

Theses of the Ph.D. dissertation

**Identification of genes associated with the induction of
embryogenic competence in leaf-protoplast-derived
alfalfa cells and characterization of the “Oxprot” gene**

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INTRODUCTION

Embryogenesis in plants is not restricted to the fertilized egg cell but can be naturally or artificially induced in many different cell types, including somatic cells. In all forms of plant embryogenesis certain criteria have to be fulfilled before initiation. The species or genotype has to possess the genetic potential to form embryos from somatic cells and one or a few cells of the plant/explant have to be competent to receive a signal (endogenous or exogenous) that triggers the pathway of embryogenic development (commitment) leading to embryo formation even in the absence of further signals.

Competent cells can respond to a variety of conditions by the initiation of embryogenic development. In general, these conditions include alterations in auxin (exogenous and/or endogenous) levels and evoke stress responses. It can be hypothesized that the initiation of somatic embryogenesis is a general response to a multitude of parallel signals (including auxin and stress factors). This response includes, in addition to cellular and physiological reorganization, the extended remodelling of the chromatin and a release of the embryogenic programme otherwise blocked in vegetative cells by chromatin-mediated gene silencing.

Alfalfa leaf protoplasts represent a rather homogenous and synchronized system that allows detailed investigation both at the single cell and the cell population level (Fehér et al. 2005). A further advantage of the system is that the development of the cells is dependent on the 2,4-D concentration: 1 μ M 2,4-D results in the formation of elongated vacuolated cells (non-embryogenic cell type, NE), while small, cytoplasmically rich, embryonic cells are formed at a tenfold higher concentration (embryogenic cell type, EM) (Dudits et al. 1991). This latter type of cells could develop into proembryogenic cell clusters and sequentially into somatic embryos under appropriate conditions.

Many tissue culture systems use 2,4-D as an efficient inducer of somatic embryogenesis. 2,4-D is a trigger of the cell fate switch, which fact is emphasized by experiment with special *Medicago* cell culture (microcallus suspension culture)

maintained in the presence of naphthylacetic acid (NAA) (Dudits et al. 1991; Györgyey et al. 1991). If a high concentration (100 μ M) of 2,4-D is applied to the cells for one hour before the transfer to hormone-free medium, the cells will develop into somatic embryos. First embryos can be observed on the surfaces of the calli 2-3 weeks following the treatment. These observations indicate that 2,4-D is required for the initiation of a programme that can further proceed on its own. Removal of 2,4-D from the induction medium can be important to allow the establishment of cellular polarity, which is one of the first cytological events underlying embryogenic development (Samaj et al. 2003).

The best characterized gene that is associated with embryogenic competence is the gene coding for the somatic embryogenesis receptor kinase (SERK) (Schmidt et al. 1997). Ectopic expression of the *AtSERK* gene could facilitate the formation of somatic embryos (Hecht et al. 2001). SERK expression is therefore widely used as a marker of embryogenic competence (Baudino et al. 2001; Nolan et al. 2003). Another embryo-specific gene which plays an important role in somatic embryogenesis is the one coding for the LEC1 transcriptional factor. Ectopic expression of LEC1 in transgenic plants induces the formation of somatic embryo-like structures on the surface of the leaves (Lotan et al. 1998).

We identified a gene associated with the induction of embryogenic competence in leaf-protoplast-derived alfalfa cells, that showed homology to the human oxidation resistance (*OXR1*) gene. This gene has the ability to suppress oxidative DNA damage if expressed in *Escherichia coli*. *OXR1* is an evolutionarily conserved gene as its homologues are present in many eukaryotic organisms from yeast to humans. To date, only little is known about its biological function. Deletion of the *OXR1* gene in *Saccharomyces cerevisiae* (scOXR1) results in sensitivity to hydrogen peroxide damage (Volkert et al. 2000). Human hOxr1 was capable to complement the hydrogen peroxide sensitivity of the yeast *oxr1_* mutant if it was targeted to the yeast mitochondria (Elliot et al. 2004).

RESEARCH OBJECTIVES

In order to reveal gene expression changes during the establishment of embryogenic competence, we aimed to compare alfalfa leaf protoplast derived embryogenic and non embryogenic cell types using a PCR-based cDNA subtraction approach. During the experiment our main goals were:

- to identify genes induced under the establishment of embryogenic competence
- to understand what the role of 2,4-D is during the induction of embryogenic development according to the gene expression profile

One of the genes identified during the above mentioned process was selected for further characterization on the basis of its potential function. We named this gene as MsOxprot, and refer to its Arabidopsis homologue as AtOxprot. In reference to these genes the aim of our experiments was:

- to discover whether proteins coded by MsOxprot and AtOxprot genes are capable to complement the peroxide sensitivity of the yeast *oxr1_* mutant strain
- to determine relative gene expression changes of Oxprot genes under different kinds of stress treatments
- to reveal the intracellular localization of the MsOXPROT protein
- to discover whether changes in gene expression result in phenotypical changes of transgenic or mutant plants

METHODS

- Plant and cell culture maintenance, stress induction
- Alfalfa leaf protoplast isolation and maintenance
- Plant mitochondria isolation
- Transgenic plant and cell culture generation
- Genetic engineering methods, GATEWAY technology

- RNA isolation, cDNA library construction, cDNA subtraction
- Real-time polymerase chain reaction (RTQ-PCR)
- Bacterial protein expression and purification, antibody production
- Plant protein purification, Western blot analysis, immunolocalization
- Yeast two hybrid system, protein-protein interaction test
- Fluorescence microscopy

RESULTS

1. Thirty-eight transcripts, up-regulated by 10 μ M 2,4-D, were identified during the induction of embryogenic competence in leaf-protoplast-derived alfalfa cells using a PCR-based cDNA subtraction approach.
2. The corresponding genes/proteins were annotated and 11 representatives of various functional groups were selected for more detailed gene expression analysis.
3. Gene expression patterns confirmed 2,4-D inducibility for all but one of the 11 investigated genes as well as for the positive control leafy cotyledon1 (MsLEC1) gene in 2,4-D-treated alfalfa leaves.
4. The gene expression profiles of selected genes were characterized during the early and late phases of somatic embryogenesis in a microcallus suspension culture system using real-time quantitative PCR.
5. Eight genes exhibited similar expression levels of the control actin gene (within one order of magnitude) indicating strong expression.
6. It was evidenced that alfalfa and Arabidopsis OXPROT proteins could complement the peroxide sensitivity of a yeast *oxr1_* mutant strain.
7. MsOxprot and AtOxprot genes were induced by different kind of stress treatments (Paraquat, Wounding, Heatstress, Drought, Hypoxia)
8. Mitochondrial localization of the MsOXPROT protein was revealed by Western blot analysis and immunofluorescence experiments.

9. In a yeast two-hybrid screen 18 potential interacting protein partners of the AtOXPROT protein were identified.
10. *Arabidopsis* T-DNA insertion mutant plants of the AtOxprot gene failed to show phenotypical differences under different stress conditions in comparison to the wild type plants.

DISCUSSION

Induction of somatic embryogenesis in protoplast-derived cells is a complex process that includes the recovery of cells from isolation stress, synthesis of a new cell wall, cell division and the acquisition of the embryogenic cell fate. It is supposed that increasing the 2,4-D concentration in the culture medium affected all of these parameters. This is supported by the identification of genes up regulated in response to the embryogenic 2,4-D concentration in the *Medicago* leaf protoplast-derived cells.

Thirty-eight 10 μ M 2,4-D up-regulated transcripts were identified during the induction of embryogenic competence in leaf-protoplast-derived alfalfa cells. The predicted proteins coded by the identified cDNA clones were classified into functional categories based on their sequence homology to *Arabidopsis* proteins. Eleven of the identified sequences representing various functional groups (stress-related; intracellular transport-related; translation-related; transcription/chromatin-related; hypothetical proteins) were selected for further gene expression analysis.

In order to verify the dependence of the up-regulated gene expression on 2,4-D, whole leaves were vacuum infiltrated and maintained in 1 or 10 μ M 2,4-D containing liquid medium for 4 days. All except one of the investigated genes exhibited the expected increased expression in response to the higher as compared to the lower 2,4-D concentration at the the fourth day in this experimental system. The delayed (four days) activation of the genes indicates that the genes do not

respond to 2,4-D directly, but their expression is increased as a consequence of the higher 2,4-D concentration-induced cellular changes.

In order to dissect the direct effect of 2,4-D on gene expression from the process of somatic embryogenesis, a time course experiment was carried out using a cell culture system where 1 h treatment with 100 μ M 2,4-D was followed by washing and culturing the cells in hormone-free medium for 6 weeks. In this system, somatic embryos appeared after 3 weeks of culture. Gene expression was studied during the establishment of embryogenic competence (first day) and during the appearance of somatic embryos (1–6 weeks). In response to the short 2,4-D pulse, six genes showed increased relative expression during the first day. Higher relative expression of the stress genes might be attributed to the stress-inducing effect of 2,4-D, the manipulation of the cultures and transferring the cells to hormone-free conditions. The high relative expression of the intracellular transport related genes may indicate an increase in endocytic processes.

Interestingly, some of the genes isolated as responsive for high 2,4-D concentration during the first day, exhibited also increased relative expression at the time of somatic embryo differentiation, 3–6 weeks after the 1 h 2,4-D treatment, under hormone-free conditions. The expression pattern of these genes may be related to the developmental transition of the cells (differentiated-to-dedifferentiated during the first day in the microcallus suspension or in the protoplast-derived cells, and dedifferentiated-to-differentiated at 3–6 weeks following the 2,4-D shock).

The obtained data underline the complexity of cellular processes during the transition of somatic cells to a dedifferentiated and embryogenic state. It is also revealed, that a short (1 h) 2,4-D treatment results in direct (within 1 day) and indirect (after 3 weeks) gene expression changes indicating that this treatment is sufficient to alter the developmental program of the cells. This is also well demonstrated by the expression of the LEC1 embryogenesis-associated transcription factor gene.

Although the expressions of the identified genes might be used as markers of certain stages during the acquisition of embryogenic competence and the formation of

somatic embryos, the verification of their functional significance during somatic embryogenesis requires further experimentation.

On the basis of its potential function we started to further characterize one of the genes (named as Oxprot) identified during the establishment of embryogenic competence. It was evidenced that alfalfa and *Arabidopsis* OXPROT proteins can complement the peroxide sensitivity of a yeast *oxr1_* mutant strain. Additionally, mitochondrial localization of MsOXPROT protein was confirmed by immunoblot and immunofluorescence experiments. These results support the claim that MsOXPROT and AtOXPROT proteins are functional homologues of the yeast and human Oxr1 proteins. Our findings suggest that both OXPROT proteins may be part of a mitochondrial stress response pathway. Since MsOXPROT and AtOXPROT proteins are capable to protect yeast cells from oxidative damage when localized to the mitochondria, it is likely that they play a similar role in oxidative stress resistance in plant cells as well.

Abiotic as well as biotic stress inducers have some common signaling and response pathways in plants and thereby have the potential to moderate the effect of each other through crosstalk (Hong-bo et al. 2006). Alfalfa and *Arabidopsis* Oxprot genes can be induced by various stress treatments (drought, wounding, paraquat, heatstress, hypoxia), therefore are excellent candidates to mediate this stress signaling crosstalk. Especially as we observed that stress induced activations of the Oxprot genes are transient, and therefore they may rather have a role in signal transduction than in ROS detoxification. We managed to identify 18 interaction partners of the AtOXPROT protein in a yeast two-hybrid screen. There were several stress-related genes among them, which fact further supports the potential role of the OXPROT protein in stress responses.

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