The intellectual property landscape for gene suppression technologies in plants

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Reviewing the major features in the patent landscape of RNA-mediated gene suppression may aid the development of patent strategies that will support the next generation of genetically modified crops.

RNA-mediated gene suppression is a powerful technology to suppress the expression of targeted genes within plants, as well as most other organisms. In 2002, RNA interference (RNAi) was proclaimed by *Science* as the "breakthrough technology of the year" and by *Fortune* as a "billion dollar breakthrough." The recognition of RNAi-mediated gene suppression as an important experimental tool and its potential commercial application is further reflected in the patent landscape related to RNAi-mediated gene suppression, with an increasing number of patent applications seeking exclusive rights to RNAi-based discoveries. Recent publications summarizing the RNAi-based patent thicket in applications to human medicine point out legal uncertainties over who will own key RNAi intellectual property (IP) and the apprehension that this has created among investors[1,2].

Although a commercial human RNAi-based therapeutic is yet to be released, RNA-mediated gene suppression was used to produce the very first commercial genetically modified (GM) crop, the FLAVR SAVR tomato, in 1994. Here, we examine the scientific evolution of RNA-mediated gene suppression technologies used in agricultural biotech and the associated patent landscape. There is current and emerging IP in the United States with broad claims that are likely to influence the freedom to operate (FTO) for RNA-mediated gene suppression technologies used in the development of GM plants. However, early patented methods of RNA-mediated gene suppression, including antisense and co-suppression, are nearing the end of their patent life. As this IP approaches expiration it opens gaps in the patent landscape that may offer greater FTO. This survey of the major landmarks in the patent landscape of RNA-mediated gene suppression is one step in informing IP strategies that can support the next generation of genetically modified crops.

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Discovery and application of RNA-mediated gene suppression

Antisense RNA-mediated gene suppression. Gene suppression triggered by naturally occurring antisense RNA was first identified in bacteria in 1983, suggesting the possibility for applying this or similar strategies to suppress gene expression in other organisms[3]. Ecker and Davis were the first to use antisense technology to induce transient inhibition of exogenous gene expression in plant cells[4] (**Fig. 1**). Subsequent studies used antisense transcripts to suppress constitutive expression of exogenous genes in whole tobacco plants[5,6]. Rothstein and colleagues also showed that gene suppression was heritable, suggesting this technology had practical applications in generating stable transgenic crops with improved agronomic traits. Subsequent scientific landmarks included the demonstration that antisense RNA could also be used to modulate expression of endogenous plant genes including those encoding chalcone synthase to modify flower pigmentation in petunia[7], polygalacturonase to modify fruit-ripening characteristics in tomato[8,9] and the photosynthetic RuBisCo in tobacco[10]. Although the mechanism of antisense RNA-mediated gene suppression was not clearly understood in these early experiments, the hypothesis suggested that the formation of an RNA-RNA hybrid destabilized the mRNA, resulting in its rapid degradation.

Antisense RNA-mediated gene suppression was quickly adopted by plant scientists as a broadly applicable method to downregulate expression of target genes. Antisense RNA was embraced in plant research because reverse genetic approaches used in other biological systems, such as homologous recombination and gene-tagging mutagenesis, were either not applicable or not yet well developed in plants. Antisense RNA-mediated gene suppression was quickly shown to be a powerful tool for both basic transgenic research and for the development of commercial products, which highlighted the value of this technology (**Box 1, Table 1**).

Co-suppression of gene expression. In 1990, an RNA-based gene suppression phenomenon, termed co-suppression, was serendipitously discovered in plants when researchers attempted to generate new purple varietals of petunia flowers by overexpressing pigment genes[11–13] (**Fig. 1**). The unexpected results of white variegated flowers were proposed to be the result of not only post-transcriptional suppression of the endogenous pigment gene but also the suppression of the exogenous overexpressed homologous gene, hence, the term co-suppression. This was the first reported demonstration that sense transgenes, like antisense transgenes, could induce gene silencing in plants.

Co-suppression and antisense RNA-mediated gene suppression technologies, however, faced similar technical limitations. The technologies did not silence gene expression completely and as a result, large numbers of transgenic plants had to be screened to identify those with desirable levels of gene suppression. The elucidation of the mechanisms underlying co-suppression and the induction of what is now termed RNA

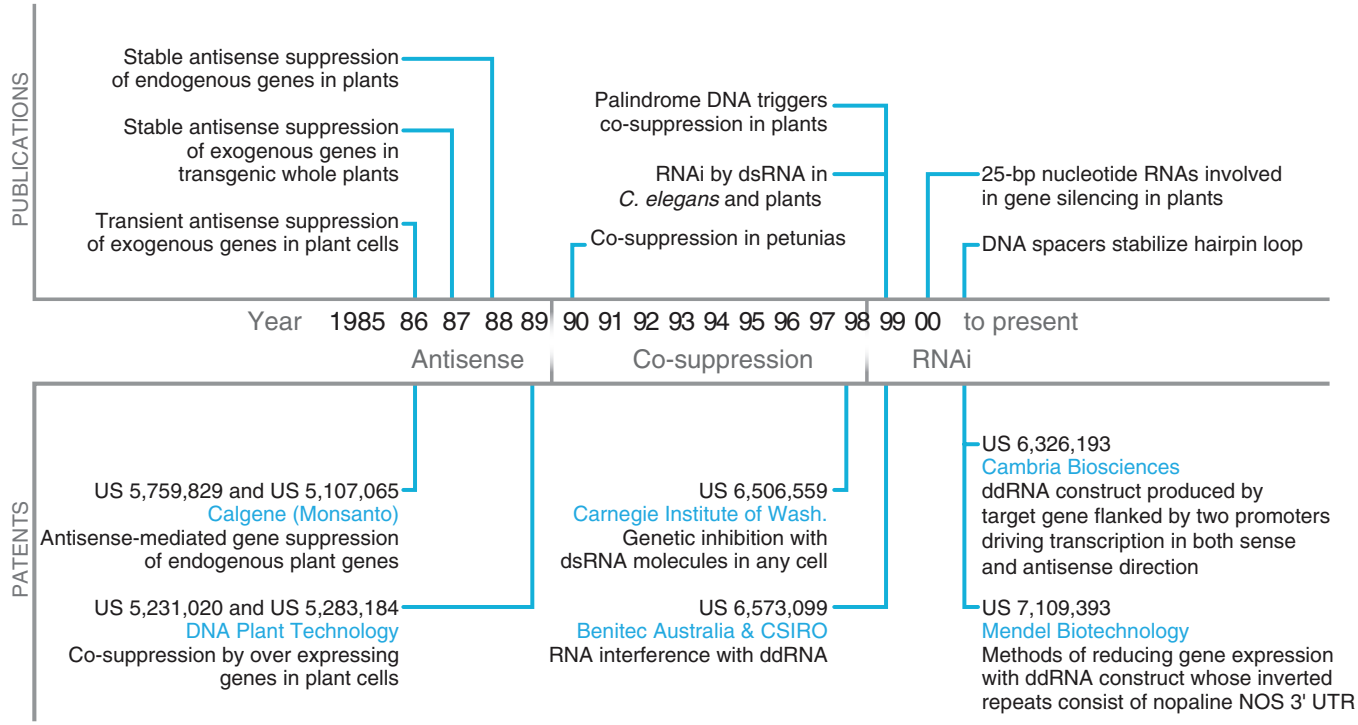

Figure 1 Scientific and US intellectual property milestones in RNA-mediated gene suppression in plant biotech. NOS, nopaline synthase.

interference led to the improvements required to efficiently and reproducibly suppress gene expression.

RNA interference. The term 'RNA interference' was coined in 1998 by Nobel laureates Fire, Mello and colleagues to describe a gene-silencing phenomenon induced by double-stranded RNA (dsRNA)[14]. The mechanism of RNAi-induced gene silencing was defined after several seminal discoveries that unveiled a common gene suppression pathway in plants, animals and nematodes. Fundamental to deciphering the RNAi mechanism was identifying RNA as the trigger for gene silencing. Central to this discovery was the unconventional observation that plant viral resistance, in some cases, could also be instigated by the coat-protein mRNA and not the translated viral coat protein[15,16]. The role of mRNA as a gene-silencing trigger was additionally established independently while the co-suppression phenomenon in petunia was further investigated[17,18]. Both Metzlaff and Stam proposed that the aberrant mRNAs paired with complementary endogenous mRNA and triggered RNA cleavage resulting in post-transcriptional gene silencing. Together, the data demonstrated that co-suppression was a consequence of the rapid degradation of mRNA that shared a high degree of sequence homology. Although it was proposed that the complementary RNA bound with targeted RNA and triggered cleavage, the role of dsRNA in triggering cleavage was unproven.

In 1998, Fire, Mello and colleagues and Waterhouse and colleagues independently demonstrated definitively that dsRNA was required to induce gene silencing in nematodes and co-suppression in plants, respectively (**Fig. 1**). Fire and colleagues injected purified sense and antisense oligonucleotides separately or together into *Caenorhabditis elegans*. Co-introduction of sense and antisense strands, which formed dsRNA, resulted in gene suppression two orders of magnitude higher than when the oligonucleotide strands were introduced separately[14,19]. Waterhouse and colleagues hypothesized that co-suppression was triggered by dsRNA that was formed by the hybridization of complementary transgene mRNAs or complementary regions of the same transcript[20]. They also theorized that the presence of complementary regions in the same transcript could be mimicked by the insertion of multiple transgene copies in a palindromic orientation resulting in a read-through transcript similar to that observed by Stam[18]. To test this hypothesis, they designed a plasmid such that a single transcribed RNA would form a double-stranded hairpin structure. Transformation of rice by this dsRNA plasmid into suppressed target gene expression significantly more than plasmids encoding a single gene in the sense or antisense orientation[20]. This powerful technology is now known as DNA-directed RNA (ddRNA). Hamilton and Baulcombe further defined the mechanism of RNAi when they discovered small RNA species, of ~25 nucleotides, in plants undergoing co-suppression, that were absent in nonsilenced plants[21] (**Fig. 1**). They also noted these species were complementary to the silenced gene. These short interfering RNAs (siRNAs) are now known to be the functional form of RNAi. Collectively, these foundational findings became the basis to develop tools to efficiently trigger gene silencing in plants and animals.

Gene-silencing DNA constructs and plasmids. The deduction that dsRNA triggers RNAi led to the development of methods to produce dsRNA and efficient gene silencing. Using synthetic oligonucleotides, as Fire and Mello did, induces transient gene suppression but is dependent on efficient transformation of the plant cell by the oligonucleotides, a traditionally inefficient process. However, *in vivo* transcription of the dsRNA can potentially yield stable gene suppression. Waterhouse and colleagues demonstrated that ddRNA

Box 1 RNA gene suppression in the agbiotech pipeline

The discovery and application of gene-suppression strategies, such as antisense and RNAi, to modify phenotypes led companies to use these strategies to develop transgenic crops. In 1994, the use of antisense RNA–mediated gene suppression technology gave birth to the first commercial transgenic crop approved by the US and other international regulatory agencies: Calgene's FLAVR SAVR tomato[32]. Engineered to express the endogenous polygalacturonase gene in the antisense orientation, FLAVR SAVR showed reduced expression of this cell wall–degrading enzyme, consequently delaying the softening of tomatoes. FLAVR SAVR's international commercial release was a historic milestone marking antisense technology's usefulness in applied research. Other transgenic plants in the US regulatory pipeline that use co-suppression or antisense to downregulate gene expression are listed in **Table 1**. Currently, there are no commercial GM crops that use RNAi. With the recent advent of optimized methods to efficiently trigger RNAi gene silencing and interest in quality crop traits that require precise regulation of steps in metabolic pathways, we anticipate new RNAi-based, genetically modified cultivars will enter the commercialization pipeline. However, the extent to which this will be observed depends on the FTO within the patent landscape.

Table 1 A summary of commercial developments in agbiotech developed with RNA-mediated gene suppression.

Company	Crop	Trait gene	RNA-based gene suppression approach	Regulatory approval (animal feed, human food and/or environmental)	Phenotypic description
Calgene (now Monsanto)	Tomato (FLAVR SAVR)	Polygalacturonase	Antisense	US, Canada, Mexico, Japan	Delayed fruit ripening
Zeneca London, UK	Tomato	Polygalacturonase	Antisense and co-suppression	US, Canada, Mexico	Delayed fruit ripening
DNA Plant Technology	Tomato	Aminocyclopropane cyclase	Co-suppression	US, Canada, Mexico	Delayed fruit ripening
Vector Tobacco Durham, NC	Tobacco	Quinolinic acid phosphoribosyltransferase	Antisense	US	Reduced nicotine levels
DuPont Canada Agricultural Products Ontario, Canada	Soybean	Fatty acid desaturase	Co-suppression	US, Canada, Japan, Australia	High oleic acid soybean
Florigene Pty. Ltd.	Carnation	1-aminocyclopropane-1-carboxylic acid	Co-suppression	Australia, European Union	Longer vase life
US Department of Agriculture	Plum	Plum pox virus coat protein	Co-suppression	US	Viral resistance

Data source: Agbios (http://www.agbios.com)

that encodes an RNA hairpin could suppress target gene expression by up to 90% in transgenic plants[20]. The level of gene suppression was further increased to almost 100% by inserting a DNA spacer (intron) between the complementary inverted sequences to stabilize the hairpin loop structure[22] (**Fig. 2**). Additionally, Brummell and colleagues, using complementary inverted nopaline synthase 3′ untranslated transcription region (UTR), demonstrated that the inverted complementary regions used to form a hairpin could be composed of DNA sequences other than those of the target gene[23]. This ddRNA construct is suitable for high-throughput cloning as only a single copy of the target gene needs to be present (**Fig. 2**).

DsRNA can also be produced by transcribing individual strands of sense and antisense transcripts. Individual strands of RNA can be produced by transcribing the RNA from separate plasmids or from a single plasmid using either two promoters to drive expression of a sense or antisense DNA, or two opposing promoters to drive the transcription of the sense and antisense RNA (**Fig. 2**). A number of plasmids based on these various structural arrangements are now available to facilitate cloning of RNAi plasmids for gene silencing in plants. The ongoing improvement and development of new biotech methodologies continues to fuel the evolution of plasmids for RNA-based gene suppression.

Landscape of RNA-mediated gene suppression patents in the United States

Antisense RNA-mediated gene suppression. The antisense RNA-mediated gene suppression technology used to create the FLAVR SAVR tomato was also the basis for one of the first patents with broad claims in the field of gene suppression. Calgene (now St. Louis-based Monsanto) was awarded two US patents, US 5,107,065 and US 5,759,829 (refs. 24,25) (**Fig. 1**). These are dominant patents in the RNA-mediated gene suppression landscape because of their broad claims, which describe antisense-mediated suppression of any gene indigenous to the plant cell. At the time, the mechanism of gene suppression was thought to occur through complementary hybridization of the antisense and endogenous, sense mRNA transcripts. However, in 2005, Monsanto, knowing the mechanism of RNAi-mediated gene silencing, performed a retrospective examination of the transgene insertions that comprised the FLAVR SAVR tomato[26] and the example cited in US 5,107,065 and US 5,759,829. The structural analysis of the inserted transgenes revealed the presence of tandem T-DNA insertions resulting in the possible formation of an inverted double-stranded loop (**Fig. 2**), which is now known as a structure that triggers RNAi-induced gene silencing. Monsanto's antisense patents were awarded in the early 1990s and expired in 2009.

Co-suppression of gene expression. Prior to the identification of dsRNA as a trigger for co-suppression in plants, the method to induce co-suppression, although unreliable, was to overexpress a sense gene in plants. DNA Plant Technology (Oakland, CA, USA) was awarded two US patents, US 5,231,020 and US 5,283,184 (refs. 27,28), both with broad claims to achieve endogenous gene silencing by overexpressing a sense gene in any plant

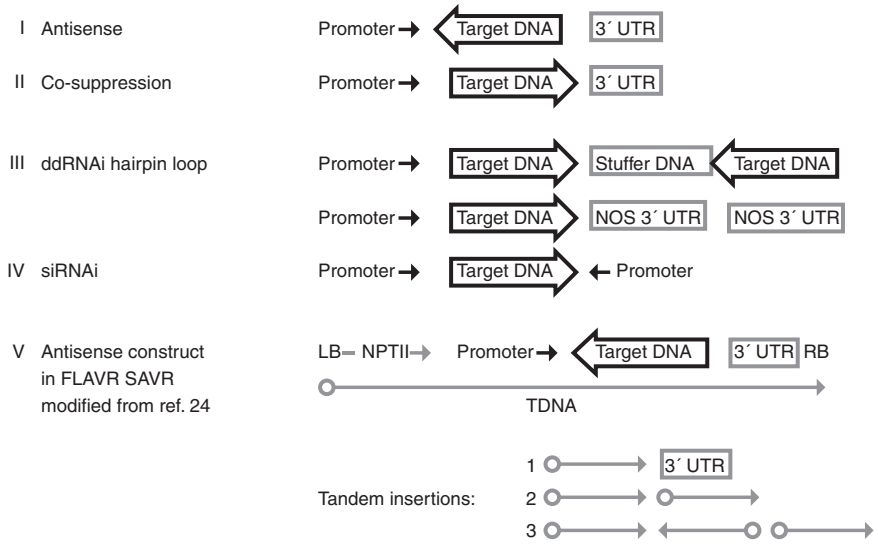

Figure 2 RNA Gene-silencing constructs commonly used in plant genetic modification. NOS, nopaline synthase.

(**Fig. 1**). Both patents specify altering the phenotype of the plant by suppressing endogenous genes in a plant cell. Like Monsanto's antisense IP, these broad co-suppression patents were filed in the early 1990s and are either expired or will expire in 2010.

RNAi-induced gene suppression. RNAi can be induced by the delivery of siRNAs or DNA constructs encoding complementary RNA into cells. Fire, Mello and colleagues at the Carnegie Institute of Washington (Washington, DC, USA) were awarded a broad patent, US 6,506,559 (ref. 29), also known as the Carnegie patent, which is now widely considered the most fundamental patent in the RNAi field. The patent encompasses the general method of using dsRNA formed with complementary copies of the target gene to downregulate gene expression in a cell (plant or animal) *in vitro*. Based on the construction of the claims, it is arguable that the exclusivity of the claims is limited to gene suppression *in vitro*. It is unclear if the patent rights include applications of dsDNA to suppress gene expression *in vivo*, such as would be the case in living transgenic plants.

Fire and Mello's fundamental patent also spawned related-child patent applications including one US application serial no. 10/283,267 with pending claims specific for use of dsRNA in plants. The examination of this application was suspended. However, the subject matter has the potential to be dominant in the plant biotech patent landscape. The United States Patent and Trademark Office (USPTO) recently published three additional patent applications submitted by Carnegie in 2007 (serial nos. 11/826,385; 11/905,449; 11/905,368). These applications encompass the use of dsRNA *in vivo* in plant and animal cells, which, if awarded, may substantially affect the RNAi IP landscape.

RNAi DNA constructs. The development of the ddRNA technology by Waterhouse and colleagues, unlike synthetic siRNA, enabled the stable production of dsRNA in a cell and stable gene suppression in transgenic plants. This research formed the basis of one of the most pertinent US patents in animal biotech fields, US 6,573,099 (ref. 30). This patent was co-awarded to Benitec Australia (Melbourne) and the Commonwealth Scientific and Industrial Research Organization (CSIRO, Canberra) and described the use of ddRNA constructs in animal cells. Benitec's US 6,573,099 patent family encompasses 11 patent applications. Three of the current applications, including serial nos. 09/646,807, 11/218,999 and 11/180,928 contain pending claims that describe the process of using ddRNA plasmids to suppress gene expression in eukaryotic cells and plants.

Indicative of the potential dominance of Benitec's US 6,573,099 within the US RNAi landscape, this key patent has been challenged in the USPTO. In 2004, after Benitec sued Nucleonics (Horsham, PA, USA) and two other companies for alleged infringement of their patent, Nucleonics aggressively challenged Benitec's IP by requesting two successive reexaminations of US 6,573,099 by the USPTO. In April 2008, Benitec cancelled several claims and the remaining claims were rejected by the USPTO as being obvious in view of prior art, including the Carnegie patent US 6,506,559, which Nucleonics licensed (**Fig. 2**). In response to the USPTO, Benitec/CSIRO claims that they were the first to invent. Though most international patent systems follow the first-to-file priority, the USPTO follows the first-to invent priority. Until the IP issues are resolved by the USPTO, uncertainty remains on one of the most critical patent estates for deploying RNAi in animals and plants.

To expedite DNA cloning manipulations in generating RNAi constructs, Brummell and colleagues devised a strategy amenable to high-throughput gene-silencing experiments. This method is the subject of Mendel Biotechnology's (Hayward, CA, USA) US patent 7,109,393. The patent claims a method to suppress gene expression in a plant cell by expressing a cassette that encodes two inverted nopaline synthase 3′ UTRs interrupted by spacer DNA; this structure forms a loop at the 3′ termini of the target gene to be silenced (**Fig. 1**). The patent's subject matter is not specific to a particular target gene and thus broadly encompasses any target gene. However, the invention's claims are limited to the use of inverted repeats from the gene encoding nopaline synthase, in particular the 5′ and 3′ UTRs, leaving open the possibility of using other inverted repeat sequences to achieve the same effect in suppressing expression of a target gene. Mendel's patent issued in 2006 and is expected to expire in 2020.

A novel plasmid designed to produce dsRNA using dual promoters (US 6,326,193) was patented by Cambria Biosciences (Woburn, MA, USA). The specified inventions include a plasmid that contains a single DNA segment that can be transcribed in both directions by separate promoters placed in opposite orientations (**Fig. 2**). Other narrower patent claims describe the use of the expression plasmids as a biological pest control agent. The term of this patent is expected to expire in 2019.

Discussion

Scientific research advancements are often nurtured by a highly synergistic environment, as was evidently the case for the deduction of the mechanism of RNAi-mediated gene suppression (**Fig. 2**). In contrast, when claiming patent rights, patent law favors clear and well-defined invention boundaries. Failure to invent around or license technologies claimed in patents may trigger legal repercussions and even prevent product commercialization. Thus, IP due diligence to evaluate potential legal risks is an important step in the commercialization of products and requires both

technical and legal expertise[31]. A key aspect of this process is assessing the FTO of a product, that is, does the developed product infringe on third-party proprietary IP? Because IP laws vary between countries and patents have national boundaries, it is essential to perform an FTO IP analysis for each country in which products will be developed and deployed. Here, we presented an overview of the scientific development and IP landscape of RNA-mediated gene suppression technologies used in agbiotech in the United States. The application of RNA-mediated gene suppression to produce GM organisms evolved from strategies based on expression of target genes in antisense orientation, to co-suppression by overexpressing sense transcripts and then to producing dsRNA. There now exist both emerging as well as expiring patents in the United States for the general use of RNAi in plants, and DNA constructs that mediate dsRNA production.

Currently, there is substantial FTO for the use of RNA-mediated gene suppression in plants in the United States. The broadest US patent in RNAi is awarded to the Carnegie Institute of Washington, US 6,506,559. Patent rights were awarded for the *in vitro* use of dsRNA in controlling gene expression in plants and animals. It is unclear if the breadth of the claims would also encompass the *in vivo* use of RNAi. However, a conservative IP strategy would be to consider licensing this patent, which Carnegie offers nonexclusively. Both the patent families of Benitec/CSIRO and Carnegie Institute contain related pending patent applications seeking to gain patent rights toward very similar subject matter related to the production and use of dsRNA molecules for gene suppression in plants. Owing to the potential for overlapping subject matter and the commercial potential of this technology, the prospect of ongoing opposition to these pending applications is likely. Until the patent application prosecution processes are completed, it is difficult to assess the repercussions of these developing claims in the RNAi patent landscape.

Gene-suppression technologies expected to have greater FTO in the near future are the RNAi predecessors technologies: antisense and co-suppression. Though there is broad IP issued to these technologies, their patent lives are expired or nearing expiration. The broadest patent claims on RNA-based gene suppression based on antisense-suppression was awarded to Calgene (now Monsanto) and expired in 2009. Co-suppression by overexpression of sense genes in plant cells was another novel invention aimed at suppressing expression in plant cells. Broad patent claims for this technology were awarded to DNA Plant Technology in the 1980s and are scheduled to expire soon. Although antisense-based and co-suppression induced gene suppression methods were considered technically less optimal than dsRNA, modern high-throughput genetic screening methods and the expiration of these patents may make these alternatives more attractive.

This RNAi patent landscape highlights the legal complexities in any given technology space. It illustrates some of the FTO IP considerations developers must consider in generating new agricultural or pharmaceutical products. Evolving patent landscapes create a great deal of uncertainty in making product development and investment decisions that rely on a realistic FTO assessment. Currently the average processing time for US patent applications is 40 months; however, in emergent technology areas this time frame can be prolonged. This is the case for the patent application by Carnegie Institution and Benitec/CSIRO, which remain under prosecution 10 years after the initial filing date. RNAi is continuing to develop as a fundamental tool in both plant and animal biotech and an ongoing assessment of the patent landscape will be important to equip scientists and investors with knowledge for evaluating FTO in this technology sector.

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