RNA MOLECULES AND VECTORS FOR GENE SILENCING

Inventors: David Charles Baulcombe, Norfolk (GB); Andrew John Hamilton, Helensburgh (GB)

Assignee: Plant Bioscience Limited, Herts (GB)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

Appl. No.: 11/013,315
Filed: Dec. 17, 2004

Prior Publication Data
US 2005/0102709 A1 May 12, 2005

Related U.S. Application Data
Division of application No. 10/805,804, filed on Mar. 22, 2004, which is a division of application No. 09/491,549, filed on Jan. 26, 2000, now Pat. No. 6,753,139.

Foreign Application Priority Data
Oct. 27, 1999 (GB) 9925459.1

Int. Cl.
C07H 21/04 (2006.01)
C07H 21/02 (2006.01)
A61K 48/00 (2006.01)

U.S. Cl. 536/24.5; 536/23.1; 536/24.31; 536/24.3; 536/24.33; 514/44

Field of Classification Search None
See application file for complete search history.

References Cited
U.S. PATENT DOCUMENTS
5,998,203 A 12/1999 Matule-Adamic et al.
6,107,094 A * 8/2000 Crooke 435/455
6,506,559 B1 1/2003 Driver et al.
2002/0086356 A1 7/2002 Tuschi
2002/016216 A1 10/2002 Beach
2003/0084471 A1 5/2003 Bench
2003/0157030 A1 8/2003 Davis
2003/0198627 A1 10/2003 Arts
2003/0204318 A1 10/2003 Feldman
2004/0020777 A1 1/2004 Taira
2004/0018993 A1 1/2004 Beach
2004/0086884 A1 5/2004 Beach
2004/0253694 A1 12/2004 Lin
2005/0020525 A1 1/2005 McSwiggen
2005/006278 A1 2/2005 Tuschi

FOREIGN PATENT DOCUMENTS
EP 1 080 208 B1 * 2/1999 435/320.1
EP 1 080 208 B1 3/2001
EP 1 144 623 10/2001
WO 94/10150 A1 1/1994 514/44
WO 01/75164 A2 11/2001

OTHER PUBLICATIONS

Primary Examiner — Amy Bowman
Attorney, Agent, or Firm — Morrison & Foerster LLP

ABSTRACT
The invention is directed to compositions for gene silencing by providing short RNA molecules to cells.

15 Claims, No Drawings
OTHER PUBLICATIONS


Manche et al., Interactions between Double-Stranded RNA Regulators and the Protein Kinase DAI, 1992, Molecular and Cellular Biology, vol. 12, No. 11, pp. 5238-5248.*

Stratagene pBluescript II Phagemid Vectors Instruction Manual for Catalog # 212207, downloaded from the Stratagene, Inc. website on Jan. 11, 2007.*

Basic Local Alignment Search Tool (BLAST) analysis available through NCBI, of nucleic acid sequence “ceggtaagacgatgtgtce” completed on Jan. 11, 2007.*


Cullen, RNAs the natural way, 2005, Nature Genetics, vol. 37, No. 11, pp. 1163-1165.*


Lee et al., Cell (1993) 75:843-854.


Overheads from talk given by one of the inventors on Feb. 27, 1999 at EMBO workshop on “Post-transcriptional regulation of gene expression in plants”; Feb 25-28, 1999 conducted at Lesin, in Switzerland.

Poster given at meeting: Molecular Plant Microbe Interactions (MPMI), 9th International Congress, Jul. 25-30, 1999.


* cited by examiner
RNA MOLECULES AND VECTORS FOR GENE SILENCING

CROSS-REFERENCE TO RELATED APPLICATIONS


TECHNICAL FIELD

The present invention relates generally to methods and materials for use in achieving and detecting gene silencing, particularly post-transcriptional gene silencing, in an organism.

PRIOR ART

Methods of detecting and efficiently achieving gene silencing are of great interest to those skilled in the art.

Post-transcriptional gene silencing (PTGS) is a nucleotide sequence-specific defence mechanism that can target both cellular and viral mRNAs. PTGS occurs in plants and fungi transformed with foreign or endogenous DNA and results in the reduced accumulation of RNA molecules with sequence similarity to the introduced nucleic acid (1, 2).

PTGS in plants can be suppressed by several virus-encoded proteins (6) and is closely related to RNA-mediated virus resistance and cross-protection in plants (7, 8). Therefore, PTGS may represent a natural antiviral defence mechanism and transgenes might be targeted because they, or their RNA, are perceived as viruses. PTGS could also represent a defence system against transposable elements and may function in plant development (9-11). To account for the sequence specificity, and post-transcriptional nature of PTGS it has been proposed that antisense RNA forms a duplex with the target RNA thereby promoting its degradation or interfering with its translation (12).


DISCLOSURE OF THE INVENTION

The present inventors have investigated PTGS of target genes initiated by a variety of silencing mechanisms in different organisms, and have established that in every case a previously uncharacterised species of antisense RNA complementary to the targeted mRNA was detected. These RNA molecules were of a uniform length, estimated at around 25 nucleotides, and their accumulation required either trans-
RNA is used herein, it will be understood by those skilled in the art that the comments would apply equally in the event that the SRMs do not have this precise length.

Indeed the precise length may not be important, since the disclosure herein permits the identification, isolation and utilisation of SRMs in any case.

In performing the invention, it may be preferred to analyse or otherwise utilise short antigene RNA molecules (SARMs) rather than short sense RNA molecules (SSMRs). Nonetheless, where reference is made herein to SARMs (except where context clearly suggests otherwise) it will be appreciated by those skilled in the art that the SSMRs may also be used.

In particular, the SSMRs methodology may be used as an indicator of PTGS. As is well known to those skilled in the art, PTGS occurs post-transcriptionally: i.e. the transcription rates of the suppressed genes are unaffected. The term 'gene' is used broadly to describe any sequence which is suitable for translation to a protein.

Thus the presence of SSMRs can be used as a diagnostic test for the existence of PTGS.

In one embodiment of this aspect there is disclosed a method of detecting or identifying the silencing of a target gene in an organism, which method further comprises characterising any SSMRs which are present. It should be noted that PTGS effects are very dominant. In principle the presence of SSMRs may indicate the silencing of more than one gene, providing that they have sufficient homology.

'Characterised' and 'characterising' does not necessarily imply complete sequencing, although this may be preferred. In order to detect silencing of a known sequence, the SSMRs may be fully or partially sequenced, or sequence identity or similarity may be inferred from e.g. blotting.

Applications for such a diagnostic test will depend on the organism in question. For instance, in plants, since PTGS is the basis for a lot of pathogen derived resistance (PDR), GM field crops (e.g. individuals, or populations) engineered for PDR could be monitored "in field" by checking for the existence of 25 nt RNA to make sure that the PDR was still operating prior to the attack by the virus.

Similarly, crops depending upon co-suppression for the knockout of a particular plant gene to achieve a specific modified trait could be assayed for the continued function of PTGS by checking the presence of 25 nt RNA against the intended target. Such an assay may be particularly useful in view of evidence that transgenes have a tendency to become transcriptionally inactivated over the generations. PTGS depends upon transcription of the initiating transgene to function and so if this gets reduced the PTGS will begin to fail. Monitoring 25 nt RNA provides a quick way to test the lines.

Non-limiting examples of silenced genes which could be monitored in this way include any of those which have already been shown to be suppressible by PTGS in the literature. These may include, for example, chalcone synthase of petunia or polygalacturonase of tomato (Jorgensen, R. A. (1995), Science, 268: 686-691, Hamilton, A. J., et al (1995), Current Topics In Microbiology and Immunology, 197: 77-89).

It is also possible that the process of PTGS underlies certain plant developmental processes. If there are plant genes which are being targeted naturally as a result of PTGS in order to satisfy some plant developmental programme, a 25 nt RNA corresponding to sequences from these genes may be detectable.

Thus, in this embodiment, the SSMRs may be used to identify and isolate an unknown target. This could be achieved by analysing the 25 nucleotide fraction of RNA from a plant, tagging it with a marker (e.g. a radioactive one) and then using this radioactive RNA to probe a library of plant genes. This probe will anneal to genes which are undergoing PTGS in the plant, which genes can then be further analysed or characterised if required. Such genes, inasmuch as they are novel, represent a further aspect of the present invention.

In a further aspect of the present invention, there is disclosed a process for producing or isolating short RNA molecules. As discussed above, SSMRs may not be readily detected by routine RNA analyses, particularly those which include a step in which such molecules are "lost" (for instance SSMRs may not be efficiently retained on silica columns which are used to isolate longer molecules such as mRNAs). A preferred process is set out in the Examples hereinafter.

Broadly speaking, the processes provided divide into two parts: extraction/purification and detection.

For extraction, initial steps may be performed using conventional RNA extraction methods and kits appropriate to the organism in question, modified as required to ensure that SSMRs are retained at each step.

In order to enhance purification of short RNAs, the extraction may optionally be followed by one or more of the following steps:

(i) filtration (e.g. through Centricron 100 concentrators (Amicon) or similar),
(ii) differential precipitation (e.g. with 5% polyethylene glycol(8000)/0.5M NaCl)
(iii) ion exchange chromatography (e.g. using Qiagen columns).

These steps enrich and purify the short RNAs to greater degrees than is obtained with the routine rRNA extraction method alone, and may be performed in conventional manner using, if required, proprietary reagents.

It should be noted that there is no requirement that the short RNAs be purified to homogeneity, provided only that they are capable of detection.

Regarding detection, because of their small size the method for this is not the usual one for "RNA gel blot analysis" although the principle is the same i.e. separation of the RNA molecules according to size by electrophoresis through a gel.

Preferably the gel is a 15% polyacrylamide gel containing 7M urea as a denaturant and TBE (0.5×) as a buffer.

The RNAs are preferably transferred to a hybridisation membrane by electrophoresis (rather than the more conventional capillary blot). Once the RNA is on the membrane, it is covalently attached to it by UV irradiation. The membrane is then placed in "prehybridisation solution" for a short time.

Radioactive probe may be prepared using standard techniques. However, preferably, it is made as a single stranded RNA molecule transcribed in vitro from an appropriate plasmid DNA template. The length of the probe may, preferably, be shortened by limited hydrolysis before adding to the pre-hybridisation solution; this may reduce non-sequence specific binding of probe to the membrane.

The hybridisation of the probe to its target is allowed to proceed at a stringency level (specific temperature, salt concentration and the concentration of formamide in the prehybridisation solution) appropriate to the requirements of the process. For instance low temperature, high salt, no formamide equals a low stringency, which may permit short probes or probes with imperfect homology to the target to hybridise with the target. Conversely high temperature, low salt and formamide mean high stringency with only lengthy duplexes stable under these conditions. Preferred conditions are 45% formamide, 7% SDS, 0.3M NaCl, 0.05M Na2HPO4/
Na₂HPO₄ (pH 7), 1x Denhardt’s solution, and single stranded heterologous nucleic acid (e.g. derived from salmon sperm).

This is one preferred process of purifying (or partially purifying) SRMs for the purpose of detection and/or further characterisation e.g. for sequencing. However it should be understood that the present invention is in no way limited to this particular format, and others methods for SRMs analysis, such as those which may be devised in the future, will also be encompassed.

The process described above may form part of a more extensive process for producing or isolating a silencing agent for a target gene, which silencing agent is a preferably a SRM, the process comprising the steps of:

(i) silencing a target gene in an organism,

(ii) performing a process as described above in order to isolate a SRM appropriate for that gene.

“Silencing agent” in this context may be one or more of an inducer, signal, or specificity determinant of gene silencing, particularly PTGS. Preferably this will be a SARM (as opposed to a SSRM). Isolated silencing agents obtained or obtainable by this method, inasmuch as they are novel, form a further aspect of the present invention.


In a further aspect of the present invention there is disclosed a method for identifying or selecting a target region of a gene, which gene it is desired to silence, which method comprises:

(i) silencing the target gene in an organism,

(ii) performing a process as described above in order to isolate a SRM appropriate for that gene,

(iii) identifying a region in the sequence of the gene which corresponds to the sequence of the SRM.

The region may be identified most readily by comparing the sequence of the SRM with the sequence of the gene; however any appropriate method may be used (e.g. RNAase protection). If several SRMs are isolated, then several target regions may be identified.

As described in the introduction, this method provides an alternative to e.g. computational analysis in order to identify the most suitable site on e.g. an mRNA corresponding to a target gene, for targeting for silencing e.g. with an anti-sense construct. With the information obtained using the methods and processes herein about, more efficient antisense reagents (not necessarily RNAs) may be produced which are tailored such that they would be recognised and used by the PTGS machinery of the organism.

In a further aspect of the present invention there is disclosed a method of silencing a target gene in an organism which utilises the methodology described above.

“Silencing” in this context is a term generally used to refer to suppression of expression of a gene. The degree of reduction may be so as to totally abolish production of the encoded gene product, but more usually the abolition of expression is partial, with some degree of expression remaining. The term should not therefore be taken to require complete “silencing” of expression. It is used herein where convenient because those skilled in the art will understand this.

In one embodiment, the method comprises introducing anti-sense molecules [SARMs] appropriate for the target gene into the organism in order to induce silencing. This could be done, for instance, by use of transcribable constructs encoding the SARMs.

In a related embodiment, the silencing may be achieved using constructs targeting those regions identified by the SRMs-based method disclosed above. Such constructs may e.g. encode anti-sense oligonucleotides which target all are part of the identified region, or a region within 1, 2, 3, 4, 5, 10, 15 or 20 nucleotides of the identified region.

Suitable target genes for silencing will occur to those skilled in the art as appropriate to the problem in hand. For instance, in plants, it may be desirable to silence genes conferring unwanted traits in the plant by transformation with transgene constructs containing elements of these genes.

Examples of this type of application include silencing of ripening specific genes in tomato to improve processing and handling characteristics of the harvested fruit; silencing of genes involved in pollen formation so that breeders can reproducibly generate male sterile plants for the production of F1 hybrids; silencing of genes involved in lignin biosynthesis to facilitate paper making from vegetative tissue of the plant; silencing of genes involved in flower pigment production to produce novel flower colours; silencing of genes involved in regulatory pathways controlling development or environmental responses to produce plants with novel growth habit or (for example) disease resistance; elimination of toxic secondary metabolites by silencing of genes required for toxin production. In addition, silencing can be useful as a means of developing virus resistant plants when the transgene is similar to a viral genome.

As described above, the disclosure herein provides evidence that SRMs may be a common mediator of PTGS in both plants and higher organisms. These new findings can be utilised, inter alia, in that it now appears that induction of SRMs (particularly SARMs) with an appropriate specificity in one organism (say, a plant) may be used to silence an appropriate target gene in another organism (say, a predator) which comes into contact with that plant.

In one aspect of the invention there is provided a method for targeting a gene in a first organism, which method comprises generating a SARMs silencing agent in a second organism, and introducing the SARMs into the first organism.

The SARMs may be generated by any appropriate silencing method. Preferably the target gene will be one which is not an endogenous gene in the second organism (but preferably is endogenous to the first). The “contact” may be ingestion, injection, or any other method of administration. How precisely, the method is performed will depend on the organisms and genes involved.

For instance, in the case of plants and plant predators, it is known that the systemic signal of PTGS travels out of plant cells into the phloem (sap) of plants and induces silencing in previously non-silencing parts of the plant. In the light of the present disclosure it is clear that, since plant parasitic nematodes feed directly upon the sap and contents of plant cells, they will ingest the signal and inducer of PTGS (i.e. SARMs) in the plant.

As shown in the Examples below, it appears that the same type of SARMs are present in C. elegans which are undergoing PTGS induced by the ingestion of dsRNA. This implies that the mechanism of PTGS in plants and nematode is similar.
if not identical. Thus plant SARMs may trigger the PTGS of any similar sequences present in the worm. Therefore when the nematode feeds on the plant, and eats the PTGS signal, if there is homology between the plant’s transgene from which the PTGS signal derived and a nematode gene, PTGS of that gene ought to be triggered in the worm.

Where the targeted gene is an essential gene, this method provides a means of controlling or killing plant predators or pests. Naturally, more than one gene can be targeted at once.

It may be desirable that the targeted gene is one which is either not present, or not important, in the wild-type plant or other potential consumers of the plant i.e. is nematode specific gene, such as a nematode protease gene. This gives the method a high degree of specificity.

Interestingly *C. elegans* is a nematode distantly related to the nematodes that parasitise plants. Since dsRNA induced PTGS is conserved between nematodes, protozoa and insects it is likely that these other organisms which support PTGS may be susceptible to SARMs.

dsRNA interference has also been shown to work in insects and transgene induced PTGS works in fungi, so it is likely that this is a mechanism that is broadly conserved across the kingdoms. This implies that any organism that directly feeds off plant cellular contents or extracellular components such as sap could ingest PTGS specific SARMs. If these have sequence homology with genes resident in the parasite, PTGS of these genes could be initiated.

Thus insect specific genes (e.g. from aphids) represent a further target. Most preferable would be those insect genes or sequences not found beneficial insects, such as ladybirds.

Other targets include genes specific for plant parasites of plants which feed off the host plant.

Specifically regarding higher animals (e.g. mammals, fish, birds, reptiles etc.) methods of the present invention include, inter alia:

(i) methods for detecting or diagnosing gene silencing, or silencing of particular genes, in the animal by using SARMs as described above,

(ii) methods for identifying silenced genes in the animal by using SARMs as described above,

(iii) methods for selecting target sites on genes to be silenced using SARMs as described above,

(iv) method for silencing a target gene in the animal, either directly, or through an animal-derived transgene in a second organism (e.g. a plant) as described above.

Generally speaking target genes in animals may be those whose functional impairment being therapeutic benefits. Typical genes of interest may be (for instance) those involved in apoptosis, cancer, cell-cycle regulation, neurological processes, signal transduction etc. Examples and references can be found in the Oncogene Research Products 1999 General Catalog, pp 21-265, available from Oncogene Research Products, 84 Rogers Street, Cambridge, Mass. 02142, U.S. Preferred examples include oncogenes, transcriptional regulators, pocket proteins, members of the MHC superfamily (to produce allootypic organs) etc.

Some further aspects and applications for the present invention will now be discussed.

According to one aspect of the present invention there is provided, preferably within a vector suitable for stable transformation of a plant cell, a DNA construct in which a promoter is operably linked to DNA for transcription in a plant cell to generate either:

(i) a SARM as described above, or

(ii) an anti-sense RNA molecule selected to target a region identified by the SRM-based methods discussed above.

Generally speaking, such constructs may be used to silence genes within plants, or within organisms predating or being administered material from plants, in the terms discussed above.

Anti-sense partial gene sequences selected in accordance with SRM-based methods may be used analogous to those previously used in the art. See, for example, Rothstein et al., 1987; Smith et al., (1988) *Nature* 334, 724-726; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588; English et al., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496. Generally the selected sequence will be less than 50, 40, 30, 25, or 20 nucleotides. It may be preferable that there is complete sequence identity in the targeting (e.g. foreign) sequence in the construct and the target sequence in the plant, although total complementarity or similarity of sequence is not essential.

Again, generally speaking, plants and associated methods and processes which form a part of the present invention are either those which:

(i) are transformed with the ‘targeting’ anti-sense vectors such as those described above, for instance so as to silence an (endogenous) target gene in the plant or perhaps a viral gene, or

(ii) are transformed with transgenes taken from other organisms such as to induce transgene silencing and thereby generate SARMs which can be used to silence a target gene in that other organism, or

(iii) are transformed with vectors which encode SARMs directly, which can be used for either purpose.

The general methodology discussed below will be applicable to all of these applications.

A vector which contains the construct may be used in transformation of one or more plant cells to introduce the construct stably into the genome, so that it is stably inherited from one generation to the next. This is preferably followed by regeneration of a plant from such cells to produce a transgenic plant. Thus, in further aspects, the present invention also provides the use of the construct or vector in production of a transgenic plant, methods of transformation of cells and plants, plant and microbial (particularly *Agrobacterium*) cells, and various plant products.

The function of the promoter in the construct is to ensure that the DNA is transcribed into RNA containing the viral sequences. By “promoter” is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3′ direction on the sense strand of double-stranded DNA). A promoter “drives” transcription of an operably linked sequence.

“Operably linked” means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter.

Preferred promoters may include the 35S promoter of cauliflower mosaic virus or the nopaline synthase promoter of *Agrobacterium tumefaciens* (Sanders, P. R., et al (1987), *Nucleic Acids Res.*, 15: 1543-1558). These promoters are expressed in many, if not all, cell types of many plants. Depending on the target gene of amplicon gs, other promoters including those that are developmentally regulated or inducible may be used. For example, if it is necessary to silence the target gene specifically in a particular cell type the construct may be assembled with a promoter that drives transcription only in that cell type. Similarly, if the target gene is to be silenced following a defined external stimulus the construct may incorporate a promoter that is to be activated specifically by that stimulus. Promoters that are both tissue specific and inducible by specific stimuli may be used. Suitable promoters
may include the maize glutathione-S-transferase isof orm II (GST-II-27) gene promoter which is activated in response to application of exogenous safener (WO93/01294, IC1 Ltd).

An additional optional feature of a construct used in accordance with the present invention is a transcriptional terminator. The transcriptional terminator from nopaline synthase gene of *Agrobacterium tumefaciens* (Depicker, A., et al. 1982). *J. Mol. Appl. Genet.* 1: 561-573 may be used. Other suitable transcriptional terminators will be well known to those skilled in the art.

Those skilled in the art are well able to construct vectors (including those based on ‘naked’ DNA) and design protocols for recombinant gene expression. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.


For introduction into a plant cell, the nucleic acid construct may be in the form of a recombinant vector, for example an *Agrobacterium* binary vector. Microbial, particularly bacterial and especially *Agrobacterium*, host cells containing a construct according to the invention or a vector which includes such a construct, particularly a binary vector suitable for stable transformation of a plant cell, are also provided by the present invention.

Nucleic acid molecules, constructs and vectors according to the present invention may be provided isolated and/or purified (i.e. from their natural environment), in substantially pure or homogeneous form, or free or substantially free of other nucleic acid. Nucleic acid according to the present invention may be wholly or partially synthetic. The term “isolate” encompasses all these possibilities.

An aspect of the present invention is the use of a construct or vector according to the invention in the production of a transgenic plant.

A further aspect provides a method including introducing the construct or vector into a plant cell such that the construct is stably incorporated into the genome of the cell.

Any appropriate method of plant transformation may be used to generate plant cells containing a construct within the genome in accordance with the present invention. Following transformation, plants may be regenerated from transformed plant cells and tissue.

Successfully transformed cells and/or plants, i.e. with the construct incorporated into their genome, may be selected following introduction of the nucleic acid into plant cells, optionally followed by regeneration into a plant, e.g. using one or more marker genes such as antibiotic resistance. Selectable genetic markers may be used consisting of chimeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorosulfuron, methotrexate, gentamycin, spectinomycin, and imidazolinones and glyphosate.

When introducing a nucleic acid into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material should occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants. Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711-8721 1984), particle or microprojectile bombardment (U.S. Pat. No. 5,100,792, EP-A-444882, EP-A-434616) microinjection (WO 92/00696, WO 94/00583, EP 331085, EP 175966, Green et al. 1987) *Plant Tissue and Cell Culture, Academic Press*, electroporation (EP 295039, WO 8706614 Gelvin Debyser—see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, U.S. Pat. No. 4,684,611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990)). Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotec. Adv.* 9: 1-11.


Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with *Agrobacterium* coated microprojectiles (EP-A-486234) or microprojectile bombardment to induce wounded following by co-cultivation with *Agrobacterium* (EP-A-486235).
Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practicing the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Also according to the invention there is provided a plant cell having incorporated into its genome a DNA construct as disclosed. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the construct into a plant cell. Such introduction should be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. RNA encoded by the introduced nucleic acid construct may then be transcribed in the cell and descendants thereof, including cells in plants regenerated from transformed material. A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, so such descendants should show the desired phenotype.

The present invention also provides a plant comprising a plant cell as disclosed.

A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders’ Rights.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed.

The present invention may be used in plants such as crop plants, including cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea and other root, tuber or seed crops. Important seed crops are oil seed rape, sugar beet, maize, sunflower, soybean and sorghum. Horticultural plants to which the present invention may be applied may include lettuce, endive, leafy brassicas including cabbage, broccoli and cauliflower, and carnations and geraniums. The present invention may be applicable to tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum, poplar, eucalyptus and pine.

In relation to use in mammals or other higher animals, DNA vectors (including naked DNA suitable for expression in mammals) of the present invention encode either:

(i) a SARM as described above, or

(ii) an anti-sense RNA molecule selected to target a region identified by the SARM-based methods discussed above.


Also provided by the present invention is an organism, preferably a non-human mammal, comprising cells in which a target gene is subject to PTGS by use of the SARM-based methods or materials disclosed herein. Particularly preferred is a rodent e.g. murine organism. In this embodiment the invention provides an alternative to known methods of producing ‘knock out’ mammals in which specific gene activities have been impaired (see e.g. Boerrigter et al (1995) Nature 377: 657-659, or Gossen and Vijk (1993) Trends Genet 9: 27-31.)

The invention will now be further described with reference to the following non-limiting Examples describing work of the inventors. The results are also discussed, and suggestions made as to the origin of the SRM of the present invention. However it will be appreciated by those skilled in the art that the materials, methods and processes in the present disclosure may be usefully applied irrespective of the precise underlying mechanisms involved.

All references discussed herein, inasmuch as they may be required to supplement the present disclosure, are incorporated herein by reference.

EXAMPLES

Example 1

Detection of SRMs in Silenced Plants

Analyses were performed to detect low molecular weight antisense RNA in four classes of PTGS in plants using the following general methods.

Total RNA was extracted from leaves of tomato, tobacco and *N. benthamiana* as described previously (E. Mueller, J. E. Gilbert, G. Davenport, G. Brigneti, D. C. Baulcombe, *Plant J.* 7, 1001 (1995)). From these preparations, low molecular weight RNA was enriched by ion exchange chromatography on Tiagen columns following removal of high molecular weight species by precipitation with 5% polyethylene glycol (8000)/0.5M NaCl (for tobacco and *N. benthamiana*) or (for tomato) by filtration through Centricon 100 concentrators (Amicon). Low molecular weight RNA was separated by electrophoresis through 15% polyacrylamide/7M urea/0.5x TBE gels, transferred onto Hybond N filters (Amersham) and fixed by UV crosslinking. Prehybridization was in 45% formamide, 7% SDS, 0.3M NaCl, 0.05M NaH₂PO₄/NaHPO₄ (pH7), 1x Denhardt’s solution, 100 mg.ml⁻¹ sheared, denatured, salmon sperm DNA at between 30°C and 40°C. Hybridization was in the same solution with single stranded RNA probes transcribed with a-³²P-labelled UTP. Before addition to the filters in the prehybridization solution, probes were hydrolysed to lengths averaging 50 nucleotides. Hybridization was for 16 hours at 30°C. (ACO probes), 35°C (GUS probe) or 40°C (GFP and PVX probes).

Sizes of RNA molecules were estimated by comparison with ³¹P phosphorylated RNA oligonucleotides run on the same gels but imaged separately. Additionally, samples from different types of PTGS including those discussed were frequently run on the same gel. Alignment of the filters following hybridization with different specific probes confirmed that the PTGS specific signals were identical in size. The probes
used are in each case sequence specific. We have observed no cross-hybridization between 25 nt signals in different PTGS systems using either filter hybridisation or RNAase protection.

We do not have an exact measurement of amount of 25 nt per cell, but given the short exposure times routinely used to detect these molecules and taking into account their size, they are likely to be very abundant in cells exhibiting PTGS.

Co-Suppression

The first class tested was transgene-induced PTGS of an endogenous gene (“co-suppression”). We used five tobacco lines (T2.1, T2.2, T5.1, T5.2, T5.3), each transformed with a tobacco 1-aminocyclopropane-1-carboxylate oxidase (ACO) cDNA sequence placed downstream of the cauliflower mosaic virus 35S promoter (35S). Two lines (T5.2, T5.3) exhibited PTGS of the endogenous ACO mRNA when amplified by RT-PCR and detected by hybridization with labelled ACO cDNA.

Low molecular weight nucleic acids purified from the five lines were separated by denaturing polyacrylamide gel electrophoresis, blotted, and hybridized to an ACO sense (antisense-specific) RNA probe. More specifically, the low molecular weight RNA and a 30-mer ACO antisense RNA oligonucleotide were fractionated, blotted and hybridized with either ACO sense RNA or antisense RNA transcribed from full length ACO cDNA. The low hybridisation temperature permitted some non-specific hybridization to rRNA and small tRNA species which constitute most of the RNA mass in these fractions. A discrete, ACO antisense RNA of 25 nucleotides (nt) was present in both PTGS lines but absent from the non-silencing lines. 25 nt ACO RNA of sense polarity and at the same abundance as the 25 nt ACO antisense RNA was also present only in the PTGS lines.

The 25 nt ACO antisense signal was completely abolished by pretreatment with either RNAaseOne or NaOH.

Transgene Silencing

PTGS induced by transgenes can also occur when a transgene does not have homology to an endogenous gene (1). Therefore we tested whether this type of PTGS was also associated with small antisense RNA. We analysed three tobacco lines carrying 35S-b-glucuronidase (GUS) transgenes. Two of these lines, T4 (15) and 6b5 (16) exhibited PTGS of GUS. The third line (6b5×271) tested was produced by crossing 6b5 with line 271 (17) in which there is a transgene suppressor of the 35S-promoter in 6b5. There was no PTGS of GUS in 6b5×271 due to the transcriptional suppression of the 35S GUS transgene (18).

Hybridization with a GUS-specific probe revealed that low molecular weight GUS antisense RNA was present in T4 and 6b5 but absent from line 6b5×271. 25 nt GUS antisense RNA was detected by hybridization with hydrolysed GUS sense RNA transcribed from the 3’ 700 bp of the GUS cDNA. The amount of antisense RNA correlated with the degree of PTGS: line 6b5 has stronger PTGS of GUS than line T4 (18) and also had more GUS antisense RNA. It appears that 25 nt antisense GUS RNA is dependent upon transcription from the 35S promoter.

As for PTGS of ACO in tobacco, the GUS antisense RNA was a discrete species of approximately 25 nt.

Systemically Induced Transgene Silencing

In some examples of PTGS, silencing is initiated in a localized region of the plant. A signal molecule is produced at the site of initiation and mediates systemic spread of silencing to other tissues of the plant (19, 20). We investigated whether systemic PTGS of a transgene encoding the green fluorescent protein (GFP) is associated with 25 nt GFP antisense RNA. PTGS was initiated in Nicotiana benthamiana expressing a GFP transgene by infiltration of a single leaf with *Agrobacterium tumefaciens* containing GFP sequences in a binary plant transformation vector.

More specifically, lower leaves of untransformed *N. benthamiana* and *N. benthamiana* carrying an active 35S-GFP transgene (35S-GFP) were infiltrated with *A. tumefaciens* containing the same 35S-GFP transgene in a binary vector. Two to three weeks following this infiltration, the GFP fluorescence disappeared due to systemic spread of PTGS as described previously (11, 20).

RNA from upper, non-infiltrated leaves of these plants and from equivalent leaves of non-infiltrated plants was hybridized with GFP sense RNA transcribed from a full length GFP cDNA. We detected 25 nt GFP antisense RNA in systemic tissues exhibiting PTGS of GFP. It was not detected in equivalent leaves of plants that had not been infiltrated or in non-transformed plants that had been infiltrated with the *A. tumefaciens* i.e. only the transgenic *N. benthamiana* infiltrated with the *A. tumefaciens* accumulated 25 nt GFP antisense RNA.

RNA-Mediated Defence Against Viral Infection

A natural manifestation of PTGS is the RNA-mediated defence induced in virus infected cells (8). Therefore we investigated whether virus-specific, 25 nt RNA could be detected in a virus-infected plant.

A high titre, synchronised PVX infection on leaves of untransformed *N. benthamiana*. was initiated by infiltration of single leaves with *A. tumefaciens* containing a binary plasmid incorporating a 35S-PVX-GFP sequence. Once transcribed, the PVX RNA replicon is independent of the 35S-PVX-GFP DNA, replicates to high levels and moves systemically through the plant. The *A. tumefaciens* does not spread beyond the infiltrated patch and is not present in systemic leaves (20). The GFP reporter in the virus was used to allow visual monitoring of infection progress. We have obtained similar signals with wild type PVX inoculated as virions in sap taken from an infected plant.

RNA was extracted from inoculated leaves after 2, 4, 6 and 10 days and from systemic leaves after 6 and 10 days. RNA was extracted from mock inoculated leaves after 2 days. 25 nt PVX antisense RNA was detected by hybridization with PVX sense RNA transcribed from a full length PVX cDNA. 25 nt RNA complementary to the positive strand (genomic) of potato virus X (PVX) was detected 4 days after inoculation of *N. benthamiana* and continued to accumulate for at least another 8 days in the inoculated leaf. 25 nt PVX RNA was not detected in mock inoculated leaves.

Discussion

Thus, 25 nt antisense RNA, complementary to targeted mRNAs, accumulates in four types of PTGS. We have also detected 25 nt RNA in other examples of PTGS as follows: *N. benthamiana* (spontaneous silencing of a 35S-GFP transgene), tomato (35S-ACO containing an internal direct and inverted repeat), petunia (co-suppression of chalcone synthase transgenes and endogenes) and *Arabidopsis thaliana* (PTGS of 35S-GFP by a 35S-PVX-GFP transgene).

However the 25 nt RNA has never been detected in the absence of PTGS. This correlation and the properties of 25 nt RNA are consistent with a direct role for them in PTGS induced by, for instance, transgenes or viruses (12). 25 nt RNA species also serve as molecular markers for PTGS. Their presence could be used to confirm other examples of e.g. transgene or virus-induced PTGS and may also serve to identify endogenous genes that are targeted by PTGS in non-transgenic plants. The 25 nt antisense RNA species are not degradation products of the target RNA because they have antisense polarity. A more likely source of these RNAs is the
transcription of an RNA template. This is consistent with the presence of the 25 nt PVX RNA in PVX infected cells that do not contain a DNA template. In a further experiment, low molecular weight RNA was extracted from plants containing silencing (S) or non-silencing (NS), 35S-ACC-oxidase (ACO, tomato) or 35S-GFP (N. benthamiana) transgenes. Each was hybridized with 32P-labelled RNA probes transcribed in the sense orientation from ACC-oxidase and GFP cDNAs and single stranded RNA then removed by digestion with RNAaseONE (Promega). The remaining protected RNA molecules were denatured, separated by electrophoresis on a 15% polyacrylamide/7M urea 0.5x TBE gel. The gel was dried and imaged by autoradiography. “+” and “−” consist of each probe incubated alone with or without subsequent digestion with RNAaseONE. With the ACO probe, protected fragments are obtained only with RNA from the ACO silencing tomato plants and with the GFP probe only with RNA from the GFP silencing plants illustrating the sequence specificity of the signal. The short RNA species detected in this assay correspond to the 25 nt RNA detected by northern analysis but are more discrete because of RNAase digestion at the ends of the RNA. Some higher molecular weight signals were also obtained, possibly as a result of incomplete digestion of single stranded regions.

The dependency of 25 nt GUS antisense RNA accumulation on sense transcription of a GUS transgene also supports the RNA template model. An RNA-dependent RNA polymerase, as required by this model, is required for PTG in Neurospora crassa (23). With the present data, we cannot distinguish whether the antisense RNA is made directly as 25 nt species or as longer molecules that are subsequently processed. The precise role of 25 nt RNA in PTG remains to be determined conclusively. However, as they are long enough to convey sequence specificity yet small enough to move through plasmodesmata, it is probable that they are components of the systemic signal and specificity determinants of PTGS.

Example 2
Detection of SRMs in Silenced Nematodes

RNA from Caenorhabditis elegans was obtained from Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, Md. 21210, USA. RNA was extracted by standard methods known in the art and was concentrated by ethanol precipitation and redissolved in formamide prior to analysis here.

Nematodes were selected which showed either PTG by ingestion of E. coli which synthesises double stranded GFP RNA or non-silencing of a GFP transgene.

Northern analysis of this RNA was performed generally as described above. RNA was fractionated by electrophoresis through a 15% polyacrylamide gel containing 7M urea and 0.5x Tris Borate EDTA buffer and electrophoretically transferred onto a Hybond N filter (Amersham) The membrane was placed on three layers of MM (Whatman) filter paper saturated with 20x SSC for 20 minutes and then allowed to dry at room temperature. The RNA was covalently linked to the membrane by Ultraviolet radiation crosslinking (“autoradiolink” setting in “Stratalinker” apparatus (Stratagene). The membrane was prehybridized 45% formamide, 7% SDS, 0.3M NaCl, 0.05M NaHPO4/Na2HPO4 (pH 7), 1x Denhardt’s solution, 100 mg.ml−1 sheared, denatured, salmon sperm DNA at 40° C. Hybridization was in the same solution with a single stranded RNA probe transcribed in the sense orientation with 32P-labelled UTP from the entire coding sequence of GFP. Before addition to the filter in the prehybridization solution, the probe was hydrolysed to lengths averaging approximately 50 nucleotides by incubation in 100 mM Na2HCO3/NaHCO3 (pH 10.2) at 60° C for 3 hours. Hybridization was for 16 hours 40° C. The membrane was washed at 50° C in 2xSSC/0.1% SDS and the radioactive signal imaged by a phosphorimager.

As in the previous example, 25 nt anti-sense RNA was detectable in the silenced material.

References

The invention claimed is:
1. A composition for introduction into a cell to effect gene silencing, consisting essentially of isolated short antisense RNA molecules (SARMs) and isolated short sense RNA molecules (SSRMs), collectively short RNA molecules (SRMs), wherein the SSRMs and the SARMs consist of 21-30 nucleotides;
2. wherein said SARMs are complementary to, and can base pair with, a target RNA, which target RNA is transcribed from a gene that is silenced when said SRMs are present in a cell containing said gene, and said SSRMs correspond to the target RNA; and
3. wherein said gene is endogenous to an organism selected from the group consisting of a plant, a mammal, an avian organism, a reptile, an insect, and a protozoan, or said target RNA is generated by a pathogen.
2. The composition of claim 1 wherein said SRMs are unmodified.

3. The composition of claim 1 wherein each SSRM and each SARM consists of 25 nucleotides.

4. The composition of claim 1 wherein each SSRM and each SARM consists of 26 nucleotides.

5. The composition of claim 1 wherein each SSRM and each SARM consists of 27 nucleotides.

6. The composition of claim 1 wherein each SSRM and each SARM consists of 28 nucleotides.

7. The composition of claim 1 wherein each SSRM and each SARM consists of 29 nucleotides.

8. The composition of claim 1 wherein each SSRM and each SARM consists of 30 nucleotides.

9. A composition for introduction into a cell to effect gene silencing, which composition comprises at least one vector which, when introduced into a cell, produces short antisense RNA molecules (SARMs) and short sense RNA molecules (SSRMs), said SARMs and SSRMs designated, collectively, short RNA molecules (SRMs), wherein the SSRMs and SARMs consist of 20-30 nucleotides;

10. The composition of claim 9 wherein said SARMs are complementary to, and can base pair with, a target RNA, which target RNA is transcribed from a gene that is silenced when said SRMs are present in a cell containing said gene, and said SSRMs correspond to the target RNA;

11. The composition of claim 9 wherein said gene is endogenous to an organism selected from the group consisting of a plant, a mammal, an avian organism, a reptile, an insect, and a protozoan, or said target RNA is generated by a pathogen.

12. The composition of claim 9 wherein each SSRM and each SARM consists of 25 nucleotides.

13. The composition of claim 9 wherein each SSRM and each SARM consists of 26 nucleotides.

14. The composition of claim 9 wherein each SSRM and each SARM consists of 27 nucleotides.

15. The composition of claim 9 wherein each SSRM and each SARM consists of 28 nucleotides.

* * * * *