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Review article

Genetic use restriction technologies: a review

Luca Lombardo*

Department of Crop Systems, Forestry and Environmental Sciences, University of Basilicata, Potenza, Italy

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**Correspondence* (Tel +39 3408691477;
fax +39 0971205378;
email lombluca@yahoo.it)

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Summary

Genetic use restriction technologies (GURTs), developed to secure return on investments through protection of plant varieties, are among the most controversial and opposed genetic engineering biotechnologies as they are perceived as a tool to force farmers to depend on multinational corporations' seed monopolies. In this work, the currently proposed strategies are described and compared with some of the principal techniques implemented for preventing transgene flow and/or seed saving, with a simultaneous analysis of the future perspectives of GURTs taking into account potential benefits, possible impacts on farmers and local plant genetic resources (PGR), hypothetical negative environmental issues and ethical concerns related to intellectual property that have led to the ban of this technology.

Introduction

Genetic use restriction technologies (GURTs) are the name given to experimental methods, described in a series of recent patent applications and providing specific genetic switch mechanisms that restrict the unauthorized use of genetic material (FAO, 2001a) by hampering reproduction (variety-specific V-GURT) or the expression of a trait (trait-specific T-GURT) in a genetically modified (GM) plant.

Variety-GURT (also known as suicide/sterile seed/gene technology, or terminator technology) is designed to control plant fertility or seed development through a genetic process triggered by a chemical inducer that will allow the plant to grow and to form seeds, but will cause the embryo of each of those seeds to produce a cell toxin that will prevent its germination if replanted, thus causing second generation seeds to be sterile and allowing manufacturers to maintain their intellectual property rights and avoid concerns related to GM seed dispersal.

Considered by some authors as the second generation of V-GURT (Fisher, 2002), T-GURT (ironically known as traitor technology) is designed to switch on or off a trait (such as herbicide/cold/drought/stress tolerance, pest resistance, germination, flowering, ripening, colour, taste and nutritional qualities of the plant, defence mechanisms, or production of industrial or pharmaceutical compounds) using inducible promoters regulating the expression of the transgene through induced gene silencing (e.g., by antisense suppression) or by excision of the transgene using a recombinase (FAO, 2001a). In this case, the genetic modification is activated by a chemical treatment or by environmental factors such as heat (Jefferson *et al.*, 1999), enabling farmers to maintain the value-added traits of seeds (Eaton and van Tongeren, 2002).

Both the nicknames 'terminator' and 'traitor' for these technologies were coined by the Canadian-based nongovernment organization Rural Advancement Foundation International (RAFI; today Action Group on Erosion, Technology and Concentration, ETC).

History of GURTs

The first patent applications related to a biological switch mechanism regulated by external inducers date back to the first years of the 1990s. In 1991, DuPont filed a patent application, granted in 1994 (U.S. 5,364,780), entitled 'External regulation of gene expression by inducible promoters' that described a method 'utilized to transform plants and bring the expression of the gene product under external chemical control in various tissues of monocotyledonous and dicotyledonous plants'. In 1992, Zeneca (today Syngenta, after the merger with Novartis Agribusiness in 2000) filed a technology application entitled 'Improved plant germplasm' published by WIPO (World Intellectual Property Organization) in February 1994 (WO9403619A2, where the letter A indicates the request for approval), providing 'a gene switch which is inducible by external application of a chemical inducer and which controls expression of a gene product which affects expression of a second gene in the genome'; the second gene could encode a cytotoxic molecule fatal to the plant or a desirable characteristic that may be excised selectively by applying or withholding chemical application.

The true watershed was marked when Melvin Oliver, a British researcher, was assigned (1990) by the United States Department of Agriculture (USDA) to develop together with the Delta & Pine Land (DPL) Company a seed-embedded protection technology. The challenge was to create a cultivar that would become sterile only in farmers' fields by means of an external stimulus to protect the varieties developed by biotech companies, thus preventing farmers from seed saving. The conception of this 'genetic switch' was realized with the filing of a patent application on 7 June 1995. It was registered at WIPO in 1996 under the number WO 9604393 and finally, on 3 March 1998, the United States Patent and Trademark Office (USPTO) granted the joint application of Delta & Pine Land Corporation and the U.S. Department of Agriculture's Agricultural Research Service and issued the patent U.S. 5,723,765 entitled 'Control of plant gene expression' (Oliver *et al.*, 1998). Under a research agreement with the USDA, the Delta & Pine Land Co had the exclusive rights to license the new technology to other parties. In

accordance with its original intention, in the promotional communications the holders of the patent called this invention 'Technology Protection System' (TPS), whereas in the scientific publications and discussions of international institutions, it was eventually called 'Genetic Use Restriction Technology' with reference to the limitations imposed on its users.

The adoption of neutral definitions for this new technology did not prevent it from drawing the attention of the whole world. Fierce protests raged worldwide as many saw it as a very disadvantageous and unethical mechanism for poor farmers, especially in developing countries where saving seeds (also known as 'brown-bagging') is a common practice, and as an advantage for multinational companies that would have thus increased the dependence of indigenous and rural communities worldwide on their GM seeds. These objections are borne out by the fact that seed saving is estimated to account for between 15% and 20% of the world's food supply, practised by 100 million farmers in Latin America, 300 million in Africa and 1 billion in Asia (IIPTA, 2012). In June 1999, as a result of the great opposition to this technology by the public opinion, nongovernmental organizations and farmers, Zeneca announced that they would not market terminator seeds. Four months later (October 1999), Monsanto's CEO Robert Shapiro, under the advice of Gordon Conway, president of the Rockefeller Foundation (Shapiro, 1999; Vidal, 1999), pledged not to commercialize gene protection systems that render seeds sterile to avoid compromising the public image of the company (technically at that time Monsanto did not possess GURT patents, as it acquired Delta & Pine Land Co. along with its patents only in 2007; however, the announcement that the two companies would merge was made in May 1998). In 2000, D&PL claimed that they would continue trials for commercializing the technology protection system (Collins, 2000), and in 2005, Monsanto opened the possibility of using terminator technology in nonfood crops such as cotton and grass.

The invention was greeted with caution even by the scientific community. The Report of the 8th Meeting (October 1998) of the Consultative Group on International Agricultural Research (CGIAR) regarding the 'Implications of the embryo viability terminator mechanism' affirmed that the CGIAR, supported by 16 research institutes engaged in breeding new crop varieties for resource-poor farmers, 'will not incorporate into its breeding materials any genetic systems designed to prevent seed germination. This is in recognition of (a) concerns over potential risks of its inadvertent or unintended spread through pollen; (b) the possibilities of the sale or exchange of inviable seed for planting; (c) the importance of farm-saved seed, particularly to resource-poor farmers; (d) potential negative impacts on genetic diversity and (e) the importance of farmer selection and breeding for sustainable agriculture'.

In June 1999, at its fourth meeting in Canada, the Subsidiary Body on Scientific, Technical and Technological Advice of the UN Convention on Biological Diversity (CBD) recommended that, 'in the current absence of reliable data on genetic use restriction technologies [...] and in accordance with the precautionary approach, products incorporating such technologies should not be approved by Parties for field testing until appropriate scientific data can justify such testing and for commercial use until appropriate, authorized and strictly controlled scientific assessments with regard to, interalia, their ecological and socio-economic impacts and any adverse effects for biological diversity, food security and human health have been carried out in a transparent manner, and the conditions for their safe and beneficial use validated'. This official document used for the first time the term 'GURT' (Jefferson *et al.*, 1999). The same guidelines were maintained in the CBD Decision V/5 section III of the Fifth Conference of the Parties (COP5) held in Nairobi in June 2000, which imposed a *de facto* global moratorium on this technology. As a consequence of the moratorium and of the rising farmers' alarmism, in 2001, the Indian Parliament ratified the 'Protection of plant varieties and farmers' rights act' banning the registration of seeds containing terminator technology (Section 18, Point 1, C). Similarly, in Brazil, Article 6 of law number 11.105 of 24 March 2005 prohibited (point VII) 'utilization, marketing, registration, patenting and licensing of use restricted genetic technologies', whereas in Canada, the 2009 Bill C-353 was introduced as an 'Act to prohibit the release, sale, importation and use of seeds incorporating or altered by variety-genetic use restriction technologies (V-GURTs)'.

The first session of the FAO Panel of Eminent Experts on Ethics in Food and Agriculture (2001b) unanimously stated that the 'terminator seeds are generally unethical, as it is deemed unacceptable to market seeds whose offspring a farmer cannot use again because the seeds do not germinate. GURTs are not inherent in genetic engineering. While corporations are entitled to make profits, farmers should not be forced to become dependent on the supplier for new seeds every planting season'. The final document also recognized that GURTs may be justified where there is concern for possible outcrossing with GM crops that could damage wild plant populations.

In the memorandum prepared by the office of International Union for the Protection of New Varieties of Plants (UPOV) on the Genetic Use Restriction Technologies (2003a), it was argued that a GURT 'prevents access to germplasm, hampers research and breeding progress and sustainability and limits benefits to society'. However, these claims were afterwards partially retracted (UPOV, 2003b).

An attempt to undermine the moratorium was introduced during the Fourth Meeting of the Ad Hoc Open-Ended Inter-Sessional Working Group on Article 8(J),[1] and related provisions of the Convention on Biological Diversity held in Granada, Spain, in January 2006 because the final report recommended that the Conference of the Parties at its upcoming eighth meeting, in addition to reaffirming its decision V/5, section III, 'invites Parties, other Governments and relevant organizations and stakeholders to promote cooperation and synergies between agencies and experts in order to undertake further research and studies on potential impacts and other aspects of genetic use restriction technologies, including their ecological, socio-economic and cultural impacts on indigenous and local communities, including on a case-by-case risk assessment basis with respect to different categories of genetic use restriction subject to the precautionary approach'. Nevertheless, the introduction of this distinction between different types of restriction of GURTs, with the consequent 'case-by-case risk assessment' strategy, was rejected and the moratorium was upheld in March 2006 during the eighth Ordinary Meeting of the Conference of the Parties (COP8) held in Curitiba, Brazil, so that to date no plant with these characteristics is yet commercially available.

[1]Providing to respect, preserve and maintain the knowledge, innovations and practices of indigenous and local communities relevant for the conservation of biological diversity and to promote their wider application with the approval of knowledge holders and to encourage equitable sharing of benefits.

State of the art

To date, there are over 40 granted or submitted patent families (groups of patents that include identical or similar applications and filed by the same applicants) related to GURTs, resumed in Appendix S1. The holders of the patents include universities and, especially, multinational companies such as Syngenta, Bayer, Monsanto (D&PL), Ceres, Pioneer and BASF. The number of patent applications started to increase in the late 1990s, with the peak in 2006 but with a decline thereafter, that is, after the confirmation of the ban of these technologies (Figure 1). The moratorium, and the expiry of the first patents, which will occur in 2018, most likely pushed companies and universities to invest in the development of alternative strategies to prevent the unauthorized use of patented seeds and plant varieties.

Although several variations on the basic design have been proposed (see below) the general molecular construction, similar for both T- and V-GURTs, provides the use of (i) a repressor gene (the gene switch) that is responsive to an external stimulus; (ii) a recombinase gene (the trait activator gene), the expression of which is blocked by the repressor; and (iii) a target gene (Figure 2). With respect to the inducing substance, mostly of chemical origin, it should be biodegradable, nontoxic for the ecosystem, directly applicable in the field or in seeds, and capable of being absorbed by the involved plant, and its catalytic action should be specific for the target genetic system. The induced genetic system should be sensitive to small doses of inducer and the induction should be highly specific. Both T- and V-GURTs can be applied to any type of seed, independently from the contemporary genetic manipulation for the introduction of a trait of interest such as herbicide tolerance, pest resistance or nutritional improvement.

V-GURTs

For V-GURTs, essentially three different restriction mechanisms have been proposed (Visser *et al.*, 2001). The first mechanism of action is that described in the patent (U.S. 5,723,765) by the USDA and Delta & Pine Land (nominally the first V-GURT). This GURT is based on the transfer of a combination of three genes (transgenes), two derived from bacteria and one from another plant, into a plant's cells:

1. a gene coding for a cytotoxic protein (the terminator or lethal gene), under control of a late embryogenesis abundant (LEA) promoter linked to a DNA spacer (blocking) sequence flanked by specific excision sites (*lox* sequence) that prevents the activation of the terminator gene. In the '765 patent, the cytotoxic protein is the ribosome inactivating protein (RIP), otherwise known as saporin derived from *Saponaria officinalis*, which prevents plant cells from synthesizing proteins (Jiang *et al.*, 2008). The source of each gene and the transformation method were not divulged in the patent.
2. a phage P1 site-specific recombinase gene under the control of a constitutively active promoter (e.g., CaMV 35S) containing one or more tet operons that is subject to repression by the Tet repressor. This gene encodes a protein (Cre) that cuts the specific excision sites flanking the blocking sequence linked to the toxic gene;
3. a Tn10 tet repressor gene under the control of a constitutive promoter and encoding a protein that binds to the tet operon, preventing the expression of the recombinase gene. The presence of an external stimulus (inducer) prevents binding of the repressor to the operon. The external stimulus can be chemical inducers such as agrochemicals, in most cases produced by seed companies possessing the same restriction technologies, and antibiotics. In the case of U.S. patent number 5,723,765, the chemical inducer is the antibiotic tetracycline (Jefferson *et al.*, 1999), although subsequently DPL stated that the tetracycline-inducible expression system (in a patent on *Escherichia coli*) is not the most suitable choice (Working Group on Article 8(j), 2006).

Before being sold to the consumer (in most cases, to the farmer), these seeds are exposed to the inducer that inhibits the function of the repressor, which causes transcription of the Cre recombinase gene, which produces Cre that recognizes the Cre blocking sequence in the *lox* sequence and splices *lox* from the genome, thus placing the ribosomal inactivating protein under the direct control of the late embryogenesis abundant promoter. Genes under the control of the LEA promoter are only transcribed during late embryogenesis when the seed accumulates most of its storage oil and protein and is drying down in preparation for the dormant period (Hundertmark and Hincha, 2008). During late embryogenesis, the ribosomal inactivating protein (the terminator gene) is expressed, leading to the abortion of all embryos. Thus, the seeds purchased by farmers will be able to germinate in the field, and the culture will develop normally. However, the seeds

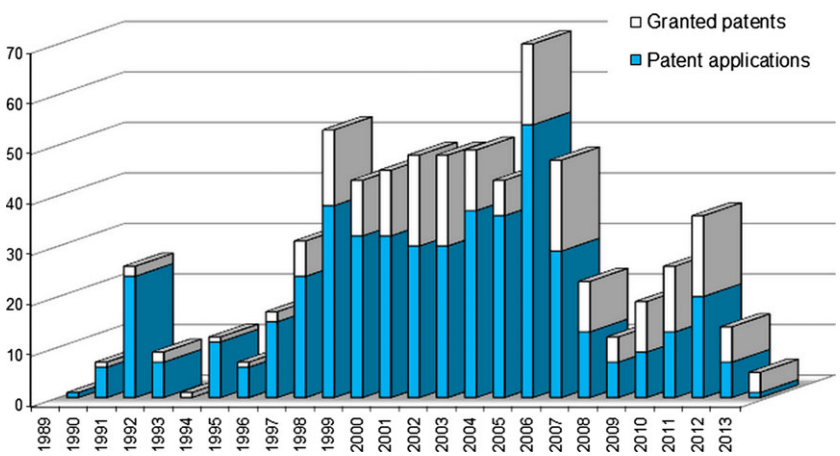

Figure 1 Granted and applied GURT patents.

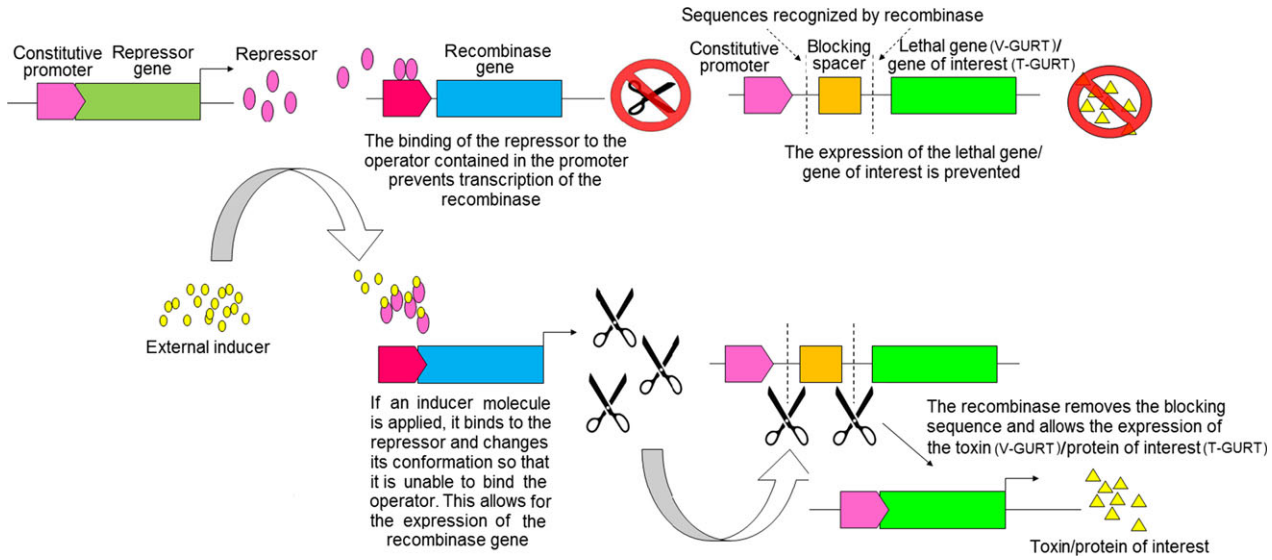

Figure 2 Schematic representation of the mechanism of GURTs. In a variant of this scheme, the repressor proteins are normally unable to bind to the pertinent DNA and the inducer is applied to bind the repressor that undergoes a change in its conformation so that it can bind to the DNA and activate transcription.

produced in the harvest will be sterile and thus cannot be stored for later cropping. According to the patent, the RIP is nontoxic to organisms other than plants, although some doubts have been raised (Crouch, 1998).

In a simplified model, the recombinase gene is directly linked to an inducible promoter (Oliver *et al.*, 1998).

This technology was designed specifically for pure line seed production in self-pollinated crops; the genes introduced into separate transgenic founder lines were then cross-pollinated to provide a genome with the full suite of TPS genes in the target crop (Oliver and Velten, 2001). The production of 'terminator hybrid seeds' has also been proposed (Gupta, 1998; Lehmann, 1998; Pendleton, 2004). The pollen carries the dominant protein synthesis inhibitor gene and thus the traits cannot be passed onto closely related weedy species (Oliver and Velten, 2001).

Similar strategies within the same transgene construct have been proposed, using alternative stimuli such as temperature or osmotic shock to regulate the mechanism, or by using abnormal levels of plant hormones as the cytotoxic element leading to cell destruction (Daniell, 2002). Potential inducers include ethanol, hormones (e.g., dexamethasone), salicylic acid, pesticides and metals such as copper (Gatz, 1996; Padidam, 2003). Nevertheless, despite the strong opposition received, to date, the idea remains theoretical because none of these variants has yet been fully implemented into practical applications.

The second mechanism of action of V-GURT is based on a reversed process because it is characterized by the presence of a gene encoding a disrupter protein permanently active in the seed, which makes it sterile. The gene promoter is under the control of a specific operator sequence. A further repressor protein, whose gene is under control of a chemically inducible promoter, can bind to the operator, inhibiting the expression of the disrupter protein. In the absence of the exogenous chemical inducer, no repressor protein is expressed; therefore, the breeder must apply the specific chemical inducer throughout the process of seed multiplication to inactivate the disrupter gene that causes sterility, interrupting the application only at the time of selling the seeds. This type of mechanism, incorporated into transgenic tobacco plants that are not commercially available, has been patented by the English agricultural company Zeneca Ltd. under the titles of 'Hybrid Seed Production' in 1992 (patent AU 621195 B2) and 'Plant Gene Construct Comprising Male Flower Specific Promoters' in 1998 (patent US 5,808,034 A).

A further technology assimilable to this 'second type' V-GURT, but specifically designed for gene flow control in transgenic plants, is the so-called recoverable block of function (RBF). The RBF, developed in tobacco by Kuvshinov *et al.* (2001), consists of a blocking sequence (encoding a barnase) linked to the gene of interest and a recovery sequence (encoding a barstar), expressed under control of sulfhydryl endopeptidase (SH-EP) and heat shock (HS) promoters, respectively, and all contained in a single insert. The natural expression of the barnase in embryos and sprouts confers cell death or prevents sexual reproduction of the transgenic plant (by blocking mRNA synthesis and germination) in the natural environment. The expression of the recovery sequence is induced by an artificial external stimulus such as a heat shock treatment (in the case of the cited study, the developing seeds were subjected to prolonged heating to 40 °C in a greenhouse) or chemical application; recovery of the blocked function results in the 'restoration' of the viable/fertile phenotype (Gleba *et al.*, 2004). This regulatory mechanism does not occur under natural conditions; therefore, any seed formed from hybridization between wild relatives and the GM crops that contain the RBF will be unable to germinate because of the action of the blocking sequence. The first patent for this technology was granted in 2005 (US 6849776 B1) to the Finnish biotechnology company Unicrop specialized in the development of therapeutic proteins, which also owns a patent (US 7495148 B2) for the Double Recoverable Block Of Function (Kuvshinov *et al.*, 2005) granted in 2009. The ETC group (2007) incisively dubbed the RBF as 'Zombie technology', demonstrating once again the communicative effectiveness of opponents to GURTs.

The third strategy is applied to vegetatively reproduced species, such as tuber and root crops and ornamental plants, or plants'

organs such as the cotyledons, leaves and stem, where growth is prevented during the period in which they are stored to increase the 'shelf life' of the product. This mechanism patented by Zeneca (Syngenta) in 2001 involves a permanently active gene able to block the vegetative growth of the plant, preventing the multiplication of the seeds. This default-expressed blocking gene can eventually be suppressed by application of a chemical activating a second gene allowing the plant to develop.

A further strategy for creating selectively terminable transgenic plants without the insertion of protein encoding genes has been described by Lin *et al.* (2008). A RNA interference cassette was introduced in tandem with the glyphosate tolerance 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene in transgenic plants. The RNA interference cassette consists of the CaMV35S promoter and an inverted repeat sequence of the cytochrome P450 gene CYP81A6 encoding the enzyme responsible in rice for detoxification of bentazon (Lin *et al.*, 2008) or the inverted repeat sequence of the nicosulfuron detoxifying enzyme gene CYP81A9 in corn (Li *et al.*, 2013). All of the glyphosate-tolerant transgenic plants were selectively and efficiently killed by spraying of bentazon or nicosulfuron, respectively. In a variant of this strategy, a second transgene expressing the Bt insecticidal protein Cry1Ab is introduced (Liu *et al.*, 2012).

For completeness, the so-called 'repressible seed-lethal system' proposed by Schernthaner *et al.* (2003), which provides a single repressor containment system based on the simultaneous insertion at the same locus on homologous chromosomes of a seed-lethal gene linked to a novel trait (SL-NT) and a repressor gene (R), is mentioned. When the parental lines are crossed, the offspring will present viable seeds with the genotype SL-NT/R. Upon outcrossing, the two alleles will be separated and when gametes carrying the SL-NT allele are introduced into a non-GM plant, in the absence of the R element, the seed lethality gene is activated in the seed embryo and thus any seed containing the novel trait will not germinate. However, this strategy for producing sterile seeds technically differs from V-GURTs as it lacks the use of an external inducer.

T-GURTs

Regarding T-GURTs, there are two mechanisms by which they work (FAO, 2001a). In the first one, a gene cassette is expressed in the seed and programmed so that the gene responsible for the production of a toxin/disrupter protein is instructed to undo a particular plant trait of interest, without, however, killing the embryo. Thus, a desirable characteristic may be excised selectively by applying or withholding chemical application before being sold to farmers; consequently, the first generation plant is capable of expressing the trait of interest, but the second generation is not (e.g. Zeneca patent WO 9403619 titled 'Improved Plant Germplasm'). In the second mechanism of action, the gene encoding the trait of interest is kept silent, but it can be activated by the farmer through the application of a chemical inducer to the plant or seed. In the subsequent fertile generations, the gene is inherited in the inactive state, so that the chemical must be purchased each year that farmer needs the trait to be expressed (Shi, 2006). A variant of the latter mechanism has been hypothesized by Shoemaker *et al.* (2001) on behalf of the USDA, providing that the gene of interest can be activated by the farmer spraying a 'standing crop' with an activator only at the occurrence of an unfavourable event (e.g., a pest disease). This focused strategy may help in reducing the build-up of resistance in the target population, whereas the flexible (and timely) interventions would allow the grower to save on the purchase of chemicals (Boettiger *et al.*, 2004).

However, transgene activation or inactivation by means of an artificial stimulus is already feasible through the use of inducible promoters. The stimuli can include suitable transacting factors (receptor-like proteins that binds the ligand), heat shock, and chemical inducers such as steroid-related molecules (dexamethasone and estradiol), the inducer of pathogen-related proteins benzothiadiazol, antibiotics (e.g., tetracycline), copper, ethanol, herbicide safeners, the insecticide methoxyfenozide and other organic molecules (Borghi, 2010).

Another promising (although not field-validated) technology that implies the restricted use of the inserted traits is the 'GM-gene-deletor' system developed to remove all functional transgenes from pollen, seeds, fruits and other edible parts of GM crops when transgene presence may cause concerns (Luo *et al.*, 2007). Basically this invention has a 'combined step' strategy based on the use of two site-specific recombination systems to remove unneeded DNA after site-specific integration as described by Srivastava and Ow (2004) and the innovative use of a site-specific recombinase to remove all transgenes from target organs to prevent gene flow, as proposed by Keenan and Stemmer (2002). The system functions through combination and interactions of the recombination systems Cre/*lox* from bacteriophage P1 and FLP/*FRT* from *Saccharomyces cerevisiae*. The transgenes and the FLP or Cre recombinase gene, under control of a tissue- or developmental stage-specific promoter, are inserted within two fused *loxP-FRT* recognition sequences because it was observed that the two direct repeat *loxP-FRT* fusion sequences enhance FLP or Cre recombinase efficiency. The expression of the recombinase will lead to the deletion of all functional transgenes between the two *loxP-FRT* fusion sites, including the recombinase gene. The use of the pollen- or seed-specific promoter PAB5 from *Arabidopsis* limits the *FLP* or *Cre* expression and the consequent transgene excision exclusively to these cells. Eventually, the deleted sequences will be destroyed by nonspecific nucleases in the cell (Srivastava and Ow, 2003). The production of nontransgenic pollen and seed from GM plants on the one hand may eliminate (or at least strongly reduce) the concerns related to the spread of transgenes and on the other hand would require farmers to annually purchase new seeds if they want to maintain the genetically modified trait such as insect or herbicide resistance. Such technology would also be a step forward compared with the terminator technology as it eliminates the ethical implications.

The PAB5-FLP system remains genetically stable in vegetatively or artificial seed-propagated plants, but has been proposed (Li, 2012; Srivastava and Ow, 2003) to be applicable even in sexually propagated crops through introduction of a chemically inducible RNAi-FLP gene cassette into the provided gene deletor system to prevent the deletion of transgenes in pollen and seeds. *FLP* expression leads to the deletion of all the functional transgenes in pollen and seeds unless an inducer (e.g., ethanol) is applied during pollen or seed development to activate RNAi-FLP gene repression, in turn resulting in site-specific expression. Accordingly, the transgene will be expressed in the pollen or seeds in that generation, but further application of the inducer will be required in the subsequent generation to prevent *FLP* expression from deleting the desired trait. These features allow the deletor system to be categorized as a T-GURT.

Finally, the functional alteration of the genetic code defined 'Genetic encryption', described in the granted patent US 8592199 B2 (Ardell, 2013), has been classified by its own author

as a GURT. This invention assumes that altering the anticodon-stem loop (ASL) templates of tRNA genes *in situ* by genetic engineering or whole genome synthesis changes the codon reading specificity of the tRNA produced. Protein-coding genes engineered according to this altered genetic code are encrypted, that is, they can only produce proteins with an intended structure when translated within the context of that specifically engineered organism or *in vitro* translation system. When encrypted genes are translated into an unencrypted genetic background (a natural genetic background, *in vitro* translation system, or any other translation system that is not itself engineered correspondingly), the misfolded proteins produced will in almost all circumstances have a disrupted amino acid sequence and are likely to be nonfunctional.

Rationales behind GURTs

The main goal for which GURTs were designed is the technological protection of genetic resources and innovations (Garí, 2002); however, their possible application would be further useful for preventing undesired transgene flow and obtaining specific agronomic/economic benefits.

Intellectual property protection

The intention driving the development of the terminator technology is clearly expressed in the statements of the representatives of the patent holders of the first V-GURT:

Harry Collins, at that time Vice President of the Technology Transfer Department of the Delta & Pine Land Co., in the article 'New Technology and Modernizing World Agriculture' distributed during the formal FAO meeting, held in Rome in October 1998, declared that 'The centuries old practice of farmer saved seed is really a gross disadvantage to third world farmers who inadvertently become locked into obsolete varieties because of their taking the 'easy road' and not planting newer, more productive varieties'. Willard Phelps, official spokesman of the U.S. Department of Agriculture (USDA), in an interview with *New Scientist* (March 29, 1998) declared that: 'Our system is a way of self-policing the unauthorized use of American technology. It's similar to copyright protection', adding that: 'This technology is designed to increase the value of proprietary seed owned by US seed companies and to open up new markets in Second and Third World countries'. His words were echoed by Melvin J. Oliver, inventor of the technology, who, in March 1998, said: 'My main interest is the protection of American technology. Our mission is to protect US agriculture, and to make us competitive in the face of foreign competition. Without this, there is no way of protecting the technology'. In fact, although intellectual property protection is granted at the local level in the form of patents or plant varietal protection (PVP), also 'plant breeder's rights' (PBR), and at the international level by the UPOV (International Union for the Protection of New Varieties of Plants) and by the WTO Trade-Related Aspects of Intellectual Property Rights (TRIPS) Agreement (Article 27.3b[2]), the monitoring of patent right infringement by unauthorized use of seeds, if not impossible, is at least time-consuming and expensive. Moreover, there are several countries (for the most part developing countries) where plant varieties and/or biotechnological inventions are not protected or protected with an ineffective or very expensive intellectual property rights (IPR) system. In this sense, GURTs, giving a perpetual form of physical protection, would be an effective mechanism to bypass, either at local, national or international level, the intellectual property regulatory framework and other related judicial systems that provide, among the other things, an expiration date for the patents or the licenses (generally 20 years). This latter aspect would maintain the relevance for industrial/biotech research as the GURT technology would be protected during the life of the patent. Thus, the intellectual property protection granted by GURTs has a double target as it ensures that farmers cannot reuse saved seeds or exploit a valuable trait without purchasing a (patented) chemical and also prevents competitor biotech industries from using seeds in their own breeding programmes. Eventually, as suggested by Pendleton (2004), a company could use the prospect of the commercial use of GURTs in negotiations with governments or customers as leverage to achieve greater legal protections, better enforcement, or contractual concessions.

A commonly managed form of restriction use is the hybrid seed technology, where the outcrossing occurring in the second and every other generation will produce a significantly lower performance of the plants (insofar as the first rationale of hybridization is to obtain more valuable plants by incorporating desired traits). However, hybridization may be infeasible or ineffective for many self-fertilizing crops such as rice, wheat, soya bean, cotton and horticultural crops (Jefferson *et al.*, 1999), whereas GURTs could potentially be applied to all seed-propagated crops (Lehmann, 1998). Nevertheless V-GURTs, would not prevent the clonal propagation of plants such as some grass species, shrubs, and trees (Committee on the Biological Confinement of Genetically Engineered Organisms, 2004).

Transgene containment

Genetic use restriction technologies could be used for the environmental containment of transgenic seeds (V-GURT) or transgenes (T-GURT), thus solving or marginalizing one of the greatest concerns associated with GM crops (Collins and Krueger, 2003; FAO, 2001b). According to Dunwell and Ford (2005) seed lethality is the only strategy at present that prevents transgene movement via seeds; however, GURTs may generally prevent unwanted gene flow from transgenic to nontransgenic varieties (including wild relatives) because it is argued that pollen carries the dominant allele of the lethal/inhibiting protein. Thus, the GURT would most likely be transferred along with the desired trait in the hybrid through cross-pollination (Jefferson *et al.*, 1999; Lamkey, 2002; Lee and Natesan, 2006). Accordingly, GURTs may help the breeding companies to address any legal liabilities if the transgenic crop has the ability to cross with other commercial varieties or introgress into wild relatives (Hills *et al.*, 2007), thus making it particularly attractive in the case of biopharm crops (Oguamanam, 2005).

As an indirect effect, GURTs could reduce or remove the need for buffer zones for gene containment and drastically limit the eventuality of volunteer plants by preventing volunteer seeds from germinating (V-GURTs) or from expressing the GM trait (T-GURTs). Additionally, according to Budd (2004), V-GURTs would be useful to effectively reduce the risk of creating 'superweeds' by reducing the presence of the GM crop in subsequent years.

[2]Plants and animals other than micro-organisms, and essentially biological processes for the production of plants or animals other than non-biological and microbiological processes. However, Members shall provide for the protection of plant varieties either by patents or by an effective sui generis system or by any combination thereof.

There are several proposed methods for transgene containment in plants, such as physical containment (in greenhouses, growth rooms and bioreactors), partial genome incompatibility, harvesting before flowering, parthenocarpy, stenospermocarpy, reduced shattering, inhibition of seed dormancy, apomixis, plastid transformation (transplastomic approach), cleistogamy, induced triploidy, conditional lethality, male sterility, inducible promoters, complete sterility by nonflowering, transgene excision, transgene mitigation (TM), inteins and auxotrophy (Kausch *et al.*, 2010; Liu *et al.*, 2013). Many of these methods including some degree of genetic use restriction; however, none of the strategies currently available blocks all avenues for transgene spread (de Maagd and Boutilier, 2010). In particular, regarding other gene containment methods based on genetic engineering, several crucial points still need to be overcome. Plastid transformation is not suited to all traits or crops and does not offer complete containment; in fact, the transmission of plastid DNA via pollen has been described through passage from the pollen tube into the zygote along with the sperm cell during fertilization (Cummins, 1998; Wang *et al.*, 2004). The rare phenomenon of paternal inheritance of plastids is known in rye (Mogensen and Rusche, 2000) and tobacco (Avni and Edelman, 1991). A method to reduce the risk of plastome gene outflow may include the combination of male sterility with transplastomic traits (Rotteveel *et al.*, 2006). Male sterility has limitations in preventing escape via seeds for fruit/seed-bearing crops (although coupling parthenocarpy to male sterility could offset this risk); moreover, hybrid seeds produced from male-sterile GM crops by cross-pollination from weeds may produce fertile pollen (Daniell, 2002). The use of inteins, that is, controlled excision of the transgene and insertion of genes to reduce hybrid fitness, requires further development (Daniell, 2002; Dunwell and Ford, 2005; Gils *et al.*, 2008). Mechanisms to control transgene expression via conditional lethality, inducible promoters, 'GM-gene-deletor' systems, transgene mitigation and auxotrophy to prevent floral or seed development need to be validated in the field. Methods to control flowering and fruit development such as apomixis and cleistogamy are, to date, limited because the genes controlling and regulating these conditions have not been identified or fully characterized; furthermore, these strategies will not prevent transgene escape via seeds (de Maagd and Boutilier, 2009). Recently, Hague *et al.* (2012) demonstrated that the Zm13 promoter can drive pollen-specific expression of the cytotoxic gene barnase in stably transformed rice, resulting in plants with pollen sterility. As it is pollen-specific and inherited as a single Mendelian trait, pollen sterility could be an effective strategy for gametophytic transgene confinement but it would not be effective against transgene escape through seed scatter and vegetative propagation. According to another approach, Mlynárová *et al.* (2006) obtained transformed Arabidopsis and tobacco plants that produce transgene-free pollen by introducing loxP-embedded cassettes with a plant intron containing a recombinase gene driven by the NTM19 promoter, highly specific for the microspores at early uninucleate stage, and linked to a gene of interest. This construct leads to recombinase-mediated auto excision of all transgenes during microsporogenesis, but is only valid for the production of GM plants as hemizygous lines. Similarly, Moon *et al.* (2011) described a method for transgene excision in pollen via the nonreversible site-specific CinH-*RS2* recombination system, based on a codon-optimized serine resolvase recombinase (CinH) that recognizes recombination sites with a specific 119-base sequence. Further recombination systems for transgene excision in plants are derived from the serine resolvase family, for example, ParA-*MRS* (Thomson *et al.*, 2009) and Bxb1-*att* (Blechl *et al.*, 2012), or from the tyrosine integrase family, for example, R-*RS* (e.g., Darwish *et al.*, 2014) and the previously cited Cre-*lox* and FLP-*FRT*, which exploit the catalytic tyrosine's hydroxyl group for a nucleophilic attack on a phosphodiester bond of the target DNA site (Lyznik *et al.*, 2007). The introduced recombinase removes itself and the transgene by auto excision (Konig, 2003; Mlynárová *et al.*, 2006) or, in GURTs, by spraying with chemicals (Zhang *et al.*, 2003) or heat shock (Hoff *et al.*, 2001). Other promising genome-editing approaches to knockout genes and potentially prevent transgene flow with far greater efficiency than traditional strategies include the use of artificially engineered nucleases: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), the CRISPR/Cas system and site-specific meganucleases (Liu *et al.*, 2013). In particular, Kausch and Dellaporta (2011) described a method to create 'synthetic fertility lethality' by inducing stable knockout mutations in genes required for floral development in male and female lines that when crossed will produce hybrid sterile plants. Synthetic lethality is realized by using zinc-finger nucleases, which are synthetic endo-restriction enzymes generated by fusing a zinc-finger DNA-binding domain (composed of three to six fingers) to a nonspecific DNA-cleavage domain of the bacterial FokI restriction endonuclease. ZFNs are capable of generating double-stranded breaks in specific DNA sequences, which will be imperfectly repaired (in the absence of a homologous template) by nonhomologous end-joining (NHEJ), resulting in deletion or insertion of base-pairs that is exploited to precisely alter specific genomic loci. In another patent application (Russel and Petolino, 2011), one or more ZFNs (zinc-finger nucleases) were used to remove transgenes from specific plant tissue at a particular developmental stage as a means of reducing gene flow into non-GM crops. However, if the zinc-finger domains are not specific enough for their target site or if they do not target a unique site within the genome of interest, off-target cleavage may occur (Durai *et al.*, 2005). An alternative, nontransgenic approach (Marton *et al.*, 2010) relies on the use of viral-based (tobacco rattle virus, TRV) vectors, capable of systemically infecting the host plant, for transient expression of ZFNs in plant (growing and developing) tissues and cells.

In the near future, the possibility of applying these techniques alone or in conjunction with GURTs could open up a new phase in the use and acceptance of genetically modified plants.

Other possible benefits

The major agronomic benefits deriving from this technology are related to T-GURTs because they could be used to switch a desired trait on or off in favourable or unfavourable situations, such as drought and salt stress or pest attack (FAO, 2001a), whereas V-GURTs could be used to prevent preharvest sprouting (Budd, 2004; Pilger, 2002) and, according to Louwaars *et al.* (2002), when combined with apomixis, they could allow seed suppliers to produce seeds with hybrid vigour at reduced cost while protecting the investment.

Genetic use restriction technologies may increase competition by encouraging private companies to enter the market of self-fertilizing cultivars, especially in countries where seed saving is a common activity (FAO, 2002). Breeders would obtain their economic return through the sale of seeds. The resulting boosted investment in research and development in the plant breeding sector, favoured by the lower costs resulting from cover contracts and intellectual property laws (Smyth *et al.*, 2002), could

eventually increase productivity and (paradoxically) agricultural biodiversity where the breeders would be able to use a much wider gene pool or develop more varieties (Louwaars *et al.*, 2002). Eaton and van Tongeren (2002) suggested that even governments may benefit from GURTs through reduced investment requirements for breeding and fewer enforcement costs for plant variety protection.

Moreover, against the increased costs to buy seeds (or chemicals to activate the seeds/traits), farmers could profit from the new (improved) varieties providing higher yield potentials and improved pest resistance (Mukherjee and Senthil Kumar, 2014). These benefits may also have a secondary positive impact on consumers, leading to lower food costs (Eaton and van Tongeren 2002).

Nevertheless, in the forecast realized by Goeschl and Swanson (2003) on the possible outcomes deriving from the application of GURTs in a 20-year horizon, it is suggested that the most developed countries would stand to benefit most, whereas the least developed countries would stand to lose (especially in the short term).

Concerns

Agrarian sustainability

The main arguments put forward against GURTs, particularly against terminator technology, are the impacts on biodiversity, sustainable agricultural development, and farmers' access to and use of genetic resources through the inability to save and re-sow seeds.

Regarding the impact on agrobiodiversity, the first concern is that the introduction of new, uniform, GURT-protected varieties would replace the adapted or selected (possibly less productive) autochthonous cultivars and wild relative species, resulting in the erosion of genetic diversity in fields, adverse effects on local germplasms (or at least the landraces), and effects on the coevolution of crops at the farm level (FAO, 2001a; Visser *et al.*, 2001). This would be at odds, for example, with the European directive 2008/62/EC aimed at protecting seed varieties of agricultural crops threatened by genetic erosion and providing that 'landraces and varieties which are naturally adapted to local and regional conditions and threatened by genetic erosion (conservation varieties) should be grown and marketed even where they do not comply with the general requirements as regards the acceptance of varieties and the marketing' of seeds.

Genetic use restriction technologies-transformed crops may also produce low quantities of autotoxic compounds with negative impacts on nontarget organisms, induce competition with wild species, and eventually, as food/feed, transfer allergenicity and antibiotic resistance (Working Group on Article 8(j), 2006);. Similarly, the chemicals used to treat the seeds each year may have negative impacts on the environment where a massive use of antibiotics such as tetracyclines, although harmless to humans and plants, may have a detrimental effect on soil ecology, particularly on microflora and fauna, and increase the prevalence of antibiotic-resistant bacteria (Mariani, 2001; Mukherjee and Senthil Kumar, 2014). Moreover, Giovannetti (2003b) suggested that it cannot be excluded that suicide genes could be suddenly activated at different times and in different parts of the plant other than the seed, with disastrous effects on ecosystems and life itself, whereas the application of GURTs that would prevent the formation of pollen in plants could have a detrimental ecological impact on some pollen feeding insects.

From a socioeconomic point of view, GURTs would limit the fair and equitable sharing of benefits arising from their utilization provided by the Nagoya Protocol of the Convention on Biological Diversity (and by Article 10 of the International Treaty on Plant Genetic Resources for Food and Agriculture) because of the increased dependency on 'industrial' costly seeds and chemical inducers that would create a companies' monopoly over markets (with an unbalanced distribution of benefits) and a subsequent reduction of the so-called 'food sovereignty'. Moreover, the 2003 report of the Ad Hoc Technical Expert Group (AHTEG) on the potential impacts of GURTs on smallholder farmers, indigenous and local communities and farmers' rights listed various possible negative impacts including:

1. reduction and limitation of traditional seed exchange practices and participatory plant breeding;
2. reduction of the traditional knowledge and innovation capacity for informal crop genetic improvement, local agrobiodiversity protection and food security;
3. displacement of local farming systems and the social, cultural and spiritual dimensions associated with them.

A very common issue is that this technology would favour large multinational corporations and would have a negative impact on the employment of small farmers (Mukherjee and Senthil Kumar, 2014). In addition, according to Garí (2002), GURTs would tend to concentrate breeding efforts and options, rather than widening them, setting limits to the effective adherence to the international policy framework on plant genetic resources and thus restricting poor farmers' access to new varieties and technologies and preventing them from making crosses to develop valuable and locally adapted varieties.

Eventually, the introduction of GURT-transformed crops could be counterproductive for companies that export to the European market, which is traditionally hostile to genetically modified organisms (GMOs) (IIPTA, 2012).

Risk of transgene escape

Some drawbacks are related to the real effectiveness of GURTs in preventing gene flow, but more generally to the real feasibility of these mechanisms. Whereas partial V-GURT efficiency, that is, causing the reduction of the germination rate, would be enough to force farmers to buy seeds from companies each year (Sang *et al.*, 2013), the prevention of flower or seed development and the inducible expression of the GM trait would require a 100% effective application of a chemical inducer to prevent the escape of a nonfunctioning transgene via both seed and pollen. In fact, some seeds may not respond or may not take up enough inducer to activate the recombinase, thereby producing fertile GM plants (Lemaux, 2009; Van Acker *et al.*, 2007) able to transmit the inserted trait and causing exactly the opposite effect to the one intended.

Other technical issues have been raised regarding the escape of genes over generations, the mutation of genes, the accidental switching on of sleeper genes, the instability of the promoters and the horizontal flow of genetically modified pollen to nontarget organisms (e.g., birds, insects and soil biota) (FAO, 2002; Working Group on Article 8(j), 2006);. It is further possible that inducer-blocked/activated expression of a GURT trait could naturally or artificially (either voluntarily or involuntarily) occur in response to related compounds (Pendleton, 2004; Working Group on Article 8(j), 2006).

Several criticisms have been specifically levelled at terminator technology. Unresolved questions remain regarding:

1. proper segregation of multiple genes because it is important that the three genes, that is, the toxic protein gene, the recombinase gene and the repressor gene, segregate together during reproduction (Daniell, 2002); otherwise, the technique would not be effective;
2. gene silencing because the LEA promoter may be subject to silencing, resulting in malfunction of the system (RIP would not be produced), and introgression of a GM trait would become possible (Daniell, 2002);
3. presence of transgenic pollen, as the insect- or wind-mediated flow of pollen containing the ready-to-act toxin gene could lead to the production of infertile seeds in adjacent non-GM fields, causing economic losses for 'blameless' farmers (Giovannetti, 2003a). However, 'the probability may be low, given the multiple gene recombination events that would need to accompany outcrossing' (FAO, 2001a).

FAO also warns that GURT-modified seeds, introduced through commercial channels or as food aid, could mingle with conventional varieties with the risk of contaminating them and inhibit their fertility (FAO, 2001b).

Discussion

Despite several forecasts (FAO, 2001a; Louwaars *et al.*, 2002; Shoemaker *et al.*, 2001) that assumed GURTs to be functional in the short-medium term, it is difficult to predict the development of GURTs in the near future because they seem still to be very far from commercialization. Actually, T-GURTs could be received by public opinion as a favourable innovation as they would allow farmers to decide whether, and possibly when, to activate a valuable trait, so that their practical application should not require too much time. Furthermore, T-GURTs would not impede plant viability and would not affect the traditional conservation practices and exchange of seeds, offering at the same time a solution to the problem of genetic pollution by preventing the spread of the engineered traits. In contrast, the ethical concerns against V-GURTs that led to the global moratorium remain to date too strong to overcome and will surely play a pre-eminent role in the future political debate to decide whether to use or not use these technologies. After all, over one billion people, the majority of whom live in developing countries, depend on seed saving and exchanging of seeds with their neighbours, whereas these technologies are conceived (and perceived) as a means to protect multinational corporations and their patents. A crack at this absolute ban can be represented by the two proposed bills (PL 268/2007 and PL 5575/2009) pending (but constantly postponed) in the Brazilian House of Deputies that would prohibit the marketing of seeds containing V-GURTs, except in the case of seeds/plants genetically modified to produce proteins or substances intended primarily for the industrial or therapeutic use. Accordingly, Dunwell and Ford (2005) suggested that such technology as a gene containment strategy could be particularly useful and most likely more acceptable in phytoremediation and in bio-pharming, where environmental dissemination is to be avoided and in situations where the seeds are not intended for human and animal consumption.

The alternative form of V-GURT under study provides reversible sterility could represent a first step towards the acceptance of this technology.

In the final analysis, to date several GURTs have been patented but none has been put into practice because of strong opposition. Nevertheless, the cases presented above demonstrate how the interest in the development of GURTs is still alive as they have the potential to represent (positively or negatively) a real new agrarian revolution if they can be accepted by farmers and consumers.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1 Granted or applied patent families V- and T-GURTs published under the Patent Cooperation Treaty (PCT) from 1989 to date.