Regulatory DNA binding peptides as novel tools for plant functional genomics

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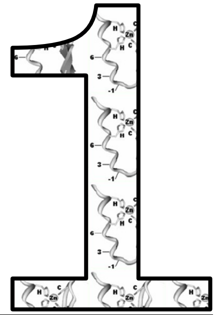
Chapter



General introduction

GENERAL INTRODUCTION

In multi-cellular organisms, every cell contains the same genomic DNA, meaning that every cell contains the same set of genes. Nevertheless, cells take on different specialized roles during their life span. To fulfil these roles, the amounts of transcripts and proteins that are generated from a particular gene in a given cell type per unit of time, commonly regarded as gene expression, is tightly regulated. For each of the many thousands of genes present within the complex genomes of multicellular eukaryotic organisms, means to control its expression in a spatiotemporal manner are required. This is a truly remarkable fact and, as such, gene regulation has been and still is a prime area of molecular biological research.

Over time more insight has been obtained regarding the mechanisms that confer regulation of gene expression (Blazek *et al.,* 2005; Szutorisz *et al.,* 2005). Transcription of the genetic information into RNA molecules forms the basis of gene expression. In the large majority of cases these RNA molecules (mRNAs) are translated into proteins, which can then be considered as the actual determinants for the biochemical or structural function of genes. Post-translational modification of proteins might further determine whether or not the protein will be in the right state to exert its function, thus whether or not one considers the gene to be really expressed. In such a chain of events, multiple regulatory steps can determine the final level of gene expression. Apart from the lifetime of the different types of molecules, the rate at which they are generated will be of utmost importance. Transcription of DNA into (m)RNA is is commonly regarded as the key determinant for the whole process. In higher eukaryotes, transcription of DNA into protein encoding mRNAs is brought about by RNA polymerase II, a multi subunit enzyme. For accurate transcription, RNA polymerase II needs to bind to the promoter region of the gene, a DNA sequence directly preceding the transcribed region and in many occasions encompassing a so-called TATA-box. While binding of RNA polymerase II will suffice for transcription initiation on 'purified 'naked' DNA, the situation in living cells is much more complicated as the DNA is embedded in chromatin, a complex of DNA, RNA and proteins. In particular the positively charged histones, which constitute the nucleosomes around which the DNA is wrapped (Figure 1), can be a barrier for assembly of the transcription machinery. The key principle that is now being regarded as governing transcription initiation is 'recruitment' of a large series of different proteins at or nearby the promoter region of a gene, whereby the exact order of the events might very well be of secondary

importance (Szutorisz *et al.*, 2005). A subset of proteins that seem to be present during the majority of transcription initiations have been designated general transcription factors, but apart from these a wealth of other proteins can be involved in the final recruitment of all the factors needed to initiate the transcription of a particular gene. These additional transcription factors can directly or indirectly lead to a change in chromatin which is more conducive for transcription or, on the contrary, lead to a state of the chromatin where transcription is further repressed. In the first case, an 'activator' of gene expression is said to have bound to an 'enhancer' element in the vicinity of the promoter or even much further away. When gene repression is at hand, a repressor has apparently bound to a 'silencer' element. The most straightforward examples of activation and repression result from remodelling of the strength of the DNA-nucleosome association by enzymatic modification of histone tails by addition or removal of acetyl groups, which form hallmarks for increased and decreased transcription respectively (Berger, 2002).

For RNA polymerase II, a highly complex assembly of proteins called the 'mediator' (Figure 1) seems to integrate and interpret all the different molecular signals in the vicinity of the promoter or even much further away in terms of the number of successful transcription initiation events (Blazek et al., 2005). Once that an RNA polymerase II molecule has departed for the process of gene transcription, remaining information in terms of transcription factors and chromatin modifications that are still present can repetitively facilitate the recruitment of other RNA polymerase II molecules to the promoter and thus enhance transcription initiations from this very site. Whatever the order of events that leads to transcription initiation or repression thereof, it is evident that information regarding the transcription rates of particular genes or chromosomal loci must result from DNA sequence-specific binding of those proteins that are commonly regarded as activators or repressors (see Figure 1). During life's history, a large variety of different types of DNA-binding domains have evolved. Most of these domains are modular in structure, meaning that they can be combined with structural protein domains or enzymatic activities. Extant DNA binding domains can be classified in a large number of groups, each group being characterized by a particular protein structure that is aimed to establish the contact with the DNA. The types of protein-DNA contacts cover a spectrum ranging from non-specific (as for histones, mentioned above) to highly specific for particular stretches of DNA sequences (as for the zinc finger containing domains mentioned below).

The fundamental insights in the naturally occurring mechanisms underlying regulation of gene expression as mentioned above have begun to

lead to experimental methods to control the expression of endogenous genes of an organism at will. Such methods are required for gaining a better understanding of gene function, but also for a broad spectrum of biotechnological applications. A detailed discussion of all methods is far beyond the aim of this chapter. Those methods that have in particular been proven to be valuable for plant research include RNAi-based approaches for down-regulation of gene expression (for review see Meins *et al.*, 2005) and the generation of artificial transcription factors which can be used for both up- and downregulation of gene expression (for review see Ansari and Mapp, 2002). Especially the development of artificial transcription factors has recently accelerated, mainly due to the progress made in the field of zinc finger technology. This chapter and this thesis focuses on the development of artificial transcription factors, in particular zinc finger-based artificial transcription factors, and their implications for plant research.

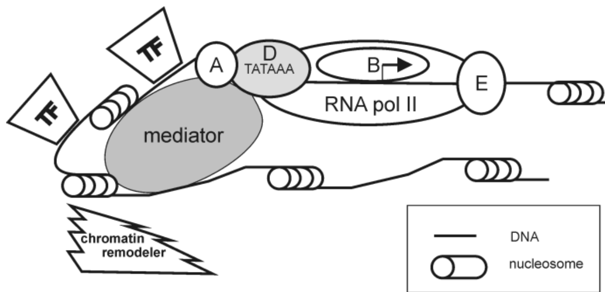


Figure 1: Simplified model of factors involved in transcription initiation. General transcription factors TFII A, B, D and E (indicated by their respective letter), in concert with the action of the mediator complex, recruit RNA polymerase II to the promoter. Additional transcription factors (TF) can act as activators or repressors of the assembly of the complete transcriptional machinery at the promoter. The arrow indicates the site and direction of transcription. See text for further details.

Artificial transcription factors

Transcription factors contain a modular structure with distinct domains for DNA binding and regulation of transcription. It has been well established that DNA binding domains and regulatory domains can be regarded as portable elements that are in principle interchangeable, thus enabling the construction of artificial transcription factors. The first artificial transcription factors that were used in plants were based on the design of the GAL4 transcriptional activator (Ma *et al.*, 1988) that was known to mediate galactose-inducible gene expression in *Saccharomyces cerevisiae* (budding yeast) after switching of carbon source (Stone and Sadowski, 1993). Fusion proteins of the GAL4 DNA binding domain

with a transcription activation domain of the C1 protein of maize (normally involved in anthocyanin biosynthesis) or the herpes simplex virus-derived activation domain VP16 were found to be able to activate expression of genes via synthetic promoters carrying the appropriate binding site (Goff *et al.*, 1991; Wilde *et al.*, 1994; Aoyama and Chua, 1997; Guyer *et al.*, 1998). The well studied GAL4 DNA binding domain has now become the basis for a number of technologies, like inducible gene expression systems and yeast two-hybrid screenings (Young, 1998; Padidam, 2003). Besides using artificial transcription factors for activation of gene expression, artificial transcription factors can also be used to repress the transcriptional activity of target genes when the effector domain fused with the DNA binding domain exerts a negative effect upon transcription. For instance, in plants a protein motif called EAR (ERF-associated amphiphilic repression motif) has been utilized as a dominant repressor domain when fused with an existing transcription factor that normally activates transcription (Ohta *et al.*, 2001; Hiratsu *et al.*, 2002).

Studies concerning artificial transcription factors based on natural transcription factors have thus clearly demonstrated that it is possible to change the expression profile of a gene of interest in a specific manner. However, not every gene of interest can be regulated using such types of artificial transcription factors since their use typically depends on the presence of a particular DNA recognition sequence nearby the transcriptional start site of a gene. Thus, to target any gene of interest it will be necessary to create artificial transcription factors containing a DNA binding domain based upon the genomic sequence that needs to be targeted, combined with an regulatory domain of choice.

Polydactyl zinc fingers

As outlined above, for customized specificities of artificial transcription factors, DNA binding domains are needed that can be designed for a specific target sequence. Cys_2His_2 zinc finger domains, a particular class of DNA binding domains containing two pairs of conserved cysteine and histidine residues held together by a zinc ion, occur in a wide variety of natural transcription factors and have been found to be very suitable for this purpose (Beerli and Barbas, 2002). Each Cys_2His_2 zinc finger domain consists of 30 amino acid residues that form a short two-stranded anti-parallel β-sheet followed by an α-helix, with the zinc ion required to stabilize the conformation. The X-ray crystal structure of a zinc finger protein bound to DNA was first elucidated for a protein called Zif268 which contains three consecutive zinc finger domains (Elrod-Erickson *et al.*, 1996; Pavletich and Pabo, 1991). From the structure it was clear that the α-helix is

located within the major groove of the DNA double helix and predominantly interacts with three contiguous DNA bases via the amino acid residues at positions -1, 3 and 6 relative to the start of the α-helix (Figure 2). The amino acid residue at position 2 exhibits cross strand interaction with a 4th base at the other strand, which can result in target site overlap with a second zinc finger domain (Pavletich and Pabo, 1991; Choo and Klug, 1994; Suzuki *et al.*, 1994). When more zinc finger domains are fused, they constitute a polydactyl zinc finger domain which will recognize an extended DNA target sequence.

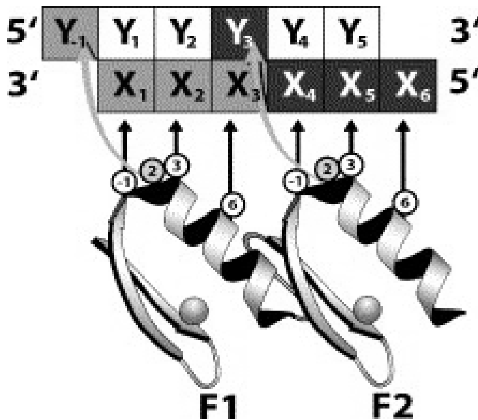


Figure 2: Model of zinc finger DNA recognition. Amino acid residues at positions -1, 3 and 6 with respect to the start of the α-helix make base contacts with contiguous bases (X1-3 for F1 and X4-6 for F2). The amino acid residue at position 2 interacts with a 4th base on the opposite strand (Y-1 for F1 and Y3 for F2) (reprinted with permission from Papworth *et al.*, 2006).

Much effort has been put in systematic characterization of the DNA binding properties of zinc finger domains, most notably by the group of Barbas at the Scripps Research Institute (La Jolla, USA). This systematic characterization started off with selection of zinc finger domains that bind specifically to 3-bp DNA sequences that start with a guanine nucleotide residue (G) (Segal *et al.*, 1999). This study involved construction of phage display expression libraries encoding three fingered zinc finger proteins that were variable in the -1 to 6 region of the α-helix sequence of the second finger, thus variable in binding to the middle three base pairs within a 9-bp target sequence. After multiple rounds of selection, three fingered zinc finger proteins were isolated in which the second zinc finger moiety interacted specifically with a particular member of the series of 16 triplets starting with G. The selected zinc fingers were further analysed for specificity and affinity (Segal *et al.*, 1999). After elucidation of this "GNN-code",

recognition helices for triplets starting with an A or a C followed based on the developed methods (Dreier *et al.*, 2001; Dreier *et al.*, 2005). These studies have resulted in a sort of lexicon for zinc finger-mediated DNA recognition which states the crucial α-helix sequences that are needed for specific DNA binding (Figure 3). It should be mentioned that the methodological approach of zinc finger selection followed by the Barbas laboratory has been a reason for some concern. Since the outer two zinc fingers of a three fingered zinc finger domain were fixed, DNA-binding specificity for the second zinc finger was optimized within a given context. Sera and Uranga have therefore proposed a slightly different code for zinc finger recognition (Sera and Uranga, 2002). However, their recognition code is predominantly based upon theoretical grounds.

G		third base			
		G	A	T	C
second base	G	RSDKLVR	QRAHLER	TSGHLVR	DPGHLVR
	A	RSDNLVR	QSSNLVR	TSGNLVR	DPGNLVR
	T	RSDELVR	QSSSLVR	TSGSLVR	DPGALVR
	C	RSDDLVR	QSGDLRR	TSGELVR	DCRDLAR

Figure 3: Zinc finger lexicon for triplets starting with G. Each zinc finger domain binds 3-bp DNA sequences through specific amino acid residues present in its α-helix. The table lists the most optimal amino acid sequences (-1 to 6 of the α-helix) for specific triplets (according to Segal *et al.*, 1999).

Irrespective of the precise selection criteria used to formulate zinc finger lexicons, the possibility to exploit their modular nature allows the construction of more complex polydactyl zinc fingers which recognize an extended stretch of nucleotides. At the current date, successful application of polydactyl zinc finger domains for specific DNA recognition in living cells has by now been described in a growing series of papers (for reviews Beerli and Barbas, 2002; Blancafort *et al.*, 2003; Papworth *et al.*, 2006), also in those forming this Thesis.

Polydactyl zinc finger-containing artificial transcription factors

The first successful experiments with zinc finger artificial transcription factors were reported in mammalian cell cultures where it was shown that the expression of specific genes could indeed be regulated at will (reviewed in Segal

and Barbas, 2001; Beerli and Barbas, 2002). Like with many emerging techniques, possible therapeutic applications stimulated a wave of research activities within the medical field (Gommans *et al.*, 2005; Klug, 2005). During the preparation of this thesis zinc finger technology also entered the plant research arena, initially by work initiated at the Scripps Research Institute together with the Skaggs Institute. By means of transient expression assays in tobacco protoplasts it was demonstrated that a previously thoroughly characterized six-fingered polydactyl zinc finger protein fused with strong transcriptional activators, like VP16 and its tetramer VP64, was able to up-regulate expression of a reporter gene containing six binding sites for the polydactyl zinc finger domain (Ordiz *et al.*, 2002). Also in stably transformed plants the generated zinc finger-based transcription activators stimulated expression of the same reporter gene (Ordiz *et al.*, 2002). Since then also others have utilized VP16 and VP64 domains in combination with polydactyl zinc fingers to activate specific (endogenous) target genes in plants, either via transient or via stable expression (Guan *et al.*, 2002; Sanchez *et al.*, 2002; Stege *et al.*, 2002). More recently also the activation domain of the maize transcription factor C1 has been utilized in combination with polydactyl zinc fingers for targeted gene activation in plants (Van Eenennaam *et al.*, 2004; Holmes-Davis *et al.*, 2005).

For mammalian cells, published attempts aimed at repression of transcription activity by means of zinc finger artificial transcription factors are about as common as those regarding transcriptional activation, probably due to the availability of well characterized portable repression domains. Repression domains from plant origin have not yet been exploited in combination with polydactyl zinc fingers in plants, but several of the mammalian repression domains were shown to be active in plants, such as the SID (Guan *et al.*, 2002; Stege *et al.*, 2002) and the KRAB domain (Sanchez *et al.*, 2006). There thus seems to be no reason to postulate that application of zinc finger technology in plants would be less flexible than in mammalian systems. The successful use of polydactyl zinc finger domains fused with histone methyl transferases, histone de-acetylases or other histone modifying proteins that are implicated in gene repression (Snowden *et al.*, 2002; Fuchs *et al.*, 2006) can thus be foreseen in plants as well.

Other polydactyl zinc finger applications

In addition to the use of polydactyl zinc finger domains for regulating the expression of specific genes, zinc finger-based technology has by now been applied for several other purposes. For instance, polydactyl zinc fingers were

shown to be active as an antiviral agent by inhibiting viral replication through high-affinity binding to the binding site of the viral replicase (Sera, 2005; Mino *et al.*, 2006). Another application resides in the use of polydactyl zinc fingers to target the endonuclease domain of the prokaryotic enzyme *FokI* to specific sites within a complex genome, thereby creating DNA double stranded breaks at specific genomic locations (reviewed in Durai *et al.*, 2005). This possibility is very exciting and might prove to be an invaluable tool for gene therapy or for gene targeting purposes.

Outline of this thesis:

At the start of this project, application of zinc finger technology in plants had not yet been reported. However, the already published GNN (Segal *et al.*, 1999) and ANN (Dreier *et al.*, 2001) recognition codes provided ample opportunities to explore several interesting possibilities of zinc finger technology in plants. Apart from some technical issues, like choosing suitable expression systems as well as effector domains to be fused with polydactyl zinc finger domains, the concept of zinc finger-mediated DNA recognition should not depend on a particular host organism. Moreover, the relative ease of obtaining transgenic organisms should make application of the technology in an easily transformable model plant like *Arabidopsis thaliana* extra exciting. This thesis describes various experiments aimed to further develop zinc finger technology for plant molecular biology and to use it as a tool to find novel mutants with enhanced rates of homologous recombination. Although our focus was mainly on the development of zinc finger technology for plant research and to explore potential applications, several other model organisms like yeast and mouse were used to address key questions and hypotheses.

Chapter 2 describes experiments that were required to unequivocally determine which type of polydactyl zinc finger design would be the most robust for generating artificial transcription factors that are effective on chromatin-embedded DNA sequences. Despite the impressive amount of data generated within the rapidly developing field of zinc finger technology such information was lacking or ambiguous. In literature, controversy existed about the precise molecular structure of the linker amino acid sequence between the different zinc finger moieties that form a polydactyl zinc finger domain (Kim and Pabo, 1998; Moore *et al.*, 2001). To sort out this issue we embarked upon a systematic approach with two sets of polydactyl zinc fingers to be tested both *in vitro* (binding assays) and *in vivo* (regulation of a chromosomal reporter locus) in yeast. Polydactyl zinc fingers were constructed in which the spacing between

individual zinc finger moieties was varied using flexible linkers. Different types of polydactyl zinc fingers were fused with an activation domain to obtain artificial transcription factors that were tested for their *in vivo* performance in yeast strains carrying a chromosomal reporter gene with binding sites for the polydactyl zinc finger in the promoter. Polydactyl zinc fingers without effector domain were additionally evaluated for *in vitro* binding affinity. It was found that polydactyl zinc fingers that show strong affinity to their binding site *in vitro* do not necessarily perform well when tested for regulation of an integrated reporter gene. In contrast to what has been suggested in literature, our experiments clearly demonstrated that six-fingered zinc fingers constructed exclusively with canonical amino acid linkers with the sequence TGEKP were superior over other designs with longer linkers.

In Chapter 3 we have tested the hypothesis whether polydactyl zinc finger proteins can be used as a labelling device in living cells. In principle this would allow live cell imaging of repetitive sequences in living cells, which can have major implications for research related to chromosome dynamics or the regulation of chromatin status. To test our hypothesis we have constructed several GFP-tagged polydactyl zinc fingers aimed to bind short 9-bp target sequences that occur in a repetitive fashion in the genome. By targeting the centromere repeats in Arabidopsis we were able to visualize the corresponding region in living Arabidopsis roots. We show that this live cell imaging method can also be applied in mouse cell cultures via expression of a GFP-tagged polydactyl zinc finger which is designed for binding a specific 9-bp sequence within the mouse major satellite repeat. Furthermore we describe a relatively simple quantification method for GFP signals, by using commercially available fluorescent beads which were calibrated against single GFP molecules.

Chapter 4 focuses on the question whether zinc finger artificial transcription factors can be used for "mutagenesis" of Arabidopsis plants. We hypothesised that low complexity zinc finger artificial transcription factors with only three zinc finger domains have in principle the ability to regulate a large number of genes in the Arabidopsis genome. Altered gene expression of one or several genes may lead to a certain phenotype of interest, after which the responsible library member can be identified and used again to reconfirm its action. The strength of this approach resides in the large number of genes that are potentially regulated by one specific artificial transcription factor. In this way almost saturating coverage of the genome can be reached. While our experiments were in progress, this 'phenotype-fishing approach' was reported for mammalian cells and prokaryotes (Blancafort *et al.*, 2003; Park *et al.*, 2003; Lee *et al.*, 2004; Park *et al.*, 2005a; Park *et al.*, 2005b; Kwon *et al.*, 2006), but in this

chapter we investigated whether it is also applicable at the level of a multi-cellular eukaryote like Arabidopsis. We searched for mutants with altered frequency of homologous recombination, which are therefore of fundamental scientific interest in relation to the mechanisms of DNA repair but also in relation to much desired possibilities for gene targeting. For our experiments we constructed three-fingered zinc finger artificial transcription factor libraries which were transformed into an homologous recombination indicator Arabidopsis line. After selecting mutants with increased homologous recombination frequency, we were able to link such a phenotype to a specific zinc finger artificial transcription factor.

More in depth analysis of mutants expressing this specific zinc finger artificial transcription factor, which was named VP16-HRU, is described in Chapter 5. Phenotypic characterization showed that VP16-HRU expressing plants are somewhat delayed in development, but develop in a normal fashion. Shortly after germination, recombination events in mutant seedlings are most frequently detected in cotyledons while they are almost absent in roots. Transcriptome profiling revealed that numerous genes turned out to be regulated through VP16-HRU. Among differentially expressed genes, also genes known to be involved in DNA repair like *Rad51* and *PARP2* were present. Several genes that were found to be strongly regulated were selected for an assay to investigate whether their overexpression could be correlated with an increase in somatic homologous recombination. When 10-day-old primary transformants were evaluated, ectopic expression of two out of nine HR-inducing candidate genes, resulted in a 4-fold increase in somatic homologous recombination. In 20-day-old primary transformants it was found that the presence of several other gene-constructs positively influenced the homologous recombination frequency. We have thus shown that zinc finger-mediated mutagenesis is a powerful tool for mutagenesis and that subsequent analysis of mutants can lead to the discovery of novel genes involved in the regulation of somatic homologous recombination.

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