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A rapid and scalable approach to build synthetic repetitive hormone-responsive promoters

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Summary

Advancement of DNA-synthesis technologies has greatly facilitated the development of synthetic biology tools. However, high-complexity DNA sequences containing tandems of short repeats are still notoriously difficult to produce synthetically, with commercial DNA synthesis companies usually rejecting orders that exceed specific sequence complexity thresholds. To overcome this limitation, we developed a simple, single-tube reaction method that enables the generation of DNA sequences containing multiple repetitive elements. Our strategy involves commercial synthesis and PCR amplification of padded sequences that contain the repeats of interest, along with random intervening sequence stuffers that include type IIS restriction enzyme sites. GoldenBraid molecular cloning technology is then employed to remove the stuffers, rejoin the repeats together in a predefined order, and subclone the tandem(s) in a vector using a single-tube digestion–ligation reaction. In our hands, this new approach is much simpler, more versatile and efficient than previously developed solutions to this problem. As a proof of concept, two different phytohormone-responsive, synthetic, repetitive proximal promoters were generated and tested *in planta* in the context of transcriptional reporters. Analysis of transgenic lines carrying the synthetic ethylene-responsive promoter *10x2EBS-S10* fused to the *GUS* reporter gene uncovered several developmentally regulated ethylene response maxima, indicating the utility of this reporter for monitoring the involvement of ethylene in a variety of physiologically relevant processes. These encouraging results suggest that this reporter system can be leveraged to investigate the ethylene response to biotic and abiotic factors with high spatial and temporal resolution.

Introduction

With the recent advances in single-cell genomics, *in vivo* and *in vitro* high-throughput quantification of DNA and RNA regulatory elements' activities, and increasingly powerful machine-learning approaches, the synthetic biology goal of designing programmable regulatory sequences that confer the desired expression characteristics is now in the foreseeable future (reviewed in Bhandari *et al.*, 2021; Schmitz *et al.*, 2022; Vazquez-Vilar *et al.*, 2023; Yu *et al.*, 2023). The plant biology community has been experimenting with relatively simple versions of these types of synthetic promoters for decades, making important advances and identifying bottlenecks. Three notable examples of these types of synthetic regulatory sequences are the *DR5* (direct repeats with a 5-bp spacer) and its derivatives (Liao *et al.*, 2015; Ulmasov *et al.*, 1997), *TCS* (two component output sensor) and its variants (Müller and Sheen, 2008; Steiner *et al.*, 2020; Zücher *et al.*, 2013), and *EBS* (ETHYLENE INSENSITIVE3 (EIN3) binding site) (Stepanova, 2001; Stepanova *et al.*, 2007) promoters and their derivatives that specifically respond to the plant hormones auxin, cytokinin and ethylene, respectively. These synthetic promoters are not only more sensitive to the triggering stimuli than most native genes, but more importantly, they lack additional regulatory sequences potentially present in the corresponding native promoters that could be sources of unwanted interference or unpredictable response (Butel *et al.*, 2021; Fagard and Vaucheret, 2000; Leibovic and Gronau, 2021). In these hormone-responsive promoters, multiple copies of the consensus DNA-binding sequence of a transcription factor (or a family of transcription factors) specifically activated by the desired stimulus are placed upstream of a minimal promoter driving a reporter gene in response to auxin, cytokinin or ethylene. Increasing the number of *cis*-elements in a synthetic promoter has been shown to lead to stronger reporter activation (Ulmasov *et al.*, 1997; Zücher *et al.*, 2013), with *cis*-element stacking expected to lead to greater transcription factor binding, enhanced recruitment of the general transcriptional machinery, and more efficient transcription initiation. Nonetheless, even for these relatively simple synthetic promoters, the expression levels and patterns of the reporter gene are strongly affected not only by the specific consensus sequence(s) used and their copy number, but also by the arrangement of the DNA elements (direct, inverted or everted tandems) and their spacing (Grabczyk and Usdin, 1999; Jiang *et al.*, 1996; Pandey *et al.*, 2019). Thus, experimental testing of different promoter architectures is

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central to deciphering the rules governing gene regulation and optimizing the design of synthetic promoters.

In the past few years, DNA synthesis has become very affordable, and it is now common practice in biological research to commercially synthesize DNA fragments of interest. The ability to order desired sequences from a vendor has opened doors to the field of synthetic biology by removing the requirement for a suitable DNA template and making it possible to synthesize unnatural DNA sequences. Despite the burst in demand for commercial DNA synthesis, sequences that are repetitive, contain homopolymers, are highly (>80%) GC- or AT-rich, or are prone to forming secondary structures are notoriously difficult to synthesize (reviewed by Hoose *et al*., 2023). In fact, although direct synthesis seems the obvious answer for the rapid generation of synthetic promoters, most vendors decline DNA synthesis requests for sequences containing *cis*-element tandems characteristic of the aforementioned synthetic promoters as they fall into the 'complex sequence' categories. It is, therefore, common for synthetic biologists to use alternative approaches that allow for the assembly of these highly repetitive sequences that cannot be synthesized directly. Thus, for example, pairs of complementary oligos containing a single DNA element can be assembled into large arrays by introducing a few nucleotides at their flanks that would produce compatible overhangs upon oligo hybridization or restriction enzyme digestion (Figure S1). Although this and related approaches have been successfully used (Atanassov *et al*., 2009; Horton *et al*., 1989; Jiang *et al*., 1996; Scior *et al*., 2011; Williams and Coster, 2022), they have some important limitations. Thus, for example, undesired artefacts can be produced when annealing oligonucleotides to generate inverted, everted, or palindromic repeats. In addition, this strategy involves several time-consuming and inefficient steps such as size fractionation, gel purification, and classical restriction digest and ligation cloning strategies (Figure S1) (Blachinsky *et al*., 2004; Boë and Masson, 1996; Jobbagy *et al*., 2002; Kim and Szybalski, 1996; Scior *et al*., 2011; Yasmeen *et al*., 2023).

One potential alternative consists of generating several individual, non-identical-repeat entry clones and combining them via Type IIS restriction enzyme-based cloning, such as MoClo (Weber *et al*., 2011), Golden Gate (Engler *et al*., 2008) or GoldenBraid (GB) (Figure S2) (Sarrion-Perdigones *et al*., 2011, 2013), in an ordered fashion through the use of unique and sequentially compatible overhangs solving the insert directionality and targeted sequence variation dilemma. However, such an approach is also labour intensive as each element needs to be 'domesticated' into the corresponding entry vectors and then combined in the desired destination vector. Fragment domestication involves the addition of vector-compatible restriction enzyme sites at the flanks of the DNA fragment and their removal from the interior of the fragment via site-directed mutagenesis or commercial DNA synthesis prior to the subcloning of the fragment into an entry vector (Figure S2A). Working with a single Type IIS site-flanked insert source clone, on the other hand, is less cumbersome than making a series of several sequence-divergent clones, but it does not allow for repeat variation (Hillson, 2011; Kim and Szybalski, 1996). Finally, scarless DNA assembly methods like Gibson (Gibson *et al*., 2009), SLIC (Li and Elledge, 2012), SLiCE (Zhang *et al*., 2014) or CPEC (Quan and Tian, 2009) all rely on homology-based DNA fragment annealing and thus are not generally suitable for generating highly repetitive sequences (Hillson, 2011).

Herein, we worked on solving the problem of generating complex, repetitive sequences and developed a universal approach that should be applicable to the production of extremely complex and otherwise not directly synthesizable sequences while overcoming some of the key limitations of other existing technologies. One of the main advantages of the proposed approach is its simplicity and ease by which repetitive DNA sequences composed of short element tandems can be generated using a combination of commercial DNA synthesis and GB cloning methodology. We initially synthesized the desired repetitive DNA fragments with random stuffers strategically positioned between individual repeats. The addition of these stuffers diversified the repetitive sequences facilitating standard commercial synthesis of relatively large sets of repeats and enabling PCR amplification of one or several of the repeats. We then generated the stuffer-free versions of these repetitive sequences by removing the DNA stuffers and ligating the repeats in a desired order using a modified single-tube GB cloning reaction.

The need for the development of this technology arose from the desire to build a new ethylene reporter. The most widely adopted ethylene reporter currently in use, *EBS:GUS*, was generated over 20 years ago based on limited information on the binding of the transcription factor EIN3 to the promoter of the ethylene-inducible *ETHYLENE RESPONSE DNA-BINDING FACTOR1* (*EDF1*) gene, *At1g25560* (Stepanova, 2001; Stepanova *et al*., 2007). To make *EBS:GUS*, five copies of an experimentally validated EIN3-binding site (aka *EBS*) from *EDF1* were placed upstream of the *35S*(−46) core promoter (Odell *et al*., 1985) driving the *β-glucuronidase* gene *GUS*. Although this reporter has proven very useful (reviewed in Fernandez-Moreno and Stepanova, 2020), it is based on a single native *EBS* (Song *et al*., 2015; Stepanova, 2001; Stepanova *et al*., 2007) which may or may not be optimal for EIN3 binding. A more comprehensive analysis of the *in vitro* binding properties of another *EBS cis*-element derived from the promoter of the ethylene-inducible *ETHYLENE RESPONSE FACTOR1 (ERF1)* gene, *At3g23240*, suggested that EIN3 can bind DNA as a dimer and identified the spacing of 10 nucleotides separating the two everted half-sites of the *EBS* motif as most optimal (Solano *et al*., 2015; Song *et al*., 2015).

Similarly, information derived from the crystal structure analysis of two divergent AUXIN RESPONSE FACTORs (ARFs) and corresponding protein-binding arrays has been used to develop an auxin reporter *DR5v2* that partially overlaps and, in some tissues, expands the expression domains of the classical *DR5* auxin reporter (Liao *et al*., 2015). To explore the possibility of improving the performance of existing ethylene and auxin reporters, we used the information derived from the aforementioned studies to design *2EBS-S10* and hybrid *2DR5-2DR5v2*. *2EBS-S10* consists of 10 copies of the spacing-optimized dual DNA binding site for the EIN3 dimer, whereas *2DR5-2DR5v2* contains 10 copies of alternating dual *DR5* and *DR5v2* DNA elements.

To overcome the challenges of generating these highly repetitive *2EBS-S10* and *2DR5-2DR5v2* distal promoters, we employed GB. We argue that the potential utility of this approach extends beyond the construction of synthetic repetitive promoters, for example, to the generation of complex combinatorial libraries of *cis*-regulatory elements that can help in deciphering the rules governing gene regulation in eukaryotes.

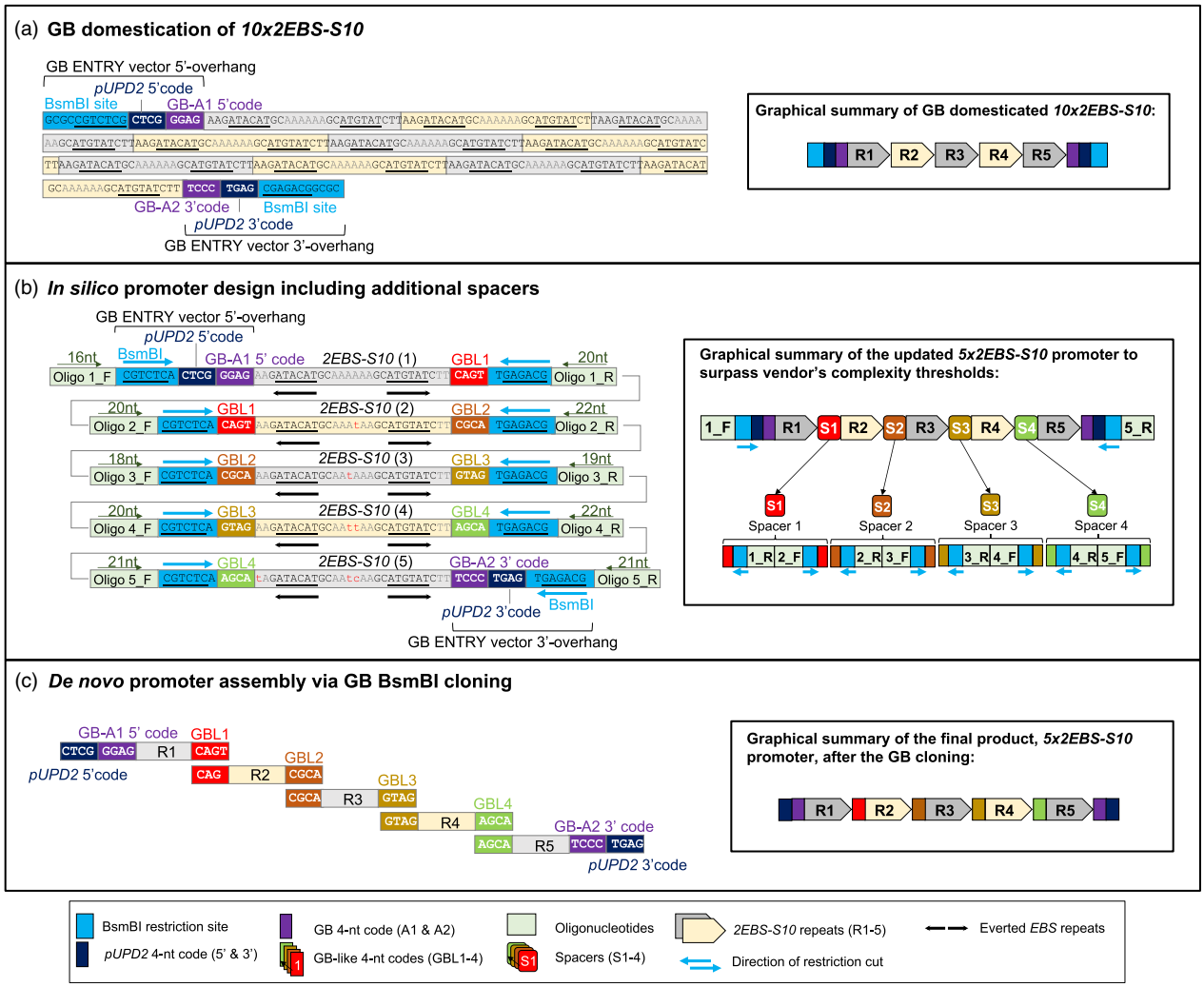

Figure 1 *In silico* design for *de novo* ethylene-responsive *10x2EBS-S10* promoter commercial synthesis. Original *10x2EBS-S10* sequence design failed to pass sequence complexity filters for commercial DNA synthesis, whereas the modified *5x2EBS-S10* sequence design was compatible with commercial DNA synthesis. (a) Ten repeats of the synthetic *2EBS-S10* element (Song *et al.*, 2015) were stacked in tandem for DNA synthesis. Each repeat (grey and yellow) consisted of two everted sequences with a 10 bp spacer. *pUPD2*-compatible GB entry vector overhangs (dark blue) were added at the tandem flanks to allow for the subcloning of the *10x2EBS-S10* synthetic fragment. Directional GB codes (purple) with (A1-A2) grammar were included to enable the use of this fragment in transcriptional unit assemblies as a distal-proximal promoter. The recognition sequence of the *BsmBI* restriction site (cyan) and the EIN3 binding site are underlined. (b) The synthetic DNA fragment harbours five non-identical repeats of the *2EBS-S10* sequence, with each repeat flanked by the four-nucleotide codes for standard GB grammar (purple) or newly designed GBL codes (code 1 in red, code 2 in brown, code 3 in mustard and code 4 in green), followed by the *BsmBI* restriction site (cyan) and a forward or reverse stuffer oligonucleotides (light green) strategically selected from our group's existing primer collection (see Figure S8 for the full DNA sequence). The first and last repeats also contain a four-nucleotide code compatible with the *pUPD2* vector (dark blue) between the GB grammar and the *BsmBI* restriction site. (c) Digestion of the synthetic DNA fragment shown in panel b with *BsmBI* enzyme liberates the individual repeats flanked by compatible four-nucleotide GBL codes enabling directional assembly. The GB grammar for a distal-proximal promoter element [GGAG (5′-A1) – TCCC (3′-A2)] allows for subcloning of this fragment into *pUPD2*. A graphical summary of the different elements is shown on the right side of each panel. A legend listing different types of DNA elements is included on the bottom.

Results

De novo assembly of the ethylene-inducible *10x2EBS-S10* synthetic promoter

We set out to generate a novel ethylene-inducible synthetic proximal promoter on the basis of an *in vitro*-optimized *2EBS-S10* [AAGATACATGCAAAAAAGCATGTATCTT] DNA element characterized by Song *et al.* (2015) and chosen for its efficient recruitment of the ethylene-regulated transcription factor EIN3 in electrophoretic mobility shift assays. This DNA sequence element harbours two everted *EBS cis*-elements separated by a 10-nucleotide randomly chosen spacer (Song *et al.*, 2015).

Our strategy consisted of synthesizing a DNA fragment harbouring 10 of those dual *2EBS-S10* elements flanked by standardized GB codes to enable the fragment's subsequent assembly with other gene parts via GB technology (Sarrion-Perdigones *et al.*, 2013, 2014; Figure 1a) to ultimately build a new ethylene-regulated reporter. The flanking sequences on both sides of the tandem contain three essential elements: a restriction site for the Type IIS enzyme *BsmBI* (cyan and underscored), a

directional four-nucleotide code (dark blue) compatible with the 5′ or 3′ ends of *BsmBI*-linearized *pUPD2* GB entry vector, and a four-nucleotide code (purple) with the standard GB grammar (Patron *et al*., 2015; Sarrion-Perdigones *et al*., 2014; Vazquez-Vilar *et al*., 2015) of a distal-proximal promoter (A1-A2) category [GGAG – TCCC] (see colour-coded parts in Figure 1a). Due to the repetitiveness of the sequence tandem, this synthetic DNA fragment did not pass the sequence complexity filters for the commercial synthesis by several different vendors and needed to be redesigned. The spacer sequence within each dual repeat was modified and nucleotide-diversified stuffer sequences were inserted between the dual repeats. Specifically, the fifth and/or sixth adenine nucleotide in the original 10-nucleotide internal spacer of each everted *2EBS-S10* repeat (red lowercase nucleotides, Figure 1b) was changed and each dual repeat was flanked by 18- to 22-bp-long oligonucleotide stuffers (Figure 1b, Table S1) to generate unique inter-repeat buffer sequences that were about twice as long as each individual repeat. In addition to diversifying the otherwise very repetitive sequence to meet the vendor's sequence complexity requirements, these stuffer sequences could also be used to amplify each individual repeat, or a subset of repeats, in case the amplification of the full DNA sequence fails. To enable the post-synthesis removal of these new stuffer sequences prior to the final construct assembly, each of the stuffers was flanked by four-nucleotide GB-like (GBL) codes (different from those in the standard GB parts grammar described in Figure S2A, shown in red, brown, mustard and green) and a *BsmBI* restriction site (cyan and underscored) placed between the oligonucleotide stuffer and the four-nucleotide GBL code (Figure 1b). This design allows for the enzymatic removal of the inter-repeat stuffers during the GB domestication reaction using *BsmBI* and preserves the directionally of the assembly of the dual repeats upon rejoining of overlapping GBL codes (Figure 1c). With these additional modifications, up to five repeats could be accommodated in a DNA fragment and still pass the vendor's sequence complexity filters for DNA synthesis at Integrated DNA Technologies (IDT) (Figure 1b).

The resulting commercially synthesized IDT gBlock containing this repetitive DNA fragment with stuffers (Figure 1b) was PCR amplified using a proofreading polymerase, purified and used in a typical single-tube digestion–ligation GB domestication reaction using the type IIS restriction enzyme *BsmBI* (optimal activity at 37 °C), the T4 DNA ligase (optimal activity at 16 °C) and the *pUPD2* entry vector (Sarrion-Perdigones *et al*., 2013, 2014; Vazquez-Vilar *et al*., 2015). An additional final *BsmBI* digestion step (37 °C for 5 min with fresh enzyme) was included at the end of the digestion–ligation reaction in order to enrich for circularized constructs containing the desired stuffer-free *5x2EBS-S10* promoter by linearizing the residual stuffer-containing clones. The screening of white colonies was done by *NotI* digestion of plasmid DNA, which liberated the insert from *pUPD2* (Sarrion-Perdigones *et al*., 2013, 2014), and the size was determined by gel electrophoresis. Upon confirming the fidelity of a stuffer-less *5x2EBS-S10* clone by Sanger sequencing, we next aimed to assemble the desired full-length *10x2EBS-S10* synthetic fragment by combining two *5x2EBS-S10* fragments. For that, we designed two GB code-converting oligonucleotide primers to change the GB-A1 5′ code [GGAG] or the GB-A2 3′ code [TCCC] of the *5x2EBS-S10* (A1-A2) synthetic promoter by GB-A2 5′ code [TGAC] or the GB-A1 3′ code [TGAC], respectively, to generate two compatible and independent *5x2EBS-S10*-A1 [GGAG – TGAC] and *5x2EBS-S10*-A2 [TGAC – TCCC] promoters. Thus, to convert the **GGAG**-*5x2EBS-S10*-**TCCC** (A1-A2) promoter into **GGAG**-*5x2EBS-S10*-**TGAC** (A1) promoter, we designed a primer with two nucleotide changes in the 3′ GB code: [TCCC > TGAC] (Figure 2a). Similarly, to convert the original (A1-A2) promoter into a **TGAC**-*5x2EBS-S10*-**TCCC** (A2) promoter, we designed a new primer harbouring two nucleotide changes in the 5′ GB code: [GGAG > TGAC] (Figure 2a).

The aforementioned cloning procedure of *5x2EBS-S10* (A1-A2) was followed to subclone *5x2EBS-S10* into two independent *pUPD2* plasmids as (A1) and (A2) GB elements (Figure 2b). Thus, the original stuffer-containing IDT gBlock was used as a PCR template for the amplification with the new oligos containing the modified GB-codes, *2EBS-S10-A1R* and *2EBS-S10-A2F* (Table S1). The resulting amplicons were subcloned into *pUPD2* following the same strategy as described above. Stuffer-less clones were then analysed via *NotI* restriction digest and confirmed as error-free and containing the converted GB codes by Sanger sequencing for both *5x2EBS-S10* (A1) and *5x2EBS-S10* (A2) reactions (Figure 2c).

Functional validation of *10x2EBS-S10* as an ethylene-inducible proximal promoter

To test the functionality of *5x2EBS-S10* in plants, the two GB elements, (A1) and (A2), of this DNA sequence were combined via a standard alpha-level GB assembly procedure (Figure S2B) (Sarrion-Perdigones *et al*., 2014; Vazquez-Vilar *et al*., 2015) to generate a reporter construct containing a full *10x2EBS-S10* proximal promoter, minimal *35S*(−46 bp) promoter (Odell *et al*., 1985), nuclear localization signal (*NLS* or *3xNLS*) (Hicks and Raikhel, 1993), reporter gene (*1xYPet*, *3xYPet*, or *GUS*) (Brumos *et al*., 2020; Eudes *et al*., 2008; Jefferson *et al*., 1987; Nguyen and Daugherty, 2005; Zhou *et al*., 2011) and the *35S* terminator (Franck *et al*., 1980) in a single-tube digestion–ligation reaction (Figure 3a). A *BASTA* selectable maker (Rathore *et al*., 1993) was then introduced into these reporter constructs via standard omega-level GB cloning (Figure S2C) (Sarrion-Perdigones *et al*., 2014; Vazquez-Vilar *et al*., 2015) and all three final modules were stably transformed into Arabidopsis.

The screening of T2 seedlings harbouring the *10x2EBS-S10* driving the expression of either *1xYPet*, *3xYPet* and *GUS* revealed that one of 36, 14 of 18 and 19 of 50 T1 lines showed ethylene-mediated reporter induction respectively. All 15 ethylene-inducible *YPet* lines (including those from both *10x2EBS-S10:35Smp:NLS:YPet:35Sterm* and *10x2EBS-S10:35Smp:3xNLS:3xYPet:35Sterm* transgenic lines) showed weak and patchy but detectable expression in the nuclei of root tip cells of three-day-old dark-grown Arabidopsis seedlings germinated in the presence of the ethylene precursor ACC (1-aminocyclopropanecarboxylic acid), but not in control media (Figure S3A). In addition, the 14 lines expressing the *3xYPet* transgene also exhibited a relatively strong expression in the nuclei of lower hypocotyl cells and of root cells above the elongation zone in ACC-treated seedlings, but again displayed no detectable fluorescence in control conditions (Figure 3b, Figure S3B). Unfortunately, in later generations, silencing was observed in all of the *YPet* lines, with the reporter activity no longer reliably detectable by the T3 or T4 generation (Figure S3C). Similarly to the *YPet* lines, the 19 positive lines harbouring the *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* transgene showed a robust ethylene-inducible expression in roots and hypocotyls of three-day-old T2 Arabidopsis seedlings germinated in the presence of ACC, but not in control conditions (Figure S4). Importantly, the pattern of induction of the *10x2EBS-S10:GUS* reporter persisted in later generations, as shown for two

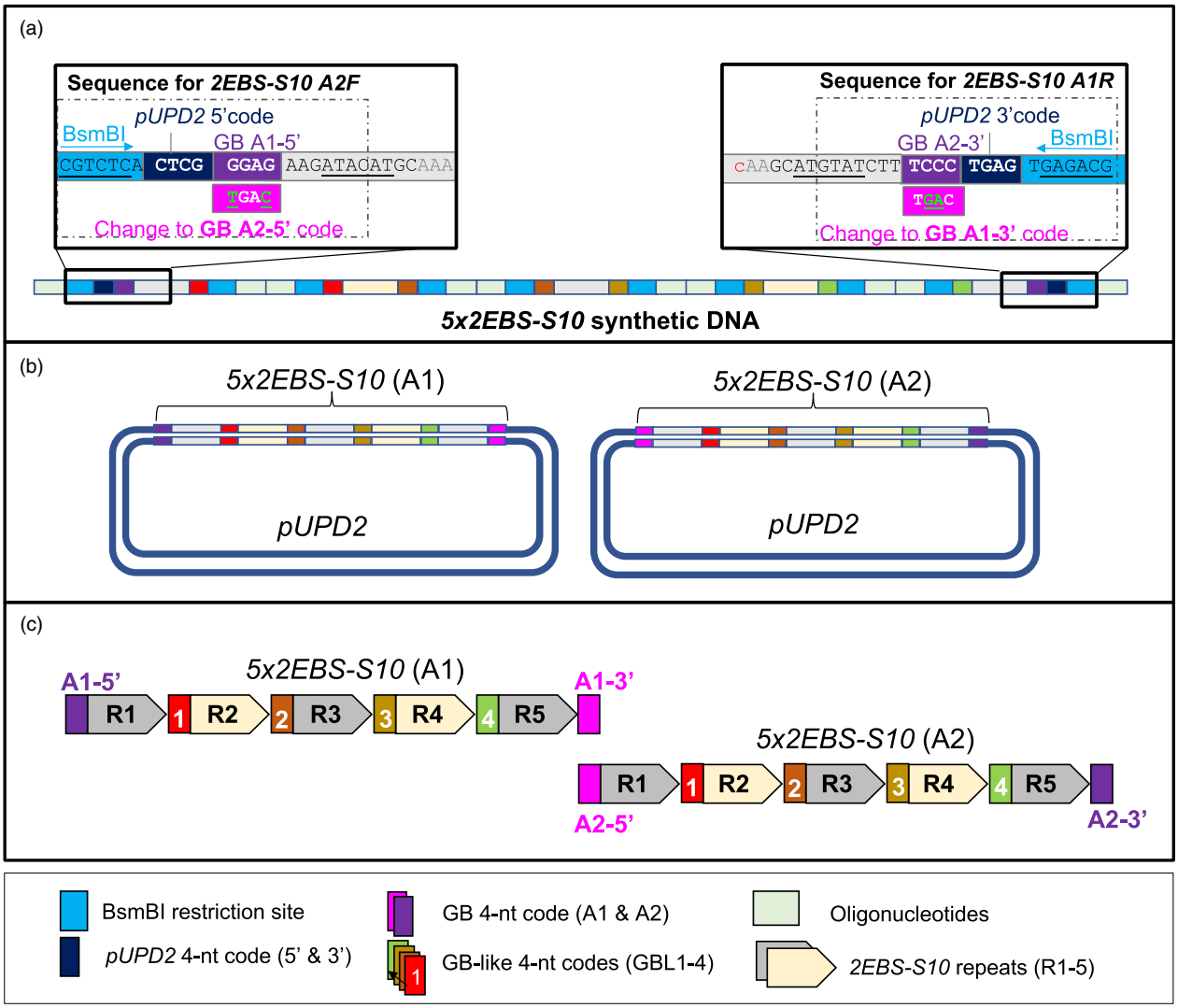

Figure 2 The strategy for generating the *10x2EBS-S10* synthetic promoter. (a) Schematic colour-coded representation of the *5x2EBS-S10* synthetic DNA fragment described in Figure 1b, with two upper insets showing GB entry vector overhang sequences. The grammar for the A1-A2 distal-proximal promoter GB element, GB-A1 5′ code [GGAG] and GB-A2 3′ code [TCCC], was modified by site-directed mutagenesis using *2EBS-S10_A1R* or *2EBS-S10_A2F* oligos (Table S1) with two nucleotide changes in either A1 or A2 GB code (light green nucleotides in the pink box) to generate the [TGAC] grammar in either the A1 3′ code or the A2 5′ code (pink). (b) To generate the *10x2EBS-S10* promoter, two *5x2EBS-S10* copies were cloned in independent *pUPD2* vectors. One vector harboured a *5x2EBS-S10* (A1) part [GGAG – TGAC] and the other carried a *5x2EBS-S10* (A2) part [TGAC – TCCC]. (c) The compatibility of the [TGAC] grammar (pink) makes it possible to combine the (A1) and (A2) parts via GB cloning in a destination vector of interest to generate a *10x2EBS-S10* synthetic promoter. A legend listing different types of DNA elements is included on the bottom. See also Figure 1 for colour coding description.

independent *GUS* lines, #18 and #21 (Figure 3c) grown in the presence of ethylene gas. No prominent GUS expression was observed in control conditions, confirming the ethylene-inducibility of this new reporter.

Although some transgenic lines displayed ethylene-triggered GUS staining in apical hooks and/or cotyledons, the primary domain of *GUS* reporter activity encompassed only the hypocotyls and roots, with the upper part of most seedlings showing no or little GUS activity in ethylene or ACC in a majority of transgenic lines (Figure S4, Figures 3c, 4). This pattern clearly differs from that of the classic *EBS:GUS* reporter (Stepanova, 2001, Stepanova *et al.*, 2007) that harbours five copies of an unpaired EIN3-binding element from the promoter of the *EDF1* (*At1g25560*) gene and is predominantly expressed in cotyledons, apical hooks and root tips (Figure 3d), suggesting that the two synthetic promoters may recruit alternative EIN3-containing transcriptional complexes that have different tissue-specific prevalence.

In contrast with the aforementioned widespread silencing of the *YPet* and *3xYPet* lines, the robust inducibility of the new *10x2EBS-S10:GUS* reporter persisted until at least the T4 generation without any signs of silencing, as shown for line 18 (Figure 4a). Sanger sequencing was performed to confirm the structural integrity of the *10x2EBS-S10* promoter of lines 18 and 21, with all 10 dual *2EBS-S10* elements found to be intact (Figure 4b). In contrast, a loss of four of 10 dual *2EBS-S10* everted repeat elements was discovered in the promoter of line 17, with

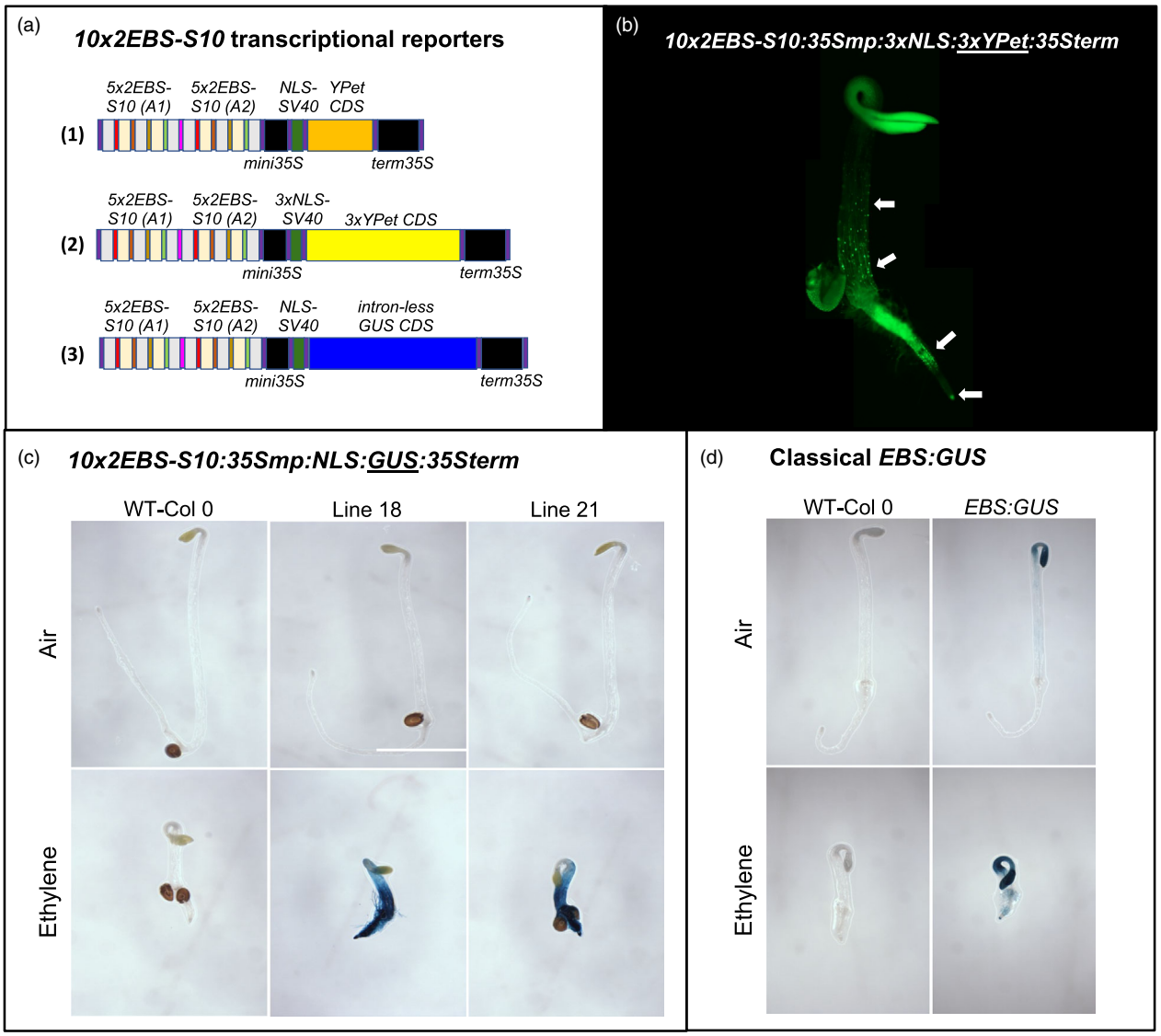

Figure 3 Ethylene-inducible expression of *10x2EBS-S10* reporters in Arabidopsis. (a) Schematic representation of three *10x2EBS-S10* reporter constructs harbouring the *10x2EBS-S10* proximal and *35S*(−46 bp) minimal promoters, a *NLS-SV40* nuclear localization signal, the *YPet*, *3xYPet* fluorescent proteins or *GUS* coding region, and a *35S* terminator. (b) A representative three-day-old dark-grown *10x2EBS-S10:35Smp:3xNLS:3xYPet:35Sterm* T2 seedling germinated in the presence of the ethylene precursor ACC. Fluorescence in the nuclei of lower hypocotyl, root and root tip cells is marked with white arrows. (c) Four-day-old *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* T4 seedlings grown in the dark in air or in the presence of ethylene gas (10 ppm). (d) Three-day-old Arabidopsis seedlings carrying the classical *EBS:GUS* reporter grown in the dark in air or 10 ppm ethylene.

the deletion encompassing repeat #5 from the (A1) fragment and repeats #1 to #3 from the (A2) fragment (Figure 4b). A detailed comparison of the expression patterns in the intact line 18 and the truncated line 17 showed that, on average, line 17 displayed a much stronger transgene expression in apical hooks, hypocotyl vasculature, and root tips relative to line 18, but less GUS activity elsewhere in the hypocotyls of three-day-old seedlings germinated in the presence of ACC. Although it is tempting to suggest that these differences could be due to the truncated promoter, it is also possible that they are caused by the differential positional effects of the insertion (Matzke and Matzke, 1998) or tissue-specific transcriptional or post-transcriptional gene silencing (Butel *et al.*, 2021).

The activity of the *10x2EBS-S10:GUS* reporter was also examined in the inflorescences of soil-grown adult plants for the promoter-intact lines 18 and 21. The *10x2EBS-S10:GUS* expression was primarily observed in the anthers and pollen (Figure 5), implicating ethylene in pollen development (Mira *et al.*, 2015). Interestingly, in older plants that have experienced stress due to mild soil toxicity, some *10x2EBS-S10:GUS* activity was also observed in mature ovules and young embryos (Figure S5). The pollen-enriched expression of *10x2EBS-S10:GUS* is in sharp contrast with the notable lack of any activity in these tissues for the classical *EBS:GUS* reporter, which in our growth conditions appears to be excluded from both the pollen and a majority of the pistil (Figure 5). In the flower, *EBS:GUS* is most active in the sepals, nectaries, petal vasculature and stamen filaments, with milder expression also detected in the pedicels, styles, transmitting tracts and the septums of young developing siliques. Noteworthy, non-transgenic wild-type Col-0 Arabidopsis

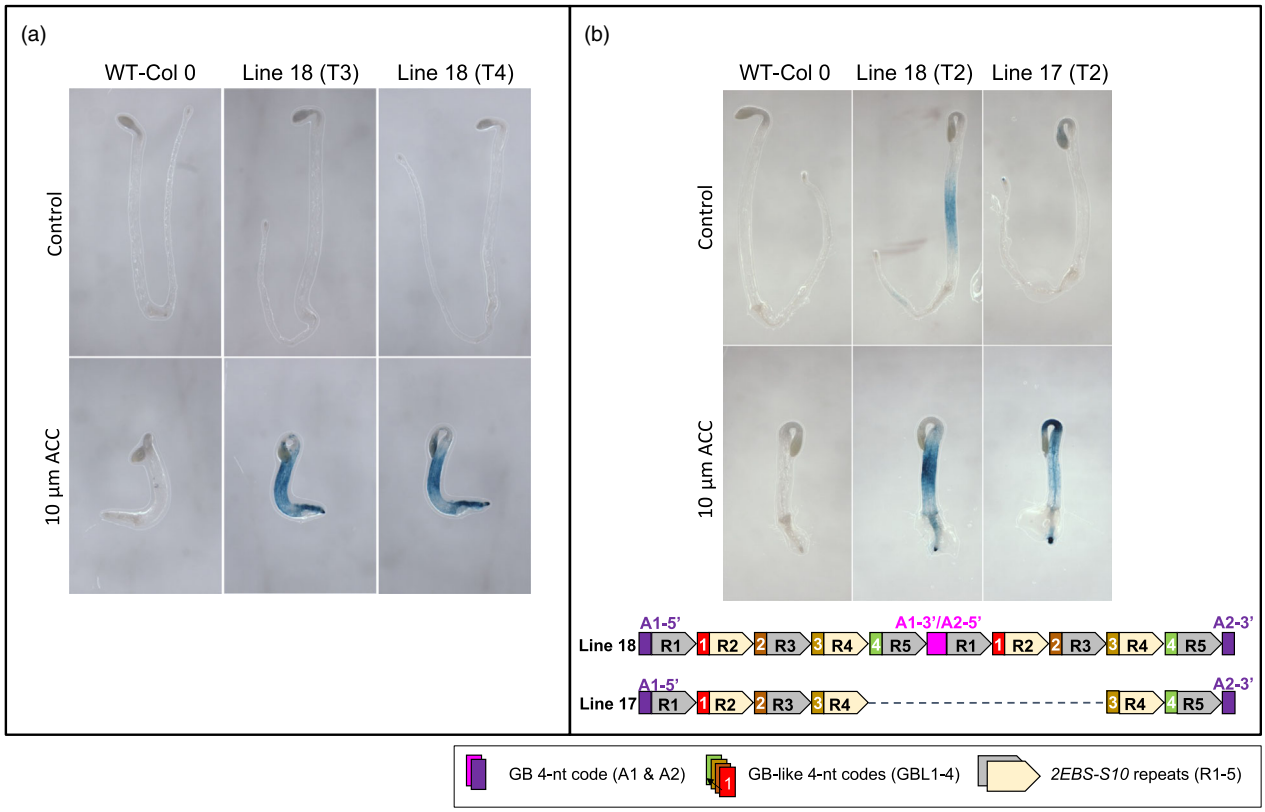

Figure 4 Stability of the *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* transgene in the Arabidopsis transgenic lines. (a) Whole-plant images of four-day-old dark-grown Col-0 (left panels), representative *10x2EBS-S10:35Smp: NLS:GUS:35Sterm* transgenic T3 (central panels) and T4 (right panels) seedlings from line 18 germinated in control media or media supplemented with 10 µM ACC and stained for GUS overnight. (b) Sanger sequencing uncovered four missing *2EBS-S10* repeats in line 17 relative to the repeat-intact line 18. A legend listing different types of DNA elements is included on the bottom.

plants also showed some basal GUS activity in immature anthers and nectaries of mature flowers (Figure 5), consistent with prior reports of the Arabidopsis genome harbouring *GUS*-like genes (Bruno *et al.*, 2023; Eudes *et al.*, 2008).

In summary, our data demonstrate that the *10x2EBS-S10* promoter is functional in stably transformed plants and shows robust ethylene-inducible expression. This finding backs the utility of our novel method of building sequence tandems with temporary stuffers as an effective way to lower the overall sequence complexity allowing for its commercial synthesis. The characterized *10x2EBS-S10:GUS* lines represent a new and powerful tool to examine the activation of the ethylene response at a high spatial and temporal resolution.

De novo assembly and functional validation of the *10x2DR5-2DR5v2* synthetic promoter

To demonstrate the broad applicability of our repetitive sequence generation method beyond making *10x2EBS-S10*, we designed another hormone-responsive proximal promoter, in this case, a new version of an auxin-responsive synthetic sequence that harbours alternating AUXIN RESPONSE FACTOR (ARF)-binding sites, *DR5* [TGTCTC] (Ulmasov *et al.*, 1997) and *DR5v2* [TGTCGG] (Liao *et al.*, 2015). The *DR5* and *DR5v2* elements are both functional *in planta* and their respective reporters show different spatial distributions (Liao *et al.*, 2015; Ulmasov *et al.*, 1997). We strategically chose to combine both auxin responsive elements in hopes of increasing the sensitivity of the novel synthetic proximal promoter. Thus, each *2DR5-2DR5v2* repeat of the new auxin-specific synthetic promoter consisted of two inverted *DR5* elements followed by two inverted *DR5v2* elements, with 7 base pair spacers placed within and between these DNA elements (Figures 6a, Figure S6). Each *2DR5-2DR5v2* repeat (52 bp, 57.69% GC) is about twice the size and GC content of the *2EBS-S10* repeat (28 bp, 28.57% GC) and, accordingly, has greater sequence complexity (Figure 6A).

Similar to the design of the padded *5x2EBS-S10* sequence (Figure 1b), we flanked each *2DR5-2DR5v2* repeat by four-nucleotide GB (purple and pink) or GBL (red, brown, mustard and green) codes, *BsmBI* restriction sites (cyan), and oligonucleotide stuffer sequences (light green) (Figure 6b, Figure S6). Additionally, the *pUPD2* GB code (dark blue) was incorporated at the beginning and at the end of the DNA fragment. As before, only a tandem of five repeats was able to pass the sequence complexity filters for its synthesis with IDT (Figure 6b).

Following the same steps as for the generation of *10x2EBS-S10*, we amplified either the *5x2DR5-2DR5v2* (A1) or (A2) fragments (Figure 5b) from the corresponding IDT gBlock template and used the purified PCR products to assemble these promoters into *pUPD2* using the same procedures as for the *10x2EBS-S10* promoter (Figure 6c). Similarly, *NotI* restriction enzyme was used to identify positive clones with insert bands of expected sizes that were later confirmed by Sanger sequencing.

To rapidly test the activity of the *10x2DR5-2DR5v2* proximal promoter *in planta*, we generated a *YPet* transcriptional reporter

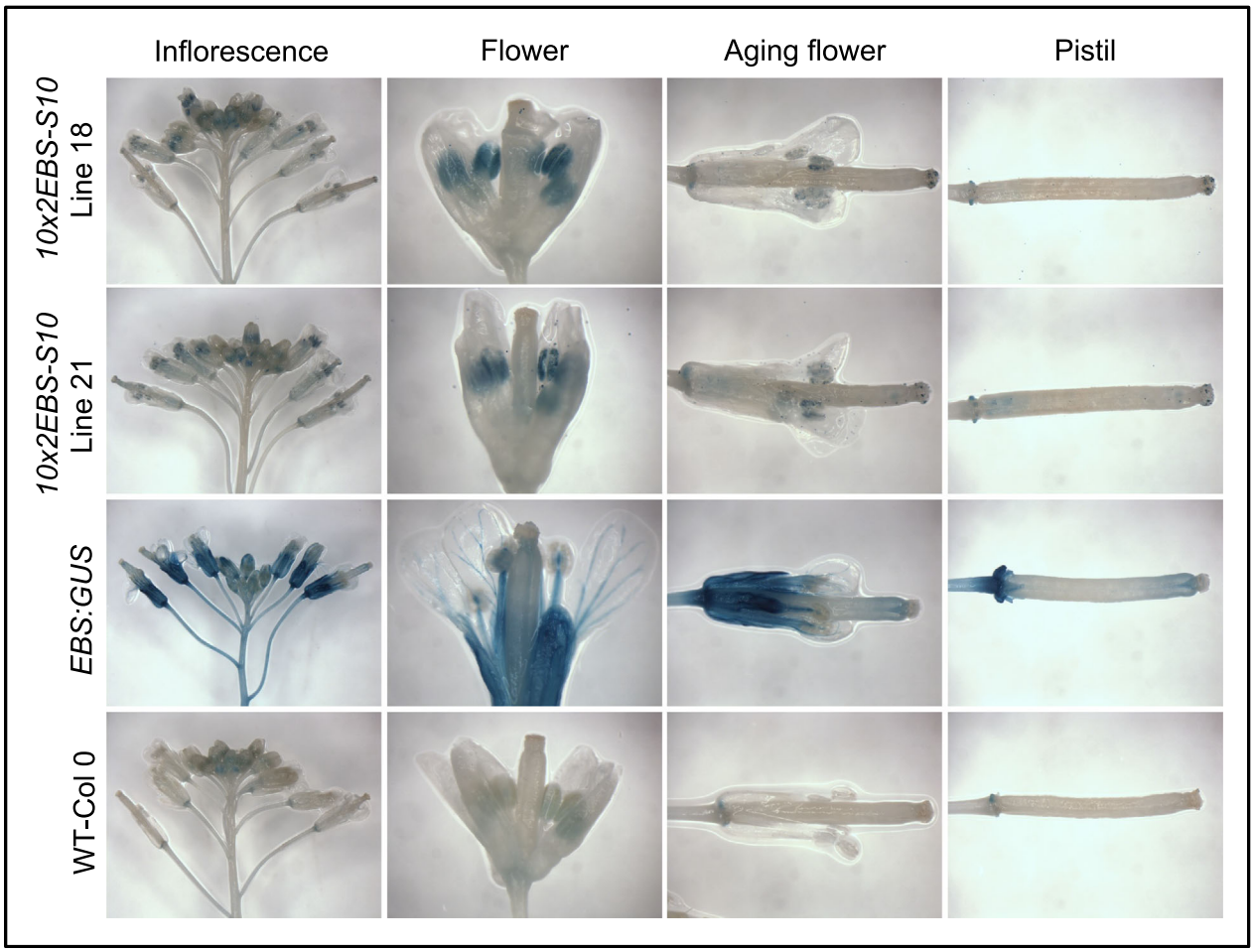

Figure 5 Expression of *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* and *EBS:GUS* in the reproductive organs of soil-grown plants. Images were taken at different magnifications: inflorescence at 1×, flower at 5×, aging flower at 2.5× and pistil at 2.5×. Pistils imaged are the same pistils as in the aging flower images, but with stamens, sepals, and petals removed.

via an alpha-level GB cloning reaction. The resulting construct harbours a minimal *35S*(−46) promoter, a single copy of *YPet*, a nuclear localization signal *NLS-SV40* and a *35S* terminator (Figure 6d). The reporter construct was agroinfiltrated into *Nicotiana benthamiana* leaves treated with 50 μM 1-naphthaleneacetic acid (NAA), a synthetic auxin analogue (Figure 6e). Consistent with our expectations, YPet was detected in the nucleus and nucleolus of infiltrated plants, indicating the ability of *10x2DR5-2DR5v2* to support expression of the reporter construct in NAA-treated plants. These results confirmed that our approach can be successfully used to assemble highly repetitive sequences from commercially synthesized, padded DNA fragments containing intervening stuffers that are then enzymatically removed during the assembly of the repetitive sequences in a single GB cloning reaction.

Discussion

Repetitive sequence generation

In this study, we developed a simple procedure that bypasses current limitations of commercial DNA synthesis to generate highly repetitive sequences (Figure S7). Temporary inclusion of random stuffers in the DNA template reduces the overall repetitiveness of the sequence and allows for the selective amplification of the desired repeat or group of repeats from synthesizable DNA fragments. By lowering the overall sequence complexity, sequence padding helps with passing vendor's complexity filters and makes it possible to synthesize DNA fragments containing sets of repetitive sequences commercially and amplify them by PCR. By including Type IIS recognition sites in the stuffers and designing the cut sites in a way that unique complementary overhangs are formed upon cutting, we make such padded DNA sequences compatible with standard GB cloning. A typical single-tube digestion–ligation reaction leads to stuffer removal and ordered rejoining of up to five repeats directly in an entry vector of interest. An extra digestion step at the end of the digestion–ligation cycle eliminates the clones that retained any of the stuffers. Successful cloning events are then verified by restriction digest through insert sizing by gel electrophoresis or by colony PCR (with the caveat that some highly repetitive sequences may not be directly amplifiable by PCR (Hommelsheim *et al.*, 2014; Riet *et al.*, 2017)) and ultimately confirmed by Sanger sequencing of plasmid DNA or PCR products (Figure S7).

While all the work described in this study was carried out on relatively simple, repetitive sequences, padding approaches can be extended to even more complex promoter architectures

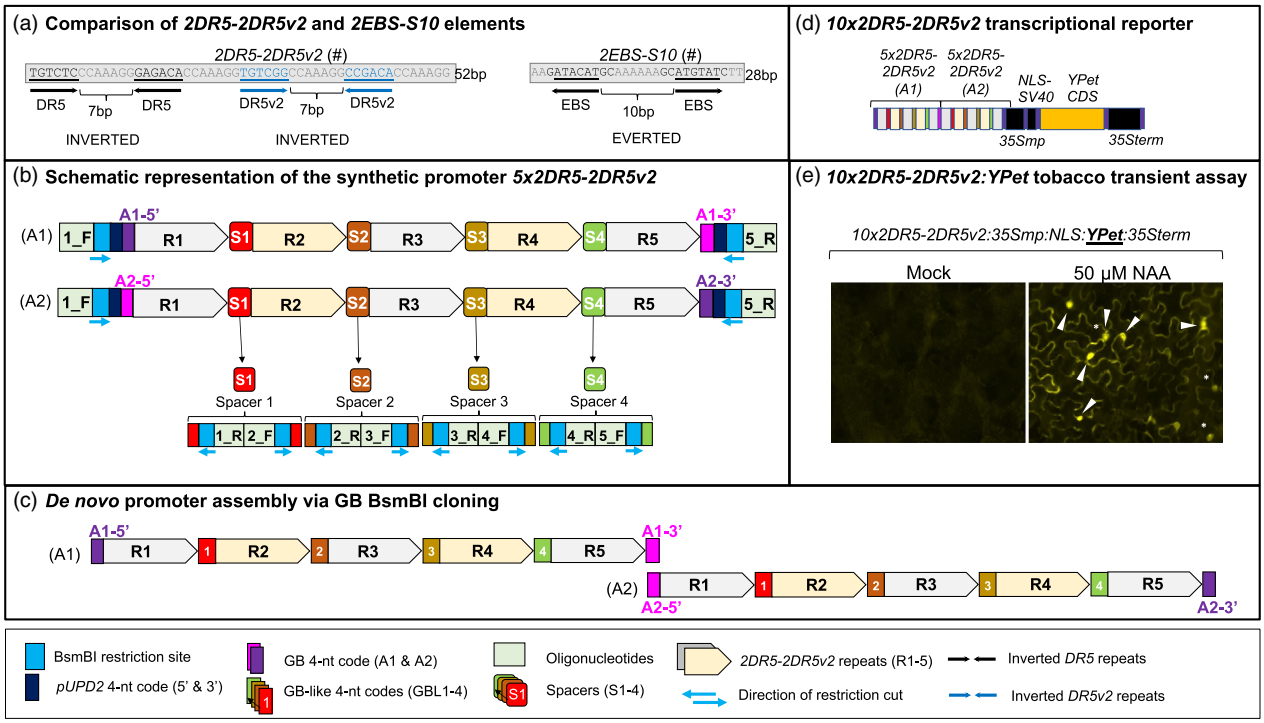

Figure 6 Cloning and functional testing of the *10x2DR5-2DR5v2* synthetic promoter. (a) Comparison of the *2DR5-2DR5v2* and *2EBS-S10* DNA element structures. (b) Schematic representation of the amplified *5x2DR5-2DR5v2* synthetic (A1) and (A2) fragments that pass vendor's sequence complexity thresholds (details of the sequence are shown in Figures S6 and S9). (c) Digestion of the synthetic *5x2DR5-2DR5v2* (A1) and (A2) DNA fragments with *BsmBI* enzyme facilitates the directional assembly of the repeats flanked by compatible four-nucleotide GBL codes. The GB grammar for the (A1) [GGAG (5′) – TGAC (3′)] and the (A2) [TGAC (5′) – TCCC (3′)] promoter elements allows for their compatible subcloning in tandem into the GB alpha-level vectors when building the reporter transcriptional unit, generating the *10x2DR5-2DR5v2* promoter. (d) Schematic representation of the *10x2DR5-2DR5v2:35Smp:NLS:1xYPet:35Sterm* fluorescent reporter construct. (e) *Nicotiana benthamiana* leaf agroinfiltration confirmed the induction of the reporter in 50 µM NAA-treated plants relative to the mock-treated leaves. The arrowheads point to the nuclei expressing the new auxin-inducible reporter. Asterisks mark the nucleoli visible inside of some nuclei. A legend listing different types of DNA elements is included on the bottom.

comprising multiple copies of two or more synthetic transcription factor-binding sites or even for generating libraries of randomly assorted *cis*-elements. Furthermore, adding GC-rich stuffers to an otherwise AT-rich template (or vice versa) would normalize its GC content. Likewise, interrupting homopolymers with occasional random stuffers would diversify the sequence, allowing for their direct synthesis and improving otherwise inefficient PCR amplification. By strategically inserting removable stuffers in the DNA region that tends to fold into secondary structures, the formation of those structures can be minimized, and the likelihood that the sequence can be synthesized commercially increased. Furthermore, the GB-like codes used for the sequence padding could be designed to be part of the stuffers, allowing for a scarless sequence assembly.

Although our approach provides a universal strategy to deal with short, repetitive sequences, it remains to be determined on a case-by-case basis whether stuffers can be safely enzymatically removed, as ultimately, the user is still left working with complex sequences that may be difficult to handle *in vitro* and propagate faithfully *in vivo*. In the case of the repetitive sequences we worked with in this project, we encountered a number of technical difficulties including inefficient PCR amplification of all stuffer-less promoters, mild toxicity of the *10x2DR5-2DR5v2* construct in *E. coli* and occasional repeat loss of *10x2EBS-S10* upon extended culturing in *E. coli*. Poor PCR amplification (Hommelsheim *et al.*, 2014; Riet *et al.*, 2017) and spontaneous loss of repetitive sequences in bacteria have been widely reported (Blackwood *et al.*, 2010; Bzymek and Lovett, 2001; Metzgar *et al.*, 2001), and some of these problems could be at least partially bypassed by, for example, using special *E. coli* strains such as Stbl4 or improved PCR protocols (Assad *et al.*, 2021; Riet *et al.*, 2017). In this project, one of the three sequence-verified *10x2EBS-S10:35Smp:3xNLS:3xYPet:35Sterm* Arabidopsis transgenic lines was found to be missing four dual *2EBS-S10* repeats (Figure S3D). Likewise, one of 19 PCR-positive *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* transgenic lines, #17, that was Sanger sequenced had a truncation of four of 10 dual *2EBS-S10* repeats (Figure 4b). Given that a majority of transgenic plant lines generated with an *Agrobacterium* strain have intact repeats and assuming that the transformed *Agrobacterium* cell line had initially acquired a single copy of the intact plasmid, the truncation of some of the repeats must have happened in *Agrobacterium* or in the plant. Interestingly, the truncated promoter *GUS* line exhibited a different pattern of expression in ACC relative to that of the intact promoter lines (Figure 4b). In fact, the activity pattern of the *2EBS-S10* line #17 appears to be similar to the expression pattern of the original *EBS:GUS* reporter line (Figure 3d), but the reason for that phenomenon is currently unclear.

Our repetitive sequence padding approach also allows for the assembly of non-identical repeats (that are still highly similar and thus incompatible with Gibson and related scarless,

homology-based DNA assembly methods) in a desired order. This important advantage of the proposed method further broadens the downstream utility of the resulting constructs, for example, to recruit entire transcription factor families with divergent DNA-binding preferences to a synthetic, non-identical-repeat promoter of interest or, alternatively, to make the promoter sequence-specific to a select subset of transcription factor family members. In the era of synthetic biology, padded commercially synthesized DNA fragments, in combination with enzymatic stuffer removal, will be a critical solution to DNA synthesis limitations until commercial manufacturing of complex sequences enters mainstream.

Surprisingly, the recent market trends have been shifting in the opposite direction, with some vendors increasing, rather than decreasing, the stringency of the complexity filters for commercial DNA synthesis, making the ordering of repetitive DNA fragments harder than before. In fact, we recently re-submitted the desired original (as well as padded) stuffer-containing DNA sequences of *5x2EBS-S10* and *5x2DR5-2DR5v2* to different vendors. No vendors were willing to synthesize the non-padded repetitive sequences and only one of the vendors, Twist Biosciences, was willing to proceed with the synthesis of the padded *5x2EBS-S10* sequence. IDT, the vendor we originally ordered from, was no longer able to accept our *5x2EBS-S10* and *5x2DR5-2DR5v2* order, with even the padded versions of these sequences now failing to pass the new, more restrictive complexity filters. These stricter cut-offs in the sequence complexity thresholds recently adopted by DNA synthesis companies make our approach even more relevant, as even moderately repetitive sequences can no longer be directly synthesized. As a workaround, with our approach, repetitive sequences can be synthesized in different DNA fragments (e.g., one or two repeats per fragment, possibly combined with other, unrelated sequences in the same gBlock), selectively amplified using the flanking stuffers, and assembled together in the desired order by means of the *BsmBI* restriction sites and GB codes present in the stuffers.

Novel hormone-responsive promoters

While this work highlights primarily the development of a suitable method for generating repetitive sequences that are not amenable to direct DNA synthesis using current commercial technologies, the ethylene and auxin reporters made in this work represent a useful resource for the plant community. While several auxin-responsive synthetic promoters have been described in the plant biology literature and multiple transcriptional reporters, degradation-based and FRET-based biosensors are available for auxin (reviewed by Zhao *et al.*, 2021), only one version of the synthetic ethylene reporter, *EBS:GUS* (reviewed by Fernandez-Moreno and Stepanova, 2020), has been adopted by the plant research community, and additional tools are urgently needed. Our group is working on developing alternative synthetic ethylene-responsive promoters, with the *2EBS-S10*-based version being of great interest due to its high performance in EIN3 binding gel-shift assays *in vitro* (Song *et al.*, 2015). Limited reporter construct testing included in this work shows that the *10x2EBS-S10* promoter may experience repeat instability and that the *YPet* versions of the construct undergo silencing. This may not be surprising given that the new synthetic promoter harbours four times as many EIN3 binding sites as does *EBS:GUS*. However, since the *GUS* lines driven by the *10x2EBS-S10* promoter show consistent expression until at least the T4 generation, the silencing issues observed in the equivalent *1xYPet* and *3xYPet* lines may at least in part be associated with the *YPet* sequence and not with the highly repetitive *10x2EBS-S10* promoter alone. The use of alternative fluorescent reporters may provide a simple solution to the observed silencing problem observed in the *YPet* lines.

Regardless, given their unique expression pattern and the apparent lack of silencing in the *10x2EBS-S10:NLS:GUS* transgenic lines, we believe that these reporter lines represent an important new genetic tool complementary to the original *EBS:GUS*. Not only does the new reporter provide an *in vivo* validation of the synthetic EIN3-binding *2EBS-S10* sequence previously tested only *in vitro* or in transient expression assays in protoplasts (Song *et al.*, 2015), but it also enables monitoring of ethylene-regulated processes in tissues beyond those where the classic *EBS:GUS* is responsive to ethylene. For example, to study the role of ethylene in pollen development or in hypocotyl- or root-localized processes or events (e.g., to explore the role of ethylene in pollen sterility under heat stress, or to understand the contribution of ethylene to plant host invasion by haustoria of parasitic fungi or plants), this new *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* reporter may prove to be better suited than the classical *EBS:GUS*.

From the basic science perspective, the difference in the seedling as well as flower expression patterns between *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* and *EBS:GUS* suggests that in Arabidopsis EIN3 may exist in alternative transcriptional complexes that show different tissue-specific distribution and divergent preferences for binding to specific architectures of the *EBS* (Figure 7). Thus, *10x2EBS-S10* carries 10 copies of dual everted *EBS* sites ATACAT(nx10)ATGTAT and may recruit EIN3 (and potentially also its paralogues EILs) as dimers that in the presence of ethylene are predominantly active in the hypocotyls and roots of etiolated seedlings (Figure 7a). The classical *EBS* harbours five copies of a single *EBS* site caaaggggggATGCACt from the promoter of the *EDF1* (*At1g25560*) gene and may recruit monomeric EIN3/EILs or, alternatively, heterodimers of EIN3 (or EILs) with sequence-unrelated proteins, that, according to the *EBS:GUS* reporter staining, may be most abundant in the cotyledons, apical hooks and root tips of the ethylene-treated plants (Figure 7b).

While *EBS:GUS* has been reported as functional in Arabidopsis, tomato and the parasitic plant *Triphysaria versicolor* (reviewed in Fernandez-Moreno and Stepanova, 2020), thus far, we have tested *10x2EBS-S10* only in Arabidopsis. To our knowledge, neither *10x2EBS-S10* nor *EBS:GUS* have been used in monocots or outside of flowering plants, so it remains to be determined if either of these two reporters and their respective sequence-divergent EIN3 binding elements are functional in a wider range of plant species beyond a handful of dicots.

The second version of the hormone-responsive promoter we have built, *10x2DR5-2DR5v2*, was successfully validated only by transient assays. We did transform the *10x2DR5-2DR5v2:35Smp:NLS:1xYPet:35Sterm* construct into Arabidopsis and generated stable transgenic lines. However, as with the *10x2EBS-S10:NLS:YPet*, no reliable expression was observed with or without auxin, beyond the T1 generation, suggesting transgene silencing. It would be interesting to determine if, like with the *2EBS-S10* constructs, the use of the *GUS* reporter results in more stable expression of the transgene. Alternatively, the sequences of individual repeats could be diversified with the goal to minimize the incidence of silencing. To bypass some of the problems associated with the *10x2DR5-2DR5v2* promoter-based expression in plants, our group is now developing a series of novel auxin

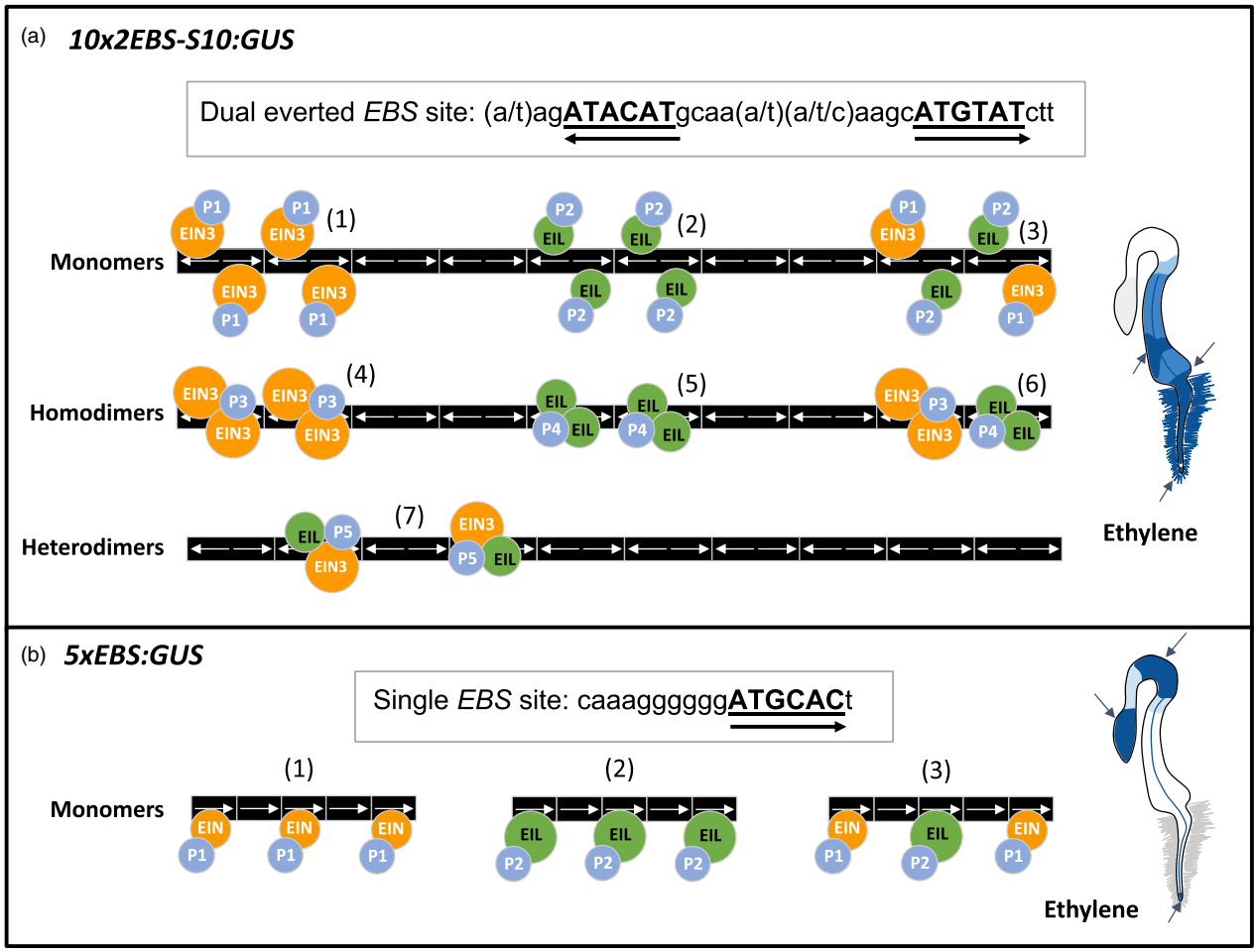

Figure 7 Possible mechanisms behind the different expression patterns of *10x2EBS-S10:GUS* and *5xEBS:GUS*. (a) In the *10x2EBS-S10* reporter lines, the presence of 10 everted *EBS* repeats may allow the recruitment of EIN3 in monomeric (1) or dimeric (4) forms. Potentially, these repeats may also recruit EIN3 paralogues, EILs, as monomers (2) or dimers (5). Finally, EIN3 and EIL may also be co-recruited together as monomers (3), homodimers (6) or heterodimers (7). (b) In the *5xEBS:GUS* reporter lines, the presence of single (unpaired) direct *EBS* repeats may recruit only the monomers of either EIN3 (1), EIL (2) or both (3), although with different affinity due to the sequence differences between the repeats in the *10x2EBS-S10* and the *5xEBS:GUS* constructs. The differential affinity of different EIN3/EIL family members for the specific sequences in the *10x2EBS-S10* and the *5xEBS:GUS* is reflected in the figure by different sizes of the EIN3 and EIL circles. EIN3/EILs may be recruited to these DNA elements in the context of different protein complexes depending on the cell type, developmental stage, etc. These different protein complexes may work as transcriptional activators or repressors and are illustrated as blue numbered 'P' circles (where numbers indicate the different nature of these proteins) that preferentially interact with different monomers and/or dimers. The panels on the right show a schematic representation of typical expression patterns of these two ethylene reporters in three-day-old, etiolated seedlings treated with 10 ppm ethylene. Areas with the strongest induction of the reporter are coloured dark blue and marked with arrows. Areas with lower ethylene-inducible expression are shown in lighter shades of blue, and areas where the reporter is not expressed in ethylene-treated plants are left blank or light grey.

reporters with different heteromeric synthetic promoter architectures. Another reasonable strategy for minimizing the probability of construct silencing and repeat instability would be to optimize the number of *cis*-elements being stacked in an attempt to strike a better balance aiming for a greater level of transgene expression (the more repeats, the better) yet lower construct silencing and instability (the fewer repeats, the better).

In conclusion, the repetitive sequence generation method we have developed in this study provides a simple strategy for dealing with high-complexity sequences that are not amenable to direct commercial DNA synthesis and/or PCR amplification. The new technology is not limited to producing repetitive promoter element tandems for monitoring plant hormones, but rather can be applied to building synthetic, high-complexity sequences for a variety of *in vitro* and *in vivo* applications. We foresee that this hack will prove useful to a broad range of basic and applied scientists interested in applying synthetic biology tools to their favourite biological questions.

Experimental procedures

Design of repetitive DNA fragments for *de novo* synthesis

Benchling (https://www.benchling.com) was utilized to design and build the synthetic DNA fragments for *5x2EBS-S10* (A1-A2), *5x2DR5-2DR5v2* (A1) and *5x2DR5-2DR5v2* (A2) GB distal-proximal promoter elements, and to test *in silico* their amplification and cloning prior to their independent synthesis by

IDT (https://www.idtdna.com/pages) (Figure S7A). Previously described *cis*-elements, ethylene-responsive *2EBS-S10* recognized by EIN3 *in vitro* (Song *et al*., 2015) and auxin-responsive *DR5* (Ulmasov *et al*., 1997) and *DR5v2* (Liao *et al*., 2015) ARF binding sites, were leveraged in the design of these promoters. The 12 standard GB grammar codes were utilized for the assembly of the canonical gene parts (Patron *et al*., 2015, https://gbcloning.upv.es), while random four-nucleotide GBL codes, different from the standard GB codes, were used for the removal of the stuffers (Figure S7B). Full padded sequence of the synthesized stuffer-containing *5x2EBS-S10* (A1-A2) fragment is shown in Figure S8. The equivalent sequences for *5x2DR5-2DR5v2* (A1) and *5x2DR5-2DR5v2* (A2) fragments are displayed in Figure S9. All primers and oligonucleotide stuffer sequences used in this work are compiled in Table S1.

Assembly of synthetic DNA fragments using GB methodology

GB domestication, stuffer removal and subcloning of repetitive DNA fragments

Amplification of stuffer-containing DNA fragments from the three aforementioned synthetic IDT gBlock templates was done with the proofreading iProof High-Fidelity DNA Polymerase (Bio-Rad) following manufacturer's recommendations at the annealing temperature of 58 °C (Figure S7C). The three synthetic DNAs, *5x2EBS-S10* (A1-A2), *5x2DR5-2DR5v2* (A1) and *5x2DR5-2DR5v2* (A2), were amplified with the common flanking primers *Oligo 1_F* and *Oligo 5_R* (Table S1). In order to create a *10x2EBS-S10* synthetic promoter, the *5x2EBS-S10* (A1-A2) template was amplified with *Oligo 1_F* and *2EBS-S10_A1R* (Table S1) to introduce targeted nucleotide changes in the 3′ GB grammar of *5x2EBS-S10* (A1-A2) fragment and to generate the *5x2EBS-S10* (A1) fragment. In parallel, *2EBS-S10_A2F* and *Oligo 5_R* (Table S1) were used to change the 5′ GB grammar of the *5x2EBS-S10* (A1-A2) fragment to create the *5x2EBS-S10* (A2) fragment. PCR products (50 μL) were extracted with 50 μL chloroform (1:1 v:v) and precipitated with ethanol. Air-dried pellets were resuspended in 15 μL sterile distilled water and further diluted to 40 ng/μL. One μL of the purified DNA was used for the domestication of the PCR fragment into the GB platform. Standard GB cloning procedures to clone PCR products into the *pUPD2* entry vector (Figure S2A) (Sarrion-Perdigones *et al*., 2013, 2014; Vazquez-Vilar *et al*., 2015) were followed with minor modifications: 25 cycles of digestion/ligation with *BsmBI* restriction enzyme (NEB) at 37 °C for 3 min and ligation with T4 DNA ligase (NEB) at 16 °C for 5 min were performed, followed by a final digestion step at 37 °C for 5 min with 0.5 μL of fresh *BsmBI* to linearize the residual clones with unremoved stuffers (Figure S7D). Three μL of each GB reaction were transformed into 50 μL of home-made chemically competent TOP10 *E. coli* cells. The cells containing the desired *pUPD2*-derived plasmid were selected in LB-agar plates supplemented with 25 μg/mL chloramphenicol (GoldBio) and 20 μg/mL X-gal (GoldBio) for 16 h at 37 °C. White colonies were resuspended in 10 μL of sterile distilled water and 1 μL of each sample was used to test the insertion size by colony PCR [96 °C – 2 min; (96 °C – 30 s, 58 °C – 30 s and 72 °C – 1 min) × 35 cycles; 72 °C – 10 min; hold at 4 °C] with *pUPD2_NewF2* and *pUPD2_NewR2* primers (Table S1). 7 μL of water suspensions of PCR-positive colonies were inoculated into 4 mL of liquid LB supplemented with 25 μg/mL chloramphenicol (GoldBio). Cell cultures were grown for 16 h at 37°C with shaking. Plasmid DNA was extracted using alkaline lysis (Alonso and Stepanova, 2014) and digested with *NotI* (NEB) for 2 h at 37 °C, an enzyme that cuts twice in *pUPD2* at the flanks of the insert. Clones containing the desired DNA inserts were chosen by restriction digest- and gel-electrophoresis-based insert size analysis (Figure S7D) and Sanger sequencing using *pUPD2*-specific primers *pUPD2_NewF2* and *pUPD2_NewR2* (Table S1). Sequencing results were analysed in Benchling.

GB domestication of additional DNA fragments

GB assembly strategy (Sarrion-Perdigones *et al*., 2014; Vazquez-Vilar *et al*., 2015) was used to build hormone-responsive reporters (Figure S2). The previously published coding DNA sequence of the histochemical marker gene *β-glucuronidase* (GB0208-*pGUS*) (Eudes *et al*., 2008; Jefferson *et al*., 1987), *35S* terminator (GB0036-*pT35S*) (Franck *et al*., 1980) and the transcriptional unit of the *BASTA* resistance gene (GB0023-*pUPDtNOS:BASTA:pNOS*) (Table S2) (Rathore *et al*., 1993) were utilized to build the ethylene and auxin reporters. Ten additional entry clones were domesticated into *pUPD2*: the distal promoters *5x2EBS-S10* (A1-A2), *5x2EBS-S10* (A1), *5x2EBS-S10* (A2), *5x2DR5-2DR5v2* (A1) and *5x2DR5-2DR5v2* (A2); the minimal *35S* promoter (−46, UTR) (Odell *et al*., 1985); the nuclear localization signal *NLS-SV40* (Hicks and Raikhel, 1993) and its triple version (*3xNLS*) generated in our lab using nucleotide diversification; and the coding DNA sequence of either the single yellow fluorescent protein, *1xYPet* (Nguyen and Daugherty, 2005; Zhou *et al*., 2011), or the triple *AraYPet* yellow fluorescent protein, *3xYPet* (Brumos *et al*., 2020; Zhou *et al*., 2011) (Table S2). Both the original entry vector, *pUPD*, and its updated version, *pUPD2*, are interchangeable during the assembly process in *pDGB3alpha*- or *pDGB3omega*-level vectors (Sarrion-Perdigones *et al*., 2014; Vazquez-Vilar *et al*., 2015) and were used in parallel.

GB-alpha level assembly into transcriptional units

The GB alpha-level assembly (Figure S2B) (Sarrion-Perdigones *et al*., 2013; Vazquez-Vilar *et al*., 2015) was performed by combining the *pUPD* and *pUPD2* parts of interest into *pDGB3alpha*-level vectors to generate the following five individual transcriptional units: three ethylene-responsive reporters, *pDGB3alpha1_10x2EBS-S10:35Smp:NLS:1xYPet:35Sterm*, *pDGB3alpha1_10x2EBS-S10:35Smp:NLS:3xYPet:35Sterm*, and *pDGB3alpha1_10x2EBS-S10:35Smp:NLS:GUS:35Sterm*, one auxin-responsive reporter, *pDGB3alpha1_10x2DR5-2DR5v2:35Smp:NLS:1xYPet:35Sterm*, and a herbicide-resistant marker *pDGB3alpha2_35Sterm:BASTA:35Sp* (Table S3). Three μL of the different GB cloning reactions were transformed into 50 μL of chemically competent TOP10 *E. coli* and grown overnight in LB-plates supplemented with Kanamycin (GoldBio) 50 μg/mL and X-gal 20 μg/mL (GoldBio). White colonies were then analysed by colony PCR using the following oligo pairs (Table S1): *YPet-internal_F* and *UniversalpDGB3_R* for the *1xYPet* fluorescent reporter, *3xYPet-internal_F* and *UniversalpDGB3_R* for the *3xYPet* fluorescent reporter, *GUS-3′end_F* and *UniversalpDGB3_R* for the *GUS* reporter, and *UniversalpDGB3_F* and *BASTA-3′end_R* for the *BASTA* reporter. Construct identity was subsequently confirmed by restriction digests with *EcoRI* (NEB) which cuts the alpha-level vectors twice, releasing the full insert from the plasmid backbone.

GB-omega level assembly into gene modules

The GB omega-level assembly (Figure S2C) (Sarrion-Perdigones *et al*., 2013, 2014; Vazquez-Vilar *et al*., 2015) was carried out to

combine individual hormone-responsive transcriptional reporters with the *BASTA* marker in *pDGB3omega1* (Table S3). Primers *BASTA-5′end_F* and *UniversalpDGB3_R* (Table S1) were utilized to screen the positive clones of *1xYPet*, 3x*YPet* or *GUS* reporters in the *pDGB3omega1* vector. To further corroborate the assembly of each reporter unit with *BASTA*, additional primer combinations were employed: *YPet-internal_F* and *BASTA-3′end_R* primers for two *1xYPet* modules (harbouring *10x2EBS-S10* or *10x2DR5-2DR5v2* distal promoters); *3xYPet-internal_F* and *BASTA-3′end_R* or *35Smp_F* and *3xYPet-internal_R* for the *3xYPet* module; and *GUS-3′end_F* and *BASTA-3′end_R* primers for the *GUS* module (Table S1). Three μL of the GB cloning reactions were transformed into 50 μL of chemically competent TOP10 *E. coli* cells and grown overnight in LB-plates supplemented with Spectinomycin (GoldBio) 100 μg/mL and X-gal (GoldBio) 20 μg/mL. Construct identity in white colonies was subsequently validated by restriction digests with *BamHI* (NEB) which cuts the omega-level vectors twice, releasing the full insert.

Functional testing of the GB-assembled reporter DNA constructs

Transient agroinfiltration of Nicotiana benthamiana leaves

Omega-level plasmids harbouring the desired modules were transformed into home-made electrocompetent *Agrobacterium tumefaciens* GV3101 cells as described (Alonso and Stepanova, 2015) and confirmed by colony PCR using the same primer combinations as the ones used for *E. coli* colony validation. Positive agro clones were then tested in *N. benthamiana* leaf agroinfiltration assays, performed as described (Wang *et al*., 2015). For testing the *10x2DR5-2DR5v2:35Smp:NLS:1xYPet:35Sterm* reporter, 50 μM NAA (Sigma-Aldrich) was gently painted over the agro-infiltrated leaf area with a soft paint brush (Brumos *et al*., 2018) 1 h before microscopy imaging. Fluorescent imaging of agro-infiltrated leaf discs was done with a DFC365 FX camera and a Zeiss Axioplan microscope. A 20× objective (100% intensity, 587.5 ms Exposure, 1.5 Gain) was utilized.

In-planta hormone inducibility assays in transgenic Arabidopsis lines

Stable *Arabidopsis thaliana* transformation with the ethylene reporters *10x2EBS-S10:35Smp:NLS:1xYPet:35Sterm*, *10x2EBS-S10:35Smp:NLS:3xYPet:35Sterm* and *10x2EBS-S10:35Smp:NLS:GUS:35Sterm*, as well as with the auxin reporter *10x2DR5-2DR5v2:35Smp:NLS:1xYPet:35Sterm*, was performed via floral dip (Clough and Bent, 1998), and transgenic lines were screened as described (37, 38) in AT plates (4.33 g/L Murashige & Skoog media (PhytoTech Labs), 10 g/L sucrose, pH 6.2, 6 g/L Bactoagar) supplemented with 20 μg/mL phosphinothricin (PPT, GoldBio). T2, T3 and/or T4 seedlings were germinated at 22 °C in the dark in AT media with or without 10 μM ACC (PhytoTechnology Lab) supplementation, flow-through 10 ppm ethylene (Airgas), or 50 μM NAA (Sigma-Aldrich) for 3 days prior to fluorescence imaging as described above or for 4 days prior to GUS staining as described (Brumos *et al*., 2020). Adult transgenic plants harbouring the *10x2EBS-S10:35Smp:NLS:GUS:35Sterm* reporter were grown in soil (50:50 mix of Sun Gro Horticulture Professional Growing Mix and Jolly Gardner PRO-LINE C/B Growing Mix) in a walk-in growth chamber under led lights in ambient air without ethylene supplementation at a 16 h/8 h light/dark cycle. Inflorescences and emerging siliques were collected into cold 90% acetone, washed, and stained for GUS overnight for 20 h, then kept in 70% ethanol at 4 °C for several days to remove chlorophyll before being imaged as described (Brumos *et al*., 2018). Zen (Zeiss, https://www.zeiss.com) and ImageJ (ImageJ, https://imagej.net/Fiji/Downloads) software (Schneider *et al*., 2012) were used to process and adjust the brightness of the yellow fluorescence images.

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Conflict of interest

We declare no conflict of interest in this work.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

References

Alonso, J.M. and Stepanova, A.N. (2014) Arabidopsis transformation with large bacterial artificial chromosomes. *Methods Mol. Biol.* **1062**, 271–283.

Alonso, J.M. and Stepanova, A.N. (2015) A recombineering-based gene tagging system for Arabidopsis. *Methods Mol. Biol.* **1227**, 233–243. https://doi.org/10.1007/978-1-4939-1652-8_11

Assad, L., Matjošaitis, K. and Gross, H. (2021) Genome sequence *of Escherichia coli* Stbl4, a versatile genetic tool for heterologous expression. *Microbiol. Resour. Announc.* **10**, e00823-21.

Atanassov, I.I., Atanassov, I.I., Etchells, J.P. and Turner, S.R. (2009) A simple, flexible and efficient PCR-fusion/Gateway cloning procedure for gene fusion, site-directed mutagenesis, short sequence insertion and domain deletions and swaps. *Plant Methods* **5**, 14.

Bhandari, N., Khare, S., Walambe, R. and Kotecha, K. (2021) Comparison of machine learning and deep learning techniques in promoter prediction across diverse species. *PeerJ Computer Sci.* **7**, e365.

Blachinsky, E., Marbach, I., Cohen, R., Grably, M.R. and Engelberg, D. (2004) Procedure for controlling number of repeats, orientation, and order during cloning of oligonucleotides. *BioTechniques* **36**, 933–936.

Blackwood, J.K., Okely, E.A., Zahra, R., Eykelenboom, J.K. and Leach, D.R.F. (2010) DNA tandem repeat instability in the *Escherichia coli* chromosome is stimulated by mismatch repair at an adjacent CAG·CTG trinucleotide repeat. *Proc. Natl. Acad. Sci. USA* **107**, 22582–22586.

Boë, F. and Masson, J.M. (1996) Selective cloning of a defined number of tandem DNA repeats in *Escherichia coli*. *Nucleic Acids Res.* **24**, 2450–2451.

Brumos, J., Robles, L.M., Yun, J., Vu, T.C., Jackson, S., Alonso, J.M. and Stepanova, A.N. (2018) Local auxin biosynthesis is a key regulator of plant development. *Dev. Cell* **47**, 306–318.

Brumos, J., Zhao, C., Gong, Y., Soriano, D., Patel, A.P., Perez-Amador, M.A., Stepanova, A.N. *et al*. (2020) An improved recombineering toolset for plants. *Plant Cell* **32**, 100–122.

Bruno, L., Ronchini, M., Binelli, G., Muto, A., Chiappetta, A., Bitonti, M.B. and Gerola, P. (2023) A study of GUS expression in *Arabidopsis* as a tool for the evaluation of gene evolution, function and the role of expression derived from gene duplication. *Plan. Theory* **12**, 2051.

Butel, N., Yu, A., Le Masson, I., Borges, F., Elmayan, T., Taochy, C., Gursanscky, N.R. *et al*. (2021) Contrasting epigenetic control of transgenes and endogenous genes promotes post-transcriptional transgene silencing in *Arabidopsis*. *Nat. Commun.* **12**, 2787.

Bzymek, M. and Lovett, S.T. (2001) Instability of repetitive DNA sequences: the role of replication in multiple mechanisms. *Proc. Natl. Acad. Sci. USA* **98**, 8319–8325.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Engler, C., Kandzia, R. and Marillonnet, S. (2008) A one pot, one step. Precision cloning method with high throughput capability. *PLoS One* **3**, e3647.

Eudes, A., Mouille, G., Thévenin, J., Goyallon, A., Minic, Z. and Jouanin, L. (2008) Purification, cloning and functional characterization of an endogenous beta-glucoronidase in *Arabidopsis thaliana*. *Plant Cell Physiol.* **49**, 1331–1341.

Fagard, M. and Vaucheret, H. (2000) (Trans)gene silencing in plants: How many mechanisms? *Annu. Rev. Plant Physiol.* **51**, 167–194.

Fernandez-Moreno, J.-P. and Stepanova, A.N. (2020) Monitor ethylene in plants: genetically encoded reporters and biosensors. *Small Methods* **4**, 1900260.

Franck, A., Guilley, H., Jonard, G., Richards, K. and Hirth, L. (1980) Nucleotide sequence of Cauliflower Mosaic Virus DNA. *Cell* **21**, 285–294.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., III and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345.

Grabczyk, E. and Usdin, K. (1999) Generation of microgram quantities of trinucleotide repeat tracts of defined length, interspersion pattern, and orientation. *Anal. Biochem.* **267**, 241–243.

Hicks, G.R. and Raikhel, N.V. (1993) Specific binding of nuclear localization sequences to plant nuclei. *Plant Cell* **5**, 983–994.

Hillson, N.J. (2011) Chapter 14 – DNA assembly method standardization for synthetic biomolecular circuits and systems. In *Design and Analysis of Biomolecular Circuits*(Koeppl, H., Setti, G., di Bernardo, M. and Densmore, D., eds), pp. 295–314. New York, NY: Springer.

Hommelsheim, C.M., Frantzeskakis, L., Huang, M. and Ülker, B. (2014) PCR amplification of repetitive DNA: a limitation to genome editing technologies and many other applications. *Sci. Rep.* **4**, 5052.

Hoose, A., Vellacott, R., Storch, M., Freemont, P.S. and Ryadnov, M.G. (2023) DNA synthesis technologies to close the gene writing gap. *Nat. Rev. Chem.* **7**, 144–161.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: ?-Glucoronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Jiang, S.W., Trujillo, M.A. and Eberhardt, N.L. (1996) An efficient method for generation and subcloning of tandemly repeated DNA sequences with defined length, orientation and spacing. *Nucleic Acids Res.* **24**, 3278–3279.

Jobbagy, Z., Ward, J.L., Toan, S.-V., Leung, G.P.H. and Tse, C.M. (2002) One-Step unidirectional cloning of tandem repeats of DNA fragments: An application for fusion protein production. *Anal. Biochem.* **303**, 104–107.

Kim, S.C. and Szybalski, W. (1996) Amplification of cloned DNA as tandem multimers using *Bsp*MI-generated asymmetric cohesive ends. *Gene* **71**, 1–8.

Leibovic, Z. and Gronau, I. (2021) *Eliminating unwanted patterns with minimal interference. ArXiv*. https://api.semanticscholar.org/CorpusID:236987203

Li, M.Z. and Elledge, S.J. (2012) SLIC: A method for sequence- and ligation-independent cloning. In *Gene Synthesis. Methods in Molecular Biology*, Vol. **852** (Peccoud, J., ed), pp. 51–59. Totowa, NJ: Humana Press.

Liao, C.Y., Smet, W., Brunoud, G., Yoshida, S., Vernoux, T. and Weijers, D. (2015) Reporters for sensitive and quantitative measurement of auxin response. *Nat. Methods* **12**, 207–210.

Matzke, A.J.M. and Matzke, M.A. (1998) Position effects and epigenetic silencing of plant transgenes. *Curr. Opin. Plant Biol.* **1**, 142–148.

Metzgar, D., Thomas, E., Davis, C., Field, D. and Wills, C. (2001) The microsatellites of *Escherichia coli*: rapidly evolving repetitive DNAs in a non-pathogenic prokaryote. *Mol. Microbiol.* **39**, 183–190.

Mira, M.M., Adel, E.-A. and Stasolla, C. (2015) Ethylene is integrated into the nitric oxide regulation of *Arabidopsis* somatic embryogenesis. *J. Genet. Eng. Biotechnol.* **13**, 7–17.

Müller, B. and Sheen, J. (2008) Cytokinin and auxin interplay in root stem-cell specification during early embryogenesis. *Nature* **453**, 1094–1097.

Nguyen, A.W. and Daugherty, P.S. (2005) Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat. Biotechnol.* **23**, 355–360.

Odell, J.T., Nagy, F. and Chua, N.H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**, 810–812.

Pandey, B., Prakash, P., Verma, P.C. and Srivastava, R. (2019) Chapter 10 – Regulated gene expression by synthetic modulation of the promoter architecture in plants. In *Current Developments in Biotechnology and Bioengineering*(Singh, S.P., Pandey, A., Du, G. and Kumar, S., eds), pp. 235–255. Cambridge, MA: Elsevier.

Patron, N.J., Orzaez, D., Marillonnet, S., Warzecha, H., Matthewman, C., Youles, M., Raitskin, O. *et al*. (2015) Standards for plant synthetic biology: a common syntax for exchange of DNA parts. *New Phytol.* **208**, 13–19.

Quan, J. and Tian, J. (2009) Circular polymerase extension cloning of complex gene libraries and pathways. *PLoS One* **4**, e6441.

Rathore, K.S., Chowdhury, V.K. and Hodges, T.K. (1993) Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. *Plant Mol. Biol.* **21**, 871–884.

Riet, J., Ramos, L.R.V., Lewis, R.V. and Marins, L.F. (2017) Improving the PCR protocol to amplify a repetitive DNA sequence. *Genet. Mol. Res.* **16**, 1–11.

Sarrion-Perdigones, A., Falconi, E.E., Zandalinas, S.I., Juárez, P., Fernández-del-Carmen, A., Granell, A. and Orzaez, D. (2011) GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. *PLoS One* **6**, e21622.

Sarrion-Perdigones, A., Palaci, J., Granell, A. and Orzaez, D. (2014) Design and construction of multigenic constructs for plant biotechnology using the GoldenBraid cloning strategy. In *DNA Cloning and Assembly Methods. Methods in Molecular Biology*, Vol. **1116** (Valla, S. and Lale, R., eds), pp. 133–151. Totowa, NJ: Humana Press.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J. *et al*. (2013) GoldenBraid2.0: A comprehensive DNA assembly framework for Plant Synthetic Biology. *Plant Physiol.* **162**, 1618–1631.

Schmitz, R.J., Grotewold, E. and Stam, M. (2022) Cis-regulatory sequences in plants: their importance, discovery, and future challenges. *Plant Cell* **34**, 718–741.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.

Scior, A., Preissler, S., Koch, M. and Deuerling, E. (2011) Directed PCR-free engineering of highly repetitive DNA sequences. *BMC Biotechnol.* **11**, 82. http://www.biomedcentral.com/1472-6750/11/87

Solano, R., Stepanova, A.N., Chao, Q. and Ecker, J.R. (2015) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* **12**, 3703–3714.

Song, J., Zhu, C., Zhang, X., Wen, X., Liu, L., Peng, J., Guo, H. *et al*. (2015) Biochemical and structural insights into the mechanism of DNA recognition by *Arabidopsis* ETHYLENE INSENSITIVE 3. *PLoS One* **10**, 0137439.

Steiner, E., Israeli, A., Gupta, R., Shwartz, I., Nir, I., Leibman-Markus, M., Tal, L. *et al*. (2020) Characterization of the cytokinin sensor *TCSv2* in arabidopsis and tomato. *Plant Methods* **16**, 152.

Stepanova, A.N. (2001) *Nuclear Events in Ethylene Signalling in Arabidopsis Thaliana. PhD Thesis, University of Pennsylvania. Dissertation available from ProQuest. AAI3015380*.

Stepanova, A.N., Yu, J., Likhacheva, A.V. and Alonso, J.M. (2007) Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. *Plant Cell* **19**, 2169–2185.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T.J. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic Auxin Response Elements. *Plant Cell* **9**, 1963–1971.

Vazquez-Vilar, M., Sarrion-Perdigones, A., Ziarsolo, P., Blanca, J., Granell, A. and Orzaez, D. (2015) Software-assisted stacking of gene modules using GoldenBraid 2.0 DNA-assembly framework. In *Plant Functional Genomics. Methods in Molecular Biology*, Vol. **1284** (Alonso, J. and Stepanova, A., eds), pp. 399–420. New York, NY: Humana Press.

Vazquez-Vilar, M., Selma, S. and Orzaez, D. (2023) The design of synthetic gene circuits in plants: new components, old challenges. *J. Exp. Bot.* **74**, 3791–3805.

Wang, G.F., Ji, J., El-Kasmi, F., Dangl, J.L., Johal, G. and Balint-Kurti, P.J. (2015) Molecular and functional analyses of a maize autoactive NB-LRR protein identify precise structural requirements for activity. *PloS Pathog* **11**, 1004674.

Weber, E., Engler, C., Gruetzner, R., Werner, S. and Marillonnet, S. (2011) A modular cloning system for standardized assembly of multigene constructs. *PLoS One* **6**, e16765.

Williams, S.L. and Coster, G. (2022) Cloning and expansion of repetitive DNA sequences. *Methods Cell Biol.* **182**, 167–185. https://doi.org/10.1016/bs.mcb.2022.10.013

Yasmeen, E., Wang, J., Riaz, M., Zhang, L. and Zuo, K. (2023) Designing artificial synthetic promoters for accurate, smart, and versatile gene expression in plants. *Plant Commun.* **4**, 100558.

Yu, X., Liu, Z. and Sun, X. (2023) Single-cell and spatial multi-omics in the plant sciences: Technical advances, applications, and perspectives. *Plant Commun.* **4**, 100508.

Zhang, Y., Werling, U. and Edelmann, W. (2014) Seamless Ligation Cloning Extract (SLiCE) cloning method. In *DNA Cloning and Assembly Methods. Methods in Molecular Biology*, Vol. **1116** (Valla, S. and Lale, R., eds), pp. 235–244. Totowa, NJ: Humana Press.

Zhao, C., Yaschenko, A., Alonso, J.M. and Stepanova, A.N. (2021) Leveraging synthetic biology approaches in plant hormone research. *Curr. Opin. Plant Biol.* **60**, 101998.

Zhou, R., Benavente, L.M., Stepanova, A.N. and Alonso, J.M. (2011) A recombineering-based gene tagging system for Arabidopsis. *Plant J.* **66**, 712–723.

Zücher, E., Tavor-Deslex, D., Lituiev, D., Enkerli, K., Tarr, P.T. and Müller, B. (2013) A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in planta. *Plant Physiol.* **161**, 1066–1075.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Traditional cloning strategy for building tandems of identical repeat sequences in a vector from short oligonucleotides.
Figure S2 GoldenBraid multiplexing platform.
Figure S3 Characterization of the *10x2EBS-S10 1xYPet* and *3xYPet* lines.
Figure S4 Robust expression of the *10x2EBS-S10:GUS* reporter across individual Arabidopsis T2 lines.
Figure S5 GUS staining of flowers and young siliques of *10x2EBS-S10:GUS* transgenic Arabidopsis plants that had experienced stress due to mild soil toxicity.
Figure S6 Synthetic DNA fragments harbouring the A1 and A2 versions of the *5x2DR5-2DR5v2* repetitive sequences with intervening stuffers.
Figure S7 Schematics of the pipeline involved in building *de novo* synthetic repetitive promoters.
Figure S8 DNA sequence for the *5x2EBS-S10* IDT gBlock and assembled stuffer-less promoters.
Figure S9 DNA sequences of the A1 and A2 *5x2DR5-2DR5v2* IDT gBlocks and assembled stuffer-less promoters.
Table S1 Oligonucleotides used in PCR amplification and as DNA stuffer sequences.
Table S2 DNA fragments and GB entry clones utilized in the construction of hormone-responsive reporters.
Table S3 Clones harboring transcriptional units (*pDGB3alpha*-level) and modules (*pDGB3omega*-level) for the hormone-responsive reporters.