport during embryo patterning in *Arabidopsis*. Within the early embryo (Fig. 3A), PIN7 has been shown to be localized at the apical side of the basal cell directing auxin transport upwards into the embryo proper. However, by the globular stage (Fig. 3D), PIN7 is localized at the basal membrane of the suspensor cell directing auxin transport downwards into the developing root (Friml *et al.*, 2003; Jenik & Barton, 2005). It was therefore proposed that PAT causes surges in auxin that result in localized gene activation and formation of the apical-basal pattern of the embryo (Jenik & Barton, 2005). Notably, it has been suggested that PIN-mediated auxin transport loop regulates the expression of the *PLETHORA 1* and *2* (*PLT1* and *PLT2*) genes which promote the development of the root stem cell niche in *Arabidopsis* (Blilou *et al.*, 2005; Jenik & Barton, 2005). Furthermore, existing evidence suggests that auxin transport regulates the expression of the *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) genes which together with *SHOOT MERISTEMLESS* (*STM*) redundantly promote the formation of the SAM and separation of the cotyledons (Aida *et al.*, 2002; Jenik & Barton, 2005; Möller & Weijers, 2009).

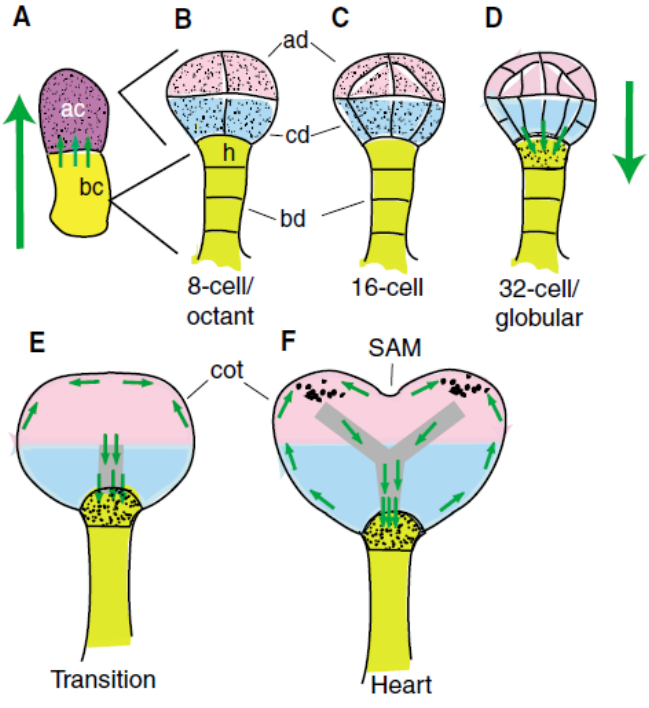


**Figure 3**. Auxin transport relative to early events in *Arabidopsis* embryo patterning. (A) An early *Arabidopsis* embryo, consisting of an apical cell (ac) and a basal cell (bc). Green arrows indicate the direction of auxin transport; stippling indicates regions with high auxin levels. (B) Eight-cell/octant-stage embryo. (Cell numbers used to stage embryos reflect the number of cells in the apical cell lineage.) The apical domain (pink) and the central domain (blue) both derive from the apical cell and each consists of four cells. The basal domain (yellow) derives from the basal cell. (C) A 16-cell stage, early globular embryo. (D) In a 32-cell stage globular embryo, auxin transport has shifted direction (green arrows), and auxin now accumulates in the hypophyseal lineage. The hypophyseal lineage is derived from the hypophysis (h) – the suspensor cell closest to the embryo proper. This lineage gives rise to a portion of the root meristem, specifically the quiescent center and the central columella with associated stem cells. (E) A transition stage (transitioning between globular and heart stage) embryo. Auxin transport in the apical domain is directed toward the center of the cotyledon primordial (cot). (F) An early heart-stage embryo, showing the emergence of cotyledons and a cleft where the shoot apical meristem (SAM) will form. Gray indicates regions of vascular development. Reproduced with permission from The Company of Biologists: Jenik and Barton (2005), *Development* 132, 3579.

Larsson *et al.* (2008) examined the role of auxin during somatic embryogenesis by treating different stages of Norway spruce embryos with 1-N-napthylphthalamic acid (NPA), a polar auxin transport inhibitor. The NPA treated embryos exhibited apical and basal abnormalities including: fused

cotyledons, abnormal shoot meristems and split basal regions. Interestingly, the NPA-treated embryos of Norway spruce exhibit similar phenotypes as the auxin transport mutants of *Arabidopsis* (Aida *et al.*, 2002), suggesting that polar auxin transport is important for embryo patterning in angiosperms and gymnosperms (Larsson *et al.*, 2008).

### 1.3.3 Regulation of embryo development by extracellular components

So far, chapters 1.3.1 and 1.3.2 have addressed the regulation of embryo development by gene expression and plant hormones, respectively. However, a growing body of evidence shows that embryo development is also under the control of extracellular components, a few of which are discussed below.

**Sugars** are involved in several physiological processes during plant growth and development. The role of sugars during embryo development has been studied using somatic embryogenesis as a model system. In conifers, the addition of exogenous sugars (such as sucrose, maltose or fructose) to culture medium is an essential prerequisite for somatic embryogenesis, suggesting that sugars play an important role during embryo development (Tremblay & Tremblay, 1991; Nørgaard, 1997; Li *et al.*, 1998; Niskanen *et al.*, 2004). Although sugars are primarily considered as sources of carbon and energy for the embryogenic cultures, other lines of evidence indicate that sugars may also act as signaling molecules that control gene expression and developmental processes (Rolland *et al.*, 2002). For example, the availability of sucrose or glucose has been shown to differentially regulate mRNA levels of *Arabidopsis* cyclin D2 (CycD2) and cyclin D3 (CycD3) genes, thereby controlling the plant cell cycle (Riou-Khamlichi *et al.*, 2000). Additionally, somatic embryos of oil palm (*Elaeis guineesis*) treated with 175 and 263 mM sucrose were found to have increased abundance of transcripts for the *GLO7A* gene which encodes a 7S globulin storage protein (Morcillo *et al.*, 2001). Furthermore, studies in broad bean (*Vicia faba*) have reported the existence of glucose gradients across developing embryos and also established a correlation between the concentration of glucose and mitotic activity (Borisjuk *et al.*, 1998). These observations suggest a mechanism in which sugars acting as morphogens may provide positional information to several development programs in plants (Rolland *et al.*, 2002).

**Arabinogalactan proteins (AGPs)** are a family of proteins which have been implicated in several processes associated with plant growth and development including cell division, programmed cell death, secondary wall deposition and embryogenesis (Egertsdotter & von Arnold, 1998; Seifert & Roberts, 2007; Ellis *et al.*, 2010). Arabinogalactan proteins are mainly found in plasma membranes, cell walls and in secretions (e.g. to intercellular spaces

and culture medium) (Egertsdotter *et al.*, 1993; Showalter, 2001; Ellis *et al.*, 2010). Structurally, AGPs consist of a hydroxyproline-rich protein backbone which is O-glycosylated by arabinose and galactose-rich polysaccharide units (Showalter, 2001; Seifert & Roberts, 2007). In addition to the hydroxyproline-rich and O-glycosylated protein backbone, AGPs contain other unique features such as a glycosylphosphatidylinositol (GPI) lipid anchor and the ability to react with β-glucosyl Yariv reagent, a synthetic chemical reagent that specifically binds to AGPs (Showalter, 2001; Hu *et al.*, 2006; Seifert & Roberts, 2007; Ellis *et al.*, 2010). Besides the use of β-glucosyl Yariv reagent, the other technique for studying the function and localization of AGPs involves the use of AGP-specific antibodies which react with the carbohydrate moieties of AGPs. These antibodies include: JIM8, JIM13, JIM14, LM2, CCRC-M7 and MAC 207 (Hu *et al.*, 2006; Seifert & Roberts, 2007).

From experimental studies, it's clear that AGPs play an important role during embryogenesis in several plant species [reviewed by Showalter (2001)]. In *Arabidopsis* zygotic embryos, localization of AGPs by immunofluorescence labeling with JIM13 showed that AGPs were mainly distributed in the embryo and basal part of the suspensors (Hu *et al.*, 2006). In the same study, addition of β-glucosyl Yariv reagent to *in vitro* ovule cultures of *Arabidopsis* resulted in inhibition of embryo development, shoot meristem formation and cotyledon differentiation. However, these abnormalities were reversed by removal of β-glucosyl Yariv reagent indicating a role for AGPs during embryo differentiation and shoot meristem formation (Hu *et al.*, 2006).

Arabinogalactan proteins have also been implicated in the development of somatic embryos in Norway spruce. Particularly noteworthy, AGP fractions of concentrated extracellular proteins and seed extracts stimulated less developed embryos to develop further into aggregated somatic embryos comprising of large, densely packed embryonic regions and well-defined suspensor regions (Egertsdotter *et al.*, 1993; Egertsdotter & Arnold, 1995). Although the underlying mechanism for the stimulatory role of AGPs during embryo development is not known, it has been postulated that AGPs may promote the adhesion and association of cells with other molecules [see Egertsdotter and Arnold (1995), and references therein]. It has also been suggested that AGPs may be the source of oligosaccharide signals that are involved in development and differentiation [see Egertsdotter and von Arnold (1998), and references therein].

**Chitinases** (EC 3.2.1.14) are enzymes that are found in plants, fungi and bacteria and, they are involved in pathogen defense, plant-microbe interactions, abiotic stress responses and developmental aspects of plants (e.g. embryogenesis and programmed cell death) (Collinge *et al.*, 1993;

Kasprzewska, 2003; Wiweger *et al.*, 2003; Grover, 2012). Chitinases catalyze the hydrolysis of glycosidic bonds which are present in biopolymers of N-acetylglucosamine, mainly in chitin and also the deacetylated form of chitin, which is known as chitosan. The alternative substrates for chitinases include lipochitooligosaccharides, peptidoglycan, glycoproteins and arabinogalactan proteins containing N-acetylglucosamine (Kasprzewska, 2003; Grover, 2012). Chitinase proteins have a primary structure which comprises of two domains, an *N*-terminal chitin-binding domain (CBD) and a catalytic domain at the C-terminal (Raikhel *et al.*, 1993; Kasprzewska, 2003). The CBD consists of a highly-conserved, cysteine-rich region of approximately 40 amino acid residues whereas the catalytic domain consists of about 200-230 amino acid residues. Based on sequence similarity, chitinases are divided into seven classes (Class I-VII) and two families [glycosyl hydrolase family 18(GH18) and glycosyl hydrolase family 19 (GH19)] [see Ellis *et al.* (2010), and references therein].

There is a wealth of data supporting the view that chitinases stimulate the development of embryos in plants. An experiment with cell cultures of carrot (*Daucas carota*) found that the wild type cultures secreted a 32-kD endochitinase into the culture medium. In the same study, the addition of the endochitinase to the medium of a temperature-sensitive (ts) carrot cell mutant (*ts11*) which is arrested at the globular stage, promoted the transition from globular to heart-shaped stage under non-permissive temperature conditions (De Jong *et al.*, 1992). In *Arabidopsis*, expression of the chitinase gene (*AtchitIV*) was detected in leaves in response to abiotic stresses and in zygotic embryos from torpedo stage until full maturation (Gerhardt *et al.*, 2004). Based on the expression pattern of the *AtchitIV* gene, it was suggested that this particular *Arabidopsis* chitinase is involved in plant defense and embryogenesis (Gerhardt *et al.*, 2004). In Norway spruce, a class IV chitinase gene (*Chia4-Pa*) was found to be up regulated upon the withdrawal of PGRs in embryogenic cultures. Accordingly, it was suggested that *Chia4-Pa* regulates the differentiation of somatic embryos from PEMs by promoting programmed cell death (Wiweger *et al.*, 2003).

# 2 Aim and objectives

The overall aim of this thesis was to identify and characterize metabolic and biochemical events that are involved in regulation of embryo development in Norway spruce. The objectives were to:

- Identify the metabolic changes during embryo development.
- Establish the metabolic events which are necessary for normal development of embryos.

After the metabolic study (Paper I), it was found that elevated levels of endogenous fructose were associated with abnormal and blocked development of embryos in embryogenic cultures of Norway spruce. Therefore, we investigated the regulatory role of fructose during development of Norway spruce embryos. Furthermore, we examined the Maillard reaction**** as a possible mechanism through which fructose may inhibit embryo development (Paper III).

Finally, we also examined the biochemical effect of carbohydrates and osmoticum during maturation and germination of embryos of Norway spruce (Paper II).

****The Maillard reaction is described in chapter **3.1.1**

# 3 Material and methods

This section provides an overview of the plant material, experimental design and the principles of the methods which were used in paper I, II and III. The procedures on how the methods were used to perform the experiments are presented in the methods section of each paper.

## 3.1 Plant material and Experimental design

**Plant material and experimental design for paper I and II**

Three embryogenic cell lines of Norway spruce (denoted as: 09:73:06, 06:28:05 and 06:22:02) were used to perform the gas chromatography–mass chromatography (GC-MS)-based metabolite profiling experiment. The first two digits of each cell line represent the year in which the cell line was established. All three cell lines were initiated from seeds from elite crossings at the Forest Research Institute in Sweden (Skogforsk) and they are not genetically related to each other. For proliferation, the embryogenic cultures of each cell line were sub-cultured with a two week interval on solidified half-strength LP medium supplemented with 9.0 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 4 µM $N^6$-benzyladenine (BA) as the plant growth regulators (von Arnold & Eriksson, 1981). Subsequently, the cultures were transferred to half-strength LP medium lacking PGRs for one week to stimulate the differentiation of early embryos from PEMs. For further development and maturation of embryos, the cultures were transferred to DKM medium (Krogstrup, 1986) supplemented with ABA (29.0 µM) for eight weeks. Samples of embryogenic cultures were collected at the end of four developmental stages denoted as: (i) proliferation; (ii) embryo differentiation; (iii) late embryogeny; (iv) maturation (Fig. 4). The aforementioned developmental stages were observed and photographed using a Zeiss AX10 microscope and Zeiss STEMI 2000-C microscope equipped with a Canon PowerShot G9 camera (Canon, Japan). All samples were subjected to GC-MS analysis and significant metabolites at each developmental stage were determined using the multivariate data analysis approaches in section 3.2.1.

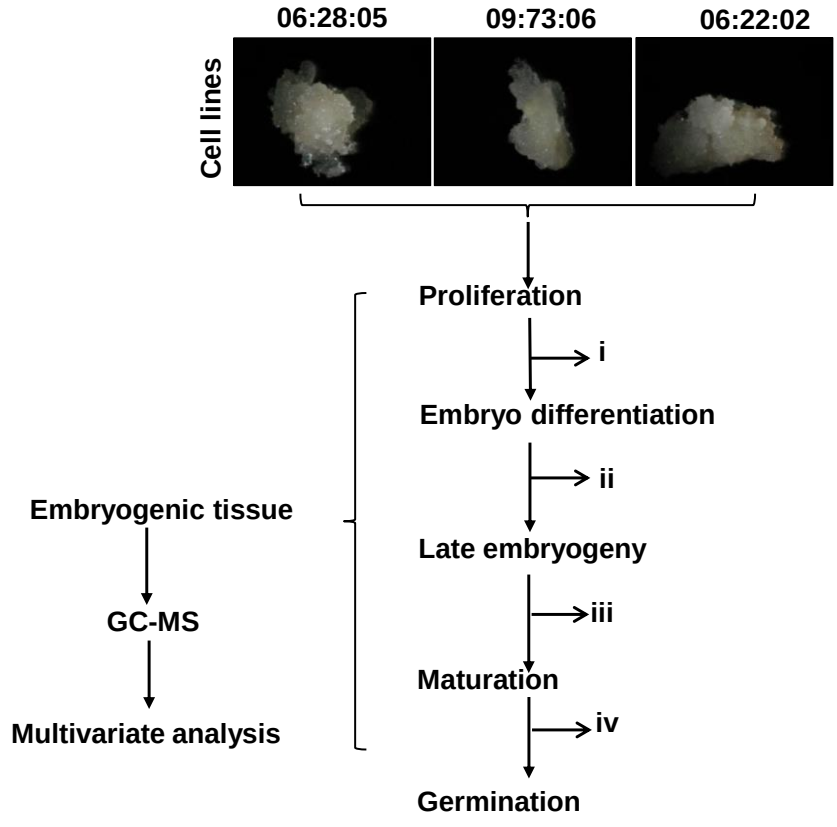


**Figure 4.** Overview of the experimental design for paper I. Embryogenic samples for (GC-MS)-based metabolite profiling were collected at time points: i, ii, iii and iv. The time points correspond to the end of PEM proliferation, embryo differentiation, late embryogeny and maturation.

In the study described in paper II, two embryogenic cell lines of Norway spruce denoted as 09.77.17 and 09.77.03 were used to investigate the biochemical effect of carbohydrates and osmoticum during maturation and germination of embryos. During PEM proliferation and early embryo differentiation, the embryogenic cultures were grown on half-strength medium containing and lacking PGRs (2, 4-D and BA), respectively. For late embryogeny and maturation, the cultures were grown on DKM medium containing: (I) 3% (w/v) sucrose; (II) 3% (w/v) maltose and 7.5% (w/v) PEG (Fig. 5). The mature somatic embryos from each treatment were used to evaluate the accumulation of storage reserves, and the maturation and germination frequencies of the embryos.

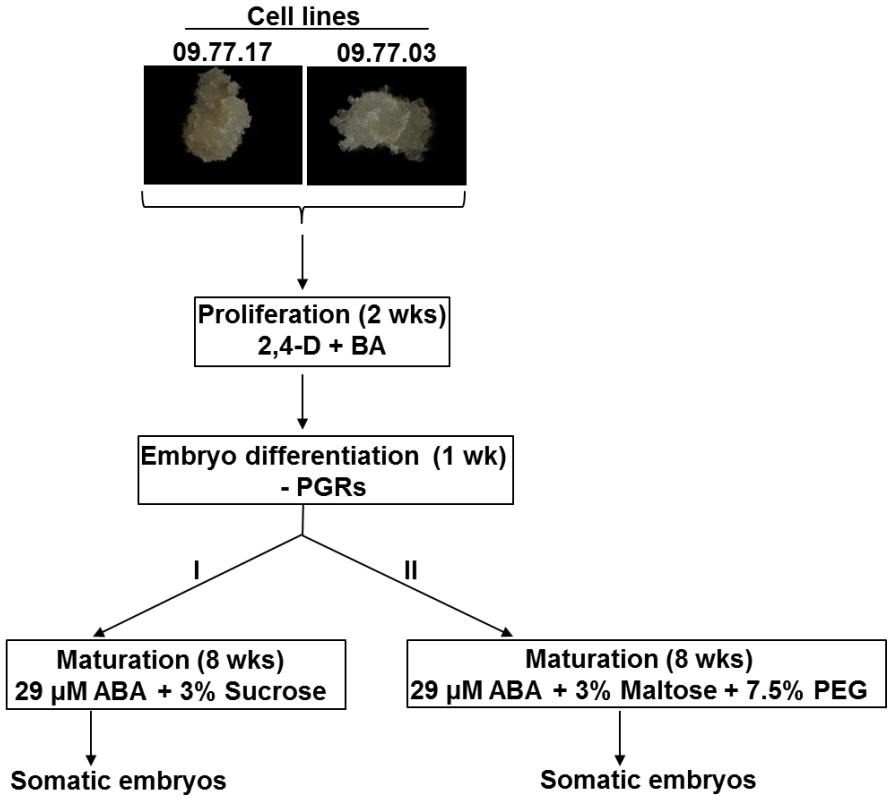


**Figure 5.** Overview of the experimental design for paper II. The cell lines were subjected to two maturation treatments referred to as: (I) 3% sucrose and (II) 3% maltose + 7.5% PEG. The mature embryos from each treatment were used for analyzing storage reserves, embryo maturation and germination frequencies.

### 3.1.1 Experimental back ground (paper III)

### 3.1.2 The Maillard reaction

In 1912, Louise-Camille Maillard, a French chemist and physician with an interest in the interactions between amino acids and sugars discovered that a reaction between an amino acid and a reducing sugar resulted in the manifestation of a brown colour. This reaction would later be named the Maillard reaction. The Maillard reaction (Fig. 6) involves a sequence of chemical reactions which are initiated by a reaction between a reducing sugar and amino groups in proteins, nucleic acids and lipids. The end products of the reaction are known as advanced glycation end-products (AGEs) or advanced Maillard products (Fig. 6) (Monnier, 1990; Schalkwijk *et al.*, 2004).

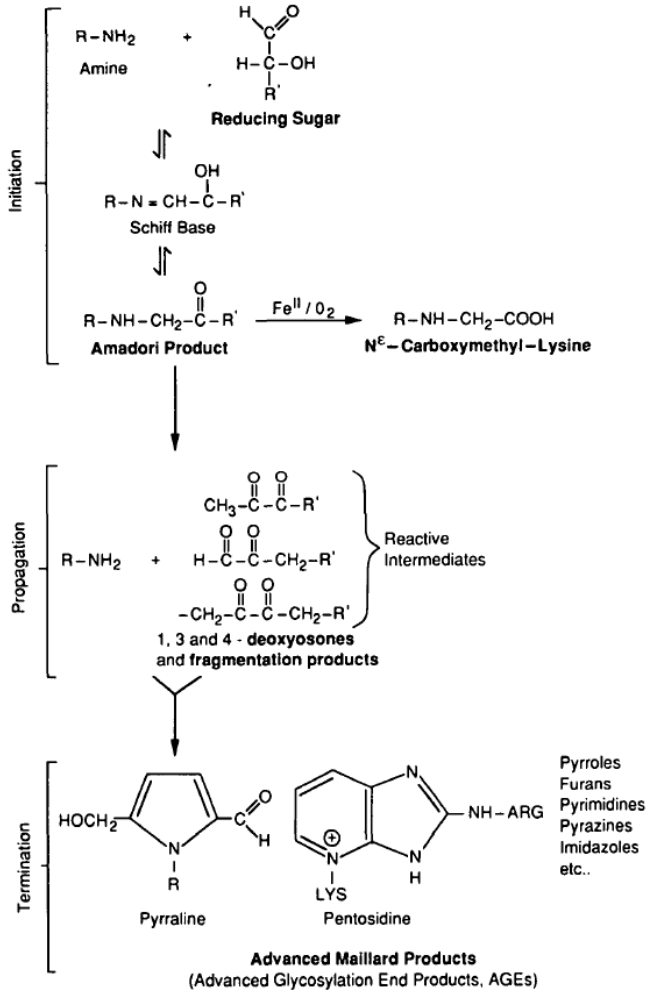


**Figure 6.** General scheme of the Maillard reaction. The initial step is generally referred to as nonenzymatic glycosylation or glycation. Reproduced with permission from Oxford University Press: Monnier (1990), *Journal of Gerontology: Biological Sciences* 45, B106.

### 3.1.2.1 Basic chemistry of the Maillard reaction

The Maillard reaction (glycation or non-enzymatic glycosylation) occurs in three sequential steps which may be referred to as initiation, propagation and termination (Fig. 6) (Monnier, 1990). The first step involves non-enzymatic condensation between the carbonyl group of a reducing sugar and a free amino group to form a Schiff base or glycosylamine. Structurally, Schiff bases are characterized by a functional group which contains a carbon-nitrogen double bond. Additionally, the nitrogen atom is connected to an alkyl or aryl group but

not a hydrogen atom. For that reason, Schiff bases are chemically unstable and undergo isomerization to form a stable Amadori or Heyns product (Fig. 6). The formation of an Amadori or Heyns product depends on whether the reducing sugar involved in the reaction is an aldose or ketose respectively (Monnier, 1990; Ames, 1992; McNaught & Wilkinson, 1997; Ojala *et al.*, 2000).

In the second step of the Maillard reaction, the stable protein Amadori or Heyns product is fragmented to form highly reactive intermediates (Monnier, 1990; Dills, 1993; Strelec *et al.*, 2008). Next, the reaction is terminated by the formation of advanced Maillard or glycation end-products (AGEs) (Fig. 6). Most AGEs are active under ultraviolet (UV) light and exhibit browning, fluorescent and polymeric characteristics (Monnier, 1990; Dills, 1993). A key feature of the Maillard reaction is that it causes a loss of amino acid residues and reduction in protein digestibility which in turn leads to deterioration of protein quality and irreversible molecular damage (Monnier, 1990; Dills, 1993).

Since its discovery, most studies have focused on the role of the Maillard reaction in food processing [reviewed by Martins *et al.* (2000) and Friedman (2005)] and age-related diseases such as: diabetes (Chevion *et al.*, 2000), Alzheimer's disease and rheumatoid arthritis (Berlett & Stadtman, 1997). Nevertheless, the Maillard reaction has also been implicated in developmental aberrations in plants such as: loss of seed vigor, lipid peroxidation and browning of seed coats in Soybean (*Glycine max*), Mung bean (*Vigna radiata*) and snap bean (*Phaseolus vulgaris*), respectively (Sun & Leopold, 1995; Murthy & Sun, 2000; Taylor *et al.*, 2000).

In the study presented in Paper I, we found that cell lines of Norway spruce with elevated levels of endogenous fructose also exhibited abnormal and blocked development of embryos. Moreover, the blocked cell line also exhibited severe browning of tissue (Businge *et al.*, 2012) which has also been associated with the Maillard reaction in seeds of snap bean (*Phaseolus vulgaris*) (Taylor *et al.*, 2000). Therefore, we examined the Maillard reaction as a possible mechanism through which fructose may inhibit development of embryos in Norway spruce (paper III). For this study, we used two embryogenic cell lines (denoted as 09.73.06 and 09.77.03) of Norway spruce. Both cell lines had previously been found to exhibit normal development of embryos when grown on half-strength LP and DKM medium containing sucrose (Businge *et al.*, 2012; Businge *et al.*, 2013). It should be noted that since glucose is the most prevailing endogenous and exogenous monosaccharide in living organisms, most glycation related studies have focused on the reaction between glucose and the amino acids of biomolecules (Levi & Werman, 2001; Semchyshyn *et al.*, 2011). Therefore, we monitored

changes in protein fluorescence, a marker of the Maillard reaction (Bosch *et al.*, 2007), in two cell lines of Norway spruce which were grown on media containing sucrose (control), glucose or fructose. Furthermore, the changes in DNA damage, fructose, glucose, glutathione (GSH) and protein carbonyl content during embryo development were analyzed by biochemical assays.

During proliferation, the cultures were grown on half-strength LP medium containing 29.2 mM of sucrose, glucose or fructose. Thereafter, the cultures were transferred to DKM medium containing 87.6 mM of sucrose, glucose or fructose. For biochemical assays (Chapter 3.3), embryogenic samples were collected at the start of the experiment (week zero). Thereafter samples were collected at the end of PEM proliferation (week two), early embryogeny (week three), late embryogeny (week seven) and maturation (week eleven). These developmental stages were also observed and photographed using a Zeiss AX10 microscope and Zeiss STEMI 2000-C microscope equipped with a Canon PowerShot G9 camera (Canon, Japan).

The samples for all experiments were flash frozen in liquid Nitrogen and stored at $^{-}$ 80$^{o}$C until the analyses were performed. Before analysis, the samples were homogenized by shaking with a stainless bead using a bead mill (MM400, Retsch GmbH, Germany) at a frequency of 30 Hz for 2-3 minutes.

## 3.2 Analytical aspects of metabolomics

Biological systems contain a lot of complex information which can be analyzed using different omics strategies such as: proteomics (protein translation), transcriptomoics (gene expression) and metabolomics (metabolic networks) (Fiehn, 2002). Metabolomics involves comprehensive qualitative and quantitative analysis of the low molecular weight molecules (metabolites) in a biological system (metabolome). Metabolites such as amino acids, fatty acids, carbohydrates and lipids are produced by different cellular processes, and their levels can be used to elucidate the regulation of developmental events. Furthermore, changes in levels of metabolites can be used as indicators of plant responses to biotic and abiotic events (Fiehn, 2002; Dunn & Ellis, 2005; Dettmer *et al.*, 2007). The results presented in Paper I were obtained using a metabolomics approach known as metabolite profiling. Metabolite profiling involves the identification and quantification of selected metabolites which are related to a particular metabolic pathway (Dunn & Ellis, 2005). Gas chromatography coupled with mass spectrometry (GC-MS) is a powerful analytical method for plant metabolite profiling (Fiehn *et al.*, 2000). In particular, GC-MS offers high chromatographic peak resolution, sensitivity and mass spectral libraries for identification of metabolites (Sumner *et al.*, 2003;

Dettmer *et al.*, 2007). For the study in paper I, we used GC-MS to perform metabolic profiling of embryogenic tissue from cell lines of Norway spruce. Therefore, the next section will provide an overview of the steps for GC-MS based metabolite profiling in plants:

**Extraction and derivatisation of metabolites:** the metabolites are extracted from 20-25 mg fresh weight (FW) of plant tissue using an extraction mixture consisting of chloroform, methanol and water (6: 2: 2). The extraction mixture also contains stable isotope reference compounds (internal standards) which are used for normalization of metabolomics data (Bravo *et al.*, 2002; Schauer *et al.*, 2005). Prior to GC-MS analysis, the metabolites are derivatised in order to increase their volatility and thermal stability. Derivatisation is performed by methoxyamination and trimethylsilyation using methoxyamine hydrochloride in pyridine and *N*-methylsilyltrifluoroacetamide (MSTFA) respectively (Kopka *et al.*, 2004; Dettmer *et al.*, 2007).

**Gas chromatography (GC)** involves gaseous separation of the components of a mixture. During GC, a known volume of the sample is injected into a sample inlet leading into a heated hollow tube referred to as the column (Fig. 7; part 5). The column contains a thin layer of non-volatile material (stationary phase) which is coated onto the walls of the column. A carrier gas (mobile phase) moves the vaporized sample from the injection inlet into the column. Inert gasses such as helium and argon are used as carrier gasses to avoid interactions between the sample and mobile phase, and adsorption by the stationary phase. The components of the sample are separated depending on how they interact with the stationary phase. For instance, components that don't interact with the stationary phase will emerge first from the column. Conversely, components with greater solubility in the stationary phase will be retained longer and emerge from the column at a later time. Accordingly, the time that a substance requires to move through the column is known as its retention time (RT) (Kitson *et al.*, 1996; Watson & Sparkman, 2007).

**Mass spectrometry (MS)** is an analytical technique that provides the information for identifying and determining the content of the analyte. The mass spectrometer used in paper I was a time-of-flight (TOF) mass spectrometer. Time-of-flight mass spectrometry is based on measuring the time that an ion requires to travel from an ion source to a detector. Figure 7 illustrates the major components of the mass spectrometer; i.e., ion source (for generating the gas-phase ions), mass analyzer (mass separator; TOF) and a detector. During mass spectrometry, the effluent from the column is fed into the ion source and ionized by electron impact (EI). Next, the ions are discharged with similar kinetic energy and directed towards the mass analyzer.

Due to differences in their mass-to-charge ratio (*m/z*), the ions will travel at different velocities resulting into their separation. A detector situated at the end of the mass analyzer records the number of ions for a given *m/z* value and produces a graphical depiction (mass spectrum) of the ions. On the mass spectrum, the x and y axes represent the mass-to-charge and intensity scales respectively (Kitson *et al.*, 1996; Kopka *et al.*, 2004; Watson & Sparkman, 2007).

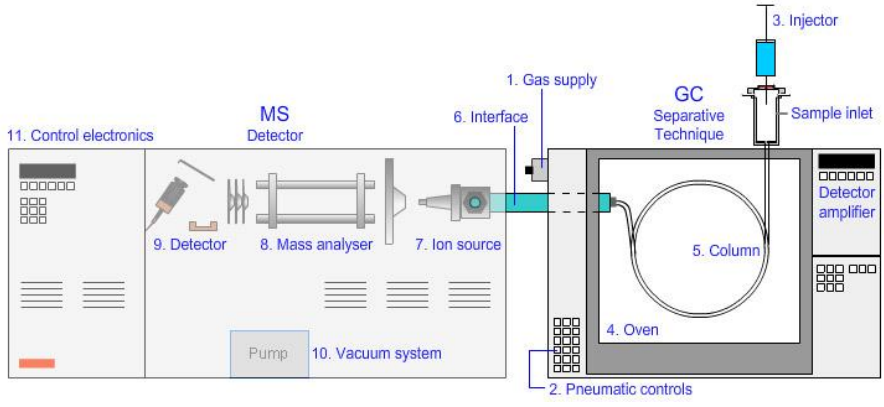


**Figure 7.** Setup of a GC/TOFMS system. Reproduced with permission from CHROMacademy (www.chromacademy.com).

**Data export and pre-processing:** the goal of data export is to convert GC-MS data into a standard and instrument independent file format. In contrast, pre-processing is aimed at obtaining a list of detected peaks with peak areas for quantification and mass spectra for indentification of compounds (Fiehn, 2008). The pre-processing procedures include:

(i) Baseline correction - involves the correction of inaccuracies within the data due to systematic drift or misalignment between samples.

(ii) Peak alignment - is aimed at ensuring that peaks from the same metabolite appear at the same retention time.

(iii) Deconvolution – is aimed at detecting and resolving co-eluted peaks into pure peaks that give peak areas and mass spectra (Dunn & Ellis, 2005; Liland, 2011; Thysell *et al.*, 2012).

**Indentification of metabolites** is performed by using a database search engine to compare the sample spectra with spectra of analytes in a library. The National Institute of Standards (NIST) mass spectral (MS) search program (version 2.0) was used to perform the metabolite searches in paper I. The searches were performed in the: Umeå Plant Science Center (UPSC), Max

Planck Institute and NIST98 MS libraries. Figure eight shows an identity search result of a metabolite (4-Aminobutyric acid) obtained using the NIST MS search program. In order to confirm the search as a hit, the sample and library spectrum must be identical to each other. Furthermore, a numerical value (retention index) is used to indicate whether the sample and library compounds match each other. The retention index (RI) is defined as the retention time of a compound after it has been normalized to the retention times of adjacently eluting *n*-alkanes (Kovats, 1958; Desbrosses *et al.*, 2005; Watson & Sparkman, 2007).

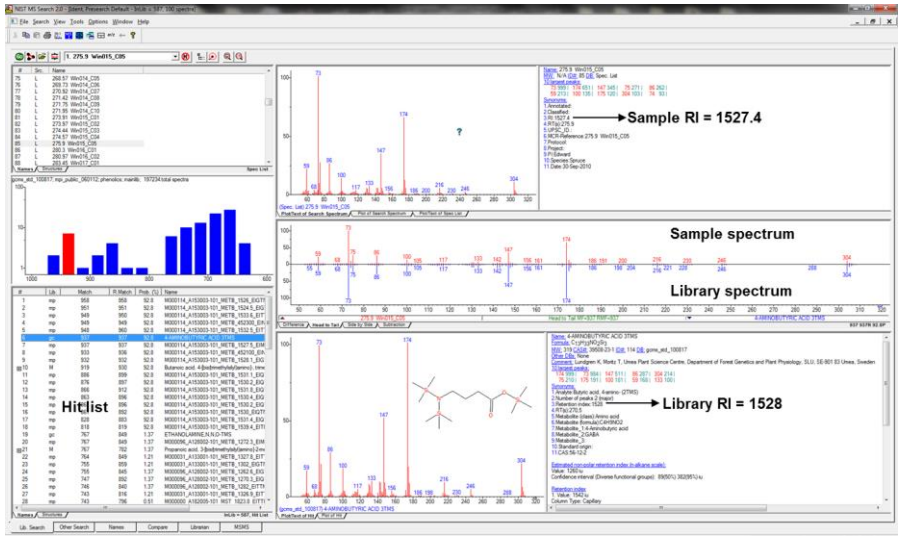


**Figure 8.** The National Institute of standards (NIST) mass spectra search program library tab. The hit list (bottom left corner) consists of library spectra that closely match the sample spectrum. The hits are arranged in descending order according to their *m/z* values. Visual comparison is performed by checking how well the sample and library spectrum match each other. Furthermore, the spectra match is assessed by comparing the retention index (RI) of the sample spectrum to that of the library hit. In this case, the sample and library spectrum closely match each other. Additionally, the retention index (RI) of the sample (1527.4) closely matches that of the library hit (1528). Therefore, the identity search is confirmed as 4-Aminobutyric acid.

### 3.2.1 Multivariate analysis for metabolomics data

The data obtained using GC-MS is massive in size (mostly in gigabytes), complex and comprises of numerous variables. Such data is difficult to analyze using the traditional univariate statistical approaches. In view of that, multivariate analysis (MVA) software comprises of various statistical

approaches which can be used to analyze data with more than one variable. Therefore, MVA approaches are ideal for extracting useful information from GC-MS data. The multivariate projection methods used in papers I and II were principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) (Eriksson *et al.*, 2001; Bylesjö *et al.*, 2006).

**Principal component analysis (PCA)** is an unsupervised method for summarizing and simplifying multivariate data. The term unsupervised indicates that PCA is performed without using any pre-known information about the data (Madsen *et al.*, 2010; Liland, 2011). Much of PCA concerns itself with finding new variables called principal components (PCs) that describe the bulk of the variation in the data. The PCs make it possible to describe the data using fewer variables than was present in the beginning. Therefore, the operating principle of PCA is that the first principal component (PC1) describes the largest variation in the data. The second principal component (PC2) which is also orthogonal to PC1 describes some of the remaining variation in the data. Once the PCs have been specified, the observations (samples) are projected onto them and the co-ordinate values or scores of each observation along PC1 and PC2 are obtained. When the score vectors are plotted against each other; the groups, trends, patterns and outliers within the observations can be graphically illustrated using a scores plot in which each dot represents a sample (Davies & Fearn, 2004; Liland, 2011). Figure nine shows a score plot for embryogenic samples of a Norway spruce collected at different stages of somatic embryogenesis.

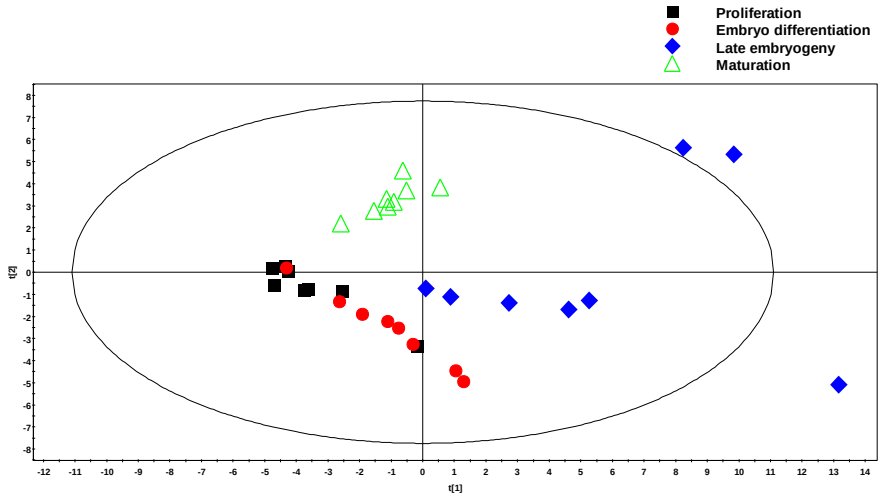


**Figure 9.** Principal component analysis (PCA) score scatter for SE samples of Norway spruce. The samples are colour coded according to developmental stage. Samples with similar characteristics are usually grouped together. The three samples (blue) outside of the circle are referred to as outliers. The orthogonal lines *t1* and *t2* represent the first and second principal components respectively.

**Orthogonal projections to latent structures discriminant analysis (OPLS-DA)** is a supervised method which is used to distinguish between sample classes such as: wild type and mutant, proliferating and early embryogeny samples. The term supervised indicates that OPLS-DA is performed using pre-known information about the data. During OPLS-DA, a regression model is computed between a response variable (class) and the multivariate data. The OPLS-DA approach utilizes a single component (predictive component) for class prediction. The predictive component describes the variation between the classes. Therefore, when all the samples are projected onto the predictive component, their predictive scores can be obtained. Consequently, the separation between sample classes can be illustrated using an OPLS-DA score scatter (Fig. 10) and the variables (metabolites) causing the separation of the samples can be indentified from the corresponding loadings plot. The loadings represent the weights of the original variables which are used to obtain the component scores (Trygg & Wold, 2002; Bylesjö *et al.*, 2006; Wiklund *et al.*, 2008; Westerhuis *et al.*, 2010).

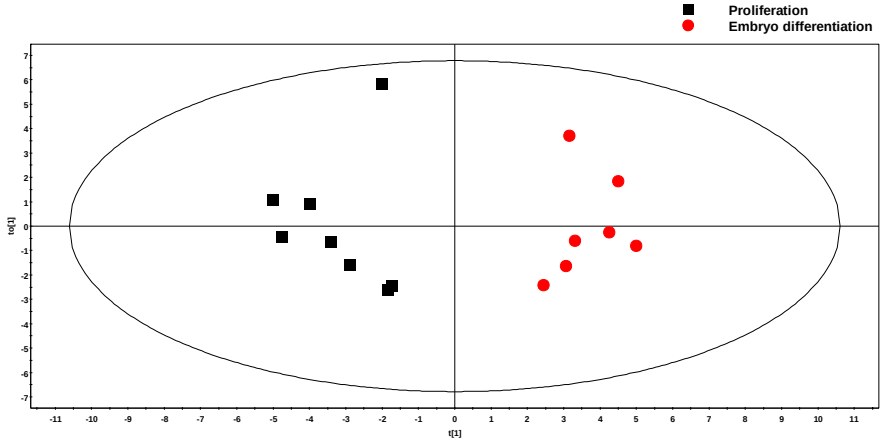


**Figure 10.** An OPLS-DA score scatter showing the separation of two Norway spruce sample classes. In OPLS-DA, the samples are separated along a discriminatory direction *t(1)* which makes it easy to identify the variables (metabolites) responsible for the sample separation.

## 3.3 Biochemical assays

### 3.3.1 Enzymatic analysis of sugars

In the studies described in paper II and III, the content of sugars in mature embryo and embryogenic samples of Norway spruce was determined by enzymatic digestion of glucose, fructose and sucrose (Stitt *et al.*, 1989). Enzymatic quantitation of sugars utilizes enzymes that catalyze specific reactions and a calorimetric method for measuring the concentration of the reaction products. The enzymatic reaction is supported by a reaction buffer containing: adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and glucose-6-phosphate dehydrogenase (G6PDH) (Brummer & Cui, 2005).

**Glucose (Glc)** content is determined by adding hexokinase (HK) to the reaction mixture. In the presence of ATP, hexokinase phosphorylates the glucose in the sample to form glucose-6-phosphate (G6P) and adenosine diphosphate (ADP) (Eq. 1). Subsequently, glucose-6-phosphate reacts with G6PDH to form 6-phosphogluconate (6PG) and $NADP^+$ is reduced to NADPH in the same reaction (Eq. 2).

$$Glc + ATP \xrightarrow{HK} G6P + ADP \quad (\textbf{Eq. 1})$$

$$G6P + NADP^{+} \xrightarrow{G6PDH} 6PG + NADPH \quad (\textbf{Eq. 2})$$

**Fructose (Fru)** content is determined by adding phosphoglucose isomerase (PGI) to the reaction mixture. In the presence of ATP, hexokinase phosphorylates the fructose in the sample to form fructose-6-phosphate (F6P) (Eq. 3). When PGI is added to the reaction mixture, fructose-6-phosphate is isomerized to glucose-6-phophate (Eq. 4). Subsequently, G6PDH oxidizes glucose-6-phosphate to form 6-phosphogluconate (6PG) and $NADP^{+}$ is concomitantly reduced to NADPH (Eq. 5).

$$Fru + ATP \xrightarrow{HK} F6P + ADP \quad (\textbf{Eq. 3})$$

$$F6P \xrightarrow{PGI} G6P + ADP \quad (\textbf{Eq. 4})$$

$$G6P + NADP^{+} \xrightarrow{G6PDH} 6PG + NADPH \quad (\textbf{Eq. 5})$$

**Sucrose (Suc)** content is determined by adding invertase (Inv) to the reaction mixture. When invertase is added to the reaction mixture, the sucrose in the sample is hydrolyzed to fructose (Fru) and glucose (Glc) (Eq. 6). Fructose and glucose are then converted to fructose-6-phosphate and glucose-6-phosphate by hexokinase and phosphoglucose isomerase (Eq. 6). The glucose-6-phosphate is subsequently oxidized to 6-phosphogluconate by G6PDH while $NADP^{+}$ is reduced to NADPH (Eq. 7).

$$Suc \xrightarrow{Inv} Fru + Glc \xrightarrow[PGI]{HK} F6P + G6P \quad (\textbf{Eq. 6})$$

$$G6P + NADP^{+} \xrightarrow{G6PDH} 6PG + NADPH \quad (\textbf{Eq. 7})$$

After addition of each enzyme, the reaction is allowed to proceed and the concentration of NADPH is spectrophotometrically measured at a wave length of 340 nm. In all cases, the amount of NADPH is stoichiometric with the content of glucose, fructose and sucrose in the sample. Consequently, sample absorbance maxima at 340 nm are converted to µg of sugar using a glucose

standard curve (Hendriks *et al.*, 2003; Brummer & Cui, 2005; Smith & Zeeman, 2006).

### 3.3.2 Protein carbonyl assay

In the study presented in paper III, the carbonyl content of proteins was determined using a protein carbonyl assay (Cayman Chemical Company, Item No. 10005020). Protein carbonylation involves oxidative modification of proteins through the introduction of carbonyl groups such as ketones and aldehydes on the side chains of the proteins (Levine *et al.*, 2000; Dalle-Donne *et al.*, 2003; Suzuki *et al.*, 2010). Therefore, the protein carbonyl assay utilizes a carbonyl reagent (2, 4-dinitrophenylhydrazine, DNPH) to derivatize the carbonyl group which results in the formation of a Schiff base and a corresponding stable 2, 4-dinitrophenyl (DNP) hydrazone. The concentration of the stable hydrazone (2, 4-dinitrophenyl DNP) is spectrophotometrically measured at 370 nm (Levine *et al.*, 1994; Levine *et al.*, 2000; Dalle-Donne *et al.*, 2003). Eventually, the carbonyl content of proteins is determined using the following equation:

$$\text{Protein Carbonyl (nmol/ml)} = [(\text{CA})/ (*0.11\ \mu M^{-1})]\ (500\ \mu l/200\ \mu l))$$

***Where***

- CA is the corrected absorbance of the sample.
- 0.11 $\mu M^{-1}$ is the adjusted extinction coefficient for DNPH at 370 nm.
- 500 µl is the amount of guanidine hydrochloride used to resuspend the samples.
- 200 µl is the amount of sample used at the start of the assay.

### 3.3.3 Glutathione (GSH) assay

Glutathione (ɣ-glutamylcysteinylglycine) is a low molecular weight thiol and a major antioxidant in plant and animal cells (Noctor *et al.*, 2012). In the study presented in paper III, the content of glutathione in the control and test samples was determined using a glutathione assay (Cayman Chemical Company, Item No. 703002). Prior to the assay, the sample extracts are deproteinated to prevent interferences from particulates and sulfhydryl groups on the proteins. Cayman's GSH assay uses a recycling method involving glutathione reductase to quantify glutathione. During the assay, the sulfhydryl group of GSH reacts with DNTB (5, 5'-dithio-*bis*-2-(nitrobenzoic acid) to produce a yellow compound (TNB; 5-thio-2-nitrobenzoic acid) and a mixed disulfide (GSTNB) between GSH and TNB. Subsequently, GSTNB is reduced by glutathione

reductase to recycle GSH and produce more TNB. The rate of TNB production is directly proportional to the recycling reaction which in turn is proportional to the concentration of GSH in the sample. Therefore, measurement of the absorbance of TNB at 405-414 nm gives a precise estimation of the GSH in the sample. The concentration of GSH in the sample is determined using the following end point method equation:

Total GSH = [(Absorbance at 405-414 nm) – (Y-intercept)/Slope] x 2* x SD

***Where***

- Y-intercept and slope are derived from the GSH standard curve.
- 2* is the adjustment for sample deproteination with metaphosphoric acid (MPA).
- SD is sample dilution.

### 3.3.4 DNA damage-AP (apurinic/apyrimidinic) sites assay

An abasic (AP) site is a location within DNA without a purine and pyrimidine base. Abasic sites are indicative of DNA damage caused by chemical modification of bases, repair of oxidized bases and free radicals (Kubo *et al.*, 1992; Boiteux & Guillet, 2004). To investigate the relevance of the Maillard reaction to blocked development of embryos (Paper III), we used a DNA damage-AP site calorimetric assay (Abcam, ab65353) to check for abasic sites in the DNA of control and test samples of Norway spruce. The assay utilizes an aldehyde reactive probe (ARP) to detect AP sites in DNA. The ARP reagent reacts with aldehyde groups, and tags the AP sites with biotin residues. The number of biotin-tagged AP sites is then quantified calorimetrically by an enzyme-linked immunosorbent assay (ELISA)-like assay using avidin-biotin coupled with horse radish peroxidase (HRP)-Streptavidin (Kubo *et al.*, 1992).

# 4 Results and discussion

The studies described in this thesis address some fundamental aspects of embryo development in plants. In particular, the studies seek to understand how metabolic and biochemical events regulate the development of embryos in Norway spruce. Norway spruce is of particular interest because it is the most dominant and economically important coniferous species in northern and central Europe (Svobodova *et al.*, 1999). Therefore, a detailed understanding of the regulation of embryo development in Norway spruce is necessary in order to develop clonal propagation and breeding programs for trees with elite traits.

We used somatic embryogenesis as a model system for studying the regulation of embryo development because of the close similarities between somatic and zygotic embryogenesis in Norway spruce (Suarez *et al.*, 2004). The studies in Papers I, II and III were performed using a total of five embryogenic cell lines of Norway spruce. It should be noted that cell lines of Norway spruce are characterized into two types: A and B. The characterization is based on the morphology of the somatic embryos and capability of the cell lines to form embryos (Egertsdotter *et al.*, 1993; Egertsdotter & Arnold, 1995). Morphologically, somatic embryos of type A cell lines are characterized by large embryonic regions comprised of densely packed cells. Type A embryos are capable of forming mature somatic embryos when treated with ABA (Egertsdotter & Arnold, 1995). In contrast, somatic embryos of type B cell lines are characterized by small embryonic embryos regions comprised of loosely packed cells. Type B embryos are incapable of forming mature somatic embryos when treated with ABA (Egertsdotter & Arnold, 1995). From an investigative perspective, the aforementioned differences in developmental capabilities between cell lines can be exploited to study the regulatory mechanisms of embryo development in Norway spruce.

## 4.1 Development of Norway spruce embryos (Paper I)

In order to provide a framework for studying the metabolic regulation of embryo development, we first used manual time-lapse photography to monitor morphological changes during development of somatic embryos in three cell lines (09:73:06, 06:28:05, 06:22:03) of Norway spruce (Fig. 11).

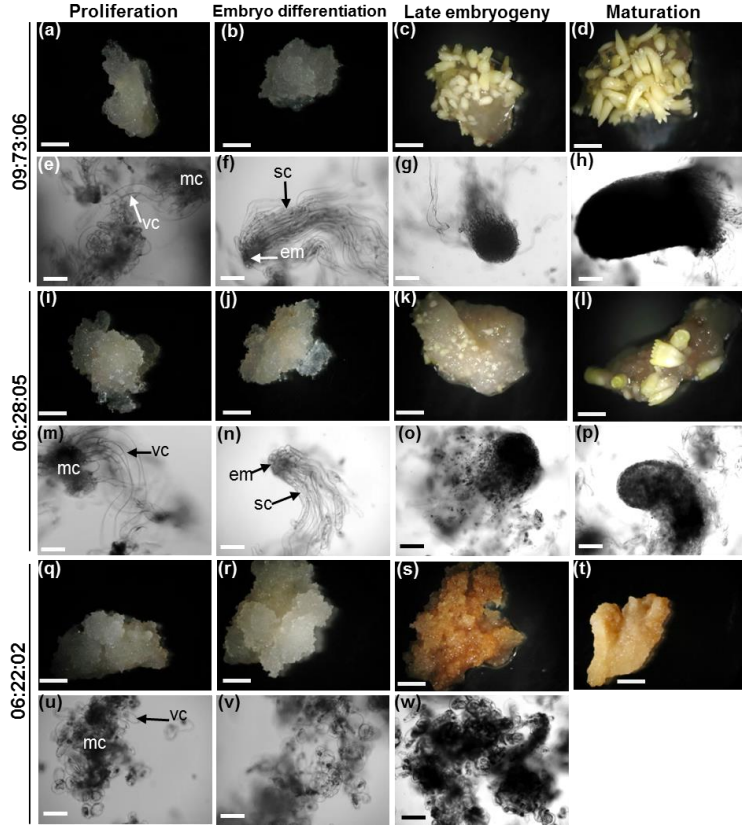


**Figure 11.** Development of somatic embryos of Norway spruce (*Picea abies*). Embryogenic cultures: cell lines 09:73:06 (top rows), 06:28:05 (middle rows) and 06:22:02 (bottom rows). (a, e, i, m, q, u) Proliferated embryogenic cultures after 2 weeks on medium with auxin and cytokinin. (b, f, j, n) Early embryos 1 week after withdrawal of PGRs. (r, v) Lack of embryos after withdrawal of PGRs. (c, g, k, o) Late embryogeny after 4 weeks on medium containing ABA. (s, w) Browning, tissue death and lack of late embryos after 4 weeks on medium containing ABA. (d, h) Fully mature cotyledonary embryos after 8 weeks on medium containing ABA. (l, p) Mature aberrant embryos with fused embryos after 8 weeks on medium containing ABA. (t) Lack of mature embryos after 8 weeks on medium containing ABA. mc, meristematic cells; vc, vacuolated cells; em, embryonal mass; sc, suspensor cells. Bars, 100 µm.

Development of embryos progressed from proliferation of PEMs to early embryos with a polar structure and fully mature cotyledonary embryos in cell line 09:73:06 (Fig. 11; top rows). The PEMs consisted of meristematic cells (mc) with a dense cytoplasm and vacuolated cells (vc) that were highly elongated (Fig. 11e). During embryo differentiation, the early embryos exhibited a polar structure comprised of an apical embryonal mass (em) and basal vacuolated suspensor cells (sc) (Fig. 11f). After 8 weeks on medium containing ABA, cell line 09:73:06 formed fully mature embryos with a normal set of split cotyledons (Fig. 11d). Thus, the progression of embryo development in cell line 09:73:06 was identical to normal development of embryos in Norway spruce as previously reported by Larsson *et al.* (2008). The 06:28:05 cell line developed aberrantly and formed embryos with fused cotyledons (Fig. 11l) whereas cell line 06:22:02 exhibited blocked development of embryos (Fig. 11t). Taken together, these observations indicate that 09:73:06 is a type A cell line while 06:28:05 and 06:22:02 are type B cell lines.

### 4.1.1 Metabolic regulation of embryo development

Samples for GC-MS-based metabolite profiling were collected over a period of eleven weeks covering four stages of embryo development including: PEM proliferation, embryo differentiation, late embryogeny and maturation. In total, we collected 72 samples from the three cell lines and detected 52 compounds within these samples. Eight of the compounds were unknown while the remaining 44 could be assigned metabolite identities. The identified metabolites were categorized as: amino acids and derivatives, carbohydrates, sugar alcohols, organic acids and other metabolites (Table 1 in Paper I).

By employing a combination of multivariate (PCA and OPLS-DA) and univariate (*t*-test) statistical approaches, we were able to determine significant metabolites at each of the aforementioned developmental stages for the three cell lines (Tables 2, 3 and 4 in Paper I). In order to explain the observed variations in embryo developmental patterns among the cell lines (Fig. 11), we focused on the unique metabolites at each developmental stage. By using this approach, we were able to highlight the relevance of specific metabolites to normal development of embryos in Norway spruce.

***Role of sucrose during proliferation of proembryogenic masses (PEMs)***

Sucrose was the only metabolite detected in samples from proliferating cultures of our normal cell line, 09:73:06 (Table 2 & Fig. 3 in Paper I). Sucrose is believed to have a positive effect on proliferation and maturation of somatic

embryos in conifers (Schuller & Reuther, 1993). Indeed, presence of endogenous sucrose during proliferation has been positively linked with the capability of cultures to develop normal mature embryos in embryogenic cell lines of Loblolly pine (*Pinus taeda*) (Robinson *et al.*, 2009). In addition, studies of Pullman and Buchanan (2008) with *P. taeda* revealed that sucrose contributes to the osmotic environment during early and late development of seeds. Another plausible link between sucrose and normal development of embryos is that by acting as a signaling molecule, sucrose may well induce the expression of genes which are involved in development programs such as the cell-division cycle (Riou-Khamlichi *et al.*, 2000; Rolland *et al.*, 2002).

***Embryo differentiation: an inhibitory role for fructose?***

During somatic embryogenesis, withdrawal of PGRs stimulates the differentiation of early somatic embryos from PEMs (Filonova *et al.*, 2000a). We found that fructose, threonic acid and unknown compound #7 were the shared compounds in the embryo differentiation samples of the abnormal (06:28:05) and blocked (06:22:02) cell line (Tables 3 & 4 in Paper 1). A comparison of the metabolite contents revealed relatively higher levels of fructose in the embryo differentiation samples of cell line 06:28:05 and 06:22:02 (Figs 4 & 5 in Paper I). At elevated levels, fructose is potentially detrimental as it can cause aberrations that lead to arrested development. Recently,Cho and Yoo (2011) showed that treatment of *Arabidopsis* seedlings with a high concentration of fructose induced premature developmental arrest in the form of repressed cotyledon expansion and inhibited growth of roots and hypocotyls. There is also evidence showing that fructose-grown cells of yeast (*Saccharomyces cerevisiae*) exhibit marked reduction in reproductive ability and enhanced mortality (Semchyshyn *et al.*, 2011). Based on the aforementioned studies, we hypothesized that accumulation of fructose during embryo differentiation may have led to abnormal and blocked development of embryos in cell line 06:28:05 and 06:22:02, respectively (Paper I). Also, since the Maillard reaction has previously been associated with browning of seed coats of *Phaseolus vulgaris* (Taylor *et al.*, 2000), we sought to examine whether the Maillard reaction mediates the inhibitory effect of fructose with regard to embryo development. These hypotheses were investigated in a subsequent study whose results are presented in paper III and discussed in chapter 4.2.

***Late embryogeny and maturation: roles of pinitol, 4-Aminobutyric acid, maltose and inositol***

Transfer of early embryos to medium supplemented with ABA and osmoticum prompts further development of embryos and maturation into cotyledonary embryos (Filonova *et al.*, 2000b; Stasolla *et al.*, 2002). More specifically, the combination of ABA and osmoticum prevents premature germination of embryos and promotes the accumulation of storage compounds (Kermode, 1990; Misra *et al.*, 1993). Recently,Vestman *et al.* (2011) studied the global changes in gene expression during the early stages (proliferation, early embryo differentiation, late embryogeny) of somatic embryo development in Norway spruce. The authors recognized osmotic stress adaptation events in the form of up-regulation of several osmotic stress response genes during the transition from early to late embryogeny. In our study, we detected similar adjustments at the metabolic level in the late embryogeny samples of the normal and abnormal cell lines (Tables 2 and 3 in Paper I). In particular, the normal cell line contained differential levels of two metabolites (pinitol and 4-Aminobutyric acid) which are associated with osmotic tolerance (Fig. 3 in Paper III). Pinitol (3-*O*-methyl-D-*chiro*-inositol) is a major plant soluble carbohydrate while 4-Aminobutyric acid (γ-gamma-Aminobutyric acid, GABA) is an amino acid derivative and product of polyamine catabolism (Guo & Oosterhuis, 1997; Shelp *et al.*, 1999; Dowlatabadi *et al.*, 2009). Pinitol and 4-Aminobutyric acid are known osmoprotectants and existing evidence suggests that these metabolites accumulate and perform osmoprotective functions in Douglas fir (Robinson *et al.*, 2007), loblolly pine (Pullman *et al.*, 2003), white spruce (*Picea glauca*) (Dowlatabadi *et al.*, 2009) and cultured cells of tomato (*Lycopersicon esculentum*) (Handa *et al.*, 1983). The osmoprotective role of pinitol and 4-Aminobutyric acid may be attributed to their ability to act as endogenous osmolytes which balance exogenous osmotic pressure thereby enabling plant cells to survive periods of osmotic stress (Vernon & Bohnert, 1992).

We also detected relatively high levels of maltose in the late embryogeny samples of the normal cell line (Fig. 3 in Paper I). Nørgaard (1997) and Tremblay and Tremblay (1991), working with embryogenic cultures of *Abies nordmanniana* and Red spruce (*Picea rubens*), reported that addition of maltose to the culture medium promoted the formation of a high number of somatic embryos respectively. Scott *et al.* (1995) suggested that the stimulatory effect of maltose is due to nutrient stress in the form of low hexose levels caused by slow hydrolysis of maltose resulting in restricted cellular carbon nutrition. Blanc *et al.* (2002) further suggested that a limited supply of hexoses

in the presence of maltose is perhaps the biochemical trigger that reorients metabolism towards somatic embryogenesis, via starch catabolism.

Inositol was the only compound with known identity that we detected in the maturation samples of the normal cell line (Table 2 in Paper I). Recent experimental evidence from our lab indicates a positive effect of inositol on maturation of Norway spruce embryos *in vitro*. Egertsdotter and Clapham (2011) found that embryos cultured on inositol exhibit more synchronized development and maturation compared to when cultured under standard conditions. These findings prompted the authors to propose that the positive effect of inositol is osmotic in nature, and that inositol induces an osmotic effect responsible for preventing precocious embryo germination and supporting synchronized development of embryos.

## 4.2 Fructose and glucose have an inhibitory effect on embryo development (Paper III)

The study in Paper III seeks to establish whether the Maillard reaction may contribute to abnormal and blocked development of embryos that was previously found to be associated with fructose accumulation in the early embryos (Businge *et al.*, 2012). In this study, we monitored the changes in protein fluorescence, a marker of the Maillard reaction (Bosch *et al.*, 2007) in embryogenic cultures of Norway spruce which were grown on media containing sucrose (control), fructose or glucose. Initially, we found that the sucrose-grown cultures exhibited normal development of embryos while the fructose- and glucose-grown cultures did not develop mature embryos (Figs 1, 2 and S1in Paper III). In addition, the fructose-grown cultures exhibited significant increases in endogenous levels of fructose throughout the growth period (Fig. 3a, b in Paper III). In contrast, the glucose- and sucrose-grown cultures exhibited significant increases in endogenous levels of glucose throughout the growth period (Fig. 3c, d in Paper III). One question that arises from the above findings is what is/are the underlying mechanism/s of the apparent inhibitory effect from fructose and glucose on embryo development?

### 4.2.1 Relevance of the Maillard reaction to blocked development of embryos in Norway spruce

Measurements of protein fluorescence at different excitation and emission wave lengths have previously been used as an index of protein modification by Amadori and Maillard reactions during seed storage (Sun & Leopold, 1995; Murthy & Sun, 2000; Murthy *et al.*, 2003). Thus, we also indirectly examined the modification of proteins through the Maillard reaction by measuring the

fluorescence of the desalted proteins from the cultures grown on media containing sucrose, glucose or fructose. For both cell lines, the protein fluorescence in the fructose-grown cultures significantly increased from 16-27 initially to 58-62% by the end of the maturation period. In contrast, the protein fluorescence in the glucose-grown cultures showed fluctuations throughout the eleven week growth period (Fig. 4a, b in Paper III). Correlation analysis revealed that the protein fluorescence in the glucose-grown cultures was not well correlated to the endogenous levels of glucose (Fig.5c, d in Paper III). This finding suggests that the inhibitory effect of glucose with respect to embryo development likely involves mechanisms other than the Maillard reaction. This is supported by findings from studies in *Arabidopsis* which implicate cross talk between glucose and phytohormone signaling in the regulation of early events of plant development, such as seed germination and seedling development (Price *et al.*, 2003; Dekkers *et al.*, 2004).

The Maillard reaction seems to be involved in the mediation of fructose inhibition of embryo development because the protein fluorescence in the fructose-grown cultures was highly correlated to the endogenous levels of fructose (Fig.5a, b in Paper III). Thus, accumulation of Maillard products may be suggested as the basis for the observed browning of tissue in the fructose-grown cultures (Figs 1 and 2 in Paper III). The proposed association of the Maillard reaction with the inhibitory effect of fructose may perhaps be explained by the reducing capacity of fructose and reactive nature of its products. In particular, it has been suggested that the stronger reducing capacity of fructose makes it a more powerful initiator of the glycation reaction compared to glucose (Semchyshyn *et al.*, 2011). Moreover, the highly reactive Heyns products from ketones are quickly converted to advanced glycation end products compared to the Amadori products from aldoses (Suarez *et al.*, 1989).

### *Embryogenic cultures grown on fructose exhibit higher levels of protein carbonyl content and DNA damage than cultures grown on glucose or sucrose*

Protein carbonylation, a marker of protein oxidation, involves irreparable damage of the protein amino-acid residues and leads to impairment of protein function, increased susceptibility of proteins to proteolysis and cellular deterioration (Berlett & Stadtman, 1997; Dukan *et al.*, 2000; Job *et al.*, 2005; Nyström, 2005). Figure 6 in paper III shows that the protein carbonyl content was always highest in the fructose-grown cultures. The possible mechanism as to how protein carbonyls may repress embryo development remains unclear. In yeast (*Saccharomyces cerevisiae*), higher levels of carbonyl groups in proteins

and reactive oxygen species were detected in cells grown on fructose compared to glucose. Consequently, it was proposed that buildup of reactive carbonyls (RCS) and oxygen species (ROS) may lead to development of carbonyl/oxidative stress (Semchyshyn *et al.*, 2011; Semchyshyn, 2013). Miyata *et al.* (1999) defined carbonyl stress as "a situation resulting from either increased oxidation of carbohydrates and lipids (oxidative stress) or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and nonoxidative chemistry". In this context, it is an intriguing possibility that long-term growth of cultures on fructose could lead to perturbation of intracellular oxidative homeostasis, which can lead to cell damage and repression of embryo development.

The Maillard reaction is suggested to mediate a wide spectrum of DNA damage, including single strand breaks and mutations (Hiramoto *et al.*, 1997; Baynes, 2002). In vitro studies in *Escherichia coli* reveal that fructose and its phosphate metabolites can modify DNA faster than glucose and its phosphate metabolites (Levi & Werman, 2001). In view of this, we compared the DNA damage (apurinic/apyrimidinic (AP) sites) in embryogenic cultures grown on media containing sucrose, fructose or glucose. The fructose- and glucose-grown cultures exhibited the highest and lowest levels of DNA damage, respectively (Fig. 7a, b in Paper III). Clearly, fructose has a profound influence on the integrity of cell culture DNA; however, we don't know whether this is due to a direct chemical modification of the DNA or an indirect effect, due to accumulation of reactive compounds induced by fructose.

### *The changes of glutathione (GSH) content*

In addition to generation of reactive carbonyls (RCS) and oxygen species (ROS), the Maillard reaction has been implicated in alteration of glutathione content (Yen *et al.*, 2002; Semchyshyn *et al.*, 2011). Glutathione is an intracellular thiol that plays an important role against oxidative stress and its cellular levels are maintained by glutathione reductase (Xiang *et al.*, 2001; Chavan *et al.*, 2005; Cairns *et al.*, 2006; Mhamdi *et al.*, 2010). In *Arabidopsis*, embryo development is influenced by availability of glutathione as revealed by the observation that GSH-deficient mutants exhibit an embryo lethal phenotype (Cairns *et al.*, 2006). Also, addition of glutathione to culture medium enhances the embryo-forming capacity of white spruce (*Picea glauca*) embryogenic cultures (Belmonte & Yeung, 2004). To further address the possible mechanism of sugar mediated inhibition of embryo development; we measured the glutathione content in the cultures after the different embryo development stages. The fructose- and glucose-grown cultures displayed almost similar developmental variations, with particularly low glutathione content after the

period of PEM proliferation, late embryogeny and maturation (Figure 8a, b in paper III). The highest content of glutathione was detected in the sucrose-grown cultures; however, glutathione content in these cultures was found to be cell line dependent (Figure 8a, b in paper III). The possible mechanism for the alteration of glutathione content by nonenzymatic reactions has thus far been described in mammalian cells. Yen *et al.* (2002) working with lymphocyte cells reported that Maillard products derived from a reaction between fructose and lysine reduced the content of glutathione and the activity of glutathione reductase. In this light, further investigation is required to determine whether the changes of glutathione content in our embryogenic cultures may involve interference with glutathione biosynthesis.

## 4.3 Biochemical effect of carbohydrates and osmoticum during maturation and germination of embryos (Paper II)

As mentioned earlier, carbohydrates and osmoticum are important compounds for embryo maturation in angiosperms and gymnosperms. During maturation, seeds prepare for germination by accumulating storage reserves such as carbohydrates, lipids and proteins. These storage reserves are later used for inducing desiccation tolerance and providing nutrients during embryo germination and plantlet growth (Crowe *et al.*, 1992; Morcillo *et al.*, 2001; Stasolla *et al.*, 2003; Coelho & Benedito, 2008). The study presented in Paper II was intended to investigate the biochemical effects from carbohydrates and osmotic compounds which are present during early embryo development to the subsequent accumulation of storage reserves and germination. To that end, we used two cell lines of Norway spruce and subjected them to two maturation treatments containing: (I) 3% sucrose. (II) 3% maltose and 7.5% polyethylene glycol (PEG).

***Maltose and PEG restrict the accumulation of compounds taking part in acquisition and maintenance of desiccation tolerance in embryos***

Figure 1 in Paper II shows the number of mature embryos (maturation frequency) obtained with treatment I and II. In general, the maltose and PEG treatment resulted in significant increment of maturation frequency. As a result, this treatment has previously been applied for optimizing the existing protocols for clonal propagation of Norway spruce via somatic embryogenesis. Unfortunately, subsequent analyses have revealed that the maltose and PEG-treated embryos exhibit significantly lower germination frequencies and poor root development compared to the embryos that matured on medium containing sucrose (Fig 2 in Paper II). Existing evidence suggests that PEG-

treated embryos of Norway spruce are incapable of germinating due to PEG-induced morphological aberrations such as intercellular spaces in the shoot pole, root cap and meristem region (Find, 1997; Bozhkov & von Arnold, 1998). However, the effect of these compounds on acquisition of desiccation tolerance in developing Norway spruce embryos remained unknown. For that reason, we examined the effect of carbohydrates and osmotic compounds on the accumulation of compounds that are required for acquisition and maintenance of desiccation tolerance in embryos.

Somatic embryos acquire desiccation tolerance during the maturation phase of embryogenesis. In addition, it is well known that the desiccation-tolerant state in seeds is associated with high levels of sucrose, late embryogenesis abundant (LEA) proteins and raffinose family oligosaccharides (RFOs) (Blackman *et al.*, 1992; Thomas, 1993). Our metabolic and proteomic analyses revealed that the maltose and PEG-treated embryos contained significantly lower levels of sucrose, LEA proteins and raffinose compared to the sucrose-treated embryos (Figs 3, 4 & 8 in Paper III). Due to their non-reducing nature, sucrose and RFOs induce desiccation tolerance through the so called "water replacement hypothesis" by substituting for water to maintain the hydrophilic interactions necessary for membrane and protein stabilization (Koster & Leopold, 1988; Crowe *et al.*, 1992). Alternatively, sucrose and RFOs form a viscous glassy state that serves as a physical stabilizer and averts deteriorative reactions during desiccation (Vertucci & Farrant, 1995; Minorsky, 2003). The LEA proteins participate in desiccation tolerance by maintaining the innate structure of proteins and interacting with sucrose and RFOs to sustain the glassy state (Blackman *et al.*, 1992; Vertucci & Farrant, 1995; Hoekstra *et al.*, 2001). A plausible interpretation of our data is that the low germination frequency of the maltose and PEG-treated embryos may be due to their susceptibility to desiccation damage. This premise is strengthened by the finding that the germination frequency of maltose and PEG-treated somatic embryos of Loblolly pine can be improved by substituting desiccation with a washing and cold conditioning treatment (Nehra *et al.*, 2005).

### *Sucrose, maltose and PEG-treated embryos exhibit differential accumulation of storage compounds*

We also investigated the effect of the sucrose, maltose and PEG treatments on accumulation of storage compounds in mature embryos of Norway spruce. Storage proteins are the major source of amino acids and nitrogen during seed germination and plantlet germination (Shewry *et al.*, 1995). Our proteomics data showed that regardless of the maturation treatment, the mature embryos

accumulated 2S seed storage, legumin and vicilin-like storage proteins (Table 1 in Paper II). The 2S albumins are water soluble proteins characterized by a high content of asparagine, glutamine and arginine (Youle & Huang, 1981). Legumin proteins are synthesized in precursor form during maturation and later broken down into mature globulins while vicilins are oligomeric proteins characterized by a distinctive lack of cysteine residues (Shewry *et al.*, 1995; Shutov *et al.*, 1995). Of particular interest in our proteomics results is the observation that all the detected storage proteins were mostly abundant in the embryos that matured on the medium containing maltose and PEG (Fig. 7 in Paper II). Increases in the content of storage proteins have also been observed in somatic embryos of White spruce (*Picea glauca*) in response to PEG (Misra *et al.*, 1993). Stasolla *et al.* (2002) found that PEG-treated somatic embryos of White spruce displayed elevated transcript levels of the storage protein synthesis enzymes glutamine synthetase and glutamate synthase. However, we did not perform gene expression analysis to clarify whether the aforementioned genes are up regulated in the maltose and PEG-treated somatic embryos of Norway spruce.

Storage reserve accumulation also involves the deposition of energy-rich compounds such as starch. Starch is the major form of storage carbon and it is utilized as a substrate for biosynthesis of lipids and free sugars such as sucrose (Leprince *et al.*, 1990; Luthra *et al.*, 1991). Enzymatic analysis of sugars showed that starch content varied significantly between the sucrose, and maltose/PEG-treated embryos; the sucrose-treated embryos showed elevated levels of starch compared to the maltose and PEG-treated embryos. It has previously been shown in detached ear experiments of wheat (*Triticum aestivum* var Cardena) that PEG may influence starch biosynthesis by repressing the activities of ADP-glucose pyrophorylase (AGPase) and starch synthase (Ahmadi & Baker, 2001). This observation calls for further investigations into the activities of the aforementioned enzymes during maturation of Norway spruce embryos in presence of sucrose, maltose and PEG.

# 5 Conclusions and Future Perspectives

Metabolic profiling of cell lines with different capabilities of embryo development allowed for the indentification of metabolites which are associated with normal and abnormal development of embryos in Norway spruce. We found that the early stages of embryo development appear to benefit from endogenous sucrose for subsequent normal late embryo formation to take place (Paper I). The metabolite profiles also show that osmotic stress tolerance is necessary during late embryogeny and maturation as indicated by the high levels of osmoprotectants (4-Aminobutyric acid and pinitol) in the late embryogeny samples of the normal cell line. Moreover, our results show that fructose is associated with aberrant and blocked development of embryos (Paper I). Further studies are required to elaborate on the underlying molecular mechanisms linking specific metabolites to normal and abnormal development of embryos in Norway spruce. Furthermore, it would be of interest to elucidate the structural characteristics of the unknown compounds (#1-8). These studies could help us to understand why or how these unknown compounds influence development of Norway spruce embryos.

The study presented in paper III investigates whether the Maillard reaction mediates the inhibitory effect of fructose with regard to embryo development in Norway spruce. We found that growth of embryogenic cultures on medium containing sucrose (control) was characterized by normal development of mature embryos whereas the embryogenic cultures which were grown on media containing glucose or fructose did not develop mature embryos. Furthermore, glucose or fructose induced significant alterations in DNA damage, protein carbonyl and glutathione content. Glutathione is of particular importance because it was shown to be essential for embryo development and proper maturation of seeds in *Arabidopsis* (Cairns *et al.*, 2006). In future studies, it would be of interest to examine the viability of the fructose- and glucose grown cultures. A spectrophotometric analysis of extracts (water-insoluble red formazan) from tetrazolium-treated cultures may help to elucidate

the impact of fructose or glucose on the metabolic activity of the embryogenic cultures. Also, application of the methylene blue dye reduction test (MBRT) may help to evaluate cell mortality within the fructose- and glucose-grown cultures.

Finally, our findings also demonstrate the regulatory role of carbohydrates and osmoticum during maturation and subsequent germination of embryos in Norway spruce. In particular, the results suggest that maltose and PEG impede embryo germination by restricting the accumulation of compounds (sucrose, LEA proteins and RFOs) which are necessary for acquisition and maintenance of the desiccation tolerant state. Moreover, the results also indicate differences in accumulation of storage reserves in response to carbohydrates and osmotic compounds (Paper II). There are some questions that remain unanswered from this study. For instance, the regulatory mechanisms of sucrose, maltose and PEG need to be investigated further at the gene expression and enzymatic activity level. These studies could help in enhancing our understanding of embryo development in Norway spruce and optimization of the existing protocols for clonal propagation of conifers by somatic embryogenesis.

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