University of South Bohemia

Faculty of Science

Molecular characterisation of selective proteins from plant photosystem II

Master thesis

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Annotation:
This qualification work is trying to shed a little bit more light on some proteins present in higher plants, which structure and function in photosynthetic reaction remain unclear. In particular it treats proteins of photosystem II, called PsbR, PsbW and PsbX that are responsible for photosynthetic reaction optimization. This thesis contains data about proteins acquisition and their sequences elucidation.
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Prohlašuji, že svoji diplomovou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

V Českých Budějovicích dne 18. září 2012

Bc. Jiří Heller

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Here, I would like to thank very much to my supervisor Mgr. Jaroslava Kohoutová Ph.D. for her undisputed enthusiasm and fervour, with which she was giving me precious advice and helping hand for almost two years. I do not have to omit also my guarantor Associated Professor RNDr. Miroslav Oborník CSc. for his kind attitude, and the fact that he always found a spare time to spend with me when I needed more information. Subsequently I highly appreciate the effort of all members of the Institute of Physical Biology, and School of complex systems from Faculty of Fisheries and Protection of Waters, who were also very kind-hearted to me and never hesitated to grant me an aid. At last, I would like to thank all my family members for their understanding and financial support. My special thanks is dedicated to my girlfriend Šárka for her encouragement during these sometimes hard times.

SUMMARY

1. FOREWORD

In this part of my thesis I would like to tell my reasons which have brought me to this field of study, and which have caused that I chose this subject for my master's study qualification work.

In the very beginning I have to admit to the avid reader that I am not a complete novice in this branch of research. I was dealing with one protein in my bachelor's thesis that was closely connected with my current topic. Namely, it was PsbQ protein from *Spinacia oleracea* from higher plants, where we were trying to clarify his role in functioning in oxygen-evolving complex of photosystem II. That was done thanks to two one-point mutations, which were performed to replace one amino acid with another in PsbQ protein sequence, and then we tried if because of this one key amino acid change it would have an impact on the functioning of our recombinant protein, or not. The result was quite satisfactory and I personally think that it gave me a lot of experience and enough laboratory know-how. Not only that it enabled me to finish my bachelor's studies and gave me an insight into those type of studies, but also the best thing of all that work was the fact that it allowed me to get a job in the Institute of Soil Biology ASCR, v. v. i. in České Budějovice, where I was working for approximately fifteen months under the kind supervision of Ing. Václav Krištůfek Csc., and RNDr. Alica Chroňáková Ph.D.. My work there was really interesting. I was doing genetic screening of *Actinomycetes*, which are soil gram-positive bacteria strains responsible for infections of cultural plants. For example in potatoes they are responsible for so called potato scab. That working experience was very beneficial as I learned for me some new methods used in molecular biology and microbiology, which are nowadays widely and commonly used. To give an example I could mention bacterial conjugation, southern blotting or extraction of secondary metabolites from those bacterial strains mentioned above. But as time was passing by a feeling of certain unease seized me, and I took a decision to make a change and to close that working chapter of my life for once and for all. I decided to start again visit the university and to continue my studies, because it did not appeared anymore like a good idea for me that I had left them two years ago to take a working gap. To tell the truth, I was indecisive between two subjects, because genetics and also molecular biology appealed both very much to me. I found them extremely promising for the future. Then I decided for cell and evolutionary biology, because it seemed for me a slightly more convenient than the pure genetics, and after my dream being accomplished and I being accepted at this faculty I started to look for my supervisor and the concrete

subject of my diploma thesis. Finally, I made up my mind to continue my studies with dedication to areas connected with bioenergetics, because I found photosynthesis and all things connected with that extremely remarkable and without doubts also indispensable for the future. I would like to make a contribution to current level of knowledge and I would also like to manage that my work will be worth of all time and money, which were consumed on its creation.

This work has been done partly in the Institute of Physical Biology, which was on 31. 12. 2011 terminated as individual part of the University of South Bohemia in České Budějovice. Afterwards, we continued with our research in the part of the Institute of Physical Biology, which formed a new scientific division called the School of complex systems. This new unit is now a part of the Faculty of Fishery and Water Protection, which is also a part of the University of South Bohemia in České Budějovice. Specifically I was working in the crystallization laboratory, which is headed by Associated Professor Mgr. Ivana Kutá-Smatanová Ph.D.. People who are working in this laboratory have focused their strenghts on discovery of unknown structures of proteins, which play essential roles in photosynthesis of higher plants. They obtained constructs of PsbP, PsbQ and PsbO proteins of OEC and are trying to crystalize them to be eventually capable to discover their mutual relationship, which remains still mystery. Here I would also like to accent that it is a very difficult task, because for example the complete structure of photosystem II from higher plants was not yet, despite the effort of thousands of people crowned with success. My job was to obtain a homogenous sample of PsbR protein, to pass it on other people to make crystallization trials, and to determine sequence of PsbW and PsbX, other two proteins of photosystem II, which protein sequences in *Pisum sativum* are not known. All proteins were obtained from *P. sativum*, and all our research has been done in laboratories in Nové Hrady, a small town situated near the Czech-Austrian border in the South Bohemia region, where these two institutions have had facilities and background in a local old château and biotechnological hall. The research on molecular level has had there more than 10-year-old history.

In the end of this short preface I would like to tell something about our work in the laboratory, and also what I judged important to communicate to the people reading this writing.

In the laboratory we tried to use laboratory kits as much as possible. I think that it is because everybody of our crew was accustomed to them, and although that the yields or price may be sometimes better in manually prepared procedures we think that it worths,

because of time saving and their reliability. For this reason I did not find it significant to mention the chemicals used in these kits on the list of chemicals in this work as everybody can find them on the internet, or with its producer. I did not mention used equipment too, as it is a common part of each laboratory, and it is very well known to everybody, who is working in the same or related domain. Only *Escherichia coli* was used in experiments. The work, which we have done was very varied. We have performed many things, but because of their unimportance, or because they did not belong in this division of science they are not even mentioned. For example crystallization trials, cultivation of plant material for DNA/RNA, or some experiments during the summer school. Also sometimes occured some problems, because of the sensitivity of the material, which had to be resolved. Like a classical example I could mention thylakoid membranes, where we had to change three things. Firstly, the procedure of isolation, then we had to adjust SDS-PAGE electrophoresis conditions, and finally we had to tune up the western blotting procedure. Sometimes we procceded almost like using the approach attempt, fail or attempt, success. This thesis is all written in english, because although I was not obliged to do it I found it better for me, then to try to make some hair-raising translation.

1.1. AIMS OF MASTER THESIS AND RECOMMENDED LITERATURE

AIMS

1**. To determine the sequence of PsbR protein from *Pisum sativum,* and prepare construct of recombinant PsbR protein from higher plants (*Pisum sativum* and *Spinacia oleracea*).**

2. **To determine sequences of PsbX and PsbW proteins from *Pisum sativum*.**

RECOMMENDED LITERATURE

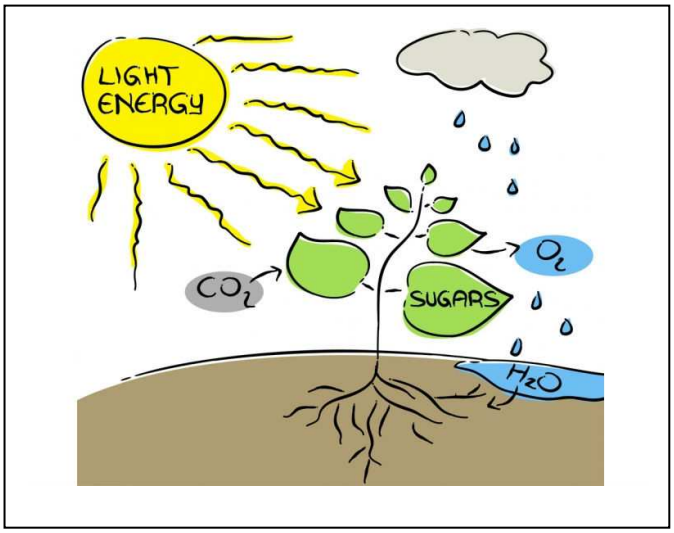
All actual publications corresponding to given issue.

2. INTRODUCTION

2.1. PHOTOSYNTHESIS

Photosynthesis is a scientific term, which is over one hundred years old. Before this word was utilised the scientists used the expression assimilation, which was used both for plants, but also for the designation of animal metabolism. We can trace its origin back to 1893, when it was utilized for the very first time by Charles Barnes (1858 – 1910), who considered the old shape not very suitable and outdated. He suggested that the process of synthesis of complex carbon compounds in the presence of chlorophyll and under the action of light should be described as either photosynthesis (a compound word, which comes from the Greek words *photo*, which means light, and *synthesis* which means composition or putting things together) or photosyntax (which means the same like photosynthesis). Although that the author itself preffered the second term it was the first one, which came as the term of choice into ordinary usage after many tumultuous years of debates (Gest, 2002).

Figure n. 1 **Schematic photosynthetic reaction**



http://www.obrazky.cz/?q=fotosynt%C3%A9za&fulltext

Between photosynthetic organisms we can classify those that are capable to obtain the energy needed for life from the sun light and carbon atoms needed for anabolic reactions from carbon dioxyde thanks to the photosynthetic reaction. It is one of the oldest processes in the Earth (3,500 millions of years), which is used mainly by green plants, but also by other organisms like red (*Rhodophyta*), green (*Chlorophyta*), brown (*Chromophyta*) algae, cyanobacteria (*Cyanobacteria*), dinoflagellates (*Dinophyta*), cryptomonads (*Cryptophyta*), euglenozoa (*Euglenophyta*) and bacteria. In this complicated, and more stepped process the

light energy of the sun is captured and transformed into the energy of chemical bonds. This energy can then be used by organism itself, or serve like a source of food for other species.

The general equation for photosynthesis:

$6\ CO_2 + 12\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$

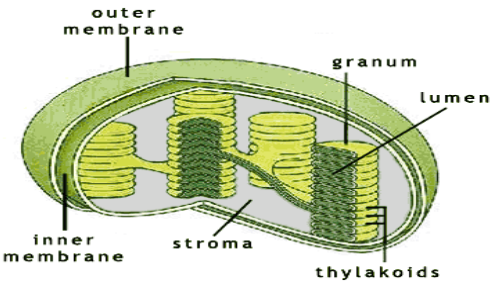
This equation is often simplified to this form: **$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2$**

Some of the authors think that the photosynthesis is the most important biochemical process on our planet, and to tell the truth I think so too. Biosphere would be without it not at all, or very little supplied with organic matter (chemoautotroph bacteria). These organic substances are used by heterotrophic organisms, for example also by us, humans, in their diet. Photoautotrophic organisms capture each year approximately 10^{71} kJ of energy and produce roughly 14×10^{11} tons of organic matter, while releasing 15×10^{11} tons of oxygen and fixating 20×10^{11} tons of carbon dioxyde from air and oceans. Our industry, which is using fossil fuels (carbon, oil, gas) everyday is also very dependent on this chemical reaction. Opposite reaction to photosynthesis is respiration, during which is the energy stored in chemical bonds released. The ATP is produced and the oxygen is consumed in this reaction. The ATP then serves like the major player in energy dependent anabolic reactions (Buchanan, 2000).

2.1.1. THYLAKOID MEMBRANES

A thylakoid is a membrane-bound compartment inside cyanobacteria and chloroplasts. Its name comes via Latin from Greek *thylakos* meaning *sac* or *pouch*. Therefore *thylakoid* means *sac-like* or *pouch-like*. They are indispensable for their role in the light-dependent reactions of photosynthesis and are embedded into the chloroplast stroma (http://en.wikipedia.org/wiki/Thylakoid). As it can be inferred from the figure n. 2, a thylakoid is composed of two parts. They are a thylakoid lumen, which is surrounded by a thylakoid membrane. It means that chloroplasts have amazing 3 membranes, instead of mitochondria having only 2. This is in conformity with endosymbiotic theory, according to that photosynthetic bacteria were absorbed by heterotrophic organisms. Another part of the chloroplast is a granum, which is not anything else than a stack of thylakoids joined together.

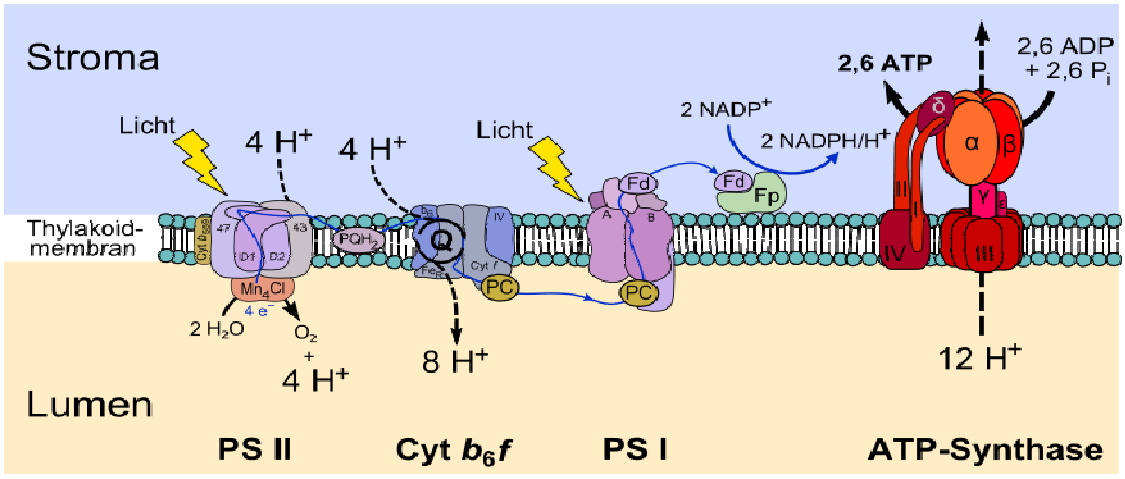
Figure n. 2 **Thylakoids inside a chloroplast**



http://withfriendship.com/user/sathvi/thylakoid.php

Chloroplasts can contain from 10 – 100 grana, which enable them to obtain larger area surface at the expense of volume ratio (which is desirable). The thylakoid lumen is essential for its role of photophosphorylation during photosynthesis, when protons are pumped across the thylakoid membrane into the lumen (as it can be seen on the next figure n. 3).

Figure n. 3 **Thylakoid membrane with proteins used in photosynthetic reaction**



http://commons.wikimedia.org/wiki/File:Thylakoid_membrane.svg

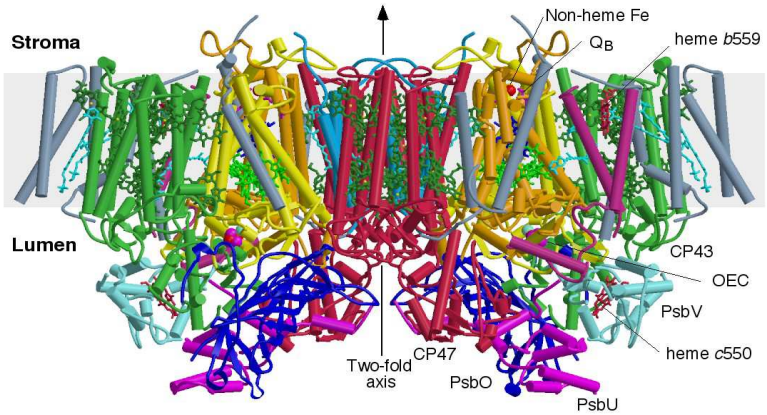
The thylakoid membrane is the home of photosynthetic pigments, where the light-dependent reactions of photosynthesis take place. Higher plants have their thylakoid membranes mainly consisting of phospholipids and galactolipids, which are asymmetrically arranged allong and across the membranes. I do not have to omit to mention that also important molecules of NADPH are made from $NADP^+$ in priming reaction to serve as coenzymes in biosynthetic anabolic reactions, which require NADPH as a reducing agent. This anabolic reaction is in plants for example the Calvin cycle, which uses NADPH to assimilate carbon dioxyde from air. In thylakoid membrane are 4 major complexes, PS II, Cyt b_6f, PS I and ATP-Synthase. The first of them, Photosystem II was for us very

important, because in it reside proteins of our interest, and is therefore the subject of the next chapter (Buchanan, 2000).

2.1.1.1. PHOTOSYSTEM II

Photosystem II is a huge pigment-protein complex, which is composed from more than 25 protein subunits. They are denoted PsbA - Z owing to the genes, which encode them. Most of them are embedded in the thylakoid membrane (Barber, 2003). PS II is located in thylakoids of higher plants, cyanobacteria and algae, where it reduces plastochinon using water like donor of electrons in the light dependent reaction, which produces like a by-product molecular oxygen (Balsera, 2003 ref. herein). The real complexity of PS II remains to be revealed, and the knowledge of the structure of photosystem II is a key prerequisite for us, if we want to understand the functioning of the molecular mechanism of the oxygen-evolving reaction. The importance of this reaction is crucial, because all oxygen needed for life on this planet is released in it. It is very probable that the origin of this oxygenic photosynthesis was dependent on the genesis of the chlorophyll. We believe so, because the bacteriochlorophyll, the predecessor of today's chlorophyll is not capable to achieve enough high redox potential to cleave the water (Barber, 2003). Here, I would like to to remind again that the complete structure of the PS II in higher plants is not known and thus we have to be content with the following figure n. 4, which comes from cyanobacteria, where it has been acquired by X-ray crystallography (Zouni *et al.*, 2001). The structure in higher plants may be, or not may be so much of similarity.

Figure n. 4 **Crystal structure of PSII systhem from *Thermosynechococcus elongatus* (3.0 Å)**



Loll *et al.*, 2005

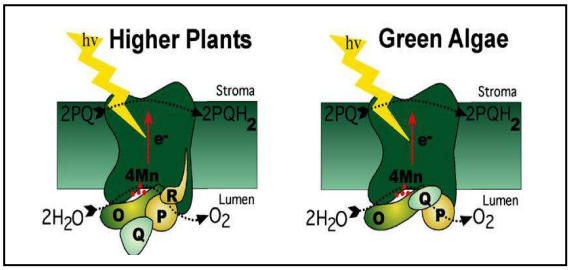
It is evident that PS II, where the oxygen-evolving complex of photosynthetic organisms takes place contains a breathtaking large number of low molecular weight proteins, and thus its true complexity rests to be established in higher plants (Cerdán, 2008).

This photosystem II is in the thylakoid membranes of algae, cyanobacteria and higher plants, where it is embedded and contains approximately 30 proteins, which molecular weights varies from 3 to 47 kDa (it is not in concordance with Barber, 2003 who found only 25 subunits). It is not a surprising fact that these proteins are greatly conserved among all PS II having organisms from ancient cyanobacteria to modern plants, what indicates their important function of keeping the essence of PS II light driven reactions from prokaryotes to eukaryotes (Kashino *et al*., 2002; Thornton *et al*., 2005). Another indisputable fact is the evidence that these small membranespanning proteins have in more than half of cases only a single transmembranespanning helix, and the most common values (roughly 50%) are oscillating around molecular weights of 10 kDa (Shi, 2004).

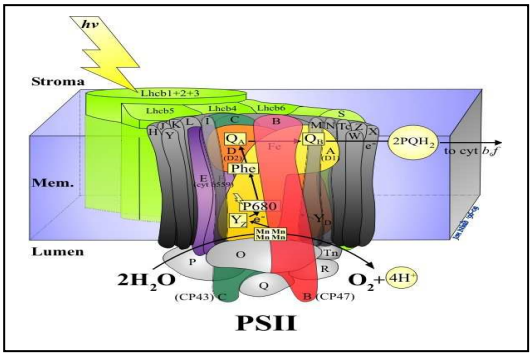
This fact is misfortunate, because of it it is very difficult to distinguish and separate the proteins one from another. Another surprising event was for the scientists a discovery that some of the proteins of this immense complex could not be found in the chloroplast genome, which implies that are nuclearly encoded. For example PsbX, PsbW and PsbY1/Y2 are nuclear. Unfortunatly the evidence are not consistent, and when I was doing my research I did not discover the exact number of these nuclear genes, because various articles contained various numbers of different proteins. On the contrary, what is in concordance is a conjecture that this moving into the nucleus was playing and still plays an important role in the evolution, allowing organisms to get more control about its own regulation of photosynthetic activity and more precise regulation. It is obvious that during that process of evolution the reaction center had to undergo many changes. The most noteworthy from them was the increase of complexity and number of involved proteins. This indispensable occurence was without doubts required to protect the organisms from the highly toxic oxygen, which is created in the photosynthetic processes. The number of involved proteins rose from three or four proteins present in the anoxygenic reaction center to more than thirty subunits and thus formed the photoystem II as we know it today. Although that in the wide majority of examples the evolutionary sources are enigmatic, gene duplication events are suspected to play the key roles in this improvement of complexity (Raymond, 2008).

Now, at the end of this chapter I would like to mention OEC, which means the oxygen-evolving complex, to tell something about it, because I think it is significant after so many allusions being made to oxygen evolution.

Figures n. 5 and 6 **The extrinsic proteins of OEC in higher plants: PsbP (23 kDa), PsbO (33 kDa), PsbQ (17 kDa) and PsbR (10 kDa)**



De Las Rivas, 2007



www.bio.ic.uc.uk/research/barberúps IIimages/PSII.html

Also the PsbR protein, which was my main topic in this thesis may interact with this protein complex containing manganese ions, where the water is cleaved. To tell the truth, we even do not know, if these PsbP, PsbQ and PsbO proteins forming this complex are mutually dependent, or not, because of being a part of PS II we dont know its exact structure. The sure thing is that PsbR protein is in the close vicinity with them and has to help them somehow. Proteins of oxygen-evolving complex are very important for adjustment of optimal conditions in water cleaving reaction, which takes place during the photosynthetic reaction. The water-splitting reaction of oxygen-evolving complex is estimated to develop roughly 2.5 billion years ago from anoxygenic photosynthesis systems in primordial cyanobacteria (De Las Rivas, 2007).

2.1.1.1.1. PsbR, PsbW AND PsbX PROTEINS

PsbR protein

The first mention about the PsbR protein is over 25 years old, concretely from 1986, when it was for the very first time isolated as a 10 kDa polypeptide from thylakoid membranes of the higher plant PS II. Unfortunately that protein was very unstable and they even did not succeed to sequence it to determine its nucleotide sequence. They also discovered that in the visible region of the spectrum it did not contain any metallic cofactors or substances with absorption. Furthemore the protein showed some hydrophobic properties.

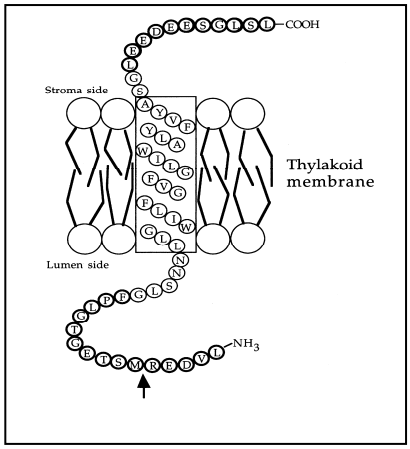
They made a suggestion that this 10 kDa polypeptide could provide a binding-site for the extrinsic 23-kDa protein in the thylakoid membrane (Ljunberg, 1986). Only three years later it was discovered on wheat that this 10 kDa polypeptide related with the oxygen-evolving complex had a putative C-terminal non-cleavable thylakoid transfer domain (Webber, 1989). Owing to transgenic potatoes plants with the anti-sense RNA gene efficiently inhibiting a formation of the 10 kDa polypeptide of photosystem II it was found out that even the drastic reduction of the 10 kDa protein did not altered the accumulation of other associated polypeptides from photosystem II at both the protein and RNA levels. On one hand no dramatical changes were observed in phenotype of those modified potatoes plants with regard to the ultrastructure of the chloroplasts, growth rate, or habitus, but on the other hand a general disorder into the PS II complex was distinguished (Stockhaus, 1990). Nowadays we know, thanks to the recent studies, more. The PsbR protein is present in green algae and higher plants, but still remains a little bit mysterious one. It has quite unusual primary structure and like the other OEC components it has a lumenal targeting sequence, which is shorter than the sequences present in other extrinsic proteins. We assume that its non-cleavable C-terminal region serves as a signal for lumen transport, whereas its N-terminal sequence navigates PsbR directly into the chloroplast, and is afterwards cleaved, thus leaving PsbR protein without the N-terminal hydrophobic loop. In its N-terminus it has lumenally exposed 70 amino acids. We presume that the presence of the PsbR protein is important for optimization of water oxidation and electron transfer, but its precise function still remains hidden. Experiments showed that the lack of the PsbR polypeptide in mutant plants caused decrease in oxygen evolution and increase of PS II excitation pressure. We also believe that it can play an important role in PsbP docking and stable assembly of PsbP and PsbQ proteins, which are all together with PsbO protein other extrinsic proteins of OEC in higher plants, as could be seen in the pictures n. 5 and 6 (Roose 2007, ref. herein). Because of some functional studies we are also aware of the fact that the PsbR protein from thylakoid membranes of *Arabidopsis thaliana* containing an insertion mutation showed about 34% less evolution of oxygen than the unaffected ones, which corresponds with the amounts of PS II estimated (Allahverdiyeva, 2007). Another confirmation of its important function in PS II complex is the founding that PsbR is in a relationship with manganese cluster, where evolution of oxygen takes place (De Las Rivas, 2007). A laboratory colleague of Allahverdiyeva one year before concluded from her research on *Arabidopsis thaliana* that both sides (acceptor and donor) of PS II complex were significantly modified in the lack of PsbR protein (Suorsa, 2006). One of the latest papers propose a posibility that in the absence

of the PsbR protein occur some functional defects, which are owing to faulty binding or assembly of the PsbP and PsbQ components to PS II and thus corroborating all the evidence about its relevant role (Haijun, 2009).

PsbW protein

The PsbW protein is a protein present in higher plants, which molecular mass is 6.1 kDa. Like the PsbX protein is also nuclearly encoded, which could suggest a method how to affect the photosynthetic activity in the partly autonomous chloroplast and possible evolution in environment with more quantity of oxygen. It is a typical transmembrane protein with a hydrophobic transmembrane segment with the length of 54 amino acid residues as can be inferred from the picture n. 7. Both the PsbW mRNA levels and the PsbX mRNA levels are light regulated, but instead of PsbX, PsbW protein was as well present in etiolated spinatch plants (Lorković, 1995). Investigation of its transversal orientation and lateral distribution showed that the main part (around 80%) was present in thylakoid membranes, specifically in the grana region. These observations vindicated the earlier findings of close association of PsbW with PS II. Enzyme linked immunosorbent assay revealed that the N-terminus was exposed to the lumenal side of the thylakoid membrane, whereas C-terminus was exhibited on the stromal side (Shi, 1997).

Figure n. 7 **Schematic drawing of the PsbW protein. The arrow indicates the trypsin cleavage site, bold marked amino acids indicate the epitopes.**



Shi, 1997

Experiments with *Arabidopsis thaliana* were made 12 years ago to discover its function and role in higher plants. Exactly 2000 seedlings of *A. thaliana* were transformed with an antisense gene to the PsbW of PS II, and transgenic plants with more than 96% reduced contents of PsbW protein were obtained. These plants were used for photosynthetic

functional studies and it was found out that although there were only slight differences in phenotype between the wild type and antisense mRNA type plants the dimeric PS II supracomplex could be found out only in the wild type plants. As well as analysis of isolated thylakoids indicated the decrease by 50% of oxygen-evolving rate in antisense plants. The amount of other PS II proteins was transformed to dissimilar quantities in the absence of the PsbW. The results proved the reduction of PS II complexes and their diminished stability in the lack of PsbW protein. We presume that in the biogenesis and photosynthetic activity regulation is the PsbW protein a key player (Shi, 2000). An organello import assay combined with a blue-native gel electrophoresis system showed that the PsbW is directly assembled in dimeric PS II supercomplexes (Thidholm, 2001). Recently, a paper on completely knock-outed transgenic lines of *A. thaliana* was published. When compared with the previous ones we can see clearly that its results are in concordance with that of more than 96% reduced contents of PsbW plants from the previous article. Transgenic lines exhibited no significant phenotype, although their photosynthetic performance, PS II core protein phosphorylation and photoinhibition recovery was slightly decreased. Despite the fact that the PS II antennae size was not changed, their connectivity was reduced. Scientists concluded that the PsbW protein is critical for the stability of the PS II - LHC II supercomplexes, and it is involved in the following areas of activities. It affects energy transfer between the various parts of PS II complex. Regulates the state of transition and energy distribution. Optimize the interaction between PS II and the plastoquinone pool. Facilitates the PS II protein phosphorylation, and help to reduce the damage and time recovery from high light levels, which were observed in knock-outed transgenic lines. To terminate, this protein is not essential in photosynthetic eukaryotes as the plants are capable of photo-autotrophic growth in its absence, but is highly probable that provides a fine-tuning of assembly of PS II - LHC II supercomplexes and their stabilization (Cerdán, 2011).

PsbX protein

From all three here treated proteins it is the PsbX protein, which is the least well characterised. When doing my research I was capable to find out only a few articles dealing with this issue. The PsbX protein was firstly identified in *Spinacia oleracea* and it was discovered that consists of 38 – 42 amino acids, with molecular mass of 4.0 – 4.2 kDa (Ikeuchi *et al.*, 1989). Now we know that it is present in various plants and cyanobacteria (Shi *et al.*, 1999; Funk, 2000). In *Arabidopsis thaliana* it is the PS II protein, because of its detection only in association with PS II and it is a thylakoid membrane protein, because of its presence in the granal extract. This protein is not a part of OEC, but the evidence are that

is associated with large (α) subunit of cyt 559 within the PS II core. The light seems to play an indispensable role in its development induction, because in etiolated plants neither protein, nor mRNA was found. Also it could be found only in some parts of plants, where the chloroplasts were expected to develop and not in parts like for example the roots. We guess that it is rather an integral-membrane protein, which function is not clear at the moment. Some scientists think that it could have a function in electron transfer within PS II, but its close vicinity to PS II core could also indicate linking of the inner PS II reaction centre with outer protein antennas complexes. Even more, this protein does not contain histidine in its sequence, which suggests that chlorophyll is not attached to it. Therefore it does not seem to be directly involved in the energy transfer (Shi *et al*., 1999). Another team of researchers discovered thanks to the antisense inhibition of the PsbX protein in *Arabidopsis thaliana* that this protein plays an important role in PS II integrity, and thus is important for accumulation of functional PS II. In transgene plants PS II complexes were impaired and showed 30% - 40% reduction in the contents of PS II complexes and severe reduction of the phosphorylation levels in the remaining PS II complexes. Although that the PS II turnover was not affected by the reduced amount, which showed that the light-harvesting antennas are not affected, the PS II complexes showed destabilized charge separation. All this indicates the fact that the PsbX protein is partly needed, but not vital for proper assembly and functioning of the photosystem II core complex in plants with highly reduced PsbX amounts (Cerdán, 2009). To sum up, it is also like the PsbW protein one of the proteins, which are not encoded by plastid DNA, and are therefore transported from the nucleus. It is very probable that this adaptation evolved during the evolution, when PsbX was transferred to the nucleus (Shi, 2004; Thornton *et al.,* 2005).

3. MATERIAL AND TECHNICAL EQUIPMENT

3.1. MATERIAL

3.1.1. CHEMICALS AND LABORATORY STRAINS

CHEMICALS	
ALKALINE PHOSPHATASE	FERMENTAS
ALKALINE PHOSPHATASE BUFFER	FERMENTAS
ACETIC ACID	PENTA
ACRYLAMIDE/BIS-ACRYLAMIDE 30%	SIGMA
AGAROSE	AMRESCO®
AMPICILIN	DUCHEFA BIOCH.
ANTIBODIES:	
PsbR PRIMARY ANTIBODY	AGRISERA
PsbW PRIMARY ANTIBODY	AGRISERA
PsbX PRIMARY ANTIBODY	AGRISERA
SECONDARY ANTIBODY	THERMO SCIENT.
APS	SERVA
BACTO AGAR	ROTH
BROMPHENOL BLUE	SIGMA
CALCIUM CHLORIDE	LACHNER
DMSO	SIGMA
DTT	DUCHEFA
EDTA	SIGMA
ETHANOL	PENTA
EZ-VISION™ ONE SAMPLE	AMRESCO®
ETHIDIUM BROMIDE	SIGMA
GLYCINE	SERVA
HYDROCHLORIC ACID	PENTA
IPTG	DUCHEFA BIOCH.
KANAMYCIN SULPHATE	SIGMA
LB MEDIUM HIGH SALT	DUCHEFA BIOCH.
MAGNESIUM CHLORIDE	PENTA

MAGNESIUM SULPHATE	PENTA
MANGANESE CHLORIDE	ANALYTIKA®
MASS RULER LOW/HIGH RANGE	FERMENTAS
METHANOL	PENTA
PCR REACTION COMPONENTS:	
dNTP (25 mM)	FERMENTAS
PRIMERS	SIGMA-ALDRICH®
Pfu DNA POLYMERASE	PROMEGA
Pfu DNA POLYMERASE BUFFER	PROMEGA
Taq DNA POLYMERASE	TOP-BIO
Taq DNA POLYMERASE BUFFER	TOP-BIO
PROTEIN MARKER (PRESTAINED, UNSTAINED)	BIORAD
SDS	BIO-RAD LAB.
SODIUM CHLORIDE	PENTA
SUCROSE	SΛFC™
RBS-250	SIGMA
RESTRICTION ENDONUCLEASES AND BUFFERS:	
BamHI	FERMENTAS
BamHI UNIQUE BUFFER	FERMENTAS
EcoRV	NEW ENGL. BIOL.
EcoRV BUFFER	NEW ENGL. BIOL.
NdeI	NEW ENGL. BIOL.
NcoI	NEW ENGL. BIOL.
XmnI	THERMO SCIENT.
TANGO TM 1X BUFFER	FERMENTAS
RNAse A	NEW ENGL. BIOL.
TEMED	SIGMA-ALDRICH®
TETRACYCLINE	DUCHEFA BIOCH.
TRICINE	SIGMA
TRIS	SERVA
T4-DNA LIGASE	FERMENTAS
T4-DNA LIGASE BUFFER	FERMENTAS
T4-POLYNUCLEOTIDE KINASE	FERMENTAS
T4-POLYNUCLEOTIDE KINASE BUFFER	FERMENTAS

UREA ANALYTIKA®

WESTERN BLOTTING COMPONENTS:

PVDF TRANSFER MEMBRANE GE HEALTHCARE

TWEEN® 20 SIGMA

WB PRESTAINED PROTEIN LADDER THERMO SCIENTIFIC

WHATMAN FILTER PAPER GE HEALTHCARE

LABORATORY STRAINS

BL21 (DE3α)

F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3) STRATAGENE

XL1-BLUE MRF' SUPERCOMPETENT CELLS

Δ*(mcrA)183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*q*ZΔM15* Tn*5* (Kanr)] STRATAGENE

3.1.2. STOCK SOLUTIONS AND CULTIVATION MEDIA

STOCK SOLUTIONS

ANTIBIOTICS AND OTHERS

AMPICILIN

100 mg/ml in distilled water

ETHIDIUM BROMIDE

10 mg/ml (has to be stored in the dark at 4 °C)

KANAMYCIN SULPHATE

50 mg/ml in distilled water

TETRACYCLINE

1.25 mg/ml in ethanol

WASHING SOLUTION FOR DNA ISOLATION

Ethanol 70%

COMPETENT CELLS SOLUTIONS AND INDUCING REAGENTS

$CaCl_2$ SOLUTION

$CaCl_2$ 0.10 mol/dm^3

TRIS 0.10 mol/dm^3

pH = 8.00

$MgCl_2$ + $CaCl_2$ SOLUTION

$MgCl_2$ 0.08 mol/dm^3

$CaCl_2$ 0.02 mol/dm^3

IPTG SOLUTION

IPTG 1 mol/dm^3

BUFFERS FOR SONIFICATION

PHOSPHATE BUFFER

$NaH_2PO_4 \times 12\ H_2O$ 0.20 mol/dm^3

$Na_2HPO_4 \times 2\ H_2O$ 0.20 mol/dm^3

pH = 7.50

TRIS BUFFER

TRIS 0.02 mol/dm^3

NaCl 0.15 mol/dm^3

EDTA 0.001 mol/dm^3

pH = 7.50

SDS-PAGE ELECTROPHORESIS BUFFERS

APS SOLUTION

APS 10.0%

GEL BUFFER (FOR SEPARATION GEL)

TRIS-HCl 1.50 mol/dm^3

pH = 8.80

GEL BUFFER (FOR STACKING GEL)

TRIS-HCl 1.00 mol/dm^3

pH = 6.80

ELECTROPHORESIS RUNNING BUFFER

TRIS 0.025 mol/dm^3

Glycine 0.192 mol/dm^3

SDS 0.1%

pH = 8.30

LOADING BUFFER

DTT	0.1%
Glycerol	25.0%
SDS	5%
TRIS	0.6%

pH = 8.00

SDS SOLUTION

SDS	10.0%

STAINING WITH BRILLIANT BLUE – COOMASIE R250

DESTAINING SOLUTION

Acetic acid	7.0%
Methanol	20.0%

STAINING SOLUTION

RBS–250	0.1%
Methanol	45.0%
Acetic acid	10.0%

50 x TAE

TRIS	2.00 mol/dm^3
EDTA	0.05 mol/dm^3

pH = 7.80

THYLAKOID MEMBRANES ISOLATION BUFFERS

SOLUTION A

Tricine	0.03 mol/dm^3
NaCl	0.015 mol/dm^3
Sucrose	0.3 mol/dm^3

pH = 8.00

SOLUTION B

Tricine	0.005 mol/dm^3

pH = 8.00

SOLUTION C

Tricine	0.005 mol/dm^3
NaCl	0.05 mol/dm^3

pH = 8.00

SOLUTION D

Tricine	0.02 mol/dm^3
Glycerol	20%
$CaCl_2$	0.001 mol/dm^3
$MnCl_2$	0.001 mol/dm^3

pH = 8.00

WESTERN BLOTTING BUFFERS AND DETECTION WITH ANTIBODIES

BLOCKING BUFFER

TRIS	0.01 mol/dm^3
NaCl	0.15 mol/dm^3
TWEEN 20	0.2%

pH = 7.60

1x BLOTTING BUFFER (Can be used repeatedly)

Methanol	400 ml
10x BL. B.	200 ml
dH_2O	1400 ml

10x BLOTTING BUFFER

TRIS	0.25 mol/dm^3
Glycine	1.92 mol/dm^3

pH = 8.30 (do not adjust)

10x TBS BUFFER

TRIS	0.1 mol/dm^3
NaCl	1.5 mol/dm^3

pH = 7.60

BUFFER FOR ANTIBODY DETECTION AND WASHING BUFFER

TRIS	0.01 mol/dm^3
NaCl	0.15 mol/dm^3
TWEEN	0.05%

pH = 7.60

CULTIVATION MEDIA

BACTO AGAR

Components for 1 litre of medium (25 g in 1 litr of distilled water)

Yeast extract 5.0 g

NaCl 10.0 g

Trypton 10.0 g

Agar 1% (10 g in 1 litre of LB medium)

LB MEDIUM HIGH SALT

Components for 1 litre of medium (25 g in 1 litr of distilled water)

Yeast extract 5.0 g

NaCl 10.0 g

Trypton 10.0 g

3.1.3. COMMERCIAL KITS AND VECTORS

COMMERCIAL KITS

CN/DAB SUBSTRATE KIT THERMO SCIENTIFIC

DNA CLEAN & CONCENTRATOR™ – 25 KIT ZYMO-RESEARCH

InnuSPEED RNA KIT (ISOLATION OF RNA FROM PLANTS)
ANALYTIK JENA

PLASMID MIDI AX (KIT FOR ULTRAPURE PLASMID DNA PURIFICATION) A&A BIOTECHNOLOGY

PLASMID MINI (FOR HIGH COPY NUMBER PLASMID DNA ISOLATION)
A&A BIOTECHNOLOGY

SUPER SCRIPT® III FIRST STRAND SYNTHESIS SYSTEM FOR RT-PCR
INVITROGEN™

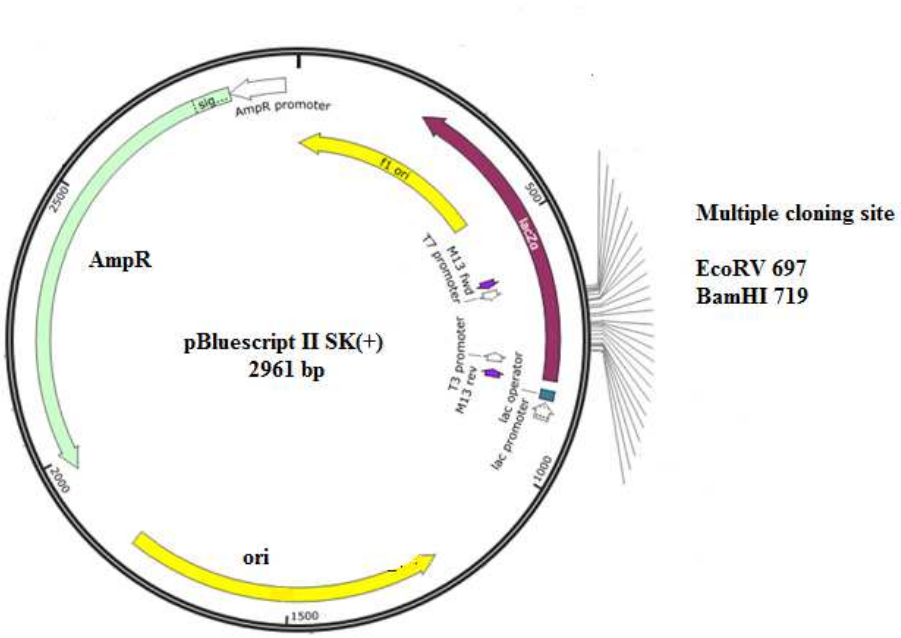
ZYMOCLEAN™ GEL DNA RECOVERY KIT ZYMO-RESEARCH

3’ RACE SYSTEM FOR RAPID AMPLIFICATION OF cDNA ENDS
INVITROGEN™

5’ RACE SYSTEM FOR RAPID AMPLIFICATION OF cDNA ENDS
INVITROGEN™

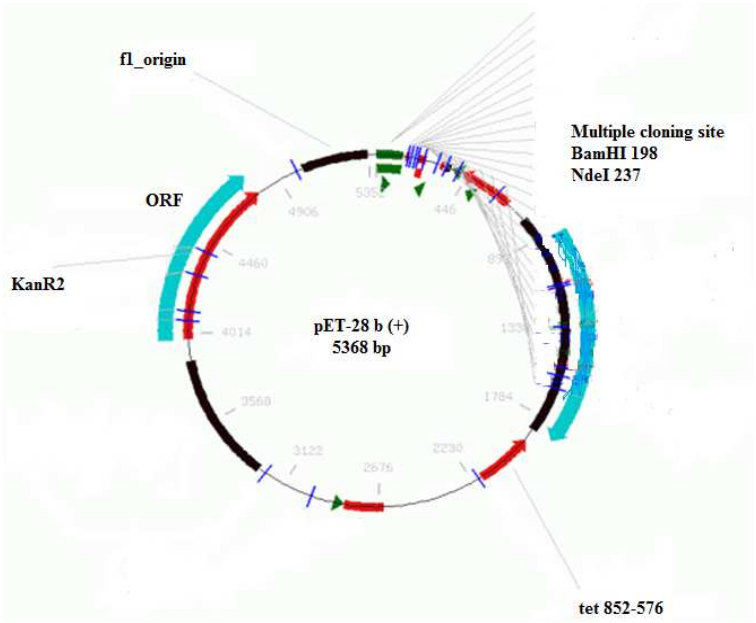
VECTORS

Figure n. 8 **pBluescript II SK(+)**



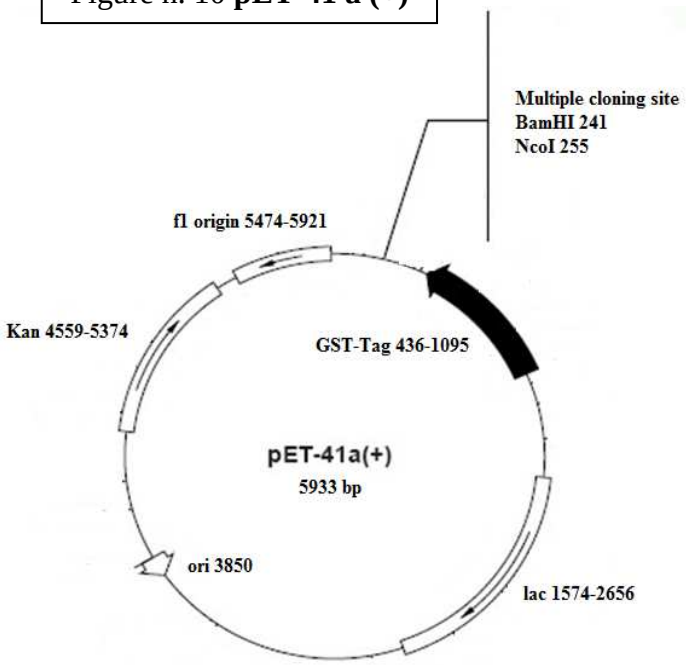
http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pBluescript_II_SK(+)/

Figure n. 9 **pET-28 b (+)**



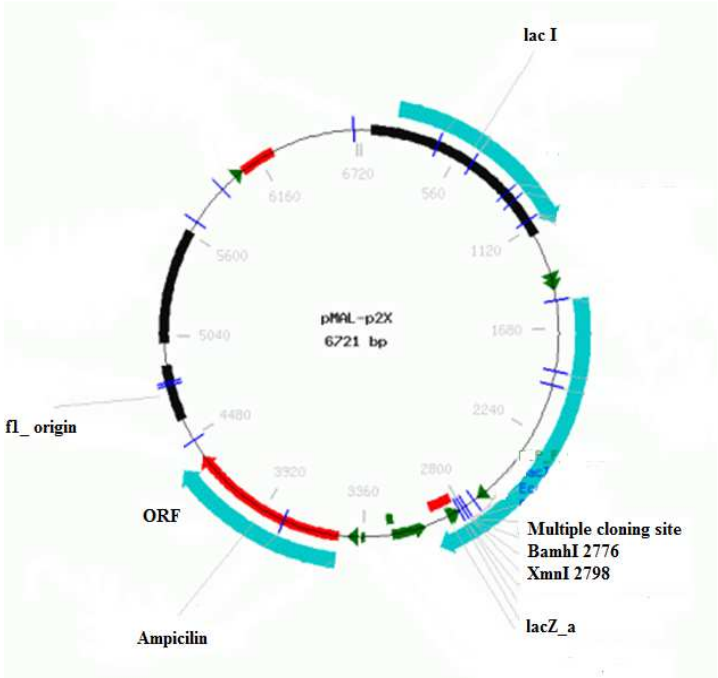
https://www.lablife.org/g?a=seqa&id=vdb_g2.eMqKcsKCvhGh09YXbrRJjhk2iLA-_ sequence_d372486e710e333a23a5bf699ae943b40336d909_10

Figure n. 10 **pET-41 a (+)**



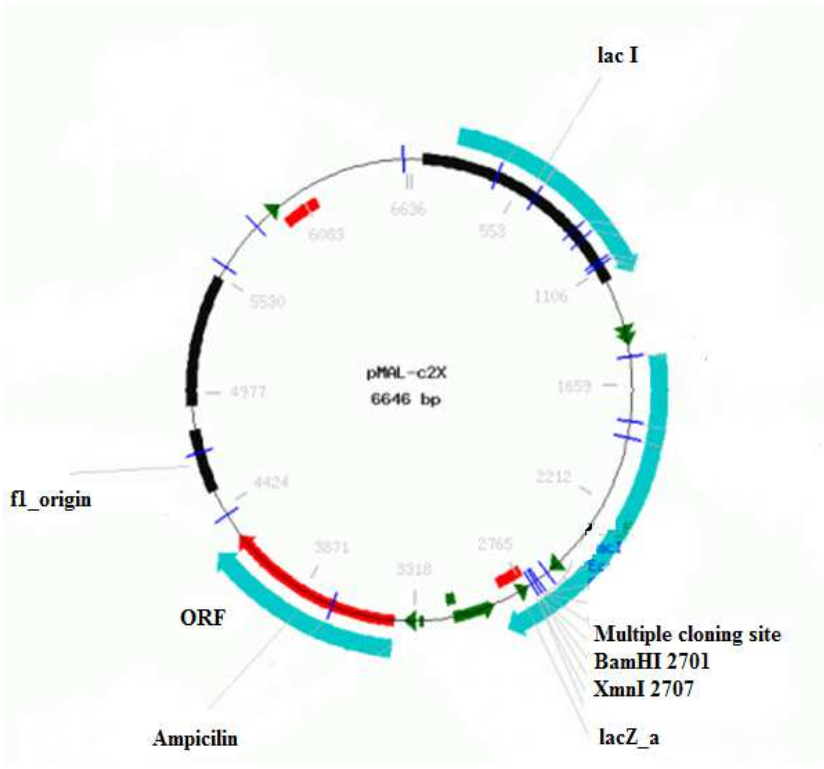
http://ecoliwiki.net/colipedia/index.php/pET-41a(%2B)

Figure n. 11 **pMAL-p2X**



https://www.lablife.org/g?a=seqa&id=vdb_g2.IJiBCIx_5U3JqnRKkNwr8bCvOSk-_sequence_7798f39cb8a7f39a93923ef8ddde85a13559d93a_10

Figure n. 12 **pMAL-c2X**



https://www.lablife.org/g?a=seqa&id=vdb_g2.EHBOxirdwxdkziWnuZpJgRxA.hE-_sequence_58f4805fbd3b34e20c0843369ceb4ae9fc4a4b07_10

3.2. TECHNICAL EQUIPMENT

ACCURATE SCALES ABJ 120-4M	KERN
ACCURATE SCALES U20-3NM	KERN
ANALYTIC SCALES PB153-S	METTLER
ALARM CLOCK	EPPENDORF
ALARM CLOCK	BIOTECHNOLOGIE
AUTOCLAVE	FEDEGARI
CENTRIFUGE PICO 21	THERMO EL. CORP.
CENTRIFUGE 3L-R	HERAEUS
DEEP FREEZER ULTF 80	TRIGON-PLUS®
DIGITAL CAMERA OLYMPUS B202	OLYMPUS
DISINTEGRATOR	EMULSIFLEX
ELECTROPHORETIC APARATURE CAST™ B1	THERMO SCIEN. LTD.
ELECTROPHORETIC APARATURE MULTI SUB. MINI	CLEAVER SCIENTIF.
GAS BURNER	BUNSEN
DIGESTER	FLORES VALLES
ICEMAKER	BREMA®
LAMINAR FLOW CABINET	LABOX
MICROWAVE OVEN	BRAVO
MINICENTRIFUGE MINISPIN PLUS	EPPENDORF
MIXER	PHILIPS
PCR THERMOCYCLER	EPPENDORF
PH METER PH 720	INOLAB
PHOTOMETER BIO PLUS	EPPENDORF
PIPETTES	EPPENDORF
POWER SOURCE EASY	THERMO SCIENTIFIC
POWER SUPPLY EV 231	CONSORT
REFRIGERATOR + FREEZER	ARDO
SCANNER HP SCANJET G3110	ENERGY STAR
SHAKER	TRIGON-PLUS®
SHAKER KS 260 BASIC	IKA®
SPECTROPHOTOMETER UV-2401 PC	SHIMADZU
STIRRER AND HEATER	FISHER SCIENTIFIC

STIRRER MAGNETIC 1000	HEIDOLPH
THERMOSTAT	TRIGON-PLUS®
THERMOSTAT PLUS	EPPENDORF
THERMOSTAT (BUILT IN) TS 606	WTW
TRANSILLUMINATOR ECX-F26-MX	VILBERT-LOURMAT
TRANSILLUMINATOR TCP-20.LC	VILBERT-LOURMAT
ULTRACENTRIFUGE L-90K	OPTIMA
VORTEX MR 100	HEIDOLPH

4. METHODOLOGY

4.1. PROTEIN PsbR FROM *S. OLERACEA*

4.1.1. ISOLATION OF PLANT TOTAL RNA FROM *S. OLERACEA*

This procedure was done with InnuSPEED RNA Kit, which contained all necessary solutions, and also specific columns with matrix, where the RNA was trapped. The procedure was the same for *S. oleracea* like for *P. sativum*.

About 50 mg of fresh leaves were homogenized with 100 µl of PL solution in lysis tube for 3 minutes. Then 350 µl of PL solution was added and the sample was incubated for 10 minutes at room temperature with continuous shaking. Then the sample was centrifuged (1 min.; 14,500 rpm; 20°C). Spin filter D was inserted into receiver tube and obtained supernatant was added to this spin filter D. Followed centrifugation (2 min.; 12,000 rpm; 20°C). Spin filter D was discarded and 400 µl of 70% ethanol was added to the filtrate. Filter R was inserted into new spin receiver tube. The sample was added to spin filter R and centrifuged (2 min.; 12,000 rpm; 20°C). After 500 µl of HS solution was added and centrifuged (1 min.; 12,000 rpm; 20°C). Then 650 µl of LS solution was added and centrifuged (1 min.; 12,000 rpm; 20°C). This last step was once repeated. The filtrate was discarded. Spin Filter was added to the receiver tube and centrifuged (2 min.; 14,500 rpm; 20°C). Spin filter was added to an elution tube and 50 µl of elution buffer was added. Followed incubation 1 minute at room temperature. Finally the RNA in 50 µl of sterile DEPC treated water was obtained by centrifugation (1 min.; 8,000 rpm; 20°C) and acquired RNA was stored in a freezer at -20°C.

All laboratory equipment had to be cleaned with special solution to destroy RNAses and special RNAse-free tips and gloves were used too.

4.1.1.1. PREPARATION OF cDNA USING SUPER SCRIPT® III FIRST STRAND SYNTHESIS SYSTEM FOR RT-PCR

The following procedure was designed to convert 1 pg to 5 µg of total RNA or 1 pg to 500 ng of poly(A)+ RNA into first-strand cDNA. Each component was mixed and briefly centrifuged before being used. The following components were combined in a 0.2 ml tube.

Primer:

oligo(dT)20 5‘ – TTTTTTTTTTTTTTTTTTTT

Component	**Amount**
up to 5 μg total RNA	2.5 μl
50 μM oligo(dT)20	1 μl
10 mM dNTP mix	1 μl
DEPC-treated water to	10 μl

The sample was incubated at 65°C for 5 minutes, then was placed on ice for at least 1 minute. The following cDNA synthesis mix was prepared, adding each component in the indicated order.

Component	**1 Rxn**	**10 Rxns**
10X RT buffer	2 μl	20 μl
25 mM $MgCl_2$	4 μl	40 μl
0.1 M DTT	2 μl	20 μl
RNaseOUT (40 U/μl)	1 μl	10 μl
SuperScript™ III RT (200 U/μl)	1 μl	10 μl

Firstly, 10 μl of cDNA synthesis mix was added to each RNA/primer mixture. All was gently mixed and collected by brief centrifugation. It was incubated 50 minutes at 50°C. The reaction was terminated at 85°C for 5 minutes and chilled on ice. The reactions were collected by brief centrifugation. 1 μl of RNAse H was added to the tube and incubated for 20 minutes at 37°C. Finally cDNA synthesis reaction was used for PCR and stored in the fridge at -20°C (Invitrogen™ manual kit).

4.1.1.1.1. ISOLATION OF PsbR GENE FROM *S. OLERACEA* USING PCR

***S. oleracea* primers:**

FW - PsbR SO *NcoI* 5‘ – CCCATGGGAAGCGGTGTCAAGAA [pET28 b (+) - PsbR, pET41 a (+) -GSTPsbR]

FW - PsbR SO *NdeI* 5‘ – CATATGATGAGCGGTGTCAAGAA (pET28 b (+) - HisPsbR)

FW - PsbR SO *XmnI* 5‘ – GAAGGATTTCAGGTGTCAAGAAGA (pMAL-c2X, pMAL-p2X -MBP-PsbR)

RW - PsbR SO *BamHI* 5‘ – GGATCCTTATTGTGCCAAAGCA

Reaction mixture:	**50 µl**
Buffer for *Taq* DNA polymerase	5 µl
dNTP	0.4 µl
Taq DNA polymerase	0.5 µl
DNA (*S. oleracea*)	2 µl
FW primer	5 µl
RW primer	5 µl
Sterile dH_2O	32.1µl

Temperature-time profile of PCR:

Initial denaturation	94°C	5 minutes	
Denaturation	94°C	1.5 minutes	
Annealing(explanation under)	-°C	2 minutes	35 cycles
Extension	72°C	2 minutes	
Elongation	72°C	10 minutes	

As it can be inferred from the temperature-time profile, the PCR conditions were the same, only the annealing temperature was different for various primers.

For: FW - PsbR SO *NcoI* *61.9*°C

FW - PsbR SO *NdeI* *65*°C

FW - PsbR SO *XmnI* *63*°C

Indefinite hold was 4°C, until the samples were removed. The PCR products were analysed with electrophoretic division in 1.3% agarose gel.

4.1.2. PREPARATION OF CONSTRUCTS WITH PsbR FROM *S. OLERACEA*

4.1.2.1. LIGATION

Three cleaving mixtures were made, and both the vectors and PCR products were cleaved with:

1. ***BamHI*** and ***NcoI*** [**pET28 b (+), pET41 a (+)**]
2. ***BamHI and NdeI*** [**pET28 b (+)]**
3. ***BamHI*** and ***XmnI*** (**pMAL-c2X, pMAL-p2X**)

Vector DNA cleaving:

Cleaving mixture	**10 µl**
DNA	1 µl
BamHI	0.5 µl
Buffer (uniqe)	1 µl
Sterile dH_2O	7.5 µl

DNA (PsbR) cleaving:

Cleaving mixture	**10 µl**
DNA	5 µl
BamHI	0.5 µl
Buffer (uniqe)	1 µl
Sterile dH_2O	3.5 µl

Cleaving conditions were incubation for 1h at 37°C in water bath. After the cleaving the enzyme *BamHI* was inactivated by 20 minutes incubation at 70°C. The mixture was shortly spun and applied to 1.3% agarose gel, from which DNA fragments of our interest were excised with a scalpel and processed using Zymoclean™ Gel DNA Recovery Kit.

Zymoclean™ Gel DNA Recovery Kit procedure:

Firstly 3 volumes of ADB Buffer were added to each volume of our sample (excised agarose gel). It was incubated at 50°C for 5 – 10 minutes (until the agarose was not completely dissolved). Then the melted agarose solution was added into Zymo-Spin column, which was placed into a collection tube. The sample was centrifuged (30 sec.; 14,500 rpm; 20°C) and collection tube was emptied (if necessary). After 200 µl of wash buffer was added to the column and centrifuged (30 sec.; 14,500 rpm; 20°C). This wash step was once repeated. The Zymo-Spin column was placed into a new 1.5 ml tube. Finally 8.5 µl of sterile dH_2O was added directly to the column matrix and spun (30 sec.; 14,500 rpm; 20°C) to elute the DNA.

Followed cleaving with another restriction enzyme *NcoI*, *NdeI* or *XmnI* (as explained above).

Vector DNA cleaving:

Cleaving mixture	10 µl
DNA (in dH_2O)	8.5 µl
Enzyme	0.5 µl
TANGO TM 1X BUFFER	1 µl
Sterile dH_2O	0 µl

DNA (PsbR) cleaving:

Cleaving mixture	10 µl
DNA (in dH_2O)	8.5 µl
Enzyme	0.5 µl
TANGO TM 1X BUFFER	1 µl
Sterile dH_2O	0 µl

Cleaving conditions were incubation for 1h at 37°C in water bath. After this step we were not obliged to use electrophoresis again and we performed only cleaning of our samples using DNA Clean & Concentrator™ – 25 Kit.

DNA Clean & Concentrator™ – 25 Kit procedure:

In the beginning 2 volumes of DNA binding buffer were added to each volume of DNA sample. It was vortexed. Sample was applied into a Zymo–Spin column and this

column was placed into a 2 ml collection tube. Followed centrifugation (30 sec.; 14,500 rpm; 20°C). The flowthrough was discarded. Then, 200 µl of wash buffer was added to the column and spun (30 sec.; 14,500 rpm; 20°C). Zymo–Spin column was placed into a new 1.5 ml tube. The amount of 10 µl of sterile dH_2O was added directly to the column matrix and spun (30 sec.; 14,500 rpm; 20°C) to elute the DNA. Obtained cleaved vectors and the DNA were used in the ligation reaction.

Ligation mixture was prepared and the samples were left overnight in the refrigerator at 4°C (We tried also ligation at room temperature for 1 hour, but because of not satisfactory results and time schedule we mostly preferred the overnight incubation).

Our ligation mixture 10 µl		**Recommended reaction 10 µl**	
DNA (vector)	2 µl	vector DNA	100 ng
DNA insert (PsbR)	5 µl	insert DNA	17 ng
T4 DNA ligase	0.5 µl	T4 DNA ligase	0.1 – 1 µl
T4 DNA buffer	1 µl	T4 DNA buffer	1 µl
Sterile dH_2O	1.5 µl	Sterile dH_2O to	10 µl

The concentration of vectors and PsbR were measured and adjusted to be in concordance with recommended reaction. The samples were incubated overnight in the refrigerator at 4°C (Sambrook *et al*., 2001).

4.1.2.2. PREPARATION OF XL1 AND BL21 *E. coli* COMPETENT CELLS

(For both bacterial strains the procedure was the same, between the centrifugation the cells had to be left on ice.)

To prepare overnight culture one colony of XL1/BL21 was inoculated into 5 ml of LB medium, and also 50 µl of TET was added.

Into 25 ml of LB medium was inoculated 250 µl of overnight culture of BL21 or XL1 *Escherichia coli* strains. The solution was let grown at 37°C to OD 580 = 0.3 – 0.4. Then the cells were left on ice for 5 minutes and centrifuged (10 min.; 2,700 x g; 4°C). The supernatant was poured away and 5 ml of solution ($MgCl_2$ + $CaCl_2$) was added to the pellet and the cells were resuspended. Followed centrifugation (10 min.; 2,700 x g; 4°C). The supernatant was discarded. Afterthat 1 ml of solution (100 mM $CaCl_2$ + 70 µl DMSO) was added and the cells were resuspended. In the end 100 µl aliquots were made and were stored in a deep freezer at -80°C.

4.1.2.3. TRANSFORMATION OF *E. coli* XL1 WITH CONSTRUCTS

Before the proper transformation the ligation mixture had to be inactivated (10 min., 70°C) in water bath and shortly spun.

Competent cells XL1 (100 µl aliquots) were added to the eppendorf tube with inactivated ligation mixture. The whole sample was left on ice for 30 minutes. After the tube was submerged into the water bath set on 42°C for 45 seconds (heat shock) and left on ice for 1 – 2 minutes. Then 400 ml of LB medium was added, and the whole mixture was incubated for 1 hour at 37°C. Followed centrifugation (5 min.; 2,700 x g; 20°C). After 300 ml of supernatant was removed and the cells were resuspended in the rest of supernatant. Petri dishes with appropriate antibiotic (AMP, or KAN) were inoculated with these transformed cells (depend on type of plasmid see 3.1.3. COMMERCIAL KITS AND VECTORS). Petri dishes were left overnight at 37°C, and the next day were examined for possible positive colonies, if contained colonies were stored in the refrigerator at 4°C.

4.1.2.4. ISOLATION OF VECTORS WITH PLASMID MINI ISOLATION KIT (FOR HIGH COPY NUMBER PLASMID DNA ISOLATION)

To prepare overnight culture each colony of XL1 was inoculated into 5 ml of LB medium and 5 µl of apropriate antibiotics (AMP, KAN) was added too.

Bacterial cell pellet was obtained by centrifugation (2 min.; 14,500 rpm; 20°C) from 3 ml of overnight culture of XL1. Then these cells were resuspended in 200 µl of solution L1. After 200 µl of solution L2 was added and the suspension was gently mixed by inverting the tube 5 - 6 times. The mixture was incubated for 3 minutes at room temperature. After this step 400 µl of Solution GL3 was added and the tube was gently 6 - 8 times inverted. The sample was centrifuged (10 min.; 14,500 rpm; 20°C). The supernatant was applied to the designed column. Followed centrifugation (1 min.; 14,500 rpm; 4°C). The filtrate was removed and the column was washed with 500 µl of solution W. Followed centrifugation (1 min.; 14,500 rpm; 20°C). The filtrate was removed and the column was washed with 600 µl of Solution A1. Followed centrifugation (1 min.; 14,500 rpm; 20°C). To elute the DNA the dry column was transferred into a new tube and 60 µl of sterile dH_2O was added directly onto the column membrane. The sample was incubated for 3 minutes at room temperature and centrifuged (1 min.; 14,500 rpm; 20°C). The column was removed and discarded and the plasmid DNA sample was stored in a freezer at -20°C. The result of isolation was analysed

with electrophoretic division in 0.7% agarose gel and if being promising sent to be sequenced (A&A BIOTECHNOLOGY).

4.1.2.5. ISOLATION OF VECTORS WITH PLASMID MIDI AX ISOLATION KIT (KIT FOR ULTRAPURE PLASMID DNA PURIFICATION)

Plasmid midi isolation procedure was performed to obtain bigger and more pure amount of our vectors with desired gene DNA insert. To prepare overnight culture 0.5 ml of XL1 was inoculated into 50 ml of LB medium and 50 µl of apropriate antibiotik (KAN, AMP) was added too.

Firstly 50 ml of overnight bacterial culture of XL1 was centrifuged (10 min.; 4,000 rpm; 4°C). Then the bacterial pellet was resuspended in 5.0 ml of L1 solution. Then 5.0 ml of L2 solution was added and the tube was gently mixed by inverting few times. The mixture was incubated in room temperature for 5 minutes. After 5.0 ml of L3 solution was added and the tube was mixed gently, but thoroughly by inverting few times. The whole mixture was loaded into filtration tube and centrifuged (5 min.; 1,500 x g; 4°C). The filter was removed and the clear lysate flowthrough was applied onto Plasmid 200 column, which was placed in 50 ml tube. The whole volume solution was allowed to pass by gravity. The empty column was washed with 20 ml of K2 washing solution. When the K2 solution stopped pass through the rasin the column was transferred to a 15 ml precipitation tube and the plasmid DNA was eluted by adding 6 ml of K3 elution solution. To condense 25 µl of precipitation enhancer and 5.0 ml of isopropanol was added to the eluate, and the whole contents was mixed. The sample was centrifuged (10 min.; 11,000 x g; 4°C). The supernatant was carefully discarded. The pellet was washed with 2.5 ml of 70% ethanol. The sample was centrifuged (3 min.; 11,000 x g; 4°C). Finally the supernatant was carefully discarded. The tube was placed upside down and the pellet air dried for 5 – 10 minutes at room temperature. The plasmid DNA was dissolved in 500 µl of sterile dH_2O and the plasmid DNA sample was stored in a freezer at -20°C. The result of isolation was analysed with electrophoretic division in 0.7% agarose gel (A&A BIOTECHNOLOGY).

4.1.2.6. CLEAVING OF VECTORS WITH RESTRICTION ENZYMES

The procedure was the same like in the paragraph named: **4.1.2.1. LIGATION**.

The result of linearisation (cleaving with one enzyme) was analysed with electrophoretic division in 0.7% agarose gel. After the detection of possible successful colonies the samples were sent to be further verified by sequencing.

Sometimes it was better to cleave with two restriction endonucleases to cleave out PsbR gene fragment.

Reaction mixture	**10 µl**
Plasmid DNAwith PsbR	2.0 µl
Buffer TANGO 1X TM	1 µl
Enzymes	0.5 + 0.5 µl
dH_2O	6.0 µl

The result of isolation was analysed with electrophoretic division in 0.7% agarose gel. Buffer TANGO 1X TM was used for his ability being compatible with all three enzymes.

4.1.3. ISOLATION OF RECOMBINANT PROTEIN PsbR FROM *S. OLERACEA*

4.1.3.1. TRANSFORMATION OF *E. coli* BL21 WITH PsbR CONSTRUCTS

This procedure was the same like in the paragraph named: 4.1.2.3. TRANSFORMATION OF *E. coli* XL1 WITH CONSTRUCTS.

4.1.3.2. INDUCTION TEST

The samples had to be left on ice when working with them. To prepare overnight culture 50 µl of bacterial cells BL21 containing plasmids pET41 a(+), pET 28 b(+), pMAL-p2X and pMAL-c2X with PsbR inserts were inoculated into 5 ml of LB medium and 5 µl of apropriate antibiotic AMP/ KAN was added too.

Firstly 3 ml of overnight bacterial cells BL21 containing plasmids pET41 a(+), pET 28 b (+), pMAL-p2X and pMAL-c2X with PsbR inserts were added into 300 ml of LB medium with 300 µl of KAN/AMP antibiotic and let grown at 37°C. When optical density reached the value 0.6 by λ = 580 nm the expression of recombinant PsbR protein was triggered by adding of 300 µl from stock 1M IPTG solution. Bacterial cells were harvested after 18h incubation at 20°C. Then the bacterial culture was centrifuged (10 min.; 10,000 rpm; 4°C). Then the cells were homogenized in 1M TRIS/phosphate buffer (pET 28 b (+)

phosphate buffer, all three remaining TRIS buffer). To break them the cells were 10 minutes sonificated and samples were centrifuged (10 min.; 10,000 rpm; 4°C) to be consequently analysed with SDS-PAGE electrophoresis. The rest of unused samples was stored in a deep freezer at -80°C.

In this procedure we tried also to use the homogenizator instead of sonicator, to discover which method was better at obtaining lysated cells. Sonicator proved to have better results in small volumes – to 2 ml, and homogenizator could be used over the 10 ml, in small scale we continued with sonicator.

4.1.3.3. ANALYSIS OF SAMPLES WITH SDS-PAGE ELECTROPHORESIS

According to the manual was built up an electrophoresis aparature. Then followed preparation of a gel with the content of acrylamid in separation gel 15% and in stacking gel 5%. After the gel polymerysation were applied samples solubilizated in solubilization solution (for proteins). Separation of proteins lasted 60 minutes at 25 amperes (for 1 gel). For analysis was used prestained or unstained protein marker.

10 ml separation gel 15%

3.3 ml sterile dH_2O

4.0 ml 30% AA

2.5 ml gel buffer (for separating gel)

0.1 ml 10% SDS

0.1 ml 10% APS

10 µl TEMED

3 ml stacking gel 5%

2.1 ml sterile dH_2O

0.5 ml 30% AA

0.38 ml gel buffer (for stacking gel)

0.03 ml 10% SDS

0.03 ml 10% APS

3 µl TEMED

4.1.3.4. STAINING WITH COOMASIE-BLUE R250

Polyacrylamid gel was after the electrophoresis inserted into staining solution containing Coomasie-Blue for a period of 10 minutes. The incubation in destaining solution enabled us to analyze protein samples.

4.1.3.5. WESTERN BLOTTING

WB had to be run on ice to prevent proteins degradation during that process, when quite high amount of the heat is released. Two type sof membranes were used. At first we used nitrocelulose membrane, but the visualisation was better on PVDF membrane, which is suitable for MALDI TOFF analysis.

After the electrophoresis the gel was transferred into the WB aparature, which was built up following the manual (Sambrook *et al.*, 2001). Filtration paper and blotting membranes were prepared during the electrophoresis. WB cassette was opened and was put together with the rubber into the small box containing a blotting buffer. Filtration paper was added. Followed the PVDF membrane, which was before it equilibrated by dipping in methanol and water for 1 – 2 minutes. Into the centre of this membrane was inserted electrophoresis gel, which was covered into the sandwitch with another filtration paper. Then the second piece of the ruber was added and the whole sandwitch was gently squeezed to get rid of bubbles. The cassette was closed and inserted into the aparature with electrodes containing blotting buffer.

The procedure was performed at 100 V for 30 minutes. Then the aparature was dismantled, and the membrane was blocked for at least 1h in the blocking buffer, (where it could be left for a longer period of time).

4.1.3.6. DETECTION OF PsbR AND HisPsbR WITH ANTIBODIES

At first the membranes were incubated for at least 2h at room temperature in the primary PsbR antibodies. Followed washing in the buffer for antibodies and washing buffer (the same buffer), where the membranes were rinsed for 10 minutes. This washing step was once repeated. Then the membranes were incubated in the secondary antibodies for at least 45 minutes. Followed washing in the buffer for antibodies and washing buffer, where the membranes were rinsed for 10 minutes. This washing step was once repeated. Washing

solution was discarded and the membranes with bounded secondary antibodies were developed with CN/DAB SUBSTRATE KIT.

Mixture total volume:	**2 ml**
CN/DAB substrate 10x	0.2 ml
Substrate buffer 1x	1.8 ml

This amount was sufficient for approximately 10 x 10 cm PVDF membrane.

4.2. PREPARATION OF PsbR FROM *P. SATIVUM*

4.2.1. ALIGNMENT OF PsbR SEQUENCES

Nucleotide sequences of classes of higher plants were looked up thanks to NCBI database and nucleotide blasts were made to find the most conserved sequences. After, the most conserved sequences were used to design primers for PsbR gene from *P. sativum*.

4.2.1.1. ISOLATION OF PLANT TOTAL RNA FROM *P. SATIVUM* AND PREPARATION OF cDNA USING SUPER SCRIPT® III FIRST STRAND SYNTHESIS SYSTEM FOR RT-PCR

The plant RNA from pea and cDNA was prepared according protocol described in in the paragraph named: 4.1.1. ISOLATION OF PLANT TOTAL RNA FROM *S. OLERACEA* and in the paragraph named: 4.1.1.1. PREPARATION OF cDNA USING SUPER SCRIPT® III FIRST STRAND SYNTHESIS SYSTEM FOR RT-PCR.

4.2.1.1.1. ISOLATION OF PsbR GENE FROM *P. SATIVUM* USING PCR

According to alignment of PsbR proteins from *Arachis hypogaea, Trifolium pratense, Glycine max* and *Prosopis juliflora* were designed degenarated and nondegenerated primers.

***P. sativum* primers:**

Forward primers:

NON DEGENERATE: ATGGC C TC T TC A GT G ATGGC A TC

DEGENERATE: ATGGC(CTG)TC(TC)TC(AT)GT(GT)ATGGC(AT)TC

FW - PsbR PS NONDEG 5‘ – ATGGCCTCTTCAGTGATGGCATC

FW - PsbR PS DEG 5‘ – ATGGCBTCYTCWGTKATGGCWTC

Reverse primers:

Reverse complements of:

NON DEGENRERATE: TACAACAC A AGTGC T T T G G C G CAAT

DEGENERATE: TACAACAC(ATC)AGTGC(TC)(TC)T(GT)(GT)C(AG)CAAT

RW – PsbR PS NONDEG 5‘ – ATTGCGCCAAAGCACTTGTGTTGTA

RW - PsbR PS DEG 5‘ – ATTGYGMMARRGCACTDGTGTTGTA

Reaction mixture:	**50 µl**
Buffer for *Taq* DNA polymerase	5 µl
dNTP	0.4 µl
Taq DNA polymerase	0.5 µl
DNA (*P. sativum*)	2 µl
FW DEG/NONDEG primer	5 µl
RW DEG/NONDEG primer	5 µl
Sterile dH_2O	32.1µl

Temperature-time profile of PCR:

Initial denaturation	94°C	3 minutes	
Denaturation	94°C	1 minutes	
Annealing(explanation under)	58-67°C	2 minutes	35 cycles
Extension	72°C	2 minutes	
Elongation	72°C	10 minutes	

Four PCR reactions were made with: FW DEG/ RW DEG, FW NONDEG/ RW NONDEG, FW DEG/ RW NONDEG AND FW FW NONDEG/ RW DEG. Gradient PCR was used with temperature range from 58 - 67°C. Indefinite hold was 4°C, until the samples were removed. The PCR products were analysed with electrophoretic division in 1.3% agarose gel.

4.2.1.1.2. LIGATION INTO pBluescript II SK(+) VECTOR

The PCR product of PsbR gene from *P. sativum* was cloned into the vector pBluescript II SK(+) by method blunt-end cloning.

Linearization and dephosphorylation of vector:

Vector DNA cleaving:

Cleaving mixture	10 µl
DNA (in dH_2O)	2 µl
Enzyme EcoRV	0.5 µl
Buffer for EcoRV	1 µl
Sterile dH_2O	7.5 µl

Incubation at 37°C for 1 hour.

Cleaned from gel by Zymoresearch recovery kit.

Dephosphorylation:

Total volume	15 µl
DNA linearized	10 µl
Buffer fo AP 10x	1.5 µl
AP (alkaline phosphatase)	0.5 µl
Sterile dH_2O	3 µl

Incubated at 65°C for 30 min.

Cleaned by Clean and concentrator kit.

Preparation of PsbR PCR for ligation:

Phosphorylation of PCR:

PCR	5 µl (20ng)
Kinase buffer 10x	1 µl
10mM ATP	1 µl
T4 Polynucleotide kinase	2 µl (2U)
Sterile dH_2O	7.5 µl

Incubation for 1 hour at 37°C. Stoped reaction at 70°C for 10 min.

Cleaned by kit Clean and concentrator.

Our ligation mixture 10 µl		**Recommended reaction 10 µl**	
DNA (vector)	2 µl	vector DNA	100 ng
DNA insert (PsbR)	5 µl	insert DNA	17 ng
T4 DNA ligase	0.5 µl	T4 DNA ligase	0.1 – 1 µl
T4 DNA buffer	1 µl	T4 DNA buffer	1 µl
Sterile dH_2O	1.5 µl	Sterile dH_2O to	10 µl

The samples were incubated overnight in the refrigerator at 4°C. After that were competent cells *E.coli* XL1 transformed by ligation mixture.

4.2.1.1.3. DEFINING OF PROTEIN SEQUENCE USING TRANSLATE TOOLS

To analyse data from sequenator we used Expasy org. translator program, which enabled us to translate our nucleotide sequence into protein sequence (http://web.expasy.org/translate/).

4.2.2. THYLAKOID MEMBRANES ISOLATION

Thylakoid membranes were isolated using the protocol from (Ben-Shen, 2003). All this procedure had to be performed under the green light to prevent excitation of chlorophyll and in cooled recipients.

To 50 g of freshly harvested leaves was added 100 ml of buffer A. This compound was mixed for 15 sec. in a mixer. Then the homogenized suspension was left to flow down by gravity by cheesecloth. The filtrate was centrifuged (7 min.; 1,500 x g; 4°C) to get in pellet chloroplasts. To the pellet was added 80 ml of buffer B and the pellet was homogenized. To remove the starch granules the suspension was centrifuged (2 min.; 500 x g; 4°C). This last step was once repeated. The supernatant was taken and centrifuged (15 min.; 20,000 x g; 4°C). The pellet was resuspended in 40 ml of buffer C and centrifuged (15 min.; 20,000 x g; 4°C). This last step was twice repeated. The pellet was resuspended in 4 ml of buffer D. The spectra were mesured to discover their photosynthetic activity, and the chlorophyll concentration was adjusted by adding of buffer D to 3 mg/ml and the sample was freezed at -80°C.

4.2.3. SDS-PAGE, WB AND DETECTION WITH ANTIBODIES

These methods were the same like in: 4.1.3.3. ANALYSIS OF SAMPLES WITH SDS-PAGE ELECTROPHORESIS, 4.1.3.4. STAINING WITH COOMASIE-BLUE R250, 4.1.3.5. WESTERN BLOTTING and 4.1.3.6. DETECTION OF PsbR AND HisPsbR WITH ANTIBODIES.

4.2.4. MALDI TOFF ANALYSIS

To determine mature PsbR peptide was used method MALDI TOFF. Thylakoid membranes were given onto the SDS gel with total content of acrylamide 15% in separating gel (protocol in paragraph 4.1.3.4. ANALYSIS OF SAMPLES WITH SDS-PAGE ELECTROPHORESIS). Subsequently WB with PsbR antibodies was performed (protocol in paragraph 4.1.3.5. WESTERN BLOTTING), and the PsbR band from *P. sativum* was sent to MALDI TOFF analysis (collaborative group of Assoc. prof. Zbyněk Zdráhal from Brno), where it was digested by proteases (trypsin, chymotrypsine, pepsine, Arg-C), and obtained peptids were subjected to MALDI- MS/MS analysis using Ultraflex III mass spectrometer.

The MS/MS data were searched against NCBI protein sequence database and compared with corresponding PsbR sequence to confirm the presence of the protein and to indicate extent of the signal sequence.

4.2.5. 3' RACE KIT FOR COMPLETING *P. SATIVUM* PsbR SEQUENCE

To determine end of the PsbR sequence from *P. sativum* was used method 3'RACE PCR. This kit consisted from 2 protocols. First of them was: First Strand cDNA Synthesis, which we were not obliged to do, because we did it with SUPER SCRIPT® III FIRST STRAND SYNTHESIS SYSTEM FOR RT-PCR as was mentioned above. The second protocol in this kit was: Amplification of the Target cDNA, which was performed. To a cooled 0.5 ml microcentrifuge tube was added the following:

Primers:

GSP/PsbR/FW/PS/3 5' – CGAATGGCTTCTTTGAGTCTC

AUAP 5' – GGCCACGCGTCGACTAGTAC

Component	**Volume**
10X PCR buffer	5 µl
25 mM $MgCl_2$	3 µl
autoclaved dH_2O	36.5 µl
10 mM dNTP mix	1 µl
GSP (prepared as 10 µM solution)	1 µl
AUAP (10 µM)	1 µl
Taq DNA polymerase (2 to 5 units/µl)	0.5 µl

The amount of 2 µl from the cDNA synthesis reaction was added to the tube. It was gently mixed and the reaction was collected briefly by centrifugation. The reaction was incubated for 3 minutes at 94°C.

Temperature-time profile of PCR:

Initial denaturation	94 °C	3 minutes	
Denaturation	94 °C	30 secondes	
Annealing	58 °C	1 minute	30 cycles
Extension	72 °C	2 minutes	
Elongation	72 °C	10 minutes	

Indefinite hold was 4°C, until sample was removed.

Temperature-time profile of PCR:

Initial denaturation	94 °C	3 minutes	
Denaturation	94 °C	30 secondes	
Annealing	58 °C	1 minute	30 cycles
Extension	72 °C	2 minutes	
Elongation	72 °C	10 minutes	

Indefinite hold was 4°C, until sample was removed.

The result of amplification was analysed with electrophoretic division in 1.3% agarose gel (INVITROGEN™). Samples were sent to be sequenced and the results were translated using Expasy org. translator program (http://web.expasy.org/translate/).

4.2.6. PREPARATION OF CONSTRUCTS OF RECOMBINANT PsbR PROTEIN FROM *P. SATIVUM* USING pMAL-c2X AND pET-41 a (+) VECTORS

From MALDI-TOFF analysis was possible to determine mature peptid sequence and according it to design primers for *P. sativum* PsbR. The sequence from beginning of the protein is the same as in *P. sativum* as in *S. oleracea*, so the primers for *S. oleracea* could be used for *P. sativum*. The end of PsbR sequence is similar, but not identical, so revers primer had to be designed.

FW - PsbR SO *NcoI* 5‘ – CCCATGGGAAGCGGTGTCAAGAA [pET28 b (+) -PsbR, pET41 a (+) -GSTPsbR]

FW - PsbR SO *XmnI* 5‘ – GAAGGATTTCAGGTGTCAAGAAGA (pMAL-c2X, pMAL-p2X -MBP-PsbR)

RW - PsbR SO *BamHI* 5‘ – GGATCCTTATTGCGACAGGGCA

Constructs of recombinant protein PsbR from *P. sativum* with MBP and GST anchor were prepared like constructs of recombinant PsbR from *S. oleracea* (protocols in paragraph 4.1.2.1. LIGATION to 4.1.2.3. TRANSFORMATION OF *E. coli* XL1 WITH CONSTRUCTS).

4.2.7. 5' RACE KIT FOR COMPLETING THE BEGINNING OF *P. SATIVUM* PsbR SEQUENCE

Primer:

GSP1 5‘ – TTGCGACAGGGCACTTGTGT

First strand cDNA synthesis

GSP1	2,5pmoles (10 to 25 ng)	0.6 µl
Sample RNA	1-5 ng	3 µl
DEPC-treated water		11.9 µl

The mixture was incubated for 10 minutes at 70°C to denature RNA. Then it was chilled 1 minute on ice and collected by brief centrifugation. The following was added in the order given:

10X PCR Buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
10 mM dNTP mix	1 µl
0.1 M DTT	2.5 µl
Final volume	8.5 µl

The final volume of these two steps was 24 µl. The reaction was gently mixed and collected by short centrifugation. It was incubated for 1 minute at 42°C. After was added 1 µl of SuperScript^{TM} II RT. The reaction was gently mixed and incubated for 50 minutes at 42°C. To terminate the previous reaction it was incubated at 70°C for 15 minutes. Followed centrifugation from 10 to 20 secondes and the mixture was placed at 37°C. Finally was added 1 µl of RNase mix, it was gently but thoroughly mixed, and incubated for 30 minutes at 37°C. The reaction wa collected by brief centrifugation and placed on ice.

S.N.A.P. Column Purification of cDNA

The binding solution was equilibrated to the room temperature. 100 µl of sterilized dH_2O was equilibrated at 65°C. Then 120 µl of binding solution (6M NaI) was added to the first strand reaction. The cDNA/NaI solution was transferred to a S.N.A.P. column. It was centrifuged (20 sec.; 13,000 x g; 20°C). The cartridge insert was removed from the tube and the flowtrough was transferred to a microcentrifuge tube. The solution was saved until the recovery of cDNA was confirmed. The cartridge insert was placed back into the tube. The amount of 0.4 ml of cold (4°C) 1x wash buffer was added to the spin cartridge. It was centrifuged (20 sec.; 13,000 x g; 20°C). The flowthrough was discarded and this wash step was repeated three additional times. The cartridge was washed with 400 µl of 70% ethanol

and centrifuged (20 sec.; 13,000 x g; 20°C). This washing step was once repeated. After removing the final 70% ethanol wash from the tube it was centrifuged (1 min.; 13,000 x g; 20°C). The spin cartridge insert was transferred into a fresh sample recovery tube. In the end 50 µl of sterilized, dH_2O (preheated to 65°C) was added to the spin cartridge. Followed centrifugation (1 min.; 13,000 x g; 20°C) to elute the cDNA.

TdT tailing reaction

The following components were added to a tube and mixed gently.

Component	Volume
DEPC-treated water	6.5 µl
5X tailing buffer	5.0 µl
2 mM DTP	2.5 µl
S.N.A.P.-purified cDNA sample	10.0 µl
Final volume	24.0 µl

It was incubated for 2 - 3 minutes at 94°C. Then 1 minute chilled on ice. The contents of the tube were collected by brief centrifugation and placed on ice. 1 µl of TdT was added, it was gently mixed and incubated for 10 minutes at 37°C.

PCR of dC-tailed cDNA

The thermal cycle block was equilibrated to 94°C. To a 0.2 ml thin-wall PCR tube sitting on ice was added the following.

Primers:

GSP2/PsbR/RW/PS/5 5‘ – CATCTGTTCCTCACGTGGTG

AAP 5‘ – GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG

Component	**Volume**
Sterilized dH_2O	31.5 µl
10X PCR buffer	5.0 µl
25 mM $MgCl_2$	3.0 µl
10 mM dNTP mix	1.0 µl
Nested GSP2 (prepared as 10 µM solution)	2.0 µl
Abridged Anchor Primer (10 µM)	2.0 µl
dC-tailed cDNA	5.0 µl
Final Volume	49.5 µl

At last 0.5 µl of Taq DNA polymerase was added and the tube was immediately mixed. The tube was transferred directly from ice to the thermal cycle preequilibrated to 94°C and PCR reaction performed.

Temperature-time profile of PCR:

Initial denaturation	94°C	3 minutes	
Denaturation	94°C	30 secondes	
Annealing	55°C	30 secondes	30 cycles
Extension	72°C	1 minute	
Elongation	72°C	7 minutes	

Indefinite hold was 4°C, until sample was removed. The PCR product was analysed with electrophoretic division in 1.3% agarose gel.

Nested amplification

At first 5 µl aliquot of the primary PCR product was diluted into 495 µl of TE buffer. The thermal cycle block was equilibrated to 94°C. The following was added to a 0.2 ml thin-wall PCR tube sitting on ice.

Primers :

nGSP/PsbR/FW/PS/5 5‘ – GTCGATCTTCTGGCCGATCT

AUAP 5‘ – GGCCACGCGTCGACTAGTAC

Component	**Volume**
Sterilized dH_2O	33.5 µl
10X PCR buffer	5.0 µl
25 mM $MgCl_2$	3.0 µl
10 mM dNTP mix	1.0 µl
Nested GSP (10 µM)	1.0 µl
AUAP (10 µM)	1.0 µl
Dilution of primary PCR product	5.0 µl
Final Volume	49.5 µl

It was very important to have the reaction mixture ice cooled to avoid nonspecific binding and extension of primers. To the reaction 0.5 µl of DNA *Taq* polymerase was added and the mixture was immediately mixed and shortly centrifuged. A tube was transferred directly from ice to thermal cycler preequilibrated to 94°C and PCR performed.

Temperature-time profile of PCR:

Initial denaturation	94 °C	3 minutes	
Denaturation	94 °C	30 secondes	
Annealing	55 °C	30 secondes	30 cycles
Extension	72 °C	1 minute	
Elongation	72 °C	7 minutes	

Indefinite hold was 4°C, until sample was removed. The PCR product was analysed with electrophoretic division in 1.3% agarose gel. All this procedure was done using INVITROGEN™ 5' RACE SYSTEM FOR RAPID AMPLIFICATION OF cDNA ENDs KIT.

4.3. ANALYSIS OF PsbW AND PsbX

For analysis of PsbW and PsbX proteins were used nucleotide sequences of classes of higher plants by using NCBI database, and nucleotide blasts were made to find the most conserved sequences. Both proteins did not have conserved sequences to design primers and MALDI TOFF analysis was the possible way to detect amino acids of these proteins. For MALDI TOFF analysis were used thylakoid membranes (paragraph 4.2.2. THYLAKOID MEMBRANES ISOLATION) and firstly send bands from SDS – PAGE with different content of acrylamide in separation gel (gradient gel 12% + 16%, 12% + 18%). SDS-PAGE electrophoresis had to be optimized because of low molecular mass of both proteins. The acrylamide content in separation gel and the conditions of running of gel had to be optimized.

10 ml separation gel 12%	**10 ml separation gel 16%**
4 ml sterile dH_2O	3.1 ml sterile dH_2O
3.3 ml 30% AA	4.2 ml 30% AA
2.5 ml gel buffer (for separating gel)	2.5 ml gel buffer (for separating gel)
0.1 ml 10% SDS	0.1 ml 10% SDS
0.1 ml 10% APS	0.1 ml 10% APS
10 µl TEMED	10 µl TEMED

3 ml stacking gel 5%	**10 ml separation gel 18%**
2.1 ml sterile dH_2O	2.7 ml sterile dH_2O
0.5 ml 30% AA	4.7 ml 30% AA
0.38 ml gel buffer (for stacking gel)	2.5 ml gel buffer (for separating gel)
0.03 ml 10% SDS	0.1 ml 10% SDS
0.03 ml 10% APS	0.1 ml 10% APS
3 µl TEMED	10 µl TEMED

The amount of sample given into one well was 5 µl (the total chlorophyll concentration 2 ng). Finaly it was shown that suitable method for analysis of bands for MALDI TOFF is Western blotting, which was performed according to protocol (paragraph 4.1.3.5. WESTERN BLOTTING). That procedure was the same like in paragraph: 4.1.3.6. DETECTION OF PsbR AND HisPsbR WITH ANTIBODIES, only different primary antibodies were used. For PsbX protein were used antibodies anti-PsbX and for PsbW

protein were used antibodies anti-PsbW. Finally the samples were sent to be examined using mass spectrometry.

5. RESULTS

5.1. PCR PRODUCT OF PsbR FROM *S. OLERACEA*

Obtained PCR product of PsbR from spinach should have 296 bp.

1. **2.**

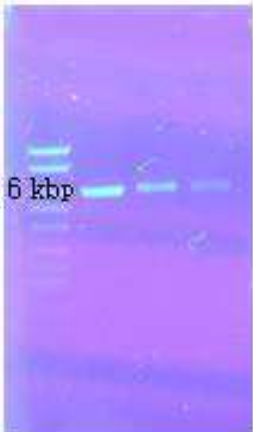
Figure n. 13: **PCR PsbR from *S.oleracea***

1. MASS RULER LOW RANGE
2. PsbR PCR from *S. oleracea*

5.1.1. CONSTRUCTS OF PsbR FROM *S. OLERACEA*

Vectors pET28 b (+) with cloned PCR products PsbR (*NcoI*) and PsbR (*NdeI*) were sent to automated DNA sequenator. Colonies with PsbR sequence were used for other analysis.

5.1.1.1. pET-41 a (+) WITH INSERTED PsbR (*NcoI*)



1. **2.** **3.** **4.**

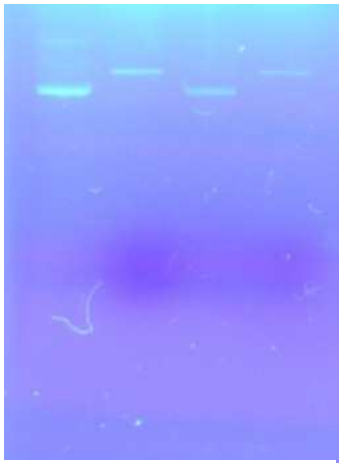
Figure n. 14: **Analysis of plasmids (pET 41 a (+))**

1. Mass RULER HIGH RANGE
2. pET41 a (+) (control) cleaved with *NcoI*
3. – 4. pET41 a (+) + PsbR cleaved with *NcoI*

Samples 1. and 2. were in comparison with the control bigger, so their concentration was measured, and they were sent to be sequenced to verify successful insertion of PsbR fragment. Both of them contained PsbR sequence and were used for transformation of competent cells *E.coli* BL21 and for overexpression.

5.1.1.2. OPTIMIZATION RESULTS AND pMAL-p2X AND pMAL-c2X WITH INSERTED PsbR

To prepare constructs pMal-p2X and pMAL-c2X with PsbR gene from *S. oleracea* we had to do optimization of DNA amount (vectors) in cleaving reaction, type of buffer and the amount of restriction endonuclease.

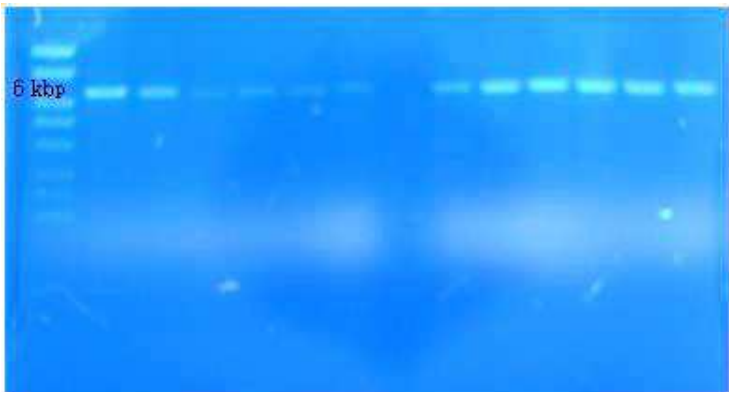


1. **2.** **3.** **4.**

Figure n. 15: **Cleaving of pMal-p2X and pMAL-c2X with *BamHI* (method optimization)**

1. pMAL-p2X (control)
2. pMAL-p2X cleaved with *BamHI*
3. pMAL-c2X (control)
4. pMAL-c2X cleaved with *BamHI*

We continued with ligation and transformation of these vectors after optimization. For analysis by restriction enzymes were taken five colonies.



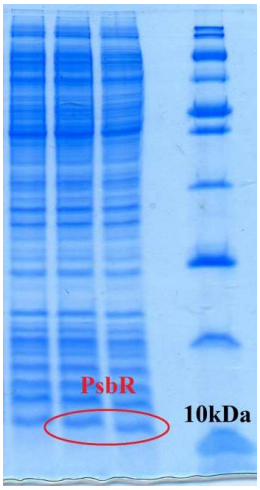
1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13.

Figure n. 16: **Analysis of plasmids** (**pMAL-p2X and pMAL-c2X)**

1. MASS RULER HIGH RANGE
2. pMAL-p2X (control) cleaved by *BamHI*
3. – 7. pMAL-p2X + PsbR cleaved by *BamHI*
8. pMAL-c2X (control) cleaved by *BamHI*
9. – 13. pMAL-c2X + PsbR cleaved by *BamHI*

Only colonies pMAL-p2X number 5 and pMAL-c2X number 2 and 3 were sent to automated sequenator and it was shown that colonies contained desired inserts and were preserved to continue experiments with them (transformation of *E.coli* BL21 and overexpression).

5.1.2. INDUCTION OF HisPsbR, GST PsbR, pMAL-p2X AND pMAL-c2X WITH IPTG



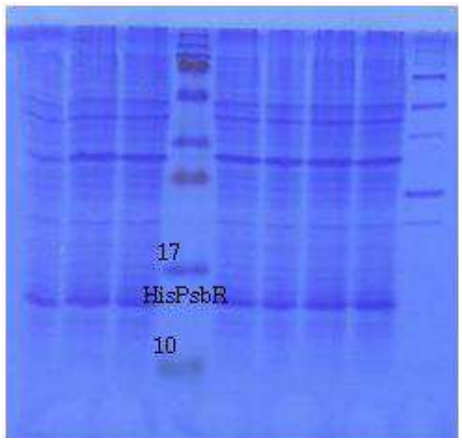
1. 2. 3. 4.

1. – 3. PsbR protein

4. PROTEIN MARKER prestained

Figure n. 17: **SDS-PAGE: Overexpression of PsbR protein in different temperatures 15°C, 30°C and 37°C (pET 28b+ +PsbR(*NcoI*))**

As can be seen from the gel above we wanted to induce overexpression of HisPsbR in pET28 b (+) vector by using different temperature conditions. This was done using 1M IPTG. As it can be seen we achieved this induction in three lines, where thick bands can be seen.



1. 2. 3. 4. 5. 6. 7. 8.

1. – 3. HisPsbR overexpression

4. PROTEIN MARKER prestained

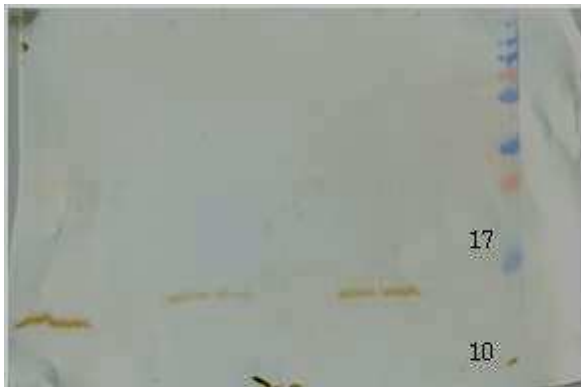
5. – 8. HisPsbR overexpression

9. PROTEIN MARKER unstained

Figure n. 18: **SDS-PAGE: Overexpression of HisPsbR at 30°C 7 different colonies (pET28b+ + PsbR (*NdeI*)=HisPsbR**

Overexpression of PsbR protein cloned in vector pET28 b (+) in form PsbR and HisPsbR showed not good overexpression of recombinant protein. The Western blot analysis was performed to detect presence of PsbR proteins in both constructs.

5.1.3. DETECTION OF PsbR AND HisTag PsbR WITH ANTIBODIES

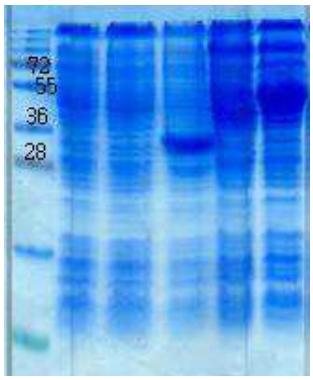


1. 2. 4. 5. 7. 8. 10.

Figure n. 19: **Western blot analysis (anti-PsbR)**

1. – 2.	PsbR – overexpression at 15°C and 30°C
4. – 5. and 7. – 8.	HisPsbR –overexpresion at 15°C and 30°C-two different vectors contained PsbR sequence
10.	PROTEIN MARKER prestained

The results of Western blotting showed that the constructs contained PsbR sequence, but overexpression could not be improved by different temperatures. A problematic constructs could not be used for isolation of recombinant PsbR protein used for other structural studies because of low yield of protein.



1. 2. 3. 4. 5. 6.

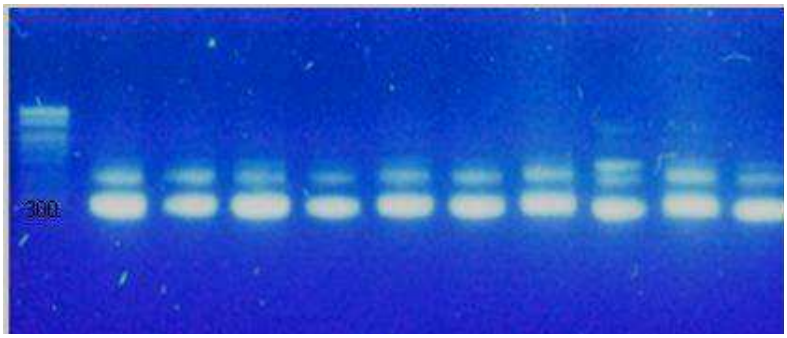
Figure n. 20: **SDS-PAGE: Overexpression of *S. oleracea* PsbR protein in different vectors at 30°C**

1. PROTEIN MARKER prestained
2. HisPsbR (pET28 b (+)) overexpression
3. PsbR (pET28 b (+)) overexpression
4. GST PsbR (pET41 a (+)) overexpression
5. MBP-PsbR (pMAL p2x)
6. MBP-PsbR (pMAL c2x)

We compared overexpression of PsbR protein at 30°C in different constructs. From the results is obvious that MBP-PsbR (pMAL-c2X) appeared very promising on the gel with its molecular mass corresponding to the expected values and also a very strong expression, which made it an excellent adept for our next trials. The overexpression of MBP-PsbR (pMAL-p2X) was not very successful. The size of GST-PsbR corresponded to expected molecular mass, and the overexpression of proteins was successful. The stability of fusion proteins, optimization steps of purification protocol and expense of all procedure will be other factors to determine suitable constructs used for purification of recombinant proteins PsbR.

5.2. *P. SATIVUM* RESULTS

5.2.1. PCR PRODUCTS OF PsbR FROM *P. SATIVUM*



1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11.

Figure n. 21: **Gradient PCR (58°C - 67°C) of PsbR from *P. sativum***

1. MASS RULER LOW RANGE

2. – 11. PCR products of PsbR from *P. sativum* (gradient of temperature)

The size of PsbR gene is around 400 bp. Successful PCR reaction for PsbR from *S. oleracea* was by using primers: RWDEG/FWNONDEG. The result PCR product was cloned into the pBluescript vector.

5.2.1.1. pBluescript WITH INSERTED PsbR

1. 2. 3.

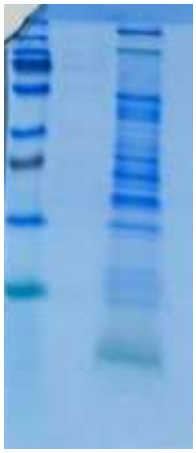
Figure n. 22: **Analysis of plasmids (pBluescript SK II(+))**

1. MASS RULER HIGH RANGE
2. pBluescript SK II(+) (control) cleaved by *BamHI*
3. pBluescript SK II(+) + PsbR cleaved by *BamHI*

This PCR product was ligated into pBluescript II SK(+) to obtain nucleotide sequence of the PsbR gene. The PCR products were sent to be sequenced and then used using Expasy org. translator tool, publicaly disponible on: http://web.expasy.org/translate/, where our sequence was inserted to discover the protein sequence of mature peptide of the PsbR protein from *P. sativum*.

Met A S L S L K P T P F T V Q K S S V K G L P S I S R P F R V V A S G V K K I K T D T P Y G T G G G **Met** D L P N G L D A S G R K Q R G K G V Y Q F V D K Y G A N V D G Y S P I Y E P K E W S A T G D V Y A G G T T G L A I W A V T L A G L L A G G A L L V Y N T S A L S Q

5.2.2. ISOLATION OF THYLAKOIDS MEMBRANE AND SDS-PAGE ELECTROPHORESIS OPTIMIZATION



1. **2.**

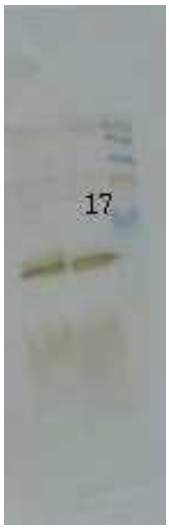
Figure n. 23: **Thylakoid membranes**

1. PROTEIN MARKER prestained

2. sample of separated proteins from thylakoid membranes

We have to optimized concentration of acrylamide content in separating gel in SDS-PAGE and concentration of samples given on the gel. The gel does not have to be over-loaded, but the ammount has to be sufficient.

5.2.2.1. DETECTION OF PsbR FROM THYLAKOID MEMBRANES



1. 2. 3.

Figure n. 24: **PsbR protein on PVDF membrane**

1. – 2. PsbR hybridized with secondary antibodies

3. PROTEIN MARKER prestained

On this PVDF blotting membrane can be well seen PsbR. Pieces of membrane with hybridised antibodies were excised and sent to MALDI TOFF analysis. Samples provided high-quality MALDI-MS, MALDI-MS/MS and LC-MS/MS data.

Trypsin/chymotrypsin - bez imunodetekce

```
  1 MASLSLKPTP FTVQKSSVKG LPSISRPFRV VASGVKKIKT DTPYGTGGGM
 51 DLPNGLDASG RKQRGKGVYQ FVDKYGANVD GYSPIYEPKE WSATGDVYAG
101 GTTGLAIWAV TLAGLLAGGA LLVYNTSALS Q
```

Arg-C - bez imunodetekce

```
  1 MASLSLKPTP FTVQKSSVKG LPSISRPFRV VASGVKKIKT DTPYGTGGGM
 51 DLPNGLDASG RKQRGKGVYQ FVDKYGANVD GYSPIYEPKE WSATGDVYAG
101 GTTGLAIWAV TLAGLLAGGA LLVYNTSALS Q
```

Pepsin - bez imunodetekce

```
  1 MASLSLKPTP FTVQKSSVKG LPSISRPFRV VASGVKKIKT DTPYGTGGGM
 51 DLPNGLDASG RKQRGKGVYQ FVDKYGANVD GYSPIYEPKE WSATGDVYAG
101 GTTGLAIWAV TLAGLLAGGA LLVYNTSALS Q
```

Arg-C - bez imunodetekce

```
  1 MASLSLKPTP FTVQKSSVKG LPSISRPFRV VASGVKKIKT DTPYGTGGGM
 51 DLPNGLDASG RKQRGKGVYQ FVDKYGANVD GYSPIYEPKE WSATGDVYAG
101 GTTGLAIWAV TLAGLLAGGA LLVYNTSALS Q
```

By MALDI TOFF analysis was determined sequence of mature peptide of *P. sativum* PsbR protein.To determine full sequence the method 3'RACE PCR was choosen.

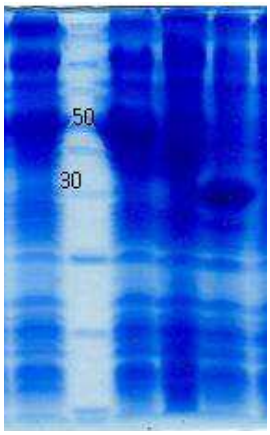
5.2.3. 3' RACE KIT FOR COMPLETING *P. SATIVUM* PsbR SEQUENCE

The results were obtained using Expasy org. translator tool, publicaly disponible on: http://web.expasy.org/translate/, where our sequence was inserted to discover the sequence of 3' end of the PsbR protein from *P. sativum*. From six possible results was chosen the right sequence with 5'3' reading frame 1.

F R V V A S G V K K I K T D T P Y G T G G G **Met** D L P N G L D A S G R K Q R G K G V Y Q F V D K Y G A N V D G Y S P I Y E P K E W S A T G D V Y A G G T T G L A I W A V T L A G L L A G G A L L V Y N T S A L S Q **Stop**

As it can be inferred from the sequence above, these data verified our sequence and assured us about 3' end, which ends with L,S and Q proteins placed before the stop codon. These results were in accordance with the results from MALDI TOFF analysis and we were able to design primers for preparation of MBP-PsbR and GST-PsbR

5.2.4. INDUCTION OF MBP-PSBR AND GST-PsbR WITH IPTG



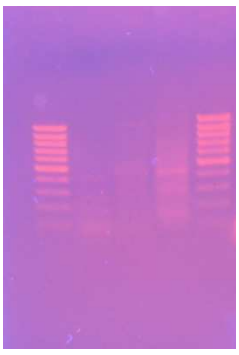
1. 2. 3.

Figure n. 25: **SDS-PAGE: Overexpression of pea PsbR (MBP-PsbR and GST-PsbR) at 30°C**

1. MBP-PsbR (pMAL-c2x) induction
2. PROTEIN MARKER unstained
3. GST-PsbR (pET41a+) induction

The preparation of constructs of recombinant protein PsbR from pea was successfully determined with overexpression of GST-PsbR and MBP-PsbR.

5.2.5. 5' RACE KIT FOR COMPLETING *P. SATIVUM* PsbR SEQUENCE



1. 2. 3. 4. 5

Figure n. 26: **5'RACE PCR products**

1. and 5. MASS RULER LOW RANGE
2. and 3. 5'RACE PCR product
4. nested 5'RACE PCR product

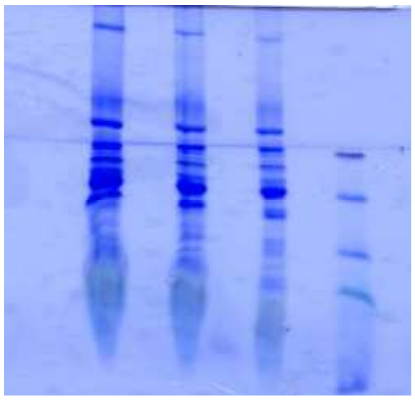
This method has to be optimized, because sequenced PCR products were not good.

5.3.PsbW AND PsbX PROTEINS

5.3.1. SDS-PAGE ELECTROPHORESIS

SDS-PAGE OF THYLAKOID MEMBRANES

Because of low molecular mass of proteins PsbX and PsbW, the SDS-PAGE had to be optimized, not only by different contained of acrylamide in separating gel. We have to optimized conditions of running gels.

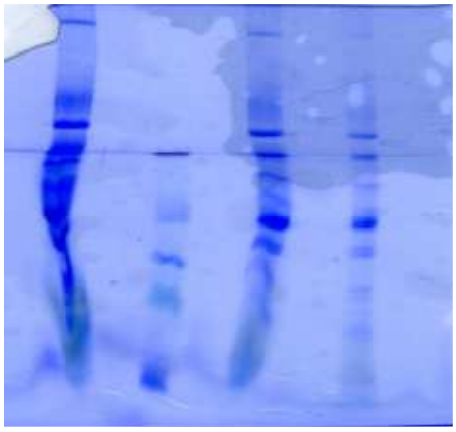


1. 2. 3. 4.

1. PROTEIN MARKER prestained

2. – 4. lines with free thylakoid membranes samples

Figure n 27: **SDS – PAGE: Thylakoid membranes gradient gel (12 and 16%)**



1‘. 2‘. 3‘. 4‘.

1. PROTEIN MARKER prestained

2‘. – 4‘. Lines with free Thylakoid membranes samples

Figure n 28: **SDS – PAGE: Thylakoid membranes gradient gel (12 and 18%)**

Here we can see two SDS-PAGE gels with different concentrations of acrylamid. We tried which density would be better for protein separation. In this case the first gel with SDS

16% seems better than the second one with kontent of SDS 18%. It was shown, that we have to run gel not at 25mA like for PsbR detection, but at 13mA.

5.3.2. DETECTION OF PsbW AND PsbX WITH ANTIBODIES AND OPTIMIZATION

1. 2. 3.

Figure n. 29: **Western blot analysis (anti-PsbW)**

1. – 2. PsbW protein

3. PROTEIN MARKER prestained

1. 2. 3.

Figure n. 30: **Western blot analysis (anti-PsbX)**

1. – 2. PsbX protein

3. PROTEIN MARKER prestained

On these PVDF blotting membranes can be well seen PsbW protein, PsbX is not very well visible. Primary antibodies were sitting in the upper part (predicted binding with other

proteins of photosystem II) and to the lower part (expected proteins). Bands were sent to MALDI TOFF analysis, but from bands of low molecular mass was not possible to make all analysis and from bands with high molecular mass were detected some fragments of studied proteins, but all method is now in progress.

Optimization of purification sample from plants, optimization of SDS-PAGE and Western blot method were time consuming procedures, which did not allow us to determine the sequence of these two proteins.

6. CONCLUSION

The first aim of this diploma thesis was to determine the sequence of PsbR protein from *Pisum sativum* and prepare construct of recombinant PsbR protein from higher plants (*Pisum sativum* and *Spinacia oleracea*). Higher plants PsbR protein is small 10 kDa polypeptide with C-terminal membrane part, which could cause problematic purification. The preparation of recombinant *S. oleracea* PsbR protein in form of PsbR and HisPsbR showed problematic overexpression, which was eliminated by preparation of fusion proteins GST-PsbR and MBP-PsbR with successful overexpression. Simultaneously it was possible to determine protein sequence of mature peptide of *P. sativum* PsbR and subsequently to prepare constructs of recombinant protein PsbR (MBP-PsbR, GST-PsbR). The full sequence of transit part of this protein is not yet confirmed, and the method called 5'RACE PCR will be provided to determine the full part.

The second goal of this writing was to determine sequences of PsbX and PsbW proteins from *Pisum sativum*. It is still under construction. The methods of isolation of native proteins and MALDI TOFF analysis were optimized, but unfortunately time consuming analysis did not allow yet to determine sequences of both proteins.

7. DISCUSSION

This diploma thesis was provided in the laboratory of crystalography leaded by Assoc. Prof. Ivana–Kuta Smatanova. One of the project of this group are structural studies of higher plants photosystem II and photosynthetic proteins. First aim of this work was to prepare recombinant protein PsbR for structural studies (NMR, X-ray and interaction studies with other extrinsic proteins). Model organisms were *P. sativum* and *S. oleracea*. Crystallization is the method, which is very specific and unpredictible. It may happen that two proteins, which are very similar do not react (crystallize) in the same way under the resembling conditions. It is the reason for which we worked with these two plants at the same time.

Without problems we obtained RNA from our two samples and were capable to transform it into cDNA and afterthat into DNA, which was used in PCR reaction with our primers. PCR reaction from *S. oleracea* was successful, but from *P. sativum* had to be optimized (gradient PCR). Resulting PCR products were firstly cloned into the pBluescript for better sequencing in the case of *P. sativum* and for better ligation to expression vector in the case of *S. oleracea*. Finally it was shown that the step of ligation into the pBluescript can be omitted and PCR product can be cloned immediately into the expression vectors. We continued with ligation and tranformation with our vectors, and we managed to obtain five vectors with PsbR *S. oleracea* gene. From those five candidates we found out, that the most suitable for our next experiment would be pMAL-c2X and pET41 a (+), which were subsequently used also for *P. sativum*. In the case of PsbR and HisPsbR we had some problems with protein induction, where we discovered that high temperatures were not good for recombinant protein production. But even the decrease to 15°C did not have wanted effect, so these two vectors were rejected. By using all described methods we were able to prepare constructs like fusion protein with good overexpression - GST-PsbR and MBP-PsbR from both model organisms.

The sequence of mature peptide from *P. sativum* PsbR protein was determined successfully by method MALDI TOFF. Native PsbR protein is very unstable, and up to now we were not able to prepare pure, stable sample in solution for MALDI TOFF analysis. For this case has to be provided analysis from PsbR bands cutted from SDS-PAGE. During MALDI TOFF analysis it was shown that this band contained not only our PsbR protein, but also degraded products of other proteins, and it was really hard to analyse this band. So we had to try all possible protocols to isolate plant material suitable for this analysis. We tried

preparation of different modifications of so called KM membranes (according to protocol Kuwabara and Murata), then BBY membranes (protocol of Berthold, Babcock and Yocum), but finally we had to prepare plant material by modification of protocol described in this diploma thesis. The studies of PsbR protein by MALDI TOFF was useful for work with other photosynthetic proteins too. After optimization of purification protocol and western blotting we were able to get all sequence of mature peptide of *P. sativum* PsbR protein. Time consuming experiments allowed us to determine only the sequence of mature peptide. So called 3´RACE PCR was achieved with good results for the very first time and thus the 3´end remains clear. To determine all sequence of *P. sativum* PsbR the method 5´RACE PCR was choosen. Unfortunately, we did not optimized condition of this method in case of *P. sativum* PsbR and it is now in progress.

The second goal of this project was determination of the protein sequence of PsbW and PsbX proteins from *P. sativum*. These two proteins are small proteins with molecular mass around 4 kDa. Their molecular mass is problematic for isolation of bands from PVDF membrane for MALDI TOFF analysis. In the case of PsbW protein it was not possible to find homologs to design primers, and in the case of PsbX protein there are a lot of uncertain information in NCBI database, for which we finally decided to verify its sequence. The isolation of plant material was the same problem like in the case of PsbR protein, but it was not the last problematic step. The representation of these two proteins in so called thylakoid membranes is not very high, and the problem of resolution in SDS-PAGE had to be resolved. SDS-PAGE electrophoresis is the method widely and commonly used to separate proteins. Various polyacrylamid contents and running profiles can be used to optimize conditions of proteins isolation. Also the amount of proteins in each well is very important. Low amounts are poorly, or not at all visible, but on the contrary high amounts of the proteins are not well separated. This method had to be adjusted. With lower concentration of thylakoid membranes given on the gel was nicely resoluted the bands, but it had too low concentration of both proteins for other analysis. With higher concentration of thylakoid membranes were bands around 4 - 8 kDa not well separated and proteins with higher molecular weight caused smart band. For this case we had to try several different types of gradient gels (caused good separation of bigger proteins), optimization of running time and amper value. The western blot analysis showed presence of fragments of both proteins on the proteins with higher molecular weight, but after MALDI TOFF analysis was shown that those fragments were too small to determine bigger parts of protein sequence. After detection with antibodies the small bands were not very well visible on the PVDF membranes, but this problem could be solved

by adding more lines, what could help for MALDI TOFF analysis. To this time we were not able to verify the sequence of PsbX, and compare it with information from database. All method of MALDI TOFF and determination of content of amino acids in protein PsbX and PsbW is in progress.

8. LIST OF UTILIZED ABBREVIATIONS

INTRODUCTION:

ATP	adenosine-5'-triphosphate
DNA	deoxyribonucleic acid
LHC II	light harvesting complex II
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OEC	oxygen-evolving complex
PAGE	polyacrylamid gel electrophoresis
PCR	polymerase chain reaction
PS II	photosysthem II
RNA	ribonucleic acid

LABORATORY WORK:

AA	acrylamid
AMP	ampicilin
APS	ammonium persulfate
ASB 14	3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate
AUAP	abridged universal amplification
Å	angström
B	G + T + C
Bis	bis acrylamid
bp/kbp	base/kilobase pairs
C	concentration
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CH_3COOH	acetic acid
CN/DAB	(4-chloro-1-naphthol)/(3,3'-diaminobenzidinetetrahydrochloride)
D	G + A + T
dC	deoxy-cytosine nucleotides
Deg primer	degenerated primer
DEPC	diethyl pyrocarbonate
dH_2O	distilled water

DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
dT	deoxy-thymine nucleotides
DTP	deoxycytidine triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FW	forward primer
GSP	gene-specific primer
GSTPsbR	glutatione S-transferase PsbR
H	A + T + C
HCl	hydrochlorid acid
HisPsbR	His-tag PsbR
HS	high-salt
IPTG	isopropyl-β-D-1-thiogalactopyranoside
K	G + T
KAN	kanamycin
kJ	kilojoule
LB	Luria Broth medium
LC	liquid chromatogramy
M	A + C
MALDI	matrix-assisted laser desorption ionization
MBP-PsbR	maltose binding protein PsbR
$MgCl_2$	magnesium chloride
MgSO4	magnesium sulphate
min.,	minute
$MnCl_2$	manganese chloride
MS	mass spectrometry
N	A + C + G + T
NaCl	natrium chloride
NaI	sodium iodide
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance
Nondeg primer	nondegenerated primer

O.D.	optical density
PM	precipitation mix
PVDF	polyvinylidene fluoride
R	A + G
RACE	rapid amplification of cDNA ends
RBS-250	Brilliant Blue-Coomasie R250
RNAse H	family of endonucleases
rpm	rotates per minute
RT	real time (PCR)
RW	reverse primer
RXN/RXNS	reactions
S	G + C
SDS	sodium dodecyl sulfate
sec.,	second
S.N.A.P.	type of column used in 5' RACE kit
TAE	tris-acetate buffer
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-Tetramethylethane-1,2-diamine
TET	tetracycline
TOF	time-of-flight mass spectrometer
TRIS	1,1,1-TRIS-(hydroxymethyl)-aminoethane
TWEEN 20	polysorbate 20 (detergent, emulsifier)
UREA	carbamide/urea
UV	ultraviolet light
V	A + C + G
V	volt
W	A + T
WB	western blotting
x g	gravitational acceleration
Y	C + T
λ	wave lenght

9. LIST OF USED LITERATURE AND INTERNET REFERENCES

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