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RNAi for revealing and engineering plant gene functions

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RNA interference (RNAi) is now widely used in plant biotechnology, both as a useful tool for discovering or validating gene functions as well as a quick way of engineering specific reductions in expression of chosen genes. Although the amazing popularity of RNAi as a biotechnology tool is certainly justified, the underlying biology is still being worked out and the relative advantages and disadvantages of the approach are only now becoming clear. Recent breakthroughs in elucidating the multiple pathways of RNA-based post-transcriptional control and preliminary results from the first large-scale uses of RNAi in plants will make it easier to gauge the usefulness of the technique. To fully capitalize on the potential of RNAi, we need to become better at predicting and controlling its effects.

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Introduction

Gene-based biotechnology has three basic phases: a gene discovery phase, where the genes responsible for desired characteristics are identified; a validation phase, where the expression of a chosen gene is modified to check that the desired result is obtained; and an implementation phase, where the desired alterations are introduced into the germplasm to be used commercially. RNA interference (RNAi) is a quick, easy, sequence-specific way to 'knock-down' the expression of chosen genes. In principle, it allows the scientist to quantitatively reduce the expression of any specific target gene or group of genes at will, and as such it shows enormous promise for all three phases of gene-based biotechnology. So far, its primary use has been for gene discovery and validation of gene function. This review will cover the latest research on RNAi from the viewpoint of potential uses in plant biotechnology, rather than the underlying science. The review concentrates on general aspects of RNAi technology rather than on examples of individual applications and should be helpful to any researchers and biotechnologists keen to employ RNAi in their projects.

Mechanisms of RNAi

It is now clear that many genes in most eukaryotes are regulated to some extent by transcriptional and post-transcriptional silencing mediated by small interfering RNAs (siRNAs) produced from double-stranded templates by enzymes of the Dicer family [1]. These 21–24 nucleotide RNAs confer sequence specificity to various inhibitory effector complexes that can cleave or sequester mRNAs, preventing their translation, or inhibit transcription by altering chromatin configuration and/or methylation status around the siRNA-binding site. This whole area of research is still in an exciting state of flux as new components and pathways are discovered; the complexity of the processes involved has been a great surprise, particularly in plants. A couple of excellent recent reviews have been published that cover the range of inhibitory pathways shown or suspected to exist [2•,3]. From the point of view of the various biotechnological uses of RNAi, several points, all revealed by fundamental research into the mechanisms of RNAi, need to be borne in mind when planning experiments (Box 1).

RNAi screens in other organisms

RNAi has been used on a genome-wide scale for gene discovery in those model organisms where delivery of siRNAs is easy. The best examples come from *Caenorhabditus elegans* where feeding bacteria expressing double-stranded RNA is sufficient to trigger RNAi in many cases; this extremely simple delivery system has prompted multiple large-scale screens with spectacular success (e.g. [4]). In other animals, delivery is more difficult and usually consists of transfection with plasmids capable of expressing short hairpin (hence double-stranded) RNAs or direct transfection with synthetic siRNAs. Several large libraries of such resources are now available [5,6] and are being used with some success (e.g. [7]), although problems with specificity have arisen (discussed later).

RNAi delivery in plants

Plant cells do not eat bacteria and the cell wall prevents easy delivery of siRNAs into the cell. Thus, delivery of siRNAs into plants has almost always been achieved by expressing hairpin RNAs that fold back to create a double-stranded region that acts as substrate for the Dicer-like enzymes. Such hairpin RNAs are potent inducers of RNAi and give rise to copious siRNAs derived from the double-stranded region. The RNAi 'craze' in

Box 1 Points to consider when planning RNAi experiments.

The sequence specificity of RNAi cannot exceed about 20 nucleotides and is often less than this

Multiple inhibitory mechanisms exist, such that reduction in expression can be obtained without an obvious effect on steady-state mRNA levels

The silencing 'signal' can in some cases be transmitted from cell to cell and even over long distances throughout the plant

Some pathways involve RNA-dependent RNA polymerases such that siRNAs can, under some circumstances, self-propagate, leading to a potential loss in control over the intended silencing signal

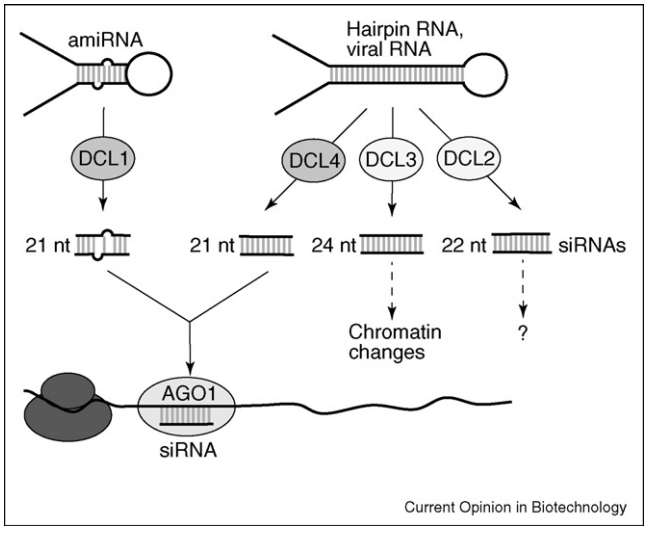
Many endogenous genes are regulated by natural RNAi pathways, so perturbations in these processes can lead to pleiotropic effects

plants was launched by Smith *et al.* [8] and many popular and widely used protocols have been developed from this early work [9•–11•]. Since then, the complexity of the various RNAi pathways in plants has slowly become apparent, and much work has gone into discovering the mode of action of RNAi induced by long hairpin RNAs. It is probable that several different pathways are activated, but the major route appears to be via the DCL4 pathway (Figure 1) [12••,13••]. This RNAi pathway is also activated by viral RNAs and is a major line of defence against RNA viruses. In fact, modified viruses have been used to trigger RNAi and virus-induced gene silencing (VIGS) is a promising approach to knock down gene expression. VIGS has the advantage that it negates the need to introduce a transgene into the target plant genome, and thus permits RNAi to be carried out in plant species that are recalcitrant to genetic transformation (reviewed in [14,15]). The direct delivery of siRNAs into protoplasts or of double-stranded RNA into cells by biolistics or wounding is also possible, but it is not practicable on a large scale and thus is little used. The different RNAi delivery platforms in plants have been reviewed quite recently [16].

A promising new approach is to more closely mimic natural microRNAs (miRNAs). miRNAs are natural, endogenous siRNAs produced by DCL1 from specific hairpin precursor transcripts. They play important, often evolutionarily conserved roles in the control of expression of a significant number of plant genes (reviewed in [2•,3]). It has recently been shown that by deliberately modifying the sequence of miRNA precursors, so-called artificial miRNAs (amiRNAs) can be generated that will efficiently cleave different target transcripts [17••]. This approach still requires genetic transformation of the plant, but the amiRNAs produced by the transgene will be processed by the DCL1 pathway (Figure 1). The greater precision and strand-specificity of the processing is expected to lead to improved targeting specificity and reproducibility.

Most uses of RNAi to date have generated constitutive expression of the inhibiting siRNAs, but some of the most exciting uses of RNAi will rely on controlling the expression of the triggering hairpin RNAs or amiRNAs to achieve tissue-specific or inducible inactivation of expression of the target gene. Several 'proof-of-principle' examples have been published [18–21], but none of the possible approaches has yet taken the scientific community by storm.

Figure 1



The multiple pathways of RNAi in plants. RNAi can be induced in plants by expressing artificial microRNAs (amiRNA), long hairpin RNAs, modified viral RNAs or by directly introducing synthetic small interfering RNAs (siRNAs). amiRNAs are processed by the enzyme Dicer-like 1 (DCL1), whereas hairpin RNAs and viral RNAs are predominantly processed by DCL4. The siRNAs produced are loaded into the RNAi silencing complex of which AGO1 is a major component. The AGO1–siRNA complex inhibits the expression of mRNAs carrying complementary sequences by cleaving the target mRNA or preventing its translation. Some of the sometimes unpredictable effects of RNAi can result from the production of siRNAs through the DCL2 and DCL3 pathways; for example, the 24 nt siRNAs produced by DCL3 cause chromatin modifications that can lead to transcriptional silencing of the target gene or of the hairpin-producing transgene. Mutations in *DCL3* exacerbate RNAi phenotypes [13]. The subcellular location of the various processes indicated here are not always entirely clear [2•,3]. Processing by DCL enzymes takes place in the nucleus and AGO1-mediated cleavage is predominantly cytosolic, although there may be exceptions. siRNAs produced by DCL3 function in the nucleus. How siRNAs are transported around plant cells (particularly between the nucleus and cytosol) is not yet worked out in detail. As yet, there is no reason to think that any of these RNAi processes occur within plant mitochondria or chloroplasts.

Advantages of RNAi for gene discovery in plants

On the basis of the plant genomes sequenced so far, the minimal set of plant genes is likely to number at least 20,000 and many plants will have significantly more than this owing to alloploidy or genome duplication. Discovering the functions of all of these genes is a huge undertaking and an explicit target of the scientific community. Current estimates, even for a plant as well-studied as

Box 2 Advantages of using RNAi to determine gene function.

RNAi is sequence-specific and thus can be targeted, requiring only a few transformants per target gene

RNAi is dominant, so phenotypes can be observed in the T1 generation

RNAi often leads to partial knockdown and thus to a range of phenotypes of differing severity; this facilitates the study of essential genes whose inactivation would lead to lethality or extremely severe pleiotropic phenotypes

RNAi can be controlled in a tissue-specific or time-dependent manner

RNAi can be quickly and easily used in a wide range of genotypes or even species, whereas insertion mutant collections are limited to just a few due to the effort involved

RNAi can be used to reduce the expression of several related genes in parallel by targeting conserved regions of the genes, facilitating the study of redundant gene functions

Arabidopsis, suggest that for the majority of genes we have only sequence and expression data, and no true experimental evidence of function. Genetic approaches are strongly favoured in plants for identifying gene function, but obtaining mutants in every gene using untargeted approaches such as chemical mutagenesis or insertional mutagenesis will require massive effort [22,23]. RNAi offers an easy, cost-effective approach to generate 'phenocopies' of genetic mutants. In theory, there are numerous advantages to RNAi with respect to the highly popular and widely used insertional mutagenesis approaches (Box 2). These theoretical advantages have led to several large-scale studies designed to employ hundreds or thousands of RNAi constructs to examine their efficacy in high-throughput gene function screens of the type so successfully employed in model animal systems (see Box 3). The results of these studies should be available soon and will hopefully provide definitive answers to the questions that remain over the use of this technology in plants.

Box 3 Large-scale studies employing RNAi to determine gene function.

AGRIKOLA (www.agrikola.org) a project funded by the European Union to produce resources for targeting up to 25 000 *Arabidopsis* genes by RNAi [10•]. About 3000 of these constructs are being used to transform plants and the phenotypes examined.

ChromDB (ChromDB.org) is a project funded by the National Science Foundation (NSF) that is generating hundreds of RNAi lines in *Arabidopsis* and maize with reduced expression of specific chromatin-associated proteins [11•].

The Medicago truncatula RNAi database (www.medicago.org/rnai/) is an NSF-funded project planning to silence 1500 genes involved in symbiosis in this model legume.

amiRNA Central (http://2010.cshl.edu/scripts/main2.pl) is a new NSF-funded project to provide a comprehensive resource for knockdown of *Arabidopsis* genes.

Disadvantages and pitfalls of RNAi

Despite the impressive list of potential advantages, RNAi is not without a similarly long list of potential disadvantages that need to be borne in mind (and preferably eliminated or worked-around).

Off-target effects

It has become clear from the large-scale screens in animals that significant effects on the expression of genes that were not the predicted targets of RNAi can occur [24–27]. The major difficulty is the limited sequence specificity of siRNAs — as few as seven nucleotides of sequence complementarity between an siRNA and an mRNA can lead to the inhibition of expression [28]. The possibility of productive siRNA–mRNA interactions despite scattered mistmatches in the paired region makes the prediction of potential off-target effects very difficult. It has been often repeated that such problems are much less frequent in plants, but this might be simply because no systematic studies have yet been completed. The isolated reports to date conclude that RNAi in plants exhibits much greater sequence specificity [29]. A second specificity problem can occur via 'transitive silencing', whereby RNAi against a gene-specific sequence 'spreads' into neighbouring sequences conserved between the target mRNA and mRNAs from related genes, which become silenced in turn [30–32]. Such 'transitive silencing' results from the action of RNA-dependent RNA polymerases that maintain and amplify the RNAi signal in some pathways. However, transitive silencing appears to occur rarely even when suitable conserved sequences exist in the targeted RNAs.

A completely different type of off-target effect can occur owing to the inhibition of natural miRNA or siRNA regulation through saturation of the pathways with exogenous or transgene siRNAs. This has again been observed in animal systems [33], but there are no published reports so far of this effect in plants. In fact, even when specifically searched for, no off-target effects of any type were observed, even when very strong promoters were used to drive hairpin RNA production [34].

Inefficacy and instability

A mutation at the DNA level (base change, deletion or insertion) is almost always irreversible (except in the special case of some natural transposon insertions) and the effect on the function of the affected gene generally predictable (i.e. premature stop codons or insertions into the middle of a gene usually lead to null phenotypes). By contrast, RNAi inhibition can have widely varying effects depending on the target gene, the region of the transcript that is targeted and even between sibling plants carrying identical RNAi constructs [35]. The reasons for this variability are multiple and need to be considered when interpreting RNAi phenotypes. Firstly, short siRNAs

might target a part of the mRNA that is masked by secondary structure or bound proteins, thus reducing efficacy through inaccessibility of the target site to siRNAs. Secondly, inefficacy could result from an inability to reduce translation to the point where a phenotype ensues; for example, some genes can have their expression reduced drastically without generating the phenotype. Lastly, instability can result from silencing of the transgene — long hairpin transgenes appear to be particularly sensitive to transcriptional silencing leading to a loss of RNAi phenotypes over several generations.

The extent of these problems is still uncertain in plants, but the high-throughput screens mentioned earlier should give definitive answers. Preliminary results from the AGRIKOLA project suggest that up to 50% of *Arabidopsis* genes are difficult to silence using long hairpin RNAi constructs. Workarounds to each of these problems exist, for example, comparing results between two or more constructs and using transformation protocols that limit the number of hairpin transgenes introduced to help avoid transgene silencing [11•].

Validation of RNAi knockdown

Given these potential problems with RNAi, careful thought needs to be given to validating the results obtained. Typically, genetic mutations are validated by complementation with a wild-type gene, but this is impossible for RNAi mutants as the wild-type transgene would be silenced in turn. A variant of this process can be used in conjunction with the amiRNA approach by complementing with a gene modified to change the amiRNA binding site without changing the encoded amino acid sequence [17••]. For long hairpin RNAi, at a minimum, one should examine multiple independent lines to check for a reproducible phenotype and attempts should also be made to check that genes related to the target gene are not being affected by off-target effects. In many cases, RNAi is best used as an easy initial screen for interesting phenotypes that then need to be validated by other methods.

Uses of RNAi in applied plant biotechnology

In species where reverse genetics is difficult owing to a lack of suitable mutant collections (and that means almost all plant species except *Arabidopsis* and rice), RNAi is a popular approach for validating the function of candidate genes predicted to be involved in interesting traits on the basis of homology or identified by genetic mapping. RNAi has also been used to deliberately engineer desired characteristics in various plants, generally as a 'proof-of-principle' demonstration rather than as an intended commercial release. Many reports (far too numerous to list here) describing the successful use of RNAi for these purposes have been published (e.g. see [36,37] for reviews). TILLING [38] should be preferred to RNAi for irreversibly reducing or eliminating the expression of target genes in commercial crop plants, as it avoids genetic transformation and will provide more stable, predictable lines. RNAi will find uses, however, in providing pathogen resistance by targeting pathogen RNAs (e.g. [39]) and in manipulating cells cultured to produce high-value secondary metabolites [40]. It will also be invaluable where tissue-specific or inducible reductions of expression are required. An excellent example of this is the production of cotton plants capable of producing oil free of gossypol toxin [41••].

Conclusions

The study of RNAi has led to a revolution in the understanding of gene expression, as underlined by the recent award of a Nobel prize on the topic. Scientists were quick to apply RNAi as a new tool and the resources are now available for relatively quick, cheap screens of gene function in easily transformable plants. Currently, problems with efficacy, stability and validation limit the uses of RNAi for both scientific and commercial applications, but the rapid pace of discovery will lead to continuous improvements in biotechnological uses of RNAi. New approaches such as amiRNA promise to bring more precision and predictability to the technology, and we have yet to make full use of tissue-specific and inducible RNAi that offer potentially unparalleled control over plant gene expression.

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