Plant Science 201–202 (2013) 52–65

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Proteomic and histological analyses of endosperm development in *Cyclamen persicum* as a basis for optimization of somatic embryogenesis

Jenniffer Wamaitha Mwangi[a], Christina Rode[a], Frank Colditz[a], Christin Haase[a], Hans-Peter Braun[a], Traud Winkelmann[b,*]

[a] Institute of Plant Genetics, Leibniz Universitaet Hannover, Herrenhaeuser Str. 2, D-30419 Hannover, Germany
[b] Institute of Floriculture and Woody Plant Science, Leibniz Universitaet Hannover, Herrenhaeuser Str. 2, D-30419 Hannover, Germany

ARTICLE INFO

Article history:
Received 18 September 2012
Received in revised form
13 November 2012
Accepted 15 November 2012
Available online 28 November 2012

Key words:
Cyclamen persicum
Endosperm
Mass spectrometry
Proteomics
Seed development

ABSTRACT

The endosperm plays an important role for the development of zygotic embryos, while somatic embryos lack a seed coat and endosperm and often show physiological disorders. This study aims at elucidating the cellular and physiological processes within the endosperm of the ornamental species *Cyclamen persicum* Mill. Histological analyses were performed from 0 to 11 weeks after pollination (WAP). At 3 WAP, a syncytium was clearly visible with a globular zygotic embryo. From 4 WAP, cellularization of the endosperm, at 5 WAP a small torpedo shaped embryo, and from 7 WAP cell expansion was observed. By 11 WAP the endosperm appeared fully differentiated. Total soluble proteins were extracted from the endosperm at 4, 5, 7, 9 and 11 WAP and resolved using two dimensional isoelectric focussing/sodium dodecyl sulphate–polyacrylamide gel electrophoresis (2D IEF/SDS–PAGE). A shift from high-molecular-mass proteins to low-molecular-mass proteins during endosperm development was observed. A total of 1137 protein spots/gel were detected in the three protein fractions extracted at 7, 9 and 11 WAP. Mass spectrometry analysis of the 48 predominant protein spots in endosperm at 7, 9 and 11 WAP resulted in the identification of 62 proteins, ten of which were described for the first time in *Cyclamen*. Additionally, 186 proteins were identified using the *C. persicum* embryo proteome reference map. Proteins involved in abscisic acid signalling and oxidative stress responsive proteins were found to be important for seed development in *Cyclamen*. The new insights into endosperm physiology including storage compounds are discussed.

1. Introduction

Within many plant seeds, the endosperm functions mainly to provide nutrients to the embryo, thereby supporting its development and also its later germination [1–4]. In addition, the endosperm insulates the embryo from mechanical pressure imposed by the seed coat [5], and plays a role in signalling towards the embryo [4]. Recently, the function of the endosperm especially as an integrator of seed growth and development based on signalling between endosperm and embryo as well as mechanical barrier has been emphasized [6].

The endosperm, depending on the species, may be a transient tissue which is largely reabsorbed during late seed development e.g. in *Arabidopsis*, or it may be enlarged and persistent even upon seed maturity e.g. in *Cyclamen* and cereals. In the case of *Arabidopsis*, the cotyledon offers a comparable storage function as represented by the endosperm [3,4]. Endosperm development generally progresses through several characteristic stages, i.e. syncytium formation by several nuclear divisions, cellularization, growth and differentiation and finally maturation including accumulation of storage compounds, development of desiccation tolerance and dormancy [7]. Plants are able to accumulate carbohydrates [8], proteins [9] and fatty acids [10] as storage compounds in their endosperm. Therefore the endosperm functions as the storage organ and developmental control unit for the embryo and for the germinating seed [2,11].

Cyclamen persicum is a popular ornamental crop with high economic relevance. For commercial propagation, the F_1 hybrid cultivars are of predominant meaning. However, relatively high costs for seed production due to inbreeding depression of parent lines and intensive manual work are challenging problems. Therefore, there is an interest in an alternative vegetative propagation system. Somatic embryogenesis has been reported to represent an efficient in vitro propagation technique in *Cyclamen* [12–15]. However, somatic embryos often show physiological disorders,

Abbreviations: CBB, Coomassie Brilliant Blue; IEF, isoelectric focussing; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; 2D, two-dimensional.

* Corresponding author. Fax: +49 511 762 3608.
E-mail address: traud.winkelmann@zier.uni-hannover.de (T. Winkelmann).

0168-9452/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2012.11.004

asynchronous development and misshaping during development. In addition, unlike zygotic embryos, somatic embryos lack a seed coat and the endosperm. One marked difference being the availability of storage compounds i.e. carbohydrates, proteins and lipids in the endosperm.

For *Cyclamen* seeds, the storage polysaccharide xyloglucan [16,17], the storage proteins 11S and 7S globulin [17] and truncated forms of enolase proposed to function as storage proteins [18] have been reported. But so far, a profound knowledge about the components and physiological processes within the endosperm development of *Cyclamen* seeds is lacking.

Proteomic studies have been shown to be a powerful tool for monitoring the present physiological status of cells and tissues under specific developmental conditions [19] and during development [20,21] and have been performed successfully for seeds and seed compartments in many crops recently, e.g. in maize [22], coffee [23] or rice [24].

In this study, we aimed (i) to identify the key events during endosperm development at the cellular level by histological analyses, and (ii) to elucidate on the basis of alterations in the protein profiles of *C. persicum* endosperm during seed development the predominant role of the endosperm for the embryo development.

2. Materials and methods

2.1. Plant material

The diploid *C. persicum* commercial F_1 cultivar 'Maxora Light Purple' bred by the company Varinova (Berkel en Rodenrijs, Netherlands) was grown in the greenhouse at a heating set point of 17 °C and a ventilation set point of 20 °C. Closed flower buds were emasculated and self-pollinated after 48 h. Fruits were harvested weekly from 0 (before pollination) to 11 weeks after pollination (WAP). Three biological replications were collected for each stage.

2.2. Histological analyses

Histological analyses were performed from day 0 (before pollination) to 11 WAP. For each stage, histological analyses were repeated twice to ensure two biological and technical replications. The samples were fixed in FAA (formaldehyde acetic acid) solution containing 67% ethanol, 20% H_2O, 1.8% formaldehyde and 5% glacial acetic acid for at least 24 h and then stored at 4 °C. The tissues were dehydrated in a vacuum using graded alcohol series (70–96% ethanol and 100% isopropanol) and embedded in paraffin wax [25].

Sections of 3–8 μm were cut using a microtome and stained using FCA (Fuchsine Chrysoidine Astra blue, Morphisto, Germany) solution according to Hoenemann et al. [25] and Morphisto histology manual (www.morphisto.de). The slides were visualized using a light microscope (Carl Zeiss, Germany) and the photographs were taken using AxioCam MR3 and edited with the AxioVision software (Carl Zeiss, Germany).

2.3. Proteomic analyses

2.3.1. Phenolic protein extraction

Proteomic analyses were performed for tissue harvested at 4, 5, 7, 9 and 11 WAP. Three biological replicates were collected for each stage. The samples were prepared under a stereo microscope (Carl Zeiss, Germany) directly frozen in liquid nitrogen and stored at μ80 °C until the time of analysis.

Total proteins were extracted and precipitated according to Hurkman and Tanaka [26] protocol modified by Colditz et al. [27]. Whole seeds were used for proteome analyses at 4 and 5 WAP as the endosperm was still liquid (Fig. 1a and b). For 7, 9 and 11 WAP, endosperm and seed coat were collected (Fig. 1c). 60 mg (9 and 11 WAP) and 80 mg (4, 5, and 7 WAP) of endosperm tissue were pulverized in a bead mill and homogenized in extraction buffer (700 mM sucrose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, 2 mM PMSF and 2% (v/v) (-mercaptoethanol, pH 8.0). Saturated phenol (pH 6.6/7.9) was added to the samples and proteins were precipitated in the phenolic phase with 100 mM ammonium acetate in methanol at −20 °C overnight.

2.3.2. Two dimensional (2D) IEF/SDS–PAGE

For first dimension isoelectric focussing (IEF), immobilized dry strips (18 cm) with pH gradients 3–11 were rehydrated with protein samples in rehydration buffer (8 M urea, 2% (w/v) CHAPS, 100 mM DTT, 0.5% (v/v) IPG buffer). Isoelectric focussing was done using IPGphor system (GE Healthcare). IPG strips were equilibrated in equilibration solution I (30% (v/v) glycerol, 50 mM Tris–HCl pH 8.8, 6 M urea, 2% (w/v) SDS, a trace of bromophenol blue, 0.01 g DTT ml^{-1} (w/v)) and equilibration solution II (same compounds like equilibration solution I, but DTT substituted by 0.025 g iodoacetamide ml^{-1}).

For second dimension of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) IPG strips were fixed horizontally onto SDS–tricine–polyacrylamide gels of 12% acrylamide. Electrophoresis was carried out for 20 h at 30 mA mm^{-1} using Biorad Protean IIXL gel system (Biorad, München, Germany). Gels were subsequently stained overnight using colloidal Coomassie Brilliant Blue (CBB-250 G, Merck, Darmstadt, Germany) after treatment with the fixing solution (40% (v/v) methanol, 10% (v/v) acetate) for at least 2 h [28,29].

2.3.3. Quantitative gel evaluation

The gels were scanned on an ImageScanner III (GE Healthcare, Freiburg, Germany) and evaluated using Delta 2D software, version 4.0 (Decodon, Greifswald, Germany) with three replicates for each group (4, 5, 7, 9 and 11 WAP). Spots detection was done automatically and occasionally corrected manually. In gel normalization was performed using the Delta 2D software for the overlays of three replicate gels each. Spots with a relative spot volume of less than 0.005% were deleted. Significant changes in spot patterns of the different endosperm groups were determined using Student's *t*-test (confidence interval ≥95%) based on the relative spot volume.

2.4. Mass spectrometry

Protein spots were cut out from the 2D IEF/SDS gel using a manual spot picker (Genetix; spot diameter, 1.4 mm) and were assigned the unique spot number identifier according to Delta 2D software. Mass spectrometry analyses were carried out according to [30]. The gel pieces were de-stained by washing in water, dried under vacuum and dehydrated using acetonitrile and then incubated with 0.1 M NH_4HCO_3. Trypsin digestion (2 mg/ml resuspension buffer [Promega] in 0.1 M NH_4HCO_3) was performed at 37 °C overnight. The resulting tryptic peptides were extracted by incubation with 5% formic acid in 50% acetonitrile for 15 min at 37 °C. The supernatant was set aside and a second extraction with 100% acetonitrile was performed. Finally, the supernatant was combined with the first one. Extracted peptides were dried by vacuum centrifugation and stored at μ20 °C. The peptides were analysed via LC–MS/MS using a Proxeon Easy – nLC (pre-column: 100 μm diameter/2 cm length, main column: 75 μm diameter/10 cm length; Acclaim® PepMap, Germany) and a micrOTOF – QII ESI-MS/MS (Bruker Daltonics, Bremen, Germany). Spectra were generated using the "HyStar compass post processing" software (Bruker Daltonics, Bremen, Germany). Proteins were identified using the MASCOT search algorithm against the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov, RefSeq collection (Release 47, May 2011), plant sub-database),