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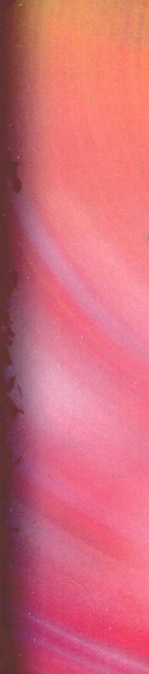
Gene Silencing

Theory, Techniques and Applications



GENETICS
RESEARCH AND ISSUES

Anthony J. Catalano
Editor

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GENETICS – RESEARCH AND ISSUES

GENE SILENCING: THEORY, TECHNIQUES AND APPLICATIONS

ANTHONY J. CATALANO
EDITOR

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GENE SILENCING: THEORY, TECHNIQUES AND APPLICATIONS

GENETICS – RESEARCH AND ISSUES

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Preface

Gene silencing is a general term describing the epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification. This new book reviews research in the study of gene silencing including RNA silencing in transgenic plants and mycorrhizal research, gene silencing in the CNS and on the most extensively studied systems to mediate siRNA and shRNA delivery into the brain, siRNA delivery strategies as a therapeutic tool in gene therapy, galectin-3 epigenetics and effective methods for selecting siRNA sequences by using the average silencing probability and a hidden Markov model.

Chapter I - This review aims to describe the state of and progress in the knowledge of RNA silencing in transgenic plants, including the experimental applications and new perspectives opened by the most recent studies. Modern plant breeding involves new technical approaches, and genetic transformation is undoubtedly a powerful tool in plant biology and plant pathology. However, genetic engineering does not always result in efficient transgene expression, and often transgene copy number does not correlate with transgene expression level. Research in the past decade has shed light on the importance of RNA silencing as a mechanism of virus resistance in transgenic plants. Several plants that are resistant to viruses have been obtained, and some have commercially been applied for crop protection on fields. Transgene silencing is part of a broad host defence system called RNA silencing, a process that leads to homologous RNA degradation, which has widely been observed in animals, plants, and fungi. A key feature of RNA silencing is the presence of small RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are processed by a member of the RNAse III-like enzyme family, known as DICER. In plants, several distinct RNA silencing pathways operate to repress gene expression at transcriptional or post-transcriptional level. Transcriptional silencing is associated with DNA methylation, in which DNA homologous to a dsRNA is methylated *de novo*. In addition to defence responses against viruses and transposons, short RNAs have been demonstrated to have a role in a diverse range of functions, including regulation of gene expression, development and chromatin structure. RNA silencing is also a powerful tool for functional genomic studies in several species. Transgene-mediated gene silencing through tissue-specific, partial and/or total gene inactivation is a convenient approach to study target genes functions, particularly in species for which mutant collections are not available. The authors review various strategies for small RNA-based gene silencing: viral expression vectors (virus-induced gene silencing,

VIGS), transgenes containing hairpin RNA structures and a recently introduced approach, based on artificial microRNAs (amiRNAs).

Chapter II - Mycorrhiza is a mutualistic association between fungi and the roots of the vast majority of terrestrial plants. In natural ecosystems the plant nutrient uptake from the soil takes place via the extraradical mycelia of these mycosimbionts. While most herbaceous plants and tropical trees form endomycorrhiza-type interactions, trees of boreal and temperate ecosystems are typically ectomycorrhizal (ECM). These species include the majority of ecologically and economically important trees and the fungal symbionts are predominantly filamentous basidiomycetes.

The symbiotic phase in the life cycle of ECM basidiomycetes is the dikaryon. Hence, studies on symbiotic relevant gene functions would require the inactivation of both gene copies in the dikaryotic mycelium.

RNA silencing is a sequence homology-dependent degradation of target mRNAs based on an ancient cellular mechanism believed to have evolved as protection of eukaryotic cells against alien nucleic acids. In different eukaryotic organisms, including fungi, the RNA silencing pathway can be artificially triggered to target and degrade gene transcripts of interest, resulting in gene knock-down. Most importantly, RNA silencing can act at the cytosolic level affecting mRNAs originating from several gene copies and different nuclei, and it can thus offer an efficient way for altering gene expression in dikaryotic organisms.

Laccaria bicolor, the first symbiotic fungus with its genome sequenced, has rapidly turned into a model fungus in ectomycorrhizal research. *Laccaria* possesses a complete set of genes known to be needed for RNA silencing in eukaryotic cells. The authors have demonstrated that RNA silencing is functional in *L. bicolor* and that it can be triggered via *Agrobacterium*-mediated transformation. Moreover, targeted gene knock-down in dikaryotic mycelium can result in functional phenotypes altered in the symbiotic capacity confirming that RNA silencing is a powerful way to study symbiosis- regulated genes. These findings have now initiated the RNA silencing era in mycorrhizal research, a field that has been hindered by the lack of proper genetic tools.

Chapter III - RNA interference (RNAi) has recently emerged as a powerful tool in functional genomic studies, allowing dissection of entire signalling pathways and elucidation of the molecular mechanisms of neurobiological processes, thereby facilitating rapid identification and validation of possible therapeutic targets. Moreover, RNAi holds great therapeutic potential since application of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) may allow specific knockdown of selected toxic proteins, even when allele-specific silencing is needed, as in the case of dominantly inherited disorders.

Nevertheless, the development of RNAi-based therapeutics for *in vivo* application faces the same challenge common to all classes of drugs: achieving an efficient and sustained distribution into the target tissue at sufficient concentrations to accomplish a therapeutic effect. Although significant progress has been made regarding the safety and stability of siRNAs and shRNAs, a major limitation for the *in vivo* application of RNAi technology concerns the inability of these molecules to cross cellular membranes. Multiple delivery methodologies, including viral and non-viral vectors, have been developed with different degrees of success for the introduction of siRNAs and shRNAs into cells, both *in vitro* and *in vivo*.

This review is focused on the available strategies to achieve gene silencing in the CNS and on the most extensively studied systems to mediate siRNA and shRNA delivery into the

brain. Moreover, the authors summarize the most important studies concerning RNAi application in the context of neurodegenerative diseases and other neurological disorders.

Chapter IV - Small interfering RNAs (siRNA) are emerging as promising therapeutic agents for the treatment of inherited and acquired diseases, as well as research tools for the elucidation of gene function. Since the molecules undergo rapid enzymatic degradation and have poor cellular uptake, there is a need to design a delivery system which can protect and efficiently transport siRNA to the target cells. Polymeric nanoparticles have emerged as systems of choice with reduced cytotoxicity and enhanced efficacy. These systems not only protect siRNA from enzymatic degradation by forming condensed complexes but also leads to tissue and cellular targeting, improve cellular penetration, release the siRNA in the right intracellular compartment. Nanoparticles prepared from polycationic polymers like polyethylenimine, chitosan have been widely investigated due to ease of manipulatibility, stability, low immunogenicity, low cost and high flexibility regarding the size of transgene delivered. This chapter presents an overview of siRNA delivery strategies employing polymeric nanoparticles, with emphasis on self-assembled polymeric nanoparticles with promising potential to evolve as therapeutic tool in gene therapy.

Chapter V - With the aim in view to improve physicochemical and biological properties of natural oligonucleotides, several types of DNA analogues and mimics were designed, particularly negatively charged PNA-like mimics. Among them, two types of DNA mimics representing hetero-oligomers constructed from alternating monomers of phosphono peptide nucleic acids and monomers on the base of *trans*-1-acetyl-4-hydroxy-L-proline (HypNA-pPNAs) as well as oligomers constructed from chiral analogues of peptide nucleic acids with a constrained trans-4-hydroxy-N-acetylpyrrolidine-2-phosphonic acid backbone (pHypNAs) were developed. Their physico-chemical and biological properties were evaluated in the comparison with natural oligonucleotides, classical peptide nucleic acids and morpholino phosphorodiamidate oligonucleotide analogues. The results obtained in a set of experiments revealed a high potential of these phosphonate-containing PNA derivatives for a number of biological applications, such as diagnostic, nucleic acids analysis and inhibition of gene expression. HypNA-pPNA and pHypNA mimics combine high hybridization and mismatch discrimination characteristics with good water solubility and biological stability as well as the ability to penetrate cell membranes. Their effectiveness to provide the specific knockdown of a target protein production was demonstrated in research involving *in vitro* systems, living cells and intact organisms. As their effect lasts over a period of several days, due to their high stability in living cells, it represents a very potent technology for administrating antisense- or antigene-based drugs for future therapeutic applications.

Chapter VI - Insects are organisms of considerable interest for comparative biology and medicine, therefore it is not surprising that several publications referred to them as model organisms. Insect and vertebrate evolution diverged more than 500 million years ago, but the molecular bases of several fundamental biological functions, including innate immune response, were already established in their common progenitor and have been conserved. Consequently, starting from information collected in insects, new insights into human biology and pathology were gained. Gene silencing includes several powerful methods, such as the production of loss-of-function mutants and RNA interference. These procedures, in particularly when performed in models for which molecular databases are already available, allow the genetic dissection of several immune-related processes and pathways. In the present review, we will concentrate our attention on the information derived from gene silencing

techniques on insect immune signalling with particular attention for *Drosophila melanogaster* and *Anopheles gambiae*.

Chapter VII - Sequencing of plant genome and expressed sequence tag (EST) have provided abundant sequence information in several plant species. Elucidating function(s) of all of these genes is a huge undertaking. Even in well-studied plants like Arabidopsis, function is not known for majority of genes. Hence, a powerful tool that can be widely used to understand gene function is necessary. Several functional genomics tools were developed in the recent past to achieve this goal. RNA interference (RNAi) is one such tool widely used to analyze gene function. RNAi is also proved to be a tool for plant researchers to produce improved crop varieties.

First part of this review is focused on three RNAi based concepts that has potential applications in plant functional genomics and agriculture. These concepts are tissue specific silencing, inducible silencing and host delivered RNAi (hdRNAi) during plant-pest interaction. Tissue specific promoters driving RNAi constructs can induce gene silencing in a particular organelle or tissue. Also, RNAi constructs with stress or chemical inducible promoters can be used to induce gene silencing only when required. These two concepts together can be used to achieve temporal and spatial control of gene silencing in plants. In the hdRNAi, dsRNA generated in an RNAi transgenic plant is delivered to interacting target organism (pest), activating gene silencing in the target organism. A comprehensive review pertaining to these areas is presented.

Second part of the review deals with applications of RNAi in agriculture, animal husbandry and biofuel industry. As suppression of gene expression by RNAi is inheritable, this has been a tool for developing transgenic crop plants for resistance against disease, pests, drought and in other areas of agriculture. This review summarizes developments in these areas with major emphasis on application of RNAi for development of biotic stress tolerant crops. We also note limitations of RNAi technology and ways to overcome the same.

Chapter VIII - Protein-carbohydrate interactions play significant role in modulating cell-cell and cell-extracellular matrix interactions, which, in turn, mediate various biological processes such as growth regulation, immune function, cancer metastasis, and apoptosis. Galectin-3, a member of the β-galactoside-binding protein family, is found multifunctional and is involved in normal growth development as well as cancer progression and metastasis, but the detailed mechanisms of its functions or its transcriptional regulations are not well understood. Besides, several regulatory elements such as GC box, CRE motif, AP-1 site, and NF-κB sites, the promoter of galectin-3 gene (*LGALS3*) contains several CpG islands that can be methylated during tumorigenesis leading to the gene silencing. This review discusses the galectin-3 epigenetics, which represents a novel regulatory mechanism of its transcription.

Chapter IX - Chronic infection with hepatitis B virus (HBV) occurs in approximately 6% of the world's population and is often complicated by cirrhosis and hepatocellular carcinoma (HCC). Existing therapy rarely has durable effects and improving treatment to counter the infection remains an important medical priority. Although harnessing the RNA interference (RNAi) pathway to achieve therapeutic HBV gene silencing holds promise, precise regulation of the expression of silencing sequences is critically important for safe application of this approach. Earlier work from our laboratory demonstrated that pri-miR-31- and pri-miR-122-based anti HBV shuttles were capable of potent, safe antiviral activity and can be used in modular multimeric arrangements. To advance this approach, and limit the potential problems caused by extrahepatic expression of anti HBV RNAi activators, these sequences were placed

under control of liver specific transcription control elements, viz. the human Factor VIII (FVIII), alpha-1-antitrypsin (A1AT), HBV preS2 and HBV basic core (BCP) promoters. Using a luciferase reporter gene assay optimal liver-specific transcription control was observed with A1AT and BCP regulating sequences. These elements were then incorporated into pri-miR-expression cassettes and were tested for antiviral efficacy in cell culture and a murine model of HBV replication. Results showed that silencing of HBV replication was achieved. Importantly there was no evidence for disruption of endogenous miR function, which is a significant advantage over use of stronger and constitutively active RNA polymerase (Pol) III promoter RNAi expression cassettes. The use of anti HBV pri-miR shuttles in the context of liver-specific Pol II promoters is likely to have usefulness for therapeutic HBV knockdown, and should also have general applicability to silencing of pathology causing genes in the liver.

Chapter X - Short interfering RNA (siRNA) has been widely used for studying gene functions in mammalian cells but varies markedly in its gene silencing efficacy. Although many design rules/guidelines for effective siRNAs based on various criteria have been reported recently, there are only a few consistencies among them. This makes it difficult to select effective siRNA sequences in mammalian genes. This chapter first reviews the recently reported siRNA design guidelines and then proposes a new method for selecting effective siRNA sequences from many possible candidates by using the average silencing probability on the basis of a large number of known effective siRNAs. It is different from the previous score-based siRNA design techniques and can predict the probability that a candidate siRNA sequence will be effective. The results of evaluating it by applying it to recently reported effective and ineffective siRNA sequences for various genes indicate that it would be useful for many other genes. The evaluation results indicate that the proposed method would be useful for many other genes. It should therefore be useful for selecting siRNA sequences effective for mammalian genes. The chapter also describes another method using a hidden Markov Model (HMM) to select the optimal functional siRNAs and discusses the frequencies of the combinations for two successive nucleotides as important characteristics of effective siRNA sequences.

Chapter XI - Small RNA-mediated gene silencing as a natural defense mechanism against viruses, transposons, and other invading nucleic acids or a means of regulating plant endogenous genes is a powerful tool and is being employed to down-regulate the expression of the targeted genes. Such a small RNA-mediated gene silencing has many different applications in a variety of organisms including humans and animals to control disease as a therapeutic agent, as well as plants to alter plant phenotypes. This silencing platform works through RNA-directed degradation or translational repression of target mRNA and has been devised towards a high-throughput approach for the gene suppression. In particular, sequence-specific control of gene expression by these non-coding RNAs has gained a significant amount of importance in plant biotechnology to influence specific plant phenotypes over the past years. It has been demonstrated that crops that were transformed with RNAi constructs, introduced stable modifications to the biochemical pathways. This can open new avenues in the improvement of crop productivity and quality. Here, the authors review the role of small RNA-directed gene silencing in plant biotechnology. The review will focus on the application of a gene silencing approach mediated by three subclasses of small RNAs for improved oil quality, reduced allergen, virus resistance, and other agronomical

traits. The advantages and drawbacks of each gene silencing approach are also discussed with regard to crop improvement.

Chapter XII - Several abiotic stress specific functional and regulatory genes have been cloned, and a number of EST databases representing stress specific genes are available for many plant species. These sequences have to be translated into functional information, necessitating the need for potential functional genomic approaches. Post transcriptional gene silencing (PTGS) is one of approaches to characterize functional relevance of stress responsive genes. Virus-induced gene silencing (VIGS) and developing stable gene knock down plants using hairpin RNA interference (hpRNAi) constructs (referred here as RNAi) are two important PTGS methods. Over a period, these methods are becoming integral part of plant stress functional genomics. Among these two methods, use of VIGS for characterizing abiotic stress responsive genes is still an emerging approach while RNAi has been widely used.

This review is focused on VIGS vector resources, brief methodology of VIGS and application of gene silencing to identify/characterize genes involved in drought-, salinity-oxidative-, high light-, and nutrient-stress management. VIGS can be used as fast forward genetic screening method to identify genes involved in stress tolerance and also an effective reverse genetic tool to validate the relevance of genes identified from high-throughput screening. Further, VIGS can be effectively integrated with abiotic stress imposition and response of gene silenced plants can be quantified using suitable techniques. We describe here an comprehensive approach to silence large number of cDNA clones and characterize the silenced plants under abiotic stresses. We also discussed application of other PTGS based methods like RNAi and artificial micro RNA (amiRNA) in abiotic stress functional genomics.

We propose that PTGS is an useful technology for translational genomics to assign function to large number of abiotic stress responsive genes. Even with their current limitations, gene silencing techniques are set to revolutionize plant abiotic stress functional genomics. Limitations and future directions for these techniques are also briefly discussed.

Chapter XIII - Small regulatory RNAs including short interfering RNAs (siRNAs) and microRNAs (miRNAs) are crucial regulators of gene expression at the posttranscriptional level. Recently, additional roles for small RNAs in gene activation and suppression at the transcriptional level were reported; these RNAs were shown to have sequences that closely or completely match to their respective promoter regions. However, no global analysis for identifying target sequences for miRNAs in the promoter region have been carried out in the human genome.

We performed a genome-wide search for upstream sequences of mRNA transcription start sites where miRNAs are capable of hybridizing with high complementarity. We identified 219 sites in the 10-kb upstream regions of transcription start sites with complete complementarity to 94 human mature miRNAs. Furthermore, the mismatched positions and nucleotides in near-completely matched sites were highly biased, and most of them appear to be possible target sites of miRNAs. The expression of downstream genes of miRNA target sites were examined following transfection of each miRNA into three different human cell lines. The results indicate that miRNAs dynamically modulates gene expression depending on the downstream genes and the cell type.

Chapter XIV - Gene silencing is an exciting field of functional genomics. It has been used as a research tool to discover or validate the functions of genes. It involves short sequence of nucleic acid that can bind to RNA of the gene and interferes the process of its

expression. It is diverse in occurrence as well as in applications. This phenomenon occurs from nematodes to fungi and can cause gene silencing in plants, animals and human beings. The core aspects of the mechanisms and functions of gene silencing include co-suppression, RNA-mediated virus resistance and RNA-directed DNA methylation (RdDM). The applications of gene silencing cover a wide spectrum in plants, from designer flower colors to plant-produced medical therapeutics. These functions are achieved by two types of approach such as protection of the plant against attack and fine-tuning of metabolic pathways. RNA-mediated gene control mechanism has already provided new platforms for developing molecular tools for gene function studies and crop improvements. We are now exploring this technology for commercially focused applications in plants. Here, we review the theory of gene silencing discovery and the mechanism of this technique in plants. Further, we discuss the potential use of this technique in plant science particularly in crop improvements.

Chapter XV - RNA interference (RNAi) has been utilized in a variety of applications to target specific gene silencing mediated by small-interfering RNA (siRNA) over the last few years. Cell-penetrating peptides (CPPs) were proven to be able to traverse cell membranes and deliver biological macromolecules into living cells. Here, we provided an efficient and safe method for the delivery of siRNA into mammalian cells mediated by CPPs noncovalently. We first established a GC-EGFP cell line stably expressing enhanced green fluorescent protein (EGFP) from human gastric cells. CPPs were demonstrated to interact with and deliver siRNA into GC-EGFP cells, and the internalized dsRNA tended to localize in the perinuclear region within cells. The sulforhodamine B (SRB) assay further confirmed CPPs were nontoxic to cell viability. Finally, our results showed that siRNA fulfilled its targeted *egfp* gene silencing. In the future, CPPs may provide a useful and nontoxic tool for the delivery of siRNA into mammalian cells.

Chapter XVI - RNA interference (RNAi) has become an indispensible technology for biomedical research and promises to usher in a brand new class of therapeutics that work by silencing disease genes. Until recently, the paradigm for gene silencing in mammalian cells has relied on a small symmetrical RNA structures containing a 19-base-pair duplex with 2 nucleotide overhangs at each 3' end: the standard siRNA structure. The standard siRNA scaffold is based on structures generated by Dicer digestion of a double stranded RNA, and is considered to be the fundamental template for designing RNAi inducers. In fact, early studies suggested there was only very limited flexibility regarding the length and symmetry of the siRNA structure in order to maintain optimal gene silencing. Recent studies, however, have demonstrated that gene silencing siRNAs with duplex lengths shorter than 19 bp or asymmetric structures can trigger specific gene silencing in mammalian cells. Importantly, asymmetric siRNA structures can ameliorate several sequence-independent, nonspecific effects triggered by the canonical siRNA structure. These findings demonstrate the structural flexibility of RNAi inducers in mammalian cells.

Contents

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Chapter I

Transgene Silencing in Plants: Mechanisms, Applications and New Perspectives

Chiara Pagliarani[1], Irene Perrone[1], Andrea Carra[2] and Giorgio Gambino[3],*

[1]Department of Arboriculture and Pomology, University of Torino. Via L. da Vinci 44, I-10095, Grugliasco (TO), Italy

[2]Research Unit for Wood Production Outside Forests, CRA, Strada Frassineto 35, I-15033, Casale Monferrato (AL), Italy

[3]Plant Virology Institute, National Research Council, Grugliasco Unit, Via L. da Vinci 44, I-10095 Grugliasco (TO), Italy

Abstract

This review aims to describe the state of and progress in the knowledge of RNA silencing in transgenic plants, including the experimental applications and new perspectives opened by the most recent studies. Modern plant breeding involves new technical approaches, and genetic transformation is undoubtedly a powerful tool in plant biology and plant pathology. However, genetic engineering does not always result in efficient transgene expression, and often transgene copy number does not correlate with transgene expression level. Research in the past decade has shed light on the importance of RNA silencing as a mechanism of virus resistance in transgenic plants. Several plants that are resistant to viruses have been obtained, and some have commercially been applied for crop protection on fields. Transgene silencing is part of a broad host defence system called RNA silencing, a process that leads to homologous RNA degradation, which has widely been observed in animals, plants, and fungi. A key feature of RNA

* Author for correspondence. Phone: +39-011-6708666; Fax: +39-011-6708658; E-mail: g.gambino@ivv.cnr.it

silencing is the presence of small RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are processed by a member of the RNAse III-like enzyme family, known as DICER. In plants, several distinct RNA silencing pathways operate to repress gene expression at transcriptional or post-transcriptional level. Transcriptional silencing is associated with DNA methylation, in which DNA homologous to a dsRNA is methylated *de novo*. In addition to defence responses against viruses and transposons, short RNAs have been demonstrated to have a role in a diverse range of functions, including regulation of gene expression, development and chromatin structure. RNA silencing is also a powerful tool for functional genomic studies in several species. Transgene-mediated gene silencing through tissue-specific, partial and/or total gene inactivation is a convenient approach to study target genes functions, particularly in species for which mutant collections are not available. We review various strategies for small RNA-based gene silencing: viral expression vectors (virus-induced gene silencing, VIGS), transgenes containing hairpin RNA structures and a recently introduced approach, based on artificial microRNAs (amiRNAs).

Introduction

RNA silencing, a process leading to the degradation of homologous mRNAs, has been widely observed in animals, plants, and fungi [1]. In the early '90s, gene silencing phenomena were first noted through a surprising observation that occurred during the course of plant transformation experiments: the introduction of transgenes inside the genome led to silence both transgenes and their homologous endogenes. Napoli and collaborators [2], with experiments on a chimeric chalcone synthase (*chs*) gene over-expressed in petunia (*Petunia hybrida*) petals, discovered a co-suppression process of both the transgene and the homologous mRNA endogenous sequence. In fungi, Romano and Macino [3] described transient gene expression inactivation events in *Neurospora crassa* transformants and called the discovered silencing process "quelling"; in animals, Lee and colleagues [4] carried out experiments on the nematode *Caenorhabditis elegans*, finding that the gene *lin-4* encodes for a small RNA that strongly regulates larval developmental transitions. Since those results, several improvements have been made to achieve a whole comprehension of RNA silencing pathways; today, most functions involved in this biological process have been described. A key feature of RNA silencing is the presence of small RNAs, which were first observed in plants [5], such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are processed from double-stranded RNAs (dsRNAs) by a family of the RNAse III-like enzyme, known as DICER [6]. RNA silencing is an important mechanism used by plants to defend themselves against viral infections and transposons. In addition, short RNAs have been demonstrated to have a role in a diverse range of functions, including regulation of gene expression, development and chromatin structure.

As in co-suppression, genetic engineering does not always result in efficient transgene expression levels. Several cases have been reported, where the transgene copy number does not correlate with the level of transgene expression [7]. Two types of events contribute to the realization of transgene silencing. The first is linked to the specific genomic site in which the T-DNA integrates itself [8], whereas the second closely depends on the configuration of the integrated T-DNAs. Indeed, it is possible that multiple T-DNAs, integrated at one locus, bind

to each other to form complex T-DNA structures [7]. Over the last decade, transgene silencing has no longer become just an unforeseen consequence of genetic transformation, but it has been induced with high efficiency through various strategies, using viral expression vectors (virus-induced gene silencing, VIGS), transgenes containing hairpin RNA structures, and a recently introduced approach based on artificial microRNAs (amiRNAs).

There are numerous possible applications for transgenic silencing in plants. Initially, the main research field was focused on enhancing disease resistance. Several plants resistant to viruses have been obtained to date, and some have commercially been used for crop protection on fields. Gene silencing has been applied to change the chemical composition of plant products, for industrial use or to improve fruit quality, and to increase the nutritional value. In parallel, other applications have been tested to reduce the production of plant-derived substances, as in the case of allergens, which can be harmful to human health if present in nutritious foods. In recent years, functional genomic studies have been gaining more and more importance, as they can provide interesting insights about genetic regulation of plant functions and support novel tools for isolating and characterizing genes. Transgene silencing through tissue-specific, partial and/or total gene inactivation is a convenient approach to study target gene functions, in particular in species for which mutant collections are not available.

This review focuses on the main molecular mechanisms involved in plant RNA silencing, with particular attention to transgene-mediated gene silencing. We report the major applications and future perspectives of this technology applied to the most important woody and herbaceous crop plants.

Mechanisms of Gene Silencing

RNA silencing evolved in eukaryotes to regulate gene expression, to control transposable elements and to fight against pathogens. In plants, several distinct RNA silencing pathways operate to repress gene expression at the transcriptional (transcriptional gene silencing, TGS) or post-transcriptional level (post-transcriptional gene silencing, PTGS) [9]. RNA silencing pathways are characterized by the production of double stranded RNAs (dsRNAs) from endogenous or exogenous transcripts, or by the production of self-complementary foldback RNAs. These dsRNAs are digested by a family of RNAse III-like enzymes, termed DICER, into short RNA duplexes of 21-24-nt length, referred to as short interfering RNAs (siRNAs) or microRNAs (miRNAs) [10]. Once generated, siRNAs and miRNAs guide the sequence-specific inactivation of a target mRNA via an RNA-induced silencing complex (RISC), which includes a member of the Argonaute (AGO) proteins, a family of enzymes with endonucleasic activity. RISC mediates cleavage or translational repression of target mRNAs. RNA silencing also involves RNA-directed DNA methylation (RdDM), in which DNA homologous to a dsRNA triggering gene silencing is methylated *de novo* [11]. Small RNAs guide the RNA-induced transcriptional gene silencing (RITS) complex to direct chromatin modifications and DNA methylation of the homologous DNA sequences [12]. In mammals, DNA methylation occurs almost exclusively on cytosines in the symmetric di-nucleotides CpG, whereas in plants, cytosine methylation occurs both at symmetric sites (CpG and CpNpG, where N is A, T or C) and at asymmetric sites (CpNpN).