Regulatory DNA binding peptides as novel tools for plant functional genomics

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Summary

Just as naturally occurring transcription factors, artificial transcription factors (ATFs) are operationally defined as proteins that influence the number of transcription events per unit of time for a particular gene. Typically, ATFs contain a DNA-binding domain (DBD) for specific interaction with a DNA sequence in the vicinity of the transcriptional start site of a gene and a regulatory or effector domain (ED) for exerting its effect upon transcription of that gene. When an ATF can be constructed, it is theoretically possible to use this protein for gene regulation on demand. The development of ATFs has accelerated greatly with the progress made in the field of Cys_2His_2 types zinc fingers (ZFs). Such ZFs are small sequence-specific DNA-binding proteins that can be linked together in a so-called polydactyl zinc fingers (PZF). In a PZF, each ZF domain binds a consecutive 3-bp target sequence, thus a PZF consisting of 3ZFs binds specifically to a 9-bp sequence. It is this modularity that makes ZFs extremely suitable for constructing sequence-specific DBDs that can be used for assembling ATFs. It is therefore not surprising that much effort has been made to characterize the DNA-binding characteristics of ZF domains with the aim to determine a lexicon for ZF-mediated DNA recognition. A large part of this lexicon has become available and can indeed be used as a starting point to create custom DNA-binding domains for various purposes.

The main goal of the research described in this thesis was to explore the potential of PZF technology for plant research. Besides the utility of PZFs as DBDs in ATFs, experiments were performed to explore possibilities for additional applications of PZFs, such as PZF-based cytogenetics and PZF-based mutagenesis. A first key question was to determine a proper design for PZFs based upon a part of the zinc finger lexicon that had been published (Chapter 2). A vector system was developed that allowed efficient assembly of ZF modules and a variety of PZFs was constructed. Next to proteins that possessed short canonical linkers between all constituting ZFs, PZFs were generated with longer linkers between two three-finger units and between three two-finger units. Fusions of these PZFs with the VP16 transcriptional activation domain were tested for their gene activating potential in yeast on a repressed genomic locus containing contiguous or noncontiguous zinc finger binding sites. Based upon these experiments it was concluded that short canonical linkers provided a sound base for PZF design for regulation of genomic loci at will. In general, as was also evident from the results described in Chapter 2, PZF domains with six ZF moieties (6ZF) possess higher affinity for their cognate DNA binding sequence than PZF domains with three ZF moieties (3ZF). Possibly for this reason, transcriptional activation of a repressed genomic locus in yeast could not be achieved 3ZF-ATFs fused with VP16. In literature, however, successful

application of 3ZF-ATFs for regulation of genomic loci had been reported for several occasions in mammalian cells, but not on an actively repressed locus. It can thus be concluded that high affinity ZF-ATFs are needed to overcome active repression of an endogenous locus.

In Chapter 3 experiments were designed to investigate whether 3ZFs, routinely constructed according to the protocol developed in Chapter 2, could bind to their chromosomal target sites in living plant cells and thereby also serve as a platform for cytogenetic applications. While several techniques were already available to study chromosomes or chromosomal domains in nuclei of chemically fixed cells, methods to directly detect DNA sequences in living cells were extremely limited and of very little practical use as they were based upon interactions between a previously introduced DNA sequence and either the well-known Tet- or Lac-repressor domain for *E. coli* bacteria. It was thus examined whether GFP-tagged PZFs aimed to recognize specific target sites within repetitive DNA sequences were able to bind *in vivo* to their target locus in such a way that live cell imaging via confocal microscopy could be achieved. In Arabidopsis, centromeric 180-bp repeat regions were readily visualized using this method. Similarly, in mouse cells a GFP-tagged 3ZF protein was targeted to a 9-bp sequence in the centromeric major satellite repeat. This proved that our types of 3ZF domains are well suited for *in vivo* DNA interaction. To quantify the number of molecules that were located in the targeted region in Arabidopsis, a novel method was developed that allows quantification of fluorescent signals with near single molecule precision. The number of GFP-tagged PZF molecules per centromere approximated the number of expected binding sites.

The third aim in this research was to determine whether PZFs could be instrumental for mutagenesis in plants, in this case Arabidopsis (Chapter 4). A PZF-mediated screening method was designed and evaluated in a screen for Arabidopsis mutants exhibiting enhanced homologous recombination (HR) frequencies, a phenotype that can greatly enhance our understanding of DNA repair mechanisms. A library of genes for PZF-ATFs was generated by fusion of DNA sequences encoding 3ZF domains with the VP16 activation domain under control of the promoter of the ribosomal protein gene *RPS5A* from Arabidopsis. Given the size of the Arabidopsis genome and the 9-bp length of the PZF recognition site, each PZF-ATF could potentially regulate up to 800 genes, in this way genome wide coverage is expected to be reached with a relative small collection of different PZF-ATFs. After introduction of a PZF-ATF library representing nearly 4000 different members into an Arabidopsis HR indicator line, primary transformants exhibiting multiple somatic HR events were selected. PCR-mediated rescue of PZF sequences and reconstitution PZF-ATFs led to the

identification of a PZF-ATF that caused a 200 -1000-fold increase in somatic HR upon reintroduction into the HR indicator line. A mutant plant line expressing the HR-inducing PZF-ATF exhibited increased resistance to the DNA-damaging agent bleomycin and was more sensitive to methyl methanesulfonate (MMS), a combination of traits not described previously. The results thus demonstrated that the use of ZF-ATF pools is highly rewarding when screening for novel dominant phenotypes in Arabidopsis.

Chapter 5 elaborates on mutants containing the identified HR stimulating PZF-ATF, which was named VP16-HRU. The macroscopically visible phenotype of plant with *RPS5A* promoter-mediated expression of VP16-HRU was mild, mutant seedlings only exhibited slightly epinastic leaves and increased anthocyanin production under high light intensity and flowered one week later than the parental line. Transcriptome profiling revealed that numerous endogenous genes turned out to be regulated through *RPS5A::VP16-HRU*, whereas the number of genes differentially regulated upon short induced expression of *VP16-HRU* was far lower. The HR phenotype seen in *RPS5A::VP16-HRU* plants thus seems to depend on *RPS5A*-specific expression of VP16-HRU, further supported by the finding that induced expression of VP16-HRU does not lead to the extremely increased frequency of somatic HR. Some of the differentially regulated genes in *RPS5A::VP16-HRU* plants encoded proteins that were implicated to be involved in DNA repair, such as RAD51 and PARP2. Based on the transcriptome profiles, a number of genes were selected that were tested for their individual effects upon somatic HR. From the nine gene constructs that were analysed *in planta*, two had a stimulatory effect upon HR frequency in ten-day-old seedlings. When twenty-day-old seedlings were analysed, it was evident that several other gene constructs were also capable of elevating the HR frequency.

Taken together the data presented in this thesis demonstrate that PZF-technology is highly valuable for a variety of applications in (plant) molecular biology. PZFs can be used to generate ATFs that act as potent inducers of transcription of endogenous genes, even when such genes are actively repressed (Chapter 2), for generating fluorescent tags that allow live cell imaging of endogenous chromosomal loci (Chapter 3), for novel mutant screens in a multicellular organism and recovery of dominant transcription factors exerting the effect (Chapter 4), and for creating possibilities to unravel genetic networks underlying a mutant phenotype (Chapter 5). In the near future, the HR-inducing factor VP16-HRU will be instrumental for further HR research.