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TECHNICAL ADVANCE

Employing libraries of zinc finger artificial transcription factors to screen for homologous recombination mutants in Arabidopsis

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Summary

A library of genes for zinc finger artificial transcription factors (ZF-ATF) was generated by fusion of DNA sequences encoding three-finger Cys_2His_2 ZF domains to the VP16 activation domain under the control of the promoter of the ribosomal protein gene *RPS5A* from *Arabidopsis thaliana*. After introduction of this library into an Arabidopsis homologous recombination (HR) indicator line, we selected primary transformants exhibiting multiple somatic recombination events. After PCR-mediated rescue of ZF sequences, reconstituted ZF-ATFs were re-introduced in the target line. In this manner, a ZF-ATF was identified that led to a 200–1000-fold increase in somatic HR (replicated in an independent second target line). A mutant plant line expressing the HR-inducing ZF-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. Our results demonstrate that the use of ZF-ATF pools is highly rewarding when screening for novel dominant phenotypes in Arabidopsis.

Keywords: zinc finger transcription factor libraries, Cys_2His_2 zinc finger, homologous recombination, mutagenesis, Arabidopsis, VP16.

Introduction

Zinc finger artificial transcription factors (ZF-ATFs), consisting of a polydactyl zinc finger (PZF) DNA-binding domain linked to a protein domain that either activates or represses gene expression near the PZF binding site, have recently received considerable attention (Blancafort *et al.*, 2004, 2005; Eberhardy *et al.*, 2006). ZF-ATFs are generally constructed using Cys_2His_2 ZF domains, the most thoroughly characterized ZF moieties to date. Apart from being small, just 30 amino acids, Cys_2His_2 ZF domains typically bind three contiguous DNA bases per ZF and are highly modular. These features allow construction of more complex PZF fusion proteins, which in principle can recognize unique sites within a complex genome. For GNN, ANN and recently also CNN target sites, where N represents any of the four bases, the most optimal zinc finger designs have been characterized, making ZF-ATF construction possible for almost any target site (Dreier *et al.*, 2001, 2005; Segal *et al.*, 1999). In Arabidopsis, it has been demonstrated that a ZF-ATF containing six ZF domains, designed for the endogenous *APETELA* (*AP3*) promoter, was able to specifically regulate the expression of a promoter–GUS fusion (*AP3::GUS*) *in planta* (Guan *et al.*, 2002). Recently, PZF domains consisting of only three ZF domains and thus designed for a 9 bp binding site were also shown to be useful for gene regulation in Arabidopsis (Holmes-Davis *et al.*, 2005; Van Eenennaam *et al.*, 2004).

Apart from being instrumental for interfering with expression levels of specific genes, ZF-ATFs can in principle also be used for random mutagenesis in order to reveal novel phenotypes. To this end, pools consisting of large numbers of ZF-ATFs were introduced into *Escherichia coli* and yeast cells, as well as various mammalian cell lines, to screen for phenotypes including thermotolerance, osmotolerance, increased protein yield and drug resistance (Blancafort *et al.*, 2003; Kwon *et al.*, 2006; Lee *et al.*, 2004; Park *et al.*, 2003, 2005a,b). The ZF moieties used for these purposes pos-

sessed modest complexity, and, as such, unique genomic target sites are unlikely. This, however, is at the same time the strength of ZF-mediated mutagenesis: with a relatively low number of ZF-ATFs, saturating coverage of the genome is assured due to the large number of potential DNA binding sites. So far, this application of pools of ZF-ATFs for mutagenesis has been restricted to the cellular level and has not been reported in multi-cellular higher eukaryotes such as whole Arabidopsis plants, for example.

Here we describe the use of pools of three-finger (3F) ZF-ATFs to obtain homologous recombination (HR) mutants in Arabidopsis. An understanding of the regulation of HR events is of great interest, not only from a purely scientific perspective, but also because HR is required for the introduction of novel traits at a precise locus via genetic engineering and controls the process of meiosis. Unfortunately, in higher eukaryotic organisms and especially in plants, almost all incoming DNA molecules that integrate into the genome do so apparently at random positions via non-homologous recombination (NHR) (Offringa *et al.*, 1990; Paszkowski *et al.*, 1988). Efficient procedures for gene modification in plants thus require either a suppressed NHR pathway and/or a strongly activated HR pathway. Apart from these practical aspects, phenotypes related to HR will be instrumental in the investigation of intriguing aspects of genome stability and genome evolution. In this study, we used an *in planta* recombination assay (Swoboda *et al.*, 1994) to select for mutants upregulated in HR after transformation of Arabidopsis plants with pools of ZF-ATFs. In this manner, we were able to isolate a novel mutant exhibiting a dramatic increase in the frequency of HR events. Our findings demonstrated that ZF-ATF technology is readily applicable for identification of novel types of mutants even at the level of the multicellular organism.

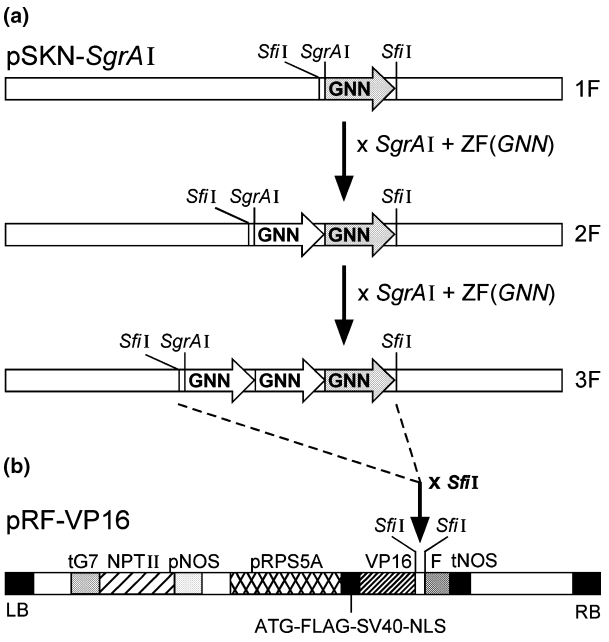

Figure 1. Schematic representation of the construction of 3F ZF-ATF pools.
(a) Annealed oligonucleotide pairs encoding ZF domains were ligated in a directional manner into the *Sgr*AI site of vector pSKN-*Sgr*AI, resulting in one-finger (1F) and two-finger (2F) constructs. Three-finger (3F) pools with a complexity of 256 3Fs per pool were each formed separately by introduction of a third ZF domain into an equimolar mix of 16 *Sgr*AI-digested 2F constructs. The resulting 3F pools were named after the identity of the first ZF cloned (shaded 'GNN' arrow). In this manner, 15 of the possible sixteen 3F pools were constructed.
(b) The combined 3F sequences of each pool were isolated as *Sfi*I fragments and cloned in a directional manner into T-DNA vector pRF-VP16 for *in planta* expression of fusion proteins via the *RPS5A* promoter (pRPS5A). When expressed, the N-terminus of the 3F domains will be preceded by a FLAG tag (directly following the ATG translational start codon), a SV40 nuclear localization signal (SV40-NLS) and the VP16 transcriptional activator domain (VP16). A translational stop codon is provided after a 37 amino acid C-terminal sequence of the VirF protein of *Agrobacterium tumefaciens* (F), and transcriptional stop is provided by the nos terminator (tNOS). The *NPTII* gene is present as a plant selectable marker. LB, left border; RB, right border.

Results

Construction of 3F pools for plant transformation

A schematic overview of the procedure to obtain 3F ZF-ATF pools is given in Figure 1. After cloning the first 16 oligomers encoding GNN-recognizing ZF moieties into pSKN-*Sgr*AI, we invested in the systematic cloning and sequencing of all possible two-finger (2F) constructs (for details regarding cloning procedure and oligomer sequences, see supplementary material in Neuteboom *et al.*, 2006). In this manner, 256 2F-encoding plasmids were obtained, divided into 16 pools of 16 members, and each pool labeled according to the first ZF domain cloned. As substrate for addition of the third consecutive ZF, we used restriction enzyme-digested mixtures of equimolar amounts of plasmids belonging to a pool. This protocol, although more elaborate than random multimerization and shotgun cloning of ZF moieties, was chosen for several reasons. Firstly, a better control of successful 3F pool generation is ensured. Secondly, having the total collection of 3F-containing plasmids in several non-overlapping pools of lower complexity offers the possibility of screening each of the sixteen 256-member 3F pools to different extents. This is of great advantage when, instead of screening at the cellular level, mutant phenotypes have to be selected at the level of the complete organism and handling of large numbers of transgenic individuals in a single experiment is generally unfeasible. Sub-dividing the larger pool of potentially mutagenic transcription factors allows much easier recovery of interesting mutants in case an initial molecular analysis fails. For 15 out of the possible 16 different 3F pools, the construction was successful. For unknown reasons, the 2F clones designed for 5′-GAA-GNN-3′ binding sites frequently possessed errors, and as these 2F clones are needed for preparation of the GAA 3F pool, this pool was not further developed. All other libraries were

finally introduced as *Sfi*I fragments into the wide host range vector pRF-VP16 (Figure 1b). Once cloned within this vector, the 3F moieties were fused with an N-terminal VP16 domain, preceded by a FLAG tag and a SV40 nuclear localization domain, and linked to the *RPS5A* promoter, which normally drives expression of the ribosomal protein S5A in Arabidopsis (Weijers *et al.*, 2001). The choice of the *RPS5A* promoter resides in the extremely early onset of its activity in the zygotic stage of embryo formation, as well as its continued expression in meristematic tissues of plants. Such an expression pattern ensures that all cells present in a transformed seedling have experienced a period during which the transgene is expressed, even when the transgenic seedling directly stems from the initial transformation by the floral dip procedure. In differentiated older cells, *RPS5A* activity sharply declines (Weijers *et al.*, 2001), which avoids prolonged expression of a transgene that might be detrimental for seedling survival and would therefore diminish the chance of successful characterization of the 3F sequence that led to the phenotype of interest.

Screening for Arabidopsis mutants with increased homologous recombination frequencies

To monitor HR frequencies in Arabidopsis seedlings, we have used target line 1406 (Gherbi *et al.*, 2001), containing an HR substrate in the form of an interrupted GUS reporter gene that can be restored after an HR event within a direct repeat of the GUS coding sequence (Swoboda *et al.*, 1994). Individual recombination events can be visualized as blue spots or sectors upon histochemical staining of seedlings. For each separate pool of pRF-VP16-3F plasmids present in *Agrobacterium*, a practically realistic compromise for the number of transgenic seedlings to be generated and screened had to be defined. With each of the 15 pRF-VP16-3F pools having a theoretical complexity of 256 different members, screening almost all (>95%) constituent *VP16:3F*-encoding genes for their potential to enhance HR would require the analysis of more than 750 primary transformants for each of these pools ($N = \ln(1 - 0.95)/\ln(1 - 1/\text{library size})$). As indicated in Table 1, the average number of transformants screened per pool was around 400, which corresponds to a fair chance of about 80% of any member of the pool being represented in a transgenic seedling.

Although we observed a variety of morphological aberrations within the collection of transformed seedlings (not shown), we focused on those seedlings that exhibited more somatic HR events than the parental line and the majority of transgenics. With a spontaneous HR frequency in line 1406 of about 0.012 events per 10-day-old seedling, those transgenic seedlings with three or more HR events were considered to be candidates for *VP16:3F*-induced HR mutants. Within a total of 6400 transgenic seedlings, three met this requirement; two seedlings with three spots in pools GTC and GCA, and a seedling with eight spots in pool GTG (Table 1).

Table 1 Homologous recombination events in 10-day-old seedlings of Arabidopsis line 1406 transformed with the different pRF-VP16-3F pools

		No. seedlings		
Pool[a] name	No. seedlings	1 spot	2 spots	≥3 spots[b]
GGG	402			
GGA	405			
GGT	382	3		
GGC	459			
GAG	440	6		
GAA	ND			
GAT	72	2		
GAC	359	3		
GTG	686	2	1	1 (8)
GTA	553	5		
GTT	384			
GTC	634	10		1 (3)
GCG	143	6		
GCA	415	1		1 (3)
GCT	701	2		
GCC	365			
Total	6400	40	1	3
Control	167 (1406)[c]	2		

[a]Pools are named after the first cloned ZF domain.
[b]Number of spots in parentheses.
[c]Untransformed seedlings of line 1406.
ND, not done

Identification of ZF-ATFs in transgenic seedlings and verification of HR-inducing potential

During preliminary experiments, several GUS staining protocols were tested for accurate determination of somatic HR events while at the same time being compatible with further molecular analysis of 3F sequences. The GUS staining method involving acetone pre-treatment (see Experimental procedures) provided excellent penetration of substrate throughout the seedling and led to sharply stained cells. DNA extracted from these stained seedlings was in most cases still suitable for PCR analysis of the 3F region of the transgene. From the seedling with eight somatic HR events, a PCR product of correct size was readily obtained, but not from the two plants with three recombination events. Whether this reflects imperfectness of the DNA recovery protocol or a true absence of an intact transgene in the other two putative mutants remains uncertain. A vital staining procedure would have allowed the generation of more biomass from putative mutants, but such procedures proved to be too ineffective to reveal single stained cells.

Sequence analysis of the PCR product obtained from the seedling with eight HR events verified the presence and identity of a 3F domain, which was subsequently recloned as

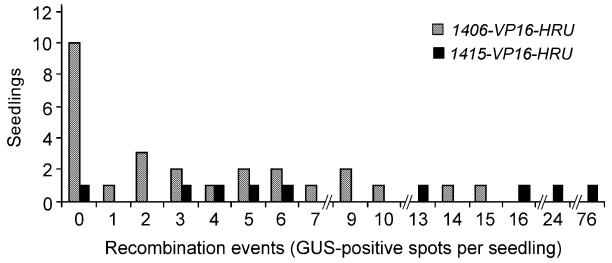

Figure 2. Distribution of recombination frequency in individual pRF-VP16-HRU primary transformants in lines 1406 and 1415.
Recombination frequency in 10-day-old primary transformants was determined as number of GUS-positive spots per seedling.

a *Sfi*I fragment into pRF-VP16 and introduced into *Agrobacterium*. According to the GNN recognition code (Segal *et al.*, 1999), the most optimal binding site for this 3F should be 5′-GTGGAGGCT-3′ (for oligomer sequences, see Neuteboom *et al.*, 2006). Most transgenic seedlings obtained with the resulting *Agrobacterium* strain again possessed a high number of HR events, proving that the particular *VP16-3F* factor was the causal agent of the original phenotype. Of the 27 primary transformants that were stained for GUS activity, one plant exhibited one event, while at least two events were present in 16 out of the total 27 stained seedlings, ranging from 2 to 15 HR events per seedling (Figure 2). With an average number of 3.8 HR events per seedling within the group of 27 primary transformants, the mean HR frequency after transformation with this VP16-3F factor was increased approximately 300-fold compared to the parental line, which had an HR frequency of 0.012 spots per seedling. For some individual primary transformants, those with ten or more GUS positive spots, the HR frequency apparently increased by a factor of 1000 or more. The HR-inducing ZF-ATF was designated VP16-HRU, and the resulting up-regulated HR phenotype in transgenic plants was designated HRU.

The effect of VP16-HRU *is independent of the HR reporter locus*

To rule out the possibility that the HRU phenotype observed in reporter line 1406 after transformation with *VP16-HRU* is dependent on the precise genomic location of the reporter gene and/or on the layout of the reporter locus itself, this construct was also used to transform an independent second target line for intra-chromosomal homologous recombination. In this line 1415 (Gherbi *et al.*, 2001), the substrate for homologous recombination also comprises an interrupted GUS reporter gene, but arranged as an inverted repeat. Although the frequency of spontaneous recombination in this line is higher than in line 1406 (0.083 events per seedling for line 1415 versus 0.012 events per seedling for line 1406 under our conditions), the effect of *VP16-HRU* was indisputable. From nine randomly picked primary transformed seedlings, only one did not reveal any GUS-positive spots, while for the remaining seedlings HR frequencies as high as 76 events per 10-day-old seedling were observed (Figure 2). On average, 16.3 recombination events per seedling were seen in this set of nine, indicating that the mean HR frequency had risen about 200-fold after transformation with pRF-VP16-HRU, a value comparable to that observed in line 1406. Therefore, we can conclude that the increase in HR frequency is independent of the precise location of the reporter locus and is seen both with direct and indirect GUS repeats. Morphological aberrations were not observed in the seedlings with the HRU phenotype. Later in development, HRU plants did have relatively long and somewhat narrower leaves than the parental line and flowering time was slightly delayed. HRU plants were fertile and produced a normal amount of seeds.

Several T_1 HRU mutants in the backgrounds 1406 and 1415 were allowed to self-pollinate. T_2 populations showing a 3:1 segregation of the kanamycin resistance gene, indicating a single transgenic locus, were maintained to obtain homozygous T_3 lines. In both types of lines, designated *1406-VP16-HRU* and *1415-VP16-HRU*, the HRU phenotype remained stable up to the T_3 generation (data not shown).

VP16-HRU *expression leads to increased tolerance to the genotoxic agent bleomycin*

Although it was clearly demonstrated that *VP16-HRU* triggers a higher recombination frequency in Arabidopsis seedlings in a dominant fashion, the molecular mechanism underlying the HRU phenotype is not immediately evident. On one hand, HRU seedlings might possess a directly activated HR pathway and as such be subject to an enhanced frequency of recombination events. On the other hand, the presence of *VP16-HRU* might induce a more general kind of genotoxic stress that triggers DNA repair and thereby also HR events, such that HR is activated in an indirect manner. Experiments in which plants are treated with DNA-damaging agents can in principle discriminate between these possibilities. Bleomycin is known to cause double-strand breaks (DSBs) in genomic DNA (Charles and Povirk, 1998; Favaudon, 1982; Menke *et al.*, 2001; Norskov-Lauritsen *et al.*, 1990), which are repaired either by HR or NHR. If in HRU plants the HR pathway is constitutively up-regulated prior to inflicting extra DSBs, the HRU plants will most likely be more tolerant to bleomycin treatment than the parental line, at least in as much as the already up-regulated HR pathway can repair the sudden increase in DSBs. Alternatively, if HRU plants are already experiencing genotoxic stress, a further increase of this stress by bleomycin should more readily cause severe problems. In the latter case, plants are expected to be hypersensitive to bleomycin.

As shown in Figure 3(a), a 24 h bleomycin treatment led to a 16.5-fold induction of HR frequency in the parental line

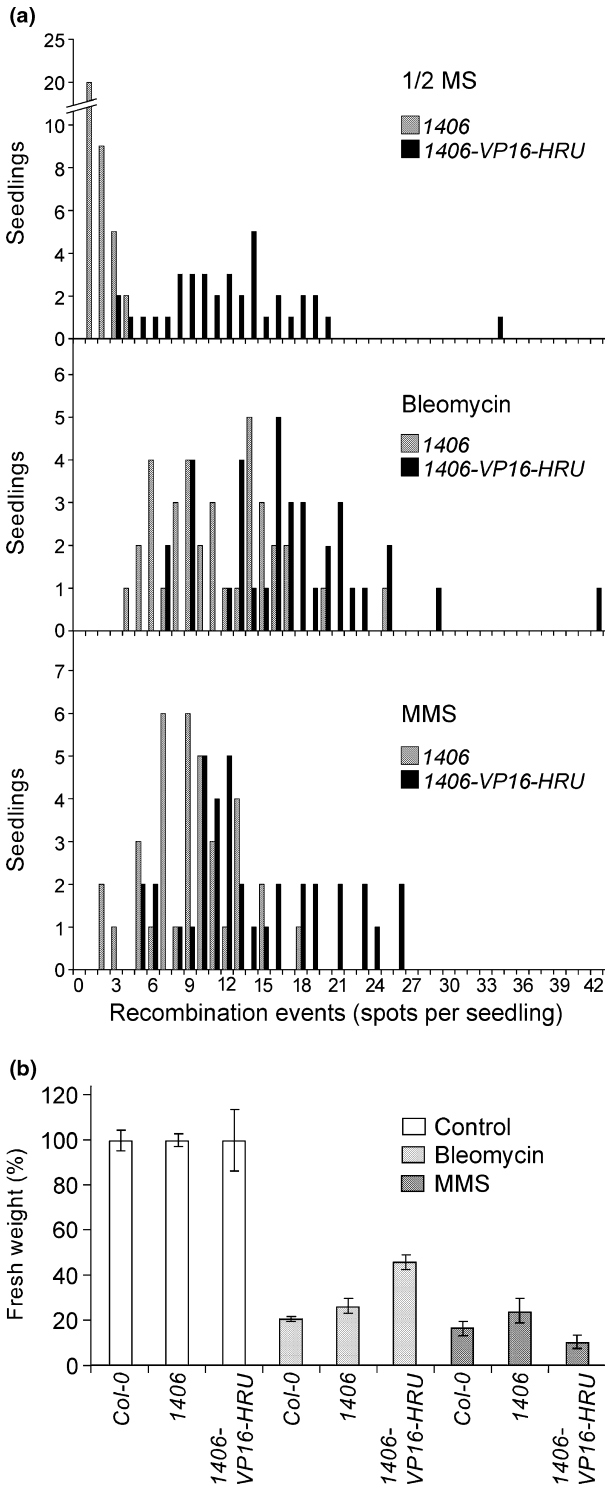

Figure 3. Effect of genotoxic chemicals on HR frequency and fresh weight development in parental control (1406) and *VP16-HRU*-expressing seedlings.
(a) Distribution of HR frequency per seedling in mock-treated seedlings (top) and seedlings exposed for 24 h to bleomycin (middle) or MMS (bottom). HR events were determined 5 days after treatment as the number of GUS-positive spots per seedling.
(b) Effect of long-term treatment (2 weeks) with bleomycin or MMS on fresh weight development of wild-type Columbia (Col-0), 1406 and 1406-*VP16-HRU* seedlings.

1406, which is comparable with published data (Molinier *et al.*, 2005). The HR frequency in HRU seedlings rose from a basal 11.3 events per seedling to 17.1 after bleomycin treatment, an increase of about 1.5-fold (50%). Remarkably, the basal frequency of HR events in HRU seedlings was virtually identical to the frequency observed in bleomycin-treated 1406 seedlings (11.4 HR events per seedling). In order to assess different levels of bleomycin resistance, the fresh weight of seedlings was determined after 2 weeks of continuous treatment. Compared with wild-type Columbia seedlings and the parental line 1406, seedlings expressing *VP16-HRU* gained more fresh weight in the presence of bleomycin, thus demonstrating increased tolerance to this genotoxic compound (Figure 3b).

In addition to bleomycin, we also investigated the effect of long- and short-term treatments with the alkylating compound methyl methanesulfonate (MMS). Genotoxic stress caused by DNA alkylation is mainly repaired via the nucleotide excision repair (NER) pathway (Lundin *et al.*, 2005). Short-term MMS treatment increased the frequency of HR events per seedling of line 1406 11.7-fold, corresponding to 8.1 events per seedling, while for HRU seedlings the frequency increased only by about 15% to 13.0 events per seedling. When analyzed in terms of fresh weight development after 2 weeks of treatment, HRU plants were slightly less resistant than controls (Figure 3b).

Discussion

Although a wide variety of mutagenic treatments exists to manipulate higher eukaryotic organisms, the recently emerging technique using ZF-ATFs to induce dominant mutations had so far only been used at the cellular level (Blancafort *et al.*, 2003; Lee *et al.*, 2004; Park *et al.*, 2003, 2005a,b). In this paper, we investigated the possibility of employing ZF-ATF-mediated mutagenesis in an efficient manner for isolation of mutant phenotypes at the level of individual plants; in this particular case, Arabidopsis mutants altered in intra-chromosomal recombination frequency. Rather than generating huge numbers of transgenic organisms in order to find a desired mutation, we sought to exploit the potential robustness of ZF-ATF mutagenesis. The use of sub-pools of ZF-ATFs with relatively low complexity, combined with the large number of genomic target sites for each zinc finger, should allow an unbiased screening at almost saturating level. Assuming that all base pairs occur at equal frequency, a complete pool of 3F DNA binding domains recognizing 9 bp targets with sequence 5′-GNNGNNGNN-3′ $(GNN)_3$, where N represents any of the four bases will find a target site every 32 bp in a double-stranded genome. On average, any specific 3F domain will encounter the optimal cognate 9 bp target site once within 131 000 bp ($4^9/2$ bp for dsDNA). Given the size of the Arabidopsis genome, each specific (GNN)3 target site occurs on

average about 800 times within the genome. Even when only a minority of the possible DNA target sites will be available for 3F binding, the possibility that at least one member of a 3F ZF-ATF pool has access to a binding site at a position from which the transcriptional activity of a particular gene can be influenced should be quite large. Consequently, even by generating a relative small number of transgenic organisms, efficient screening for novel mutations in complete multicellular organisms, such as plants, should be feasible. In this respect, 3F ZF-ATF-mediated mutagenesis would be a highly attractive method to investigate whether or not the generation of a particular mutant phenotype lies within the scope of possibilities of the genome of an organism, given that an altered regulatory network of gene expression signals could be brought about. Although T-DNA activation tagging (Weigel *et al.*, 2000) in principle acts in a similar manner, the number of independent transgenic organisms required for a saturated screening via this method depends on the genome size in a linear fashion. For plant species with small genomes for which efficient transformation procedures exist and/or tagged collections are available, such as for Arabidopsis, the benefits of 3F ZF-ATF-mediated mutagenesis over T-DNA activation tagging might be limited. However, it should be realized that most plant species possess much larger genomes, often combined with inefficient and impractical transformation procedures. As larger genomes contain a correspondingly higher number of 3F target sites, the chance of finding a potential dominant phenotype via 3F ZF-ATF-mediated mutagenesis within a limited number of transgenic plants is the same as in Arabidopsis. In the majority of plant species, 3F ZF-ATF-mediated mutagenesis will thus be a highly attractive option.

Our experiments, aimed at identifying mutants with upregulated HR frequencies, clearly indicate that 3F ZF-ATF-mediated mutagenesis can be an efficient means to find a particular phenotype in higher plants. Within just 6400 primary transformants, we positively identified a 3F ZF-ATF (named VP16-HRU) that strongly up-regulates intra-chromosomal HR frequencies in Arabidopsis. Although they vary in the specific nature of the mutants found, several other studies describing the isolation of HR mutants have been reported (Schuermann *et al.*, 2005), thus allowing a tentative comparison between different methods. The *atino80*-1 mutant, a semi-dominant mutation leading to reduced HR frequency, was identified after screening 20 000 T-DNA insertion mutants (Fritsch *et al.*, 2004). A hyper-recombinogenic phenotype due to reduced expression of At *CEN2* was found after screening 4200 T_1 plants harboring a T-DNA activation tagging construct (Molinier *et al.*, 2004). Based on these data, the efficiency of 3F ZF-ATF-mediated mutagenesis of Arabidopsis seems to be comparable with that of activation tagging. As outlined above, this strongly indicates that, for larger plant genomes, 3F ZF-ATF-mediated mutagenesis can be a highly rewarding and efficient method to investigate whether or not particular mutant phenotypes can be found within the species studied.

In our study, it was clearly demonstrated that the 3F ZF-ATF gene *VP16-HRU* acts as a dominant factor, causing 200–1000-fold increase in HR frequencies in two different HR reporter lines, 1406 and 1415. These frequencies are much higher than reported previously using the same or related types of reporter loci; the most dramatic HR increase described so far being 36-fold for the At*CEN2* mutant (Molinier *et al.*, 2004). The optimal 9 bp recognition sequence (5′-GTGGAGGCT-3′) for the VP16-HRU protein is not present within the sequences of the HR reporter loci, and it is very unlikely that the flanking genomic regions of both randomly integrated reporter loci are related. For this reason, it can be postulated that VP16-HRU activates essential regulatory components of an HR pathway rather than acting directly on the reporter loci. As HRU mutant seedlings were resistant to the DSB-inducing agent bleomycin, HRU plants most likely possess an HR system that is already activated, leading to more efficient HR-based DSB repair. In contrast, MMS sensitivity seemed to be increased in HRU mutants (Figure 3b), supporting the view that the genotoxic damage caused by MMS is repaired via another pathway, the nucleotide excision repair (NER) pathway (Lundin *et al.*, 2005). It is tempting to speculate that up-regulation of HR-mediated DSB repair is accompanied by a reduction in NER activity. In addition to the observed increase in HR events, the observed changes in tolerance to genotoxic compounds further corroborate that the 3F transgene *VP16-HRU* acts upon crucial processes in DNA metabolism.

While the *VP16-HRU* gene acts as an artificial exotic regulator of HR-related events in Arabidopsis, a variety of endogenous genes and physiological conditions have been described that may be involved in the frequency of recombination events. Mutations within the *RAD50*, *RAD17*, *RAD9*, *CENTRIN2*, *RECQl4A* and *BRU1* genes result in hyper-recombinogenic phenotypes (Bagherieh-Najjar *et al.*, 2005; Gherbi *et al.*, 2001; Heitzeberg *et al.*, 2004; Molinier *et al.*, 2004; Takeda *et al.*, 2004). Stress in the form of pathogens or genotoxic treatments have also been correlated with increased somatic HR events (Gherbi *et al.*, 2001; Lebel *et al.*, 1993; Molinier *et al.*, 2005). Mutants showing altered frequencies of somatic HR are generally hypersensitive to genotoxic stress and/or UV irradiation (Dubest *et al.*, 2002, 2004; Masson and Paszkowski, 1997; Masson *et al.*, 1997; Mengiste *et al.*, 1999). In this respect, the effect of expression of *VP16-HRU* in Arabidopsis is very interesting, as these plants show a higher degree of resistance to bleomycin. To our knowledge, such a phenotype, in particular in combination with the unprecedented up to 1000-fold increase in HR, is novel to the field. We intend to use gene expression profiling to check for differentially expressed genes due to the VP16-HRU artificial transcription factor. As VP16-HRU

seedlings are refractory to a further induction of recombination events by bleomycin, it will be of particular interest to compare the expression pattern of these seedlings with published data regarding changes in gene expression patterns due to bleomycin or other genotoxic compounds (Chen et al. 2003; Molinier *et al.*, 2005).

In conclusion, our findings demonstrate that the use of 3F ZF-ATF pools for mutagenesis at the organism level is feasible and highly rewarding. Especially for rapid assessment of potential dominant phenotypes in species with a large genome size, ZF-ATF-mediated mutagenesis might be a highly attractive method. The 3F pools in *Agrobacterium* are available upon request and will be deposited to the Nottingham Arabidopsis Stock Center (NASC).

Experimental procedures

Construction of plant expression vector pRF-VP16

Plant expression vector pGPTV-KAN (Becker *et al.*, 1992) was modified in order to obtain pRF-VP16. Briefly, *Not*I and *Sfi*I sites within the vector backbone were removed via sequential digestion and treatment with Klenow enzyme and T4 DNA polymerase, respectively. These modifications did not hamper the frequency of plant transformation. The promoterless GUS coding sequence was replaced by a 1.7 kb *Xma*I–*Sac*I fragment containing the *RPS5A* promoter (Weijers *et al.*, 2001), after which the plasmid was digested with *Sac*I followed by insertion of a sequence providing the vector with an ATG translational start codon, a FLAG tag, a SV40 nuclear localization signal and *Not*I and *Xho*I sites. An *Xho*I–*Sac*I fragment encoding the 37 C-terminal amino acids of the VirF protein of an octopine strain of *Agrobacterium tumefaciens* was added to the vector. Although this domain was of no further specific relevance for this study, the DNA fragment provided a translational stop codon for the fusion proteins produced *in planta*. The unique *Not*I site was used for introduction of the VP16 transcriptional activation domain (Sadowski *et al.*, 1988), and *Sfi*I sites were used for directional cloning of zinc finger domains. The most relevant features of the resulting plant expression plasmid pRF-VP16 are shown in Figure 1(b). The plasmid sequence is available upon request.

Construction of 3F ZF-ATF pools

Zinc finger modules designed for 5′-GNN-3′ or 5′-$(GNN)_2$-3′ binding sites were constructed in pSKN-*Sgr*AI as described previously (Neuteboom *et al.*, 2006), using annealed oligonucleotide pairs each encoding an optimal zinc finger sequence as established by Segal *et al.* (1999). Once a sequence-verified series of constructs encoding two-finger (2F) proteins sharing the C-terminal finger was established, the third finger was added in a controlled manner (Figure 1a). For each 2F series, the sixteen 2F constructs were grown overnight in LC medium containing carbenicillin at 100 mg l^{-1} for plasmid selection, and glucose (20 mM) in order to repress any untimely expression of the ZF proteins via the *lac* promoter. A larger volume (100 ml) of the same medium was subsequently inoculated with equal amounts of each of the 16 bacterial strains belonging to the series (corresponding to 0.5 ml of bacterial culture with an OD of 1.0 at 600 nm) and grown for an additional 4 h. Plasmid DNA was isolated, digested with *Sgr*AI, and subdivided for 16 separate ligation reactions with one specific ZF-encoding oligonucleotide pair present at 1000-fold molar excess relative to vector molecules. After ligation and heat inactivation of the enzyme, the reactions were pooled. After gel electrophoresis linear plasmid molecules containing additional ZF oligonucleotides at each end were isolated from gel. Upon a denaturation–renaturation procedure as described previously (Neuteboom *et al.*, 2006), circularized annealed plasmid DNA was transformed into *E. coli* DH5α to obtain the 3F pool. Each pool consisted of at least 2000 independent colonies to ensure that complexity of 256 different 3F modules per pool was maintained. Colonies belonging to a pool were scraped from solid medium and grown together for an additional 5–6 h, after which the 3F-containing plasmids were isolated. The plasmid pool was digested with *Sfi*I to obtain 325 bp fragments containing 3F sequences, which were subsequently ligated into the *Sfi*I-digested plant expression vector pRF-VP16 and transformed into *E. coli* DH5α. pRF-VP16 3F pools typically consisted of at least 2000 independent colonies with >90% recombinant plasmids. The colonies were scraped from plates and grown briefly in LC under kanamycin selection. The resulting pools were each mobilized to *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) via triparental mating (Ditta *et al.*, 1980). The resulting masses of *Agrobacterium* colonies were scraped from selection plates (kanamycin, rifampicin and carbenicillin at 100, 10 and 75 mg l^{-1}, respectively). After briefly growing pools at 28°C in selective liquid medium, 1 ml aliquots of *Agrobacterium* cultures were frozen in bactopeptone medium with 17% glycerol and stored at −80°C to start bacterial cultures to be used for plant transformation.

Plant transformation and homologous recombination assay

Plants of line 1406 (Gherbi *et al.*, 2001) were transformed with each of the pRF-VP16-3F pools by floral dip (Clough and Bent, 1998; Lazo *et al.*, 1991), and primary transformants (T_1 seedlings) were selected on MA medium (Masson and Paszkowski, 1992) lacking sucrose and containing kanamycin, timentin and nystatin (50, 100 and 100 mg l^{-1}, respectively). Ten-day-old primary transformants were stained for GUS activity in order to reveal HR events as GUS-positive spots or sectors, essentially as described previously (Swoboda *et al.*, 1994) but including treatment with 95% acetone for 1 h at −20°C followed by three rinses with phosphate buffer (pH 7.2), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, prior to a 14–16 h incubation in 1 mg ml^{-1} X-gluc in the same buffer. Recombination events were determined as the number of spots per seedling.

DNA isolation and PCR analysis for identification of ZF-ATFs from T_1 mutants

DNA was isolated from single GUS-stained T_1 seedlings essentially as described previously (Murray and Thompson, 1980), and dissolved in a total volume of 20 μl 1/4-strength TE buffer. For each PCR-mediated rescue of the ZF sequence, 2 μl of the DNA sample was used in a PCR analysis with primers pRF-uni-fw (5′-GAAGCGTAAGGTCGAGC-3′) and pRF-2pol-rev (5′-CTCGCGAATGCATCGAG-3′), amplifying a 676 bp PCR product containing the sequence encoding the 3F domains. PCR was performed in a total volume of 25 μl with 0.4 μM of each primer, 0.4 μM dNTPs and 1.25 units *Pfu*Turbo® DNA polymerase (Stratagene, La Jolla, CA, USA). Part of the PCR product was analyzed by gel electrophoresis; the remaining part was digested with *Sfi*I for cloning into *Sfi*I-digested pSKN-*Sgr*AI. The 3F sequence was determined after sequencing with primer M13R (5′-CAGGAAACAGCTATGACCATGA-3′).

Treatment with genotoxic chemicals

All treatments were performed in a growth cabinet at 21°C with a 16 h photoperiod and under continuous gentle shaking when seedlings were incubated in liquid medium. Seeds were sown on solidified 1/2-strength MS medium. Five days after germination, groups of three seedlings were transferred to each well of six-well plates (Greiner bio-one, Alphen a/d Rijn, The Netherlands), each well containing 4 ml liquid 1/2-strength MS medium with or without the genotoxic agents bleomycin (0.125 mg l^{-1}) or MMS (0.007%). For the recombination assay, seedlings were treated for 24 h, and were further incubated for 5 days in liquid medium without genotoxic agents prior to GUS staining. To monitor developmental effects, treatment of seedlings was continued for 2 weeks. After this period, fresh weight reduction compared with controls was determined by weighing the seedlings in batches of 18 seedlings in triplicate.

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