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CONTENTS

RNAi-mediated Gene Silencing and Its Implications for Agriculture

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Background

RNA interference (RNAi) has emerged as a powerful genetic tool for scientific research over the past several years. RNAi is known as a conserved integral part of the gene regulation processes present in all eukaryotes that utilizes small RNAs (sRNAs) to direct the silencing of gene expression at i.a. the post-transcriptional level. Post-transcriptional gene silencing (PTGS) starts with the initial processing or cleavage of a precursor double-stranded (ds)RNA into short 21–25 nucleotide small-interfering RNA (siRNA) duplexes by an RNaseIII-like enzyme called Dicer. Double-stranded siRNAs are incorporated into an RNA-induced silencing complex (RISC). The activated RISC subsequently unwinds the siRNA in an ATP-dependent reaction, thereby generating an antisense (or guide) strand that targets complementary mRNA transcripts via base-pairing interactions. Subsequent degradation of the targeted mRNA causes inhibition of protein biosynthesis[1]. The consequence of RNAi is a loss of function phenotype that, ideally, is identical to that of a genetic null mutant. Therefore RNAi has been utilized not only in fundamental research for the assessment of gene function, but also in various fields of applied research, such as human and veterinary medicine and agriculture. The first investigations on the potential of RNAi in agriculture research focused on the "proof of concept" that lethal outcomes are possible by gene silencing through RNAi signals *in vitro*. To that end agricultural pests such as insects, nematodes, and fungi underwent feeding, soaking, and microinjection experiments where RNAi signals were exogenously applied with overwhelming results. Encouraged by the fact that agricultural pests can be killed by exogenously supplied RNAi targeting their essential genes, some scientists have done pioneering work on the level of *in vivo* RNAi and found first evidence that plant predation can be controlled by lethal RNAi signals generated *in planta*. We owe it to them that today the RNAi technique has provided possibilities for improved plant production in more efficient agricultural systems.

RNAi as tool for metabolic engineering

Given current trends in increasing numbers of human food allergies, it will be necessary to find a consumer-friendly alternative instead of abandonment of a product. In plants, RNAi strategies have the potential to be used as a molecular tool for breeding high-value crops. For example, RNAi technology has been used to enhance or reduce the accumulation of specific metabolites in food and feed crops, thereby altering their nutritional value[2]. Apart from modifying the nutritional value, RNAi strategies have a great potential for generating plants with high medical value, such as plants that are hypoallergenic or that exhibit reduced auto-immunogenic activity, for example hypoallergenic carrots and low gluten wheat, respectively[3,4]. Such strategies at the intersection of plant biotechnology and medicine especially bear hope for a vast number of patients suffering from food allergies, and thus may also have a great economic impact. There are several examples of RNAi applications to reduce allergenic proteins and toxic compounds[2].

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In addition to metabolic engineering, RNAi technology has been employed to develop plants with improved resistance to abiotic and/or biotic stresses.

Why do we need alternatives in plant protection?

Given current trends in both food and energy demands of a growing population, as well as the changing climate, it will be necessary to greatly improve crop yields worldwide during the coming years. Meeting this challenge will require developing agronomic solutions that promote sustainable plant production systems despite conditions of increased biotic and abiotic stresses, while significantly reducing negative side effects on the environment.

Plant diseases caused by microbial pathogens are a serious risk to plant culture, with estimated annual losses commonly of 30 to 50% of the attainable production, depending on the crop and the treatment[5]. Worldwide pre-harvest losses of six major food and cash crops (rice, wheat, maize, potatoes, soybean, and cotton) caused by animal pests (arthropods, nematodes, rodents, birds, slugs, and snails) amount to an average of 10.8%[6]. More than that, over 500 species of insects and mites are resistant to one or more insecticides. Diseases of cereal crops such as Fusarium head blight (FHB) and crown rot (FCR) caused by phytopathogenic fungi of the genus *Fusarium*, such as *Fusarium graminearum* (teleomorph *Gibberella zeae*), *Fusarium culmorum* and *Fusarium avenaceum*, exert great economic and agronomic impact on global grain production and the grain industry. In addition to considerable yield losses, food quality is detrimentally affected by grain contamination with mycotoxins, which are produced by the fungi during plant infection. These contaminants represent a serious threat to human and animal health. To protect consumers from mycotoxicosis, many countries, including the European Union Member States, have established maximum allowed levels for the most prevalent Fusarium mycotoxins in cereals and cereal products. Plant protection and toxin reduction strategies are presently mediated by chemical treatments, resistance breeding strategies, biological control, and genetic engineering. The latter relies on the use of antifungal transgenes, such as chitinase, defensins, polygalacturonase, and the use of mycotoxin detoxifying enzymes. However, the use of antifungal traits has not provided convincing practical solutions in terms of efficiency and reliability under agronomical practice. Currently, the application of systemic fungicides, such as sterol demethylation inhibitors (DMIs), is essential for controlling Fusarium diseases and thereby reaching the attainable production level of modern high-yield cultivars. DMI fungicides, such as tebuconazole, triadimefon, and prochloraz, act as ergosterol biosynthesis inhibitors because of cytochrome P450 lanosterol C-14α-demethylase (CYP51) binding, which subsequently disturbs fungal membrane integrity. Because of a shortage of alternative chemicals, DMIs have been used extensively in the field since their discovery in the 1970s. Therefore, it is hardly surprising that reduced sensitivity, or even resistance to DMI fungicides, has begun to develop in many plant pathogenic fungi.

These alarming developments demonstrate that novel strategies in pathogen and pest control are urgently required. Agrobiotechnological strategies such as *in planta* mediated RNAi has an immense potential for pest and disease control.

Host plant-induced gene silencing (HIGS)

Plants and other eukaryotes have evolved RNA silencing machineries that not only regulate developmental programs, but also provide protection from invasion by foreign nucleic acids, such as viruses. This natural phenomenon can be exploited

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to control agronomically relevant plant diseases, based on the demonstration that *in vitro* feeding of dsRNA can signal PTGS of target genes in various plant pests and pathogens, such as insects, nematodes and fungi. Indeed, expression of such dsRNAs in the corresponding host plant conferred protection from predation or infection. This biotechnological method, termed host-induced gene silencing (HIGS), has emerged as a promising alternative in plant protection because it combines high selectivity for the target organism with minimal side effects, as compared with chemical treatments.

Since the first publications in the early 90s, a vast number of studies on host derived gene silencing in plant-pathogen interactions have been conducted, which at least reflect the rapid progress of RNAi and its great relevance for agriculture[2].

In the last few years, several studies were published that used HIGS to control microbial pathogens, such as: insect pests (rootworms, bollworms, planthoppers, and aphids), parasitic nematodes (root knot nematodes and cyst nematodes), parasitic plants (Striga, Cuscuta, and Orobanche), bacteria (*Agrobacterium tumefaciens*) and fungi/oomycetes (Fusarium, Blumeria, Puccinia, and Phytophthora)[2].

Recently, it was demonstrated that HIGS of essential fungal ergosterol biosynthetic genes is a highly efficient strategy for controlling the growth and development of the phytopathogenic fungus *Fusarium graminearum*[7]. We assessed the potential of HIGS by targeting the fungal CYP51 genes via CYP3RNA, a 791 nucleotides (nt) dsRNA complementary to CYP51A, CYP51B, and CYP51C. Expression of the dsRNA in Arabidopsis and barley rendered susceptible plants highly resistant to fungal infection. The results demonstrate that HIGS of the fungal CYP51 genes is an efficient method for inhibiting fungal mycelium formation and plant infection. The results also demonstrate the potential of RNAi to at least partly supplant application of azole fungicides for control of fungal diseases.

Conclusion

Biotic stress disproportionately affects farm productivity around the world with immense annual yield losses. For these reasons, control of microbial pathogens continues to be an agronomic and scientific challenge, and innovative and ground-breaking strategies are required to meet the requirement of a growing population. Recent work suggested that novel RNAi-based plant protection strategies may provide new opportunities for improving the world's food supplies and thus can have a huge impact on world's economy. A great number of basic research studies have enabled the rapid increase of knowledge in dsRNA-mediated silencing of target genes. Whereas the first investigations focused on the use of model organisms, it is now becoming possible to apply this knowledge towards modifying specific traits in agriculturally relevant crop plants. In addition to metabolic engineering and HIGS-mediated enhancement of disease resistance, RNAi strategies may be used to improve food safety by controlling the growth of phytopathogenic, mycotoxin-producing fungi. More research is required to optimize practical application strategies and to assess safety aspects; work to resolve these issues is ongoing and, when fully developed, should allow HIGS approaches to at least partly replace traditional chemical protection measures.

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Combining Genome-wide Association and QTL Analysis: Opportunities and Challenges

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Introduction

Soybean is an important oilseed crop worldwide, contributing more than 50% of globally consumed edible oil. But nowhere is it more important than in the Americas where close to 85% of the world soybean production is concentrated, a production that reached close to a quarter billion tons in 2013. The ability of soybean to fix nitrogen from the air obviates the need to apply nitrogen fertilizers (major sources of greenhouse gases), making it an attractive crop for increasing the sustainability of our agricultural systems. In addition, this highly versatile legume is an exceptional source of high quality oils and proteins for human food, a key element in ensuring a more sustainable and healthy diet. The oil content in soybean seed varies considerably, from as little as 8.1% to as much as 27.9%. Similarly, the seed protein content varies between 34.1% and 56.8%. A high correlation has been observed among seed oil content and different traits, including a highly negative correlation between oil and protein content in the seed. This makes it extremely challenging to improve one of these traits without compromising the other. Finally, these seed traits have been found to be governed by many loci and highly influenced by environment.

The identification of genomic loci that govern complex traits has been facilitated by the development of quantitative trait locus (QTL) mapping approaches. Conventionally, QTL mapping is performed on segregating populations derived from biparental crosses. In such an approach, low-density marker coverage (ca. 100 – 200 markers) on one to a few hundred lines has usually proved sufficient to allow the identification of QTLs for different traits in different mapping populations. Typically, however, these roughly estimated QTL intervals extend over several cM, a genetic distance that translates into large genomic regions with dozens, if not hundreds, of candidate genes.

This intrinsic limitation of the traditional QTL mapping approach can be overcome through genome-wide association studies (GWAS) in which a panel of unrelated genotypes is used. In essence, these lines are characterized both phenotypically and genetically with a large number of markers (many thousands at least). It is only recently that developments in high-throughput genotyping techniques have provided an opportunity to obtain the required marker coverage on many hundreds of lines, either through the use of SNP genotyping arrays (e.g., Song et al., 2013[1]) or via a genotyping-by-sequencing approach (e.g., Sonah et al., 2013[2]). Such GWAS have proved useful for the identification of candidate loci associated with numerous traits in animal as well as plant species[3]. In Arabidopsis, GWAS and QTL mapping performed together have been found to be complementary by mitigating each other's limitations[4], but such a combined approach has seldom been used in soybean. In the work described here and in Sonah et al. (2014)[5], we have jointly used GWAS and QTL mapping to precisely map and validate loci underlying eight agronomic traits in a collection of soybean lines.

Materials and Methods

Plant materials, phenotyping and genotyping

A total of 304 soybean lines mostly belonging to early maturity groups (MG000 – II) were used for initial evaluation. A subset of 139 "core" lines spanning the genetic diversity of the complete set (based on the analysis of population structure) was phenotyped for three qualitative traits (flower, hilum, and pubescence color) and five quantitative traits (maturity, plant height, seed weight, seed oil content, and seed protein content) at three sites in Eastern Canada over two years (2012 and 2013). In addition to this association panel, a population of 141 RILs derived from the cross between a Canadian cultivar (Majesta) and a Korean accession (Hikmok sorip; PI372415A) was also used in the present study.

DNA was extracted using a commercial kit (Qiagen) and SNP genotyping was performed using a GBS approach as described in Sonah et al. (2013). Single-end sequencing of multiplex (96x) GBS libraries was performed on an Illumina HiSeq2000 and read processing, mapping, SNP calling and genotyping were performed using the IGST-GBS pipeline. Vcftools and several in-house scripts were used to obtain good quality SNPs. Imputation of missing data was performed with fastPHASE 1.3.

Population structure and QTL mapping

Population structure was estimated by using either STRUCTURE or principal component analysis (PCA). Relatedness among individuals was calculated either using the VanRaden (K) or EMMA method (K*). Genome-wide analyses were performed using TASSEL3.0 and GAPIT. A general linear model (GLM) was used with or without the covariate P from principal component analysis (PCA) and the covariate Q obtained from STRUCTURE. Compressed mixed linear models (CMLM) incorporating a kinship matrix (K or K*) along with P or Q were also tested. The negative log(1/n) was used to establish a significance threshold. Finally, genotypic and phenotypic data for the Majesta x Hikmok sorip RILs were used for QTL mapping. This RIL population was also genotyped using GBS and a linkage map of 778 SNPs was derived (Iquira et al., in prep.). QTL mapping was performed using the QTL IciMapping software (v3.3).

Results

Number and distribution of SNPs in the soybean genome

Sequencing of the GBS libraries yielded approximately 450 million reads in total and ~87% of the reads were successfully mapped to the soybean reference genome. In total, 47,702 SNPs including 2,744 InDels were identified and the average depth was observed to be 13x. The physical distribution of SNPs was fairly uniform with only seven gaps of greater than 500 kb, and all of these gaps occurred within centromeric or pericentromeric regions.

On average for the entire genome, 50 SNPs/Mb were found, and this varied between a minimum of 37 SNPs/Mb on Gm01 and a maximum of 59 SNPs/Mb on Gm16. Among the core set of 139 soybean lines selected for association analysis, a total of 17,172 SNPs were retained for the GWAS after excluding SNPs with more than 20% missing data and/or a minor allele frequency less than 5%.

GWAS on qualitative traits

A total of seven different models, from a naïve model devoid of any correction for confounding to models incorporating a correction for both population structure (covariates P or Q) and/or kinship (covariates K or K*), were tested on all traits. Models that took into account kinship were found to provide the best fit. GWAS was first performed for three simple traits, namely flower color, pubescence color, and hilum color, for which the causal genes are known. For flower color, a single region on chromosome Gm13 showed many significant marker-trait associations with *p*-values as low as 3.4 x 10^{-17}, including a SNP marker located less than 10 kb from the *W1* locus (coding for a flavonoid 3',5' hydroxylase) known to control flower color. Similarly, for pubescence color a very strong ($p < 10^{-16}$) marker-trait association was found within 100 kb of the T locus (coding for a flavonoid 3' hydroxylase) known to underlie this phenotype. In the case of hilum color, two strong associations ($p < 10^{-12}$) were found on Gm08 and Gm09 (**Figure 1**, top panel), including markers located within 100 kb of the *I* and *R* loci (a chalcone synthase and a R2R3 MYB, respectively). In this case, the difference in hilum color between Majesta and Hikmop sorip allowed us to perform QTL mapping in the progeny of this cross. The same two genomic regions exhibited very high LOD scores (>20) when interval mapping was performed on the population of segregating RILs (**Figure 1**, bottom panel), with each QTL explaining a very significant portion of phenotypic variance (65.6% for *qHC8* and 15.4% for *qHC9*).

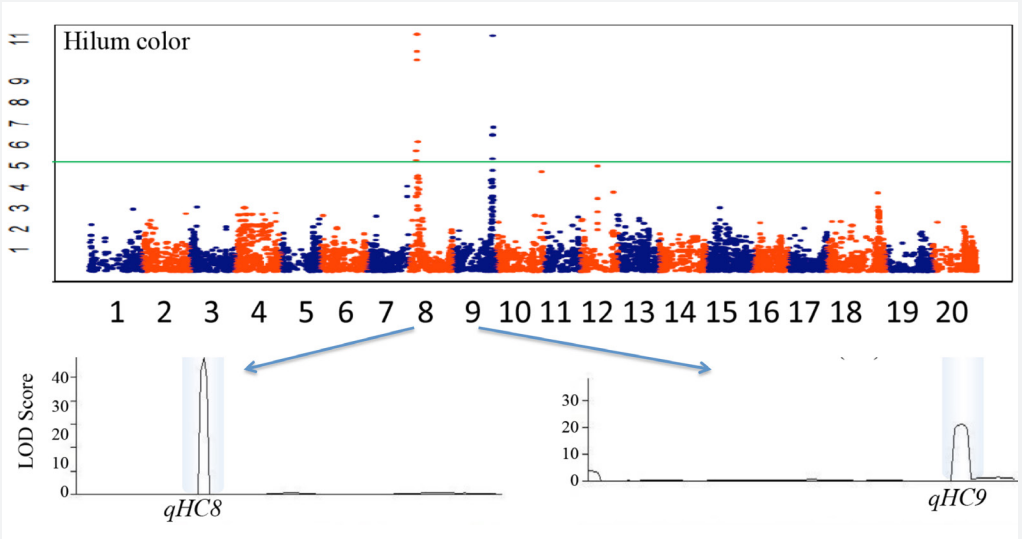

Figure 1. QTLs for hilum color. Manhattan plot showing the genome-wide marker associations with hilum color (top panel). LOD score plots (for Gm08 and Gm09) derived from QTL for hilum color in a collection of 141 RILs derived from a cross between Majesta and Hikmok sorip (bottom panel).

GWAS and QTL mapping on quantitative traits

Similarly, we conducted association analyses on five quantitative traits, namely plant height, maturity, hundred seed weight, and seed oil and protein content. A region on Gm19 known to harbor the earliness gene *E3* (coding for phytochrome A3) was highly associated with both maturity and plant height, two positively correlated traits. For hundred seed weight, a total of three significant regions, one each on Gm02, Gm13 and Gm20, were associated with the trait. QTL mapping using the Majesta x Hikmok sorip RILs also identified a QTL (*qHSW13*) within the same region (**Figure 2**); it had a LOD score of 5.5 and accounted for 17.6% of the phenotypic variance. Although the GWAS QTL on Gm20 showed a similarly strong association, it was not detected in the RILs. Finally, a total of eight regions significantly associated with protein content were identified via GWAS and these regions were practically identical to those described for oil content with six loci spanning the exact same intervals, and the other two regions (on Gm05 and Gm19) overlapping. Furthermore, five of eight peak SNPs were identical. A single QTL (*qOC10*) for oil content was identified using biparental QTL mapping. This QTL was located on Gm10 with a LOD score of 3.7 and explained 11.8% of the phenotypic variance for oil. None of the QTLs identified for oil content using GWAS overlapped with the single QTL identified using the biparental population. For protein content, a single QTL (*qPC19*) on Gm19, with a LOD score of 2.95 and accounting for 10.3% of the phenotypic variance, was identified and, again, was distinct from the GWAS QTLs for protein content on this chromosome.

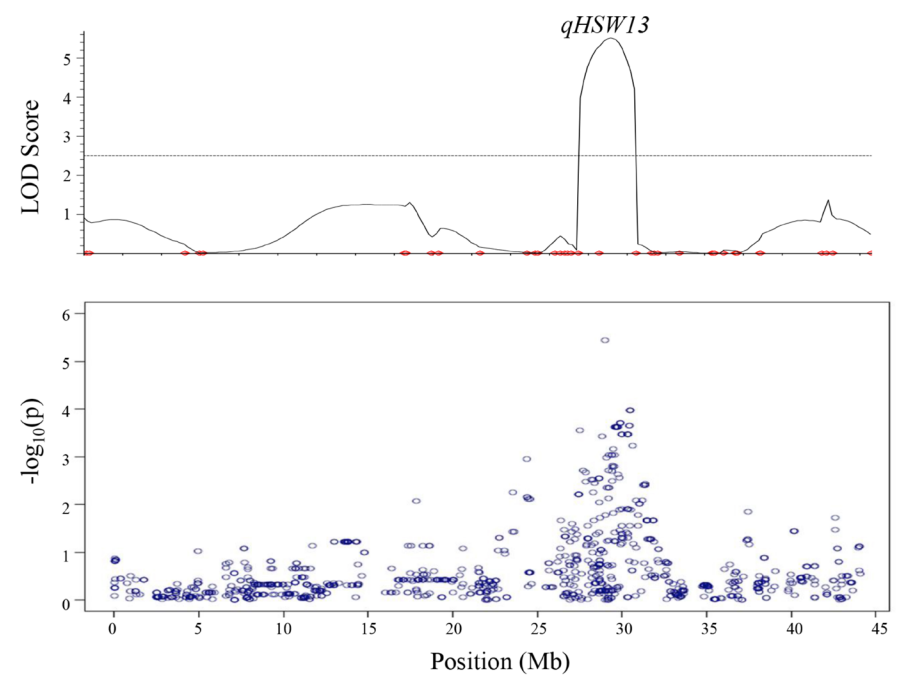

Figure 2. QTL mapping of hundred seed weight (HSW) on chromosome Gm13. Manhattan plot showing the marker associations with hundred seed weight on Gm13 (top panel). LOD score plot for a QTL contributing to hundred seed weight (LOD = 5.50; r2 = 17.6%) detected among a collection of 141 RILs derived from a cross between Majesta and Hikmok sorip (bottom panel).

Discussion

GWAS is not yet well established in soybean despite the availability of a high-quality, well annotated reference genome. Even though inbreeding in soybean has resulted in long-range linkage disequilibrium, as compared to an outcrosser such as maize, the number of markers needed to provide extensive genome coverage and detection power has been estimated to be in the tens of thousands[6,7]. Thus, it is only recently that high-throughput genotyping platforms capable of meeting this requirement have been developed[1,2]. We demonstrate here that a GBS approach, even on a narrow collection of 139 very early soybean lines, allowed us to capture over 17,000 informative markers that were suitable for GWAS. At approximately 20$ per sample (for library preparation and sequencing), this represents tremendous value for the dollar.

When performing a GWAS, it can be useful to analyze simple traits for which the causal locus is known to validate both the genotypic and phenotypic data sets as well as the statistical model used. In addition, such analyses can also be useful to evaluate the measure of proximity obtained between significantly associated markers and the causal locus at the level of marker density achieved. In this work, three qualitative traits controlled by a total of four known loci were identified with a high degree of confidence, and highly associated markers were typically within 100 kb of the known causal locus. If one did not know the underlying locus controlling a trait, this level of proximity and resolution would be very helpful in limiting the number of candidate loci that one would have to examine.

Compared to conventional

QTL mapping in a biparental population, association studies are thought to offer improved resolution and to better capture a broader and more relevant set of alleles, all the while being prone to an increased risk of false positives[3]. Thus, QTL mapping can be viewed as a natural extension of GWAS aiming to confirm the validity of candidate QTLs initially revealed via GWAS. In this work, we demonstrated that the two genomic regions associated with hilum color were independently identified through the analysis of a set of RILs segregating for this trait. In other cases, however, many candidate QTLs uncovered in the GWAS could not be confirmed via QTL mapping. This underscores some of the challenges in performing such validation work.

When the genetic architecture of the trait is simple, the loci controlling a trait (possibly even the alleles) will be the same between a GWAS panel and any mapping population segregating for this trait. In this case, there should be no difficulty in thoroughly validating candidate QTLs. For more complex traits, however, the identification of a suitable mapping population can be challenging, especially when working with multiple traits. In this work, although three genomic regions showed an association with hundred seed weight, only one of these could be validated in the population of RILs. As the GWAS panel was composed of Canadian soybean lines, while the mapping population was derived from a cross between a Canadian and a Korean line, it is possible that the parents were fixed for the same allele at two of these candidate QTLs and were segregating only at other loci (including the one validated QTL on Gm13). This is even more striking in the case of the seed oil and protein content QTLs where none of the candidate QTLs identified through the GWAS could be confirmed in the RILs. Because a large number of QTLs (>50) have been reported for these seed traits, it is not at all unlikely that different sets of QTLs control oil and protein content in the association panel compared to the biparental population. Thus, numerous different populations will likely be needed to capture, within QTL mapping populations, the diverse loci and alleles that can be identified in a single GWAS.

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Genetic Use Restriction Technologies: Good for Seed Companies and Bad for Farmers?

Luca Lombardo

Introduction

The term "Genetic Use Restriction Technologies" (GURTs), coined in 1999 by the Subsidiary Body on Scientific, Technical and Technological Advice of the UN Convention on Biological Diversity (CBD), relates to a series of experimental methods aimed at restricting the unauthorized use of genetic material by controlling gene expression in genetically engineered (GE) plants at the variety level (V-GURTs) or at the trait level (T-GURTs). The general design provides the insertion in plants of a "genetic switch" activated (or inactivated) by an external – chemical or physical – inducer to prevent germination in V-GURTs, or, in the case of T-GURTs, to turn on/off a value-added trait such as tolerance to herbicides or biotic and abiotic stresses, pest resistance, etc.

Despite the very first patent application (granted to DuPont in 1994 – U.S. patent 5,364,780) containing GURT concepts dates back to 1991, it took seven years for these technologies to catch the world's attention, with the grant of the patent U.S. 5,723,765 entitled "Control of plant gene expression" jointly owned by the United States Department of Agriculture (USDA) and Delta & Pine Land Company. The invention described in the specification was the first V-GURT, thereafter best known as "terminator technology," and quickly became one of the most opposed genetic engineering biotechnologies. In fact, while the holders of the patent considered the possibility for seed companies to realize transgenic plants producing only sterile seeds as a "technology protection system," public opinion, non-governmental organisations and smallholder farmers' associations saw it as a disadvantageous and unethical mechanism to force farmers to purchase new seeds every year, preventing the practice of seed saving (also "brown-bagging") estimated to account for between 15% and 20% of the world's food supply involving 1.4 billion people[1].

As a consequence of the strong protests all over the world, the CBD Decision V/5 section III of the Fifth Conference of the Parties (COP5) held in Nairobi in June 2000 imposed a *de facto* global moratorium on this technology, ratified by specific national laws in India, Canada and Brazil. 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, in spite of the over 40 granted or submitted patent families (groups of patents that include identical or similar applications) related to GURTs, no plant with these characteristics has been either approved or marketed anywhere in the world.

Variety level GURTs

The molecular construction described in the patent U.S. 5,723,765 provides the use of (i) a lethal gene, (ii) a repressor gene (the gene switch) that is responsive to an external stimulus, and (iii) a Cre recombinase gene – the expression of which is blocked by the repressor – linked together in a gene cassette. The lethal (or terminator) gene codes for the cytotoxic protein Ribosome Inactivating Protein (RIP, or saporin) and is under control of a Late Embryogenesis Abundant (LEA) promoter – so that it is only transcribed during late embryogenesis – linked to a DNA spacer (blocking) sequence flanked by specific excision sites (lox sequence) that prevent the activation of the terminator gene. The repressor gene, a Tn10 tet repressor gene, is under the control of a constitutively active promoter (e.g., CaMV 35S) and encodes a protein that binds to the tet operon(s) contained in the constitutive promoter of the Cre recombinase gene, preventing its expression. Before being sold to the farmer, the seeds are exposed to an external chemical inducer (tetracycline) that prevents binding of the repressor to the operon. This causes transcription of the Cre Recombinase that cuts the specific excision sites flanking the blocking sequence linked to the toxic gene. Consequently, during late embryogenesis, the lethal 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, but the seeds produced in the harvest will be sterile and thus cannot be saved for later cropping.

Other technologies have subsequently been developed for producing sterile seeds. The patent AU 621195 B2 owned by Zeneca describes an embodiment in which a gene encoding a disrupter protein is permanently active in the seed, making 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

sequence controlling the promoter of the disrupter protein gene, inhibiting its expression. The breeder must apply the chemical inducer during the process of seed multiplication, interrupting the application only at the time of selling the seeds.

The recoverable block of function (RBF) developed in tobacco by Kuvshinov et al.[2] exploits the barnase/barstar gene system. The barnase gene, under control of a sulphydryl endopeptidase promoter active at the time of seedpod development, encodes a potent ribonuclease that in embryos and sprouts confers cell death or prevents sexual reproduction. In the RBF, barnase is inserted in an artificial intron within the gene of interest; the same insert contains a second inducible sequence: a barstar, which is a strong repressor gene that prevents barnase action.This recovering action is induced by an external stimulus such as chemical application or heat shock; therefore, if seeds are exposed to the chemical, they will express the normal phenotype, otherwise they will die. Because of this reversible sterility, activists dubbed RBF "zombie technology."

A further strategy patented by Zeneca (Syngenta) in 2001 was designed to increase the "shelf life" of vegetatively reproduced species such as tuber and root crops and ornamental plants through the insertion of a permanently active gene able to block the growth of the plants or plants' organs.

RIDL technology

The technically but not conceptually equivalent version of the terminator technology in the animal kingdom is the so-called RIDL (Release of Insects carrying a Dominant Lethal) technology. This technique, under development by Oxford Insect Technologies (Oxitec), was employed for creating sterile offspring in insect vectors of diseases (such as dengue fever and malaria) or agricultural insect pests. The RIDL system requires that a strain of the target insect carries a conditional, dominant, sex-specific lethal gene activated by the expression of the tetracycline-repressible transactivator fusion protein (tTA), in turn, under the control of a suitable (constitutive, female-specific, embryo-specific, etc.) promoter[3]. tTA is a hybrid, synthetic transcription factor resulting from the fusion of the prokaryotic (bacterial) Tet repressor TetR – a sequence-specific DNA binding protein – with a eukaryotic transcriptional transactivation domain (most widely used so far is the acidic domain of herpes simplex virus VP16). In the absence of tetracycline, tTA binds, by means of its tetR domain, a short, specific, DNA sequence called tet operator (tetO) and acts as a transcriptional activator of the lethal gene through the VP16 transcriptional activation domain, leading the insect to death. Tetracycline, generally added in the diet, prevents the tTA-tetO bond, allowing the insects to be reared in manufacturing facilities. When the transgenic tTA insects are released in the environment, the permissive condition – tetracycline – is not encountered by the wild population; consequently, the expression of the lethal gene will cause mortality in the early developmental stages of the heterozygous progeny but will not affect the viability of the GE parent, provoking an effective control of the wild pest population.

Trait level GURTs

Several methods have been proposed to switch on or off a trait by means of chemical treatment or environmental factors. The mechanism described in the Zeneca patent WO 9403619 is quite similar to the one described in the terminator technology patent. The gene responsible for the production of a toxin/disrupter protein can be selectively activated or inactivated without killing the embryo by applying or withholding the inducer chemical before the seed is sold. Consequently, the first generation plant is capable of expressing the trait of interest, but the second generation is not. This is why T-GURTs are known as "traitor technologies." In another method, the application of a chemical inducer activates the expression of a silenced gene, e.g., by anti-sense suppression, encoding the trait of interest.

The transgene can also be excised using recombination systems like ParA-MRS, Bxb1-att, R-RS, Cre-lox, or FLP-FRT. The introduced recombinase removes itself and the transgene after chemical or heat shock treatment.

Eventually, Ardell[4] classified his own patent application entitled "Genetic encryption" as a GURT. This invention assumes that genetically altering the anticodon-stem loop (ASL) templates of tRNA genes in situ changes the codon reading specificity of the deriving tRNA, inducing a functional alteration of the genetic code able to produce proteins with an intended structure only when translated within the context of that specifically engineered organism or in vitro translation system.

Possible benefits

As previously written, GURTs were basically conceived to protect genetic resources and innovations by preventing seed saving. Indeed, hybridisation is a commonly used biological means to prevent seed saving practices, as the

out-crossing that occurs in the subsequent generations will (generally) generate plants with lower vigour or uniformity. However, in many self-fertilising crops such as rice, wheat, cotton, etc., the protection of genetic resources via hybridization may be infeasible, while GURTs could potentially be applied to all seed-propagated crops. Similarly, despite the fact that intellectual property protection of new plant varieties is granted both at the local (by patents or plant varietal protection – PVP) and at the international level (by the International Union for the Protection of New Varieties of Plants – UPOV – and by the WTO Trade-Related Aspects of Intellectual Property Rights – TRIPS – Agreement), and despite the additional fact that the purchasers of seeds containing a patented GE trait (e.g., in Roundup Ready crops by Monsanto) are required to sign a contract agreeing not to save second-generation seeds for replanting, these social means to restrict the unauthorized use of genetic material have been often proved to be ineffective or at least time- and money-consuming. Moreover GURTs would guarantee that farmers would be dependent on the purchase of new seeds and chemical inducers even after the expiry of licenses; at the same time, farmers would benefit from the improved new plant varieties. GURTs would also prevent competitor biotech industries from using seeds in their own breeding programmes, which could encourage investment in research and development in the plant-breeding sector of the seed companies that could favour a decrease in selling prices and food costs, and, paradoxically, increase agricultural biodiversity.

Other possible advantages of GURT technology are related to the environmental containment of transgenic seeds (V-GURT) or the prevention of unwanted transgene flow (T-GURT), which are among the greatest concerns associated with GE crops, thus facilitating their public acceptance. Furthermore, GURTs might allow the breeding companies to circumvent any legal disputes due to transgene introgression into wild populations, either for food or biopharm crops.

Concerns

The principal complaint against GURTs is that farmers would be forced to buy seeds and the chemicals required to switch on the value-added trait each year, which could further impoverish poor farmers and would increase farmers' dependence on multinational seed corporations. This could also reduce farmers' food security and erode their traditional knowledge and capacity for innovation of informal crop genetic improvement, since the adapted or selected autochthonous cultivars would be replaced by the new GURT-protected varieties, resulting in loss of the local genetic diversity. GURTs could limit access to genetic resources and the fair and equitable sharing of benefits arising from their utilization enshrined in the Nagoya Protocol and would cause the displacement of local farming systems and the social, cultural, and spiritual dimensions associated with them.

The replacement of diverse ecotypes with a few uniform varieties, as well as the potentially massive use of chemicals to treat the seeds each year might have a detrimental effect on soil microflora and fauna, and increase the prevalence of antibiotic-resistant bacteria. GURT-transformed crops might also produce low quantities of autotoxic compounds with negative impacts on non-target organisms, and eventually, as food/feed, transfer allergenicity and antibiotic resistance.

Further issues relate to the effectiveness of GURTs in preventing transgene escape. In fact, since the GURT engineered seeds and plants would require a 100% effective application of the chemical inducer to prevent the escape of a non-functioning transgene via both seed and pollen, some seeds and plants may not respond or may not take up enough inducer to activate the repressor gene, thereby producing fertile GM plants.

Other technical criticisms[5] of the GURT technology refer to the proper segregation of multiple genes during reproduction, escape of genes over generations, involuntary response to natural or artificial related compounds, accidental switching on of sleeper genes, the instability of the promoters, and the horizontal flow of genetically modified pollen to non-target organisms.

Conclusion

To date, GURTs are only a theoretical design; nevertheless, in the light of the hypothetical pros and cons discussed in the text, it is difficult to make a definitive judgment on them. On the one hand, T-GURTs could allow farmers to decide whether, and possibly when, to activate a valuable trait and so 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. On the other, the ethical concerns against V-GURTs that led to the global moratorium seem too strong to overcome and will surely play a preeminent role in the future social and political debate.

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