University of South Bohemia

Faculty of Science

Genetic engineering of *psbA* gene in *Nicotiana tabacum*

Master's thesis

Bc. Dagmar Hucková

Supervisor: Mgr. David Kaftan, Ph.D.

Consultant: Assoc. Prof. RNDr. Jindřich Bříza, CSc.

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Annotation

Transformation vector with mutated *psbA* gene and selective *aadA* gene was created and transferred into *Nicotiana tabacum* living cells using Biolistic bombardment. Due to homologous recombination, transformed plant lineage carrying D1-A209, D1-C-212 instead of D1-S209, D1-S212 in D1 protein in PS II was obtained. Seeds from transformed plant were harvested and homoplasmy of the first generation was tested. These mutations caused higher thermostability in *Synechocystis* sp. PCC6803 so the transformed plant is expected to be the first step in the study of PS II thermostability in higher plants.

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I would like to thank my supervisor, Mgr. David Kaftan, Ph.D., and my consultant, Assoc Prof. RNDr. Jindřich Bříza, CSc. It was honour to work with them.

1. Introduction

1.1 Chloroplast genome

The chloroplast is a semiautonomous organelle found within the interior of photosynthetically competent cells of higher plants and in eukaryotic algae. Acquired by a unique endosymbiotic act, chloroplasts share their common ancestor with extant cyanobacteria (Raven and Douglas, 2003) unlike mitochondria that derive their ancestry from proteobacteria (John and Whatley, 1975).

Due to their origin, chloroplasts preserved their DNA in the form of a 120-160 kb circular chromosome coding for sequences of approximately 130 genes (Buchanan *et al*., 2000) which however evince both prokaryotic and eukaryotic features. For instance, sequence homology coding for some tRNA, rRNA, protein coding genes as well as homology of regulatory sequences and gene clusters can be found within the chloroplast chromosome and prokaryotic genomes. On the other hand, some chloroplast genes contain introns as in eukaryotes (Shinozaki *et al*., 1986). Some genes (800 – 2000) present in the nucleus are homologous with these in the cyanobacterial genome indicating that a part of the chloroplast genome was transferred into nucleus. The products of more than half of these genes are not directed back into the chloroplasts but they participate in other pathways in different organelles (Martin *et al*., 2002). In *Nicotiana tabacum*, the frequency of gene transfer from chloroplast to nuclear genome is approximately one transposition in 16 000 pollen grains (Huang *et al*., 2003). These nuclear genes originating from cpDNA are regulated at the transcription level (Wanner and Gruissem, 1991), with regulation of cpDNA genes being realized post transcriptionally (Sugita and Sugiura, 1996). Active chloroplasts depend on nuclear encoded proteins , regulatory factors (Sugita and Sugiura, 1996) and transcription of chloroplast originated genes in the nucleus (Oelmüller and Mohr, 1986; Rapp and Mullet 1991). Higher plants chloroplasts are composed of two identical inverted repeats separated by two single copy regions (Dyer, 1984).

The chloroplast genome of *N. tabacum*, is made of a single circular chromosome of 155 939 bp coding for approximately 105 genes. The chloroplast has no inverted repeats, with about half of all genes coding for proteins and enzymes taking part in photosynthetic reactions while the rest are genes for the genetic system, and a minor part belongs to enzymes for biosynthetic pathways (e.g. lipid synthesis; Fig. 1; Wakasugi *et al*. 1998).

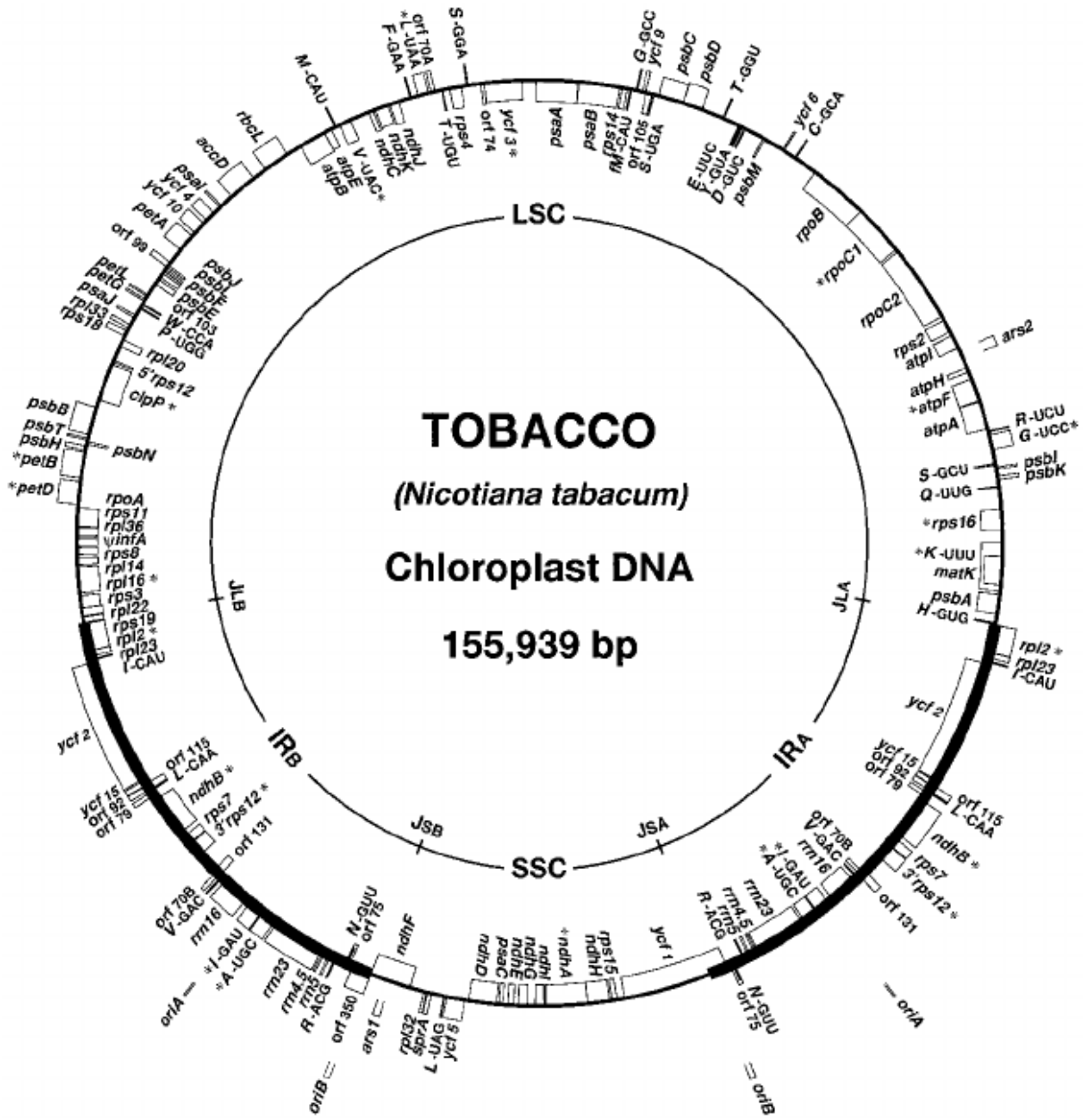

Figure 1: Chloroplast genome of Nicotiana tabacum (Wakasugi *et al*., 1998).

1.2 Photosynthesis

Plants, algae and photosynthetic prokaryotes are cable of capturing and utilizing light energy to produce organic material in a process called photosynthesis. Photosynthesis comprises of a series of reactions terminating with CO_2 conversion into carbohydrates accompanied with water oxidation into molecular oxygen (Blankenship, 2002).

The entire process takes place with in the chloroplast internal membrane system, termed thylakoids (light dependent reactions) and chloroplast stroma (carbon fixation/light

independent reactions). Several protein complexes are involved in the photosynthetic reactions – photosystem II, photosystem I, cytochrome b_6f complex and ATP synthase. The reaction centre of PS II is located in the thylakoid grana membranes together with light harvesting complexes (LHC) while PS I locates to the stroma exposed thylakoid membranes (Anderson and Melis, 1983). PS II acts as a light driven water/plastoquinone oxidoreductase. Functionally, PSII is composed of a peripheral and inner light harvesting antennae and a reaction centre that performs the primary steps of light energy conversion. Here, two core proteins D1 and D2 coordinate all the cofactors that are responsible for charge separation (P680 - pair of chlorophyll a molecules) and stabilization (accessory chlorophyll molecules, pheophytins, primary quinone Q_A; Blankenship, 2002).

1.3 *psbA* variants

All photosynthetic organisms notwithstanding the higher plants had to adapt to variable environmental conditions that differ, especially in temperature. These changes occur on diverse temporal scales from seasonal changes during a year down to fluctuations in light/temperature within seconds.

The PS II core complex contains the D1 and D2 protein heterodimer encoded by chloroplast genes *psbA* and *psbD* (Suorsa and Aro, 2007). Because the D1 protein is under constant highly oxidative stress, it is permanently degraded and re-synthetized by photo-damage and the repair cycle described in Figure 2 (Baena-González and Aro, 2002). Chloroplasts of higher plants contain one copy of *psbA* gene but many cyanobacteria have more than one copy of the *psbA* gene which are differentially expressed depending on environmental conditions. Here, one isoform is typically dominant and expressed under standard conditions, in contrast to structurally different isoform(s) that are expressed under stress (Mulo *et al*., 2009). The various D1 isoforms facilitate, for example, the different tolerances of PSII to high light (Kulkarni and Golden, 1994; Campbell *et al*., 1996). Under light stress or UVB radiation, alternative *psbA* genes are expressed while the higher rate of D1 synthesis is important to increase the balance between protein synthesis and degradation (Li and Sherman, 2000). These isoforms often differ in primary nucleotide sequence resulting in differing protein sequence and putatively a different structure of the final protein. Evidence has been presented (Shlyk *et al*. 2006) that thermophilic cyanobacteria consistently carry two changes in amino acid sequence within the essential part of the D1 protein. The two serine residues found at the D1-

209, D1-212 positions in mesophiles are occupied by alanine (D1-A209) and cysteine (D1-C212). Genetic engineering of a wild type mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 (D1-S209, D1-S212) into a strain carrying the thermophilic sequence D1-A209, D1-C-212 resulted in a thermotolerant phenotype (Dinamarca *et al*. 2011).

In higher plants, the psbA gene sequence is highly conserved and no alternative copies are present in the chloroplast genome. The sequence of the psbA carries the serines in the D1-209, D1-212 position (Su et al., 2017).

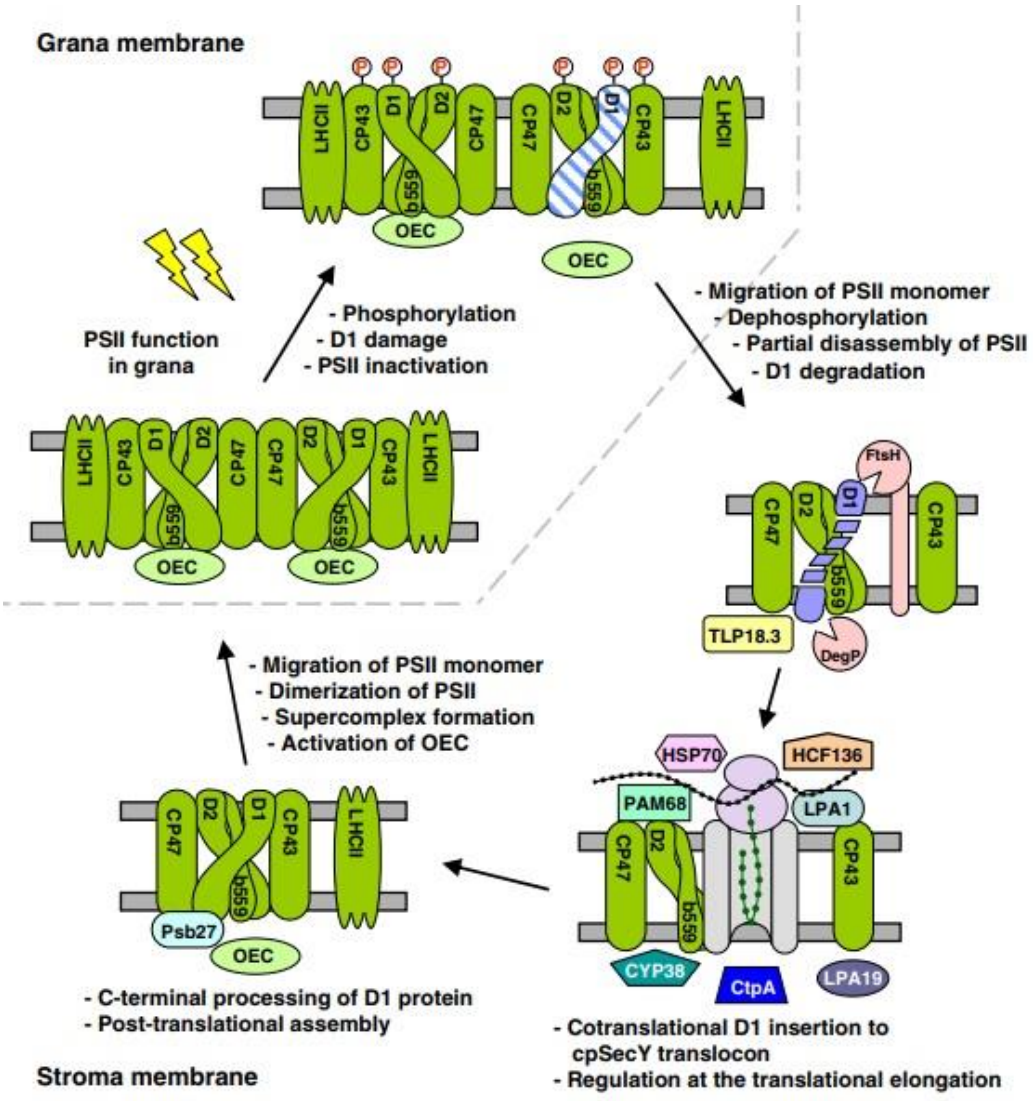

Figure 2: Repair cycle of PS II: damaged D1 protein is degraded in the stroma thylakoids, Newly synthetized D1 protein is incorporated into thylakoid membrane (Mulo *et al*., 2012).

1.4 Biolistics

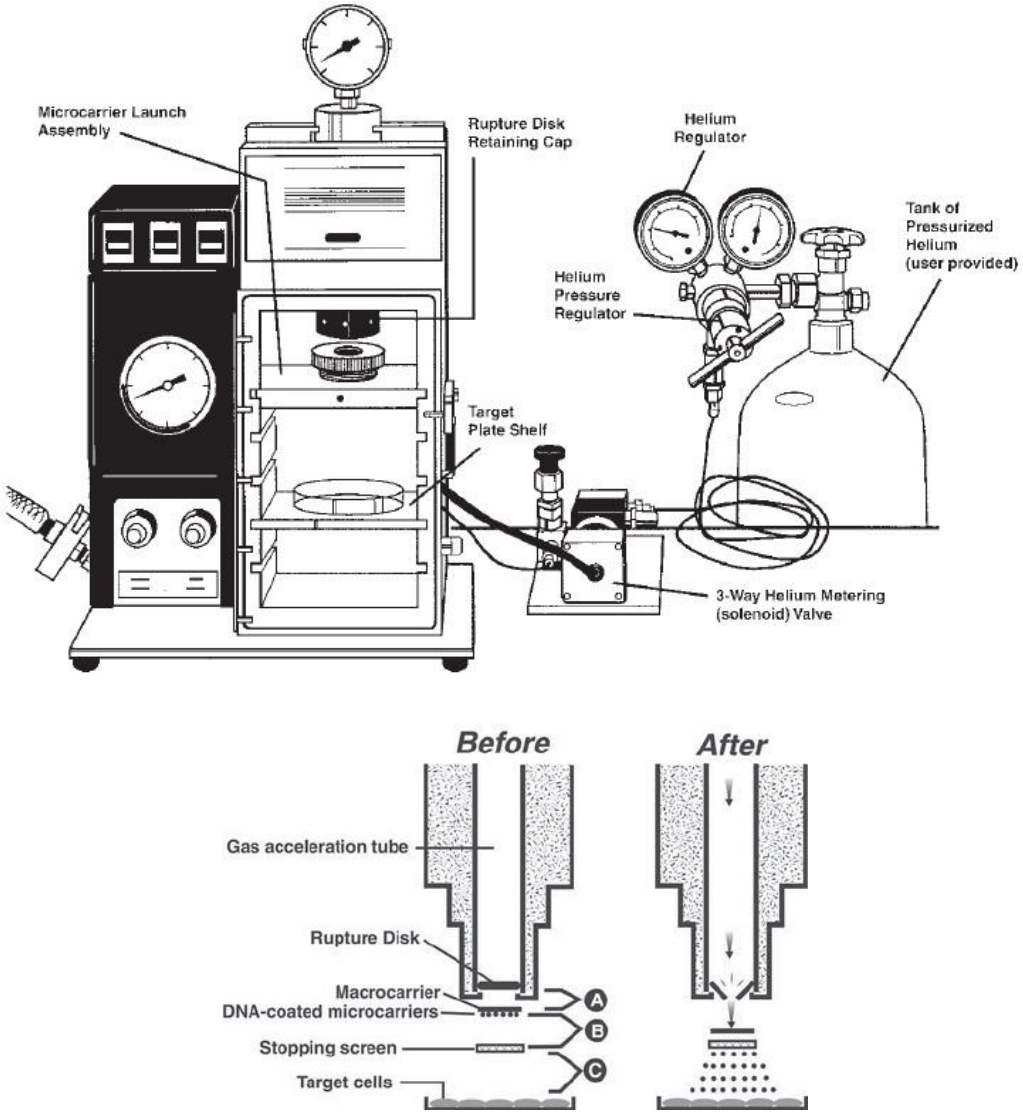

Figure 3: A figure showing the Biolistic® PDS-1000/He particle delivery system machine (top) and the detail of the process inside the chamber (bottom). A – distance between rapture disk and macrocarrier, B – distance between macrocarrier and stopping screen, C – distance between stopping screed and target cells. (Drawing courtesy of Bio-Rad Laboratories, Hercules, CA.)

Many methods of genetic transformation in plants exist – PEG mediated protoplast transformation, DNA transfer mediated by *Agrobacterium tummefaciens* (Gram-negative soil

bacterium causing plant tumors by Ti-plasmid insertion into infected plant nuclear genome) and particle bombardment system or biological ballistics, shortly Biolistics.

Although, *Agrobacterium*-mediated transformation is a low cost method compared with Biolistics, Biolistics offers significant advantages. One of them is the absence of another organism during particle bombardment. Whilst there is high risk of insertion of unwanted extraneous plasmid sequences together with the gene of interest during the transformation using *Agrobacterium*, in Biolistics, transferred plasmid DNA can be reduced in size or can even be linearized (but the linearization of transformation vector has shown low efficiency in tobacco ptDNA engineering [Bříza *et al*., 2013]). Also, *Agrobacterium* can cause oversensitivity, causing plant cell death (Perl *et al*., 1996; Hansen and Wright, 1999). For ptDNA transformation, Biolistics is widely used (Taylor and Fauquet, 2002) but still low efficiency of this method is described in many plant species (Sidorov et al., 1999; Sikdar et al., 1998; Khan and Maliga, 1999) and PEG mediated protoplast transformation is preferred sometimes (Eibl *et al.*, 1999).

Biolistics enables delivering of foreign DNA (as well as RNA or proteins) precipitated onto high-velocity microparticles into living cells through the cell wall and membranes. Afterwards, if particles penetrate the nucleus, fragments can be incorporated into host cell DNA and expressed. The greatest advantage on this process is its effectivity, thousands of cells can be affected in one step (Sanford *et al*., 1987). Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad; Fig. 3) is presently the most widely used device enabling DNA transformation by this method. Microcarriers (mostly 0.7–1.0 μm gold particles) with coated DNA are transferred into a cell using high-pressure release into a vacuum chamber with the cell sample. To create the high pressure, helium is used to rupture the disk. Microcarriers loaded onto macrocarrier are pushed by the pressure increase. The macrocarrier is prevented from carrying on the sample by a stopping screen while the microcarriers continue downwards to the target cells at high speed. The speed is influenced by the helium pressure, the distance between macrocarrier and stopping screen, the distance between stopping screen and target cells and the degree of vacuum inside the chamber. For example, a vacuum of -28 inches Hg (6.5 kPa), a helium pressure of 1100 psi (7.5 MPa), a macrocarrier travel distance 6 – 10 mm are generally optimal conditions for most plant DNA transformation (Kikkert, 1993).

Besides physical parameters, biological ones are important in the process of particle bombardment. At first, gene constructs containing promoter, gene and terminal sequence need

to be designed with respect to the nature of the target cells as well as tissue type, age and fitness. These parameters influence the ability of the delivered gene to be expressed and of the transformed cells to regenerate (Southgate *et al*., 1995). The ptDNA transformation is simplified due to homologous recombination, which enables precise insertion of foreign DNA (Paszkowski, 1994).

1.5 ptDNA transformation and its advantages

The first transformation of living *Escherichia coli* cells by plasmid artificially composed of two different DNA plasmid segments was performed by Cohen *et al*. (1973) who proved that a functional plasmid can be constructed from two plasmid fragments generated by restriction digestion and carry features of both individuals. Morrow *et al.* (1974) later demonstrated that plasmids with *in vitro* inserted DNA sequences from eukaryotic species is able to replicate after transformation into *E. coli* cells and replicate. These techniques are used till today to generate transformation vectors.

The first cpDNA transformation was described by Boynton et al. in 1988 in *Chlamydomonas reindhartii* and was accomplished using tungsten microparticle bombardment. Chloroplast DNA transformation of higher plants was achieved in tobacco two years later also using tungsten microprojectile bombardment (Svab *et al.,* 1990), transient expression of vectors introduced into the chloroplast was confirmed in tobacco the same year by Daniel *et al.* (1991) using also particle bombardment. After that, ptDNA transformation of many other higher plants was described, for example in the potato plant (Sidorov *et al.,* 1999), *Arabidopsis* (Sikdar *et al*., 1998), rice (Khan and Maliga, 1999) or wheat (Daniell *et al*., 1991). Regenerated *Arabidopsis* was sterile after reaching homoplasmy, stable transformation of rice or wheat was not achieved. However, transformation of ptDNA has a high success rate only in tobacco. Per one shot, 5-15 independent transformed cells are described (Daniell *et al*., 2001; Fernandez-San Millan *et al.,* 2003; Dhingra and Daniell, 2004) compared to for example tomato ptDNA transformation in which only one successful transformation occurs per ten shots on average (Ruf *et al.,* 2001). High efficiency of ptDNA transformation in tobacco is the reason why this plant is still popular in genetic manipulation.

Plastids are organelles where many essential pathways take place (such as photosynthesis, photorespiration or metabolism of nutrients important for the whole cell; Klaas and Baginsky, 2011) and transformation of genes participating in these pathways can significantly affect their

function and expression. Apart from this fact, there are several reasons why cpDNA transformation is more advantageous than nuclear DNA transformation.

Primarily, transformation of plastid DNA is derived from homologous recombination mediated by a bacterial-like RecA-based system. This phenomenon (rare in nuclear DNA transformation; Sodeinde and Kindle, 1993), enables the controlled insertion of transformed DNA directly into selected loci of the plastid genome and eliminates position effects which can result in a decrease in gene expression or total gene silencing (Paszkowski, 1994).

Plastid genome in higher plants is presented in up to 10 000 copies per one cell. This causes a possibility of high-level expression of inserted genes (Oey *et al*., 2009). In addition to that, results of plant studies accomplished by Faye and Daniell (2006) expose the limitation of proteolytic pathways in chloroplast so proteins introduced into ptDNA are protected from degradation and protein expression is higher and neither epigenetic changes nor gene silencing naturally occurring in nuclear genome was observed in the chloroplast genome (Daniell *et al*., 2005).

Plastids generally show uniparental heredity which means, probability of the gene flow of the transformed cpDNA caused by pollen grain is very low. In addition, tobacco has tendency to self-pollination so cross-fertilization frequency would be low. This ability to fertilize a wild type plant with transgenic pollen rapidly decreases with distance (with 10 m separation of wild type and genetically modified plants, the probability of cross-fertilization decline to 1%; Ruf *et al*., 2007). Due to this, genetically transformed plastids are excluded from transmission by pollen even to related species and hence are unable to create “superweeds” or cause other genetical issues.

1.6 Plastid transformation vector

Genetic engineering producing transgenic plants requires separate steps – design of the transformation vector, the introduction this vector into cells *in vivo*, callus organogenesis and regeneration of vital plants and to select plants carrying the integrated genes (Scotti et al., 2011).

In order to reach homologous recombination in ptDNA transformation, the vector directed to ptDNA contains two 1-2 kb flanking fragments homologous to plastid sequences in the site of insertion. To provide expression of the inserted gene into the chloroplast, regulatory sequences

need to be joined, for example strong PEP promoters are usually used to reach high mRNA transcription (promoters of the 16S rRNA and *psbA* gene; Koop and Herz, 2007; Maliga, 2003). In view of the fact that cpDNA originates from prokaryotic organisms and transcripts are processed polycistronically (Westhoff and Herrmann 1988), selective genes can be co-transformed in one cluster with the gene of interest.

The high copy number of chloroplast DNA per one cell could also be disadvantageous . Only one or very few of them are transformed in the first step. Additional regeneration on selective medium is needed to keep regenerated cells under constant selective stress to gain homoplastic plants (Fig. 4). To gain a successfully transformed stable plant culture, transformation is only the initial step followed by a long state of passaging the plants and seed harvesting (Scotti et al., 2011).

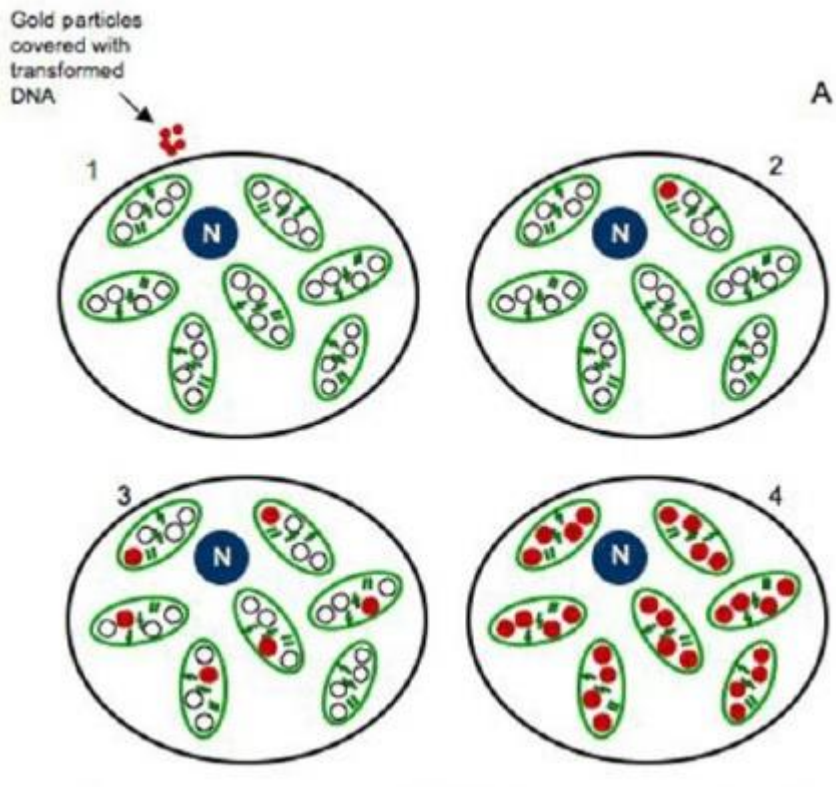

Figure 4: The principle of homoplasmy process. 1 – Wild type cells are bombarded with microparticles with coated DNA. 2 – One of cpDNA is transformed. 3 – The mixture of wild type and mutated genome occurs. 4 – Homoplasmy is achieved under selectable stress (Scotti *et al.*, 2011).

1.7 *aadA* gene as a selectable marker

The *aadA* (aminoglycoside resistance protein) gene encodes aminoglycoside 3"-adenyltransferase, enzyme belong to transferase family. This gene causes resistance to antibiotics in bacteria (spectinomycin and streptomycin) by catalysing their decomposition (Chinault *et al*., 1986). This gene is used in plant genetic engineering as a selectable marker since it offers resistance to these antibiotics when transferred into the tobacco nucleus DNA (Svab *et al*., 1990) or *Chlamydomonas reindhartii* chloroplast DNA (El-Sheekh, 2000). Experiments using aadA gene as a selectable marker in tobacco cpDNA transformation showed high efficiency after biolistic delivery of plasmid pZS197 with chimeric *aadA* sequence into *N. tabacum* (Svab and Maliga, 1993). That is the reason why the *aadA* gene was chosen as a selectable marker in this study.

2. AIMS OF THE STUDY

- The construction of a DNA vector for biolistic transformation of *psbA* gene encoded by chloroplast DNA carrying mutations in D1-S209, D1-S212 to version of D1 protein from thermophilic cyanobacteria (D1-A209, D1-C-212) which resulted thermotolerant phenotype in mesophilic cyanobacterium *Synechocystis* sp. (Dinamarca *et al*. 2011).
- The biolistic transformation of *Nicotiana tabacum* var. Samsun plants with constructed vector.
- The selection of successfully transformed calli on spectinomycin and the generation of the first generation from seedlings.

3. MATERIAL AND METHODS

3.1 Plant material

Nicotiana tabacum var. Samsun was chosen as a plant material for transformation because the ptDNA transformation in *N. tabacum* is well-established and provides the highest transformation yields known among higher plants (Verma and Daniel; 2007). Plants were cultured *in vitro* in 140 mm high OS140boxes (Duchefa, NL) on MS medium (Murashige and Skoog, 1962) solidified with agar (6 g per litre of medium) with the addition of vitamins and sucrose (20 g per litre of medium). Transgenic plants were cultured in MS medium supplemented with 500 mg L^{-1} of spectinomycin. All plants were grown at a constant temperature of 25 °C and under continuous light of 150 μmol *photons* m^{-2} s^{-1} provided by fluorescent tubes.

3.2 Used primers

Primers used during the experiments are listed in the Table 1. Their actual usage is described in the appropriate chapters.

Table 1: Used primers, their sequences and source.

Primer	Sequence	Citation
aadA1	5'-GAAGCGGTTATCGCCGAAG-3'	Goldschmidt-Clermont; 1991
aadA2	5'-TTATTTGCCAACTACCTTAGTGATC-3'	Goldschmidt-Clermont; 1991
FP2A	5'-AACTAGCATATTGGAAGATCAATCGGC-3'	This study
Mut-F2	5'-AGTGAATTTCTAGAGGGAAGTTGTGAGC-3'	This study
Mut-F4	5´-AGTGAATTCACCCATGTGGTACTTC-3´	This study
Mut-F5	5´-TGTAAACAAAAAATTCGCCGTCGTTCAATGAG-3´	This study
Mut-F6	5´-CGGTAGAGTGTCTATGTAAGTAAAATAC-3´	This study
Mut-R3	5'-TGACCATGAGCAATTTTAGAGAGACGCG-3'	This study
Mut-R5	5´-CTTACATAGACACTCTACCGATTGAGTTAC-3´	This study
Mut-R7	5´-CAATATGCTAGTTTCAACAACTCTCG-3´	This study
Mut-R8	5´-TCACTCATTAGGCACGCAATTTTAGAGAGACGCGAAAGC-3´	This study
psbA R2.1	5'-ATTTTCTGTAGAGAAGTCCG-3´	This study
psbA R2.4	5'-GATCTACCCAATTGGTCAAGG-3´	This study
SB-F	5´-AAG GAG CAA TAG CAC CCT CTT G-3´	This study
UC-R4	5´-AGTACCACATGGGTGAATTCACTGGCCGTCGTTTTAC-3´	This study
UC-F4	5´-TCTCTCTAAAATTGCTCATGGTCATAGCTGTTTCCTG-3´	This study
UC-R5	5´- TAATGCAGCTGGCACGACAGG-3´	This study

3.3 Used plasmids

For transformation of *N. tabacum*, pUC19 plasmid cloning vector isolated from *Escherichia coli* was used (Yanisch-Perron *et al*.; 1985). This plasmid enables the selection of transformed cells on substrate containing ampicillin or tetracycline antibiotics. Insertion site was modified by deleting the *lacZ* alfa promoter in order to prevent transcription of the inserted *psbA* gene in bacteria because of the toxicity of the *psbA* gene product in *E. coli* cells. Plasmid pCB0773A (Bříza *et al*.; 2013) served as a template for isolation of the *aadA* gene carrying spectinomycin resistance used for the selection of the transformed plants.

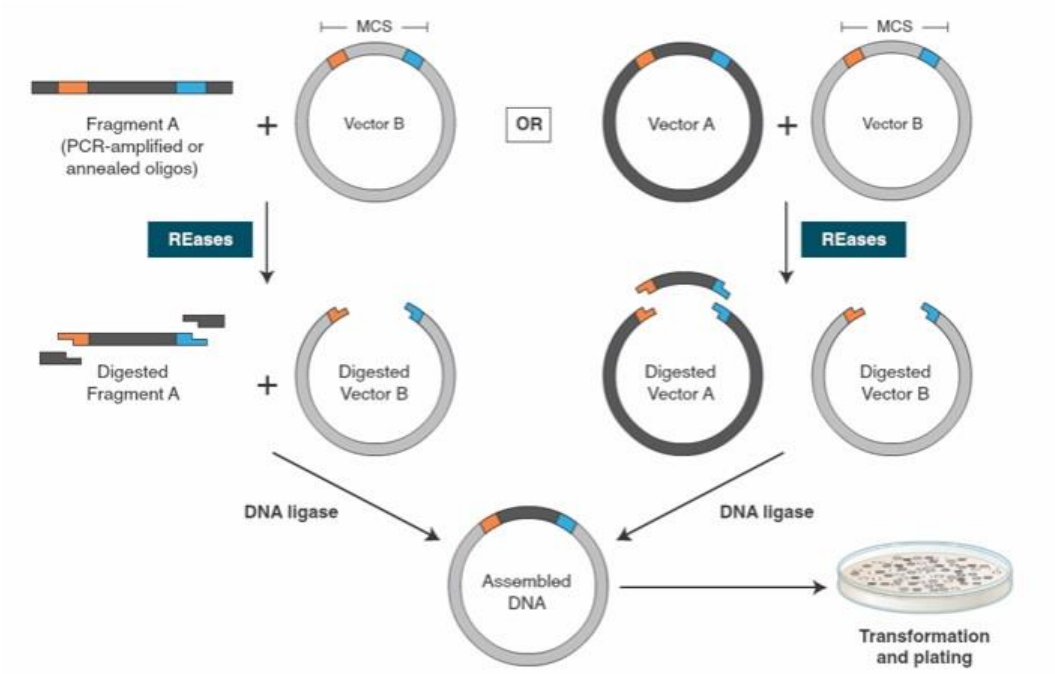

Figure 5: Figure of DNA assembly, consisting of cloning vector and fragment (PCR-amplified or digested from another vector).

3.4 Gibson assembly

Gibson assembly was used to assemble transformation vector from five DNA fragments – pUC19, left homological region, selective cassette with *aadA* gene, *psbA* gene part 1 (standard sequence) and *psbA* gene part 2 (with required point mutations). Both fragments of the *psbA* gene served simultaneously as a right homology region (designed by Assoc. Prof. RNDr. Jindřich Bříza, CSc.).

Fragment 1: Template - WT N. tabacum gDNA
Left homological region
Lenght - 1039 + 8 + 12 = 1059 bp

Mut-F4 5´-AGTGAATTCACCCATGTGGTACTTC-3´
Mut-R4 5´-AACGACGGCGAATTTTTGTTTACATTATAG-3´

Fragment 2: Template - plasmid pCB0773A
Selective cassette with aadA gene
Lenght - 1353 + 13 + 11 = 1377 bp

Mut-F5 5´-TGTAAACAAAAAAATTCGCCGTCGTTCAATGAG-3´
Mut-R5 5´-CTTACATAGACACTCTACCGATTGAGTTAC-3´

Fragment 3: Template - WT N. tabacum gDNA
psbA mut first part
Lenght - 406 + 9 + 8 = 423 bp

Mut-F6 5´-CGGTAGAGTGTCTATGTAAGTAAAATAC-3´
Mut-R7 5´-CAATATGCTAGTTTCAACAACTCTCG-3´

Fragment 4: Template - plasmid psbA mut
psbA mut second part
Lenght - 782 + 15 + 17 = 814 bp

Mut-F8 5´-GAGAGTTGTTGAAACTAGCATATTGGAAG-3´
Mut-R8 5´-TCACTCATTAGGCACCGCAATTTAGAGAGACGCGAAAGC-3´

Fragment 5: Template - pUC19 plasmid
pUC19, transformation vector
Lenght - 2536 +15 + 15 = 2566 bp

UC-F4 5´-TCTCTCTAAAATTGCTCATGGTCATAGCTGTTTCCTG-3´
UC-R4 5´-AGTACCACATGGGTGAATTCACTGGCCGTCGTTTTAC-3´

Total lenght of transformation vector: 2536 + 1039 + 1353 + 406 + 782 = 6114 bp

Figure 6: Figure of Gibson assembly describing the position and length of five fragments, their source, together with the position of used primers and their sequence.

Fragment 1 was amplified from WT *N. tabacum* gDNA using Mut-F4 and Mut-R4 primers and served as a left homological region in *N. tabacum* plasmid DNA. Fragment 2, the selective gene *aadA*, was amplified from plasmid pCB0773A using Mut-F5 and Mut-R5 primers. Fragment 3 was amplified from WT *N. tabacum* gDNA as the first part of the *psbA* gene using Mut-F6 and Mut-R7 primers. Fragment 4 was commercially produced part of *psbA* gene with target mutations and was amplified using Mut-F8 and Mut-R8 primers. Plasmid pUC19 was modified by deleting its *lacZ* promoter via PCR using primers UC-F4 and UC-R4 (Fig. 6). Inactivation of the promoter prevented transcription of the *psbA* gene product that is toxic for *E. coli* cells.

For DNA fragments ligation based on sequence homology using three enzymatic reactions are required - exonuclease activity to create a single stranded 3' overhangs, DNA polymerase extension activity to fill gaps within annealed fragments and DNA ligase activity to seal assembled DNA (Fig. 7). These reactions are possible to accomplish in one tube with Gibson Assembly® Master Mix (New England BioLabs Inc., USA).

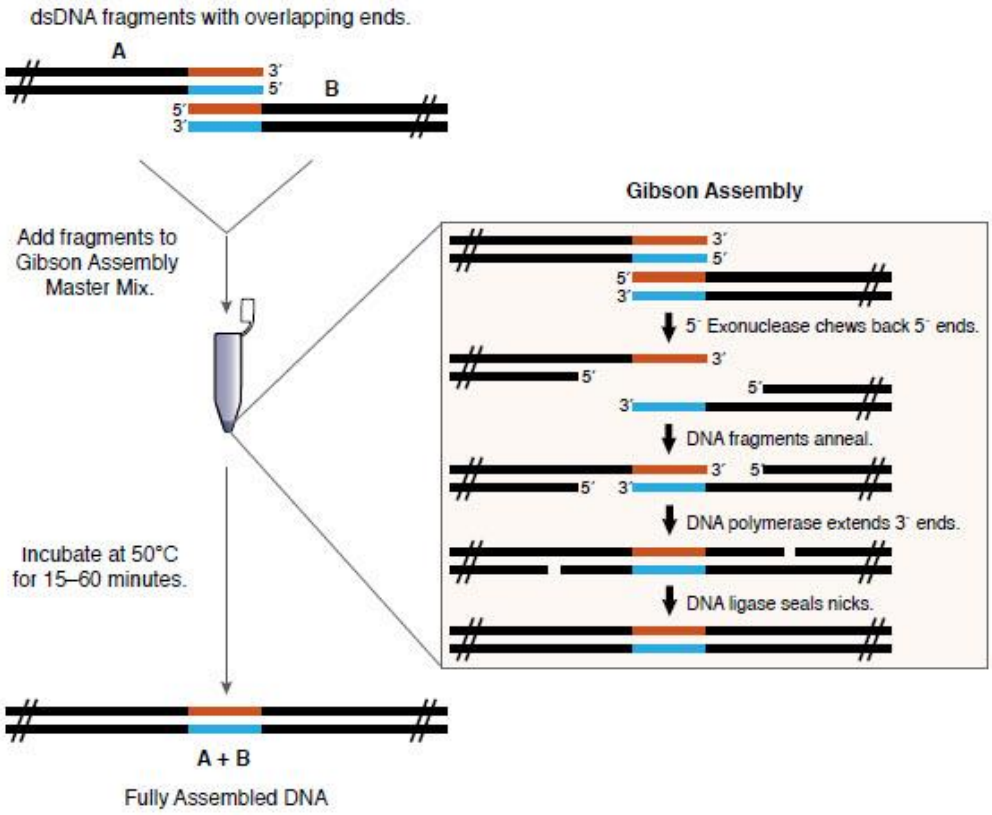

Figure 7: Figure showing the three enzymatic reactions in Gibson assembling (www.neb.com).

Reaction mixture was prepared according to the manufacturers protocol for 4-6 fragment assembly (0.2 – 1 pmol of total DNA fragments, 10 μL Gibson Assembly Master Mix (2x), dH_2O to total volume of 20 μL). Samples were incubated at 50 °C for 60 minutes.

2 μL of assembled product were added to 50 μL of competent cells in a 2 mL microtube and mixed by pipetting. Samples were incubated on ice for 30 seconds. After heat shock at 42 °C for 30 seconds, samples were cooled on ice for 2 minutes and afterwards were placed with 950 μL of SOC media on an orbital shaker for 60 minutes at 37 °C and shaken vigorously. Meanwhile, Petri dishes with LB medium (Bertani; 1951) containing 100 $mg.L^{-1}$ ampicillin (AMP) were preheated to 37 °C. Onto every Petri dish, 100 μL of mixture was spread and incubated at 37 °C overnight. Bacterial colonies growing on AMP were transferred with a sterile stick to a new Petri dish.

3.5 PCR template preparation

DNA extracts from bacterial cultures (and thereafter from plant samples) were prepared according to Klimyuk *et al*. 1993 for a fast gain of DNA material for PCR. Samples from bacterial colonies (or a small plant segment) were transferred using a toothpick into 20 μL of 0.25 M NaOH, incubated for 30 seconds at 100 °C and transferred to ice immediately. Twenty μL of 0.25 M HCl and 10 μL 0.5 Tris pH 8.0 (with 0.25% Nonidet 40 detergent) were added and samples were incubated at 100 °C for 2 minutes. Prepared samples were stored on ice and used for PCR as a DNA extract or stored at -20 °C. In the case of using frozen samples, samples were incubated at 100 °C for 2 minutes again.

3.6 Detection of presence of the aadA gene

Presence of the *aadA* gene in each bacterial colony was verified by PCR using aadA1 and aadA2 primers (expected fragment size was 787 bp). A sample of each colony was used for PCR template preparation. Total volume of each reaction without template was 25 μL (Tab. 2).

Table 2: PCR protocol for verifying the presence of *aadA* gene in bacterial colonies.

Storage concentration	Per one reaction [μL]	Final concentration
Premix rTaq 2x	12.5	1x
10% PVP	2.5	1 %
10% BSA	0.25	0.1 %
aadA1 primer (20 μM)	0.5	0.4 μM
aadA2 primer (20 μM)	0.5	0.4 μM
dH_2O	8.75	-
DNA extract	0.5	-

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min; samples were held at 4 °C at the end of the program.

Plasmid pCB0773A was used as a positive control. The samples were loaded onto 1% agarose gel with 1x TAE buffer (400 mM Tris base; 1 mM Na_2 EDTA pH 8.0; 20 mM glacial acetic acid) and separated in Wide Mini-Sub Cell GT vertical electrophoresis system (Biorad, USA) to verify the expected size of fragment.

3.7 Fragment order in plasmid DNA

To check the presence, order and orientation of inserted fragments in plasmid DNA of *E. coli*, four amplification reactions were designed. Reaction with primers Mut-F4 and Mut-R8 probed the total length of the insert in plasmid pUC19 with an expected amplicon length of 3600 bp. Reaction with primers Mut-F4 and Mut-R5 checked the reciprocal orientation of the left homological region and the *aadA* gene with expected amplicon length of 2400 bp. Reaction with primers Mut-F5 and Mut-R7 checked the reciprocal orientation of the *aadA* gene and the first part of the *psbA* gene with expected length 1800 bp. Reaction using primers Mut-F6 and Mut-R8 checked the reciprocal orientation of the first and the second part of the *psbA* gene with the expected length of 1200 bp.

DNA extraction of AMP resistant colonies was conducted according to Klimyuk *et al.* (1993). Each reaction was run in a total volume of 25 μL (Tab. 3).

Table 3: The composition of amplification reactions to identify order and orientation of inserted fragments within the plasmid.

Storage concentration	Per one reaction [µL]	Final concentration
2x Premix rTaq	12.5	1x
10% PVP	2.5	1 %
10% BSA	0.25	0.1 %
Primer 1 (20 µM) *	0.5	0.4 µM
Primer 2 (20 µM) *	0.5	0.4 µM
SDW	8.7	-
DNA extract	0.5	-

* Combination of primers P1 and P2 was selected to map the whole inserted region (P1 – P2: Mut-F4 – Mut-R8; Mut-F4 – Mut-R5; Mut-F5 – Mut-R7; Mut-F6 – Mut-R8).

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 3 min; samples were held at 4 °C at the end of the reaction program.

The samples were loaded onto 0.8% agarose gel with 1x TAE buffer along with a 1 kb ladder (New England Biolabs, USA) as a DNA length standard and separated using electrophoresis. The length of all amplicons was inspected to exclude the colonies with an incorrect order of fragments. One of the suitable colonies was selected for the following experiments.

3.8 Plasmid DNA isolation for biolistics

Plasmid DNA from *E. coli* was isolated using NucleoBond® Xtra Midi (Macherey-Nagel) according to the manufacturers protocol as follows. The selected bacterial colony was inoculated into three Erlenmeyer flasks with 100 mL of LB medium supplemented with 100 mg L^{-1} ampicillin and incubated overnight at 37°C on an orbital shaker spinning at 250 revolutions per minute. Cultures were pooled together and optical density at 600 nm was measured using a Lambda 35 spectrophotometer (Perkin Elmer, USA). The bacterial suspension was centrifuged in 8 aliquotes of 40 mL at 6000 g in an Avanti J25 centrifuge (Beckman Coulter) for 10 minutes at 4°C. Each pellet was resuspended in 3 mL of RES buffer with RNAse A and the whole suspension was separated into two falcon tubes (each with

approximately 12 mL of suspension). Twelve mL of LYS buffer was added to the cell suspension, was gently vortexed and then incubated at room temperature for 5 minutes. Afterwards, 12 ml ofNEU buffer was added into each falcon flask immediately followed by a short gentle vortexing.

Two extraction columns were equilibrated with 12 mL of EQV solution each. The suspension of bacteria was transferred onto the columns and both of them were washed with 5 mL of EQV solution followed by 8 mL of WASH buffer. To elute the DNA, 5 mL of ELU buffer heated to 50°C were applied into each column. Three and half of mL of absolute isopropanol were added into each eluate. This solution was transferred into ten 2 mL microtubes and centrifuged at 17000 g at 24 °C for 20 minutes. Pellets were washed with 0.75 mL of 70% ethanol for 5 minutes and centrifuged at 17000 g for 5 minutes. After drying, the pellets were resuspended in 10-20 μL of SDW per one microtube and DNA concentration of entire sample was measured using a Qubit® 2.0 Fluorometer (Invitrogen).

3.9 Sequencing of inserted *psbA* gene plasmid DNA

The correct sequence of *psbA* gene inserted into the transformation vector was finally verified by sequencing the whole gene together with closely adjacent sequences. Three amplification reactions were designed to cover this region. Primers SB-F and psbA R2.4 were used for reaction 1 with an expected amplicon length of 672 bp, primers FP2A and UC-R5 for reaction 2 with an expected amplicon length of 875 bp, primers FP2A and psbA R2.4 for reaction 3 with an expected length of 317 bp (Tab. 4). High-fidelity polymerase was used to result precise sequence of amplified fragments without mutations.

Table 4: The composition of amplification reactions for the sequencing of the inserted *psbA* gene.

Storage concentration	Per one reaction [µL]	Final concentration
5x Q5 Phusion buffer	10	1x
dNTPs (2.5 mM each)	4	200 µM each
Primer 1 (20 µM)	1.25	0.5 µM
Primer 2 (20 µM)	1.25	0.5 µM
Q5 DNA Polymerase (2 U μL^{-1})	0.5	0.02 U μL^{-1}
SDW	33	-
Plasmid DNA	1 ng	0,02 ng μL^{-1}

* Combination of primers P1 and P2 were selected to map the whole inserted region (P1 – P2: SB-F – psbA R2.4; FP2A – UC-R5; FP2A – psbA R2.4).

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min; samples were held at 4 °C at the end of the program.

PCR fragments were loaded onto a 1.25 % agarose gel with 1x TAE buffer. After electrophoresis, the fragments were cut out of the gel and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel). Briefly, DNA fragments were cut out of the agarose gel and the gel slices were transferred into a clean tube with NT1 Buffer (200 µL Buffer NTI per each 100 mg of gel containing the DNA fragments). Samples were incubated at 50 °C until the gel completely dissolved. The total volume of the solution was transferred onto a clean column with a collection tube and centrifuged for 30 s at 11000 g using a Eppendorf MiniSpin centrifuge. The silica membrane was washed twice with 700 µL of Buffer NT3 and centrifuged for 30 s at 11000 g. Dry columns were centrifuged for 1 min at 11000 g to remove buffer completely. DNA from membranes was eluted using 15 µL of Buffer NE heated to 70 °C followed with centrifugation for 1 minute at 11000 g.

Concentration of DNA of each purified sample was measured using a Nanodrop 2000. Mixture for sequencing consisted of 200 ng of DNA (in a variable volume according to DNA concentration in the eluate), 5 µL of 5 µM primer and SDW to the total volume of 10 µL. Each of the three PCR fragments was sequenced from both directions, i.e. 6 sequencing reactions were performed together. Such a strategy resulted in the overlapping of the obtained

sequences. The beginning as well as the rear of the *psbA* gene sequences were obtained 2 times while the central part of the gene (containing point mutations of interest) could be checked up to 6 times. Sequencing was provided by a commercial company (www.gatc-biotech.com).

3.10 Biolistic bombardment

To deliver transgene plasmid into plant cells, the biolistic process with a PDS-1000/He Particle Delivery System (BioRad, USA) was used as a successful tool for transformation of ptDNA (Daniell *et al*., 1990; Sanford *et al*., 1993).

Using ultraclean chemicals and carrying on with all procedures under sterile conditions in a flowbox became an imperative necessity. Before the actual procedure, every part of the Biolistic PDS-1000/He system was pre-sterilized. The appropriate number of macrocarriers, rupture disks and stopping screens were sterilized together

with the metal components of the Biolistic PDS-1000/He system using an autoclave. Plastic parts were sterilized in 70% ethanol for 30 minutes within the sterile hood. The 1100 psi rupture discs were sterilized directly before using absolute isopropanol inside the sterile hood.

An approximately 2 x 2 centimetre fragment of *N. tabacum* var. Samsun leaf was cut, under sterlie conditions, per one shot and put face down on a sterile Petri dish with RMOP medium (Svab *et al*., 1990).

Microcarriers with golden particles and plasmid DNA were prepared before the biolistic bombardment according to following protocol detailed in Table 5. Onto every macrocarrier in a holder, 6 μL of prepared microcarriers in an absolute ethanol were loaded and left to dry in a Petri dish with a silica gel.

Parts of the chamber were assembled together with a sterile stopping screen, a rupture disk and a new macrocarrier with loaded golden particles before every shot. The distance between rupture disk and a target shelf was 12 centimetres. An opened petri dish with a prepared leaf was put inside the chamber on a target shelf. The atmospheric pressure was reduced by 28 inches of Hg (to approximately 6.5 kPa). When a low vacuum level was reached, helium pressure was turned on to gradually increase the pressure on rupture disk until it burst, projecting the golden microcarriers against the leaf, penetrating the leaf cells.

Table 5: Microcarier preparation protocol with volume of chemicals per one experiment (24 shots).

Per one shot	
12 3g 0.6 μm Au microcarriers	wash microcarriers in 750 μL of absolute ethanol
	vortex vigorously for 5 minutes
	leave overnight
	spin for 2 seconds
	clean pellet in 750 μL of absolute ethanol
	spin for 2 seconds
	dry on air
200 μL 50% glycerol	gently resuspend in an appropriate amount of 50% glycerol
20 μg plasmid DNA (1 μg μL^{-1})	add appropriate amount of plasmid DNA
200 μl of 2.5 M $CaCl_2$	
80 μl of 0.1 M spermidine	add $CaCl_2$ and spermidine all at once
	gently mix by hand
	let stay for 5 minutes
	spin for 2 seconds
	clean pellet in 750 μL of 70% ethanol
	spin for 2 seconds
	clean pellet in 750 μL 100% ethanol
	let stay for 1 minute
	spin for 2 seconds
144 μL of 100% ethanol	add an appropriate amount of absolute ethanol

Petri dishes with bombarded leaves were sealed with parafilm and kept under a shading filter paper at 25°C and continuous light for 2 days. Afterwards, they were fragmented into 6-8 pieces and transferred on a new Petri dish with RMOP medium containing 500 μg mL^{-1} of spectinomycin and kept at 25°C and under continuous light.

3.11 Plant regeneration

Spectinomycin resistant cells successfully transformed with plasmid DNA regenerated within a few weeks into new green undiversified calluses. Under sterile conditions these were separated from the rest of the leaf fragments and transferred into a sterile plastic bucket with MS medium containing spectionomycin (500 μg mL^{-1}) and sucrose (20 mg mL^{-1}). Vital calluses differentiated into complete tobacco plants. These independent transformants were cultured *in vitro* at 25 °C and under continuous light.

3.12 *psbA*^{mut} detection via restriction digestion

To identify the inserted version of the mutated *psbA* gene in the selected transformants, restriction digestion was designed using restriction enzyme ApaI recognizing the GGGCC^C site. This site, created by one silent substitution mutation was included in the immediate vicinity of two introduced mutations of interest. In this way, we were able to discriminate transgenic individuals harbouring point mutations of interest in the gene from those carrying the *psbA* gene from those without these mutations. Two amplification reactions were prepared, one to confirm the presence of *aadA* gene and to exclude the individuals without the inserted plasmid (absence of *aadA* gene concomitant with spontaneous resistance to spectinomycin) and to amplify the part of the *psbA* gene for subsequent restriction reaction. Samples for PCR (Tab. 6) were prepared according to Klimyuk *et al.* (1993).

Table 6: Two amplification reactions – reaction A for detection the presence of *aadA* gene, reaction B for amplification the part of *psbA* gene for subsequent restriction digestion analysis.

Storage concentration	A - per one reaction [µL]	B - per one reaction [µL]	Final concentration
10x Takara buffer	2.5	5	1x
dNTPs (2.5 mM each)	2	4	200 µM of each
10% PVP	2.5	5	1%
10% BSA	0,25	0.5	0.1%
Primer 1 (20 µM)	0.5 (aadA1)	1 (Mut-F2)	0.4 µM
Primer 2 (20 µM)	0.5 (aadA2)	1 (Mut-R3)	0.4 µM
rTaq DNA polymerase	0.25	0.5	
SDW	16.5	33	-
DNA extract	0.5	1	-

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min; samples were held at 4 °C at the end of the program.

Original plasmid DNA was used as a positive control and the wild type *N. tabacum* leaf as a negative control. Reaction for detection of *aadA* gene was prepared in a total volume of 25 µL while for the amplification of the part of *psbA* gene a total volume of 50 µL was used.

Samples were loaded onto a 1% agarose gel with 1x TAE buffer and 100 bp ladder (New England Biolabs, USA). Expected size of *aadA* gene amplicon was 787 bp and the one of *psbA* gene amplicon was 1036 bp.

Samples positive for the presence of the *aadA* gene in A reaction were selected and appropriate bands after electrophoresis of B reaction were purified from gel using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel).

Concentration of DNA of each sample was measured using a Nanodrop 2000. Reactions were prepared for each selected sample in 1.5 mL microtubes: 5 µL CutSmart buffer (New England Biolabs, USA), 1 µL ApaI (25 U μL^{-1}; NEB), 250 ng DNA and appropriate amount of SDW to fill up to 50 µL. The reaction mixtures were incubated in microtubes at 25°C for 3 hours.

Afterwards, the whole volume of 50 µL was loaded onto 0.9% agarose gel with 1x TAE buffer. Amplicons from successfully transformed individuals carrying mutations of interest were

digested into two fragments of size 410 and 626 bp whilst samples originating from plants without mutations of interest remained uncut. Positively restricted individuals were selected as well as few plants with inserted *aadA* gene but without mutations of interest in the *psbA* gene as a control.

3.13 Transgene detection via Southern blot

DNA isolation

DNA of selected samples (wild type, positively restricted individuals and two control individuals) were isolated according to Tai and Tankslay (1991). Leaves were detached from source plants to gain approximately 1-2 grams of fresh material from every individual. Mortar and pestle were pre-cooled with liquid nitrogen, each leaf sample was submerged in liquid nitrogen inside the mortar and thoroughly homogenized. Leaf powder was transferred into a clean 200 mL centrifugation tube with 15 mL of extraction buffer (100 mM Tris-HCl pH 8; 50 mM EDTA pH 8; 500 mM NaCl; 1.25% SDS; 8.3 mM NaOH; Na-bisulfite 0.38 g per 100 mL; Na-diethyldithiocarbamate 0.38 g per 100 mL). Centrifugation tubes were gently vortexed and then incubated at 65°C. After 15 minutes, 4.65 mL of 5 M potassium acetate was added and after gentle vortexing the centrifugation tubes were kept on ice for 20 minutes.

Subsequently, tubes were centrifuged at 4000 rpm (1940 g) for 10 minutes at 4°C in an Avanti J25 centrifuge (Backman Coulter) using a JA20 rotor. Supernatant was filtered into a clean 50 mL tube using Miracloth and 14 mL of absolute isopropanol were added. After 10 minutes, tubes were centrifuged at 4000 rpm (2640 g) for 10 minutes at 4°C in a Hettich Universal 32 R centrifuge and supernatants were discarded. The pellet was washed with 2 mL of 70% ultraclean ethanol, centrifuged for 5 minutes at 5000 rpm (3300 g), dried on a filtration paper and transferred into a clean microtube with 0.84 ml T5E buffer (50 mM Tris pH 8; 10 mM EDTA pH 8). When dissolved, 0.36 ml of 10 M ammonium acetate was added and left for 10 minutes on ice without shaking.

Afterwards, microtubes were centrifuged at 13500 rpm (17320 g) for 5 minutes, each samples supernatant was then separated into two new microtubes and suplemented with 0.5 mL of absolute isopropanol. After 5 minutes the precipitated DNA was extracted from the isopropanol using a glass hook and washed with 70% ultraclean ethanol in a new microtube. Samples were centrifuged at 13500 rpm (17320 g) for 1 minute and rewashed with 70%

ethanol. Pellets were dried on a sterile filter paper and afterwards each was resuspended in 100-200 mL sterile ultraclean water. Finally, each sample was treated with 5 uL of RNase A (0.1 ug uL^{-1}) per 100 uL of sample. Samples were incubated at 37°C for 15 minutes allowing full rehydration and afterwards stored at -20 °C.

Probe preparation

Two probes were used for hybridization, the first to determine the presence of the selection gene *aadA* and a second to determine if the *psbA* gene is present in either mutated form or as in the original wild type (Fig. 8). Genomic DNA of the wild type was used as a template to prepare a 388 bp long *psbA* probe using SB-F and RP2A primers whilst, plasmid pCB0773A was used as a template to prepare a 787 bp long *aadA* probe using the aadA1 and aadA2 primers. The PCR protocol is described in Table 7. High-fidelity polymerase was used to result precise PCR fragment sequence without mutations.

Table 7: PCR protocol for *aadA* and *psbA* probe preparation.

Storage concentration	Reaction volume [µL]	Final concentration
5x Q5 Phusion buffer	10	1x
dNTPs (2.5 mM each)	4	200 mM each
Primer 1 (20 µM)	1.25	0.5 µM
Primer 2 (20 µM)	1.25	0.5 µM
Q5 DNA polymerase (2 U μL^{-1})	0.5	0.2 U μL^{-1}
SDW	33	-
DNA	1 ng	-

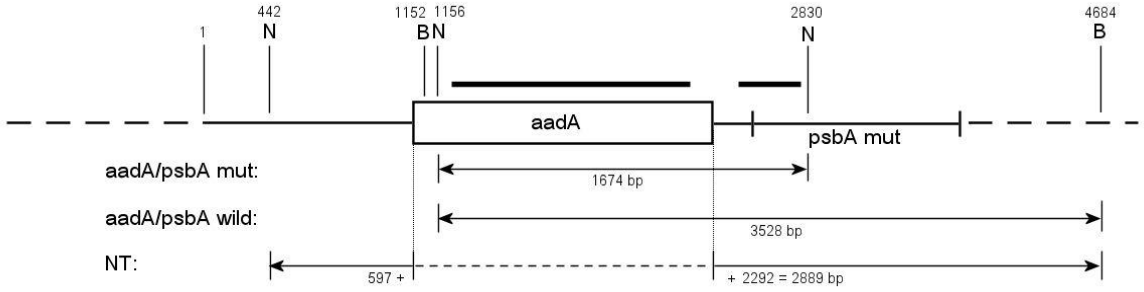

Figure 8: Figure showing the positions of used *psbA* and *aadA* hybridization probes. Dashed line – the sequence of *N. tabacum* ptDNA not included in transformation vector, full line – ptDNA included in vector, *aadA* – selective gene cassette inserted into ptDNA sequence, *psbA* mut – *psbA* gene with 2 mutations of interest and one silent mutation facilitating site for restriction digestion by NcoI enzyme, N and B – restriction site for NcoI and BamHI enzymes (used restriction enzymes to digest *N. tabacum* gDNA), thick lines – the position of used hybridization probes. The lengths of visualized fragments will be the same irrespective of the probe use (*psbA* or *aadA*). No fragment is to be detected in non transformed *N. tabacum* plants.

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min; samples were held at 4 °C at the end of the program.

The PCR products were loaded onto 1% agarose gel with 1x TAE buffer and electrophoretically separated. The DNA was purified from gel using NucleoSpin® Gel and PCR Clean-up kit (Macherey_Nagel). Concentration of both probes was measured spectrofluorometrically using a Qubit 2.0 (Thermo Fisher Scientific).

Restriction digestion of genomic DNA

Genomic DNA fragmentation as well as choice of suitable restriction enzyme(s) to accomplish diverse lengths of probe hybridization fragments for each expected genotype are essential for successful Southern blotting. Each of our samples was simultaneously digested by two

different restriction enzymes BamHI-HF (New England Biolabs, USA) and NcoI-HF (New England Biolabs, USA) overnight at 37 °C. High-fidelity enzymes were used to result precise digestion. To check the success rate of restriction digestion, 0,5 µl of each sample were electrophoresed on a 0,8% agarose gel.

Table 8: Restriction digestion

Storage concentration	Reaction volume [µl]	Final concentration
CutSmart buffer	20	1x
BamHI-HF (20U μL^{-1})*	1.5	0.15 (0.45)* U μL^{-1}
NcoI-HF (20U μL^{-1})**	1.5	0.15 (0.45)** U μL^{-1}
SDW	To 50	-
gDNA	15 ng	-

* After 2 and 4 hours, respectively, 1.5 µl *Bam*HI-HF enzyme was added to each sample.
** After 4 and 6 hours, respectively, 1.5 µl *Nco*I-HF enzyme was added to each sample.

Digested DNA was purified by 40 µL ammonium acetate and 300 µL 96% ethanol. After vortexing and 45 minutes incubation at -70 °C, samples were centrifuged for 7 minutes at 13500 rpm (17320 g). Sediments were washed with 1 mL of 70% ethanol. After drying all residual ethanol, each of the samples was resuspended in 30 µL of SDW.

Agarose gel preparation

All digested samples, each with 4 µL of 10x loading buffer, were fully loaded onto 1% agarose gel prepared with 1x TAE buffer. As a DNA size marker, 500 ng of 1 kb ladder (New England Biolabs, USA) was used. Gel electrophoresis was run with 1.3 V cm^{-1} overnight (approximately 17 hours). Afterwards the gel was stained with ethidium bromide (1 mg L^{-1}) in 1x TAE buffer for 30 minutes to check the success rate of DNA restrictase digestion as well as for discarding of unnecessary parts of the gel.

The gel was soaked in three various solutions while gentle shaking. Firstly, in depurination solution (0.25 M HCl) for 15 minutes, afterwards in denaturation solution (0.5 M NaOH; 1.5

M NaCl) for 30 minutes and at last in neutralizing solution (1.5 M NaCl; 0.5 M Tris-HCl pH 7.5) for 30 minutes.

The prepared gel was transferred onto a filter paper bridge (Whatman 3MM) immersed in 10x SSC buffer (1.5 M sodium chloride; 0.15 M trisodium citrate) and covered with a nylon membrane (Hybond-N+; GE Healthcare Life Sciences), two Whatman 3 MM filter papers soaked in 10x SSC and a stack of cellulose. The gel was weighted down with a load of about 0.75 kg and left overnight at room temperature.

The next day the blot system was carefully dismantled. To dispose the agarose and salt the membrane was briefly washed in 2x SSC. The DNA was bound onto the membrane by baking the membrane at 80°C for 10 minutes followed by irradiation with UV light at 120 mJ cm^{-2} using a UV crosslinker UVC 500 (Hoefer). The membrane was stored between filter papers, in a plastic bag at room temperature.

Hybridization

Southern hybridization was performed according to Church and Gilbert (1984). Both probes and 1 kb DNA ladder (New England Biolabs, USA) were labelled using Random Primer DNA Labelling Kit Ver. 2 (TaKaRa, Japan). Sixty ng of template DNA was mixed with 2 µL of random primers and filled to a total volume of 14 µL. This solution was heated in microtubes at 95°C for 3 minutes and cooled on ice for 5 minutes. Afterwards, 2.5 µL of 10x buffer, 2.5 µL of dNTP mixture, 1 µL of Klenow fragment and 5 µL of [α-^{32}P]dCTP (3000 Ci $mmol^{-1}$) were added to each tube and incubated at 37°C for 10 minutes. For enzyme inhibition, 2 µL of 1.6 M EDTA (i.e. to final concentration of 30 mM) was added into probe tubes and 200 µL TE buffer into ladder tube.

Success rate of labelling was estimated using DEAE (diethylaminoethyl) papers: 1 µL of labelled DNA was dropped on small piece of DEAE paper inside 20 mL scintillation vial and washed two times with 4 mL of 0.5 M $Na_2HPO_4 \cdot$. $12H_2O$. Radioactivity in the vial was measured with a dosimeter before as well as after each wash out.

Immediately before use, the probes were denatured by heating to 95°C for 3 minutes followed by chilling on ice for 5 minutes.

The membrane was transferred into a hybridization tube with 50 ml of hybridization buffer (400 mM NaP buffer pH 7.24; 1 mM EDTA pH 8.3; 1% BSA; 7% SDS) and kept spinning at

65°C for 30 minutes to pre-hybridize. Subsequently, the full volume of labelled denatured prepared *psbA* probe and 0.25 µl of labelled denatured ladder probe were added and left to spin overnight at 65°C in a hybridisation oven HB-1D (Techne).

The following day the membrane was washed three times with 50 mL of washing solution (1% SDS; 100 mM NaP buffer pH 7.2; 1 mM EDTA) at 65°C for 30 minutes. Afterwards, the wet membrane was sealed into a plastic bag and autoradiographed for about 1.5 h using a phosphoimager Typhoon system (Amersham Pharmacia Biotech).

Before hybridisation with the other probe, the membrane was stripped using 500 mL of boiling 0.1% SDS and left cool to room temperature while gently shaken.

3.14 Seeds

Obtained regenerated shoots/plants are characterized by integration of the transgene into a few plastid genome copies only. To eliminate untransformed plastids, i.e. to achieve a homogeneous population of plastid genomes, 25 to 30 cell divisions under selection pressure have to be realized (Verma and Daniel, 2007). To verify homoplasmicity we first generated a seed generation by self-pollination each transgenic plant and sowing the seeds onto MS medium supplemented with 500 mg L^{-1} of spectinomycin. In the case of homoplasmy, all seedlings (about 400) were green and able to grow on such medium.

3.15 Sequencing of *psbA* gene from the 1st generation of transformed plants

The insertion of the *psbA* gene sequence in transformants was controlled by sequencing two parts of the *psbA* gene overlapping in the site of the target mutations. Primer pairs for PCRs were: primers SB-F and *psbA* R2.4 were used for reaction 1 with an expected amplicon length of 672 bp, primers FP2A and *psbA* R2.1 for reaction 2 with an expected amplicon length of 988 bp (Tab. 9). High-fidelity polymerase was used to result precise sequence of amplified fragments without mutations.

Table 9: The protocol of amplification reactions for sequencing the inserted region of *psbA* gene region in the 1st generation of transformed plants.

Storage concentration	Per one reaction [µl]	Final concentration
5x Q5 Phusion buffer	10	1x
dNTPs (2,5 mM each)	4	200 µM each
Primer 1 (20 µM)	1,25	0,5 µM
Primer 2 (20 µM)	1,25	0,5 µM
Q5 DNA Polymerase (2 U/µl)	0,5	0,02 U/µl
SDW	33	-
DNA extract	1	-

* Combination of primers P1 and P2 were selected to map inserted region of *psbA* gene with overlap over the target mutations (P1 – P2: SB-F – psbA R2.4; FP2A – psbA R2.1).

PCR conditions using BioEr XP thermocycler were as follows: An initial denaturation step at 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min; samples were held at 4 °C after the end of the program.

Samples after PCR were subjected to electrophoresis on 1.25 % agarose gel with 1x TAE buffer. Fragments were purified from the gel using NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel).

Mixture for sequencing consisted of 200 ng of DNA (in variable volume according to DNA concentration in eluate), 5 µL 5 µM primer and SDW to fill the total volume of 10 µL. Each of the three PCR fragments was sequenced from both directions. Sequencing was provided by a commercial company (www.gatc-biotech.com).

4. Results

4.1 Plasmid preparation

The initial step of the chloroplast transformation project was the successful generation of a transformation plasmid. To accomplish that, five fragments from different sources needed to be amplified and joined: 1) left homological region (base number 155308-155943 and 1-403 in ptDNA sequence according to Shimad and Sugiura, 1991), 2) selective cassette aadA, 3) terminal part of *psbA* gene withouth mutations of interest (base number 404-809 according to Shimad and Sugiura, 1991), 4) followed by the sequence of the *psbA* gene with point mutations of interest (base number 810-1594), 5) plasmid pUC19 x with origin of replication but without promoter region in acceptor site of plasmid.

After the spectrophotometric measurement of DNA concentration in all five fragments, the molar concentration of each of the fragments was computed for the assembly reaction in two different concentrations of DNA (1A and 2A) according to the Gibson Assembly® Master Mix protocol (New England BioLabs, USA). The final reaction mixture needed to have a maximum volume of 10 μL. Because the success rate of assembly decreases with the number of assembled fragments in the Gibson Assembly® Master Mix, two additional assembly reactions (1B and 2B) were prepared with only four fragments – 3, 4, 5 and a new fragment 6 constructed by fusion of fragments 1 and 2 (left homological region and *aadA* gene cassette) using PCR to increase the probability of assembling (Tab. 10).

Competent *E. coli* cells were transformed with constructed plasmid from all four assembling reactions. Each *E. coli* suspension was inoculated onto ten petri dishes with solid LB medium with the addition of AMP. Because the plasmid pCB0773A contains a gene for AMP resistance, successfully transformed *E. coli* cells grew on the medium with this antibiotic (except for those carrying a spontaneous resistance).

Table 10: Four reactions with different DNA concentration, reaction 1A and 2A with five DNA fragments, reaction 1B and 2B with fragment 3, 4, 5 and with new fragment 6 made from fragment 1 and 2.

Fragment	1A	2A
1	250 ng = 0.36 pmol = 3.3 µL	125 ng = 0.18 pmol = 1.7 µL
2	300 ng = 0.34 pmol = 3.0 µL	150 ng = 0.17 pmol = 1.5 µL
3	100 ng = 0.37 pmol = 0.85 µL	50 ng = 0.18 pmol = 0.42 µL
4	100 ng = 0.06 pmol = 0.65 µL	50 ng = 0.03 pmol = 0.33 µL
5	200 ng = 0.38 pmol = 1.1 µL	100 ng = 0.19 pmol = 0.55 µL
Total	**1.51 pmol = 8.9 µL + 1.1 µL SDW**	**0.75 pmol = 4.5 µL + 5,5 µL SDW**
Fragment	**1B**	**2B**
3	100 ng = 0.37 pmol = 0.85 µL	200 ng = 0.74 pmol = 1.7 µL
4	100 ng = 0.06 pmol = 0.65 µL	200 ng = 0.12 pmol = 1.3 µL
5	200 ng = 0.38 pmol = 1.1 µL	400 ng = 0.76 pmol = 2.2 µL
6	100 ng = 0.06 pmol = 0.73 µL	200 ng = 0.12 pmol = 1.5 µL
Total	**0.87 pmol = 3.33 µL + 6.7 µL SDW**	**1.74 pmol = 6.7 µL + 3.3 µL SDW**

*pmols = (weight in ng) x 1 000 / (base pairs x 650 daltons) (NEB)

From reaction A, nine *E. coli* colonies grew in total: five colonies from reaction A1 and four colonies from reaction A2. These nine colonies were transferred to a new petri dish with solid LB medium and AMP for further growth. The presence of the *aadA* gene in these clones was analysed to eliminate clones with spontaneous resistance to AMP. PCR with primers aadA1 and aadA2 was performed and the amplified fragments were loaded on 1% agarose gel (expected amplicon length was 786 bp). Five colonies (1, 2, 4, 5 and 6) were positive to the presence of *aadA* gene (Fig. 9).

From reactions B1 and B2, tens of AMP resistant colonies grew. Because transformation with the assembled plasmid from reaction A showed to be successful and sufficient for our purpose, *E. coli* colonies from reaction B were never analysed.

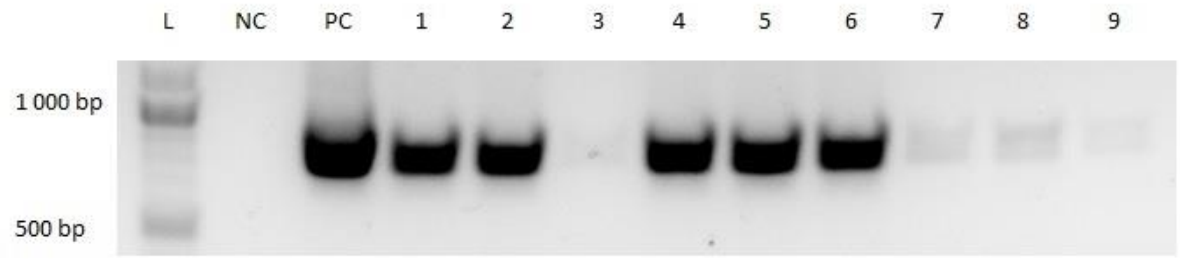

Figure 9: The electrophoretogram of eight AMP resistant *E. coli* colonies analysed for the presence of *aadA* gene. 1-9 – analysed colonies, NC – PCR mix without DNA. Colonies 1, 2, 4, 5, 6 contained *aadA* gene, colony 6 was chosen for next experiments. Expected fragment length – 786 bp, L – 100 bp DNA ladder (NEB).

A plasmid with correctly assembled fragments was essential for the next experiment. Using PCR, the presence, order and orientation of inserted fragments in plasmid DNA were checked in all five *E. coli* colonies positive for the *aadA* gene (Fig. 10).

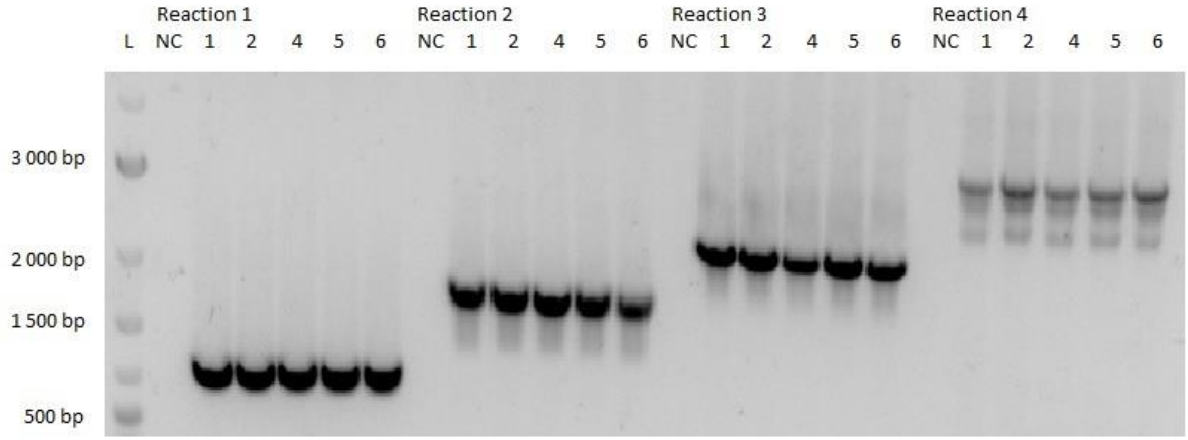

Figure 10: The electrophoretogram of four amplification reactions verifying presence, order and orientation of inserted fragments in five *E. coli* colonies positive for the *aadA* gene. 1, 2, 4, 5 and 6 – the number of colonies with *aadA* gene, NC – PCR mix of each reaction. All colonies contained plasmids assembled from fragments in the correct order and orientation. Expected fragment length – reaction 1: 1 200 bp, reaction 2: 1 800 bp, reaction 3: 2 400 bp, reaction 4: 3 600 bp; L - 1 kb DNA ladder (NEB).

Reaction 1 using primers Mut-F6 and Mut-R8 verified reciprocal orientation of first and second part of the *psbA*^{mut} gene (expected amplicon length of 1 200 bp), reaction 2 using primers Mut-F5 and Mut-R7 verified the reciprocal orientation of the *aadA* gene and the first part of the *psbA*^{mut} gene (expected amplicon length of 1 800 bp), reaction 3 using primers Mut-F4 and Mut-R5 verified the reciprocal orientation of the left homological region and the *aadA* gene (expected amplicon length of 2 400 bp) and reaction 4 using primers Mut-F4 and Mut-R8 was used to verify the length of the whole insert in pUC19 (expected amplicon length of about 3 600 bp). Because of the specific length of these four amplified fragments in the designed plasmid, colonies containing this required plasmid could be unequivocally identified (see Figure 6).

The presence, correct order and orientation of inserted parts of E. coli plasmid was confirmed in all five selected *E. coli* colonies. Colony number 6 was randomly chosen to produce enough bacterium to gain sufficient amount of plasmid DNA for the following isolation.

E. coli cells from colony number 6 were grown in three 500 mL Erlenmeyer flasks with 100 mL of LB medium. Cell suspension from all cultivation flasks were pooled together and optical density at 600 nm was measured to estimate the amount of bacteria. Because the optical density was acceptable (OD_{600} = 2.6), plasmid DNA was isolated from the harvested cells using NucleoBond® Xtra Midi (Macherey-Nagel).

Concentration of isolated plasmid DNA was measured using a Qubit® 2.0 Fluorometer (Invitrogen) was 91.6 μg μL^{-1}. The purity and size of the isolated plasmid was inspected by electrophoresis of the sample on a 0.5% agarose gel (Fig. 11). The storage solution of plasmid DNA was diluted to 1 μg μL^{-1} according to the concentration suitable for Biolistics.

The correct sequence of the *psbA* gene in the transformation vector, i. e. presence of all mutations of interest and concomitantly the absence of any kind of undesired mutations needed to be verified. Therefore, the whole *psbA* gene including closely adjacent parts was sequenced. To cover this region, three amplification reactions were performed, each of them in two reaction tubes to gain a sufficient amount of DNA for sequencing – primers SB-F and psbA R2.4 were used for reaction 1 (expected amplicon length of 672 bp), primers FP2A and psbA R2.4 for reaction 2 (expected length of 317 bp) and primers FP2A and UC-R5 for reaction 3 (expected amplicon length of 875 bp). Amplified fragments were separated electrophoretically in 1.25% agarose gel (Fig. 12).

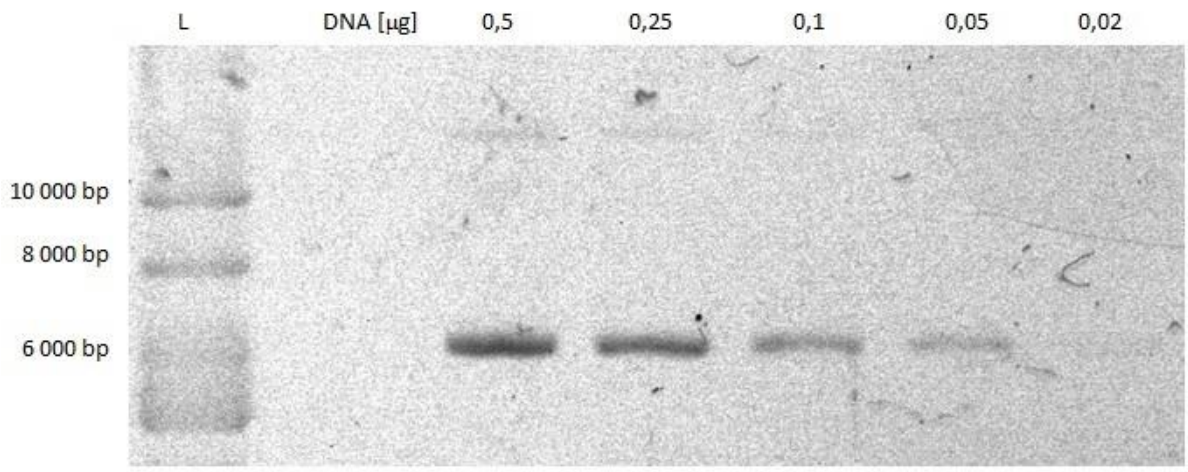

Figure 11: The electrophoretogram of isolated plasmid M6. A different amount of DNA was loaded in particular wells. Expected length – 6114 bp; L – Supercoiled DNA Ladder (NEB).

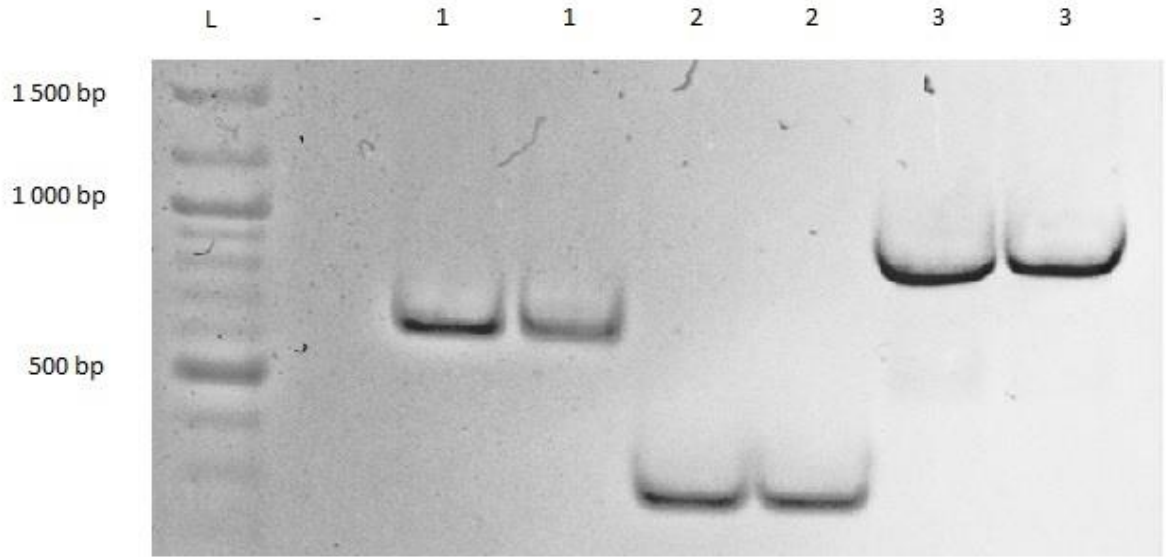

Figure 12: The electrophoretogram of three amplification reactions (each in two PCR tubes) for following sequencing of the whole inserted *psbA* gene and closely adjacent sequence. Expected fragment length – reaction 1: 672 bp, reaction 2: 317 bp, reaction 3: 875 bp; L – 2-Log DNA Ladder (NEB).

Sequencing proved that the plasmid M6 contained both introduced mutations of interest as well as two silent mutations creating two restriction sites needed for identification of transgenic plants harbouring *psbA*^{mut} and for Southern analysis, respectively. The comparison with the expected sequence of the designed vector exposed the deletion of three nucleotides at the beginning of the inserted *psbA*^{mut} gene. These three nucleotides code the second amino acid in the native sequence of the *psbA* gene. Except for this difference, plasmid M6 sequence copied the sequence of designed vector (see Appendix 1).

4.2 The successful rate of Biolistic bombardment

The Biolistic process using a PDS-1000/He Particle Delivery System (Bio-Rad) was used to transform *N. tabacum* ptDNA with plasmid M6. This system enables the transmission of isolated plasmid DNA into living leaf cells of wild type plants.

Three biolistic experiments were realized with intervals of approximately three weeks to achieve the highest probability of successful transformation by the complete homologous recombination of wild type *N. tabacum* ptDNA and constructed plasmid M6. The total number of shots was 68. We achieved 73 independent transformation events (i. e. 73 registered counts of green calli or shoots, Fig. 13), almost half of them successfully regenerated into complete *N. tabacum* plants (Fig. 15, Tab. 11) with differing degrees of vitality.

Figure 13: Example of fragmented leaf with callus (red circle, left) and the detail of callus with incipient organogenesis (right).

Tab. 11: The results of three biolistic transformation experiments describing the number of successfully regenerated plants from the total number of transformation events, together with the number of transformed plants with all mutations of interest.

	Shooting 1	Shooting 2	Shooting 3	Total
n. of shots	24	24	20	68
n. of successful shots	15	12	8	35
n. of independent SPE resistant calluses	31	28	14	73
n. of successful plant regenerations	18	13	5	36
n. of *aadA*$^{+}$/*psbA*^{mut} plants	4	1	0	5

Finally, 36 independently regenerated viable plants were retrieved. All of them were tested for the presence of the *aadA* gene using PCR with primers aadA1 and aadA2 (expected amplicon length was 786 bp). All of the 36 analysed individuals were positive for *aadA* gene presence (*aadA*$^{+}$) indicating that none of the individuals were spontaneously resistant to SPE in MS medium.

Because the introduced *psbA* gene simultaneously served as a homological region, homologous recombination could proceed anywhere within the sequence of the first and second part of the *psbA*^{mut}. For that reason, the transgenic plants may or may not contain the desirable mutations of interest. Thus, the presence of both desirable mutations in the *psbA* gene of all regenerated plants needed to be confirmed. Because a silent mutation in the *psbA*^{mut} introduced a *Apa*I restriction site (GGGCC^C) immediately after two target mutations, restriction digestion with this enzyme could discriminate individuals with the native version of the *psbA* gene from the mutated one (native *psbA* did not contain silent mutation hence restriction did not occur).

To accomplish that, the respective part of the *psbA* gene was amplified from all those individuals carrying the *aadA* gene using primers Mut-F2 and Mut-R3 (expected amplicon length of 1 036 bp). These fragments were then cleaved with the ApaI restriction enzyme and results were inspected by loading the samples onto a 0.9% agarose gel and separated by electrophoresis. Fragments from individuals with native versions of the *psbA* gene were uncut and those from individuals with mutations of interest were cut in two fragments with distinct sizes (expected length 410 and 626 bp; Fig. 14).

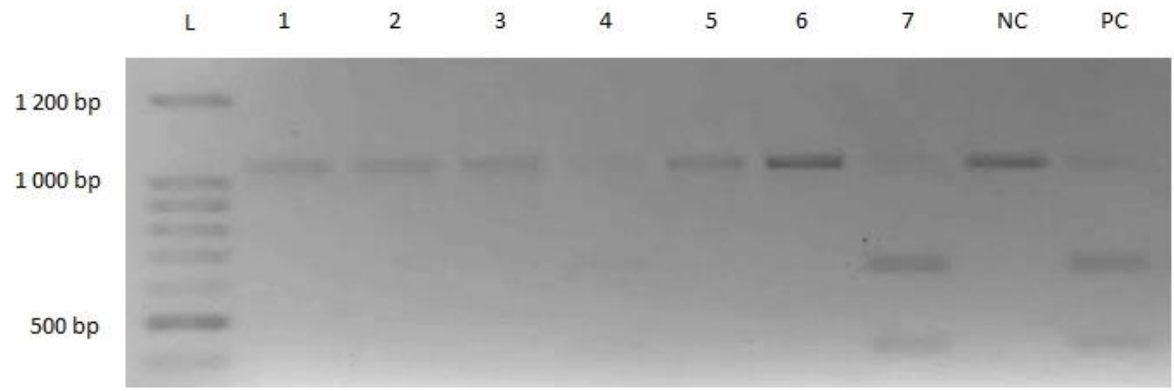

Figure 14: An electrophoretogram of restriction analysis with the *Apa*I enzyme displaying which individuals have the mutated version of the *psbA* gene and which carry the native one. 1-7 – regenerated plants, NC – wild type, PC – transformation plasmid DNA (*aadA*$^{+}$/*psbA*^{mut}). Only sample number 7 was digested. Expected fragment length – psbA: 1 036 bp, *psbA*^{mut}: 410 and 626 bp; L – 100 bp DNA ladder (NEB).

Restriction digestion revealed six individuals with introduced mutations of interest in the *psbA* gene. Additionally, different heteroplasmic/homoplasmic phases were expected to be present in individual plant lineages. The estimation of the state of homoplasticity could be theoretically based on the detected presence of the unrestricted fragment in the samples. However, unrestricted fragment residue was observed even in the positive control sample (M6 plasmid) where all DNA present in the sample only contains the *psbA*^{mut} gene. To assess if an individual still contains chloroplasts with the native form of the *psbA* gene (*psbA*^{wild}), Southern hybridization was performed.

Individuals with the *psbA*^{mut} gene were chosen (*aadA*$^{+}$/*psbA*^{mut}) as well as several ones with hte *aadA* gene but with the native *psbA* (*aadA*$^{+}$/*psbA*^{wild}) as control plants.

Figure 15: An example of plant regenerated from calli. Left – transformaned plant growing on MS medium supplemented with spectinomycin, right – transformated plant growing on hydroponic system.

4.3 Southern analysis

Southern analysis offers precise evidence if the searched sequence is presented in the analysed DNA sample using probe hybridization. With suitably designed probes, all genotypes can be uncovered (Tab. 12; see Figure 8).

Table 12: Summary of expected genotypes with the length of probe hybridized fragments.

Expected genotype	*aadA* probe hybridization	*psbA* probe hybridization
Wild type	none fragment	2 889
$aadA^{+}/psbA^{wild}$	3 528	3 528
$aadA^{+}/psbA^{mut}$	1 674	1 674

Obtaining sufficient amounts of gDNA (15 µg from each individual) as well as successful gDNA restriction digestion is the primary requirement of successful Southern analysis. To obtain a sufficient amount of gDNA, DNA was isolated twice from three individuals. Because of the low vitality of one of the five *aadA*$^{+}$*/psbA*^{mut} individuals, only four of them were analysed. Three *aadA*$^{+}$*/psbA*^{wild} individuals were chosen at random as a control together with wild type. The final amount of DNA from all analysed individuals is displayed in the Table 13.

Tab. 13: The summary of analysed individuals with information about total amount of gDNA obtained. PC – wild type, T1-T4 – *aadA*$^{+}$*/psbA*^{mut}, C1-C3 – *aadA*$^{+}$*/psbA*^{wild}.

Code	Weight of leaves [g]		Concentration [µg/µl]		Total amount of gDNA [µg]
	1. isolation	2. isolation	1. isolation	2. isolation	
WT	1,1	1.0	0.08	0.08	32
T1	2.0	1.2	0.15	0.20	55
T2	2.0	1.0	0.15	0.12	39
T3	1.4	-	0.23	-	23
T4	1.1	-	0.27	-	27
C1	1.2	-	0.11	-	11
C2	1.1	-	0.26	-	26
C3	2.1	-	0.16	-	16

Restriction enzymes (*Bam*HI-HF and *Nco*I-HF) were chosen to obtain different sites of probe hybridization due to different fragment size in all possible genotypes (see Figure 8 in chapter 3.13). The restriction digestion of gDNA was performed simultaneously with both enzymes. The success rate of gDNA digestion was checked by electrophoresis of samples loaded onto a 0.8% agarose gel. Digestion was successful in all samples and therefore probe hybridization was performed.

Both of the two probe hybridizations were successful and confirmed particular genotypes in all individuals which were estimated using restriction digestion. All four individuals with expected genotype *aadA*$^{+}$*/psbA*^{mut} were confirmed as well as three controls with genotype *aadA*$^{+}$*/psbA*^{wild}. Sample T4 probably contains chloroplasts with both analysed transformed genotypes (*aadA*$^{+}$*/psbA*^{wild}, *aadA*$^{+}$*/psbA*^{mut}) because the *aadA* probe hybridized in both sites for these two genotypes (Fig. 16).

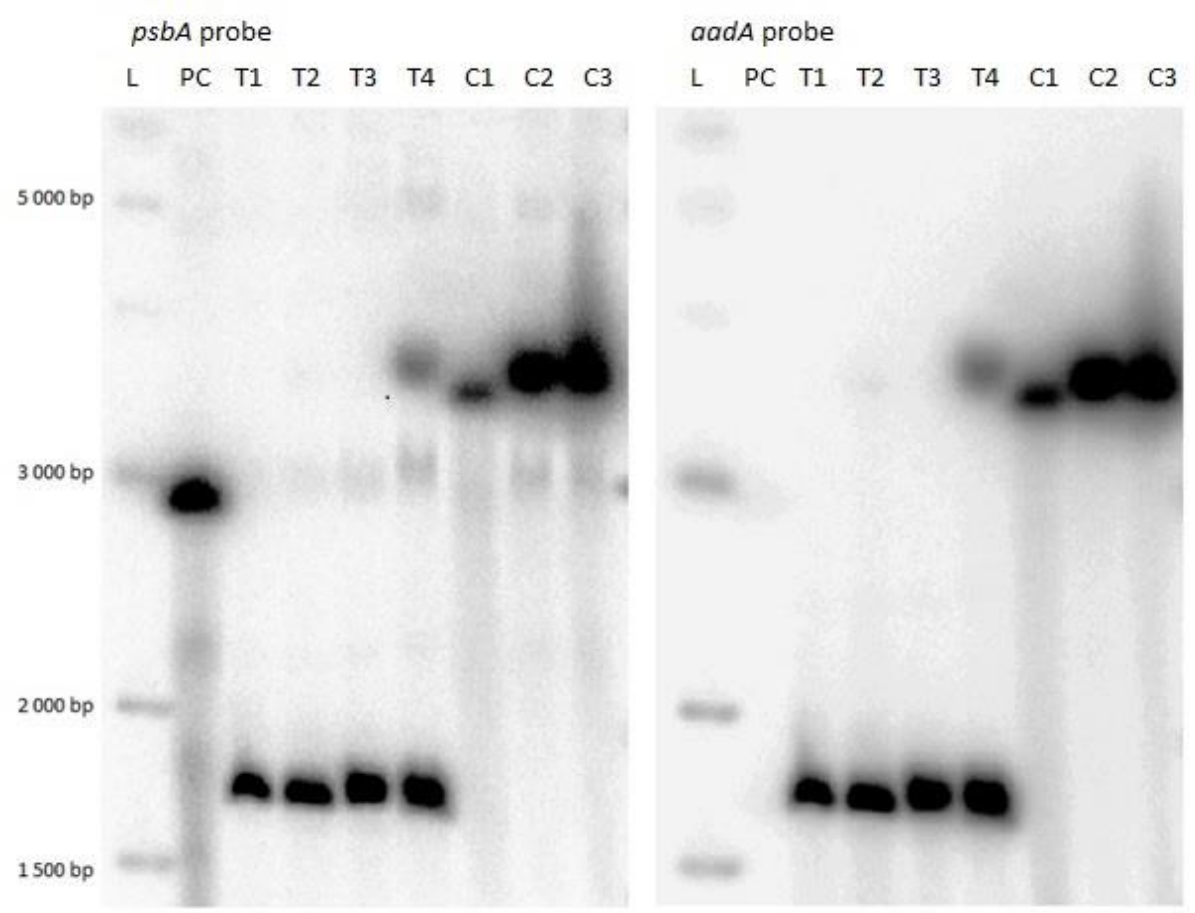

Figure 16: The electrophoretogram of Southern hybridization with *psbA* probe (left) and *aadA* probe (right). PC – wild type, T1-T4 - *aadA*$^{+}$/*psbA*^{mut} genotype, C1-C3 - *aadA*$^{+}$/*psbA*^{wild} genotype. Sample T4 is heteroplastic individual with chloroplasts. L - 1 kb DNA ladder (NEB).

4.4 Seeds sowing

To obtain homoplastic individuals, the first seed generation was obtained by self-fertilization. One *aadA*$^{+}$/*psbA*^{mut} transformant and one *aadA*$^{+}$/*psbA*^{wild} as a control were picked and grew in a hydroponic system. After flowering and self-pollination of these individuals, seeds were harvested and homoplasmy was tested using the germinability test. Four hundred seeds from each sample were sown on petri dishes with MS medium and SPE, as well as four hundred seeds from wild type *N. tabacum.* Seeds, germinated seeds and new seedlings that successfully grew, including discrimination between green and white seedlings were counted (Fig. 17).

92% of *aadA*$^{+}$/*psbA*^{mut} seeds and 94% of *aadA*$^{+}$/*psbA*^{wild} seeds germinated. All germinated seedlings were green and grew on SPE in contrast to wild type seedlings which were white. The germinability of wild type seeds on medium without SPE was 96%.

Figure 17: Seed harvesting (left) and petri dish with MS medium supplemented by SPE with seedlings of *aadA*$^{+}$/*psbA*^{mut} plant (wild type as a negative control in red circle, right).

5. DISCUSSION

5.1 Plasmid preparation

The designed transformation vector was constructed from five fragments – left homological region amplified from *N. tabacum* gDNA, selective cassette *aadA* amplified from pCB0773A (Bříza *et al*.; 2013), terminal part of *psbA* gene without mutations of interest amplified from *N. tabacum* gDNA, followed by the sequence of *psbA* gene with point mutations, commercially produced and plasmid pUC19 with origin of replication but without promoter region in acceptor site of the plasmid. Fragments were assembled using Gibson assembling.

For Gibson assembling, four reactions were performed. Two concentrations of fragment DNA were computed for two reactions (1A and 2A) according to the manufacturers protocol (New England BioLabs Inc., USA). To increase the probability of successful assembly, two reactions (1B and 2B) were added in which the number of fragments was decreased to four by joining fragment 1 and 2 using PCR. The reason was inexperience with the success rate of this method. *E. coli* cells were transformed by each reaction separately.

Even though the number of AMP resistant bacterial colonies was significantly higher in *E. coli* transformed by plasmid assembled in reactions B, only colonies from reactions A were analysed. Optimizing the efficiency of different parameters in Gibson assembly was however not the main purpose of this study. Nevertheless, in case none of the bacterial colonies from reaction A had contained the correct plasmid sequence, petri dishes with colonies from reactions B were kept.

Five bacterial colonies from reaction B grown on MS medium with AMP contained *aadA* gene (thus the transformation plasmid DNA was successfully incorporated into the cell), the remaining four were spontaneously resistant. Presence, correct order and orientation of five assembled fragments in the transformation vector in these five colonies were tested by PCR using specifically selected primers. Observed amplified fragment length was compared with expected fragment length. All five bacterial colonies contained plasmids assembled from all five fragments in the correct order. One of bacterial colonies was randomly chosen to produce the plasmid DNA.

The isolated plasmid was sequenced in three reactions covering the entire region of *psbA* gene to verify the presence of all mutations of interest and the absence of any kind of unwanted mutation changes. All mutations were confirmed at their respective planed sites according to

the planned design. The deletion of three nucleotides in the beginning of the *psbA* gene coding the second amino acid in the native sequence of *psbA* gene was exposed. Because the complete *psbA* gene was not required for effective homologous recombination, this plasmid could be used for Biolistics.

5.2 Biolistics

Biolistic bombardment was used as a transformation method because the success rate of biolistic transformation is high despite its low transformation efficiency (Lumbreras and Purton, 1998). This method is used for ptDNA transformation in higher plants since 1990 (Svab *et al.*, 1990) and is widely used today (Baron et al., 2009; Bříza et al., 2013).

All leaves for Biolistics were chosen with respect to the vitality and fitness of the entire plant and the particular parts of leaves because the stress during and after the process is very high (vacuum chamber, particle bombardment, antibiotics).

Three biolistic experiments were performed with 36 independent transformation events (Tab. 11). All individuals were positive for the *aadA* gene, only 5 of had the genotype $aadA^{+}/psbA^{mut}$. The last experiment had a low success rate with only 5 independent transformation events (18 in the first experiment, 13 in the second experiment). In comparison with reported results (5-15 independent transformed cells were described; Daniell *et al*., 2001; Fernandez-San Millan *et al.*, 2003; Dhingra and Daniell, 2004), a success rate in this study was low. The greatest variability in the success rate of this method is in the loading the golden particles onto the microcarriers. These need to be loaded in a very thin layer with all particles dispersed to avoid undesired excessive damage to the cells. Also, it is virtually impossible to load the same amount of gold particles in the same layer for every experiment (Sanford *et al.*, 1993). To compare the efficiency of all three shootings, a higher number of shots would have to be performed. Approximately zero to three calli were reached in every successful shot (in tobacco, 5-15 are described; Daniell *et al*., 2001; Fernandez-San Millan *et al.*, 2003; Dhingra and Daniell, 2004). This proves that the ground source of the variability in the success rate of this method lies in the practical experience of the operator(s).

Because the transformation vector included the selective gene *aadA*, cells with the incorporated plasmid DNA were SPE resistant and grew on MS with this antibiotic. Some

calli were not regenerated and organogenesis did not appear. The vital calli were transferred onto MS medium and were later analysed.

Some calli were not successfully regenerated and organogenesis was never reached. Biolistics is burdened with many stressful situations, for example, plant leaves are exposed to pressure increase inside a vacuum chamber which causes a loss of turgor,

5.3 Plant analysis

Because the inserted gene served as a homologous region for the recombination at the same time, homologous recombination in cpDNA could appear within the entire *psbA* part of the transformation vector - before or after mutations of desire. In view of this fact, individuals selected on SPE and carrying the *aadA* gene could have two genotypes, $aadA^{+}/psbA^{mut}$ or $aadA^{+}/psbA^{wild}$. To simply recognize individuals with the desired genotype, the restriction digestion of amplified *psbA* gene fragments was performed. One silent mutation directly after two missense mutations created the site recognized by the *Apa*I restriction enzyme to enable fast detection if homologous recombination was reached before mutations of interest or after them and if chloroplasts in the plants carried only the *aadA* gene without a mutated *psbA* gene or if all mutations were inserted.

Firstly, individuals without the *aadA* gene (spontaneously resistant) needed to be eliminated. None of those were found. Therefore, the part of *psbA* gene of all plants was amplified and restricted with the *Apa*I restriction enzyme. Because the *Apa*I site occurred only once in the amplified fragment sequence, only two possible results were expected - restricted to two fragments ($aadA^{+}/psbA^{mut}$) or not ($aadA^{+}/psbA^{wild}$).

Only five samples of the 36 exhibited positive restriction. In these, a thin unrestricted band was observed. The reason could be that the plants were heteroplastic, but the same band was visible even in the positive control (plasmid M6) carrying only a mutated version of *psbA* gene. The reason for this effect is due to an apparent limited efficiency of the restriction enzyme. Various effects could be accounted for in such a result, for example salt inhibition, not enough units of enzyme, short time of incubation (New England BioLabs Inc., USA).

Southern blot is a useful tool to recognize heterosplastic individuals using probes homologous to sequences of the sample. After restriction digestion of gDNA with suitably chosen enzymes, fragments of different length for probe hybridization are created, one length typical for each

of studied genotype. Because one of the five *aadA*$^{+}$*/psbA*^{mut} plants had lower fitness and vitality, only four individuals were analysed. The regenerant possessed a lower fitness (pale, narrow, elongated leaves, retarded growth) possibly due to incorrect somatic regeneration after the biolistic bombardment. Three *aadA*$^{+}$*/psbA*^{wild} plants were chosen as control samples. Two probes were hybridized separately because fragment length for hybridization was similar. All bands corresponded with expected fragment length of probe hybridization.

Genotype *aadA*$^{+}$*/psbA*^{wild} was confirmed in three control plants. Three *aadA*$^{+}$*/psbA*^{mut} plants were homoplastic. The fourth individual contained a mixture of chloroplasts with two different genotypes - *aadA*$^{+}$*/psbA*^{wild} and *aadA*$^{+}$*/psbA*^{mut} exhibiting a clear heteroplastic phenotype (Fig. 16). This heteroplastic genotype could originate in two possible ways. Two unique transformation plasmids coated on a golden particle(s) injected during Biolistics into a single tobacco cell caused two independent transformation events within this one cell. One transformation event took place before mutations of desire and the second one after them. Eventually, the same could happen in two neighbouring cells which fused and created one callus. In the second scenario, correct recombination was achieved and transformed cpDNA had a *aadA*$^{+}$*/psbA*^{mut} genotype. This mutation spread after selection stress but before it completely replaced the wild type psbA gene, *aadA*$^{+}$*/psbA*^{wild} genotype in one of them was achieved by homologous recombination between wild type and *aadA*$^{+}$*/psbA*^{mut} cpDNA. Because both genotypes (*aadA*$^{+}$*/psbA*^{wild} and *aadA*$^{+}$*/psbA*^{mut}) contain the gene for SPE resistance, chloroplasts with both genotypes are preserved. It is more probable explanation because homologous recombination of ptDNA within one cell is not a rare process (Puchta *et al.*, 1994).

To achieve homoplastic plant lineage, several rounds of regenerations (2-3) are suggested (Scotti et al., 2011) along with constant antibiotic selection pressure, in the contrary to Finally, obtaining the seeds by self-pollination provides the first generative generation of transformed plants (one *aadA*$^{+}$*/psbA*^{mut} genotype, one *aadA*$^{+}$*/psbA*^{wild} genotype). After flowering and seed harvesting, homoplasmy and germinability were tested. The germinability of plants with all genotypes (comparing by sowing approximately 400 of each) was comparable in each (92% of *aadA*$^{+}$*/psbA*^{mut} seeds, 94% of *aadA*$^{+}$*/psbA*^{wild} seeds and 96% of wild type seeds germinated). Only green seedlings were observed contrary to described results from Bříza *et al.* (2013) when 29 non green seedlings from sample of 200-400 were observed. Homoplasmy was confirmed.

6. CONCLUSION

As reported in Dinamarca *et al.* (2011), mesophilic cyanobacterium *Synechocystis* sp. PCC6803 with sites D1-S209, D1-S212 mutated into D1-A209, D1-C-212 corresponding locally to a version of D1 protein from thermophilic cyanobacteria evince higher thermostability. The aim of this experiment was the construction of transformation vector for particle bombardment to achieve these mutations in higher plant (*Nicotiana tabacum*). Vector was successfully assembled from five fragments and its correct sequence with mutations of desire was confirmed.

Vector carrying *psbA* gene with D1-A209, D1-C-212 mutations was successfully transferred into tobacco living cells and mutations were incorporated into cpDNA during homologous recombination together with selective *aadA* cassette. Spectinomycin resistant calli were successfully selected and rooted. Two genotypes were obtained - $aadA^{+}/psbA^{wild}$ in 35 individuals and $aadA^{+}/psbA^{mut}$ in 4 individuals. One heteroplatic plant with chloroplast mixture of both genotypes was confirmed using Southern analysis.

One individual of each genotype was grown on hydroponic system. After flowering, harvested seeds were tested to homoplasmy. All seedlings were green so homoplasmy was confirmed.

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8. Appendix

AAGGAGCAATAGCACCCTCTTGATAGAACAAGAAAATGATTATTGCTCCTTTCTTTTCAAAACCTCCT
*F52:*TCTTGATAGAACAAGAAAATGATTATTGCTCCTTTCTTTTCAAAACCTCCT
*F37:*TCCT

*ATAGACTAGGCCAGGATC*TTATCCATTTGTAGATGGAGCTTCGATAGCAGCTAGGTCTAGAGGGAAGT
ATAGACTAGGCCAGGATCTTATCCATTTGTAGATGGAGCTTCGATAGCAGCTAGGTCTAGAGGGAAGT
ATAGACTAGGCCAGGATCTTATCCATTTGTAGATGGAGCTTCGATAGCAGCTAGGTCTAGAGGGAAGT

TGTGAGCATTACGTTCATGCATAACTTCCATACCAAGGTTAGCACGGTTAATGATATCAGCCCAAGTA
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AACGAGAGTTGTTGAAACTAGCATATTGGAAGATCAATCGGCCAAAATAACCATGGGCGGCTACGATG
*F50:*ATATTGGAAGATCAATCGGCCAAAATAACCATGGGCGGCTACGATG
F38:GAAGATCAATCGGCCAAAATAACCATGGGCGGCTACGATG

TTATAAGTTTCTTCCTCTTGACCGAATCTGTAACCTTCATTAGCAGATTCATTTTCTGTGGTTTCCCT
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TTATAAGTTTCTTCCTCTTGACCGAATCTGTAACCTTCATTAGCAGATTCATTTTCTGTGGTTTCCCT
F45:GTTTCTTCCTCTTGACCGAATCTGTAACCTTCATTAGCAGATTCATTTTCTGTGGTTTCCCT
*F48:*CTTCCTCTTGACCGAATCTGTAACCTTCATTAGCAGATTCATTTTCTGTGGTTTCCCT

GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACaGAATAGGGccCCGCCGAATACACCAGCTA
GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA
GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA
GATCAAACtAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA
GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA
GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA
GATCAAACTAGAAGTTACCAAGGAACCATGCATAGCACAGAATAGGGCCCCGCCGAATACACCAGCTA

CGCCTAACATGTGAAATGGGTGCATAAGGATGTTGTGCTCAGCCTGGAATACAATCATGAAATTGAAA
CGCCTAACATGTGAAATGGGTGCATAAGGATGTTGTGCTCAGCCtgGAATACAATCA
CGCCTAACATGTGAAATGGGTGCATAAGGATGTTGTGCTCAGCCTGGAATACAATCATGAAATTGAAA
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GTACCAGAGATTCCTAGAGGCATACCATCAGAAAAACTTCCTTGACCAATTGGGTAGATCAAGAAAAC
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TGCGGTAGCAGCTGCAACAGGAGCTGAATATGCAACAGCAATCCAAGGTCGCATACCCAGACGGAAAC
TGCGGTAGCAGCTGCAACAGGAGCTGAATATGCAACAGCAATCCAAGGTCGCATACCCAGACGGAAAC
TGCGGTAGCAGCTGCAACAGGAGCTGAATATGCAACAGCAATCCAAGGTCGCATACCCAGACGGAAAC

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TAAGCTCCCACTCACGACCCATGTAACAAGCTACGCCAAGTAAGAAGTGTAGAACAATTAGTTCATAA
TAAGCTCCCACTCACGACCCATGTAACAAGCTACGCCAAGTAAGAAGTGTAGAACAATTAGTTCATAA

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GGACCACCGTTGTATAACCATTCATCAACGGATGCCGCTTCCCAGATTGGGTAAAAATGTAAACCTAT

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GTCAATAAGGTAGGGATCATCAAAACACCAAACCATCCAATGTAAAGACGGTTTTCAGTGCTAGTTAT

CCAGTTACAGAAGCGACCCCATAGGCTTTCGCTTTCGCGTCTCTCTAAAATTGCAGTgtgcctaatga
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CCAGTTACAGAAGCGACCCCATAGGCTTTCGCTTTCGCGTCTCTCTAAAATTGCxxxgtgcctaatga

gtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgcca
gtgagctaactcaca
gtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgc

gctgcatta

Green - expected *psbA* region sequence within pUC19 plasmid
Red - deletion in plasmid DNA
Yellow - mutations in D1-S209, D1-S212 to D1-A209, D1-C-212
Blue - silent mutation - restriction site for *Apa*I enzyme
Pink - silent mutation - restriction site for *Nco*I enzyme

F52 F37 - sequence from PCR fragment (SB-F - psbA R2.4)
F38 F45 - sequence from PCR fragment (FP2A - psbA R2.4)
F50 F48 - sequence from PCR fragment (FP2A - UC-R5)

Appendix 1: Expected psbA region sequence within pUC19 plasmid compared with obtained sequence from 3 PCR (Tab. 4).