A proteomic dissection of embryogenesis in *Cyclamen persicum*

Von der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover zur Erlangung des Grades Doktorin der Gartenbauwissenschaften
Dr. rer. hort.
genehmigte Dissertation

von
Dipl.-Ing. agr. Christina, Rode
geboren am 09.12.1980 in Sima
(September 2011)

.

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Tag der Promotion: 31. August 2011

Was gilt das Wort vor der Natur, der Zauberin?
Wie lässig, frei und leicht mit welchem feinen Sinn
Hat sie rings Schönheit in der Welt verstreut,
Verschiedenheit und Gleichheit ausgeglichen.

W. Shukowskij, 1783-1852
(Übersetzung aus dem Russischen von M. Schreiber)

Abstract

Cyclamen persicum is an economically important and a popular ornamental crop. Somatic embryogenesis is well established for *Cyclamen* and thus could supplement the elaborate propagation via seeds. However, the use of somatic embryogenesis for commercial large scale propagation is still limited due to physiological disorders and asynchronous development within emerging embryos. To overcome these problems, a profound knowledge of the physiological processes in *Cyclamen* embryogenesis is essential.

In this study a gel-based proteomic approach has been applied to characterize biochemical pathways in somatic and zygotic embryos. Protein isolation and separation via IEF-SDS PAGE have been optimised for the tissues of interest resulting in high quality gels resolving over 1000 spots. A novel software named GelMap was developed (www.gelmap.de) for the creation and presentation of digital proteome reference maps. A proteome reference map of somatic and zygotic *Cyclamen persicum* embryos was established with this software and is publically available at www.gelmap.de/cyclamen. This reference map includes 247 protein spots identified via mass spectrometry representing 90 distinct proteins. The proteomes of somatic and zygotic embryos were compared. The most striking results of this comparison are: i) glycolysis plays a key role in zygotic and somatic embryogenesis, ii) serine/glycine metabolism differs in zygotic and somatic embryos, iii) somatic embryos are more stressed than their zygotic counterparts, iv) in zygotic embryos, storage proteins are more abundant. Small enolase forms probably lacking the enzymes active sites were found to accumulate in zygotic embryos. They have been identified as candidates for a novel group of major storage compounds in seeds. Proteome structures following the pathway in somatic embryogenesis were analysed - from embryogenic callus to torpedo-shaped embryo. Especially, the ubiquitin-26S proteasome pathway was identified to be essential at the switches from callus to globular embryos as well as from globular to torpedo-shaped embryos. Development specific isoelectric point shifts of catalases have been reported for the first time for somatic embryogenesis in plants. In addition, abscisic acid treatment and high sucrose concentration in the culture medium improved maturation and consequently the quality of somatic embryos in *Cyclamen persicum*.

Zusammenfassung

Cyclamen persicum ist eine ökonomisch bedeutende und beliebte Zierpflanze. Die somatische Embryogenese ist für *Cyclamen* etabliert und stellt eine Alternative zu der bisherigen, sehr aufwändigen Vermehrung über Samen dar. Die Nutzung der somatischen Embryogenese für die kommerzielle Massenvermehrung ist aufgrund von Entwicklungsstörungen und einer ungleichmäßigen Differzierung immer noch begrenzt. Um diese Probleme zu überwinden, sind grundlegende Kenntnisse der physiologischen Prozesse der Embryogenese in *Cyclamen* essentiell.

In dieser Studie wurden die Proteome von zygotischen und somatischen Embryonen mittels eines gelbasierten Ansatzes untersucht, um die biochemischen Stoffwechselwege in beiden Geweben zu charakterisieren. Die Proteinaufreinigung wurde für die untersuchten Gewebe optimiert und die darauffolgende zweidimensionale Auftrennung mittels IEF-SDS PAGE resultierte in hochauflösenden Gelen mit über 1000 Proteinspots. Eine neue Software - GelMap - wurde zur Etablierung und Präsentation von digitalen Proteomreferenzkarten entwickelt (www.gelmap.de). Mittels GelMap wurde eine Proteomreferenzkarte für somatische und zygotische Embryonen von *Cyclamen persicum* erstellt (www.gelmap.de/cyclamen). In dieser Referenzkarte wurden 247 mittels Massenspektrometrie identifizierte Proteinspots annotiert, die 90 nicht-redundante Proteine repräsentieren. Ein Vergleich der Proteome von somatischen und zygotischen Embryonen zeigte folgende Ergebnisse: i) Der Glycolyse kommt eine Schlüsselfunktion in der Entwicklung der somatischen und zygotischen Embryonen zu, ii) der Serin/Glycin Stoffwechsel ist in beiden Embryotypen unterschiedlich stark ausgeprägt, iii) somatische Embryonen sind erhöhten Stressbedingungen ausgesetzt und iv) in zygotischen Embryonen sind Speicherproteine stärker abundant. Kleine Enolase Formen, denen möglicherweise das enzymatisch aktive Zentrum fehlt, wurden als Kandidaten für eine neue, wesentliche Gruppe von Speicherproteinen in Samen identifiziert. Die Entwicklung der Proteomprofile während der somatischen Embryogenese wurde ausgehend von embryogenem Kallus bis zu torpedoförmigen somatischen Embryonen untersucht. Hierbei wurde die essentielle Rolle des Ubiquitin/26S-Proteasom Stoffwechselregulationssystems für Entwicklung von Kallus zu globulären Embryonen und von globulären Embryonen zu torpedoförmigen Embryonen herausgearbeitet. Eine Verschiebung des isoelektrischen Punktes von Catalasen während spezifischer Entwicklungsstufen wurde zum ersten Mal für die pflanzliche Embryogenese aufgezeigt. Die Behandlung mit Abscisinsäure sowie die Kultivierung von somatischen Embryonen auf Nährmedium mit hohem Zuckergehalt resultierten in einer verbesserten Reifung und Qualität von somatischen Embryonen.

Schlagworte: *Cyclamen*, Embryogenese, Proteomics
Key words: *Cyclamen*, embryogenesis, proteomics

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Abbreviations

2D	two-dimensional
ABA	abscisic acid
BN	blue native
DIGE	differential gel electrophoresis
DNA	Deoxyribonucleic acid
EST	expressed sequence tag
ESI	electrospray ionization
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ICA	independent component analysis
IEF	isoelectric focusing
kDa	kilo daltons
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PGR	plant growth regulator
SDS	sodium dodecyl sulfate
TOF	time-of-flight

1 General introduction

1.1 Project objectives

This study aimed at generating in-depth details on the physiology of embryogenesis in *Cyclamen persicum* using a proteomic approach. Information presented here are of great importance for understanding embryogenesis in *Cyclamen* as well as optimising the production system of somatic embryos in vitro. Additionally, a novel tool for creation, publication and evaluation of protein reference maps was developed, helpful for future projects in gel-based proteomics.

The investigation focussed on six major objectives:

i) Improvement of protein isolation and two-dimensional protein separation of the *Cyclamen persicum* embryo proteome in order to obtain high quality two-dimensional gels

ii) Establishment of proteome reference maps for somatic and zygotic embryos of *Cyclamen persicum*

iii) Elucidation of proteins of differential and similar abundance within the proteomes of zygotic and somatic embryos of *Cyclamen persicum*

iv) Characterisation of major storage proteins and enzymes involved in storage compound metabolism of *Cyclamen persicum*

v) Analysis of major proteins of different developmental stages of somatic embryogenesis of *Cyclamen persicum*

vi) Optimising the production system of somatic embryos in a way that their proteome resembles the one of the zygotic embryos

1.2 Biology and horticultural impact of *Cyclamen persicum*

Taxonomy and geographical destribution

The genus *Cyclamen* includes about 20 species (Anderberg et al., 2000; Grey-Wilson, 2003; Compton et al., 2004). These are mainly native to the Mediterranean basin, but also extend as far as Caucasus Mountains, tropical Africa as well as alpine regions of Central Europe (Grey-Wilson, 2003; Yesson and Culham, 2006). Wild forms of *Cyclamen persicum* are endemic in Greece, Turkey, Cyprus, Lebanon, Israel, Algeria and Tunisia (Grey-Wilson, 2003). The genus *Cyclamen* was proposed to be part of the family of *Primulaceae* till the late 1990ies (Schwarz and Lepper, 1964; Grey-Wilson, 2003) but evidence from chloroplastic DNA sequence data suggests that it is closely related to members of family *Myrsinaceae* (Källersjö et al., 2000; Compton et al., 2004). However, the taxonomy of *Cyclamen* on the family level is still not finally resolved. Recent electron microscopic analysis of seeds within members of the families *Primulaceae* and *Myrsinaceae* published by Morozowska et al. (2011) proposed *Cyclamen* to exhibit significant characteristics of *Primulaceae*. Table 1 illustrates the taxonomy of *Cyclamen*.

Table 1 Taxonomy of *Cyclamen persicum*

Class	***Dicotyledonae***
Order	***Ericales***
Family	***Primulaceae*** [1] ***or Myrsinaceae*** [2]
Genus	***Cyclamen***
Species	***persicum***

[1] Schwarz and Lepper, 1964; Grey-Wilson, 2003; Morozowska et al., 2011
[2] Källersjö et al., 2000; Compton et al., 2004

Morphology

The globose tuber of *Cyclamen persicum* develops from the hypocotyl and can reach a diameter of 15 cm in a mature plant (Grey-Wilson, 2003). The dark green heart-shaped leaves are tuber born with a toothed margin and often an eye catching pattering at the upper leaf side in different shades of green, silver, cream or white (Grey-Wilson, 2003). The pedicels, born in the axils of the basal leaves, are erect to ascending, 13-32 cm long, green, brown or purple in colour (Grey-Wilson, 2003). The flowers exhibit

Figure 1 ***Cyclamen persicum*** **cultivar**
The *Cyclamen persicum* cultivar 'Maxora Light Purple' bred by the company Varinova.
Photo: Traud Winkelmann

five petals mainly colored by anthocyans, with shades of pink, purple, red and white (Grey-Wilson, 2003). The mature fruit is brown and globose with a diameter of 1-2 cm containing up to 40 seeds (Grey-Wilson, 2003). The seed have a diameter of 2-4 mm and contain a small torpedo-shaped embryo with a single cotyledon enclosed in a thick endosperm layer (Grey-Wilson, 2003 and own observations). A photo of the *Cyclamen persicum* cultivar 'Maxora Light Purple' bred by the company Varinova B.V. (Berkel en Rodenrijs, Netherlands) is given in Figure 1. This cultivar was object of the present study.

Economic importance

Cyclamen persicum is an economically important and popular ornamental crop especially in the Netherlands, Germany, Italy and Japan (Grey-Wilson, 2003; Takamura, 2007). It represents 8 % of the market volume for potted plants in Germany, which is about 100 million € (Zentrale Markt- und Preisberichtstelle, 2008). The worldwide production is estimated to be about 200 million plants per year, of which approximately 150 million plants are grown and sold in Europe (Schwenkel, 2001). Beside *C. persicum* there are also few cultivars of *C. coum, C. hederifolium* and some further *Cyclamen* species on the market mostly utilised as perennial plants for outdoor use.

Breeding and propagation

Cultivation of *Cyclamen persicum* started in 17th century in Western Europe. The arising economic boom of the late 19th century, associated with increasing wealth in broad levels of the human society promoted the flower business and thus selling of *Cyclamen* (Grey-Wilson, 2003). Today, commercial nurseries produce *Cyclamen* plants within 7-8 months from time of sowing to flowering. In addition, interest is increasing for highly uniform diploid F1 hybrid cultivars and for small diploid cultivars, the so-called ‘mini *Cyclamen*’. Nowadays, breeders focus on new flower colours, resistance to pests and diseases as well as frost and heat tolerance (Grey-Wilson, 2003). Unfortunately, the propagation process of *Cyclamen* via seeds is labour and cost intensive due to manual work, inbreeding depression of parents and genetically heterogeneous offsprings. For example, one *Cyclamen* seed can cost up to 0.20 € (Schwenkel, 2001). Vegetative propagation of *Cyclamen* via tuber cutting etc. is limited and not applicable for high throughput production. Somatic embryogenesis referring to a vegetative micro propagation technique, is well described for *Cyclamen*. Protocols for the *in vitro* production of embryos are established (see chapter 1.4), by which healthy and genetically uniform plants can be produced. Therefore, large scale propagation of *Cyclamen* via somatic embryogenesis is obvious.

1.3 Plant embryogenesis

General aspects of zygotic embryogenesis

The generative reproduction pathway of higher plants starts with the fusion of two haploid gametes forming the first cell of the next generation referred to as a zygote. The zygotic embryo then evolves within the maternal ovule undergoing specific developmental stages. Embryogenesis of angiosperms differs in certain aspects from embryogenesis observed in gymnosperms (Von Arnold, 2008). Describing embryo development within angiosperms, Goldberg et al. (1994) divided embryogenesis into tree major categories based on studies performed with *Arabidopsis* and *Capsella*:

i) The postfertilization-proembryo stage is characterised by asymmetric cell division and the formation of a suspensor and embryo proper. The first division of the zygote results in a smaller and a larger cell, where the smaller terminal cell develops into the embryo proper via further cell division. The embryo and the resulting plant are derived from this cell. The larger cell divides and forms the suspensor (Goldberg et al., 1994), which is an organ connecting the embryo to the vascular system of the mother plant, providing maternal nutrient compounds

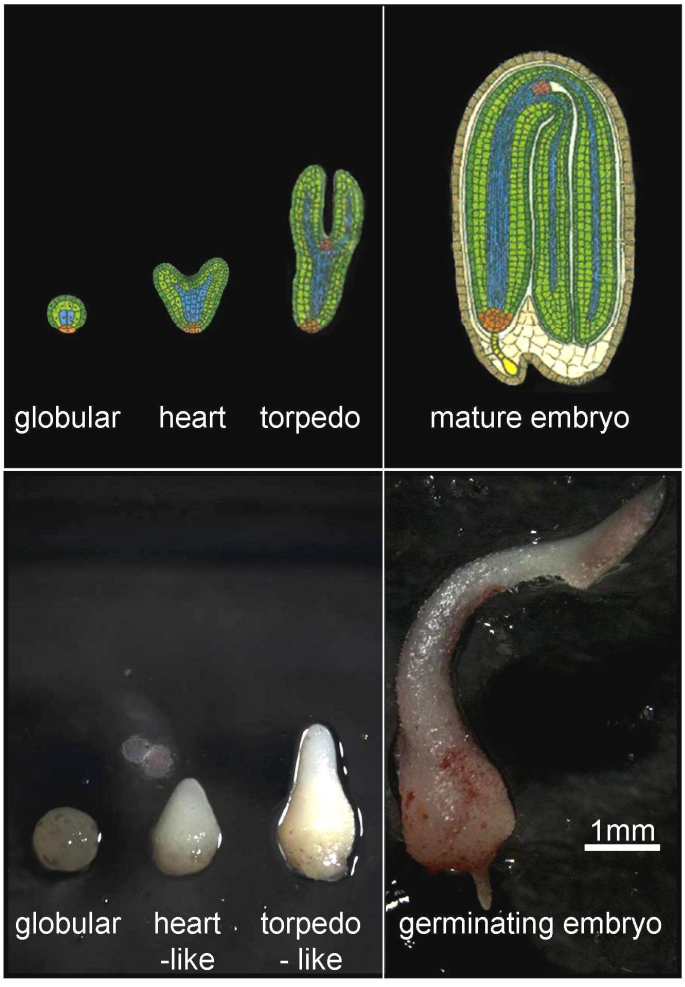

Figure 2 Overview of developmental stages in plant embryogenesis
Upper part: Scheme of the four major developmental stages of zygotic embryogenesis in *Arabidopsis* (taken from Goldberg et. al., 1994, modified). The mature embryo is embedded in endosperm, surrounded by a seed coat and will germinate after a maturation and dormancy period.
Lower part: Corresponding somatic embryo developmental stages of *Cyclamen persicum*: Since the development of one cotyledon is suppressed *in Cyclamen*, the heart and torpedo stages differ morphologically from those observed in *Arabidopsis*. Somatic embryos of *Cyclamen* germinate directly after embryogenesis without a dormancy period. A germinating somatic embryo is shown on the right side of the figure.

and phytohormones especially in the early stages of embryogenesis (Yeung and Meinke, 1993). In late stages the suspensor becomes degraded (Yeung and Meinke, 1993).

ii) The globular-heart transition stage is characterised by the formation of organs and tissue types of the embryo. At the sixteen-cell stage two distinct types of tissues are present in the embryo proper: the protoderm (outer cell layer) and the hypophysis (at the top of the suspensor) (Goldberg et al., 1994). In the subsequent development stages, the globular embryo emerges, including specified cells forming the later procambium and ground meristem. In the following heart-shaped stage the cotyledons and the root meristem appear and the embryo becomes torpedo-shaped after further cell division and elongation processes (Goldberg et al., 1994). Globular, heart and torpedo shaped embryos of *Arabidopsi*s are shown in Figure 2 and are compared to the corresponding developmental stages of *Cyclamen persicum* somatic embryos.

iii) The organ expansion and maturation stage is characterised by enlargement and differentiation of the embryo as well as accumulation of storage compounds followed by controlled desiccation, prevention of premature germination and finally dormancy (Goldberg et al., 1994). The composition of the major storage organ and the quality of storage molecules differ species-specific. For example, within legumes and *Brassicaceae* the embryo cotyledons contain large amounts of storage proteins and lipids while in cereals the endosperm is the main storage organ accumulating mainly starch (Shewry, 1995; Murphy et al., 1993; James et al., 2003).

The zygotic embryo of angiosperms is embedded in the endosperm. This triploid tissue evolves from fusion of the diploid central cell and a haploid sperm cell derived by the same ovule and pollen tube as the embryo within the process of double fertilisation (Dumas and Rogowsky, 2008). The endosperm is a nutrient tissue for the developing and germinating embryo. It provides phytohormones (Maheshwari and Rangaswamy, 1965) and recent studies indicated its active role in the coordination of the maternal tissue and embryo development (Garcia et al., 2003) as well as embryo abortion (Richard and Kang, 1998) and downstream of signalling pathways so far not well characterised (for review see Berger et al. 2006). In the mature seed the endosperm can form either the predominant part (e.g. cereals) or the minor part (e.g. *Arabidopsis*) (Berger 2003).

It is estimated that 20,000 different genes expressed during the entire process of plant embryogenesis (Thomas, 1993). Those encoding on one hand, the house-keeping proteins present in all growing cells but also embryo and embryo developmental stage-specific proteins (Von Arnold, 2008). Here, some major players within the complex molecular

network of embryo morphogenesis are introduced. The majority of those genes were elucidated via *Arabidopsis* mutant experiments. Meyer et al. (1991) identified nine genes essential for early embryo morphogenesis. The genes *GURKE, FACKEL, MONOPTEROS* and *GNOM* are shown to encode proteins necessary for apical-basal pattering; *KEULE* and *KNOLLE* are required for radial pattering while *FASS, KNOPF* and *MICKEY* are essential for the shape change of the embryo (Mayer et al., 1991). *BODENLOS* (Hamann et al., 1999) is a further gene essential for the basal and vascular pattering. Interestingly, *MONOPTEROS* and *BODENLOS* encode proteins involved in the response to auxin (Meyer et al., 1991; Hamann et al., 1999). *MONOPTEROS* and *BODENLOS* deficient mutants are auxin insensitive and their defects in the root and vascular system formation can be traced back to the lack of auxin-mediated pathways. *PIN* genes encode proteins which control the auxin distribution within the embryo and thus responsible for organ specific auxin levels: high auxin levels in the basal part promote root differentiation while low levels in the apical part promote shoot and cotyledon differentiation (Blilou et al., 2005). The expression of *CUP-SHAPED COTYLEDON* genes is promoted by relatively low auxin levels. Those genes encode transcription factors essential for division of the two cotyledons and establishment of the shoot apical meristem (Takada et al., 2001). *WUSCHEL*s are a further group of transcription factor encoding genes, essential for the formation of the shoot apical meristem (Mayer et al., 1998).

In the late stages of embryogenesis the tissue patterning program is replaced by maturation and beginning dormancy. On the genetic level four transcription factors termed the "master regulators" have been identified to regulate the transcription of seed storage proteins (Verdier and Thompson, 2008). Those are: *LEAFY COTYLEDON* (*LEC*) 1 and 2, *ABSCISIC ACID INSENSITIVE3* (*ABI3*) and *FUSCA3* (*FUS3*). *FUS3* and *ABI3* have been shown to regulate seed filling gene expression. Mutants deficient of these genes accumulate less storage compounds and thus show a decreased desiccation tolerance. The two LEC transcription factors are regulating gene expression maintaining the cotyledon identity and the complementation of the seed filling process. In *LEC* deficient embryos the cotyledons are transformed to leaves and the embryos exhibit a reduced desiccation tolerance and accumulation of storage compounds (Verdier and Thompson, 2008). The phytohormone abscisic acid (ABA) and its ratio to gibberellin as well as sucrose/hexose- mediated signalling are also involved in the regulation of the complex gene expression networks during embryo maturation (Gutierrez et al., 2007). In *Arabidopsis* seeds, high sucrose/hexose ratios promote the expression of the sucrose transporter AtSUC5 in the endosperm, which is essential for transition to the storage accumulation phase (Baud et al., 2005).

Altogether, many genes and especially those encoding transcription factors are essential for the embryo morphogenesis (for review, see De Smet et al., 2010; Gutierrez et al., 2007). In the early stages the phytohormone auxin is a major player controlling tissue patterning, while in the late stages abscisic acid and sucrose-mediated pathways facilitate seed maturation and dormancy (Gutierrez et al., 2007).

Even though, the maternal signals as well as the suspensor and the endosperm are important for embryogenesis (see above), it has been shown that zygotic embryos can develop into fertile plants without these organs outside the fruit e.g. after an in vitro fertilisation of egg cells (Kranz and Lorz, 1993; Kranz and Dresselhaus, 1996) or after preparing a developing embryo out of the seed (Sharma et al., 1996) and cultivating those embryos in vitro. This leads to a conclusion that the embryo has the potential to develop autonomously. However, the in vitro culture media may partly mimic the impact of the mother plant and the endosperm.

General aspects of somatic embryogenesis

Interestingly, also somatic cells can be stimulated to develop into embryos completely outside the maternal context via the so-called somatic embryogenesis. The technique is established for many model and crop plants (Zimmerman, 1993; Hohe et al., 2010) with the first reports on plant somatic embryogenesis for carrots published in 1958 (Steward et al. 1958; Reinert 1958). Currently, this plant is an established model for somatic embryogenesis research. Additionally, somatic embryogenesis has also been established for important horticultural crops aiming to facilitate their propagation. These include, food crops such as grapevine (Stamp and Meredith, 1988), peanut (Gill and Saxena, 1992), melon (Lou and Kako, 1995) as well as economically important ornamental plants such as cultivars of *Rosa*, *Chrysanthemum*, *Tulipa*, *Phalaenopsis*, *Anthurium* and *Pelargonium* (for review, see Hohe et al., 2010). Plant propagation via somatic embryogenesis is of great horticultural interest, since it has the potential to generate genetically identical plants in high numbers, can be used for propagation of inbred parental lines as well as a regeneration system after genetic modification of plant cells. Despite the establishment of somatic embryogenesis protocols for a large variety of ornamental crops, today this technique is still hardly applied for the commercial mass propagation of these plants (Hohe, 2010). In contrast, multiplication of high-value clones via somatic embryogenesis has been realised for the forestry conifers *Picea* and *Pinus* by specialised nurseries (e.g., CellFor, Vancouver, Canada; Carter Holt Harvey Limited, Auckland, Australia).

Von Arnord (2008) divided the in vitro regeneration of plants via somatic embryogenesis in five steps: i) Initiation of embryogenic cultures, ii) proliferation of embryogenic cultures, iii) pre-maturation of somatic embryos, iv) maturation of somatic embryos and v) regeneration of plants.

i and ii) Initiation and proliferation of embryogenic cultures: These first two steps require culture media with the plant growth regulator (PGR) auxin often in combination with cytokinin (Von Arnold, 2008). Auxin has been described above to be a major player within embryogenesis, due to its impact on pattern formation. However, its role in initiating embryogenic callus is different since no pattern formation takes plays. It has been shown, that phytohormones as well as application of different kinds of stresses can induce the reprogramming of gene expression (Lo Schiavo et al., 1989) with especially auxins influencing DNA methylation (Dutis et al., 1995). Consequently, embryogenic callus of Ginseng as well as *Pinus* expressed lower DNA methylation rates as compared to their non-embryogenic counterparts (Chakrabarty et al., 2003; Noceda et al., 2009). The phenylurea compound thidiazuron mimics the effects of both auxin and cyctokinin at the same time, and is also used for the induction of embryogenic calluses (Murphy et al., 1993). However, even by applying the required PGRs, not all somatic cells develop into embryogenic callus. The type of the explant (e.g. leaves or parts of zygotic embryos) as well as the genotype the explant was isolated from, influence the embryogenic potential of the resulting callus (von Arnold, 2008). *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) encoding genes have been shown to represent markers for embryogenic callus cells. Those genes are highly expressed in callus but also in somatic embryos up to the heart stage (Schmidt et al., 1997; Hecht et al., 2001).

iii-v) Embryo differentiation, maturation and plant regeneration take place on PGR-free medium in most systems (von Arnold, 2008). With the depletion of auxin, genes are transcribed necessary for the globular stage and further development (Zimmerman, 1993). The emerging embryos resemble the developmental stages expressed by their zygotic counterparts (see Figure 2) and also are supposed to share the above described genetic networks. Maturation of somatic embryos can be facilitated by application of abscisic acid and water stress e.g. via high sucrose concentrations in the culture medium (Dodeman et al., 1998). Maturation promoting effects of these two factors have been demonstrated in some recent studies. Sucrose improved maturation in somatic embryos of pine, *Cyclamen* and date palm (Klimaszewska et al. 2004; Winkelmann et al. 2006; Sghaier-Hammami et al. 2010) and abscisic acid in larch, oak, walnut and date palm (Gutmann et al. 1996; Garcia-Martin et al.

2005; Vahdati et al. 2008; Sghaier-Hammami et al. 2010). After completion of embryogenesis somatic embryos start to germinate and form small plants similar to seedlings. These plants can be transferred to soil after obtaining a critical size and acclimatisation period at high humidity grown at conventional, species-specific cultivation conditions (von Arnold, 2008). Somatic embryogenesis-like morphogenesis also occurs in natural habitats e.g. on the leaf margins of the *Kalanchoe*, section *Bryophyllum* (Garcês et al., 2007).

Somatic embryogenesis in *Cyclamen persicum*

Somatic embryogenesis is well established for *Cyclamen persicum* (Wicart et al. 1984; Kiviharju et al. 1992; Schwenkel and Winkelmann 1998; Winkelmann and Serek 2005). Figure 3 shows flowering *Cyclamen persicum* plants in a greenhouse propagated via somatic embryogenesis. Embryogenic callus can be obtained from various plant organs but unpollinated ovules have been shown to represent the best explant type due to the high quality of emerging callus, low contamination rates as well as easy access without damaging the mother plant (Winkelmann et al., 1998; Schwenkel and Winkelmann, 1998; Winkelmann et al., 2000). Callus can either be cultivated on solid Murashige and Skoog medium (1962)

Figure 3 ***Cyclamen persicum*** **plants propagated via somatic embryogenesis**
Cultivar: 'Libretto pink with eye' bred by the company Syngenta.
Photo: Traud Winkelmann

containing 2,4-dichlorophenoxy acetic acid and 6-(γ, γ-dimethylallylamino) purine or more efficiently in liquid systems (Hohe et al., 2001; Schwenkel, 2001). Transfer to PGR-free medium results in differentiation of somatic embryos within few weeks of cultivation in dark conditions (see Figure 2). Torpedo-shaped embryos are separated and transferred to fresh, PGR-free medium. The germinated plants are placed in the light after the cotyledon reaches a length of 1 cm. When in vitro *Cyclamen* plants develop a tuber, roots and two or three leaves, they are transferred to soil (Schwenkel and Winkelmann, 1998; Winkelmann, 2010). The high potential for mass propagation of *Cyclamen persicum* via somatic embryogenesis was demonstrated in two studies: Schwenkel (2001) calculated the production of 6,000 - 8,000 young plants within 42 weeks starting from callus derived from unpollinated ovules of one bud and cultivated on solid medium. Hohe et al. (2001) estimated the production of 27,000 young plants within 38 weeks starting from 1 l suspension of embryogenic callus in a liquid culture system. However, physiological disorders in a relevant portion of emerged embryos as well as asynchronous development (described e.g. by Schmidt et al., 2006) limit the commercial application so far. To overcome these problems, a profound knowledge of the physiological processes in *Cyclamen* embryogenesis is essential. The molecular background of embryogenesis in *Cyclamen persicum* with a focus on proteomics is presented in chapter 1.4.

1.4 Physiological analyses via proteomics

The term "proteome" is quite new, it was suggested in the 1990s by Wilkins (Wilkins et al., 1996). A proteome includes the total set of proteins expressed in a specific tissue under defined conditions. Proteins are essential for life. They are enzymes, structure and storage compounds. The protein composition of a cell is dynamic and changes in specific ways depending on external triggers (e.g. biotic or abiotic stresses, change of seasons), internal processes (e.g. development stages) and the function of the cell within the organism. Thus a global investigation of the proteome closely reflects the current biochemical processes in the analysed sample. Proteomic analysis can be performed for single cell types but also for tissues, organs or whole organisms (for reviews see, Rose et al., 2004; Takáč et al., 2011). Consequently, the analysed protein fractions are highly complex and efficient methods have to be applied for resolving them.

Two-dimensional gel electrophoresis represents a basic tool in protein biochemistry. Since its invention by O'Farrel and Klose in 1975 (O'Farrel 1975, Klose 1975) has been extensively used for high resolution in protein separations. The workflow of a gel-based proteomic approach combined with mass spectrometry is illustrated in Figure 4. For the two dimensional

isoelectric focusing - sodium dodecyl sulfate polyacrylamide gel electrophoresis (IEF-SDS PAGE) proteins are focussed depending on their isoelectric point on a gel-strip with an immobilised or slightly mobile pH gradient in the first dimension. Subsequently those proteins are separated according to their molecular weight in the second dimension. After staining, each protein fraction is visible in form of a more or less intensively stained spot (Mihr and Braun, 2003). These spot patterns are specific for the analysed fraction and can be evaluated visually or more professionally by using specialised software. When comparing two or more samples different protein abundances can be observed this way. However, gel based techniques are limited to water soluble proteins. Nowadays 2D techniques are combined with protein identification using mass spectrometry (Rose et al., 2004). Complex protein samples of a specific tissue can also be analysed by so-called 'shotgun proteomics' without using gel electrophoresis system but separated via liquid chromatography (LC) followed by mass spectrometry (Wienkoop et al., 2006; Brechenmacher et al., 2009).

Prior to mass spectrometric analyses single spots or complex protein fractions have to be digested with a site-specific protease (commonly trypsin) in order to obtain ionisable peptides. Subsequently the peptides are prefragmented via LC, ionized by an ion source (matrix-assisted laser dissociation/ionization (MALDI) or electrospray ionization (ESI)) and analysed in a mass spectrometer of choice e.g. quadrupole, ion trap, time-of-flight (TOF), and collision-induced dissociation, which can be used separately or in combination (Domon and Aebersold, 2006). Using an electrospray ionization tandem mass spectrometry system (ESI MS/MS) results in amino acid sequences of peptides. For protein identification the set of peptides identified in one single spot is compared with known protein sequences including translated genomic and transcriptomic sequences in databases using algorithms like MASCOT, SEQUEST and OMSSA. The best fitting proteins are ordered in a table providing scores for the probability of identification. The identification of proteins for non-model organisms like *Cyclamen* is challenging (Rose et al., 2004). For *Cyclamen* only few sequence informations are available and there are no closely related plants within the pool of sequenced organisms. *Arabidopsis*, *Vitis* and *Populus* are the closest relatives with a sequenced genome. Unfortunately, the three of them only share the taxonomic level of class (*Dicotyledonae*) with *Cyclamen*. Even though, protein identification can be performed based on the sequence information available for other plants e.g. by searching against the "Greenplant" database provided by the National Center for Biotechnology Information (NCBI). Nevertheless, the identification of species specific proteins or modified forms of house-keeping enzymes can fail. Fortunately, for *Cyclamen* embryos an EST-database was established by Rensing et al.

(2005) and further analysed by Hönemann et al. (2010) which benefits *Cyclamen* specific protein identification.

Two-dimensional gels coupled with information on the identity of the separated proteins are referred to as "reference maps" because they allow protein identification of analogous protein fractions by simple "spot pattern" comparison. These maps can be published in papers or on-line via a digital proteome reference map where spot specific MS-based information can be browsed by clicking on the spot of interest. Those maps are publicly accessible and the large numbers of identified proteins becomes more easily explorable than endless tables in manuscripts. Currently, about seventy 2D PAGE databases are listed on the WORLD-2DPAGE portal at ExPASy (http://expasy.org/ch2d/2d-index.html) including reference maps for various animal and plant tissues as well as for microorganisms.

Proteomic studies on somatic embryogenesis with a focus on *Cyclamen persicum*

As described above proteomic approaches can nicely be utilised for determination of the physiological status quo within the development of plants. This is also true for somatic embryogenesis. A proteomic dissection of somatic embryogenesis has been performed for some major crops in the last years (*Quercus ilex*: Mauri and Manzanera 2003; *Medicago*: Imin et al. 2005; *Picea*: Lippert et al. 2005; soybean: Hajduch et al. 2005; *Vitis*: Marsoni et al. 2008; date palm: Sghaier-Hammami et al. 2010). For *Cyclamen*, initial studies comparing the transcriptomes (Rensing et al., 2005; Hönemann et al., 2010) and proteomes of somatic and zygotic embryos (Winkelmann et al. 2006; Bian et al. 2010) as well as embryogenic and non-embyogenic callus (Lyngved et al. 2008) have been performed previously. Rensing et al. (2005) established an EST (expressed sequence tag) database for embryogenic callus and somatic embryos of *Cyclamen* including 2083 ESTs. Hönemann et al. (2010) found 417 transcripts to be differentially expressed between somatic and zygotic embryos of *Cyclamen persicum*. Especially genes encoding proteins of the extracellular compartment (apoplast) were significantly overrepresented in somatic embryos. Lyngved et al. (2008) found about 25% of the proteins resolved via two-dimensional differential gel electrophoresis (DIGE) to be differentially abundant between embryogenic and non-embryogenic callus of *Cyclamen persicum*. The majority of the proteins differentially abundant in the cell lines were involved in protein processing, cell proliferation, and stress response. Winkelmann et al. (2006) resoluted more than 200 spots on an IEF-SDS PAGE and reported that 74% of the proteins expressed in zygotic embryos were found in similar abundance in somatic embryos grown in 60 g l^{-1} sucrose. In somatic embryos the storage protein 7S globulin was underrepresented and glycolytic enzymes were higher in abundance. This may correlate with inhomogenous

development and premature germination. In accordance, a lack of storage proteins and proteins involved in maturation have been described previously for somatic embryos (e.g. carrot: Dodeman et al. 1998; alfalfa: Krochko et al., 1994; oil palm: Morcillo et al., 1998; maize: Thijssen et al., 1996). However, a large scale proteomic comparison of somatic and zygotic embryos, a proteomic view on different stages of this developmental process from callus to embryo as well as evaluation of the effects of maturation treatments on the proteomes of somatic embryos of *Cyclamen persicum* until now are missing and they form the content of the present thesis.

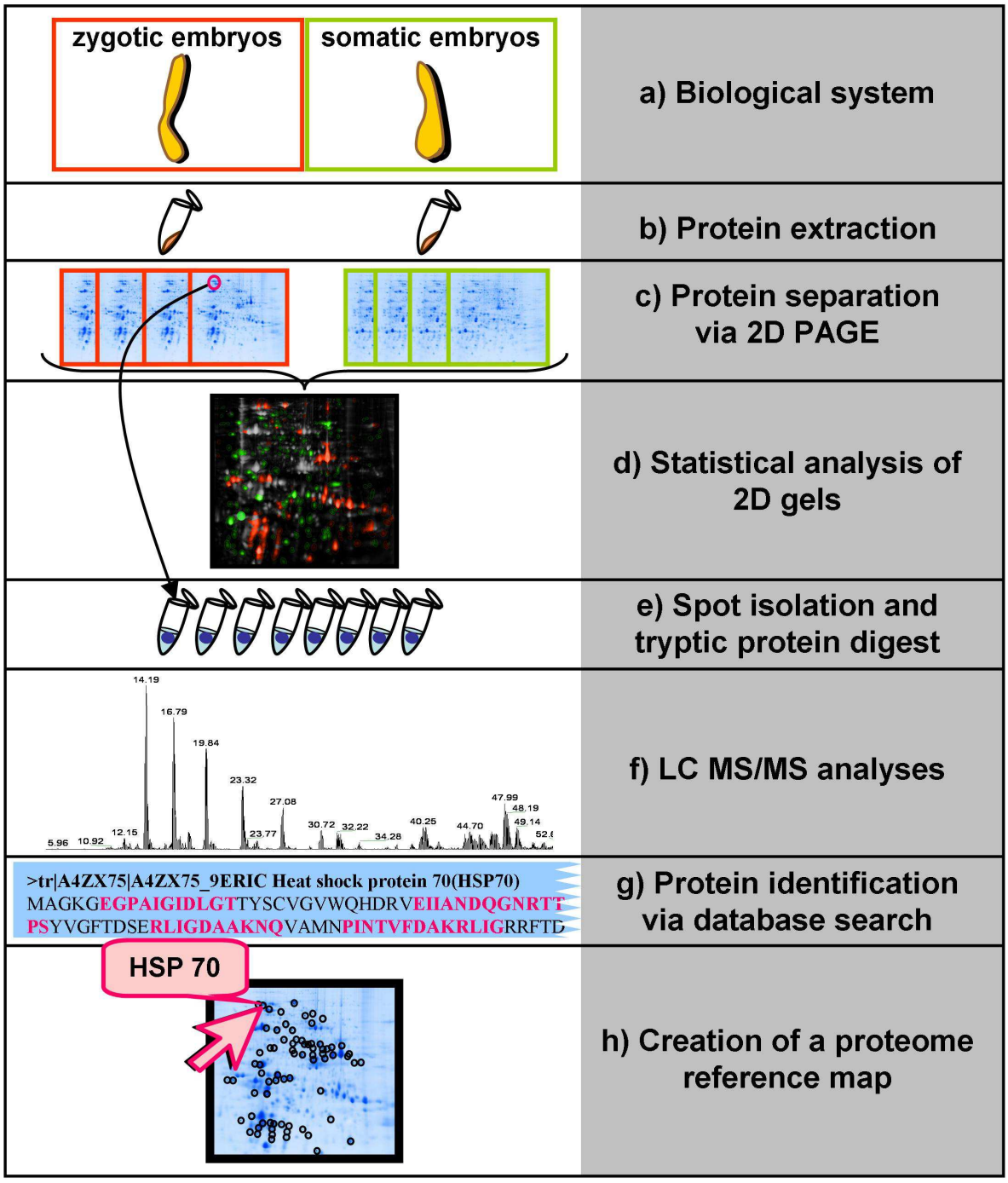

Figure 4 Workflow of a gel-based proteomic approach combined with mass spectrometry
The biological system represents one or more samples to be analysed via a gel based proteomic approach. In the example given in this diagram, proteomes of zygotic and somatic embryos of *Cyclamen persicum* are analysed and compared (a). Therefore, total proteins are extracted from each tissue (b) and separated via IEF-SDS PAGE (c). To perform statistical analyses with gels of different tissues, at least a set of three replicates for each tissue is required. Spots that differ significantly in abundance are labelled (green and red) in an overlay image of all gels analysed (d). Proteins of interest, e.g. differentially abundant proteins, are isolated from 2D gel and subsequently a tryptic protein digest is performed (e). The resulting peptides are separated via liquid chromatography (LC) before tandem mass spectrometry analyses (f). Protein identification is performed based on resulting peptide sequences (pink) via a database search matching to known sequences using a specific software (g). Finally, a digital proteome reference map can be designed indicating all identified proteins (h). Using a gel-free shotgun approach, the steps (c) and (d) are replaced by digestion of a complex protein sample which is then further analysed.

2 Publications and Manuscripts

In the following four manuscripts are presented. In the first manuscript "Analyses of plant proteomes by DIGE" (Chapter 2.1) an optimised protocol for protein purification and two-dimensional separation of plant tissues by Differential Gel Electrophoresis (DIGE) is presented based on the preparation of *Cyclamen* embryo tissue. For this manuscript I have tested and evaluated four protein purification protocols with different amounts of *Cyclamen* embryos and wrote the manuscript in cooperation with Dr. Frank Colditz and Prof. Dr. Hans-Peter Braun. The DIGE gel displaing the proteomes of somatic and zygotic embryos of *Cyclamen persicum* shown in the figure of this manuscript was performed by myself. This manuscript is in press within the book "Differential Gel Electrophoresis".

In the second manuscript "GelMap - A novel software tool for the creation and presentation of proteome reference maps" (Chapter 2.2) the new proteomic software GelMap is introduced and applications are demonstrated. GelMap was developed by Michael Senkler and myself. Michael Senkler performed the programming of GelMap while I submitted the proteomic input and the layout. I have written the manuscript, which was subsequently corrected by Prof. Dr. Hans-Peter Braun and the other co-authors. I have designed all figures and tables. This manuscript has been submitted to the scientific journal "Journal of Proteomics" and is now in the process of review.

In the third manuscript "Enolases: storage compounds in seeds? Evidence from a proteomic comparison of zygotic and somatic embryos of *Cyclamen persicum* Mill." (Chapter 2.3*) the proteomes of the somatic and zygotic embryos are compared and major physiological pathways of *Cyclamen persicum* embryogenesis are discussed. Additionally, a *Cyclamen persicum* embryo proteome reference map was established using GelMap facilitating further investigations. I have performed all experimental work in the laboratory, except of the protein identification which was realised by Dimitri Heinz and co-workers at Strasbourg University, France. I have evaluated the gel-based and the proteomic data in, established the digital proteome reference map, written the manuscript and designed the figures. The resulting data sets were interoperated together with Prof. Dr. Traud Winkelmann and Prof. Dr, Hans-Peter Braun, who also corrected the manuscript. This work was published in the scientific journal "Plant Molecular Biology" (75, 305-319) in 2011 and the article was selected as "cover story" of this issue.

In the fourth manuscript "From callus to embryo - a proteomic view on the development and maturation of somatic embryos in *Cyclamen persicum*" (Chapter 2.4) proteome structures following the pathway in somatic embryogenesis were analysed. In addition, the effects of

abscisic acid and high sucrose on the proteomes of somatic embryos were investigated. For this manuscript I have supervised the experimental work in the laboratory, evaluated the data, wrote the manuscript, which was corrected by Traud Winkelmann and other co-authors, and designed all figures and tables. The analyses concerning the effects of abscisic acid and sucrose on the proteomes of somatic embryos are part of the B.Sc. thesis performed by Kathrin Lindhorst, who is a co-author of this manuscript. Her thesis was supervised by Prof. Dr. Traud Winkelmann, Prof. Dr. Hans-Peter Braun and myself. Data interpretation for this manuscript was realised together with Prof. Dr. Traud Winkelmann. The manuscript has been submitted to the scientific journal "Planta" and is now in the process of review.

Analyses of plant proteomes by DIGE

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Summary

Two-dimensional differential gel electrophoresis is an invaluable technique for the analysis of plant proteomes. However, preparation of protein fractions from plant tissues is challenging due to the special features of plant cells: a robust cell wall, large vacuoles which often contain high concentrations of organic acids and a broad range of secondary metabolites like phenolic compounds and pigments. Therefore, protein preparation for DIGE analyses has to be adapted. Here we describe both, a phenolic protein extraction method for plant tissues and an adapted protocol for DIGE labelling of the generated fractions.

Key words: Plant cell disruption, plant protein extraction, DIGE labelling

Abbreviations: 2-D: Two-dimensional, DIGE: Differential gel electrophoresis, DMF: dimethylformamide, IEF-SDS PAGE: Isoelectric focusing - Sodium dodecyl sulfate Polyacrylamide gel electrophoresis, PMSF: phenylmethylsulfonyl fluoride, SE: Somatic embryos, ZE: Zygotic embryos

1. Introduction

The 2-D differential gel electrophoresis (2-D DIGE) technology allows separation of two differentially labelled protein samples on one gel, thus eliminating gel-to-gel variability. A major advantage of this technique is the possibility to identify even very small or marginal differences in protein migration patterns of two different fractions (occurring at the p*I* or *Mr* level) which are hardly detectable by 2-D gel electrophoresis on the basis of separate gels *(1)*. Furthermore, the fluorophores used in differential protein labelling not only are very sensitive but also allow precise protein quantification. In addition, this labelling allows a very sensitive detection of proteins on gels.

In plant research 2-D DIGE was applied successfully for analyses of protein samples from various species and different tissues. For instance, the technique was used to investigate different plant developmental processes and organogenesis *(2-4)*, plant organelle proteomes *(5, 6)*, plants under abiotic stress conditions *(7, 8)*, analyses of plants in associations to symbiotic and/or pathogenic microbes *(9-13)* and posttranslational protein modifications in plants (*14*).

Due to the presence of a cell wall and very special biochemical properties, disruption of plant cells and isolation of protein fractions are challenging but of major importance to obtain high resolution gels. To find a very efficient protein purification technique, four different protein extraction protocols were tested in our laboratory: (i) A rapid protocol without precipitation as described by Gallardo et al. *(15)*, (ii) a protocol utilising TCA precipitation in combination with acetone *(16)*, (iii) a protocol described by Corcke and Roberts *(17)* which relies on boiling of protein fractions in Laemmli buffer *(18)* and subsequent protein precipitated with acetone, and (iv) an extraction method using phenol which is combined with an ammonium acetate in methanol precipitation as described by Hurkman and Tanaka *(19)*, modified by Colditz et al. *(20)*. Optimal protein purification and resolution on IEF-SDS gels was achieved using the modified Hurkman and Tanaka *(19)* protocol even for low amounts of tissue.

2. Materials

All buffers, solutions and reagents are given in the order of usage according to the methods protocols. Prepare all solutions freshly using analytical grade chemicals in combination with pure de-ionized water.

2.1. Buffers, solutions and reagents for protein extraction with phenol

1. Extraction Buffer: 700 mM Sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, pH 8.0 (HCl). Directly before usage, 2 % ß-mercaptoethanol and 2 % PMSF are added. Extraction buffer is stored at 4 °C or frozen at -20 °C in aliquots.
2. PMSF stock solution (200 mM), stored at 4 °C for not longer than 8 weeks.
3. Water-saturated phenol (pH 6.6/7.9; Amresco, Solon, USA), stored at 4 °C.
4. Precipitation Solution: 0.1 M Ammonium acetate in methanol, stored at 4 °C.

2.2. Buffers, solution and reagents for DIGE sample preparation

1. Lysis buffer: 8 M Urea, 4 % [w/v] CHAPS, 40 mM Tris base, 3.75 mg DTT (added directly before usage), 0.2 mM PMSF (added directly before usage). Lysis buffer is stored at – 20 °C (see **Note 1**).
2. PMSF stock solution: 200 mM, stored at 4 °C for not longer than 8 weeks.
3. CyDye™ Fluor minimal labeling reagents. Cy2™, Cy3™ and Cy5™ (GE Healthcare, Munich, Germany). The fluorophores (400µM per labelling reaction) are diluted in DMF according to the manufacturers instructions. Diluted CyDyes are stored at -20 °C and should be used within 3 months.
4. Lysine stock solution: 10 mM, stored at 4 °C.
5. Rehydration buffer: 8 M Urea, 2 % [w/v] CHAPS, a spatula-tip of bromophenol blue, DTT 20-100 mM (added directly before usage), 0.5 % [v/v] IPG buffer (added directly before usage; corresponding to the IPG strip used for IEF (GE Healthcare, Munich, Germany)). Rehydration buffer is stored at -20 °C.

3. Methods

3.1. Protein extraction with phenol

This protocol is based on the protein extraction method according to Hurkman and Tanaka *(19)* modified by Colditz et al. *(20)*. Freshly ground samples are transferred to 2 ml Eppendorf vessels and directly frozen in liquid nitrogen. For subsequent 2-D DIGE analysis, proteins from two different plant samples are extracted in parallel.

1. Take 200 mg pulverized plant material (see **Note 2**) from the two protein fractions to be compared and add 750 µl extraction buffer. Incubate the samples for 10 minutes on ice.
2. Add 750 µl of water-saturated phenol (4°C) to each sample and vortex. Incubate the samples for 30 min on a table mixer (Eppendorf Thermomixer Compact; Eppendorf, Hamburg, Germany) at 1000 rpm at room temperature (see **Note 3-5**).
3. Centrifuge samples at 11.000 x g for 10 min, 4 °C.
4. After centrifugation, transfer the phenol phase (generally the upper phase) of each sample into a new 2 ml Eppendorf vessel and dilute it in an equal volume of ice-cold extraction buffer. Vortex samples (see **Note 6**).
5. Centrifuge the samples at 11.000 x g for 10 min, 4 °C.
6. Collect again the phenol phase (the upper phase) of each sample and transfer it completely into a new pre-weighted 2 ml Eppendorf vessel (see **Note 7**). Add ice-cold precipitation solution up to a final volume of 2 ml to each sample. Invert the vessels several times back and forth and let the proteins precipitate for at least 4 h at - 20 °C.
7. Centrifuge the samples at 17.000 x g for 3 min, 4 °C. Discard the supernatants and dilute the protein pellets in 1 ml of ice-cold precipitation solution.
8. Repeat **Step 7** two times, then dilute the protein pellet of each sample in 1 ml of ice-cold 80 % [v/v] acetone. Centrifuge the samples at 17.000 x g for 3 min, 4 °C. (see **Note 8**).
9. Discard the supernatants and dry the protein pellets under an extractor hood at room temperature (see **Note 9**).
10. Finally, determine the weight of the vessels including the pellets and subtract the value of the empty vessels for calculation of the pellet weights (see **Note 10**).

3.2. Sample preparation for DIGE

The following protocol relies on the comparative analyses of two related protein fractions using the CyDye^{TM} Fluor labeling reagents (GE Healthcare, Munich, Germany). Both fractions should contain 100 µg protein and should be labeled with different fluorophors. Afterwards, the samples are combined and loaded onto a single protein gel. Aditionally, a 1:1 mixture of the two protein samples is labeled with a third CyDye fluorophor and is used as an internal standard to allow a comparison of relative protein spot intensity between both individual protein samples analyzed. The following protocol uses 2 D IEF / SDS PAGE for protein separation.

1. Resuspend protein samples, 100 µg each, separately in a minimum volume of 30 µl of CyDye labeling-compatible lysis buffer (pH 8.5) in absence of DTT. Shake the samples for 30 min at room temperature (see **Note 11**).

2. Centrifuge the samples at 17.000 x g for 5 min, 4 °C. Collect the supernatant.

3. Labeling reaction: For CyDye™ labeling reaction, 100 µg protein of each sample is labeled with one CyDye. Therefore, transfer a volume corresponding to the appointed protein amount (usually in the range of 10 to 20 µl) of each sample separately to a new 1.5 ml Eppendorf vessels. A minimal volume of 10 µl for each protein sample is required for labeling reaction. For the internal reference sample (third sample), prepare a mixture of the two samples by combining half of the sample volume from each individual sample. Add 1 µl of diluted CyDye solution to each sample. Label the reference sample with the remaining third CyDye (e.g. Cy2™). Centrifuge them briefly and incubate for 30 min on ice in the dark (see **Note 12**).

4. Stop reaction: Add 10 mM lysine stock solution (one tenth volume with respect to the individual samples) to each labeling reaction to obtain a final concentration of 1 mM lysine per sample (e.g. add 1.1 µl of 10 mM lysine solution to 10 µl sample labeled with 1 µl CyDye). Incubate the samples for 10 min on ice in the dark.

5. Add to each sample the equal volume of lysis buffer containing the double amount of DTT (e.g. 12.1 µl lysis buffer to 12.1 µl CyDye-labeled sample).

6. Combine all three CyDye-labeled protein samples in one reagent tube (e.g. 3 x 24.2 µl results in 72.6 µl sample volume).

7. Add the remaining volume of rehydration buffer containing DTT and IPG buffer to the final volume required for IEF separation.

8. Perform 2-D DIGE in the dark. For second dimension SDS PAGE, the Laemmli or the Schägger protocols can be applied *(18, 21)*.

9. After finishing the electrophoretic separation, the gel is immediately scanned using a Fluorescence Scanner (Typhoon Fluorescence Scanner, GE Healthcare, Munich, Germany). Keep the gel at 4 °C and in the dark before starting the scanning procedure. Using the CyeDye fluorophores, gels have to be scanned at 50-100 micron resolution at the appropriate excitation wavelengths (488 nm for Cy2™, 532 nm for Cy3™ and 633 nm for Cy5™). Digital gel images can be visualized using the Image Quant analysis software (GE Healthcare, Munich, Germany) (**Fig.1.**). Quantification of relative differences of individual protein abundances can be carried out using specific software (e.g. Delta 2-D (Decodon, Greifswald, Germany) or DeCyder™ (GE Healthcare, Munich, Germany)).

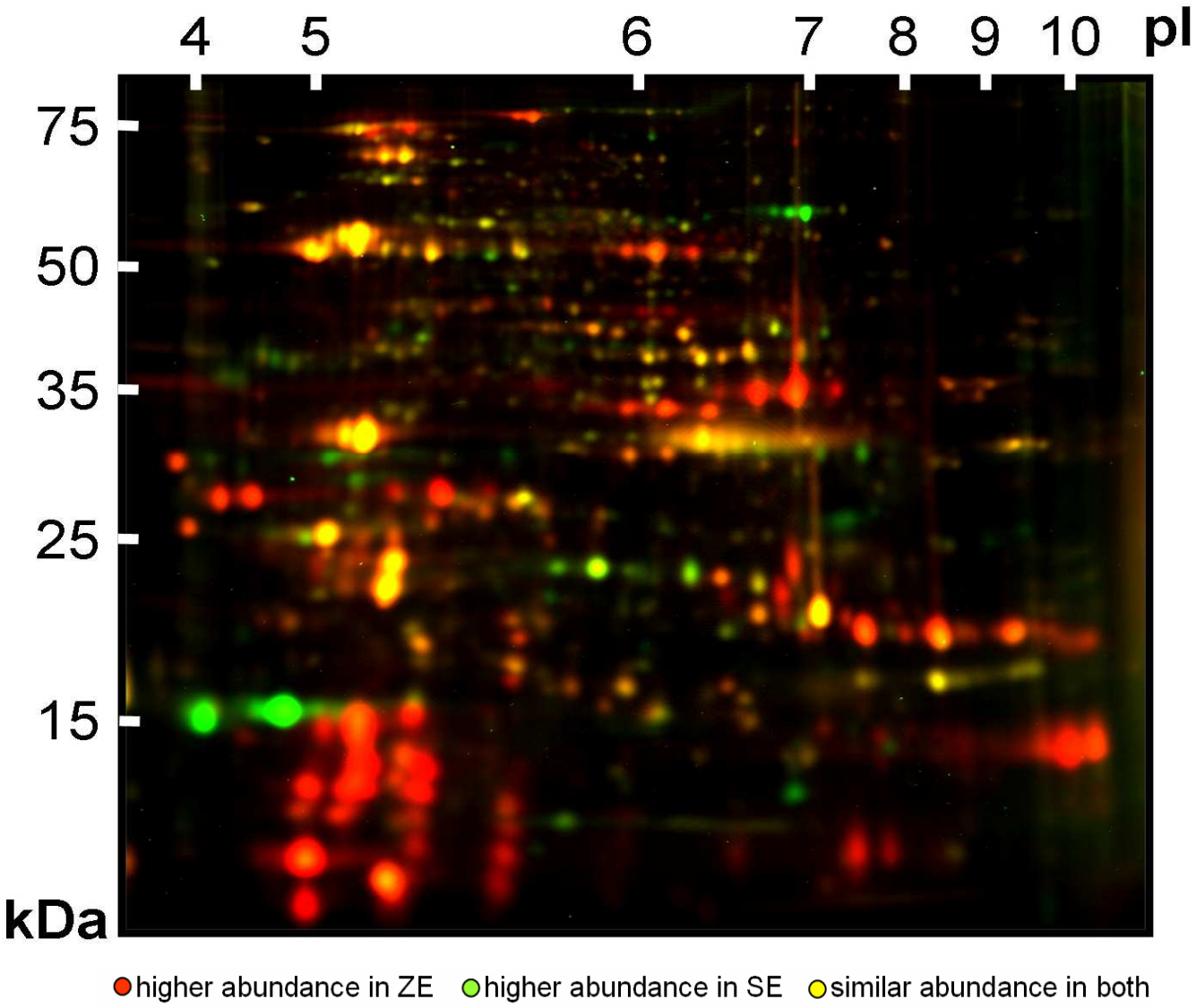

Fig. 1. Two-dimensional DIGE analysis of total protein extracts from zygotic embryos (ZE) and somatic embryos (SE) from *Cyclamen persicum*.
Proteins of each tissue were extracted as described in this chapter. The total protein fractions of ZE and SE were loaded onto one gel, 100 µg of each fraction. The ZE protein fraction was labeled with Cy3™ (red), the SE protein fraction with Cy5™ (green). Spots with similar abundance in both tissues are colored yellow. Internal standard was not included (Rode, unpublished).

4. Notes

1. Referring to **Step 1** of the protocol (3.2.), a lysis buffer without DTT addition is used. At **Step 5**, a lysis buffer containing the twofold amount of DTT is used. Since the concentration of DTT in the lysis buffer is variable (20-100 mM), addition of 7.5 mg DTT ml-1 is adequate at this step.

2. Protein amount can extremely vary in different plant tissues. Thus, the amount of tissue used for sample preparation has to be adjusted: In our hands, proteins were extracted from 500 mg *Medicago* root tissue *(20)*, 200 mg *Vigna* leaf tissue *(22)* and 80 mg *Cyclamen* seeds *(4)*. A thoroughly prepared sample obtained by grinding at low temperature (< 4 °C) improves the quality of protein extraction. The use of a bead mill (eg: Retsch MM 400, Retsch, Haan, Germany) for the pulvarisation of plant material is recommended. However, grinding time and volume of grinding tubes has to be optimized for specific plant material. Low amounts of soft plant material like leaves, roots, embryos require short grinding times (1-5 min) and can be performed directly in Eppendorf vessels using steal balls. Large amounts of hard tissue may be grinded for longer time-periods (up to 30 min). Long storage of frozen plant samples even at - 80°C conditions before protein preparation minimizes the yield of extracted total protein.

3. The 2 ml Eppendorf vessels should be filled up to three-quarter with fine ground plant tissue powder. While adding the extraction buffer and subsequently the phenol, the volume of the sample decreases.

4. All work with ß-mercaptoethanol should take place under the extractor hood!

5. The quality of the phenol is of high importance. Phenol of insufficient quality can cause dramatic losses in protein yield. When using a two-phase phenol, it is important to take up the liquid phase only from the lower phenol phase. Do not shake the phenol in order to mix both phases. Work with phenols only should take place under the extractor hood!

6. After centrifugation (**Step 3**), normally an aqueous phase is obtained at the bottom of the Eppendorf vessels, and a phenolic phase at the top. Plant cell walls and membrane compounds precipitate at the interface. In rare cases, aqueous and phenol phases are inverted after centrifugation. In this situation, the upper phase should be carefully removed with a pipette before collecting the phenol phase. Generally, transfer the phenol phase in several small volume steps (e.g. in 2 to 6 steps à 100 µl).

7. The second uptake of the phenol phase (**Step 6**) should be achieved without contamination of the liquid phase. It is recommended also to perform it via several small volume steps (e.g. à 75 µl).

8. To obtain a very pure pellet, the washing step can be repeated for several times (**Step 7**).

9. Do not dry the protein pellet for too long under the extractor hood, because otherwise its re-suspension in the lysis buffer might become difficult (1-5 min are usually sufficient). The final yield of pellet should be approximately between 5 and 20 mg protein; the protein yield can be determined using a common protein quantification method.

10. The weight of the dried protein pellet gives an indication about the amount of protein extract.

11. For an equal protein amount in the labeling reaction, the volume for re-suspension may vary according to different protein amounts of the samples.

12. CyDye labeling most effectively takes place if the pH value of the lysis buffer is in the range of 8.5. The pH of the sample in lysis solution can be tested via pH test strips. In case the pH value is significantly below 8.5, a lysis buffer of higher pH values (e.g. pH 9.0) should be used for re-suspension of proteins. Always use CyDyes from one reaction kit diluted with dimethylformamide (DMF) of one batch to assure comparative labeling conditions. Consume the CyDyes efficiently after diluting them in DMF within three months.

Acknowledgements

The authors would like to thank Jennifer Klodmann, Institute for Plant Genetics, Leibniz Universität Hannover, for critically reading the manuscript.

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GelMap - A novel software tool for the creation and presentation of proteome reference maps

Christina Rode, Michael Senkler, Jennifer Klodmann, Traud Winkelmann, Hans-Peter Braun

Journal of Proteomics, 74, 2214-2219

Enolases - storage compounds in seeds? Evidence from a proteomic comparison of zygotic and somatic embryos of Cyclamen persicum Mill.

Christina Rode, Sebastien Gallien, Dimitri Heintz, Alain Van Dorsselaer, Hans-Peter Braun, Traud Winkelmann

Plant Molecular Biology, 75, 305-319

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From callus to embryo - a proteomic view on the development and maturation of somatic embryos in *Cyclamen persicum*

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Planta, 2011, DOI: 10.1007/s00425-011-1554-1
(there is no volume number available, since the manuscript was still in press when this dissertation was published)

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3 Confirmation of the six objectives

The six main objectives of this thesis were described in Chapter 1.1. For all objectives of the thesis considerable progress was achieved providing new insights into the physiology of *Cyclamen persicum* embryogenesis. Below the objectives are listed again and the level of their achievement is explained.

i) Protein isolation and two-dimensional protein separation of the *Cyclamen persicum* embryo proteome were improved

To find a protein extraction method which allows very efficient protein purification and at the same time is compatible with high-resolution protein separation, four protocols were tested with somatic embryo tissue of *C. persicum*: A protocol utilising TCA precipitation in combination with acetone (Damerval et al., 1986), a protocol described by Corke and Roberts (1996), a rapid protocol without precipitation as described by Gallardo et al. (2002) and an

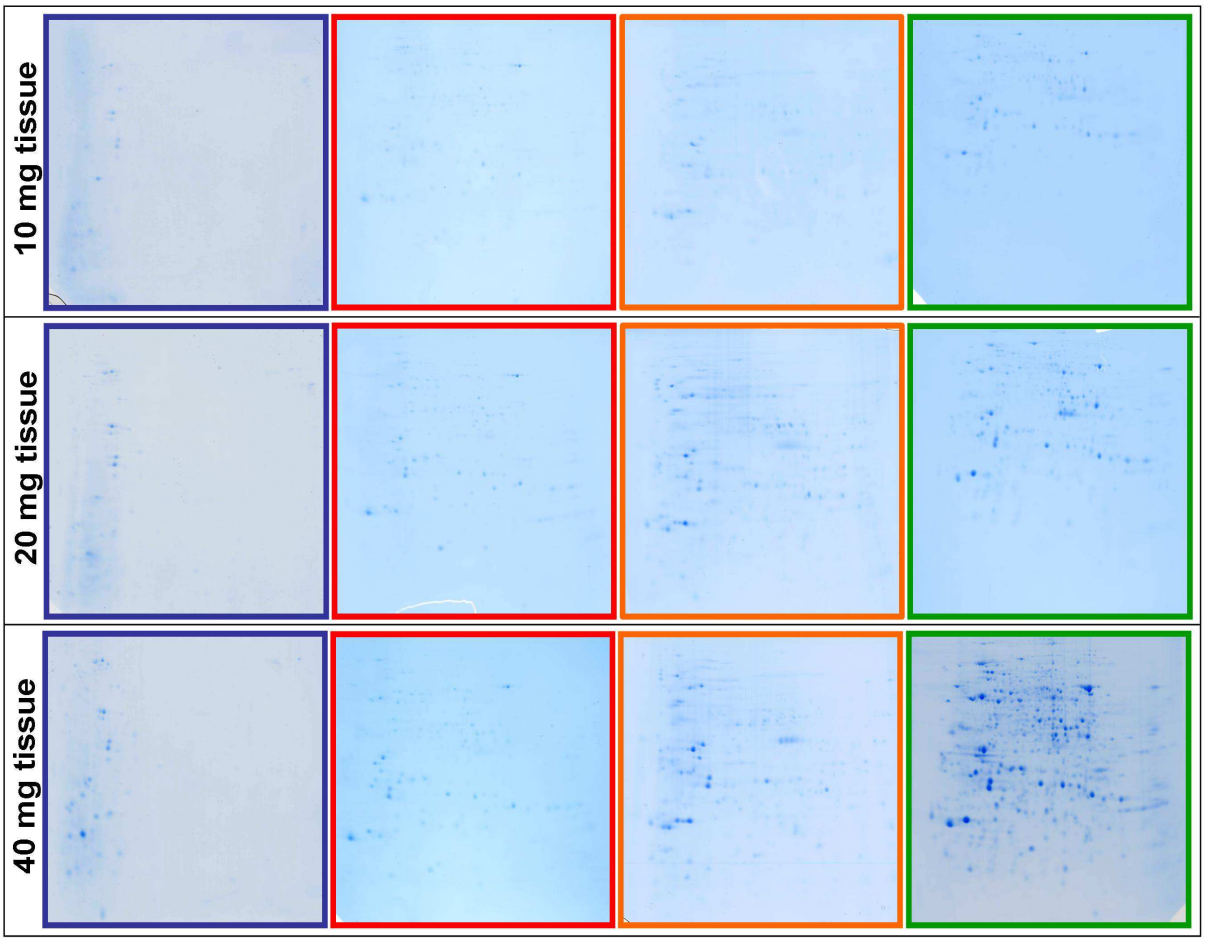

Figure 5 Comparison of four protein extraction methods
Proteins were extracted out of 10, 20 and 40 mg fresh somatic embryos using four different protein extraction methods, each.
Blue-framed gels: proteins were extracted following Damerval et al. (1986)
Red-framed gels: proteins were extracted following Corke and Roberts (1996)
Orange-framed gels: proteins were extracted following Gallardo et al. (2002)
Green-framed gels: proteins were extracted following Hurkman and Tanaka (1986) with modifications as described by Colditz et al. (2004)

extraction method using phenol combined with ammonium acetate in methanol precipitation (Hurkman and Tanaka, 1986, modified by Colditz et al., 2004). Representative IEF-SDS gels loaded with protein fractions extracted from 10, 20 and 40 mg embryo tissue using the four different protocols are given in Figure 5. Optimal protein purification and resolution were achieved using the protocol of Hurkman and Tanaka (1986) modified by Colditz et al. (2004) with 40 mg tissue. For further investigations this method was used and the amount of tissue was doubled to 80 mg (corresponding to 240 embryos) to obtain even more protein spots. Thus on IEF-SDS and DIGE gels more than 1000 protein spots could be resolved. Compared to the previous study (Winkelmann et al. 2006) resolution was five-fold increased. The DIGE technique allows separation of two protein samples in one gel system via different fluorescence labelling of the two protein samples before the gel electrophoresis. Using this tool, the protein samples of zygotic and somatic embryos were analyzed allowing identification of even small differences in the protein patterns of both tissues. The improved protein isolation and 2D separation not only improved the quality of the gels but also facilitated subsequent protein identification via mass spectrometry. See Chapter 2.1 and 2.3

ii) Proteome reference maps for somatic and zygotic embryos of *Cyclamen persicum* were established

For the establishment of a digital proteome reference map a novel software named GelMap was developed (www.gelmap.de). GelMap represents a user-friendly tool that offers a professional graphical user interface for high end creation, publication and evaluation of protein reference maps. Filters can be used to assign proteins on three levels of functional categories or other criteria like differential abundance during comparative proteome projects. As a result, GelMap provides new evaluation tools as compared to so far available 2D reference map software. The proteome reference map for zygotic embryos of *Cyclamen persicum* was created with GelMap and is publiclly available at www.gelmap.de/cyclamen. This reference map includes 247 protein spots identified via mass spectrometry isolated from the zygotic and the somatic embryos proteomes. See Chapter 2.2 and 2.3

iii) Proteins of differential and similar abundance within the proteomes of zygotic and somatic embryos of *Cyclamen persicum* were identified

More than 1,000 protein spots of zygotic and somatic embryos of *Cyclamen persicum* have been characterised and differences between both proteomes were visualised using Coomassie stained IEF-SDS PAGE and DIGE. Statistical analyses revealed 246 protein spots of at least 1.5-fold higher or lower abundance within one of the analysed tissues representing 24% of all spots detected in both tissues. Mass spectrometry analysis of the 300 most abundant protein

spots in gels of both tissues resulted in the identification of 247 proteins, which represent 90 distinct protein species. The most striking results of the comparison of somatic and zygotic embryos are: a) Glycolysis plays a key role in zygotic and somatic embryogenesis, b) serine/glycine metabolism differs in zygotic and somatic embryos, c) somatic embryos are more stressed than their zygotic counterparts, d) in zygotic embryos storage proteins are more abundant. Remarkably, the glycolytic enzyme enolase was the protein most frequently detected and thus is supposed to play an important role in *Cyclamen* embryogenesis. Enolase forms of significantly lower molecular mass than theoretically expected were present in both embryo types but highly abundant in zygotic embryos and endosperm. Peptide structure of those "small enolases" indicated a lack of the central amino acid sequence part including the active site. Thus the small enolases are supposed to be not enzymatically active and may be stored in large amounts in cells without adverse effects on the primary metabolism. The role of small enolase as storage proteins has not been reported until now. To elucidate the specific role of this protein in *Cyclamen* embryo cells, furthers investigations on subcellular localisation, functional arrangement and amino acid sequences are necessary. See Chapter 2.3.

iv) Major storage proteins and enzymes involved in storage compound metabolism of *Cyclamen persicum* were characterised

Small enolases are candidates for a novel class of major storage proteins in *Cyclamen* embryos. Additionally the storage protein 7S globulin, common in different plant species (Shewry, 1995), was highly abundant in zygotic but also present in lower amounts in mature somatic embryos. The Em-protein, an osmoprotective molecule of the late embryogenesis abundant (LEA) class (Swire-Clark and Marcotte 1999) was also high abundant in zygotic and mature somatic embryos. See Chapter 2.3 and 2.4.

v) Major proteins of the different developmental stages of somatic embryogenesis of *Cyclamen persicum* were elucidated

The proteome structures of six developmental stages from callus to torpedo-shaped embryo were analysed and proteins were identified using the digital reference map of *Cyclamen persicum* zygotic embryos. In callus, enzymes related to energy supply were especially highly abundant, most likely due to energy demands caused by fast growth and cell division. The switches from callus to globular embryo as well as from globular to torpedo-shaped embryo were associated with controlled proteolysis via the ubiquitin-26S proteasome pathway. Storage compound accumulation was first detected 21 days after transfer to plant growth regulator (PGR)-free medium in early torpedo shaped embryos. Increase in abundance of auxin-amidohydrolase during embryogenesis indicates an exceptional increase in auxin

release in the late embryo stages of *Cyclamen*. A development stage specific isoelectric point shift of catalases has been reported for the first time for somatic embryogenesis. See Chapter 2.4

vi) First factors improving the production system of somatic embryos in a way that their proteome structure resembles the one of the zygotic embryos were identified

Somatic embryos cultivated on Murashige and Skoog (1962) medium with high sucrose levels and treated with ABA expressed a proteome structure with increased similarity to the one of zygotic embryos. Especially the accumulation of storage compounds was promoted by these two factors. See Chapter 2.4.

4 Discussion and Outlook

Major physiological processes within somatic and zygotic embryos have been characterised in this study by comparing the proteomes of zygotic and somatic embryos (Chapter 2.3) and by analysing the proteome structures of different developmental stages -from callus to embryo- during somatic embryogenesis (Chapter 2.4). The most striking results of the proteomic comparisons of zygotic and somatic embryos were (i) the elucidation of glycolysis as a key pathway in zygotic and somatic embryogenesis, (ii) that somatic embryos are more stressed than their zygotic counterparts and (iii) that storage proteins are more abundant in zygotic embryos. In addition "small" 15 kDa forms of the glycolytic enzyme enolase have been identified as candidates for a novel group of storage proteins in plants. In a time-course proteomic analysis of the different developmental stages during somatic embryogenesis, an essential role of the ubiquitin-26S proteasome pathway for the switch from callus to globular embryo as well as from globular to torpedo-shaped embryo was identified. Moreover, a developmental stage -specific isoelectric point of catalase (Chapter 2.4) was observed. Abscisic acid supplementation and cultivation of embryos on medium with high sucrose content promoted the desirable maturation of somatic embryos (Chapter 2.4). The results of this work are discussed in detail in the corresponding publications and manuscripts in Chapter 2.

4.1 Discussion

In this section, findings of the current study are discussed especially in comparison to those results obtained in the four recent works regarding somatic embryogenesis of *Cyclamen* at the proteomic and transcriptomic level (Rensing et al., 2005; Winkelmann et al., 2006; Lyngved et al., 2008; Hönemann et al., 2010).

Rensing et al. (2005) analysed 2083 expressed sequence tags (ESTs) from seven different developmental stages within *Cyclamen* embryogenesis starting with callus. In total, 116 of those transcripts have been shown to be directly involved in embryogenesis. Of special interest were gibberellin oxidases and somatic embryogenesis receptor-like kinases (*SERK*), encoding proteins essential for somatic embryogenesis (Mitsuhashi et al., 2003; Schmidt et al., 1997; Nolan et al., 2003). Histone H3 and a histone deacetylase as well as a DNA methyltransferase all involved in the DNA methylation pathway were identified within the *Cyclamen* transcripts. Since low levels of DNA methylation are supposed to improve the embryogenic potential of cell lines (Chakrabarty et al., 2003; Noceda et al., 2009; see also chapter 1.3), those proteins are of special interest and should be further investigated to

improve protocols for the screening of highly embryogenic cultures. In addition, genes encoding proteins involved in reactive oxygen species detoxification were found to upregulated in the nearly stages of embryogenesis by Rensing et al. (2005). In the study presented in chapter 2.4 also stress-related proteins were detected in the early developmental stages as compared to callus, namely heat shock protein 20, heat shock protein 70 and catalase. Heat shock proteins are molecular chaperones, often highly expressed under stress conditions but also necessary for normal plant development in unstressed cells (Hendrick and Hartl 1993; Waters et al. 1996), while catalases are directly involved in reactive oxygen species detoxification (Chelikania et al., 2004). However, in the study presented in chapter 2.4 other heat shock proteins as well as proteins involved in reactive oxygen species detoxification were also identified in later developmental stages (21-56 days after transfer to PGR-free medium). Thus, in contrast to Rensing et al. (2005) no exclusive stress situation could be detected for the early developmental stages (Chapter 2.4).

Hönemann et al. (2010) compared somatic and zygotic embryos of *Cyclamen persicum*, analysed the development of somatic embryos and elucidated the differences between an embryogenic and non-embryogenic cell line using a transcriptomic approach based on the data published by Rensing et al. (2005) in combination with histological analysis. In total the expression of 1,216 transcripts was investigated by Hönemann et al. (2010). The comparison of zygotic and somatic embryos revealed that somatic embryos have been found to express more genes encoding the oxidative stress-related proteins catalase and superoxide dismutase. In accordance with the study presented in chapter 2.3, somatic embryos were also described to express more stress-related pathways as compared to the zygotic embryos especially catalases. Interestingly, the isoelectric point of these catalases changed during somatic embryogenesis and becomes more acidic with proceeding development. Torpedo shaped somatic embryos cultivated on MS medium containing high sucrose concentration (Chapter 2.4) accumulated more catalases as compared to embryos of the same age cultivated on medium containing the standard sucrose concentration (Cahpter 2.4). Most likely, the increased osmotic pressure caused by the high sucrose level in the medium not only triggered the accumulation of storage proteins like “small enolases“ and globulin but also evoked stress response pathways. Superoxide dismutases were also identified in the study presented in chapter 2.3. By contrast to the study of Höhnemann et al. (2010) these proteins were not specific for one of the compared embryo type. In addition Hönemann et al. (2010) found the epidermis of somatic embryos to be more ruffled and assumably lignified. Lignification is a further hint for increased stress metabolism in somatic embryos (Dixon and Paiva, 1995).

Thus, Hönemann et al. (2010) suggested a reduction of stress sources occuring during somatic embryogenesis in vitro, e.g. by adding activated charcoal to the culture medium and further pointed out the importance of pathways involved in cell wall synthesis/degeneration for the early stages of embryogenesis.

Genes encoding chitinase, important for a proper protoderm formation (De Jong et al., 1992), and peroxidase, which plays a role in cell size expansion and cell wall synthesis (Cordewener et al., 1991; Takeda et al., 2003), were upregulated in early pre-globular stages. In the study presented in Chapter 2.3 and 2.4 none of these enzymes were identified. However, three spots were identified as polygalacturonase, an enzyme depolymerising pectin bounds within the cell walls. Interestingly, these proteins were low abundant in callus (see Chapter 2.4) and increased in abundance with processing development of somatic embryos. Two of the three polygalacturonase protein spots were higher abundant in zygotic embryos (see Chapter 2.3).

Winkelmann et al. (2006) published the first proteomic study on somatic embryogenesis in *Cyclamen*, where about 300 protein spots were resolved via IEF-SDS PAGE per gel. In total 23 proteins were identified from 83 analysed spots extracted from gels of zygotic embryos, somatic embryos, germinating embryos and endosperm (representing 28% identification rate). Winkelmann et al. (2006) estimated that 26 % of the proteins present in the 2D gels of somatic and zygotic embryos were of different abundances. This ratio is comparable to the study presented in chapter 2.3, where 24 % of the proteins resolved via IEF-SDS PAGE were of different abundances between both embryo types, even though the protein resolution was five times increased compared to the previous work. Based on the present study, Winkelmann et al. (2006) observed that somatic embryos cultivated on medium containing 30 g l-1 sucrose accumulated less storage proteins than those cultivated on medium containing the double sucrose concentration. The "small enolases" protein spot group proposed as putative storage proteins in chapter 2.3 were also present in the gels published by Winkelmann et al. (2006) and postulated to represent storage proteins. However, Winkelmann et al. (2006) identified one spot among the protein spots group including more than ten spots. This spot was identified not as enolase but as the storage protein vicillin. A ClustalW multiple alignment (Thomson et al., 1994) of the complete amino acid sequences of enolase and vicillin revealed no sequence homologies of these two proteins (data not shown).

Höhnemann et al. (2010) as well as Winkelmann et al. (2006) found xyloglucan endotransglycosylase to be highly expressed/abundant in torpedo shaped embryos and endosperm, an enzyme synthesising or hydrolysing xyloglucans (Rose et al., 2002). Xyloglucans are oligosaccharids which represent on one hand major compounds of cell walls

and on the other hand can be accumulated as storage carbohydrates in seeds (Fry, 1989; Braccini et al., 1995). In the studies presented in Chapter 2, no enzymes involved in the xyloglucan biosynthesis were identified. Probably, this was due to the limited number of analysed spots.

Lyndgved et al. (2008) analysed the proteomes of embryogenic and non-embryogenic callus of *Cyclamen persicum* via DIGE and 1,000 protein spots were resolved per gel. About twenty percent of these spots were differentially abundant in the two distinct cell lines and 38 proteins were unique to one of the analyzed tissues. The differentially abundant proteins as well as the unique proteins were analysed via mass spectrometry leading to identification of 128 protein spots representing 52% identification rate. Nearly 90% of the identified proteins were assigned to three major groups: i) metabolism (68%), ii) protein processing (14%) and iii) stress response (4%). Interestingly, those three groups also included the majority of the proteins identified in the current study (see Chapter 2.3) in comparable portions: i) metabolism (54%), protein processing (23%) and stress response (9%). It is also interesting, that in the above mentioned proteomic studies as well as in the study presented in Chapter 2.3 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) represented one of the most often identified proteins: Winkelmann et al. (2006) found three GAPDH spots representing 13% of all identified proteins, Lyndgved et al. (2008) identified seven GAPDHs representing 6% of all identified proteins and in the current study 20 protein spots were identified as this enzyme, representing 8% of all identified proteins. There are two possible explanations for the high number of identified GAPDHs: Whether this protein is especially well detectable via a gel based approach coupled to mass spectrometry due to its biochemical properties and GAPDH sequences within the protein databases are well matching to amino acid chain present in *Cyclamen* specific version. Alternatively, this protein may play a special role during embryogenesis. Like enolase, GAPDH is a glycolytic enzyme, which also was found as a structure protein in the eye-lens of vertebrates (Piatigorsky, 2003). Thus also GAPDH might be utilised as storage protein in *Cyclamen*, like discussed for enolases in chapter 2.3. Some interesting preliminary results concerning the native structure of this protein are presented in chapter 4.2.

In conclusion, similar tendencies concerning the physiology of embryogenesis in *Cyclamen persicum* were detected in the discussed studies: Somatic embryos seem to be more stressed and accumulate less storage compounds compared to their zygotic counterparts. It has been shown in chapter 2.4 that proteomes of both embryo types can be aligned to a certain level by abscisic acid treatment and high sucrose concentration within the culture medium. SERK and

histones analysed by Rensing et al. (2005) and Hönemann et al. (2010) are of special interest for embryogenesis research (see above and chapter 1.3), however those proteins are hardly detectable via a gel based proteomic aproach like performed in this study (Chapter 2.3 and 2.4). These two proteins have a hydrophobic character not compatible with the water based gel systems applied. To analyse those hydrophobic proteins a gel free approach should be more appropriate. Indeed, in a preliminary gel free shot gun analysis of *Cyclamen persicum* embryos a histone H4 could be identified. Thus gel free shot gun protein analyses revealed to be a complementary technique to a gel based approach and should provide further interesting results to elucidate the physiology of embryogenesis in *Cyclamen* (see Chapter 4.2 in "Gel-free proteome profiling via shotgun").

4.2 Outlook

For a global validation and interpretation of data observed in the current work, there were still questions left unresolved. Therefore, our future studies address these questions in the second part of this chapter (4.2). In some cases preliminary experiments have already been performed and first results are presented here.

Is the digital reference map of zygotic embryos applicable for other tissue types or somatic embryos of different developmental stages?

The digital reference map established for torpedo shaped zygotic embryos was created in order to facilitate protein identification for subsequent projects. Via this map proteins can be identified not only for somatic embryos of the same developmental stage but also for embryogenic callus, early globular embryos, as well as matured somatic embryos (see Chapter 2.4). However, protein spots on the same gel position but of distinct tissues could represent different proteins. In other cases, a protein present in the reference map can be overlapped by a more abundant protein present in a different developmental stage. Thus, the distinction/difference between the proteins indicated on the reference map and the proteins present in the gels of the developmental stages of interest should be validated. Therefore, five representative spots have been isolated from IEF-SDS gels of all developmental stages analysed (see Chapter 2.4) and protein identification is ongoing. Depending on the results, either the recent reference map will be approved for the other tissues of interest or further reference maps have to be generated for far distinct tissues like callus.

How to improve GelMap?

GelMap is an excellent tool for the establishment and publication of proteome reference maps (Chapter 2.2). However, the version of this software as described in chapter 2.2 only allows

annotation of one protein per analysed spot and only the physiological function, abundance, etc. of this protein can be included into the physiological clustering and into to the filter systems. For example, in the reference map presented in chapter 2.3 (see also: http://www.gelmap.de/cyclamen/) only the best protein hits regarding probability score and sequence coverage are included in the reference map, while minor protein hits for all 247 identified spots are listed in a separate table. Those "minor" hits are neither included in the physiological clustering nor in the filter systems of GelMap. This is somehow an unsatisfying solution, since on the one hand the minor hits may also play an important role within biology of the analysed samples and on the other hand it is not in each case clearly to ascertain what is the best hit, if for example the probability scores and sequence coverages of two or more hits are quite similar. Thus GelMap was further developed to display all protein hits for each spot of interest, which are all included into the filtering system. This modification is described in the manuscript by Klodmann et al., 2011, which is in the process of review since March 2011. In addition, an embedding of GelMap into large proteomic platforms like ExPASy and UniProt would be desirable in future.

Are the recent data transferable to other *Cyclamen* genotypes?

The proteomic studies were performed with callus and somatic embryos derived from one genotype, the commercial *C. persicum* cultivar 'Maxora Light Purple' bred by the company Varinova B.V. (Berkel en Rodenrijs, Netherlands), and with zygotic embryos derived from self pollinated flowers and thereof developed seeds of the same genotype. To transfer the new findings to *Cyclamen* in general, embryos of other genotypes and different *Cyclamen* species have to be analysed. Winkelmann et al. (2006) and Bian et al. (2010) published IEF-SDS gels of *Cyclamen persicum* embryos using different cultivars as well as different protein isolation protocols. Even though major protein patterns within those gels were common to the gels produced in this study. For *Cyclamen* species other than *C. persicum* proteomic studies are not available. However, protocols for the in vitro production of somatic embryos are published for *C. coum*, *C. mirabile*, *C. alpinum* and *C. graecum* (Prange et al. 2010a, b). Thus, material and techniques for a complementary study with a wild *Cyclamen* species e.g. *Cyclamen coum* are present at the campus and could be performed in near future.

The somatic embryos investigated in this study (Chapter 2.3 and 2.4) were derived from an F1 cultivar and thus also represented the F1 genotype. In contrast, the zygotic embryos were isolated from self pollinated F1 plants resulting in F2 offspring genotypes. Consequently the two embryo types compared in Chapter 2.3 were closely related but not absolutely genetically identical. The crossing between the two parental inbred lines of 'Maxora Light Purple' is in

progress, but so far not enough F1 seeds were developed to perform 2D gels. However, due to the high number of embryos necessary for a sufficient protein amount to load on one gel (240 embryos/gel), the effects of the slightly different genotypes might be quite low in the current study.

What is the role of the endosperm in embryo development?

Zygotic embryos are enveloped by an endosperm providing not only protection, but also regulatory and nutrition compounds (see also chapter 1.3). Probably the asynchronous development and physiological disorders found in somatic embryos are caused by missing endosperm born triggers or substances. Reinhardt (2006) reported proteins, carbohydrates and lipids to be accumulated in *Cyclamen* seeds. The storage polysaccharide xyloglucan (Braccini et al., 1995; Winkelmann et al., 2006) and the storage proteins 11S globulin and 7S globulin were identified in the endosperm of *Cyclamen* (Winkelmann et al., 2006). However, still little is known about the physiology of endosperm and its role in embryogenesis for *Cyclamen*. First comparisons of IEF-SDS gels of 11 weeks old endosperm and embryos of the same age are presented here. The protein purification and IEF-SDS PAGE were performed like described in Chapter 2.3 for 50 mg of endosperm tissue. Figure 6 shows IEF-SDS gels of endosperm in comparison to IEF-SDS gels of zygotic and somatic embryos. Major groups of endosperm proteins were found to be also present in the zygotic embryos but exhibit low abundance in somatic embryos. This suggests either a similar protein expression in endosperm and zygotic embryos or a transport of these proteins most likely from endosperm to embryo. Further investigations on the physiology and development of the endosperm are ongoing.

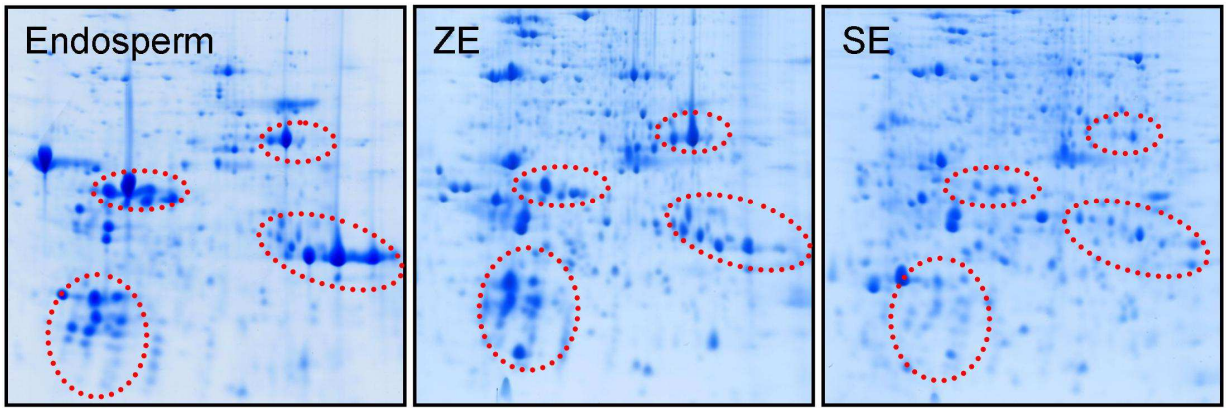

Figure 6 Alterations in protein abundance in the proteomes of endosperm, zygotic and somatic embryos
IEF-SDS gels of total soluble protein fractions extracted from fresh endosperm or embryo tissue. Gels were stained with CBB-G 250. Molecular mass range: 100–5 kDa (top to bottom). Non-linear separation: pI 3-11 (left to right). Red labelled spot groups are characteristic for endosperm and zygotic embryos but significantly lower abundant in the somatic embryos proteome. ZE = zygotic embryo; SE = somatic embryo

Is there an alternative to gel based proteomics?

The proteomic results of this study are based on two-dimensional gel electrophoresis. As described in Chapter 1.4 this technique represents a basic tool in protein biochemistry but is more or less limited to hydrophilic proteins. As a result the characterised protein population predominantly consists of cytosolic proteins (e.g. proteins of the glycolysis) but hydrophobic proteins as well as proteins of very low abundance (e.g. transcription factors) are underrepresented or even not detectable. For example SERK-proteins, which are supposed to play a significant role in the initiation of somatic embryogenesis, contain two strongly hydrophobic regions (Hecht et al., 2001; Schmidt et al., 1997) and thus their analysis via gel-based proteomics is challenging. A shotgun approach allows fast protein identification without separation on a gel. In comparison to a gel-based experiment different protein species can be identified, especially hydrophobic proteins and proteins with an extreme isoelectric point (basic or acidic). Brechenmacher et al. (2009) analysed the proteome of soya root tips via gel-based and shotgun proteomics and thus identified 1,492 different proteins, whereof only 169 proteins were identified with both methods. Thus the combination of both, gel-based and gel-free techniques, allows the identification of large numbers and different kinds of proteins and thus enables a deep view into the physiology of the analysed tissue. Shot gun proteomics is carried out with highly sensitive mass spectrometers resulting in very accurate masses for the analysed peptides. This on the one hand facilitates protein identification when searching against databases and on the other hand allows database-independent evaluation of the complex samples which is especially helpful for the non-sequenced *Cyclamen*. Hoehenwarter et al. (2008) performed a shotgun analysis followed by database independent data interpretation for different potato cultivars and could identify genotype-specific marker peptids. A similar aproach was performed with *Cyclamen* embryos and endosperm and is decribed below.

Gel-free proteome profiling via shotgun

Shotgun analyses of somatic and zygotic embryos as well as endosperm were initiated in cooperation with Dr. Stefanie Wienkoop, Vienna University (Austria). Proteins were extracted following the protocol of Hurkman and Tanaka (1986) modified by Colditz et al. (2004) from 20 mg of somatic and zygotic embryos as well as from endosperm in three replicates, each. After tryptic digestion samples were separated via nanoLC in a monolithic capillary and mass spectrometric analyses were performed using the ESI-LTQ-Orbitrap system (Thermo Scientific, Bremen, Germany) as described by Hoehenwarter et al. (2008). Two sets of data were obtained from these analyses: i) MS data, including accurate masses

(mass/charge ratios) and ii) MS/MS data including peptide sequences derived from selected, fragmented masses.

I. Database independent approach: The MS data were utilised for the database independent approach. An independent component analysis (ICA) of the 2.000 non-redundant masses resulted in an ICA plot clearly discriminating all three investigated tissues (Figure 7). The 30 masses corresponding to the highest rate of separation within the ICA plot (highest loadings) were selected for de-novo sequencing using the Peaks software (Bioinformatics Solutions, Waterloo, Canada). Hereof eight peptide sequences could be generated representing markers for the three analysed tissues (Table 2). Database alignments of these peptides resulted in the identification of three proteins, which are named in table 2. The five remaining peptides are supposed to represent *Cyclamen* specific proteins not yet known for other plants. They are of special interest, since their distinct appearance in the three compared tissues could be utilised e.g. as standard peptides for tissue specific protein quantification with mass spectrometers.

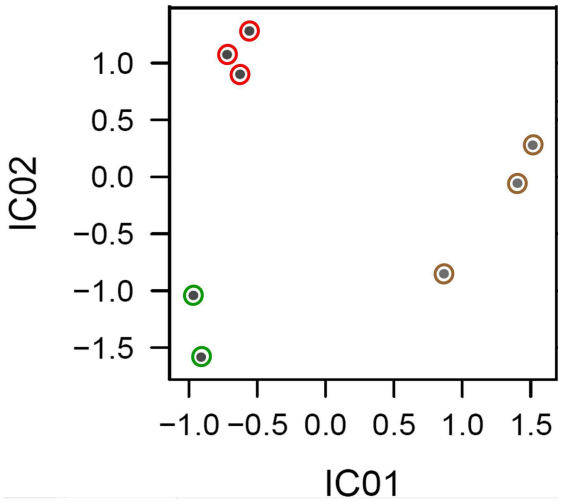

Figure 7 ICA plot based on 2000 unique masses obtained from zygotic embryos, somatic embryos ad endosperm of *Cyclamen persicum*
Red labelled spots represent complex samples from zygotic embryos, green labelled spots from somatic embryos and brown labelled spots from endosperm.

II. Database dependent approach: Based on the peptide sequences derived from the MS/MS mode protein identification was carried out using the SEQUEST algorithm (Thermo, Bremen, Germany). A search against the "Green plant" database provided by NCBI resulted in the identification of 150 unique proteins and a subsequent search against the *Cyclamen* EST database (Rensing et al., 2005) resulted in 89 unique proteins. A first comparison of the proteins identified in the gel-based study (Chapter 2.3) and the proteins identified via the shotgun approach resulted in an overlap of 70%. This was unexpectedly high. Brechenmacher et al. (2009) reported only 11% overlap between proteins identified with these two methods for soybean root hair. Most likely, this is due to the limited sequence information fitting to the peptides identified for *Cyclamen*. However, about 50 proteins could be identified which so far have not been characterised for the analysed tissues, e.g. the hydrophobic histone H4, which has also been identified in the transcriptomic study of Rensing et al. (2005) and proposed to play an essential role in *Cyclamen* embryogenesis, and a "cup shaped cotyledon ortholog"

with a molecular weight over 200 kDa, which is involved in shoot apical meristem formation (Takada et al., 2001, see also chapter 1.3). A manuscript based on the results of the shotgun study is in preparation and will be published in near future.

Table 2
Masses with the higest ranks within the ICA plot, their corresponding peptides and proteins

Mass[1]	ICA rank[2]	De-novo peptide[3]	Protein[4]
723.89	1 (ICA1)	LNLWYVDGTPLR	no hit
811.91	4 (ICA1)	CDSPELLEAAFNTGR	no hit
842.90	7 (ICA1)	EFSTLWSADDWATR	no hit
601.81	11 (ICA1)	EDAGGVVSAELR	hypothetical protein
698.37	14 (ICA1)	DVDDLLLSTYNK	protein tyrosine phosphatase
617.82	4 (ICA2)	SPEAVGSQSGSTK	no hit
616.83	7 (ICA2)	DNVLAMLLDTK	pyrovate kinase

[1] unique mass/charge ratio in Thomson (only masses where de-novo sequencing was succesful are given)
[2] rank of the mass within the ICA analysis (1 = highest rank)
[3] corresponding peptide sequence derived from de-novo sequencing using the Peaks software
[4] corresponding protein identified via databa sesearch (Green plant).

How to investigate physiological pathways of special interest in more detail?

Imuno-histology to investigate the subcellular localisation of "small enolases"

To elucidate the specific role of ‘‘small enolases’’ in *Cyclamen* embryo cells, an immunohistological approach would give essential information pertaining to functional arrangement. Storage proteins often are secreted and stored in protein bodies (Shewry, 1995), the occurrence of which was described in *Cyclamen* endosperm especially in close proximity to the embryo and the testa (Reinhardt 2006). To investigate the subcellular localisation of enolases an immunohistological experiment like described by Eticha et al. (2005) would be helpful. Eticha et al. (2005) visualised pectins coupled to fluorescence labelled pectin-antibodies in histological slides of maize roots using a confocal laser scanning microscope. Enolases in the eye-lances of vertebrates were analysed by a similar immunohistological approach by Wistow et al. (1988). Here, turtle embryos were embedded in paraffin, subsequently sectioned using a microtome and resulting slides were incubated with fluorescence labelled antibodies against enolase. As a result enolases were identified as highly abundant structure proteins in the eye-lens of turtles. For *Cyclamen*, histological slides of embryos and endosperm could be incubated with small enolase specific antibodies coupled to a fluorescent dye, and examined under a confocal laser scanning microscope or a fluorescence microscope in case of paraffin embedded samples.

BN-SDS PAGE to investigate storage protein complexes

Storage proteins are of special interest due to their impact on maturation and dormancy. These processes have been shown to be less developed in the somatic embryos of *Cyclamen* compared with zygotic embryos (Chapter 2.3; Winkelmann et al., 2006) but are essential for a more uniform development and desirable long term conservation. Various storage proteins are packed in specialised vacuoles and form therefore protein complexes (Shewry, 1995; Hermann and Larkin., 1999). BN-SDS PAGE (Wittig et al., 2006) coupled with mass spectrometry has been shown to be a powerful tool for the determination of protein complex composition (Dudkina et al., 2005; Klodmann et al., 2010). Klodmann et al. (2010) resolved the internal architecture of plant mitochondrial comlex I, which is part of the respiratory chian, via low SDS treatment of the native complex and analysis of the resulting subcomplexes by BN-SDS PAGE in combination with mass spectrometry. Those data were integrated into the electron microscopic images for this protein obtained by Dudkina et al. (2005). By performing BN-SDS PAGE proteins are separated in the first dimension in their native form due to their native molecular mass in presence of mild detergents. In the second dimension SDS is added resulting in the degradation of the native complexes into their subunits. First experiments resolving the water-soluble native protein fractions of *Cyclamen* embryos via BN-SDS PAGE have been started. 2D gel electrophoresis and protein identification via mass spectrometry were performed as described previously by Klodmann et al. (2010). Figure 8 shows a BN-SDS gel of the somatic embryos proteome. Protein spots indentified by mass spectrometry are indicated. Of special interest are the subunits identified

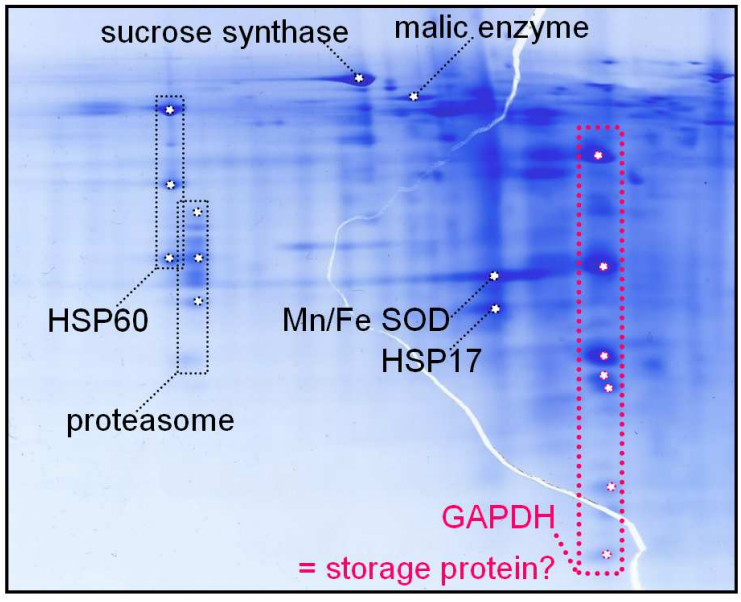

Figure 8 Protein complexes in somatic embryos of *Cyclamen persicum*
Coomassie-stained BN-SDS gel of the total native water-soluble proteome of somatic embryos of *Cyclamen persicum*. Indicated spots were identified via mass spectrometry.
Abbreviations: HSP = Heat shock protein, Mn/Fe SOD = Manganese/Iron dependent superoxide dismutatse, GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

for GAPDH suggesting an aggregation of this glycolytic enzyme in large protein complexes. Eventually the glycolytic enzyme GAPDH is recycled as a storage protein similar to the glycolytic enzyme enolase (Chapter 2.3).

Determination of posttranslational modification

Which proteins are ubiquitinated and phosphorylated during developmental decision from callus to globular embryo and from globular to torpedo-shaped embryo? Are there stage specific isoforms of catalase? To resolve the new questions concerning post translational modifications a gel-base approach like performed by Trapphoff et al. (2009) could be helpful. In this study, phosphorylation site specific antibodies were used to blot IEF-SDS gels of pathogen-infected and non-infected *Medicago truncatula* root cells. As a result phosphorylated protein spots could be detected in both treatments and differences in phosphorylation could be observed between infected and non-infected roots. Since phophorylated proteins are supposed to be activated forms of enzymes, Trapphoff et al. (2009) could identify those proteins actively involved in pathogen response. A comparable experiment could be performed to determine the differentially phosphorylated proteins within the developmental stages of somatic embryos analysed in chapter 2.4. Using an ubiquitin specific antibody in a comparable approach also the proteins “labelled” for proteolytic degradation could be identified on the IEF-SDS gels of the different developmental stages of *Cyclamen* embryos. For a gel-free large scale screening of phosphorylated proteins a technique as presented by Grimsrud et al., (2010) could be applied. In this study more than 1,600 phosphorylation sides whithin *Medicago truncatula* root proteins could be identified and have been made publicly available. However, this approach required a mass spectrometer including an ETD (Electron Transfer Dissociation)-function, which rarely is available in common mass spectormeters.

Metabolomic analyses to investigate major metabolites and auxin levels during somatic embryogenesis

The metabolome of an organism or a specific tissue includes "the complete complement of small molecules" like carbohydrates, lipids and secondary compounds below a molecular mass of 15 kDa (Hall, 2006). For plant tissues the presence of 100,000-200,000 of those small molecules has been estimated (Oksman-Caldentey & Inzé, 2004). The extracted metabolites can be separated via High Performance Liquid Chromatograpy (HPLC), analysed by mass spectrometry and subsequently identified by database search (Hall, 2006). Metabolomics is successfully applied to investigate different research objectives in plant science including not only the elucidation of physiological processes but also for biomarker discovery as well as

qualitative analysis of plants within breeding programmes (Hall, 2006). Robinson et al (2009) identified 47 metabolites within five embryognic lines of *Pinus taeda* which supposed to be markers for the prediction of their embryogenic productivity. They found metabolites correlated to stress response, mainly osmoprotectants like sorbitol, sugar alcohols arabitol, serine and proline to be more abundant in the cell line with decreased embryo productivity. For a better understanding of the physiology of somatic embryogenesis in *Cyclamen* a metabolic profiling would provide essential insights, especially within the carbohydrates and lipids metabolisms, which could not be investigated by the present transcriptomic or proteomic approaches. In addition, a metabolic profiling of auxin levels in the different stages of embryogenesis of *Cyclamen* could elucidate the dynamics of this plant hormone, its correlation to auxin amidohydrolase and auxin-storage conjugates. High levels of auxin-amidohydrolase were typical for the older developmental stages of *Cyclamen* embryos and this enzyme also exhibited an increased abundance in embryos grown on high sucrose MS medium. High auxin levels are not characteristic for late embryos (e.g. Michalczuk et al., 1992, see also chapter 1.3) and the (putative) increase of endogenous auxin evoked by auxin-amidohydrolase in these developmental stages require further investigation in *Cyclamen*.

4.3 Conclusions

In this research work, major physiological aspects of embryogenesis in *Cyclamen persicum* were elucidated and factors promoting the quality of somatic embryos were identified. Additionally, the software GelMap was established and further developed to create, present and evaluate proteomic reference maps. Future studies should focus on further optimisation of the in vitro culture and unravel the newly arised fascinating questions regarding the physiology of embryogenesis. Furthermore, the extension of the plant proteomic portal by including high quality reference maps of various plant tissues and species designed with GelMap is desirable to support proteomic research. Therefore a more user defined upload file as well as the integration of GelMap generated maps into meta-databases would be helpful.

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Appendices

Curriculum Vitae

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University education

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Education

1988-1995	Primery School Sajansk, Russia and Gifhorn, Germany
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Awards

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Publications

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Conference Contributions

Lectures

Rode C. (2009) Erstellung von Proteom-Referenzkarten für somatische und zygotische Embryonen von *Cyclamen persicum* zur Beschreibung und Optimierung der somatischen Embryogenese als Vermehrungssystem. Gemeinsame Tagung der Gesellschaft für Pflanzenbiotechnologie und des Arbeitskreises Deutscher in-vitro-Kulturen, Rostok, Germany

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Posters

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Danksagung

Ich bedanke mich bei Prof. Dr. Traud Winkelmann und Prof. Dr. Hans-Peter Braun für die engagierte Betreuung während der gesamten Zeit meiner Doktorarbeit.

Dr. Frank Colditz danke ich für die fachliche und kritische Beratung sowie für die Korrektur meiner Manuskripte.

Kathrin Lindhorst danke ich für ihre kompetente Arbeit im in vitro Labor, für die Herstellung zahlreicher Gele sowie für ihre Loyalität.

Bei Joachim Klöhn und Diana Wimmer bedanke ich mich für die technische Hilfe im Labor im ersten Jahr der Promotion.

Leonard Kiirika danke ich für die Korrektur der Englischen Sprache in dieser Arbeit.

Meinen Bürokollegen Jennifer Klodmann, Katrin Peters und Christof Behrens danke ich für den freundschaftlichen Umgang, die vielen klugen Tipps im Labor- und Büroalltag und für die Versorgung mit heißen und kalten Getränken.

Ich bedanke mich bei allen Mitarbeitern der Arbeitsgruppen Braun und Winkelmann für ein angenehmes Arbeitsklima. Insbesondere gilt mein Dank Michael Senkler, Dagmar Levejohann, Katja Luck, Dr. Melanie Bartsh und Dr. Annika Prange für ihre praktische Hilfe im Labor und am Schreibtisch.

Dr. Stefanie Wienkoop danke ich für die Einladung an die Universität Wien, Departement für Systembiology und für die gemeinsame Durchführung der shot gun Experimente. In diesem Zusammenhang danke ich auch Dr. Wolfgang Höhenwarter für die Hilfe bei der Auswertung der shot gun Daten.

Dr. Daniel Gerhard vom Institut für Biostatistik, Universität Hannover danke ich für die statistische Auswertung der in vitro Experimente sowie für weitere statistische Beratung.

Dr. Ann-Kathrin Lühmann danke ich für die moralische Unterstützung und Freundschaft am Anfang meiner Promotion.

Evelin Berger vom Helmhotzzentrum für Infektionsforschung in Braunschweig danke ich für die Einführung in die Software Delta2D.

Chrstin Lebing von der Firma Decodon in Greifswald danke ich für die geduldige und kompetente Beratung bei der Auswertung von zweidimensionalen Gelen.

Meinen Eltern Irina und Peter, meinem Bruder Gabriel, meinem Freund Frank, Tante Nelly und Opa Martin danke ich für ihre Liebe und Solidarität.

Ich bedanke mich bei der Deutschen Forschungsgemeinschaft (DFG) für die Finanzierung meiner Stelle, einer Hiwistelle sowie der nötigen Sach-, Reise- und Publikationsmittel (Projektnummer WI2002/3-1).