Gene expression analyses during somatic embryogenesis in *Cyclamen persicum*

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

von

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geboren am 01.08.1977 in Bremen

2012

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Tag der Promotion: 05.01.2012

Abstract

Somatic embryogenesis (s.e.) enables a clonal propagation which is especially desirable for valuable crops. Particularly for plant species where clonal propagation by cuttings is not feasible, s.e. offers great potential for achieving mass propagation. This includes the economically important ornamental crop, *Cyclamen persicum,* that has been established as a model plant for s.e. In spite of its potential for encouraging high rates of propagation, the application of s.e. to commercial plant propagation is very low. This can be mainly attributed to the insufficient reproducibility of empirically developed propagation protocols as well as the restrictions imposed by the frequent occurrence of developmental aberrations and non-embryogenic callus.

To date, it has been standard practice to change protocols empirically to improve the outcome. But the present approach pursued the idea of using the information obtained from gene expression profiling to better understand the underlying processes and thereby develop proposals on the targeted improvement of the existing protocol for *in vitro* s.e. For this purpose, putative underlying physiological processes were discussed and hypotheses on the improvement of the *in vitro*-protocol were deduced based on the data achieved by a cDNA microarray (1,216 transcripts).

Selected hypotheses on the impact of putative glutathione S-transferases (GST) and pectin-modifying enzymes were analysed in more detail by qRT-PCR. Therefore, qRT-PCR protocol have been established and optimised for *Cyclamen,* especially with regard to the reference genes. Both validation approaches demonstrated that in their cases the initial hypothesis could not be supported. However, the approach involving putative GST homologues gave insights into the responsiveness of a putative GST homologue to abiotic stress in embryogenic cell cultures of *Cyclamen* and the study on putative genes of pectin modifying enzymes identified their correlation with the texture of the callus.

Zusammenfassung

Die somatische Embryogenese (S.E.) ermöglicht eine klonale Vermehrung, was insbesondere für wertvolle Kulturpflanzen erstrebenswert ist. Vor allem für Pflanzenspezies, bei denen eine klonale Vermehrung über Stecklinge nicht möglich ist, bietet die S.E. ein großes Potential, eine Massenvermehrung zu verwirklichen. Das schließt auch die wirtschaftlich bedeutende Zierpflanze *Cyclamen persicum* ein, die bereits als Modellpflanze der S.E. etabliert wurde. Trotz dieses hohen Potentials, hohe Vermehrungsraten zu fördern, findet die S.E. in der kommerziellen Pflanzenvermehrung kaum Anwendung. Das ist vor allem auf die schlechte Reproduzierbarkeit empirisch entwickelter Vermehrungs-protokolle sowie die Einschränkungen durch das häufige Auftreten von Fehlentwicklungen und nicht-embryogenem Kallus zurückzuführen.

Bisher ist die empirische Änderung von Protokollen zur Verbesserung der Ergebnisse das übliche Verfahren. Dieser Ansatz hingegen verfolgt die Idee, Genexpressionsinformationen zu nutzen, um die zugrunde liegenden Prozesse besser zu verstehen und dadurch Ansätze zu entwickeln, das bestehende Protokol für die S.E. *in vitro* gezielt zu verbessern. Zu diesem Zweck wurden vermeintlich unterliegende Prozesse diskutiert, und basierend auf den Daten eines cDNA-Microarrays (1216 Transkripte) wurden Hypothesen zur Verbesserung des *in vitro*-Protokolls abgeleitet.

Ausgewählte Hypothesen zum Einfluss von Genen putativer Glutathione S-Transferasen (GST) und pektinmodifizierender Enzyme wurden mittels qRT-PCR detaillierter untersucht. Hierfür wurde ein qRT-PCR Protokoll etabliert und für *Cyclamen* optimiert, insbesondere hinsichtlich der Referenzgene. Beide Validierungsansätze haben gezeigt, dass in diesen Fällen die ursprünglichen Hypothesen nicht bestätigt werden konnten. Allerdings ermöglichte die Untersuchung zu den putativen GST Homologen Erkenntnisse zur Empfindlichkeit einer putativen GST auf abiotischen Stress in embryogenen Zellkulturen. Die Studie zu putativen Genen pektin-modifizierender Enzyme konnte deren Korrelation mit der Kalluskonsistenz zeigen.

Keywords

In vitro-protocol optimisation, glutathione S-transferase, pectin modifying enzymes

Schlüsselwörter

In vitro-Protokolloptimierung, Glutathione S-Transferase, pektin-modifizierende Enzyme

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Abbreviation	Explanation
2,4-D	2,4-tichlorophenoxyacetic acid
2iP	6-[γ,γ dimethylallyl amino]-purine
AMI	Agrarmarkt Informations-Gesellschaft
AtSERK	*Arabidosis thaliana* SERK
BAC	bacterial artificial chromosome
bn	billion
bp	base pair
CCD	charge-coupled device
cDNA	complementary DNA
Cy3/Cy5	cyanine dyes
DNA	desoxyribonucleic acid
Ef-Tu	elongation factor thermo unstable
EgAP2-1	*Elaeis guineensis* APETALA2-1
EST	expressed sequence tags
GEO	Gene Expression Omnibus
GFP	green fluorescent protein
GO	Gene Ontology
GST	glutathione S-transferase
GUS	β-glucuronidase
IZE	immature zygotic embryo
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LEC2	LEAFY COTYLEDON 2
MGED	Microarray Gene Expression Data
MIAME	Minimum Information About a Microarray Experiment
miRNA	micro RNA
mRNA	messenger RNA
NGS	next generation sequencing
PCA	principal component analysis
PCR	polymerase chain reaction
PGR	plant growth regulator
qRT-PCR	quantitative realtime-PCR
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
s.e.	somatic embryogenesis
SERK	somatic embryogenesis receptor-like kinase
siRNA	small interfering RNA
smRNA	small modulatory RNA
SNP	single-nucleotide polymorphism
XTH	xyloglucan endotransglucosylase/hydrolase

1. General foreword

1.1 Summary of the project objectives

The results described below were part of the DFG project "Expression analysis for the molecular physiologically founded progression of somatic embryogenesis in the horticultural model culture *Cyclamen persicum*" (A.H. (HO 2100/2-1) and S.A.R. (RE 1697/3-1)).

The project was essentially divided into two parts:

A In the first part, a cDNA microarray had already been developed. For this purpose 1,216 already functionally annotated transcripts from *C. persicum* embryogenic cell cultures were used. These transcripts represented mainly candidate genes for probable involvement in somatic embryogenesis that were identified in a preceding EST-project (Rensing et al. 2005). The expression of the represented genes was investigated during the development of zygotic and somatic embryos under standard conditions on the one hand and in cultures with raised malformations or loss of embryogenic competence on the other. Based on the resulting data, hypotheses on the potential roles of different genes and functional gene groups were deduced.
All microarray analyses were conducted in cooperation with the Faculty of Biology of the Albert-Ludwigs-University Freiburg (PD Dr. Stefan A. Rensing).

B In the second part, exemplary results of the microarray were initially validated by quantitative realtime-PCR (qRT-PCR). For this purpose, this method first had to be established, or rather modified, for *C. persicum*.
Selective microarray data were analysed in more detail using qRT-PCR to deduce hypotheses that identify possible reasons for malformations and/or the loss of embryogenic competence. Furthermore, these hypotheses should be used to modify the existing micropropagation-protocol in a more

target-oriented manner. For selective functional gene groups (focusing on glutathione S-transferases and pectin modifying enzymes), detailed quantitative expression analyses were carried out using qRT-PCR.
Developmental stages were analysed histologically using differentiating staining methods.

The basic idea of the presented approach was to better understand the underlying processes that lead to malformation or non-embryogenic cell cultures. Understanding these underlying processes should enhance the development of suggestions for targeted improvements of the propagation protocol.

1.2 Introducing *Cyclamen persicum*

Cyclamen is a genus of plants that, according to (Grey-Wilson 2003), contains 22 species. Traditionally, they were classified as part of the *Primulaceae* family (Grey-Wilson 1997) but Källersjö et al. (2000) re-classified them into the *Myrsinaceae* family. In the wild, their distribution is centred around the Mediterranean, making them native to parts of Europe, Western Asia and parts of North Africa (Grey-Wilson 1997).

The genus also provides florist varieties in the form of cultivars based on *Cyclamen persicum*. This is the species of highest economical importance and one of the most popular ornamental plants, especially in the Netherlands, Germany and Italy (Grey-Wilson 1997).

According to the last survey of the Statistisches Bundesamt, about 22 million plants were produced in Germany in 2008 (Statistisches Bundesamt 2008). Annual worldwide production is estimated to be around 150 to 200 million plants (Winkelmann 2010). Figure 1 shows that in Germany *Cyclamen* are ranked at number 3 in the Top 10 of flowering potplants.

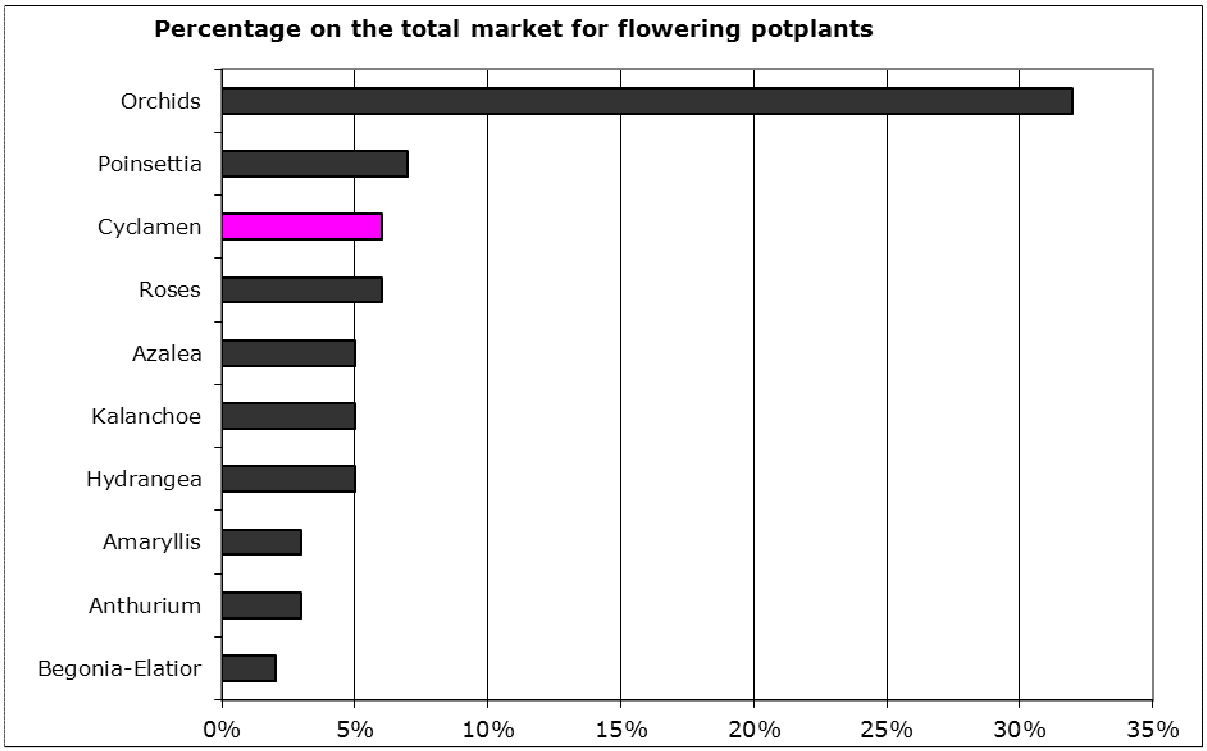

Figure 1: Germany's flowering potplants' Top 10 for 2009 (total market (private and commercial consumption): € 1.23 bn). Reproduced from Hanke 2010 (AMI Berechnungen, Figure 2).

1.3 Propagation of *Cyclamen persicum*

Traditionally, *Cyclamen persicum* have been propagated via seeds for commercial and private use. But this is still cost intensive due to manual pollination (up to € 0.20 per seed) (Winkelmann 2010). With the increasing popularity of *Cyclamen*, problems such as inbred depression, inhomogeneity in some cultivars, and the high costs of manual labour involved in seed production have become concerns for commercial cyclamen growers (Winkelmann et al. 2004).

Vegetative propagation of *Cyclamen persicum* is more difficult than with many other potplants. *Cyclamen* tubers have few growing points, making mass propagation by cuttings or tuber division impractical (Winkelmann 2010). Takamura and Miyajima (1997) reviewed that the first report on *in vitro* regeneration via organogenesis from tuber segments of Cyclamen was described by (Mayer-Hörster 1956). Because tuber tissues contain systematic microorganisms, micropropagation of these explants is very difficult (Takamura

and Miyajima, 1997). Additionally, Schwenkel and Grunewaldt (1988) reported that also other investigations were carried out to propagate *Cyclamen* using *in vitro* methods (Geier 1977; Fersing et al. 1982; Hoffmann and Preil, 1987). Nevertheless, it was observed that fundamental problems remain, namely highly contaminated explants, low regeneration rates, the regeneration of single leaves without meristems at the basis, and pronounced genotypic specificity for *in vitro* propagation ability. An alternative propagation system for *Cyclamen* is somatic embryogenesis (Wicart et al. 1984; Kiviharju et al. 1992; Kreuger et al. 1995; Schwenkel and Winkelmann 1998).

1.4 Somatic embryogenesis

Somatic embryogenesis is an asexual form of plant propagation that almost replicates the process of zygotic embryo formation. They are bipolar and bear typical embryonic organs (von Arnold 2008). In this process, somatic cells develop into plants through a series of characteristic morphological changes (de Jong et al. 1993). Although somatic embryos undergo most of the stages that are observed with zygotic embryos, differences also exist:

- the lack of endosperm
- a missing or retarded suspensor development
- no embryo desiccation or dormancy and
- the embryogenic competence of a somatic cell is achieved prior to the initiation of embryo development
  (Fehér et al. 2003).

Naturally, it occurs to a limited extent in species such as *Malaxis paludosa*, where somatic embryos form spontaneously on the leaf tips (Taylor 1967) or in some species of the genus *Kalanchoë*, that form somatic embryos when placed under stress, or others that form them spontaneously on leaves (Garcês et al. 2007). Also, this process can be induced by the experimental manipulation of tissues and cells *in vitro*. Reinert (1958) and Steward et al. (1958) are widely credited for describing somatic embryogenesis in *Daucus carota* for the first time, but according to Merkle et al. (1995) this recognition should instead be given to

Levine (1947). Levine (1947) reported the recovery of carrot "seedlings" from tissues exposed to low levels of α-naphthaleneacetic acid. Since then, *Daucus carota* has become a model plant for researching somatic embryogenesis. In recent years, somatic embryogenesis has been described in a large number of plant species, including those from plant families such as *Ranunculaceae*, *Rutaceae*, *Apiaceae* and *Poaceae* (Pierik 1997). Among the dicotyledonous herbaceous plants, about 180 species from 37 families have developed somatic embryos *in vitro* (Brown et al. 1995) and to date more will have been followed.

Somatic embryos can either differentiate directly out of a single cell of organised tissue, such as a leaf or stem segment, ovules, from protoplasts or microspores without any intervening callus phase, or indirectly via an intermediary step of callus culture (Williams and Maheswaran 1986).

Some of the most important factors required for successful plant regeneration are the culture medium and environmental incubation conditions as well as the explant type and genotype. Plant growth regulators (PGR) in the tissue culture medium are used to induce callus formation and are often subsequently changed to induce embryos to form from the callus. The ratio of different PGR that is required to induce callus or embryo formation varies depending on the plant species or genotype. Plant regeneration via somatic embryogenesis in *Cyclamen* according to the protocol of Schwenkel and Winkelmann (1998) comprises the following three steps:

1. Embryogenic cultures are initiated by culturing the primary explant on a medium supplemented with PGRs (2.0 mg/l 2,4-D and 0.8 mg/l 2iP).
2. Embryogenic cultures are proliferated by subculturing on a semi-solid or in a liquid medium that is supplemented with PGRs as in the initiation medium.
3. Somatic embryo development is initiated by transfer to a PGR-free medium.

In vitro somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement, as well as for mass propagation (Santacruz-Ruvalcaba et al. 1998).

Since somatic embryogenesis was first recognised, a substantial amount of information has become available. Regardless of this, the successful induction of somatic embryos and the subsequent development of viable plants is not efficient for the majority of plant species (Merkle et al. 1995). Likewise, Hohe (2010) summarised that even though today the knowledge of the process of somatic embryogenesis is great, in fact, almost no ornamental plants have been propagated through somatic embryogenesis to date.

It is well known that the development of embryogenic or non-embryogenic cell types can be influenced by several factors (e.g. stress, pH, PGRs and genotype) (Fehér et al. 2002). However, little is known about the detailed biological background (Fehér 2008). Kennedy and Norman (2005) collected the current 125 most important scientific questions that in general point to the gaps in knowledge. Among the 25 questions they highlighted was also the one asked by Vogel (2005) : *"How Does A Single Somatic Cell Become A Whole Plant?".* Even if there was great progress in the identification of key molecular factors involved in this process, the clarification of *how* dedifferentiation leads to cellular totipotency, and *why* it is expressed only in certain cells or genotypes remains elusive (Fehér 2008). Similarly, also processes leading to physiological disorders, asynchronous development and malformed embryos have not been completely understood until now. Based on current knowledge, Fehér (2008) arranged a schematic description of his hypothesis on the induction of somatic embryogenesis that is given in Figure 2.

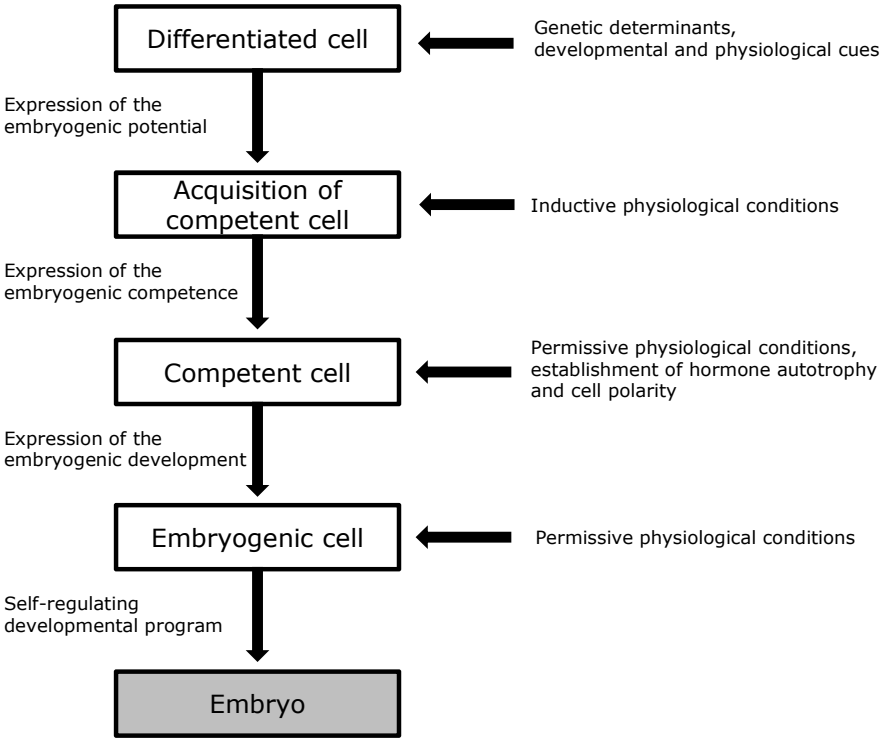

Figure 2: Schematic description of a hypothesis on the induction of somatic embryogenesis. Although plant cells are likely to have the capability for s.e. in general, the acquisition of embryogenic competence depends on many circumstances (reproduced from Fehér 2008, Figure 1).

Gene expression profiling has shown to be a promising tool for generating hypotheses that identify key molecular factors and their functions in somatic embryogenesis. In recent years, an increasing number of articles have analysed gene expression pattern in s.e. (e.g. Imin et al. 2008; Che et al. 2006; Thibaud-Nissen et al. 2003).

1.4.1 Gene expression during somatic embryogenesis

Several systems have been investigated to assist with understanding the mechanisms of gene regulation during the developmental process of somatic embryogenesis (Chugh and Khurana 2002). These include model plants (e.g. *Arabidopsis* (Hecht et al. 2001; Magioli et al. 2001), cotton (Wu et al. 2009), maize (Che et al. 2006), *Medicago* (Imin et al. 2008), soybean (Thibaud-Nissen et al. 2003) and wheat (Singla et al. 2007)) as well as non-model plants (e.g.

alfalfa (Domoki et al. 2006), chicory (Lucau-Danila et al. 2010) and *Elaeis guineensis* (Lin et al. 2009)).

Various studies have concentrated on how the general expression profiles of genes that are potentially involved in somatic embryogenesis were generated (e.g. Thibaud-Nissen et al. 2003; Che et al. 2006; Imin et al. 2008; Wu et al. 2009; Lucau-Danila et al. 2010). In their study on gene expression patterns during the somatic embryogenesis of maize Hi II callus cultures, Che et al. (2006) identified genes that might be used as developmental markers or targets to optimise the regeneration procedure. Imin et al. (2008) described transcriptional differences between the *M. truncatula* super-embryogenic line 2HA and its non-embryogenic progenitor Jemalong. Their data facilitated the mapping of regulatory metabolic networks involved in gaining totipotency and regeneration capacity and it provided candidate genes for functional analysis (Imin et al. 2008). They were able to demonstrate that the two different cell lines did not only differ in many aspects of biochemical pathways but also in their response to auxin and cytokinin (Imin et al. 2008). Microarray analysis of two different chicory genotypes differing in their responsiveness to s.e. induction permitted Lucau-Danila et al. (2010) to identify several putative key genes related to the process of s.e. in chicory. These studies show the high potential of gene expression profiling for generating hypotheses on the mechanisms of the process of somatic embryogenesis. Nonetheless, as Chugh and Khurana (2002) have already summarised in their review on recent advances in gene expression during somatic embryogenesis, the precise mechanisms controlling plant gene expression and the detailed steps by which these genes direct the process of somatic embryogenesis are still not clarified by this observation.

However, other studies (e.g. Hecht et al. 2001; Malinowski et al. 2004; Aberlenc-Bertossi et al. 2008; Stone et al. 2008; Chiappetta et al. 2009) focused on single genes or aspects. The first gene specifically identified as being involved in s.e. was a somatic embryogenesis receptor-like kinase (SERK) (Schmidt et al. 1997). In *Arabidopsis* it has been shown that the AtSERK1 gene was highly expressed during somatic embryo formation and early embryogenesis of zygotic embryos while it was no longer detectable in the mature somatic embryo or in any part of the developing seed (Hecht et al. 2001). It was also concluded that an increased AtSERK1 level was sufficient to confer embryogenic competence (Hecht et al.

2001). Another gene regulating many distinct aspects of embryogenesis is LEAFY COTYLEDON2 (LEC2) (Stone et al. 2001). Stone et al. (2008) hypothesised that LEC2 induces s.e. through two mechanisms: (1) By activating other genes that are also expressed during the maturation phase, LEC2 appears to increase the embryogenic competence of cells; (2) LEC2 seems to promote auxin activity and thereby induces s.e. in competent cells. Besides, other genes were also identified for their role in embryogenesis, for example, in *Vitis vinifera*, the WUSCHEL-related homebox (WOX) genes (Gambino et al. 2010). Thus, a series of genes have now been identified as being involved in s.e. In the review of Chugh and Khurana (2002), various genes were represented to influence s.e. in higher plants (e.g. hormone-responsive genes, housekeeping genes, signal transduction, homeeotic genes, genes coding for extracellular proteins and maturation genes).

Studies aiming to improve the protocol of mass propagation by the use of gene expression profiles are still rare. An approach for establishing a connection between gene expression profiles and the development and optimisation of the protocol of s.e. induction was represented by Stasolla et al. (2003) and Stasolla et al. (2004). Both studies analysed gene expression patterns in response to medium supplementation with the aim to improve the s.e. maturation in *Pinus glauca* (Stasolla et al. 2003; Stasolla et al. 2004).

In *Cylamen persicum* the process of s.e. was analysed by various proteomic studies (Winkelmann et al. 2006; Lyngved et al. 2008; Bian et al. 2009; Rode et al. 2010; Rode et al. 2011). Whilst in the proteomic study of Winkelmann et al. (2006) somatic and zygotic embryos of *Cyclamen* were compared, Lyngved et al. (2008) analysed embryogenic and non-embryogenic callus cultures before s.e. induction. Bian et al. (2009) analysed the proteome of somatic embryos in different developmental stages along with non-embryogenic callus and zygotic embryos. Rode et al. (2010) first established proteome reference maps of somatic and zygotic embryos. Later they conducted a comparative analysis of the proteomes (Rode et al. 2011) to obtain a profound knowledge of the physiological processes in *Cyclamen* embryogenesis for overcoming problems such as physiological disorders and asynchronous development. They supposed that the glycolytic enzyme enolase plays an important role in *Cyclamen*

embryogenesis because it was detected most frequently (Rode et al. 2011). Nevertheless, none of these studies primarily aim to improve the *in vitro* culture system and still deal with fundamental aspects of embryogenesis in *Cyclamen*. Therefore, this represented gene expression study was conducted based on a cDNA microarray representing transcripts that were identified in a preceding EST analysis (Rensing et al. 2005). Hence, this study was restricted to a number of genes known to be expressed during the embryogenesis of *Cyclamen*. By analysing different stages of somatic and zygotic embryos, as well as embryogenic and non-embryogenic callus lines, along with different cultivated callus lines, this study offers the potential to identify key physiological pathways that are fundamentally involved in *Cyclamen* embryogenesis as well as to generate hypotheses for improving the *in vitro* protocol.

1.5 Gene expression profiling

One of the key regulatory mechanisms of living cells to sustain and execute their individual function is the control of gene expression (Aharoni and Vorst 2002). Therefore, measuring mRNA levels has proved to be a valuable molecular tool even though the final activity of a gene is determined by the encoded protein (Aharoni and Vorst 2002).

Gene expression profiling is the measurement of the simultaneous activity (expression) of thousands of genes and holds enormous promise for analysing regulatory mechanisms and transcriptional networks that underlie biological processes (Alba et al. 2004). Extensive transcriptome analysis should enable the identification and dissection of complex genetic networks that underlie processes that are critical to physiology, development and responses to different stimuli. Further characterisation of these gene networks in plants will help in the understanding of the molecular basis of plant processes (Alba et al. 2004).

In recent years, expressed sequence taqs (ESTs), microarrays, large-scale gene expression (transcriptome) profiling and associated informatics technologies are rapidly becoming routine in the plant sciences (Alba et al. 2004). These methods have been established to analyse the expression of hundreds or even thousands

of genes in a single experiment (Lee and Tranel 2008). By now, microarrays have become the standard method for gene expression experiments (Galbraith and Birnbaum 2006).

1.5.1 Microarrays

Microarrays were developed by Schena and co-workers in the early 1990s at Stanford University (Schena 2003). The basic principle is the detection of hybridisation of complementary nucleic acid strands (see Figure 3). Phimister (1999) recommended a nomenclature for referring to the hybridisation partners: A "probe" is the tethered nucleic acid whereas a "target" is the free nucleic acid. Before, there existed two nomenclature systems in the literature: What one described as "probes", others described as "target", and vice versa (Phimister 1999). In spite of this recommended nomenclature, the usage of "probes" and "targets" is still contentious. In this study, the nomenclature recommended by Phimister (1999) will be used throughout.

Microarrays are physical substrates, such as glass slides, 96- or 384-well plates or quartz chips, on which many samples of biological probes are spotted (Boutros 2006). Even if largely nucleic acids are spotted on microarrays, proteins are still used too (a thousand times more papers using nucleotide than protein assays exist) (Boutros 2006). Similarly, fluorescent detection is the most common method, but additionally, a variety of fluorophores and radioisotopes are used (Boutros 2006).

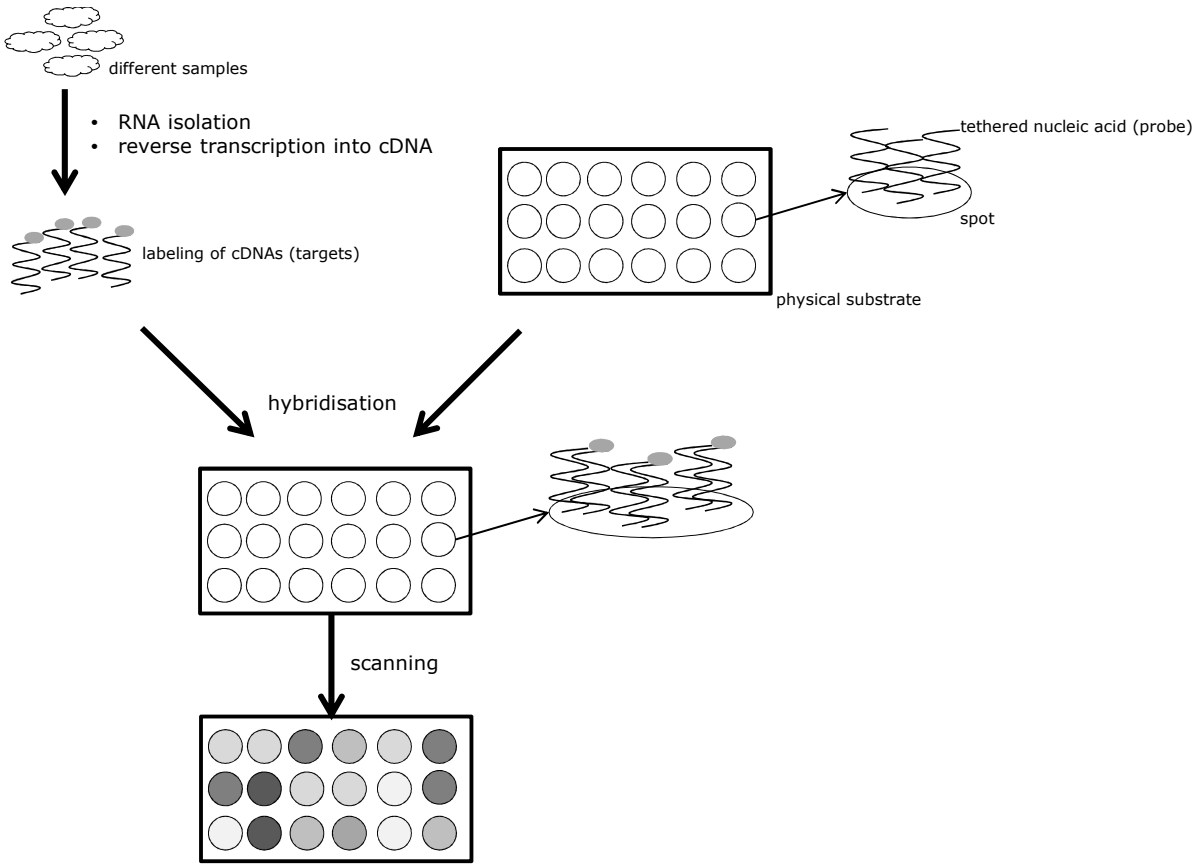

Figure 3: Schematic description of the events of a cDNA-microarray experiment: For target preparation, RNAs of the samples are isolated to synthesise cDNA. cDNAS are labelled with a fluorescence dye. Templates of genes of interest (probe) are then amplified and spotted to a physical substrate (e.g. glass microscope slides). Subsequently, targets and probes are hybridised and scanned to measure intensity. Each spot binds specifically and glows with an intensity proportional to the expression of each gene. This figure was reproduced in parts from Schena 2003 (Figure 1.3) and further aspects were added.

Typically, competitive fluorescent hybridisation is used. Here, two different cDNAs (e.g. sample and reference) are labelled in discrete labelling reactions with different fluorescence dyes (Cy3/Cy5) and are hybridised on a chip. For each gene, the signal ratio of both fluorescence dyes (Cy3/Cy5) is indicated. For visualisation these data are represented by overlaying red (Cy5) and green (Cy3) picture elements. Therefore, a green signal represents a gene that is up-regulated under one condition (e.g. control/reference condition) more than under another (e.g. test situation). Consequently, a red signal represents a gene that is expressed under contrary conditions whilst the signal appears yellow in the case of the same transcript abundances of a gene under the compared conditions (Twellmeyer 2007).

There are different experimental designs for microarrays available as the two examples show in Figure 4. Currently, no clear statement is available in the literature as to which design is preferred (depending on the experiment's objectives and conditions) (Twellmeyer 2007). In the present study, a common reference design, as proposed by Dudley et al. (2002), was used that is represented schematically in Figure 4 b.

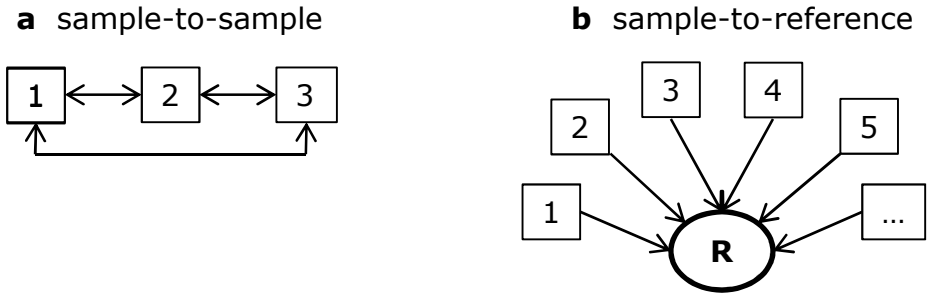

Figure 4: Schematic description of two examples of microarray designs. Every chip is represented by an arrow. In the sample-to-sample (**a**) design, all samples are compared with each other directly whilst in the sample-to-reference, or common reference design, (**b**) every sample is compared to a reference (fluorescence labelled antisense oligonucleotide).

To quantify the expression levels, the principle is used that the amount of fluorescence measured is directly proportional to the amount of nucleic acid with the complementary sequence of the analysed sample (Tarca et al. 2006). Microarrays were evolved to compare relative transcript abundances between different samples, but they should not be applied to compare transcript abundances between different genes within the same sample (Clarke and Zhu 2006).

Depending on the material placed onto the slides, microarrays are specified as, for example, DNA microarray, RNA microarray or protein microarray, but DNA microarrays are the most commonly used (Hovatta et al. 2005). Principally, the classification of microarrays can be performed by using at least three criteria: length of the probes, manufacturing method, and number of samples that can be profiled on one array simultaneously (Tarca et al. 2006).

cDNA microarrays include a collection of gene sequences, usually each spot includes the cDNA clone of a known gene (PCR product), ranging in size from

100 to 2,000 bp (Murphy 2002). These PCR products are applied individually on a solid matrix (e.g. glass or nylon) (Murphy 2002). In contrast, oligo microarrays comprise synthesised single-stranded probes on the basis of sequence information available in databases and thus no PCR-product is needed (Murphy 2002). The oligo microarray industry leader is Affymetrix (http://www.affymetrix.com/estore/) (Murphy 2002). The major advantage of Affymetrix GeneChips is that there is no need to prepare and verify the probes, such as PCR products or cDNAs, and thus they are "ready to use" (Murphy 2002). Admittedly, these kinds of chips are only available for model organisms (e.g. *Arabidopsis*, *Medicaco*, cotton). However, it could be demonstrated that some microarrays could be used for related species. Van Zyl (2002) conducted a heterologous array analysis in *Pinaceae* and hybridised *Pinus tadea* cDNA microarrays with cDNA from needles and embryogenic cultures of *P. taeda*, *P. sylvestris* and *Picea abis*. Because of the high level of sequence conservation between *Pinus* and *Picea,* they investigated the use of arrays from one genus for gene expression studies in the other (van Zyl 2002). It has been concluded that cDNA microarrays from loblolly pine were useful for gene expression studies in other pines or spruces (van Zyl 2002). Similarly, Bar-Akiva et al. (2010) was able to demonstrate the use of cross-species hybridisation *Brunfelsia* cDNAs to potato cDNA microarrays in their study on metabolic networking in *Brunfelsia calycina* petals after flower opening.

The methods for analysing microarray data are constantly being refined, but the increasing amount of microarray experiments has demanded general guidelines, which were formulated by (Brazma et al. 2001) in the so-called MIAME Standards (Minimum Information About a Microarray Experiment). Meanwhile, most journals are calling for adherence to these standards. Numerous statistical methods have been developed to improve the validity of microarray results, but nevertheless it is still recommended that the microarray data are randomly verified using another testing method such as quantitative realtime-PCR (qRT-PCR).

Over recent years, the use of microarrays has expanded enormously. For several plant species, large amounts of genomic data have been generated. The number of publications retrieved by the PubMed search engine rose from about 500

articles in 1998 to about 2,200 in 2004 when using the keywords "gene expression" AND "clustering" OR "classification" (Asyali et al. 2006). Today (March 2011), the same keywords produce more than 550,000 results. This clearly underlines how rapidly this method is developing and has become increasingly important.

1.5.2 Quantitative realtime-PCR (qRT-PCR)

Quantitative realtime-PCR (qRT-PCR) has become an outstanding tool for the quantitative analysis of nucleic acid sequences (Kubista et al. 2006). This technique represents a refinement of the original PCR developed by Kary Mullis and co-workers in 1983 (Dorak 2006). Higuchi et al. (1993) first reported a method of realtime PCR. They used ethidium bromide intercalation and a modified thermocycler that irradiated the samples with ultraviolet light and then detected the fluorescent signal with a CCD camera (Higuchi et al. 1993). Today, other dyes, especially SYBR® Green I, are predominantly applied because of superior signal-background-ratio (Mülhardt 2008). Overall, the detection chemistries applied to qRT-PCR can be divided into two basic groups: non-specific chemistries and target-specific chemistries. Non-specific chemistries detect fluorescence of DNA binding dyes (e.g. SYBR® Green I) whilst target-specific chemistries utilise fluorescent probes and/or primers (e.g. TaqMan® or Molecular Beacons) (Anonymous 2009).
In 1996 qRT-PCR became commercially available when Applied Biosystems released the 7700 instrument (Heid et al. 1996). By then, some other companies had also developed qRT-PCR thermocyclers. The major differences concern the excitation and emission of available wavelength, and the speed and number of reactions performed in parallel (Kubista and Zoric 2004). By now, many people have access to this technology due to the increasing number of suppliers and the decreasing prices of qRT-PCR thermocyclers as well as of reagents (Ginzinger 2002).

qRT-PCR is the reliable detection and measurement of PCR products generated during each cycle of the PCR process until an end point that is set after a predefined number of cycles (Ginzinger 2002). The fluorescence signals of every

reaction are transferred to a numerical value for every probe (Dorak 2006). The schematic description in Figure 5 outlines the general steps performed during qRT-PCR, from RNA isolation to data analysis.

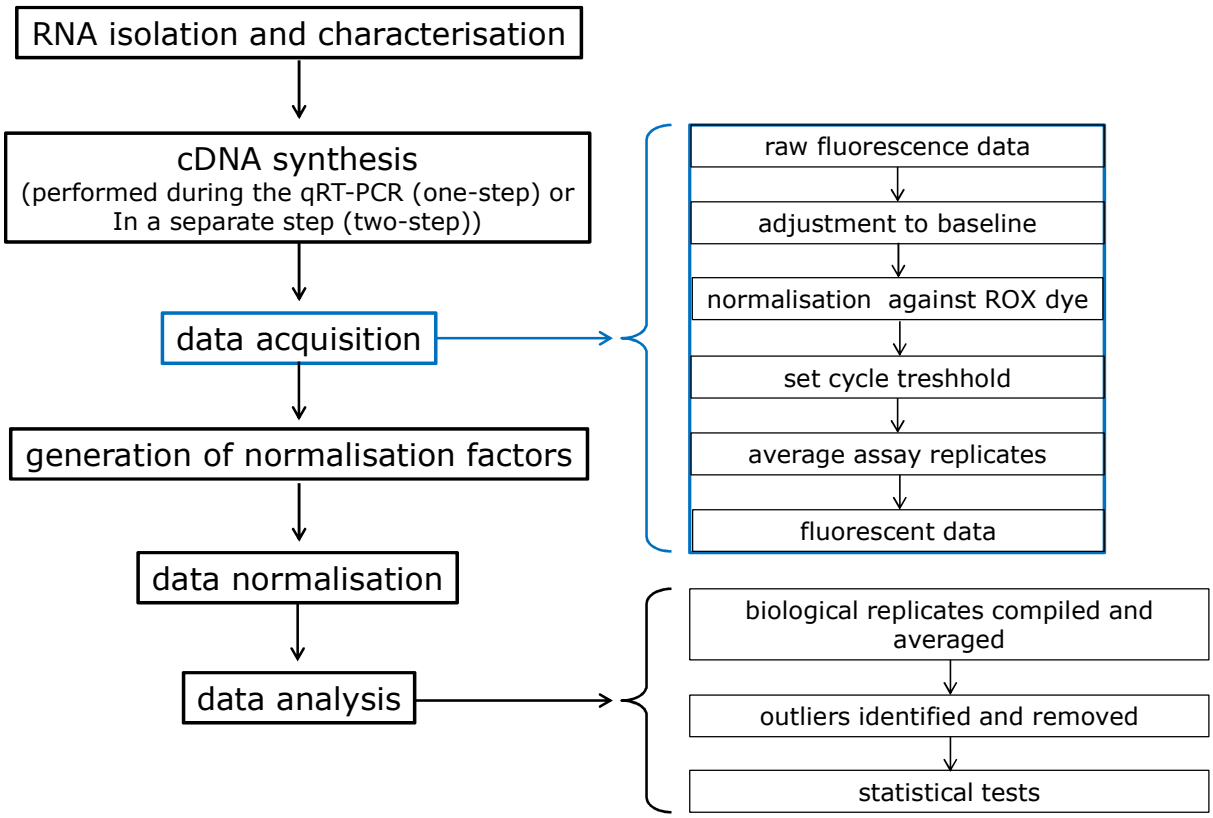

Figure 5: Schematic description of the steps performed when measuring gene expression using qRT-PCR. Firstly, RNA is isolated and characterised for quantity and integrity. That is followed by the cDNA-synthesis. If performing a one-step reaction, reverse transcription occurs during the PCR run while performing a two-step reaction, cDNA is synthesised in a separate step and then used as a PCR template. The steps carried out in the realtime-PCR-Cycler are highlighted in blue: the raw fluorescence data are collected, adjusted and manipulated to generate output data for further analysis. For the normalisation of the results with reference genes, a normalisation factor has to be calculated for each individual sample (reproduced from Wong & Medrano (2005, Figure 1).

Since first mentioned by Higuchi et al. (1993), qRT-PCR has been continually improved and refined. In particular, this includes the requirements for methodological standardisation associated with the selection of an appropriate normalisation strategy (Bustin et al. 2009). Initially, most studies used potentially constitutively expressed control genes without further verification of their transcript abundance stability (Vandesompele et al. 2002). It has been demonstrated that this may have generated incorrect results (Vandesompele et

al. 2002; Pfaffl et al. 2004). Besides, it has been shown that the normalisation of gene expression with a single reference gene may cause defective results because even the expression levels of housekeeping genes can vary significantly by the effect of different developmental or environmental conditions (Jain et al. 2006; Hong et al. 2008; Silveira et al. 2009). Thus, the verification of expression stability of every potential reference gene, as well as the normalisation with multiple reference genes, has recently established a new standard for qRT-PCR (Tong et al. 2009; Artico et al. 2010; Lee et al. 2010; Maroufi et al. 2010).

In the following study, the approach will be to at first focus on the evaluation of the microarray data and from there, hypotheses on what may explain some of the underlying processes of s.e. as well as deducing how the directed improvement of the *in vitro* protocol (chapter 2.1) may be achieved. Furthermore, according to the increased requirements of methodological standardisation of qRT-PCR, a protocol optimised for *Cylamen* should be established (Section 2.2). In addition, selective hypotheses should be validated by further detailed analyses (Section 2.3).

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Affymetrix:

http://www.affymetrix.com/estore/

2. Related publications and manuscripts

The main topics of this thesis are:

- Generating of hypotheses for improvement of somatic embryogenesis in *Cyclamen* by microarray based expression profiling (chapter 2.1),
- improvement of a qRT-PCR protocol for *Cyclamen* cell cultures (chapter 2.2.) and
- detailed examination of selected hypotheses (chapter 2.3).

The following peer reviewed publications and manuscripts that have been submitted referring on these topics.

The manuscript **Large impact of the apoplast on somatic embryogenesis in *Cyclamen persicum* offers possibilities for improved developmental control *in vitro*** was published in BMC Plant Biology (doi:10.1186/1471-2229-10-77). The publication as well as the additional files are accessible online at http://www.biomedcentral.com/1471-2229/10/77.

The manuscript **Selection of reference genes for normalization of quantitative real-time PCR in cell cultures of *Cyclamen persicum*** was published in Electronic Journal of Biotechnology (doi: 10.2225/vol14-issue1-fulltext-8). The publication is accessible online at http://www.ejbiotechnology.info/content/vol14/issue1/full/8/index.html.

The manuscript **Gene expression of a putative glutathione S-transferase is responsive to abiotic stress in embryogenic cell cultures of *Cyclamen persicum*** was submitted to Electronic Journal of Biotechnology (July 2011).

The manuscript **Expression analysis of putative genes of pectin modifying enzymes in different callus lines of *Cyclamen persicum* and their correlation to the texture of callus** was submitted to Scientia Horticulturae (October 2011).

2.1 Generating of hypotheses for improvement of somatic embryogenesis in *Cyclamen* with the use of expression profiling

Hoenemann *et al. BMC Plant Biology* 2010, **10**:77
http://www.biomedcentral.com/1471-2229/10/77

BMC Plant Biology

RESEARCH ARTICLE Open Access

Large impact of the apoplast on somatic embryogenesis in *Cyclamen persicum* offers possibilities for improved developmental control *in vitro*

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Abstract

Background: Clonal propagation is highly desired especially for valuable horticultural crops. The method with the potentially highest multiplication rate is regeneration via somatic embryogenesis. However, this mode of propagation is often hampered by the occurrence of developmental aberrations and non-embryogenic callus. Therefore, the developmental process of somatic embryogenesis was analysed in the ornamental crop *Cyclamen persicum* by expression profiling, comparing different developmental stages of embryogenic cell cultures, zygotic vs. somatic embryos and embryogenic vs. non-embryogenic cell cultures.

Results: The analysis was based on a cDNA microarray representing 1,216 transcripts and was exemplarily validated by realtime PCR. For this purpose relative transcript abundances of homologues of a putative receptor kinase, two different glutathione S-transferases (GST), a xyloglucan endotransglycosylase (XET) and a peroxidase (POX) were quantitatively measured by realtime PCR for three different comparisons. In total, 417 genes were found to be differentially expressed. Gene Ontology annotation revealed that transcripts coding for enzymes that are active in the extracellular compartment (apoplast) were significantly overrepresented in several comparisons. The expression profiling results are underpinned by thorough histological analyses of somatic and zygotic embryos.

Conclusions: The putative underlying physiological processes are discussed and hypotheses on improvement of the protocol for *in vitro* somatic embryogenesis in *Cyclamen persicum* are deduced. A set of physiological markers is proposed for efficient molecular control of the process of somatic embryogenesis in *C. persicum*. The general suitability of expression profiling for the development and improvement of micropropagation methods is discussed.

Background

Plant micropropagation on a commercial scale has developed since the 1960s and gained high impact during the last centuries for clonal mass propagation especially of ornamental crops [1,2]. The method with the potentially highest multiplication rate is regeneration via somatic embryogenesis (s.e.), which was initially described in 1958 for *Daucus carota* [3,4]. Since then, somatic embryogenesis systems have been developed for a multitude of plant species, but despite the large number of published protocols, only very few systems are actually used in commercial plant propagation. This can be put down to the fact that many protocols are inadequately reproducible, a differing fraction of the embryos shows developmental aberrations and non-embryogenic callus frequently arises during the use of indirect embryogenesis systems. Due to the often insufficient reproducibility, these problems are difficult to solve by empirical protocol changes. Yet, efficient propagation by somatic embryogenesis would be the method of choice for plant species that do not allow clonal propagation by cuttings, including the ornamental crop *Cyclamen persicum*.

In the last decade a series of genes have been identified that play a role in the s.e. of seed plants (for review see e.

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g. [5,6]). The expression of single genes has frequently been investigated in the course of somatic and zygotic embryogenesis and the importance of certain gene products has been proven for individual stages of development in different plant species. Developmental aberrations, however, can rarely be attributed to single or few genes in the course of s.e. Instead, it can be assumed that the whole expression pattern is changed during the course of the culture. Thus, in problem-oriented approaches, microarray-based expression analyses might give a more complete picture of the cultures' physiology that subsequently allows molecular physiologically founded progression of propagation protocol development.

During the last five years a steadily increasing number of studies has been published, analysing the process of somatic embryogenesis by gene expression profiling (e.g. in *Glycine max*: [7], *Picea abies*: [8], *Oryza sativa*: [9]; *Zea mays*: [10]; *Gossypium hirsutum*: [11,12], *Cichorium intybus*: [13], *Triticum aestivum*: [14], *Elaeis guineensis*: [15]). However, only a few studies aimed at an improvement of the protocol for mass propagation. In this context Stasolla et al. [16,17] have been the first to establish a connection between gene expression studies in s.e. and application-oriented work on protocol development and optimisation by analysing gene expression patterns in response to medium supplementation for improvement of maturation of somatic embryos of *Pinus glauca*.

S.e. in *Cyclamen persicum* represents a well established system very much resembling that in *D. carota* [18]. In contrast to *D. carota*, an efficient clonal propagation method for *C. persicum* is highly desired in the horticultural industry. Following publication of the original protocol, the system was developed further by establishing suspension and bioreactor cultures [19-22] and developing methods for desiccation and cryoconservation of the somatic embryos [23,24]. However, major problems caused by development of non-embryogenic cell lines, absence of a maturation phase and occurrence of malformed embryos could not be solved to date [25,26].

Recently, two proteomic studies have been conducted to analyse the process of s.e. in *C. persicum*. Winkelmann et al. [27] compared the proteome of somatic and zygotic embryos of *C. persicum*, whereas Lyngved et al. [28] analysed embryogenic and non-embryogenic callus before induction of somatic embryo development. Both studies were of fundamental character, not primarily aiming at improvement of the *in vitro* culture method. Therefore, we conducted an expression profiling study based on a cDNA microarray representing 1,216 transcripts identified in a preceding EST analysis using a normalised cDNA library prepared from embryogenic cell cultures and young somatic embryos [29]. Thus, in contrast to the proteomic studies, our expression analyses were restricted to a group of pre-selected genes expressed during embryogenesis. Due to the normalisation process also low expressed signalling genes were included. The overall goal of the present study was to identify key physiological pathways that are (i) fundamentally involved in s.e. in *C. persicum* (ii) prone to cause aberrant development and (iii) accessible for manipulation by *in vitro* culture. Therefore, the microarray was hybridised with cDNA generated from a selection of different embryogenic and non-embryogenic cell cultures as well as from zygotic embryos. These data were evaluated with the aim of generating new hypotheses for improving the micropropagation protocol using the expression of specific genes as physiological markers. A more general goal of our study was to prove the suitability of expression profiling analyses as a molecular physiologically based approach for development and improvement of to date mainly empiric *in vitro* culture methods.

Results and Discussion

Global expression profiling results

We analysed the expression of 1,216 transcripts (Additional file 1) during somatic and zygotic embryogenesis in *C. persicum* using a cDNA microarray derived from annotated transcripts of a previous analysis [29]. The overall aim of our study was to develop new hypotheses to improve the protocol of s.e. Therefore, we analysed gene expression during different stages of induction and development of embryogenic cell cultures as well as in mature somatic and zygotic embryos.

In total, 417 genes were found to be differentially expressed in 21 experiments comparing 17 different tissues or conditions ($p \leq 0.005$) (Additional file 1). After pairwise analysis (Figure 1) we selected eight experiments comparing ten different tissues for detailed interpretation (Additional file 2). These comparisons have been selected since they allow to draw interesting conclusions about the process of s.e. and provide indications for the improvement of propagation protocols. Within this reduced set of experiments a total of 279 genes were found to be differentially expressed. These comparisons were evaluated with regard to different questions as given in Figure 1, i.e. development of the somatic embryos (marked in red), putative reasons for developmental arrest in the globular stage (marked in blue), difference between embryogenic and non-embryogenic cell cultures (marked in magenta), difference between somatic and zygotic embryos (marked in yellow) as well as of the difference between a diploid and a tetraploid callus line (marked in green). In order to find key physiological pathways that are (i) fundamentally involved in s.e. in *C. persicum* (ii) prone to cause aberrant development and (iii) accessible for manipulation by *in vitro* culture, we subjected our data to Gene Ontology (GO) annotation [30] (Figure 2). It was tested which GO

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terms were significantly over- or underrepresented among the 279 differentially expressed genes as compared to the complete set of genes on the chip. From the summary of these analyses (Table 1) it can be deduced that predominantly processes of stress response located in the apoplast are important for s.e. in *C. persicum*. Therefore, these are shown and discussed in detail in the following paragraphs.

In order to confirm the microarray data, the expression of ten randomly chosen differentially expressed genes was validated by realtime PCR. Here, the qualitative results of the microarray were proven in nine out of the ten transcripts, moreover for eight transcripts the results of the two methods corresponded as well quantitatively (Figure 3). Therefore, we regard the microarray data as generally reliable. A principal component analysis (PCA) demonstrates the high reproducibility of the three independent biological replicates (Figure 4). The first two Eigen items contribute 18.7 and 14.4% of the variance, respectively, and are sufficient to separate the data according to the broad developmental stage of the tissues (Figure 4). Additional Eigen items result in an ever better resolution of developmental stages (data not shown). The PCA results nicely reflect the tissue/experiment selection described above.

Development of somatic embryos

Three days after induction

The total number of genes differentially expressed when comparing cells at induction and later developmental stages increased with ongoing periods of development (Figure 1: red marked comparisons). Four hours after induction only seven genes were differentially expressed as compared to the cells prior to induction. In contrast, three days later 79 genes showed differential expression as compared to four hours after induction. Two out of these 79 genes encode homologues of a chitinase (CYC12T7_E02) and a peroxidase (POX) (CYC04T7_G04) and belong to the GO term "cellular component"/"cell wall" that was overrepresented in this comparison (Table 1). A second POX (CYC11T7_B02) homologue was regulated similarly, although not annotated within this GO category. All three genes were up-regulated three days after induction as compared to four hours after transfer to growth regulator free medium.

It has been found that s.e. in *C. persicum* resembles that of *D. carota* in terms of transcripts involved [29]. In *D. carota*, a mutant cell line has been identified in which somatic embryo development is arrested in the pre-globular stage due to incorrect protoderm development [31]. This line morphologically resembles the somatic embryos in our study that - although partially developing beyond

Table 1: Gene Ontology terms significantly overrepresented among the differentially expressed genes comparing tissue 1 with tissue 2 (Fisher's exact test, $p \leq 0.05$)

Comparison		Gene Ontology		
Tissue 1	**Tissue 2**	**Biological Process**	**Cellular Component**	**Molecular Function**
zygotic embryo (ID 1.3)	somatic embryo (ID 2.1.14)		Chloroplast	
embryogenic callus (ID 2.1.1)	non-embryogenic callus (ID 2.3.1)	response to stress, response to biotic stimulus	extracellular region	
embryogenic tissue, 4 h after induction (ID 2.1.11)	embryogenic tissue, 3 d after induction (ID 2.1.12)		cell wall	catalytic activity
embryogenic tissue, 3 d after induction (no torpedo-shaped embryos generated) (ID 2.1.9)	embryogenic tissue, 3 d after induction (torpedo-shaped embryos generated) (ID 2.1.12)	response to abiotic stimulus	cell wall, cytosol	
embryogenic tissue, 3 d after induction (ID 2.1.12)	Torpedo-shaped somatic embryos, 3 weeks after induction (ID 2.1.14)	response to abiotic stimulus		

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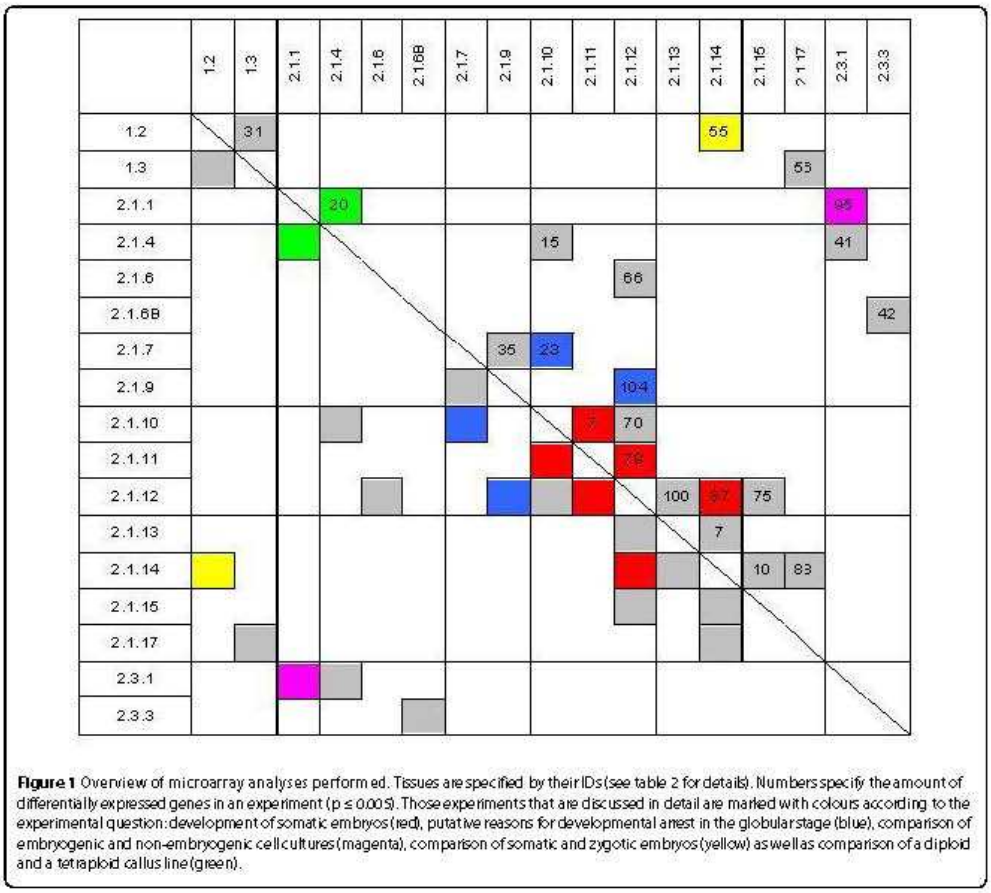

Figure 1 Overview of microarray analyses performed. Tissues are specified by their IDs (see table 2 for details). Numbers specify the amount of differentially expressed genes in an experiment ($p \leq 0.005$). Those experiments that are discussed in detail are marked with colours according to the experimental question: development of somatic embryos (red), putative reasons for developmental arrest in the globular stage (blue), comparison of embryogenic and non-embryogenic cell cultures (magenta), comparison of somatic and zygotic embryos (yellow) as well as comparison of a diploid and a tetraploid callus line (green).

the (pre)-globular stage - displayed an aberrant epidermal cell layer (Figure 5b ii and 5c ii). De Jong et al. [31] were able to rescue the cell cultures by addition of an endochitinase. From this they deduce an essential role of the endochitinase in formation of a proper protoderm in the pre-globular stage that in turn is a prerequisite for transition to subsequent embryo stages. The expression of a chitinase in induced embryogenic cultures in our study supports this hypothesis. Arabinogalactan proteins, known to be active compounds in so-called conditioned culture medium, have been suggested to be the substrate of this chitinase in *D. carota* [32].

In addition, a cationic peroxidase (POX) has been identified as being essential for pre-globular somatic embryo development for the first time in *D. carota* [33]. This corresponds to our results showing up-regulation of a POX homologue (CYC04T7_G04) three days after induction. Cordewener et al. [33] concluded from their results that cationic POX activity prevented cell size expansion, thus causing development of small cytoplasm-rich cells as a prerequisite for embryo development. Takeda et al. [34] analysed the expression and function of a cell wall-bound cationic POX during somatic embryogenesis in *Asparagus officinalis*. In this study, transcripts of putative POX genes were also most abundant during early s.e. and depletion of lignin precursors is discussed as a possible function in cell differentiation.

In this comparison 27 transcripts were found to be up-regulated between four hours and three days after induction and repressed again three weeks after induction as compared to the expression level three days after transfer to growth regulator-free medium (Additional file 2). Thus, these genes seem to be specific for induction and early developmental processes. One of those encodes a

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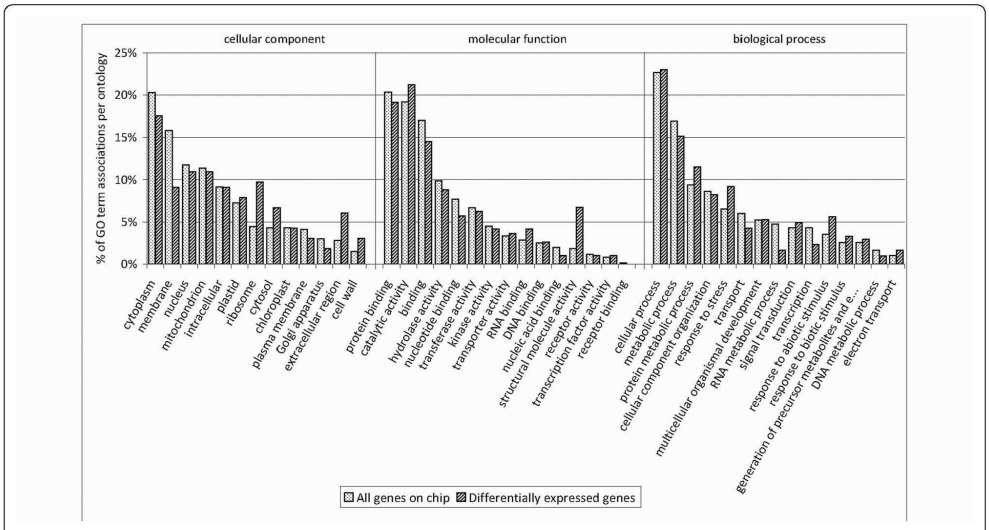

Figure 2 GO annotation. GO annotation of all transcripts present on the microarray in comparison to the differentially expressed genes in the eight selected experiments. The annotation is shown in percentage of GO associations per gene set and GO category.

homologue of a glutathione S-transferase (GST) (CYC32T7_B11). GSTs exist in many isoforms in plants and are active in cellular detoxification processes, in which the different isoforms are specific for different substrates [35]. Since two transcripts of cytochrome P450 homologues (CYC14T7_A10 and CYC32T7_D03) were also highly abundant specifically three days after induction, cellular detoxification seems to be important for this early developmental process. Whereas other authors deduce a central role of GST in the regulation of somatic embryogenesis from its auxin-inducibility [14], the GST homologue in our study was in fact up-regulated in response to auxin removal. However, two out of the five GST homologues (CYC01T7_E12 and CYC33T7_F07) in our study were repressed upon auxin-removal, whereas the remaining two (CYC16T7_B04 and CYC29T7_E07) did not show differential expression when the cells were transferred to auxin-free medium. This is in line with results from Pan et al. [36] demonstrating differential regulation of three GST homologues during somatic embryogenesis of *Citrus sinensis*. These authors argue that GST, together with other enzymes involved in oxida-

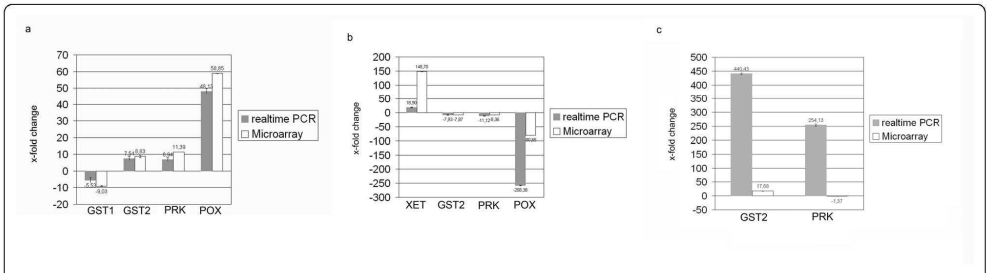

Figure 3 Validation of microarray results using realtime PCR (arithmetic mean of fold change +/- average absolute deviation (AAD)). a: GST1 (CYC01T7_E12), GST2 (CYC32T7_B11), putative receptor kinase (PRK) (CYC04T7_G06) and POX (CYC04T7_G04) in the comparison embryogenic suspension 4 h (2.1.11) vs. 3 d after induction (2.1.12). b: XET (CP_59_C1), GST2 (CYC32T7_B11), putative receptor kinase (PRK) (CYC04T7_G06) and POX (CYC04T7_G04) in the comparison embryogenic suspension 3 d after induction (2.1.12) vs. selected torpedo-shaped somatic embryos 3 weeks after induction (2.1.14). c: GST2 (CYC32T7_B11) and putative receptor kinase (PRK) (CYC04T7_G06) in the comparison: zygotic embryos (1.2) vs. selected torpedo-shaped somatic embryos 3 weeks after induction (2.1.14)

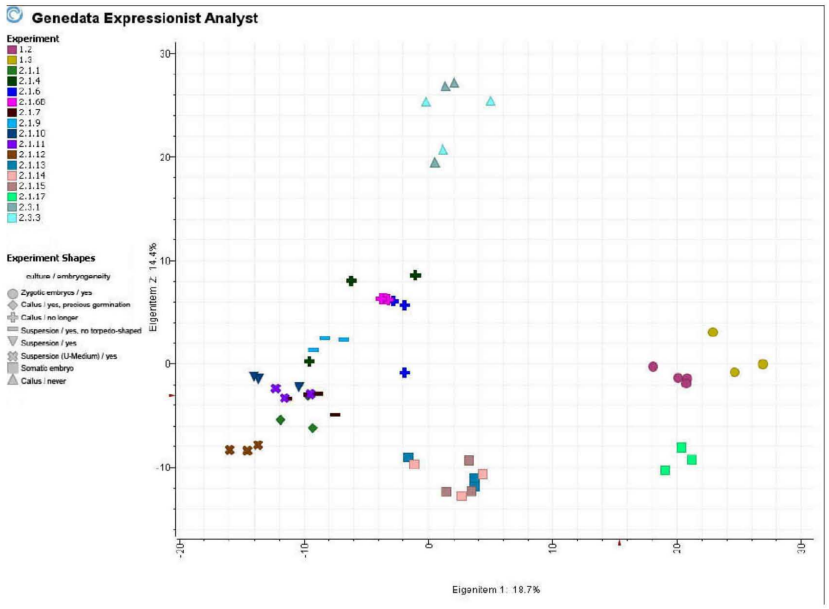

Figure 4 PCA showing differences in gene expression for the total of 17 tissues under different conditions. Differences between treatments as well as high reproducibility of the biological replicates are demonstrated. Tissues not interpreted in detail are marked in grey, all tissues selected for detailed analysis are coloured. (1.2: zygotic embryos, 2.1.1: embryogenic callus (diploid), 2.1.4: embryogenic callus (tetraploid) 2.1.7: embryogenic suspension (blocked in the globular stage) before induction, 2.1.9 embryogenic suspension (blocked in the globular stage) 3 d after induction, 2.1.10: embryogenic suspension before induction, 2.1.11: embryogenic suspension 4 h after induction, 2.1.12: embryogenic suspension 3 d after induction, 2.1.14: selected torpedo-shaped embryos 3 weeks after induction, 2.3.1: non-embryogenic callus before induction)

tive stress reactions, might play a role in regulation of redox changes critical for triggering s.e.

Three weeks after induction

Three weeks after induction torpedo-shaped somatic embryos were present in the culture. Here, 87 genes were differentially expressed as compared to the cultures three days after induction. Two of the genes specifically up-regulated at this stage encode homologues of a xyloglucan endotransglycosylase (XET, an enzyme synthesising or hydrolysing xyloglucans) (CYC16T7_D07 and CYC32T7_F01). Xyloglucans are common cell wall compounds and their regulation has thus also been analysed in the context of somatic embryogenesis [37]. In addition, they also serve as cell wall-bound seed storage compounds as in the case of members of the Primulaceae [38] and also in *C. persicum* [39]. Winkelmann et al. [27] observed high abundance of XET in the endosperm of *C. persicum* seeds in a proteomic study comparing somatic and zygotic embryos. In contrast, our study showed a high abundance of XET homologue transcripts in three week old somatic embryos as compared to cultures three days after induction. Likewise, one of the XET homologues (CYC32T7_F01) was also up-regulated in somatic embryos as compared to their zygotic counterparts. Thus, somatic embryos might accumulate storage compounds that are confined to the endosperm in zygotic embryogenesis. It remains to be investigated whether this is a prerequisite for successful development of somatic embryos (that necessarily lack an endosperm) or whether this might be a reason for abnormal development of somatic embryos and can be avoided by changes in growth media composition.

Differences between cell lines

Developmental arrest in the pre-torpedo stage

In the current study we also compared two cell cultures that differed in their ability to develop torpedo-shaped embryos (Figure 1: marked in blue). The culture in standard medium was developmentally arrested in the globular stage and lost its ability to form torpedo-shaped

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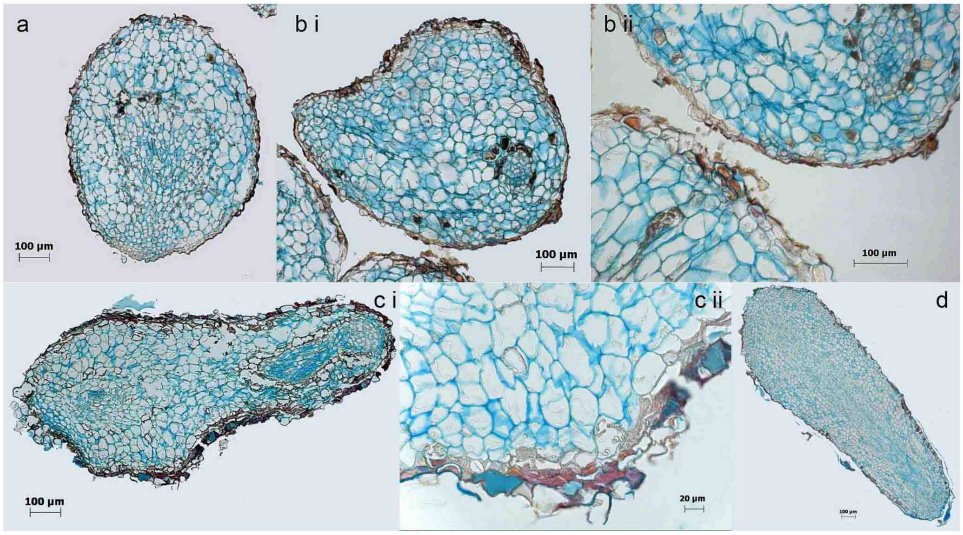

Figure 5 Histological analyses of different stages of somatic embryos 3 weeks after induction by transfer of the cells to growth regulator free medium using FCA staining. Cell walls are stained in blue, lignified or cutinised cell walls in orange to brownish red and nuclei in light purple. Globular shaped embryos display an irregular anatomical organization (a and b). Torpedo-shaped embryos, although displaying a regular outer shape, can be anatomically abnormal (c). Orange stained cell walls are found within globular shaped embryos (a and b). A rough and irregular epidermis structure is typical for somatic embryos (b ii and c ii). However, morphologically and anatomically normal somatic embryos also have been detected (d).

embryos after the 75th subculture. In contrast, when the same culture was transferred to the richly supplemented U-medium, globular embryos developed further into torpedo-shaped embryos. These two cultures differed in gene expression before induction with regard to only 23 genes. However, three days after induction, 104 genes were found to be differentially expressed, which is the highest number of differentially expressed genes in any pairwise comparison of our study. At this time the deviating phenotype (development of torpedo-shaped embryos 14 d after induction) was not yet visible. Thus, while some of the differences might be due to the differing media, the differential expression of some of these genes might be causal for the developmental arrest, resp. developmental progression. Interestingly, genes belonging to the GO term "cellular component"/"cell wall" again were found to be significantly overrepresented among the genes differentially expressed in this comparison (Table 1). Among these were the two early responding POX (CYC04T7_G04) and chitinase (CYC12T7_E02) transcripts, homologues of which have been identified in *D. carota* as causing developmental arrest [33,31]. In our study both were induced in the suspension culture that later on developed torpedo-shaped embryos as compared to the culture arrested in the globular stage. Since our culture was rescued by transfer to richly supplemented U-medium, we assume one of the supplements as a necessary factor. As our cultures lost their ability to develop torpedo-shaped embryos over time, one might hypothesize that this was caused by depletion of a factor that was not present in sufficient concentration in the standard medium, e.g. microelements that serve as co-factors of stress-related enzymes.

Embryogenic and non-embryogenic cell cultures

In the comparison of gene expression between an embryogenic and a non-embryogenic cell line before induction, 95 genes were found to be differentially expressed (Figure 1: marked in magenta). As found within the comparison of suspension cultures developing torpedo-shaped embryos vs. being arrested in the pre-torpedo stage, the differential expression pattern was eminent before the differing phenotypes were realised. Thus, part of the 95 genes might be regarded as causal for the ability of s.e. and not just correlative with the phenotype. However, one has to keep in mind that these two cell lines were of similar but not of identical genotype, since they have been established from different plant individuals and were of different age.

Regarding the GO category "cellular component", again the term "extracellular region" was significantly overrep-

resented among the genes that were differentially expressed in this comparison (Table 1). This is in line with research on somatic embryogenesis in other species, showing that the extracellular matrix is an active component of signal transduction [40-42]. In our study, one of the genes of the corresponding ontologies, that was found to be up-regulated in embryogenic callus, was again the chitinase homologue (CYC12T7_E02) discussed above, which once more supports the relevance of this enzyme in early somatic embryogenesis that has been reported e.g. for *D. carota* [31,32]*Picea glauca* [43] and *Pinus caribea* [44]. Besides chitinase, also a group of three genes encoding homologues of pectin modifying enzymes (Pectinesterase: CYC26T7_G03, Pectate lyase: CYC26T7_E05 and CYC26T7_E10) belonging to the GO term "extracellular region" as well, were up-regulated in embryogenic as compared to non-embryogenic callus. Also, a pectinacetylesterase (CYC14T7_B05) as a fourth enzyme within this group showed a similar expression pattern, yet this enzyme was not annotated within the "extracellular region" GO category. Pectins are major components of the middle lamella and important for intercellular adhesion. In this context, it has already been shown that the inhibition of the transport of pectins to the cell wall caused morphological embryo defects in zygotic embryos of *A. thaliana* [45]. Bouton et al. [46] described two allelic *A. thaliana* mutants (qua1-1 and qua1-2) that carry T-DNA insertions in a gene encoding a putative glycosyltransferase that is involved in biosynthesis of pectic polysaccharides. These mutants also show reduced cell adhesion and a dwarf phenotype. Likewise, in *Nicotiana plumbaginifolia* a mutant (nolac-H18) deficient in a glycosyltransferase that is involved in pectin biosynthesis lost its ability to form tight intercellular attachments in callus cultures and also their ability to regenerate adventitious shoots [47]. Moreover, in some plant species embryogenic and non-embryogenic cell lines display differences in pectin composition, and it has been concluded that a pectin-conferred cell adhesion is a prerequisite for s.e. [48,49]. Verdeil et al. [50] showed that the acquisition of embryogenic competence in callus of *Cocos nucifera* was linked to the appearance of a fibrillar material containing pectin, coating the embryogenic cells. In our study we find homologues of genes encoding enzymes involved in pectin degradation and methylesterification to be up-regulated in the embryogenic cell line, which is less friable than the non-embryogenic one. This hints at pectin degradation and modification possibly being necessary for continuous remodelling of middle lamellas during active pre-embryogenic callus growth in *C. persicum*. In contrast, in the non-embryogenic cell line the much looser cell adhesion might be caused by reduced pectin content, which might be an important factor for the loss of embryogeneity due to reduced cell adhesion. Therefore, the *in vitro*-culture practice of selecting friable callus lines for better cultivation and establishment of suspension cultures [19] might in the case of *C. persicum* result in the selection of non-embryogenic cell lines. This hypothesis needs to be proven by detailed analyses of multiple embryogenic and non-embryogenic cell lines.

Another striking difference in gene expression between the embryogenic and the non-embryogenic cell line was the expression of three GST homologues, which were already discussed within the context of early s.e. Two were up-regulated in the embryogenic cell line (CYC01T7_E12 and CYC32T7_B11) in contrast to the non-embryogenic one, whereas a third one was repressed (CYC16T7_B04). This confirms the hypothesis that these GST homologues might be crucial for early somatic embryo development. One might also speculate on differences in auxin-responsiveness of these two cell lines, since auxin-dependent induction of GST-transcription has been reported in other plant s.e. systems [14]. In fact, in our study one of the GST homologues (CYC01T7_E12), that had been shown to be repressed upon auxin-removal, was also repressed in the non-embryogenic cell line (in contrast to the embryogenic one), although the latter cells were cultivated on auxin-containing medium.

Another differentially expressed gene (CYC14T7_H01) in this context that was specifically up-regulated in the embryogenic cell line belongs to the SERK family, which is also well known to be involved in s.e. in other plants. A SERK was the first gene specifically identified to be involved in s.e. [51]. Receptor protein kinases such as SERKs play a role in several signal transduction pathways that elicit a developmental response to exogenous input [52]. In *D. carota*, SERK was found to be expressed in embryogenic suspension cells and in early stages of both somatic and zygotic embryos [51]. Expression analyses of SERK1 in *Arabidopsis thaliana* revealed a slightly different expression pattern, since here the gene was already expressed during megasporogenesis and in late embryo vascular strands [53,54]. A correlation between SERK gene expression and somatic embryogenesis has been demonstrated for many other plant species in recent years, e.g. in *Helianthus annuus* [55], *Theobroma cacao* [56], *Oryza sativa* [57], or *Vitis vinifera* [58]. Out of the five genes in our study that are homologous to genes annotated as SERK [29], only one (CYC14T7_H01) was found to be differentially expressed in any of our experiments. This is in line with other studies showing that SERK expression is not restricted to the regulation of embryogenesis (either somatic or zygotic) but also plays a role in other developmental or physiological processes, e.g. adventitious shoot regeneration in *Helianthus annuus* [55] or host defence response in *Oryza sativa* [57,59].

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Interestingly, in the non-embryogenic cell line, a putative argonaute (AGO) homologue (CYC33T7_C02) was significantly up-regulated that was not found to be differentially expressed in any other comparison. AGO proteins are part of the RNA-induced silencing complex (RISC) involved in posttranscriptional gene silencing via RNA interference (RNAi) [60]. Tahir et al. [61] have identified a gene of the AGO family in *Picea glauca* that proved to be essential for normal somatic embryo development. Similarly Takahata [62] has demonstrated differential expression of an AGO homologue during s.e. in *D. carota* and deduces that RNAi controlled gene expression is required for s.e. We regard our result as an indication that RNAi processes might also be involved in the common phenomenon of generation of embryogenic and non-embryogenic cell lines from identical explants or the loss of embryogenic competence in tissue culture.

Comparison of zygotic and somatic embryogenesis

The anatomy of developing zygotic and somatic embryos is shown in Figures 5 and 6, respectively. Figure 6a and 6b show ovules before and ten days after pollination. The micropyle and the embryo sac can clearly be identified. Thirty days after pollination, the first tiny globular embryos were detected (Figure 6c). At this stage, the endosperm was at the transition stage to become cellular. Fifty days after pollination, embryos were in the globular stage (Figure 6d) and after 60 days a torpedo-shaped morphology developed (Figure 6e: transversal section, f: longitudinal section). Finally, 75-100 d after pollination the embryos reached their final size before maturation (Figure 6g and 6h). These periods of development are consistent with our former morphological analyses [25]. Most striking in the anatomical pictures presented here are the large size differences of zygotic embryo and endosperm cells: 423 +/- 61 μm^2 versus 1649 +/- 694 μm^2 (arithmetic mean +/- standard deviation).

In comparison, cells of somatic embryos in all developmental stages (Figure 5) are much larger (1484 +/- 508 μm^2), comparable to the size of the cells of the endosperm.

In contrast to the strictly synchronised development of the zygotic embryos, three weeks after induction of the somatic embryos all stages were present simultaneously. Another striking difference between zygotic and somatic embryos appears to be the structure of the epidermis. Whereas zygotic embryos displayed a plain and unruffled outer cell layer (Figure 6g and 6h), the epidermis of somatic embryos was not that smooth (Figure 5b ii and 5c ii). As discussed above, abnormal formation of epidermal cell layers has been identified in *D. carota* as a reason for developmental arrest in the globular stage [31]. Likewise Tokuji and Kuriyama [63] identified malformation of the epidermis, and s.e. in the context of uniconazole (a gibberellic acid inhibitor) induced malformation of *D. carota* somatic embryos. Histological staining in our study also revealed the presence of cells with cutinised and/or lignified cell walls within globular-shaped embryos (Figure 5a and 5b) as well as in the epidermis of torpedo-shaped embryos (Figure 5c). This is another hint on the impact of the extracellular matrix on somatic embryo induction and development postulated also in other studies [40-42].

These differences in the anatomy of somatic and zygotic embryos of *C. persicum* were corroborated by the corresponding gene expression analyses (Figure 1: marked in yellow). Since in this comparison, diploid (zygotic) and tetraploid (somatic) material was compared, we interpret only those data that were not among the homologues of genes differentially expressed in a comparison of diploid and tetraploid callus (Figure 1: marked in green). Three of the transcripts that were highly abundant specifically in somatic embryos are involved in oxidative stress response (homologues of GST (CYC32T7_B11), superoxide dismutase (CYC25T7_A12) and catalase (CYC13T7_H06)). As discussed above, up-regulation of genes involved in oxidative stress response is a typical reaction for early s.e. However, in our study, this comparison has been made three weeks after induction, using selected torpedo-shaped embryos. Therefore, we interpret these data as a hint that the signals inducing embryogenesis might linger, so that the cultures are prone to undergo secondary embryogenesis (which is in fact often observed in our cultures). From these data we deduce a necessity to more effectively remove auxins from the culture upon induction. This might be realised by supplementation of the medium with activated charcoal or even auxin inhibitors.

Conclusions

From the expression profiling data presented here, we can determine differentially expressed genes that are intermediate and later responders of the developmental process under investigation. Therefore, the data give valuable insights and constitute a basis for new hypotheses on how the process of s.e. in *C. persicum* might be improved *in vitro*:

1. During cell line selection more attention should be paid to cell adhesion, since this might be a factor promoting s.e. in *C. persicum*. Detailed analyses using different embryogenic and non-embryogenic cell lines are planned for the future in order to generate reliable data on the impact of pectin-mediated cell adhesion. To support proper cell line selection, the use of expression profiling of genes involved in pectin degradation and remodelling as a physiological marker will be tested.
2. High expression levels of AGO, GST and SERK homologues are additional putative indicators for the

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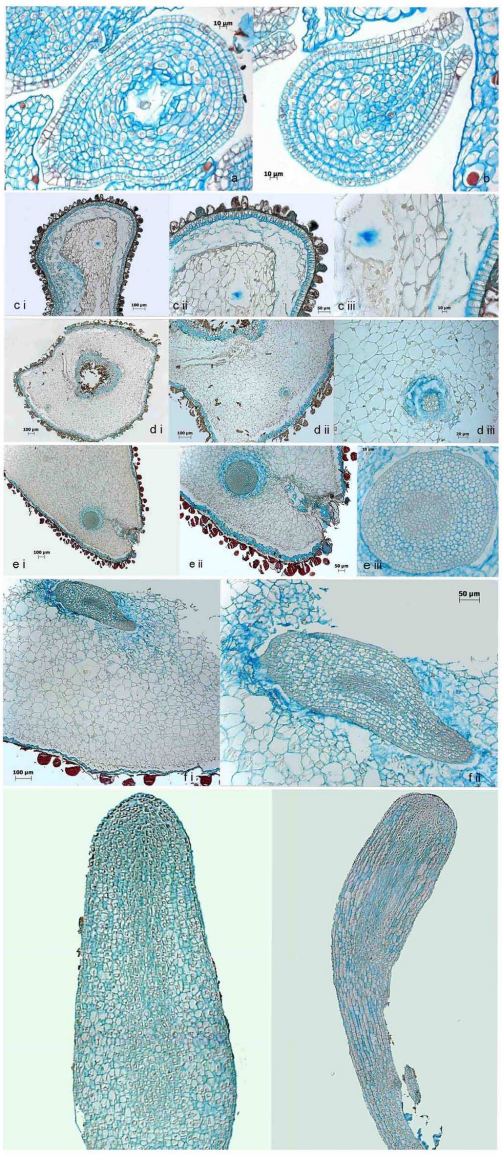

Figure 6 Histological analyses of the development of zygotic embryos using FCA staining. Cell walls are stained in blue, lignified or cutinised cell walls in orange to brownish red and nuclei in light purple. Zygotic embryo development starts within the ovules after pollination. Early stages (a: 0 d after pollination, b: 10 d after pollination) show ovules within the surrounding ovary tissue. The embryo sac and the micropyle are clearly visible. 30 d after pollination the endosperm becomes cellular and tiny multicellular embryos can be detected (c i to iii). 50 d after pollination globular shaped embryos have been formed (d i to iii). 60 d after pollination embryos become torpedo-shaped (transversal: e i to iii and longitudinal: f i to ii). 75 to 100 d after pollination zygotic (g and h, showing excised embryos) embryos reach their full size and maturation starts.

identification of embryogenic vs. non-embryogenic callus, respectively. This will be validated by screening of different cell lines under inductive and non-inductive conditions. A possible involvement of RNAi in the loss of embryogenic competence will be investigated as well.

3. Proper epidermis formation in early embryonic stages might be a prerequisite to avoid embryo malformation. Chitinase and POX activity will be checked for suitability as a physiological marker.

4. During *in vitro* culture more attention should be paid to buffering or controlling the pH as a means to influence redox homeostasis and specific enzyme activity. Putative differential expression of GST, POX, and chitinase homologues are of special interest in this context.

5. The effect of richly supplemented U-medium on overcoming the developmental arrest at the globular embryo stage might be analysed by studying expression of the early responding genes GST, POX and chitinase in response to supplementation of the standard medium with individual compounds of the U-medium.

6. The effect of rich carbohydrate supplementation of the medium - a technique well known from other plant species to promote embryo maturation - on cell size and expression of XET in somatic embryos will be analysed in order to check whether incorrect storage compound accumulation might cause malformation of embryos.

7. Secondary embryogenesis might be another major reason for formation of aberrant embryos. Therefore, protocol changes should aim at preventing the auxin signal to linger after auxin removal. This might be achieved by addition of activated charcoal. Moreover, antagonistic signals might be given by addition of gibberellic acid or even auxin inhibitors upon auxin removal.

Thus, we were able to develop new hypotheses on *in vitro* protocol improvement based on the molecular physiological knowledge we gained by gene expression profiling. Future work will focus on in-depth analyses of these hypotheses.

The following limitations of this work should be taken into account when interpreting the data:

1. Different genotypes of the cell lines could add bias to comparative expression profiling.

2. Detection limit of cDNA microarray analysis could prevent some expression changes to be detected. The realtime PCR analysis showed that at least for some genes the sensitivity of the cDNA microarray to detect differential expression was lower in comparison.

3. The mixture of cell types of some analysed tissues could limit sensitivity as well, since small expression changes in only single cell types would be masked.

4. The fragmentary nature of the EST sequences might limit their correct annotation.

5. Only about 4% of the transcripts have been analyzed by microarray analysis.

However, since the suggested protocol improvements rely on a much more detailed physiological knowledge than the common development process of propagation protocols, we expect this strategy to be more effective in terms of time and reliability. Novel cost-effective sequencing technologies will also enable the expansion of the current studies to the complete transcriptome of *C. persicum*.

Methods

Tissue culture

The cell lines of the genotype "3" were established as described by Schwenkel and Winkelmann [18] from unfertilised ovules of a single plant from the cultivar 'Sierra Purple Flame' in May 2003 and a second time in August 2005. During continuous propagation, different subtypes developed as described in detail in Table 2. Cell line "12G" was established in March 1991 from the cultivar 'Purple Flamed' (genotype 3738, [64]), which was non-embryogenic from the beginning. The cell lines were cultivated on MS based growth regulator-containing medium ("standard medium") as described by Schwenkel and Winkelmann [18] and propagated by transfer to fresh standard medium every four weeks. Suspension cultures were established as described by Winkelmann et al. [19] and propagated by transfer to fresh standard medium every two weeks. Embryo development was induced by transfer of the cells to plant growth regulator-free medium [18], either growth regulator free standard medium or growth regulator free "U-medium" [65], which is a rich supplemented medium containing additional organic compounds and micro-elements.

For RNA isolation cell material was collected 0 h, 4 h and 3 d after transfer to growth regulator-free medium. Selected globular- and torpedo-shaped embryos as well as torpedo-shaped embryos with roots were harvested 20 to 22 d after transfer to growth regulator-free medium. A part of the torpedo-shaped embryos was subjected to controlled slight desiccation over saturated salt solutions (4 d 97% relative humidity and 3 d 92% relative humidity, as described in Seyring and Hohe [23], prior to RNA isolation (for details see Table 2). In order to obtain zygotic embryos, cloned plants of genotype "3" were selfed and zygotic embryos were prepared out of the ovules 84 to 85 d after pollination for early stage torpedo-shaped embryos and 99 to 101 d after pollination for late stage torpedo-shaped embryos at the onset of desiccation. Due

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Table 2: Description of tissues

tissue ID	cell line	culture	medium	developmental stage	embryogeneity	ploidy
1.2	F1 out of 3-2-0503	zygotic embryo	in planta	84-85 d after pollination	yes	diploid
1.3	F1 out of 3-2-0503	zygotic embryo	in planta	99-101 d after pollination	yes	diploid
2.1.1	3-14-0805	callus	standard	0 h before transfer to standard media without hormones	yes, but precocious germination	diploid
2.1.4	3-43-0503	callus	standard	0 h before transfer to standard media without hormones	no longer	tetraploid
2.1.6	3-43-0503	callus	standard without growth regulators	0 h before transfer to standard media without hormones	no longer	tetraploid
2.1.6B	3-45-0503	callus	standard without growth regulators	0 h before transfer to standard media without hormones	no longer	tetraploid
2.1.7	3-75-0503	suspension	standard	0 h before transfer to standard media without hormones	yes, but no torpedo-shaped s.e.	tetraploid
2.1.9	3-75-0503	suspension	standard without growth regulators	3d after transfer to standard media without hormones	yes, but no torpedo-shaped s.e.	tetraploid
2.1.10	3-76-0503	suspension	standard	0 h before transfer to standard media without hormones	yes, torpedo-shaped s.e.	tetraploid
2.1.11	3-76-0503	suspension	U without growth regulators	4 h after transfer to U media without hormones	yes, torpedo-shaped s.e.	tetraploid
2.1.12	3-76-0503	suspension	U without growth regulators	3d after transfer to U media without hormones	yes, torpedo-shaped s.e.	tetraploid
2.1.13	3-76-0503	somatic embryo out of suspension	U without growth regulators	20-22d - globular-shaped somatic embryo	yes, torpedo-shaped s.e.	tetraploid
2.1.14	3-76-0503	somatic embryo out of suspension	U without growth regulators	20-22d - torpedo-shaped somatic embryo	yes, torpedo-shaped s.e.	tetraploid
2.1.15	3-76-0503	somatic embryo out of suspension	U without growth regulators	20-22d - torpedo-shaped somatic embryo, precocious root germination	yes, torpedo-shaped s.e.	tetraploid
2.1.17	3-76-0503	somatic embryo out of suspension	U without growth regulators	20-22d - torpedo-shaped somatic embryo (following two step desiccation: 4d 97% and 3d 92% relative humidity)	yes, torpedo-shaped s.e.	tetraploid
2.3.1	3738-12G	callus	standard	0 h before transfer to standard media without hormones	never	diploid
2.3.3	3738-12G	callus	standard without growth regulators	3d after transfer to standard media without hormones	never	diploid

to spontaneous tetraploidisation of the embryogenic cell line during the study (Table 2), additional diploid and tetraploid callus lines were included when comparing zygotic and somatic embryos (Figure 1) in order to eventually reveal genes differentially expressed due to the ploidy status alone. Each analysed tissue was represented by three independent biological replicates.

Microarray design

In a previous study, we generated an EST library from embryonic cell cultures of different developmental stages of *C. persicum* containing 1,980 assembled EST sequences [29]. Out of this, 1,216 annotated transcripts were used to generate a cDNA microarray (Additional file 1). *Escherichia coli* colonies containing the respective *C. persicum* cDNAs (> 0.5 kbp) cloned into pBluescript SK (+) were inoculated from glycerol cultures into overnight cultures in 96-well plates and plasmids were amplified from 1 μl culture using the Illustra TempliPhi Kit (GE Healthcare, München, Germany). The cDNA inserts were PCR-amplified in three 100-μl PCR reactions in GeneAmp 9600 thermocyclers (Perkin Elmer, Rodgau, Deutschland) with modified M13 primers complementary to 40 bp of the vector backbone of the cDNA clones (M13f-40, AGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGT; M13r-40, TGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGAC). The PCR products were cleaned and concentrated using Montage PCRμ96 Filter (Millipore, Schwalbach, Germany) and validated via conventional agarose gel electrophoresis. cDNAs of two human (factor IX (gi:183979970), vascular epithelial growth factor (gi:284172448)) and four *Saccharomyces cerevisiae* (YKL013c, YJR136c, YPL252C, YNL260C) transcripts were PCR-amplified to serve as heterologous negative control genes on the microarray. Each amplified cDNA was spotted four times onto NexterionE slides (Schott, Mainz, Germany) using an Omnigrid 100 arrayer (GeneMachines, CA, USA).

The Microaaray design has been desposited at ArrayExpress [66] and is accessible through the accession number E-TABM-837.

RNA extraction and hybridisation

Total RNA was isolated from each biological replicate of the tissue samples according to the method described by Chang et al. [67] and purified using RNeasy mini columns (QIAGEN, Hilden, Germany). For microarray hybridisation we used a common reference design as proposed by Dudley et al. [68], in which a fluorescence labelled antisense oligonucleotide (complementary to a sequence tag present in all spotted microarray probes) is hybridised together with the labelled cDNA of interest. First strand cDNA was synthesised from 30 μg of total RNA using 100 Units Expand Reverse Transcriptase (Roche, Mannheim, Germany) and oligo-(dT) primer in the presence of 3 nmol Cy5-dUTP. Unincorporated nucleotides were removed using Microcon YM-30 (Millipore). Each labelled cDNA was pooled with 10.5 pmol Alexa555-labelled M13f-40 primer as antisense reference oligonucleotide and hybridised over night at 42°C to the microarray slide. The slides were washed three times successively in 2× SSC and 0.5% (w/v) SDS at 42°C, 0.5× SSC, and 0.1× SSC at room temperature for 5 min. The slides were scanned at 10 μm resolution with an ArrayWorXe scanner (Applied Precision, Washington, USA) and two-channel images were obtained for subsequent quantification of both Cy3 and Cy5 fluorescence intensities. Median pixel intensities of the spots were collected using GenePix Pro v. 6.1.0.2 (Molecular Devices, CA, USA). Local Background intensities were obtained from the median of all pixels within a defined area surrounding each spot (three times the diameter of the spot, excluding a three pixel margin surrounding any spot areas). Defective spots and areas were excluded manually.

Analysis of gene expression data

Processing and statistical analysis of gene expression data was performed using Expressionist Pro v4.5 (Genedata, Basel, Switzerland), if not indicated differently. The quality of the expression data was assessed using the Refiner module of Expressionist. Only hybridisations were considered with less than three percent defective spots and yielding a quality of 96.5 or higher using the "Contrast" function of Refiner, which relies on signal-to-noise data. Hybridisations that did not fulfil these criteria were manually inspected in log-log plots of replicate arrays as well as by hierarchical clustering of the full data set and included in case of correct distribution of the data. Hybridisations were repeated until at least three microarrays passed this QC analysis for each analysed tissue. For tissues 1.2 and 2.1.7. data of four microarray slides was used. Background subtraction was performed using the Bayesian background subtraction method, which permits negative background-subtracted intensities (BSIs). Ratios between the BSIs from RNA and antisense oligonucleotide were calculated for each spot to obtain a measure of transcript abundance. Replicate spots per microarray slide were summarised by calculating the arithmetic mean (Microsoft Excel 2003). Summarised expression data of all arrays was subjected to Lowess normalization (f = 0.1) [69] to remove non-linearities in log-log plots of abundance values from different arrays. Differential gene expression was assessed using the regularised Bayesian unpaired t-test CyberT [70] and genes with p-values ≤ 0.005 were considered to be differentially expressed. Targets showing abundance values below the median of the heterologous negative controls in both conditions of the pairwise comparison were filtered out.

This data has been deposited at ArrayExpress [66] and is accessible through the ArrayExpress accession number E-TABM-837.

Principal component analysis

Principal component analysis (PCA) was utilised to identify trends, clusters or outlying samples. PCA was performed on the normalised microarray data using the Expressionist Pro v5.1 software (Genedata, Basel, Switzerland) with genes as variables.

Gene ontology annotation

Annotation of the *C. persicum* sequences using Gene Ontology and pathway mapping was carried out with the Blast2GO suite [71] and the KEGG Automatic Annotation Server [72]. The BLAST searches (BLASTX) were performed using the following parameter settings. Searches against Swiss-Prot (rel. 56) were performed with default parameters except that E-value cut-off was set to 1.0E-5. All sequences which did not yield a hit in the previous round were subjected to an additional search, with the same parameter settings, against the NCBI nr database (rel. 169). The annotation step was performed using the default parameters and followed by an InterProScan run. The resulting GO terms were merged and underwent the validation and augmentation step. The resulting GO annotation was mapped to GO slim terms using the Blast2GO internal mapping function with the goslim_tair.obo ontology set. It was tested which GO terms were significantly over- or underrepresented among the differentially expressed genes in the chosen experiments, as compared to the complete set on the chip. To test for significant bias, Fisher's exact test ($p \leq 0.05$) was performed using the Expressionist 5.1 software (Genedata, Basel, Switzerland).

Realtime PCR

Ten selected microarray results were validated by realtime PCR. For this purpose, the relative transcript abundance of homologues of a putative receptor kinase, two different GST, XET and/or POX (Table 3) were quantitatively measured in 3 different comparisons (Figure 3). PCR amplification was performed in a Stratagene Mx3000P realtime PCR System (Stratagene, La Jolla, CA, USA) using ABsolute QPCR SYBR Green ROX Mix (ABgene, Epsom, Surrey KT19 9AP, UK). First strand cDNA was synthesised from up to 1 µg of total RNA using QuantiTect® Reverse transcription Kit (QIAGEN, Hilden, Germany). PCR reactions were carried out in a total volume of 25 µl, consisting of 2 ng cDNA, 400 nM forward primer, 400 nM reverse primer and 12.5 µl ABsolute QPCR SYBR Green ROX Mix. The realtime PCR program consisted of an initial denaturation step at 95°C for 15 min, 40 cycles of amplification with denaturation at 95°C for 15 sec, primer annealing for 1 min at 59°C and elongation at 72°C for 1 min. To complete the protocol, a melting range analysis with one cycle at 95°C for 1 min, 59°C for 1 min and 95°C for 30 sec with continuously measured fluorescence was performed. The reactions were performed in triplicate for each of three independent biological samples. All primer sequences are specified in Table 3. Standard curves were calculated for evaluating primer efficiency and all passed successfully.

The values measured were normalised to the mean value of the reference gene (Ef-Tu) in each sample. The reference gene was selected because microarray results for this gene showed stable values over all tissues. The relative amount of PCR product generated from each primer set was determined on the basis of the cycle threshold (Ct) value. The relative quantity (RQ) was calculated by the ΔΔCt-method. The calculated relative quantity for one tissue is expressed as the ratio (fold change) to the tissue to which it was compared. If this number was less than one the (negative) reciprocal is given. The reported fold changes represent the arithmetic mean of the three independent experiments and three biological replicates. Differential gene expression was statistically assessed using a two-samplet-test ($p \leq 0.05$).

Histological analysis

Different stages of ovules, zygotic and somatic embryos were analysed anatomically. Materials were fixed in FAA solution containing 67% ethanol, 20% H_2O, 1.8% formaldehyde and 5% glacial acetic acid for 24 h. The tissues were dehydrated by ethanol series and embedded in paraffine (J.T. Baker, Deventer, The Netherlands). Sections of 3-5 µm were prepared using a rotary microtome (RM 2155, Leica instruments, Nussloch, Germany). All samples were stained with FCA solution according to Etzold (New Fuchsine-Chrysoidine-Astra blue: 1000 ml dH_2O, 0.1 g New Fuchsine, 0.143 g Chrysoidin, 1.25 g Astra blue and 20 ml glacial acetic acid) (Morphisto, Frankfurt, Germany). Pictures were taken using a light microscope (Zeiss, Axio Imager, Jena, Germany). Cell sizes were determined using the AxioVision v4.7.2.0 software (Carl Zeiss Imaging solutions, Jena, Germany). For each tissue 50 cells in 5 different samples were measured. Cell size of somatic embryos was analysed three weeks after induction, samples of zygotic embryos and of the endosperm were measured 60 days after pollination, i.e. before start of seed desiccation. Only diploid genotypes were used for cell size determination.

List of abbreviations

AGO: Argonaute; Ef-Tu: elongation factor thermo unstable; GO: Gene Ontology; GST: glutathione S-transferase; SERK: somatic embryogenesis receptor kinase; PCA: principal component analysis; POX: peroxidase; s.e.:

Table 3: Primer sequences for realtime PCR

Name	Gene product	Forward primer (5'-3')	Reverse primer (5'-3')
CYC32T7_B02	Ef-Tu	CGCCATACTGCCTTTTTCTC	CTCCCGGCATAACCATCTTA
CYC01T7_E12	GST1	CATCCTGGGAGAACAATGTG	ACCCCCAAAGTAGGGTTTGT
CYC32T7_B11	GST2	GCTCGGGATTTTGCTAGAAG	TTCCCTGATGACAGAGCAAT
CYC04T7_G06	putative receptor kinase	CGTGGTGAGAGAAGAATGGA	GCATTTTAGGCCTCTTTTCG
CYC04T7_G04	POX	AAATCTCCAGCAAGGCAAAG	GCCGTGATAAAGGGACTGGTT
CP_59_C1	XET	TTCCGTGCAGGCTAAGTTCT	AGCGGAGCCTTCTGTATTGA

somatic embryogenesis; XET: xyloglucan endotransglycosylase.

Additional material

Additional file 1 Annotation, fold changes and p-values of 1,216 transcripts present on the microarray

Additional file 2 Expression pattern of differentially expressed genes in the selected eight comparisons. The symbol "+ -" in table indicates lower expression of a gene in the second tissue as compared to the first tissue of a comparison given in the column header. The symbol "- +" indicates higher expression of a gene in the second tissue as compared to the first tissue of a comparison given in the column header. Transcripts annotated as being homologous to genes previously described to be involved in somatic embryogenesis in other plants (NCBI Entrez query "somatic embryogenesis") are marked in turquoise and correspond to the annotation given in Additional file 1.

Authors' contributions

CH designed, performed and analysed the realtime PCR experiments and analysed the microarray results. SR planned and carried out the microarray experiments and evaluated their results. KK realised the histological experiments and carried out the tissue culture work. ADZ analysed the microarray data. AH conceived of and supervised part of the project and layed out the manuscript. SAR conceived of and supervised part of the project and analysed the microarray data. All authors contributed to writing of the manuscript and have seen and approved the final manuscript.

Acknowledgements

We are grateful for funding by the DFG to A.H. (HO 2100/2-1) and S.A.R. (RE 1697/3-1). We thank Stefan Wölfl for providing four *S. cerevisiae* negative control transcripts.

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Received: 30 October 2009 Accepted: 28 April 2010
Published: 28 April 2010

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doi: 10.1186/1471-2229-10-77
Cite this article as: Hoenemann *et al.*, Large impact of the apoplast on somatic embryogenesis in Cyclamen persicum offers possibilities for improved developmental control in vitro *BMC Plant Biology* 2010, **10**:77

2.2 Improvement of qRT-PCR protocol for *Cyclamen*

Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue1-fulltext-8 TECHNICAL NOTE

Selection of reference genes for normalization of quantitative real-time PCR in cell cultures of *Cyclamen persicum*

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Received June 17, 2010 / Accepted December 3, 2010
Published online: January 15, 2011

Abstract As a prerequisite for gene expression analyses in cell cultures of the ornamental crop *Cyclamen persicum* basic parameters for quantitative real-time polymerase chain reaction (qRT-PCR) have been established including the selection of reference genes using the software tools 'geNorm' and 'NormFinder'. Five potential reference genes have been tested (elongation factor tu (Ef-Tu), putative ABC transporter ATPase, putative conserved oligomeric Golgi (COG) complex component, V-ATPase G subunit 1 and Histone H3-K9 methyltransferase 4 (H3-K9-HMTase 4)). 'NormFinder' as well as 'geNorm' identified Ef-Tu to be the least stable reference gene while the ranking of the most stable genes differed depending on the algorithm. According to 'NormFinder' COG complex component displayed the most stable expression whereas 'geNorm' indicated V-ATPase G subunit 1 and a putative ABC transporter ATPase to be the most reliable reference genes. Hence, we concluded to use a normalization factor calculated from the four reference genes V-ATPase G subunit 1, ABC transporter ATPase, Histone H3-K9 methyltransferase 4 (H3-K9-HMTase 4) and COG complex component for normalization of qRT-PCR in cell cultures of *Cyclamen persicum*.

Keywords: gene expression analysis, *in vitro* propagation, primer design, somatic embryogenesis

INTRODUCTION

Quantitative real-time polymerase chain reaction (qRT-PCR) has become a very powerful technique to determine steady-state transcription levels of selected genes of interest (GOI) (Bustin, 2002; Ginzinger, 2002). The technique of qRT-PCR has first been documented in 1993 (Higuchi et al. 1993). Since then it has been continually improved and refined so that it serves as a routine tool in molecular biology today with a wide range of applications (Bustin, 2002). Parallel to the technical improvements and the drastically rising applications, requirements for methodological standardization and quality control have evolved (Bustin et al. 2009). The accuracy of qRT-PCR depends on several specific features of this novel technique, but it also suffers from the problems inherent in reverse transcription (RT) and PCR in general (Pfaffl, 2004). Two major concerns in this context are primer design and concentration as well as the selection of an appropriate normalization strategy.

Assays using SYBR Green may suffer from unspecific amplification (since SYBR Green is staining any double stranded DNA) and from the formation of primer-dimers, *i.e.* primer molecules that have hybridized to each other resulting in amplification of primer sequences instead of the target transcript (Morrison et al. 1998). To differentiate primer-dimers from the specific amplicon a melting curve analysis should be performed. After amplification the temperature is continuously increased resulting in denaturing of the DNA double strands which releases the SYBR Green dye so that the fluorescence decreases. The melting temperature is highly specific for different PCR products. Pure and homogeneous products produce a single, sharply defined melting curve with a narrow peak, while - in contrast - primer-dimers melt at relatively low temperatures and generate broader peaks (Ririe et al. 1997).

With regard to calculation of quantities, relative quantification evolved as the method of choice for most applications, *i.e.* to compare the transcript abundance (commonly referred to as the 'gene expression level') of the gene of interest with that of reference genes whose expression levels do not change under various experimental conditions. Many studies make use of potential constitutively expressed control genes without proper validation of their stability of expression (Vandesompele et al. 2002). However, the use of reference genes without prior verification of the stability of their transcript abundance might generate incorrect results (Vandesompele et al. 2002; Brunner et al. 2004; Pfaffl et al. 2004).

In this study, the basic steps (RNA isolation, cDNA synthesis, primer design and concentration determination) of establishing qRT-PCR in *Cyclamen persicum* are presented including the evaluation of five potential reference genes. *C. persicum* has evolved as a model plant for studying the effect of *in vitro* culture procedures on the process of somatic embryogenesis on the molecular level (Rensing et al. 2005; Winkelmann et al. 2006; Lyngved et al. 2008; Bian et al. 2010). For the horticultural industry vegetative propagation *via* somatic embryogenesis would be highly desirable in this crop. However, the protocol (Schwenkel and Winkelmann, 1998) is not reproducible and robust enough for use in routine propagation (Seyring and Hohe, 2005). Gene expression studies have been initiated in order to better understand the underlying physiological processes (Rensing et al. 2005; Hoenemann et al. 2010).

MATERIALS AND METHODS

Plant material

Embryogenic cell cultures of *Cyclamen persicum* have been established and maintained according to Schwenkel and Winkelmann (1998), Winkelmann et al. (1998) and Hoenemann et al. (2010). Five different tissues (specified in Table 1) have been selected for the current analysis.

Isolation of RNA and cDNA synthesis

RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and modifications as described by Dhanaraj et al. (2004). The protocol was conducted using the original buffers and modified 'RLT' lysis buffer (Dhanaraj et al. 2004) which were supplemented with 0.5% (v/v) beta-mercaptoethanol, 1.25% (w/v) polyvenylpyrrolidone-3000 (PVP 3000) and 2% (w/v) sodiumsarcosyl. For eliminating genomic DNA contamination the on-column DNase digestion was performed according to the manufacturer's instructions. RNA was quantified using the Qubit fluorimeter (Invitrogen). First strand cDNA was synthesised from up to 1 µg of total RNA using the QuantiTect Reverse transcription Kit (Qiagen).

Selection of potential reference genes

Five putative reference genes (elongation factor thermo unstable (Ef-Tu) (CYC16T7_A05), putative ABC transporter ATPase (CYC09T7_H06), V-ATPase G subunit 1 (CYC21T7_D01), conserved

Table 1. Plant and in vitro culture material of *Cyclamen persicum*.

Cell Line	Culture	Developmental Stage	Embryogeneity
F1 out of 3-2-0503	zygotic embryo	84-85 days after pollination	yes
3-43-0503	callus	0 h before transfer to standard media without hormones	no longer
3-76-0503	suspension	4 hrs after transfer to U media without hormones	yes, torpedo-shaped s.e.
3-76-0503	somatic embryo out of suspension	20-22 days - torpedo-shaped somatic embryo	yes, torpedo-shaped s.e.
3738-12G	callus	0 h before transfer to standard media without hormones	never

oligomeric Golgi (COG) complex component (CYC34T7_F01) and H3-K9 methyltransferase 4(H3-K9-HMTase 4) (CYC02F2_C10); sequences available from http://www.cyclamen-est.de) have been selected on the basis of preceding comprehensive microarray data including a wide range of tissues under various conditions (Hoenemann et al. 2010). Genes which are known to have a housekeeping function and which displayed stable transcript abundances in all microarray experiments have been selected for analysis (Table 2).

Primer design and primer concentration determination

PCR primer sequences were designed using Primer3 (Rozen and Skaletsky, 2000, available from the internet: http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The optimal primer concentrations were analyzed in ranges of 100 to 500 nM of each primer. Primer sequences and conditions for amplification are given in Table 2.

qRT-PCR methodology

The transcript abundances of five selected putative reference genes were quantitatively measured by qRT-PCR in five different tissues each. PCR amplification was performed in a Stratagene Mx3000P qRT-PCR System (Stratagene, La Jolla, CA, USA) using ABsolute QPCR SYBR Green ROX Mix (ABgene, Epsom, Surrey KT19 9AP, UK). PCR reactions were carried out in a total volume of 25 µl, consisting of 1 ng cDNA, 100 to 500 nM forward primer, 100 to 500 nM reverse primer and 12.5 µl ABsolute QPCR SYBR Green ROX Mix. The qRT-PCR program consisted of an initial denaturation step at 95°C for 15 min, 40 cycles of amplification with denaturation at 95°C for 15 sec, primer annealing for 1 min at 59°C and elongation at 72°C for 1 min. The amplification steps were ensued by a melting range analysis with one cycle at 95°C for 1 min, 59°C for 1 min and 95°C for 30 sec with continuously measured fluorescence. All experiments were performed for a set of three biological replicates in triplicate. For evaluating amplification efficiencies standard curves were calculated according to common methods (Livak and Schmittken, 2001; Pfaffl, 2004).

Table 2. Primer sequences, primer concentrations, product sizes and annealing temperatures.

Abbreviated Gene Designation	Putative Gene Product	Genbank	Forward Primer (F) Reverse Primer (R) 5' → 3'	Product Size (Bp)	Annealing Temp (°C)	Primer Concentration (Nm)
Ef-Tu	Elongation factor tu	AJ886626	F: TATCCAGAGGGGGATGGTT R: TGCCTACCTCCCTCTTCCT	102	59	200
ABC transporter ATPase	ABC transporter ATPase	AJ886326	F:TGGCGAAACGTATTGAGAA R:AGTTGCTGGGGTTAGCATTT	98	59	100
V-ATPase G subunit 1	V-ATPase G subunit 1	AJ887447	F: AGATCGGGTGCTAATGTGA R: AATCCCAGCATCGTTTTTCT	79	59	400
COG complex component	putative conserved oligomeric Golgi (COG) complex component	AJ887962	F:TATCCAACGCCGACAAAATA R:GGAATGCTTCGATTTTTGCT	105	59	200
H3-K9-HMTase 4	Histone H3-K9 methyltransferase 4	AJ885940	F: GGTGTGAGATCTTGGGATT R: GTCAAGCCCCTTCATTGTTT	148	59	200
PRK	putative receptor kinase	AJ886123	F: GTGGTGAGAGAAGAATGGA R: GCATTTTAGGCCTCTTTTCG	107	59	400

Data analysis

For each of the five reference genes (in each of the five tissues) the cycle threshold (C_t) was determined, *i.e.* the number of PCR cycles after which the fluorescence from a sample crosses the threshold (automatically amplification based). These C_t values have been transformed to relative quantities by subtraction of the lowest C_t value, *i.e.* the C_t value from the tissue with the highest transcript abundance from the C_t values of all tissues for each transcript measured, resulting in five delta C_t values for each transcript (according to the five different tissues). Subsequently each data point was transformed according to the formula $2^{(-\text{delta Ct})}$ resulting in raw reference gene quantities which are the required input data for 'geNorm' (Vandesompele et al. 2002) and 'NormFinder' (Andersen et al. 2004). 'geNorm' is an application tool for Microsoft Excel (Vandesompele et al. 2002). Data was loaded to the 'geNorm' macro file (geNorm.xls, available from internet: http://medgen.ugent.be/~jvdesomp/genorm/). 'NormFinder.xla' is an Add-In for Microsoft Excel which adds the 'NormFinder' (Andersen et al. 2004) functionality directly to the Microsoft Excel software package (available from internet: http://www.mdl.dk/publicationsnormfinder.htm).

RESULTS AND DISCUSSION

Primer concentration determination

In SYBR Green-based qRT-PCR the sensitivity of the assay should be maximized in order to minimize the formation of non-specific amplification products. Therefore, it is necessary to use the lowest concentration of primers possible without compromising the efficiency. Thus, the primer concentrations that yielded the lowest C_t values, *i.e.* resulted in the highest target amplification, were identified. For Ef-Tu (CYC16T7_A05), COG complex component (CYC34T7_F01) and Histone H3-K9-HMTase 4 (CYC02F2_C10) the optimal primer concentration proved to be 200 nM, while 100 nM was optimal for ABC transporter ATPase (CYC09T7_H06) and 400 nM for V-ATPase G subunit 1 (CYC21T7_D01). All primers passed efficiency evaluation calculated by standard curves. Besides, it was ensured that the primer concentrations chosen also minimized the presence of non-specific amplification products by dissociation curve analysis. Efficiencies were calculated with the Mx3000P Software (MxPro). Investigated transcripts showed high efficiency rates of 99% (ABC transporter ATPase and V-ATPase G subunit 1) and 101% (COG complex component and Histone H3-K9-HMTase 4) for the investigation range from 2.0 to 0.125 ng cDNA input with high linearity for the optimal primer concentration reported.

Validation of reference genes

The average expression stability M of the tested genes was calculated using the software 'geNorm' (Vandesompele et al. 2002), which is based on the "pairwise comparison strategy". The M value is defined as the average pairwise variation of a particular gene with all other reference genes within a given group of cDNA samples. The average M values of the examined reference genes are plotted in Figure 1. The gene with the lowest M value is considered to have the most stable expression, while that one with the highest M value has the least stable expression. As shown in Figure 1a, the average expression stability value (M) of V-ATPase G subunit 1 (CYC21T7_D01) and ABC transporter ATPase (CYC09T7_H06) ranked lowest whereas that of Ef-Tu (CYC16T7_A05) turned out to be the highest. These results indicate that V-ATPase G subunit 1 (CYC21T7_D01) and ABC transporter ATPase (CYC09T7_H06) displayed the most stable transcript abundance while that of Ef-Tu (CYC16T7_A05) was most variable.

Expression levels of ideal reference genes should not be affected by different developmental or environmental conditions. However, already numerous studies have described that also the expression level of housekeeping genes can vary considerably with experimental conditions (Jain et al. 2006; Hong et al. 2008; Cruz et al. 2009; Silveira et al. 2009). This is also supported by our data showing that the expression levels of all analyzed reference genes were not totally stable.

Consequently, normalization of gene expression with a single reference gene can trigger erroneous data and therefore would cause misinterpretation of experiment results. Thus, normalization with multiple reference genes has recently evolved as new standard in the analyses of quantitative PCR data as already reported by various studies in other biological systems (Tong et al. 2009; Artico et al. 2010; Lee et al. 2010; Maroufi et al. 2010).

The minimum number of reference genes included in the normalization factor has been determined by calculation of the pairwaise variation of two sequential normalization factors (Vn/n+1) containing an increasing number of genes using the 'geNorm' software (Vandesompele et al. 2002). Here, the inclusion of a fourth gene (V 3/4) resulted in the lowest pairwise variation V of 0.22, while there was an increase in instability with the addition of a fifth gene (V 4/5). Although Vandesompele et al. (2002) recommended 0.15 as a cut-off value, other studies (Kuijk et al. 2007; Silveira et al. 2009) also resulted in higher pairwise variations. Therefore we suggest to calculate a normalization factor in our case using the four least variable reference genes V-ATPase G subunit 1 (CYC21T7_D01), ABC transporter ATPase (CYC09T7_H06), Histone H3-K9-HMTase 4 (CYC02F2_C10) and COG complex component (CYC34T7_F01).

In addition to the analysis by 'geNorm' the data were also evaluated with the 'NormFinder' algorithm (Andersen et al. 2004). While 'geNorm' selects two genes with a low intra-group variation and approximately the same non-vanishing inter-group variation, 'NormFinder' takes into account the best two genes with minimal combined intra- and intergroup expression variation for normalization factor (NF) calculations (Andersen et al. 2004). Results generated by the 'NormFinder' calculation are presented in Figure 1b. This result differed from the ranking generated by 'geNorm', which could be

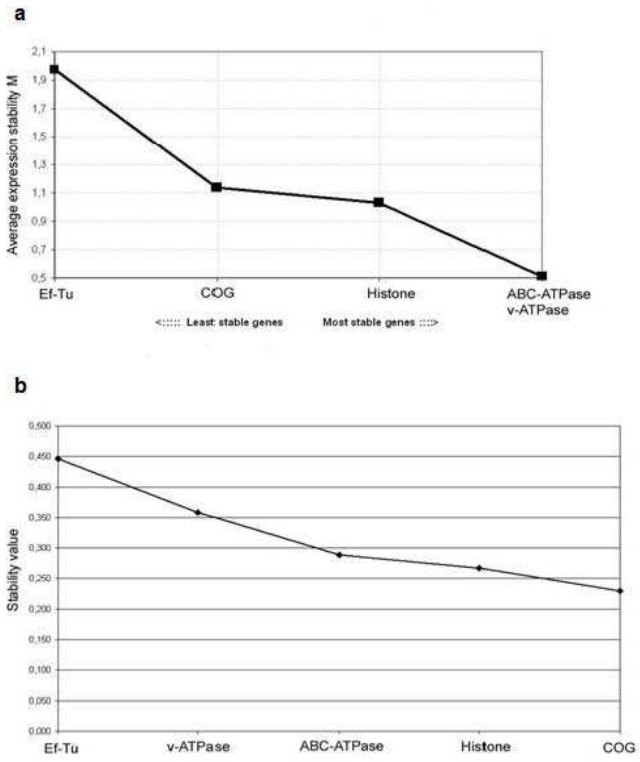

Fig. 1 Comparison of 'geNorm' and 'NormFinder' outcome: (a) 'geNorm' (Average expression stability value M of reference genes starting from the least stable gene at the left and ending with the most stable genes at the right). (b) NormFinder (Stability values for each gene starting from the least stable gene at the left and ending with the most stable gene at the right).

expected since both software tools are based on distinct statistical algorithms. However, Ef-Tu (CYC16T7_A05) ranked poorest in both algorithms and should be avoided as internal control when doing gene expression studies in embryogenic cell cultures of *C. persicum*.

The evaluation of different algorithms for reference gene selection allows a better validation of the most reliable controls and has thus become a routine tool for selection of reference genes in a wide range of organisms in the recent years (Tong et al. 2009; Artico et al. 2010; Lee et al. 2010). If different algorithms generate differing results the decision should depend on the application. An advantage of 'geNorm' is to identify the appropriate number of reference genes while 'NormFinder' is less sensitive towards co-regulation, because it examines the stability of each single reference gene independently (Lee et al. 2010).

The four identified reference genes were used for normalization of the gene expression level of a gene of interest (putative receptor kinase (PRK), CYC04T7_G0, Table 2). Exemplarily the expression levels of this gene were compared in zygotic and somatic embryos. It was shown that the transcript abundance was 3.2-fold increased in somatic embryos compared to zygotic embryos. Receptor protein kinases play an important role in several signal transduction pathways that elicit a developmental response to exogenous input (Becraft, 2002). Thus our result might be interpreted as a hint on the special impact of this gene in the process of somatic embryogenesis that is strongly dependent on exogenous triggers (Hoenemann et al. 2010).

Hence for our studies in the experimental system of cell cultures of *C. persicum*, we concluded to use a normalization factor calculated as the geometric mean of the four reference genes V-ATPase G subunit 1 (CYC21T7_D01), ABC transporter ATPase (CYC09T7_H06), Histone H3-K9-HMTase 4 (CYC02F2_C10) and COG complex component (CYC34T7_F01) for normalization of qRT-PCR.

ACKNOWLEDGMENTS

The authors thank Katja Krueger for excellent technical assistance and Stefan Rensing (University of Freiburg) for fruitful discussions on gene expression studies.

Financial support: The results were obtained within a project funded by the DFG to Annette Hohe (HO 2100/2-1) and Stefan A. Rensing (RE 1697/3-1).

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2.3 Detailed examination of selected hypotheses

2.3.1 Expression of putative genes of several glutathione S-transferases

Gene expression of a putative glutathione S-transferase is responsive to abiotic stress in embryogenic cell cultures of *Cyclamen persicum*

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Financial support:
The results were obtained within a project funded by the DFG to Annette Hohe (HO 2100/2-1) and Stefan A. Rensing (RE 1697/3-1). The works were conducted at the IGZ.

Keywords: auxin, callus, micropropagation, semisolid medium, suspension culture

Abbreviations:
Ct – cycle treshold
GST – glutathione S-transferase
MS – Murashige & Scoog
PGR – plant growth regulator

qRT-PCR – quantitative realtime PCR

2iP - 6-(gamma,gamma-dimethylallylamino) purine

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

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Abstract

Gene expression of five putative glutathione S-transferase (GST) homologues in embryogenic cell cultures of *Cyclamen persicum* were examined in liquid and semisolid culture systems using quantitative PCR. Transcript abundances were analysed with special regard to the responsiveness to auxin regulation and abiotic stress during the crucial culture step of transfer to plant growth regulator (PGR) free medium. The most distinct results were displayed by the transcript abundances of *CpGST1* which were manifold increased in the samples 4 h after transfer to PGR-free medium compared to samples 0 h after medium exchange. Comparing samples 4 h and 72 h after transfer the expression of *CpGST1* was clearly repressed during continuing culture. Hence, in contrary to preliminary data, no auxin-dependent regulation of *GST* transcript abundances could be demonstrated. Rather mechanical or drought stress imposed by the subculture procedure might be responsible for the strong and transitional induction of *CpGST1*.

Introduction

Cyclamen persicum is an ornamental potted plant with international economic importance. Because of the plant habitus and the regeneration characteristics conventional vegetative propagation (e.g. via cuttings) is not possible. Therefore, *Cyclamen* are traditionally propagated through seeds although this is associated with problems regarding variability (in case of population cultivars) inbreeding depression (in case of generation of F1-parent lines) and high costs (in case of production of F1-seed) (Ruffoni et al. 2000). Accordingly the establishment of a vegetative propagation system in vitro is highly desirable. In *Cyclamen* one of the most efficient micropropagation systems is a protocol for somatic embryogenesis starting from unfertilised ovules (Schwenkel and Winkelmann 1998). From these explants callus develops on medium containing 2,4-D and 2iP as plant growth regulators (PGRs). Callus can be propagated by regular sub-culturing on identical medium. Besides, Winkelmann et al. (1998) described suspension cultures in liquid medium of the same composition (lacking Gelrite). The differentiation of somatic embryos is initiated by transfer of callus to PGR-free medium. In order to learn more about the developmental processes of this propagation system a microarray analysis has been undertaken investigating the expression of 1,216 genes in various stages and tissues (Hoenemann et al. 2010, Rensing et al. 2005).

In this analysis a group of five putative genes of glutathione S-transferases (GSTs) displayed differential expression during important steps of the micropropagation protocol. GSTs are enzymes that catalyse the conjugation of the tripeptide glutathione to a wide variety of

hydrophobic, electrophilic and cytotoxic substrates. Many GSTs also act as glutathione-dependent peroxidases by catalysing the reduction of organic hydroperoxide to the less toxic monohydroxy alcohols (Marrs 1996). GSTs were found to be present at every stage of plant development from early embryogenesis to senescence and in every tissue type examined (Jain et al. 2010; McGonigle et al. 2000; Sari-Gorla et al. 1993). However, the function and temporal-spatial appearance of the different GST isoenzymes is highly specific (e.g. Moons 2003; Sari-Gorla et al. 1993). GSTs have been subdivided into eight distinct classes, of which seven (namely phi, tau, zeta, theta, lambda, dehydroascorbate reductase (DHAR) and tetrachlorohydroquinone dehalogenase (TCHQD)) classes are soluble (cytoplasmatic) whereas the eighth is microsomal (Basantani and Srivastava 2007). Plant GSTs have been intensively studied for their ability to detoxify herbicides (Reade et al. 2004). Besides, a major focus of research on GSTs in plants aims at its roles in phytohormone physiology, especially auxin (Bilang and Sturm 1995; Jones 1994). Moreover, they play an important role in the response to biotic and abiotic stresses (e. g. Marrs 1996). Due to their complex field of action on the cellular level they are also important for many developmental processes. E.g. GSTs are known for their relevance during somatic embryogenesis of plants as described by Pan et al. (2009) for *Citrus sinensis*.

Our study aims at analysing transcript abundances of different *GST* homologues in the process of somatic embryogenesis of *C. persicum* during the crucial step of transfer to PGR-free medium which triggers the realisation of embryo development. By this we want to learn more about the role of different in vitro manipulation steps, e.g. auxin removal and mechanical stress.

Materials and Methods

Tissue culture

The cell line of the genotype “56/2” (kindly provided by Traud Winkelmann, Institute of Floriculture and Woody Plant Science, Leibniz University Hannover) was established as described by Schwenkel and Winkelmann (1998) from unfertilised ovules of a single plant of the cultivar ‘Maxora Light Purple’ (Varinova, Berkel en Rodenrijs, The Netherlands). The cell line was cultivated on MS medium containing 2,4-D and 2iP as plant growth regulators (in the following called “standard medium”) as described by Schwenkel and Winkelmann (1998). Subculturing was performed by transfer to fresh standard medium every four weeks. As described by Winkelmann et al. (1998) suspension cultures were established and maintained by transfer to fresh standard medium every two weeks. Embryo development was

induced by transfer of the cells to PGR–free standard medium (Schwenkel and Winkelmann 1998). Subculturing of callus as well as of cell suspension comprised severe mechanical manipulations by disintegration of cell clumps (callus) and sieving through a 500 µm-mesh (cell suspension). Therefore, mechanical manipulations were performed four days before transfer to PGR-free medium (with continuing culture on fresh standard medium for four days) in order to separate mechanical manipulations from medium change. After four days cells were gently transferred to either solid or liquid standard medium lacking PGRs. In case of cell suspensions, cells were collected on a 200 µm-mesh and have subsequently been washed three times in PGR-free medium. Culture density on PGR-free medium was standardised to 500 mg/jar for cultures on semisolid medium and to a packed cell volume of 4% for cell suspensions. A flow chart of the experimental procedure is given in Fig. 1.
Cell material was collected 0, 4 and 72 h after transfer to PGR-free medium for RNA isolation. Each analysed tissue was represented by three independent biological replicates.

Isolation of RNA and cDNA synthesis

For RNA isolation the RNeasy Plant Mini Kit (Qiagen) was used according to the manufacturer's instructions except for modifications as described by Dhanaraj et al. (2004). The protocol was carried out using the original buffers including a modified 'RLT' lysis buffer (Dhanaraj et al. 2004) which was supplemented with 0.5% (v/v) beta-mercaptoethanol, 1.25% (w/v) polyvenylpyrrolidone-3000 (PVP 3000) and 2% (w/v) sodiumsarcosyl. According to the manufacturer's instructions the on-column DNase digestion was applied for eliminating genomic DNA contamination. RNA was quantified using the Quibit fluorimeter (Invitrogen).
The QuantiTect Reverse transcription Kit (Qiagen) was used to synthesise first strand cDNA from up to 1 µg of total RNA.

Quantitative realtime PCR

Partial sequences of five putative *GST* genes have been obtained by EST sequencing (Rensing et al. 2005; http://www.cyclamen-est.de: CYC01T7_E12, CYC32T7_B11, CYC29T7_E07, CYC16T7_B04, CYC33T7_F07). In the following the putative *GST* genes are named *CpGST1 - 5* (table 1). Specific primers for each of the genes have been designed using Primer3 (Rozen and Skaletsky 2000, available from the internet: http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

PCR amplification was performed with a Stratagene Mx3000P realtime PCR System (Stratagene, La Jolla, CA, USA) using SYBR Green as described by Hoenemann and Hohe (2011). Primer concentrations for optimal amplification results have been determined for each gene (table 1). The reactions were performed in triplicate for each of three independent biological samples. For evaluating primer efficiency standard curves were calculated with the Mx3000P Software (MxPro) and all passed successfully.

The values measured were normalised to the mean value of the reference gene (a putative *Ef-Tu*) as described by Hoenemann et al. (2010). The relative quantity was calculated according to Hoenemann et al. (2010) by the ΔΔCt-method (Livak and Schmittgen 2001). The calculated relative quantity for one tissue is given as the ratio (fold change) to the tissue to which it was compared. If this value was less than one, the (negative) reciprocal is given. Every reported fold change represents the arithmetic mean of three independent experiments and three biological replicates. Differential gene expression was statistically calculated using a two-sample t-test ($p \leq 0.05$).

Results and Discussion

In a broad microarray experiment comparing the expression of 1,216 transcripts in various stages of somatic embryogenesis in *Cyclamen persicum* genes of five putative GSTs were found to be differentially expressed in at least one of the experiments that are specified with tissue IDs in figure 1. Regarding the crucial step of transfer to PGR-free medium (which triggers the development of somatic embryos) a previous study showed that two out of the five *GST*-homologues diplayed a reduced transcript abundance in cells 72 h after transfer to PGR-free medium as compared with cells right before the transfer (Hoenemann et al. 2010). This might be interpreted as a hint on auxin-regulated gene expression which should be validated by additional experiments.

In the microarray experiments expression of genes in cells right before the transfer to PGR-free medium was compared to that of cells 4 h and 72 h after transfer. Here, it had been concluded that major changes in transcript abundances did only occur between 4 h and 72 h after transfer to PGR-free medium (Hoenemann et al. 2010). Therefore, in a first additional experiment gene expression of the five different *GST*-homologues 72 h after transfer to PGR-free standard medium (tissue 3.3.1) has been compared to that only 4 h after transfer (tissue 3.2.1). Fig. 2 shows that especially the transcript abundance of the *CpGST1* was extensively decreased after transfer to the PGR-free medium (fold-change: 99). This result supports the initial hypothesis that transcription of this gene was induced by auxin and repressed upon

auxin-removal. The GST enzyme group is quite diverse and some GSTs are considered to be auxin induced (Domoki et al. 2006; Flury et al. 1995; Singla et al. 2007; Van der Kop et al. 1996). Moreover, this hypothesis has been supported by a sequence comparison to *GST*-genes of other plants showing high similarities of the here presented *CpGST1* to *GST*s from *Malva pusilla* (NCBI Acc. No. AAO61854), *Ricinus communis* (NCBI Acc. No. XP_002532823 and XP_002515772), *Populus trichocarpa* (NCBI Acc. No. ADB11373 and ADB11374) and *Glycine max* (NCBI Acc. No. AAG34800). According to the data given by NCBI all these GSTs are related to the tau-class, which is described to be auxin induced (Marrs 1996). Singla et al. (2007) also described a strong repression of the expression of *GST*-homologues upon auxin removal in tissue cultures of *Triticum aestivum*, however they do not relate the GSTs encoded by these genes to one of the different GST classes as described by Basantani and Srivastava (2007).

In order to validate the putative auxin-responsive gene expression of *CpGST1* it is necessary to check changes of the transcript abundance over time. This has been done in an additional experiment comparing the expression of the *GST*-homologues in cells 4 h and 72 h after transfer to PGR-free medium to that right before transfer. The results are presented in Fig. 3. Again, transcript abundances of *CpGST*1 displayed the most distinct results while changes in the transcription level of other putative *GST*-transcripts were much less pronounced. Indeed, the expression of all putative GST-transcripts was repressed in the period between 4 h and 72 h after auxin removal (Fig. 3b, white bars) which is highly comparable to the preceding experiment (Fig. 2). However, comparing samples 0 h and 4 h after transfer to PGR-free medium transcript abundance of *CpGST*1 was manifold increased in the samples 4 h after the medium exchange regardless whether cells were transferred to liquid or to solidified medium. This observation clearly contradicts our initial hypothesis about the auxin-responsiveness of the expression of *CpGST*1, because in this case transcript abundance was expected to be reduced upon auxin removal. Thus – in order to avoid misinterpretations - it seems to be very important in this context to analyse a time period (Fig. 3) rather than randomly chosen time points (Fig. 2), especially since transcript abundances of the *GST*-homologues change rather fast. This is in line with results of Zhu et al. (2008) who analysed *GST* expression during initial cellular dedifferentiation in cotton seedlings. They described high *GST* expression levels only within a time period from 6 h to 24 h after induction by PGR treatment. These results of Zhu et al. (2008) imply that also in our experiments the high expression levels of *CpGST1* are probably not the result of stored auxins or of a late reaction to the exchange of PGR-containing medium 4 d before transfer to PGR-free medium. Therefore, we expect other

factors than auxin to be responsible for the pronounced changes in transcript abundance of *CpGST1* upon transfer to the PGR-free medium.

Levine et al. (1994) and Tenhaken et al. (1995) mentioned the function of *GST*-homologues in oxidative ore mechanical stress response. As described we tried to separate severe mechanical manipulations from the change of medium by a sequential subculture procedure. However, even during the gentle procedure of medium change, mechanical stress cannot be completely avoided, since upon transfer of cell suspensions to the PGR-free medium, the cells have been collected on a 200 µm-mesh and have subsequently been washed three times in PGR-free medium before they were inoculated into the new flasks. This procedure might impose stress by the mechanical treatment as well as by sudden exposure of the submeresly growing cells to dry clean bench air. Accordingly, a new hypothesis on induction of *CpGST1*-expression has been formulated saying that it might be induced by mechanical and/or drought stress. Therefore, transcript abundances of the *GST*-homologues have also been compared in cells growing in different culture systems, i.e. on semisolid medium in petri dishes and as suspension culture in shake flasks (Fig. 4). Except for *CpGST3* all other putative *GST*-transcripts were downregulated in the cells cultured in liquid medium (tissue 3.1) compared to the cells on semisolid medium (tissue 2.1). However, whereas fold-changes were only marginal for *CpGST2-5*, transcript abundance of the *CpGST1* was reduced 60-fold in shake flask compared to petri dish culture. Whereas mechanical stress is only imposed on cells in shake flask culture, cells transferred to petri dish culture have to cope with sudden exposure to dry air. The same comparison has been made 4 h after transfer to PGR-free medium (Fig. 4b). Again all putative GST-transcripts were repressed in suspension culture compared to the tissue cultivated on semisolid medium. However, the fold-change of *CpGST1* amounts only to factor 7 in this comparison. Since these cells have undergone the washing procedure 4 h before the samples were taken, we believe that in this experiment, the large effect of this treatment (Fig. 3) has masked a part of the differences induced by the different culture systems. Nevertheless, again the transcript abundance of *CpGST1* was increased in petri dish culture compared to suspension culture.

If expression of *CpGST1* was induced by drought stress, no significant changes in the transcript abundance should occur, if only cells from semisolid media of different culture stages are compared, because these cells do not undergo environmental humidity changes when transferred to different media. Therefore transcript abundances in cells that have only been cultured on semisolid medium have been compared right before the transfer to PGR-free

medium as well as 4 and 72 h later (Fig. 5). it becomes apparent how inhomogeneous the callus cultures were compared to cells in suspension culture.

Responsiveness of *GST* gene expression to abiotic stresses has been demonstrated in other plant systems. In *Arabidopsis thaliana* seedlings a drought-associated oxidative stress induced accumulation of a *GST* transcript that is a homologue of an extremely conserved subgroup of tau GSTs (Bianchi et al. 2002). This is of interest as also in our study *CpGST1* shows homologues to *GST* genes of other plants that belong the tau-class (see above). Diao et al. (2010) demonstrated that the expression *LbGST1* was differentially regulated by various abiotic stresses in *Limonium bicolor*. Transcript levels of a tau class *GST* from *Oryza sativa* were shown to vary significantly in response to chlorodinitrobenzene, hydrogen peroxide and atrazine treatments, indicating diverse regulation mechanisms in response to abiotic stresses (Yang et al. 2009). In the study of Jain et al. (2010) the overlap of response of *GST* genes to various stimuli (hormones, abiotic and biotic stresses) and developmental processes were analysed and provided evidence for the role of GSTs in mediating crosstalk between various stresses and hormone response.

Therefore, we tend believe that our results demonstrate responsiveness of *CpGST1* to abiotic stress in vitro, namely especially drought stress. It has long been discussed that suspension culture of plant cells might impose strong mechanical stress, because plant cells are comparatively large and possess rigid cell walls (e. g. Dunlop et al. 1994; Namdev and Dunlop 1995), whereas other authors showed that the mechanical sensitivity of plant cells was less pronounced than expected (e.g. Scragg 1995). These inconsistent conclusions might be attributed to the use of different experimental systems but also to effects of unconsidered factors. Our results might be interpreted as a hint that other abiotic stresses, e.g. possibly drought stress upon subculturing of cell suspensions and transfer to semisolid culture, are more important with regard to the comparison of different culture systems in plant tissue culture than has been expected.

Acknowledgements

The authors thank Katja Krüger for excellent technical assistance, Traud Winkelmann (Leibniz University Hannover) for providing the cell line and Stefan Rensing (University of Freiburg) for fruitful discussions on gene expression studies.

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Table 1: Primer sequences, primer concentrations, product sizes and annealing temperatures for amplification of putative *Cyclamen persicum GST* genes.

abbreviated gene designation	putative gene product	GenBank	No. Cylamen EST databased	Forward Primer (F) Reverse Primer (R) 5' → 3'	Product size [bp]	Primer concentration [nM]
CpGST1	**Probable glutathione S-transferase parA**	**AJ886042**	CYC01T7_E12	F: CATCCTGGGAGAACAATGTG **R: ACCCCCAAAGTAGGGTTTGT**	**118**	**200**
CpGST2	**Probable glutathione S-transferase**	**AJ886910**	**CYC32T7_B11**	**F: GCTCGGGATTTTGCTAGAAG R: TTCCCTGATGACAGAGCAAT**	**109**	**200**
CpGST3	**Glutathione S-transferase GST 20**	**AJ887722**	**CYC29T7_E07**	**F: GTTGGGACCGATCGAAGTA R: CAAGTGGAAGCTCGAGGAA**	**101**	**200**
CpGST4	**Glutathione-S-transferase/glutaredoxin**	**AJ887143**	**CYC16T7_B04**	**F: AAGGGCATGAGGTGGATTT R: CTCATCAGCCTCGCTATGG**	**98**	**100**
CpGST5	**Putative glutathione S-transferase**	**AJ886941**	**CYC33T7_F07**	**F: TGTGAAGCTGCTCGATGAA R: TGGGATCGCATTTTTCACT**	**91**	**100**
Ef-Tu	**Elongation factor tu**	**AJ886626**	**CYC16T7_A05**	**F: TATCCAGAGGGGGATGGTT R: TGCCTACCTCCCTCTTCCT**	**102**	**200**

Figure 1: Flow chart of the experimental procedure. Samples taken for further analysis are encircled by boxes and are specified with tissue IDs.

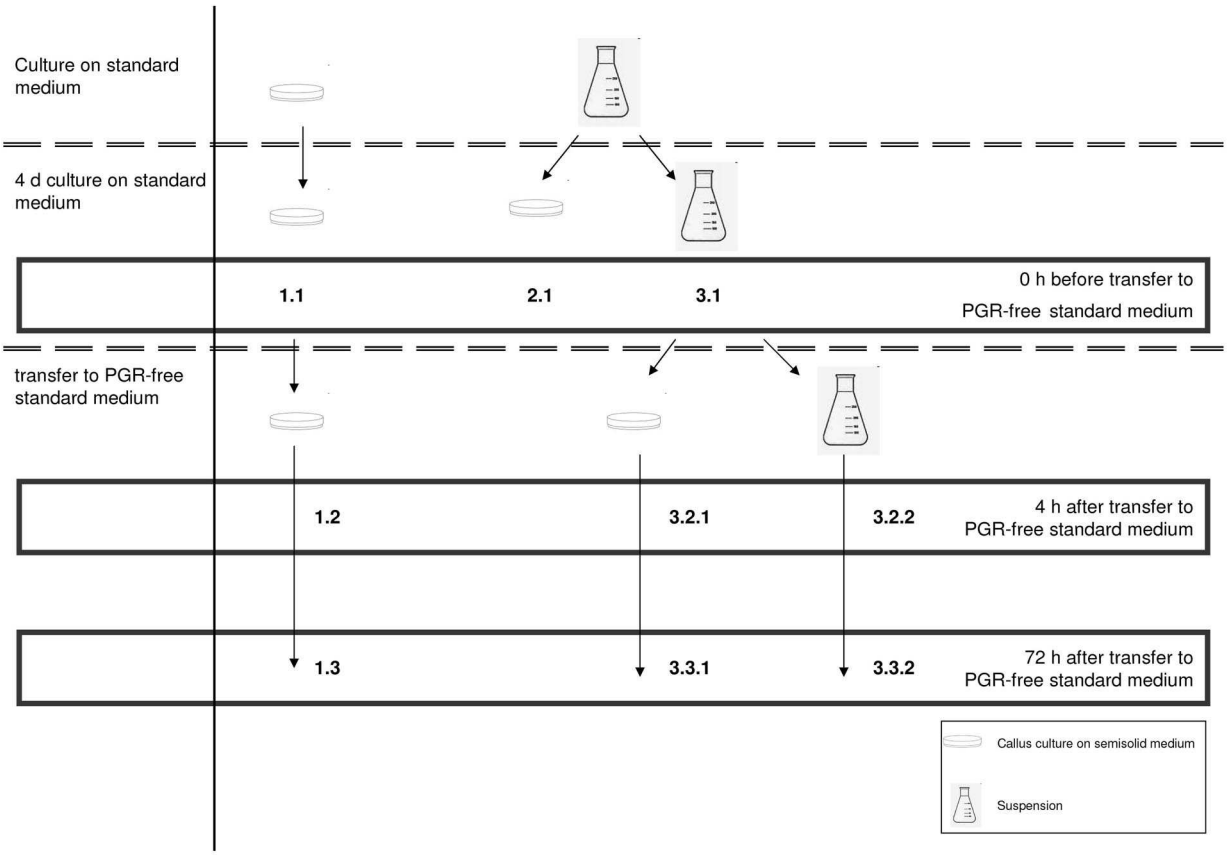


Figure 2: Transcript abundances of *GST* homologues in samples 72 h after transfer to PGR-free standard medium (tissue 3.3.1) compared to 4 h (tissue 3.2.1) presented as x-fold-change. Positive values describe up-regulation while negative values describe down-regulation.

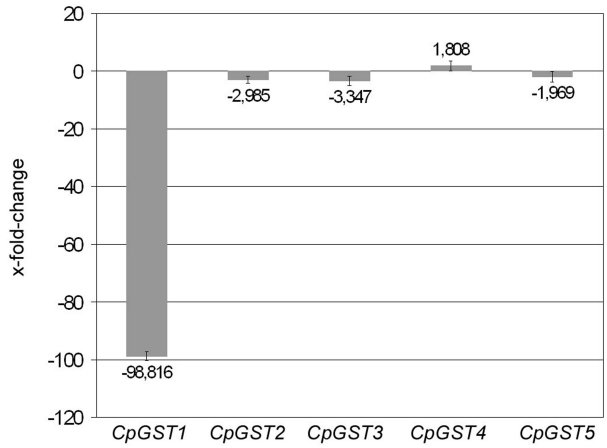


Figure 3: Gene expression of *GST* homologues 0, 4 and 72 h after transfer to PGR-free medium. a: culture on semisolid medium; b: suspension culture.
(dark grey bars: 4 h compared to 0 h after transfer to PGR-free standard medium, light grey bars: 72 h compared to 0 h after transfer to PGR-free standard medium, white bars: 72 h compared to 4 h after transfer to PGR-free standard medium). Positive values describe up-regulation while negative values describe down-regulation.

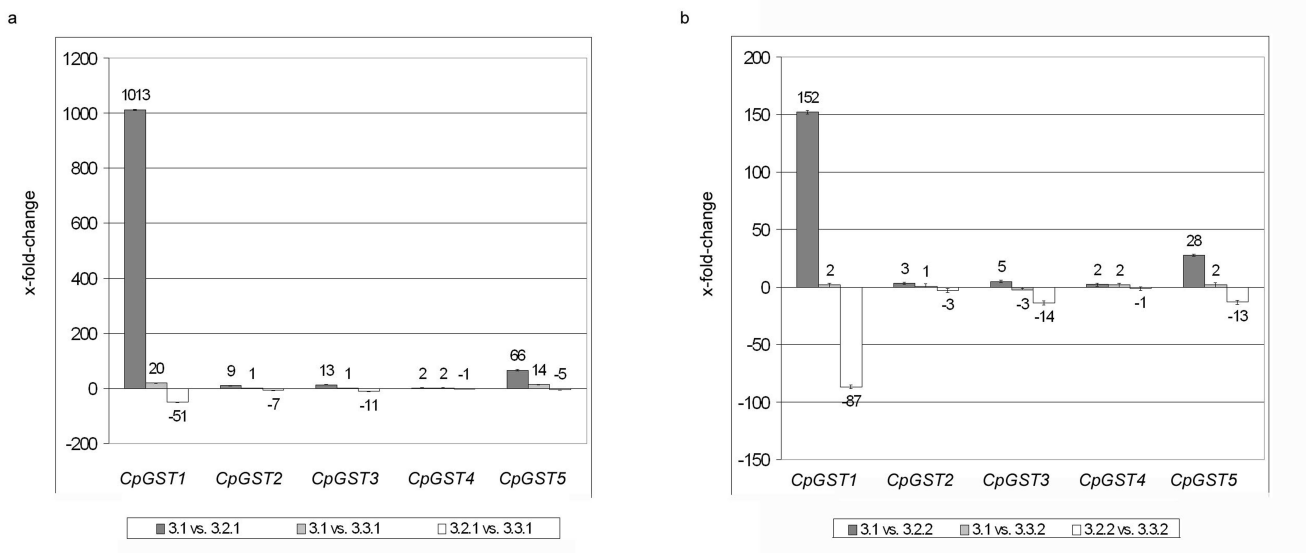


Figure 4: Gene expression of *GST* homologues in cells growing in suspension culture compared to cells on semisolid medium presented as x-fold-change (a: before transfer to PGR-free medium; b: 4h after transfer to PGR-free medium). Positive values describe up-regulation while negative values describe down-regulation.

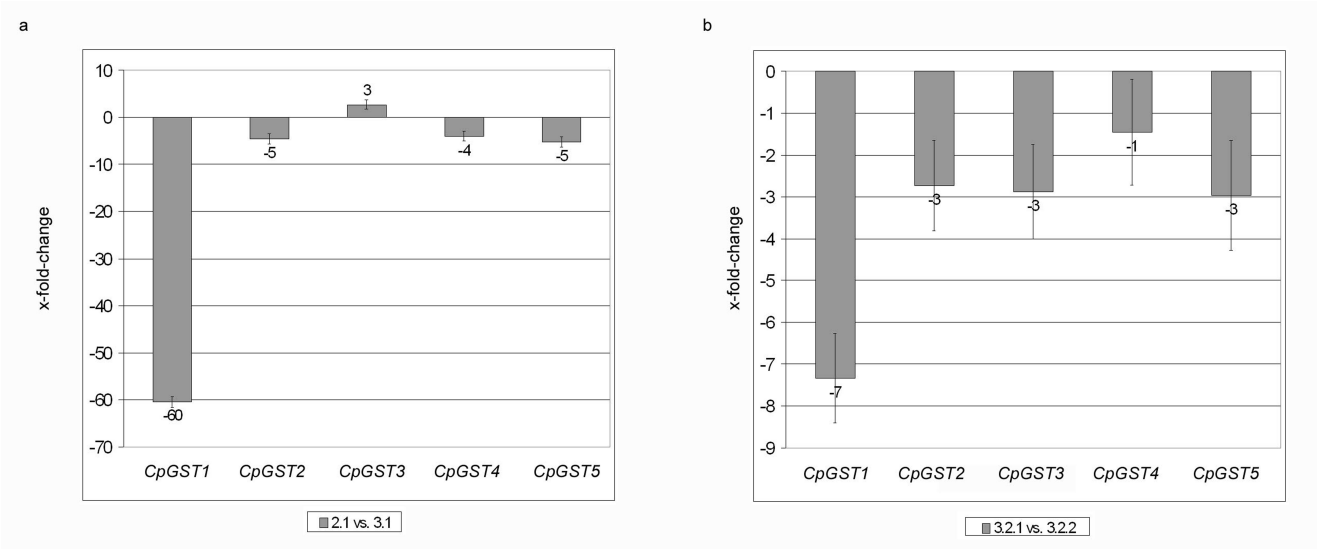
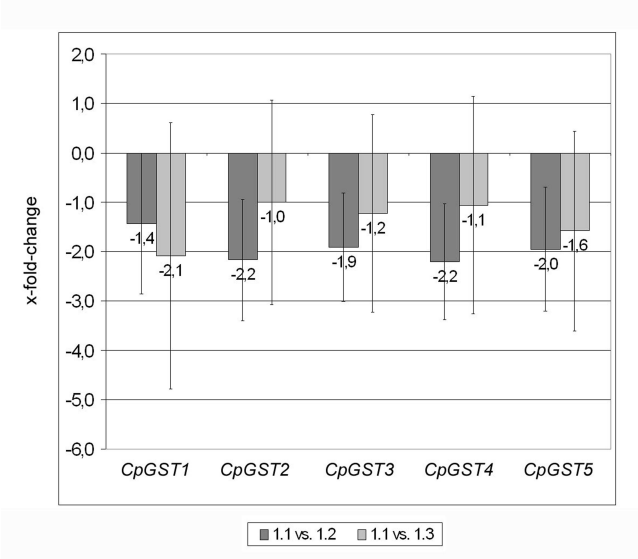


Figure 5: Gene expression of *GST* homologues in callus cultured for 4 h (dark grey bars, tissue 1.2) or for 72 h (light grey bars, tissue 1.3) compared to cells before transfer to PGR-free medium (tissue 1.1) presented as x-fold-change. Positive values describe up-regulation while negative values describe down-regulation.



2.3.2 Expression analysis of putative genes of pectin modifying enzymes

Expression analysis of putative genes of pectin modifying enzymes in different callus lines of *Cyclamen persicum* and their correlation with the callus texture

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Abstract

Gene expression of four different pectin modifying enzymes (a putative *pectinacetylesterase* (*PAE*) homologue, a putative *pectinesterase* (*PE*) homologue and two different putative *pectate lyase* (*PL*) homologues) was examined in different callus cell lines of *Cyclamen persicum* as well as in the corresponding explants using quantitative PCR. Transcript abundances were analysed with special regard to potential correlations with callus texture and embryogenic competence. No correlation has been detected between gene expression of the putative pectin modifying enzymes and the embryogenic competence of the different cell lines. However, expression of the putative *PAE* homologue correlated with the callus texture: transcript abundances of the putative *PAE* homologue were higher the more friable the tissue was, in callus as well as in explant the tissue. Histological analyses supported the conclusion that callus texture was influenced by the explant type.

Keywords: cell wall adhesion, explant type, histological analysis, somatic embryogenesis

Abbreviations:
pectate lyase - PL
pectinacetylesterase - PAE
pectinesterase - PE
plant growth regulator - PGR
ruthenium red - RR
somatic embryo – s.e.

Introduction

In indirect somatic embryogenesis callus, i.e. cells in morphologically unorganised growth, develops from the explant. Subsequently, either spontaneously or because of a change of the growth conditions, callus cells start to differentiate into somatic embryos. Such systems exhibit a high potential as very efficient propagation systems. However, the development of different callus lines with variable characteristics represents a major problem for use of indirect somatic embryogenesis in commercial plant propagation. In this context, particularly the development of embryogenic and non-embryogenic cell lines is of interest. Differences between embryogenic and non-embryogenic cell lines from the morphological to the molecular level have been analysed in various systems (e. g. in *Daucus*: Feirer and Simon 1991; Kikuchi et al. 1995; in *Medicago*: Martin et al. 2000; Pouisen et al. 1996; in *Picea*: du Jardin et al. 1996; in *Cyclamen*: Winkelmann et al. 1998).

One of the characteristics of special interest in this regard is cell adhesion, i.e. callus texture. More friable callus is often selected due to better mangeability. On the other hand cell adhesion is also an important physiological factor. For organisation of plant tissues a spatial and temporal control of intercellular attachment and communication is essential and therefore critical for normal development and morphogenesis (Iwai et al. 2002). Cell wall structure is continually modified to accommodate to the developmental stage and the environmental condition. The primary cell wall is supposed to contribute significantly to cell wall structural integrity, cell adhesion and signal transduction. The major fractions of the primary cell wall are pectic polysaccharids (Caffall and Mohnen 2009). The strength of intercellular contacts depends on cell wall structures, which include polysaccharide components, in particular pectin that is localised mainly in the primary cell wall, middle lamella and cell corners (Iwai et al. 2002). Principally, pectin consists of the pectic polysaccharides homogalacturonans (HGs), substituted HG (rhamnogalacturonan II, xylogalacturonan and apiogalacturonan) and rhamnogalacturonan I (Mohnen 2008). Compared with cellulose and hemicellulose little is known about the synthesis and assembly of pectin (Iwai et al. 2002).

A potential causal connection between cell adhesion conferred by pectin characteristics of the middle lamella and embryogenity of different cell lines has been discussed by several authors (e. g. Suzuki et al. 1990; Kikuchi et al. 1995). Besides analyses of pectin characteristics in particular, also callus texture in general has been discussed with regard to embryogenic competence: In carrot suspension culture Iwai et al. (1999) demonstrated that carrot embryogenic callus forms larger and tighter cell clusters than non-embryogenic callus does.

Quiroz-Figueroa et al. (2006) confirmed a correlation between callus appearance and embryogenic competence in *Coffea arabica*. Here embryogenic callus could be identified by brown colour and compact texture while non-embryogenic callus was pale and friable.
In a preceding microarray analysis (Hoenemann et al. 2010) four different pectin modifying enzymes were differentially expressed in the comparison of an embryogenic and a non-embryogenic cell line of *Cyclamen persicum*. From these results it has been inferred, that gene expression of pectin modifying enzymes might be correlated with the embryogenic competence. This hypothesis as well as a putative correlation between gene expression of pectin modifying enzymes and callus texture has been investigated in detail in the current study.

Methods

Tissue culture

The callus lines 3-0805 and 3738-12G are the same cell lines as given by Hoenemann at al. (2010) (tissue IDs in the latter publication: 2.1.1 and 2.3.1).
New callus lines were established as described by Schwenkel and Winkelmann (1998) from unfertilised ovules, placenta and peduncles, respectively, from genotypes 3 and 17 of the cultivar 'Sierra Purple Flame'. Callus was cultivated on MS based plant growth regulator (PGR) -containing medium ("standard medium") as described by Schwenkel and Winkelmann (1998), transferred to fresh standard medium after eight weeks and cultivated there for another four weeks. Somatic embryo development was induced by transfer of the cells to PGR–free standard medium (Schwenkel and Winkelmann 1998). Development of somatic embryos and callus texture were evaluated three weeks after transfer to PGR–free standard medium. For RNA isolation cell material was collected right before transfer to PGR-free medium. Each analysed tissue was represented by three independent biological replicates at three different dates.

Quantitative realtime PCR

Isolation of RNA and cDNA synthesis for qRT-PCR was carried out as described by Hoenemann and Hohe (2011).

PCR amplification was performed in a Stratagene Mx3000P realtime PCR System (Stratagene, La Jolla, CA, USA) using ABsolute QPCR SYBR Green ROX Mix (ABgene, Epsom, Surrey KT19 9AP, UK). PCR reactions were carried out according to Hoenemann and Hohe (2011). The reactions were performed in triplicate for each of the three independent biological samples. All primer sequences are specified in table 1. Standard curves were calculated for evaluating primer efficiency and all passed successfully.
The values measured were normalised to the mean value of four reference genes as recommended by Hoenemann and Hohe (2011). The relative amount of PCR product generated from each primer set was determined on the basis of the cycle threshold (Ct) value. The relative quantity was calculated using the the ΔΔCt-method (Livak and Schmittgen 2001). The calculated relative quantity for one tissue is expressed as the ratio (fold change) to the tissue to which it was compared. If this number was less than one the (negative) reciprocal is given. The reported fold changes represent the arithmetic mean of the three independent experiments and three biological replicates. Differential gene expression was statistically assessed using a two-sample t-test ($p \leq 0.05$).

Histological analysis

Different tissues (callus as well as explants) were fixed in FAA solution containing 67% ethanol, 20% H_2O, 1.8% formaldehyde and 5% glacial acetic acid for 24 h. The tissues were dehydrated using ethanol series and embedded in paraffine (J.T. Baker, Deventer, The Netherlands). Sections of 3 µm were prepared using a rotary microtome (RM 2155, Leica instruments, Nussloch, Germany). All samples were stained with W3A solution according to Wacker (Wacker 2006) (Morphisto, Frankfurt, Germany) and ruthenium red (RR) solution (Johansen, 1940) (Morphisto, Frankfurt, Germany). Pictures were taken using a light microscope (Zeiss, Axio Imager, Jena, Germany).

Results and discussion

Microarray analyses of various embryogenic and non-embryogenic tissue cultures of *Cyclamen persicum* revealed differential expression of four different putative genes of pectin modifying enzymes in the comparison of embryogenic and non-embryogenic callus lines (Hoenemann et al. 2010).
In order to possibly verify a putative link between expression of pectin modifying enzymes, pectin content, callus texture and embryogenic competence in the system of *Cyclamen*

persicum we first analysed histologically the two cell lines 3-0805 (embryogenic, tissue ID 2.1.1) and 3738-12G (non-embryogenic, tissue-ID 2.3.1 compared by Hoenemann et al. (2010)(Fig. 1). W3A-stainings confirmed structural differences of the callus lines. The embryogenic cell line showed tight and relatively big cell clusters whereas the non-embryogenic line displayed a loose structure (Fig. 1 ai and bi). Moreover, a more intense orange to red staining of the cell walls in the embryogenic cell lines might be a hint on lignification. RR-staining has been applied to visualise the pectin content and distribution. Here, cell walls of the embryogenic cell line in fact looked more red indicating a higher pectin content (Fig. 1 aii and bii). These data seem to support the hypothesis on a correlation between pectin-conferred cell adhesion and embryogenic competence. However, these two cell lines did not have the same genetic background.

As Schwenkel and Winkelmann (1998) described the development of cell lines showing variation in embryogenic competence according to the explant tissue used for induction, new callus lines have been induced in two different embryogenic genotypes (namely 3 and 17) starting from three different explant types (placenta, ovule, peduncle). The texture of the callus was evaluated visually as well as the development of somatic embryos three weeks after transfer to PGR-free medium (table 2). Regarding the texture of the callus the results were very consistent and in line with previous data. Callus originating from ovules was friable, while callus resulting from placenta was even more friable. In contrast all cell lines developing from peduncle segments displayed a quite compact structure. However, in opposite to the original hypothesis, no general correlation between callus texture and embryogenic competence was detected.

Nevertheless, it should be checked whether gene expression of the pectin modifying enzymes (a putative *pectinacetylesterase* (*PAE*) homologue, a putative *pectinesterase* (*PE*) homologue and two different *pectate lyase* (*PL*) homologues) was at least correlated with the callus texture. Therefore, expression of the putative genes was investigated by qRT-PCR before transfer to PGR-free medium in order to verify if their expression could be causal for callus texture. Moreover, callus texture was analysed by histological staining.

Comparing gene expression data (genotype 17, Fig. 2) with those of the microarray (Hoenemann et al. 2010) the hypotheses deduced from the microarray results could not be reproduced except for the putative *PAE* homologue: In the microarray transcripts of putative *PE* and *PL* homologues were more frequent in the compact cell line, whereas in the actual analysis this was only true for the comparison of callus derived from ovules and peduncle segments. However, comparing cell lines derived from placenta tissue, which were even more

friable than those derived from ovules, transcripts of putative *PE* and *PL* homologues were more frequent in the friable callus. In contrast, expression of the putative *PAE* homologue correlated well with the callus texture: In both analyses transcripts of the putative *PAE* homologue were more abundant the more friable the callus was.

Histological analyses of the callus lines originating from the different explants (genotype 17) confirmed the differences in texture that have been evaluated visually (Fig. 3): Whereas the cell structure of callus originating from peduncles was very dense and the W3A staining indicated lignification (Fig. 3 a i), callus originating from placenta tissue displayed a very loose structure and nearly no lignification (Fig. 3 c i). However, no clear difference in the pectin content as indicated by RR staining could be detected (Fig. 3 a ii – 3 c ii). Thus, RR-staining did not show the expected effect and did not confirm the assumed differences in pectin content. On the other hand, RR might stain a relatively large amount of different polysaccharide compounds and is therefore not entirely specific for pectins (Shevell et al. 2000).

To date, only a few plant PAEs have been functionally investigated. Pilatzke-Wunderlich and Nessler (2001) analysed transcripts of PAE and PL homologues in opium poppy. Here, transcripts of the *PAE* homologue were detected only in the latex, whereas the *PL* homologue showed very high expression levels in latex as well, but also low expression levels in other tissues. Deduced from the proposed role of a *PAE* in mung bean by Breton et al. (1996), Pilatzke-Wunderlich and Nessler (2001) suggest, that PAE might be involved in cell wall degragation and expansion. Likewise, in *Erwinia chrysanthemi* PAE action probably favors pectin degragation by making the substrate more readily available for cleavage by pectate lyases that are responsible for the symptom of maceration (Shevchik and Hugouvieux-Cotte-Pattat 2003). In our study the expression of a putative *PAE* homologue was highly increased in friable compared to more compact callus which might be a hint on the involvement of PAE in the generation of loose cell-to-cell contact in these cell cultures.

Hence, the possible correlation between transcript abundance of the putative *PAE* homologue and callus texture has been rechecked in the cell lines originating from explants of genotype 3 (Fig. 4). As in genotype 17, expression of the putative *PAE* homologue was repressed in the more compact callus lines, regardless if it was compared to callus originating from ovules or from placenta tissue. These observations support our hypothesis that the expression of the putative *PAE* homologue correlated with the callus texture.

It is assumed that the differences in transcript abundances have been conferred to the callus lines from the explant tissue. Therefore, also the explants (ovules, placenta and peduncles of

genotype 3) were analysed with respect to transcript abundances of the putative *PAE* homologue (Fig. 5). Due to their similarity in callus texture, tissues of ovules and placenta were analysed as a pooled sample. Differences in transcript abundance of the putative *PAE* homologue in the explant tissues correlated well with those in the deduced callus lines, so that this correlation might be causal.

However, histological analyses of callus lines and the corresponding explant tissues (Fig. 6 and 7, genotype 3) only partially confirm these similarities: Longitudinal sections of peduncles showed the presence of lignification, indicated by vermilion staining (Fig. 6 a i) Likewise callus originating from peduncle explants showed more red to orange stained cells, indicating the presence of lignin, than callus originating from ovules (Fig. 7 b i) and placenta tissue (Fig. 7 c i). Likewise, differences in cell size of the explant tissues were reflected by those in the originating callus lines. This was especially obvious in the comparison of callus originating from placenta tissue (Fig. 7 c), whose cells were relatively large, with callus originating from ovules (Fig. 7 b) and peduncles (Fig. 6 b). However, RR staining did not show any essential differences in the amount of pectin between the callus lines originating from different tissues (Fig. 6 b ii, 7 b ii and 7 c ii), whereas there was a clear difference in the amount of red stained pectin comparing ovules and placenta tissue (Fig. 7 a). Again, this might be a consequence of poor specificity of the RR-staining (Shevell et al. 2000).

Other studies about differences in callus texture mainly concentrate on the influence of different media and PGR supplementations (e.g Akande et al. 2009; Akbaş et al. 2009; Hassan et al. 2009; Koli et al. 2009; Sharma and Nautiyal 2009; Tiwari and Tripathi 2005). In addition Karimi et al. (2010) report an impact of the genotype and the explant position on the callus texture of *Cereus peruvianus*. In Indian cotton cultivars Tripathy and Reddy (2002) observed different callus textures depending on the explant type, the genotype and the medium composition. Comparing different explants (leaf, node and internode) Sadeak et al. (2009) concluded from their studies in *Stevia rebaudiana* that the callogenic response and therefore the callus texture depends amongst other factors on the type of explant. None of these studies specified the tissue texture of the explant. Accordingly, our results cannot be compared directly to these studies with regard to the question if the tissue structure of the explant has any impact on the texture of the resulting callus. Nevertheless, our results support the conclusion of other studies (Sadeak et al. 2009; Tripathy and Reddy 2002) that callus texture is influenced by the explant type.

Summing up, it has to be stressed, that many more factors not tested in our study affect pectin composition, cell wall adhesion and most notably the embryogenic competence of a specific

cell line. Moreover, due to the complexity of the system, it seems improbable to identify a "master switch" or a general physiological marker. On the other hand, the *PAE* expression level might be another important piece in the huge puzzle.

Acknowledgements

The authors thank Stefan Rensing (Unversity of Freiburg) for fruitful discussions on gene expression studies.

Financial support

The results were obtained within a project funded by the DFG to Annette Hohe (HO 2100/2-1) and Stefan A. Rensing (RE 1697/3-1). The works were conducted at the IGZ.

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Table 1: Primer sequences, primer concentrations, product sizes and annealing temperatures.

gene	putative gene product	GenBank	Forward Primer (F) Reverse Primer (R) 5' → 3'	Product size [bp]	Primer concentration [nM]
Pectinacetylesterase (PAE)	Homolog of pectinacetylesterase family protein	AJ887083	F: ACAATGTTTCTTCCCCCAAT R: CGTATGCTGAGTTCCCACAC	72	500
Pectinesterase (PE)	Pectinesterase 3 precursor	AJ886797	F: ACTGACAGCCCCTTGCTAAT R: ATGCCGGTGTTTTGGTTAAT	76	400
Pectate lyase 1 (PL1)	Probable pectate lyase P18 precursor	AJ887596	F: GCGAATGGAGGACATGGAA R: TTAAGCTCGAAGCCCTAGCA	115	200
Pectate lyase 2 (PL2)	Pectate lyase	AJ887577	F: GCGAATGGAGAACATGGAA R: AGAGCCCACGAGCGAAGAT	144	200
ABC transporter ATPase	ABC transporter ATPase	AJ886326	F: TGGCGAAACGTATTGAGAA R: AGTTGCTGGGGTTAGCATTT	98	100
V-ATPase G subunit 1	V-ATPase G subunit 1	AJ887447	F: GAGATCGGGTGCTAATGTGA R: AATCCCAGCATCGTTTTTCT	79	400
COG complex component	putative conserved oligomeric Golgi (COG) complex component	AJ887962	F: TATCCAACGCCGACAAAATA R: GGAATGCTTCGATTTTTGCT	105	200
H3-K9-HMTase 4	Histone H3-K9 methyltransferase 4	AJ885940	F: GGGTGTGAGATCTTGGGATT R: GTCAAGCCCCTTCATTGTTT	148	200

Table 2: Texture and embryogenic competence of different cell lines depending on the explant type

explant	**texture**		**embryogenic competence**	
	genotype 3	genotype 17	genotype 3	genotype 17
ovule	friable	friable	low	moderate
placenta	very friable	very friable	none	high
peduncle	compact	compact	low	none

Fig. 1: Histological analysis of embryogenic (cell line 3-0805) (a) and non-embryogenic (cell line 3738-12G) (b) callus stained with W3A (a i and b i) or with ruthenium red (a ii and b ii).

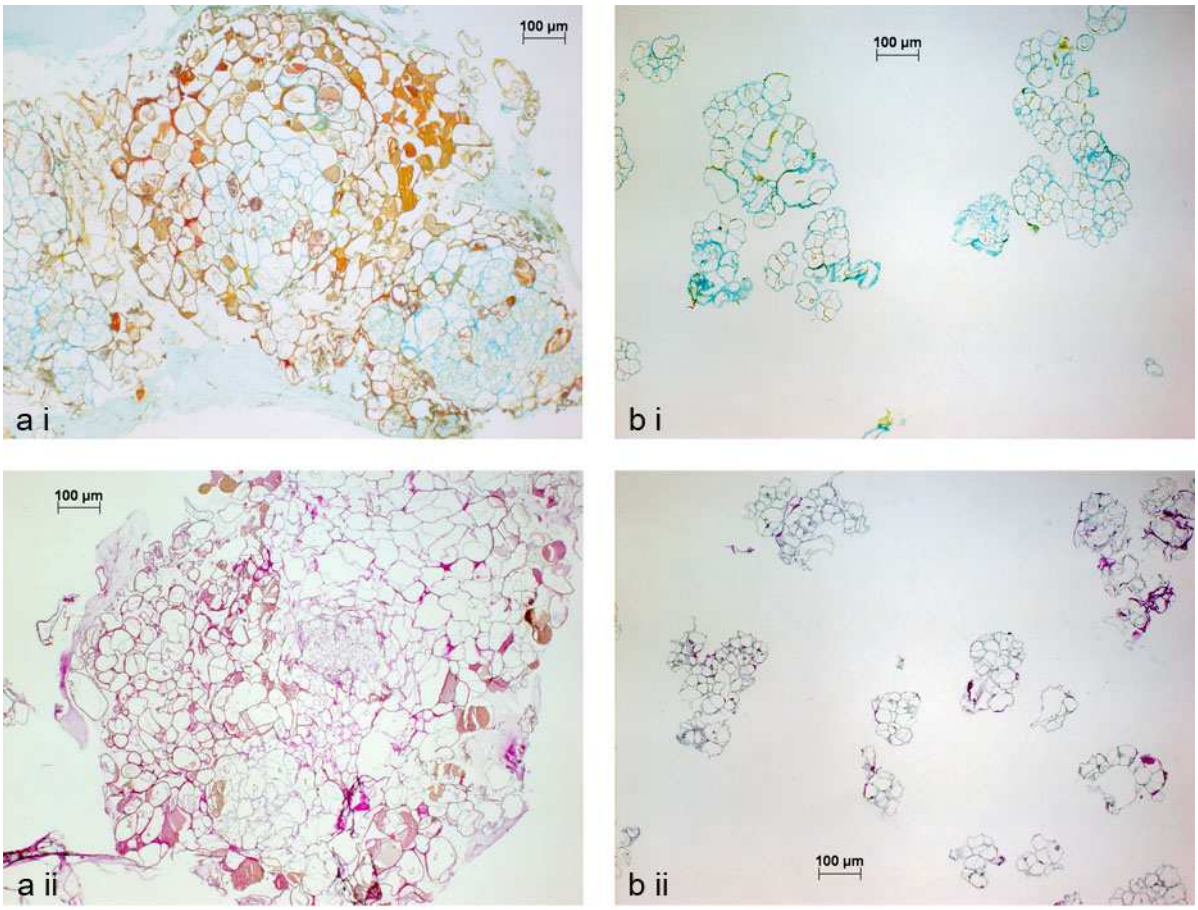


Fig. 2: Relative expression levels of putative genes of different pectin modifying enzymes in callus lines that were induced on various explants of genotype 17. White columns show the results obtained in microarray experiments published (Hoenemann et al., 2010) for better comparison. Positive values describe up-regulation while negative values describe down-regulation.

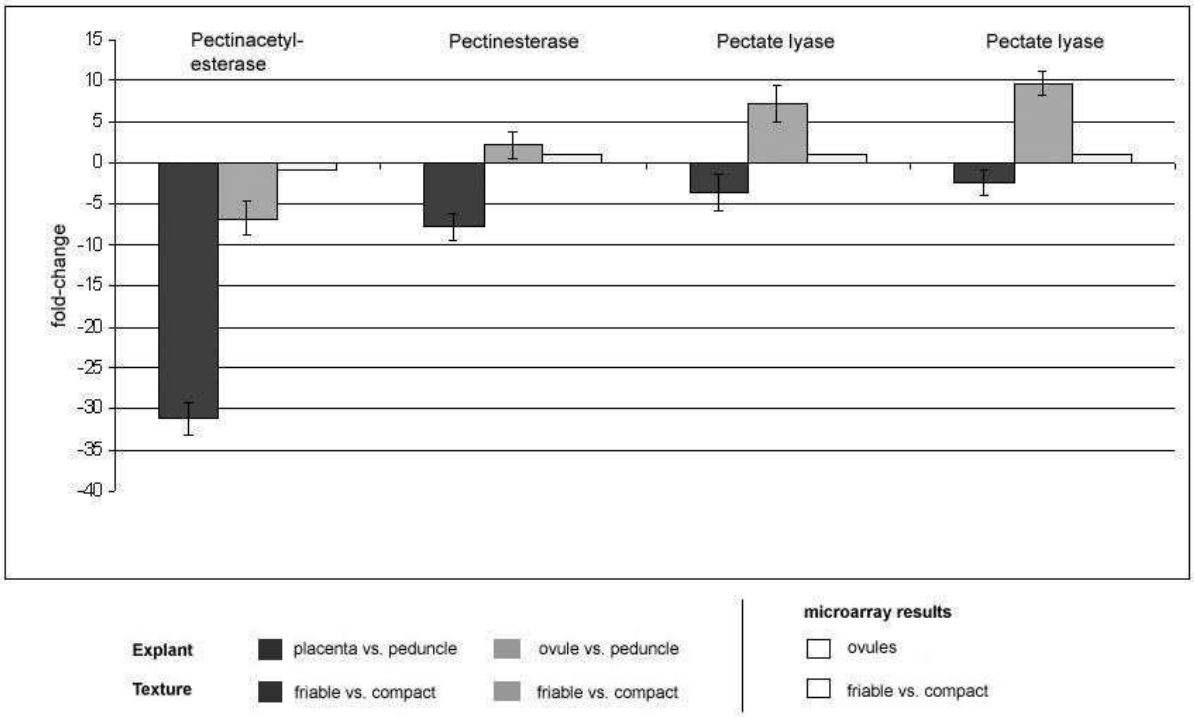


Fig. 3: Histological analysis of callus developing from peduncles (a) ovules (b) and placenta (c) stained with W3A (a i, b i and c i)) or with ruthenium red (a ii, bii and c ii) (genotype 17).

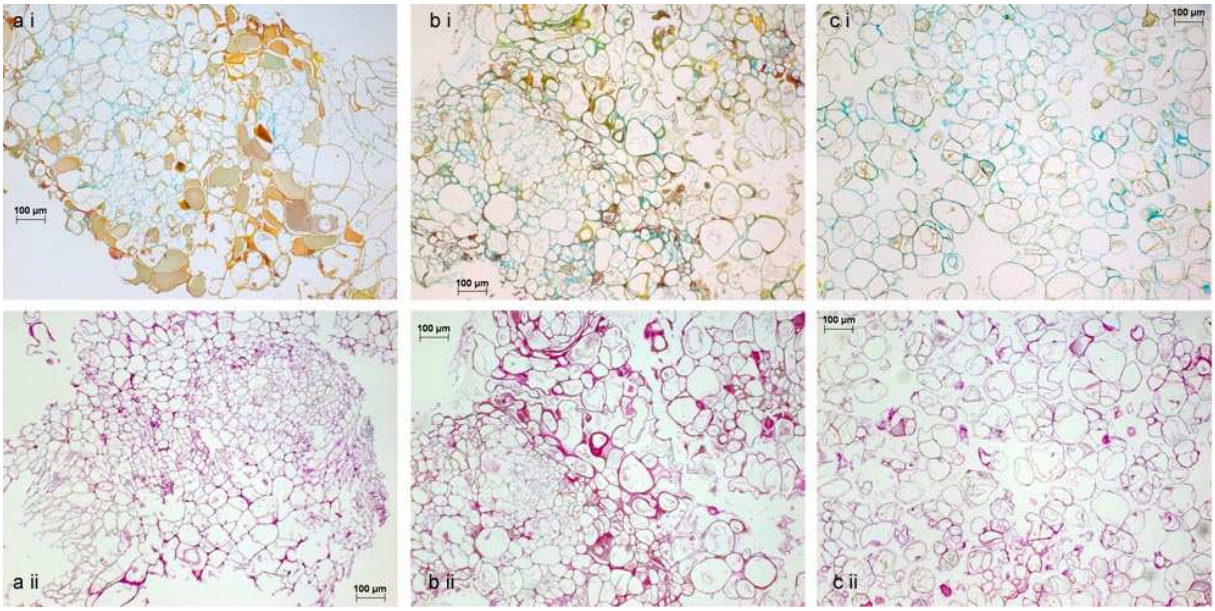


Fig. 4: Relative gene expression levels of a putative gene of a pectinacetylesterase (PAE) in callus lines developing from different explants of genotypes 3 and 17. Positive values describe up-regulation while negative values describe down-regulation.

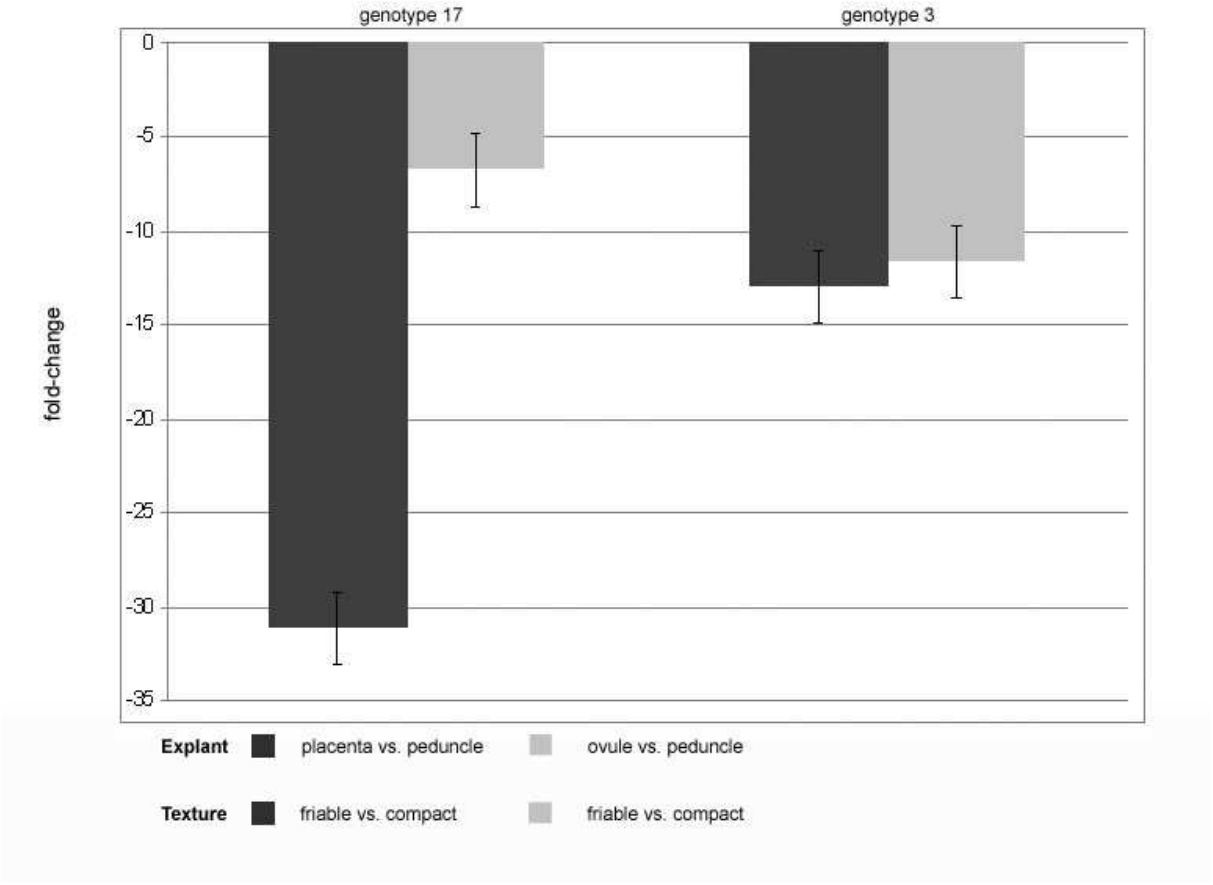


Fig. 5: Relative gene expression levels of a putative gene of a pectinacetylesterase (PAE) in callus lines developing from different explants compared to the relative gene expression levels in the corresponding explants. Positive values describe up-regulation while negative values describe down-regulation.

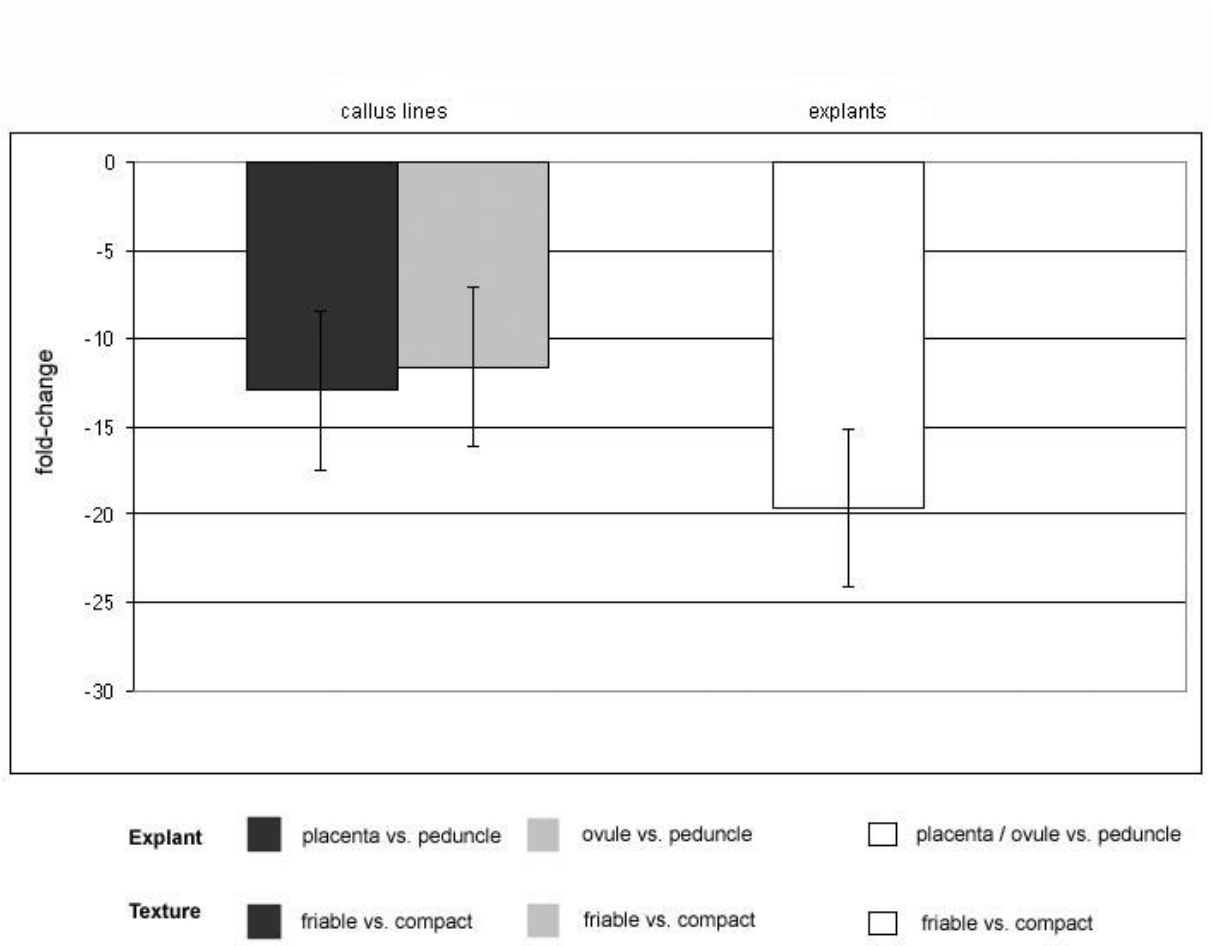


Fig. 6: Histological analysis of peduncles (explant, longitudinal section) (a) and corresponding callus (b) stained with W3A (a i and b i) or with ruthenium red (a ii and b ii) (genotype 3).

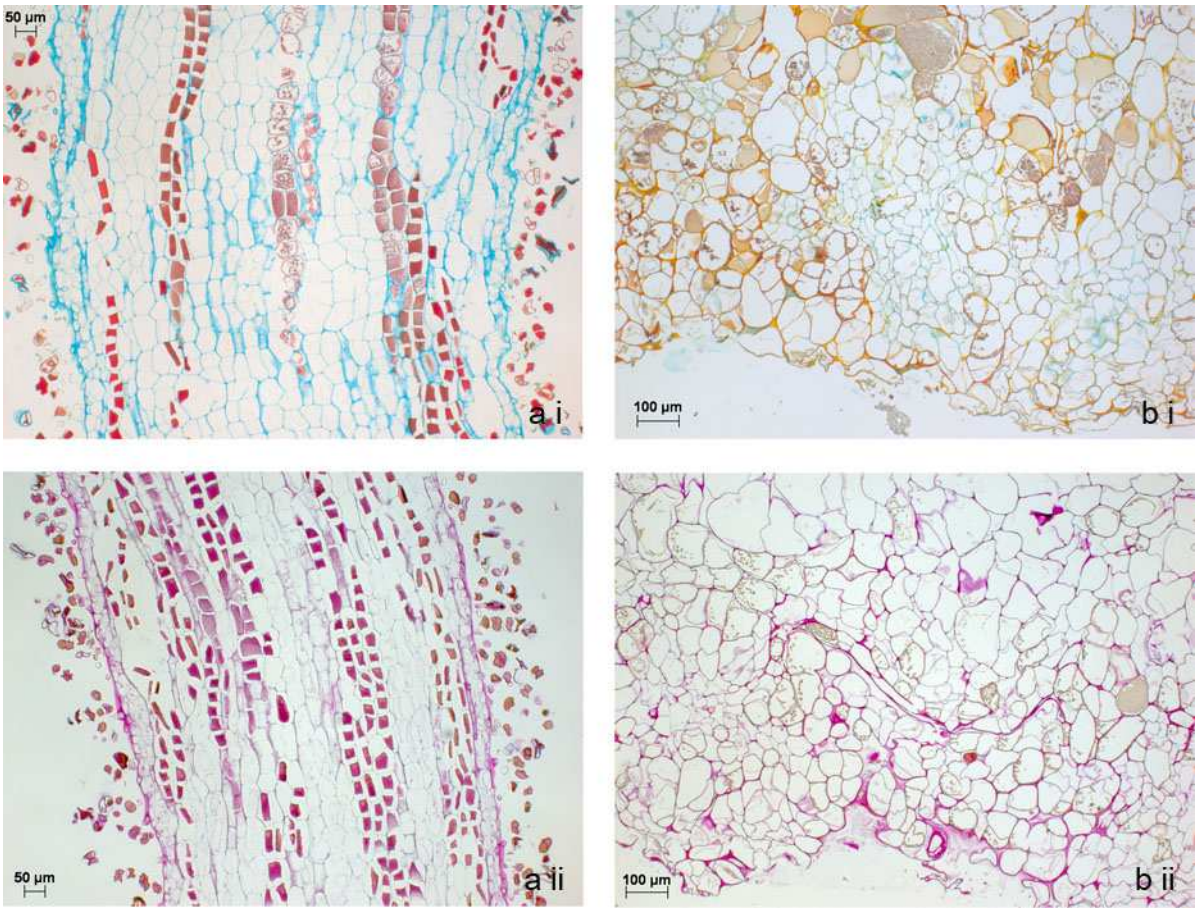
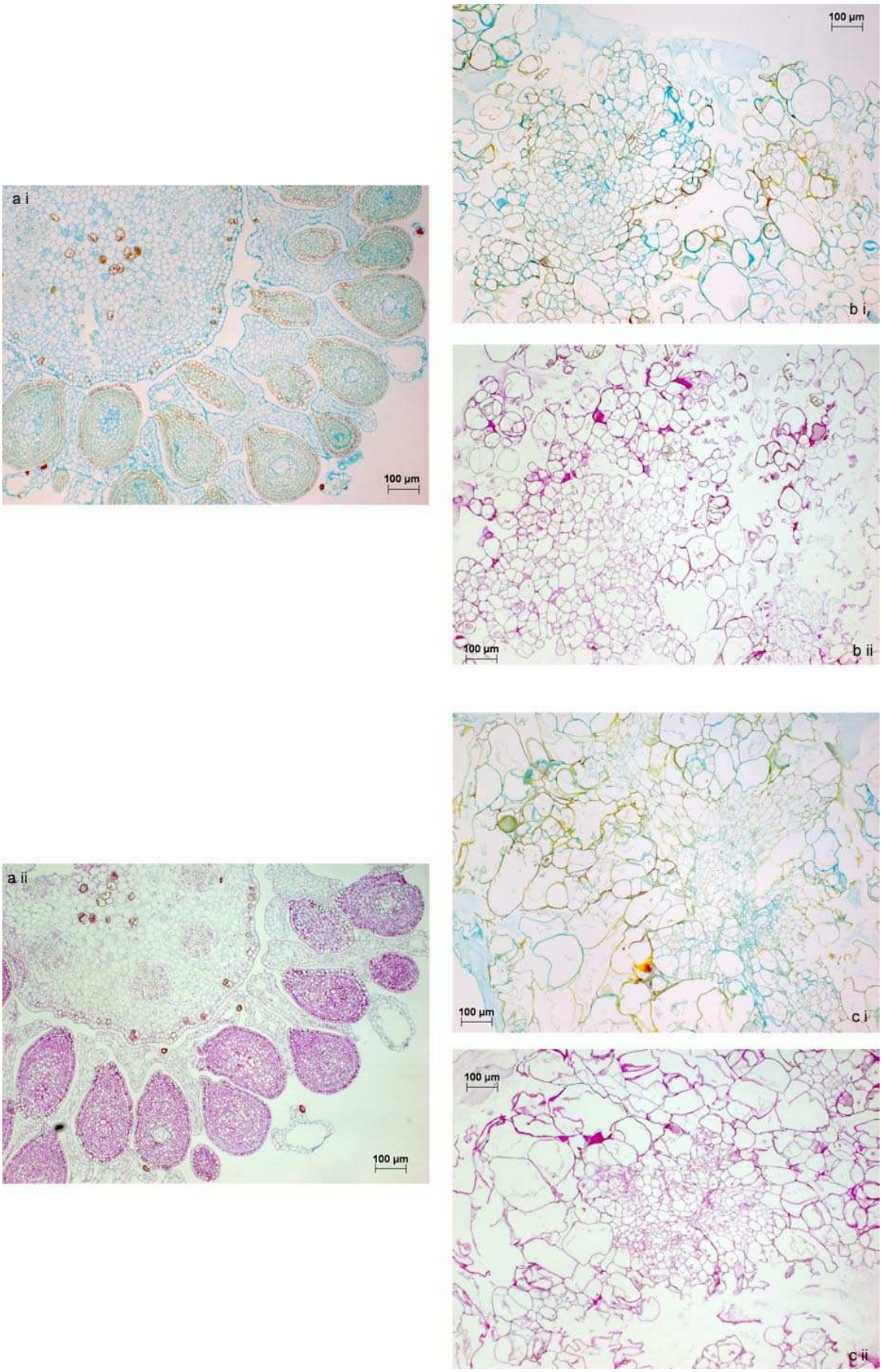


Fig. 7: Histological analysis of ovules and placenta (explant) (a) and corresponding callus of ovules (b) and placenta (c) stained with W3A (a i, b i and c i)) or with ruthenium red (a ii, bii and c ii) (genotype 3).



3. General Discussion

3.1 Methodology

3.1.1 Summary of results

The starting point for the microarray development was a preceding EST-study (Rensing et al. 2005). From this, 1,216 transcripts were chosen for further microarray analysis. The prior aim of this analysis was the generation of new hypotheses to optimise the present protocol for somatic embryogenesis (s.e.) in *Cyclamen persicum*.

In order to achieve this, a total of 21 experiments comprising 17 different tissues and covering a range of different conditions have been compared (Figure 1 in Section 2.1). Of these, eight experiments comparing ten different tissues were selected for further detailed analysis. Across the complete set, a total of 417 genes were revealed as being differentially expressed, whilst within the reduced set this was found to be the case in a total of 279 genes.

The identification of pathways was carried out by subjecting the achieved data to Gene Ontology (GO) annotations. There was further analysis of which GO terms were either significantly overrepresented or underrepresented among the 279 differentially expressed genes in comparison to the complete set of genes represented on the array (Figure 2 in Section 2.1).

On the basis of GO-annotations, as well as on single observations of genes in individual comparisons, a total of seven hypotheses have been deduced from the expression data for a putative improvement of s.e. in *Cyclamen* (Hoenemann et al. 2010). These include, amongst others, potential physiological markers such as pectin modifying enzymes, chitinase and POX activity with regard to their impact on cell adhesion or proper epidermis formation. Additionally, other putative indicators for determining embryogenic, or rather non-embryogenic callus, for example, AGO, GST or SERK could be identified. As has already been

preliminarily mentioned, it has been demonstrated that there is a high level of correlation between, especially SERK gene expression (Section 1.4.1), and s.e. Therefore, it is worth reiterating that out of a total of five genes that are homologues to genes annotated as SERK (Rensing et al. 2005), in this study, only one was found to be differentially expressed (Hoenemann et al. 2010). Also, as already noted, (Section 2.1, Hoenemann et al. 2010), POX has already been identified as having an essential role in the s.e. development of *D. carota* (Cordewener et al. 1991) and other authors can demonstrate the important role of GST in the regulation of s.e. of *Triticum aestivum* (Singla et al. 2007) and *Citrus sinensis* (Pan et al. 2006). In the cases of differential gene expression of GST, POX and chitinase homologues under different experimental conditions, hypotheses were formed focusing on aspects of the *in vitro* protocol (pH-control, media composition).

In order to examine the reliability of the microarray results, ten differentially expressed genes were chosen randomly and validated by qRT-PCR. As shown in Figure 3 in Section 2.1, nine of these were confirmed qualitatively while for eight, the results also corresponded quantitatively. Consequently, the microarray data were agreed to be reliable. The high reproducibility of the three independent biological replicates could be approved by the demonstration of a principal component analysis (PCA) (Figure 4 in Section 2.1).

This reproducibility could only be observed when using the probe material of the microarray experiment. When repeating parts of the experiment with the same cell line, several subcultures later, or even with a different cell line, the transcript abundances of genes of putative gluthatione S-transferases (Hoenemann et al., submitted in July 2011, Section 2.3.1) and putative pectin modifying enzymes (Hoenemann et al., submitted in October 2011 chapter Section 2.3.2) obtained by qRT-PCR diverged when compared to those achieved with the microarray.

Initially, the reference gene for normalisation of qRT-PCR was selected because microarray results showed stable expression values for this gene (*Ef-Tu*) in all tissues. Responding to the increasing number of publications recommending further examination of the reliability of reference genes, an optimisation of the initial protocol for normalisation was carried out. It has been demonstrated that

there are other and even better alternatives (see Figure 1 in Section 2.2). Moreover, it was shown that it is better to use more than one single reference gene.

3.1.2 Discussion and Conclusions

3.1.2.1 Microarrays

Microarrays have become widely accepted for gene expression analysis during recent years because they are an important tool able to generate information quickly. This method enables the presentation of thousands of genes of an organism in parallel (Wong and Chang 2005). They are mainly used for generating expression patterns:

- Where within the organism are all these genes expressed?
- In what way do these expression profiles influence the organism? (Murphy 2002)

Consequently, microarrays present a method for locating genes, gene regulation and identification of gene expression that defines disease states as well as assisting with research into drugs (Murphy 2002). However, the identification of biological relationships out of the mass of up and down regulated genes is usually challenging. The challenge is in interpreting which biological processes the alteration of expression of so many genes underlies and which of the analysed genes assume a key function (Wong and Chang, 2005). Thus, further bioinformatic strategies (e.g. Cluster, Gene Ontology) have been developed to provide tools that enable easier identification of regulatory networks.

In spite of all the benefits of microarray technology, there are also some drawbacks. Errors can occur due to:

- a lack of standardisation and reproducibility,
- variability of sources,
- sequence errors,
- degradation of RNA during isolation,

- insufficient computer-based tools,
- statistical problems,
- inadequate sensitivity,
- effects of probe length,
- cross-hybridisation and
- variability of outcome.

(Hinton et al. 2004; Kareem 2004; Lee et al. 2003)

It becomes apparent, therefore, that every stage in the progress of the experiment is susceptible to error and that this should be considered when interpreting the results. There have been particular developments in the optimisation of computer tools and statistics over recent years. Nevertheless, other methods should be explored for validating microarray data such as quantitative realtime-PCR (qRT-PCR).

In addition to the specified benefits and drawbacks of microarray technology in general, there are also some depending on the kind of microarray (cDNA- or oligo(nucleotid) microarray) being used. In cDNA-microarrays, each spot includes the cDNA clone of a known gene (PCR product). Hence, any sample can be used to spot on the array and therefore enables a flexible and customised design (Fält 2006; Murphy 2002). On the other hand, this also has the effect that cDNA-arrays never being as uniform as oligo arrays (Fält 2006). Furthermore, samples have to be synthesised, cleaned and stored, which can lead to errors (Murphy 2002). Moreover, this method is relatively accessible and cost effective (Murphy 2002). Because of their long length, cDNA microarrays allow a high level of specificity. By analysing this longer length, the likelihood of finding differences is enhanced and thus the differentiation of two transcripts is straightforward (Boutros 2006). However, cDNA arrays are unable to differentiate between transcripts which are very similar and are therefore less sensitive (Boutros 2006).

In contrast, oligo arrays are based on sequence information and thus no PCR product is necessary, which reduces the chances of samples being mixed up. Other major advantages of short oligonucleotide platforms such as the Affymetrix

arrays are, amongst others, that the manufacturing technique used tends to produce highly reproducible results and also the array-to-array variation is lower compared to cDNA microarrays (Boutros 2006). There is also some degree of internal replication because each feature is represented by multiple sequences (Boutros 2006). Nevertheless, oligo arrays present some disadvantages. It is necessary to revert to commercial manufacturer because special equipment is required. Hence, they are more expensive and customisation of the design is relatively restricted (Murphy 2002). Since the individual sequences are relatively short, it is possible to use them to interrogate the expression of small features (e.g. single exons, mutational status of specific SNPs) (Boutros 2006). Thus, they provide a finer resolution and therefore a higher sensitivity than cDNA microarrays (Boutros 2006). Even if the short length produces greater sensitivity, the sequences might be too short to provide sufficient specificity (Boutros 2006).

By comparing the two platforms it becomes apparent that both have their advantages as well as disadvantages and that these should be considered in the context of the questions to be answered and functions to be fulfilled. The starting point (e.g. whether sequence information is available or not) will be a crucial criterion for selecting the platform. Barrett and Kawasaki (2003) summarised that some authors (e.g. Li et al. 2002) expect better results with oligo microarrays but others (e.g. Yuen et al. 2002) prefer cDNA microarrays.

According to the Affymetrix homepage (http://www.affymetrix.com), GeneChips are available for various plants such as *Arabidopsis*, barley, *Citrus*, *Medicago*, cotton, maize, rice, soybean, tomato, sugar cane and wheat. In any case, these arrays are only available for relatively few organisms (Ophir et al. 2010). Other commercial manufacturers currently provide customised (e.g. Agilent and Nimblegen) oligo arrays (Ophir et al. 2010). These customised oligo arrays have great potential as a useful tool for other agricultural or horticultural crops, as demonstrated by Ophir et al. (2010) in their study on high throughput marker discovery in melons.

As only a fraction of the great volume of data produced by microarray experiments can be evaluated by a single workgroup, public repositories (e.g.

ArrayExpress or Gene Expression Omnibus (GEO)) were established to collect expression data. Therefore, strict standardisation of the experimental conditions is required. But – depending on the complexity of the biological system – not all experimental factors are amenable to standardisation. The Microarray Gene Expression Data Group (MGED group) addressed these problems and released relatively strict guidelines for the submission of microarray data for publication (Minimum Information About a Microarray Experiment (MIAME)) (Anonymous 2002; Brazma et al. 2001). Since that time, an increasing number of journals insist on compliance with these rules as well as on the release of the data to a repository such as ArrayExpress or GEO. The introduction of common standards for data input, annotation, information about the experimental design and data normalisation should assist with providing the resulting data in a comprehensible format (Mah et al. 2004). This will be extremely helpful in removing much of the confusion that occurs when attempting to interpret microarray data (Barrett and Kawasaki 2003).

Furthermore, the question is raised whether results generated from different platforms (oligo and cDNA) are comparable. Mah et al. (2004) evaluated whether the results obtained by using oligo and cDNA microarrays match with regard to the genes identified as expressed using RNA obtained from human colonic mucosa. Whilst the overlap of the results of the two platforms was just moderate with respect to the tagging of genes, the expression levels obtained for genes represented on both microarrays did not match at all (Mah et al. 2004). Differences between the two platforms in all stages of the experiment, from platform design, experimental conditions, spot quantitation, to the processing of data may have contributed to this divergence of the results (Mah et al. 2004). Mah et al. (2004) thus concluded that the platforms may be too different to be expected to give global expression results that can be directly correlated. Generally, even if the same platforms are compared, there might, nevertheless, be substantial differences between experiments such as, for example, slight and often unknown differences between materials and methods practiced in different laboratories (Mah et al. 2004). This conclusion is also supported by the results of this thesis. Initially, the data obtained from the cDNA microarray could be validated by qRT-PCR when using the same materials. Later, when repeating parts of the experiment with other probe materials, great discrepancies were

observed. This meant that when using the same cell line several subcultures later, or even with a different cell line, the transcript abundances of selected genes obtained by qRT-PCR diverged when compared to those achieved with the microarray.

Guidelines such as MIAME and repositories such as ArrayExpress or GEO guarantee that the presentation of microarray data is in a standard format that makes the design of microarray experiments comprehensible to other working groups (Mah et al. 2004). However, even when these tools are employed, they do not eliminate the difficulties with respect to reproducibility of results, as described above. Therefore, comparisons between different experiments have to be examined very critically.

3.1.2.2 Quantitative realtime-PCR (qRT-PCR)

In recent years it has become good experimental practice to validate the reliability and reproducibility of microarray experiments by quantitative realtime-PCR (qRT-PCR). qRT-PCR is an appropriate method for the validation of expression data because it is quantitative, fast and needs 1000-fold less RNA than other assays (Rajeevan et al. 2001). It has been estimated that large parts of expression changes observed with microarrays are often different when compared to those of other technologies (e.g. qRT-PCR) (Tarca et al. 2006). Generally, microarray data show reduced fold-changes in comparison to those derived from qRT-PCR (Tarca et al. 2006). Alike, Rajeevan et al. (2001) demonstrated differences in expression levels measured with qRT-PCR when compared to the microarray data: out of 14 validated and confirmed differentially expressed genes, 10 genes showed higher expression than in the microarray. In the comparison of qRT-PCR results to *Arabidopsis* Affymetrix chips, qRT-PCR proved to be much more sensitive (Czechowski et al. 2004). With the Affymetrix chip, transcript levels were estimated to be about 100 times lower compared to the qRT-PCR results. In addition, the array data were less accurate especially with respect to low expressed genes (Czechowski et al. 2004). In the study of Yuen et al. (2002), a commercial oligo as well as a custom cDNA microarray for the endocrine cell line LβT2 were compared with regard to measurement

accuracy. It could be demonstrated that both platforms underestimate the relative changes of mRNA expression when compared to qRT-PCR (Yuen et al. 2002). For cDNA microarrays the fold-changes were 250-fold less than the fold-changes observed with qRT-PCR (Yuen et al. 2002). Accordingly, microarrays are more suitable for measuring ratios than absolute expression levels (Tarca et al. 2006).

In particular where qRT-PCR is applied for validation of results obtained by other technologies and because of its sensitivity, it becomes apparent that special attention should be directed to accuracy and robustness. Therefore, the overall method, or rather the most decisive steps of the technique, should be critically analysed for possible improvements. One crucial factor is the choice of reference genes, because the stability of their expression is crucial to the outcome of the experiment. Initially in many studies (e.g. Chiappetta et al. 2009; Castellarin et al. 2007; Domoki et al. 2006), potential reference genes were selected on the basis of their predicted housekeeping function without further validation of their actual expression stability. Subsequently it became commonplace to establish only one reference gene for normalisation (Vandesompele et al. 2002). However, this practice increases the risk of obtaining biased or false results (Vandesompele et al. 2002; Brunner et al. 2004; Pfaffl et al. 2004) because it has been revealed that even the transcript abundances of housekeeping genes may vary in response to different experimental conditions (Nicot et al. 2005; Jain et al. 2006; Hong et al. 2008; Silveira et al. 2009). Hence, the only conclusion could be to validate the adequacy for every reference gene in each experiment. Furthermore, the results from a single reference gene should not be regarded as being reliable: during recent years, normalisation with multiple reference genes has become the accepted standard. This is especially apparent with regard to the great number of publications focusing on this subject, particularly over the last two to three years (e.g. Tong et al. 2009; Artico et al. 2010; Lee et al. 2010; Maroufi et al. 2010).

During the course of this research, the established qRT-PCR technology has been improved with respect to the application of reference genes. For validation of the microarray results (Hoenemann et al. 2010) (Section 2.1), as well as in the study about expression of putative glutathione S-transferase genes (Hoenemann et al., submitted in July 2011, Section 2.3.1), the normalisation of qRT-PCR was carried

out using only a single reference gene (*Ef-tu*). This one was selected because of its known suitability as a housekeeping gene and as it had displayed stable expression levels in the microarray experiments. It should be noted that, until then, stability of expression levels had not been proven by qRT-PCR. Because of the increasing number of publications introducing the use of normalisation factors based on multiple reference genes, this practice had been reconsidered. Vandesompele et al. (2002) recommended that potential reference genes should be validated specifically as they may be suitable under certain special conditions but this may not be transferable to other conditions and therefore should be validated individually. This has been borne out by our study (Hoenemann and Hohe 2001, Section 2.2), because of all the potential reference genes examined, *Ef-tu* proved to be the least reliable.

3.1.2.3 Complexity of the biological system

Another important methodological constraint of the studies in this thesis is also caused by the complexity of the biological system. This complexity can be regarded on several levels:

1. The complexity of the 'embryogenesis' developmental process itself
2. A multitude of variable factors in the microarray experiment
3. The instability of the system throughout the study.

The pattern formation in the early plant embryo have been analysed at the molecular level in several studies (e.g. De Smet et al. 2010; Peris et al. 2010; Tebbji et al. 2010). Peris et al. (2010) reviewed formative events during plant embryogenesis and discussed the molecular mechanisms regulating these processes focusing on *Arabidopsis*. De Smet et al. (2010) mentioned, amongst others, the important role of various phytohormones in embryogenesis but stressed in particular that their intricate cross-talk should be clarified in more detail. In general, the study of De Smet et al. (2010) brought the research a step forward by elucidating on some basic regulatory processes that control embryogenesis in *Arabidopsis*. Nevertheless, there are still many aspects left to investigate before the developmental and physiological mechanisms that control embryogenesis are better or even completely explained (De Smet et al. 2010).

Important developmental processes in this context are, for example, the establishment of polarity, specification and maintenance of cell identities, determination of the organismal axes and the control of orientation of cell division planes (De Smet et al. 2010). Although these studies focused on zygotic embryogenesis, the discussion about complexity is compatible with the current research.

A consolidated view of all the factors analysed indicates how complex the biological system was that has been dealt with in this study. A large number of different factors influenced the experiment, the most significant are:

- different levels of ploidy,
- different media compositions,
- various developmental stages,
- somatic as well as zygotic embryos and
- developmental aberrations (loss of the ability to develop torpedo-shaped embryos; loss of embryogenic competence).

This multitude of factors clearly reveals the basic reasons for the complexities. When drawing comparisons, it was observed that more than one factor had changed (e.g. ploidy and developmental aberrations).

Moreover, one has to take into account the instability of the system throughout the studies: a change of the ploidy level as well as the loss of the ability to develop torpedo-shaped embryos occurred spontaneously, just shortly before the beginning of the experiments. In addition, the embryogenic competence varied during the course of a study, as had been described in other systems as well. Changes in embryo shape have already been described by Hadfi et al. (1998) in zygotic *Brassica juncae* embryos and they concluded that this represented arrests in different auxin-regulated steps. In *Triticum aestivum* callus cultures, Jiménez and Bangerth (2001) observed a loss of embryogenic competence due to prolonged culture times that occurred concomitantly with a reduction in free IAA concentrations, comparable to concentrations detected in non-embryogenic callus. In *Coffea arabica,* Etienne and Bertrand (2003) observed increasing somaclonal variations with cell suspension age. Zhang et al. (2006) measured

the DNA-content of 35 citrus calli of different genotypes over a period of four years and demonstrated that 71.4 % of the genotypes showed a progressive increase in cells with varied DNA content but correlation analysis suggested that there was no significant correlation between the percentages of cells with varied DNA content and embryogenic competence. This result corresponds with the data presented by Borchert et al. (2007) in *Cyclamen*, demonstrating that also embryos with altered DNA content might have the ability to develop into adult plants.

Correspondingly, the complexity of this biological system impedes the reproducibility of results, especially if experiments are repeated with other genotypes months, or even years, later. Thus, it appears that it is not exceptional that even if the microarray could be reproduced with qRT-PCR when using the same samples, as shown in Hoenemann et al. (2010), this was not possible when repeating parts of the experiment with a different cell line or even with the same cell line only several subcultures later. The factors discussed probably contribute to the divergence of results obtained for analyses of transcript abundances of genes of putative gluthatione S-transferases (Hoenemann et al., submitted in July 2011, Section 2.3.1) and pectin modifying enzymes (Hoenemann et al., submitted in October 2011 Section 2.3.2) by qRT-PCR compared to those achieved with the microarray.

3.2 Suitability of the approach for *in vitro*-protocol optimisation

3.2.1 Summary of results

Two of the hypotheses for improving the process of s.e. in *Cyclamen* were analysed in more detail. As one of the major problems in the Cyclamen-s.e.-system concerns the development of non-embryogenic cell lines, genes that were differentially expressed in the comparison of embryogenic and non-embryogenic cell lines were chosen.

The first approach focused on glutathione S-transferases (GST) to evaluate the hypothesis that GST homologues might be crucial for early somatic embryo development. Accordingly, this hypothesis had to be validated by the screening of a different cell line and under different conditions. Special attention was paid to the responsiveness of the transcript abundances of five putative *GST* homologues to auxin regulation and abiotic stress during the transfer to a medium free of plant growth regulators (PGR). By presenting the experimental procedure, the flow chart shown in Figure 1 of Section 2.3.1 illustrates that various experimental conditions and dates of sampling were compared. However, this detailed analysis with a cell line other than that used in the array could not validate the initial observations of the microarray with regard to the responsiveness of the expression of putative *GST* homologues to auxin supplementation. Rather, it seems to be responsive to mechanical or drought stress caused by the subculture procedure.

The second approach concentrated on genes coding for pectin modifying enzymes to evaluate the hypothesis of an impact of pectin-mediated cell adhesion on the embryogenic competence of a cell line. Again, the detailed analysis of this hypothesis using newly established cell lines could not validate the original hypothesis. Already the evaluation of callus texture and embryogenic competence, as presented in Table 2 of Section 2.3.2, showed that the anticipated correlation between callus texture and embryogenic competence could not be proven. Likewise, gene expression analysis of four different pectin modifying enzymes (pectinacetylesterase, pectinesterase and two pectate lyases) using qRT-PCR did not confirm any correlation between transcript abundances of the genes of these pectin modifying enzymes and the embryogenic competence (see Figure 2 in Section 2.3.2). However, a correlation between the expression level of the putative gene of a pectinacetylesterase and callus texture had been revealed (Figures 4 and 5 in Section 2.3.2).

Both approaches to validating hypotheses on improving or better understanding the process of s.e. in *Cyclamen* failed with regard to proving the initial hypothesis. Largely, the microarray results could not be reproduced when repeating analogous experiments with different cell lines or even with the same cell line after additional subcultures. Thus, even if the microarray data enabled

the deduction of multiple hypotheses on possible aspects for improving the process of s.e. in *Cyclamen*, selected detailed approaches could not lead to an improvement in the existing protocol. Likewise, it was not possible to reveal substantial physiological interrelationships in the process of s.e. in cyclamen.

3.2.2 Discussion and conclusions

The major aim of the project was to improve the *in vitro* protocol through targeted protocol changes based on revealing the underlying physiological processes by expression profiling. In summary, this ambitious aim has not been achieved. Although several hypotheses for optimising the existing protocol have been generated (Hoenemann et al. 2010, Section 2.1), the two selected for validation could not be proven.

The complexity of the biological system has already been discussed and it is this crucial factor that limits the suitability of the expression profiling approach for *in vitro* protocol optimisation. In retrospect, the combination of so many variants was unfeasible, particularly because the number of putative genes covered by the microarray was comparatively small. Biological systems are generally complex and the reduction on single genes is accordingly problematic.

Grandpierre (2005) worked on an approach to shed light on the complexity of biological systems. Initially, it had been reflected if perhaps just simple physical rules control the appearance of such outstanding complexity that has perhaps not been discovered until now (Grandpierre 2005). Finally, it has to be assumed that biology cannot be explained through physics because its genetic, algorythmic and symbolic information content is much higher than that of physics (Grandpierre 2005). Nevertheless, an aim is to develop approaches that can evolve theoretical biology into a science that has an exactness almost matching that of physics (Grandpierre 2005).

Furthermore, the high level of complexity of the present system also determined the poor reproducibility when repeating parts of the experiments with other genotypes or even subcultures some time later. Indeed, the microarray

experiment included a multitude of different tissues but this involved mainly different kinds (e.g. somatic or zygotic embryo) and stages of tissues as well as different culture conditions (e.g. media composition or culture method) to cover a wide range of aspects of s.e. However, the number of different cell lines analysed by the microarray was relatively low and the impact of this became apparent when regarding the poor reproducibility of the results with other genotypes.

The relatively low number of genes covered by the microarray genes is mainly due to the available technical options when starting the study with EST sequencing in 2004. This is also the reason for being limited to protein coding genes that represent the smallest part of DNA. Only 3 to 5 % of RNA is mRNA, whilst most of the total cytoplasmic RNA is rRNA (Fält 2006). Furthermore, a group of non-coding RNAs have been identified: siRNA and miRNA that can bind on the complementary mRNA as antisense RNA and then cause degradadation of the RNA or translational repression (Fält 2006). The great impact of miRNAs and siRNAs in plants has already been assessed by Sunkar and Zhu (2007) in their review focusing on this subject in plants. Current studies increasingly analyse the expression of miRNA and siRNA (e.g. (Schmitz and Chu 2011; Song et al. 2011; Yang et al. 2011; Varkonyi-Gasic et al. 2010; Zanca et al. 2010). For example, one of these studies analyses a correlation of the expression of several miRNA families with the so-called cropping syndrome that compromises the harvest of *Rehmannia glutinosa* plants (Yang et al. 2011). Potential targets of the 32 differentially expressed miRNAs are mainly involved in transcription, plant development and signal transduction (Yang et al. 2011). Indeed, Song et al. (2011) have demonstrated that 82 % of the miRNA targets in their study were transcription factors (e.g. auxin-response, growth regulator factors) and that miRNA was the key regulator of these genes. It could be shown that miRNA abundances were organ specific and miRNAs regulate gene expression not only by mRNA cleavage but also by repression of translation (Song et al. 2011). Alike, Varkonyi-Gasic et al. (2010) were able to show differential expression of miRNAs when comparing different apple tissues. In addition, many miRNA families showed a line and species specificity (Varkonyi-Gasic et al. 2010).

The possible involvement of RNAi processes in the generation of embryogenic and non-embryogenic cell lines from identical explants and the loss of embryogenic competence have also been suggested in Section 2.1. When comparing embryogenic and non-embryogenic cell cultures, a putative *argonaute* homologue was significantly up-regulated in the non-embryogenic cell line.

3.3 Perspectives

Major drawbacks of this study were the relatively small number of genes covered by the microarray and being limited to protein coding genes. The relative new technologies – next-generation sequencing (NGS) – seem to have great potential for overcoming these drawbacks. These new technologies have been developed during the last five years (Shendure and Ji 2008). According to Eckardt (2009), first analyses of the transcriptome in *Arabidopsis* using NGS were performed by Meyers et al. (2004) and Lu et al. (2005). Advantageously, costs for sequencing are reduced by two orders of magnitude and results are obtained in just a few days that would have previously taken months to generate using the classical Sanger approach (Dechamps and Campbell 2010). These platforms have become accessible in high dimensions and enable individual analyses (Shendure and Ji 2008).

Even if various approaches for NGS exist that fundamentally differ, they do pursue similar strategies:

1. Shearing of DNA at random (via nebulisation or sonication)
2. Ligation of universal adapters at both ends of the DNA fragments
3. Immobilisation and amplification to generate clustered amplicons

(Deschamps and Campbell 2009).

Although the output is higher and costs are considerably reduced, the error rates of these techniques are (still) ten times higher than with Sanger sequencing (Deschamps and Campbell 2009). In addition, another difference compared to Sanger sequencing is the quality because DNA sequences produced by NGS are much shorter (Pop and Salzberg 2008). However, the massive output produces plenty of sequence data that could be used to screen for sequence errors and separate them from real variations (Hillier et al. 2008). The progress of these

new technologies took place extremely rapidly, including the development of robust protocols for the construction of libraries of data, new approaches for data analysis as well as continuously rethinking the designs of experiments (Shendure and Ji 2008). NGS were shown to have much potential for accelerating biological and biomedical research because an extensive analysis of genome, transcriptome and interactome is made possible through a systematic and well-priced approach (Shendure and Ji 2008). Direct sequencing methods such as NGS offer the potential for higher sensitivity (detection of more new and low-abundance transcripts) than conventional EST-sequencing or microarray analysis (Eckardt 2009).

The new technologies make possible the discovery of hitherto unknown DNA-methylations, contexts and levels of the methylation as well as the observation of local sequence effects caused by methylation (Lister et al. 2008). The most essential objective of functional genomics is the understanding of regulatory networks that control genome activity, promoting the development of organisms and responses to environmental influences more than identifying and revealing when and where genes are expressed (Eckhardt 2009). For this purpose, understanding the epigenome and "small regulatory RNAs" as components of the transcriptome are important (Eckhardt 2009). Direct sequencing methods have demonstrated their usefulness in exploring these regions of the genome (Eckhardt 2009). Thus, NGS allowed new insights into the identification, biogenesis and function of "smallRNA" in various plant and animal species (Eckhardt 2009). Through the simultaneous study of three interrelated phenomena (genomic distributions of methylcytosins, smRNAs and transcripts) in wild type plants and mutants defective in DNA methyltransferase or demethylase, Lister et al. (2008) could illuminate interactions existing between the localisation of smRNAs and DNA methylation in *Arabidopsis*. This led to the altered transcript abundances of hundreds of genes, transposons and not-annotated intergenic transcripts due to modifications of the DNA-methylation status (Lister et al. 2008). Analysis of the impact of DNA-methylation on the somatic embryogenesis of *Medicago* showed that treatment with a demethylation drug caused the loss of the regenerative capacity of an embryogenic line (Santos and Fevereiro 2002). Therefore, it was concluded that embryogenic competence was correlated with DNA-demethylation and that the formation of somatic

embryos depended on a certain level of DNA-methylation (Santos and Fevereiro 2002). According to their own account, Harismendy et al. (2010) presented the first study using NGS not only for analysing coding regions but also contiguous genomic intervals and could therewith discover DNA variants for two candidate genes. Even if the study by Harismendy et al. (2010) was not to focus on plant biology, but instead was applied to the human body mass index, their conclusions, such as discovering of variants will be helpful in advancing the level of sensitivity in genetic studies, can be transferred to other biological systems.

NGS offers much potential for drastically increasing the availibilty of sequence data, which is an interesting proposition, particularly for non-model plants where genome sequence information is absent (Desgagné-Penix et al. 2010). However, model plants such as *Arabidopsis thaliana* do not cover all plant genes. Hence, economically important species should be examined individually (Hsu et al. 2011). Mardis (2008) reviewed a subset of studies using different NGS-platforms to evaluate their potential. He summarized that early studies already demonstrated the power of these new methodologies, but time and ingenuity and associated limiting factors will prompt the modification of genetic technologies (Mardis 2008). Undoubtedly, microarrays and conventional sequencing will continue to contribute essential information, at least in the near future (Eckardt 2009). Through the new technology, more data, as well as new kinds of data are provided (Pop and Salzberg 2008). Eckardt (2009) summarized that studies such as that of Wang et al. (2009) using NGS for analysing the maize epigenome and its relationship to mRNA and smRNA transcriptomes has already demonstrated its usefulness. Further optimisation of costs and efficiency, as well as of the computer and bioinformatical analysis of the sequence data, will encourage the further promotion of this method (Eckardt 2009).

Besides broadening the number of genes analysed by NGS technologies, for example, also the precise localisation of gene expression might improve the insights gained by expression analyses. A traditional technique in this context is the use of reporter genes such as GUS, GFP or luciferase fused to the promoter of the gene of interest. By transformation of embryogenic cultures of *Arabidopsis thaliana* with *AtSERK1::GUS*, (Salaj et al. 2008) were able to report on the precise spatial localisation of the AtSERK1 gene expression during the early

stages of somatic embryogenesis. Kwaaitaal and de Vries (2007) analysed the GUS expression of *SERK1* in Arabidopsis too and were able to observe a tissue specific expression in the presence of 2,4-D. These examples show that these approaches represent useful tools for developing a better insight into the process because they are not only able to describe the expression level of a gene, but also enable the precise localisation. Clearly, this requires a functional transformation protocol. Another possibility for specifying the localisation of gene expression is by applying in-situ hybridisation. Malinowski et al. (2004) examined spatial and temporal expression patterns of Xyloglucan endotransglucosylase /hydrolase (XTH) during somatic embryogenesis in cucumbers with in-situ hybridisation to study their involvement in somatic embryo formation. Thus they were able to show the localisation of two XTH genes in different stages of cucumber somatic embryos and revealed that *Cs-XTH1* transcripts were largely accumulated within the presumed cotyledon primordia of somatic embryos (Malinowski et al. 2004). Using this method, Morcillo et al. (2007) were able to investigate the expression patterns of *EgAP2-1* (a transcription factor) in somatic and zygotic embryos of *Elaeis guineensis* (oil palm) in more detail. Thus, they revealed that *EgAP2-1* expression was concentrated in proliferating tissues (Morcillo et al. 2007). On sunflower immature zygotic embryos (IZE), either organogenesis or s.e. can be induced, depending on the culture conditions (Thomas et al. 2004). In their study, Thomas et al. (2004) compared the spatial expression of a sunflower *SERK* of s.e. and shoot organogenesis during the induction phase using qRT-PCR and in situ hybridisation. Thereby, it could be demonstrated that IZE cultured on the organogenic medium were still able to form somatic embryos when transferred onto the highly embryogenic medium as long as *SERK* transcripts were accumulated in their morphogenic zone (Thomas et al. 2004).

The combination of several methods and technologies was shown to be a beneficial approach for producing versatile genetic information. In their study on orchids, Hsu et al. (2011) used an approach that combined multiple tools (cDNA-microarray, BAC library and bombardment assay) and thus offered a fast, easy and comfortable strategy for generating useful genetic information. Transient transformation offers information about biological processes and is a helpful tool for identifying the subcellular localisation of proteins (Briesemeister et al. 2010).

Meanwhile, Desgagné-Penix (2010) applied an integration of NGS and enhanced LC-MS/MS analysis to establish a deep coverage of transcriptome and proteome in their study on the reaction of the opium poppy (source for several pharmaceutical benzylisoquinoline alkaloids such as sanguinarine) on the treatment with a fungal inductor. Through this, they provided an effective platform to catalogue the components of secondary metabolisms and to identify the genes coding uncharacterised enzymes and thus their approach supports a better integration between genes, enzymes and pathway components (Desgagné-Penix et al. 2010).

In order to understand cellular processes, it is necessary to integrate proteomic, gene expression and other biomolecular data (Fagan et al. 2007). Certainly, the linkage between the expression of a gene and its protein remains complicated because of post-transcriptional and translational regulations (Fagan et al. 2007). This also becomes apparent in the study by Griffin et al. (2002) which used an integrated genomic and proteomic approach in the yeast *Saccharomyces cerevisiae* growing under two different conditions. It was observed that, in many cases, the response measured at the mRNA level correlated with the response at the protein level (Griffin et al. 2002). Nevertheless, in other cases they found significant differences (Griffin et al. 2002).

The high complexity of the represented system may result in misinterpretations when focusing on a small number of cell lines or genotypes. This has become apparent when repeating parts of the experiment with another genotype or even the same cell line just a few subcultures later. By analysing a multitude of genotypes and cell lines, their gene expression can be examined in parallel. Thus, the reproducibility of gene expression under a certain aspect can be investigated in various genotypes in parallel. Through the discovery of similarities in all of the analysed genotypes, general mechanisms may be uncovered that regulate the process of s.e.

In conclusion, it can be summarised that the microarray data was useful for generating a number of hypotheses for optimising the s.e. protocol in cyclamen. However, results obtained in the microarray analysis could not be reproduced by subsequent detailed quantitative gene expression studies. Hence, these

hypotheses turned out to be unsuitable for providing approaches for improving protocols. However, the hypotheses generated using the microarray data that have not been analysed in more detail should not be altogether discarded. It is possible that one or another hypothesis might represent a useful basis for protocol improvement. However, because of the great complexity of this biological system, it seems more reasonable to investigate selected single aspects of the system with expanded genotypes as well as a greater number of genes. A broadening of genes can be enabled by the use of NGS technologies because much more sequence data will become available than was the case in the present approach. Furthermore, analysis of the precise localisation of gene expression might provide useful information. Last but not least, the integration of transcriptomics methods with proteomics approaches might result in producing more reliable data.

3.4 References

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4. Appendix

4.1 Acknowledgments

First of all, I would like to thank PD Dr. Annette Hohe for providing me the chance to do this research, her constant interest and excellent scientific support. Furthermore, I would like to thank Prof. Dr. Thomas Debener for the scientific discussion and supporting this external Ph. D. thesis.

I am also especially thankful to Prof. Dr. Stefan A. Rensing, Dr. Sandra Richardt, Dr. Daniel Lang and Andreas Zimmer at Freiburg University for the microarray developing and performance, their academic support as well as for the fruitful discussions on gene expression studies.

In addition, I am deeply grateful to Katja Krüger her skilled technical assitance as well as her continuous friendly encouragement in every circumstance. I am also thankful to Dr. Thomas Borchert and Dr. Yvonne Klopotek for their valuable interest in my research, scientific discussions and constant motivation. Moreover, I would like to thank the former B. Sc. Student Juliane Ambold for her valuable work that contributed essential parts to gene expression anaylsis of putative GST homologues. Of course, I am also thankful to every employee or student apprentice of the IGZ that is not mentioned in particular who participated and supported the investigations of this project.

My special thanks are extended to Prof. Dr. Traud Winkelmann, Prof. Dr. Hans-Peter Braun and Christina Rode for scientific exchange on *Cyclamen* somatic embryogenesis.

Finally, I am especially grateful to my family and friends, but foremost Mario who encouraged me in every imaginable way.

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Manuscripts (submitted)

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