THESES OF PHD THESES

STUDY OF ARTIFICIAL NITROGEN FIXING ASSOCIATIONS OF *IN VITRO* STRAWBERRY - BACTERIUM ASSOCIATION AND A UNICELLULAR GREEN ALGA - BACTERIUM - FUNGUS MODELL SYSTEM

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Budapest
2011

Introduction

It is hard to imagine life and evolution without symbiosis. Symbiosis is a long-living association between two or more individuals of different species of organisms. The stability of the association is permanent only under well-balanced conditions. The basis of the cooperation/interdependence is metabolic and/or genetic. Metabolic interactions facilitate the formation of booth looser and closer associations. Interdependency is based on the complementation of the main metabolic pathways, such a photosynthetical CO_2 assimilation and atmospheric nitrogen fixation.

Nitrogen, a component of many bio-molecules, is essential for growth and development of all organisms. Most nitrogen exists in the atmosphere, and utilisation of this source is important as a means of avoiding nitrogen starvation. However, the ability to fix atmospheric nitrogen via the nitrogenase enzyme-complex is restricted to some bacteria. Such bacteria are termed diazotrophs, as they obtain all their nitrogen by fixing molecular nitrogen. Eukaryotic organisms are only able to obtain fixed nitrogen through their symbiotic interactions with nitrogen-fixing prokaryotes.

Our aim was to study the artificial bipartite association between strawberry (*Fragaria* x *ananassa*) and the diazotrophic bacteria *Azotobacter vinelandii*, and the artificial tripartite symbiosis involving a green alga (*Chlamydomonas reinhardtii*), a bacterium (*Azotobacter vinelandii)* and a fungus (*Alternaria infectoria*).

Materials and Methods

Partners of the associations

Bacterium: *Azotobacter vinelandii* [DSM 85] , *gfp* gene containing, maintained after (Newton et al., 1953), at 25 ^{o}C, on solid agar plate.

Plant: strawberry (*Fragaria x ananassa*), maintained on MS (Murashige and Skoog, 1962) media, supplemented with 100 mg/l inozit, 0,5 mg/l BAP, 0,1 mg/l GA_3, 5 mg/l IBA.

Alga: wild strain of *Chlamydomonas reindhardtii* 187, maintained on solid TAP medium (Sager and Granick, 1953).

Fungus: *Alternaria infectoria*, isolated from the co-culture and kept as a pure culture on MMN media (pH=5.8) (Kovács et al., 2003).

Examinations with biolistic gun

Incorporation of bacteria into the plant tissues were carried out by biolistic gun (Genebooster®) which is used for genetic transformation. *Azotobacter vinelandii* cells adhered to tungsten particles were shot directly into young leaves and regenerating shoot tips. 7,5 µl of the microrpojectile - consisted of tungsten particles (3 µm average size, Biorad M25), bacterium cells and spermidin- were shot by 30 bar nitrogen.

Scanning electron microscopy

For scanning electron microscopy, small parts of leaves were fixed in 4% (v/v) paraformaldehyde for 2 h. After dehydration with increasing concentrations of ethanol, samples were placed in amyl acetate. The intermediate fluid was removed from the samples by critical point drying. Before investigation, specimens were coated with gold evaporation. Observation was made using a Hitachi S-2360 N scanning microscope.

Fluorescence microscopy

The *gfp* gene containing bacteria in the plant tissue were detected with fluorescence microscopy. Detection of the biolistic treated leaves were made with Olympus BH2-RFCA epifluorescence microscope and Zeiss Axiovert 135 TV laser scanning microscope. The excitation wavelenght was 488 nm. We used filters between 515-525 nm and above 560 nm. The magnification was 40x and 100x.

Degreening and regreening of the algal cultures

For the degreening process the algal cultures were passed onto nitrogen free TAP medium. For the regreening process the degreened algal cultures were passed onto complete TAP medium. Samples were collected for pigment extraction, for fluorescence spectroscopy, for the detection of oxygen evolution and for electron microscopy in different times.

Pigment extraction and determination of the chlorophyll content

The chlorophyllous pigments were usually extracted from algal and the associative cultures with 80% (v/v) acetone. Absorption was measured with a Perkin Elmer Lambda 25 UV/VIS spectrophotometer. The chlorophyll content of the samples was determined according to Porra et al. (1989). Data represent the means of three independent measurements and are given as cell number of algae in the symbioses, counted them in a Bürker chamber.

Fluorescence spectroscopy

The 77 K fluorescence spectra were recorded in the range 580-780 nm with a Fluoromax-3 (Jobin Yvon-Horiba, France) spectrofluorometer. Samples were frozen in liquid nitrogen under the measurement. The excitation wavelength was 440 nm. The integration time was 0.1 s and the data frequency was 0.5 nm. For each sample, the average of three spectra was automatically calculated. Five-point linear smoothing and baseline correction were performed.

Detection of respiration and photosynthetic oxygen evolution

The respiration intensity and photosynthetic oxygen evolution of the algal and the associative culture samples dissolved in 50 mM Tricin-NaOH (pH 7.8) buffer were measured using a Hansatech oxy Lab oxygen electrode. The oxygen consumption and the evolution of the samples were given in μmol O_2 ml^{-1} min^{-1}. The samples were illuminated with 900 μmol foton m^{-2} sec^{-1} light intensity. Data represent the means of three independent measurements.

Transmission electron microscopy

For transmission electron microscopy the algal and the associative cultures were fixed in 2% (v/v) glutaraldehide for 2h and postfixed in 1% (w/v) OsO_4 for 2h in 70 mM K-Na phosphate (pH 7.2). Samples were embedded in Durcupan ACM epoxy resin (Fluka Chemie AG). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi 7100 electron microscope.

Detection of amino acids

The samples (liquid media of bacteria, algae, fungus, algal-bacterial 131 and tripartite associations) were collected from 2-week-old suspension cultures and filtered consecutively to eliminate through 0.22 μm membrane filters the cells. The free amino acid contents of the filtrates were determined with an automatic amino acid analyser (AAA400, Check Republic) equipped with a cation-exchange resin column (220 x 3.7 mm); detection with postchromatographic derivatization using the ninhydrin reagent reaction at a temperature of 135°C and spectrophotometric measurement at λ= 570 and 440 nm (for Pro). Data represent the means of three independent measurements.

Results and Conclusions

1. Establishment and investigation of *Fragaria* x *ananassa* – *Azotobacter vinelandii* artificial association

1a. Bombardment of bacterial cells into plant tissues

In our laboratory artificial association was established between strawberry and a diazotrophic bacteria *Azotobacter vinelandii* under *in vitro* circumstances. Biolistic gun was used for the direct introduction of nitrogen-fixing bacteria into plant tissues. After bombardment injuries can be seen on the surface of the leaves, which contain large amount of bacteria. Bacterium cells could be landed in two positions: in the intercellular spaces of the target tissue or inside the cells. The plantlets regenerating on the sites of injuries likely contain the bombarded bacteria. The regenerated plants can be maintained on nitrogen free media that prove the nitrogen fixing activity of bacteria in the leaf tissues. Our results show that the biolistic method made possible the delivery of living bacteria into plant tissues.

1b. Detection of the injuries after bombardment with scanning electron microscope

Scanning electronmicroscopy was used to follow the fate of the the bombarded plant cells, injuries and surface bacteria from 30 minutes to 29 days after the biolistic treatment. Our results show that damaged tissues regenerated quickly, two days after bombardment. After 24 hours the site of the bombardment could be seen well but cell-regeneration began in the deeper layers. Ont he second day the injuries cuold not be detected. Two-three weeks later the only bacteria on the leaf epidermis showed the site of the injuries. Multiplication of *Azotobacter*s on the leaf surface showed that bacteria were able to survive the biolistic treatment. We assume that bacteria in the intercellular spaces also survived the treatment and were able to spread into the plant tissues.

1c. Detection of the plant regeneration

Plant regeneration from tissue culture is widely used for example during creation of artificial symbiosis/associations between *Fragaria* and nitrogen fixing bateria. Leaves were bombarded by biolistic gun with living *Azotobacter* cells and plants were regenerated after it. The way of the regeneration process is very important to know because this influences the incorporation and spread of bacterium cells into the intercellular spaces of the regenerated plants. Therefore

steps of regeneration from leaf inocules was followed up by scanning electronmicroscopy. As the results show the plantlets are regenerated in two ways: via somatic embryogenesis and organogenesis.

1d. Detection of the bacteria with fluorescence microscopy

The *gfp* gene containing *Azotobacter vinelandii* cells were detected with epifluorescence and laser fluorescence microscope. We detected the presence of the bacteria int he deeper layer of the plant tissues. The three dimensional micrograph of laser scanning microscopical investigation also proved the presence of the bacteria int he bombarded tissues.

Our results show that the elaborated technique made possible the introduction of living bacteria into plant tissues. Particle bombardment seems to be an efficient alternative method for establishing artificial nitrogen-fixing associations because it can ensure the delivery of diazotrophic bacteria in sufficiently high numbers into the target tissues.

2. **Organization and function of chloroplast and photosynthetic apparatus in the unicellular green alga *Chlamydomonas reinhardtii* during degreening on nitrogen-free and regreening on nitrogen-containing media**

Our aim was to study the cultures of the unicellular green alga, *Chlamydomonas reinhardtii,* maintaining on nitrogen-free medium. During the 3 week-long degreening process the chlorophyll content of the cells, changes in chlorophyll-protein complexes and photosynthetic activity of the cultures were measured as well as ultrastructure of the single chloroplast was studied. Upon supplying N source, the regreening of *Chlamydomonas* cells was faster, than the previous yellowing, and (regarding the proportion of the parameters) was not simply the reversion of the sequence of events.

2a. Degradation of the algal cells on nitrogen free medium

Investigation of the photosynthetic parameters

Visually detectable yellowing of the cultures on nitrogen-free medium was the result of gradual loss of chlorophyll content. Rate of decline was quicker at the beginning as chlorophyll content was about 60% on the 1st day and 40% on the 2nd day then decrease turned slower. After 18 days the algal cells practically left their chlorophyll (1.5%). Paralelly with the loss of chlorophyll content, lack of nitrogen caused gradual decline of/in photosynthetic activity. Similar tendency characterized both processes, oxygen production also was

decreased in the highest degree in the first 5 days. We experienced a close correlation between the decrease of chlorophyll amount and PSII activity, as measured by O_2 evolution. The decay of the two photosystems followed different dynamics; at 77K the fluorescence maximum around 712 nm (attributed to PSI) started to decrease, then after 15 days the decrease of the band at 687 nm (related to PSII) became more prominent, so their ratio changed.

Electron microscopy

Electron microscopic investigations confirmed that the well developed thylakoid system characteristic of the control cells was gradually degraded. This could be clearly observed on the 4th day, by which time the grana-like structures were largely disintegrated. From the 1st day of the degreening process, accumulation of starch grains could be detected. At the onset of N-starvation autophagic vacuoles with partly digested cell content were visible in the cytoplasm. Concomitantly, lipid globules appeared, then their number and size increased progressively. After two weeks the original structure of a typical *Chlamydomonas* cell was hardly recognizable, as the lipid globules occupied the greater part of the cell. Grana-like membrane stacks could not be found at all, the few thylakoids occurred on the periphery of the plastid.

2b. Regreening of the algal cells on nitrogen-containing medium

Investigation of the photosynthetic parameters

During the regreening process the chlorophyll content already increased in the first hours and its amount was 20% in the 15^{th} hour. The chlorophyll content of the 1-day-old cells was quarter of the control cells and after 2 days this value was more than 60% of the control. Oxigen production increased in a lower amount at the beginning, than a quick growth occured between the 2^{nd} and 4^{th} hours. The photosynthetic activity was 50% of the control after 1 day and 80% on the 4^{th} day. The leading parameter of regreening was the regeneration of PSII, as shown by O_2 evolution and by the appearance of fluorescence at 77K in the spectral region close to 687 nm. While this maximum was gradually shifted to 687 nm and its relative height exceeded that of the 0 day control, the O_2 production saturated at about 80%. The increase in chlorophyll content initially lagged behind this (unlike during degreening), but after 4 days exceeded 100%, and saturated at 120%.

Electron microscopy

Passing the 21-day old degreened *Chlamydomonas* cells onto N-containing medium caused the recovery of the thylakoid membranes. The reorganization of the thylakoid system began in

the 5th hour, and after the 14th hour distinct lamellae stacked in pairs could be observed. After 1 day a well-organized membrane system with stacked thylakoids could be detected, the degree of stacking and the amount of thylakoids increased on the next few days. The regeneration of the chloroplast was almost complete on the 2nd day, and by this time the starch content decreased to the normal level. Lipid globules decreased remarkably in the cytoplasm during the first 24 hours, then disappeared by the 2nd day.

The background of the conspicuous yellowing process is complex: in addition to the decline of the chlorophyll content, qualitative and quantitative changes in photosynthetically active chlorophyll-protein complexes were also detected. Ultrastructural degradation of the chloroplast included the disintegration of the thylakoid membranes and accumulation of lipid bodies and starch grains. During the regreening process on nitrogen-containing medium chloroplasts were regenerated when formation of chlorophyll was followed by gradual increase of photosynthetic activity. Chlorophyll-protein complexes were reorganized. Lipid bodies and starch grains were degraded. This process was much quicker then the degradation, total greening of the cells occured in 4 days.

3. Investigation of the alga-bacterium-fungus association

A long-living artificial tripartite symbiosis involving a green alga (*Chlamydomonas*), a bacterium (*Azotobacter*) and a fungus (*Alternaria*) was established on carbon- and nitrogen-free medium. The functioning and continuous subsistence of our associations maintained on nitrogen- and carbon-free medium is ensured by the organic nitrogen and carbon compounds (mainly amino acids and sugars) excreted by the partners into the medium.

3a. Investigation of the photosynthetic parameters

The most striking characteristic of the artificial symbioses is their spectacular green color, although the chlorophyll content of the alga cells is only 38 % of that of the control cells. Fluorescence emission spectra recorded at 77 K show that the chlorophyll molecules assembled into pigment–protein complexes similarly to the control algae. The increase in the amplitude ratios of PSII and PSI fluorescence bands in the spectra of the tripartite association can be explained, at least partly, by changes in energy transfer. The slight parallel increase in the chlorophyll *a*/*b* ratio suggests that more core complex was synthesized in the algal cells of the associations than in those of the control alga culture. This assumption is in good

agreement with the relatively high oxygen evolution of *Chlamydomonas* in the tripartite association, which proves that the algal cells were physiologically active.

3b. Ultrasructural investigations

Electron microscopic investigations of parts of a tripartite association confirmed that the algal cells were surrounded by bacterial cells and fungal hyphae. The vegetative cells of *C. reinhardtii* were healthy, having well developed chloroplasts with stacked lamellae. Compared with control algal cells grown on nitrogen-containing medium, they did not accumulate significantly more starch granules or lipid globules, which were characteristic of degreened *Chlamydomonas* cells under nitrogen-deficient conditions.

3d. Analysis of amino acids

The tripartite symbiosis excreted far fewer types of amino acids than the pure cultures, so the amino acids absent from associations maintained on nitrogen- and carbon-free media are produced by the pure cultures under optimum conditions. The lack of these molecules could have two possible causes. The partners in the co-culture may utilize all the amino acids, or the biosynthetic pathways may be inhibited by nitrogen deficiency. This assumption is supported by the fact that the partners in the symbiosis do not produce complex, branched-chain amino acids (Leu, Ile, Tyr, Phe), except for 5-carbon-atom Val, while in pure cultures these are excreted. Interestingly, Pro could not be detected in the association, whereas algal and fungal strains excreted it in high quantities in the normal medium. Cystathionine, which is a common intermediate in the sulfur-containing amino-acid metabolism, was produced in high quantities by the tripartite symbiosis, mainly as a result of fungal metabolical activity.

The functioning photosynthetic apparatus of the *Chlamydomonas* cells in associations grown on nitrogen- and carbon-free media led to the conclusion that the *Azotobacter* partner provided a nitrogen source that was effectively used for building up a well-functioning photosynthetic apparatus. This is supported by the ultrastructural investigations, as the chloroplasts in the symbiont algal cells, similarly to the control cells, contained a well-developed, stacked thylakoid membrane system without extreme starch or lipid accumulation characteristic to the pure algal cells maintained on nitrogen-free medium. The role and function of the fungal partner in this tripartite symbiosis is currently unclear, presumably ensure an additional source of nitrogen.

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List of publications containing the results of this thesis:

A. Papers in refereed journals:

Lőrincz, Zs., Preininger, É., Kósa, A., Pónyi, T., Nyitrai, P., Sarkadi, L., Kovács, G.M., Böddi, B. and Gyurján, I. (2010) Artificial tripartite symbiosis involving a green alga (*Chlamydomonas*) a bacterium (*Azotobacter*) and a fungus (*Alternaria*): morphological and physiological characterisation. Folia Microbiologica 55: 393-400

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B. Conference Proceedings:

Lőrincz, Zs., Preininger, É., Kristóf, Z. és Gyurján, I. (2006) Vizsgálatok génpuskával létrehozott mesterséges nitrogénkötő szimbiózisokon. XII. Magyar Növényanatómiai Szimpózium Sárkány Sándor emlékére – Budapest, 2006. június 22-23. Kiadvány 58-62. oldal, szerkesztő: Mihalik Erzsébet

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