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

Ph.D. Thesis

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LIST OF ABBREVIATIONS

cab	chlorophyll a/b binding protein
chl	chlorophyll
cyt	cytochrome
ER	endoplasmic reticulum
F_0	Fluorescence yield of open PSII reaction centers in dark adapted state
F_M	Maximum fluorescence yield of closed PSII reaction centers in the dark adapted state
F_V	Variable fluorescence (F_M-Fo)
HPLC	High Pressure Liquid Chromatography
Lhc	Light harvesting complex
LNA	Lock nucleic acid
mRNA	messenger RNA
ODN	Oligodeoxynucleotide
pds	Phytoene desaturase
PS	phosphorothioate
PSI	photosystem I
PSII	photosystem II
PNA	Peptide nucleic acid
RNase H	Ribonuclease H
shRNAs	short hairpin RNAs
siRNAs	small interfering RNAs

1. INTRODUCTION

After the completion of the sequencing of numerous plant genomes, the functional analysis of all these plant genes has become one of the most challenging tasks of plant biology. For establishing sequence function relationships, selective silencing of a chosen gene to suppress gene activity at the level of RNA expression is available as an alternative tool beside the classical genetic methods. It was shown in early studies, that the introduction of an antisense construct into plants reduced the level of the corresponding endogenous mRNA. This method was called antisense RNA suppression and later it became an example of the RNA interference (RNAi) approaches, in which double stranded RNAs play a central role in triggering sequence specific RNA degradation (Frizzi and Huang, 2010). RNAi technology has been used and is being used in a huge number of investigations of almost all kinds of cellular processes.

An exciting discovery was published by Zamecnik and Stephenson (1978). These authors demonstrated that simple short oligonucleotides of synthetic origin can be successfully used to inhibit viral replication in cell free systems and in cell cultures. At that time, the finding did not lead to a widespread application of these oligonucleotides, because of the lack of readily available synthetic DNA oligomers and known RNA sequences (Zamecnik, 1996). After a latency period of almost ten years, the potency of the antisense oligonucleotide approach was recognized and an exponential development started which has led to an unprecedented number of applications.

1.1.1 Antisense oligodeoxynucleotide (ODN) technology

Antisense oligodeoxynucleotides (ODNs) are short synthetic strands of DNA that consist of typically 15-25 nucleotide units. They specifically target their complementary stretches of RNA by duplex formation and they inhibit protein biosynthesis. In principle, ODNs are able to interfere with each step of nucleic acid metabolism, but preferentially with transcription, splicing and translation (Crooke, 2004; Ravichandran et al., 2004; Gleave and Monia, 2005).

Antisense ODNs possess a number of desirable features compared to alternative technologies such as short hairpin RNA (shRNA) and artificial microRNA. ODNs can be taken up directly by the leaf/plant whereas shRNAs and artificial microRNAs have to be inserted into a plasmid before they can be introduced into the cell (Scherer and Rossi, 2003;

Sandy et al., 2005; Behlke, 2008). This means as well that the inhibition level of gene expression depends on the expression level of the plasmid inside the cell. Antisense ODNs, in contrast, are ready to use in a dose dependent manner (Kurreck, 2003). Their properties and function can be tuned by chemical modifications. Another technology, synthetic siRNAs, can also be introduced directly into the cell; however, compared to ODNs they have several disadvantages: 1. their design and synthesis is more complicated and 2. the ODN target-RNA interaction can tolerate a 1-2 nucleotide mismatch and still be (at least partially) effective whereas this would make siRNAs completely ineffective. Moreover, non specific off target effects (non-antisense effects due to interactions with structures other than gene transcripts) form a bigger risk when using siRNAs (Vickers et al., 2003).

Further advantages of antisense ODNs are that they have a broad applicability, allow direct utilization of sequence information, are low cost and have a high probability of success (Scherer and Rossi, 2003). Finally, homologous sequences can be targeted with a single antisense ODN; this means that a single antisense ODN can inhibit more than one gene from the same gene family (Bennett and Cowsert, 1999).

Sequence selective inhibition of gene expression is applied extensively for the elucidation of complex gene expression patterns or validation of results gained from high throughput genomic approaches such as DNA arrays (Gleave and Monia, 2005; Rayburn and Zhang, 2008). Antisense ODNs have gained great interest, not only as genomic tools, but also as possible therapeutic entities (Gewirtz et al., 1998; Dagle and Weeks, 2001; Hu et al., 2002; Shi and Hoekstra, 2004; Yang et al., 2004). ODNs are capable of interfering with the expression of the targeted gene, thus enabling selective and rational design of genomic or therapeutic agents (Gleave and Monia, 2005; Rayburn and Zhang, 2008). Potentially, antisense ODNs could be used to cure any disease that is caused by the undesired expression of a deleterious gene, e.g. due to viral infections, cancer growth or inflammatory diseases. Since the demonstration by Zamecnik and Stephenson (1978) that antisense ODNs can be successfully used to inhibit viral replication in cell cultures a number of therapeutic applications has been reported (Scherer and Rossi, 2003).

In spite of their general applicability, the antisense ODN technology has not yet been exploited in plant tissue, although it was observed already two decades ago that plant cell suspension cultures are capable of taking up single stranded ODNs (Tsutsumi et al., 1992). Similarly, it has been suggested that pollen tubes can take up antisense ODNs (Moutinho et al., 2001a,b). One advantage of applying ODNs in plants would be that the function of vital genes can be studied and pleiotropic effects minimized, which represent common problems

when creating mutants by genetic transformation. In itself, it is also an advantage that ODN treated plants would not be genetically modified organisms; therefore, no special measures would be required during cultivation and transportation. Sun et al. (2005, 2007) were the first to apply ODNs to green leaves; unmodified antisense ODNs (natural structure) were used to inhibit the expression of SUSIBA2, a transcription factor involved in starch synthesis. The direct application of nucleic acids in plant tissues was then suggested to open the way for high throughput screening for gene function (Roberts, 2005), but the use of antisense ODNs in plant science, so far, has fallen short of expectations.

1.1.2 Novel mechanisms for antisense mediated regulation of gene expression

In the absence of antisense ODN, the normal gene and protein expression pattern is maintained (Fig. 1.1: (1)). Hybridization of antisense ODNs to their target mRNA via Watson–Crick base pairing can result in specific inhibition of gene expression by various mechanisms, depending on the chemical structure of the antisense ODN and the hybridization site on the mRNA. This results in reduced levels of translation of the target transcript (Gewirtz et al., 1998; Crooke, 2004). Antisense ODN can hybridize with target mRNA in the cytoplasm and nucleus (Chan et al., 2006). Formation of an antisense ODN–mRNA heteroduplex induces activation of RNase H (Fig. 1.1: (2)), the RNA:DNA heteroduplex is recognized by this enzyme leading to RNA cleavage. In this case, the antisense ODN remains intact like a catalyst, and can induce subsequent rounds of cleavage of other RNA molecules. An alternative mode of action for antisense ODNs is blocking the ribosome translocation mechanism sterically by hybridization, which is called hybridizational arrest (Fig. 1.1: (3)). Both actions will result in target protein knockdown. Beside these two major mechanism, the antisense ODN can also enter the nucleus and regulate mRNA maturation by inhibition of mRNA splicing (Fig. 1.1: (5)). Antisense oligonucleotides bound to the splicing site of pre-mRNA interfere with the maturation of mRNA by preventing the binding of a spliceosome to pre-mRNA and activation of RNase H (Fig. 1.1: (4-6)). Antisense oligonucleotides bound to encoding or non-encoding regions of pre-mRNA can accelerate the RNase H mediated degradation of the mRNA, or interfere with polyadenylation or cap formation (Shi and Hoekstra, 2004; Chan et al., 2006).

Nevertheless, progress in carefully defining the intracellular mechanisms of processing and action of antisense ODNs has been particularly slowed down by the technical inability to

verify the localization of the relatively small amounts of antisense ODNs active within the cell, and the limited recovery by isolation of targeted mRNA or protein fragments.

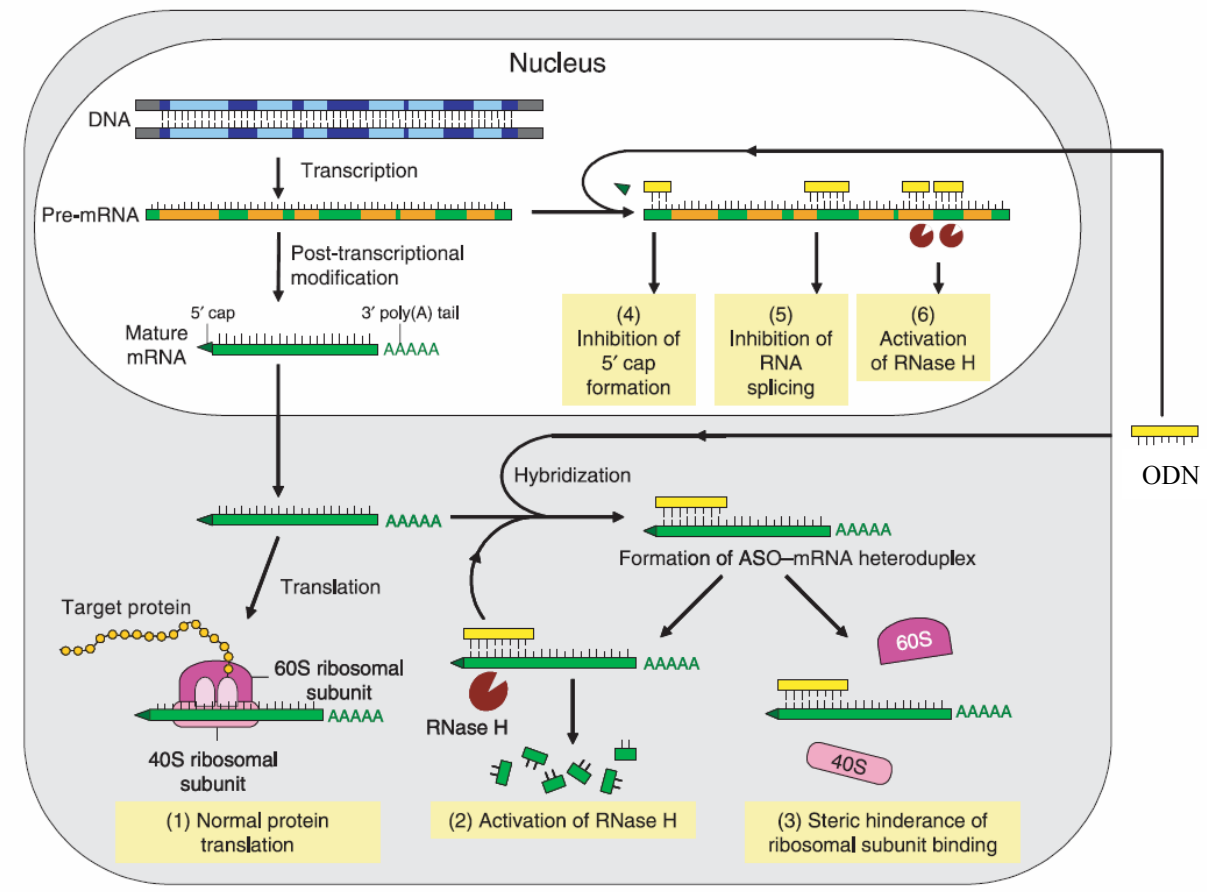


Figure. 1.1. *Major sites of action of antisense oligonucleotides. (1) protein translation, (2) RNase H activation, (3) translational arrest, (1-3) ODN action in the cytoplasm, (4) inhibition of 5' cap formation, (5) inhibition of the splicing of pre-mRNA, (6) RNAse H activation, (4-6) pre-mRNA destabilization in the nucleus (taken from Chan et al., 2006).*

1.1.3 Antisense ODNs and the selection of target sequences

Although on the basis of the simple mechanistic picture shown above, the whole (pre)mRNA sequence can potentially be targeted by antisense agents, early studies showed that only a fraction of them were really effective, their inhibitory efficacy varied significantly depending on the target site. Therefore, selection of an appropriate antisense ODN for a target gene is a crucial step and remains a major challenge in the successful application of antisense technology. The mRNA molecules form complex secondary and tertiary structures, therefore, in antisense technology, the identification of mRNA sites that can be targeted efficiently is the

key to success (Stein, 2001). A variety of strategies has been developed to successfully design ODNs (Sohail and Southern, 2000). The interaction between the antisense ODN and its complementary target mRNA depends on the thermodynamic factors of hybridization, the secondary structure of the target mRNA transcript and the proximity of the hybridization site to functional motifs on the designated transcript, such as the 5` CAP region or translational start site. At least four parameters in antisense ODN design need to be considered in order to increase the 'hit rate': (1) prediction of the secondary structure of the RNA; (2) identification of preferred RNA secondary local structures; (3) motif searching; and (4) semi-empirical calculation of free binding energy (ΔG) (Chan et al., 2006). In principle, antisense oligodeoxynucleotides are targeted to the "open" regions on the mRNA. Nowadays, a search for the presence of accessible single stranded loops in the mRNA secondary structure using the Mfold program (Zuker, 2003) is the most accepted approach. An example is shown in Fig. 1.2. In order to increase the potency of the antisense ODN design, locally conserved structures among various predicted optimal and suboptimal mRNA secondary foldings are considered (Fig. 1.2).

Primer analysis software, such as e.g. the *oligo5* programme (W. Rychlick, National Bioscience Inc., Plymouth, MN, USA) are used to determine if the base sequence of the designed antisense ODNs leads to hairpin or duplex formation (Fig. 1.3). Strong harpin or duplex formation would decrease the inhibitory efficiency of the ODNs.

Matveeva et al. (2000) analysed data collected from >1000 experiments using phosphorothioate (PS) modified antisense ODNs (see below) and found a positive correlation between antisense ODN mediated mRNA knockdown and the presence of CCAC, TCCC, ACTC, GCCA and CTCT motifs in the antisense ODNs. Conversely, the presence of GGGG (G-quartet formation), ACTG, AAA and TAA motifs in antisense ODNs weakened antisense activity. GC content is strongly correlated with the thermodynamic stability of the antisense ODN–mRNA duplexes and RNase H activity. Strong inhibition by antisense ODNs was observed if the antisense ODNs contained a minimum of 11 G or C residues per 20 bps, whereas poor inhibition was observed for antisense ODNs having nine or fewer G or C residues (Ho et al., 1996)

After confirmation of the accessible locally conserved secondary structures, the chosen antisense ODN sequences were screened for some well defined activity enhancing motifs and for GC content, discarding the ODNs containing activity decreasing motifs. Finally, an extensive database search was performed in GenBank for each antisense ODN sequence to avoid significant homology with other mRNAs.

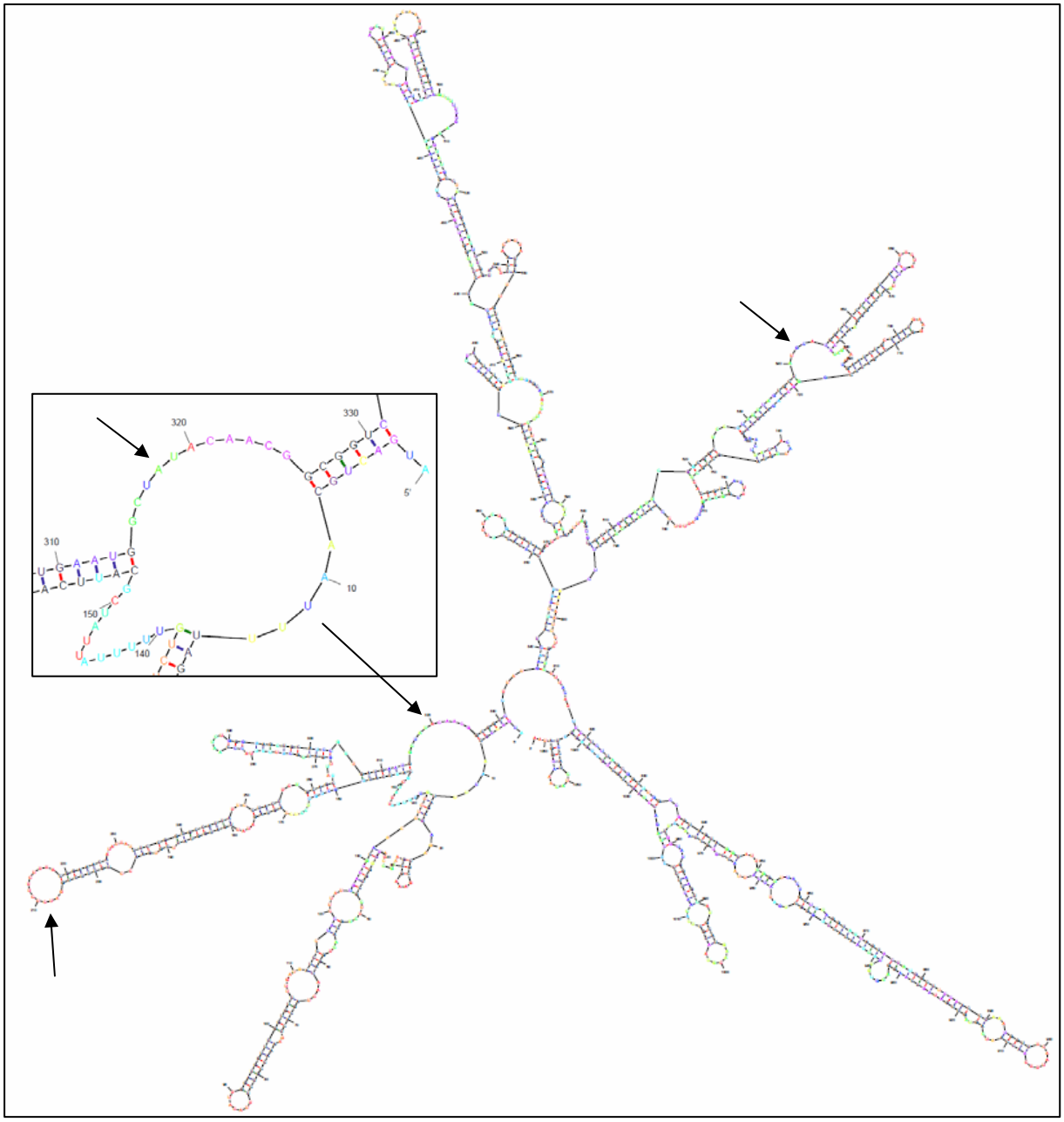


Figure 1.2. *Possible secondary structure of psbA Arabidopsis mRNA created with the Mfold programme (Zuker 2003). The arrows indicate potential target sites for ODNs.*

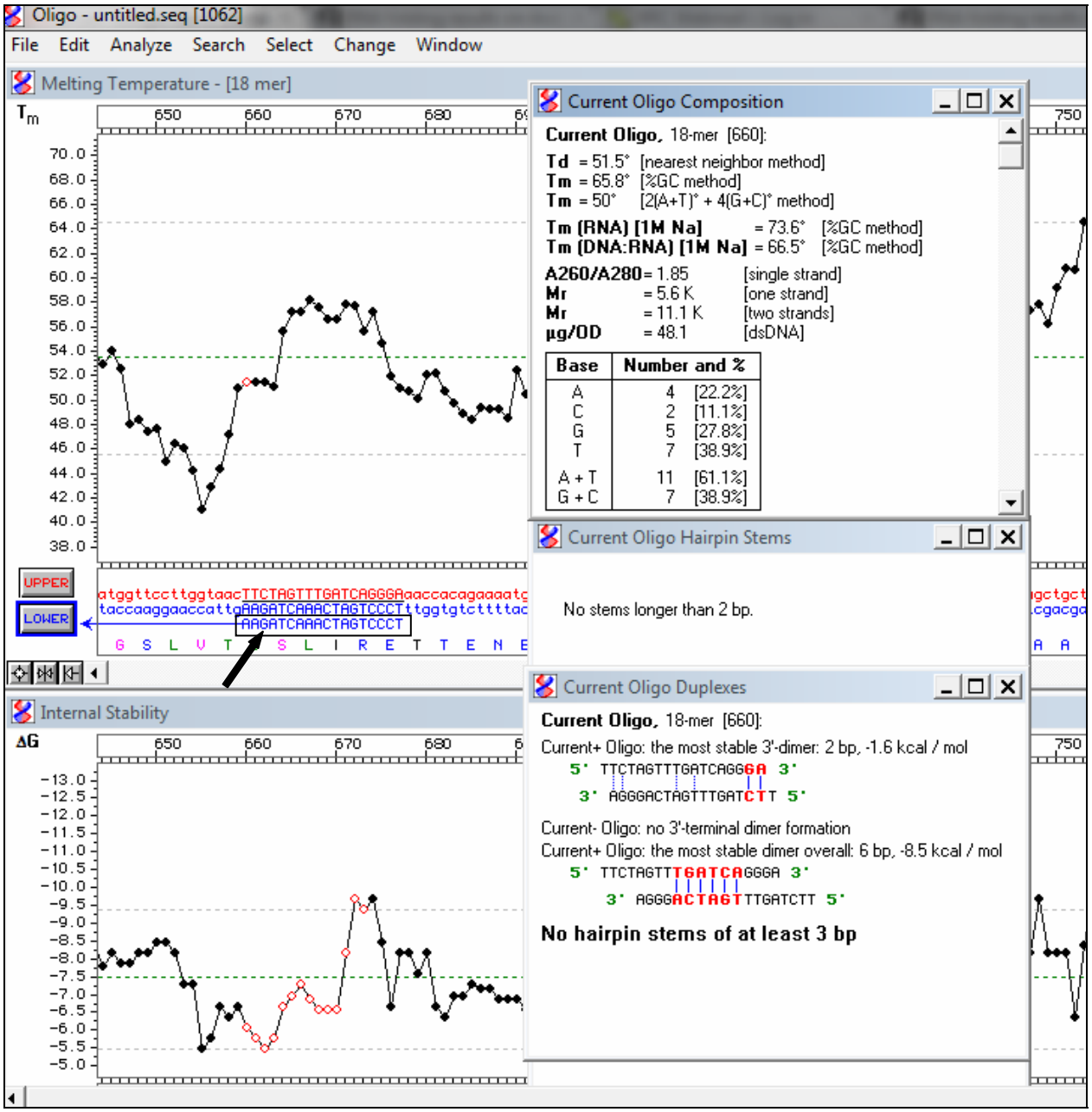


Figure 1.3. *Detailed analysis of an Arabidopsis psbA antisense ODN using the oligo5 programme.*

1.1.4 Chemical modifications of antisense oligonucleotides

Another crucial step towards a successful application of antisense technology is the improvement of the stability of antisense molecules and enhancement of the affinity of the ODNs to the targeted mRNA. Oligodeoxynucleotides having a natural structure may be rapidly degraded by cellular nucleases of the host organism (Kurreck 2003). Introduction of

chemical modifications of either the nucleobases or the sugar phosphate backbone aims to extend the biological lifespan of ODNs and improve the interaction with their complementary mRNA sequences. A number of chemical modifications was introduced successfully into antisense ODNs during the last two decades (Chan et al., 2006; Lebleu et al., 2008, Fig. 1.4).

The scope of nucleobase modifications is rather limited as the requirements for base pairing must be respected in order to maintain the specificity of the antisense strategy. Nevertheless, there are a number of base modified oligonucleotides that have been shown to be potentially useful (Luyten and Herdewijn, 1998; Herdewijn, 2000).

The first target of nuclease action is the internucleotide phosphate, and therefore the first attempted modifications involved the backbone of the ODNs (Matsukura et al., 1987). Phosphorothioate (PS) oligodeoxynucleotides are the major representatives of first generation DNA analogs that are the best known and most widely used antisense ODNs to date. In this class of ODNs, one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulfur (Eckstein, 2000; Kurreck, 2003) (Fig. 1.4). The PS modification is particularly popular because it provides sufficient stabilization against nucleolytic degradation, whereas the PS-ODN-RNA duplex is still recognized by cellular RNase H, which is not the case for most other modifications (Stein, 1993). The cleavage of target mRNA by RNase H is considered an important factor determining the efficacy of antisense oligonucleotides (Stein, 1995).

Certain chemical modifications can still induce RNAse H cleavage of the target RNA but others like 2` O-alkyl modification or peptide nucleic acids (PNAs, Fig. 1.4) can be targeted to mRNA but it is not recognized by or it is not a substrate of RNase H (Murray et al., 2012). In a cell free translation system it has been shown that these ODNs can interfere with polypeptide chain elongation resulting in the biosynthesis of truncated protein fragments (Arzunamov et al., 2001; Dias et al., 2002). Similarly, steric occupancy of the 5'cap region by 2'-O-methoxy-ethyl modified ODNs interferes with the assembly of the 80S translation initiation complex, and with the expression of target mRNA (Baker et al., 1997).

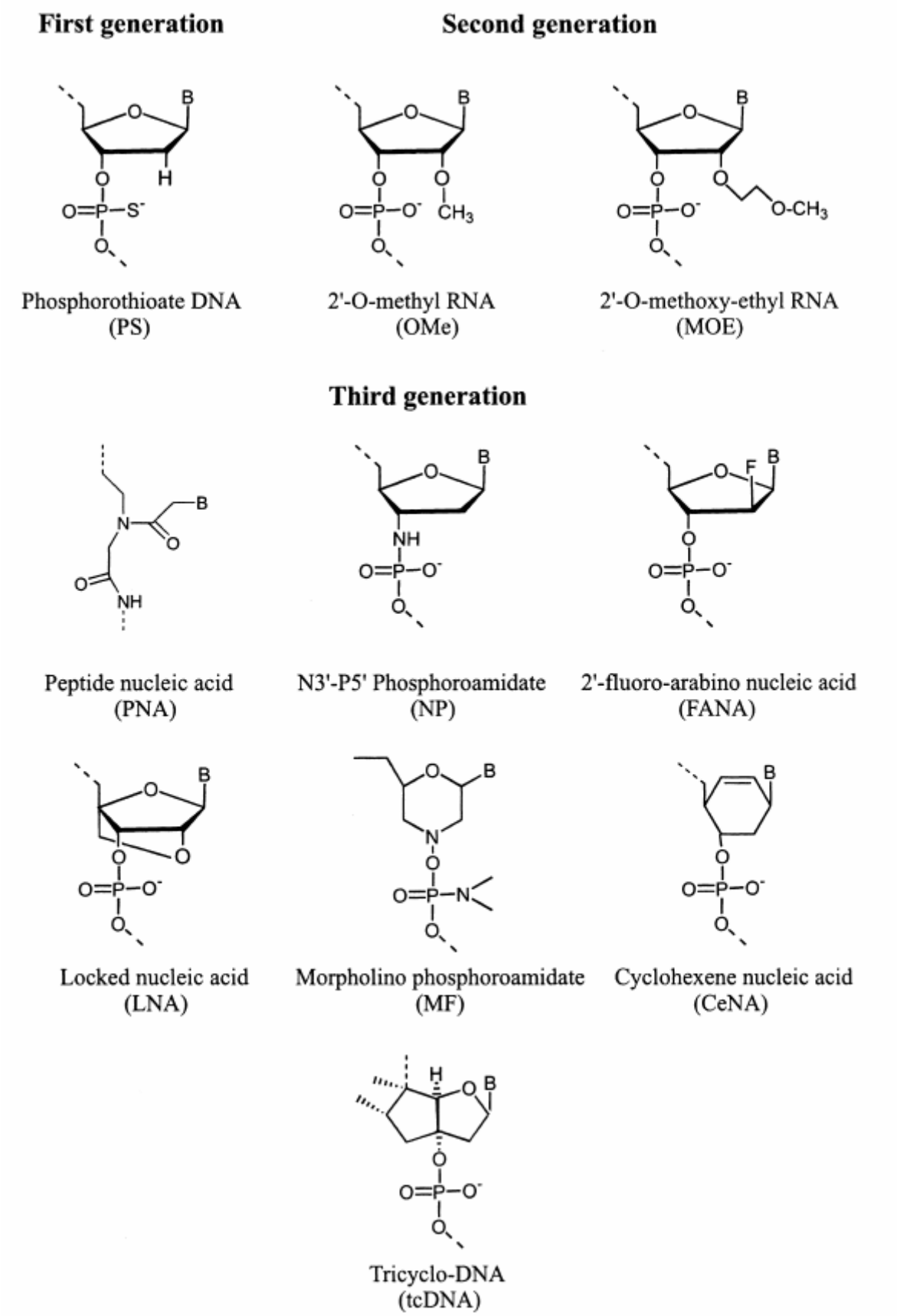


Figure 1.4. *The most often used chemical modification of antisense oligonucleotides. B denotes one of the bases of adenine, guanine, cytosine or thymine (Kurreck, 2003).*

1.1.5 Delivery of antisense oligodeoxynucleotides

Following the choice of an antisense molecule with high stability and good affinity to its target mRNA, the next question is how to direct the antisense molecule to the target mRNA and interfere with its functions in the cells. Systemically applied ODNs have to reach the target mRNA in a cell. The mRNAs transcribed from the DNA are edited in the nucleus, and then transported to the endoplasmic reticulum (ER) in the cytosol as a template for protein synthesis. Thus, to come into contact with their target mRNA, ODNs need to be transferred into the cytoplasm so they can reach the nucleus or interact with the mRNAs on their way to the ribosome, which is either free in the cytosol or attached to the ER. In tissue culture, ODNs are poorly internalized by cells so delivery vectors (several are commercially available, including lipofectin, lipofectase, cytofectin (serum stable), Star-Burst dendrimers of several generations, cationic porphyrins, and others) have been used to enhance the cellular uptake in mammalian studies (Stein 1999).

Delivery of the ODN to the plant is an even harder problem. The plant cell wall is an impenetrable barrier and this may have been the most important reason, why plant studies have been so far behind mammalian ones. Wounding of the plant tissue seems to be unavoidable. That is the reason, why Sun et al. (2005) used detached wheat leaves for their studies.

1.2 The photosynthetic apparatus

Although ODNs have a general applicability, their effects have to be detected and monitored. The expression of genes related to the photosystems can be relatively easily monitored using biophysical techniques. In this section of the 'Introduction' a short overview of the properties of the photosynthetic machinery relevant to my study is given. In addition, the biophysical techniques used to monitor the effects of the ODNs will be introduced.

1.2.1 The organization of plant thylakoid membranes

Photosynthesis is the process by which the energy of sunlight is converted into chemical energy. Aerobic photosynthetic organisms: higher plants, algae and cyanobacteria, which provide us with a source of organic carbon molecules and oxygen that is essential for life on earth. The light reactions take place in the thylakoid membrane. The main protein

complexes of the photosynthetic electron transport chain are photosystem II (PSII), photosystem I (PSI), the cytochrome b_6f complex, (Cyt b_6f), and ATP synthase. These complexes are embedded in the thylakoid membranes (Fig. 1.5). Both the PSI and PSII reaction centers (RCs), in which the charge separations occur and where the light driven electron transfer is initiated, are surrounded by light harvesting antenna systems, which consist of pigment-protein complexes (Dekker and Boekema, 2005).

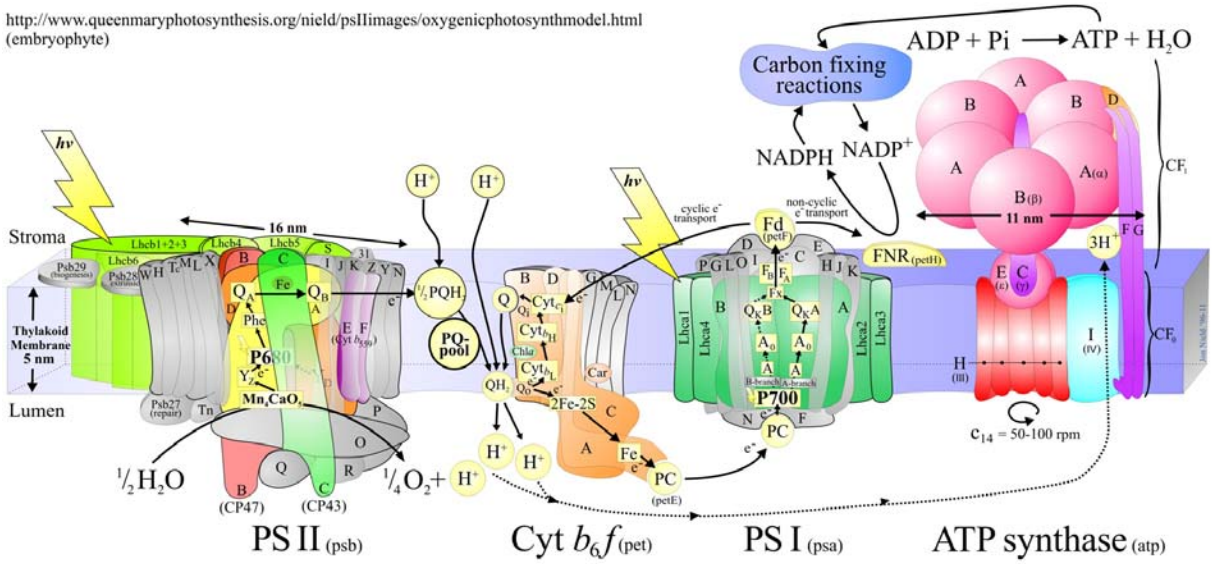


Figure 1.5. *Schematic model of the photosynthetic electron transport chain including structural information on the organization of the protein complexes involved in electron and proton transport within the thylakoid membrane of green plants. Based on structural information from e.g. Ferreira et al. (2004; PSII); Stroebel et al. (2003; Cyt b_6f); Jordan et al. (2001; PSI); Ben-Shem et al. (2003; LHCI-PSI).*

PSII is an integral pigment-protein complex embedded in the thylakoid membrane. The core of this membrane protein is formed by three proteins: D1, D2 and cytochrome b_{559} (Cyt b_{559}). Similarly to PSII, the core of the PSI reaction center is also a heterodimer, formed by two subunits, the PsaA and PsaB proteins (Fig. 1.5).

The light harvesting protein complexes contain hundreds of pigment molecules, essentially chlorophylls *a* and *b* and carotenoids. The antenna system consists of an inner antenna (containing only chl *a*), located close to the reaction center and a peripheral antenna system (containing both chl *a* and *b*) (Dekker et al. 2002). The inner antenna of PSII consists

of two subunits: CP43 (PsbC) and CP47 (PsbB). The peripheral antenna system consists of several subunits (Fig. 1.6; Dekker and Boekema 2005).

The peripheral antenna of PSI, LHCI, in higher plants consists of subunits Lhca1, Lhca2, Lhca3 and Lhca4 that form a half circle around the PSI core. Under conditions where LHCII becomes phosphorylated, trimers of Lhcb1/Lhcb2/Lhcb3 may be connected as well to the PSI reaction center core (Dekker and Boekema, 2005).

Plant photosynthesis is driven essentially by light of 380-740 nm. The first step in the conversion of light energy into chemical energy is the absorption of photons by the antenna system. The majority of the pigments serve as an antenna complex collecting light and transferring the energy to the reaction centers of the PSII and PSI, where the excitation energy can induce charge separations.

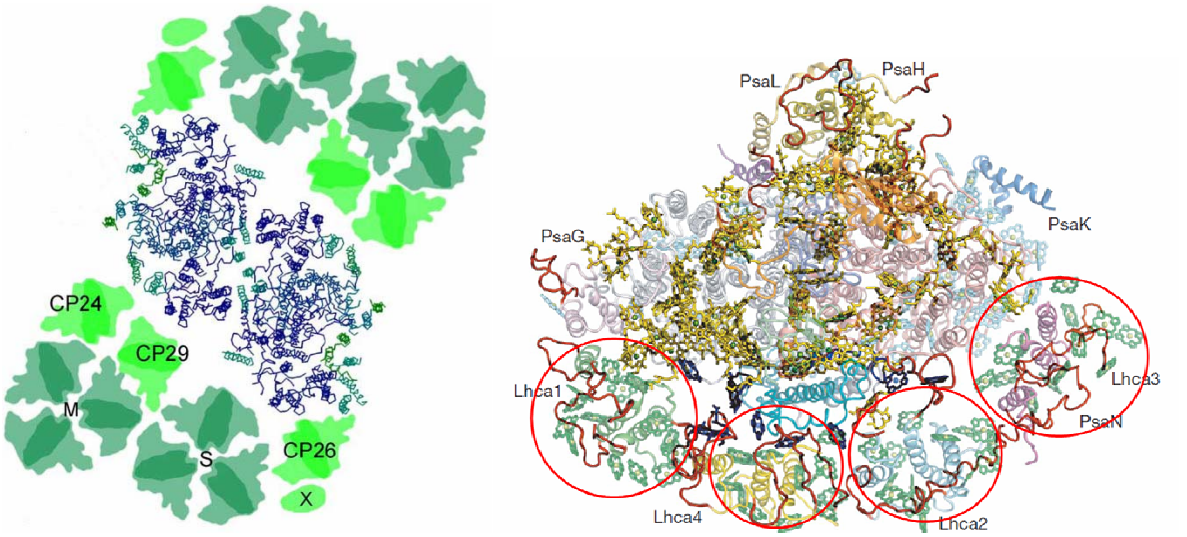


Figure 1.6. *Schematic representation of the antenna structure of photosystem II and I (taken from Dekker and Boekema, 2005 and Amunts et al., 2007). (S and M refer to trimers of LHCII with different binding affinities)*

1.2.2 Carotenoid synthesis

Carotenoids represent a class of pigments that play multiple roles in photosynthesis. Carotenoids can act both as light harvesters and as light dissipaters and they also play key roles in photoprotection. Binding of carotenoids is also necessary to maintain Lhc proteins in their proper conformation and in the absence of carotenoids the accumulation of Lhc proteins is strongly reduced (Plumley and Schmidt, 1987; Lokstein et al., 2002).

Carotenoid biosynthesis begins with the synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway using glyceraldehyde-3-phosphate (GAP) and pyruvate as initial substrates. IPP and DMAPP are used to form the central intermediate geranylgeranyl diphosphate (GGDP) for carotenoid and other isoprenoid biosynthesis via the general isoprenoid biosynthetic pathway. The synthesis of phytoene by condensation of two molecules of GGDP represents the first committed step in the carotenoid biosynthetic pathway (Lu and Li., 2008). In Fig. 1.7 a scheme of the carotenoid biosynthesis pathway is given.

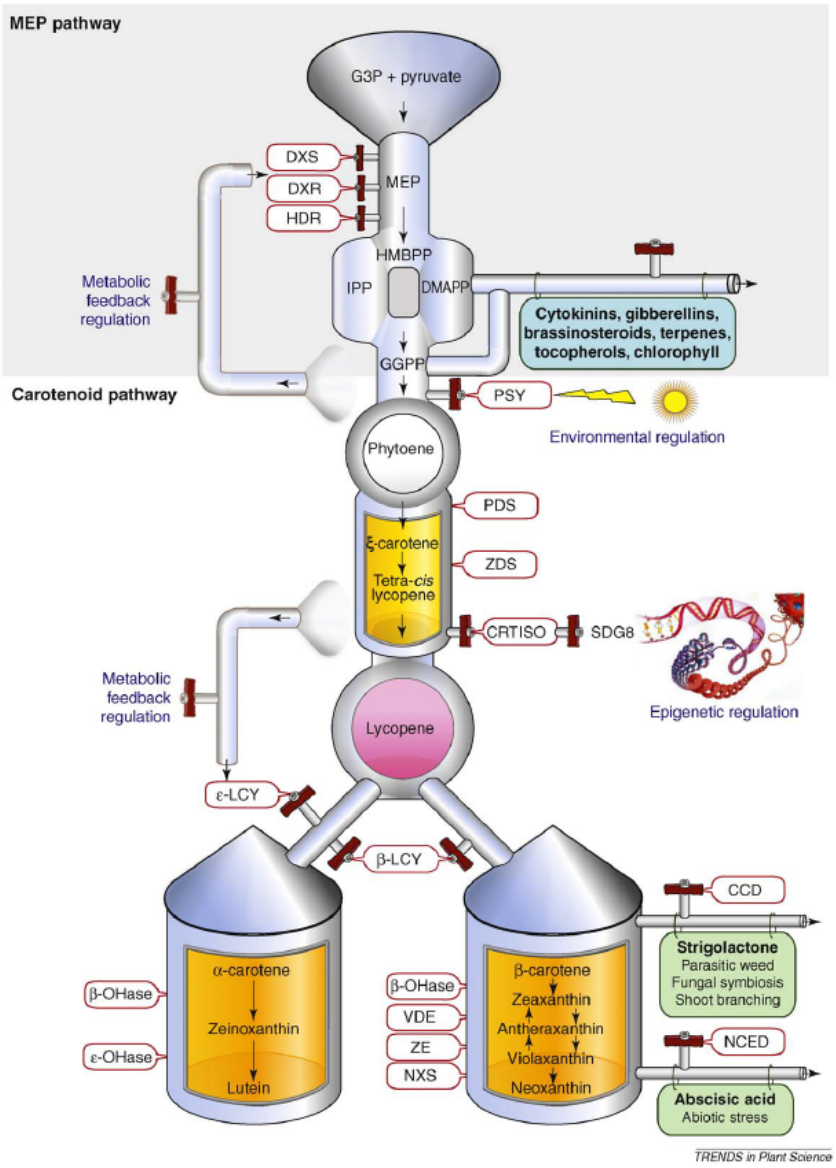


Figure 1.7. *Carotenoid metabolic pathway in plants (taken from Cazzonelli and Pogson 2010)*

1.2.3 Chl *a* fluorescence

In photosynthesis research, chl *a* fluorescence is one of the most widely used methods, both in basic and ecophysiological studies. Chl *a* fluorescence is the light emitted by chl *a* molecules upon excitation by light. It is a non-invasive and very sensitive method, though chl *a* fluorescence represents only 2-10% of the absorbed energy. It can be applied to any kind of photosynthetic sample from global scale down to PSII particles without the need of standardized or complicated preparations (Papageorgiou and Govindjee 2004).

Photosynthesis (the induction of stable charge separations) is in competition with heat emission and chl *a* fluorescence. The intensity of chl *a* fluorescence is largely determined by the redox state of Q_A, though it has been shown recently that a conformational change occurring in PSII also determines a part of the fluorescence yield (Schansker et al., 2011). If Q_A is oxidized (the reaction center is open; F_0) in about 90% (or maybe more) of the reaction centers, the absorbed energy will be used for photosynthesis; about 1-2% is re-emitted as chl *a* fluorescence and the remaining ~8% is re-emitted as heat. However, if Q_A is reduced (the reaction center is closed; F_M), no stable charge separation can be induced and photosynthesis uses 0% of the absorbed energy. In this case about 10% of the absorbed energy is re-emitted as chl *a* fluorescence and ~90% is emitted as heat.

The probability that the energy is re-emitted as fluorescence or heat depends on the lifetime of this energy package (molecular exciton). The exciton moves very rapidly from one chl *a* to the next. On each transfer there is a small risk that the energy is lost as heat or fluorescence. If the energy cannot be used for photosynthesis the exciton will keep going from one chl *a* till the next until it is lost as heat or fluorescence. In other words, in a closed reaction center, the lifetime of an exciton is longer and the probability that the energy is lost as fluorescence is proportionally greater.

The increase of the probability that the energy is lost as chl *a* fluorescence from 1-2% to 10% as the reaction centers gradually close is the basis for the fluorescence measurements used in this thesis. As shown in Fig. 1.8, fluorescence induction (the fluorescence rise from F_0 to F_M) is not monophasic. It has been shown that the different phases in the fluorescence rise on a dark-to-light transition follow the different phases of the reduction of the electron transport chain, with the OJ phase being a reflection of the reduction of the acceptor side of PSII, the JI rise being a reflection of reduction of the PQ pool and the IP phase being a reflection of the reduction of the electron acceptors around PSI (Schansker et al., 2005).

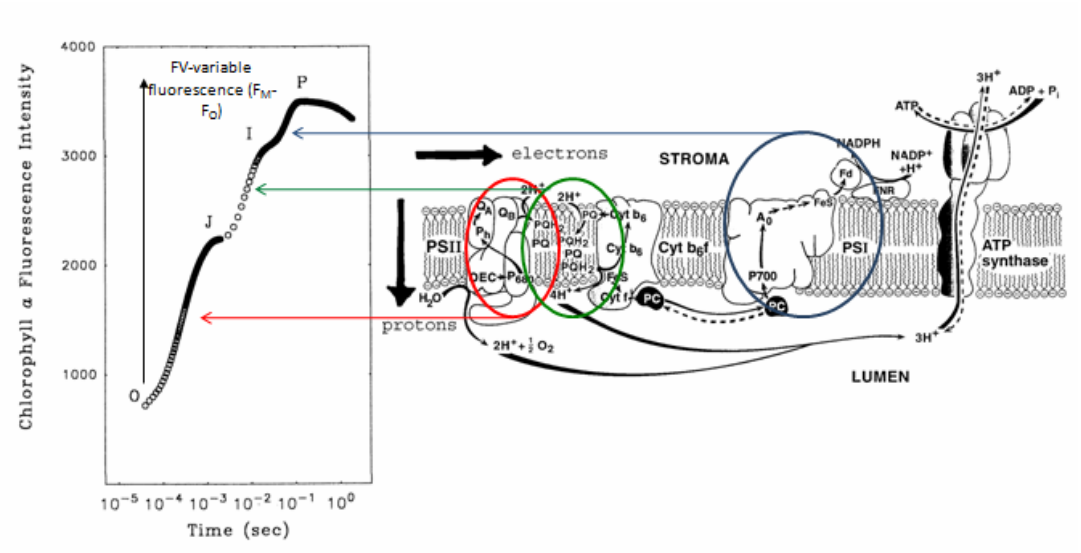


Figure 1.8. *Fast chl a fluorescence (OJIP) transient: The kinetics of the OJIP transient form a reflection of the different phases of the reduction of the photosynthetic electron transport chain (based on Schansker et al., 2005).*

1.2.4 Greening

Seedlings that are grown in darkness remain white, because chloroplast differentation does not occur in darkness (von Lintig et al., 1997; Park et al., 2002). On transferring etiolated seedlings from darkness to light initiates chloroplast differentation and leads to a greening of the plants. During greening, first the reaction centers of the photosystems and their core antennae are formed and subsequently the peripheral antennae (Srivastava et al., 1999). This process is also accompanied by the stacking of the thylakoid membranes and the segregation of PSII (in the grana) and PSI (in the grana end membranes and the stroma lamellae). Greening not only affects the measured fluorescence intensity, it also affects the rise kinetics of the fluorescence transients. Some of these changes may be due to changes in the PSII to PSI ratio and other properties of the electron transport chain, but dimerization of PSII may also have an effect on the kinetics of the OJIP transients.

1.2.5 Photoinhibition, the D1 protein and the repair cycle

The D1 protein (the gene product of the chloroplast encoded *psbA* gene) has a high turnover rate compared to other photosynthetic genes (Aro et al. 1993). The D1 and D2 proteins together with the Cyt b_{559} form the reaction center core of PSII (Fig. 1.1). Each

excitation of PSII has a certain, very low, probability to lead to inactivation of PSII and damage of the D1 protein. The inactivation of PSII/damage of the D1 protein has been shown to be dose dependent (Park et al., 1995; Tyystjärvi and Aro, 1996). The repair mechanism is normally rapid enough to prevent the symptoms of photoinhibition from appearing under optimal growth conditions, however, at any moment a small percentage of the PSII reaction centers breaks down and is recycled by the D1 repair cycle (Fig. 1.9).

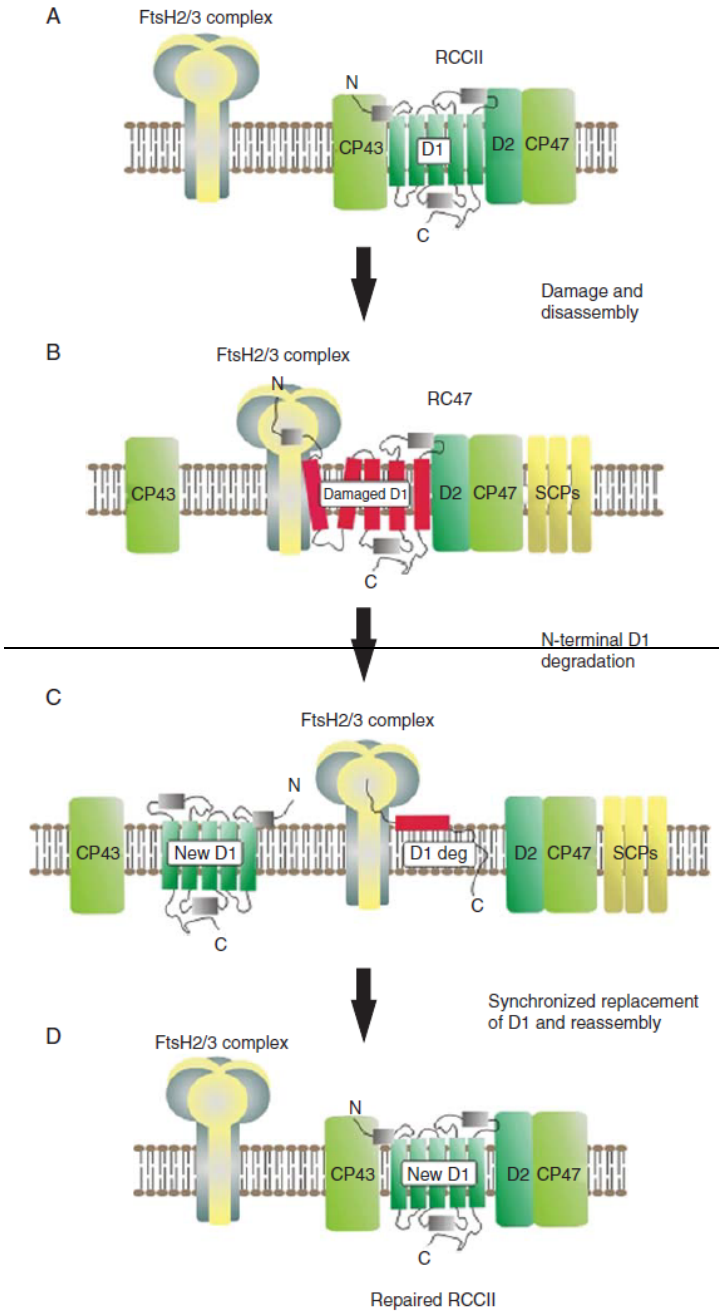


Figure 1.9. *Schematic representation of the repair cycle of PSII in which the damaged D1 protein is replaced by a newly synthesized D1 protein (taken from Nixon et al., 2010).*

1.3 Model organisms and targeted genes

To demonstrate the applicability of the antisense ODN technology in plants, we chose the nuclear encoded *phytoene desaturase* (*pds*), the *chlorophyll a/b binding protein* (*cab*) genes and chloroplast encoded *psbA* gene as model genes using wheat, tobacco and Arabidopsis as model organisms.

1.3.1 *Phytoene desaturase* (*pds*) as a model gene in tobacco and wheat

The *pds* gene has been used as a marker in several studies on gene silencing in plants (Chamovitz et al., 1993; Kumagai et al., 1995; Lindgren et al., 2003; Tao and Zhou, 2004; Wang et al., 2005; Wang et al., 2009). *Phytoene desaturase* encodes a key enzyme in carotenoid biosynthesis, which converts phytoene to the colored ξ-carotene in a two step desaturation reaction (Bartley and Scolnik, 1995) (Fig. 1.6). Low expression of the *pds* gene results in a general suppression of carotenoid biosynthesis (Wetzel and Rodermel, 1998).

1.3.2 *psbA* gene coding for the D1 protein of PSII in *Arabidopsis thaliana*

As a chloroplast encoded model gene, we choose the *psbA* gene, which encodes the D1 protein of the photosystem II reaction center (Fig. 1.5). The D1 protein has the highest known turnover rate of among photosynthetic genes and its turnover rate is light dose dependent (Park et al., 1995; Tyystjärvi and Aro, 1996).

1.3.3 *Chlorophyll a/b binding protein (cab)* genes in wheat

The genes coding for the chlorophyll *a*/*b* binding proteins (*cab* genes) was used here as a model gene family. The CAB proteins (also called light harvesting complexes (LHCs)) form a family of nuclear encoded thylakoid proteins. At least ten distinct types of Lhc proteins have been recognized in higher plants. Some are found in the antenna of photosystem I (PSI) and some in the antenna of PSII. *Lhca* genes encode the polypeptides of light harvesting complex I (LHCI), while *Lhcb* genes encode PSII antenna complexes; in particular *Lhcb1*, *Lhcb2* and *Lhcb3* genes encode the polypeptides of trimeric LHCII. The Lhcb4, Lhcb5 and Lhcb6 proteins (often called CP29, CP26 and CP24, respectively) are monomeric proteins linking the LHCII trimers to the reaction center core (Jansson, 1999).

2. AIMS OF THE STUDY

The major aim of this thesis is to extend the applicability of antisense ODNs in leaves of dicotyledonous and monocotyledonous plant species.

To achieve this we focused on the following points:

- To find methods for efficient uptake of ODNs by different types of leaves
- To test if the stability and efficiency of antisense ODNs is increased by 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).

3. MATERIALS AND METHODS

3.1 Design and synthesis of antisense ODNs

Oligodeoxynucleotide sequences were selected based on a search for freely available single stranded loops in the mRNA secondary structure (Mfold web server for nucleic acid folding and hybridization prediction *http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form,* Zuker, 2003). Sequences showing a strong hairpin and/or duplex forming ability were excluded. A genome wide nucleotide BLAST (Basic local alignment search tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search for highly similar sequences was performed with each chosen target sequence. Those exhibiting significant homology to any known gene were also discarded.

Synthesis of ODNs was performed using an Expedite 8909 DNA synthesizer (Applied Biosystems, Foster City, CA) by standard cyanoethyl phosphoramidite chemistry at a nominal scale of 3 μmol. All of the reagents for the automated ODN synthesis were also from Applied Biosystems. The ODNs were purified on Poly-PAK cartridges (Glen Research, Sterling, VA) yielding >97% full sized ODNs as shown by analytical ion exchange HPLC. Phosphorothioates were built in with >98% efficiency by using EDITH sulphurizing reagent (Link Technologies Ltd., Lanarkshire, UK) according to the manufacturer's instructions.

In the experiments a random nonsense control was used. A nucleotide BLAST search of the whole GenBank database with this sequence did not yield a single hit of a sequenced angiosperm gene. For uptake experiments, random nonsense ODNs were labelled with Fluorescein Cyanoethyl Phosphoramidite (6-FAM, Link Technologies; 494 nm excitation, 525 nm green emission) at 5' position and purified by HPLC.

3.2 ODNs used in this thesis

In tobacco: *pds* antisense ODN(8) 5'-TTCTGAGTCACTACGATTT-3' (19 mer), ODN(9) 5'-GCTTCGCTAGTTCCTTC-3' (17 mer) and their PS analogs 5'-$T_ST_SC_STGAGT_SCAC_STAC_SGA_ST_ST_ST$-3' and 5'-$G_SC_ST_ST_SC_SGC_STAGT_ST_SCC_ST_ST_SC$-3' were used, respectively. The ODNs were complementary to tobacco *pds* mRNA sequence (GenBank accession number EU165355.1) at the positions 1643-1624 and 1677-1660, respectively. The label '$_S$' in the sequence string denotes the position of the PS linkages.

In wheat: *pds* antisense ODN(5) 5'-GGCCAAGTAAGCATTTCA-3', ODN(10) 5'-ATCAACTGGTGCTGCAA-3' (18 mers) and ODN(11) 5'-CAAGGTTCGCAGTTCGGG-3' (17 mer): and their PS analogues having the structures: 5'-$G_SG_SC_SCAAGT_SAAG_SCA_STT_ST_SC_SA$-3'; 5'-$A_ST_SC_SAAC_STGGT_SGC_STG_SC_SA_SA$-3'; 5'-$C_SA_SA_SGGT_STC_SGC_SAGT_STC_SG_SG_SG$-3' were applied. Sequences were directed against the wheat *pds* mRNA sequence (GenBank accession number BT009315) at the nucleotide positions 179-161, 624-607 and 1040-1022, respectively. As controls, a 17 mer random nonsense 5'-GGCGGCTAACGCTTCGA-3' ODN and its respective PS 5'-$G_SG_SC_SGGC_STAAC_SGC_STT_SC_SG_SA$-3' ODN was used.

In Arabidopsis: *psbA* antisense ODN(1) 5'-$C_SA_ST_SAGGC_STT_ST_SCG_SCT_ST_ST_SC$-3' (17 mer), ODN(2) 5'-$T_SC_SC_SC_STGAT_SCAAAC_STA_SG_SA_SA$-3' (18 mer), ODN(3) 5'-$G_SC_SC_SC_SGA$-$A_STC_STGT_SAA_SCC_ST_ST_SC$-3' (19 mer), ODN(4) 5'-$G_ST_ST_SGT_SGAGC_SAT_STACG_ST_ST_SC$-3' (18 mer) were used. ODNs were complementary to the Arabidopsis *psbA* mRNA sequence (GenBank accession number NC000932) at the positions 41-25, 677-660, 721-703, 1014-997. As controls, a 17 mer random nonsense PS 5'-$G_SG_SC_SGGC_STAAC_SGC_STT_SC_SG_SA$-3' ODN was used.

In wheat (*Triticum aestivum*) ODN(1) 5'-TATGGTGTGTTGTCCCTGT-3' (18 mer), ODN(2) 5'-ACTTATGGTGTGTTGTCC-3' (18 mer), ODN(3) 5'-GGAAAGAGACATGGTGG-3' (17 mer), ODN(4) 5'-AGTTCTTGACGGCCTTGC-3' (18 mer), ODN(5) 5'-GACGAGGGCAAGTTCTT-3' (17 mer), ODN(6) 5'-AGAGCACACGGTCAGAG-3' (17 mer), ODN(7) 5'-GAAGGTCTCAGGGTCAG-3' (17 mer), ODN(8) 5'-TCGCTGAAGATCTGAGAG-3' (18 mer), ODN(9) 5'-ACGGCCCCCATGAGCACAA-3' (19 mer), ODN(10) 5'-GCGAGGCGGCCATTCTTG-3' (18 mer) chl *a*/*b* binding protein (cab) antisense ODNs were used. ODNs were complementary to a deposited wheat *cab* mRNA sequence (Gen Bank accession number M10144.1) at the positions 42-24, 46-28, 82-65, 115-97, 126-109, 227-210, 331-314, 476-458, 568-549 and 716-698. As control, a 17 mer random nonsense 5'-GGCGGCTAACGCTTCGA-3' ODN was used.

3.3 Plant material and growth conditions

Tobacco plants (*Nicotiana benthamiana*) were grown in a greenhouse at 20-25 °C. Supplemental light was provided by metal halide lamps for 12 h a day and the light intensity

at the plant surface was about 200 µmol photons m^{-2} s^{-1}. Expanding leaves of two months old plants were used for the experiments.

Arabidopsis plants (*Arabidopsis thaliana,* genotype Columbia-0) were grown in a greenhouse under short day conditions (8 h light, 16 h dark), at approximately 100 µmol photons m^{-2} s^{-1} during the light period. The temperature was kept between 20 °C and 24 °C. Six-week-old plants were used for the experiments.

Wheat seeds (*Triticum aestivum,* genotype CY-45) were sown in pots filled with a mixture of one part sand and two parts soil. The pots were kept in a growth chamber in total darkness for 11 days at 19 °C, 70% humidity.

Barley (*Hordeum vulgare*) wild type and the *Chlorina f2* mutant plants were grown on perlite in a greenhouse, under short day conditions. The temperature was about 20 °C during the day and 18 °C during the night. Seedlings were watered twice with Knopp's solution and they were used when they were 7 to 10 days old.

3.4 Antisense ODN treatment of leaves

The first and probably biggest problem of the treatment of plant organs is the introduction of oligonucleotides into plant tissues. Wounding of the leaves was necessary for a successful ODN application (Fig. 3.1).

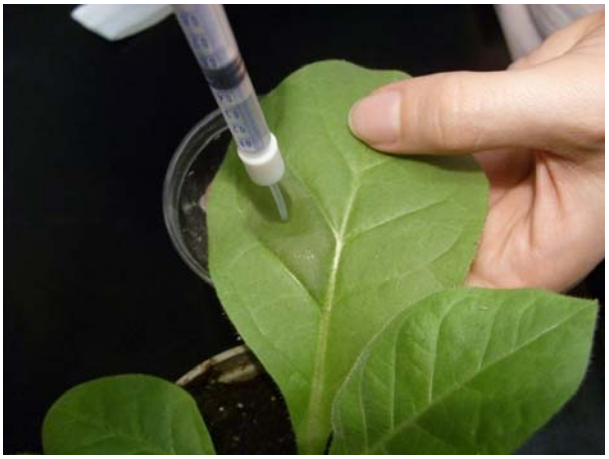
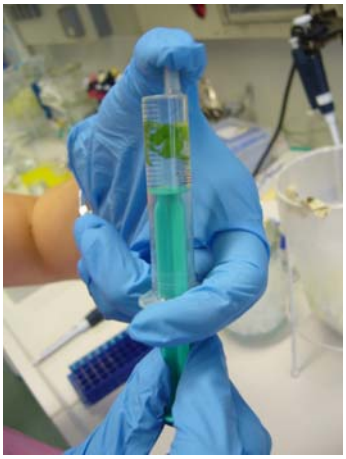
A.  B.  C. 

Figure 3.1. *Antisense ODN treatments of leaves. A: Infiltration into a tobacco leaf segment. B: Vacuum infiltration of Arabidopsis leaves and C: Passive uptake by detached wheat leaves*

For each of the three plant species a specific method was applied. In the case of tobacco, ODNs were introduced into the leaves with the help of a syringe (Fig. 3.1A) as described for transient plasmid uptake (Sparkes et al., 2006). In the case of the small Arabidopsis leaves a vacuum infiltration method was used (Fig. 3.1B). 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 (Fig. 3.1C).

3.4.1 Antisense ODN treatment of tobacco leaves

Antisense ODNs and random nonsense ODNs were dissolved in sterile distilled water at a concentration of 10 μM and infiltrated in intact tobacco leaves as described above (Fig. 3.1A). The ODN treatments were carried out between 8 and 10 a.m. After 24 h, fast chl *a* fluorescence transients were measured and treated leaf discs were cut and stored at -80 °C until further use. Beside the random nonsense ODNs, we also used water controls, i.e. treating the leaves with distilled water. Four independent experiments were carried out from which at least eight measurements were derived.

3.4.2 Antisense ODN treatment of Arabidopsis leaves

Antisense ODNs and random nonsense ODNs were dissolved in sterile distilled water at a concentration of 10 μM and vacuum infiltrated into detached Arabidopsis leaves. After 12 h of incubation in the dark, leaves were illuminated at 250 μmol photons m^{-2} s^{-1} for 48 h. After 8 h, 24 h and 48 h, fast chl *a* fluorescence transients were measured and leaf discs were cut and stored at -80 °C until further use. Eight independent repetitions were made for each ODN.

3.4.3 Antisense ODN treatment of wheat leaves

10 cm long leaf segments of etiolated wheat seedlings were prepared. The leaves were cut under water to avoid the formation of air bubbles in the veins and the lower 1-2 cm part was submerged in 10 μM water solution of *pds* and *cab* antisense ODNs, random nonsense ODNs and pure water in a 1.5 ml Eppendorf tube. The tubes were sealed with Parafilm in order to avoid evaporation of solution. After 12 h of incubation in the dark, leaves were illuminated at 100 μmol photons m^{-2} s^{-1} for 24 h. Based on preliminary experiments using fast

chl *a* fluorescence transients (see below), we chose the 8 h and 24 h illumination times for a more detailed study. After 8 h and 24 h of illumination fluorescence measurements were performed and leaf segments were harvested and stored at -80 °C until further use. Four independent repetitions were made for each ODN.

3.5 Confocal laser scanning microscopy

Microscopy was performed using an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Life Science Europa GmbH, Hamburg, Germany). The microscope configuration was as follows: objective lenses: LUMPLFL 40x (water, NA:0.8) and LUMPLFL 60x (water, NA:0.9); sampling speed: 8 µs/pixel; line averaging: 2x; zoom: 1x and 3x; scanning mode: sequential unidirectional; excitation: 488 nm; laser transmissivity: 5%; main dichroic beamsplitter: DM405/488; intermediate dichroic beamsplitter: SDM 560; FAM was detected between 500-555 nm and chl *a* fluorescence was detected between 650 and 750 nm. Unlabeled leaves were used to determine the level of green autofluorescence and the laser intensity/detector voltage parameters were set accordingly, so that no green autofluorescence was visible in unlabeled leaves.

Photographs of wheat leaves were taken with an Olympus 'Camedia C7070 Wide Zoom' digital camera.

3.6 Fast chl *a* fluorescence (OJIP) measurements

Fluorescence measurements were carried out at room temperature with a Handy PEA instrument (Hansatech Instruments Ltd, UK). Leaf samples were illuminated with continuous red light emitted by three LEDs (3,500 µmol photons m^{-2} s^{-1}, 650 nm peak wavelength; the spectral half-width was 22 nm; the emitted light is cut off at 700 nm by a near infrared short pass filter). The first reliably measured point of the fluorescence transient is at 20 µs, which was taken as F_0. The length of the measurements was 1 s. To monitor the effects of antisense ODNs on the photosynthetic machinery, the minimum fluorescence (F_0), maximum fluorescence (F_M) and the variable fluorescence ($F_V = F_M-F_0$) parameters were used.

3.7 Quantitative real time reverse transcription PCR (QRT-PCR)

Total RNA was isolated from wheat, tobacco and Arabidopsis leaves using TRI reagent (Sigma, St. Louis, MO) according to the supplier's instructions. First strand cDNA synthesis of 2 µg total RNA in a final volume of 20 µl was performed with RevertAid H minus M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) according to the supplier's protocol using random hexamer primers. Control reactions were performed by omitting reverse transcriptase. For QRT-PCR, 9 µl of 1:45 diluted cDNA was mixed with Brilliant® II SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA), 5 pmol of forward primer, 5 pmol of reverse primer (as listed below) in a final volume of 20 µl in three replicates. No-template controls were included. QRT-PCR was done using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following protocol for wheat and Arabidopsis: 95 °C for 10 min, 45 cycles at 95 °C for 15 s followed by 60 °C for 1 min; for tobacco: 95 °C 10 min, 45 cycles at 95 °C for 15 s followed by 64 °C for 1 min. The specificity of the QRT-PCR amplification was confirmed by the following criteria: (1) a single peak in the melting temperature curve analysis of real time PCR amplified products (ABI Prism Dissociation Curve Analysis Software); (2) a single band on an agarose gel. Primer pairs were designed to detect relative expression levels of the *pds* genes of wheat and tobacco (see below). The expression level of each group was normalized to (1) ubiquitin as a housekeeping gene and (2) the initial amount of *pds*, *cab*, *psbA* transcripts in the control samples of wheat, tobacco and Arabidopsis. The relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

For QRT-PCR four pairs of primers were used: wheat *ubiquitin* forward, 5'-CTGGCGAGGATATGTTCCAT-3'; wheat *ubiquitin* reverse, 5'-TCGGATGGAAGACCTTTGTC-3'; wheat *pds* forward, GAAATACCTGGCTTCCATGG; wheat *pds* reverse, CGGGACAGCATCTTAGAATCC; wheat *cab* forward, GATCGTCGACCCACTCTAC; wheat *cab* reverse, ACAATGGCCTGCACAAAG; tobacco *ubiquitin* forward, TCCAGGACAAGGAGG GTATCC; tobacco *ubiquitin* reverse, TAGTCAGCCAAGGTCCTTCCAT; tobacco *pds* forward, CAGATTTCTTC AGGAGAAACATGGTTCA; tobacco *pds* reverse, CCACAATCGGCATGCAAAGTCTC; Arabidopsis *ubiquitin* forward, AACCCTAACGGGAAAGACGATTA; Arabidopsis *ubiquitin* reverse, TGAGAACAAGATGAAGGGTGGAC; Arabidopsis *psbA* forward, TGATTGTATTCCAGGCTGAGCA; Arabidopsis *psbA* reverse, TGCCCGAATCTGT-AACCTTCAT.

3.8 Determination of the carotenoid and chlorophyll contents

Analysis of total carotenoid in plant tissue was performed in N,N-dimethylformamide (DMF) (Inskeep et al., 1985). The OD values were measured at 663, 646 and 470 nm using a spectrophotometer (Hitachi U-2900, Japan) and the chl content was determined according to Porra (2002). The total carotenoid content was determined according to Lichtenthaler (1987).

3.9 Western blot analysis

Leaf segments equivalent to total area of 4 cm^2 cut from wheat leaves and Arabidopsis leaf discs were frozen in liquid nitrogen and ground to a fine powder and then homogenized in 500 µL Laemmli buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.1% BPB, 10% glycerol, 200mM DTT, 5mM PMSF). The homogenates were incubated at 90 ºC for 5 min followed by 20 min incubation at 37 ºC, and then the proteins were separated by 15% denaturing SDS PAGE. The proteins were blotted on nitrocellulose membranes using a semi-dry blotting system with a methanol containing buffer. The nitrocellulose membranes were blocked using 5% skim milk powder in Tris buffered saline plus Tween 20 (TBST) buffer (10 mM TRIS pH 8.0, 0.15 M NaCl, 0.1% Tween 20) for 2 h and incubated with primary antibodies raised against Lhcb1, -2, -3 and -6 and Lhca1, -2 and -3 and PsbA for 2 h in TBST buffer with 5% skim milk powder. The membranes were washed three times for 5 min in TBST buffer and incubated with goat anti rabbit IgG horseradish peroxidase conjugate (Millipore) at a 1:5,000 dilution in TBST buffer with 5% skim milk powder for 2 h. Immunoblotted membranes were incubated for 5 min in ECL (= enhanced chemiluminescence) plus horseradish peroxidase substrate (GE Healthcare Bio-Sciences) and chemiluminescence was detected on Hyperfilm ECL photographic film (GE Healthcare Bio-Sciences). The developed film was digitized and analyzed by the 1D Scan software package.

4. RESULTS

4.1 Uptake and transport of ODNs in plants as investigated by confocal microscopy

Oligodeoxynucleotides were introduced into intact tobacco leaves by infiltration with a syringe, as described for plasmid uptake by Sparkes et al. (2006) and into detached wheat leaves through their vascular system (see 3.4.1 and 3.4.3). To monitor the presence of ODN uptake in wheat and tobacco leaf cells, a 17 mer ODN representing a random nonsense sequence was synthesized with a 6-FAM fluorescent dye covalently attached to the 5' end. The localization of ODNs was analyzed by confocal laser scanning microscopy. In cross sections of leaves, after 24 h of feeding, the fluorescence intensity was high in the veins of the wheat leaves (Fig. 4.1A). Oligodeoxynucleotides accumulated as well in the epidermal and parenchymal cells indicating the transport of ODNs inside the leaves. High resolution confocal laser scanning microscopy showed that the ODNs accumulated both in the cytoplasm and the chloroplasts of parenchyma cells of wheat (Figs. 4.1D and F) and tobacco (Figs. 4.1G and I). Chl *a* fluorescence emission was used to localize the position of the chloroplasts (Figs. 4.1B, E, H and K). Oligodeoxynucleotides accumulated also in the nucleus (Fig. 4.1J). These data indicate that ODNs efficiently penetrate through cell walls and plasma membranes.

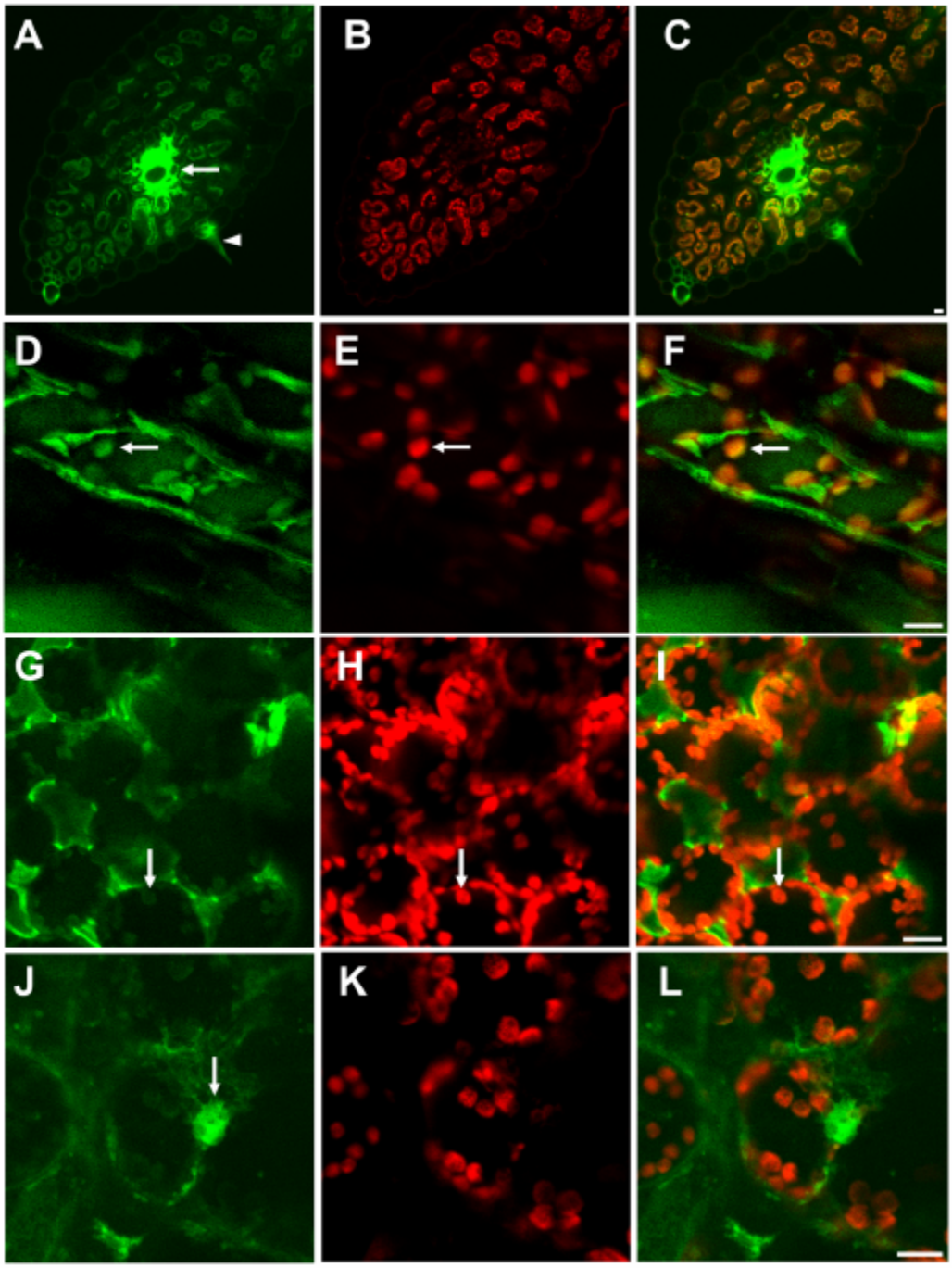


Figure 4.1. *Cellular distribution of fluorescein labelled antisense ODNs (green) and chl autofluorescence (red), in wheat (A-F) and tobacco leaves (G-L). Low magnification imaging of a leaf blade cross section is shown in panels A-C where the arrows indicate strong accumulation of antisense ODNs in the veins of the wheat leaf. The arrowhead in panel A points to a trichome containing fluorescent ODNs. Merged images are shown in the last column. Arrows in panels D-I show accumulation of antisense ODNs in chloroplasts. The arrow in (J) shows a nucleus filled with fluorescent antisense ODNs. Scale bars: 10 μm.*

4.2 *Phytoene desaturase* (*pds*) as a model gene to test the efficacy of antisense ODNs in wheat and tobacco

For the targeted *pds* mRNA, ten different antisense ODNs were designed and synthesized both for wheat and tobacco, with their natural phosphodiester and PS structures. It has been shown that a decrease in the carotenoid content due to the suppression of the *pds* gene results in a decrease of the chl content and in a decrease of the photosynthetic efficiency as well (Wang et al., 2010), which is probably due to the instability of the synthesized chl binding protein complexes in the absence of carotenoids (Plumley and Schmidt, 1987). Therefore, in order to select the most efficient ODNs and also to determine the time points where the largest differences are found between the control and the ODN treated plants, we used the fast chl *a* fluorescence (OJIP) transient technique, a non-invasive and very sensitive method for measuring photosynthetic efficiency (Govindjee, 2004; Schansker et al., 2005, Lazár and Schansker, 2009). For monitoring the effects of antisense ODNs, we used the variable fluorescence parameter (F_V) (maximum fluorescence *minus* minimum fluorescence (F_M-F_0)). Based on the F_V parameter, two pairs of antisense ODNs were selected in preliminary experiments for tobacco, which we called 8T, 8N, 9T and 9N (where T stands for the PS structure and N indicates the natural, phosphodiester structure). For wheat, three pairs of antisense ODNs were selected, called 5T, 5N, 10T, 10N, 11T and 11N. The preliminary experiments also showed that the largest differences between the F_V values of the antisense ODN treated and control samples were observed after 8 h of ODN treatment; beside the 8 h illumination time we also chose the 24 h time point in order to investigate the effects of ODNs at a later stage of greening.

4.2.1 The effects of antisense ODNs on *pds* in young tobacco leaves

To investigate the effects of *pds* antisense ODNs on a dicotyledonous species, young leaves of two months old tobacco plants (*Nicotiana benthamiana*) were used. Solutions containing *pds* antisense ODN at a concentration of 10 μM were infiltrated into intact leaf segments with the help of a syringe (Sparkes et al., 2006) and samples were taken 24 h after the infiltration.

The 24 h of antisense ODN treatment resulted in 5-10% lower F_V values compared to the random nonsense controls (Fig. 4.2A). This could be attributed to the decrease in the transcript level of the *pds* gene, which was suppressed by about 25-35% compared to the

random nonsense control (Fig. 4.2B). This inhibition led to a 5-10% decrease in the carotenoid content (Fig. 4.2C), a decrease similar to the one observed for F_V. The data also show that the antisense ODN(8) with PS modification (8T) was somewhat more efficient than its natural counterpair (8N). The relatively small effect of the antisense ODNs on the mRNA level, the carotenoid content and F_V value can be explained by the slow turnover of the PDS enzyme and the high background levels of the pigments and photosynthetic complexes. We, therefore, carried out experiments on greening leaves, i.e. during the synthesis of the photosynthetic machinery.

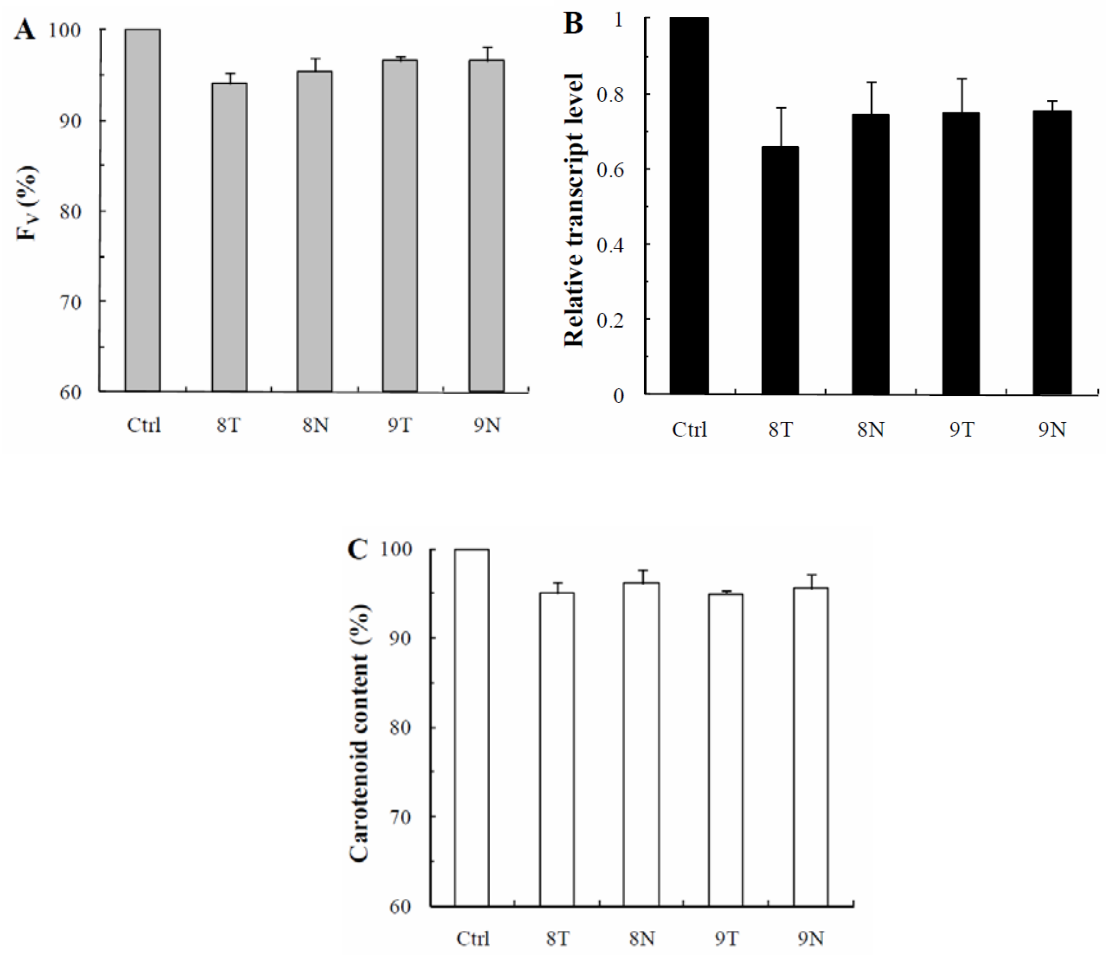


Figure 4.2. *Effects of pds antisense ODNs on the variable chl a fluorescence, F_V (A), the relative transcript level (B) and the carotenoid content (C), expressed in % of the control in young tobacco leaves 24 h after the infiltration with ODNs. Control - random nonsense sequence; T, phosphorothioate structure, N, phosphorodiester (natural) structure. The values are averages (±SD) of at least eight measurements derived from four independent experiments.*

4.2.2The effects of *pds* antisense ODNs during greening of wheat leaves

It is well known that etiolated leaves contain no chl, whereas lutein and violaxanthin can be found at low levels when compared with light grown plants and they contain only traces of other carotenoids (Park et al., 2002). When etiolated leaves are exposed to light, chl biosynthesis starts and the carotenoid content increases (von Lintig et al., 1997). Based on these observations we expected that *pds* antisense ODNs could efficiently slow down or inhibit the de-etiolation process. Etiolated leaves were detached and their basal part was submerged for 12 h in a 10 μM *pds* antisense ODN solution in the dark. Subsequently, they were illuminated with white light of 100 μmol photons m^{-2} s^{-1} for 24 h. The carotenoid content of etiolated leaves was low compared to green leaves and remained constant during the 12 h incubation in the presence of antisense ODNs in the dark (data not shown).

After 8 h of illumination of wheat leaves, the F_V value was 12-30% lower in the antisense ODN treated plants (Fig. 4.3A) compared with controls, i.e. the random nonsense ODNs. At the same time, the expression level of the *pds* gene was suppressed dramatically, by about 50 to 70% (Fig. 4.3B). This inhibition led to a significant decrease in the carotenoid content: 18-24% depending on the antisense ODN applied (Fig. 4.3C; the total carotenoid content of the control was about 0.2 μg mg^{-1} FW). It is important to note that the differences in all these three parameters were more pronounced in plants treated with PS modified ODNs compared to the ODNs with a natural structure; the largest difference was found between ODNs 11T and 11N.

These data show that *pds* ODNs slow down the photomorphogenesis in leaves. Indeed, the chl content was also lower in the antisense ODN treated plants (by about 25-42%, depending on the ODN applied, data not shown). This is consistent with the well established fact that the stability of pigment-protein complexes depends on the presence of carotenoids (Plumley and Schmidt, 1987; Lokstein et al., 2002). It is interesting to note that following an 8 h de-etiolation, the chl *a*/*b* ratio was higher (typically between 10 and 16) in ODN treated plants than in the control plants (chl *a*/*b* ratio around 8), suggesting a much slower synthesis of the peripheral (chl *a* and *b* containing) antennae in the ODN treated leaves. This difference had disappeared after 24 h of illumination, which is in agreement with the results of Wang et al. (2009), who showed that in mature transgenic plants the silencing of the *pds* gene does not change the chl *a*/*b* ratio.

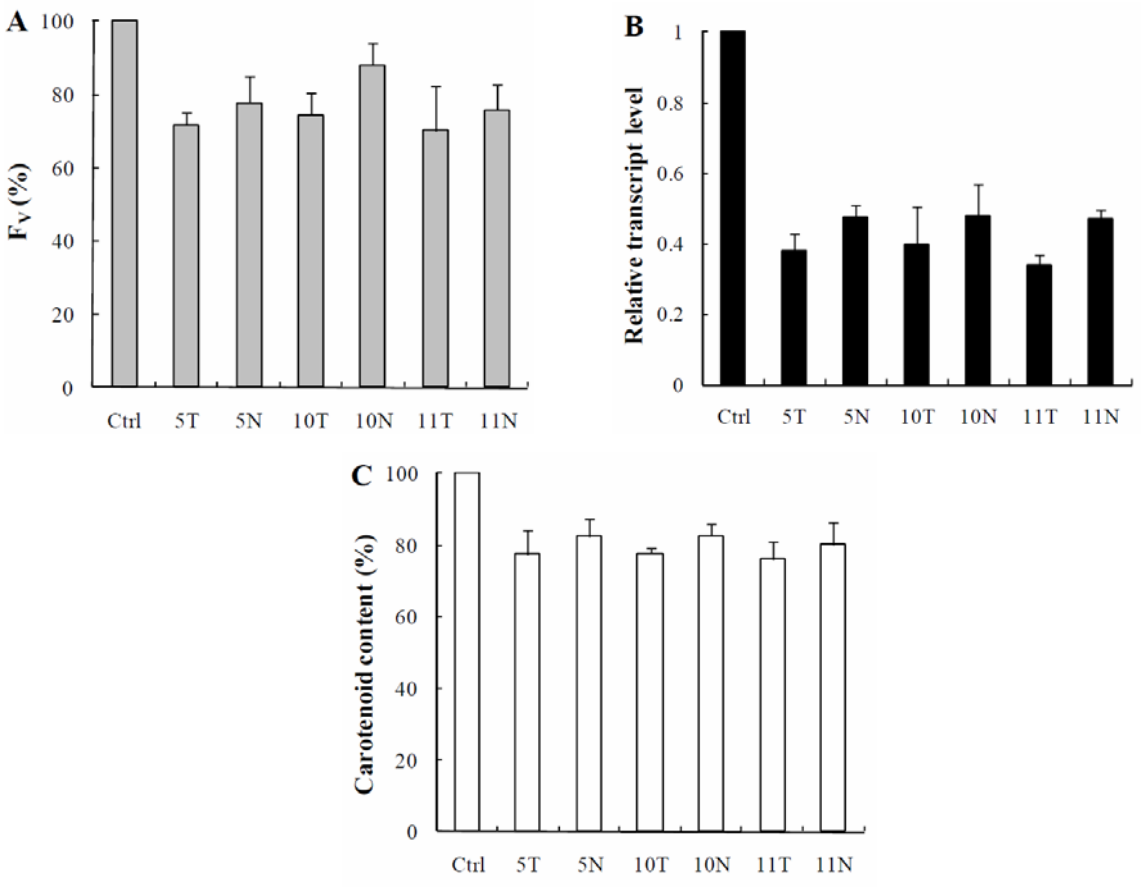


Figure 4.3. *Effects of pds antisense ODNs on the variable chl a fluorescence, F_V (A), the relative transcript level (B) and the carotenoid content (C) expressed in % of the control in etiolated wheat leaves after 8 h of illumination. Control, random nonsense sequence; T, phosphorothioate structure, N, phosphorodiester (natural) structure. The values are averages (±SD) of eight measurements derived from two independent experiments.*

Seeing the interference of ODN treatments with early greening events, we also looked at wheat leaves in a more advanced stage of their photomorphogenesis. After 24 h of de-etiolation, the effect of ODNs on the F_V value was less than 10% (Fig. 4.4A). Concerning the transcript level of the *pds* gene, the decrease induced by the antisense ODNs was found to be about 25% at this later phase of de-etiolation (Fig. 4.4B), while these values varied between 50 and 70% after 8 h (Fig. 4.3B). Also, the reduction in the carotenoid content became considerably smaller (Fig. 4.4C; the total carotenoid content was about 0.27 µg/mg FW in the control leaves) compared to leaves that had been treated at an earlier stage of greening. This result is in accordance with the notion that ODNs can only inhibit *de novo* synthesis.

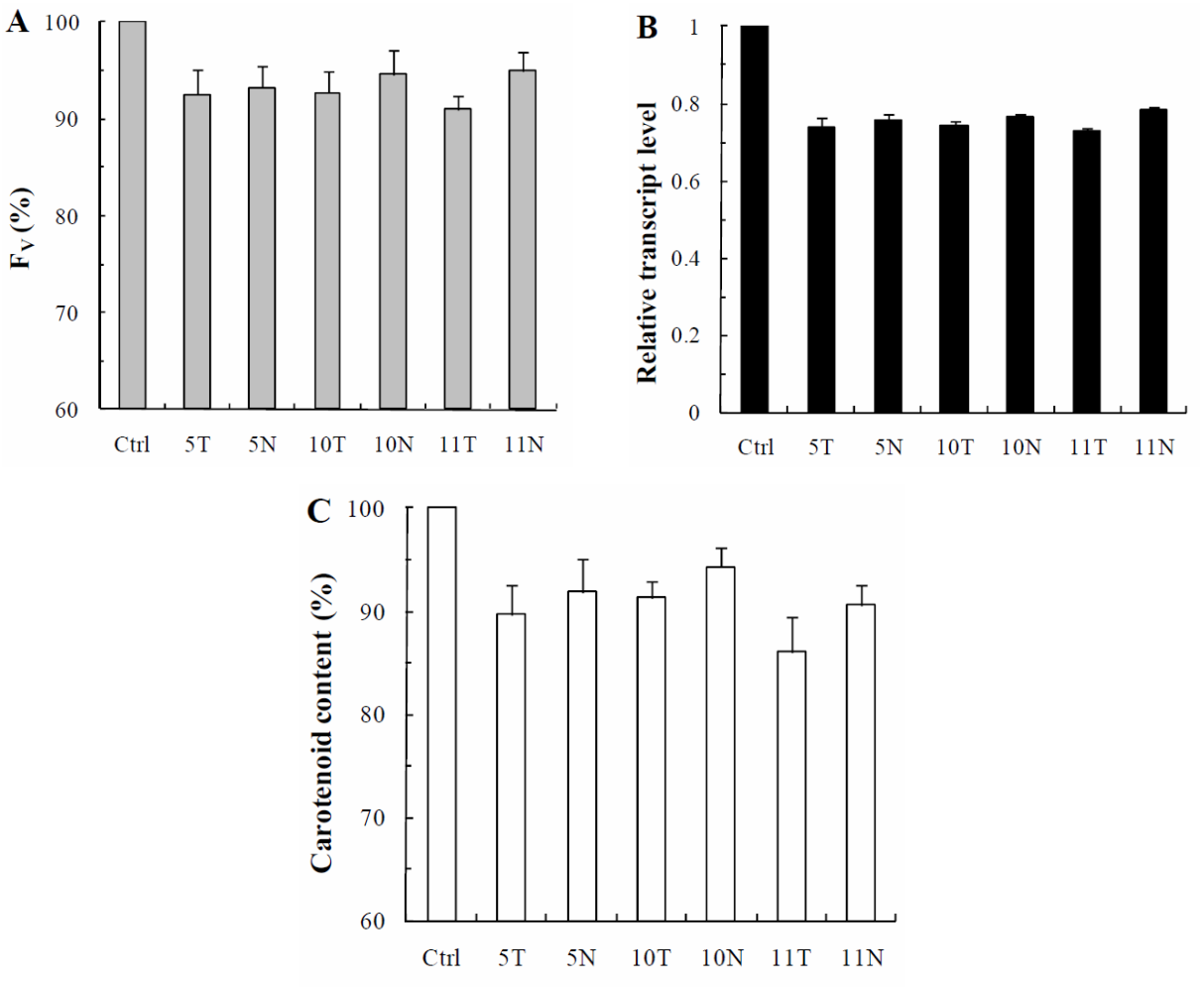
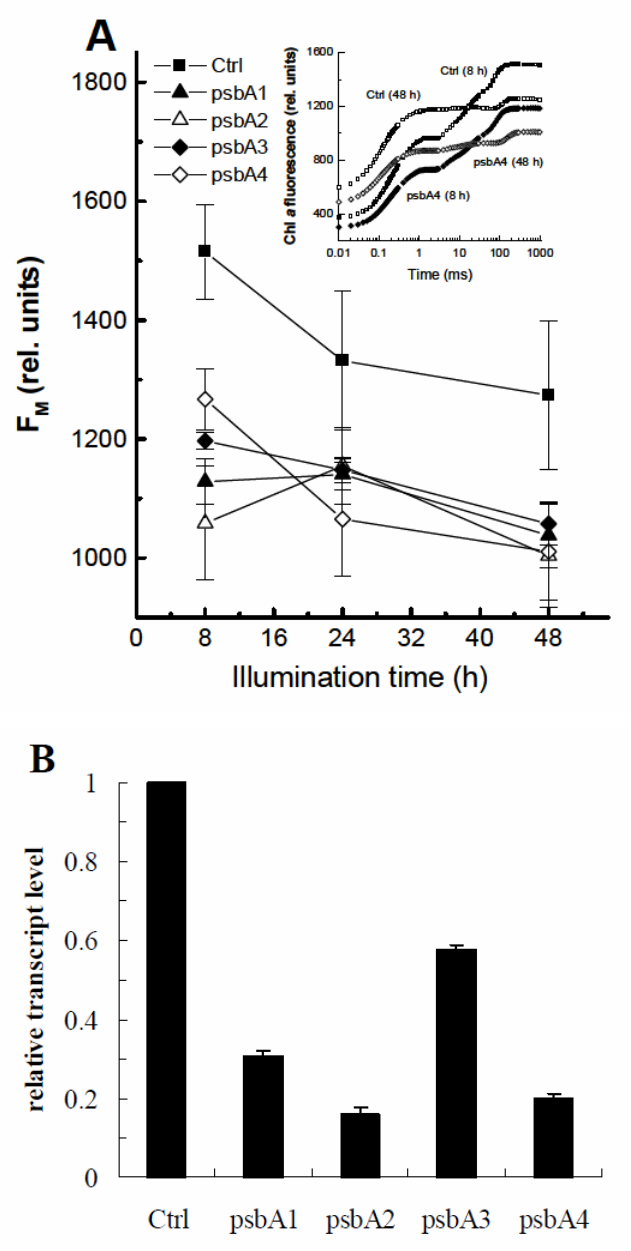


Figure 4.4. *Effects of pds antisense ODNs on the variable chl a fluorescence, F_V (A), the relative transcript level (B) and the carotenoid content (C) expressed in % of the control in etiolated wheat leaves after 24 h of illumination. Control, random nonsense sequence; T, phosphorothioate structure, N, phosphorodiester (natural) structure. The values are averages (±SD) of eight measurements derived from two independent experiments.*

4.3 Effects of antisense ODNs on *psbA* in *Arabidopsis thaliana* leaves

For the inhibition of the *psbA* gene of Arabidopsis four different antisense ODNs were designed and synthesized using the PS structure, based on the single *psbA* mRNA sequence of *Arabidopsis thaliana* in the GenBank database. Fluorescence measurements were made after 8, 24 and 48 h of illumination and of each fluorescence transient the F_M value was determined (Fig. 4.5A). The inset of Fig. 4.5A shows fluorescence transients of control and psbA4 treated leaves after 8 and 48 h of illumination. Both the control and the ODN treated leaves had a

more reduced PQ pool after 48 h of illumination, which is indicated by a high fluorescence intensity after 2 ms of illumination (J step) (cf. Schansker et al., 2005). Since the F_M value is not affected by the redox state of the photosynthetic electron transport chain it was used to assess the effect of the ODNs in Fig. 4.5A. The main difference between the control and the ODN treated leaves is already observed after 8 h of illumination. After 48 h of illumination, the four ODNs were inhibiting the *psbA* gene by 42% for the least effective ODN up to 85% for the most effective ODN (Fig. 4.5B). We performed Western blot analyses for the D1 protein. The psbA2 and psbA4 antisense ODNs gave the strongest inhibition of the D1 protein: 72 and 64% inhibition, respectively (Fig. 4.5C).



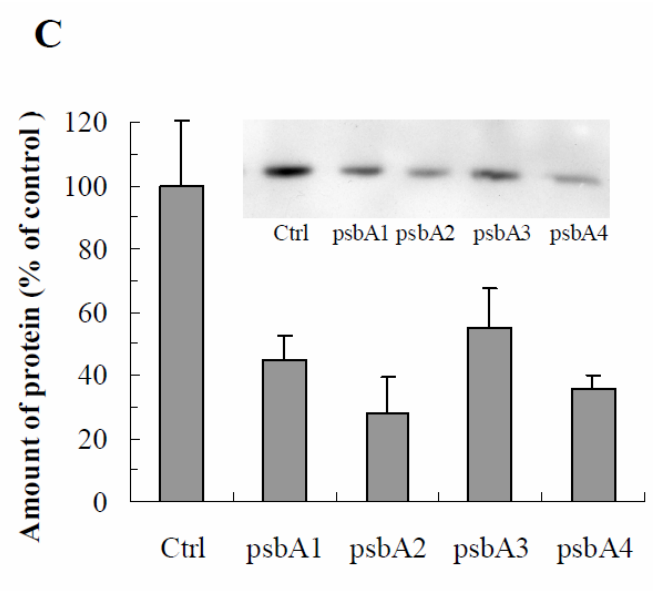


Figure 4.5. *Effects of psbA antisense ODNs on the maximum chl a fluorescence, F_M (A), the relative transcript level (B) and the amount of D1 protein in ODN treated Arabidopsis leaves after 48 h of illumination (C). Control leaves were treated with random nonsense ODNs. The insert of panel A shows OJIP transients of control and psbA4 treated leaves. Representative western blots are shown in the insert of panel C. The averages are given with their standard deviation.*

In order to increase the inhibitory effect, a photoinhibitory treatment preceding the experiment was used. The ODN dose dependency was also tested in this measurement using 10 and 30 µM ODN (Fig. 4.6). In Fig. 4.6 it is shown that in the presence of the psbA4 ODN a 30 min treatment with high light (1300 µmol photons m^{-2} s^{-1}) caused a stronger decrease of the F_V/F_M than observed for control leaves. Increasing the ODN concentration from 10 to 30 µM caused an even stronger decrease of the F_V/F_M value. The subsequent recovery of the F_V/F_M values under room light conditions (~10 µmol photons m^{-2} s^{-1}) occurred at the same rate in the absence and presence of ODN. However, the extent of recovery differed and depended on the ODN concentration.

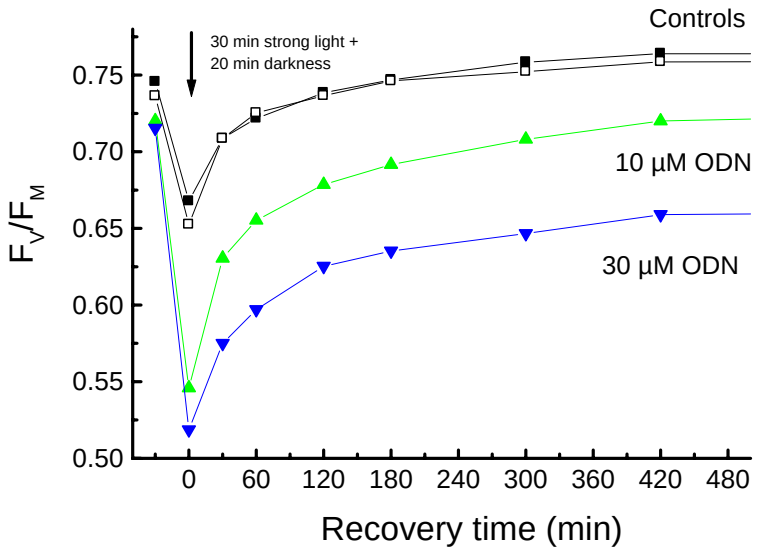


Figure 4.6. *The effect of the psbA4 ODN on photoinhibition (1300 µmol photons m^{-2} s^{-1}) and recovery in room light (~10 µmol photons m^{-2} s^{-1}) of Arabidopsis thaliana leaves. Data from a representative experiment are shown.*

4.4 The effect of *chlorophyll a/b binding protein (cab)* gene antisense ODNs on antenna development during the greening of wheat leaves

For the inhibition of the *cab* gene mRNAs of wheat, ten different antisense ODNs were designed and synthesized with a natural (phosphodiester) structure based on the single mRNA sequence found in the GenBank database for wheat (*Triticum aestivum*). Although all ODNs were able to inhibit the *cab* genes to some extent, the two most effective ODNs were selected using the fast chl *a* fluorescence transients as a selection criterion. The fluorescence data also indicated that the maximum effect of the ODN treatments was obtained after 8 h of greening. Fig. 4.7A, a photograph of etiolated wheat seedlings treated with *cab* ODNs also demonstrates very clear effects after 8 h of greening. After 8 h of illumination, the F_M values were 36 to 41% lower in the antisense ODN treated plants than in the controls (Fig. 4.7B). At the same time, the expression levels of the *cab* genes were suppressed by about 59 to 66% (Fig. 4.7C). The inhibition led also to a significant decrease in the chl contents of the leaves (chl *a*, 41 % and chl *b*, 59%, Figs. 4.7D and 4.7E).

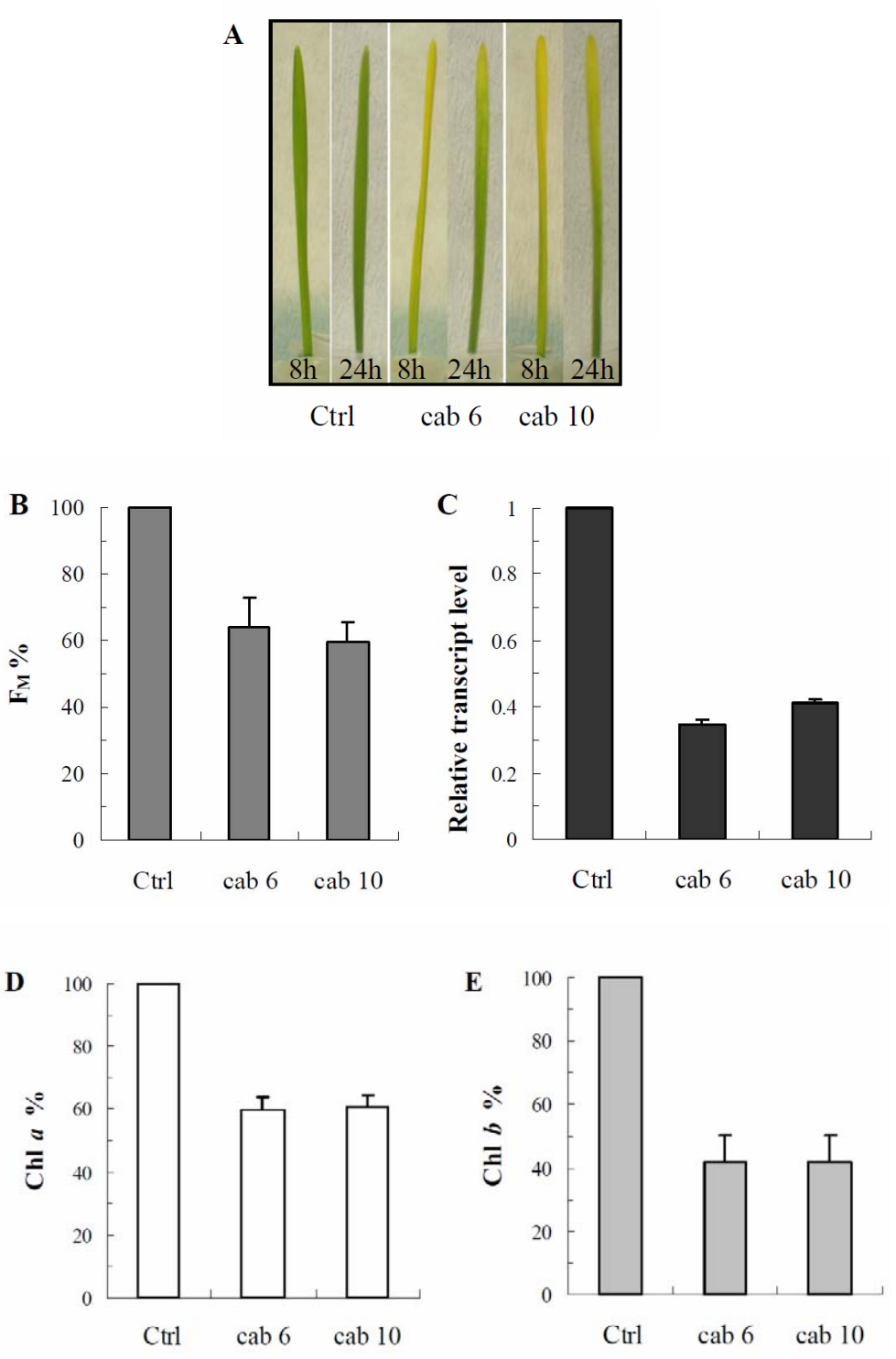


Figure 4.7. *The effects of two different cab antisense ODNs, cab6 and cab10, on wheat seedlings upon de-etiolation of the leaves. Photographs of 8 and 24 h de-etiolated leaves (A) and effects on the maximum chl a fluorescence, F_M (B), the relative transcript level (C), the chl a content (D), and the chl b content (E) after 8 h of greening. Values are expressed in % of the control; control, random nonsense sequence. The values are averages (±SD) of 8 measurements from two independent experiments.*

Western blot analyses were performed for the components of the trimeric LHCII components (Lhcb1, Lhcb2 and Lhcb3 proteins) and for one of the monomeric light harvesting complexes of PSII, the Lhcb6 protein (CP24), as well as for three components of the PSI light harvesting antenna complexes (Lhca1, Lhca2 and Lhca3). In the case of the cab6 antisense ODN, out of these complexes, the most affected proteins were Lhcb3, Lhcb6, Lhca1 and Lhca2 (66-72% inhibition, Fig. 4.8). The cab10 antisense ODN inhibited the synthesis of Lhcb3 with a very high efficiency (~88% compared to the control), whereas the other proteins analyzed were less affected. The least affected proteins were Lhcb1, Lhcb2 and Lhca3 for both ODNs tested (Fig. 4.8).

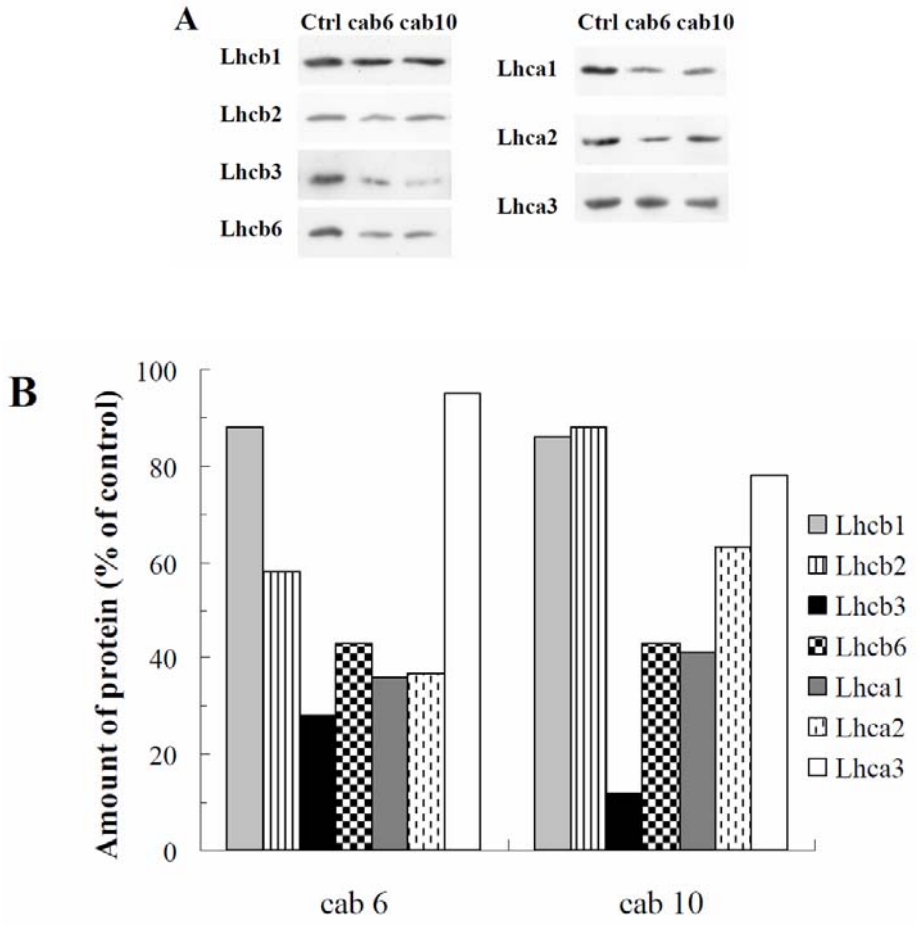


Figure 4.8. *The amounts of Lhcb1, -2, -3 and -6 and Lhca1, -2 and -3 proteins in control and ODN treated (cab6 and cab10) wheat leaves after 8 h de-etiolation, determined by Western blot analyses. Typical blots (A) and band intensities determined by densitometry (B) are presented.*

Up till now, the applicability of antisense ODNs in tobacco (*Nicotiana benthamiana*), Arabidopsis (*Arabidopsis thaliana*) and wheat (*Triticum aestivum*) was demonstrated. Chosen model genes: *phytoene desaturase* (*pds*), the D1 protein (*psbA*) and the *chlorophyll a/b binding protein genes* (*cab*).

4.5 Application of antisense ODNs to the study of a biological problem: The effects of changes in the leaf chlorophyll content and chl *a*/*b* ratios on chl *a* fluorescence parameters

In this section, ODNs were applied to understand the relationship between the fluorescence intensity (F_0 and F_M) and chl content and antenna size, respectively (Dinç et al. 2012). This is a continuation of an earlier study (Ceppi 2010) in which sugar beet plants grown under low sulphate or magnesium deficient conditions had been studied. These mineral deficiencies led to a massive loss of leaf chlorophyll, but the antenna size remained nearly unaffected (little change in the chl *a*/*b* ratio). Under these conditions, little effect on the fluorescence intensity (F_0 and F_M) was found and this led to the question if antenna size was a more important determinant of the fluorescence intensity. The effect of antenna size could be studied using etiolated wheat leaves treated with 10 different antisense ODNs that were allowed to green for either 8 or 24 h. This led to the creation of a wide range of chl *a*/*b* ratios (i.e. differences in the antenna size). The ODNs were in this case a source of biological variability.

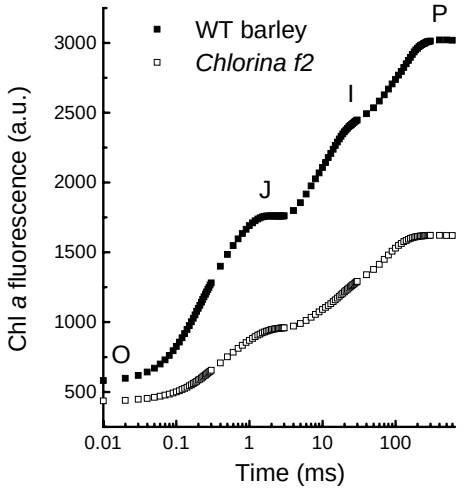


Figure 4.9. *OJIP transients measured on leaves of WT barley plants and the Chlorina f2 mutant of barley.*

The *Chlorina f2* mutant of barley lacks chl *b*. As a consequence no assembly of the peripheral antenna occurs (Preiss and Thornber, 1995). Comparing OJIP transients measured on WT and *Chlorina f2* barley leaves shows that the amplitude of these transients is much smaller than those of control transients (Fig. 4.9). The results of the mineral deficiency experiment (Ceppi 2010) allow the suggestion that the observed lower fluorescence intensities in the *Chlorina f2* plants are due to the smaller antenna size and not due to the lower leaf chl content.

To study the effect of antenna size on the fluorescence intensity in more detail a greening experiment was carried out on wheat seedlings, using antisense ODNs targeting cab mRNA. By inhibiting the synthesis of the CAB proteins to different extents, biological variability was induced. Detached wheat leaves were treated with antisense ODNs for 12 h in darkness, which was followed by a 24 h illumination period (100 μmol photons m^{-2} s^{-1} of white light).

In Fig. 4.10C and D, OJIP transients of wheat leaves treated with antisense cab ODNs after 8 h (panel C) and 24 h (panel D) of greening, respectively, are shown. After 8 h of greening, the transients of the untreated wheat leaves and the leaves treated with the antisense cab ODNs that had the least effect on the synthesis of the CAB proteins already showed the typical OJIP kinetics. Transients measured on wheat leaves treated with the antisense ODNs that had the strongest effect on the F_M value did not only have a smaller fluorescence amplitude, they also lacked the IP phase. The IP phase has been shown to reflect electron flow through PSI (Schansker et al., 2005) and its amplitude has been associated with PSI content (Ceppi, 2010; Oukarroum et al., 2009). Double normalizing the transients between O and I demonstrates that the OJ rise is much slower for the antisense ODN treated leaves indicating that these had a smaller effective antenna size (inset of Fig. 4.10C). After 24 h of greening the fluorescence kinetics both for the control and antisense ODN treated leaves had changed considerably. There were still differences in the amplitudes of the transients between the different ODN treatments but the OJIP kinetics had become qualitatively very similar and the IP phase had become considerably more prominent. In addition, the JI phase had a small relative amplitude compared to mature green leaves.

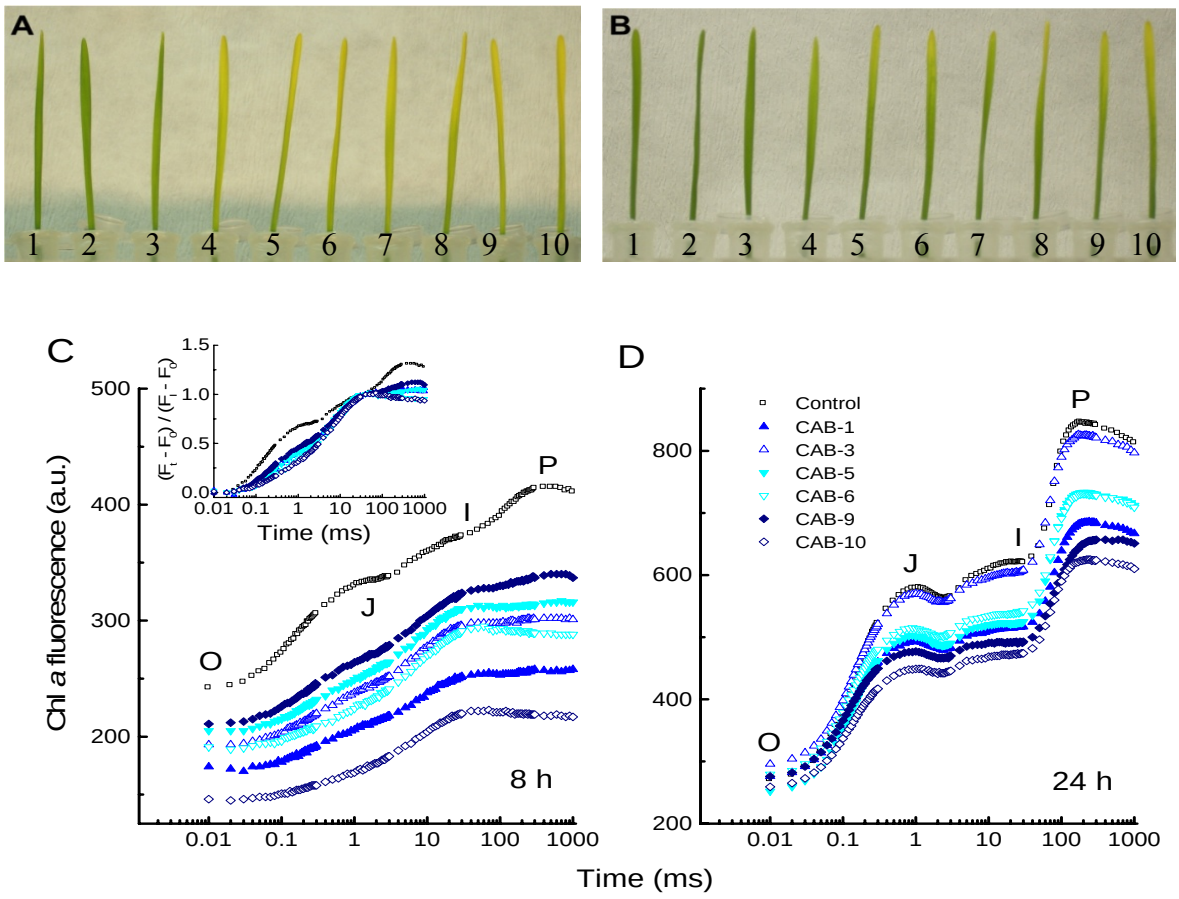


Figure 4.10. *Effects of 8 and 24 h of greening on etiolated wheat leaves. Photos of antisense ODN treated wheat leaves after 8 h (panel A) and 24 h (panel B) of greening; selection of fluorescence transients measured on wheat leaves treated with cab ODNs after 8 (panel C) and 24 h (panel D) of greening. The order of the leaves in panels A and B is: 1. control (pure water), 2-3. control (random nonsense sequence), 4. cab3 antisense ODN, 5. cab5 antisense ODN, 6. cab6 antisense ODN, 7. cab7 antisense ODN, 8. cab7T antisense ODN, 9. cab8 antisense ODN, 10. cab10 antisense ODN treated etiolated wheat leaves. In the inset of panel C, the fluorescence transients were double normalized between O and I to allow a comparison of the shapes of the OJIP transients. The chl a/b ratios associated with the measurements, following the order given by the legend, were 7.6, 13.6, 12.2, 10.1, 12.6, 11.8 and 14.7, respectively after 8 h of greening and 4.4, 5.6, 4.8, 4.3, 5.0, 5.2 and 5.7, respectively after 24 h of greening. Each transient represents the average of four independent measurements.*

The combination between the treatments with the different antisense ODNs and the two time points at which the measurements were made yielded a broad range of chl contents. For the chl *b* content a ten-fold difference between the leaves most affected by the antisense ODNs and the controls was obtained (Fig. 4.12B). In Figs. 4.11 and 4.12 the variability induced by the antisense ODN treatments was used to analyze the relationship between fluorescence intensity (F_0, F_M) and chl content (chl *a*, chl *b* and chl *a*/*b* ratio).

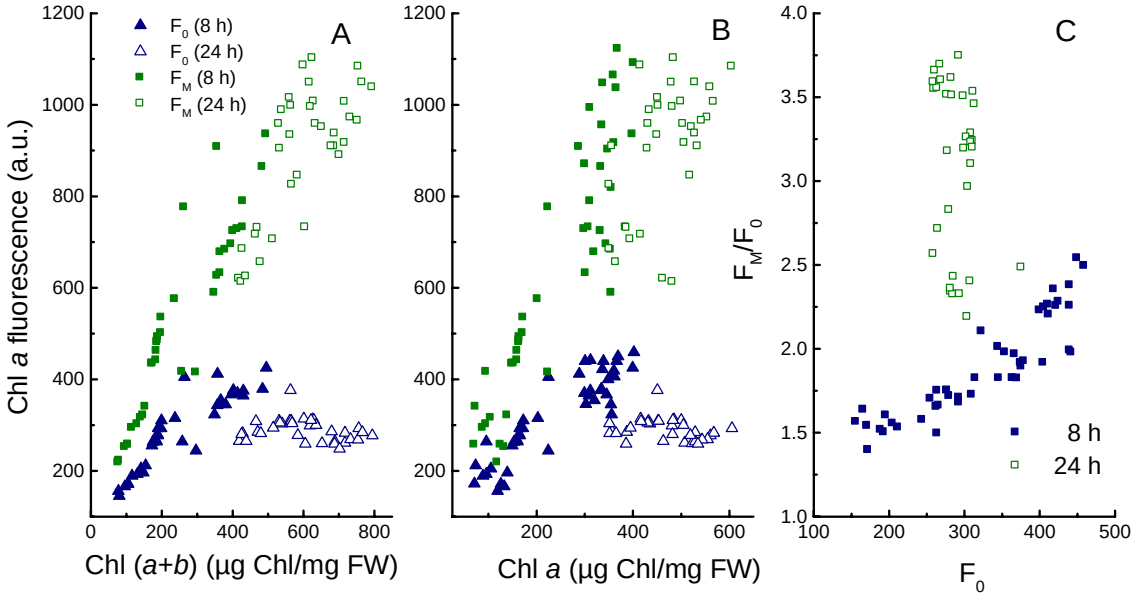


Figure 4.11. *Relationship between the chl content and chl a/b ratio and the chl* a *fluorescence intensity (F_0 and F_M) for greening wheat leaves treated with ten different antisense ODNs designed to interfere with the translation of cab mRNA, measured after 8 and 24 h of greening: relationship between chl (a+b) content and the F_M and F_0 values (panel A); relationship between chl* a *content and the F_M and F_0 values (panel B); relationship between the parameters F_0 and F_M/F_0 (panel C). The data were derived from three independent experiments.*

Fig. 4.11A demonstrates that after 8 h of greening an increase of the chl (*a*+*b*) content correlated with an increase of the F_0 and F_M intensities. After 24 h of greening the correlation between the F_0 value and the chl (*a*+*b*) content was lost (Figs. 4.11A and B). The dependence of the F_M on the chl content after 24 h of greening is more easily interpreted on the basis of Fig. 4.11B, where the F_M values are shown as a function of the chl *a* content. For both the 8 h

and 24 h data an approximately linear relationship is observed between the F_M and chl *a* content. However, after 24 h of greening a given F_M value correlates with a higher chl *a* content than after 8 h of greening, i.e. the points have shifted towards the right side of the panel. To illustrate the dependence of the F_0 and F_M values on the greening time (8 or 24 h), the parameter F_M/F_0 is given as a function of the F_0 value in Fig. 4.11C. After 8 h of greening there was an approximately linear relationship between the F_0 values and the F_M/F_0 ratio (Fig. 4.11C). This relationship was completely lost after 24 h of greening where changes in the F_M value were no longer accompanied by changes in the F_0 value.

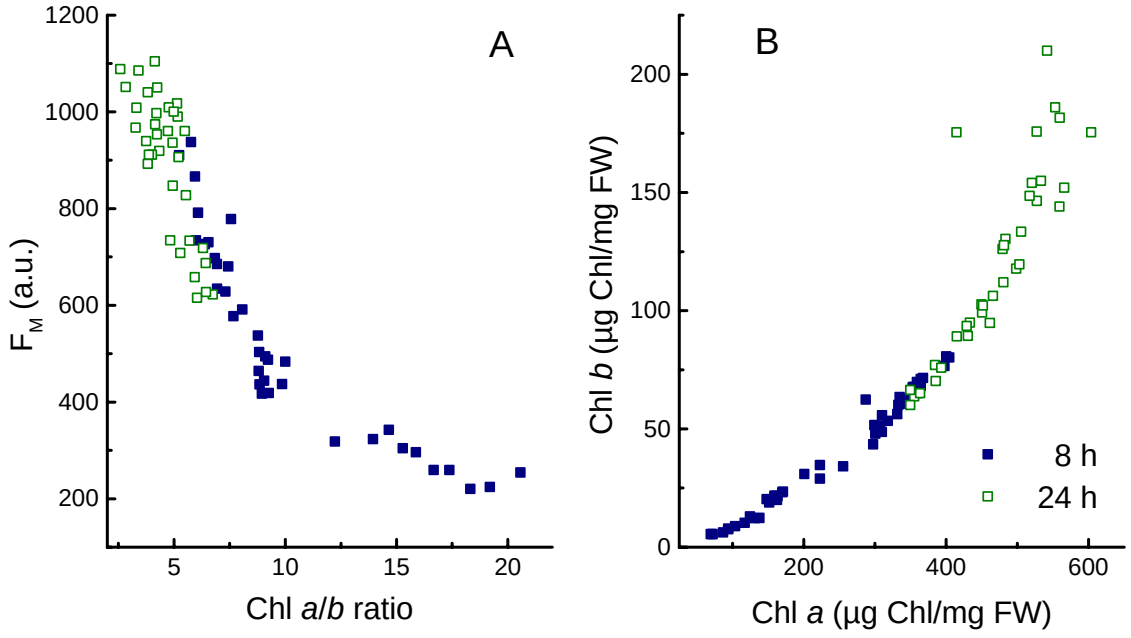


Figure 4.12. *Relationship between the F_M value and the chl a/b ratio (panel A) and the relationship between the chl b and chl a content (panel B) of antisense ODN treated wheat leaves after 8 and 24 h of greening.*

Fig. 4.12A demonstrates that, although the relationship between the F_M value and the chl *a*/*b* ratio as a whole may be best described by a hyperbole, the relationship can be described also quite well by a linear regression for chl *a*/*b* ratios up to 10. It may be noted that the naturally observed range of chl *a/b* ratios is quite limited. For example for pea plants grown at different light intensities values ranging from 2.1 (plants grown at 10 µmol photons m^{-2} s^{-1}) to 3.2 (plants grown at 840 µmol photons m^{-2} s^{-1}) were observed (Leong et al., 1984). The data in Fig. 4.12A suggest that the antenna size is an important determinant for the F_M

value. The Lhcb1-3 proteins contain 8 chl *a* and 6 chl *b* molecules (Liu et al., 2004; Standfuss et al., 2005). On the basis of the LHCII trimers a slope of 0.75 would be expected if chl *b* is presented as a function of chl *a*. However, this is an upper limit, and the slope for the average of all Lhca and Lhcb complexes together will be probably be somewhat lower although 0.5 is a likely lower limit since, as noted above, a chl *a*/*b* ratio of 2.1 was observed for pea leaves grown under low light conditions (Leong et al., 1984). Fig. 4.12B shows that initially the chl *a* content increases much faster than 1.33 to 2 x chl *b* indicating that after 8 h of greening the relationship is still dominated by the synthesis of new PSI and PSII complexes. Only after 24 h of greening for the least inhibited samples the slope of the data in Fig. 4.12B is becoming steeper suggesting that in these leaves the enlargement of the antenna started to dominate the increase of the chl content.

5. DISCUSSION

Key to identifying the functions of genes and for target validation is the ability to perturb the function of a particular gene in a given biological system. In plant science, several techniques have been applied for assigning a function to genes and proteins that include the creation of mutants, and the knockout or knockdown of certain genes, e.g. by antisense cloning. Expression libraries, small molecule inhibitors, and peptide or antibody inhibitors are also common techniques for assigning functions to genes.

Here, we have demonstrated that a sequence specific transient gene inhibition induced by antisense ODNs in tobacco and wheat leaves can be used to study gene function. Three experimental approaches were used: infiltration of ODNs into undetached leaves of tobacco plants, the passive uptake of ODNs by detached etiolated wheat leaves and vacuum infiltration of detached Arabidopsis leaves. Three model genes were chosen: *pds*, the *cab* genes and the *psbA* gene.

In undetached tobacco leaves a clear effect of the *pds* ODNs could be observed (Fig. 4.2), though it was not as pronounced as during greening of etiolated wheat leaves. In tobacco leaves carotenoids were already present at the start of the treatment and, therefore, a smaller effect was expected than in the case of de-etiolation. It should be noted that the uptake of the ODNs was not continuous when they were introduced by infiltration into the leaves. Since antisense ODNs can inhibit only the *de novo* biosynthesis a larger effect is to be expected in leaves in which the pigment content is still increasing, preferably from a low level.

In the case of wheat leaves, the inhibitory effect of *pds* ODNs on the carotenoid content after 8 h of de-etiolation was striking (Fig. 4.3C). After 24 h of greening the effects were less pronounced but still noticeable (Fig. 4.4C), similar to the effects in juvenile tobacco leaves (Fig. 4.2C). The fact that the effects of antisense ODNs were less pronounced after 24 h of greening compared to 8 h greening is a consequence of the process studied. During greening the light harvesting complexes of PSII and PSI and their associated chlorophylls and carotenoids accumulate. The ODNs targeting the *pds* and *cab* genes slow this accumulation process down, but do not prevent it completely. Therefore, the difference between the control and the ODN treated leaves will gradually disappear. Indeed, in an unreported experiment (E. Dinç, unpublished observation), in which the greening process was followed for 72 h, the difference disappeared completely.

Although the antisense ODNs applied to inhibit *cab* protein synthesis had only a natural structure and carried no phosphorothioate modification, their inhibitory effect was

strong (Figs. 4.7, and 4.8). For example, in the case of the *cab*10 antisense ODN the protein level of Lhcb3 was decreased by about 88%. Our results also show a strong differential effect on the different members of the CAB protein family (Fig. 4.8). This illustrates that it is possible to design ODNs targeting a gene family, which is a clear advantage over creating mutants. For wheat, presently, only one *cab* sequence is available in the GenBank database and it was, therefore, not possible to design antisense ODNs specific for a particular *cab* gene. With information on the whole wheat genome it would be possible to align the *cab* genes, which can then be used as a starting point for the design of ODNs that target one gene or several genes that show a high sequence homology. Nevertheless, our data show that already on the basis of a single gene sequence, ODNs could be designed that suppress the expression of several light harvesting proteins during de-etiolation of detached leaves.

The third target gene was *psbA*, which is a chloroplast encoded gene. The inhibition of *psbA* by ODNs was as powerful as the inhibition of the *cab* genes, suggesting that ODNs are as effective in chloroplasts as in the cytoplasm/nucleus. This experiment also shows that ODNs work as well in *Arabidopsis thaliana* as in wheat or tobacco.

5.1 ODN application and delivery

In this thesis it is shown that ODNs dissolved in distilled water are efficiently taken up by and transported in plant leaves (Fig. 4.1). When testing the effect of different sugars we found no evidence for enhanced uptake. The ODNs in the sucrose solutions were not taken up well and led to stress of the leaves. The leaves showed wilting and slight discoloration (E. Dinç, unpublished observation). A possible explanation for this is that the CY-45 spring cultivar of wheat has a low tolerance to osmotic stress (Dr. László Erdei, personal communication). This result contrasts with the observation of Sun et al. (2005, 2007) that the uptake by barley leaves of the SUSIBA2 transcriptional factor antisense ODNs was promoted by high concentrations of sucrose (200 mM).

The application method was adapted to the type of plant used. Tobacco leaves are too large for an efficient passive uptake of ODNs from a solution. In the literature it has been shown that fluorescent fusion proteins (Sparkes et al., 2006) and fluorescent sensors of reactive oxygen species (Hideg et al., 2002) can be injected into the leaf. We show here that this method also works for ODNs (Fig. 4.1 panels G-L). Arabidopsis leaves are too small for passive transport or injection, thus we used vacuum infiltration to treat detached leaves with ODNs.

A special feature of the application of ODNs is the timing. The expression of both the *pds* and the *cab* genes is under circadian control (Simkin et al., 2003; Staiger, 2002). The circadian rhythm does not play a role yet in etiolated wheat leaves. However, for the experiments with juvenile tobacco leaves, the same daily schedule has been followed to avoid effects of the circadian rhythm.

5.2 Phosphorothioate modification of antisense ODNs

The most frequently used way to enhance the efficiency of the inhibition of gene expression by antisense ODNs is to increase the stability of ODNs by the introduction of different chemical modifications. Phosphorothioate modification (Eckstein, 1985; Campbell et al., 1990; Agrawal et al., 1991; Ghosh et al., 1993; Uhlmann et al., 2000) was used in several studies to protect ODNs against exo- and endonucleases, of which the most active enzymes are the 3'-exonucleases (Uhlmann and Peyman 1990; Hoke et al., 1991; Shaw et al., 1991). Capping of the 3' end, or both the 3' and 5' ends by PS linkages protects the ODN against exonuclease degradation (Shaw et al., 1991; Gillardon et al., 1995), but these PS end-capped ODNs are still subject to endonuclease degradation. The combination of the end-capping technique and protection at internal pyrimidine residues, which are the major sites of endonuclease attack, has proven to be optimal (Uhlmann et al., 2000; Samani et al., 2001).

Phosphorothioate modified antisense ODNs are highly water soluble (Levin, 1999), and can efficiently recruit RNase H to cleave the target RNA (Zamaratski et al., 2001; Murray et al., 2012). All these properties make PS ODNs superior to the natural phosphodiester ODNs in antisense activity (Ravichandran et al., 2004).

In this thesis, a combination of end-capping and internal pyrimidine protection by PS linkages was used that indeed resulted in a significantly higher inhibition in the transcript level compared to the natural structure in the case of the *pds* gene (Figs. 4.2B, 4.3B, 4.4B). For tobacco leaves the influence of the PS modification could be detected in the case of the 8T-8N ODNs on the mRNA levels. A similar but smaller effect existed for the 9T-9N ODN pair (Fig. 4.2B). Studying the wheat *pds* antisense ODNs with PS modification the inhibition was about 15% higher compared to the natural structures (Fig. 4.3B). These data suggest that nucleases are active in plant cells and that PS modification can efficiently protect ODNs. Moutinho et al. (2001a, b) have already used ODNs having PS modifications in the three bases adjacent to each terminus in a pollen tube model system, but to our knowledge this is the first time that it is demonstrated that PS modification increases the efficacy of ODNs in

leaves of higher plants. New generations of modified ODN structures, bearing e.g. a 3`-O substitution or locked nucleic acids (LNA), provide higher hybridization efficiencies and cellular stability. It is, therefore, to be expected that there will be a further increase in the inhibitory efficiency of antisense ODNs in future studies.

5.3 Mechanism of the knockdown of gene expression by antisense ODNs

In the literature two major mechanisms for the inhibitory effect of ODNs are discussed. The first proven mechanism of antisense ODN inhibition was the so called hybridizational arrest or steric block mechanism in which the ODN sterically blocks the ribosome translocation (Crooke, 1999; Kurreck, 2003). However, the formation of an RNA antisense ODN duplex through complementary Watson Crick base pairing, leading to RNase H mediated cleavage of the target mRNA is the predominant mechanism for ODN action in mammalian cells (Crooke, 1999; Wu et al., 2004; Galarneau et al., 2005). RNase H is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA/DNA duplex. Oligonucleotide-assisted RNase H dependent reduction of targeted RNA expression in mammalian cells can be quite efficient, reaching 80-95% down regulation of protein and mRNA expression. This fact can be understood when we consider the catalysis-like nature of RNase H cleavage.

The importance of RNase H induced cleavage of mRNA has been demonstrated in several systems (e.g. Minshull and Hunt, 1986; Cazenave et al., 1993; Giles et al., 1995). The RNase H mechanism works in the case of ODNs that have a natural phosphodiester structure and PS modifications as well (Wilds and Damha, 2000; Damha et al., 2001; Murray et al., 2012).

In plants nothing is known of the inhibition mechanism of ODNs. For further studies we considered a special approach of making use of two cycles of photoinhibition to discriminate between the two mechanisms. Photoinhibition causes an increased turnover of D1 protein, one of the core proteins of the PSII reaction center. Via a repair cycle, active PSII reaction centers are regenerated and this recovery process can be followed if the plants are transferred to low light intensity (Aro et al. 1993). We would expect that in the case of hybridizational arrest the inhibition of the rate of repair would be higher on the first cycle than on the second, since newly synthesized target mRNA will overcome gradually the ODN effect in the cell. In the case of the catalytic RNase H mechanism it would be expected that the

inhibition of the rate of repair would be approximately the same for the first and the second recovery phase.

A more straightforward approach would be to work with modified ODNs those allow hybridizational arrest but are be insensitive to the RNase H mechanism like LNA-ODNs. LNA oligonucleotides containing a 5-8 nucleotide RNAse H gap in the middle with phosphodiester, phosphorothioate and 2'-O-methyl nucleotides were already applied in such studies (Fluiter et al., 2009), In these chimeric structures terminal LNA stretches provide the uniform high target affinity, while different types of modifications can be inserted into the central gap in order to study the RNAse H action. Such chimeras could be appropriate tools for judging this question and are subjects of our further studies.

5.4 Antisense ODNs as a tool to create biological variability

It was also demonstrated that by treating plants with specifically designed antisense ODNs it is possible to induce a considerable amount of biological variability, allowing the study of the relationships between parameters like fluorescence intensity and chl content (section 4.5). The peripheral antennae of PSII and PSI consist of approximately ten different members of the *cab* gene family. On the basis of QRT-PCR and Western blots, we showed for two of the antisense ODNs used here (Fig. 4.7B, 4.8) that these antisense ODNs targeted simultaneously the mRNA of several *Lhcb* and *Lhca* genes and most probably also the other antisense ODNs had multiple targets.

A potential complication of the Western blot analysis is the possibility that the treatments with the antisense ODNs give rise to a fraction of free antenna subunits not energetically connected to the reaction centers. However, with respect to the two antisense ODNs analysed by us, no unexpected decreases of the F_M/F_0 values were observed (see Fig. 4.11C). The study in section 4.5 is a global study in which the precise effects of individual ODNs were ignored. The fact that meaningful correlations between the different parameters that were studied were obtained implies that the approach worked. However, two effects described in the literature are worth mentioning here. In the first place, the knockout of Lhcb6 (CP24) and the antisense suppression of Lhcb4 (CP29) have been shown to have a strong effect on exciton migration through the antenna due to their importance for the connection between the LHCII trimers and the reaction center (van Oort et al., 2010). ODNs that would strongly affect these two members of the CAB family could have a much stronger effect on the fluorescence intensity than would have been expected on the basis of the effect on the chl

content. The other effect is the ability of the chloroplast to compensate the inhibition of some of the CAB members. Using antisense mutants it was shown that for PSII in the absence of Lhcb1 and Lhcb2, trimers consisting of either two Lhcb5 (CP26) and one Lhca3 (Ruban et al., 2003, 2006) or three Lhcb5 (Ruban et al., 2006) were formed. A loss of Lhcb3 was shown to be compensated for by increases in Lhcb1 and Lhcb2 (Damkjaer et al., 2009). The organization of the antenna of PSI on the other hand was shown to be more rigid with the only exchange described being the replacement of Lhca4 by Lhca5 (Wientjes et al., 2009). The fact that we obtained a range of antenna sizes indicates that these compensatory mechanisms did not overcome all the inhibitory effects of the antisense ODNs and this may be due to the fact that the antisense ODNs used targeted several members of the CAB family.

5.5 The relationship between antenna size and fluorescence intensity studied using antisense ODNs

In Figs. 4.11A and B it is shown that after 8 h and 24 h of greening the F_M value changes proportionally to the chl content but after 24 h the relationship between F_M and chl content had shifted to higher chl contents. This suggests that on the one hand there is an increase of the fluorescence intensity reflecting the maturation of the chloroplasts and the photosystems. On the other hand, the data also seem to suggest that as the chl content increases, no longer all chl molecules are monitored by the measurement (screening effect). This would be in agreement with the insensitivity of the F_M and F_0 values to changes in the chl content as observed for the mineral deficient sugar beets plants (Ceppi 2010). The Lhcb1-3 complexes have been shown to bind 8 chl *a* and 6 chl *b* each (Liu et al., 2004; Standfuss et al., 2005). The literature on the chl *a*/*b* ratio of the other Lhca and Lhcb protein complexes is contradictory. With respect to Lhca1-4 the crystal structure of PSI of pea plants suggests that all 4 protein complexes bind 14 chlorophylls just like Lhcb1-3 (Amunts et al., 2007). In vitro studies suggest that Lhca1-4 bind fewer chls (Croce et al., 2002, Storf et al., 2005), but this may also be due to a loss of more loosely bound chls during the preparation of these protein complexes. Despite these uncertainties it is possible to associate a ratio of 1.33 to 2 chl *a* per chl *b* with a predominant increase of the antenna size and much higher values with an important contribution of the synthesis of PSII and PSI reaction center to the greening process. Since the chl *a* content initially increased more than the 1.33 to 2 x chl *b* which would have been expected for a predominant increase of the antenna size (Fig. 4.12B), the data suggest that two phases can be distinguished during the greening process: 1. increase of

the number of PSI and PSII complexes and 2. enlargement of the antenna. This observation was also made by Srivastava et al. (1999) who used intermittent light grown plants instead of etiolated leaves as starting material for their greening experiment. If we interpret Fig. 4.12B in this way, then the data of Fig. 4.11C suggest that the F_0 intensity is sensitive to the increase of the number of PSII complexes (dominant process after 8 h of greening), but essentially insensitive to the antenna size (dominant process after 24 h of greening). In contrast, the increase of the antenna size was an important factor determining the F_M intensity.

The results from the greening experiment (Figs. 4.10-4.12) are in agreement with the results of the mineral deficiency experiment (Ceppi 2010). The relationship between F_M and the chl *a* content (Fig. 4.11B) shifted on going from 8 to 24 h of greening where more chl had accumulated in the leaves, suggesting that chloroplasts in the upper cell layers of the greening leaf started to hinder the passing of light through these cell layers, effectively screening the excitation light and the fluorescence. The same screening effect, but then in reverse, can explain as well the insensitivity of F_M and F_0 to the chl content during the stress induced chl loss (Ceppi 2010).

6. CONCLUSIONS AND PERSPECTIVES

In this thesis, it was shown that ODNs can be efficiently transported within leaves of vascular plants and that they reach the chloroplasts in monocotyledonous and dicotyledonous plant species. Antisense ODNs were used to knockdown the expression of the nuclear encoded *pds* and *cab* genes and the chloroplast encoded *psbA* gene. We have also shown that the efficiency of the inhibition of a target gene, the *pds* gene, can be increased by PS modification of the antisense ODNs. Although the effect of antisense ODNs is transient and it may be impossible to achieve full inhibition, which may be a disadvantage in many applications, these features can also be regarded essential in certain other important applications. For instance, in order to enhance the hydrogen evolution in green algae Melis et al. (2000) used sulphur deprivation to transiently inhibit the *de novo* synthesis of the PSII reaction center complex protein D1. An additional advantage is that ODNs can be designed to target multiple genes at the same time. I propose, on the basis of these results, that antisense ODN technology can after all be used as a high throughput screening method for studies on the role of genes and proteins with yet unknown function. It is also conceivable that interaction of genes can be studied with the help of antisense ODNs since ODNs can be applied in combination. As was shown here, photosynthesis is one of the research areas to which this method can be successfully applied. A special advantage of the study of photosynthetic genes is the availability of a range of sensitive, non-invasive techniques, such as time resolved chl *a* fluorescence and fluorescence lifetime measurements (Krumova et al., 2010), from which information can be derived on the operation of PSII and variations in the light harvesting antenna system, as well as different time resolved absorption measurements at various wavelengths providing information on e.g. the operation of the PSI reaction center, the Cyt b_6/f complex or on the ability of the thylakoid membranes to generate and maintain a transmembrane electric field (Junge, 1977; Kramer et al., 2004). These techniques can be applied on small leaf areas and for some of these techniques even on individual cyanobacterial or algal cells and chloroplast elements as small as μm sized voxels (volumetric pixels).

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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 (Crooke, 2004; Ravichandran et al., 2004; Gleave and Monia, 2005). 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 (Tsutsumi et al., 1992). Similarly, pollen tubes have also been suggested to take up antisense ODNs (Moutinho et al., 2001a,b). 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 (Kurreck 2003). This modification I used as well (Dinç et al., 2011). In the case of the phosphorothioate modification, one of the non-bridging oxygen atoms of the phophodiester 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 (Stein, 1995). 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 (Dinç et al., 2011)

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 (Dinç et al., 2011). 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 (Dinç et al., 2011). 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 (Dinç et al., 2011). 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 (Tyystjärvi 1996, Park 1995). 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 (Dinç et al., 2011)

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.

DOKTORI ÉRTEKEZÉS TÉZISEI

Antiszenz oligodezoxinukleotidok- A géncsendesítés új eszközei magasabbrendű növényekben

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BEVEZETÉS

A gének funkcionális vizsgálatának leggyakrabban használt módszerei közé tartozik a mutánsok létrehozása és a specifikus gátlószerek alkalmazása. Az antiszenz oligonukleotidok (ODN) egy lehetséges alternatív technikát képeznek a mutagenezis-módszerek mellett. Az antiszenz oligonukleotidok 15-20 nukleotidból álló, természetes szerkezetű vagy kémiai módosítást hordozó, egyes szálú nukleinsav szakaszok. Bázisszekveniájuk komplementer szerkezetű egy kiválasztott, hírvivő RNS megfelelő szakaszával. Ezáltal képesek kölcsönhatásba lépni a célzott molekulával, és azzal kettős szálat képezve specifikusan gátolni annak fehérjeként történő kifejeződését. Az antiszenz oligonukleotidok elméletileg képesek hatást kifejteni a nukleinsav metabolizmus bármely lépésében, különösen a transzkripció, az

RNS-szerkesztés és a transzláció során (Crooke, 2004; Ravichandran et al., 2004; Gleave & Monia, 2005), és ily módon hatékonyan gátolni a célzott gén kifejeződését.

Annak ellenére, hogy az antiszenz oligonukleotid technika hatásmechanizmusa révén általánosan alkalmazható az egész élővilágban, és emlős, illetve humán kutatásokban ma már jól bevett technikának számít, növényekben történő felhasználásával csak néhány közlemény foglalkozott mostanáig. Növényi sejtszuszpenziókban már húsz éve kimutatták az egyes szálú oligonukleotidok felvételét (Tsutsumi et al., 1992). Pollentömlők esetében ugyancsak igazolták az antiszenz oligonulkeotidok bejutását (Moutinho et al., 2001a,b).

Az antiszenz oligonukleotidok növényekben történő alkalmazásával létfontosságú gének funckiója is vizsgálható lenne, mivel a pleiotróp hatások kisebb mértékbennyilvánulhatnakmeg. Klasszikus mutánsokon létfontosságú gének funckiójának vizsgálata nem lehetséges, illetve a mutánsok fejlődése, növekedése során gyakran lépnek fel pleiotróp hatások. További előnyt jelent, hogy az antiszenz ODN-kezelt növények nem sorolhatók a genetikailag módosított organizmusok sorába, ezért különleges óvintézkedések nem szükségesek.

Jóllehet Michael R. Roberts már 2005-ben javasolta a nukleinsavak, illetve oligonukleotidok széles körű és nagy hatékonyságú alkalmazását a növényi génfunkciók felderítésére (Roberts 2005), mindez ideig nem történt számottevő előrelépés ezen a területen.

CÉLKITŰZÉSEK

A doktori munka alapvető célja az volt, hogy bizonyítsuk az antiszenz oligonukleotid technika általános alkalmazhatóságát kétszikű és egyszikű növények leveleiben, és ezáltal elősegítsük, hogy az általunk vizsgált módszer az általánosan elfogadott és alkalmazott géncsendesítési eljárások részévé váljék a növénybiológia területén is.

A fő cél elérése érdekében az alábbi feladatok megoldását terveztük:

- Módszerek keresése az oligonukleotidok hatékony bejuttatására különböző típusú levelekbe.
- Az oligonukleotidok által kifejtett gátlás mérése különböző módszerekkel.
- A csendesítő hatás növelése kémiai módosítást hordozó oligonukleotidok alkalmazása révén.

- Annak bizonyítása, hogy egyetlen antiszenz oligonukleotid képes több azonos géncsaládba tartozó gén kifejeződésének egyidejű gátlására is.
- Egy fotoszintetikus alapprobléma vizsgálatán keresztül igazoljuk, hogy az antiszenz oligonukleotid technika sikeresen alkalmazható valóságos biológiai kérdések megválaszolásában.

ANYAGOK ÉS MÓDSZEREK

- Az antiszenz oligonukleotidok kiválasztása, tervezése és kémiai szintézise. Kiválasztott gének: *fitoén deszaturáz* (pds), *klorofill a/b-kötő fehérje* (cab), és a D1 protein *psbA* génje
- A kísérleti növények leveleinek kezelése antiszenz oligonukleotidokkal. Kísérleti növények: dohány (*Nicotiana benthamiana*), lúdfű (*Arabidopsis thaliana*), búza (*Triticum aestivum*).
- Konfokális pásztázó lézer mikroszkópia.
- Gyors klorofill-a fluoreszcencia-mérés (OJIP).
- Valós idejű kvantitatív reverz transzkripciós PCR (QRT-PCR).
- Karotenoid és klorofill tartalom meghatározás.
- Western blot.

EREDMÉNYEK

A doktori munka során tanulmányoztuk az antiszenz oligonukleotidok kiválasztott növényekbe történő felvételét, optimalizáltuk génexpressziót gátló hatását, vizsgáltuk az alkalmazhatóság feltételeit és korlátait. Molekuláris biológiai és biofizikai technikák célszerű kombinációjával meghatároztuk a célzott kloroplasztisz génekre kifejtett csendesítő hatás mértékét. Modell génként a sejtmagban kódolt *fitoén deszaturáz* (pds), amely a karotenoid bioszintézis egyik kulcsenzime, az ugyancsak sejtmagi *klorofill a/b-kötő fehérje* (cab), és a kloroplasztiszban kódolt *psbA*, a D1 protein, a II fotokémiai rendszer központi fehérjéjének génje szolgált. A *pds* és a *psbA* gátlására foszforotioát módosítást hordozó oligonukleotidokat alkalmaztunk, hogy megnöveljük azok sejten belüli élettartamát. Az antiszenz ODN-ek fiatal dohánylevélbe történő infiltrálása 25-35%-os csökkenést váltott ki a megfelelő hírvivő RNS szintjében, kb.5% csökkenést lehetett kimérni a karotinoidok mennyiségében és a II. fotokémiai rendszer változó fluoreszcenciájában (F_V). Tőben lemetszett etiolált búzalevelek

esetén 8 óra zöldülés után a mRNS, a karotinoidok és az F_V szintjében 75, 25, illetve 20% csökkenést tapasztaltunk. Etioált búzalevelekben az ODN kezelés a kontrollhoz viszonyítva 66% *cab* mRNS csökkenést eredményezett, a protein szint 85%-ra esett, a klorofill *b* szint 59%-ra redukálódott, és a maximális klorofill fluoreszcencia szintje 41%-kal esett vissza. Arabidopsis leveleken a *psbA* mRNS és a D1 protein szintjét optimális esetben 81, illetve 72%-kal sikerült csökkenteni. Kísérleteink során az oligonukleotid molekulatervezés, a hatékony kezelés és a gyors, roncsolásmentes fizikai vizsgálatok kombinációjával igazoltuk, hogy az antiszenz technika hatékony eszköz lehet a fotoszintézis kutatásokban (Dinc et al. 2011).

Az antiszenz oligonukleotidok alkalmazhatóságát egy valós tudományos probléma megoldásában is felhasználtuk. A búza cab géncsalád gátlására 10 különböző oligonukleotidot terveztünk. Az ODN-ek a várakozásnak megfelelően erősen eltérő mértékű expresszió gátlást mutattak. Ennek megfelelően az így előállított mintákban a fotoszintetikus antenna méretek is erősen eltérőek voltak. A minták tanulmányozása lehetővé tette a fluoreszcencia mérések során kapott F_0 és F_M értékek és az antenna méret összefüggésének tisztázását. Ily módon igazoltuk, hogy a fotoszintetikus antenna méretének változása az F_M értéket erősen befolyásolja, míg a levél összes klorofill tartalma csak igen kevéssé (Dinc et al. 2012).

AZ EREDMÉNYEK MEGVITATÁSA

Az értekezés elsődleges célja az volt, hogy az antiszenz oligonukleotid technika alkalmazhatóságát bizonyítsuk, felhasználásának körülményeit tisztázzuk kétszikű és egyszikű növények körében. Három modell növény - dohány (*Nicotiana benthamiana*), lúdfű (*Arabidopsis thaliana*) és búza (*Triticum aestivum*) - esetén bemutattuk a lehetséges alkalmazást.

A kísérletek során tisztáztuk az alkalmazás feltételeit is. Minden célzott mRNS esetén 10-10 oligonukleotidot választottunk ki és szintetizáltunk meg. Az egyes oligonukleotidok gátló hatásában igen nagy változatosságot tapasztaltunk, ami arra utal, hogy célzott génszekvencia helyes megválasztása meghatározó jelentőséggel bír ebben a vonatkozásban. Az oligonukleotidok spontán módon eltérő hatékonyságát aknáztuk ki olyan mintasorozat előállítására, amelyekben a fénybegyűjtő komplex (Lhc) szintézise különböző mértékben gátlódott (Dinc et al. 2012).

A génexpresszió-gátlás maximalizálása a kutatás fontos elemét képezte. A természetes szerkezetű oligonukleotidok a sejtes környezetben erősen ki vannak téve az exo- illetve az

endonukleázok lebontó hatásának. A molekulák biológiai élettartamának növelésére általában olyan kémiai módosítások bevezetését javasolják, amelyek nem befolyásolják az oligomerek kettős-szál képző tulajdonságát. A legáltalánosabban használt módosítás a foszforotioát kötés (PS) kiépítése (Kurreck 2003), amely a foszfát-csoport egyik nem áthidaló oxigénjének kénatomra történő cseréjét jelenti. Az egyszerűség, és az emlős sejtekben mutatott közel tízszeres stabilitás-növekedés alapján munkánk során ezt a módosítást alkalmaztuk (Dinç et al., 2011). Különösen előnyös a foszforotioát oligonukleotidok azon tulajdonsága, hogy amellett, hogy megnövelik a molekula stabilitását, nem akadályozzák meg az RN-áz H működését. Ez az enzim jól felismeri és hasítja a mRNS és a foszforotioát oligonukleotidok által alkotott kettős szálat is. Az RN-áz H mechanizmus által kiváltott gátlás igen lényeges komponens az antiszenz hatás kialakításában (Stein, 1995). Méréseink azt mutatták, hogy a nukleáz hatás növényi levelekben is csökkenti az antiszenz gátlás intenzitását és a hatás időtartamát, és a PS módosítás bevitele hatékonyan megvédi a molekulát a lebontástól. Az antiszenz pds szekvenciák természetes és foszforotioát -módosított változatainak összehasonlítása igazolta, hogy a foszforotioát beépítése hatékonyan növeli az elérhető gátlás mértékét (Dinç et al., 2011).

Az oligonukleotidok hatékony bevitele ugyancsak igen fontos problémát jelentett. A három különböző faj levelére három specifikus módszert dolgoztunk ki. Mivel a dohánylevelek elég nagyok, ezért ezek egy-egy levélszegmensébe infiltrálással juttatuk be az oligonukleotidokat. Ezt az eljárást eredetileg vírusfertőzés bevitelére írták le. Az eljárás előnye, hogy a szomszédos, nem kezelt levélszegmens közvetlen kontrollként alkalmazható. A búza leveleket tőből levágtuk és oligonukleotid oldatba merítettük, így az oligonukleotidok passzív transzporttal jutottak el a sejtekhez. Az apró Arabidosis levelek esetében a vákuum infiltráció tűnt a legalkalmasabb eljárásnak. Az oligonukleotid felvétel hatékonyságát fluoreszceinnel jelölt oligonukleotid felhasználásával, konfokális pásztázó mikroszkópia útján ellenőriztük. Igazoltuk, hogy a az fluoreszcein-jelölt oligonukleotid intenzíven mozgott a szövetekben, bejutott a sejtekbe, megjelent a sejtmagban és a kloroplasztiszban egyaránt.

Az antiszenz technika lényeges eleme a célgén helyes megválasztása. Első modell génként a fitoén-deszaturázt (*pds*) választottuk. A pds gén egy, a karotinoidok bioszintézisében kulcsszerepet játszó enzimet kódol. Hiánya esetén a karotinoidok mennyiségének csökkenése, következésképp a fénybegyűjtő komplex összeszerelésének gátlása következik be, ami pedig klorofill fluoreszcencia mérésekkel jól követhető. A kísérletek során kiderült, hogy a pds modell génnek nem optimális, mert a gén és az enzim felépülési-lebomlási ciklusa, valamint a karotinoidok szintézise meglehetősen lassú. Az

antiszenz gátlás csak az újonnan szintetizálódó géntermékekre hat, a meglévő fehérjékre nincs hatással. Ezek következtében az enzim szintjében, illetve a termékek mennyiségében kimérhető gátló hatás csekély volt annak ellenére, hogy a mRNS szintjében tekintélyes csökkenést lehetett detektálni. A fenti példa rámutat arra, hogy optimális hatékonyság rövid életidejű vagy indukálható gének és fehérjék esetében érhető el. Ebben a tekintetben másik két vizsgált esetünk optimálisnak tekinthető. Az etiolált levelek zöldülése fényindukált folyamat, és a zöldülés során a korábban hiányzó *lch* géntermékek nagy sebességel szintetizálódnak, ezért az *lhc* géncsalád képezte második vizsgált esetünk tárgyát. A Western-blot vizsgálatok segítségével igazoltam, hogy egyetlen oligonukleotid képes egy géncsalád több tagjának szintézisét szimultán módon gátolni. A harmadik célzott gén a *psbA* volt. Míg a *pds* és az *lhc* sejtmagban kódolt gének, a psbA génje a kloroplasztiszban lokalizálódik. A psbA géntermék, a D1 protein igen rövid élettartammal rendelkezik. A D1 protein a D2-vel együtt képezi a II. fotokémiai rendszer (PSII) reakciócentrumát. Nagy intenzitású megvilágítás növeli a PSII és a D1 protein degradációját. A fotoinhibíciónak nevezett folyamat, vagyis a PSII degradáció sebessége a fényintenzitással arányos (Tyystjärvi 1996, Park 1995). Intenzív fénykezelés és antiszenz oligonukleotidok együttes alkalmazásával hatékony D1 protein szintézis gátlást tudtunk elérni. Ez az eredmény tekinthető egy kloroplasztiszban kódolt gén antiszenz oligonukleotidokkal történő gátlása első példájának.

Amíg a Dinc et al. 2011 közlemény az elmélet bizonyítását adta, az azt követő publikáció (Dinc et al, 2012) már valós biológiai probléma megoldásáról számol be az antiszenz oligonukleotidok alkalmazása révén. Tudományos előzményként Ceppi és munkatársainak az az eredménye szolgált, amely szerint hidropónikos kultúrában nevelt cukorrépa növényeken magnézium és kénmegvonás hatására a klorofill mennyiség jelentősen csökkent, amely nem járt a fotoszintetikus antenna méretének megváltozásával, nem okozott fluoreszcencia intenzitást változást sem az F_0, sem az F_M érték vonatkozásában. Az etiolált búzaleveleken végzett független kísérleteinkben a 10 cab-antiszenz oligonukleotid segítségével előállított sorozat mérése igazolta, hogy az F_M érték erősen függ a fotoszintetikus antenna méretétől. Ugyanakkor bizonyítást nyert, hogy az F_0 és az F_M érték nincs összefüggésben a minta klorofill tartalmával.

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ABSTRACTS AND POSTERS

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