ABSTRACT

Transcription activator-like effector (TALE) DNA binding proteins show tremendous potential as molecular tools for targeted binding to any desired DNA sequence. Their DNA binding domain consists of tandem arranged repeats, and due to this repetitive structure it is challenging to generate designer TALEs (dTALEs) with user-defined specificity. We present a cloning approach that facilitates the assembly of multiple repeat-encoding DNA fragments that translate into dTALEs with pre-defined DNA binding specificity. This method makes use of type IIS restriction enzymes in two sequential cut-ligase reactions to build dTALE repeat arrays. We employed this modular approach for generation of a dTALE that differentiates between two highly similar DNA sequences that are both targeted by the Xanthomonas TALE, AvrBs3. These data show that this modular assembly system allows rapid generation of highly specific TALE-type DNA binding domains that target binding sites of predefined length and sequence. This approach enables the rapid and flexible production of dTALEs for gene regulation and genome editing in routine and high-throughput applications.

INTRODUCTION

DNA binding domains that can be tailored to interact with user-defined DNA sequences are crucial tools for molecular biology (1). Bacterial transcription activator-like effector proteins (TALEs) from the bacterial pathogen Xanthomonas target DNA via a novel type of DNA binding domain that is composed of tandem-arranged 33–35 amino acid repeat-modules, with each repeat binding to one base (2,3). Base preferences of individual repeats are specified by residues 12 and 13, known as the repeat variable diresidues (RVDs), that determine preferential pairing with A (NI), C (HD), G (NK) and T (NG) nucleotides, respectively (2–5). In principle, the usage of this TALE code facilitates the assembly of repeat arrays that bind to any desired DNA sequence that is preceded by a T nucleotide (2,6,7). Recent studies have also shown that TALE repeats function as sequence specific targeting modules not only in the context of a transcription factor but also when fused to a FokI nuclease domain (4,8–10). This suggests that the TALE DNA binding domain enables applications comparable to zinc finger (ZF) technology (11). ZFs that are assembled into an array are known to influence the DNA specificity of adjacent ZFs (12) and, due to this context dependency, ZF arrays of desired DNA specificity require experimental validation. In contrast, there is no evidence so far that base-preferences of TALE repeats are context dependent and recent studies have demonstrated that in vitro assembled repeat arrays target the pre-defined nucleotide sequences (4,5,8,10,13).

A major hurdle to the routine application of the TALE DNA binding domain is that the assembly of genes encoding tandemly arranged repeats is difficult to achieve via standard cloning approaches. To address this need, we developed a rapid, efficient, low-cost approach for engineering TALE-type DNA binding domains with custom specificities, which involves fusing individual TALE repeats into a desired array. Functional repeat arrays can be cloned directly into an expression vector or alternatively into a Gateway-compatible entry vector that facilitates recombination based transfer into any desired Gateway-compatible expression vector. We also demonstrate that TALE repeat arrays, which have been assembled by this cloning approach, target in vivo the pre-defined DNA sequences with high sequence specificity.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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MATERIAL AND METHODS

Vector construction

The BsaI recognition site in pUC57 was mutagenized with primers RM1 and RM2 to obtain pUC57ΔBsaI (for primer sequences see Supplementary Table S1). Repeat-modules were amplified from pENTR-D-avrBs3 with primers adding BsaI recognition and restriction sites at the 5′- and 3′-end and were blunt end cloned into pUC57ΔBsaI (sequences of repeat modules are provided in Supplementary Figure S1; primer sequences are provided in Supplementary Table S1). RVD encoding nucleotides were modified via site-directed mutagenesis to obtain all six different RVDs (for nucleotide sequence of each RVD see Supplementary Figure S1). pENTR-D-avrBs3 had been amplified with primer RM3 and RM4 to create pENTR-D-TALE-Δrep-BsaI-AC. To create pUC57-AB-DEST and BC-DEST, first the aadA gene was amplified from pGWB441 (14) and cloned by blunt end ligation into the Scal restriction site of pUC57ΔBsaI using RM5 and RM6. Second, the BsaI and BpiI restriction and recognition sites were added using primer RM7 with RM8 and RM9 with RM10, respectively, and the PCR fragment obtained was ligated to a PCR fragment that had been amplified from pGWB441 using primers RM11 and RM12. pENTR-D-avrBs3 had been amplified with primers RM13 and RM14 to create pENTR-D-TALE-Δrep-BpiI-AC. The RVDs in pENTR-D-TALE-Δrep-BpiI-AC were changed using site-directed mutagenesis. Mutations were introduced with the Phusion site-directed mutagenesis kit (New England Biolabs). All BsaI and BpiI recognition sites in pGWB5 (14) were mutagenized as described previously (15) to create pGWB5* (for sequence see Supplementary Figure S4). The set of plasmids that’s required to generate genes encoding dTALE repeat arrays has been deposited in the non-profit plasmid repository Addgene (http://www.addgene.org).

Cut-ligation cloning protocol

For cut-ligation reaction 40 fmol of each plasmid, ligation buffer (Fermentas), 15 U of either BsaI or BpiI and 15 U high-concentrated T4 DNA ligase were used in a 20 μl volume. The reaction was incubated in a thermo cycler with the following program: 5 min 37°C, 5 min 20°C, 50 cycles, 10 min 50°C and 10 min 80°C. One microliter reaction mix was added to 50 μl chemical competent TOP10 cells, incubated for 15 min on ice and transformed by heat shock. Clones were analysed by colony PCR, restriction and sequencing.

In planta analysis

All entry clones were transferred by LR recombination (Invitrogen) into the expression vector pGWB5 (14) and transformed into Agrobacterium tumefaciens GV3101 (16) for in planta analysis. Sequences and generation of all promoter constructs have been described previously (5). GUS measurements were carried out as described previously (17).

RESULTS

Assembly of TALE genes by a one-step BsaI cut-ligation

Our TALE repeat assembly kit is based on type IIS restriction enzymes that cleave outside of their recognition site and produce a 4–bp 5′ overhang (15). Since recognition and cleavage sites are spatially separated in type IIS restriction enzymes, proper construct design facilitates cleavage-mediated generation of theoretically any desired 4–bp overhang for a given DNA substrate. Thus jigsaw puzzle-like directional assembly of multiple DNA fragments is feasible (18,19). Another important aspect of type IIS mediated cloning [TIIS-cloning; synonym: Golden Gate cloning (15,18,19)] is that the desired ligation products lack the recognition sites of the given type IIS endonuclease. Thus cleavage and ligation can be carried out simultaneously (cut-ligation) rather than sequentially.

We aimed to establish a toolkit that enables TIIS-mediated assembly of genes encoding 20 or more TALE repeats. The length of the corresponding TALE target sites should facilitate specific targeting of a unique DNA sequence even in the context of a highly complex eukaryotic genome. To do so, we PCR-amplified and cloned DNA fragments encoding individual repeats (herein referred to as repeat-modules) from the TALE gene avrBs3 into a modified pUC57 cloning vector (Supplementary Figure S1). To avoid vector-derived BsaI cleavage products that potentially interfere with the envisaged multi-fragment ligation, we removed the BsaI recognition site from pUC57 by site-directed mutagenesis, yielding pUC57ΔBsaI. The cloned repeat-modules served as initial building blocks for the envisaged repeat assembly. The primers introduced BsaI sites at both termini of the repeat-module in such a way that both recognition sites are cleaved off from the repeat-module (Figure 1a). Initially we cloned 10 distinct repeat-modules each producing a different combination of terminal overhangs upon BsaI cleavage. The overhangs were designed so that each repeat-module would ligate only to specific repeat-modules in such a way as to generate a pre-defined linear repeat array. The first repeat-module of an array will ligate specifically to the 5′-end of the second repeat-module. The 3′-end of the second repeat-module will ligate specifically to the 5′-end of the third repeat-module and so on. Each repeat-module array consists of: (i) a 5′-adaptor repeat-module; (ii) a variable number of core repeat-modules; and (iii) a 3′-adaptor repeat-module. The position of each repeat-module within an array is defined exclusively by the given overlap. To get a sufficient number of different overlaps for the distinct repeat-modules, we made use of the degeneracy of the genetic code and incorporated different codons for identical amino acids at the repeat-module fusion points. In addition, the fusion point between the repeat-modules was varied (Supplementary Figure S1). Thus not every repeat-module does encode a complete 34-amino acid repeat. However, each repeat-module encodes one pair of RVDs and thus determines the base preference of the given repeat. The overlaps were designed in such a way that correctly assembled repeat-modules...
Figure 1. Assembly of dTALE arrays with 10 repeat-modules via *Bsa*I cut-ligation. (a) The cut-ligation concept is shown for two representative repeat-modules that are displayed as white boxes (rep1 and rep2). *Bsa*I recognition sites are shown as pentagons with black arrowheads pointing to the cleavage site. Coloured boxes represent *Bsa*I cleavage sites and colour identity indicates ends with compatible overlaps. The line connecting both *Bsa*I sites represents the vector backbone. *Bsa*I cleavage releases repeat-modules (left side) and creates distinct overlaps at the 5'- and 3'-ends on each repeat-module. Ligation of two repeat-modules results in ligation products that lack *Bsa*I recognition sites (far right side). By contrast, re-ligation of repeat-modules into their donor vectors results in plasmids that still contain *Bsa*I recognition sites. (b) Repeat-modules that are used for dTALE
translate into an array of tandemly arranged 34-amino acid repeats. For each type of repeat-module, we generated variants encoding the RVDs NI (A), HD (C), NK (G) NN (G/A), NG (T) and NS (A/C/G/T) by site-directed mutagenesis (Figure 1b).

Next we generated a TALE gene deletion construct that lacks the repeat array encoding region and that allows in vitro integration of multiple repeat-modules by BsaI cut-ligation thereby generating a functional, full-length designer TALE (dTALE) gene. The deletion was generated using an avrBs3 gene that is flanked by attL sites (pENTR-D-avrBs3). Thus an assembled dTALE gene can be transferred by recombination into any desired Gateway-compatible expression vector. To generate this vector, 17 of the 17.5 repeat-modules of avrBs3 were removed and in their place a cassette with two BsaI recognition sites facing in opposite directions was inserted, yielding TALE-Rep-BsaI-AC (Figure 1c). A and C denotes two distinct BsaI-generated overlaps). The cassette is designed in such a way that BsaI cleavage will release a fragment containing both BsaI recognition sites and generate overhangs that will ligate to the 5’- and 3’-adaptor repeat-modules (Figure 1c). The repeat-deficient TALE gene (TALE-Rep-BsaI-AC) was cloned into a vector containing a kanamycin selection marker, whereas all repeat-modules were cloned into vectors encoding an ampicillin resistance. Thus recovery of cloned repeat-modules can be easily avoided by using kanamycin-containing medium.

Next we carried out BsaI cut-ligations with equimolar amounts of 10 repeat-modules and the repeat-deficient TALE gene to generate a functional dTALE gene. Restriction and sequence analysis of the observed plasmids showed that 90% of the in vitro generated dTALE genes contained the pre-defined repeat arrays (Figure 1d). Since dTALEs with 10 repeats are unlikely to be long enough to bind to a unique sequence within the context of a complex genome, we carried out cut-ligations with 20 repeat-modules using a BsaI cut-ligation. However, many of the observed arrays had <20 repeats, and those that contained 20 repeats did not show the pre-defined order of repeat-modules. We anticipated that optimization of the procedure might enable us to produce repeat arrays containing 20 repeat-modules. However, given the problems that we experienced with the assembly of 20 repeat-modules it seemed unlikely that it would be simple to produce routinely arrays consisting of 30 or more repeat-modules by a single step cut-ligation with an acceptable efficiency.

Assembly of dTALE genes by two subsequent cut-ligations

Given that 10 repeats could be assembled efficiently in a BsaI cut-ligation (Figure 1d), we decided to generate repeat arrays by ligation of two BsaI-generated sub-arrays into a repeat-deficient TALE gene by a subsequent cut-ligation. The second cut-ligation is carried out with the type IIS enzyme BpiI that, like BsaI, produces 4-bp overhangs. For simplicity, we refer to the individual repeat-modules that are flanked by BsaI recognition sites as level 1 modules and to corresponding sub-arrays as level 2 modules. We generated two distinct level 2 modules containing 10 and 7 level 1 modules, respectively. Both level 2 modules are assembled with mostly identical core repeat-modules but differ in their 5’- and 3’-terminal adaptor modules. Therefore in addition to A and C, we defined a new B overlap and generated 5’ and 3’ adaptor modules that connect both level 2 modules (Figure 2a and b). For the assembly of these level 2 modules, we generated two pUC57 derivatives (pUC57-AB-DEST; pUC57-BC-DEST), herein referred to as level 2 destination vectors in which level 1 modules can be assembled by BsaI cut-ligation and from which level 2 modules can be subsequently released by BpiI cleavage. In level 2 destination vectors, BsaI and BpiI are positioned in inverse orientation relative to each other but create identical overlaps (Figure 2b). The two types of level 2 modules (AB and BC level 2 modules, see Figure 2c) produce distinct overhangs when being released by BpiI cleavage, which facilitates specific ligation of the two level 2 modules to each other. For the assembly of two level 2 modules into a functional dTALE gene by BpiI-mediated cut-ligation, we generated a construct containing a repeat-deprived TALE gene, herein referred to as level 3 destination vector. The level 3 destination vector is basically identical to the above-described repeat-deficient TALE gene construct (TALE-Rep-BsaI-AC) but contains two central BpiI instead of BsaI sites (TALE-Rep-BpiI-AC). Using a BpiI cut-ligation, two distinct level 2 modules are fused into a level 3 destination vector to encode a functional dTALE gene (Figure 2c). The level 3 destination vector also encodes the last half repeat that defines the C-terminus end of each TALE repeat array. In order to have the possibility also to select desired RVDs for this terminal half-repeat, we generated six different level 3 destination vectors that encode the RVDs NI (A), HD (C), NK (G), NN (G/A), NG (T) and NS (A/C/G/T).

In summary, with these materials in hand, we can generate any dTALE gene encoding 17.5 or 20.5 repeats with pre-defined RVD composition in just two subsequent

Figure 1. Continued

assembly are represented as boxes. 5’ and 3’ adaptor modules are shown in yellow. Core repeats are shown in white. The position (rep1–rep10), RVD (NI, HD, NK, NN, NG and NS) and overlaps (boldface font) of every module is written in the box. (e) Assembly of a dTALE gene with 10.5 repeats via BsaI cut-ligation. Adaptor and core repeats are shown as yellow and white boxes, respectively. Boldface font indicates the unique overlaps of modules created by BsaI cleavage. Red dots indicate cloned core repeats 3–8 that are not displayed. Purple boxes represent the regions encoding the N- and C-terminal parts of the TALE (N- and C-term) including the last half repeat (rep 10.5). Lines connecting the boxes represent the vector backbones that mediate either ampicillin (red line) or kanamycin (blue line) resistance. The BsaI cut-ligation assemblies repeat-modules 1–10 into the TALE-Rep-BsaI-AC vector. The assembled dTALE gene does not contain any BsaI recognition sites. Black dots indicates repeat modules of the assembled array (3–8) that are not displayed. (d) PCR was used to analyse colonies obtained in a representative BsaI cut-ligation. PCR fragments from 17 colonies (1–17) were separated on a 1% agarose gel and stained with ethidium bromide. The expected size of the PCR product was 1.3 kb and is marked with an asterisk. M: GeneRuler 1 kb DNA Ladder (Fermentas).
Figure 2. Assembly of a dTALE gene with a 17.5 repeat array by two subsequent cut-ligations. If not specified, the shapes and lines are as described in the legend of Figure 1. (a) Adaptor and core repeat-modules that are used for dTALE assembly. Distinct overlaps of repeat modules are defined in bold font (A, B, C, 1–9). (b) Two BsaI cut-ligation facilitate assembly of two distinct level 2 modules that contain 7 and 10 repeat-modules, respectively. BsaI and BpiI recognition sites are shown as pentagons with black arrowheads pointing to their cleavage sites. Level 2 destination vector (continued)
cut-ligations. Notably, the majority of naturally occurring Xanthomonas TALEs, including the well-studied AvrBs3 protein contains 17.5 repeats. Thus the chosen architecture enables us to directly compare the specificity of Xanthomonas TALEs and in vitro generated dTALEs.

We carried out a number of BsaI cut-ligations and observed that desired AB level 2 modules (10 repeat-modules) and BC level 2 modules (7 repeat-modules) were observed in 50 and 95% (Figure 3a). The subsequent BpiI cut-ligations produced on average 95% clones of correct size (Figure 3b). Sequence analysis of ~100 assembled dTALE genes that showed the correct size in gel electrophoresis did not uncover a single mutation. This extremely high level of sequence fidelity is most likely due to the fact that our approach relies on sequence-validated plasmid DNA and does not involve PCR. Thus the generation of dTALE genes by two subsequent cut-ligations worked with high efficiency and fidelity.

**Functional analysis of dTALEs with target-optimized RVD composition**

To test the functionality and specificity of in vitro generated dTALE genes, we took advantage of two sequence-related 19-bp AvrBs3 target boxes [up-regulated by TALE AvrBs3 (UPTAvrBs3) boxes] that are present in the pepper Bs3 promoter (Bs3pUPTAvrBs3 box) (6,17) and the pepper UPA20 promoter (UPA20pUPTAvrBs3 box) (20). The 19-bp AvrBs3 target sites in Bs3 and UPA20 are mostly identical but differ in four basepairs (Supplementary Figure S2). Given that AvrBs3 targets both UPT boxes, we wondered if we could generate a dTALE gene with an identical number of repeat-modules as avrBs3 that, however, due to target-adapted design of the encoded RVDS, would specifically activate promoters containing the related BpiI recognition site (Supplementary Figure S2). Given that AvrBs3 targets both UPT boxes, we wondered if we could generate a dTALE gene with an identical number of repeat-modules as avrBs3 that, however, due to target-adapted design of the encoded RVDS, would specifically activate promoters containing the related BpiI recognition site (Supplementary Figure S2).

We generated the dTALE[UPA20] via two subsequent cut-ligations and transferred it via recombination into the plant-expression vector pGWB5 (GenBank: AB289768.2). In this in planta expression vector, a given gene is driven by the constitutive cauliflower mosaic virus 35S promoter (35S).

To study target specificity of dTALE[UPA20] in vivo we used one of the two previously established GUS-reporter constructs in which the two distinct UPTAvrBs3 boxes from the Bs3 and UPA20 promoter are embedded in an identical promoter context (5). Agrobacterium tumefaciens-mediated delivery of the TALE and dTALE genes in Nicotiana benthamiana leaves showed that dTALE[UPA20] produced GUS staining only in combination with the promoter containing the matching UPA20pUPTAvrBs3 box but not with the promoter containing the highly similar Bs3pUPTAvrBs3 box. In contrast, AvrBs3 produced GUS activity with both promoter constructs (Figure 4). Thus the dTALE gene produced by modular cloning was functional in vivo. Furthermore the target-adapted RVD composition enabled us to generate a dTALE that, in contrast to AvrBs3, discriminated between two highly similar target sequences.

**A modified expression vector simplifies generation of dTALE expression constructs**

In the above described approach, functional analysis of a given dTALE gene requires recombination-based transfer from the entry into a desired expression vector. Implementation of Gateway technology provides a high level of flexibility since assembled dTALE genes can be transferred into any Gateway-compatible expression vector. However, in principle, level 2 modules that encode repeat sub-arrays can also be assembled directly by BpiI cut-ligation to a functional dTALE gene within the framework of a desired expression vector. This approach allows assembly in two rather than three steps and avoids the rather costly Gateway cloning step. Direct TII-mediated cloning requires suitable expression vectors that must by devoid of recognition sites for the TIs enzyme that is used in the assembly of level 2 modules. Inspection of the in planta expression vector pGWB5 (17 961 bp) revealed nine BpiI, and two BsaI sites. We decided to remove both, BpiI and BsaI recognition sites since this would enable us to use this expression vector in cut-ligations with BpiI or BsaI.

To remove BpiI and BsaI recognition sites, we amplified 11 subfragments of pGWB5, each with primers that overlap with internal BpiI or BsaI sites but contain single nucleotide mismatches to eliminate the given recognition sequence (Supplementary Figure S3). Each of the generated 11 pGWB5-derived PCR-fragments contained at its far end a BsaI recognition site that is cleaved off upon BsaI treatment. We designed the overlap that are generated upon BsaI cleavage in such a way that the 11 pGWB5-derived fragments would assemble in the desired order in a BsaI cut-ligation, yielding pGWB5*. The newly assembled pGWB5* is identical in its functional elements to pGWB5 but does not contain BpiI or BsaI recognition sites. We used Gateway recombination to transfer the repeat deprived TALE gene from the level 3 destination vector TALE-Δrep-BpiI-AC into pGWB5*, yielding pGWB5*-TALE-Δrep-BpiI-AC. This pGWB5*-derivative is now a level 3 destination and in planta expression vector that can be used for assembly of level 2 modules in BpiI cut-ligations (Figure 5a). Regardless of whether
the assembly of dTALE genes is in a three-step procedure (involving Gateway recombination into pGWB5) or a two-step procedure (involving BpiI cut-ligation with pGWB5*-TALE-Drep-BpiI-AC), the resulting T-DNAs within the given expression vectors are identical.

We assembled ~50 repeat arrays by BpiI cut-ligation in pGWB5*-TALE-Drep-BpiI-AC and tested generally two clones. Each of these TALE arrays showed the desired composition of repeat-modules (Figure 5b). Thus the assembly of full-length dTALE genes in an expression vector allows rapid, and cost-effective cloning into expression vectors. Using the two-step cloning procedure, we assembled a dTALE gene that was identical in its RVDs to AvrBs3. In planta analysis showed that the assembled dTALE (dAvrBs3) was functionally indistinguishable from the Xanthomonas AvrBs3 protein (Figure 5c). Thus the described two-step generation of dTALE expression constructs provides a rapid and cost-efficient approach for generation dTALE genes.

DISCUSSION
TII-s mediated assembly of repeat-modules—a highly flexible approach

We developed a rapid, simple and highly cost-efficient approach that facilitates generation of dTALE genes that translate into proteins with custom specificity. The approach involves two subsequent cut-ligations fusing individual sequence-validated cloned repeat-modules into a desired array. In the present study, we fused the repeat-modules into the context of a transcription factor. However, the developed cloning approach is highly flexible in several aspects. For example, the designed repeat-modules are flexible with respect to the context into which they can be cloned. One could easily change the level 3 destination vectors to generate TALE nucleases of desired specificity instead of dTALE transcription factors with desired specificity. Our approach is also highly flexible with respect to the size of a repeat array. We have fused two repeat sub-arrays (level 2 modules) containing 7 and 10 repeat-modules into a functional dTALE gene. However, given that 10 repeat-modules could be assembled in a cut-ligation with high efficiency, it should be possible, by creating the appropriate adaptor modules, to generate repeat arrays of 30 or more repeats by fusing multiple level 2 modules. By using identical core repeat-modules for each level 2 module, the amount of effort needed to generate large repeat arrays is limited.

The hierarchical modular cloning system that we present relies on the use of the type IIIs enzymes BsaI and BpiI that are used in subsequent cut-ligations and that produce a full-length dTALE gene. In fact the described approach also facilitates the generation of higher order constructs that can combine multiple dTALEs or other functional units in one construct. For example, terminal BsaI sites flanking the dTALE gene in level 3 destination vectors could be used to combine multiple level 3 modules into a desired level 4 vector. Given that genome editing with TALE nucleases generally requires two distinct proteins with different repeat arrays (11), this might represent a useful extension of the current approach.

Our assembly kit allows incorporation of repeats with six distinct RVDs including NK that was recently found to
interact with G bases preferentially (4,5) and NS, which has been shown to target A, C, G and T bases with almost identical affinity (2). Thus our assembly kit allows generation of dTALEs with high sequence specificity as well as degeneracy within defined positions of the given target site. Previous studies have often made use of conserved pairs of restriction enzyme sites flanking the repeat region (e.g. BamHI, SphI) to move repeat arrays into vectors containing a TALE backbone (21). These conserved pairs of restriction sites are also present in the backbone sequence used in our modular assembly kit, derived from the Xanthomonas avrBs3 gene. Thus constructs made via our approach are compatible for cloning into such existing vectors.

**Design of TALE repeat arrays—how to maximize target specificity**

Target specificity is a major issue in the generation of dTALE repeat arrays and is influenced by the repeat number and type of RVDs. The well-studied TALE AvrBs3, that binds to a 19-bp target sequence, contains three NS-type RVDs, which have been shown to target A, C, G and T nucleotides with almost identical affinity (2). We assumed that target specificity of AvrBs3 could be improved if RVDs with ambiguous target specificity are replaced with RVDs with tight sequence specificity. To test this hypothesis we made use of two similar 19-bp AvrBs3 target sequences in the pepper Bs3 and pepper UPA20 promoter that differ in four basepairs. We generated a dTALE that has the same number of repeats as AvrBs3 but that does not contain RVDs with ambiguous target specificity and that was designed to differentiate between the two similar AvrBs3 target sites in the Bs3 and UPA20 promoters. Indeed, this dTALE with optimized RVD composition discriminated between the two similar AvrBs3 target sequences and activated specifically the pepper UPA20 but not the Bs3 promoter. These data demonstrate that a target-adapted RVD composition facilitates generation of repeat arrays with high specificity. Previously, we engineered two AvrBs3 derivatives with four additional repeat units that target a 23-bp target sequence.

**Figure 5.** Direct assembly of two TALE repeat sub-arrays into a modified plant expression vector that lacks BpiI recognition sites. If not specified, the shapes and lines are as described in the legend of Figure 1. (a) A BpiI cut ligation facilitates direct assembly of two level 2 modules into an expression vector. Blue and green rectangles represent the 35S promoter (35S P) and the C-terminal epitope tag (GFP). The blue and black lines that connect the boxes represent the vector backbones that contain spectinomycin and kanamycin resistance markers, respectively. (b) Cut-ligation efficiency observed in BpiI-mediated generation of dTALE gene expression constructs. Plasmids obtained in the cloning of two level 2 modules into the level 3 destination vector were analysed by HindIII and SacI digestion. Fragments obtained from 10 colonies (1–10) were separated on a 1% agarose gel and stained with ethidium bromide. The expected sizes were 0.8, 4.3 and 14.6 kb are marked (asterisks). M: GeneRuler 1 kb DNA Ladder (Fermentas). (c) Functional analysis of a dTALE that was assembled via cut ligation into the modified in planta expression vector pGWB5*. The de novo assembled dTALE gene (davrBs3) is identical in its RVD composition to the Xanthomonas avrBs3 gene. Agrobacterium tumefaciens was transformed with either pGWB5* containing davrBs3 or pGWB5 containing avrBs3. Dashed lines mark the inoculated areas. Using A. tumefaciens transient transformation the two TALE genes were delivered into the leaf of a Capsicum annuum genotype that contains the Bs3 resistance gene (ECW-30R). Two days after infiltration, the leaves were cleared in ethanol to visualize the AvrBs3-triggered and Bs3-mediated hypersensitive response (dark areas).
box and that activate specifically either the pepper Bs3 or UPA20 promoter (5). Thus we could show that RVD composition as well as the size of the repeat array affects target specificity.

**Alternative approaches for assembly of TALE repeats**

A most recent manuscript provides an alternative protocol for modular assembly of dTALE genes that encode 12.5 repeats (13). The approach relies on four cloned repeat monomers (NI, HD, NN, NG) that are linked to type IIs recognition sites via PCR, which facilitates assembly of a desired repeat array into a TALE gene deletion construct by two sequential ligations. Thus this approach facilitates assembly of TALE arrays using four cloned repeat monomers and TALE gene deletion constructs, respectively. At first sight, the method is very attractive, because only a few gene constructs are needed and thus the upfront work is quite limited. On the other hand, the assembly procedure is rather complex and laborious and involves amplification of twelve individual repeat-modules, subsequent PCR product purification, BsaI cleavage (generates distinct 4-bp overlaps for each module), purification of cleavage products, ligation of three sub-arrays (each with four repeats), gel purification and subsequent PCR amplification of the three repeat sub-arrays, subsequent PCR product purification, cleavage of repeat sub-arrays and vector (contains TALE gene deletion that lacks the repeat array), purification of cleavage products and finally ligation of three sub-arrays into the TALE gene backbone. In summary, the approach involves two rounds of PCRs, five purification steps and two ligations. Our assembly kit facilitates assembly of 17.5 TALE repeats in two subsequent single-tube cut-ligations. Importantly our approach does not require purification or PCR steps, instead relying on ligation of sequence-validated plasmid inserts. Thus assembly of dTALE genes with this approach is less laborious and results in arrays with 17.5 instead of 12.5 repeats. Since our approach relies on ligation of sequence-validated plasmids it seems likely that the accuracy of these dTALE genes is higher as compared to TALE genes that were produced by ligation of amplification products that have undergone two subsequent PCRs. While assembly of TALE genes by our assembly kit has in many ways advantages, it needs to be emphasized that our kit required 80 gene constructs (72 repeat-modules, two level 2 destination and six level 3 destination vectors) and therefore had a substantial upfront workload. In its current state, our kit enables the production of many TALE genes quickly, at low cost and with high efficiency. Since the established assembly approach does not involve any complex manual procedures it could be carried out by a pipetting robot. This would facilitate implementation of TALE technology in high throughput applications.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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