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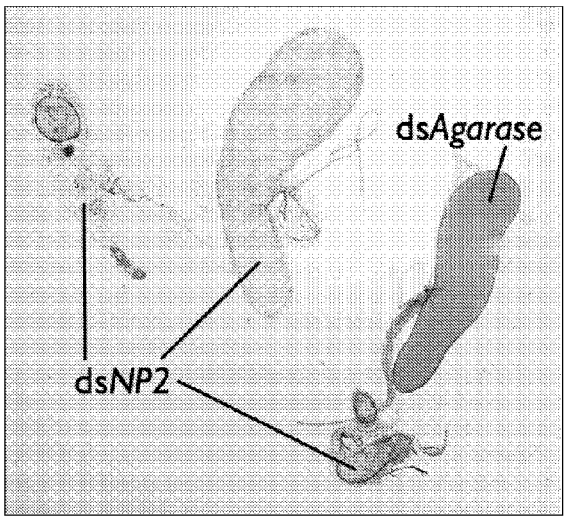

Figure-10

(57) Abstract: The invention concerns a genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of an insect vector or insect pest; characterised in that said bacterial cell is transformed to express dsRNA against at least one selected target gene of the insect vector, whereby the effect or viability of said insect is deleteriously compromised; also an insect including said bacterial cell and a method of modulating a target insect gene using said bacterial cell.

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Pest and Pathogen Control

Field of the Invention

The invention relates to a genetically transformed or transfected bacterial cell of a gut symbiont of an insect vector wherein said cell is transformed to express double-stranded RNA (dsRNA) active against at least one selected insect gene; an insect including said transformed bacterial cell and a method of pest control and/or pathogen transmission control employing the use of said bacterial cell and/or said insect.

Background of the Invention

Invertebrates and other pests are common vectors for pathogenic organisms, typically micro-organisms, that are responsible for a variety of human diseases. Arthropods, including insects, account for over 80 percent of all known animal species, and they are one of the most important disease vectors. Insects are commonly the cause for spreading a range of different bacterial, viral, and protozoan pathogens associated with different diseases. Transmission of a communicable disease from an infected host insect or group to a conspecific individual or group commonly occurs either via their bite, such as in the case of haematophagous insects, or via contaminated faeces. For example, one of the most commonly known insect-borne diseases is that of malaria which is transmitted via the *Anopheles* genus of mosquito. Other common mosquito-borne diseases include yellow fever, dengue fever, and encephalitis, and are estimated to transmit disease to more than 700 million people annually in Africa, South America, Central America, Mexico and much of Asia. Globally, 216 million cases of Malaria occur annually and it is responsible for the death of over 655,000 people, with it estimated that 6.5% of the world's population are at risk of infection. Additionally, Dengue fever is the world's fastest growing vector-borne disease. Similarly, flies constitute a large group of vectors of pathogenic micro-organisms, such as the Sandfly species and *leishmaniasis,* and the tsetse fly and protozoan trypanosomes.

American trypanosomiasis or Chagas disease is a tropical parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*. In 2010 it was estimated that 10 million people contracted the disease, resulting in greater than 10,000 deaths. *T. cruzi* is commonly transmitted to humans and other mammals by an insect vector, the blood-sucking insects of the subfamily Triatominae (family Reduviidae), most commonly species belong to the *Triatoma*, *Rhodnius*, and *Panstrongylus* genera. The disease may also be spread through blood transfusion and organ transplantation, ingestion of food contaminated with parasites, and from a mother to her fetus.

Antiparasitic treatments appear to delay or prevent the development of Chagas disease symptoms during the chronic phase of the disease, but 20–40% of chronically infected individuals will still eventually develop life-threatening heart and digestive system disorders.

Chagas disease is contracted primarily in the Americas, particularly in poor, rural areas of Mexico, Central America, and South America. It is estimated that as many as 8 to 11 million people in Mexico, Central America, and South America have Chagas disease. Large-scale population movements from rural to urban areas of Latin America and to other regions of the world have increased the geographic distribution of Chagas disease, and cases have been noted in many countries, particularly in Europe. Control strategies have mostly focused on eliminating the triatomine insect vector and preventing transmission from other sources.

It is thought that in the future many vector-borne diseases could be found in more temperate regions due to climate change providing conditions for outbreaks. There has been a worldwide resurgence of vector-borne diseases since the 1970s including malaria, dengue, Yellow fever, and leishmaniasis. This is thought to be due to a variety of reasons such as; development of insecticide and drug resistance; population growth; urbanisation; changes in agricultural practices; deforestation; climate change causing shifting or expansion of habitats and increased travel.

Consequently, there is a continued interest in developing control methods aimed at reducing or eradicating the incidence of vector-borne pathogenic diseases. This is

often achieved by targeting the vector commonly associated with transmission. Methods for controlling infestations and infection by insects have typically been in the form of; physical barriers preventing transmission; chemical compositions, such as insecticides, chemical drugs and repellents; and also biological controls such as immunisation.

There is also continued interest in developing control methods aimed at reducing or eradicating the incidence of insect pest interference in terms of healthcare, husbandry, agriculture and ecology.

Recent advances in genetics has led to a greater understanding of the development of a vast range of organisms, and paved the way for new avenues in biological pest control. The study of insect gene function provides a crucial step towards understanding physiology, behaviour, immunology and disease transmission in this very diverse and successful group of organisms. Armed with this knowledge it is possible to develop models to fight disease and develop strategies to control pest insect populations.

The publication of the first complete genome sequence has led to functional genomics, a field of molecular biology that attempts to utilise data produced by genomic projects to describe gene (and protein) functions and interactions. However, simply knowing the sequence of a gene is not enough to explain its function. Consequently, techniques termed gene silencing (epigenetic processes of gene regulation) were developed in which a particular gene can be “turned off” permitting one to determine the function of the gene as a consequence of its loss. One such technique is that of RNA interference (RNAi), a powerful technique of sequence-specific down-regulation of gene expression to interrogate eukaryotic gene function on an individual gene basis.

RNAi is a form of post-transcriptional gene silencing wherein a specific mRNA of a particular gene is destroyed or blocked, preventing translation and formation of an active gene product. RNAi occurs naturally within living cells to modulate gene activity, and is also important in defence against parasites and viral infection. For example, when a cell is injected with RNA in a double-stranded (ds) form, a protein

called Dicer (or RNase III) cleaves the dsRNA molecules into short fragments of RNA (20-25 nucleotides), termed short interfering RNA (siRNA) due to their ability to interfere with the expression of a specific gene. These siRNA molecules are unwound into single stranded (ss) RNA, whereupon the so-called guide strand is incorporated into the RNA-induced silencing complex (RISC). Often, this guide strand will base pair with a complimentary sequence of mRNA in the cell inducing its cleavage by the catalytic component of the RISC complex. The mRNA is not translated and no functional protein is produced, and therefore the effects of the gene encoding the specific mRNA are 'silenced'. This process is termed cell-autonomous RNAi, wherein gene silencing is limited to the cell in which the dsRNA is introduced. Alternatively, environmental and systemic RNAi are the two forms of non-cell autonomous RNAi, wherein the interfering effect takes place in cells/tissues different from where the dsRNA was introduced/produced. In this case, the dsRNA is either taken up into multiple cells (environmental RNAi such as in viral infections), or the silencing signal is transported from the cell in which the dsRNA is applied or expressed to other cells where the effect is observed (systemic RNAi).

By artificially synthesising dsRNA (or siRNA molecules) with a known sequence complimentary to a gene of interest, and introducing it to target cells, it is possible to understand the role of a specific gene by observing the consequences of its loss of activity. RNAi and other so-called reverse genetics techniques are thus revolutionizing biological sciences, with applications in genomics, biotechnology, and medicine.

Many of the insect species selected for genome sequencing represent species that either inflict suffering (e.g. the mosquito *Anopheles gambiae*), or provide spectacular models for animal development (e.g. the fruitfly *Drosophila melanogaster*). Indeed the dsRNA technique was rapidly adapted for use in the Anopheles mosquito.

RNAi in invertebrates is an established technology, wherein dsRNA is delivered most commonly by injection. However, this process often has high mortality rates due to injection trauma and anaesthesia, and also requires high sample numbers. Large insects also require expensive quantities of dsRNA to be synthesised. Moreover, as stated, this results in cell-autonomous gene silencing achieving transient RNAi

effects and, therefore, is not applicable for control of insect pests in the field. Typically, the latter has been the only means of delivery of dsRNA to blood-feeding insects such as malarial Anopheles species.

As a result, for efficient insect pest control non-cell autonomous RNAi is required, which has been shown to be achievable by feeding insects with a biological source such as genetically modified bacteria or plant material (Huvenne & Smagghe, 2010). This therefore begins with uptake of dsRNA environmentally into the gut lumen of the insect, where it is then spread to tissues elsewhere (systemic RNAi). Achieving RNAi depends on a reliable method to deliver, or uptake, a dsRNA copy of part of a target gene to the insect. For some insect species this has been achieved by including in their food live or dead *E. coli* cells expressing dsRNA. The bacteria are digested in the gut and the dsRNA is taken up and delivered systemically to different tissues whereupon it can mediate transient RNAi. EP2374462A2 teaches that RNAi can be introduced by ingestion of: naked dsRNA, food contaminated with *E. coli* expressing dsRNA e.g. by spraying with transformed bacteria, or genetically modified plant material expressing dsRNA. WO2011017137A2 teaches a method whereby a food bait of the insect is contaminated with genetically modified bacteria expressing dsRNA and the bait is returned to the colony to be fed on by the insects. Further, WO2011025860A1 teaches the use of RNAi against plant-feeding insects, wherein bacteria that infect specific plants are genetically modified to express specific dsRNA. Similarly, WO2011036536A2 teaches specific RNAi gene targets which are silenced by the delivery of dsRNA by spraying dead bacteria onto crop plants.

However, many of the currently developed techniques are often impractical or have poor efficacy. For example, contamination of food sources (plant or other) with genetically modified bacteria may be harmful toward other insects that may be occasional feeders. Furthermore, spraying chemical compositions containing naked dsRNA onto insects or their food source may also have implications on other non-pest organisms. More importantly, all of the existing methods only teach delivery methods whereby transient gene-silencing effects are observed. Many of the currently employed techniques only exhibit short silencing durations, which are often too transient for certain targets e.g. those for hormone and developmental studies.

Hitherto, there has been no example of a method whereby horizontal transfer of silencing constructs is observed, and therefore re-application of dsRNA (or other RNAi constructs) is often required, which is costly and time-consuming. Furthermore, as currently employed techniques are not transferrable, effective pest control has been difficult to demonstrate. Therefore the technology in its current state is inappropriate for many insect species and improved efficacy and methods of application are required. Further, to make the most of emerging insect genetic information, RNAi methods must evolve to accommodate a wider variety of species.

We have therefore developed a new RNAi delivery technique that relies on the *in vivo* synthesis of dsRNA by transgenic symbiotic gut bacteria that naturally reside in the insect host. Each insect species has its own specific gut microflora. The genetically modified symbionts therefore become established as a living population in the gut of the insect over the insect life-cycle, dividing within their hosts actively producing RNA, and can consequently mediate RNAi over prolonged periods. The dsRNA is directed against genes of the insect, leading to a knockdown effect that reveals the role of the target gene. It can also be used to target genes that disable the host (e.g. those required for survival or reproduction) in pest control. Further, we have demonstrated horizontal transmission of the transformed bacteria by coprophagy in insects, potentially representing an important mode of spread amongst the insect population.

Statements of Invention

According to a first aspect of the invention there is therefore provided a genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of an insect; characterised in that said bacterial cell is transformed to express dsRNA against at least one selected target gene of the insect.

As will be appreciated by those skilled in the art, reference herein to the term insect is reference to an organism belonging to the class Insecta. Preferably, said insect belongs to the order, although not limited to, Anoplura, Blattodea, Coloeoptera, Dermaptera, Dictyoptera, Diptera, Embioptera, Ephemeroptera, Grylloblattidae, Hemiptera, Hymenoptera, Isoptera, Phthiraptera, Plecoptera, Psocoptera, Siphonaptera, Thysanura, Strepsiptera, Thysanoptera, Trichoptera, or the like.

Ideally, said insect refers to any insect vector that transmits a pathogenic organism known to cause disease in a plant or animal host. This includes, but is not limited to, *Culicidae* spp. (mosquito), *Reduviidae* spp. (Reduviid bugs), *Phlebotominae* spp. (sand fly), *Glossinidae* spp. (tsetse fly), *Tabanidae* spp. (horsefly), Siphonaptera (fleas), Anoplura (lice), *Cicadellidae* spp. (leafhoppers), *Pentatomoidea* spp, (Shield bugs), *Aphididae* spp. (Aphids), *Membracidae* spp. (treehoppers), *Solenopsis spp.* (Ants), Coleoptera (beetles), or the like. More preferably, said insect vector belongs to the genus *Rhodnius.* More preferably still, said insect vector is *Rhodnius prolixus.*

In yet a further preferred embodiment of the invention, said pathogenic organism is to be construed as any micro-organism known to cause a disease in a plant or animal host. This includes, but is not limited to, a virus, bacterium, prion, protozoan or fungus. More preferably, said pathogenic organism is a protozoan. More preferably still, said pathogenic organism belongs to the genus *Trypanosoma*. Yet more preferably still, said pathogen is *Trypanosoma cruzi*.

In yet a further preferred embodiment of the invention still, said pathogenic micro-organism is responsible for causing disease in animals. More preferably, said pathogenic micro-organism is responsible for causing disease in humans, including, but is not limited to, Chikungunya, Japanese encephalitis, La Crosse encephalitis, Phlebotomous fever, St. Louis encephalitis, Epidemic polyarthritis, West Nile Fever, O'nyong-nyong fever, yellow fever, dengue fever, dengue haemorrhagic fever, Rift Valley fever, Chagas disease, Loiasis, Leishmaniasis, Elephantiasis, Filariasis, Bancroftian and Malayian Filariasis Onchocerciasis, Malaria, African trypanosomiasis, louse-borne typhus, murine typhus, epidemic relapsing fever, cat flea typhus, Trench fever, Plague, or the like. More preferably still, said disease is Chagas disease.

Alternatively, said insect refers to any insect that acts as a pest to man, animals, or plants, and is thus of significance in a health, husbandry, agricultural, or ecological context. This includes, but is not limited to, Isoptera (termites), *Solenopsis* spp. (Ants), *Aphididae* spp. (Aphids), *Cicadellidae* spp., Anoplura (lice), Siphonaptera (fleas), Midges, *Tabanidae* spp. (horsefly), *Calliphoridae* spp. (e.g. certain species

of blow fly), *Oestridae* spp. (e.g. warble flies and bot flies), eye moths, *Hippoboscidae* spp. (e.g. sheep ked), *Cimicidae* spp. (bed bug), Blattodea (cockroaches), certain species of Coleoptera (e.g. woodboring beetles, Darkling beetle, Spider beetle, Cigarette beetle, larder beetle, warehouse beetle, harlequin beetle, rice weevils, grain weevils), certain species of Lepidoptera (e.g Meal moths, Angoumois grain moth, cocoa moth, wine moth), Africanized honey bee, Pharaoh ant, or the like.

Preferably said insect engages in horizontal gut flora transfer or vertical flora transfer. As will be further appreciated by those skilled in the art, horizontal gut transfer is to be construed as the acquisition of gut flora bacteria by said insect vector or insect from an environmental source, for example, by ingestion. In yet a further preferred embodiment, said horizontal gut transfer is achieved by ingestion of faeces from other insects. More ideally still, ingestion of faeces is species specific whereby the parent generation of a species transfers the genetically engineered gut symbiont to an offspring generation. In this way, said insect vector or insect can acquire said transformed or transfected bacterial cell from contaminated faeces in the environment, circumventing the need for insect handling and associated mortality. Furthermore, advantageously, this permits horizontal transfer of dsRNA mediating RNAi throughout at least one insect colony and, typically, many insect colonies.

Alternatively, said transformed or transfected bacterial cell can be acquired by other means of transfer, such as, but not limited to, vertical (from adult to offspring e.g. transmission from the maternal parent to the off-spring) transfer or environmental transfer (e.g. via the ingestion of sprayed material).

Reference herein to the term gut symbiont is reference to a bacteria that successfully colonises the insect gut and, ideally, is found only in that genus or, more ideally, species, of insect at least during, but not limited to, the pathogenic part of the life cycle of the insect vector or at least during, but not limited to, the pest part of the life cycle of the insect. Therefore, preferably said gut symbiont includes but is not limited to, *Rhodococcus corynebacteriodes, Janibacter anophelis, Asaia, Rhodococcus rhodnii, Serratia marcescens, Triatoma infestans, Wigglesworthia glossinidia,*

Sodalis glossinidius, Rhodococcus opacus, Kluyvera cryocrescens, Lactococcus garviae, Achromobacter xylosoxidans, Bifidobacterium asteroides, Bifidobacterium coryneforme, Bifidobacterium indicum, Bifidobacterium bombi, Proteus vulgaris, Citrobacter freundii, Serratia liquefaciens or the like. In a further preferred embodiment of the invention, said gut symbiont is unique to a particular insect vector or insect. More preferably still, said gut symbiont belongs to the genus *Rhodococcus*. Yet more preferably still, said gut symbiont resides in *Rhodococcus rhodnii.* In this instance, a modified *dapA* gene promoter is used to generate the dsRNA.

In a preferred embodiment of the invention, said bacterial cell is transformed or genetically modified such that nucleic acid encoding said dsRNA is stably integrated into the host cell genome. This advantageously ensures long-term target gene silencing and ensures spread in insect populations. Ideally, stable integration is achieved by way of site specific integration, typically following the use of conventional site specific integration sites, and/or homologous recombination.

Those skilled in the art will appreciate that the invention is suitable for use with all invertebrates which have a gut suitable for uptake of dsRNA, and also a symbiotic gut bacteria amenable to genetic manipulation. Reference herein to a gut suitable for the uptake of dsRNA means an insect gut that can uptake dsRNA through feeding and/or digestion. The precise mechanism by which this occurs is yet to be elucidated but active channel transfer and endocytosis have been considered responsible for this process.

In a preferred embodiment of the invention, said dsRNA comprises a strand of RNA that shares 50% complementarity to at least one target gene of said insect vector or insect. It is preferred that said dsRNA comprises a strand of RNA that shares at least 75% complementarity to at least one target gene of said insect vector and, in increasing order of preference, at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% complementarity to at least one target gene of said insect vector.

As known by those skilled in the art, various models and databases, such as DEQOR (Henschel *et al.,* 2004), can be used to predict the probability that an mRNA fragment will cross-react with other genes in the cell, resulting in cross-silencing. Therefore, such models can be used to aid design of dsRNA for use in gene silencing and RNAi in this context.

In yet a further preferred embodiment of the invention, said transformed or transfected bacterial cell is also genetically engineered such that it does not produce functional RNA degrading proteins, including but not limited to, RNase I, RNase II, RNase III, RNase D, RNase E, RNase G, RNase P, RNase R, RNase T, RNase Z, RNase PH, RNase BN, RelE, MazF, Kid, PNPase, RhlB, enolase. Advantageously, this minimises the risk of enzymatic degradation of said dsRNA encoded by the transformed bacterial cell. More ideally still, said dsRNA is integrated by homologous recombination into the gene encoding the afore RNA degrading proteins. As will be appreciated by those skilled in the art, such genetic recombination will mediate RNAi against the insect vector target gene whilst simultaneously preventing the production of functional RNA degrading proteins.

In yet a further preferred embodiment of the invention, said target gene of the insect vector or insect is a gene involved in, but not limited to, growth, development, renewal or reproduction of the insect. More preferably, said gene is such that its loss results in reduced growth, renewal or differentiation, a reduction in feeding, or a reduction or loss in the capacity for reproduction. More preferably still, said target gene is such that its loss results in reduced infectivity or pathogenicity of said insect vector, for example, by targeting genes that prevent, or reduce the likelihood, of colonisation of the insect gut by the pathogen. In this way, the insect vector's capacity to transmit a pathogenic micro-organism is reduced but said insect vector remains viable such that the ecosystem in which it resides remains relatively stable, or the insect's capacity to act as a pest is reduced but said insect remains viable such that the ecosystem in which it resides remains relatively stable. Yet more preferably still, said target gene is insect-specific, more preferably still said target gene is found only in the insect vector or insect that hosts the genetically transformed gut symbiont. More preferably said gene includes, but is not limited to, genes of significance to the immune system such as *Defensin, Lysozyne,*

Transferrin, Prolixin, or alternatively those essential for viability such as the *Nitrophorins,* salivary *Lipocalins, Nitric Oxide Synthase, Vitellogenin, Juvenile Hormone, Odorant binding protein, Rhodnius Heme Binding Protein*, or the like. More preferably still said target gene is Vitellogenin or Nitrophorin 1 or Nitrophorin 2.

In use, the insect vector or insect acquires the dsRNA genetically transformed or transfected bacterial cell, wherein said bacterial cell is a gut symbiont of the insect vector or insect, from its environment through ingestion. Said bacterial cell thereby establishes itself as a living population in the gut of the insect vector or insect, wherein it divides and actively transcribes the dsRNA which it encodes. Advantageously, this therefore mediates RNAi in the insect vector or insect indefinitely. The dsRNA is targeted against a specific gene of interest of the insect vector or insect, leading to its modulation and more specifically, its down regulation. In this way the insect vector or insect is genetically compromised throughout its life cycle or at least during, but not limited to, the pathogenic transmitting component thereof or pest component thereof, respectively. Those skilled in the art will appreciate that the gene selected for RNAi targeting will be a gene that is normally active and involved in growth, differentiation renewal or reproduction of the insect vector or insect at least during, but not limited to, the phase of its life cycle that involves the transmission of a pathogenic organism or that which it acts as a pest, respectively.

According to a second aspect of the invention there is provided an insect vector that transmits a pathogenic organism characterised in that; said insect comprises a genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of said insect and is transformed to express dsRNA against at least one selected target gene of the insect vector.

Alternatively, there is provided an insect that acts as a pest characterised in that; said insect comprises a genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of said insect and is transformed to express dsRNA against at least one selected target gene of the insect.

In a preferred embodiment of the second aspect of the invention, said insect vector or insect engages in horizontal gut flora transfer.

According to a third aspect of the invention, there is provided the use of the afore described cell in a method for modulating expression of a target gene of an insect vector that transmits a pathogenic organism comprising:
contaminating a composition to be ingested by the insect vector with said bacterial cell;
whereupon ingestion of said bacterial cell by said insect vector results in said bacterial cell colonising the gut of said insect vector wherein it synthesises dsRNA against at least one selected target insect gene to modulate said insect vector gene expression.

In a preferred embodiment of the third aspect of the invention, said insect vector engages in horizontal gut flora transfer.

In yet a further preferred embodiment of the third aspect of the invention, said composition comprises a food source for the insect vector. More preferably still, said composition is the faeces of said insect vector or insect, or a food source of the adult insect such as, but not limited to, a plant source or, in the case of haematophagous insects, a contaminated blood food source

In a further preferred embodiment of the third aspect of the invention, modulating said target gene expression inhibits or reduces infectivity or pathogenicity of said insect vector, such as, but not limited to, its ability to transmit the pathogenic micro-organism.

According to a fourth aspect of the invention, there is provided the use of the afore described cell in a method for modulating expression of a target gene of an insect that acts as a pest, comprising:
contaminating a composition to be ingested by the insect with said bacterial cell;
whereupon ingestion of said bacterial cell by said insect results in said bacterial cell colonising the gut of said insect wherein it synthesises dsRNA against at least one selected target insect gene to modulate said insect gene expression.

In a preferred embodiment of the fourth aspect of the invention, said insect engages in horizontal gut flora transfer.

In a preferred embodiment of the fourth aspect of the invention, said composition comprises a food source for the insect vector or insect. More preferably still, said composition is the faeces of said insect vector or insect, or a food source of the adult insect such as, but not limited to, a plant source or, in the case of haematophagous insects, a contaminated blood food source.

As will be appreciated by those skilled in the art, said method is applicable to investigate gene function in the insect, whereupon the function(s) of a particular gene can be determined as a consequence of its down-regulation, This therefore permits the identification of further gene targets for use in the control of pests or spread of pathogenic organisms.

In a further preferred embodiment of the fourth aspect of the invention, modulating said target gene expression inhibits the activity of said insect, such as, but not limited to, its ability to feed, drink, colonise, or reproduce and in so doing reduces its impact as a pest.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprises”, or variations such as “comprises” or “comprising” is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the

accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

The present invention will now be described by way of example only with particular reference to the following figures wherein:

Figure-1. SEQID NO:1 fragment A. Nucleotide sequences 1-6 and 367-373 are recognition sites for EcoRI; nucleotide sequence 7-102 is the sequence of the modified dapA promoter; nucleotide sequences 103-108 is a recognition site for NdeI; nucleotide sequence 109-348 is the 5' end of the dagA gene of Streptomyces coelicolor; nucleotide sequences 349-354 is a recognition site for MluI; nucleotide sequences 355-360 is a recognition site for NheI; nucleotide sequences 361-366 is a recognition site for SalI.

Figure-2. SEQID NO:2 fragment B. Nucleotide sequences 1-6 and 121-127 are recognition sites for EcoRI; nucleotide sequences 7-12 is a recognition site for MluI; nucleotide sequences 13-18 is a recognition site for NdeI; nucleotide sequence 19-113 is the sequence of the modified dapA promoter; nucleotide sequences 114-120 is a recognition site for SalI; nucleotide sequences 121-126 is a recognition site for NheI.

Figure-3. SEQID NO:3 recombinant cassette in pMW101. Nucleotide sequences 1-6 and 463-468 are recognition sites for EcoRI; nucleotide sequences 7-102 and 360-456 are the sequences of the two copies of the convergent modified dapA promoter; nucleotide sequences 103-108 and 355-360 are recognition sites for NdeI; nucleotide sequence 109-348 is the 5' end of the dagA gene of Streptomyces coelicolor; nucleotide sequences 349-354 is a recognition site for MluI; nucleotide sequences 457-462 is a recognition site for SalI.

Figure-4. SEQID NO:15 RNaseIII gene sequence. Rhodococcus rhodnii RNase III gene sequence (804 bp; internal MluI site underlined).

Figure-5. SEQID NO:16 Translated RNaseIII gene of *Rhodococcus rhodnii.*

Figure-6. SEQID NO:10 Vitellogenin gene fragment. pLLMW11 insert sequence (vitellogenin sequence in upper case, NdeI sites in bold, underlined)

Figure-7. SEQIDNO:11 nitrophorin-2 gene fragment. pLLMW14 insert sequence (nitrophorin-2 sequence in upper case, NdeI sites in bold, underlined).

Figure-8. SEQID NO:12 ; nitrophorin-1 gene fragment pLLMW15 insert sequence (nitrophorin-1 sequence in upper case, NdeI sites in bold, underlined).

Figure-9. List of primers used.

Figure-10. Example of R. prolixus salivary gland phenotypes following ingestion of R. rhodnii expressing dsRNA. Control (dsAgarase) glands are cherry-red indicating normal synthesis of salivary nitrophorin proteins. Knockdown of Nitrophorin-2 (dsNP2) results in colourless glands, and in extreme cases severe tissue wastage and crystallization of the luminal contents. Knockdown insects also exhibit aberrant / abortive feeding behaviours.

Example 1

In the following text, by way of example, symbiont-mediated RNAi is shown in *Rhodnius prolixus*; a large, long-lived blood-sucking bug, which has evolved a symbiotic relationship with *Rhodococcus rhodnii* bacteria essential for development of the insect. *R. prolixus* is an important vector of Chagas disease due to transmission of trypanosomes during blood-feeds. Newly-hatched insects are free from symbiotic bacteria and must acquire them through ingestion of *R. rhodnii*-contaminated faeces from other insects. This means that dsRNA expressing bacteria have the potential to spread naturally through a colony of insects by horizontal transfer. This technique reduces insect handling and associated mortality, and is

also cost-efficient. We believe that this new technique forms the basis of a novel and highly specific pest control strategy that will target genes essential to insect survival, reproduction and transmission of parasites. It is particularly suited for application with insect vectors of tropical diseases, but is amenable to all invertebrates which have a gut suitable for uptake of dsRNA, and also a symbiotic gut bacteria amenable to genetic manipulation.

We describe the construction of recombinant *R. rhodnius* expressing dsRNA to target specific *R. prolixus* genes. In constrast to dsRNA expression in *E. coli*, in which genetic manipulations rely on using plasmid vectors that need to be maintained by constant antibiotic selection, we employ a system to stably integrate an expression cassette within the chromosome of *R. rhodnius*, obviating use of antibiotics for maintenance of expression. This is important to ensure long-term gene silencing effects and spread in insect populations.

Bacteria typically express an enzyme, RNaseIII, which specifically degrades dsRNA. Indeed we have established that dsRNA is unstable after it is expressed in R. rhodnius. To circumvent this problem, we have engineered a *R. rhodnius* mutant strain in which the gene encoding RNaseIII is disrupted and which stably expresses dsRNA.

Materials and Procedure

Expression Cassette

In vitro synthesis of the expression cassette was outsourced to a company, Eurofins MWG Operon. Two DNA fragments: SEQID NO:1 and SEQ ID NO:2 were supplied, each introduced separately by Topo-TA cloning into the *E. coli* plasmid vector pCR2.1 (Invitrogen), and supplied as pCR2.1-SEQID No:1 and pCR2.1-SEQID NO:2. SEQID NO:1 comprises a mutated copy of the promoter of the *Corynebacterium glutamicum dapA* gene (coding for dihydrodipicolinate synthase). In particular, this promoter sequence P-dapAMA16 differs from the wild-type *dapA* promoter in the sequence of the –10 hexamers (P-dapA - TAACCT; P-dapAMA16 – TATAAT), and has been demonstrated to have good activity in *Rhodococcus erythropolis* (Knoppova et al, 2007). In Fragment A, this promoter is fused to the 5' end 245 bp of the *dagA* gene of *Streptomyces coelicolor*. Expression of a double-

stranded (ds) RNA copy of this sequence was designed to act as a negative control for subsequent experiments measuring knock-down effects in the insect *Rhodnius prolixus*. Recognition sites for specific restriction enzymes (*Eco*RI, *Nde*I, *Mlu*I, *Nhe*I and *Sal*I) were included at the ends and junctions of the promoter and *dag*A sequence to facilitate subsequent cloning steps (Figure 1).

SEQID NO:2 comprises a second copy of P-dapAMA16 flanked by appropriate restriction sites (figure 2).

To construct a 468 bp recombinant cassette comprising the two convergent promoters flanking the *dagA* sequence, the two plasmids pCR2.1-SEQID NO:1 and pCR2.1-SEQID NO:2 were cut with restriction enzymes *Mlu*I and *Sal*I, the DNA's mixed and ligated with T4 DNA ligase. Following ligation, the DNA was further restricted with *Nhe*I to cut any of the original plasmids that had either been not cut to completion initially or had reformed by ligation. To recover plasmid containing the recombinant cassette, *E. coli* strain JM109 (Yanisch-Perron *et al.*, 1985) was electroporated and transformants selected by plating cells on L-agar containing ampicillin and kanamycin. Recombinant plasmids obtained this way were verified by restriction with *Eco*RI. One example was selected for further use, pMW101 containing the recombinant dsRNA expression cassette (SEQID NO:3; figure 3).

To introduce this cassette into a plasmid for use in *Rhodoccocus rhodnii*, the plasmid pMW101 was first fused with plasmid pIJ8600 (Sun et al, 1999) by cutting both with the restriction enzyme *Bam*HI, ligating the two molecules with T4 DNA ligase, and recovering recombinant plasmids after electroporation of *E. coli* JM109 and plating cells on L-agar containing apramycin and kanamycin. Recombinant plasmids obtained this way were verified by restriction with *Eco*RV. One example was selected for further use and named pMW102. To delete the majority of the pCR2.1 sequence in pMW102, this plasmid was cut with *Eco*RV, the DNA ligated and introduced by electroporation of *E. coli* JM109, selecting cells on L-agar containing apramycin. Recombinant plasmids were verified by restriction with *Eco*RV. One example was selected for further use and named pMW103 (pIJ8600 containing the expression cassette with *dagA* sequence).

***R. prolixus* gene fragments**

Fragments of *R. prolixus* genes encoding vitellogenin, nitrophorin-1 and nitrophorin-2 were obtained by PCR amplification using (respectively) primers RP11 (SEQID NO:4) & RP12 (SEQID NO:5), RP15 (SEQID NO:8) & RP16 (SEQID NO:9), and RP14 (SEQID NO: 6) & RP6 (SEQID NO:7). GTTTCT overhangs were added to primers to improve priming efficiency, and *Nde*I sites added *(with the exception of RP6, due to the presence of an* Nde*I site in the immediate vicinity of the target sequence)*. The chosen target sequences assessed as having a low risk of potential cross-silencing (Deqor online programme [ref: Henschel, A., et.al., Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W113-20] and ClustalW). The amplicons were cloned into pGEM→-T Easy (Promega) and transformed into *E. coli* JM109. Following selection of positive clones by ampicillin resistance, and sequencing of the inserts of the correct size, each insert was then excised from pGEM→-T Easy by *Eco*RI restriction, and cloned into the *E. coli* T7 expression vector pLL10 (Levashina et al 2001) by restriction of both vector and respective PCR product with *Eco*RI, calf intestinal phosphatase treatment of the vector, ligation of PCR product and vector with T4 DNA ligase, transformation of *E. coli* JM109 and selection of recombinant colonies with ampicillin. Recombinant plasmids were verified by restriction with *Eco*RI and sequencing of those with inserts of the correct size. Plasmids pLLMW11 (with the vitellogenin fragment; SEQID NO:10), pLLMW14 (with the nitrophorin-2 fragment; SEQID NO:11) and pLLMW15 (with the nitrophorin-1 fragment; SEQID NO:12) were selected for further use.

To transfer these sequences into the expression cassette, plasmid pMW103 was cut with *Nde*I and the terminal phosphates removed with antarctic phosphatase. The linearised vector minus the *dagA* sequences was then ligated with the insect gene-containing *Nde*I fragments purified from pLLMW11-15. Recombinant plasmids were recovered in *E. coli* JM109. Positive clones were verified by restriction digests with *Nde*I and, subsequently, by DNA sequencing. Plasmids pMW104 (with the vitellogenin fragment), pMW105 (with the nitrophorin-2 fragment) and pMW106 (with the nitrophorin-1 fragment) were selected for further use.

Construction of an RNase III deficient mutant of *Rhodococcus rhodnii*

Degenerate primer RR4 (SEQID NO: 13) and primer RR5 (SEQID NO: 14) were designed from ClustalW alignments of RNase III gene sequences from related bacterial species, and used to amplify a 300 bp product from *R. rhodnii*, that was verified by sequencing as a fragment of RNase III. *Sac*I digests of wildtype *R. rhodnii* gDNA were then probed by Southern blotting using RR4 (SEQID NO: and RR5 labelled with digoxigenin (Roche PCR DIG Probe synthesis kit). The probes identified an approx. 2kb fragment, which was ligated into the ampicillin-resistant pUC18 plasmid (Yanisch-Perron *et al*, 1985**)** and transformed into *E. coli* JM109 competent cells. Two clones (A28 & A43 [pMW43]) containing the 2kb insert were identified by colony hybridization, and the full RNase III gene sequence (SEQID NO: 15) was determined in both clones by sequencing of the recombinant plasmids (figure 4). A translated amino acid sequence of the RNase III gene is shown SEQID NO:16; figure 5)

The sequencing identified a *Mlu*I restriction site within the RNaseIII coding sequence. To disrupt the gene, a kanamycin-resistance cassette flanked by *Mlu*I sites was generated by PCR. The template for this was plasmid pME6 (Fernandez-Martinez et al, 2009), using as forward primer SEQID NO: 17**,** and as reverse primer SEQID NO:18 (*Mlu*I sites underlined). This amplicon was digested with *Mlu*I and ligated with pMW43 cut with *Mlu*I. The recombinant plasmid was selected after electroporation of *E. coli* JM109 and growing on media containing both ampicillin and kanamycin. The plasmid pMW44 that was derived this way was verified by restriction analysis.

pMW44 was introduced into *R. rhodnii* by electroporation (see below for method), and recombinants selected by plating the bacteria on tryptic soy agar containing kanamycin. Several recombinants were thus obtained and subsequently characterised by isolation of gGNA, restriction with *Sac*I and Southern hybridization with a probe consisting of the *Sac*I fragment containing the RNaseIII gene isolated from pMW43. All kanamycin-resistant *R. rhodnii* clones possessed the disrupted RNaseIII gene containing the kanamycin-resistant cassette.

Electroporation of *R. rhodnii*

Plasmids were introduced into *R rhodnius* cells by electroporation using a Biorad MicroPulser. To obtain electrocompetent cells, 10 ml of TSB supplemented with 0.5% (w/v) glycine was inoculated with 0.1 ml of an overnight TSB preculture and grown at 28°C for 24 h. Cells were harvested, washed twice with ice-cold HS buffer containing 7 mM HEPES and 252 mM sucrose (pH 7.0) and concentrated 10-fold in ice-cold HS buffer. Immediately before electroporation, 400 ul of competent cells were preincubated at 40°C for 10 min and mixed with DNA (final concentration 0.1 – 1.0 ug/ml). The electroporation was performed in electrocuvettes with gaps of 1 mm and the following settings: 1.8 kV, 1 pulse. Pulsed cells were immediately diluted with 4 ml TSB and regenerated at 28°C for 24 h before they were plated on TSB agar plates supplemented with 50 ug/ml antibiotic (either kanamycin for construction of the RNase III mutant, or apramycin for introduction of the expression cassette).

The backbone of the expression cassette plasmids (based on pIJ8600) contains a site-specific recombination system, whereby after introduction of a plasmid by electroporation, the plasmid then integrates site-specifically into the *R. rhodnius* genome. Consequently, the expression cassette is maintained stably without recourse to constant antibiotic selection.

Introduction of *R. rhodnii* strains into *R. prolixus*/egg sterilization

Aposymbiotic *R. prolixus* were raised from eggs sterilized for 10 minutes in iodine tincture (iodine 2.5% w/v, potassium iodide BP 2.5% w/v, ethanol 89% v/v) and rinsed twice with autoclaved water. [A 15 minute immersion in Savlon→ solution followed by 3 washes is also effective]. The eggs were transferred to sterile plastic 25 ml tubes containing autoclaved filter paper and sealed with gas-permeable plate-sealer adhesive (Thermo Scientific), which formed a microbiological barrier. Eggs and hatched insects were maintained in an incubator at 28°C, 70% RH with a 12:12 light:dark cycle, and membrane-fed at intervals of 2-3 weeks with sterile defibrinated horse blood (TCS) in a sterile flow cabinet. Non-sterile experimental insects were reared similarly, except the eggs were not sterilized and the insects were provided with soiled filter paper from the parental (non-sterile) insect colony, to ensure uptake of symbiotic bacteria by coprophagy.

For blood feeding, blood in a Petri dish was heated to 37°C on a hot plate and covered with a sheet of sterile latex cut from Ansell Gammex® PF Micro-Thin® powder-free surgical glove. The insect tubes' adhesive lids were replaced with autoclaved net fabric during feeding (to allow proboscis probing), fixed with a rubber band, and inverted over the feeding membrane.

Aposymbiotic and non-sterile insects of different ages were infected by membrane-feeding as above, by premixing transformed *R. rhodnii* with the blood. The bacteria were cultured in TSB supplemented with apramycin, with shaking, at 28°C for 3 days, washed twice by centrifugation (and resuspension in sterile water, and mixed with horse blood to a concentration of $5x10^6$/ml. Unfed (unengorged) insects were easily identified and could thus be removed from the tubes following feeding.

Two to seven days after a blood feed, insects were transferred to fresh containers under sterile conditions, and faeces in the vacated containers were assessed for the presence or absence of transformed bacteria and/or to verify sterile conditions. Tubes were rinsed with TSB, which was then spread on TSA plates +/- apramycin. Apramycin-resistant colonies were randomly sampled for the presence of cloned sequence by colony PCR using the relevant primers described above. Transformed bacteria were usually detectable in voided faeces within 48h of an infective feed. Gut contents from sacrificed insects were also monitored at regular timepoints post infective feeding.

RESULTS

Figure 10 shows an example of R. prolixus salivary gland phenotypes following ingestion of R. rhodnii expressing dsRNA against nitrophorin 2 (test) or agarase (control). Control (dsAgarase) glands are cherry-red indicating normal synthesis of salivary nitrophorin proteins. Knockdown of Nitrophorin-2 (dsNP2) results in colourless glands, and in extreme cases severe tissue wastage and crystallization of the luminal contents. Knockdown insects also exhibit aberrant / abortive feeding behaviours. This experiment thus demonstrates the effectiveness of the technology

for targeting specific genes and so interfering with the insect host phenotype in a manner deleterious to feeding and so survival.

References

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Claims

1. A genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of an insect; characterised in that said bacterial cell is transformed to express dsRNA against at least one selected target gene of the insect.

2. A bacterial cell according to claim 1, wherein said bacteria is a gut symbiont of an insect vector that transmits a pathogenic organism known to cause disease in a plant or animal host.

3. A bacterial cell according to claim 1, wherein said bacteria is a gut symbiont of an insect pest.

4. A bacterial cell according to any preceding claim, wherein said bacteria is a gut symbiont of an insect that engages in horizontal gut flora transfer.

5. A bacterial cell according to claim 4, wherein horizontal gut flora transfer is achieved by ingestion of faeces from other insects.

6. A bacterial cell according to claim 4, wherein horizontal gut flora transfer is species specific.

7. A bacterial cell according to claims 1-3, wherein said bacteria is a gut symbiont of an insect that ingests said bacteria from the environment.

8. A bacterial cell according to any preceding claim, wherein said bacteria is a gut symbiont that is species specific.

9. A bacterial cell according to any preceding claim, wherein said bacteria is a gut symbiont of *Rhodinius prolixus*.

10. A bacterial cell according to claim 9, wherein said bacteria is *Rhodococcus rhodnii*.

11. A bacterial cell according to claims 9 or 10 wherein said pathogen is a Trypanosome.

12. A bacterial cell according to any preceding claim wherein said bacterial cell is transformed such that nucleic acid encoding said dsRNA is stably integrated into the host cell genome by site-specific integration and/or homlogus recombination.

13. A bacterial cell according to any preceding claim wherein said dsRNA comprises RNA that shares 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with at least one target gene of said insect vector or insect.

14. A bacterial cell according to any preceding claim wherein said transformed or transfected bacterial cell is also genetically engineered such that it does not produce functional RNA degrading proteins.

15. A bacterial cell according to claim 14 wherein said dsRNA is integrated by homologous recombination into at least one gene encoding at least one of said RNA degrading proteins.

16. A bacterial cell according to any preceding claim wherein said target gene of the insect vector or insect is a gene involved in growth, development, renewal or reproduction of the insect.

17. A bacterial cell according to any preceding claim wherein said target gene is insect-specific and so said target gene is found only in the insect vector or insect that hosts the genetically transformed gut symbiont.

18. A bacterial cell according to claims 16 or 17 wherein said target gene is Vitellogenin or nitrophorin 1 or nitrophorin 2.

19. An insect vector that transmits a pathogenic organism or an insect that is a pest characterised in that; said insect comprises a genetically transformed or transfected bacterial cell wherein said bacteria is a gut symbiont of said insect and is transformed to express dsRNA against at least one selected target gene of the insect vector.

20. A method for modulating expression of a target gene of an insect vector that transmits a pathogenic organism comprising:
contaminating a composition to be ingested by the insect vector with a bacterial cell according to claims 1-18;
whereupon ingestion of said bacterial cell by said insect vector results in said bacterial cell colonising the gut of said insect vector wherein it synthesises dsRNA against at least one selected target insect gene to modulate said insect vector gene expression.

21. A method for modulating expression of a target gene of an insect that acts as a pest, comprising:
contaminating a composition to be ingested by the insect with a bacterial cell according to claims 1-18;
whereupon ingestion of said bacterial cell by said insect results in said bacterial cell colonising the gut of said insect wherein it synthesises dsRNA against at least one selected target insect gene to modulate said insect gene expression.

gaa

ttcggaatgt ggcttgggcg attgttatgc aaaagttgtt aggtttttg cggggttgtt 63

taacccccaa atgagggaag aaggtataat tgaactctac atatgGTGGT CAACCGACGT 123

GATCTCATCA AGTGGAGTGC CGTCGCACTC GGAGCGGGTG CGGGGCTCGC GGGTCCCGCA 183

CCCGCCGCTC ATGCCGCAGA CCTCGAATGG GAACAGTACC CCGTGCCGGC CGCCCCTGGC 243

GGAAACAGGT CCTGGCAGCT TCTCCCCAGC CATTCGGACG ACTTCAACTA CACCGGCAAG 303

CCTCAAACCT TCAGGGGCAG ATGGCTGGAC CAGCACAAGG ATGGCacgcg tgctagcgtc 363

gacgaattc

Figure-1.

gaattcacgc gtcatatgta gagttcaatt ataccttcttc cctcatttgg gggttaaac 60

aaccccgcaa aaaacctaac aacttttgca taacaatcgcc caagccacat tccgtcgac 120

gctagcgaat tc

Figure-2.

ttcggaatgt ggcttgggcg attgttatgc aaaagttgtt aggtttttg cggggttgtt 63

taacccccaa atgagggaag aaggtataat tgaactctac atatgGTGGT CAACCGACGT 123

GATCTCATCA AGTGGAGTGC CGTCGCACTC GGAGCGGGTG CGGGGCTCGC GGGTCCCGCA 183

CCCGCCGCTC ATGCCGCAGA CCTCGAATGG GAACAGTACC CCGTGCCGGC CGCCCCTGGC 243

GGAAACAGGT CCTGGCAGCT TCTCCCCAGC CATTCGGACG ACTTCAACTA CACCGGCAAG 303

CCTCAAACCT TCAGGGGCAG ATGGCTGGAC CAGCACAAGG ATGGCacgcg tcatatgtag 363

Agttcaatta taccttcttc cctcatttgg gggttaaaca accccgcaaa aaacctaaca 423

Acttttgcat aacaatcgcc caagccacat tccgtcgacg aattc

Figure-3.

ATGTGGCACCTACAAGGGTCGCCAGGTCACCGCAGCGATCTGATTCGTCGGTTTCCGGTGAA
CACCGACTCTCGCAGCCACCCCGAGCGCGGCGACGAGGGCCATGAGCCCCTCCTCGCCGCGC
TCGGCGTTACGCTCGATCCCGAACTGCTGACGCTCGCGCTCACCCACCGCTCGTACGCGTAC
GAGCACGGTGGGCTACCCACCAACGAACGCCTCGAGTTTCTCGGCGACTCGGTGCTGGGCCT
CGCCGTGACCGAGCGGCTCTATCTCGATCACCCCACTCGCCCCGAGGGCGAACTCGCGAAGA
TCCGCGCCAGCATCGTCAACATGCACGCGCTCGCCGAGGTCGCGCGGGAGCTGGGCCCCGGT
GGGCTCGGTGCCCACATCCTGCTCGGCAAGGGCGAGGAGATGACGGGCGGCCGCGACAAGCC
GAGCATCCTCGCCGACGGCATGGAGTCGCTCCTCGGCGCGATCCACCTGCAGCACGGCATCG
ACGTCGCGCGCGGCGTCGTGTCGCGGCTCTTCGGCGCCCTCCTCGAACGTGCGCCCGGACTC
GGCGCCGGCCTCGACTGGAAGACAAGCCTGCAGGAGCTGACGGCCGAACGCGGCCTCGGCGT
CCCCGCGTACGAGATCACCGCGACCGGGCCCGATCACGACAAGGAGTTCACCGCGACCGTCG
TCGTCGCGGGTGGCGGACTCGGCGTCGGCGTCGGACGCACGAAGAAGGAAGCCGAGCAGAAG
GCCGCCGCGACCGCGTGGACCGAGCTGAGCGGCGGCCCCGCCGACGTCGCCGGCGCGTAG

Figure-4.

MWHLQGSPGHRSDLIRRFPVNTDSRSHPERGDEGHEPLLAALGVTLDPELLTLALTHRSYAY
EHGGLPTNERLEFLGDSVLGLAVTERLYLDHPTRPEGELAKIRASIVNMHALAEVARELGPG
GLGAHILLGKGEEMTGGRDKPSILADGMESLLGAIHLQHGIDVARGVVSRLFGALLERAPGL
GAGLDWKTSLQELTAERGLGVPAYEITATGPDHDKEFTATVVVAGGGLGVGVGRTKKEAEQK
AAATAWTELSGGPADVAGA

Figure-5.

gaattcACTAGTGATTGTTTCT**CATATG**GCATAAACATGCACCAGAATGTAGTCACCATTATATTTGAA
TAGAGCTACAGATTCCTTAGAGAACTCTAGATTGCGGCCGTTAACGACGACTTTGCCAACTACTTCTGA
AGATCCGGTTGGGAACACCTGGACGGTGTATTTGTCGAGCAAAATCAACAACTCCTTTTGATATGAAGC
ACCTTCACGGACTAAGACACTAAAAGCGTCATCATAATAGTGAGTAGTCTGGCCTGAATAACCATATTC
AGTCTTAAAAACTGGGTGAAGCATAGCATGCCAGCATTTGCCAACTTGCACTGGATAAGAAGTGTTGTC
GAAAGTTGTGGCT**CATATG**AGAAACAATCGAATTCctgcag

Figure-6.

gctt**catatg**gaattcGATTGTTTCTCATAGGAGTCAGTGGAGACTGTAGTACAAATATCAGTCCTAAA
AGTGGTTTAGATAAAACCAAGTATTTCAGCGGTAAATGGTATGTGACTCATTTTCTAGACAAGGATCCT
CAGGTTACAGATAAATACTGCTCTAGTTTTACACCAAGAGAATCTGGTGGTGAAGTAAAAGAGGCATTG
TATCACTACAATGTAGAAAAAAAAACATCTTTTTACAATATAGGTGAAGGTAAATTGGGATCGTCTGGG
GTACAATACACTGCAAAATATAAAACTGTTGATAAAAAAAAGCTGTGTTAAAAGAAGCAGATGAGAAAA
ACTCATATACACTTACCGTTTTGGAAGCTGACGATTCGTCCGCTCTGGTCCA**CATATG**TTTGCGGGAAG
GATCAAAAGATCTTGGAGATCTCTACACTGTTTTAACTCACCAAAAAGATGCGGAATCACTAGTctgca
g

Figure -7

gaattcGATTGTTTCT**CATATG**CAGTGGGTGTAAGTGGAAAGTGTACAAAAAATGCACTAGCTCAAACT
GGTTTTAATAAAGACAAGTACTTCAATGGTGATGTATGGTACGTGACAGATTACCTAGATTTGGAACCT
GACGAGGTTCCAAAAAGATACTGCGCTGCCCTTGCAGCGGGTACAGCTAGTGGTAAATTAAAAGAAGCT
CTATATCACTACGATCCAAAAACTCAAGACACTTTTTACGATATAAGTGAACTTCAAGAGGAATCTCCC
GGAAAATACACTGCAAACTTTAAAAAAGTTGAGAAAAATGGAAATGTCAAAGTAGATGTAACGTCGGGC
AACTATTATACCTTTACCGTTATGTATGCTGACGATTCGTCTGCTCTTATCCACACTTGTTTGCATAAA
GGAAACAAGGACTTGGGAGATCTCTACGCTGTATTAAATCGCAATAAAGATACCAATGCTGGTGATAAA
GTTAAAGGCGCCGTAACTGCTGCTAGTTTAAAATTCAGCGACTTCATTTCCAC**CATATG**AGAAACAATC
ACTAGTctgcag

Figure-8

RP11 (SEQID NO:4; *Nde*I site bold & underlined)

5′-GTTTCT**CATATG**AGCCACAACTTTCGACAACA-3′

RP12 (SEQID NO:5; *Nde*I site bold & underlined)

5′-GTTTCT**CATATG**GCATAAACATGCACCAGAA-3′

RP15 (SEQID NO:8; *Nde*I site bold & underlined)

5′-GTTTCT**CATATG**CAGTGGGTGTAAGTGGAA-3′

RP16 (SEQID NO:9; *Nde*I site bold & underlined)

5′- GTTTCT**CATATG**GTGGAAATGAAGTCGCTGAA-3′

RP14 (SEQID NO: 6; *Nde*I site bold & underlined)

5′-GTTTCT**CATATG**GAGTCAGTGGAGACTGT-3′

RP6 (SEQID NO:7)

RP6 5′-CCGCATCTTTTTGGTGAGTT-3′

RR4 (SEQID NO: 13)

5′-CACCGCTCGTAYGCGTA-3′

RR5 (SEQID NO: 14)

5′-TGGAGTCGATTCTCGGCG-3′

SEQID NO: 17 (*Mlu*I site underlined)

5′ agaacgcgtAAGCGAACCGGAATTGCCAG-3′

SEQID NO:18 (*Mlu*I site underlined)

5′ caaacgcgtGAATACTCATACTCTTCCTT-3′

Figure-9.

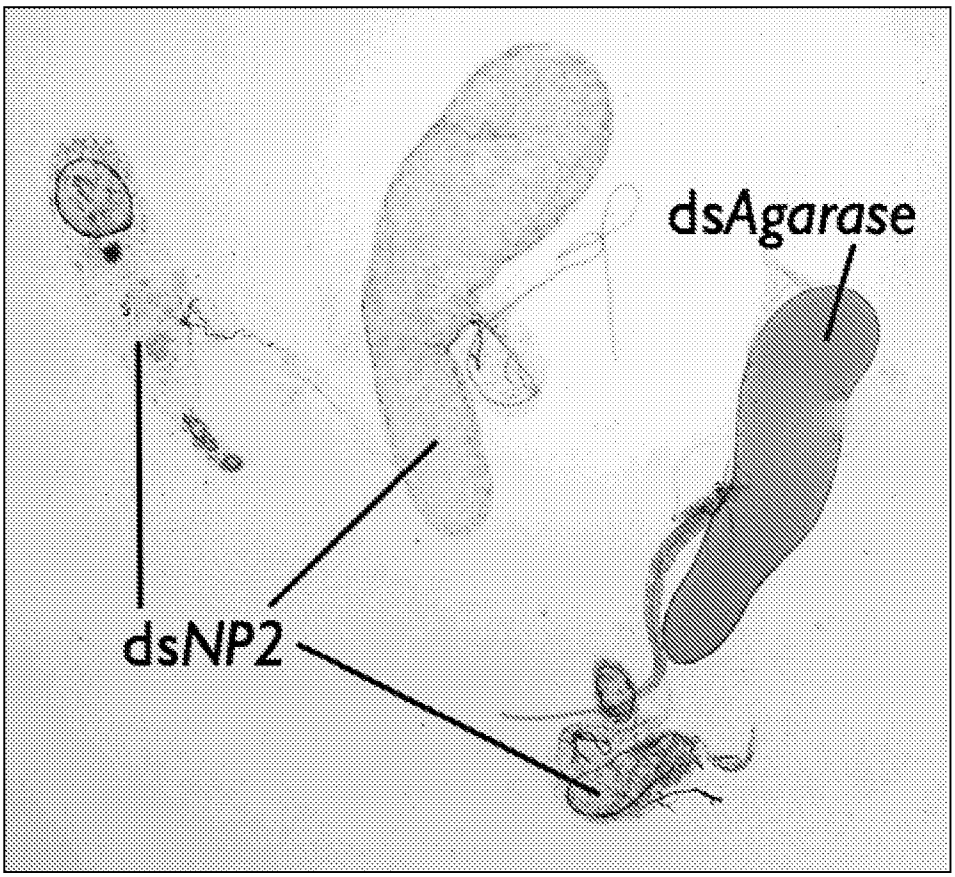

Figure-10

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050228

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 C12N15/82 A01N63/00 C12R1/01
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A01N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/110068 A2 (MONSANTO TECHNOLOGY LLC [US]; BAUM JAMES A [US]; GILBERTSON LARRY A [U) 24 November 2005 (2005-11-24)	1-8, 11-13, 16,17, 19-21
Y	page 12, line 30 - line 26 page 13, line 33 - line 36	9,10,12, 14,15,18

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2013	07/05/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilat, Daniel

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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