Zinc finger nuclease and homing endonuclease-mediated assembly of multigene plant transformation vectors

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Running title: Multigene binary vectors

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Binary vectors are an indispensable component of modern *Agrobacterium*-mediated plant genetic transformation systems. A remarkable variety of binary plasmids has been developed to support the cloning and transfer of foreign genes into plant cells. The majority of these systems is, however, limited to the cloning and transfer of just a single gene of interest. Thus, plant biologists and biotechnologists face a major obstacle when planning the introduction of multigene traits into transgenic plants. Here we describe the assembly of multi-transgene binary vectors by using a combination of engineered zinc finger nucleases (ZFNs) and homing endonucleases. Our system is composed of a modified binary vector that has been engineered to carry an array of unique recognition sites for ZFNs and homing endonucleases and a family of modular satellite (SAT) vectors. By combining the use of designed ZFNs and commercial restriction enzymes, multiple plant expression cassettes were sequentially cloned into the acceptor binary vector. Using this system, we produced binary vectors that carried up to nine genes. *Arabidopsis* protoplasts and plants were transiently and stably transformed, respectively, by several multigene constructs and the expression of the transformed genes was monitored across several generations. Because ZFNs can potentially be engineered to digest a wide variety of target sequences, our system allows overcoming the problem of the very limited number of commercial homing endonucleases. Thus, users of our system can enjoy a rich resource of plasmids that can be easily adapted to their various needs and since our cloning system is based on ZFN and homing endonucleases, it may be possible to reconstruct other type of binary vectors and adapt our vectors for cloning on multigene vectors systems in various binary plasmids.
To date, biotechnological improvement of plant species has largely been limited to the introduction of single novel traits into the genomes of target plants. However, many agronomic traits may depend on complex interactions between several proteins, and biotechnological improvement of a particular species may thus require the delivery and expression of whole, complex metabolic pathways (Halpin et al., 2001; Daniell and Dhingra, 2002; Lyznik and Dress, 2008; Naqvi et al., 2010). In addition, transgenic modification of commercially important plant species also calls for the development of novel tools for the removal, addition and replacement of existing transgenes in plant cells (Hanin and Paszkowski, 2003; Porteus, 2009; Moon et al., 2010; Weinhthal et al., 2010). Thus, two of the major challenges that are still to be addressed in plant biotechnology are the development of a technology to combine several transgenic traits in a single plant by stacking a number of genes in the same chromosomal locus into a single multigene array and the successive manipulation of this array by genome editing (Dafny-Yelin and Tzfira, 2007; Lyznik and Dress, 2008; Taverniers et al., 2008; Naqvi et al., 2010). The main strategies for the introduction of multiple genes into plant cells include re-transformation, co-transformation, sexual crossing and transformation of multigene constructs (Reviewed by Dafny-Yelin and Tzfira, 2007; Naqvi et al., 2010). While proven useful for the production of transgenic plants with novel traits, re-transformation, co-transformation and sexual crossing approaches all suffer from several flaws (Dafny-Yelin and Tzfira, 2007; Naqvi et al., 2010). Re-transformation and sexual crossing, for example, are time consuming and rely on the use of different selectable marker genes for each transformation/crossing cycle. In co-transformation, it is virtually impossible to predict the number of insertions and the distribution of the inserted genes across the plant genome. In addition, co-transformation may result in complex integration patterns which may hinder the use of such plants for commercial purposes for which single and well-characterized integration events are required. While sexual crosses may be simplified by
using marker free transgenic plants, the delivery of multiple genes as a single, well-defined, multigene array would perhaps be the simplest and most reliable method for the production of multigene transgenic plants. Furthermore, multigene arrays may also offer the advantage of simplifying successive manipulations of multigene assays in transgenic plants by genome editing technologies (Porteus, 2009; Weinthal et al., 2010). Yet, while multigene constructs have been successfully used in several studies (e.g. Bohmert et al., 2000; Bohmert et al., 2002; Wu et al., 2005; Zhong et al., 2007; Fujisawa et al., 2009), the assembly of multigene constructs remains challenging, being nearly impossible to achieve by traditional cloning methods (Dafny-Yelin and Tzfira, 2007; Naqvi et al., 2010).

Only a handful of dedicated vector assembly systems has been developed in the past several years for the assembly of multigene transformation vectors (e.g. Cheo et al., 2004; Sasaki et al., 2004; Karimi et al., 2005; Chen et al., 2006; Wakasa et al., 2006; Chen et al., 2010). Most of these vector assembly systems have a rather limited capacity and have hence been utilized for the delivery of only a small number (i.e., up to five) of transgenes in a single array. Noteworthy here is the assembly system of Lin et al. (2003), in which a combination of the Cre/loxP recombination system and two homing endonucleases is used for successive cloning of a potentially infinite number of genes onto a transformation-competent artificial chromosome (TAC)-based vector. Lin et al. (2003) used their system for delivery of eight different genes from two independent transferred DNA (T-DNA) molecules that were launched from a single binary vector into the rice genome. However, a crucial limitation of the ingenious approach of Lin et al. (2003) was that in contradiction to classical cloning by restriction enzymes, once assembled into the binary vector, new DNA fragments can no longer be removed or replaced by others.

Another important contribution to the field is the unique multiple-round in vivo site-specific assembly (MISSA) system of Chen (2010) that enables in vivo recombination-based assembly
of multigene vectors. The system was used to construct several multi-transgene binary vectors and led to the production of transgenic plants with eight transgenes (Chen et al., 2010). A number of other recombination-based cloning strategies has also been developed for the construction of multigene plant transformation vectors (Cheo et al., 2004; Sasaki et al., 2004; Karimi et al., 2005; Chen et al., 2006; Wakasa et al., 2006; Chen et al., 2010). However, here too, the irreversible nature of the recombination-based reactions does not enable the modification of existing binary vectors, and users of such systems may be required to completely rebuilt their transformation vectors to give different gene combinations. Multigene transformation vectors have also been assembled by using both traditional cloning methods. Wu et al. (2005), for example, combined the use of type-II restriction enzymes and Gateway®-mediated cloning to construct several binary vectors with up to ten different expression cassettes and used the combination to reconstitute the docosahexaenoic acid biosynthetic pathway in Indian mustard. Fujisawa et al. (2009) combined the use of type-II restriction enzymes and homing endonucleases to construct a seven-transgene-long T-DNA molecule, which they applied to genetically modify the carotenoid biosynthesis pathway in Brassica napus. Other examples include the engineering of soybean, potato, rice, Arabidopsis and several other plant species by using four- to five-transgene-long transformation vectors (reviewed in Naqvi et al., 2010; Peremarti et al., 2010). While proven successful for the metabolic engineering of various plant species, such vectors were mostly custom designed to their specific tasks, and their modification for other metabolic pathways and multigene traits may prove to be difficult or even impossible to achieve.

A versatile and modular system for the assembly of multigene binary vectors has been developed by Goderis et al. (2002) who exploited a set of homing endonucleases to construct a vector system facilitating the successive cloning of independent plant expression cassettes. The principles of the assembly system and successor pSAT vector system was previously reported.
by Tzfira et al. (2005). An important advantage of this method, over the above described approaches, is its modularity. Plant expression cassettes can easily be removed or replaced from existing binary vectors. Nevertheless, the capacity of the vector system and its successors is limited by the very small number of commercially available homing endonucleases.

We have recently shown that zinc finger nucleases (ZFNs), engineered restriction enzymes that can be designed to bind and cleave long stretches of DNA sequences (Mani et al., 2005), can be used for molecular cloning, and we have used such enzymes for the construction of dual gene binary vectors (Zeevi et al., 2008). In the present paper, we describe a modular binary vector assembly system that represents a fundamental improvement over the original design of Goderis et al. (2002) in that it supports the construction of multigene transformation vectors not only by homing endonucleases but also by designed ZFNs. We describe the design of our system and demonstrate its use by cloning nine different DNA fragments onto a modified binary transformation vector. We also show that such vectors can be used for the production of multigene transgenic plants and for transient expression of multigenes in plant protoplasts. The advantage of using ZFNs for the construction of multigene transformation systems is discussed.

Results

Design of the binary vector and novel pSATs

We followed the basic design of the pAUX (Goderis et al., 2002) and its successor pSAT (Chung et al., 2005; Tzfira et al., 2005; Dafny-Yelin et al., 2007; Dafny-Yelin and Tzfira, 2007) family of plasmids to facilitate the assembly of multigenes into a single binary plasmid. In the pSAT system, functional plant expression cassettes are individually cloned into different pSAT plasmids (e.g., geneB in pSAT2, Figure 1A), and an expression cassette from each type of pSAT plasmid can be cloned onto pPZP-RCS1- or pPZP-RCS2-based (Goderis et al., 2002) Agrobacterium binary plasmids by using matching homing endonucleases (Figure 1A). As was
the case for the original set of pAUX plasmids, the original set of pSAT plasmids was composed of seven different versions, in which the expression cassettes were flanked by Ascl (pSAT1), Ascl and I-Ppol (pSAT2), I-Ppol (pSAT3), I-Sce1 (pSAT4), I-Ceu1 (pSAT5), PI-Psp1 (pSAT6) and PI-Tli1 (pSAT7). Here we subsequently expanded this set to include pSAT10, pSAT11 and pSAT12, in which the expression cassettes were flanked by ZFN10, ZFN11 and ZFN12 (3x3 finger ZFNs), respectively (e.g., geneA in pSAT12, Figure 1A). We also constructed pRCS11.1, which is a modification of of pPZP-RCS1 that was engineered to include, in addition to the original recognition sites of Ascl, I-Ppol, I-Sce1, I-Ceu1, PI-Psp1 and PI-Tli1, recognition sites to the ZFN10, ZFN11 and ZFN12 zinc finger nucleases (Figure 1A). This system was subsequently used for the construction of several multigene binary plasmids, as described below.

Construction of multigene vectors by exploiting ZFNs

We constructed a set of pSAT plasmids carrying a wide variety of genes. Table 1 lists the different pSAT plasmids constructed, the genes they carry, and the enzymes used for their cloning into pRCS11.1. We started by cloning the constitutive expression cassette of the encoplastic-reticulum (ER)-bound chalcone synthase (CHS) gene (Pelletier and Shirley, 1996), tagged with EYFP from pSAT10.EYFP-CHS by using ZFN10 to produce pRCS11[10.EYFP-CHS]. Next we added the constitutive expression cassette of the P protein of Sonchus Yellow Net Rhabdovirus (SYNV) (Goodin et al., 2001; Goodin et al., 2002) tagged with DsRed2 from pSAT11.DsRed2-P by using ZFN11, to produce pRCS11.1[10.EYFP-CHS][11.DsRed2-P].

The key to the versatility of the original pSAT/pRCS2 multigene assembly system lies in the ability it offers not only to add but also to remove and replace DNA fragments by homing endonucleases during the construction of the various binary plasmids (Tzfira et al., 2005). Thereafter, we demonstrated that ZFNs too can also be used to remove and replace DNA
fragments from existing binary constructs. We started by adding the plasmid backbone from pSAT12.MCS into pRCS11.1[10.EYFP-CHS][11.DsRed2-P] and produced pRCS11.1[10.EYFP-CHS][11.DsRed2-P][12.AMP]. As expected, the resultant plasmid conferred resistance to ampicillin in *Escherichia coli* cells. We also observed that DNA preparation of this plasmid from an overnight *E. coli* culture resulted in much higher DNA yield than that obtained with its progenitor or with other pRCS2-based vectors, most probably due to the presence of the ColE1 origin of replication on the pSAT12.MCS backbone. We then used ZFN12 to remove the pSAT12.MCS backbone and replaced it with a constitutive expression cassette of the chromoplast-specific carotenoid-associated protein ChrD from *Cucumis sativus* (Vishnevetsky et al., 1996; Vishnevetsky et al., 1999) tagged with RFP from pSAT12.ChrD-RFP to produce pRCS11.1[10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]. As expected, this plasmid no longer conferred resistance to ampicillin in *E. coli* cells. We tested the expression of the three reporter genes from pRCS11.1[10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP] in *Arabidopsis* protoplasts. As expected, the YFP-tagged CHS associated with the rough ER throughout the cell, around theoplasts and around the nucleus (Figure 2A); DsRed2-P was observed in the cytoplasm clustered around the chloroplasts; and ChrD-RFP, which has been previously characterized as a chloroplast associated protein (Ben Zvi et al., 2008), indeed localized in the chloroplasts (Figure 2B). The clustering of DsRed2-P was probably due to the tendency of DsRed2 to aggregate in living cells. We next added a fourth plant expression cassette for the SYNV N protein and produced pRCS11.1[2.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]. Interaction of the SYNV N protein with SYNV P is required to translocate it to into sub-nuclear compartments in *Nicotiana bentamiana* cells (Goodin et al., 2001; Goodin et al., 2002). Indeed, nuclear localization and sub-nuclear compartmenting of the DsRed2-P signal was observed in pRCS11.1[2.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]-infected protoplasts (Figure 2F and 2K) but not in pRCS11.1[10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]-infected protoplasts
(Figure 2B and 2I). Our data thus show that designed ZFNs can be used for the construction of multigene vectors and that such vectors can be used to drive the simultaneous expression of several genes in plant cells.

Assembly of multigene binary vectors for stable transformation

pRCS11.1[2.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP] carried four pairs of repetitive elements (i.e., CaMV dual 35S promoters and CaMV 35S terminators). We next tested whether the four transgenes and two additional genes (i.e., a hygromycin resistance gene driven by the control of the octopine synthase promoter and terminator and the Basta resistance gene driven by the control of the Rubisco small subunit promoter and terminator) can be stably transformed and expressed in transgenic plants. Many commonly used binary vectors have been designed with the selection marker cassette cloned next to the T-DNA’s left border (van Engelen et al., 1995; Hellens et al., 2000). We elected to clone the hygromycin-resistant expression cassette near the T-DNA’s right border. This design, while having the potential to produce transgenic plants with truncated T-DNAs, allowed us to examine whether repetitive elements have a negative impact on the T-DNA structure during transformation. To achieve the above design, we first produced pRCS11.1[1.HYG][2.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP], in which the hygromycin-resistance gene expression cassette was cloned into the Ascl site of pRCS11.1[3.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]. We then added the plasmid backbone from pSAT3.MCS into the I-Ppol site to facilitate future cloning and subsequent isolation of T-DNA-plant junction sequences by plasmid rescue, and the Basta hygromycin-resistant expression cassette into the I-CeuI site of pRCS11.1[3.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]. Finally, we used the seven-transgene-long pRCS11.1[1.HYG][2.N][3.AMP][5.BAR][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP] binary plasmid (Figure 3A) to produce transgenic Arabidopsis plants. Confocal microscopy analysis
revealed that 9 of our 27 transgenic lines exhibited yellow and red fluorescence expression patterns similar to the patterns of protoplasts infected with pRCS11.1[2.N][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP] (data not shown). Molecular analysis of two randomly selected transgenic lines revealed that PCR amplification of their T-DNA regions was similar to that of the seven-transgene-long binary vector (Figure 3A); these findings indicate that our transgenic lines carried all the T-DNA molecule-encoded transgenes within their genome.

We then allowed several randomly selected transgenic lines to mature and set seed. Shown in Figure 4 are examples of hygromycin- and Basta-resistant seedlings. Collectively, our data indicate that the T-DNA-encoded seven transgenes were stability expressed and inherited.

Production of nine-transgene-long transgenic plants
We constructed a nine-transgene-long binary vector by adding the GUS and PAP1 (Arabidopsis transcription factor production of Anthocyanin pigment) (Borevitz et al., 2000; Ben Zvi et al., 2008) expression cassettes into the I-SceI and PI-PspI sites of pRCS11.1[1.HYG][2.N][3.AMP][5.BAR][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP]. In the final binary vector, pRCS11.1[1.HYG][2.N][3.AMP][4.GUS][5.BAR][6.PAP1][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP], GUS expression is controlled by the hsp18.1 heat shock promoter (Takahashi and Komeda, 1989), while PAP1 expression is controlled by CaMV dual 35S promoter. We used this vector to produce over 30 hygromycin-resistant transgenic Arabidopsis plants, of which about half exhibited PAP1-related reddish hypocotyls and cotyledons during germination. We further characterized these lines and determined whether they are likely to harbor the full T-DNA molecule by monitoring DsRed2-P expression, which was located next to the left border. Twelve T₀ plants of the PAP1 transgenic plants also expressed DsRed2-P, which indicated that they are likely to harbor at least one full T-DNA copy. Molecular analysis of several lines revealed that PCR amplification of several regions on their T-
DNA regions was similar to that of the nine-transgene-long binary vector, as demonstrated, for example, in Figure 3B. These observations indicate that the plants carried the entire T-DNA molecule-encoded genes.

We allowed seven of the transgenic lines to mature and set seed and determined the inheritance and stability of the T-DNA in the next generations. T₀ plants of lines L6, L7 and L10 exhibit a plausible single insert segregation pattern when grown on a hygromycin-containing medium (Table 2). T₁ seedlings of lines L2, L6, L7 and L10, T₂ seedlings that derived from homozygous (i.e., L6-1) and heterozygous (i.e. L6-2 and L10-2) parents, exhibited resistance to Basta, as did T₂ seedlings derived from lines L2 and L3 (Table 2 and Figure 5A). Confocal microscopy analysis revealed that all T₀ lines and their progeny (Table 2) exhibited red and yellow fluorescence, as shown, for example, for line L6-1 (Figure 5C-F). Analysis of several T₂ lines revealed that they all expressed heat-shock-induced GUS expression (Figure 5G). GUS expression was not observed in untreated plants. In addition, all T₁, T₂ and T₃ hygromycin-resistant plants exhibited PAP1 expression (Table 1), as shown, for example, for L3-3-1 (Figure 5B) and L6-2-1-1 (Figure 5F) plants. The segregation analyses suggest that a single transgene locus was obtained in some of the analyzed lines (i.e. L6, L7 and L10).

We further analyzed four transgenic lines to demonstrate the stability and integrity of the T-DNA inserts in T₃ (lines L2 and L10) and T₄ (lines L6 and L7) hygromycin-resistant plants, by PCR amplification of overlapping fragments of their T-DNA region (Figure 6). When combined with segregation analysis, our molecular data further supported the notion that our transgenic plants carry full T-DNA inserts. No less important, our data clearly show that multigene-expressing plants can be produced using our multigene transformation vectors.
Discussion

We demonstrated that by using engineered ZFNs we can overcome the limitation imposed by the small number of commercial homing endonucleases to the construction of multigene binary vectors. We used several ZFN- and homing-endonuclease-constructed binary vectors for transient and stable genetic transformation of plant cells. We then showed that transgenic plants that had been stably transformed by nine-transgene-long T-DNA stably expressed the cloned genes across several generations. To the best of our knowledge, our report is one of just a small number of studies in which a very large number of independently expressed genes were delivered using a single T-DNA molecule into plant cells and moreover it is the only report in which a multigene vector has been assembled by a modular, step-by-step construction method (reviewed in Naqvi et al., 2010). Thus, our approach represents an important technical leap in the construction of multigene plant transformation vectors.

There are three main advantages of our system over other multigene construction systems, as detailed below. First, we demonstrated that ZFNs can be used in a manner similar to the technique that relies on homing endonucleases and that ZFNs can be exploited not only for adding but also for replacing DNA fragments from existing multigene binary vectors. Thus, in contrast to many other multigene vector assembly systems (e.g. Lin et al., 2003; Karimi et al., 2005; Wakasa et al., 2006; Chen et al., 2010), our system has the dual advantage of being modular and of enabling the modification of existing plant expression cassettes at any stage during the construction of the transformation vector. In addition, the cloning capacity of existing pRCS11.1-based binary vectors can be increased by reengineering their multiple cloning sites (MCSs) for additional features (which may include, for example, recognition sites for new ZFNs and homing endonucleases or even sites for Cre/Lox and Gateway® recombination systems) not only prior to but also during the process of assembling multigene vectors. This can be achieved by cloning an expanded ZFN-recognition-MCS into the growing pRCS11.1-based
binary vectors, which can then be used for successive additions of new expression cassettes. This feature further adds to the modularity of our ZFN-based vector assembly system, as compared with the more rigid design of other binary vector assembly systems.

Second, because ZFNs can be designed to target and digest an extremely large number of target sequences (Maeder et al., 2008), novel ZFNs can be developed and used to expand our nine-gene-long system beyond its current capacity. Since most pSAT vectors share a similar basic structure (Tzfira et al., 2005), it is easy to convert existing pSAT vectors into novel vectors by creating pSAT backbones with new ZFN recognition sites. Furthermore, since we used semipalindromic ZFN recognition target sequences, it may be feasible to adapt existing ZFNs (i.e., those that have been developed for targeting experiments in various non-plant eukaryotic cells [e.g. Urnov et al., 2005; Carroll et al., 2008; Zimmerman et al., 2008; Geurts et al., 2009; Takasu et al., 2010]) for cloning purposes. Indeed, we have previously showed that ZFNs can be expressed and purified to the level of molecular-biology reagents by using relatively simple expression and purification steps (Zeevi et al., 2010; Tovkach et al., 2011). While specific modifications may be required to adapt our expression and purification system for novel ZFNs, the simplicity of the process and the availability of dedicated ZFN assembly and expression vectors for bacterial expression and for plant genome editing (Tovkach et al., 2010; Zeevi et al., 2010) may further facilitate the use of our system for assembly of multigene vector systems.

Third, our system supports the use of a very large family of pSAT- and pAUX-based plasmids. The pSAT family of plasmids is composed of plasmids that have been designed to facilitate: (i) the overexpression of target genes under the control of various promoters and terminators (Chung et al., 2005; Tzfira et al., 2005), (ii) analysis of protein-protein interactions by using the bimolecular fluorescence complementation and multi-color bimolecular fluorescence complementation assays (Citovsky et al., 2006; Lee et al., 2008), (iii) the fusion of target genes to various autofluorescence proteins (Tzfira et al., 2005), (iv) RNAi-mediated downregulation...
(Dafny-Yelin et al., 2007), (v) expression of ZFNs (Tovkach et al., 2009; Tovkach et al., 2010), (vi) Gateway®-mediated gene cloning (Tzfira et al., 2005; Chakrabarty et al., 2007) and (vii) expression of epitope-tagged proteins (Tzfira T., unpublished data). Thus, users of our system can enjoy a rich resource of plasmids that can be easily adapted to their various needs. Furthermore, since our cloning system is based on ZFN and homing endonucleases, it may be simple to transfer – or reconstruct – the ZFN and homing endonuclease MCS from pRCS11.1 into any other type of binary vectors and adapt our pSAT set of vectors for cloning on multigene vectors systems in various binary plasmids. Such binaries can, for example, be based on the TAC (Liu et al., 1999; Lin et al., 2003) and BIBAC (Hamilton, 1997; Frary and Hamilton, 2001) vectors which can facilitate the cloning and transfer of extremely large number of independent plant expression cassettes.

An important feature of our multigene vector assembly system lies in the unique structure of the final array of transgenes, in which each expression cassette or gene is flanked by pairs of unique ZFNs or homing endonucleases. This structure may facilitate genomic editing of multi-transgene arrays in transgenic plants by harnessing the cells' non-homologous end joining (NHEJ) DNA repair pathway. Both homing endonucleases and ZFNs have been used for gene targeting in plant cells (i.e., site-specific mutagenesis, gene addition deletion and/or replacement) (e.g. Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira et al., 2003; Cai et al., 2009; Shukla et al., 2009; Townsend et al., 2009). Yet, while most ZFN-mediated genome editing systems rely on homologous recombination between the donor DNA and the target genome, the presence of repetitive elements (i.e., promoter and terminator sequences) in multi-transgene arrays may hinder homologous recombination-mediated genome editing of such structures. Thus, genome editing of multi-transgene arrays, i.e., removal, replacement and addition of expression cassettes by NHEJ, can potentially be achieved by transient ZFN-expression in target cells, with or without the addition of a donor DNA molecule (Weinthal et al.,
Indeed, both I-Scel- and I-CeuI-mediated transgene addition (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira et al., 2003) and ZFN-mediated transgene deletion (Petolino et al., 2010) have been reported in plant cells. In addition, we have recently demonstrated that ZFN- and NHEJ-mediated gene replacement is also feasible in plant species (Weinthal, Taylor and Tzfira, unpublished data). Worth noting is the fact that by flanking each expression cassette with semipalindromic sequences, the editing process may be technically simplified since, in contrast to most homologous recombination-mediated gene-replacement methods, it will require the expression of just one ZFN monomer and not pairs of ZFNs. Also worth noting is that other types of engineered enzymes [i.e. engineered meganucleases (Gao et al., 2010) and TALENs (Cermak et al., 2011; Maftouz et al., 2011)] have also been used for genome editing in plant cells. Developing procedures for the expression and purification of engineered meganucleases and TALENs for cloning purposes will further extend the repertoire of enzymes suitable for assembly of multigene vector systems and their successive manipulation in plant cells.

Production of transgenic plants often results in a wide range of transgene-related phenotypes, a phenomenon that is typically attributed to a transgene positional affect (Matzke and Matzke, 1998). Thus, obtaining plants with superior transgenic performances (e.g., high and stable expression levels during the plant life span and across several generations) often calls for the production and screening of a large number of transgenic plants. Increasing the number of transgenes in plants is likely to increase the number of transgenic plants that need to be produced, screened and selected for the desired performances. This procedure may be time consuming and labor intensive, especially if the different transgenes are scattered across the genome. Linking several transgenes to the same genomic location may assist in the screening process and in maintaining superior clones across several generations. The tendency of multiple DNA molecules, derived from co-transformation of several different Agrobacterium
strains or from co-bombarded of several plasmids, to integrate into the same genomic locus (De Neve et al., 1997) has been utilized as a viable method for the production of single-locus multigene transgenic plants (reviewed in Naqvi et al., 2010; Peremarti et al., 2010). While this approach has been successfully used to produce transgenic plants in which up to 11 transgenes are co-integrated into the same locus (Chen et al., 1998; Zhu et al., 2008; Naqvi et al., 2009), it is impossible to predict the physical organization and the arrangement of co-transformed DNA molecules in the transgenic plants. Indeed, it has been suggested that future progress in multigene transformation may depend not only on driving the transgenes under the specific combinations of promoter and terminator sequences but also on organizing the transgenes in a predetermined pattern (Peremarti et al., 2010). Our modular system can thus be efficiently adapted for the analysis of various regulatory elements and gene organization patterns by shuffling between different pSAT vectors.

We observed a variety of phenotypes among our different transgenic lines, as was evident, for example, by differences in hygromycin resistance (Figure 3A) and GUS expression (Figure 5G). We also observed that transgenic lines in which all the transgenes were expressed across several generations could be obtained (Table 2). Yet, we could not identify a clear correlation between the phenotypes of different transgenes on a given T-DNA or between different lines. Thus, for example, lines that exhibited strong resistance to hygromycin did not necessarily exhibit high levels of PAP1 or GUS expression, while plants that exhibited high levels of PAP1 expression, did not yield high intensity of their fluorescence genes. Similarly, Fujisawa et al. (Fujisawa et al., 2009) reported that they could not associate the expression level of individual transgenes with the performances of three transgenic lines; similarly, they could not determine the efficiency of a specific promoter in a given multigene array due to differences in the gene expression levels, which did not correlate with their promoter types. Similarly, Chen et al. (2010) reported phenotypical variation not only between the same trait in different transgenic lines but
also between individual traits, driven under the same promoter in a given multigene array, in a selected transgenic line. It thus seems that not only may the expression levels of individual genes vary among different lines, but also that similar promoters, driving different genes, may behave differently in a particular multigene cluster. We are currently applying our multigene vector assembly system to construct a set of multigene binary vectors in which identical expression cassettes will be cloned to different organizations. We will use our vectors to produce collections of independent transgenic plants, which will be subjected to gene expression analysis in an attempt to produce the necessary data that will assist in defining the putative rules for the organization of regulatory elements within a given construct.

Material and Methods

Construction of pSAT vectors

To construct pSAT12.MCS, we PCR-amplified the pSAT6.MCS (Tzfira et al., 2005) backbone by using

5'ATAAGAATGCGGCCGCTAAGTGGTGCTGTAAGTATGGATGCACTGTAATCATGGTCATAGCTGTTTCC and

5'GACGCACCGGTAGCACCAACACTTACGTTGGTGCTGGCACTGGCCGTCGTTTTACAACG

and ligated the Agel-NotI digested PCR product to the Agel-NotI 1.2-kb fragment from pSAT6.MCS (Tzfira et al., 2005). The construction of pSAT10.MCS and pSAT11.MCS was previously described (Zeevi et al., 2008). pRCS11.1 was constructed by cloning the SmaI fragment of self-annealed pairs of primers 5'

TCCCCCGGAGACCCCACAAACTTACTTGGGGAAGCACCACACTTACGTTGGTGCTCCCGGGGGA and 5'

TCCCCCGGGAGACCCACAAACTTACGTTGGTGCTTTCCCAAGTAAGTGGTGGGAACCC

17
GGGGGA which encode the ZFN11 and ZFN12 sites and KpnI fragment of self-annealed pairs of primers 5’ GGGGTACCTGCATCCATGTAAGTATGGATGCAGGTACCCC and 5’ GGGGTACCTGCATCCATACTTACATGGATGCAGGTACCCC, which encodes the ZFN10 into the same sites of pRCS2. The ChrD-RFP expression cassette was transferred from pSAT6A.ChrD-RFP (Citovsky et al., 2006) as an AgeI-NotI fragment into pSAT12.MCS to produce pSAT12.ChrD-RFP. The construction of pSAT10.YFP-CHS and pSAT11.DsRed2-P has been previously described (Zeevi et al., 2008). To produce pSAT2.N, the nucleocapsid protein (N) of the sonchus yellow net virus expression cassette was transferred as an AgeI-NotI fragment from pSAT3.N (Tzfira et al., 2005) into pSAT2.MCS (Tzfira et al., 2005).

pSAT5A.RbcsP.BAR was constructed by transferring the BAR encoding sequence as an XmaI-XhoI fragment from pSAT1A.ocsP.BAR (Chung et al., 2005) into the same sites of pSAT5A.RbcsP.MCS (Chung et al., 2005). pSAT4.hsP.GUS was constructed by transferring the Ncol-BamHI GUS coding sequence from pRTL2-GUS into pSAT4.hsP.MCS (Tovkach et al., 2009). pSAT6A.PAP1 was constructed by transferring the KpnI-BamHI PAP1 coding sequence from pCHS3-PAP1 (Borevitz et al., 2000) into pSAT6A.MCS (Chung et al., 2005).

Assembly, expression and purification of ZFNs
The coding sequences of ZFN10, ZFN11 and ZFN12 were assembled from overlapping oligonucleotides and cloned into the bacterial expression vector pET28-XH to produce pET28-ZFN10, pET28-ZFN11 and pET28-ZFN12. The protocol for molecular assembly of ZFNs from overlapping oligonucleotides, and their expression and purification have been previously described in detail (Zeevi et al., 2010). Briefly, the ZFN DNA binding coding sequences were assembled by a single PCR reaction from a mixture of ZFN backbone primers and ZFN finger specific primers. Each PCR product was then cloned into pET28-XH, where it was fused with the FokI endonuclease domain and a 6xHis-tag. For expression in E. coli cells, the ZFN
expression vectors were transferred into BL21 GOLD (DE3) PlysS cells (Stratagene). The cells were cultured, harvested and lysed, and the extracted proteins were purified on Ni-NTA agarose beads (Qiagen) as previously described (Zeevi et al., 2010). Eluted ZFNs were stored at -20 C in 50% glycerol. Alternatively, we used the Expressway in vitro protein synthesis system (Invitrogen) for in vitro expression of ZFNs.

Construction of binary vectors
For ZFN-mediated digestion of pSAT expression cassettes and binary plasmids, about 200 ng, each, of acceptor and donor plasmids were digested with 0.05–1 µl of purified enzyme in 10 mM Tris [pH8.8], 50 mM NaCl, 1 mM DTT, 100 µM ZnCl₂, 50 µg/ml BSA and 100 µg/ml tRNA in a total reaction volume of 20–30 µl. The reaction was preincubated for 30 min at room temperature, followed by addition of MgCl₂ to a final concentration of 5 mM. The digestion reaction mixture was further incubated for 2–40 min at room temperature. For homing-endonuclease-mediated digestion of pSAT expression cassettes and binary plasmids, we followed the recommended reaction conditions for each enzyme. Cleaved fragments were separated by gel electrophoresis, purified with a GFX Gel Band Purification Kit (Amersham), dephosphorylated with shrimp alkaline phosphatase (Fermentas), ligated with T4 ligase (NEB) and transferred to chemically competent DH5α E. coli cells by using standard molecular biology protocols. The order in which the different pSAT expression cassettes were assembled into pRCS11.1 is described in the Results section. Binary vectors were transformed into chemically competent EHA105 Agrobacterium cells as previously described (Tzfira et al., 1997). pET28 ZFN-expression plasmids and the pSAT and binary plasmids described in this study are available upon request.
Protoplast transfection and production of transgenic plants

The Tape-Arabidopsis Sandwich (Wu et al., 2009) method was used for protoplast isolation and transfection by multigene binary vectors. Transfected protoplasts were cultured in W5 solution in 1% BSA-coated 6-well plates for 16-24 h at 24°C to allow expression of the transfected DNA. Transgenic Arabidopsis plants were produced by using the standard flower-dip transformation method (Clough and Bent, 1998). Transgenic plants were selected on a hygromycin-selection medium. For analysis of resistance to Basta, hygromycin-resistant seedlings were transferred to soil, allowed to grow, and then sprayed with commercial Basta.

Confocal microscopy

Protoplasts and plant tissues were viewed directly under a confocal laser-scanning microscope (TCS SP5; Leica). EYFP was excited with an argon laser at 514 nm, and fluorescence was monitored between 525 and 540 nm. DsRed2 and mRFP were excited with a helium-neon laser at 561 nm, and fluorescence was monitored between 570 and 630 nm. Chlorophyll fluorescence was monitored above 660 nm.

Acknowledgements

The work was funded by grant from US-Israel Binational Agricultural Research and Development to T.T. (grant no. US-4150-08).

References


autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57: 503-516


  Molecular cloning of a carotenoid-associated protein from Cucumis sativus corollas: homologous genes involved in carotenoid sequestration in chromoplasts. Plant J 10: 1111-1118


  Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. Nat Biotechnol 23: 1013-1017


Figure legends

**Figure 1.** General features of ZFN- and homing-endonuclease-mediated multigene binary vector assembly system. (A) The general structure of a typical pSAT vector is exemplified by pSAT6, in which the promoter is flanked by the unique AgeI and Ncol sites, the terminator is flanked by the unique Xbal and NotI sites, the gene of interest is cloned into an extended MCS, and the entire plant expression cassette is flanked by recognition sites for ZFNs (e.g., ZFN12 in pSAT12) or homing endonuclease (e.g., PI-PspI in pSAT6). The ZFN- and homing-endonuclease recognition sites are shown on the binary pRCS11.1. Using ZFNs and homing endonucleases, up to nine expression cassettes can be transferred from the pSAT vectors into the T-DNA region of the pRCS11.1. LB, left border; MCS, multi-cloning site; p, promoter sequence; RB, right border; t, terminator sequence. (B) Structure and scale of the pRCS11.1[1.HYG][2.N][3.AMP][4.GUS][5.BAR][6.PAP1][10.EYFP-CHS][11.DsRed2-P][12.ChrD-RFP] nine-transgene-long plant transformation binary vector. The general structure and direction of each expression cassette is shown. Promoters and terminators: 35S, double cauliflower mosaic virus (CaMV) 35S; Rbcs, Rubisco small subunit; ocs, octopine synthase; hs, hsp18.1 heat shock. Proteins: DsRed2-P, P protein of Sonchus Yellow Net Rhabdovirus fused to DsRed2; ChrD-RFP, chromoplast-specific carotenoid-associated protein ChrD fused to RFP; EYFP-CHS, chalcone synthase fused to EYFP; PAP1, *Arabidopsis* transcription factor production of anthocyanin pigment 1; BAR, Basta resistance encoding gene; GUS, β-glucuronidase; AMP, ampicillin resistance; N, N protein of Sonchus Yellow Net Rhabdovirus; HYG, hygromycin resistance.

**Figure 2.** Multiple gene expression in protoplasts from triple- and quadruple-gene long binary vectors. EYFP-CHS expression was targeted to the rough ER (shown in yellow in panels A and E), while ChrD-RFP was targeted to the chloroplasts (shown in red in panels B, F, I and K) and
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Figure 3. PCR analysis of multigene binary T-DNAs in transgenic plants. (A) PCR analysis of seven-transgene-long T-DNA inserts in a binary plasmid (P) and two (L1 and L2) transgenic plants. (B) PCR analysis of nine-transgene-long T-DNA inserts in a transgenic plant (L6) and in a binary plasmid (P). The general structure and the expected sizes (in kb) of the PCR bands are shown.

Figure 4. Phenotypic analysis seven-transgene-long transgenic Arabidopsis plants. (A) Hygromycin resistance in two independent transgenic T₁ (L1 and L2) Arabidopsis plants. w.t., wild type plants. (B) Basta resistance in a hygromycin-resistant transgenic (left) Arabidopsis line. On the right, wild type plants.

Figure 5. Phenotypic analysis of nine-transgene-long transgenic Arabidopsis plants. (A) Basta resistance in three independent transgenic lines. Clockwise from bottom left: offspring of T₀ L10 plants, offspring of T₁ L6-1 plants, offspring of T₁ L7 plants, wild type plants. (B) PAP1 phenotype in offspring of T₂ L3-3-1 plants (right). On the left, wild-type plants. (C-F) EYFP-CHS expression (in yellow), ChrD-RFP and DsRed2-P expression (in red), plastid autofluorescence (in blue) and merged signals in leaf of T₁ L6-1 plants. All panels are single confocal sections. (G) Heat-shock-induced GUS expression in transgenic leaves. Clockwise from top left: samples
from L2-1-1, L2-2-1, L6-1-1, L10-2-1, L10-1-1, L6-2-2. (H) PAP1 phenotype in seedlings of offspring of T<sub>2</sub> L6-2-2 plants (left). On the right, seedlings of wild-type plants.

**Figure 6.** PCR analysis of T-DNA inserts in offspring of transgenic *Arabidopsis* plants. (A) Structure of the 9-gene-long T-DNA molecule, sizes and locations of the overlapping amplified PCR fragments. (B) PCR analysis of binary plasmid, T<sub>3</sub> (lines L2 and L10) and T<sub>4</sub> (lines L6 and L7) hygromycin-resistant plants. Note the two distinct fragments in lane c, produced using a single pair of primers due to the presence of repetitive elements on the 9-gene-long T-DNA molecule. The molecular marker (M) and expected sizes of the PCR fragments are given in kb.
Table 1 Genes, vectors and enzymes used for the construction of multigene vectors, in order of their assembly in pRCS11.1

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<th>Enzyme(s)</th>
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<td>Decoration of the cytoplasm and nucleus by red fluorescence</td>
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<td>Directing DsRed2-P into subnuclear compartments</td>
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<td>Ascl</td>
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<td>3</td>
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<td>I-Ppol</td>
<td>Bacterial ampicillin resistance gene and origin of replication</td>
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<td>BAR</td>
<td>I-Ceu</td>
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<td>4</td>
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<td>I-Scel</td>
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<td>PI-Pspl</td>
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Table 2: Analysis of transgenic *Arabidopsis* plants across several generations

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a Percentage of hygromycin- resistant plantlets growing on hygromycin-containing germination medium.
b Several hygromycinresistant plantlets were transferred to soil and sprayed with Basta.
c ‘+’ denote positive phenotype.
d ND, not determined.
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