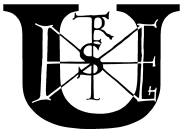

SZENT ISTVÁN UNIVERSITY

Tissue culture and genetic transformation of *Craterostigma plantagineum* Hochst. and *Ramonda myconi* Reichb. – important model plants of desiccation tolerance studies

Synopsis of the PhD thesis of

SÁNDOR TÓTH

Gödöllő

2009

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INTRODUCTION AND OBJECTIVES

The world's population is continuously growing, while cultivated land surface is more likely to decrease. One of its reasons is the greenhouse effect, which lately has become more intensive. It causes extreme weather in many countries of temperate climate as well, often resulting in drought lasting for years. Its equilibration with irrigation is becoming unfortunately more and more difficult, because the earth is running out of fresh water supplies and the available resources are rather used as drinking water. If plants able to tolerate negative stress resulting from an increased water shortage could be cultivated without any significant decrease in the yield, the tension resulting from the more intense food demand and the decreasing surface of cultivation could be mitigated.

Through physiologic and morphologic changes, plants cultivated today are trying too to avoid damages caused by water deficit, as a result of which evaporation through the leaves is significantly decreased. The most drastic and most frequent reaction is when the plant gets rid of a part of its leaves. As a result of strong sunshine and high temperature, the stomata of the remaining leaves stay closed and non-water permeable wax is more intensively secreted to the surface of the leaves. Plants sensible to water deficit operate these basic physiologic mechanisms as well, but they can only survive a 10-15 % decrease of their water content. In case of a more severe water shortage, they die.

However, a special group of the flowering plants is able to tolerate an extreme level of water loss as well. This group is made up of the desiccation tolerant plants. The fresh mass of desiccation tolerant plants is made of 70 % water, but if water supply is cut, they are able to lose 90 % of it and after 48 hours following rehydration, they reach their original metabolic activity again. Naturally, a much efficient protection mechanism is needed to survive such water shortage compared to plants sensible to water deficit. This also means that it is much easier to find the different elements of this protection mechanism and to explore the whole protection process. Thus, it is not surprising that the popular representatives of this group of plants, *Craterostigma plantagineum* and *Ramonda myconi* has become molecular genetic and biochemical models of drought resistance experimentation. During the past few years, a number of genes and genetic control elements (transcription activators, promoters and enhancer elements) have been isolated from them which have got a specific activity during dehydration and rehydration. Functional analysis of isolated genes and the exploration of regulation relations still remain to be done. One of its reasons is that the genetic transformation system of these plants is not yet

known. Our objective is therefore to elaborate the transformation method of *C. plantagineum* and *R. myconi*.

MATERIALS AND METHODS

Plant materials

Craterostigma plantagineum Hochst. plants originally collected from the wild in Namibia had been supplied to us by Prof. Dorothea Bartels, Max Planck Institute, Cologne, Germany. In order to carry out micropropagation, the surface-sterilised leaves were placed onto growth regulator-free media in plastic Green Box containers (Duchefa Biochemie) and transferred to a growth chamber maintained at 23±1°C and a 16/8-h (light/dark) regime (light intensity: approximately 4,500 lx). The micropropagation medium (MP medium) contained MS macro- and microelements (Murashige and Skoog 1962), 100 mg/l myo-inositol, 1 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 30 g/l sucrose and 8 g/l plant agar. The pH was adjusted to 5.8 using 1 *M* KOH prior to autoclaving. After 20 minutes of autoclaving, a buffered glutathione solution (1 l medium, 10 ml of potassium phosphate buffer was prepared by mixing 3.81 ml 1 *M* K_2HPO_4 and 6.19 ml 1 *M* KH_2PO_4 and 200 mg glutathione was dissolved in the 10 ml of buffer and) was filter-sterilised into the media, when the temperature of the medium cooled down to about 65°C.

Seeds of *R. myconi* (L.) Rchb. were obtained from a commercial depot of ornamental plants in the UK. Aseptic seedlings developped from the seeds constituted the basic material of the experiments, of which the sterile maintenance and propagation was the next step. From this material, we have chosen for micropropagation subjects with whole and healthy roots and leaves, with leaves growing fastly, without any brown edges or necrotic spots. The explants were incubated on *Ramonda* medium (RA medium) that was also supplemented with 200 mg/l filter sterilised glutathion (as above) and of which the pH was also stabilised with K-phosphate buffer. In the RA medium, the concentration of macro and microelements was decreased to 40% of that of the original MS medium. Micropropagated plants were grown in Vitro Vent containers (Duchefa) at 24±2 ◦C under a 16 h light (ca. 4500 lx) per 8 h dark regime and were subcultured in every 3 weeks.

Induction of calli and plant regeneration

Approximately 5-mm x 7-mm mature leave segments were cut (each having a minimum of 3 cut edges) from fast growing *C. plantagineum* plants. These explants were placed onto our own callus induction media (CI medium). Callus induction required an alternation of 16 h of moderate illumination (approximately 1,000 lx) and 8 h of darkness at 23±1°C. Explants were subcultured every 2 weeks without separation of the developing calli.

Following the callus induction phase (4–6 weeks), callus forming explants were transferred onto the corresponding plant regeneration media. The culture room conditions for plant regeneration were slightly modified: explants were incubated under a photoperiod of 16 h illumination (approximately 4,500 lx) and 8 h darkness at 23±1°C. Explants were subcultured every 2 weeks.

Vigorously growing *R. myconi* plantlets were selected for callus induction and subsequent plant regeneration. Approximately 4mm x 6mm segments were cut from 3 to 4 weeks old healthy, mature leaves of micropropagated plants and were used as starting explants in the regeneration experiments. Leaf segment explants were cultured on basal RA media supplemented with 0,2 mg/l BAP and 2 mg/l picloram. Callus induction required an alternation of 16 h of moderate illumination (approximately 1,000 lx) and 8 h of darkness at 23±1°C.

Plant regeneration took place in glass Petri dishes (10 cm in diameter) where the density of explants was six leaf segments per Petri dish. Explants were incubated under a photoperiod of 16 h illumination and 8 h darkness at 24±2 ◦C. After 3-4 weeks, shoots appeared at the surface of calli. Vigorously growing plants developed from these shoots.

***Agrobacterium tumefaciens* strain**

Agrobacterium tumefaciens strain GV2260 (Deblaere et al. 1985) with the binary vector p35SGUSINT (Vancanneyt et al. 1990) was used for genetic transformation (kindly provided by Dr. Reiner Höefgen, MPI of Molecular Plant Physiology, Golm, Germany). The T-DNA region of the p35SGUSINT vector contained a p35S*gus-intron*-3′35S transformation cassette in which the β-glucuronidase (*gus*)-coding region was interrupted by the second intron of the potato *st-ls1* gene.

Genetic transformation

C. plantagineum explants were pre-induced on CI+AS medium [CI medium supplemented with 50 μ*M* acetosyringone (AS) – method B) for 2 days, following which time they were used as starting explants for genetic transformation. Leaf segments were dried for a short period of time on sterile filter paper discs after the infection and then placed back onto the same callus induction media (CI+AS medium) for 2 additional days of co-culture. The explants were subsequently transferred onto selective CI medium supplemented with 150 mg/l kanamycin and 500 mg/l cefotaxime. After 4–6 weeks of selection, leaf-segment explants with surviving calli were transferred onto selective PR medium supplemented with 150 mg/l kanamycin and 350 mg/l cefotaxime for plant regeneration. Putative transgenic plants were then transferred onto selective MP medium (with 100 mg/l kanamycin) for micropropagation and further examination following the 4-week regeneration period.

R. myconi explants (entire leaf disks) were perforated with a sterilized needle immediately before co-culture. After 20 minutes of infection, they were dried shortly on sterile filter paper discs and were placed onto RA callus induction media for 2 additional days of co-culture. Subsequently, explants were transferred onto selective callus induction medium (basal RA medium containing 0.2mg/l BAP and 2.0 mg/l picloram) supplemented with kanamycin (1.0 mg/l) and cefotaxime (400 mg/l). Subculturing took place every 2 weeks. After 4–6 weeks of selection, leaf segment explants with surviving calli were transferred onto selective RA regeneration medium supplemented with 0.2 mg/l BAP, 1.0–2.0 mg/l NAA, as well as filter sterilized kanamycin (1.0 mg/l) and cefotaxime (350 mg/l). Putative transgenic plants were then transferred onto selective basal RA medium (contained 1.0 mg/l kanamycin) for micropropagation after the 4-week long regeneration process.

Evidence of the genetic transformation

For polymerase chain reaction (PCR) analyses, total plant DNA was extracted from the greenhouse-grown wild-type and putative transgenic plants using the Qiagen Plant Mini Kit (Qiagen Hilden, Germany), while plasmid DNA isolation from *Escherichia coli* was performed using the Qiagen Plasmid Mega Kit, according to the manufacturer's instructions.

1500 bp *gus* fragment was amplified in the PCR reaction in case of *C. plantagineum*, while 750 bp *nptII* fragment was amplified in the PCR reaction in case of *R. myconi.*

In case of Southern hybridizations, purified DNA (10 μg) was digested overnight with *Bam*HI and *Hind*III, as these enzymes cut once within the T-DNA insert, and separated by electrophoresis on 0.8% agarose gels. DNA was blotted onto nitrocellulose (Hybond-C extra, Amersham)membranes. A 1.9 kb gel-purified *Pst*I fragment from the plasmid pFF19G (Timmermans et al. 1990), representing the *gus* coding sequence, was labeled by the random priming approach (Feinberg and Vogelstein 1983) using α[32P]-dCTP. Hybridization and autoradiography was carried out according to the standard protocols (Sambrook et al. 1989) and the instructions of the manufacturers.

Gus expression was detected by immersing intact leaves of putative transgenic and wild-type plants into the substrate (1 mM X-Glca: 5-bromo-4-chloro-3-indolyl-β-dglucuronic acid cyclohexyl-ammonium) that was vacuum infiltrated into the samples. The incubation for enhancement of the specific enzyme reaction was performed as reported by Jefferson (1987) and Jefferson et al. (1987).

Statistical analysis

Examinations were repeated three to five times. The data were evaluated by mean analysis, and standard deviations were calculated from the weighted mean difference (Sváb, 1981).

RESULTS

A method has already been published for *C. plantagineum* (Furini et al., 1994), but no one has ever been able to reproduce it. We also tried to reproduce it according to the description, unfortunately without success. Than we planned the experiments to develop a well functioning, reproduceable, efficient system of tissue reproduction and transformation in case of both plants. The initial experiences showed that both plants were equally sensible to tissue culture manipulations. Under suboptimal conditions they grew slowly, often suffered from tissue necrosis, many of them became hyperhydrated, with elevated polyphenol emission. This was the case both with *C. plantagineum* and *R. myconi* species, but their micropropagation, callus induction and regeneration required different culture mediums. *C. plantagineum* is a desert plant, its life strategy has been adapted to such conditions, that is to say, long dry periods interrupted by short periods with precipitations, when the available nutrients and water have to be used quickly and efficiently. This justified to preserve a high

MS culture medium mineral substance content in the *in vitro* culture. *R. myconi,* on the other hand, lives in an upland environment, where a much slower development and metabolism has emerged because of the restricted mineral substance resources. Therefore we used MS salts only in 40 % concentration in the *R. myconi in vitro* cultures, justified by the results of the experiments during optimisation. (Figure 1, A, B).

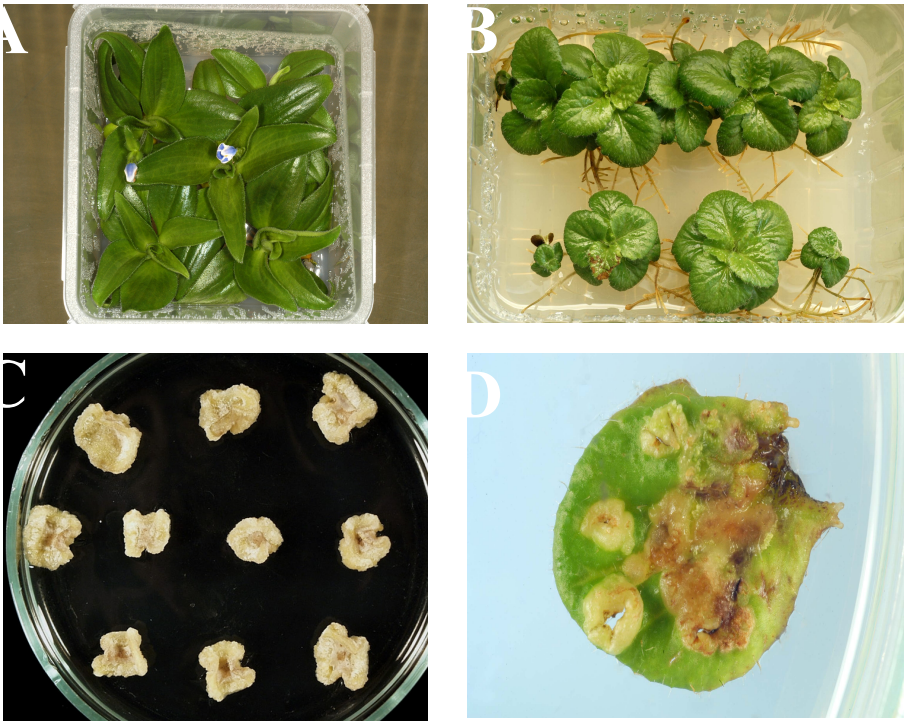

Figure 1 (A) *Craterostigma plantagineum* on the micropropagation medium. (B) *Ramonda myconi* on the micropropagation medium. (C) *C. plantagineum* callus induction. (D) *R. myconi* callus induction.

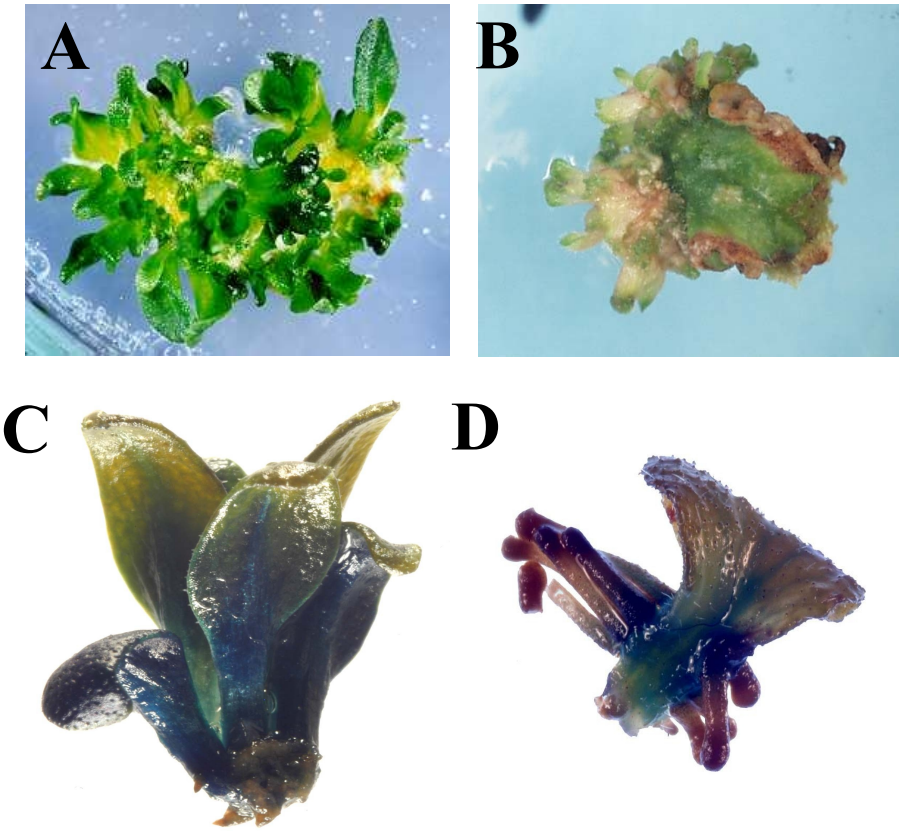

Figure 2 (A) *Craterostigma plantagineum* plant regeneration from dedifferentiated calli. (B) *Ramonda myconi* plant regeneration from dedifferentiated calli. (C) Evidence of genetic transformation with GUS analysis in case of *C. plantagineum*. (D) Evidence of genetic transformation with GUS analysis in case of *R. myconi*.

A prerequisite of genetic transformation is an efficient plant-cell-plant system, of which the first step is callus induction. In this phase, the two plants have reacted in a similar way in the experiments. In callus induction a synthetic auxin, picloram has been found the best at both plants, but it was not sufficient

alone. If besides picloram, the culture medium had not contained any cytokinin in low concentration, explants necrotised and died in the initial phase of callus induction. The experiences have justified that besides the presence of auxin (picloram), the dosage of cytokinin (BAP) to the culture medium was indispensable as well.

There were further similar elements in the *in vitro* cultures of the two desiccation tolerant plants: their efficiency of micropropagation, callus induction and plant regeneration increased when the pH of the culture medium had been stabilised with K-phosphate buffer and an antioxidant, glutathione had been added. Glutathione has decreased the proportion of necrotic and hyperhydrated plants and pH stabilisation allowed a longer lasting nutrient absorption from the aging culture medium. It is known that the ideal pH to the absorption of mineral substances is between 6 and 6,5. In the culture medium, if the initial pH is set to 5,8, it falls approximately to pH 4-5 during the first days of the tissue culture, basically affecting substance solubility, therefore a relative mineral substance shortage appears within a short period. This is probably the reason why pH-stabilisation has improved by 10-40 % the efficiency of micropropagation and plant regeneration at both plants.

We have started transformation experiments on the basis of these tissue cultures. The leaf segments selected to be the initial explants of transformation have behaved completely differently. In case of *C. plantagineum*, the method developed in the callus induction experiments could be applied without any change. An important quantity of compact callus has been formed along the cut edges of the leaf segments after the transformation. From the dedifferentiated callus cells, we have increased the proportion of transformants on a selection culture medium and we could regenerate transgenic plants from them. It has been proven that the applied physical methods (infiltration and injuries caused by microbullets) has not increased transformation efficiency, while chemical stimulation (low pH, aldose-type carbon source, organic nitrogen source, acetosyringon) has significantly increased transformation frequency in the co-culture. It has became evident as well that the young leaves used in the Furini method are much worse initial explants of transformation than the developed, mature leaves.

At the beginning of the transformation of *R. myconi*, we have found that all material applicable in the transformation and in the method have induced strong stress reactions of the plant, leading to quick death. Therefore we had to optimise the type and quantity of the antibiotics used to kill the bacteria. We had to define the quantity of antibiotics needed to reproduce transformant calli as well. We tried to decrease the strong stress reactions by applying a non-lethal

selection method using 1 mg/l kanamicyne. The advantage of this method is that transformants of normal and abnormal fenotypes are created and a visual selection can be done. However, we could not apply the callus induction method developed in the pre-experiments, because after co-culture with *A. tumefaciens,* cut edges had necrotysed in any case, which caused the death of all explants within short time. To avoid this, we have used complete leaf discs for the transformation which had been perforated with a sterile syringe right before the co-culture. Calli have been formed along the microinjuries and after selection we could regenerate transformant plants from them. The chemical stimulation applied in the case of *C. plantagineum* could be applied successfully in the case of *R. myconi* as well.

The presence and functioning of the transgene has been justified at *C. plantagineum* and at *R. myconi* as well by histochemical painting, PCR analysis and Southern-blot analysis.

The elaboration of the tissue culture and the genetic transformation system transmitted by *A. tumefaciens* of the two model plants has opened the opportunity for the functional analysis of genes isolated from desiccation tolerant plants. Through this, reactions to avoid damages caused by water shortage of not only the plants but any other living organism can be better understood, that may be used in the future in the improvement of the abiotic stress tolerance of cultivated plants. Through a better knowledge of the material and processes of desication tolerance, it can be found out how these plants defend their cell structures, proteins and hereditary material indispensable for the vital functions.

In case of *C. plantagineum* and *R. myconi* as well, we have justified the presence and the functioning of the transgene by histochemical painting (figure 2, C, D), by PCR analysis and by Southern-blot analysis.

New research findings

1., We have elaborated the method for the micropropagation of *Craterostigma plantagineum* and *Ramonda myconi*, including the followings:

- we have stopped hyperhydration caused by the low pH with K-phosphate buffer;
- we have reduced the negative effects of the plants' polifenol secretion by applying glutation as antioxidant;
- in case of *R. myconi*, the reduction of MS salts' concentration to its third in the micropropagation medium has provably increased the

number of fast growing plants with big leaves, which could be used for the following transformation.

2., We have elaborated the method for the plant regeneration from callus of *C. plantagineum* and *R. myconi*, including the followings:

- we have developed the callus induction medium for both plants;
- we have proven that an adequate efficiency of callus induction could be reached only in the presence of both cytokinin and auxin, we have determined their optimal concentration;
- we have developed the plant regeneration medium for both plants;
- we have optimised the hormone combination necessary for pant regeneration and rooting.

3., We have elaborated the method for the genetic transformation of *C. plantagineum* and *R. myconi* as well, including the followings:

- we have proven that the utilisation of mature leave segments (*mls*) has increased transformation efficiency;
- in case of *R. myconi*, we have proven that causing microinjuries has got a critical importance regarding the successful transformation;

in case of a *R. myconi*, we have optimised the type and concentration of antibiotics suitable to kill *Agrobacterium* and useable for the selection of transgenic explants.

CONCLUSIONS AND RECOMMENDATIONS

By the elaboration of the tissue propagation method of the two model plants and by the genetic transformation system transmitted by *A. tumefaciens*, the path has been opened for the functional analysis of the genes isolated from desiccation tolerant plants, through which reactions given to prevent damages due to water shortage can be better understood, not only in the case of plants, but in case of all living organisms, that could be applied later in the improvement of abiotic stress tolerance of the cultivated plants as well. Through a better knowledge of the substances and processes of desiccation tolerance, it could be found out how these plants defend their cell structures, proteins and hereditary material indispensable for the vital functions.

On the basis of the similarities found in the tissue cultures and transformation processes of the two plants, it can be concluded that these

methods, or certain elements of them, will be applied successfully in case of other, taxonomically far, desiccation tolerant plant species as well.

We are planning, in case of both plants, the physiological analysis of the mechanism of osmotic stress tolerance by applying the elaborated transformation process. According to the detailed biochemical studies, it is supposed that the increased osmotic stress tolerance of these plants is due to their special sugar metabolism (Schwall and coll., 1995, Scott, 2000). In our laboratory we have got such gene constructions, through which an extreme level of starch accumulation or saccharose overproduction of the plants can be induced. Our method is based on the effect of fructose-2,6-biphosphate (Fru-2,6-P2) on signal metabolite starch synthesis and saccharose synthesis. By the developed transformation techniques, these constructions can be taken into the plants, so that we can prove or reject our former conceptions.

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