Background

During the last few years it became evidence that nitric oxide serves as a signaling molecule in plants such as in animals. The role of NO in orchestrating plant defense against biotic and abiotic stresses is probably the best established. However, there are accumulating pieces of experimental evidence that NO is also involved in the regulation of plant growth and development. Among others, NO can stimulate overall plant growth, root, hypocotyl and mesocotyl elongation and induce adventitious root development in various plant species. These observations indicate that NO may, directly or indirectly, affect cell elongation and division, the two basic processes of plant morphogenesis. There are no reports, however, about the involvement of NO in the activation and/or the progression of the cell division cycle in cultured plant cells.

Aim of the study

Our main goal was to establish the role of nitric oxide in the regulation of cell division and in the activation of somatic embriogenesis.

As experimental approach we used the unicellular system of alfalfa leaf protoplasts and a continuously dividing cell culture from the same genotype.

During our experiments the concentration of NO in the culture media was changed by exogenous application of NO donors, NO scavenger and an inhibitor of NOS.

Materials and methods

Plant technics:

Mesophyll protoplast isolation and cultivation from alfalfa (*Medicago sativa* ssp. varia A2)

Double phosphate starvation of an alfalfa cell culture (*Medicago sativa* ssp. varia A2)

Cytological technics:

Determination of the viability, morphology and cell division of the leaf protoplast derived cells

Determination of S phase frequency of the leaf protoplast derived cells by immunological detection of BrdU (bromodesoxyuridine)

Flow cytometry

In vivo NO detection by the NO sensitive fluorescent dye DAF2 DA (4,5-diaminofluoresceine diacetate)

Protein analysis:

Protein extraction and immunoblott

CDK kinase activity assay

Gene expression studies:

RNA isolation and quantitative RT-PCR analysis

Results and discussion

NO is required for and promotes the activation but not the progression of the cell division cycle in plants.

Individual cell-wall-less plant cells, the protoplasts, isolated from leaf tissues and cultured in synthetic liquid media are probably one of the best *in vitro* experimental models to study the activation of cell division in differentiated plant cells. Although they are isolated via stressful procedure, experimental

evidences demonstrate that properly handled protoplasts quickly recover in culture and respond to hormonal as well as stress signals as do intact plant cells.

As the NOS-inhibitor (L-NMMA), as well as the NO scavenger (PTIO) decreased and the NO-donor (SNP) could increase S-phase frequency as well as the division rate of alfalfa leaf protoplast-derived cells, it is very likely that NO is involved in the signaling cascade that leads to the dedifferentiation and division of leaf cells. That the observed effects indeed depend on NO, is strengthened by the observation that the L-NMMA-mediated inhibition of BrdU incorporation as well as the activity of the Medsa;CDKA1,2 kinase could be reverted by SNP application. SNP had a concentration dependent effect on the frequency of protoplast-derived cells entering the S-phase of the cell cycle. 1 and 10 μM SNP promoted while 100 μM inhibited DNA replication. Concentration dependent action of NO is well known in both plant and animal cells. Activation of sea urchin egg cells by NO-donors is possible only in a narrow concentration range and higher concentrations are even inhibitory of sperm cell mediated activation. High level of NO has been reported to inhibit whereas low levels to enhance leaf expansion and plant growth.

During the process of dedifferentiation, the loss of differentiated functions, recovery from isolation stress, cell wall synthesis and activation of the cell cycle machinery take place in a serial, although overlapping, manner in the protoplast-derived cells. It is not clear at what step NO may accelerate the process. Shifted pulses of L-NMMA application and subsequent measurements on BrdU incorporation and CDK activity indicated, however, that protoplast-derived cells older than three days are not responsive for the presence of the NOS inhibitor. It may indicate that an L-NMMA-sensitive step operates during the first three days of the alfalfa leaf protoplast culture and this step likely precedes the entry of the cells into the S-phase of the cell cycle.

In order to address this question, the expression of cyclin genes previously reported to be activated before the S-phase entry or even at the G0-to-G1

transition of cells, have been tested in the presence of SNP and L-NMMA. The protein level of a further D-type cyclin, the strongest interactor of the Medsa;CDK;A1,2 kinase in the yeast two-hybrid system, has also been determined.

Based on the results, specific inhibition of cyclin transcription or accumulation by L-NMMA could not be established. The increased level of both cyclins in response to L-NMMA at 48 hours might indicate that the treated cells had already entered into the division cycle. Interestingly, protoplast-derived cells cultured in the presence of L-NMMA also exhibited increased CDK activity and *SERK* gene expression at around the third days of culture. It is interesting to note that peaks of endogenous indoleacetic acid level and S-phase frequency could be detected at the second and third days of culture, respectively, and embryogenic competence is also established during this period in these protoplast-derived cells. It might be speculated that L-NMMA does not block but slows down cell activation that results in the synchroneous accumulation of the cells in the G1/S-phases. The synchrony seems to be lost after the S-phase as the cells at this stage become insensitive to the presence of L-NMMA. This may result in a transiently higher trancript/protein/activity levels of the investigated genes/proteins during a certain period. Similar response could be seen in the same type of cultures when cell activation was slowed down by the buffering of the medium pH.

To validate the above hypotheses as well as the determination of the exact timing and the nature of the L-NMMA-sensitive process(es) during the dedifferentiation and division of leaf cells require further experimentation.

In agreement with the above findings described for leaf protoplast-derived cells, L-NMMA did not inhibit cell cycle progression or growth rate of continuously dividing suspension-cultured cells, but delayed re-entry of these cells to the cell division cycle after phosphate starvation or after the subculture of eleven-days-old stationary phase cells. SNP at 10-100 μM concentrations had no effect on the cell cycle progression of suspension cultured alfalfa cells, although these

cells could respond to NO as indicated by 100 μM SNP-induced and PTIO-sensitive ferritin protein accumulation. This observation indicates that the absence of the cell cycle response was not due to a general insensitivity of suspension cultured cells towards NO. Although no response to SNP could be observed on phosphate starved or stationary phase cells, it may only be the consequence of the applied experimental conditions (e.g. very rapid re-entry of phosphate starved cells to the cell cycle) that did not allow a proper resolution of cell cycle events.

In contrast to the above observations with plant cells, in the case of animals NO is a well known cytostatic agent and a regulator of the balance between cell proliferation and cell differentiation during animal development. However, the site where the cell cycle is actually stopped in response to NO varies in different cell types. Our experiments revealed that SNP-generated NO did not block cell cycle progression in cultured alfalfa cells. It can not be excluded, however, that NO is involved in the regulation of cell division and differentiation in whole plants or organs.

The observations that neither dividing leaf protoplasts nor dividing suspension cultured cells are responsive to L-NMMA support a hypothesis that the activity of L-NMMA-sensitive plant enzymes is not required to sustain cell division activity in plant cells.

NO is involved in 2,4-D-induced embryogenic cell formation from alfalfa leaf protoplasts.

The presented data indicate that although nitric oxide is not required for and does not influence cell cycle progression in exponentially dividing cultured plant cells, it may interplay with auxins linking the regulation of cell division to differentiation. Plants exhibit a remarkable developmental plasticity as compared to animals. Somatic plant cells can regain cell division capability during a process termed as „de-differentiation” and „de-differentiated” plant

cells can „re-differentiate” into whole plants under suitable conditions. Our experiments with embryogenic leaf protoplast-derived cells support the view that NO in concert with auxin can play important roles during these transitions.

The altered response of protoplast-derived cells to exogenous auxin concentration in the presence of L-NMMA or SNP, and the observation that in the absence of auxin SNP could not promote the division of protoplast-derived cells, may indicate that NO alters auxin sensitivity of the cells and/or is involved in the mediation of auxin action during these processes. Auxin and NO have also been suggested to share common steps in signal transduction pathways leading to root elongation and adventitious root formation. In cucumber explants, indoleacetic acid (IAA) treatment induced the level of endogenous NO in the region where the new root meristems developed. The effect of NO on IAA-induced root formation was shown to be dependent on intracellular cGMP levels, although cGMP-indepent pathways may also exist. The downstream events of the putative NO-dependent signaling cascade leading to mitotic activation by auxin are unknown. Whether the same NO-affected signal transduction pathways operate during the formation of dividing, embryogenic cells from leaf protoplasts and adventious root meristem formation remains also a question to be answered.

In addition to the frequency of dividing cells, both L-NMMA and SNP affected the pathway of auxin concentration-dependent development of leaf protoplast-derived cells. It has been previously shown that these cells can develop either to vacuolized, elongated cells or to small, isodiametric cells with dense cytoplasm exhibiting embryogenic competence. These developmental pathways are dependent on exogenous auxin (2,4-D) concentration or oxidative stress-inducing agents. The application of the nitric oxide donor, SNP, resulted in the formation of embryogenic-type cells at a low (1 μM) 2,4-D concentration although these type of cells otherwise appear at higher auxin concentrations (5-10 μM 2,4-D). The high level expression of the *MsSERK1* gene and the further

development of the cells under culture conditions allowing somatic embryo formation verified that SNP application could indeed alter the developmental pathway of the auxin treated cells.

The *MsSERK1* gene is the alfalfa ortholog of the carrot and Arabidopis *SERK* genes implicated both in somatic and zygotic embryogenesis. *SERK* gene expression is frequently used as a marker of embryogenic competence although its elevated expression was also associated with auxin-induced root formation and was suggested to be rather a morphogenic than only an embryogenic marker.

The application of the NOS inhibitor L-NMMA, resulted in the vacuolization and elongation of the cells at the high (10 μM) 2,4-D level in the medium. Embryogenic cell formation, however, was not stopped, only delayed in the presence of L-NMMA as indicated by *SERK* gene expression and further development of the cells. This delay was very similar to the delay observed in the case of the entry of the protoplast-derived cells into the cell division cycle. It is interesting to note, that delayed cell division activity of the same type of cells by transiently altering the medium pH, also resulted in a parallel inhibition of embryogenic cell formation.

It is well established that 2,4-D induced acquisition of embryogenic competence is associated with endogenous IAA accumulation in various types of plant cells including alfalfa leaf protoplasts. Whether auxin (in this case 2,4-D) treatment is also associated with an increase in endogenous NO level, similarly as was observed during root meristem formation, remains an interesting question to be answered.

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