Plant Biotechnology Journal

Plant Biotechnology Journal (2013) **11**, pp. 649–658 doi: 10.1111/pbi.12084

Review article

Gene use restriction technologies for transgenic plant bioconfinement

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Received 1 February 2013;
revised 3 April 2013;
accepted 9 April 2013.
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Keywords: transgenic plants, transgene escape, male sterility, embryo sterility, transgene deletion, gene flow.

Summary

The advances of modern plant technologies, especially genetically modified crops, are considered to be a substantial benefit to agriculture and society. However, so-called transgene escape remains and is of environmental and regulatory concern. Genetic use restriction technologies (GURTs) provide a possible solution to prevent transgene dispersal. Although GURTs were originally developed as a way for intellectual property protection (IPP), we believe their maximum benefit could be in the prevention of gene flow, that is, bioconfinement. This review describes the underlying signal transduction and components necessary to implement any GURT system. Furthermore, we review the similarities and differences between IPP- and bioconfinement-oriented GURTs, discuss the GURTs' design for impeding transgene escape and summarize recent advances. Lastly, we go beyond the state of the science to speculate on regulatory and ecological effects of implementing GURTs for bioconfinement.

Introduction

Transgenic crops have become an integral part of modern agriculture and have been increasingly adopted worldwide (James, 2011). While the number of transgenic plants increases, so does the possibility of adventitious presence of transgenes in the environment. Pollen- and seed-mediated gene flow from transgenic plants to nontransgenics has occurred at a low level for a few species, but with little measurable effect (Warwick *et al.*, 2009). It is natural for crops to exchange genes with their wild and weedy relatives, but regulators have focused, in particular, on gene flow–associated risks for transgenic crops. However, just because hybridization and introgression could occur, it does not follow that transgenes necessarily give a selective advantage to their weedy relatives (Gressel and Al-Ahmad, 2012). Indeed, the introgression of endogenous crop genes to wild relatives is relatively rare for most crops in most places (Ellstrand *et al.*, 2013). Even though ecological risk is generally associated with a trait, environment and mating system of the crop and wild relative, there is an apparent benefit in preventing gene flow altogether (Stewart *et al.*, 2003). This would seem to be especially the case with transgenic perennial crops (Kausch *et al.*, 2010; Kwit and Stewart, 2012; Moon *et al.*, 2010a), and when transgenes and their conferred traits, such as drought, salt or cold tolerance, are not completely understood and fitness enhancing (Warwick *et al.*, 2009). Crops producing pharmaceutical products may require bioconfinement by regulators for commercialization (Moon *et al.*, 2010b; Stewart, 2008). Thus, technologies to reduce or eliminate gene flow would be beneficial from an ecological risk and regulatory standpoint.

The biological confinement (bioconfinement) of transgenes has been a topic of vigorous debate since the inception of plant biotechnology. Currently, no bioconfined transgenic plants have been commercialized wherein pollen- and seed-mediated gene flow is completely controlled. Many technologies have been proposed (Daniell, 2002; Gressel, 1999; Moon *et al.*, 2011), including strategies for male sterility (Mariani *et al.*, 1990), maternal inheritance (Daniell *et al.*, 1998; Iamtham and Day, 2000; Ruf *et al.*, 2001), transgenic mitigation (Al-Ahmad *et al.*, 2004, 2006), transgene excision (Luo *et al.*, 2007; Moon *et al.*, 2010b, 2011) and seed-based methods (Gressel, 2010a; Oliver *et al.*, 1998, 1999a,b). Additionally, although the naturally existing bioconfinement systems, such as apomixis, ploidy barriers and genomic incompatibility, are only applicable in a limited number of species, which are less manageable for genetic engineering (Daniell, 2002; Gressel, 1999; Kausch *et al.*, 2010), they have attracted intensive research attentions with exciting advances (Hanna, 1981; Johnson *et al.*, 2006; Kannan *et al.*, 2012; Sandhu *et al.*, 2009, 2010). Each of these strategies has advantages and drawbacks. For example, one of the first technologies proposed and shown to be effective was a seed-based method (above) originally called the 'Technology Protection System' that later became known as 'GeneSafe Technologies' (Oliver *et al.*, 1998, 1999a,b), which infamously became widely known as 'Terminator'. The GeneSafe invention rendered seeds that were not capable of germination (Oliver and Hake, 2012), thus eliminating the possibility of transgene flow. The critics of GeneSafe, who widely embraced the 'Terminator' label, argued that farmers in developing countries would be at a disadvantage because it would have prevented them from saving viable seed for planting the following season. The GeneSafe critics also argued that fields growing nontransgenic crops of the same species nearby would suffer viable seed loss from cross-pollination with GeneSafe plants. Because of public pressure and other reasons, this technology has never been commercially deployed even though GeneSafe is a good candidate for preventing gene flow.

Collectively, these bioconfinement technologies have become known as gene use restriction technologies (GURTs); the name has been adopted in a report to the Commission on Genetic

Resources for Food and Agriculture of the Food and Agriculture Organization of the United Nations in 2001 (Oliver and Hake, 2012). We believe that 'GURTs' might not be the best label because the implication of purpose is mainly to restrict the use of intellectual property or traits owned by agribusinesses (Oliver and Hake, 2012). Of course, GURTs can be used to protect intellectual property, but they can also be invaluable tools in the bioconfinement of transgenes, which we believe might eventually be a more valuable purpose.

In this review, we seek to survey the various GURTs that have been developed and examine the state of the technology for use in bioconfinement. Because of the proprietary nature of GURTs, there might be some technologies that have been invented in companies, but not found in the literature; there might be technology gaps in the review. Nonetheless, we discuss GURT components, the benefits of GURTs as the technology relates to intellectual property protection and bioconfinement, and the future outlook of GURTs from regulatory and commercialization perspectives.

Gene use restriction technologies defined

Gene use restriction technologies have been categorized into two classes: V-GURTs (variety-related GURTs) and T-GURTs (trait-related GURTs) (Hills *et al.*, 2007; Van Acker *et al.*, 2007; Visser *et al.*, 2001). V-GURTs are designed to restrict the use of all genetic materials contained in an entire plant variety. Prior to being sold to growers, the seeds of V-GURTs are activated by the seed company. The seeds can germinate, and the plants grow and reproduce normally, but their offspring will be sterile (see below for details). Thus, farmers could not save seed from year-to-year to replant. In contrast, T-GURTs only restrict the use of particular traits conferred by a transgene, but seeds are fertile. Growers could replant seed from the previous harvest, but they would not contain the transgenic trait.

Components of GURTs

For all GURTs, there are three indispensable necessary characteristics: controlled functionality, spatiotemporal specificity and controlled transgene expression. To achieve these, a signal cascade is required (Figure 1) that is generally composed of the following four genetic components or modules: the target gene, the promoter associated with the target gene, the trait switch and the genetic switch.

The target gene

For all GURTs, the target gene renders the host plant to have a specific trait such as lethality or an agronomic trait ('gene 3' in Figure 1). In theory, there is any number of genes that could be used as the target, even those whose gene product is simply RNA. The nature of the target gene determines a GURT's functionality. If a gene's product interferes with fundamental biological process (es) and therefore causes death of the host cell, it serves as a disrupter gene as discussed later. Because the entire genome of the host cell will be eliminated because of cell disruption, the GURTs utilizing these disrupter genes are classified as V-GURTs. In contrast, if the expression of the target gene results in particular traits (such as pest resistance or removal of specific genomic fragment as detailed below), but not lethality, it would be a T-GURT application.

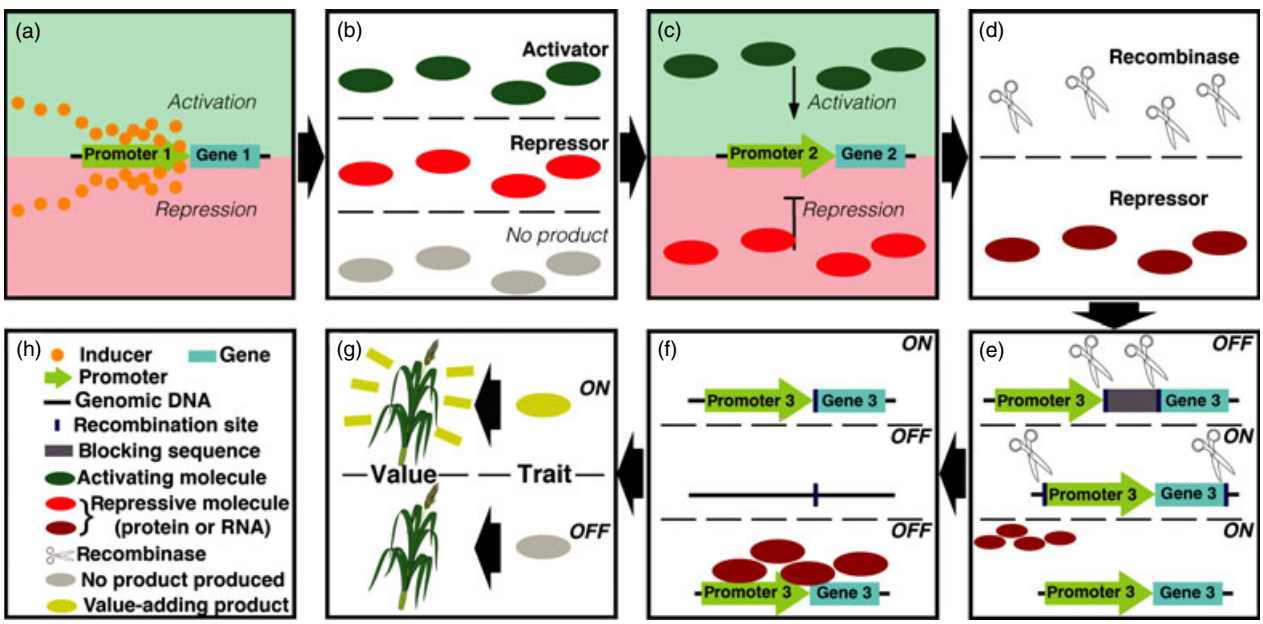

Figure 1 A schematic diagram of signal cascade of GURTs. (a) A promoter (promoter 1), whose activity is either activated or repressed by exogenously applied inducers, drives expression of the enabler gene (gene 1). (b) Output of (a) serves as input of (c). The product of the enabler gene in (a) could act as either activator (upper level) or repressor (middle level). Alternatively, there is no product generated at all (lower level) if the enabler gene is repressed in (a). (c) In response to the input from (b), the promoter 2 that drives expression of the trait switch gene (gene 2) is either activated or repressed. (d) Output of (c) serves as input of (e). The products could be either recombinases or repressor molecules. (e) Depending on the presence of the 'blocking sequence', the target gene (gene 3) could be set by default on or off. (f) The recombinases generated in (d) specifically recognize the recombination sites and remove the sequence in between, resulting in switching on or off the target gene (upper and middle levels, respectively). Alternatively, the repressors block the transcription/translation of the target gene (lower level). (g) The product of the target gene (trait) renders the host plant to express specific agronomic trait(s). (h) A list of symbols used in this figure.

However, because the expression of the target gene has to be tightly controlled in order to meet the requirement of original IPP designs or derived bioconfinement applications, other gene regulation elements (see below) are necessary.

Promoter of the target gene

A promoter ('promoter 3' in Figure 1) regulates the expression of the target gene in a spatiotemporal pattern, that is, where and when it should be conditionally expressed. To prevent unwanted pleiotropic effects, that is, damage to the plant from possible leakage of promoter activity especially in the case of V-GURTs, a 'blocking sequence' is often used to physically separate the promoter and its target gene. Thus, the interaction between the target gene and its promoter can be set by default 'on' (without blocking sequence) or 'off' (with blocking sequence) (Figure 1e). In order to deactivate or activate the desired trait, additional genetic elements, including the trait switch gene and the genetic switch components, are required.

Trait switch gene

This group of genetic elements has been previously termed 'trait activator gene' because in most cases it is used to activate the expression of the target gene. The trait switch gene ('gene 2' in Figure 1) often encodes for a recombination enzyme, such as a site-specific recombinase, that physically deletes DNA sequences between specific recognition sites (Wang *et al.*, 2011). In the cases that the target gene is set by default 'off' (i.e. there is a blocking sequence between target gene and its promoter), these activator genes' products do act as activators by removing the blocking sequence (Figure 1e,f). However, there are two proposed designs in which the target gene is instead deactivated by the switch genes (Figure 1e,f). One is when the recombinase deletes part or all of the target gene or its promoter (or both) to deactivate the target gene (Figure 1e,f). The other common design uses repressor molecules (either RNA or protein) to block the target gene's transcription/translation and therefore also function as a deactivator (Figure 1e,f). The consequence of the trait switch gene's activation would be either deletion of certain genomic fragment (upper two levels of Figure 1e,f) or repression of the target gene (bottom level of Figure 1e,f), representing permanent switch (also called physical switch) or reversible regulation, respectively. Because of the obvious advantage of physical switch over reversible regulation in terms of absolutely tight control, the former is commonly preferable in all GURT designs and recombinases as the trait switch genes.

The expression of the trait switch gene is driven by a directly associated promoter ('promoter 2' in Figure 1), which reacts to signals from the genetic switch (see below). Regardless of its role as 'activator' or 'deactivator', the trait switch gene stands between the physical switch and the genetic switch, that is, receives input from genetic switch followed by releasing output to physical switch (Figure 1).

Genetic switch

The genetic switch component is made up of an 'enabler' gene ('gene 1' in Figure 1) and its promoter ('promoter 1' in Figure 1). Through this component, the application of the exogenous inducer could either activate or repress the expression of the enabler gene that directly controls the activity of the trait switch gene component and therefore downstream signal transduction (Figure 1). Thus, as a gateway that converts exogenous input (inducer) to biological signal (the trait switch gene or more directly the target gene itself; see below), the genetic switch should further include the promoter of the trait switch gene ('promoter 2' in Figure 1) as a subcomponent, which would be apparently indispensable for all GURT designs. However, to simplify discussion and diagrams (see below and Figures 1 and 2), the promoter of the trait switch gene will still be put into the trait switch component, and the genetic switch component will be discussed as dispensable throughout the manuscript.

Functionality of GURTs: putting all the components together

The signal cascade shown in Figure 1 represents the original V-GURT GeneSafe design (Oliver *et al.*, 1998, 1999a,b) (Figure 2). The application of the inducer serves as input and the switch of the target gene's expression status as output. The three components, the genetic switch, the trait switch gene and the promoter of the target gene, transduce the signals from input to output (Figure 2a). By rearranging the genetic elements of them, the GURT system could be modified to fit other purposes. For example, the inducer-responsive promoter ('promoter 1' in Figure 1 and 'inducible promoter' in Figure 2), which originally belongs to the genetic switch (Oliver *et al.*, 1998, 1999a,b), could replace the promoter of the trait switch gene (Figure 2b). Thus, an inducer treatment would more directly regulate its expression compared to the original design (Figure 2a,b). However, based on logical reasoning rather than empirical evidence, the cost could probably be weaker induction of the trait switch gene because of the absence of signal amplification. Therefore, this design would be considered to fit the cases that require rapid but weak responses. In these two designs (Figure 2a,b), the dual switch provides controllability of the GURT system by breeders/companies via the inducible promoter, as well as spatiotemporal specificity of the target gene's expression via the natural signal-responsive promoter.

The design can be further simplified. As shown in a single switch design (Figure 2c), the inducible or natural signal-responsive promoter exclusively controls the target gene's expression so that the system can solely respond to exogenous inducers or developmental signals instead of both. This design has been widely used for bioproduction in plants, and recent advances have been reviewed by Corrado and Karali (2009).

Instead of placing all the components in one plant, the controllability could be realized by a hybridization event, that is, in trans (Oliver *et al.*, 1998, 1999a,b). In this case, the target gene and its promoter, as well as the blocking sequence in most cases, are constructed in one plant ('maintainer' line) and the trait switch gene is constitutively expressed in another ('inducer' line or 'activator' line) so that the GURT system would be triggered for the first time in F_1 hybrid plants (Figure 2d). This design also contains a dual switch.

Based on these four fundamental designs and the fact that every single component in a GURT design is exchangeable and the outcome of different combinations of these components varies considerably, GURTs could be further modified to fit particular purposes and provide value beyond simply protecting intellectual property.

Benefits of GURTs

Intellectual property protection

The original aim of GURTs was for protection from patent infringement from saving seed (Oliver *et al.*, 1998, 1999a,b). A

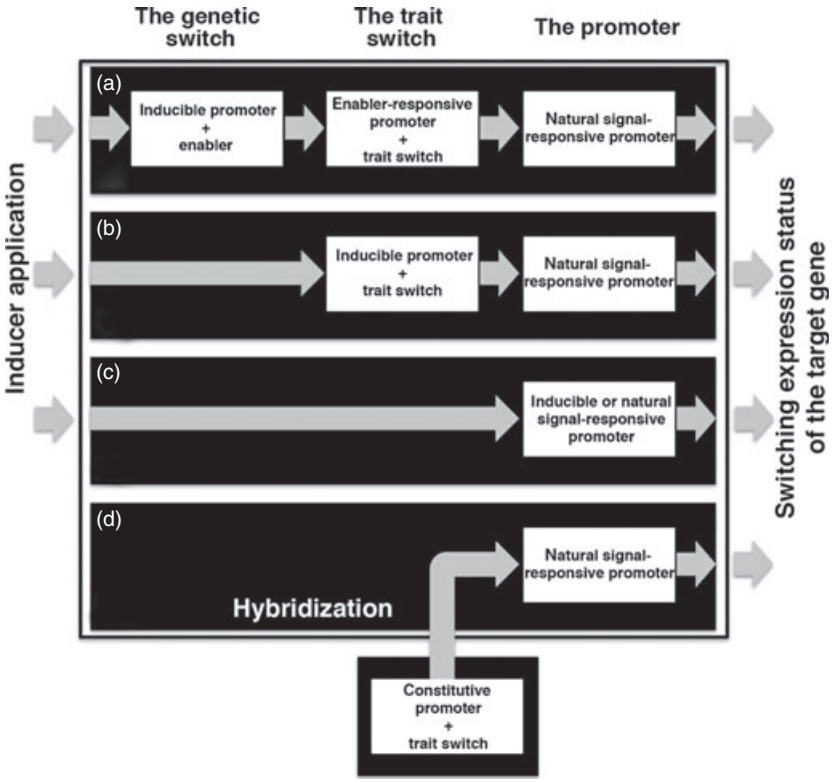

Figure 2 A schematic diagram describing fundamental designs of GURTs. Each black box indicates a genome containing components shown in white boxes. The input could be either inducer application ((a)-(c)) or hybridization event (d) that initiates the signal transduction indicated by arrows regardless of positive or negative effects leading to the toggling of the expression status of the target gene. (a) Typical GURT system containing all components (indicated above the frame) discussed in the text and Figure 1. (b) GURTs in which the genetic switch component is omitted. (c) GURTs in which the genetic switch and the trait switch components are omitted. (d) GURTs in which the target gene's expression is switched only when hybridized with the inducer line constitutively expressing the trait switch gene.

common provision in seed sales agreements to farmers disallows saving seed. Even though many of today's crop varieties are hybrids for which saving and replanting seed would not be valuable, there was an initial very intense public relations drive made by nongovernmental organizations to protest GURTs, which largely led companies to forego commercialization of the technology. No matter which side GURTs are viewed from, robust IPP would stimulate private research and development in plant breeding, as proven by the activities to create hybrid varieties (Goeschl and Swanson, 2003; Lence *et al.*, 2005). There is nothing to be gained from saving and replanting inbred GURT-protected lines and hybrids. However, farmers should profit in the long term from improvements in innovation because increased breeding efforts will, in turn, yield more productive varieties and unique novel traits. Furthermore, these innovations might help to enhance genetic diversity in many important crops, thereby providing long-term economical and environmental benefits (Van Acker *et al.*, 2007).

Bioconfinement

The benefits and risks of growing transgenic crops have been discussed over the past three decades (Conner *et al.*, 2003; Lu and Yang, 2009; Nap *et al.*, 2003; Stewart *et al.*, 2003; Warwick *et al.*, 2009). Gene flow continues to rank near or at the top of the list among debated risks. Gene flow is the transfer of genes from one population to another. In the context of biosafety of transgenic crops, the movement of transgenes from crop to wild relative and/or weedy plants (both intra- and interspecies and also known as transgene escape) is of continued concern (Conner *et al.*, 2003; Daniell, 2002; Nap *et al.*, 2003; Stewart *et al.*, 2003; Warwick *et al.*, 2009). Pollen, seed or vegetative propagules can contribute to gene flow (Husken *et al.*, 2010; Warwick *et al.*, 2009), with pollen- and seed-borne gene movement of primary concern (Daniell, 2002; Ellstrand, 2003; Heuberger *et al.*, 2010; Hills *et al.*, 2007; Husken *et al.*, 2010; Lu and Yang, 2009). It has been proposed that using GURTs would greatly impede transgene escape (Gressel, 2010b; Hills *et al.*, 2007; Kausch *et al.*, 2010). Briefly, the idea is to apply a GURT system together with the targeted transgene, so the latter will be eliminated once the GURT is activated (see below and figure 3). The definitions of V- and T-GURTs for bioconfinement would be slightly different from those for IPP. They refer to the technologies that restrict possible transgene dispersal mediated by sexual reproduction from host plants to their relatives, via elimination of the entire genome (V-GURTs) or removal of the transgene from the genome (T-GURTs). On the other hand, all GURTs, regardless of the aims, are based on similar molecular mechanism as discussed above. Below, a detailed comparison will be discussed.

Comparison of IPP- and bioconfinement-oriented GURTs

All plants containing GURTs have to be grown under two distinct conditions: (i) for research, breeding, seed bulking, (ii) in commercial fields. In the first, the GURTs are 'off' in which plants reproduce normally, and in the second, the GURTs are 'on,' meaning that the particular trait is protected (IPP GURTs) or gene flow is restricted (bioconfinement GURTs). It is important to have a switchable system so that during breeding, for example, the GURT system is inactivated for improving plant genetics.

The target tissue (determined by the natural signal-responsive promoter shown in Figure 2) of GURTs aiming for IPP could be the seed for V-GURTs or any part of the plants for T-GURTs. In contrast, the GURTs for bioconfinement are mainly focused on reproductive parts of a plant such as pollen, seed and floral organs (Gressel, 2010a; Hills *et al.*, 2007; Kausch *et al.*, 2010).

In many cases, the supposed outcomes of IPP and bioconfinement GURTs might be the same, barring some caveats noted here. In all V-GURTs, the activation of the system should result in

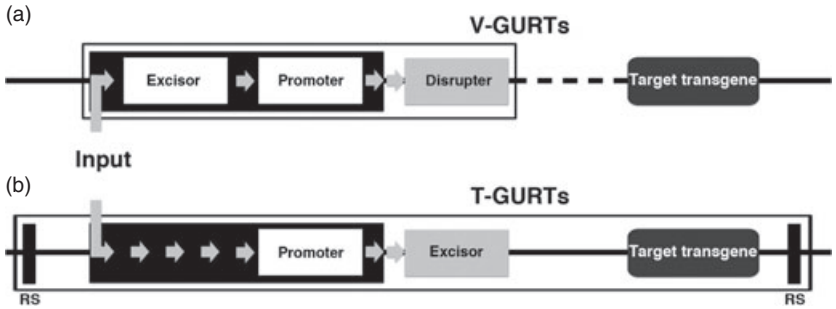

Figure 3 Schematic diagrams showing V- and T-GURTs for bioconfinement. All components belonging to a GURT design are framed. Arrows indicate the signal transductions initiated by input. The outputs are activation of disrupter gene in V-GURTs (a) or excisor gene in T-GURTs (b). The key components (see text for details) are represented by white or grey boxes. The round cornered rectangles indicate the target transgene(s) that should be eliminated upon activation of the GURT system. There would necessarily be two recognition sites (RS) in each design, but those in V-GURTs (a) are not shown to simplify the black box. Lines, with dashed line indicating any genetic distance, represent the genomic DNA. V-GURTs allow confining the target transgenes located on any chromosome(s) within a genome. T-GURTs only allow confining the target transgenes located within the T-GURTs itself.

the elimination of an entire genome of the target cells without altering the genetic material in nontarget tissues. By contrast, IPP plants that employ T-GURTs should express the desired trait but not change genome sequence in both target and nontarget tissues, whereas bioconfinement T-GURTs generate nontransgenic genome only within the target cells. When doing so, the efficiency required for efficacy varies. In the case of IPP V-GURTs, there is no need to approach 100% efficiency, because greatly reduced germination rate is enough to force farmers to continually buy seeds from companies. The requirement for IPP T-GURTs would be higher, depending on the particular nature of the desired traits. However, the efficiency requirement for any bioconfinement GURTs should approach 100%.

Designing GURTs for bioconfinement

Host plants and system design

The major similarities and differences between IPP and bioconfinement GURTs are notable when designing a strategy (Table 1). Both V- and T-GURTs could be targeted to reproductive tissues, most typically pollen and seed (or embryo) (Table 1). It is important to tailor the GURT to the target species and its reproductive system, as well as that of wild relatives that could be gene flow recipients. For those crops that are primarily self-pollinated such as wheat, rice and cotton, the best choice would be seed-specific GURTs because of the relative lower probability of pollen-mediated transgene escape. In contrast, the pollen-specific GURTs are more suitable for plants that primarily cross-pollinate (Hills *et al.*, 2007; Kausch *et al.*, 2010; Van Acker *et al.*, 2007; Visser *et al.*, 2001). In species that are primarily farmed for vegetative organs, a mechanism has been proposed to ablate the entire reproductive organs (Kausch *et al.*, 2010). Additionally, because longer-lived and more fecund species will likely have higher gene escape frequency, the perennial plants that produce abundant pollen and seeds might be in the worst-case scenarios.

An important design feature is the choice between V-GURTs and T-GURTs. In cases in which seeds are target for harvest regardless of their ability to germinate, T-GURTs may not work because excision of the transgene will result in the loss of the desired trait. One example is in the case of plants engineered to produce recombinant pharmaceutical and industrial proteins or other biomolecules. In these so-called biopharming plants, the products for harvesting are typically accumulated and stored in endosperm tissue of a seed (Mascia and Flavell, 2004; Obembe *et al.*, 2011). Thus, application of V-GURTs that disable only embryos will eliminate transgene escape without compromising production. In contrast, T-GURTs are preferred when the goal is to produce nontransgenic seed or pollen by excising transgenes from the genome in order to not interrupt natural food chains (Kwit *et al.*, 2011; Moon *et al.*, 2010b).

It is also possible to further improve the bioconfinement efficiency by combining GURTs with other bioconfinement systems. It has been demonstrated that the pollen-mediated transgene flow between transgenic apomictic tetraploid and

Table 1 Comparison of GURTs aiming to intellectual property protection and bioconfinement

	Intellectual property protection	Bioconfinement
GURTs' status	Off in research and breeding and on commercial in field (with exceptions, see text)	
Target tissue	Seed (V-GURTs); Any tissue (T-GURTs)	Reproductive tissues (commonly seed and pollen; both V- and T-GURTs)
Desired effects on target tissue	Genome eliminated (V-GURTs); Trait expressed while the genome is unchanged (T-GURTs)	Genome eliminated (V-GURTs); Nontransgenic genome generated (T-GURTs)
Desired effects on nontarget tissue	Genome unchanged* (all GURTs)	
Required efficiency	100% not necessary (V-GURTs); Higher, the better, but no need to be 100% (T-GURTs)	Approaching 100% (both V- and T-GURTs)

*A blocking sequence would be deleted from the genome in some cases, but other parts of the transgene retained.

sexual diploid bahiagrass could be as low as 0.03%, compared with 0.17% between transgenic apomictic tetraploids and nontransgenic apomictic tetraploids (Sandhu *et al.*, 2010). Because the viability of pollen from paternal plants is unaffected (Quarin, 1999; Sandhu *et al.*, 2010), additional application of pollen-targeted GURTs is probably able to further confine the gene flow mediated by pollen to an acceptable level for both public and regulatory sectors.

The linkage of V- or T-GURTs to the target transgene might be variable depending on desired outcome. V-GURTs function at any genetic distance from the trait (Figure 3a), whereas a T-GURT DNA construct has to be genetically linked to the target gene (Figure 3b). Thus, V-GURTs could be easily introduced into any existing varieties, which might already contain multiple transgenes in the genome, without greatly increasing the cost. By contrast, implementation of a T-GURT system requires that all transgenes, including T-GURTs itself, be packaged into one contiguous DNA insertion fragment. Because of the limitations of currently available DNA engineering and delivery methods (Ananiev *et al.*, 2009; Chen *et al.*, 2010; Dhar *et al.*, 2011; Gelvin, 2003; Hamilton *et al.*, 1996; Liu *et al.*, 1999; Yu *et al.*, 2007), T-GURTs are only able to restrict the movement of a few transgenes that are directly linked. Thus, T-GURTs could be more costly to implement than V-GURTs.

Choosing appropriate components

An ideal GURT system should have no unexpected phenotypic effects when off or on (Table 1). Because a physical switch (blocking sequence or DNA fragment between excision sites in Figure 1) is apparently the best way to prevent unwanted gene expression, both V- and T-GURTs for bioconfinement contain it where an excisor (recombinase in Figure 1) is required for deletion upon GURTs' activation (Figure 3). In the context of V-GURTs-based bioconfinement system, the following three factors are critical for efficacy: (i) the ability of the disrupter to ablate target cell, (ii) activity strength of the excisor gene to remove the blocking sequence, (iii) the spatiotemporal specificity that the promoter of the disrupter gene provides (Figure 3a). In contrast, when designing a feasible T-GURT system (Figure 3b), only the latter two variants play key roles. Because of the importance of these three components in a bioconfinement GURT system, we focus on them for the remainder of this section.

Disrupter gene

All disrupter genes used to date in V-GURTs are those coding for cytotoxins. These include nucleases such as barnase and ribonuclease A, which degrade either DNA or RNA, and catalytic lethal proteins such as diphtheria toxin and ribosomal inhibitor protein that prohibit RNA or protein synthesis (Burgess *et al.*, 2002; Gils *et al.*, 2008; Mariani *et al.*, 1990; Moon *et al.*, 2010a; Oliver *et al.*, 1998, 1999a,b; Zhang *et al.*, 2012). Although there is no evidence to date that the products of these genes are toxic to human and livestock, the acceptance by public might be questionable (Conner *et al.*, 2003; Gressel, 2010b; Lu and Yang, 2009; Nap *et al.*, 2003; Stewart *et al.*, 2003; Warwick *et al.*, 2009). Because of these concerns, certain cytotoxins should probably be expressed in nonfood crops or not expressed in tissues that are used for food or feed.

Excisor gene

The requirements for an ideal excisor include its (or its orthologs') absence within the host plant, specificity of recognition of target excision sites, the uniqueness of excision sites within the host plant's genome and efficient excision. There are a few recombinases, combined with corresponding excision or recombination sites, that have been tested in different plant species, which include CRE-*loxP* (Dale and Ow, 1990; Luo *et al.*, 2007; Zuo *et al.*, 2001), FLP-*FRT* (Akbudak and Srivastava, 2011; Lloyd and Davis, 1994; Luo *et al.*, 2007; Nandy and Srivastava, 2011; Rao *et al.*, 2010), R-*RS* (Khan *et al.*, 2011; Onouchi *et al.*, 1995), Bxb1-*att* (Blechl *et al.*, 2012; Thomson *et al.*, 2012; Yau *et al.*, 2011), ParA-*MRS* (Thomson *et al.*, 2009; Zhou *et al.*, 2012), PhiC31-*att* (Kempe *et al.*, 2010; Thomson *et al.*, 2010) and CinH-*RS2* (Moon *et al.*, 2011; Zhou *et al.*, 2012) systems. In addition, recently developed genome-editing technologies such as zinc finger nucleases (ZFNs) and transcriptional activator–like effectors (TALEs) fused with nucleases (TALENs) also have potential to be used as excisor genes (Bogdanove and Voytas, 2011; Petolino *et al.*, 2010; Tzfira *et al.*, 2012).

Among these, a combination of CRE-*loxP* and FLP-*FRT* systems demonstrated 100% excision efficiency when tested in tobacco pollen and seed in many, but not all, transgenic events (Luo *et al.*, 2007). CinH-*RS2* system has also been tested in tobacco pollen, and more than 99% excision events were observed (Moon *et al.*, 2011). These results suggested that both systems are powerful candidates as acceptable excisors in GURT-based bioconfinement systems. However, field data are required to determine whether such systems would be suitable for commercial applications. Secondly, and also importantly, their efficiency in crop species is yet unknown. Based on the fact that the CRE-*loxP* system showed a wide range of efficiency in several plant species (Sreekala *et al.*, 2005; Zhang *et al.*, 2006; Zuo *et al.*, 2001), it is reasonable to conclude that host plant's genome could affect transgene excision efficiency within a recombination system.

Promoter

Regardless of whether a V-GURT or T-GURT system is chosen, a promoter that provides spatiotemporal specificity is required (Figure 3). Although there have been many efforts to find candidate promoters whose activities are exclusively restricted within seed (Coussens *et al.*, 2012; Fauteux and Stromvik, 2009; Furtado *et al.*, 2008; Galau *et al.*, 1992; Hood *et al.*, 2003; Huang *et al.*, 2011a; Hughes and Galau, 1991; Kawakatsu *et al.*, 2008; Kuwano *et al.*, 2011; Qu le and Takaiwa, 2004; Qu le *et al.*, 2008; Streatfield *et al.*, 2010; Woodard *et al.*, 2003) or pollen/anthers (Anand and Tyagi, 2010; Cook and Thilmony, 2012; Gupta *et al.*, 2007; Huang *et al.*, 2011a,b; Jeon *et al.*, 1999; Kato *et al.*, 2010; Khurana *et al.*, 2012; Mlynarova *et al.*, 2006; Park *et al.*, 2006; Swapna *et al.*, 2011; Twell *et al.*, 1989; Xu *et al.*, 1999; Zhang *et al.*, 2012), most tissue-specific promoters characterized to date have been suboptimal for GURTs. Both high expression and sharp tissue specificity are needed; system tuning is needed.

Tuning GURTs

The regulation of gene expression is not limited to its promoter, but also extends to its introns and 3′-untranslated regions (3′-UTRs). In many cases, the introns of a gene could act as enhancers or alternative promoters to affect its spatiotemporal expression pattern, or elevate mRNA accumulation to increase expression strength, or even facilitate expression in heterologous species (Bartlett *et al.*, 2009; Giani *et al.*, 2009; Liu and Liu, 2008; Rose, 2008; Yang *et al.*, 2011). In addition, 3′-UTR also plays an important role in regulating gene expression at both the

transcriptional and post-transcriptional levels by affecting mRNA accumulation, stability and translation efficiency (Bashirullah *et al.*, 2001; Fabian *et al.*, 2010).

Other methods and DNA elements have been explored as gene expression regulators. For example, the disrupter protein barnase could be split into two separate but complementary peptides and synthesized under the control of two separate promoters, and thus, only cells that contained both would be ablated (Burgess *et al.*, 2002; Kempe *et al.*, 2009). By carefully selecting their promoters, it is very possible to precisely restrict the functional barnase complex within desired tissue. Recently, Zhang *et al.* (2012) demonstrated that mutant versions of barnase expressed in transgenic tobacco, pine and *Eucalyptus* (spp.) had various activities. One of these mutants, H102E, exhibited enough ability to ablate pollen with no obvious off-target effects. These results suggest that there is room to tune GURT components to increase efficiency.

Perspectives and future outlooks

Clearly, GURTs have utility for both technology/intellectual property (IP) protection and limiting gene flow, that is, for environmental and regulatory reasons. These latter reasons are probably the most imminent in the case of next-generation biofuel feedstock crops and the case of other crops in which there are special concerns about gene flow and introgression (Gressel, 2010a,b; Kausch *et al.*, 2010; Kwit and Stewart, 2012; Stewart, 2007; Strauss *et al.*, 2010). The concerns can be broken down into two general categories. First, scientists, regulators and the public are concerned about preventing gene flow in crops where hybridization, and especially introgression, could jeopardize the integrity of managed or unmanaged ecosystems (Kwit *et al.*, 2011; Stewart *et al.*, 2003). The second concern, admixture of transgenic grain containing pharmaceutical or other products into the food and feed supplies, is held mainly by consumers and regulators (Kausch *et al.*, 2010; Kwit and Stewart, 2012; Stewart, 2007). The commonality here is the ubiquitous governmental regulation of transgenic plants, which seems to be getting more stringent throughout the world. This stringency is paradoxical given the environmental and food safety record of transgenic plants as a whole.

Thus, we see a push–pull dynamic emerging with regard to research and development with the goal of eventual commercialization of GURTs. The push to improve and commercialize GURTs would have a tangible regulatory and IP benefit. We envisage the pull coming from consumers and regulators, thus begging the question: Why are GURTS not currently implemented in commercial transgenic crops? Agricultural companies have been chided for even considering GURT deployment for an IP benefit, even though it is completely reasonable for companies to diligently protect their IP. This situation could well limit the push for companies to seriously consider GURTs for ecological reasons. In addition, companies could well be weighing implementation benefits versus the potential for regulators to expect GURTs in all crops in future; that is, that GURTs could become a regulatory requirement even when it is not necessary. An additional factor affecting push and pull is that it might be quite expensive for companies to deploy GURTs given the large number of discrete units of DNA that would need to be licensed. One solution is the invention of a complete system *de novo* that could be in-licensed as a whole. A final factor affecting the regulatory pull is the tendency for regulators to desire streamlined constructs and the insertion of minimal DNA into plant genomes via biotechnology. Again, paradoxically, by installing a GURT system into a transgenic plant (more DNA), it increases the likelihood that less DNA of interest will be released from a transgenic field. In addition, it is also unknown how much testing would be required for regulatory approval. Would it be prohibitively expensive for companies to test the ecological and food safety effects of each GURT component? One last consideration revolves around nontransgenic gene flow from crops to wild and weedy relatives. There is some ecological and agricultural concern about crop genes increasing weediness of wild relatives and disrupting natural ecosystems (Warwick *et al.*, 2009). GURTs could also limit introgression of crop alleles into their wild and weedy relatives.

Thus, we see that there is a tipping point that involves complexities of economics, government regulation and consumer affairs; these go beyond the science and technology of GURTs. That said, the science plays a role in the tipping point of adoption too. The key is the creation of an extremely efficient GURT system that is environmentally robust and deployable in a number of crops. Synthetic biology will likely play an important role in the discovery, creation and implementation of key components for GURTs. Whereas plant synthetic biology lags behind that for microbial and biomedical applications, it is poised to revolutionize agriculture (Liu *et al.*, 2013). Specifically, bioinformatic and synthetic biology tools should greatly improve our ability to make very strong specifically inducible promoters to regulate key GURT components. These tissue-specific inducible promoters would likely be the key to creating effective ecological GURTs. In addition to designing promoters, synthetic biology should be able to improve the efficiency of site-specific recombination systems and also employ new tools such as TALENs to produce very effective excision systems (Liu *et al.*, 2013). Thus, we envisage synthetic biology as the key to develop gene circuits and other components to push the efficiency of GURTs to approach the 100% efficacy that could be required for bioconfinement purposes.

In conclusion, GURTs hit the scene of plant biotechnology with a bang in the late 1990s and early 2000s, but their potential has never been reached. We envisage plant synthetic biology and new crops and products as driving the research and development of GURTS towards implementation into a commercial crop pipeline within the next 10 years.

Acknowledgements

This work was supported by NIFA grants including those from the USDA Biotechnology Risk Assessment Grants program, and the University of Tennessee. The authors declare no conflicts of interest.

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