Abstract of Ph. D. thesis

In vitro regeneration system from Arabidopsis roots

Dóra Bernula

Supervisors:
Professor Dr. Attila Fehér
Head of the Department

Dr. Katalin Picherernné Gémes
Assistant professor

Biological Research Centre, Szeged
Institute of Plant Biology

University of Szeged
Ph. D. School in Biology

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Introduction

In recent decades, uncountable number of research articles reported successful *in vitro* regeneration of various plant species. Two main systems are widely used for *in vitro* plant regeneration: *de novo* organogenesis or somatic embryogenesis (SE). Both regeneration processes can occur either directly or indirectly without or with intermediate callus formation.

The success of regeneration is influenced by several factors, such as the presence of appropriate explant, environmental conditions and the proper composition of the medium. The importance of stress and plant hormones, primarily auxin and cytokinin, in plant regeneration processes have been described in several studies. While high auxin-to-cytokinin ratios promoted root regeneration, high cytokinin-to-auxin ratios resulted in shoot regeneration and high concentrations of both hormones resulted in overproliferation of explant cells and callus formation.

Somatic embryogenesis can be induced by a variety of hormone treatments, but the most prevalent inducer is the auxin. The effect of cytokinins on somatic embryogenesis is variable. In most systems, a low level of cytokinin is applied along with auxin to promote embryo induction while in other cases exogenous cytokinins were found to inhibit auxin-induced SE.

In recent years, a number of transcription factors have been identified that are involved in the process of embryo formation. The transcription factors LEAFY COTYLEDON 1 (LEC1), LEAFY COTYLEDON 2 (LEC2) and FUSCA 3 (FUS3) interact with each other to regulate the seed maturation, the identity of embryo and in some cases, the process of SE.

Wounding is also important in triggering the regenerative processes. One of the key regulators, which is rapidly activated in response to wounding is the WOUND-INDUCED DEDIFFERENTIATION 1 (WIND1) transcription factor. Wound and wound-induced WIND1 have been shown to play a central role in the increased regeneration potential of root explants.

Research on the model plant *Arabidopsis thaliana* has largely contributed to our understanding of many molecular and hormonal aspects of plant regeneration because various mutants, transgenic lines, marker lines, gene constructs, antibodies and specific information are available in large quantities. However, the opportunity to take advantage of them has so far been limited in SE research because there was no efficient and routinely used cell and tissue culture system that would have allowed this. Although the study of SE based on culturing Arabidopsis mature/immature zygotic embryos provided significant insight into this process, the use of other explants (preferably those containing more differentiated tissue than zygotic embryos) would be

useful to generalize the findings. In the early 1990s regeneration via SE or at least the appearance of somatic embryo-like structures has been reported as a result of works on Arabidopsis root cultures.

Research objectives

Although SE is widely used for *in vitro* plant propagation, the biological background of this plant-specific phenomenon is hardly known. Until now SE research in *Arabidopsis thaliana* was mostly limited to direct or indirect SE from immature/mature zygotic embryos due to the absence of a really efficient and easy system using more differentiated tissues.

In view of all this, our goal was to develop an efficient *in vitro* root-based regeneration experimental system simpler than the currently widespread system of zygotic embryo culture that provides sufficient quality and quantity of experimental material.

In our work we aim to:

- establish an efficient root-based *in vitro* regeneration experimental system that allows the induction of organogenesis and SE in the *Arabidopsis thaliana* model plant and the comparison of the two processes.
- To achieve this, our goal was to:
    - adapt the regeneration protocol described by Márton and Browse (1991);
    - characterize the Columbia (Col) ecotype in terms of SE/organogenesis competence and efficiency;
    - monitor the changes in cellular (microscopy) and molecular (RT-QPCR) levels in space and time during the induction phase of regeneration processes.

In addition, we wanted to examine:

- what may cause the difference between the roots of whole seedlings and the ability of root explants derived from seedlings to regenerate.

Materials and methods

Plant materials and culture conditions

Col wild type and heterozygous *leafy cotyledon 1* (*lec1*) mutant (*AT1G21970*) *Arabidopsis thaliana* (L.) seeds were provided by the Nottingham Arabidopsis Stock Centre.

To produce wild type seedlings, seeds were surface-sterilized with 70% ethanol for 60 sec followed by immersion in 4% commercial sodium hypochlorite solution (having 4.5% active chlorine) for 10 min. The seeds then were rinsed for 1 min five times with sterile water. 90 seeds were placed in each 120×120×17 mm square plastic Petri dishes containing full-strength Gamborg B5 Medium Including Vitamins, 1% sucrose and 1% plant agar. The pH of the medium was adjusted to 5.7. Petri dishes were placed horizontally for 1 day and fixed vertically by special holders for 6 days in a growth chamber at 21 ℃, under continuous light with an irradiance of 50 µmol m^{-2} s^{-1} provided by white fluorescent tubes.

Homozygous *lec1* seedlings were produced by culturing immature embryos on a medium containing half-strength Murashige and Skoog (MS) Medium Including Gamborg B5 Vitamins, 1% sucrose and 0.5% plant agar (pH=5.8). Germinated embryos exhibiting the *lec1* phenotype were selected and the seedlings were cultured under the same conditions as the wild type.

In vitro root-based regeneration system

7-days-old whole seedlings were placed onto a medium containing full-strength MS Basal Salt Mixture, 2 ml/l vitamix (555 mM myo-inositol, 14.8 mM thiamine-HCl, 2.4 mM pyridoxine–HCl, 4.1 mM nicotinic acid, 13.3 mM glycine, 0.2 mM biotin), 3% sucrose, 0.8% plant agar and 2.7 µM naphthaleneacetic acid (NAA) at pH 5.8 and cultured for 24 h vertically. Subsequently, each plantlets were transferred for three days to a hormone-free MS basal medium supplemented with 2 ml/l vitamix, 3% sucrose and 0.8% plant agar (pH 5.8).

To induce regeneration, roots were excised from the shoots at the junction of the shoot to the root with a scalpel under sterile conditions. Sixteen root explants were then cultured first on the ARM I medium (Arabidopsis regeneration media I; Márton and Browse 1991) including MS Basal Salt Mixture, 2 ml/l vitamix, 3% sucrose, 0.8% plant agar and different growth regulators, including 17.1 µM indole-3-acetic acid, 0.68 µM 2,4-dichlorophenoxyacetic acid, 2.68 µM 6-benzyladenine,

1.48 μM N^6-(2-isopentenyl)-adenine (2iP) for three days then on ARM IIr medium (Arabidopsis regeneration media IIr; Márton and Browse 1991), containing MS Basal Salt Mixture, 2 ml/l vitamix, 3% sucrose, 0.8% plant agar, 1.1 μM NAA, 19.7 μM 2iP. The pH was adjusted to 5.8 for both media. After four days, eight root explants were transferred to hormone-free medium containing MS salts, 2 ml/l vitamix, 3% sucrose, 0.8% plant agar (pH 5.8). Non-dissected whole seedlings were cultured in the same way and in the same number as controls.

To examine the effect of shoot-derived auxin on the regeneration processes, 5 μM auxin polar transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) was applied in a low gelling agarose droplet at the junction of the shoot to the root.

Samples were collected from the roots of untreated 7-days-old whole seedlings, from the root explants on ARM IIr medium between 1-4 days, each day, from roots of TIBA-treated whole seedlings, roots of untreated whole seedlings and from untreated root explants cultured for 5 days on hormone-free medium and cultured for 9 days continuously on the ARM IIr medium. The roots of untreated 7-days-old whole seedlings and the roots of untreated whole seedlings cultured for 5 days on hormone-free medium were used as reference. Three independent replicates were analysed in all experiments.

Applied experimental methods

- Light microscopy
- Scanning electron microscopy
- RNA isolation, cDNA synthesis
- Gene expression analyzes (real-time quantitative PCR, RT-QPCR)

Results

I. Experimental background

Regeneration via SE or at least the appearance of somatic embryo-like structures has been reported as a result of previous works on Arabidopsis root cultures. In the system of Márton and Browse (1991), embryo-like structures were formed after auxin (ARM I medium) and cytokinin

(ARM IIr medium) induction on the surface of Col root explants previously cultured in liquid culture.

However, during the adaptation of the system a different reaction was observed: root explants placed on ARM IIr medium after auxin induction initially showed intense root development and the appearance of green callus was observed subsequently instead of embryo formation. After 1 week, regenerates with trichomes appeared on the surface of the root explants. The presence (true leaf, shoot organogenesis) and the absence (cotyledon, SE) of the first leaf-like structures is often used as a morphological marker of shoot organogenesis and SE, we hypothesized that the regenerates were derived from indirect shoot organogenesis in our system.

Initially, liquid culture was used for establishing root cultures. However the age of the roots in this liquid culture was different and this negatively affected the efficiency and predictability of the system, furthermore, as there is an increased risk of various infections in these cultures, we started to the use only solid media.

II. Timely removal of cytokinin alters the regeneration pathway of Arabidopsis root explants

Morphological examinations by stereomicroscopy

The length of the period of cytokinin action required for the induction of shoot regeneration from Arabidopsis root explants was investigated. It was found that approximately 4 days were required on the high cytokinin medium to efficiently induce morphogenic foci on the root explants. Interestingly, transferring the root segments to hormone-free conditions after 4 days of cytokinin induction, callus growth was restricted and green globular structures appeared on the explants some of which developed into somatic embryo-like structures following ca. 6 more days of incubation. Additional 2 weeks on the hormone-free medium resulted in plantlet regeneration from these structures. Approximately half of the regenerated plantlets had trichome-less first leaves resembling cotyledons. Keeping the root explants continuously on the high cytokinin medium, however, we found, extensive callus formation. Culturing the explants for further 2 weeks on this medium, shoots appeared on the surface of explants. The regenerates had trichome-bearing first leaves indicating their formation via shoot organogenesis and not SE.

Gene expression analyzes

To verify whether the developmental pathway leading to trichome-less regenerates is indeed SE, the relative expression of three embryogenesis-associated genes (*LEC1*, *LEC2* and *FUS3*) was determined. It was established that culturing the cytokinin-induced root explants on hormone-free medium for 5 days resulted in the increased expression of all three investigated genes. The expression of the genes was not augmented in cultures maintained continuously on the cytokinin-rich medium strengthening that shoot organogenesis rather than SE was induced under that condition.

Next we examined the expression levels of *CUP-SHAPED COTYLEDON 1* (*CUC1*), *CUP-SHAPED COTYLEDON 2* (*CUC2*), *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*), *ENHANCER OF SHOOT REGENERATION 2* (*ESR2*) genes involved in shoot organogenesis and the relative transcript levels of *LEC1*, *LEC2* and *FUS3* marker genes involved in embryogenesis during the first 4 days of cytokinin induction. We found that while the expression levels of genes involved in shoot organogenesis were increased, the genes involved in embryogenesis showed decreased gene expression levels.

Investigation of *lec1* mutant

To further support the view that the transient cytokinin treatment indeed led to SE, root explants of the embryogenesis pathway defective *lec1* mutant was subjected to the same culture regime. It was found that culturing root explants continuously on the cytokinin-containing medium, the mutant reacted on the same way as the wild type. It formed green calli and subsequently shoots. However, if *lec1* root segments were moved to hormone-free medium after 4 days of cytokinin induction, the development of calli was restricted and the number of potentially morphogenic green foci was approximately the half as compared to the wild type. From the *lec1* roots on hormone-free medium, only shoots with trichome-bearing leaves could be regenerated.

III. Shoot-derived auxin prevents the regeneration from the roots of Arabidopsis seedlings

Morphological examinations by stereomicroscopy

Interestingly, the above described regeneration system was inefficient and no regenerates could be observed when whole seedlings were used instead of root explants. Roots of the whole seedlings that were cultured on high cytokinin medium thickened and became green but failed to regenerate calli and shoots. When the seedlings were removed to hormone-free medium after 4 days of the cytokinin treatment, the thickening and greening of the root did not take place. The potential role of shoot-derived auxin on the regeneration potential of the root was tested using the auxin transport inhibitor TIBA. 5 μM TIBA in a low gelling temperature agarose drop was applied to the shoot-to-root junction of seedlings before they were transferred to the high cytokinin medium. TIBA application restored the regeneration potential of the seedling roots. Like the excised root explants, the roots of TIBA-treated seedlings regenerated only trichome-bearing shoots on the high cytokinin medium. Approximately 50% of regenerated shoots were devoid of trichomes in case of transient (4 days) cytokinin treatment followed by hormone-free culture.

Gene expression analyzes

The trichome-less plantlets are likely the result of somatic embryogenesis. It is supported by the increased relative expression of the three embryogenesis markers (*LEC1*, *LEC2* and *FUS3*) in the TIBA-treated whole seedling roots indicating that inhibition of the transport of shoot-derived auxin to the root is sufficient to establish regenerative capacity in the roots of the seedlings and that some of the regenerates formed are the result of SE. Wounding and the wound-induced expression of the WIND1 transcription factor are considered to have a central role in the increased regeneration potential of root explants as compared to the roots of intact seedlings. The roots of intact seedlings, as we have shown above, do not regenerate shoots after auxin and cytokinin induction in our system. Overproduction of WIND1 transcription factor in the roots of intact seedlings also allows the induction of regeneration processes, similar to root explants. Therefore, the expression of *WIND1* in the root explants, roots of TIBA-treated and untreated whole seedlings was investigated. Similar to wounding (root explants), blocking the shoot-to-root auxin transport by a TIBA-

containing agarose droplet resulted in increased *WIND1* expression in the roots of whole seedlings in agreement with the increased regeneration potential. Our observation indicates that either the removal of the shoot as the auxin source or blocking the shoot-to-root auxin transport result in increased *WIND1* expression and enhanced competence for callus/shoot/embryo regeneration under appropriate inductive conditions.

Investigation of *lec1* mutant

In our system whole seedlings of *lec1* mutant without TIBA-treatment reacted on the same way as the wild type, no regeneration could be observed. The roots of TIBA-treated *lec1* seedlings regenerated only plants with trichomes on their leaves on ARM IIr medium and after transient cytokinin induction on hormone-free medium. The regeneration of TIBA-treated *lec1* seedlings did not result in trichome-less plantlets.

Summary

Our aim was to establish an *in vitro* root-culture-based regeneration system to study the initiation steps of organogenesis and somatic embryogenesis in *Arabidopsis thaliana*. Whole seedlings or root explants were used in this system. To induce regeneration processes, low auxin concentration followed by a strong temporal cytokinin treatment was used. In a possible scenario, low auxin concentration induces the formation of lateral root primordia that starts to transdifferentiate into shoot meristem in the presence of cytokinin. If the root explants were kept on the medium containing cytokinin, organogenesis occurred. However, if the root explants were transferred onto a hormone-free medium at a right time (after 4 days), approximately half of the regenerated plantlets were formed through somatic embryogenesis.

The process of somatic embryogenesis was verified by the absence of trichomes on the first leaves (which is a morphological marker of somatic embryogenesis) and the enhanced expression level of three embryogenic marker genes (*LEC1*, *LEC2*, *FUS3*). Furthermore, culturing root explants of the embryogenesis-pathway-defective *lec1* mutant under the same culture regime only shoot organogenesis could be observed.

Based on our results, we hypothesize that timely removal of exogenously applied cytokinin is a very important factor in determining the regeneration pathway. Our hypothesis is that shoot regeneration is induced initially and after removal of cytokinin, the shoot primordia

transdifferentiate into embryonic-like structures, but since the development of the shoot primordia is not synchronized, not all of them is competent for the transdifferentiation.

Interestingly, the above described regeneration system was inefficient when whole seedlings were used instead of root explants. Roots of whole seedlings cultured on high cytokinin medium thickened and became green but failed to regenerate calli and shoots. When the seedlings were removed to hormone-free medium after four-days cytokinin induction, even the thickening and greening of the root did not take place.

Root explants exhibit a high regeneration potential in contrast to the roots of whole seedlings. Therefore our attention turned to the role of wounding and the auxin transport from the shoot to the root on the regeneration processes. The potential role of shoot-derived auxin on the regeneration potential of the root was tested using the auxin transport inhibitor TIBA. 5 µM TIBA in a low gelling temperature agarose drop was applied to the shoot-to-root junction of seedlings. TIBA application restored the regeneration potential of the seedling roots cultured on hormone-free medium, and nearly half of the regenerated plantlets formed through somatic embryogenesis.

Wounding and the wound-induced expression of the WIND1 transcription factor are considered to have a central role in the increased regeneration potential of root explants (Iwase et al., 2015). In our experimental system, both the removal of the shoot as an auxin source and the blocking of auxin transport from the shoot to the root by TIBA treatment resulted in increased *WIND1* expression in the roots of whole seedlings in agreement with the increased competence for callus/shoot/embryo formation under appropriate inductive conditions. Thus, the effect of wounding on cytokinin-mediated shoot/embryo regeneration from roots could be mimicked by blocking the endogenous auxin transport from the shoot apex. The auxin transport from the shoot somehow reduces the regeneration ability and the cytokinin response of roots of intact plants.

The obtained results could help to better understand the regulatory background of plant regeneration processes making possible to set up more effective regeneration systems even in the case of economically relevant plant species.

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Reference

Márton, L., Browse, J. (1991). Facile transformation of Arabidopsis. Plant Cell Rep, 10(5):235-239.

Publications

MTMT: 10051208

Article related to this Ph.D. thesis:

Bernula, D., Benkő, P., Kaszler, N., Domonkos, I., Valkai, I., Szőllősi, R., Ferenc, G., Ayaydin, F., Fehér, A., Gémes, K. (2020). Timely removal of exogenous cytokinin and the prevention of auxin transport from the shoot to the root affect the regeneration potential of Arabidopsis roots. Plant Cell Tiss Organ Cult, 140(2):327-339. doi: 10.1007/s11240-019-01730-3. IF(2018): 2.200

Other articles:

Barna, B., Gémes, K., Domoki, M., **Bernula, D.**, Ferenc, G., Bálint, B., Nagy, I., Fehér, A. (2018). Arabidopsis NAP-related proteins (NRPs) contribute to the coordination of plant growth, developmental rate, and age-related pathogen resistance under short days. Plant Sci, 267:124-134. doi:10.1016/j.plantsci.2017.11.006. PMID: 29362091 IF(2018): 3.785

Ördög, A., **Bernula, D.**, Wodala, B. (2014). The effect of xanthan gum as an elicitor on guard cell function and photosynthesis in *Vicia faba*. Acta Biologica Szegediensis, 58(1):21-26.

Book chapter:

Fehér, A., **Bernula, D.**, Gémes, K. (2016). The Many Ways of Somatic Embryo Initiation. In: Loyola-Vargas VM, Ochoa-Alejo N (szerk.) Somatic Embryogenesis: Fundamental Aspects and Applications. Dordrecht, Hollandia: Springer, (2016) pp: 23-37. (ISBN: 978-3-319-33704-3)

Conference posters:

Borák, N., Ördög, A., **Bernula, D.**, Wodala, B. (2014). Photosynthetic activity of poplar lines under copper stress. 11th Congress of the Hungarian Society of Plant Biology, 27-29 August, 2014, Szeged, Biological Research Centre. BOOK OF ABSTRACTS, pp: 68.

Bernula, D., Fehér, A., Gémes, K. (2016). *In Vitro* Somatic Embryogenesis on Arabidopsis Root Explants. VISCEA International Conference – „Plant Cells *in Vitro*: Theory and Practice", 8-9 February, 2016, Vienna. Programme and Abstracts, pp: 36.

Bernula, D., Fehér, A., Gémes, K. (2016). *In Vitro* Somatic Embryogenesis on Arabidopsis Root Explants. National Conference of Young Biotechnologists, 21-22 March, 2016, Gödöllő, Szent István University. Book of Abstracts, pp: 84.

Bernula, D., Gémes, K., Benkő, P., Domonkos, I., Ferenc, G., Ayaydin, F., Fehér, A. (2017). Inhibitory effect of auxin on somatic embryogenesis from Arabidopsis roots. 12th Congress of the Hungarian Society of Plant Biology, 30 August – 1 September, 2017, Szeged, Biological Research Centre. BOOK OF ABSTRACTS, pp: 42. (ISBN: 978-963-12-9736-2)

Bernula, D., Gémes, K., Benkő, P., Domonkos, I., Ferenc, G., Ayaydin, F., Fehér, A. (2017). How to induce somatic embryogenesis in Arabidopsis roots? STRAUB-DAYS, 24-25 May, 2017, Szeged, Biological Research Centre.

Benkő, P., **Bernula, D.**, Domonkos, I., Fehér, A., Gémes, K. (2018). Inhibitory effect of auxin on plant regeneration from cytokinin-induced Arabidopsis roots. STRAUB-DAYS, 10-11 May, 2018, Szeged, Biological Research Centre.

Benkő, P., **Bernula, D.**, Domonkos, I., Fehér, A., Gémes, K. (2018). *In vitro* somatic embryogenesis on Arabidopsis root explants. I. The role of hormones. 3rd National Conference of Young Biotechnologists, 28-29 March, 2018, Budapest, Eötvös Loránd University, Faculty of Science. Book of Abstracts, pp: 111.

Kaszler, N., **Bernula, D.**, Szepesi, Á., Fehér, A., Gémes, K. (2018). Role of polyamines during plant regeneration of *Arabidospsis thaliana*. STRAUB-DAYS, 10-11 May, 2018, Szeged, Biological Research Centre.

Kaszler, N., **Bernula, D.**, Szepesi, Á., Fehér, A., Gémes, K. (2018). *In vitro* plant regeneration from Arabidopsis root explants. II. The effect of polyamines. 3rd National Conference of Young Biotechnologists, 28-29 March, 2018, Budapest, Eötvös Loránd University, Faculty of Science. Book of Abstracts, pp: 121.

Benkő, P., Fehér, A., Gémes, K., **Bernula, D.** (2019). Somatic embryogenesis: inhibitory effect of shoot derived auxin on cytokinin-induced Arabidopsis roots. VISCEA International Conference – „Plant Cells & Tissues *in Vitro*", 1-2 July, 2019, Vienna. Programme and Abstracts, pp: 27.

Kaszler, N., **Bernula, D**., Szepesi, Á., Fehér, A., Gémes, K. (2019). Polyamines affected regeneration processes in Arabidopsis. VISCEA International Conference – „Plant Cells & Tissues *in Vitro*", 1-2 July, 2019, Vienna. Programme and Abstracts, pp: 26.

Other scientific publications:

Wodala, B., Borák, N., **Bernula, D.**, Ördög, A. (2013). Impact of PEG-induced osmotic stress on the photosynthesis of three Poplar lines studied by gas-exchange, chlorophyll fluorescence and P700 absorbance. HUSRB/1002/214/036"OXIT", Characterization and oxidative stress tolerance in plants: from models to trees, BOOK OF FINAL REPORT, pp: 24-37.

Wodala, B., Borák, N., **Bernula, D.**, Ördög, A. (2013). Impact of zinc and copper stress on the photosynthesis of three Poplar lines studied by gas-exchange, chlorophyll fluorescence and P700 absorbance. HUSRB/1002/214/036"OXIT", Characterization and oxidative stress tolerance in plants: from models to trees, BOOK OF FINAL REPORT, pp: 38-49.