

Summary of the Ph.D. Thesis

**Antisense Oligodeoxynucleotide Technology:  
A Novel Tool for Gene Silencing in Higher Plants**

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## INTRODUCTION

To understand the function of a gene or a protein we often make use of either mutants or inhibitors. Antisense oligodeoxynucleotides (ODNs) can be designed to work as inhibitors of specific genes and thereby also share characteristics with a mutant. ODNs are short synthetic strands of DNA or analogs that consist of 15-20 nucleotides. They specifically target their complementary stretches of RNA by duplex formation and inhibit protein biosynthesis. In principle, they are able to interfere with each step of nucleic acid metabolism, preferentially with transcription, splicing and translation. In this way, ODNs act as inhibitors that target gene expression.

In spite of the general applicability of the antisense ODN approach, only few papers on the application of ODNs to plant tissue have been published. The uptake of single stranded ODNs by plant cell suspension cultures was already observed almost two decades ago. Similarly, pollen tubes have also been suggested to take up antisense ODNs. The application of ODNs in plants would allow the study of the function of vital genes while minimizing pleiotropic effects. In classical mutants the knockout of vital genes is impossible whereas pleiotropic effects can occur during the development of mutants. The fact that ODN treated plants are not genetically modified is also an advantage. It means that no special measures have to be taken during cultivation and transportation. In 2005, Roberts proposed the application of nucleic acids in plant tissues as a means for high throughput screening for gene function. However, so far there has been no breakthrough of the application of this technique in plant science.

## **AIMS OF THE STUDY**

The major aim of this thesis is to prove the applicability of antisense ODNs in leaves of dicotyledonous and monocotyledonous plant species and in this way enhance the development the antisense ODN technology to become a widely accepted and applied alternative of gene silencing also in the plant biology.

To achieve this we focused on the following points:

- To find methods for efficient uptake of ODNs by different types of leaves
- To test the inhibitory efficacy of the antisense ODN at all possible levels
- To enhance the silencing efficacy by the application of antisense ODNs bearing chemical modifications.
- To show that it is possible to target and inhibit more than one gene within the same gene family with a single ODN.
- To demonstrate that ODNs can be successfully applied to a relevant biological problem by making use of its potential to inhibit gene expression to different extents (biological variability).

## MATERIALS AND METHODS

- Design and synthesis of antisense ODNs. Targeted genes are as follows: *Phytoene desaturase* (*pds*), *chlorophyll a/b binding protein* (*cab*), *psbA* the gene for D1 protein.
- Antisense ODN treatment of tobacco, Arabidopsis and wheat leaves. Experimental species: Tobacco (*Nicotiana benthamiana*), Arabidopsis (*Arabidopsis thaliana*), wheat (*Triticum aestivum*).
- Confocal laser scanning microscopy
- Fast Chl *a* fluorescence (OJIP) measurements
- Quantitative real time reverse transcription PCR (QRT-PCR)
- Carotenoid and chlorophyll content determinations
- Western blot analysis

## RESULTS

In this thesis, the use of ODNs in different plant species is extended, optimizing the uptake, stability and efficiency of ODNs with a combination of molecular biological and biophysical techniques to transiently inhibit the gene expression of different chloroplast proteins. We targeted the nuclear encoded *phytoene desaturase* (*pds*) gene, encoding a key enzyme in carotenoid biosynthesis, the *chlorophyll a/b binding* (*cab*) protein genes and the chloroplast encoded *psbA* gene, encoding the D1 protein of photosystem II. For *pds* and *psbA*, the *in vivo* stability was increased by phosphorothioate modifications. After infiltration of ODNs into juvenile tobacco leaves we detected a 25-35% reduction in mRNA level and a ~5% decrease in both the carotenoid content and variable fluorescence ( $F_V$ ) of photosystem II. In detached etiolated wheat leaves after 8 h of

greening the mRNA level, the carotenoid content and  $F_V$  were inhibited up to 75, 25 and 20%, respectively. Regarding *cab*, ODN treatments of etiolated wheat leaves resulted in an up to 59% decrease in the amount of chl *b*, 41% decrease of the maximum chlorophyll fluorescence intensity, the *cab* mRNA levels were reduced up to 66% and the protein level was suppressed up to 85% compared to the control. The *psbA* mRNA level and protein levels in *Arabidopsis* leaves were inhibited by up to 85% and 72%, respectively. To exploit the potential of ODNs for photosynthetic genes we propose molecular design combined with fast, non-invasive techniques to test functional effects. We have also applied the method. A set of 10 antisense ODNs has been used to target the *cab* gene family. The different samples that were obtained in this experiment differed in their antenna size over a wide range. This allowed us to study the relationship between antenna size and the  $F_0$  and  $F_M$  values taken from the chl *a* fluorescence measurements. The study as a whole demonstrated that antenna size had more effect on the  $F_M$  value than the chl content of the leaf.

## DISCUSSION

The major aim of this thesis was to extend the applicability of antisense ODNs to leaves of a dicotyledonous and a monocotyledonous plant species. In practice applicability of ODNs in tobacco (*Nicotiana benthamiana*), *Arabidopsis* (*Arabidopsis thaliana*) and wheat (*Triticum aestivum*) was demonstrated.

For the application of ODNs several pre-conditions have to be met. For each selected target RNA about 10 ODNs were designed and among these ODNs there was a large variability in effectiveness showing that the chosen sequence is critical to efficacy of the ODN. In Dinç et al. (2012) we have made use of this

feature to create a series of plants in which the Lhc synthesis was inhibited to different extents.

A second challenge with respect to the application of antisense ODNs was stabilization. The natural molecular structure of ODNs is exposed to endo- and exo-nucleolytic degradation. The most widely applied modification to increase the stability of ODNs is the introduction of phosphorothioate (PS) groups. This modification I used as well. In the case of the phosphorothioate modification, one of the non-bridging oxygen atoms of the phosphodiester bond is replaced by sulfur. The PS modification is particularly advantageous because it provides sufficient stabilization against nucleolytic degradation, whereas the duplex of PS-ODN and RNA is still recognized by cellular RNase H. This is an advantage compared to most other modifications. The cleavage of target mRNA by RNase H is considered an important factor for the activity of antisense ODNs. We demonstrated for the first time that nucleases are active in plant cells and that PS modification can efficiently protect ODNs. We showed that the efficiency of the inhibition can be increased by phosphorothioate modification of the designed antisense ODNs against the *pds* mRNA.

The third challenge was the application of the ODNs to the leaf. For each of the three plants mentioned above a specific application method was developed. Tobacco leaves are big and in that case the ODNs were infiltrated in the leaf using a method developed for the introduction of viruses. The wheat leaves were allowed to take up the ODNs passively by cutting the wheat leaves and putting the ends in an ODN containing solution. In the case of the small *Arabidopsis* leaves a vacuum infiltration method was used. Uptake of ODNs was visualized using fluorescent dye labelled ODNs and confocal scanning microscopy. The confocal scanning microscopy data show that ODNs are efficiently transported within the leaf and reach the nucleus and the chloroplast.

Another important point was the choice of the target gene. As a first model gene, phytoene desaturase (*pds*) was chosen. The *pds* gene encodes a key enzyme in the carotenoid biosynthesis pathway and carotenoid loss will have an effect on the assembly of the light harvesting complexes that can be monitored with chlorophyll fluorescence measurements. The *pds* gene turned out to be a less than perfect choice as a model gene since the turnover of the enzyme as well as the synthesis of the product (carotenoids) was rather low. As a consequence the ODN induced effects were rather small despite the fact that *pds* antisense ODNs efficiently knocked down the gene expression of the *pds* gene. Given the transient nature of the effect of ODNs they work best if the protein encoded by the targeted gene has a high turnover or if the antisense ODN targets an inducible gene. The other two cases that were studied were examples of these two options. Greening of etiolated leaves is an inducible process and during greening there is a strong accumulation of the *lhc* gene products that were the second target gene family. Using antisense ODNs against the *lhc* genes and Western blot analysis I was able to confirm that it is possible to target with a single ODN several paralogous genes. The third target gene was the *psbA* gene. While the *pds* gene and *lhc* genes are nuclear encoded, the *psbA* gene is chloroplast encoded. In addition, the D1 protein (the gene product of the *psbA* gene) has a high turnover rate. The D1 and D2 proteins form the reaction center core of photosystem II (PSII) and the rate of PSII inactivation can be increased using high light intensities. The inactivation of PSII/turnover of the D1 protein has been shown to be dose dependent. Using ODNs to inhibit the *psbA* gene, encoding for the D1 protein, I show for the first time that it is possible to inhibit a chloroplast encoded gene with antisense ODN technology.

The Dinç et al. (2011) article presented a proof of principle. In a second article (Dinç et al., 2012) an example of the application of ODNs to a biological question is given. For starting point, the results of Ceppi and co-workers were

used: Using sugar beet plants grown hydroponically on sulfur and magnesium deficient solutions it was shown that the loss of chl not accompanied by a change in antenna size did not affect the fluorescence intensity (neither  $F_0$  nor  $F_M$ ). In our experiments, treating etiolated wheat leaves with a set of 10 antisense ODNs targeting the *cab* gene family followed by greening allowed us to obtain leaves containing reaction centers varying in their antenna size. This experiment demonstrated that the  $F_M$  value was quite sensitive to antenna size. This study shows that the  $F_0$  and  $F_M$  values do not have a direct relationship with chlorophyll content.

## PUBLICATIONS

**Dinç E**, Tóth S.Z, Schansker G, Ayaydin F, Kovács L, Dudits D, Garab G, Bottka S. Synthetic antisense oligodeoxynucleotides to transiently suppress different nuclear- and chloroplast-encoded proteins of higher plant chloroplasts. **Plant Physiol.** (2011) 157: 1628-1641. IF: 6.451

**Dinç E**, Ceppi MG, Tóth S.Z, Bottka S, Schansker G. The Chl *a* fluorescence intensity is remarkably insensitive to changes in the chlorophyll content of the leaf as long as the chl *a/b* ratio remains unaffected. **Biochim Biophys Acta** (2012) 1817: 770-779. IF: 5.132



## ABSTRACTS AND POSTERS

**Emine Dinç**, Szilvia Z Tóth, Győző Garab, Sándor Bottka. Application of Synthetic Antisense Oligodeoxynucleotides in Higher Plants. 4th International Symposium of the SFB 429 " Signals, Sensing and Plant Primary Metabolism" University of Potsdam, Sanssouci, October 6th - 9th, 2010. Potsdam, Germany

**Emine Dinç**, Szilvia Z. Tóth, Gert Schansker, László Kovács, Győző Garab, Sándor Bottka. Transient inhibition of the expression of wheat *chlorophyll a/b-binding protein* genes using synthetic antisense oligodeoxynucleotides. International Conference of Photosynthesis Research for Sustainability, 23-30 July 2011, Baku, Azarbaijan

## ORAL PRESENTATIONS

**Emine Dinç**, Do antisense ODNs make sense in plants? 2nd ITC Alumni Meeting, 1-3 September 2011, Szeged, Hungary

**Emine Dinç**, Antisense oligodeoxynucleotide technology for gene silencing applied to photosynthetic organisms targeting nuclear and chloroplast encoded genes. Vrije University, 10 February 2012, Amsterdam, Netherlands