Plant synthetic promoters and transcription factors

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Synthetic promoters and transcription factors have become incredibly powerful and efficient components for precise regulation of targeted plant transgene expression. Synthetic promoters can be rationally designed and constructed using specific type, copy number and spacing of motifs placed upstream of synthetic or native core promoters. Similarly, synthetic transcription factors can be constructed using a variety of DNA binding domains and effector domains. Synthetic promoters and transcription factors can provide tremendous advantages over their natural counterparts with regards to transgene expression strength and specificity. They will likely be needed for coordinated transgene expression for metabolic engineering and synthetic circuit applications in plants for bioenergy and advanced crop engineering. In this article we review the recent advances in synthetic promoters and transcription factors in plants and speculate on their future.

Introduction

Transcriptional regulation plays an important role in gene expression, which is mainly controlled via the interactions between gene promoters and transcription factors (TFs), and between TFs and chromatin-modifying machinery. TFs bind to specific promoter sequences that comprise *cis*-regulatory elements (or motifs) to determine the temporal and spatial features of gene expression (Figure 1a). Native promoters have a dispersed arrangement of motifs that are seemingly non-conserved among genes with similar expression patterns [1]. Thus, the type, copy number and spacing of motifs within a promoter can be reorganized, which is the basis of synthetic promoter construction [2-6]. Therefore, synthetic promoters offer the prospect of streamlined constructs with specified expression patterns. Whereas promoters can essentially be synthesized *de novo*, synthetic TFs have been designed using an effector (activation or repression) domain and various existing proteins with DNA binding features, in which the DNA binding function can be altered to bind to preselected genomic loci [2,7,8]. Since the early 2000s, synthetic promoters have been used in plant genetic engineering and continue to advance in design and utility in plant synthetic biology [2-4]. Synthetic TFs are more recent arrivals in plant biotechnology, but will likely be necessary to enable metabolic engineering in plants [9,10]. In this review we will discuss the current status and trends in synthetic promoters and TFs in plants. Furthermore, we will speculate on their future utility.

Synthetic promoters

A synthetic promoter consists of a core promoter and synthetic motifs for spatial and temporal control of transgene expression. Typically, a motif sequence is derived from

extant sequences, but multiplied or otherwise recombined. The core promoter sequence is set ± 50 bp with respect to the transcription start site (TSS), and contains a TATA-box, GA elements or a coreless region together with some core promoter elements (such as Y patch, CCAAT element, Inr element, CA element, etc.; [11,12]). The distribution of core promoter elements exhibit relatively positional conservation with respect to the TSSs in plants [13]. The core promoter directs accurate transcription initiation when bound by some basal transcription factors, and provides little or no basal expression level [14*,15]. The most well-known core promoter is the minimal CaMV 35S promoter, which is about 54 bp in length and provides a very low basal expression and an efficient transcription initiation in both dicots and monocots. Recently, a minimal *ZmUbi1* promoter of 126 bp in length was characterized from maize *Ubiquitin-1* with a low basal expression for use in monocots [16]. Even though there is a need to identify additional core promoters from native plant genes and viruses, synthetic core promoters can obviously be produced using different TATA-box regions and core promoter elements, as demonstrated in yeast *Pichia pastoris* [17**].

The selection, copy number and spacing of *cis*-regulatory elements determine the strength, temporal and spatial expression patterns of synthetic promoters. Selection of motifs with known functions can be conducted with the help of three main databases, i.e., PLACE [18], PlantCARE [19] and TRANSFAC [20]. Novel motifs can be characterized using synthetic motif library screening [21], bioinformatics-based *de novo* motif discovery [22], and/or experimental approaches (i.e., 5' and 3' deletion, and addition of motifs individually or in combination; [3]). As the deconstructive analysis of plant natural promoters for functional motif discovery has slowly increased during the past

three years [23-34], the effectiveness of using computational tools for *de novo* motif discovery in plants has been experimentally demonstrated in *Arabidopsis thaliana* [35] and soybean [36].

Once motifs of interest have been selected for synthetic promoter construction, motif copy number and spacing have to be optimized. Motif copy number often correlates with an increase in synthetic promoter strength as demonstrated from work in various plant species, such as *Arabidopsis* [28], tobacco [37] and rice [38]. The motif dosage effect in synthetic promoters is not surprising, since congruent findings have been observed in native promoters in *Arabidopsis* [24**] and apple [39**], even though no dosage effects have also been observed [4]. When multiple motifs are engineered into a single synthetic promoter, proper spacing among motifs is required for the hierarchical arrangement of their corresponding transcription factors in order to obtain full synergistic interactions with the RNA polymerase II complex (Figure 1; [37]). Mehrotra and Mehrotra [40] demonstrated that two copies of the ACGT motif in synthetic promoters resulted in salicylic acid-inducibility in tobacco when separated by 5 nucleotides, but were abscisic acid-inducible when separated by 25 nucleotides.

Recent advances in plant synthetic promoter engineering are making strides to generate more constitutive, bidirectional or inducible synthetic promoters for a better transcriptional regulation of transgene expression in plants (Table 1; see [2] for more plant synthetic promoters). Most synthetic promoters tested to date were either hybrids of multiple promoter parts or fusions of specific *cis*-regulatory elements with a core promoter (Figure 1b). Curran *et al.* [41**] demonstrated that functional, purely synthetic yeast promoters could be created from various known motifs with the help of a

nucleosome architecture model. Active synthetic promoters have to be associated with some chromatin signatures such as reduced nucleosome occupancy and enriched specific histone modifications (methylation or acetylation). They can also be designed with a minimal sequence similarity or no homology to any sequences in the genomes; thus homology-dependent gene silencing can be avoided [2,5,6].

Synthetic transcription factors

Synthetic TFs can be designed by fusing tailored DNA binding domains (DBDs) with effector (activation or repression) domains and nuclear-localization signals. The most widely used activation domain in plants is the acidic VP16 (or its four tandem copies VP64) from herpes simplex virus [42], which facilitates the formation of a preinitiation complex on the promoter regions by recruiting multiple components such as TFIID, TFIIF and histone acetyltransferases [43-46]. The repression domains that are most often used in plants are the moderate human SID and KRAB domains, and the strong tobacco EAR and its derivative SRDX domains, which suppress gene expression via recruitment of chromatin remodeling factors [47,48]. These repression domains are active even when fused to plant native TFs that contain their own activation domains [47,49-53] (Table 2).

The DBDs of natural plant TFs may recognize multiple motifs with variable sequences [54*], and require their preferable DNA binding sites to be pre-engineered into synthetic promoters driving target gene expression. However, synthetic DBDs provide competitive advantages over natural ones since synthetic DBDs such as the C2H2 zinc-finger (ZF) proteins and transcription activator-like effectors (TALEs) can be designed to specifically bind to virtually any (endogenous gene or transgene) promoter sequences. ZF

proteins (ZFPs) consist of a tandem array of 3-6 ZF domains with each ZF domain composed of ~30 amino acids in a $\beta\beta\alpha$ configuration targeting its specific DNA binding site that is contiguously 9-18 bp in length (Figure 2a) [55]. The DNA-binding residues are located on positions -1, 3, and 6 of each α -helix [56]. The DBDs of TALEs contain a variable number of tandemly arranged 34/35 amino acid repeats with each repeat binding to each nucleotide on their DNA binding site (Figure 2b), which is determined by its amino acids on position 12 and 13 (i.e., the repeat-variable diresidues; RVDs) [57,58]. Thus, engineering of domain specificity of these two types of DBDs only requires the alteration on those DNA-binding residues of each domain, and rearrangement of the order of domains changes the binding specificities of the whole TFs. Synthetic ZF- and TALE-TFs have been generated by fusing custom-designed DBDs to various effector domains for activation or repression of both endogenous and reporter genes in *Arabidopsis*, tobacco, maize, rice, *Brassica napus* and *Chlamydomonas reinhardtii* (Table 2). The efficiency and precision of these synthetic TFs highly depend on DNA binding domain selection, effector domain selection and the binding site position. It was observed that a maximal regulation of targeted gene expression could be obtained when the binding sites were located close to the TSSs [55,59-62], even though synthetic TALE-TFs may allow for more distant regulation than ZF-TFs [48]. It is interesting to note that synthetic ZF proteins lacking effector domains could also significantly enhance [61] or inhibit [63] reporter gene expression depending on the locations on the reporter gene promoters where they compete with other TFs for the DNA binding sites.

Compared to synthetic ZF-TFs, synthetic TALE-TFs have less context dependency, more predictable specificity, and do not require usage of expression libraries for desired

specificity. Nonetheless, both synthetic proteins could differentiate a difference of 1~3 nt between two binding sites [64,65]. They can be used for the regulation of expression of multiple genes simultaneously in plants [48,66,67], and multiple synthetic TALE-TFs have been used for synergistic regulation of targeted gene expression in mammalian cells [68*]. Both synthetic ZF- and TALE-TFs also require re-engineering of DBDs for every binding site, which is time consuming and costly. Recently, a catalytically inactive Cas9 (i.e., dCas9 with D10A H841A mutations), which is the catalytic protein of the clustered regularly interspaced short palindromic repeats (CRISPRs)-Cas9 system, was fused to either the activation domains of the EDLL and TAL effectors or the repression domain SRDX for targeted gene activation/repression in tobacco (Figure 2c) [69**]. The dCas9 was also used for the construction of dCas9-TFs for targeted gene activation in human cells when fused to the catalytic histone acetyltransferase core domain of the human E1A-associated protein p399 [70**]. Unlike synthetic ZF- and TALE-TFs, dCas9-TFs can be designed to target to any genomic DNA sequences with the help of guide-RNAs (gRNAs) which have sequence homology to the target sites (Figure 2c) [69**]. Thus, it is much easier to engineer the gRNAs of synthetic dCas9-TFs than the DBDs of synthetic ZF- or TALE-TFs for targeting to various target sites. Synthetic ZF-, TALE- and dCas9-TFs are powerful tools for the regulation of any endogenous gene expression in their native contexts as well as transgene expression in crops. They could be designed to target promoters of any endogenous genes and transgenes for transcriptional activation, repression or epigenetic modifications (i.e., methylation, acetylation, deacetylation, amination, deamination, etc.).

Coupling synthetic promoters with synthetic transcription factors

So far, constitutive promoters such as CaMV 35S and AtUBQ3 have been used to drive expression of synthetic ZF-, TALE- and dCas9-TFs for transcriptional regulation of endogenous genes and transgenes in plants (Table 2). Synthetic promoters could be used to drive transgene expression when paired with synthetic TFs that bind to these synthetic promoters. Thus, transgene expression could be limited to a very low (or high) basal expression level under the control of synthetic promoters, and be enhanced (or decreased) to an optimal expression level with the corresponding synthetic TFs binding to the synthetic promoters. Paired expression of synthetic promoters with synthetic ZF-, TALE-, and dCas9-TFs that target these promoters have been used for targeted transgene activation or repression in tobacco [61,62,69**,71-74], maize [62], and *Arabidopsis* [48,64,74] (Table 2). In these cases, the synthetic TFs were constitutively expressed for a maximal enhancement or repression of expression of target genes. These synthetic TFs could also be conditionally expressed under the control of various synthetic promoters so that their proteins would only be available to the promoters driving transgene expression under certain conditions. For example, the synthetic promoter pTPGI was used for the inducible expression of synthetic dCas9-VP64 in yeast while growing on galactose and anhydrotetracycline media, which, in turn, only targeted the minimal promoter driving a reporter gene with the help of gRNAs which had sequence homology to the minimal promoter [75].

Future uses of synthetic promoters and TFs

As plant biotechnologists desire to install more transgenes together, the transcriptional control of these genes becomes increasingly important. Engineering novel synthetic pathways and networks into plants that could function independently from or even replace endogenous networks will require novel control devices. Synthetic promoters paired with synthetic TFs could be used to provide a coordinated transcriptional control of multiple genes, which would be required for successful metabolic engineering [10] and implementation of synthetic circuits into plants [76]. One way to do this would be to engineer the same DNA binding site into different synthetic promoters for targeted activation, say, only in one tissue or cell type, whose expression would be moderated by a single synthetic TF (Figure 3). A dynamic range of transcription levels of regulatory networks would be obtained for the proper interactions between synthetic circuits and endogenous pathways. They could enable a precise, robust and specific regulation of synthetic circuits while minimizing background noise and unwanted cross-talks between synthetic circuits and endogenous networks. Even with relatively simple transgenic systems in which one or a few genes are added into a plant, we envisage the desire of governing officials and agricultural companies to focus on increasing the regulatory precision of transcription and translation [2]. Furthermore, we expect that the fundamental progress in motif discovery, modeling and optimization of synthetic promoter and TF engineering, DBD and effector domain discovery will facilitate the implementation of synthetic promoters and TFs into plant synthetic biology and biotechnology.

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241 **References and recommended reading**

242 Papers of particular interest, published within the period of review, have been highlighted
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Tables

Table 1. Synthetic promoters that have been designed for use in plants during the past three years.

Promoter Name	Expression Profile	Source(s)	Species Tested	Ref.
<i>FSgt-PFIt</i> ; <i>MSgt-PFIt</i> ; <i>PFIt-UAS-2X</i>	Constitutive	Promoter regions of <i>FSgt</i> , <i>MSgt</i> , and <i>PFIt-UAS</i> ; <i>PFIt</i> core promoter (<i>PFIt</i>)	Tobacco; Petunia; <i>Arabidopsis</i> ; Tomato; Spinach	77
<i>FsFfCBD</i>	Bidirectional	<i>FsCP</i> ; <i>CaMV 35S</i> core promoter; a tri-hybrid enhancer <i>FsEFfECE</i>	Tobacco	78
<i>p35S-PCHS-Ω</i> ; <i>p35S-LCHS-Ω</i> ; <i>pOCS-PCHS-Ω</i> ; <i>pOCS-LCHS-Ω</i>	Flower-specific	<i>CaMV 35S</i> or <i>OCS</i> enhancer; petunia <i>CHSA</i> core promoter; lily <i>CHS</i> core promoter, an omega element	<i>Torenia fournieri</i>	79
<i>pCL</i>	Tuber-specific and cold-inducible	<i>Arabidopsis cor15a</i> promoter region and potato <i>patatin</i> promoter region	Potato	80
<i>sab</i> ; <i>sba</i>	Cold-inducible	<i>Arabidopsis cor15a</i> promoter regions and	Tobacco	81

		<i>cor15b</i> promoter regions		
<i>4×RSRE</i>	General stress-inducible	<i>Arabidopsis</i> rapid stress response elements; Opaline synthase (<i>NOS</i>) core promoter	<i>Arabidopsis</i>	82
<i>4×CCTC</i>	Fungal colonization under low-Pi condition-inducible	Potato Pi transporter 3 (<i>StPT3</i>) promoter regions; <i>CaMV 35S</i> core promoter	Potato; Lotus	83
<i>4×RE;</i> <i>B4×REA</i>	Hormonal- and bacterial pathogen-inducible	Hormone-response elements; <i>CaMV 35S</i> core promoter	Tobacco; <i>Arabidopsis</i>	84
<i>4×GCC</i>	Jasmonic acid-inducible	<i>Arabidopsis PDF1.2</i> promoter; <i>CaMV 35S</i> core promoter	<i>Arabidopsis</i>	32
<i>4× ROSE1~7</i>	ROS-inducible	<i>Arabidopsis</i> ROS-responsive elements; <i>CaMV 35S</i> core promoter	<i>Arabidopsis</i>	33

631 Peanut Chlorotic Streak Virus, *PFlt-UAS*; Figwort Mosaic Virus, *FSgt*; Mirabilis Mosaic
632 Virus, *MSgt*; Figwort mosaic virus subgenomic transcript core promoter, *FsCP*.

633 **Table 2. Synthetic ZF-, TALE- and dCas9-TFs that have been designed for use in plants.**

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DNA Binding Domain	Effector Domain	Promoter used to regulate Synthetic TF	Test Species and Systems	Target Gene	Target Size (bp)	Target Distance From TSS (bp)	Effectiveness	Ref.
<i>Synthetic ZF-TFs</i>								
Designed	VP64;SID	AtUBQ3; AtAP1	<i>Arabidopsis</i> protoplasts or transgenics	<i>AtAP3::AtAP3</i> ; <i>AtAP3::Gus</i>	18	-112~ -95	↑ in AtAP3 and GUS	55
Designed	VP16;VP64	CaMV 35S; RTBV	Tobacco BY2 protoplasts; Tobacco transgenics	<i>Synthetic promo- ter::Gus</i>	18	-178~+72	5-30× or 450× ↑ in GUS	61
Designed	VP64;SID; KRAB	TMV coat protein subgenomic	Tobacco BY2 protoplasts;	<i>Synthetic promo- ter::Luc</i>	9, 18	-443~+73	20-80×↑ in LUC	62

		promoter; ZmUBI	maize HE89					
			protoplasts					
Designed	C1	CaMV 35S; BrNA1	<i>Arabidopsis</i> protoplasts & transgenics	<i>AtGMT::AtGMT</i>	9	-500~ +500	20×↑ in AtGMT	85
Designed	VP16;VP64	XVE	<i>Onion epider-</i> <i>mal cells;</i> <i>Arabidopsis</i> transgenics	<i>Synthetic promo-</i> <i>ter::Luc or GFP</i>	27	-150~+8	33×↑ in reporter gene	64
Designed	VP64; KRAB	CaMV 35S	<i>Arabidopsis</i> transgenics	<i>At4CL1::At4CL1</i>	9	-143~-124	30%↑ or 50%↓ in lignin	86
Designed	C1	CaMV 35S	<i>Arabidopsis</i> transgenics	<i>AtAdh::AtAdh</i>	9	-317~+72	20×↑ in AtAdh	87
Designed	VP16	AtRPS5A	<i>Arabidopsis</i> transgenics	<i>HR indicator line</i>	9	N.A.	200-1000×↑ in HR	66, 67
Designed	VP16	AtUbi10	<i>Brassica</i>	<i>BnKASII</i>	15	-50~0	22% ↓ in	88

			<i>napus</i>	:: <i>BnKASII</i>			palmitic acid	
			transgenics					
Designed	SID	CsVMV	Tobacco BY2	<i>RTBV promo-</i>	18	-58~+50	80%↓ in	63
			protoplasts;	<i>ter::Gus</i>			GUS	
			<i>Arabidopsis</i>					
			transgenics					
<hr/> <i>Synthetic TALE-TFs</i> <hr/>								
Designed	XcvAvrBs3;	CaMV 35S	Tobacco agro-	<i>CpBs3::CpBs3;</i>	19	-108~ -46	↑ in GUS,	74
	XcvAvrBs4		infiltration;	<i>CpBs4C::CpBs4C;</i>			NtBs4,	
			<i>Arabidopsis</i>	<i>Synthetic promo-</i>			AtEGL3,	
			transgenics	<i>ter::Gus;</i>			AtKNAT1	
				<i>NtBs4Sp::Gus;</i>				
				<i>NtBs4::NtBs4;</i>				
				<i>AtEGL3::AtEGL3;</i>				
				<i>AtKNAT1::AtKNAT1</i>				
Designed	<i>XcadHax3</i>	CaMV 35S	Tobacco agro-	<i>Synthetic promo-</i>	14	-108~ -46	↑ in GUS	73

			infiltration	<i>ters::Gus</i>				
<i>XcadHax3</i>	SRDX	CaMV 35S	<i>Arabidopsis</i>	<i>AtRD29A::Luc</i>	13	-139~-128	↓ in LUC	48
			transgenics					
RsBrg11;	XcvAvrBs3;	CaMV 35S	Tobacco agro-	<i>Synthetic promo-</i>	14	-108~ -46	↑ in GUS	71
RsHpx17;	RsBrg11		infiltration	<i>ter::RsBrg-</i>				
XcvAvrBs3				<i>11/RipTALs</i>				
Designed	VP16;VP64	CaMV 35S	Tobacco agro-	<i>Synthetic promoter::</i>	19, 25	-169~ -4	2×↑ in	72
			infiltration	<i>pporRFP</i>			pporRFP	
Designed	XooAvrXa1	XooAvrXa10	Rice leaf	<i>OsXa27::OsXa27;</i>	24	-92~ -67	↑ Resistance to	60
	0		inoculation	<i>OsXa13::OsXa13;</i>			Xoo	
				<i>OsSWEET12</i>				
				<i>::OsSWEET12</i>				
Designed	XooAvrXa7	CrRbcs2/Hsp70A	<i>Chlamydom-</i>	<i>CrARS1::CrARS1</i>	24	-79~ +1	↑ in CrARS1,	59
			<i>onas rein-</i>	<i>CrARS2::CrARS2</i>			CrARS2	
			<i>hardtii elec-</i>					

			troporation					
Designed	Designed	NbAct2	Tobacco	<i>Synthetic promoter::Gus</i> or <i>GFP</i> or <i>SlDXS2</i> or <i>NtGGPPS2</i> or <i>NtCBTS2a</i>	19	-71~-52	↑ in GUS and CBTol	89
<hr/>								
<i>Synthetic dCas9-TFs</i>								
Designed	ELLE; gRNAs	CaMV 35S	Tobacco agro-infiltration	<i>Synthetic promoter::Gus</i> ; <i>NbPDS::NbPDS</i>	20	-297~ +83	↑ in GUS and NbPDS	69**
	SRDX							

635 *Brassica rapa*, *Br*; *Capsicum pubescens*, *Cp*; Cassava Vein Mosaic Virus, *CsVMV*; *Chlamydomonas reinhardtii*, *Cr*; *Nicotiana*
636 *benthamiana*, *Nb*; *N. tabacum*, *Nt*; *Ralstonia solanacearum*, *Rs*; Rice Tungro Bacilliform Virus, *RTBV*; *Solanum lycopersicum*, *Sl*;
637 *Xanthomonas campestris* pv. *armoraciae*, *Xca*; *X. campestris* pv. *vesicatoria*, *Xcv*; *X. oryzae* pv. *oryzae*, *Xoo*.

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Figure Legends

Figure 1. Schematic representations of synthetic promoters versus natural promoters. (a) A natural promoter contains a core promoter region, a proximal region and a distal region that typically range from 500 bp to over 2000 bp [90]. The core promoter contains core promoter elements (pink) and the proximal and distal regions are composed of various *cis*-regulatory elements. Binding of multiple transcription factors to these motifs determines the complex expression profiles of these promoters. (b) Synthetic promoters could be built by fusing multiple copies of a single motif (or several motifs) together with a core promoter [i.e., a TATA-box region and several core promoter elements (pink)], or purely synthetically with the help of modelling. Only user-preferred transcription factors could bind to these motifs and activate gene expression. Compared to natural promoters, synthetic promoters provide higher (or lower) expression level with high specificity, lower basal expression level, shorter length, and less sequence homology to any host genomic sequences. The transcription initiation sites of genes-of-interest (GOI) are indicated by horizontal arrows.

Figure 2. Synthetic TFs can be built using platforms that are more often used for genome editing applications, such as ZF-, TALE- and dCas9 systems for regulation of expression of targeted genes. (a) Synthetic ZF- and TALE-TFs contain custom-designed DNA binding domains (DBDs) and effector (activation or repression) domains. The DBDs of TALE-TFs are composed of a variable number of tandemly arranged 34/35 amino acid repeats with each repeat binding to each nucleotide on their DNA binding site. The DBDs of ZF-TFs consist of 3-6 ZF domains with each domain targeting its specific DNA

binding site of contiguously 9-18 bp in length (not shown). (b) Synthetic dCas9-TFs contain a catalytically inactive Cas9 (dCas9 with D10A H841A mutations) and an effector domain, and are guided by custom-designed guide-RNAs (gRNAs; yellow) which have sequence homology to the target sites and reverse complementarily bind to the antisense strands (not shown) of the target sites. The transcription initiation sites of genes-of-interest (GOI) are indicated by horizontal arrows, and core promoter element are indicated in pink.

Figure 3. Illustration of the use of a synthetic TF driven by a synthetic promoter for coordinated expression of multiple genes for metabolic engineering or synthetic circuits in plants. The synthetic TF (syn TF) is under the control of the synthetic promoter 1 which contains a synthetic core promoter and tetramers of motif 1. The transcription factor binding site of the syn TF is M, which is tetramerized and fused to different synthetic core promoters for expression of different genes of interest (GOI1~5). Thus, conditional expression of the syn TF can regulate coordinated expression of GOI1~5, which could be stacked on a single vector.

Figure 1.

Figure 2.

Figure 3.