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Activation of embryogenic cell division in leaf protoplast-derived alfalfa cells: the role of auxin and stress

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ABSTRACT Leaf protoplast-derived cells of the embryogenic alfalfa genotype (*Medicago sativa* ssp. varia A2) follow different fate if cultured in the presence of 0.25, 1 or 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Cells grown in the presence of the highest auxin (2,4-D) concentration become embryogenic and can develop into somatic embryos if subcultured into fresh medium with the lower 2,4-D level. Cells cultured at the lower auxin concentrations from the beginning develop into elongated differentiated cells. In order to reveal physiological changes that characterize the reactivation of cell division in resting cells as well as the transition of somatic plant cells to an embryogenic state, morphological, cell division, intracellular pH and stress-related parameters have been determined during the first five days of parallel cultures at the above 2,4-D concentrations in combination with stress treatments.

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KEY WORDS

somatic embryogenesis
cell cycle reactivation
auxin
2,4-dichlorophenoxyacetic acid
stress
pH gradients

Plant ontogenesis has a remarkable plasticity with continuous post-embryogenic organogenesis during the entire life cycle. This is due to the presence of specific undifferentiated organ-forming cell files, called meristems, the activity of which is maintained, initiated or stopped by endogenous as well as environmental signals. An other characteristics of plant development is, that the differentiation of somatic cells is reversible. Under specific conditions, certain somatic plant cells are capable to form embryos following the regaining of cell division activity and totipotency through the developmental pathway of somatic embryogenesis.Revealing the key events related to the transition of differentiated plant cells to a totipotent and finally an embryogenic state can give us better insights into the cellular levels of regulation underlying the flexible developmental strategy of plants which rely on coordinated responses to complex environmental and endogenous ("hormonal") signals.

Materials and Methods

Leaf protoplasts have been isolated from axenically growing plantlets of the embryogenic alfalfa genotype *Medicago sativa* ssp. varia A2 as described elsewhere (Pasternak et al. 2000). Treatments have been applied to the protoplasts simply by supplying different 2,4-D concentrations or stress-inducing compounds to the culture medium from the beginning of culture (Pasternak et al. 2002). Intracellular pH and auxin measurements have been carried out as described by Pasternak et al. (2002). Hydrogen peroxide determination was done essentially according to Van Gestelen et al. (1998). APX (EC 1.11.1.11) activity was determined by monitoring the decrease in absorbance at 290 nm in 1 ml reaction mixture containing 50 mmol potassium phosphate buffer (pH 7.0), 0.5 mmol ascorbate, 0.1 mmol H_2O_2; 0.1 mmol EDTA and 10 μg protein (Rao et al. 1997). POD activity was measured spectrophotometrically as an increase in absorbance at 603 nm in a reaction mixture containing 20 mM Tris (pH 7.0), 2.6 mM benzidine 8 mM H_2O_2 and 1 μg protein. CAT (EC 1.11.1.6) activity was determined by monitoring the decomposition (consumption) of H_2O_2 in 1 ml reaction mixture containing 66 mM potassium phosphate buffer (pH 7.0) 12.5 mM H_2O_2 and 10 μg protein (Gosset et al. 1994).

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Results and Discussion

Studying the acquisition of embryogenic competence require a system with a well defined transition phase between somatic and embryogenic cell types in a single cell system. Leaf protoplast-derived cells of embryogenic genotypes of *Medicago sativa* L. (like the genotype A2 used in our laboratory) provide a usefull system to study the requirements for embryogenic competence (Bögre et al. 1990; Dudits et al. 1991). Growing in the presence of different concentrations of 2,4-D these cells will follow different developmental pathways. This allows the direct comparison of parallel cultures under embryogenic and non-embryogenic conditions at the single cell level in order to determine characteristics of competent and non-competent cells.

Acquisition of embryogenic competence largely rely on dedifferentiation when the existing developmental information must be stopped or altered in order to make the cells responsive for new signals. It is generally accepted that the reactivation of cell division in somatic plant cells is required for dedifferentiation (Nagata et al. 1994) and the establishement of embryogenic competence (Dudits et al. 1991, 1995; Yeung 1995). Auxin is considered to be the main plant hormone required for the activation of cell division in differentiated plant cells both *in vivo* and *in vitro*. One of the possible targets of auxin action in this respect is the induction of the expression of the cdc2 gene coding for the key regulatory protein kinase of the cell cycle. In alfalfa leaf protoplast-derived cells it was shown that auxin alone can result in the accumulation of this protein in high ammounts

but for the activation of the kinase the presence of cytokinin is required (Pasternak et al. 2000). These protoplast-derived cells enter the cell cycle faster under embryogenic than non-embryogenic conditions although it could not be correlated with increased activity of the cdc2 kinase. Under the same conditions, leaf protoplasts of embryogenic versus non-embryogenic genotypes of alfalfa have also been shown to divide faster by Bögre et al. (1990).

Auxin is considered to be the most important plant growth regulator in relation of cell division and differentiation as well as in the induction of somatic embryo-genesis. In this later process, 2,4- dichlorophenoxy-acetic acid (2,4-D) an auxin analogue also used as herbicide is especially effective. Many *in vitro* somatic embryogenesis sytems relay on the use of exogenous 2,4-D as an inducer. In alfalfa, cultures of dedifferentiated cells are initiated in the presence of 1-naphthylacetic acid (NAA) and a short 2,4-D shock (as short as one minute) is sufficient to induce embryo development under the following hormone-free conditions (Dudits et al. 1991). This indicates that 2,4-D has a specific signal role in the initiation of somatic embryogenesis unrelated to the induction of cell division. 2,4-D was shown to influence the endogenous auxin (indoleacetic acid, IAA) metabolism in carrot cells, which was hypothetised to affect somatic-embryogenic transition (Michalczuk et al. 1992). In alfalfa leaf protoplast-derived cells, endogenous indoleacetic acid (IAA) pools (both the free and conjugated forms) increase in response of 2,4-D (Pasternak et al. 2002).

More interestingly, similar effects on the endogenous IAA level could be seen when non-embryogenic cells have been cultured under stress conditions (iron stress) and this could be correlated with the characteristic morphological and physiological changes associated with embryogenic competence. Strong correlation could also be detected concerning the changes in the intracellular pH gradients caused by stress or high 2,4-D concentrations as compared to controls (Pasternak et al. 2001). Under embryogenic conditions the cytoplasmic as well as vacuolar pH increase at a higher degree during protoplast reactivation as compared to non-embryogenic conditions. It can be hypothetised that 2,4-D at relatively high concentrations (around 10 μM) acts at the same time as auxin (either itself and/or through endogenous IAA levels) and as a stressor.

This hypothesis is supported by the findings that in the presence of low, non-embryogenic 2,4-D concentration cell division and the formation of the embryogenic cell type is enhanced by different stress treatments like iron, copper, paraquat, menadion etc. Interestingly, however, the embryogenic cell type formed in response to these stressors releases significantly less H_2O_2 into the medium and possesses a much less activated stress-defence system as compared to differentiating cells growing in the presence of low 2,4-D concentrations only. Moreover, the inhibition of a putative plasmamembrane NADH-oxidase by DPI or removal of H_2O_2 by DMTU inhibits protoplast division at all 2,4-D concentrations. These data support a hypothesis that mild oxidative stress is parallel with and is required for the reactivation of leaf protoplast-derived cells but later it is suppressed by a non-enzymatic way if cells dedifferentiate and follow the embryogenic developmental pathway.

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