RNAi for Plant Improvement and Protection

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RNAi for Plant Improvement and Protection
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We also acknowledge the contributions of the authors to their chapters in this book. The authors are from a wide range of countries, organizations and disciplines and present a range of perspectives on RNAi. We thank them for imparting their experience and expertise.
1 Introduction to RNAi in Plant Production and Protection

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RNA interference (RNAi) has the potential to have a major impact on agriculture, horticulture and forestry with many different applications for plant improvement in terms of both quality of products and productivity. In addition, crop protection applications are being developed which provide ‘green’ alternatives to conventional pest control methods. RNAi is a naturally occurring process present in plants and animals, in which double-stranded RNA (dsRNA) molecules interfere with homologous RNA. It allows genes to be targeted to remove unwanted products in plants and improve plant productivity and quality of plant products. These RNAi mechanisms were only discovered and described 20 years ago and their discovery led to a Nobel prize in 2006. RNAi is now being developed within plants to silence genes often described as host-induced gene silencing (HIGS). Also, external and topical applications, such as sprays and seed treatments, are being developed to substitute for other types of pesticides or growth regulator treatments. An example is the spray-induced gene silencing (SIGS) approach for targeting pest and pathogen genes and for manipulating endogenous gene expression in plants. Examples of plant improvement applications include: improving fatty acid profiles of soybeans; delayed ripening and improved shelf life of fruits such as apples and tomatoes; or removing unwanted compounds, toxins and allergens from crop products such as decaffeinated coffee, gossypol in cotton seeds and hypoallergenic fruits and cereals.

For pest and disease control applications, dsRNA can be selected for silencing essential genes in pests, pathogens and viruses, expressed either in transformed plants or in exogenous applications. dsRNA can be very specifically targeted at genetic sequences in these targets so that off-target effects are avoided or minimized. Recent advances in genomics and transcriptomics have provided sequence data that enable the design of highly targeted dsRNAs, providing efficient silencing while minimizing the risk of effects on off-target genes or the silencing of gene expression in non-target organisms. Due to the involvement of RNA in virus replication, several virus-resistant plants have been developed (e.g. papaya, plum, squash and tomato) and many more virus control applications are in the pipeline. More recently, plant resistance
to a range of other pests and fungal diseases is being developed, including insect pests such as Colorado potato beetle (*Leptinotarsa decemlineata*) and insect vectors of viruses. The fungal disease targets include a range of diseases such as cereal rusts and *Botrytis* grey mould on fruit. In the USA, maize transformed to express a dsRNA targeting a gene in corn rootworm (*Diabrotica* spp.) has been developed and commercialized.

RNAi provides additional options for plant breeders to improve plant varieties compared with other new breeding techniques (NBTs) such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, transcription activator-like effector nucleases (TALENs). For example, RNAi provides a method for reducing gene expression (knockdown) rather than complete blocking of the expression (knockout). This is important for providing reduced levels of gene expression, or when a specific stage in a physiological process is to be targeted. Another important feature of RNAi is that dsRNA molecules can be highly mobile in plants. Therefore, dsRNA produced in part of the plant (e.g. rootstock) can have the potential to spread into the grafted parts of the plant to confer resistance to disease to the whole plant, including fruit. This results in fruits that are not genetically modified but protected by the presence of target-specific degradable small RNA molecules (Limera *et al.*, 2017). In addition, dsRNA molecules can be formulated and applied as a topical treatment to plants to change their physiology or combat pests and pathogens. This approach will avoid genetically modified organism (GMO) regulations if no GMOs are present in the products.

Research on RNAi is being conducted mainly in Europe, the USA and China. However, in Europe and some regions of the world the technology and its applications are being held back by policies and legislation on biotechnologies, by failures in the implementation of GMO regulations and by failure to develop appropriate methods for the regulation and assessment of novel plant protection products. This is inhibiting investment in research and development (R&D) on novel ‘green’ applications of RNAi, as can be seen by the reduction in patent applications in Europe. It has been shown that RNAi has the potential to make major contributions towards sustainable crop production and protection with minimal environmental impacts compared with other technologies. In regions where legislation prevents the use of RNAi technology, farmers will not have access to the technology and important options for improving productivity and economic competitiveness (Taning *et al.*, 2019; Mezzetti *et al.*, 2020). Ironically this will be at a time when governments are trying to introduce more sustainable ‘green’ agricultural practices and when food demand is increasing and food supplies are at risk from climate change, new invasive species and urbanization.

In 1971 a European Cooperation in Science and Technology (COST) programme had been created. In 2016 the iPlanta COST Action CA15223 ‘Modifying plants to produce interfering RNA’ (available at https://iplanta.univpm.it, accessed 1 November 2020) was established with the objective of bringing together experts from a wide range of fields to develop a deeper understanding of the science of RNA, the applications of RNAi, the biosafety of these applications and the socio-economic aspects of these potential applications. This book contains a series of chapters by experts from many countries, who are participating in iPlanta, to review the current scientific knowledge on RNAi, methods for developing RNAi systems in GM plants and a range of applications for crop improvement, crop production and crop protection. Chapters examine both endogenous systems in GM plants and exogenous systems where interfering RNAs are applied to target plants, pests and pathogens. The biosafety of these different systems is examined and methods for risk assessment for food, feed and environmental safety are discussed. Finally, aspects of the regulation of technologies exploiting RNAi and the socio-economic impacts of RNAi technologies are discussed.
References


2 Gene Silencing to Induce Pathogen-derived Resistance in Plants

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2.1 Introduction: Concept and Historical Overview of the Use of Pathogen-derived Resistance in Plants

The discovery and use of RNA interference (RNAi) and pathogen-derived resistance (PDR) in plants has a large history that has been previously reviewed by Gottula and Fuchs (2009), Lindbo (2012) and Rosa et al. (2018), among others. The concept of PDR was introduced by Sanford and Johnston (1985) describing the use of a pathogen’s own genome to confer resistance via genetic engineering as an alternative strategy to avoid problems in identifying and isolating host resistance genes, the polygenic control of the resistance or, directly, the lack of available resistance genes. This approach is based upon the disruption of parasite-encoded cellular functions that are essential to the parasite but not to the host. As a model, Sanford and Johnston (1985) used genes of the bacteriophage Qß to confer resistance in Escherichia coli against this bacteriophage. Before the discovery and description of RNAi, transgenic tobacco plants expressing the coat protein (CP) gene of the tobacco mosaic virus (TMV) were the first demonstration of PDR against a plant virus (Abel et al., 1986). As a result of these experiments, some transgenic lines showed no symptoms, or a delay in the development of the disease. Afterwards, numerous studies were conducted using CP genes and also other viral sequences (reviewed by Gottula and Fuchs, 2009), but the mechanism of the engineered resistance was not well understood at the time. It was suggested that the expression of the viral CP in a transgenic plant interfered with the virus replication, translation or virion assembly. Later, during an experiment to obtain plants resistant to the tobacco etch virus (TEV), transgenic lines expressing the TEV CP were obtained, and also other lines that expressed a non-translatable, sense-stranded mRNA for the TEV CP that were called RNA control (RC) lines (Lindbo and Dougherty, 1992). Surprisingly, during the TEV challenge, several of the RC lines were immune to the infection. In these plants, the accumulation of antisense RNA was responsible for this protection and not the ectopic expression of a viral protein, but, once again, at this time the cellular mechanism was not fully understood.

RNAi was first recognized in plants in the late 1980s and early 1990s. During experiments to increase the pigment content in purple petunia flowers using genetic engineering, some transgenic plant lines had flowers that were totally white or variegated (Napoli et al., 1990; van

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nder Krol et al., 1990). These authors called this phenomenon ‘cosuppression’ or ‘gene silencing’ of both the transgene and the homologous endogenous genes. However, the mechanisms involved were still unknown. Lindbo and collaborators, following their experiments with TEV-resistant transgenic plants, proposed that cytoplasmic activity targeting specific RNA sequences was responsible for the virus resistance in these plants, as transgene mRNA levels were 12- or 22-fold higher in unchallenged transgenic tissues compared with recovered transgenic plants of the same developmental stage (Lindbo et al., 1993). In this publication, the authors proposed a mechanism for post-transcriptional gene silencing (PTGS)/RNA silencing, where the RNA-dependent RNA polymerase (RdRP, also known as RDR) used the overexpressed viral transgene as a template to produce small RNAs that could rebind to new target RNA (viral and transgene) sequences. This model was further expanded by Dougherty and Parks (1995) suggesting that 10–20 nucleotide (nt) RNAs, generated from aberrant or overexpressed transgenes, were part of a cellular sequence-specific RNA targeting and degradation system. In fact, Hamilton and Baulcombe (1999) detected ~25 nt antisense RNAs, complementary to targeted mRNAs, in four types of transgene- or virus-induced PTGS in plants, that were likely synthesized from an RNA template. These authors suggested that these 25 nt antisense RNAs were components of the systemic signal and specificity determinants of PTGS.

Studies with other biological systems contributed to a deeper understanding of the mechanism of PTGS. The discovery of double-stranded RNA (dsRNA) as a potent inducer of PTGS in plants (Waterhouse et al., 1998) and nematodes (Montgomery and Fire, 1998) was a key contribution. Waterhouse et al. (1998) transformed tobacco and rice with gene constructs that produce RNAs capable of duplex formation to confer virus immunity or gene silencing to plants. In parallel, Fire et al. (1998) demonstrated that the direct injection into adult animals of dsRNA molecules was substantially more effective in producing interference effects than either strand was individually, and just a few molecules were required per affected cell. These authors described dsRNA as a potent trigger for RNAi. The use of direct dsRNA injection was suggested as a new tool for gene function studies in Caenorhabditis elegans, but also for other nematodes, other invertebrates and, potentially, in vertebrates and plants. As the genetic screens got easier, the identification of the genes required for RNAi in C. elegans, and their comparison with the ones required for gene silencing in Drosophila, plants and fungi, showed the existence of a common underlying mechanism (Mello and Conte, 2004). In 2006, Andrew Z. Fire and Craig C. Mello were awarded the Nobel Prize in Physiology or Medicine for their discovery of ‘RNA interference – gene silencing by double-stranded RNA’.

RNA silencing, or RNAi, is a conserved regulatory mechanism of gene expression in eukaryotic organisms that involves both transcriptional and post-transcriptional regulation. Different classes of small non-coding RNA molecules (sRNAs) are generated from dsRNAs by an RNAse III-like nuclease called Dicer or Dicer-like (DCL). The guide strand of the dsRNA binds an Argonaute (AGO) protein to form the mature RNA-induced silencing complex (RISC), while the passenger strand of the duplex is selectively degraded (Fang and Qi, 2016). The sRNAs function as a guide to direct RISCs to RNA or DNA targets through base-pairing. Moreover, in some eukaryotes, including plants, RDRs can convert the targeted mRNAs into dsRNAs, generating secondary sRNAs (de Felippes, 2019). This could produce an amplification of the silencing signal, both against the initial target and/or by silencing new ones. Additionally, although a basic structure of RNAi pathways is maintained throughout eukaryotes, the evolution of the DCL, AGO and RDR gene families, including gene duplication or loss, has increased the diversity of these pathways (Molnar et al., 2011). Plants seem to share a core set of primarily four DCL proteins (DCL1–4) (Rosa et al., 2018), while the number of AGO family members varies greatly in different species (10 in Arabidopsis, 15 in poplar, 17 in maize and 19 in rice) (Fang and Qi, 2016). At a broader level, Pyott and Molnar (2015) classified the RNAi mechanisms based on the source of the dsRNA initiator as endogenous (within the host genome) or exogenous (outside the host genome). By contrast, Fang and Qi (2016) explained the mechanisms according to the role of the different AGO family members involved. As a result, several sRNA species that differ in
biogenesis and functions have been characterized in plants and are discussed in the next sections.

2.2 Use of PDR for Basic Research

RNAi is widely used for functional analysis of plant genes. This approach can be achieved via generating stable transformants but also transient assays, avoiding difficult drawbacks that typically affect the stable transformation protocols. Also, this RNAi approach can be utilized for gene functional analysis in protoplasts (Zhai et al., 2009). Different types of constructs have been employed to achieve gene silencing purposes that have become more complex over time as knowledge on RNAi mechanisms has been advanced (Quintero et al., 2015; Khalid et al., 2017). Inspired by the PDR concept, the sense gene-induced PTGS strategy was the first employed in trying to confer resistance to viruses by overexpression of a viral protein. For these types of constructs, a fragment of the viral sequence was directly cloned in sense. Although resistance was successfully achieved in many cases, an RNA-mediated PTGS was actually the mechanism responsible (Lindbo et al., 1993). Also, before the RNAi mechanism was well understood, the expression of antisense viral sequences was also tested for conferring resistance. Prins et al. (1996) investigated the RNA-mediated resistance to tomato spotted wilt virus (TSWV) using randomly selected sequences (sense and antisense) of the viral genome to confer resistance.

A second generation of constructs led to the hairpin RNA-induced PTGS strategy. For this, a sense and an antisense viral fragment are cloned (separated by a fragment, usually an intron) to produce transcripts that can fold into dsRNA due to the complementarity of both fragments. This approach has been widely used for gene silencing in plants. As reviewed by Singh et al. (2019), intron-spliced hairpin RNA (ihpRNAs) constructs derived from viral proteins have been used, for instance, to confer resistance to plum pox virus (PPV) in plum, prunus necrotic ringspot virus (PNRSV) in cherry, or banana bunchy top virus (BBTV) in banana. Gaffar and Koch (2019) also provided a broad list of examples of the use of this method to control viral pathogens in different plant families, such as Solanaceae (tobacco, tomato, or potato), Cucurbitaceae (melon, cucumber), Fabaceae (soybean, common bean, cowpea and white clover), Poaceae (rice, wheat, maize and barley), Euphorbiaceae (cassava and poinsettia), or Rutaceae (Citrus macrophylla, Mexican lime and sweet orange).

Moreover, virus-induced gene silencing (VIGS) can be used as an alternative to exploit the innate plant defence system of PTGS against viral infections. The development and use of VIGS vectors have been recently reviewed by Dhir et al. (2019), and also previously by Lange et al. (2013), or Robertson (2004). Nowadays, the validation of gene functions is the major bottleneck in functional genomics. For this purpose, VIGS can be used as a fast method for screening candidate genes. To obtain a VIGS vector, a fragment of a target gene is inserted into a plant virus that upon infection of a plant host induces PTGS of the target gene. For instance, Gunupuru et al. (2019) used a barley stripe mosaic virus (BSMV) VIGS vector for functional characterization of disease resistance genes in barley seedlings. Moreover, VIGS can be used to silence genes from the host plant but also from other plant pathogens during co-infections. As an example, Lee et al. (2015) adapted the latest generation of binary BSMV VIGS vectors for functional analysis of wheat genes involved in susceptibility and resistance to *Zymoseptoria tritici*, a filamentous ascomycete fungus. Different methods to deliver the viral vectors to the plant have been employed, like agro-inoculation, or mechanical or biolistic inoculation. The utility of a virus as a VIGS vector will be determined by its ability to infect more or fewer species. For instance, Kawai et al. (2016) used the apple latent spherical virus (IALSV) vector in seven *Prunus* species, including apricot, sweet cherry, almond, peach, Japanese apricot, Japanese plum and European plum, with different efficiency depending on the species and/or cultivar used.

After microRNAs (miRNAs) became known, a new revolution began and new tools appeared. Transcription of MIR genes produces long non-coding transcripts with internal self-complementary regions that allow them to fold back and form an imperfect dsRNA stem-loop structure (primary miRNA, or pri-miRNA). They are recognized and cleaved by DCL1 to produce
a 21 nt dsRNA heteroduplex in the canonical pathway (Pyott and Molnar, 2015). From them, the guide strand is loaded into the AGO protein to produce a mature miRNA that can silence the target gene. According to Khalid et al. (2017), the artificial miRNAs (amiRNAs) are the third generation of constructs. For this purpose, the mature miRNA sequences in a natural pri-miRNA transcript are replaced with specific RNA sequences that are complementary to target viruses/templates. The first attempts to confer viral resistance using this strategy were reported in Arabidopsis and tobacco. Niu et al. (2006) modified an Arabidopsis thaliana miR159 precursor to express amiRNAs targeting viral miRNA sequences encoding the P69 of turnip yellow mosaic virus (TYMV) and the helper-component proteinase (HC-Pro) of turnip mosaic virus (TuMV). Qu et al. (2007) used an amiRNA targeting sequences encoding the silencing suppressor 2b of cucumber mosaic virus (CMV) in transient expression assays.

Later, the discovery of secondary small interfering RNAs (siRNAs) allowed the development of new tools, as recently reviewed by Carbonell (2019) and de Felippes (2019). In some cases, an miRNA-loaded RISC activity on a target transcript results in the production of dsRNA via RDR6 activity and can produce secondary siRNAs by successive DCL processing. They are called miRNA-triggered secondary siRNAs and can act by reinforcing the initial silencing signal (acting in cis) or affecting new targets (in trans) (de Felippes, 2019). The latter are known as trans-acting siRNAs (tasiRNAs). To date, four families of tasiRNA-producing loci (TAS1–4) have been described in Arabidopsis thaliana (TAS1 and TAS2 targeted by miR173, TAS3 by miR390, and TAS4 by miR828), and another six TAS genes (TAS5–10) have been described or predicted in other species (de Felippes, 2019). In order to use this process as a tool, artificial tasiRNAs (atasiRNAs), also known as synthetic tasiRNAs (syn-tasiRNAs) and miRNA-induced gene silencing (MIGS) constructs were developed (see Figure 3 in de Felippes, 2019). As described by this author, to obtain atasiRNAs, one or more of the tasiRNAs in the TAS gene was replaced by a fragment of the target gene. In the case of MIGS, constructs can be generated by placing the sequence recognized by an miRNA that can start transitivity in front of a fragment of the target gene (de Felippes et al., 2012). According to Carbonell (2019) the use of silencing tools based on secondary siRNAs in plants will continue despite the emergence of clustered regularly interspaced short palindromic repeats (CRISPR) technologies, due to their advantages such as high specificity, possibility of multi-targeting, spatio-temporal control of silencing or the ability to target genes whose complete knockout induces lethality.

2.3 Use of PDR for Commercial Purposes

According to FAO (2017), the threats posed by climate change and the upsurge in transboundary pests and diseases are part of the ten key challenges to eradicate hunger and poverty while making agriculture and food systems sustainable. Climate change is modifying the dynamics of pest populations and creating new ecological niches for the emergence or re-emergence and spread of pests and diseases. The impacts of transboundary plant pests and diseases vary from region to region and year to year. In some cases, they result in total crop failure. Recently, Savary et al. (2019) estimated that the yield losses worldwide caused by 137 individual crop pests and pathogens on five major crops (wheat, rice, maize, potato and soybean) ranged between 17% and 23% for all five crops, except rice, for which the estimate is 30%.

Crop pests and pathogens include a wide diversity of organisms, such as viruses and viroids, bacteria, fungi and oomycetes, nematodes, arthropods, molluscs, vertebrates and parasitic plants. The development and use of resistant crops are the most efficient strategies to mitigate the impact of these pests and diseases and to improve yield stability. Traditional breeding has been the way to obtain resistant varieties by classically identifying new resistance sources and introgressing them into economically important crops (Piquerez et al., 2014). However, for some cases this is not possible, as no resistant sources are available, or is too difficult, as in the case of species with a long reproductive cycle. Transgenic approaches can solve these situations and one of the strategies for that is the use of PDR RNAi, in which transgenic plants...
produce a dsRNA that silences a critical pathogen gene. Moreover, non-transformative strategies, such as the use of topical applications of dsRNA (e.g. spray-induced gene silencing) could be applied in some cases (Wang and Jin, 2017; Taning et al., 2020). The study of the molecular mechanisms underlying plant–pathogen interactions provides new opportunities to identify putative target genes (Dong and Ronald, 2019). Singh et al. (2019) reviewed the use of RNAi against viruses in perennial fruit plants, and Gaffar and Koch (2019) reviewed the potential of RNA silencing strategies to protect plants of various major plant families. Additionally, Mamta and Rajam (2017) and Liu et al. (2020) discussed the use of RNAi for crop pest control. In this section, some of the commercially approved varieties will be presented; further information is detailed in the suggested reviews. Although this approach has been widely used for basic research, the number of varieties approved for commercial production is not very high, probably due to the impressive regulation and public opinion against GMOs. Dong and Ronald (2019) reviewed the genetically engineered food crops with resistance to microbial pathogens approved by at least one international regulatory agency. Varieties approved for commercial production include squash (Cucurbita sp.), papaya, potato, sweet pepper, tomato, plum and bean, in chronological order.

In 1994, a squash variety expressing the CP genes of watermelon mosaic virus 2 (WMV2) and zucchini yellow mosaic virus (ZYMV) and, as a result, resistant to both of these potyviruses, received exemption status from the US Department of Agriculture’s Animal and Plant Health Inspection Service (APHIS) (Tricoll et al., 1995). Named as Freedom II, it was the first commercially available virus-resistant and disease-resistant transgenic crop released in the USA. Another interesting example was the development of papaya varieties resistant to the potyvirus papaya ringspot virus (PRSV) (Ferreira et al., 2002). In 1992, PRSV appeared in a district of Hawaii where 95% of the production was located. During a field trial, the transgenic papaya line 55-1, with a single insert of the CP gene of a mild strain of PRSV, showed resistance to a Hawaiian isolate of PRSV. From this line, the cultivars ‘Rainbow’ and ‘SunUp’ were developed. Their release to growers in May 1998 saved the papaya industry in Hawaii.

Another perennial fruit, the ‘HoneySweet’ plum cultivar, is resistant to sharka disease caused by plum pox virus (PPV), a very limiting factor for stone fruit production worldwide. This cultivar was originated by a transformation experiment aimed at the insertion of PPV-CP gene in the plum cultivar ‘Bluebyrd’, by using hypocotyl slices as starting explants (Scorza et al., 1994). As a result, the transgenic clone C5 (later named as ‘HoneySweet’) was highly resistant to this potyvirus. Further analysis explained that the insertion event produced a hairpin of the PPV-CP transgene, resulting in a PTGS event (Scorza et al., 2001; Hily et al., 2005). This cultivar was made freely available for fruit production and as a source of PPV resistance for plum breeding in the USA. Currently, ‘HoneySweet’ plum has not received approval for cultivation in the European Union (EU) or other locations outside the USA. However, field tests have been developed in Europe, where PPV is endemic, and the effectiveness and safety of ‘HoneySweet’ have been demonstrated (Polák et al., 2017).

Regarding DNA viruses, to date, bean golden mosaic virus (BGMV), a single-stranded DNA virus of the genus Geminivirus (family Geminiviridae), is the only example of a deregulated genetically engineered crop showing resistance to a virus with this kind of genetic material (Dong and Ronald, 2019). BGMV is the largest constraint to bean production in Latin America and causes significant yield losses (40–100%) in South and Central America, Mexico and the USA (Bonfim et al., 2007). After initial research efforts using traditional breeding techniques and different transgenic approaches (Aragão and Faria, 2009), Bonfim et al. (2007) decided to silence the rep viral gene to interfere with viral DNA replication using an intron-hairpin construct. As a result, a transgenic common bean line with superior agronomic performance in field trials was selected and registered as cultivar ‘BRS FC401 RMD’, becoming the first transgenic common bean cultivar in the world. In Brazil, the EMBRAPA researchers pointed out that this work is an example of a public sector effort to develop useful traits resistance to a devastating disease in an ‘orphan crop’ cultivated by poor farmers throughout Latin America (Aragão and Faria, 2009).
2.4 Limitations and Tools

Although RNAi technology has been widely used for functional analysis and development of crop varieties due to its many advantages, non-specific effects, often referred to as off-target gene silencing, should be considered. Senthil-Kumar and Mysore (2011) discussed the potential problems of off-target gene silencing in plants and considered possibilities that favour this effect. Du et al. (2011) also reviewed the off-target effects in mammals, classifying them into sequence-dependent effects and sequence-independent effects. The first type refers to the possibility that partial sequence homology can lead to the degradation of non-target mRNAs, while the second one refers to any unwanted effect at different steps during the PTGS pathway.

RNAi has been widely used as a reverse genetic tool for gene function characterization in plants. However, these off-target effects introduce uncertainty in gene function studies. According to Xu et al. (2006), 50–70% of gene transcripts in Arabidopsis plants have potential off-targets when used as a silencing trigger for PTGS and this can obscure experimental results. In fact, 50% of the potential off-targets identified using an siRNA Scan computational tool were actually silenced when tested experimentally. Their results suggest that a high risk of off-target gene silencing exists during PTGS in plants. This problem was also observed in 2003 by using microarray analysis in mammalian cells (Du et al., 2011). Another important aspect to consider is the possibility that the off-targeting could also affect exposed non-target organisms, causing environmental and biosafety issues.

Off-target effects can occur in different steps of the silencing process. Senthil-Kumar and Mysore (2011) indicated the steps that can or cannot be manipulated to increase specificity according to knowledge of the respective mechanism. According to these authors, the most troublesome points are the Dicer cleavage and siRNA production, the siRNA amplification and transitive silencing, and the target gene mRNA recognition and degradation. In order to prevent them, the gene fragment used for producing dsRNA (the trigger) should be chosen to be as specific as possible, taking into account that sequence complementarity of only 14 nt or less can lead to inhibition of gene expression. The use of vectors with tissue-specific and inducible promoters is another solution suggested by these authors. Moreover, excessive siRNA production could also lead to off-target effects, so that the use of appropriate promoters could be really important as well as the number of transgene copies introgressed into the host genome.

Computational prediction tools can be used to design RNAi constructs and to screen potential off-target effects. Fakhr et al. (2016) reviewed various algorithms for efficient siRNA design and listed the pros and cons of different online software, but mainly focused on mammalian siRNA design. Interestingly, some of the steps of the scoring system suggested by these authors could also be applied in plants, such as the simultaneous use of various online designing tools to identify the more favourable siRNAs, or the use of specific parameters of Basic Local Alignment Search Tool (BLAST) algorithms (Altschul et al., 1990) to take into account the alignment of small sequences. Moreover, these authors suggested the design of at least three siRNAs for any experiment to achieve the best silencing results. Regarding gene silencing specifically in plants, Ahmed et al. (2015) reviewed the most popular computational and experimental approaches. As a first step, a specific region of the target gene should be selected, and the BLAST algorithms could be used to find regions of local similarity against the whole genome of the species. The reduction in sequencing costs has made it possible to have an increasing number of complete genomes of different species that can be accessed using different public databases, like Phytozome (Goodstein et al., 2012), Plaza 4.0 (Van Bel et al., 2018), or PlantGDB (Duvick et al., 2008), among others. Regarding specific RNAi-related databases, PVsiRNAdb holds detailed information related to plant virus-derived small interfering RNAs (vsiRNAs) from 20 different viral strains infecting 12 different plant species (Gupta et al., 2018). Additionally, as sequencing costs have fallen, high-throughput sequencing has become an important tool for sRNA discovery and profiling. The UEA small RNA Workbench is a suite of tools for analysing miRNA and other small RNA data from high-throughput sequencing devices (Stocks et al., 2018).

There are a lot of online tools for siRNA design (Fakhr et al., 2016), but their main use
is in mammals. Regarding the tools designed for plants, as an example we could cite some of them, like P-SAMS (Fahlghren et al., 2016) and si-Fi21 (Lück et al., 2019). The Plant Small RNA Maker Site (P-SAMS) is a web tool for efficient and specific targeted gene silencing in plants using two applications: P-SAMS amiRNA and P-SAMS syn-tasiRNA Designers, for the simple and automated design of artificial miRNAs and synthetic trans-acting small interfering RNAs, respectively (Fahlghren et al., 2016). According to Lück et al. (2019), si-Fi21 offers efficient prediction of RNAi sequences and off-target search and it is specifically intended for long double-stranded RNAi constructs including virus-, microRNA-, and host-induced gene silencing (HIGS).

Given the interest generated by this topic, numerous reviews are available. We encourage the reading of those that have been cited here as well as other chapters of this book in order to have a deeper knowledge.

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References


Gene Silencing to Induce Pathogen-derived Resistance in Plants


3 Exogenous Application of RNAs as a Silencing Tool for Discovering Gene Function

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3.1 Introduction

RNA silencing is a powerful technique to unravel the function of genes by inhibiting gene expression at the post-transcriptional level. This technique is particularly appropriate for studying developmental processes such as fruit setting and growth that require a tight organ/tissue and time-specific regulation of target genes expression. Gene silencing in plants is usually achieved by the stable or transient expression of genetic constructs producing hairpin (hp) RNA or microRNA (miRNA). The use of exogenously applied small RNAs (sRNAs) and long double-stranded RNAs (dsRNAs) for transient gene silencing in whole plant and/or detached organs would allow a much higher number of genes to be analysed in a shorter time. The successful application of this technique requires efficient systems for sRNA delivery as well as methods to enhance RNA stability in plant cells.

3.2 Methods Used for Establishing the Function of a Specific Gene Altering Gene Expression at either the Genomic or Post-transcriptional Level

In the past decades, over 300 plant species have been sequenced, improving considerably our understanding of the overall structure and dynamics of plant genomes. However, despite the large number of genes identified, the functional role for the vast majority remains to be uncovered. The most widely used strategy to study gene function exploits reverse genetics, a gene-driven approach that links the alteration in the expression of a target gene with the full range of phenotypes controlled by the gene itself. Information on the role of a gene could be obtained by increasing its expression beyond the norm (i.e. overexpression), or by expressing the gene in a cell type and/or developmental stage or condition in which it is normally not...
expressed (i.e. misexpression), or by diminishing (i.e. knockdown) or completely abolishing (i.e. knockout) its expression. The complete suppression is generally obtained by introducing mutations at the genomic DNA level. In this regard, mutant plants have been obtained by X-rays and γ-ray irradiation, by chemical mutagens such as ethyl methane sulfonate (EMS), by targeting induced local lesion in genomes (TILLING) which couples random chemical mutagenesis with PCR-based screening, by transposon-mediated gene disruption, or by T-DNA insertion. The most recent approach for generating precise modifications of genome sequences is targeted genome editing, carried out by using either engineered nucleases such as zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN), or RNA-guided nucleases based on the naturally occurring type II Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) system (Chen et al., 2019). Besides the approaches aimed at modifying genomic DNA, tools directed to the mRNA/protein level of the gene of interest, such as post-transcriptional gene silencing (PTGS), have been extensively used for functional studies (McGinnis, 2010). The methods acting post-transcriptionally rarely cause complete loss of function of the target gene, but generally result in various degrees of downregulation with residual levels of the targeted mRNA/protein still detectable. PTGS, a natural mechanism used by the plant for protection against viruses and other invading nucleic acids and as a system to regulate gene expression, is activated by dsRNA molecules which are processed by Dicer-like (DCL) enzymes into 21–24 sRNAs (Martínez de Alba et al., 2013). The two principal classes of sRNAs are the small interfering RNAs (siRNAs) and miRNAs (Axtell, 2013). These sRNAs are loaded into Argonaute-containing silencing effector complexes and guide the sequence-specific cleavage of their mRNA targets. Thus, the expression of a dsRNA homologous to the gene of interest is sufficient to elicit the RNA silencing pathway against the target gene. Using hp or artificial miRNA-based constructs, dsRNA can be expressed in plants and used to silence both specific endogenous genes and genes of invading pathogens. The siRNA population can be increased, and the silencing signal amplified, in a process known as transitivity (Himber et al., 2003). An RNA-dependent RNA polymerase (RdRP) uses the cleavage products of the target mRNA, which include sequences outside the initial homology region present in the silencing construct, as substrate to generate a new population of siRNAs, called secondary siRNAs. The silencing signal that originates from primary and secondary siRNAs is not cell autonomous and can move to adjacent cells and systemically via the phloem (Melnyk et al., 2011). The systemic spread of the silencing seems to compromise the possibility of obtaining an siRNA silencing restricted to specific cells/tissues. However, silencing spread and amplification have been documented with viral sequences and overexpressed transgenes (Luo and Chen, 2007; Melnyk et al., 2011), whereas several studies support a tissue-specific siRNA silencing for endogenous genes when the hp construct is under the control of tissue/developmental specific-promoters (Davuluri et al., 2005; Okabe et al., 2019). For pleiotropic genes, the downregulation obtained with RNA silencing could partially unveil the activity of the target gene, whereas a complete genetic ablation would reveal the full functional role. However, the loss of a vital gene can often lead to embryo lethality as well as severe developmental abnormalities, which preclude the assessment of its role in adult vegetative and reproductive phases. To circumvent these problems, a fine-tuned downregulation of the target gene expression via PTGS could be preferable to a complete knockout.

In this regard, biological processes related to plant growth and development are under the control of complex networks of transcription factors which downstream regulate multiple signalling pathways. This implies a strict modulation of the gene expression brought about by tissue- and time-specific promoters. The use of strategies based on genomic silencing (e.g. T-DNA insertion, genome editing) to identify the function of genes implicated in developmental processes could result in complex phenotypic alterations or embryo lethality. On the other hand, PTGS constructs associated to appropriate promoters may offer a clearer phenotypical output. In this chapter, we discuss different RNAi strategies for studying the genes implicated in fruit set and growth, focusing on the use of ectopically applied sRNAs.
3.3 RNA Silencing as a Tool for Studying Genes Implicated in the Early Phases of Tomato Fruit Development

Tomato (*Solanum lycopersicum*) represents the model species for the study of fleshy fruit development and a great deal of biochemical and genetic information on the different phases of development from flowering to fruit maturation is available (Gapper et al., 2014). The tomato fruit originates from the ovary, the enlarged basal portion of the pistil. Fruit set is the earliest phase of fruit growth and represents the transition from the static condition of the ovary before fertilization to that of the rapidly growing fruit after fertilization (Fig. 3.1). The presence of fertilized ovules generally sustains the development of the ovary into a fruit, and the number of fertilized ovules usually determines the fruit growth rate (Gillaspy et al., 1993). Following fertilization, cell division is activated in the ovary and continues for about 7–10 days. After the period of cell division, fruit growth is mainly due to an increase in cell volume. During the period of rapid cell expansion, the embryos/seeds mature, showing well-developed cotyledons and established root–shoot axis. Rapid growth continues in the mature green fruit stage. The terminal stage of development is the ripening and initiates after seed maturation has been completed.

In parthenocarpic plants, fruit develops without fertilization, indicating that the ovary growth inhibitory factors have been released before fertilization. Indeed, several genes proved to be repressors of fruit set display a sharp downregulation during the transition from pre-anthesis to fertilized flowers. The genetic factors that repress ovary growth before fertilization can be identified by RNA silencing. The down-regulation of the expression of components of auxin (e.g. IAA9, ARF7) and gibberellin signalling pathways (DELLA) and auxin transport (PIN4) induced parthenocarpy (Wang et al., 2005; Goetz et al., 2006, 2007; Martí et al., 2007; Chaabouni et al., 2009; de Jong et al., 2009; Mounet et al., 2012). Other positive regulators of fruit set have been identified by RNA silencing, since their downregulation determines reduced fruit set and increased fruit abortion (e.g. AtNAOD) (Molesini et al., 2015).

Transcription regulators such as members of the Aux/IAA protein family display distinct functions in plant growth and development and some play a role both in vegetative and

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**Fig. 3.1.** Principal phases of tomato fruit development. Fruit set represents the onset of ovary growth after successful fertilization of the ovules. The subsequent fruit growth occurs by cell division and cell expansion. Fruit ripening represents the terminal stage of development.
reproductive development as for *S. lycopersicum* Aux/IAA transcription factor IAA9 (Wang *et al.*, 2005). The use of a strong and constitutive promoter (CaMV35S) to drive the expression of an IAA9 silencing construct, as well as the knockout of IAA9 obtained by CRISPR, induced the parthenocarpic development of the tomato fruit accompanied by alterations in leaf morphology (Wang *et al.*, 2005; Ueta *et al.*, 2017). Thus, a constitutive silencing can produce the same effects as a knockout mutation and can be employed as a strategy to unravel the role of a target gene when the pleiotropic effects are present in different organs and easily distinguishable. However, when multiple phenotypic alterations are manifested in the same organ, for instance when the parthenocarpic trait is associated with modifications of flower morphology (Ampomah-Dwamena *et al.*, 2002; da Silva *et al.*, 2017; Takei *et al.*, 2019), the approaches based on genome modifications or constitutive silencing of the target gene can be cumbersome. Besides, from an applied perspective, plants harbouring a desired trait but also showing unintended pleiotropic effects are not marketable.

### 3.3.1 RNA silencing obtained by either stable or transient transformation

Stable transformation of plants with siRNA-generating constructs is an efficient method for activating the RNA silencing pathway, but this procedure is time consuming and labour intensive. It includes: genetic transformation via *Agrobacterium* or through biolistic methods; regeneration and selection of stable transgenic plants; molecular analysis of the transgenic state of several independent lines; and phenotypic analysis of subsequent plant generations (T1, T2). To obtain the first hints about the function of a target gene, thus reducing the time of functional analysis, methods for transient RNA silencing have been developed. Transient gene expression is also useful, since it is not influenced by position effects of the transgene in the genome, does not require selection and can be utilized in differentiated plant tissues. It is largely applied for production of high amounts of foreign proteins and for gene silencing by PTGS. The transient transformation can be obtained by infiltration into the plant cells of *Agrobacterium tumefaciens* harbouring viral vectors or binary vectors. Concerning the study of genes implicated in the development of fruit, which is the last organ produced by a plant, strategies based on agro-injection of virus-induced gene silencing (VIGS) vectors have been developed (Liu *et al.*, 2002). A few examples are reported on the use of agro-injection of VIGS vector in studying fruit development and ripening in Solanaceae species (Fu *et al.*, 2005; Orzaez *et al.*, 2006; Wang and Fu, 2018). In these studies, fruit infiltration was performed *in planta* using a syringe to inject the bacteria into the carpogonium of tomato fruits at 10 days after pollination (Fu *et al.*, 2005), into the stalks of eggplant fruits 5 cm long (Wang and Fu, 2018) and into the stylar apex of tomato fruits at the beginning of mature green stage (20–25 days post-anthesis), respectively (Orzaez *et al.*, 2006). The phenotypes of the fruits were scored about 10–20 days post-inoculation, a temporal window that allows the evaluation of the transient silencing effect. The previous examples refer to genes whose silencing produces a visible phenotype (e.g. impaired synthesis of pigments). To apply transient silencing to genes with no expected visible phenotype and to overcome the problem of irregular distribution of VIGS, a strategy based on a visual reporter of VIGS in tomato fruit was developed (Orzaez *et al.*, 2009; España *et al.*, 2014). These methods, although effective, have some drawbacks; for example, the massive injection of bacteria or virus-derived sequences might induce unintended and not specific effects. Most importantly, the available methods seem feasible to study genes involved in later stages of fruit development and ripening but do not appear ideal to study very early phases of ovary/fruit growth. Orzaez *et al.* (2006) noted deleterious side effects after agro-injection in young ovaries/fruits (from 7 to 20 days post-anthesis) consisting of growth arrest, premature ripening and abscission. Topical application of dsRNA/siRNAs can be an appealing alternative to genetically modify crops for the functional characterization of genes involved in early stages of fruit set and growth.
3.3.2 Applications of exogenously supplied RNA silencing effector molecules to plant tissues

The possibility of exploiting exogenous RNAs for gene functional analyses in plants and for crop improvement is supported by many studies on sRNA metabolism carried out over the past few years in plants. sRNAs can move cell to cell, presumably via plasmodesmata, and over long distances through the vasculature (Brosnan and Voinnet, 2011; Melnyk et al., 2011; Brunkard et al., 2011; Melnyk et al., 2016; Wang et al., 2016). In addition, plant cells can take up exogenously supplied dsRNA and sRNAs (Koch et al., 2016; Wang et al., 2017). sRNAs and dsRNAs can enter the plant through stomata and through wounded or abraded surfaces (Wang et al., 2016) and move away from the initial point of application (Faustinelli et al., 2018). The binding of dsRNAs to nanoparticles, besides increasing their stability (Mitter et al., 2017), could facilitate their penetration (Sanzari et al., 2019). Movement of sRNAs and dsRNAs can take place also between interacting organisms (e.g. plants and fungi) in a process called ‘cross-kingdom RNAi’ (Wang et al., 2016; Cai et al., 2018).

The variables to be considered for ectopic applications of sRNA are numerous and mainly concern: the type of RNA molecules; the origin of RNA molecules (in vitro, chemically, or bacterially synthesized); the choice of the delivery method; and the use of sRNA carriers. The optimization of these parameters will vary depending on the aim of the study and the plant organ to be treated.

Many studies have proved that the topical application of RNA molecules on plant tissues represents an efficient system for inducing resistance against viruses, fungi and insects (for a comprehensive overview see Dubrovina and Kiselev, 2019; Dalakouras et al., 2020). Fewer examples have been reported on the efficacy of exogenously applied sRNA for silencing of transgenes and endogenous genes (Dubrovina and Kiselev, 2019; Dalakouras et al., 2020). In accordance with the observations made on plants stably expressing silencing constructs, the silencing capacity of ectopic RNAs seems more effective with transgenes rather than endogenous genes (Dubrovina and Kiselev, 2019). This phenomenon can be due to the higher expression of the transgenes that is usually driven by strong promoters, the high frequency of aberrant transcripts, and the absence of introns and 5’ and 3’ UTR sequences which contribute to the mRNA stabilization (Luo et al., 2007; Dadami et al., 2014).

In the studies describing the use of external RNAs for the silencing of endogenous genes, the sRNAs or dsRNAs were topically applied on leaves and roots and only in a single case on reproductive organs (Sammons et al., 2011; Numata et al., 2014; Lau et al., 2015; Li et al., 2015). The study by Lau et al. (2015) described the silencing of the MYb1 gene in flower buds of Dendrobium hybrida. The DhMYb1 gene encodes a transcription factor, expressed during flower development, which is putatively involved in flower morphogenesis. To obtain MYb1 silencing, they applied a crude lysate of RNaseIII-deficient Escherichia coli cells expressing dsRNA corresponding to 430 bp DhMYb1 cDNA, on very young flower buds (≤ 0.5 mm in length), by gently rubbing. The treatment was repeated every 5 days and the phenotype recorded 25–29 days after the first treatment. The transcript level of DhMYb1 was reduced approximately two- to fourfold in the treated flower buds as compared with that in the untreated ones. At the phenotypic level, RNA-treated and untreated flower buds appeared indistinguishable, but microscopic analyses revealed that the dsRNA treatment caused changes in the epidermal cells, which had a flattened instead of conical shape.

Interestingly, the suppression of genes involved in reproductive development can be obtained also by systemic silencing after ectopic dsRNA application. As demonstrated in the paper by Li et al. (2015). In this study, 2-week-old Arabidopsis thaliana roots were soaked with a solution containing in vitro synthesized dsRNA of 554 bp in length for the silencing of MOB kinase activator-like 1A (Mob1A). Arabidopsis Mob1A is required for organ growth and reproduction, since its suppression resulted in reduced growth of vegetative organs and defects in seed set (Pinosa et al., 2013). Two weeks after root soaking, a reduction in Mob1A expression was observed as well as impaired bolting and flowering. These results indicate that the silencing in the reproductive organs can occur because of the movement of the silencing signal from the root to the shoot via the vascular tissues.
3.3.3 Perspective on the use of exogenous sRNAs and long dsRNAs for the silencing of genes involved in fruit growth and development

In this section, we will discuss the possibility of utilizing ectopic RNAs as a fast system for the functional analysis of the genes involved in tomato fruit growth and development.

Fruit development is characterized by two important transition phases: the first, from pre-anthesis to the fertilized flower with the consequent activation of ovary growth (fruit set); and the second, from the end of the growth phase to the start of fruit ripening (Fig. 3.1). These transitions involve marked hormonal and biochemical modifications resulting from changes in the expression of many genes. The exogenous sRNAs or dsRNAs could be applied in planta on flowers and fruits or in vitro under sterile conditions on detached reproductive organs.

In this regard, Nitsch (1950) showed that auxin exogenously supplied to culture medium of pre-anthesis tomato flower buds is sufficient to guarantee the growth of the ovary/fruit up to the ripening phase. More recently, we observed that genetically engineered flower buds with increased auxin synthesis can be grown in vitro after emasculation (i.e. stamen detachment) up to fruit ripening without phytohormones in the culture medium (Pandolfini et al., 2010). Therefore, this in vitro system can be used to evaluate the genes involved both in fruit setting and in growth and ripening phases.

To assay the efficiency of the exogenous treatment, it is essential in the setting up of the experiments to include some positive controls, consisting of genes whose silencing obtained via stable transformation produces the expected phenotype (e.g. changes in pigment production or fruit set efficiency) (Molesini et al., 2009; Osorio et al., 2012).

The choice of RNA type is the first variable to be considered. Both long dsRNAs (generally 200–800 bp) and short siRNAs (22–24 nt) have been proved to induce the silencing of endogenous genes efficiently (Dubrovina and Kiselev, 2019; Dalakouras et al., 2020). dsRNA, when processed by Dicer within the cell, produces a pool of effector molecules (siRNAs) homologous to different portions of the target mRNA, thus increasing the probability that the RNAi silencing complex recognizes and cleaves the target mRNA. However, the complexity of the siRNA pool generated from a dsRNA substrate may increase the possibility of having partial and/or perfect matching with off-target mRNAs. When using a single siRNA molecule, an accurate in silico design can diminish the probability of unintended matching, but the high dosage of siRNAs needed for silencing may lead to off-target effects.

In our case, since exogenous RNAs will be applied to specific organs, pleiotropic and off-target effects are presumably limited. In addition, considering that the target mRNA structure can affect the accessibility and consequently the gene silencing ability of siRNAs (Gredell et al., 2008), the use of long dsRNAs appears to be an appropriate choice since, once processed, it generates a heterogeneous pool of siRNAs targeting different portions of the transcript.

For dsRNA production, it is preferable to use an in vitro transcription system because it guarantees high yield and purity of dsRNAs, rather than raw bacterial lysates, which contain RNA of bacterial origin besides other contaminants.

The choice of the RNA delivery system mainly depends on the anatomy of the tissue/organ being treated. In our case, the effector molecules must penetrate the ovary or the growing/mature fruit (Fig. 3.2). Regarding the ovary, one of the possible routes can be the stylus, therefore RNAs could be applied on the stigma. An alternative method could be the injection of the RNAs in the pedicel of the flower through a syringe, or by deposition after abrasion of the tissue. Direct injection into the ovary must be avoided, as it has been observed that this practice causes damage to the growing fruit. It is also possible to spray the RNAs directly on the entire surface of the flower bud, in which case the entry of sRNAs might also occur through other natural openings (e.g. stomata) of the flower organs (sepals, petals, stamens) (Fig. 3.2).

It has been observed that, after the delivery of siRNAs to the petiole of Nicotiana benthamiana as well as after the injection of dsRNAs in the trunk of Vitis vinifera (Dalakouras et al., 2018), the RNAs are transported in the xylem and restricted to the apoplast, while high-pressure spraying is effective in the delivery of siRNAs to the symplast (Dalakouras et al., 2018). This last technique could allow siRNAs to be efficiently conveyed within the...
ovary. High-pressure spraying could also be used on the leaf immediately below the flower bud, in which case sRNAs could be systemically transported to the ovary, avoiding any mechanical damage to the floral tissues (Fig. 3.2).

If in vitro cultivated flower buds are used, sRNAs can also be applied to the cutting surface of the pedicel (Fig. 3.3). In this case, to facilitate the entry of the effector molecules, the tissue could be subjected to air stress in a laminar air-flow hood (Faustinelli et al., 2018).

The application of sRNAs to young ovaries allows the functional study of the genes involved in fruit setting and in fruit development (Fig. 3.3). If the specific target of the investigation is the parthenocarpy, the methods described can be used on flower buds after stamen excision (emasculating). However, in the absence of pollination and fertilization, the tomato ovary ceases cell division and abscises in a few days, therefore sRNAs should be loaded on emasculated very young buds and the treatment should be repeated several times.

Regarding the treatment of growing or mature fruits, it should be considered that the presence of the cuticle can be an obstacle to the entry of sRNAs. Therefore, the application of sRNAs by injection appears a more suitable method than spraying. Injection in the peduncle or in the pulp of the fruit has been used several times for transient expression of transgenes without producing damage to the fruit (Fig. 3.2).

One of the major problems linked to the use of ectopically delivered sRNAs is due to the instability of naked RNA molecules (e.g., action of nucleases and/or environmental conditions such as excessive sunlight). A recent paper demonstrated that the use of dsRNAs loaded on layered double hydroxide (LDH) clay nanosheets improved the stability of the ectopically delivered dsRNA molecules, resulting in a prolonged silencing effect (Mitter et al., 2017).

As previously mentioned, the problem of sRNA penetration is critical when the target organ is the female gametophyte or the ovule, since the sRNAs loaded on the surface of the flower

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**Fig. 3.2.** Exogenous sRNA application for the in planta silencing of genes involved in fruit growth and development. (A) sRNAs can be applied to young flower buds either by spraying or by abrasion of the pedicel followed by deposition or by injection into the flower pedicel (circled in purple). Using the same application methods (circled in yellow) sRNAs can also be delivered to the young leaves just below the flower trusses. The movement of RNA silencing to the flowers located upstream occurs systemically. (B) sRNAs can be injected using a syringe into the pulp or pedicel of growing fruits. To avoid damage, the treated fruits should have a diameter greater than ~1 cm.
Fig. 3.3. sRNA application on in vitro cultivated flower buds. For the evaluation of genes putatively involved in parthenocarpy (upper image), flower buds collected before anthesis are emasculated, sterilized and cultured in vitro in a medium not supplemented with phytohormones. The sRNAs could be applied to the stigma (yellow arrow), to the cut surface of the pedicel (red arrow) and to the whole flower buds (green arrow). The growth of the ovary confirms the role of the silenced target gene as repressor of fruit setting. For the evaluation of genes putatively involved in fruit development (lower image), flower buds collected before anthesis are sterilized and cultured in vitro in a medium supplemented with auxin. The sRNAs could be applied to the cut surface of the pedicel (red arrow) and to the whole flower buds (green arrow). After approximately 30 days of cultivation, fruits start to ripen.

bud should cross several cell layers before being effective. In this case, the use of nanoparticles as sRNA carriers can be advantageous in favouring the distribution of the effector molecules, as well as sRNA stability. In fact, there is evidence that nanoparticles can passively enter natural plant openings (stomata, stigma, etc.) and those of reduced length (3–50 nm) can also pass through the cell wall (Sanzari et al., 2019). The cuticle is normally a strong barrier to the nanoparticles’ diffusion, although TiO₂ particles are reported to produce holes in the cuticle, thus favouring sRNA penetration (Larue et al., 2014).

3.4 Conclusions

The utilization of ectopic sRNAs as a tool for the discovery of gene function is in its infancy and we need future research efforts to test the efficacy of this system. However, it is an attractive perspective for the study of genes involved in developmental processes such as flowering and fruit growth. From a biotechnological point of view, the use of sRNAs not only to improve the crop’s defence against pathogens and pests but also to modulate productivity is an exciting challenge.

References


4 The ‘Trojan Horse’ Approach for Successful RNA Interference in Insects

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Abstract

Since the discovery of RNA interference in 1998 as a potent molecular tool for the selective down-regulation of gene expression in almost all eukaryotes, increasing research is being performed in order to discover applications that are useful for the pharmaceutical and chemical industry. The ease of use of double-stranded RNA for targeted in vivo gene silencing in animal cells and tissues gave birth to a massive interest from industry in order to discover biotechnological applications for human health and plant protection. For insects, RNAi became the ‘Holy Grail’ of pesticide manufacturing, because this technology is a promising species-specific environmentally friendly approach to killing natural enemies of cultured plants and farmed animals. The general idea to use RNAi as a pest-control agent originated with the realization that dsRNAs that target developmentally or physiologically important insect genes can cause lethal phenotypes as a result of the specific gene down-regulation. Most importantly to achieve this, dsRNA is not required to be constitutively expressed via a transgene in the targeted insect but it can be administrated orally after direct spraying on the infested plants. Similarly, dsRNAs can be administered to pests after constitutive expression as a hairpin in plants or bacteria via stable transgenesis. Ideally, this technology could have already been applied in integrated pest management (IPM) if improvements were not essential in order to achieve higher insecticidal effects. There are many limitations that decrease RNAi efficiency in insects, which arise from the biochemical nature of the insect gut as well as from deficiencies in the RNAi core machinery, a common phenomenon mostly observed in lepidopteran species. To overcome these obstacles, new technologies should be assessed to ascertain that the dsRNA will be transferred intact, stable and in high amounts to the targeted insect cells. In this chapter we will review a wide range of recent discoveries that address the delivery issues of dsRNAs in insect cells, with a focus on the most prominent and efficient technologies. We will also review the upcoming and novel use of viral molecular components for the successful and efficient delivery of dsRNA to the insect cell.

4.1 Introduction

In 1998 researchers first discovered that double-stranded RNA (dsRNA), instead of antisense RNA, was substantially more potent at producing RNA interference (RNAi) (Fire et al., 1998). These researchers showed that injection of Caenorhabditis elegans adults with purified antisense or sense...
RNAs targeting a wide range of selected genes had, at most, a mild effect, while on the contrary dsRNA mixtures acted with stronger efficiency and specificity against their targets (Fire et al., 1998). Only low doses of injected dsRNA were sufficient per affected cell, thus depicting that stoichiometric interference with endogenous mRNA was not necessary and suggesting the involvement of a component that catalyses or amplifies the silencing effect (Fire et al., 1998).

Follow-up studies showed that a specific nuclease activity is associated with dsRNA-mediated RNAi in Drosophila melanogaster S2 cells and that this activity is responsible for the degradation of endogenous transcripts homologous to the transfected dsRNA (Hammond et al., 2000). The nuclease was speculated to contain an essential RNA component after the discovery of small RNA species that could act as specificity agents through homology to the substrate mRNAs (Hammond et al., 2000).

Nowadays we know that RNAi is initiated by ribonucleases that generate small interfering RNAs (siRNAs) from long dsRNAs and mature microRNAs (miRNAs) from primary transcripts (Hammond, 2005). This is accomplished by the action of two RNase III enzymes: Dicer (Fig. 4.1) and Drosha. Class III RNase enzymes contain two RNase III catalytic domains, a helicase domain and a Piwi/Argonaute/Zwille (P AZ) domain. This last domain is also present in Argonaute family proteins that are essential in later steps of RNAi (Hammond, 2005), while especially in Dicer it is responsible for the recognition of the dsRNA substrate (Hammond, 2005), while especially in Dicer it is responsible for the recognition of the dsRNA substrate (Lau et al., 2012). Dicer cleaves the substrate at ~22 nucleotides (nt) from the open helicoid end (Lau et al., 2012). In the Dicer enzyme of the protzoan Giardia lamblia a ‘platform’ domain has been observed that separates the PAZ domain from the RNase III catalytic site, thereby providing structural insights for the production of small RNAs of 25–27 nt in length (Lau et al., 2012). Other eukaryotic...

Fig. 4.1. The RNAi ‘decathlon’. Insects possess all three RNAi (miRNA, siRNA and piRNA) machineries. Exogenously applied dsRNAs must overcome a series of cellular barriers in order to be processed by the RNAi core machinery. In insects, extracellular and intracellular nucleases degrade dsRNAs before and after entering cells. Intracellular uptake is being promoted by SID-1-like transporters and clathrin-mediated endocytosis. Both pathways might act in parallel in some insects, e.g. Leptinotarsa decemlineata. When taken up by endocytosis, dsRNA must undergo endosomal escape in order to interact with the RNAi core machinery. SID-1-like proteins may be responsible for endosomal escape of dsRNAs in some insects. In lepidopteran species, dsRNA may accumulate in endosomes because of inefficiency of endosomal escape. In the cytoplasm, the dsRNA is being processed by Dicer protein to 20–22 nt siRNAs. The complementary siRNA strand is then introduced into the RISC complex and mRNA degradation is initiated. In plants and worms, but not insects, initial RNAi triggers can be amplified by RdRP proteins. Viral suppressors of RNAi (VSRs) may antagonize Dicer and Ago proteins either by protein–protein interactions or dsRNA/siRNA sequestration as an antiviral defence mechanism. Extracellular transportation of siRNAs may be mediated by SID-1-like proteins and intercellular transport can be carried out by nanotubules (in Drosophila).
Dicers use a similar mechanism of molecular ruler although their products are 4 nt shorter (Lau et al., 2012). Small RNA products of Dicer are incorporated into large multiprotein processing complexes termed RNA-induced silencing complexes (RISCs) (Fig. 4.1) (Lau et al., 2012). RISC selects one strand of the small RNA duplex, such as miRNA or siRNA (Filipowicz et al., 2008). The single strand acts as a guide for RISC to recognize complementary sequences in mRNAs (Fig. 4.1). One of the proteins in RISC, called Argonaute (Ago), exhibits slicer activity and cleaves the mRNA. A complex that consists of an Ago and a single guide strand is referred to as ‘mature RISC’ or simply ‘RISC’, while the same complex is also called ‘RISC core’ in the context of considering RISC as a huge complex that includes many other components to achieve silencing, e.g. by translational repression and de-adenylation (Fig. 4.1) (Nakanishi, 2016).

In insects, three main small RNA-based silencing pathways are observed: the miRNA, siRNA and PIWI-interacting RNA (piRNA) pathways (Fig. 4.1) (Mongelli and Saleh, 2016). Although all three pathways use small RNAs (from 18 to 33 nt) to guide the sequence-specific recognition of target sequences by an Ago effector protein, the small RNAs in each pathway differ according to their biogenesis, the nature and fate of their targets, and their biological function (Mongelli and Saleh, 2016). Despite the fact that the main RNAi pathways’ operation strategies are highly conserved among several organisms, they can involve different proteins and operate via different mechanisms (Terenius et al., 2011). The primary example is the amplification of the RNAi effect in nematodes, plants and fungi through the action of a cellular RNA-dependent RNA polymerase (RdRP) that generates target gene-derived secondary siRNAs (Terenius et al., 2011). It is highly probable that RdRP is responsible for the robust effect of dsRNA-mediated RNAi in these organisms. Homologues of cellular RdRPs do not exist in insect genomes, although they have been identified in genomes of basal arthropods such as ticks (Fig. 4.1) (Terenius et al., 2011).

RNAi efficiency is relatively low in lepidopteran insects compared with many other insect species (Terenius et al., 2011; Guan et al., 2018). dsRNA degradation as well as inefficient cellular uptake and transport seem to be crucial main factors that determine the various levels of RNAi efficiency among insects (Guan et al., 2018). Previous research demonstrated that dsRNA may remain stable for much longer periods after uptake in many species of Coleoptera compared with most lepidopteran species (Terenius et al., 2011; Shukla et al., 2016; Guan et al., 2018). New studies demonstrated that Lepidoptera contain a specific nuclease (RNase) which is responsible for the digestion of dsRNA before its processing by Dicer and therefore can negatively affect the RNAi efficiency (Guan et al., 2018). In addition, degradation of dsRNA in the lumen of the gut and in the haemocoel is considered to be an important factor responsible for differences in the efficacy of RNAi between coleopteran and lepidopteran species (Shukla et al., 2016). Shukla et al. (2016) demonstrated that intracellular transport of dsRNA can be a major factor affecting the differential efficacy of RNAi that is observed between a lepidopteran (Heliothis virescens) and a coleopteran (Leptinotarsa decemlineata) species. Moreover, in sharp contrast to coleopteran species, it was observed that Lepidoptera do not efficiently process plant-originated long dsRNAs to 21 bp siRNAs (Ivashuta et al., 2015).

Numerous molecular and physiological processes may be responsible for the insufficient response of RNAi in particular insect orders such as Lepidoptera. Thus, the variability of RNAi in insects is a phenomenon that has to be addressed in order for this technique to become a widely valuable tool in efficient pest control strategies. In this chapter, we will focus on the most important causes of RNAi deficiency and will review scientific and technical methodologies to overcome them.

### 4.2 dsRNA Uptake in Insects: Molecular Mechanisms and Endosomal Escape

dsRNAs can penetrate the insect’s cells via several routes (Fig. 4.1). It has been demonstrated that two inhibitors (chlorpromazine and bafilomycin-A1) of clathrin-dependent endocytosis (Fig. 4.1) can nearly abolish or significantly diminish RNAi of the Lethal giant larvae (TcLgl) gene in Tribolium castaneum (Coleoptera) whereas methyl-β-cyclodextrin and cytochalasin-D substances that are known to have inhibitory action on other endocytic pathways, showed no effect (Xiao et al., 2015).
In addition to clathrin-mediated endocytosis, transport by the SID-1-like transmembrane channel is also considered as a major pathway for dsRNA uptake in insects (Fig. 4.1) (Cappelle et al., 2016). SID-1-like genes have been identified in the genomes of many insects, with the notable exception of Diptera. While three genes similar to sid-1 were identified in the genome of *T. castaneum*, none of these genes seem to be indispensable for systemic RNAi in this species (Yoon et al., 2017). On the other hand, two recently identified sid-1-like genes in the Colorado potato beetle *Leptinotarsa decemlineata* are necessary for an efficient RNAi response in the *L. decemlineata* cell line Lepd-SLI (Yoon et al., 2017). In *L. decemlineata*, therefore, both endocytosis and SID-1-like pathways are involved in dsRNA uptake (Cappelle et al., 2016).

Studies in lepidopteran Sf9 cells also suggest that the clathrin-mediated pathway through endosomes is used as the major route for transport of dsRNA into and within these cells (Yoon et al., 2017). However, despite efficient uptake of dsRNA, no silencing effects are observed, which seems to be caused by the ability of dsRNA to escape from the endosomes. Overexpression of *Caenorhabditis elegans* SID-1 improved RNAi efficiency in Sf9 and Bombyx mori cells, which could be related to a stimulation of endosomal escape by dsRNA. On the other hand, overexpression of the SID-1 homologue of the migratory locust was not effective (Yoon et al., 2017), indicating that not all SID-1-like homologues are involved in dsRNA transport. Interestingly, in mammals, SIDT1 and SIDT2, closely related members of the SID-1 transmembrane family, are required to transport internalized dsRNA molecules from endosomes to the cytosol to activate the innate immune response (Nguyen et al., 2019). The role for SID-1-like transmembrane channels in the regulation of endosomal escape of dsRNA molecules needs to be further investigated in the future.

In dipteran insects, fatty acid biosynthesis and metabolism may play important roles in the regulation of RNAi efficiency (Dong et al., 2017). Prior exposure to dsRNA (‘dsRNA priming’) in the fly *Bactrocera dorsalis* resulted in changes in the ratio between linoleic acid (LA) to arachidonic acid (AA) in the haemolymph and inhibition of endocytosis of dsRNA into the midgut cells. Interestingly, injection of AA resulted in an increase in the uptake of ingested dsRNA in *Drosophila melanogaster* and a facilitation of RNAi effects (Dong et al., 2017).

### 4.3 Physiological and Cellular Mechanisms that Affect RNAi Efficiency

The cotton boll weevil (*Anthonomus grandis*) is a coleopteran insect for which reports on RNAi-mediated gene silencing showed that it does not function efficiently when dsRNA feeding is used (Almeida Garcia et al., 2017). Three nucleases of the DNA/RNA non-specific endonuclease family were identified in the cotton boll weevil transcriptome (AgraNuc1, AgraNuc2, AgraNuc3) and were found to be mainly expressed in the posterior midgut region of the insect (Almeida Garcia et al., 2017). Gene silencing of *AgraNuc1-2-3* showed that *A. grandis* midgut nucleases are one of the main barriers to dsRNA delivery (Almeida Garcia et al., 2017).

Consistent with the above result, a major observation is that insects of different orders express different levels of dsRNA-degrading enzymes in both haemolymph and midgut tissues (Wang et al., 2016). In comparative RNAi studies among species that belonged to four different insect orders, the cockroach *Periplaneta americana* exhibited the best silencing response followed by the coleopteran *Zophobas atratus*, the orthopteran *Locusta migratoria* and the lepidopteran *Spodoptera litura* (Wang et al., 2016). This variability in RNAi response was correlated with the enzymatic degradation of dsRNA, which functions as a key factor that determines the effective dosage duration of inner target exposure (Wang et al., 2016).

Expression of the core RNAi machinery can vary among different developmental stages of insects (Guo et al., 2015). Developmental and growth defects associated with the silencing of the S-adenosyl-L-homocysteine hydrolase (LdSAHase) gene of *L. decemlineata* occurred with different levels of penetrance depending on the stage of the larva at which dsRNA was administered (Guo et al., 2015). In young larvae the expression levels of *LdDcr2a*, *LdDcr2b*, *LdAgo2a* and *LdAgo2b* (encoding Dicer-2 and Argonaute-2 proteins in the siRNA pathway,
respectively) were higher, which affected RNAi efficiency in *L. decemlineata* (Guo et al., 2015).

In some organisms, mRNA suppression by dsRNA is observed in many tissues throughout the body as the RNAi signal spreads between tissues, with this non-cell-autonomous RNAi response being referred to as systemic RNAi (Fig. 4.1) (Cooper et al., 2018). In the nematode model, *C. elegans*, siRNAs generated by Dicer are transported from cell to cell and their abundance is amplified by RdRP (Cooper et al., 2018). Both the recipient and donor cells must possess SID-1 channels so that cell-to-cell transport can occur (Fig. 4.1) (Cooper et al., 2018). However, vesicle transport as well as endocytosis are also involved in the systemic RNAi response (discussed above). A similar mechanism as in nematodes was hypothesized to exist in insects; however, all insect genomes lack RdRP genes and some, like dipterans, also lack SID-1 homologs (Cooper et al., 2018). In *D. melanogaster*, it was reported that nanotube-like structures can establish a systemic RNAi response that functions as an antiviral mechanism in different cell types (Karlikow et al., 2016). The nanotubes are composed of actin and tubulin, and associate with components of the RNAi machinery, including Ago-2, dsRNA and CG4572 (Fig. 4.1) (Karlikow et al., 2016).

### 4.4 Viral RNAi Suppressors

RNAi is a mechanism that is necessary for antiviral defence in insects, including vectors of human viral diseases such as mosquitoes. Viruses have evolved to escape this antiviral defence system by encoding suppressors of RNAi that function as obstacles for the elimination of viral RNAs, thus contributing to efficient viral replication (Fareh et al., 2018). It was shown that viral suppressors of RNAi (VSRs) from *Drosophila* RNA viruses antagonize Dcr-2 enzyme by safeguarding viral RNA molecules (Fareh et al., 2018). VSR proteins such as VP3 of *Drosophila* X virus and *Culex* Y virus (both of genus *Entomobirnavirus*, family Birnaviridae) and 1A protein of *Drosophila* C virus (genus Cripavirus, family Dicistroviridae) directly bind to dsRNA molecules and prevent the recognition by Dcr-2 in an irreversible manner (Fig. 4.1) (Fareh et al., 2018).

RNAi suppressors can demonstrate host-specific activities (van Mierlo et al., 2014). VP1 of *D. melanogaster* Nora virus (DmelNV) suppresses Ago-2-mediated target RNA cleavage to antagonize antiviral RNAi (Fig. 4.1) (van Mierlo et al., 2014). Ago-2 antagonists of divergent Nora-like viruses in natural populations of *D. immigrans* (DimmNV) and *D. subobscura* (DsubNV), however, cannot suppress RNAi in *D. melanogaster* S2 cells. RNAi suppressor activity of DimmNV VP1 is restricted to its natural host species, *D. immigrans* (van Mierlo et al., 2014). While DimmNV VP1 interacts with *D. immigrans* Ago-2 by suppressing slicer activity in embryo lysates from the same species, it does not interact with *D. melanogaster* Ago-2, thus presenting no suppressive effect in this species lysates (van Mierlo et al., 2014).

The possible role of RNA virus infections in inhibiting RNAi in lepidopteran insects has been investigated (Swevers et al., 2016). Several lepidopteran cell lines were found to be persistently infected by the RNA viruses Flock house virus (FHV; Nodaviridae) and Macula-like virus (MLV; related to plant viruses of the family Tymoviridae) without any apparent pathogenic effects. RNAi reporter assays failed to detect a significant interference with gene silencing in SF21 and Hi5-SF cells that were persistently infected, when compared with virus-free cells. In Hi5 cells, FHV could be easily eliminated through the expression of an RNA hairpin specific for its VSR gene, confirming that the RNAi mechanism was not inhibited (Swevers et al., 2016). Despite the above-mentioned results, functional tests indicated that the B2 gene of FHV coding for an RNAi inhibitor exhibited RNAi suppressor activity, indicating that protection against RNAi is essential for virus survival (Swevers et al., 2016). In another study using lepidopteran cell lines, overexpression of Dcr-2 and Ago-2 could delay the progression of pathogenic infection by Cricket paralysis virus (*Dicistroviridae*) while knockdown of these RNAi factors resulted in an increase in the levels of persistent infections of FHV and MLV (Santos et al., 2018). The impact of persistent virus infections on the performance of the RNAi machinery requires further study, because reports have revealed the ubiquitous presence of viruses in many insects after the applications of high-throughput sequencing techniques (Bolling et al., 2015).

Also, DNA viruses can express VSR proteins. Baculovirus (*Autographa californica* multiple nucleopolyhedrovirus, AcMNPV) infection induces an RNAi response in *Spodoptera frugiperda* cells,
as documented by the detection of a large number of viral siRNAs (Mehrabadi et al., 2015). The p35 gene in the AcMNPV genome, an established inhibitor of apoptosis, was also found to have VSR activity when tested in RNAi reporter assays that employed diverse insect and mammalian cell lines. VSR activity of p35 was not due to the inhibition of dsRNA cleavage by Dicer-2, but because of a downstream action in the RNAi pathway (Mehrabadi et al., 2015).

### 4.5 Improvement of RNAi

RNAi can be improved by identifying methodologies that overcome the biochemical, molecular and physical boundaries imposed by insect cells. Many technologies have been developed in order to confront these limitations by focusing on the improvement of dsRNA stability and penetrative ability in insect cells.

#### 4.5.1 Nanoparticle-mediated dsRNA encapsulation

The cationic polymer chitosan is able to form stable nanoparticles with anionic nucleic acids (dsRNAs) via electrostatic interactions (Fig. 4.2) that can be observed by atomic force microscopy (Ramesh Kumar et al., 2016). Chitosan/dsRNA-mediated knockdown of a reporter gene was first demonstrated in the lepidopteran Sf21 insect cell line (Ramesh Kumar et al., 2016). In subsequent studies, chitosan/dsRNA nanoparticles targeting the vestigial gene in the mosquito *Aedes aegypti* were able to cause significant mortality, adult wing-malformation and delayed growth development (Ramesh Kumar et al., 2016). Moreover, a comparative study of nanoparticles that complexed dsRNA with chitosan, carbon quantum dot (CQD) or silica showed that CQD was the most efficient carrier for dsRNA retention, delivery and concomitant gene silencing and mortality in *Ae. aegypti* (Das et al., 2015). Aerosolization of siRNA–nanoparticle complexes was described and used as a delivery method in three aphid species (*Acyrhosphion pisum, Aphis glycines* and *Schizaphis graminum*) to target genes involved in pigmentation and amino acid metabolism and it was concluded that the nanoparticle emulsion significantly increased the efficacy of gene knockdown (Thairu et al., 2017).

Particle replication in non-wetting templates (PRINT) technology (Fig. 4.2) has been investigated to be used as an alternative dsRNA-carrying technology for mosquito control (Phanse et al., 2015). Phanse et al. (2015) fabricated fluorescently labelled polyethylene glycol-based nano-complexes of specific sizes, shapes and charges and evaluated their properties both in vitro in mosquito cell culture and in vivo in *Anopheles gambiae* larvae following injection and feeding. Following direct administration into the larval body, the bio-distribution of positively and negatively charged PRINT nanoparticles of each size and shape was similar and accumulation was mainly observed in the thoracic and abdominal regions of the larvae. Positively charged nanoparticles were more likely to be associated with the gastric caeca in the gastrointestinal tract. Negatively charged nanoparticles could have been persisting through metamorphosis and were localized in adult insect organs such as head, body and ovaries (Phanse et al., 2015). During in vitro experiments, positively charged nanoparticles were more efficiently internalized into the cells and trafficked to the cytosol, while negatively charged nanoparticles accumulated in lysosomes (Phanse et al., 2015). No cytotoxic effects were observed for any of the tested nanoparticles (Phanse et al., 2015). The authors finally concluded that the excellent low cell and larval toxicity profiles, efficient internalization and widespread bio-distribution of PRINT nanoparticles rendered them as attractive candidates for dsRNA delivery in mosquitoes.

Liposomes have also been examined as a potential dsRNA delivery system (Fig. 4.2) in the German cockroach (*Blattella germanica*). Injection of non-complexed dsRNA into the abdomen of *B. germanica* caused dramatic depletion of the essential α-tubulin gene and associated mortality (Lin et al., 2016). In contrast, when the naked dsRNA was orally delivered, lower RNAi efficiency was observed, which was accounted for by the rapid degradation of the dsRNA in the midgut of *B. germanica* (Lin et al., 2016). On the other hand, continuous ingestion of dsRNA-containing lipoplexes was potent with respect to slowing down the degradation of dsRNA in the midgut and to increasing the mortality of the German cockroach.
Fig. 4.2. Molecular ‘Trojan horses’ of dsRNA delivery. Chitosan, carbon quantum dots, silica, PRINT®-based, perfluocarbon-bound, fluorescent cationic core-shell and star polycation nanoparticles as well as liposomes, guanylated polymers and PTD–DRBD peptides have been used for efficient dsRNA delivery in insects. Mechanisms include stabilization and protection of dsRNAs from nuclease degradation and enhancement of dsRNA uptake and endosomal escape. Bacteria-mediated dsRNA delivery is a low-cost method for RNAi delivery but improvements can be made in efficiency of dsRNA synthesis. VLPs loaded with dsRNAs is an alternative methodology to create potent insecticidal dsRNAs. VLP components and dsRNAs could be co-expressed in biotechnological platforms such as the baculovirus expression vector system (BEVS) for efficient packaging.
following inhibition of α-tubulin expression in the midgut (Lin et al., 2016).

In a recent work (Christiaens et al., 2018) researchers used guanidine-containing polymers to protect dsRNA against degradation in Spodoptera exigua, which has a very alkaline gut environment while it is also characterized by a strong intestinal nucleic acid-degrading activity. In this research, it was shown that polymers with high guanidine content (Fig. 4.2) proved to be highly protective against dsRNA degradation at pH 11, while the shielding effect lasted for up to 30 h (Christiaens et al., 2018). Moreover, the use of this polymer enhanced the cellular uptake in the lepidopteran CF203 midgut cells. Additionally, a synthetic cationic polymer, poly-[N-(3-guanidinopropyl)methacrylamide] (pGPMA), which mimics arginine-rich cell penetrating peptides, was found to be efficiently taken up by Sf9 cells and to drive highly efficient gene knockdown and moderate larval mortality in Spodoptera frugiperda (Parsons et al., 2018).

As well as the above-mentioned experimental cases, a wide range of nanoparticles of different compositions have been fabricated resulting in improved insecticidal properties. For example, perfluocarbon-bound siRNA nanoparticles have been administered by aerosol to aphids (Thairu et al., 2017) in order to stabilize the RNA trigger and deliver it to internal organs via the tracheoles. Using this technology, the aerosolized perfluocarbon-bound siRNA nanoparticles were transferred through tracheoles to the gut and to the haemolymph of aphids. Aerosolization of naked RNAs improved RNAi efficiency, which was even more profound using the perfluocarbon-bound siRNA nanoparticles (Thairu et al., 2017). Moreover, fluorescent cationic core-shell nanoparticles, which consisted of a fluorescent core of perylene-3,4,9,10-tetracarboxydimide chromophore (PDI) in the centre and polymer shells terminating with multiple amino groups, efficiently entered into live cells presenting low cytotoxicity as well as high gene delivery efficacy (He et al., 2013). Using this technology, researchers have efficiently silenced CHT10, a midgut-specific chitinase gene expressed in the gut peritrophic membrane of the Asian corn borer, Ostrinia furnacalis (Lepidoptera: Crambidae), leading to severe defects in larval growth and consequently to death (He et al., 2013).

Manufacturing of dsRNA-bound nanoparticles should be cost effective and convenient for industrialization. Recently, researchers have developed a star polycation (SPc) as an efficient but low-cost gene carrier for pest management (Li et al., 2019). As emphasized by the authors, the chemical sources of SPc are cheap and easily available. The chemical structure of SPc containing four arms in one core with a compact tertiary amine, confers high gene transfection efficiency. The nanoparticles can deliver dsRNAs to knock down insect gene expression and inhibit pest growth (Li et al., 2019).

4.5.2 Bacterial delivery

Bacterial dsRNA administration (Fig. 4.2) was pioneered by Timmons and Fire (1998) who showed that ingestion of bacterially expressed dsRNAs could be effective in the production of specific and potent genetic interference in C. elegans. This approach uses an RNase III-deficient Escherichia coli strain known as HT115 (DE3) (Timmons and Fire, 1998; Kourti et al., 2017). Following this methodology, cloning of the gene of interest takes place between two T7 promoters on the special RNAi plasmid L4440. For the transformation, HT115 cells are used and dsRNA is produced upon induction of T7 RNA polymerase. Following induction of dsRNA production, the cells are introduced in the worm’s growth medium and RNAi happens after a short period of incubation. In a similar way in insects, dsRNA-producing bacteria are incorporated in their artificial diets or are sprayed on plant organs that are the food for the insects, while again RNAi occurs following a period of continuous feeding. Successful bacteria-mediated RNAi has been reported in many insect species (Tian et al., 2009; Zhu et al., 2010; Kontogiannatos et al., 2013; Zhang et al., 2013; Li et al., 2014) with various results, mostly reflecting efficiency issues. In S. exigua a high dosage of dsRNA is required to efficiently kill late-instar stages because of high activity of RNases in the midgut lumen (Vatanparast and Kim, 2017). It was observed that sonication of bacterial cells before oral administration minimizes dsRNA release and causes higher larval mortality (Vatanparast and Kim, 2017). Moreover, targeting of young
larvae that possessed weak RNase activity in the midgut lumen led to significant enhancement of RNAi efficiency and insecticidal activity against *S. exigua* (Vatanparast and Kim, 2017).

A prominent approach for continuous dsRNA delivery via bacterial expression was published by Whitten *et al.* (2016). In this work, researchers genetically modified symbiotic bacteria of the blood-sucking bug *Rhodnius prolixus* and the western flower thrips *Frankliniella occidentalis* in order to constitutively express dsRNAs. When the modified bacteria were ingested, they colonized the insects, while they also successfully competed with the wild-type microflora and sustainably mediated systemic knockdown phenotypes that could be horizontally transmitted (Whitten *et al.*, 2016).

### 4.5.3 Ribonucleoprotein delivery

siRNA-based therapeutics are receiving much attention because of their promising impact in human cancer therapy. As happens in insects, the siRNA size and anionic charge are limiting factors that do not facilitate efficient penetration of the dsRNAs into mammalian cells. An efficient siRNA delivery approach was reported for the first time by Eguchi *et al.* (2009), where a peptide transduction domain–dsRNA-binding domain (PTD–DRBD) fusion protein was used. In this report it was shown that DRBDs bind to siRNAs strongly, thus bypassing the siRNAs' negative charge and allowing PTD-mediated cellular uptake. The RNAi response was quickly induced by PTD–DRBD-delivered siRNA in many types of primary and transformed cells (Eguchi *et al.*, 2009).

The use of PTD–DRBD peptides to improve insect RNAi (Fig. 4.2) was investigated in the coleopteran *Anthonomus grandis* almost 8 years after the discovery of PTD–DRBDs (Gillet *et al.*, 2017). As could reasonably be expected, the chimeric PTD–DRBD protein combined with dsRNA formed a ribonucleoprotein particle that improved the effectiveness of the RNAi mechanism in this insect. The same authors reported that the complex slows down nuclease activity in the gut of *A. grandis* and that PTD-mediated internalization in insect gut cells is achieved within minutes after plasma-membrane contact, limiting the exposure time to gut nucleases. Most importantly, the efficiency of insect gene silencing upon oral delivery presented an approximately twofold increase when PTD–DRBDs were used as a delivery method compared with naked dsRNA (Gillet *et al.*, 2017).

### 4.6 Viral Components as ‘Trojan Horses’ of Insect RNAi

#### 4.6.1 dsRNA viruses

dsRNA viruses comprise a diverse group that infect a wide range of hosts from animal, plant, fungal and bacterial kingdoms. Their genome is organized in segments numbered from 1 to 12 and their virions are all non-enveloped and possess icosahedral capsids that are differentiated by T-number, capsid layer and turret forms. The dsRNA viruses are segregated into 12 families: Amalgaviridae, Birnaviridae, Chrysoviridae, Cystoviridae, Endornaviridae, Hypoviridae, Megabirnaviridae, Partitiviridae, Picobirnaviridae, Quadriviridae, Reoviridae and Totiviridae. Of these, *Reoviridae* is the largest and most diverse family with respect to host range, with the most important members in this group being rotaviruses that cause gastroenteritis in young children and bluetongue virus, an economically important pathogen of cattle and sheep that is transmitted by mosquitoes (Louten and Reynolds, 2016).

In contrast to DNA viruses, RNA viruses typically do not penetrate an infected cell nucleus. Since they do not form a DNA intermediate, they do not need any of the host enzymes to replicate their RNA genome. However, RNA viruses still need to transcribe their mRNAs to allow host ribosomes to translate viral proteins and to form new virions. Because cells do not contain the enzymes required to transcribe mRNA from an RNA template, all RNA viruses therefore must carry and encode their own RdRP enzyme to transcribe viral mRNA. dsRNA viruses therefore typically code for and contain an RdRP that is carried into the cell within the virion (Louten and Reynolds, 2016).

dsRNA replication occurs in the cytoplasm for all dsRNA viruses that have been investigated. Transcription, known as the synthesis of a dsRNA template’s viral positive strands, occurs
in viral particles or core particles. Usually, the new positive strands are extruded from the viral particles and translated into viral proteins. The same positive strands are then packed to produce new particles or subviral particles. Negative strand synthesis on the positive strand template (replication) completes the creation of new dsRNA once the new particles or cores have been formed. For dsRNA viruses with more complicated structures, addition of new layers of protein and/or membrane completes the virus reproduction cycle (Wickner, 1993).

### 4.6.2 The dsRNA virus replication machinery

dsRNA viruses have evolved sophisticated mechanisms for cellular entry. Because they are carriers of dsRNA molecules (genome segments), it is interesting to analyse their life cycle in some detail and get an idea of how the dsRNA fragments are replicated and shielded from degradation or interaction with the RNAi machinery. Two dsRNA viruses with relatively well-characterized life cycles are discussed.

Bluetongue virus (BTV) (genus: Orbivirus; family: Reoviridae) is an arthropod-borne virus (arbovirus) that is transmitted by midges (Culicoides sp.). The typical dsRNA virus replication machinery resembles that of the BTV. As described by Lourenco and Roy (2011) and Sung et al. (2019), the BTV particle has two capsids, an outer capsid and an inner capsid, the latter of which is also called the core. The outer capsid contains proteins VP2 and VP5 to facilitate virus entry through the cellular membrane and the release of the core into the cytoplasm. The core particles do not further disassemble and are capable, using the encapsidated dsRNA genome segments as template, of producing positive strand RNA molecules that are transported to the cytoplasm through channels in the core particles. The icosahedral-shaped core principally comprises two proteins, VP7 and VP3, which are arranged in two layers. The VP3 layer encloses the viral genome of ten dsRNA segments (S1–S10). In addition, the core contains three minor proteins: the polymerase (VP1), the capping enzyme (VP4) and VP6, an essential structural protein of 36 kDa with RNA and ATP binding activity. VP6 is unique in the Orbivirus genus within the Reoviridae family. Upon entry, core particles become transcriptionally active, producing and extruding single-stranded positive sense RNAs (ssRNA) through the local channels at the fivefold axis, without further disassembly. These ssRNAs then act as mRNAs for viral protein synthesis and as templates for genomic RNA synthesis. The ten newly synthesized ssRNA segments are first combined via specific intersegment RNA–RNA interactions to form RNA complexes of all ten segments and then packaged together with VP1, VP4 and VP6 into the assembling VP3 capsid layer. Genomic dsRNA molecules are subsequently synthesized within this assembled particle (known as the ‘subcore’), prior to encapsidation by the VP7 layer, leading to robust core particle formation (Lourenco and Roy, 2011; Sung et al., 2019).

Cypoviruses (cytoplasmic polyhedrosis viruses (CPVs); genus: Cypovirus; family: Reoviridae) are widespread pathogens of insects. The type species is Cypovirus 1, which specifically infects silkworms (Bombyx mori) and negatively affects the sericulture industry (Cao et al., 2012; He et al., 2017; Zhao et al., 2019). In contrast to all other reoviruses, CPV virions consist of one layer of capsid that corresponds to the core particle of other reoviruses (discussed above for BTV). A distinctive feature of CPVs, which is shared by the DNA viruses, baculoviruses, is the production of polyhedra or occlusion bodies that protect encapsulated virions against damage and enhance viral survival in the environment (and actually can be regarded as a replacement for the outer capsid layer in other reoviruses). After feeding, CPV polyhedra are lysed because of the high pH in the lepidopteran midgut, which results in the infection of the midgut epithelium by the released virions. Interestingly, electron microscope images show that CPV virions can directly penetrate the plasma membrane of the microvilli that are localized at the apical sides of the enterocytes during midgut infection of silkworm larvae (Cao et al., 2012; He et al., 2017; Zhao et al., 2019). Functional studies also show the involvement of clathrin-mediated endocytosis for uptake of CPV virions in silkworm-derived BmN cells and the midgut epithelium (Tan et al., 2003). In the cytoplasm, CPV virions undergo activation and become capable of RNA transcription using
similar mechanisms as described for core particles of BTV (see previous section). Besides an RdRP, CPV virions contain additional processing activities that form a cap-like structure, mGpppAmpGp, to protect viral mRNAs from degradation by exonuclease enzymes. All of the dsRNA segments in the genome of Cypovirus 1 contain the conserved sequence (GUUA......GUUAGCC) at their ends which likely function as recognition signals for the RdRP complex to initiate transcription or replication but may also have a role in the binding of the mRNAs to the ribosomes or the interaction with viral structural proteins (Tan et al., 2003; Cao et al., 2012; He et al., 2017; Chen et al., 2018).

4.6.3 The use of unique viral components as molecular tools to achieve improved RNAi efficiency in insects

The unique mechanism of dsRNA virus replication is an evolutionary conserved molecular adaptation that aims to protect viral dsRNA genomes from the hostile cellular environment of their hosts. For efficient infection, dsRNA viruses must be able to: (i) penetrate efficiently the host cell’s cytoplasmic membrane; (ii) protect their dsRNA genome from RNA degradation and the RNAi response; (iii) translate the non-structural and structural proteins encoded by their genome; and (iv) multiply and construct new virions. In order to use RNAi as a potent tool for efficient pest control, applied dsRNAs must have two characteristics in common with those found in dsRNA virus infections: (i) penetration efficiency; and (ii) RNA degradation resistance. The question then arises as to whether one could mimic the molecular components of viral infection in order to improve RNAi in insects.

In the pharmaceutical industry, viruses provide an ideal basis for the development of targeted drug delivery vehicles (Yildiz et al., 2011). Interest in the exploitation of virus-based nanoparticles (VNP s) and virus-like particles (VLPs) has united efforts among researchers in different fields such as biology, chemistry, engineering and medicine (Fig. 4.2). VLPs are the genome-free counterparts of virions and are valuable because of their biocompatibility and biodegradability. Plant and bacterial VLPs have the additional advantage of being non-infectious and non-hazardous in humans and other mammals (Yildiz et al., 2011). VNPs are well-characterized, monodisperse structures that can be produced in large quantities, which also enables solving their structures at atomic resolution. VNPs have highly symmetrical structures and can be considered as one of the most advanced and flexible nanomaterials. Furthermore, the basic VNP structure can be ‘programmed’ for loading with drug molecules, imaging reagents, quantum dots and other nanoparticles, while its external surface can be changed to reveal targeting ligands that allow cell-specific delivery (Yildiz et al., 2011).

The use of VLPs for biotechnological applications in agriculture remains unexplored so far. However, the potential of RNA viruses for triggering of gene silencing and concomitant lethal effects was underscored in a recent study that employed recombinant FHV that was engineered to package foreign RNA sequences (Taning et al., 2018). Nonetheless, in this case viruses with replicating genetic material were used that can be classified as genetically modified organisms (GMOs). Because GMOs are associated with strong public and political opposition and require lengthy evaluation procedures, the approach of VLPs with inert genetic cargo (non-replicating dsRNAs) may be considered safer and more feasible from a regulatory viewpoint. For transport and delivery of dsRNAs, VLPs based on dsRNA viruses are more suitable than those of ssRNA viruses that may not package efficiently long dsRNA molecules that form strong secondary structures (Zhao et al., 2018). However, packaging of short RNA hairpins is possible, as was illustrated for VLPs based on the bacteriophage Q β that naturally packages a positive strand ssRNA genome. In this case, RNAi scaffolds consisted of fusions of the 29 nt Q β RNA hairpin packaging signal with a miRNA-based stem loop of 59 nt (Fang et al., 2016). When co-expressed in bacteria, the Q β capsid protein and the RNAi scaffold become spontaneously assembled in VLP-RNAi particles. While this example illustrates that delivery of short RNA hairpins with VLPs of ssRNA viruses is possible, VLPs of dsRNA viruses may have the advantage of packaging long dsRNAs that may have more potent silencing and insecticidal effects (Fang et al., 2016; Zhao et al., 2018).

Among dsRNA viruses, cypoviruses may constitute the basis for the development of a
biotechnological platform in agriculture for production of VLPs that carry long dsRNAs as cargo (RNAi-VLPs). Cryo-electron microscope studies established that the CPV virion consists of three major capsid proteins: (i) the capsid shell protein (also known as VP1) that spontaneously forms a thin icosahedral capsid shell of 66 nm; (ii) the turret protein (also known as VP3); and (iii) 'large protruding protein' (also known as VP5) (Cheng et al., 2011; Fang et al., 2016). The mature virion also contains a small number of copies of the A-spike protein (also known as VP2) that could be involved in cell attachment, and the transcription enzyme complex consisting of the RdRP and VP4 (Hagiwara et al., 2002; Cheng et al., 2011; Fang et al., 2016). The well-known structure of CPV virions permits the rational design of VLPs with enhanced properties such as increased stability and facilitated cell penetration. For delivery of RNAi, methods for efficient incorporation of (long) dsRNA molecules also need to be devised (Kolliopoulou et al., 2017; Zhao et al., 2018).

4.7 Conclusions

RNAi technology is one of the most appealing trends in the field of crop protection and has major advantages in comparison with chemical insecticides that are currently in use. In RNAi applications, the requirement of specific base-pairing almost guarantees the precise targeting of the intended pest with minimal repercussions on non-target species. In comparison with the non-specific detrimental effects of chemical insecticides on non-target organisms (pollinators, parasitoids, predators and vertebrates), this can be considered as a major asset. However, RNAi technology suffers from issues with efficiency and speed of killing and more research efforts are required to improve the methodology.

Currently many laboratories are investigating different dsRNA delivery methods in order to achieve better performances of RNAi in insects. Chemically synthesized nanoparticles, ribonucleoproteins, specialized bacterial strains and VLPs underline the continuous effort that the scientific community is currently taking to produce more efficient but also safer and more environmentally friendly RNAi-based pesticides. Considering the amount of basic knowledge that still needs to be acquired, RNAi research remains an ongoing process whose valuable applications will likely not be shown sooner than the ending of the coming decade.

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Biogenesis and Functional RNAi in Fruit Trees

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Abstract

In plants, genome expression is linked to the transcribed mRNAs that are synthesized by RNA polymerase. Following its move to the cytoplasm, the generated mRNA is briefly translated to the encoded protein. If transcription and translation are dependent on the family of RNA polymerase, these two phenomena could be interfered with through the process designated as gene regulation. Thus, large molecules of RNA (single-stranded or double-stranded) consequently sliced into small molecules produce nascent small interfering RNA ranging from 21 to 27 nucleotides. This chapter revisits the biogenesis of these two types of RNAi, miRNA and siRNA, and notably their involvement in plant gene regulation. Following their sequential transcription and their specific involvement, we will consider the sources and roles of RNA interference in plants and we will look at their detection in fruit crops. We discuss their applications and the risk assessment studies in fruit crops.

5.1 Brief Report about Biogenesis of RNAi in Plants

Recent progress with plant genome sequencing has increasingly led to the rapid development of gene regulation studies. In parallel with better knowledge about cellular components, knowledge has increased about plant promoters (Chow et al., 2016) and how plant genes can interact in leading to either gene knock-out or an overexpression of plant phenotypes (Baulcombe, 2004). The development of many satellite studies on gene interference has been successfully performed and among these were studies of the silencing of changes to coloured petals of petunias (Napoli et al., 1990; Jorgensen et al., 1996). These studies showed that knock-out or overexpression was due to small RNA molecules that interfere with the homologous nucleotide sequences of the encoding genes. Interfering sequences and consequent phenotypes were closely dependent (Hamilton and Baulcombe, 1999), showing that a genetic character can be either reverted or exclusively fixed (Zotti et al., 2018). Two types of RNA interference (RNAi) can be involved in plants: microRNA (miRNA) and small interfering RNA (siRNA). Whereas miRNA is single-stranded (Bartel, 2004; Carthew and Sontheimer, 2009), siRNA functions as a small dsRNA (21–27 nt) produced from cleavage of a larger double-stranded RNA (dsRNA), designated as a precursor (Nakahara and Carthew, 2004). Levels of miRNA are variable in plant cells, because the binding of the miRNA to its complementary endogenous mRNA is specifically occurring.

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and they also change either in different tissues or in some developmental stages (Carrington and Ambros, 2003; Carthew and Sontheimer, 2009). It has been shown that miRNA can bind to a specific peptide, conferring their stable function in cells through miRNA encoded peptides (miPEPs) (Couzigou et al., 2015). However, siRNA can interact with the plant enzyme RDR6 in amplifying the molecule ratios in cells that cause a robust gene interference that increasingly confers a strong phenotype (Fahlgren et al., 2006).

While a high population of RNAi is synthesized in cells, the lifespan of these molecules results in a long chain of gene regulation and interfering interactions that can be affected either spatially or temporally. It is known that a population of miRNA can be involved in regulation of several genes; however, an siRNA response is specific (Carthew and Sontheimer, 2009).

### 5.2 Diversity of RNAi in Fruit Crops

Studies of new technologies in fruit crops have lagged behind those in the annual crops that have a high economic value as foods (Dempewolf et al., 2017). In perennial fruit crops, traditional plant breeding has been the main technology for crop improvement (Peña and Séguin, 2001). This has limitations as fruit trees grow slowly and need to achieve maturity for fruiting, so that genetic improvement required patience and good management (good knowledge and a high expertise about tree physiology and cultivation). Molecular breeding as an approach developed by scientists led to the strategic discovery of new genes of interest (Wei et al., 2015). New traits that were discovered conferred virus resistance (Zuriaga et al., 2018) and plant transformation technologies permitted introduction of these new traits (Petri et al., 2011). Genetic engineering of Prunus, Citrus, Malus, Vitis and other crops brought variable benefits for fruit-tree breeders, including resistance to viruses (Scorza et al., 1994; Ravelonandro et al., 1997; Reyes et al., 2011; Scorza et al., 2013; Rubio et al., 2015). Papaya is a promising example that reflects the success of RNAi against papaya ringspot virus (PRSV) (Gonsalves, 2006). This has led to growing interest in the discovery of new gene(s) and the subsequent exploitation of gene silencing.

Many plant genes are known to control the metabolic chain of any enzymatic complex in the Krebs cycle (Senthil-Kumar and Mysore, 2010). Among the challenges was the feasibility of investigating the role of genes encoding the enzymes expressed in different plant tissue. Physical studies that focused on agronomic and horticultural traits of fruit tree species have revealed that mapping different genes helped complement full genome sequencing (Iwata et al., 2016). Hence, the ability to transform a fruit crop facilitates the introduction and regulation of engineered gene(s) in order to achieve expression of specific traits (Petri et al., 2011).

In juvenile fruit trees, the immature age of the fruit trees did not allow verification of the RNAi effects until a few years later. Once introduced into plant genomes, new engineered sequences can be verified through sequencing and molecular hybridization (Ravelonandro et al., 2019). RNAi occurs in any cellular compartment so has a key role in regulating plant life. The role of RNAi is threefold: (i) to regulate the endogenous genes; (ii) to specifically interfere with the targeted sequences; and (iii) to convert such molecular interactions in plant phenotype (Zotti et al., 2018). Focusing on fruit, RNAi appears as part of molecules enabling specific interference with genes expressed either endogenously or exogenously. The models supporting these phenomena are the apple ‘Arctic’, the ‘HoneySweet’ plum, the ‘Rainbow’ papaya and the activity against certain pests of grapevines (Nandety et al., 2015; Taning et al., 2016) (Figs 5.1 and 5.2).

### 5.3 Detection and Application in Fruit Crops

The relevance of the efficiency of RNAi and the targeted virus RNA was significantly highlighted in plum (Scorza et al., 2013) and other fruit. In ‘Arctic’ apple, four genes are silenced that control polyphenol oxidase (PPO) production (Armstrong and Lane, 2009), which causes the production of brown melanin due to oxidation following fruit damage. Consequently, the ‘Arctic’ apple differs from conventional fruit in that its flesh does not turn brown after slicing (Fig. 5.2).
Studies of healthy plums sampled from trees challenged with plum pox virus (PPV) and ‘Arctic’ apple have shown that the engineered RNAi construct functioned in whole plants (Figs 5.1 and 5.2). Studies of any other effects of the introduced gene showed homology of ‘HoneySweet’ fruits with those sampled from healthy conventional cultivars (either ‘Stanley’ or ‘Reinclod’) (Bobis et al., 2019). Enzymes involved in fruit storage and maturation function similarly and there is homologous fruit composition (Ravelonandro et al., 2013; Callahan et al., 2019). The genetic engineering of the PPV coat protein (CP) gene as an introduced sequence did not lead to any change in plum tree traits, apart from PPV resistance, showing that the RNAi is restrictively expressed to target only PPV RNA (Fig. 5.1c,d). Resistance traits against either PPV (‘HoneySweet’ plum parent) or PRSV (‘Rainbow’ papaya parent) share the same properties as those transferred in hybrids and conserved similar effects.

5.4 Biosafe Use of RNAi

This interference strategy occurring in plants can be exploited to change metabolic paths to optimize desired characteristics that could not
Fig. 5.2. (a) Sliced fruits of conventional apple showing browning (left) compared with ‘Arctic’ apple (right). (Figure courtesy of Okanagan Company, Summerland, Canada.) (b) Molecular mechanism of the silencing of the mRNA encoding the polyphenol oxidase (PPO) so that browning does not occur in ‘Arctic’ apple when sliced.
be achieved with classical breeding. However, in common with the use of new genetic techniques, the introduction of this new RNAi technology raised safety concerns, although the highly selective nature of RNA activity reduces the likelihood of off-target and non-target effects. This has been supported by the genetic and bioinformatic information obtained through next-generation sequencing (NGS), high-throughput sequencing (HTS) and other techniques (Shendure and Ji, 2008). The sources, paths and compartmental cell storage are also relevant. A number of risk assessment studies of engineered fruit trees have been conducted and have not shown any unexpected or adverse effects (Yien et al., 2011; Scorza et al., 2019). For example, oral feeding of mammals with PPV-resistant plum in experimental models showed no adverse effects on mice and no allergenic reactions. The RNAi modified papaya did not reveal any genotoxicity in any analysed gastro-organs in rats (Yien et al., 2011). These results suggest that RNAi does not elicit any unexpected toxic reactions and does not represent any bio-risk to mammals (Scorza et al., 2019).

5.5 Conclusions

The aim of this chapter has been to provide information on RNAi that can be either endogenously produced by plant cells and then accumulated in fruits (Figs 5.1 and 5.2) or exogenously applied on fruits in order to protect them against parasites (Taning et al., 2016). For RNAi technology to be firmly and clearly appreciated by consumers, it is important for us to deliver honest and relevant communications, especially in EU countries. First, during the period of development of genetically modified organisms for these past four decades, the writing of laws and rules concerning the use and release of modified plants in the environment has been a dominant factor (DeFrancesco, 2013). Secondly, the emergence of new technologies, ranging from RNAi to gene editing (Shan et al., 2013), provides a powerful platform for gene regulation that should offer potential resources in crop improvement. Further improvements in metabolomics, genetics and bioinformatics will provide further evidence (Shameer et al., 2014) to support the acceptance of RNAi.

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6 Gene Silencing or Gene Editing: the Pros and Cons

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Abstract

Research into plant genetics often requires the suppression or complete knockout of gene expression to scientifically validate gene function. In addition, the phenotypes obtained from gene suppression can occasionally have commercial value for plant breeders. Until recently, the methodological choices to achieve these goals fell into two broad types: either some form of RNA-based gene silencing; or the screening of large numbers of natural or induced random genomic mutations. The more recent invention of gene editing as a tool for targeted mutation potentially gives researchers and plant breeders another route to block gene function. RNAi is widely used in animal and plant research and functions to silence gene expression by degrading the target gene transcript. Although RNAi offers unique advantages over genomic mutations, it often leads to the formation of a genetically modified organism (GMO), which for commercial activities has major regulatory and acceptance issues in some regions of the world. Traditional methods of generating genomic mutations are more laborious and uncertain to achieve the desired goals but possess a distinct advantage of not being governed by GMO regulations. Gene editing (GE) technologies have some of the advantages of both RNAi and classical mutation breeding in that they can be designed to give simple knockouts or to modulate gene expression more subtly. GE also has a more complex regulatory position, with some countries treating it as another conventional breeding method whilst the EU defines GE as a technique of genetic modification and applies the normal GMO authorization procedures. This chapter explores the pros and cons of RNAi alongside other methods of modulating gene function.

6.1 Introduction

Blocking the expression of a (candidate) gene has long been an experimental tool for research that aims to define the cellular function of specific DNA sequences. Alongside other methods, it can provide strong evidence to support a hypothesis on the role of a gene. It can also provide novel phenotypes with useful characteristics for commercial products (see elsewhere in this text). Until recently, the options for doing this were restricted to screening individuals possessing a knockout phenotype due to random natural or induced mutations in the genome, or gene silencing that resulted in reduced protein synthesis from the gene under investigation. These two fundamentally different approaches have coexisted in research and commercial arenas over the past few decades but have various pros and cons, which are discussed below.

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The invention of gene editing offered researchers another, potentially more powerful, approach to gene suppression. CRISPR (clustered regularly interspaced short palindromic repeats) (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013) is proving to be faster, cheaper and easier to use than other existing programmable gene editing technologies, including oligonucleotide-directed mutagenesis (ODM), meganucleases (MN), zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) reviewed by Guha et al. (2017). The exponential growth in research outputs using CRISPR is testament to its utility for targeted mutation and gives researchers and plant breeders yet another route to block or fine-tune gene function and expression. This chapter compares and contrasts these various methods of modulating gene expression in plants and highlights the pros and cons of RNAi in particular.

### 6.2 Random Mutations

The earliest approach was simply to screen wild populations for natural variants that possessed random mutations resulting in a 'knockout' of gene function. While its use as a 'forward genetic' tool (with no control and often no knowledge of the genetic changes made) has been integral to plant and animal breeding for centuries, it requires long time scales and very large plant populations. It is often not a feasible approach to use when specific, pre-defined genetic mutations are sought. One way to overcome this limitation is to substantially increase the cellular mutation rate using chemicals or radiation that randomly damages DNA. Mutation breeding has exploited this approach since the 1930s, although the term 'Mutationszüchtung' (mutation breeding) was not coined until 1944 (Freisleben and Lein, 1944). The reverse genetic tool of TILLING (Targeting Induced Local Lesions in Genomes) also depends on chemically induced or natural mutations and can be used to identify individuals that carry specific genetic regions that may or may not result in gene knockout (McCallum et al., 2000). An advantage of inducing mutations is that it can generate very high numbers of genetic changes in some species. For example, in a wheat TILLING experiment, after selfing plants germinated from seeds exposed to ethyl methanesulfonates (EMS), it was estimated that individual plants carried an average of 340,000 mutations (Chen et al., 2012). TILLING also exploits high-throughput molecular methods to screen for mutations in specific genes, such as targeted sequencing or mismatch cleavage assays that utilize specific endonucleases such as CEL1 (Kurowska et al., 2011).

An alternative method to generate knockout mutants is via insertional mutagenesis, which utilizes the random integration of transgenes (often by Agrobacterium T-DNAs) to interrupt gene function. The main drawback of this method is the low efficiency of generating T-DNA insertions in functional genes. For this reason, insertional mutagenesis is fully applicable only in highly transformable plant species with small genomes, such as Arabidopsis and rice (Bolle et al., 2011). However, it also has advantages in that the resulting mutation can be tagged using a reporter gene incorporated within the T-DNA and thus can be modified to identify promoter sequences in genomes. For example, a promoterless GUS construct randomly inserted into the cereal Tritordeum identified anther-specific expression patterns (Salgueiro et al., 2002).

All the methods described above generate random changes in genomic DNA where the location and type of mutation cannot be predicted. Of these mutations, only a subset would reside in a target gene and only some of these would result in knockout phenotypes. While whole genome or other sequencing strategies may retrospectively be able to identify the sequence changes generated, there remain two major drawbacks of these methods. Firstly, because it is impossible to target the mutations, very large numbers of individuals, each carrying a very large number of mutations, must be generated. As a consequence of this, considerable cost in terms of time, labour and money must be invested to screen large populations of individuals to identify those carrying any useful mutations. In addition, to 'clean up' the desired mutation from the many unwanted DNA changes in the same individuals, back-crossing to a recurrent parent for many generations is needed.
6.3 Gene Editing

Gene editing is a set of molecular tools developed over the past few decades that aims to precisely change an organism’s genome in a targeted fashion. Although many engineered nucleases have been developed to perform this task, CRISPR coupled with a CRISPR-associated protein (CAS), which is based on a natural system found in Streptococcus pyogenes, has proved to be the most facile and popular (Martínez-Fortún et al., 2017). Other programmable nuclease systems include MN, ZFN and TALENs but, in general, these require more work to get them functioning optimally for each new target sequence. Two common features of these editing tools are the ability to scan the host genome for the predetermined short DNA sequence and the subsequent binding of an exonuclease to generate a double-strand break (DSB) at the target site. Plant cells tend to repair DSBs in nuclear DNA using the error-prone, non-homologous end-joining (NHEJ) pathway, which can introduce small insertions and deletions (indels) at the cut site. Although the location of the cut site can be precisely predetermined by the design of the guide sequence, the exact mutation resulting from erroneous repair cannot. Thus, in practice, many different gene-edited individuals must be generated and screened by sequencing or phenotype for the desired knockout or other endpoint. A more deterministic variation of gene editing is to supply a short additional DNA fragment, which may or may not have homology to the flanking regions of the cut site. This can be incorporated into the host genome in a targeted manner either by the NHEJ pathway or, if sufficient identical sequence overlap is present, by an alternative minor repair pathway, known as homology directed repair (HDR), which can be also exploited to insert a DNA fragment into the DSB site. Like conventional mutation breeding, gene editing to generate mutations can result in knockouts or the synthesis of aberrant proteins. Where these mutations are in the coding regions of genomic DNA, the altered expression will appear in all cell types at all developmental stages. Recent developments have further expanded the capacity of the CRISPR-Cas system to produce, for example, nickases that cut only one DNA strand, methods to edit many targets simultaneously and Cas variants lacking nuclease activity that instead can recruit synthetic enhancers or repressors to alter gene expression. Using specific repressors, it has been possible to achieve heterochromatin-mediated gene silencing (termed CRISPRi) (Gilbert et al., 2013). However, we still lack a full understanding of the rules by which a given guide RNA may engage and be active on a given target site (Boettcher and McManus, 2015).

Thus, while there may be theoretical approaches to use of gene editing for silencing or to give tissue-specific or developmentally regulated alterations of expression, current commercial products under development (of which the author is aware) lack the subtle control of expression possible with RNAi.

6.4 RNA Interference

Post-transcriptional gene silencing via RNAi is a series of molecular interactions that lead to the suppression of target gene translation. There are several pathways of epigenetic regulation of gene expression found in cells but double-stranded RNA (dsRNA) designed to the coding region of an endogenous gene often leads to post-transcriptional degradation of target gene mRNA. To achieve RNAi in plants, dsRNA designed to complement the target sequence is inserted into cells, where it is cleaved by Dicer, incorporated into the RNA-induced silencing complex (RISC) and acts as a guide for Argonaute to degrade mRNAs specific to the target gene (Baulcombe, 2000). Transgene-induced RNAi requires a genetic transformation step and for some species it is relatively straightforward to design the necessary plasmid constructs to produce a dsRNA sequence and to transform plants so that they routinely display silencing of the gene target. However, it is also possible to observe transient silencing in transformed tissues when stable and heritable germline expression of dsRNA molecules is not the intention. For example, RNAi has been demonstrated following high-pressure spray application of siRNA into plant cells (Dalakouras et al., 2016) and by physical...
rubbing of virus particles on to leaves as in virus-induced gene silencing (VIGS) approaches (reviewed by Robertson, 2004). In addition, feeding or soaking animals such as nematodes and certain insects with dsRNA can induce robust silencing (reviewed in Britton et al., 2012 and Christiaens et al., 2018).

The levels of gene silencing that result from transgene-induced RNAi is highly variable, ranging from no apparent effect to high levels of suppression where expression of the target gene is undetectable. The most common outcome is partial suppression and, even when the same dsRNA cassette is used, different transgenic events can be found with different levels of silencing (Eamens et al., 2008). This can be advantageous if reduced expression rather than complete knockout phenotypes is desired. By choosing tissue-specific, developmentally regulated or inducible promoters to drive the dsRNA cassette, it is possible to direct silencing to specific cells (Tuteja et al., 2004; Rao and Wilkinson, 2006). This is a significant advantage of RNAi over the genomic knockout methods described above. However, silencing has also been observed in transgenic lines where RNAi was not intended, for example in events possessing multiple copies of gene cassettes intended for expressing functional genes. This silencing is difficult to predict, being variable between lines and over time (Howarth et al., 2005).

VIGS is a particularly well used research tool that has significant advantages over other techniques used for reverse genetic analysis of gene function. It is relatively rapid, facile and has low start-up costs. The optimization of several virus vectors for different plant species makes VIGS attractive for research in many monocot and dicot crops. VIGS can be used to rapidly screen many tens or hundreds of candidate genes because it does not need the stable, germline transformation step associated with T-DNA mutagenesis or transgene-induced RNAi.

As described above, RNAi can be readily used to silence native genes to alter the biochemistry or other phenotypic characteristics in the host organism. In addition, RNA silencing has been exploited as a powerful tool for engineering pest resistance into crop plants and the strategies to achieve this via mutation breeding or gene editing are still in their infancy. For example, a range of approaches have been deployed to silence the expression of viral components in crops such as papaya, squash, banana, plum and common beans (Wang et al., 2012). There are also many examples of plant-derived and sprayable sources of dsRNA being successfully used against pest insects (Bachman et al., 2013). Although there have been attempts to adapt genome editing for control of plant viruses (Mushtaq et al., 2019), it is not clear whether this will ever become a viable alternative to RNAi (Romay and Bragard, 2017).

### 6.5 Regulatory Considerations

In addition to the scientific rationale for choosing one methodology over another, where the equivalent end point can be achieved by more than one method, there may also be regulatory factors that influence the decision (Jones, 2015).

Applications of plant-derived RNAi necessitate the generation of a genetically modified organism (GMO) and so would be captured by GMO legislation and liable for risk assessment, authorization and, in some countries, product labelling. The exact procedures vary between the competent authorities in different countries but many are costly and have long time-frames. This is a particular issue in the EU, driven by the perverse voting patterns of various member states in advisory committees, which results in considerable uncertainty regarding the outcome. Silencing resulting from dsRNA sprays or other topical applications are not yet commercially available and there is considerable discussion regarding how they will be regulated.

Several countries in North and South America, along with others in Asia, have ruled that products of simple gene editing are not GMOs and would be regulated as any other conventionally bred variety. Prior to the European Court of Justice (ECJ) ruling on mutagenesis in 2018, there was a general expectation that the EU would follow a similar path by treating gene-edited mutations in a similar manner to classical mutagenesis with both being exempted from EU GMO legislation. However, the ECJ ruled that gene editing and other new forms of mutagenesis did not fit the exemption and that even simple mutations generated by gene editing must be regulated as GMOs. Although
there is an expectation that the EC will revisit this situation sometime in the future, as of now both plant-derived RNAi and gene editing are GMOs in EU law and have similar expectations in terms of risk assessment and labelling.

New lines produced by mutation breeding, whether incorporating wild mutations found naturally in the gene pool, or induced artificially by radiation or chemical mutagens, are dealt with as any new conventional variety. The exact procedures vary from region to region but in the EU they involve national trials and listing in the plant variety catalogue. Technically, mutation breeding is defined in Directive EC 2001/18 as a technique of genetic modification but exempted from the regulation because it was considered at the time to have a history of safe use. Thus, classical mutation breeding or TILLING, a relatively rapid method to generate characterized mutations in target genes, would be advantageous from a regulatory perspective in that new varieties would not require expensive GMO authorization or labelling.

### 6.6 Conclusions

Recent years have seen great advances in developing technologies for modulating gene expression in plants. Induced mutation breeding is hampered by its lack of precision, the numbers of mutations per individual and large population sizes required. It also needs to use genetic segregation over multiple generations to remove the undesired mutations from the breeding lines. However, the significant benefit of its non-GMO status in law means that it will always have a place in some breeding programmes that lack accessible variation in target traits.

The CRISPR/Cas9 technology can be used to edit nucleotide sequences of gene coding regions, regulatory elements or other selected genomic loci in plants. Early commercial examples have been simple loss-of-function alleles, because these are straightforward to generate. In the immediate future, commercial gene editing will likely focus on traits under simple genetic control and where the results of modification are already well understood from null alleles in existing gene pools or other knockout or silencing approaches, such as induced mutations or RNA interference (Martínez-Fortún et al., 2017). In regions of the world where simple gene edits are not governed by overburdensome GMO regulations and where food from these plants has broad consumer acceptance, gene editing is likely to displace RNAi approaches for applications where complete knockout phenotypes are desired. However, RNA silencing is now a well-established and easy-to-use technology, which will continue to serve as a useful tool in gene function analysis and crop improvement. Where complete knockout of genes is undesirable or indeed lethal to plants, or where silencing is required in some cells and not others, RNAi is the preferred method. With continuing efforts in further understanding the RNA silencing mechanisms in plants, it can be anticipated that RNA silencing technologies will be further improved to overcome potential limitations, allowing for wider applications in agriculture.

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### References


7 Application of RNAi Technology in Forest Trees

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Abstract

A diverse set of small RNAs is involved in the regulation of genome organization and gene expression in plants. These regulatory sRNAs play a central role for RNA in evolution and ontogeny in complex organisms, including forest tree species, providers of indispensable ecosystem services. RNA interference is a process that inhibits gene expression by double-stranded RNA and thus causes the degradation of target messenger RNA molecules. Targeted gene silencing by RNAi has been utilized in various crop plants in order to enhance their characteristics. For forest tree species, most of the successful RNAi modification has been conducted in poplar. Over the past 20 years, successful RNAi-mediated suppression of gene expression has been achieved with a variety of economically important traits. Moreover, the stability of RNAi-mediated transgene suppression has been confirmed in field-grown poplars. In this chapter, we describe examples of successful RNAi applications mainly in poplar but also provide some information about application of RNAi in pest control in forest tree species. Advantages and disadvantages of this technology with respect to the particular features of forest tree species will be discussed.

7.1 Introduction

Forests contribute profoundly to human well-being by providing a diverse set of important ecosystem services. These services may be divided into regulating, provisioning (e.g. production of timber and non-timber products) and cultural services. The regulating ecosystem services include various vital processes such as fire-risk prevention and soil erosion control as well as water and climate regulation. Indeed, forests play a major role in the global carbon cycle by absorbing CO2 and storing it in their biomass through photosynthesis. In contrast, deforestation elevates atmospheric CO2 levels and it has been estimated that deforestation and forest degradation can account for 26% of the CO2 emissions since 1870 (Le Quéré et al., 2016). Regardless of the well-recognized importance of forests, the global forest land area continues to decline as forests are converted to other land uses (FAO, 2016), predominantly commercial and subsistence agriculture (Whiteman, 2014). The loss of natural intact forests (primary forest, see Box 7.1.) is alarming, as these forests have greater capability to adapt to environmental changes and short-term climatic anomalies than forests that have been under human influence (Watson et al., 2018). Furthermore, intact forests support globally significant environmental values such

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as conservation of biodiversity; therefore, extra efforts should be made for their preservation. Halting deforestation and restoring degraded forests are important, as loss of forests threatens sustainable development as well as human well-being (Watson et al., 2018).

Simultaneously, the demand for wood biomass and other bio-based products is increasing with the needs of growing human populations and bio-based economies. Jürgensen et al. (2014) estimated that, in the year 2012, natural forests supplied most of industrial roundwood, while production of forest plantations was 33% (562 million m³). Projections on industrial roundwood supply indicate an increase of 67% in plantation wood production over the period 2000 (624 million m³) to 2040 (1043 million m³). The area of planted forests (Box 7.1) has increased since 1990, with an average annual rate of 3.2 million ha for the period 2010–2015 (FAO, 2016). However, it is likely that climate change and food production pressures will restrict land availability for planted forests, and thus create a need for more intensive management regimes for existing forests, including improved health management (Payn et al., 2015).

Wood production can be enhanced with improved plantation management, which includes soil preparation, weed and pathogen control and fertilization in addition to utilization of improved tree varieties (Häggman et al., 2013). Production of tree varieties with improved traits (growth, stem characteristics, abiotic and biotic resistance) has been the goal of modern tree breeding programmes launched in the 1950s. Due to the characteristics of forest tree species (Fig. 7.1), tree breeding is a slow and costly process. The tree breeding cycle typically consists of selection, field testing, controlled crossings and progeny/clonal testing. As tree species have a long juvenile phase, one must wait for years before trees flower. Moreover, it also takes years to be able to assess the phenotype of the progeny: DNA-based molecular markers have not yet enabled early selection of material, because of the complex patterns of inheritance of desired tree traits (Häggman et al., 2014). For instance, the breeding cycle for Scots pine was estimated to take 40 years (Ruotsalainen, 2014) or, if progeny tests were omitted, less than 30 years (Rosvall and Mullin, 2013). Genomic selection utilizing single nucleotide polymorphism (SNP) as genome-wide markers in predicting phenotypes may speed tree domestication by accelerating breeding cycles, increasing selection intensity and improving the accuracy of breeding values (Grattapaglia et al., 2018). Isik and McKeand (2019) reported on the fourth cycle of loblolly pine breeding of the Cooperative Tree Improvement Program at North Carolina State University, initiated in 1960. The authors were positive that high-quality SNP markers and SNP array available for loblolly pine will be a major advantage and that the predictive power of SNP markers will be verified in the near future.

Forest tree breeding can also be accelerated by using genetic engineering. Genetic

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**Box 7.1. How primary and planted forests can be defined**

**Forest** is determined both by the presence of trees in a land area (≥ 0.5 ha) and the absence of other predominant land uses (FAO, 2012).

**Primary forest** is naturally regenerated forest composed of native tree species with natural forest dynamics, including species composition, occurrence of dead wood, age structure and regeneration processes (FAO, 2012). Furthermore, there should be no clearly visible indications of human activities, the area is large enough to maintain its natural characteristics and the ecological processes are not significantly disturbed.

**Planted forest** is predominantly composed of trees established through planting and/or deliberate seeding (FAO, 2012). Planted forests include plantation forests and semi-natural forests. **Plantation forests** can be defined as intensively managed planted forest aimed for commercial production of wood and non-wood forest products, or production of specific environmental service (Carle and Holmgren, 2003). **Semi-natural forest** can be defined as a managed forest having some of the principal characteristics and key elements of native ecosystems (e.g. complexity, structure and diversity) and which is predominantly composed of native species (FAO, 2002).
engineering enables expression or repression/silencing of targeted genes (recombinant or endogenous) at certain developmental stages, in different tissues or by specific environmental cues (Hernandez-Garcia and Finer, 2014). In general, the economically most significant tree characteristics have been successfully modified with genetic engineering, including wood properties and productivity as well as abiotic and biotic resistance of trees (e.g. Häggman et al., 2013, 2014; Séguin et al., 2014; Chang et al., 2018). The stability of genetic modification, in addition to issues related to flowering onset and fertility, has been demonstrated as trustworthy in greenhouse and field studies (Häggman et al., 2013, 2016). Several different genetic engineering approaches repress the expression of the target gene via the RNA interference (RNAi) process present naturally in cells containing a nucleus. RNAi inhibits gene expression by double-stranded RNA (dsRNA) and thus causes the degradation of target messenger RNA (mRNA) molecules. Moreover, RNAi includes the suppression of the transcription of the target gene and also inhibition of the target gene translation. In this chapter we cover examples of successful RNAi-mediated genetic modifications conducted with dsRNA producing gene constructs and poplar, a model woody tree species in plant science.

### 7.2 Discovery of RNAi

For a long time, RNA was believed to only act as a messenger between DNA and protein; however, discoveries in the past ten years suggest that RNA is also involved in the regulation of genome organization and gene expression. Evidence has been obtained that regulatory RNA molecules play a central role for RNA in evolution and ontogeny in complex organisms, including tree species. Regulatory RNA comprises all types of small RNA molecules (sRNAs), including micro- and small interfering RNAs (miRNAs, siRNAs) that mediate the silencing effect of RNA interference (RNAi), an antiviral defence system discovered by Andrew Fire and Craig Mello.

Andrew Fire started collaboration with Craig Mello at the Carnegie Institution in Baltimore, Maryland, in 1986. The pioneering gene expression studies were done using Caenorhabditis elegans worms and injecting mRNA (sense RNA) of a gene encoding for muscle protein production (unc-22); however, no
responses from the worms were found, neither did injecting the worms with antisense RNA cause twitching movements typical for reduction of unc-22. Twitching movements from the worms were only detected when both the sense and the antisense RNA were applied, indicating silencing of the worm unc-22 gene. These findings, published in 1998 (Fire et al., 1998), led to the Nobel Prize in Physiology or Medicine in 2006 being awarded jointly to Andrew Z. Fire and Craig C. Mello ‘for their discovery of RNA interference – gene silencing by double-stranded RNA. It turned out that the role of introns in DNA was to code for RNAi elements. These early discoveries of RNAi technology were groundbreaking for all the applications presented in this chapter.

Before the universal mechanisms of RNAi were revealed, the RNAi phenomenon had been observed 30 years ago in plants when attempts were made to overexpress chalcone synthase (CHS) in petunia in order to make the flower colour more purple (Jorgensen, 1990; Napoli et al., 1990). However, instead of CHS overexpression, the gene was suppressed in varying levels, resulting in white-purple variegated and even white-coloured petunia flowers. Since its discovery, RNAi has been found to be common in almost all organisms as a basic biological process serving protection against viral infections and disabling the spread of transposable elements within a genome. RNAi induced silencing has been used widely in basic and applied research to functionally characterize gene-of-interest by loss of function and the RNAi mechanism has also been extensively used in crop protection platforms. So far, RNAi approaches have been conventionally based on the use of transgenic plants expressing dsRNAs against selected targets. However, the use of transgenes and genetically modified (GM) organisms has raised considerable scientific and public concerns; hence the need for alternative approaches has emerged, as underlined by Dalakouras et al. (2020).

### 7.3 RNAi in Plants

RNAi enables regulation of gene expression at transcriptional and post-transcriptional level mediated via target mRNA cleavage and/or translation inhibition. RNAi-mediated post-transcriptional gene silencing (PTGS) can be achieved with different genetic engineering approaches, including artificial/synthetic miRNA-induced gene silencing (MIGS) as well as virus- and host-induced gene silencing (VIGS, HIGS), while siRNAs can be exploited in transcriptional gene silencing (TGS).

In the RNAi process, ‘exogenous’ dsRNAs, originating from viral replication, transgenes or transposons are first recognized and cleaved in a cell by Dicer-like endonucleases into 21–24 nt short siRNAs. In plants, there are several Dicer-like endonucleases producing siRNAs with characteristic 3’ and 5’ termini (Bologna and Voinnet, 2014; Borges and Martienssen, 2015). The RNase III family enzyme Dicer-like1 generates miRNA/miRNA duplexes by processing imperfect primary hairpin RNAs (pri-miRNAs) encoded by the plant microRNA (MIR) genes. Both siRNAs and miRNAs possess 3’ overhangs of 2 nt which are stabilized by methylation by the enzyme Dicer-like1 (Yu et al., 2005; Yang et al., 2006). The 5’ terminal nucleotide of siRNAs and miRNAs will determine which one of the two sRNAs is loaded on to Argonaute protein, a core component of RNA-induced silencing complex (RISC).

The loaded sRNA acts as a guide for Argonaute in a search for complementary transcripts (mRNA) that are degraded (Hamilton and Baulcombe, 1999; Mi et al., 2008). In addition, Argonaute, together with sRNAs of 22 nt, will direct RNA-directed RNA polymerase to the 3’ of the target RNA, which leads to transcription of the target and generation of dsRNA. Subsequently generated secondary siRNAs, defined as transitive RNAi, cause systemic genetic interference. Moreover, Argonaute 24-nt sRNA complexes guide DNA methyltransferases to cognate DNA or its nascent transcript, leading to methylation of cytosines in both DNA strands (RNA-directed DNA methylation) (RdDM) and suppression of transcription (Wassengegger et al., 1994; Chan et al., 2004). In plants, the RNA silencing signal spreads to adjacent cells and long-distance through the vascular system, creating systemic signalling. The exact mechanism of RNAi signal movement is still undetermined. Cell-to-cell transportation is likely to occur through plasmodesmata, while long-distance transport has been shown to involve siRNA and...
miRNAs 21–24 nt long (Dunoyer et al., 2010; Mermigka et al., 2016).

7.4 Functioning of RNAi Vectors in Poplar

RNAi involves the silencing of a target gene by introduction of dsRNA corresponding to the sequences within the target gene to be silenced. Different software can be used in the design of RNAi constructs. The efficient prediction of long dsRNA RNAi constructs can be conducted, for instance, with siDirect (Naito et al., 2009) or siRNA-Finder (Si-Fi) (Lück et al., 2019), the latter incorporated with a tool specifically intended for VIGS, HIGS and miRNA. To make dsRNA, one needs to transcribe both sense and antisense strands of RNA from a complementary DNA (cDNA) and allow them to anneal. This is achieved by utilizing a construct (vector) containing a partial sequence of the target gene which is subsequently expressed in the plant cell. There are several different plasmid vectors available for this purpose; the target sequence may be cloned to both sides of intron-containing hairpin RNA (ihpRNA) vector in antisense and sense orientation; or the target sequence may be surrounded by two promoters, as presented below.

A set of RNAi vectors was constructed and transferred to poplar by Meyer et al. (2004). To address the question of silencing, the GUS reporter gene was applied as a test system. The functionality of these dsRNA-forming vectors was then proofed in GUS-transgenic poplar in both transient assays by transforming protoplasts with the RNAi constructs and in stably transformed GUS-expressing poplar (Meyer et al., 2004). Based on the observation that the RNAi:GUS construct with the Intron290 spacer showed the strongest downregulation of the reporter gene, the authors concluded that the RNAi vectors are functional in poplar.

A novel RNAi approach without spacer but with two promoters flanking the gene to be silenced has been proposed by DNA Cloning Service (Hamburg, Germany) (Fig. 7.2). The advantage of this approach is that no cloning of sense and antisense sequences of the gene to be silenced is needed. The approach was first tested in transgenic poplar constitutively expressing the GUS gene under the cauliflower mosaic virus 35S promoter.

A modified RNAi construct carrying the GUS gene flanked by two 35S-promoters (Fig. 7.3A) was transferred to stably GUS-expressing poplar (M. Fladung, unpublished results). Silencing of the GUS gene was validated in GUS-staining experiments of chlorophyll-less leaf discs harvested from different independent GUS:RNAi-35::GUS-transgenic poplar lines. The GUS-stains ranged from slightly decreased blue to nearly completely white leaf discs (M. Fladung, unpublished results, Fig. 7.3B).

Fig. 7.2. Schematic representation of the novel RNAi approach without intron spacer but with two promoters flanking the gene to be silenced. Promoters can be constitutive, inducible, or tissue and developmental specific. T = terminator, dsRNA = double-stranded RNA. Red arrows indicate the transcription directions. (Source: DNA Cloning Service, Hamburg, Germany).
Flowering onset is very important with respect to yield in many plant species. Unravelling the interactions of genes involved in flowering time is, therefore, of high interest for crop breeders. In addition, a variety of growth factors, secondary metabolites and exogenous compounds have been shown to influence flowering time in annuals and perennial plants (Ionescu et al., 2016).

The role of genes controlling these processes or the identification of genes inducing flowering has mostly been studied in *Arabidopsis* at the beginning of this century; however, some of the genes are being analysed in the *Populus* tree model system by applying RNAi suppression. Tylewicz et al. (2015) studied the possible participation of poplar homologues of the evolutionarily conserved basic-leucine zipper (bZIP) domain transcription factor *FD* and the *Arabidopsis FLOWERING LOCUS T* (*FT*) on floral transition by using gain of function and RNAi-suppressed *FD* transgenic plants. Following the identification of two *FD*-like homologues (*FDL1* and *FDL2*) in *Populus*, the authors studied the role of both *FDL* genes by RNAi suppression. In addition to being primarily involved in flowering induction in combination with *FT*, it seems that, independently from *FT*, *FD* has dual roles in the photoperiodic control of seasonal growth and stress tolerance in trees (Tylewicz et al., 2015).

From another well-known flowering *Arabidopsis* time regulator, *GIGANTEA* (*GI*), which connects networks involved in developmental stage transitions and environmental stress responses, only a little is known about its role in poplar (Ke et al., 2017). The authors identified three *GI*-like genes in poplar, and following overexpression and RNAi suppression of these genes, *Arabidopsis GI* functions seemed to be conserved in poplar. Downregulation of the poplar *GI*-like genes by RNAi led to vigorous growth, higher biomass and enhanced salt stress tolerance in transgenic poplar plants (Ke et al., 2017).

So far, the function of the floral homeotic genes *AGAMOUS* (*AG*) and *SEEDSTICK* (*STK*) in the development of poplar catkins have been studied by Lu et al. (2019). RNAi co-suppression of both the two *AG* and the two *STK* paralogues led to modifications in poplar floral phenotypes, e.g. carpel-inside-carpel phenotypes, complete disruption of seed production, or sterile
anther-like organs (Lu et al., 2019), but without changes in biomass growth or leaf morphology. Lu et al. (2019) concluded that AG and STK gene functions are strongly conserved during poplar catkin development.

Genetically modified forest trees, including poplar, eucalyptus and pine, have been produced in many laboratories in the world and safety has been tested in the field (Walter et al., 2010). A few GM forest trees have been commercialized in China and the USA, but in Europe, market introduction is impeded by environmental concerns and political and social interference with the EU regulatory system (Custers et al., 2016). One biosafety concern regarding commercialization of GM forest trees is possible transgene flow into wild tree populations. Reducing flower fertility or induction of complete flower sterility is a containment strategy and will probably be necessary before most commercial uses of GM trees are possible.

As early as in 2001, Meilan et al. (2001) applied RNAi to downregulate genes involved in flowering to engineer sterility in poplar. Although some genes were successfully downregulated, it could not be confirmed that RNAi can be applied as a long-term containment measure that is stable in the field under natural environmental conditions, because of expression changes during tree maturation. Stability of RNAi in the field has been investigated by Li et al. (2008) by testing 56 independent poplar RNAi transgenic events over 2 years (over winter-to-summer seasonal cycles). Here, the BAR resistance transgene was targeted with two different RNAi constructs. Although the degree of RNAi suppression varied widely, the authors found that it was highly stable in each event over the two years (Meilan et al., 2001). The authors concluded that RNAi is highly effective for functional genomics and biotechnology of perennial plants. An effective containment strategy in transgenic trees was postulated by Klocko et al. (2016, 2018). Targeting of the poplar homologue of LEAFY (LFY) via RNAi resulted in a decrease in catkin size and loss of functional sexual organ development in field-grown poplar plants (Klocko et al., 2016). RNAi silencing has also been successfully used in Populus tremula × tremuloides trees to engineer sterility with constructs targeting the LFY and AGAMOUS (AG) flowering genes (Klocko et al., 2018).

Stability of RNAi in the field over several years has already been indicated by Meilan et al. (2001), Mohamed et al. (2010) and Klocko et al. (2016). A comprehensive study on stability of catkin sterility by testing over 3300 genetically engineered (GE) poplar trees and 948 transformation events in a single, 3.6 ha field trial was performed by Klocko et al. (2018). The goal was to assess modified RNA expression or protein function of floral regulatory genes, including LFY, AG, APETALA1 (AP1), SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS T (FT) for seven growing seasons in the field. All modifications induced by the RNAi or overexpression constructs revealed stability over three to five flowering seasons (Klocko et al., 2018). No somaclonal variation and no floral modification that was not related to the added transgene could be observed. This study has shown that RNAi-based sterility of catkins is stable and could be one successful containment option for transgenic forest trees (Klocko et al., 2018).

7.5 Secondary Cell Wall Formation

Modifications of lignin and/or cellulose biosynthesis have been one of the major goals of tree biotechnology and molecular biology for more than 25 years to improve biofuel production from woody biomass, because its energy largely resides in plant cell walls. However, wood is composed of 40–50% cellulose, 15–20% hemicellulose and 25–30% lignin. The complex structure of lignified cell walls makes wood largely inaccessible to cellulases for cellulose degradation and breakdown into sugars (Hisano et al., 2009). Because the presence of lignin is responsible for wood hardness, downregulation of major lignin genes could lead to reduced lignin content and, therefore, increased accessibility of celluloses for cellulose degradation. On the other hand, modification of cellulose content is also of interest to improve wood quality and strength.

Modification of lignin biosynthesis by RNAi suppression of 4-coumaroyl-CoA 3′-hydroxylase (C3′H) has been investigated by Coleman et al. (2008). C3′H catalyses the hydroxylation of 4-coumaroyl shikimate and 4-coumaroyl quinate. When downregulated, C3′H becomes a
rate-limiting step in lignin biosynthesis. RNAi suppression of C3′H led to a significant decrease in total lignin content and to a significant shift in lignin monomer composition in the accumulation of phenylpropanoid glycosides (Coleman et al., 2008). In another study, both C3′H and SHIKIMATE HYDROXYCINNAMOYL TRANSFERASE (HCT) were RNAi downregulated in transgenic poplar (Zhou et al., 2018). Wood analyses revealed that lignin content was lower in the C3′H/HCT double RNAi transgenic poplar than in the non-modified control plants. In addition, wood anatomical characteristics like cell wall thickness, diameter of fibre cells and mechanical properties were changed in the transgenic poplars (Zhou et al., 2018). Transgenic up- and downregulation of 4-coumarate:coenzyme A ligase 1 (4CL1) altered lignin content and composition in transgenic poplars (Tian et al., 2013). 4CL1 ligates 4-coumarate with CoA. There were no negative effects on growth of the transgenic plants but an enhanced growth performance could be observed. The results suggest that 4CL1 is a traffic control gene in monolignol biosynthesis in poplar (Tian et al., 2013). In another study, orthologues of cinamyl alcohol dehydrogenase (CAD) and cinnamoyl-CoA reductase (CCR) were RNAi downregulated in Populus trichocarpa (Yan et al., 2019). Suppression of PtrCAD1 in transgenics led to reduced CCR protein activity in the stem-differentiating xylem, while downregulation of PtrCCR2 caused a lower CAD protein activity. The results provide evidence for the formation of PtrCAD1/PtrCCR2 protein complexes in monolignol biosynthesis in planta (Yan et al., 2019).

In plant cell walls, members of the cellulose synthase A gene family (CesA) control cellulose biosynthesis in plant cell walls. To understand the functional role of single CesA genes in the complex pathway in P. trichocarpa, Abbas et al. (2020) RNAi downregulated PtrCesA4, PtrCesA7-A/B and PtrCesA8-A/B during wood formation. RNAi knockdown of CesA led to a dramatic decrease in cellulose content, possibly responsible for changes in phenotype, physiology and wood characteristics. CesA:RNAi poplar revealed stunted growth and narrow leaves, and the reduced mechanical strength may be due to thinner fibre cell walls (Abbas et al., 2020). Xylem vessels in the CesA:RNAi poplar were collapsed, indicating that water transport in xylem may be affected and thus causing early necrosis in leaves. The authors conclude that PtrCesA4, PtrCesA7-A/B and PtrCesA8-A/B are not only involved in wood formation but also trigger pleiotropic effects of their perturbations on wood formation (Abbas et al., 2020).

The transcription factor WOX4 regulates cell divisions in the cambium in Arabidopsis. WOX4 is a key target of the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED 41 (CLE41) signalling pathway. The functions of homologues of both genes during secondary growth were studied in P. tremula × P. tremuloides (Kucukoglu et al., 2017). In Populus, WOX4 homologues are specifically expressed only in the cambial region during vegetative growth but not after growth cessation and during dormancy (Kucukoglu et al., 2017). Transgenic trees with RNAi downregulated poplar WOX4 revealed unchanged primary growth; however, secondary growth was reduced. Further, the poplar CLE41 homologues positively regulate the poplar WOX4 homologues, indicating that regulation of vascular cambium activity between angiosperm and gymnosperm tree species is evolutionarily conserved (Kucukoglu et al., 2017).

The functional role of secretory carrier-associated membrane proteins (SCAMPS) in wood formation of Populus has been studied by Obudulu et al. (2018). SCAMPS are highly conserved 32–38 kDa proteins that are involved in membrane trafficking. An RNAi vector to downregulate SCAMP3 was constructed and transferred to Populus tremula × tremuloides (Obudulu et al., 2018). Wood harvested from SCAMP3 downregulated transgenic trees revealed increased amounts of both polysaccharides and lignin oligomers, indicating that SCAMP proteins influence accumulation of secondary cell wall components. Indeed, secondary cell walls from SCAMP3:RNAi transgenic trees deposited higher amounts of both carbohydrate and lignin (Obudulu et al., 2018).

A very important component of hemicelluloses is xylan. Xylan is abundant in plant biomass, occurs mainly in all cell walls of grasses and is the second most abundant polysaccharide in secondary cell walls of dicot wood (Lee et al., 2011; Mellerowicz and Gorshkova, 2011; Rennie and Scheller, 2014). Molecular dissection of xylan biosynthesis was performed by Li et al. (2011) through RNAi knockdown of
several candidate genes. Members of glycosyltransferase protein families GT8, GT43 and GT47 have been identified to be involved in the biosynthesis of xylan in the secondary cell walls of Arabidopsis. However, their functional role in xylan biosynthesis in poplar was largely unknown. Knockdown of poplar GT8 homologues (PtrGT8D1 and PtrGT8D2) through RNAi resulted in 29–36% reduction in stem wood xylan content (Li et al., 2011). Interestingly, xylan reduction in poplar wood had essentially no effect on cellulose quantity but caused an 11–25% increase in lignin and anatomically an increased vessel diameter and thinner fibre cell walls (Li et al., 2011). For GT43, five genes were shown to be highly expressed in the developing wood in the genome of poplar (Lee et al., 2011). Downregulation of both GT43B and FbGT8D by RNAi in hybrid poplar led to smaller cell walls and lower xylan content in wood, indicating that both genes are involved in xylan biosynthesis in poplar wood (Lee et al., 2011).

### 7.6 Seasonal Growth, Tree Architecture and Yield

Tree growth and architecture play an important role for biomass production (Teichmann and Muhr, 2015). Determinants of productivity are, among others, large leaves, sylleptic branching, narrow crown architecture, adapted activity of stomata and a compact root system. Improved trees could show tolerance towards desiccation, anaerobic conditions and high temperatures, and resistance to insect damage and diseases. Reports have been published over the past 50 years describing individuals from different tree species showing modified plant architecture named, e.g. dwarf, nana, erecta, fastigiata, pyramidalis and columnar. Induction of mutations has been a key element of mutation breeding for many plant species, including trees, for more than 70 years.

Enhanced shoot and root growth in poplar by RNAi suppression of the poplar homologues of the Short Internodes (SHI) and its closely related gene STYLISH1 (STY1) from Arabidopsis has been described by Zawaski et al. (2011). The SHI gene belongs to a gene family that includes important developmental regulators. In addition, increased fibre length and modified proportion of xylem tissue were found, indicating that both genes play an important role in the regulation of vegetative growth and wood formation (Zawaski et al., 2011). To study the role of bioactive gibberellic acid (GA) concentrations on above- and below-ground biomass growth, Gou et al. (2011) overexpressed and RNAi suppressed both paralogues of the gibberellin 2-oxidase (GA2ox) gene in poplar. PtGA2ox4 and its paralogue PtGA2ox5 are primarily expressed in aerial organs. Overexpression of PtGA2ox5 produced a strong dwarfing phenotype, while RNAi suppression of both paralogues promoted leaf growth and led to changes in wood development and to a decrease of root biomass, but did not modify the overall plant phenotype (Gou et al., 2011).

An interesting study described the effects following RNAi downregulation of central circadian clock components in P. tremula × P. tremuloides trees (Edwards et al., 2018). The circadian clock is a biochemical oscillator that regulates and coordinates physiological and biochemical factors in roughly 24 h cycles. Transgenic trees with reduced expression of two late elongated hypocotyl genes (LHY1 and LHY2) revealed reduced growth and lower biomass production than wild-type trees. Analysis of the activity of genes involved in growth regulation showed arrhythmic and misaligned expression, indicating that impaired circadian clock function leads to misregulation of cell division genes (Edwards et al., 2018).

Double knockout mutations of the flowering genes SUPPRESSOR OF CONSTANS1 (SOC1) and FRUITFULL (FUL) in Arabidopsis led to plants revealing wood formation and perennial growth (Melzer et al., 2008). Double overexpression of both SOC1 and FUL genes in poplar led to stunted plants and changes in leaf morphology. RNAi suppression of the closest poplar SOC1 and FUL homologues yielded plants with unchanged plant phenotype and wood formation (Bruegmann and Fladung, 2019). However, due to salicoid genome duplication, possibly additional paralogues with redundant function exist and not all of these paralogues were RNAi knocked out, i.e. in P. trichocarpa, three paralogues of SOC1 and two paralogues of FUL were found.

Onset of flowering, axillary meristem identity and dormancy release were studied by Mohamed et al. (2010) by modifying the expression of
CENTORADIALIS (CEN) and MOTHER OF FT AND TFL1 (TERMINAL FLOWER 1) (MFT) for 6 years in the field. Members of these subfamilies control shoot meristem identity; and loss-of-function mutations in herbaceous plants result in dramatic changes in plant architecture. RNAi downregulation of PopCEN1 and its close paralogue, PopCEN2, yielded precocious first flowering with higher number of inflorescences and changed proportion of short shoots (Mohamed et al., 2010). Strikingly, terminal vegetative meristems did not develop inflorescences, indicating that the flowering signal is transported to axillary meristems rather than the shoot apex. Thus, PopCEN1/PopCEN2 genes are involved in shoot developmental transitions correlated with age (e.g. catkin formation on adult trees).

7.7 Abiotic Stress Tolerance

Trees are exposed to a number of environmental stresses throughout their entire lifespan. Besides biotic interactions, in particular, abiotic stresses such as drought, high soil salinity, heat, cold, oxidative stress and heavy metal toxicity are the most harmful environmental conditions that affect and limit crop productivity worldwide. As trees are sessile and long-lived organisms, the responses to occasionally detrimental environmental conditions are crucial for their survival. Therefore, there is a strong need to understand how trees react against high stress severity or when multiple stresses like high temperatures, drought and diseases act on trees. Unfortunately, plant responses to these stresses are mostly very complex: thus holistic, system biology or ‘omics’ approaches allow the identification of regulatory knots in the complex network of molecular and biochemical interactions (Cramer et al., 2011).

By applying RNAi, research has revealed important information about the role of involved candidate gene families that may help tree breeders to develop abiotic stress-tolerant clones. Downregulation of poplar plasma membrane intrinsic proteins (PIPs) has led to a number of leaf physiology trait changes (Bi et al., 2015). PIPs are a subfamily of aquaporins whose primary function is the transport of water across cell membranes in response to changes in osmotic pressures. RNAi:PIP poplar leaves indeed revealed wider-opened stomata, leading to higher net CO₂ assimilation and transpiration rates. Possibly the higher transpiration caused a certain level of dehydration in the leaf, implying that leaves of RNAi:PIP plants were at risk of drought stress (Bi et al., 2015). But levels of hormones like abscisic acid (ABA), auxin and brassinosteroids were also altered. In particular, ABA is a well-known regulator of the water status in plants, controlling various abiotic stress responses such as drought. Changes in levels of ABA are therefore expected to affect drought tolerance in plants. Yu et al. (2019) overexpressed and downregulated genes involved in ABA stress signalling and photoperiodic regulation in a poplar hybrid. Poplar lines overexpressing bZIP transcription factor FD like1 (FDL1) or its close homologue FDL2 revealed drought sensitivity, whereas RNAi:FDL lines showed higher biomass allocation to roots under drought.

Ethylene responsive factors (ERFs) are also very important in responses to abiotic stress. To study the role of an ERF gene from Betula platyphylla (birch), BpERF11 was overexpressed and RNAi downregulated (Zhang et al., 2016). Overexpression of BpERF11 led to plants with higher electrolyte leakage revealing increased transpiration rates, while downregulation of this gene resulted in increase of genes involved in abiotic stress tolerance. The authors conclude that BpERF11 is a transcription factor that negatively regulates salt and severe osmotic tolerance by modulating various physiological processes (Zhang et al., 2016). Another group of transcription factor, the zinc-finger proteins (ZFPs), were analysed by Zang et al. (2015, 2017) in Tamarix hispida. ZFPs are abundant in plants and characterized by a zinc finger domain. First, Zang et al. (2015) cloned the ThZFP1 gene from T. hispida and could show that ThZFP1 responds to abiotic stress and plays a role in improving salt and drought tolerance. In a second study, Zang et al. (2017) identified ThDof1.4, a transcriptional regulator of ThZFP1, and studied its function by up- and RNAi downregulation. As expected, overexpression of ThDof1.4 increased the transcripts of ThZFP1 in T. hispida and RNAi silencing reduced its expression, indicating that ThZFP1 and its regulator are involved in responses to salt or drought stress in T. hispida (Zang et al., 2017).
Freezing tolerance in poplar was studied by Zhou et al. (2010) by up- and downregulation of the fatty acid desaturase (PtFAD2). Whereas PtFAD2 overexpressing lines revealed significant higher survival rates of cuttings after freezing treatment compared with controls, the downregulated lines showed lower survival rates. The results indicate that the level of polyunsaturated fatty acids in plant cells affect freezing in poplar (Zhou et al., 2010).

Isoprene emission has been described in many, but not all, plant species (Sharkey et al., 2008; Monson et al., 2013). Isoprene is the most abundant volatile compound emitted by vegetation (Behnke et al., 2009). Plants that emit isoprene are believed to tolerate sunlight-induced rapid heating of leaves as well as ozone and other reactive oxygen species better than non-emitting plants (Sharkey et al., 2008). On the other hand, emission of isoprene from plants is important as it affects atmospheric chemistry. Isoprene emission has appeared and been lost many times independently during the evolution of plants (Monson et al., 2013). Expression of the isoprene synthase gene can account for control of isoprene emission capacity (Sharkey et al., 2008). To better understand the regulation of isoprene emission and to retrieve new insights into the link between isoprene and enhanced temperature tolerance, Behnke et al. (2007, 2009) downregulated the expression of the isoprene synthase gene by RNAi. By applying heat stress to isoprene- and non-isoprene-emitting poplars, the non-isoprene-emitting plants showed reduced net assimilation and photosynthetic electron transport rates, but not in the absence of stress (Behnke et al., 2007). Further, the non-isoprene-emitting poplars were more resistant to ozone, as indicated by less damaged leaf area compared with isoprene-emitting wild-type poplars (Behnke et al., 2009). In the field, growth performance and biomass yield of non-isoprene-emitting poplars revealed no change for two growing seasons (Behnke et al., 2012).

### 7.8 RNAi in Forest Tree Pest Control

An interesting application of RNAi has been reported regarding insect pest control for several forest tree species. The idea behind it is based on entry of specific dsRNA delivery into the insect cell leading to the subsequent degradation of complementary mRNA of a carefully selected essential target gene, leading to insect mortality (Agrawal et al., 2003; Vogel et al., 2019). The sequence specificity of the small RNAs and the fact that, at least theoretically, any ‘mortality gene’ can be chosen makes RNAi highly attractive as a species-specific pesticide (Vogel et al., 2019).

For the very dangerous pine wood nematode, Bursaphelenchus xylophilus, which was the causal agent of pine wilt disease that killed millions of pine trees in China and the rest of eastern Asia in the past, RNAi was used to downregulate the expression of the endo-beta-1,4-glucanase gene of the nematode (Ma et al., 2011). Silencing of this gene led to reduced propagation and dispersal ability of this nematode. Another strategy was applied by Qiu et al. (2016) when blocking the function of the pectate lyase 1 gene in B. xylophilus (Bxpel1) through RNAi. B. xylophilus individuals propagated much less in a solution soaked in dsRNA than in a control solution treatment; thus, application of Bxpel1 dsRNAi to nematode-infected Pinus thumbergii trees resulted in reduced migration speed and reproduction rates of the nematodes (Ma et al., 2011). The authors concluded that Bxpel1 is a significant pathogenic factor in pine wilt disease break-out which could be the starting point for B. xylophilus control.

Also, for the interaction of the emerald ash borer (Agrilus planipennis), an invasive and destructive insect pest attacking ash (Fraxinus spp.), RNAi provides an alternative approach for insect pest management (Zhao et al., 2015). Following microinjection of the dsRNA of the AplaScrB-2 gene encoding a β-fructofuranosidase enzyme into the beetle, the expression levels of AplaScrB-2 decreased in the following days. The authors could show that RNAi is functional in the emerald ash borer A. planipennis causing ash dieback (Zhao et al., 2015). Following targeting of two essential genes, inhibitor of apoptosis (IAP) or COPI coatamer, beta subunit (COP) by RNAi, Rodrigues et al. (2017) observed insect mortality, providing evidence that RNAi could successfully be applied to counteract the dangerous ash dieback.
7.8.1 Crop protection by topical RNAi by spray-induced gene silencing (SIGS)

Plant pathogens cause serious crop losses worldwide. Studies on the pathogenic fungus *Fusarium graminearum* pathosystem (Koch et al., 2016; Wang and Jin, 2017) revealed that spraying dsRNAs (i.e. 791 nt CYP 3-dsRNA) targeting the three fungal cytochrome P450 lanosterol C-14α-demethylases, required for biosynthesis of fungal ergosterol, inhibited fungal growth in the directly sprayed (local) as well as the non-sprayed (distal) parts of detached leaves. Moreover, efficient spray-induced control of fungal infections in the distal tissue involved passage of CYP3-dsRNA via the plant vascular system and processing into siRNAs by fungal DICER-LIKE 1 (*FgDCL-1*) after uptake by the pathogen. The authors also underlined the use of target-specific dsRNA as an anti-fungal agent offering unprecedented potential as a new plant protection strategy. Song et al. (2018) studied the effect of spray-induced gene silencing (SIGS) by targeting dsRNA to *myosin5* gene of *Fusarium asiaticum* and found that the RNAi-induced silencing lasted in *Fusarium* for only 9 h, in contrast to wheat cells with efficient and longer-lasting turnover of dsRNA into secondary siRNA. This might indicate that the RNA-dependent RNA polymerases, required for the production of secondary siRNA in plants, are only transiently functional or non-functional in *Fusarium*. Thus, the authors underlined that the mechanism of SIGS is still unknown and demonstrated that secondary siRNA amplification limits the application of SIGS.

Dubrovina and Kiselev (2019) reviewed the exogenous application of RNAs (dsRNAs, hairpin RNAs and siRNAs) designed to silence important genes of plant pathogenic viruses, fungi, or insects. Plants can uptake and process exogenously applied RNAs, leading to local and systemic spread within the plant and resulting in induction of RNAi-mediated plant pathogen resistance. Furthermore, sRNAs originating from a plant host can subsequently be delivered into fungal pathogens and lead to silencing of fungal genes vital for pathogenicity. The authors summarized the studies reporting on exogenous RNA applications for downregulation of essential fungal and insect genes as well as targeting of plant viruses for increased resistance, and, in addition, reported on the suppression of plant transgenes and endogenes by application of exogenous RNAs.

7.8.2 Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses

Mitter et al. (2017) used topical application of pathogen-specific dsRNA for virus resistance in plants. This is an attractive alternative to transgenic RNAi. However, the instability of naked dsRNA sprayed on plants has been a major challenge towards its practical application. The authors showed that dsRNA can be loaded on designed, non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets. Once loaded, it showed sustained release and could be detected on sprayed leaves even 30 days after application. They found evidence for the degradation of LDH, dsRNA uptake in plant cells and silencing of homologous RNA on topical application. Significantly, a single spray of dsRNA loaded on LDH (BioClay) afforded virus protection for at least 20 days when challenged on sprayed and newly emerged unsprayed leaves. To conclude, nanotechnology can be used in crop protection as an environmentally sustainable and easy-to-adopt topical spray.

7.9 Outlook

Forests provide many benefits and services to society, including clean water and air, recreation, wildlife habitat, carbon storage, climate regulation and a variety of forest products (EPA, 2017). Climate influences the structure and function of forest ecosystems and plays an essential role in forest health. A changing climate will challenge the adaptation capacity of forest tree species and may worsen many of the threats to forests, such as pest outbreaks, fires, overexploitation and drought. Greenhouse gas emissions from human activity and livestock are a significant driver of climate change, trapping heat in the earth’s atmosphere and triggering global warming. The United Nations 2030 Agenda for Sustainable Development is a commitment made by countries
to tackle the complex challenges we face, from ending poverty and hunger and responding to climate change to building resilient communities, achieving inclusive growth and sustainably managing the Earth’s natural resources. The Agenda’s 17 Sustainable Development Goals lay out specific objectives for countries to meet within a given timeframe, with achievements monitored periodically to measure progress. Universally relevant, they call for comprehensive and participatory approaches (FAO, 2017, 2018).

In addition to human activities, forests are threatened by invasive exotic pathogens either due to climate change and/or due to long-distance trade of host- or pathogen-containing goods and climatic extremes, i.e. wildfires, droughts and storms. As an example, Finland’s Ministry of Agriculture and Forestry indicates that, at present, the health of Finnish forests is good, but climate change and immigrant species increase the risk for damage (MMM Finland, 2020). European spruce bark beetle (*Ips typographus*) caused serious damage in spruce forests in southern and south-eastern Finland during 2010–2013 and in Germany from 2015 to 2018. Spruce bark beetle benefits from dry, hot summers.

The rapid pace of climate change may exceed the ability of many species to adapt in place or migrate to suitable habitats and this fundamental mismatch raises the possibility of extinction or local extirpation. Assisted migration (AM), i.e. human-assisted movement of species in response to climate change, is one management option that is available to address this challenge (e.g. Ste-Marie et al., 2011). Winder et al. (2011) discussed the ecological constraints and consequences of AM and options for their mitigation at three scales: translocation over long distances (assisted long-distance migration), translocation just beyond the range limit (assisted range expansion) and translocation of genotypes within the existing range (assisted population migration). They concluded that, from an ecological perspective, AM is a feasible management option for tree species. However, AM needs honest considerations in each case to evaluate its potential benefits and threats for future forestry, as species may have potential to become harmful in new locations or transmit diseases to new areas (Ricciardi and Simberloff, 2009). The US Forest Service offers a comprehensive online search engine for literature about climate change and AM (US Forest Service, 2020).

In 2012 an international group of experts in silviculture, forest tree breeding, forest biotechnology and environmental risk assessment (ERA) met to examine how the ERA paradigm used for GE plants may be applied to GE trees for use in plantation forests (Häggman et al., 2013). The group pointed out that intensively managed, highly productive forestry incorporating the most advanced methods for tree breeding and application of genetic engineering has tremendous potential for producing more wood on less land. Furthermore, they emphasized the need to differentiate between ERA for confined field trials of GE trees, compared with ERA for unconfined or commercial-scale releases. In the latter case, attention should be paid to characteristics of forest trees distinguishing them from shorter-lived plant species, the temporal and spatial scale of forests, and the biodiversity of the plantation forest as a receiving environment (Häggman et al., 2013). Yet, the deployment of GE trees in plantation forests is still a controversial topic even though no indications of any risk to the environment or human health have been found in hundreds of field trials conducted with GE forest trees (Walter et al., 2010).

Klocko et al. (2018) published a paper on phenotypic expression and stability in a large-scale field study of GE poplars with sexual containment transgenes. They tested over 3300 GE poplar trees and 948 transformation events in a single 3.6 ha field trial for seven growing seasons. The trial is the largest field-based study of GE forest trees in the world. The goal was to assess a diversity of approaches for obtaining bisexual sterility by modifying RNA expression or protein function of floral regulatory genes. Modified floral traits were stable over three to five flowering seasons and they identified RNAi or overexpression constructs that either postponed floral onset or led to sterile flowers. No detectable somaclonial variation and no trees with vegetative or floral modifications were related to the transgene added. Thus, GE containment traits can be obtained which are effective, stable and not associated with vegetative abnormalities or somaclonal variation.

Regardless of the promising RNAi plants generated and potential of genetic engineering to aid the adaptation of future reforestation material to climate change, the annual number of confined field trials (CFTs) conducted with
GM plants was shown to decrease in a review by Smets and Rüdelsheim (2018). The observed decrease in CFTs during the study period 2014–2017 was especially drastic in North America and Europe, while only a slight decrease was found in Latin America. Public research institutes, i.e. not-for-profit research organizations, such as universities and government-owned institutes accounted for only 4.2% of all CFTs; in contrast, industry accounted for 95.5% of all CFTs. Three categories of trees in CFTs were discerned: poplar/aspen and eucalyptus (for timber and biofuel); fruit trees; and ornamental trees. Generally, 88% expressed marker genes, 29% virus resistance, 28% nematode resistance and 28% product quality traits. During the study period the number of recorded CFTs conducted with tree species was 216, which comprised less than 1% of the total number of CFTs (Smets and Rüdelsheim, 2018). The low number of tree CFTs may be partly explained by the strict regulation on the containment of GE material during excessive laboratory, greenhouse and field testing (Strauss et al., 2015).

References


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8 Host-induced Gene Silencing and Spray-induced Gene Silencing for Crop Protection Against Viruses

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Abstract

Since the beginning of agriculture, plant virus diseases have been a strong challenge for farming. Following its discovery at the very beginning of the 1990s, the RNA interference (RNAi) mechanism has been widely studied and exploited as an integrative tool to obtain resistance to viruses in several plant species, with high target-sequence specificity. In this chapter, we describe and review the major aspects of host-induced gene silencing (HIGS), as one of the possible plant defence methods, using genetic engineering techniques. In particular, we focus our attention on the use of RNAi-based gene constructs to introduce stable resistance in host plants against viral diseases, by triggering post-transcriptional gene silencing (PTGS). Recently, spray-induced gene silencing (SIGS), consisting of the topical application of small RNA molecules to plants, has been explored as an alternative tool to the stable integration of RNAi-based gene constructs in plants. SIGS has great and innovative potential for crop defence against different plant pathogens and pests and is expected to raise less public and political concern, as it does not alter the genetic structure of the plant.

8.1 Introduction

Plant viruses represent a major threat to global agriculture. Viruses have been found in all cultivated plant species and a wide range of wild species (see the Tenth Report of International Committee on Taxonomy of Viruses (ICTV): Lefkowitz et al., 2017). Viruses are infectious particles containing a nucleic acid core of RNA or DNA, surrounded by a protective shell made of one or more coat proteins. They are considered as obligate parasites, as they exploit the host cell machinery for their replication in living cells. In particular, during the infection process, a plant virus penetrates the plant cell through wounds made by, for example, arthropod pests or during agricultural practices (e.g. badly executed pruning); progressively, it colonizes the surrounding cells/tissues and spreads through the whole plant via the phloem. Agricultural practices such as crop rotation, precocious detection and prompt eradication of infected entities, use...
of virus-resistant varieties, virus-free certified plants, or chemical prophylaxis against insect vectors can help to contain viral infections (Hull, 2014).

Taking into consideration the serious economic damage caused worldwide by viral diseases, researches have been committed to introduce genetic resistances against viruses in plants. One of the most promising approaches relied on genetic engineering techniques, an integrative strategy to traditional breeding methods for obtaining virus resistance in several crop species.

One of the key studies in this research area, published in the mid-1980s, is commonly referred to as the pathogen-derived resistance (PDR) strategy. The idea behind this concept comprises the ability of plant cells, transformed with specific gene sequences derived from the pathogen, to interfere with the replication or the infection of the pathogen itself (Sanford and Johnston, 1985). For plant viruses, the proof of concept of PDR was reported by Abel et al. (1986). In this study, tobacco explants transformed with Agrobacterium tumefaciens carrying the coat protein (CP) gene of tobacco mosaic virus (TMV) showed a reduction of virus symptoms when inoculated with TMV. Despite several reports of overexpression of CP or other virus coding sequences, such as replicases, proteinases and movement proteins, the molecular pathways behind this induced resistance were not always clarified (Prins et al., 2008). Later studies revealed that PDR was not always linked to a deregulated synthesis of the corresponding viral proteins, or to the overexpression of dysfunctional viral proteins. Correlations between PDR events and RNA-dependent degradation mechanisms were detected in most cases. This phenomenon was later described as post-transcriptional gene silencing (PTGS) (Lindbo et al., 1993).

### 8.2 RNA Interference and Virus Resistance

Between the end of the 1980s and the beginning of the 1990s, two different groups conducting studies on the regulation of gene expression in petunia observed that the overexpression of a foreign sequence homologous to an endogenous plant gene led to specific degradation of both sequences, terming this phenomenon ‘coordinated suppression’ (co-suppression) (Napoli et al., 1990; van der Krol et al., 1990). Two years later, a non-translatable CP gene sequence of tobacco etch virus (TEV) was introduced into tobacco plants (Lindbo and Dougherty, 1992). Some of the transgenic lines expressing the TEV-CP gene transcripts developed feeble symptoms when inoculated with TEV, while some of them were symptomless. Surprisingly, the latter presented low steady-state levels of transgenic mRNA, despite highly active expression. This demonstrated the existence of a cellular-based, sequence-specific, post-transcriptional RNA degradation system induced by the transgenic mRNA, targeting both the transgene transcript and the homologous virus mRNA for degradation. This was therefore the first described PTGS-based example of virus resistance. Starting from these observations, it has been understood that in plant cells the RNA-mediated virus resistance based on PTGS is part of a natural and complex process now universally known as RNA silencing or RNA interference (RNAi) (Baulcombe, 2004).

The activating molecule of the RNAi machinery is represented by double-stranded RNA (dsRNA) precursors (Voinnet, 2008); in the cytoplasm, Dicer-like enzymes identify and specifically cut these dsRNA molecules into small RNAs (sRNAs) composed of 21–24 nt (Hamilton and Baulcombe, 1999; Bernstein et al., 2001; Elbashir et al., 2001; Baulcombe, 2004). The sRNAs sense strand, recruited by the RNA-induced silencing complex (RISC) with the help of Argonaute proteins, is used to scan the cytoplasm in order to find and degrade homologous mRNAs or compromise their translation, thus modulating gene expression (Tijsterman et al., 2002; Denli and Hannon, 2003; Ghildiyal and Zamore, 2009).

Protection against viruses and modulation of endogenous gene expression are the two main fields of activity of RNAi in plants (Vazquez et al., 2010). As a gene expression regulator, RNAi functions also in insects (Kennerdell and Carthew, 1998), fungi (Romano and Macino, 1992), animals (Fire et al., 1998) and mammals (Maillard et al., 2019). Moreover, the characteristics of the RNAi mechanism have been exploited to silence invading viral sequences in order to prevent and/or reduce their accumulation.
in plants. Two main biotech strategies based on the RNAi system have been exploited for crop defence against viruses, known as HIGS and the more recently studied SIGS method. HIGS depends on the induction of the plant RNAi biological system and is obtained by stable expression of dsRNAs specific for a target virus. As reviewed by Khalid et al. (2017), the activation of PTGS against viruses can depend on the characteristics of the gene constructs introduced in the plant to produce dsRNAs. In this chapter, we offer an excursus concerning different hairpin RNA (hpRNA)-based gene construct features and applications, which are definitively considered one of the most powerful tools to induce stable genetic resistance in crops against viruses.

On the other side, SIGS, the more recent strategy based on RNAi, relies on the exogenous application of dsRNA molecules that are homologous to the target viral sequences to trigger the natural RNAi-based defence mechanism towards plant viruses. In this chapter, we discuss the major achievements in producing dsRNA molecules on a large scale, using biofactories, and their topical application to plants. Moreover, we discuss the problems and benefits related to the efficacy and stability of SIGS, compared with HIGS, in particular for field conditions.

8.3 Host-induced Gene Silencing (HIGS) Strategy Against Viruses: hpRNA Silencing Approaches

An elegant study published in Nature by Fire et al. (1998) showed that in Caenorhabditis elegans RNAi was induced by dsRNA molecules and that these molecules were more efficient in inducing silenced phenotypes compared with single-stranded RNA molecules. At the same time, another study demonstrated increased silencing efficiency obtained by the co-expression in the host cell of sense and antisense sequences, compared with their separated expression (Waterhouse et al., 1998).

Later, the expression of dsRNAs was achieved in plants mainly by introducing hpRNA gene constructs, and these were also designed to induce PTGS against viruses. These gene constructs normally include short inverted sequences homologous to vital viral genes, usually split by a non-coding sequence, such as an intron, all under the control of specific promoters and terminators (Lemgo et al., 2013).

Such a construct strategy was described by Smith et al. (2000), who reported the increase of the silencing effect when an intron-based sequence was inserted as a junction between the sense and antisense arms of the hpRNA construct, leading to almost 100% of independently transformed tobacco lines showing silencing against potato virus Y (PVY). It has been supposed that the intron removal throughout splicing may simplify the folding of the hairpin structure and its transit from the nucleus to the cytoplasm (Wesley et al., 2001). As suggested by molecular analysis carried out on transgenic tomato plants expressing intron hpRNA-derived sRNAs and resistant to tomato yellow leaf curl virus (TYLCV), it seems that few unspliced hairpin molecules are processed by DCL 3 into 24 nt sRNAs in the nucleus and used as phloem-mobile silencing inducers. On the contrary, spliced hairpin molecules are processed in the cytoplasm by DCL 4 and DCL 2 into 21 nt and 22 nt sRNAs, respectively, and used as cell-autonomous silencing inducers of the target viral sequence (Fuentes et al., 2006, 2016; Pooggin, 2017).

Concerning the choice of the target viral genome sequence selected to build the short inverted repeats of the hpRNA construct, various aspects have to be considered. All viral genes chosen as RNAi targets for crop defence encode essential proteins necessary for the survival and the replication of the virus in the host, such as coat protein, nuclear capsid protein, replicase and replication-associated proteins (Khalid et al., 2017). Sequences of different lengths have been chosen and inserted into a wide range of plant species (Cillo and Palukaitis, 2014). In general, essential viral genome portions from 300 up to 800 nt are preferred as target regions (Simón-Mateo and García, 2011), but much smaller sequences (from 23 up to 60 nt) have also been successfully used to induce virus resistance (Thomas et al., 2001). The idea behind such preference in terms of sequence length is connected with the concept that hpRNA-mediated silencing occurs when the homologous region between the hp-derived transcripts and the target viral sequence covers more than 100 nt (Pang et al., 1997; Jan et al., 2000).
The 35S cauliflower mosaic virus (CaMV), the first plant promoter identified almost 40 years ago (Covey et al., 1981), is the most broadly exploited promoter sequence in plant biotechnology, also in the case of the hpRNA constructs design, as it causes constitutively high levels of gene expression in a large variety of plant tissues, despite being derived from a pathogenic virus.

Since the dawn of plant biotechnology, tobacco has been widely exploited as a model plant system, mainly to validate the functionality of new gene constructs due to the ease of genetic transformation and virus infection. Since the end of the 1990s, many achievements and failures in terms of RNA and DNA virus defence via hpRNA have been reported, both in model plants and in crops, including several examples where 100% of resistance to the target virus was achieved (reviewed by Khalid et al., 2017). Different hp-gene constructs against several viruses have been evaluated in the model species *Nicotiana tabacum* or *N. benthamiana*, and complete resistance was achieved in 12 cases (ten in *N. benthamiana* and two in *N. tabacum*, respectively). For example, the production of transgenic *N. benthamiana* plants resistant to citrus tristeza virus (CTV) expressing an hp-gene construct targeting P23+3’UTR sequences led to the application of the same approach in citrus (Batuman et al., 2006). However, following transformation via *Agrobacterium* of the citrus ‘Alemow’ to enable insertion of a hairpin construct (p23UI), potentially capable of inducing CTV resistance via PTGS, none of the transgenic citrus plants exhibited resistance. This example shows that a result achieved in a model plant may not be directly reproduced in a target crop, possibly since specific host factors participate in the infection process. To partially explain this outcome, it was supposed that a virus could be more virulent in its own natural host than in a different experimental host. To integrate the Khalid et al. (2017) review, it has to be mentioned that the RNAi mechanism was exploited against plum pox virus (PPV) for the first time by Pandolfini et al. (2003) who designed and introduced an hp-gene construct against PPV in the model species *N. benthamiana*. In this study, a 197 bp-long sequence of the PPV strain D genome was chosen to design the ihprolC-PP197 gene construct, placed as two inverted repeats separated by a non-coding sequence under the control of the phloem-specific rol C promoter. When the ihprolC-PP197 gene construct was employed to transform *N. benthamiana* plants, systemic PPV resistance was obtained. Systemic viral infections are common in fruit trees; thus a comparable construct could be developed to achieve PPV resistance also in *Prunus* spp. (Ilardi and Tavazza, 2015).

The RNAi-based strategy was shown to work also against viruses with a DNA genome, as reviewed in Pooggin (2017). One of the most intriguing examples of hpRNA constructs active against the geminivirus TYLCV consisted of the expression of an hpRNA construct targeting the viral replicase C1 gene (Fuentes et al., 2006). When transgenic lines expressing this construct were tested in field conditions, a long-lasting resistance was demonstrated; moreover, the authors highlighted the possibility that this strategy could induce off-target effects and modify the transcriptome of the transgenic lines, as determined by deep-sequencing approaches (Fuentes et al., 2016; Pooggin, 2017).

### 8.4 Transgrafting as a Tool to Develop Genetic Resistance Against Viruses in Crops

In worldwide farming, grafting is a very common procedure that basically consists of connecting a portion of a plant (i.e. scion) to another plant (i.e. rootstock), through the junction of their vascular systems. Essentially in a grafted plant system, the rootstock absorbs nutrients from the soil that move to the scion, while the scion synthesizes carbohydrates through photosynthesis that are translocated to the rootstock. The phloem of a grafted organism is where the traffic of plant growth factors, soluble organic compounds, nucleic acids and proteins takes place, creating a dynamic link between rootstock and scion that should lead to an improved growth and yield of the grafted plant (Aloni et al., 2010; Dinant and Suárez-López, 2012; Guelette et al., 2012; Ham et al., 2014). Plant grafting is mostly used for vegetative propagation, to induce resistance against pathogens, to alter plant vigour and increase endurance to abiotic stresses (Gonçalves et al., 2006; Kubota et al., 2008; Aloni et al., 2010; Koepke and Dhingra, 2013).
As explained by Pyott and Molnar (2015), a non-cell autonomous gene silencing signal is ‘one whose action extends beyond the cell producing the signal’. In the late 1990s, the transmission of a silencing signal in the form of dsRNA molecules over long distances was demonstrated by two key studies applied on N. benthamiana plants (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). In particular, Voinnet and Baulcombe (1997) induced the stable expression of GFP-encoding sequence in N. benthamiana plants and, through an optimized Agrobacterium infiltration protocol, a temporary GFP silencing was induced in the older leaves of the same treated plants. Probably thanks to the translocation of the silencing molecules, a GFP silenced phenotype was detected also in the upper leaves. In the same year, using a grafting procedure, Palauqui et al. (1997) joined wild-type tobacco scions onto transgenic stocks expressing nitrate/nitrite reductase (Nia/Nii) transgene. Chlorosis and reduced amounts of Nia/Nii mRNA in the scions suggested a movement of Nia/Nii silencing signals from the transformed stock to the wild-type scion.

The nature of the systemic RNA silencing signal has been an enigma for researchers. At the beginning, it was supposed that the traveling of long dsRNA precursors should take place in the phloem to achieve systemic silencing (Mallory et al., 2001, 2003), but later reports suggested that systemic RNA silencing depends almost exclusively on sRNAs as mobile molecules (Chiou et al., 2006; Buhtz et al., 2008; Martin et al., 2009; Molnar et al., 2010; Melnyk et al., 2011; Zhang et al., 2014).

The ability of the silencing molecules to move along the plant vascular system can be exploited in a transgrafting system (Song et al., 2015). In this case, transgrafting used as a method to spread sRNAs through the plant and to switch off the replication of a target virus could represent an alternative and promising approach to protect woody plant species against viral diseases. The goal of the hpRNAi transgrafting system would be to obtain a cultivar whose tissues and organs, including pollen and fruits, remain untransformed but which is resistant to one or more target viruses thanks to the translocation of sRNAs from an RNAi transgenic grafted rootstock (Lemgo et al., 2013). This approach is particularly suitable for fruit trees species, which are usually propagated vegetatively and not through seeds. For example, peach (Prunus persica L. Batsch), grapevine (Vitis vinifera) (Bouquet and Hevin, 1978) and sweet cherry (Prunus avium) (Akçay et al., 2008) plants are propagated by grafting to retain the same parental traits in terms of quality and vigour of fruit. Since 1998, transgenic rootstocks have been exploited in grafting systems for woody fruit-bearing plants (reviewed by Song et al., 2015). Two promising examples of virus resistance in non-transgenic scions grafted on transgenic rootstocks were achieved in grapevine (Vigne et al., 2004) and sweet cherry plants (Song et al., 2013; Zhao and Song, 2014); in the latter studies, resistance against prunus necrotic ringspot virus (PNRSV) relies on RNAi mechanism, activated by an hpRNAi-based gene construct integrated in the grafted transgenic rootstock.

Although the HIGS approach applied to transgrafting systems shows several advantages, especially for inducing plant virus resistance, its use is currently hindered by different issues, especially by the need to generate transgenic plants. Furthermore, this process presents several bottlenecks both from a technical point of view and for regulatory and social aspects. In fact, certain crop species are hard to regenerate in vitro and/or difficult to transform genetically (Sabbadini et al., 2019). Moreover, the inserted transgenes can be unstable in the host genome, or their expression can be silenced or suppressed in the offspring, making transformation ineffective. In addition, the generation and characterization of transgenic lines can be time consuming for some cultivated crops, making the evaluation of the effective lines unaffordable (Alt peter et al., 2016). To reduce or overcome public concerns and bypass technical difficulties to obtain stable and efficient transgenic lines, the exogenous delivery of RNAi effective molecules (sometimes termed SIGS) has been proposed as an appealing alternative for plant disease control. In this case, the plant host genome is not modified, multi-target strategies are feasible and the products of this strategy can be obtained in a relatively shorter time.

8.5 Spray-induced Gene Silencing (SIGS) Strategy Against Viruses

The first report of the successful use of exogenously applied dsRNAs against plant viruses was
that of Tenllado and Diaz-Ruiz (2001). In this pioneering work, RNA-mediated virus resistance was triggered by dsRNA molecules against three different viruses, all with a positive single-stranded RNA genome, such as pepper mild mottle virus (PMMoV), tobacco etch virus (TEV) and alfalfa mosaic virus (AMV). When these viruses were mechanically inoculated on N. benthamiana leaves with in vitro transcribed dsRNA fragments targeting the PMMoV replicase, the TEV helper component (HePro) or the AMV RNA3 (fragments of 997 bp, 1483 bp and 1124 bp, respectively), a local antiviral response was elicited, in a dose-dependent manner. However, the authors stated that a certain length of dsRNA was required to reach resistance (Tenllado and Diaz-Ruiz, 2001). Since then, this strategy has been applied on many different plant species targeting different viruses, as reviewed in Dalakouras et al. (2020). This work reviews the use of different kinds of formulations of dsRNAs that were delivered on maize plants against sugarcane mosaic virus (SCMV) CP (Gan et al., 2010) and on pea against pea seed-borne mosaic virus (PSBMV) CP (Šafárová, D et al., 2014), as well as on the orchid Brassolaeliocattleya hybrida against cymbidium mosaic virus (CymMV) CP (Lau et al., 2014). Other constructs were tested on tobacco, targeting the TMV p126 replicase (Konakalla et al., 2016) and on pea against yellow mosaic virus (ZYMV) HePro (Kaldis et al., 2018), on N. benthamiana, targeting a 2611 bp region of the replicase and MP of TMV (Niehl et al., 2018) and on papaya tree against papaya ringspot virus CP (Shen et al., 2014).

For a broad application of dsRNAs in greenhouses and fields, efficient and economically acceptable methods for their large-scale production and purification are required. The initial systems adopted to obtain dsRNAs relied on the in vitro enzymatic synthesis of two complementary ssRNA strands, followed by physical annealing (Tenllado and Diaz-Ruiz, 2001; Carbonell et al., 2008). One of the most frequently used enzymes for ssRNA synthesis is the DNA-dependent RNA polymerases (DdRPs) of the bacteriophage T7. For plant virus control, specific target sequences are transcribed by DdRPs from cDNA templates extracted from plants infected by the target virus, using specific primers that carry the T7 promoter at their 5′-end; alternatively, the in vitro transcription by DdRP can occur starting from plasmids carrying the target viral sequences cloned between two T7 promoters (Konakalla et al., 2016). Different kits are commercially available for this purpose, such as the MEGAscript® RNAi Kit (Life Technologies), Replicator™ RNAi Kit (Finnzymes) or T7 RiboMAX™ Express system (Promega, USA). The production of dsRNA molecules specifically targeting a selected pathogen region can be followed, optionally, by digestion with Dicer-like (DCL) enzymes, obtaining a heterogeneous mix of short interfering RNAs (siRNAs), of 18–25 nt in length when the ShortCut® RNase III (NEB, Ipswich, Massachusetts) kit is used or of 25–27 nt when the PowerCut Dicer (Thermo Scientific) kit is employed; the siRNA mixture can be further subjected to cleaning with the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, California) (Koch et al., 2016; Wang et al., 2016). In a more vigorous in vitro system, the ssRNA synthesis performed by the T7 RNA polymerase was coupled to a de novo primer-independent initiation, using the highly processive RNA-dependent RNA polymerase (RdRP) enzyme of bacteriophage φ6 (Makeyev and Bamford, 2000), a dsRNA virus infecting Pseudomonas syringae cells (Aalto et al., 2007).

To overcome the high costs linked to the in vitro dsRNA synthesis, in vivo approaches using bacterial cells have been developed. Both in Escherichia coli (Tenllado et al., 2003; Yin et al., 2009) and in P. syringae cells (Aalto et al., 2007; Niehl et al., 2018). In the E. coli system, a stably replicating plasmid carrying the target viral sequence cloned within two T7 promoters is introduced into bacteria; following chemical induction of the T7 DdRP gene, which is expressed by a gene cloned in a DE3 prophage or in an additional plasmid, the target sequences are transcribed in both directions; then, the newly generated ssRNA molecules anneal, yielding the desired dsRNAs. Their degradation is inhibited using RNase-III deficient strains, such as E. coli HT115 (DE3) or M-JM109lacY, the latter having also a knockout LacY permease gene. This easily scaled-up process is reported to yield about 4 μg dsRNA/ml of bacterial culture (Tenllado et al., 2004).

In a pioneering work, Aalto et al. (2007) described an in vivo dsRNA production system in P. syringae that had been engineered in order to express the RdRP of bacteriophage φ6. This
system was further improved using the stable carrier cell line amplifying RNA by means of the phage $\phi 6$ RdRP (Sun et al., 2004), finally leading to $P. syringae$ cells transformed with different plasmids that individually express the viral target sequences, the T7 RdRP, and the $\phi 6$ RdRP. The dsRNA amplification takes place within the $\phi 6$ polymerase complexes that also provide a protected environment from bacterial RNases (Voloudakis et al., 2015; Niehl et al., 2018). These bacterial dsRNA production systems can be scaled up, allowing cost-effective large-scale production of long dsRNA molecules targeting pathogen genes or genomes, suitable for application in crop protection (Niehl et al., 2018).

However, most studies reporting the delivery of dsRNAs produced in vitro or in vivo showed that the protective antiviral effect lasts for only a few days, indicating insufficient stability or efficacy of these molecules for practical use. As frequent treatments with dsRNAs would be necessary to protect plants from virus infection, especially for long-lasting crops cultivated in open fields, establishing methods ameliorating the delivery of dsRNA and their stabilization has become a major challenge. dsRNA formulations based on biocompatible and safe materials are currently being evaluated (Pérez-de-Luque, 2017; Vurro et al., 2019); these include packaging of dsRNAs into virus particles or in virion-like particles (VLPs) (reviewed in Zotti et al., 2018 and Dalakouras et al., 2020). Implementation of dsRNA formulations has been achieved by a biotech company with the ‘Apse RNA Containers’ (ARCs) system (available at https://arborjet.com, accessed 17 March 2020). Here, $E. coli$ cells express naturally occurring proteins, such as the CPs from bacteriophage MS2 that can self-assemble and form VLPs. The same cells also contain another plasmid carrying the target RNA precursor signal sequence, linked to a packaging site. During $E. coli$ growth, VLPs made of MS2 CP subunits will encapsidate the target RNA molecules.

From another perspective, an elegant breakthrough of the obstacles related to dsRNA delivery relies on the use of non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets of 80–300 nm (BioClay) that bind to dsRNAs and protect them from degradation (Mitter et al., 2017). These BioClay nanostructures are not only resistant to plant watering but also allow gradual release of dsRNAs to the plant cell, leading to more successful inhibition of the propagation of cucumber mosaic virus (Mitter et al., 2017) and bean common mosaic virus in $N. benthamiana$ and cowpea ($Vigna unguiculata$) plants, respectively (Worrall et al., 2019).

Other recently developed delivery strategies include direct trunk injection, as in the commercially available Arborjet strategy (available at https://arborjet.com, accessed 17 March 2020) described in Zotti et al. (2018) and Dalakouras et al. (2018), but their efficacy against viruses affecting woody plants remains to be evaluated. Another delivery strategy, which seems to be appropriate for inducing virus resistance in plants, consists of a high-pressure spraying method inducing a supramolecular delivery of the effective dsRNA molecules (Dalakouras et al., 2020). Indeed, this technique, first described by Dalakouras et al. (2016), can trigger both local and systemic silencing and the production of secondary siRNAs, especially when 22 nt molecules are sprayed on the plant tissues.

### 8.6 Biosafety Considerations

Although one of the major problems hindering a widespread use of the HIGS approach includes the cumbersome regulatory procedures to get governmental approval of transgenic plants, the authors would like to highlight 24 examples where all the bureaucratic processes reached a fruitful outcome, described in detail by Khalid et al. (2017). Among them was a successful case of intron hpRNA-based transgenic common bean plants resistant to bean golden mosaic virus (BGMV) accepted for commercialization in Brazil (Bonfim et al., 2007), which exhibit durable resistance in open fields, with unaltered agronomic characteristics and nutritional value (Aragão et al., 2013; Carvalho et al., 2015). Examples of virus-resistant fruit tree species approved for commercial release and generated by HIGS technique include the papaya ringspot virus (PRSV)-resistant papaya (Fitch et al., 1992) and the PPV-resistant plum (Scorza et al., 2001).

Although RNAi-based transgenic plants produce only dsRNA molecules complementary
to the target pathogen transcripts, without the synthesis of any new protein, possible off-target effects need to be evaluated. These can be caused by dsRNA molecules’ complementarity with unintended sequences in the GM plant or in non-target species (Mlotshwa et al., 2008; Auer and Frederick, 2009; Frizzi and Huang, 2010).

Regarding the use of transgrafting to obtain RNA-based virus-resistant rootstocks in arboriculture, it is expected that this technology would cause less public concern and that the risk assessment would be limited to the transgenic rootstock, as the scion, fruits and pollen maintain their genetic inheritance. These aspects could encourage, in principle, a simplified approach for their application in agriculture (Lemgo et al., 2013; Petrick et al., 2013).

From the biosafety side, the most relevant feature of SIGS relies on the fact that the exogenous application of dsRNA does not involve any modification of the plant genome. Moreover, these substances act by means of their specific nucleotide sequence, have higher specificity and a reduced tendency to induce pathogen resistance if managed appropriately. Importantly, and, contrary to chemical pesticides, dsRNAs are biocompatible and biodegradable compounds, ubiquitously occurring in natural conditions inside and outside organisms (Niehl et al., 2018). Based on the expert panel of the Toxicology Forum at its 40th Annual Summer Meeting held in 2015, local delivery of dsRNAs is considered safe for human consumption, as RNA molecules are present in all kinds of food and exogenous RNAs are considered free of residues potentially toxic for the plant, food or the environment (Sherman et al., 2015). Based on the expert panel of the Toxicology Forum at its 40th Annual Summer Meeting held in 2015, local delivery of dsRNAs is considered safe for human consumption, as RNA molecules are present in all kinds of food and exogenous RNAs are considered free of residues potentially toxic for the plant, food or the environment (Sherman et al., 2015). Nonetheless, to increase the activity and safety of these molecules, careful design and predictions by bioinformatics tools are necessary on a case-by-case basis, in order to avoid off- and non-target effects on related or non-related organisms with available genomics information (Zotti et al., 2018).

For the policy relevance of this topic, consensus views on dsRNA-based products have not yet been reached and official legislations governing their use are not yet available in Europe (Gathmann, 2019). Nonetheless, the European scientific community is currently assessing a regulatory framework for such products, as attested by the Organisation for Co-operation and Economic Development (OECD) Conference on RNAi-based pesticides held in April 2019. It is noteworthy that the safety and legislation issues for such products are generating heated debates in many countries. For example, in New Zealand, the official Decision of the Environmental Protection Authority considered that exogenous application of dsRNAs was technically out of the area of interest of the legislations on new organisms, and any environmental risk assessments of such products was unnecessary (EPA, 2018); however, this statement generated an active debate with negative reactions in the scientific community (Heinemann, 2019).

**8.7 Conclusions and Future Prospects**

In summary, we have presented the major characteristics of HIGS and SIGS strategies so far developed to inhibit the infection and spread of plant viruses. As the majority of plant viruses are transmitted by insects, the reader is also invited to refer to the specific chapters concerning the use of such strategies addressed against insect vectors.

The hpRNA-mediated HIGS strategy is suitable for targeting one or more specific viruses by the integration of one or more copies of the transgene in the plant genome (Stoutjesdijk et al., 2002). During the past 40 years, the Agrobacterium tumefaciens T-DNA-mediated gene insertion method has been deeply understood and it is routinely used to transform several plant species, also via the HIGS approach. However, some crops are recalcitrant to Agrobacterium-mediated transformation, for which alternative transformation strategies may be attempted, such as electroporation, microinjection, or particle bombardment. Despite the fact that the HIGS strategy is known to be a durable approach for virus control in agriculture, these plants still suffer from low public acceptance and strict rules for their commercialization and/or release into the environment, especially in the European Union.
The exogenous delivery of dsRNAs to trigger the RNAi mechanism against viruses in plants seems to be a reality for the future of plant disease control, considering that these RNAi effective molecules do not fall under the Directive 2001/18/EC (12 March 2001) of the European Commission or the US regulations, since the plant genome is not modified. In the expectation that regulations of small natural molecules for disease control would include these products as biopesticides, researchers are working to stabilize the formulation of dsRNA molecules suitable for field-scale applications at affordable costs.

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9 Small Talk and Large Impact: the Importance of Small RNA Molecules in the Fight Against Plant Diseases

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Abstract
This short and general chapter summarizes how plants and pathogens communicate using not only proteins for recognition and signal transduction or other metabolites but also RNA molecules where small RNAs with sizes between 21 to 40 nt are most important. These small RNAs can move between plants and a range of interacting pathogenic organisms in both directions, that is, a ‘cross-kingdom’ communication process. The first reports on RNA-based communications between plants and plant pathogenic fungi appeared about 10 years ago. Since that time, we have learnt much about sRNA biology in plants and their function in different parasitic organisms. However, many questions on the processes involved remain unanswered. Such information is crucial in order to sustain high crop production. Besides giving a brief background, we highlight the interactions between the potato late blight pathogen and its plant host potato.

9.1 Introduction
In the early 1990s, a significant breakthrough was achieved by the cloning of several resistance (R) genes against different pathogenic organisms in tomato, tobacco and, not least, in Arabidopsis. The latter is the first plant species with a sequenced genome and for which numerous genetic and molecular tools and information are now available (TAIR, 2020). This R-gene work elucidated, for example, the involvement of conserved protein domains. Two main groups of R genes are distinguished based on different N-terminal domains: (i) those with a coil–coil (CC) sequence; and (ii) those that share sequence similarity with the Drosophila Toll and human interleukin-1 receptor (TIR) domain. These domains can be combined with nucleotide binding sites (NBS) and leucine-rich repeats (LRR). Together the different domains function as cell surface or intracellular receptors, but confer also loss of susceptibility (Kourelis and van der Hoorn, 2018). In parallel, information on regulatory RNA was derived from studies on viruses demonstrating the importance of transcript regulation on RNA levels (Lindbo et al., 1993; Hamilton and Baulcombe, 1999; Baulcombe, 2004). Non-coding RNAs have emerged since then as important and ubiquitous components of eukaryotic transcriptional and post-transcriptional regulatory processes. These
molecules are entities that include both long and short small RNAs (sRNAs) (Ghildiyal and Zamore, 2009) that are commonly present in sizes ranging from 16 to 40 nt in length. Many classes of sRNAs are described today with different biogenesis, functions and targets (Axtell, 2013). One key function is RNA-mediated gene silencing. In plants, sRNAs are classified into two major categories: microRNAs or miRNAs (21–24 nt long); and short interfering RNAs (siRNAs), of which several sub-classes are known. Among these are 21 nt phased siRNAs (phasiRNAs) and the plant-specific transacting tasiRNAs that are important regulatory factors during plant defence (Deng et al., 2018).

Cross-kingdom sRNA transport refers to sRNAs capable of moving between two taxonomically unrelated organisms (Figs 9.1 and 9.2). As mentioned earlier, sRNA-mediated immunity in plants was first studied with respect to virus infections. Viruses have also evolved a counter-defence strategy by inhibiting the plant’s sRNA-mediated antiviral response. This so-called arms race based on pathogen–plant interactions is a natural evolutionary system to defeat the mechanisms that lead to

Fig. 9.1. All species are taxonomically organized in different distinct groups or kingdoms. Four kingdoms (plants, animals, fungi, protists) in eukaryotes were originally found. Today molecular-based analysis has further divided them into several new taxonomic categories. In this context, communication between plants and fungi is an example of a cross-kingdom event.
Fig. 9.2. Schematic illustration of ‘cross-kingdom’ exchange of sRNAs between plants and different intruders. Different main components in the diverse sRNA pathways are listed top right. Argonaute (AGO) has a central role in RNA silencing processes together with Dicer and RNA-directed RNA polymerase, or RNA-directed DNA methylation (RdDM). In plants, DNA is methylated via the cytosine base (meC). There are different classes of sRNAs where the miRNAs are one of the most well-studied. Several protein complexes take part in the different processes. There remains a lot to learn about the transport mechanisms between different organisms (indicated by question marks). The top left panel shows the simplified mechanism of RNA silencing-based antiviral immunity. The plant small RNA binding protein 1 works as a cargo, delivering viral-derived sRNAs to the neighbouring plant cells and therefore amplifying the antiviral immunity. The bottom panel shows sRNA trafficking between plants and two types of eukaryotic pathogens. Extracellular vesicles have been shown to mediate the sRNA movement between plants and fungi. Our current work provided proof-of-concept that potato and P. infestans exchange sRNAs during infection (Hu et al., 2020) and vice versa (Jahan et al., 2015). A P. infestans miRNA guides either potato AGO or P. infestans AGO protein to cleave the target mRNA in the host, promoting infection. Yet, the molecular mechanism of intracellular trafficking remains elusive. (Drawings modified after Zhu et al. (2019), Hudzik et al. (2020) and Yan et al. (2020). © C. Dixelius.)
reduced survival of the organisms. The most common system that is used for making transgenic plants or fungi, the Agrobacterium-based system, is per se a typical cross-kingdom system where genes of the soil bacteria can move into plant genomes under natural conditions, resulting in tumour formation (Nester, 2015).

Bacteria species have evolved different secretion systems. The type III secretion system is used by Pseudomonas syringae to inject effector molecules into the plant cell, thereby assisting in processes of suppressing plant immunity responses to promote infection and disease. Much understanding of plant immunity responses is based on P. syringae and Arabidopsis interactions (Xin and He, 2013). In this plant–pathogen system, auxin receptors were shown to be targets of miR393 but this interaction becomes repressed upon treatment with the pathogen-associated molecular pattern (PAMP) effector flagellin of P. syringae (Navarro et al., 2006). This work became the starting point of several functional studies of plant miRNAs under various stress conditions. At present, we know that plant miRNAs, taSiRNAs and phasiRNAs target R-gene regulation upon stresses. Thus, there is some sort of self-regulation of its own R genes resulting in a delicate balance between growth and defence responses. Upon pathogen infection, phasiRNAs and 22 nt miRNAs are downregulated and a subsequent activation of defence responses occurs. Here, some miRNA families seem to be more involved than others, for example miR484 (Yang et al., 2013, 2015).

9.2 Interactions Between Potato Late Blight and its Host Potato Plant

The Solanaceae plant family includes many crops that are grown in almost all countries (Fig. 9.3). One important example is the potato (Solanum tuberosum), being the third food crop in the world in terms of human consumption, exceeding 300 million tonnes in annual production (CIP, 2020). To meet the global needs of potato food products and starch, China and India have now advanced ahead of Europe and the USA in acreage and production. Africa, particularly sub-Saharan Africa, has also experienced an increased interest and cultivation of potato (FAO, 2020). Potato tubers are ‘easy’ to put in soil for multiplication; however, tuber production is sensitive to drought and flooding in the fields. Potato plants suffer from many diseases, of which the potato late blight caused by Phytophthora infestans can rapidly destroy the green parts and the tubers both at field levels and in storage (Birch et al., 2012). To prevent infection, plants are commonly protected in the field by recurring chemical sprays. Estimates of annual costs due to treatments and yield losses worldwide associated with P. infestans vary between years but can exceed €10 billion. The problem of generating durable resistance and/or efficient agrochemicals is related to how the genome of this filamentous oomycete is organized. P. infestans has a large genome, which encodes close to 1000 genes that could facilitate plant infection. These genes are located in genome regions rich in transposable elements (Haas et al., 2009). Together, these features accelerate genetic changes and adaptation to the surrounding environment imposed by use of new cultivars, chemicals and other new cultivation practices. Introduction of resistance traits often present in related wild Solanum species is possible by sexual crossings but commonly takes considerable time, due to the need to remove unwanted DNA introduced into the recipient potato genome along with the desired genes (Vleeshouwers et al., 2011). Thus, the toolbox to combat P. infestans needs to be constantly refilled and refined, preferably with different defence components to counter loss of resistance function.

In contrast to plants, P. infestans encodes few core components for functional RNA interference pathways: two Dicer-like enzymes, five Argonautes and one RNA-directed RNA polymerase (Vetukuri et al., 2011). After intensive search only one miRNA has been found, compared with plants that could encode hundreds of miRNAs (Fahlgren et al., 2013). There are no specific membrane RNA transporters in P. infestans like those found in the nematode Caenorhabditis elegans. Neither are such transporters present in plants. In plants, details on mobility of sRNAs, including intercellular, extracellular and long-range mobility, mainly derive from studies on viruses (Yan et al., 2020).
Movements of soluble compounds in the plant transport system have been elucidated from studies concerning plant virus infection (Hipper et al., 2013), because viruses use the plant vascular system to spread and colonize the host. It has long been speculated that vesicles in the cellular secretion system can contain sRNAs and be part of the mobility system. To demonstrate their function is a problem, due to their low cellular numbers and because they are therefore difficult to detect. However, this obstacle was recently overcome by the demonstration that Arabidopsis sRNAs, protected in extracellular vesicles, could target the fungal virulence genes of Botrytis cinerea (Cai et al., 2018). The ability of sRNAs to move from plants to different pathogens, including P. infestans, had been demonstrated earlier via host-induced gene silencing (HIGS) experiments (Jahan et al., 2015) without explicitly explaining the mechanism of movement from one to the other (Fig. 9.2). HIGS requires knowledge on important virulence genes or species-specific active sites in the metabolism to be targeted by sRNAs produced in the properly designed transgenic plant. Interestingly, the single miRNA from P. infestans is demonstrated to target a membrane protein localized in the tonoplast of potato, resulting in enhanced susceptibility (Hu et al., 2020). Extensive targeting of potato and pathogen-derived sRNAs to a large number of mRNAs was observed, including 206 sequences coding for R genes in the potato.

Fig. 9.3. Eggplant, pepper, tomato, potato and petunia are examples of agricultural and horticultural crop species in the Solanaceae family. Potato and tomato are closely related. For more details, see Bombarely et al. (2016). (© C. Dixelius.)
genome. Whether genome editing of target sites of these sRNAs in the R genes would generate a release of new functional genes against *P. infestans* remains to be shown, as well as the transport mechanism of sRNAs between potato and *P. infestans*.

Direct application of RNA molecules is also under development (Koch et al., 2016). This so-called spray-induced gene silencing (SIGS) has the advantage of not being a genetically modified organism (GMO) strategy but requires upscaling of sRNA quantities for use at field levels, together with overcoming other limiting factors (Song et al., 2018). Several attempts to test this approach against *P. infestans* are ongoing. Besides organism specificity, the designed molecules need to pass national legislations and such regulatory framework is presently not adapted to RNA molecules. Here, studies on environmental RNAi effects will most likely be asked for.

A new form of site-directed nuclease-related class has been developed that originally built on a bacterial defence mechanism against virus phages called clustered regularly interspaced short palindromic repeats associated system number 9 (CRISPR-Cas9). Genome editing approaches, not least by the CRISPR-Cas9 system which is based on RNA biology, may offer new possibilities to induce specific mutations, which gives hope for future crop improvements (Chen et al., 2019) (see also Chapter 6, this volume). The emphasis on resistance breeding during the past century has focused on identification and transfer of resistance genes from related or wild species to crops. However, *R*-genes are rapidly overcome when frequencies of genetic recombination are high, not least in an organism with efficient spore spreading. New technologies such as resistance gene enrichment sequencing (RenSeq) and in combination with association genetics (AgRenSeq) can now facilitate *R* gene identification in complex crop genomes (Jupé et al., 2013) as well as in wild relatives (Arora et al., 2019). We envisage the need to combine genome-wide or genome-selection technologies along with genome-editing approaches to expand the genetic potentials to control late blight disease in potato.

**References**


10 The Stability of dsRNA During External Applications – an Overview

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Abstract

The research community is deeply convinced that RNA is unstable in the environment. Its roots rise from numerous failed attempts to isolate functional cellular RNA molecules. Further support had originated from the fast turnover of RNA in the cells. The situation changed recently with the discovery that externally applied dsRNA can produce targeted gene silencing in plant-feeding insects. First results have demonstrated that external dsRNA can successfully pass the insect gastrointestinal tract and reach its final destination within the body cells. This was somewhat unexpected and sparked new interest in RNA stability in the environment and its fate in the insect organism. In this brief review we make an attempt to summarize current knowledge and to propose a model of how dsRNA can perform its function under these settings.

10.1 Introduction

Since the initial discovery of the phenomenon (Ecker and Davis, 1986; Napoli et al., 1990) and its detailed investigation (Fire et al., 1998), RNA interference (RNAi) technology has gained much attention not only for fundamental research but also for practical applications. During the following decades most components of the interference apparatus were described along with regulatory mechanisms, as well as the constantly widening field of application. Recently, another aspect of RNA interference has gradually focused scientific interest: double-stranded RNA (dsRNA) stability either in vivo or in vitro.

There were at least two reasons determining this interest. The first was related to better understanding of the regulation of RNA interference pathways in the cell. A second reason emerged upon the discovery that dsRNA might be used as a therapeutic agent in medicine or as a plant protection agent in agriculture. In this case dsRNA is released into the environment in one form or another, which raises biosafety concerns. Initially, the only sources of artificial dsRNA in the environment were transgenic plants. Since dsRNA expression levels were not very high, the risk to the environment was estimated as insignificant (see below). The situation changed radically when a novel application of RNAi appeared as an externally applied insecticide. In this case the amounts of dsRNA directly applied to plants and soil might be significantly higher than those provided by genetically modified (GM) plants. As a result, new concerns about dsRNA biosafety were raised which, in turn, renewed the interest in RNA persistence in the environment. RNA stability in the environment also became a topic of interest for

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more practical reasons: how, applied externally as an insecticide, dsRNA could survive harsh conditions on leaf surfaces and within insect gastrointestinal tracts during feeding; and how to reach the target cells.

After decades of laboratory work, RNA is generally recognized as a degradation-prone molecule both in vivo and in vitro. Special precautions are a mandatory part of almost all protocols and manuals dealing with all types of RNA studied. There is a good foundation for both chemical and biochemical reasons. RNA possesses an additional 2′-OH group that makes the molecule more reactively competent than DNA, especially under alkaline conditions. In addition, organisms produce a number of RNA-degrading enzymes, both intracellular and secreted — some of them with very high stability in the environment.

RNA plays numerous roles in the cell, such as a temporary mediator of gene expression (messenger RNA) (mRNA), a structural component of translational apparatus (ribosomal RNA (rRNA), transfer RNA (tRNA)) and regulatory functions (RNAi), to name a few. Since it is not a long-lived molecule both in vivo and in vitro. Special precautions are a mandatory part of almost all protocols and manuals dealing with all types of RNA studied. There is a good foundation for both chemical and biochemical reasons. RNA possesses an additional 2′-OH group that makes the molecule more reactively competent than DNA, especially under alkaline conditions. In addition, organisms produce a number of RNA-degrading enzymes, both intracellular and secreted — some of them with very high stability in the environment.

The fate of short RNAs involved in interference in the cell is more complicated and has received attention during the past decade. The dsRNA precursor might be bound by specific RNA binding proteins and, eventually, targeted for degradation (Heo et al., 2008) as part of regulatory mechanisms. Once loaded in the RNA-induced silencing complex (RISC), microRNA (miRNA) is relatively stable with a half-life well over that of mRNA (hours or even days).

Further degradation of small RNAs depends on several 5′-to-3′ and 3′-to-5′ miRNA-degrading enzymes. A small RNA degrading nuclease (SDN1) was identified and cloned from Arabidopsis. SDN1 uses a 3′-to-5′ exonucleolytic mechanism, yielding a final degradation product of 8–9 nt. SDN1 can degrade single-stranded RNA in the range of 17–27 nt with comparable efficiency, but not pri-miRNAs, longer RNAs, double-stranded RNA or single-stranded DNA (Ramachandran and Chen, 2008; Wang et al., 2018). Uridylation by terminal nucleotydil transferase of processed miRNA was also suggested as a degradation-targeting signal (see Ruegger and Grosshans, 2012). Recently, another mechanism for specific degradation of particular miRNA triggered by its target mRNA or another miRNA was identified (Ghini et al., 2018), which is believed to form a complicated network regulating miRNA activity in the cell (Nicassio, 2019).

Bearing in mind the complicated and extensive metabolism of RNA in the cell, the discovery that externally applied dsRNA precursors might efficiently act as an insecticide during feeding was somewhat unexpected from the very early stages (Fire et al., 1998). Since then the mechanisms of the phenomenon have received sufficient attention for both practical and theoretical reasons (Huvenne and Smagghe, 2010). Despite the extensive research, our knowledge about the mechanisms underlying the insecticidal effect of externally applied dsRNA is still fragmented. In this review we attempt to propose a model of how dsRNA acts as an insecticide.

The key questions are how dsRNA reaches its target destination and what are the major factors influencing its stability.

10.2 Challenges to dsRNA Stability in the Environment

The first major stopover of topically applied dsRNA is the leaf surface, where major determinants of its fate are environmental conditions. These can generally be divided into abiotic and biotic factors by their nature. Since little specific research has targeted the effects of these factors on the leaf surface, some hints can be taken from both in vitro experiments and data available for two main environmental compartments: water and soil.

RNA in water solution under controlled physiological conditions in vitro is a relatively stable molecule with a half-life rate of several months. Its main degradation pathway is via internal phosphoester transfer reaction, promoted by specific base catalysis (Li and Breaker, 1999). The presence of buffer compounds and, especially, Mg\(^{2+}\) ions at pH 7 and above can significantly facilitate RNA hydrolysis to half-life times in the range of minutes. The catalytic effect of Mg\(^{2+}\) can be reduced by the presence of chelating agents (AbouHaidar and Ivanov, 1999).
Ultraviolet (UV) irradiation is another factor that compromises RNA stability. Exposure to UV can lead to photochemical modifications, crosslinking, and oxidative damage of the molecule (Singer, 1971). Interestingly, the presence of Mg$^{2+}$ can reduce UV damage. Also, single-stranded RNA is more prone to UV damage than dsRNA.

These data suggest that UV irradiation and chemical microenvironment (i.e. pH and presented metal ions) can be considered as the main abiotic factors leading to RNA degradation in the environment (Albright et al., 2017).

The recently opened possibilities for using RNAi technologies in agriculture have sparked new interest in RNA stability, especially for regulatory reasons about its biosafety. One of the key questions was how long dsRNA (either externally applied or produced by transgenic plants) persists in the environment. For example, recombinant Bacillus thuringiensis (Bt) toxins produced by transgenic plants show a half-life in the range of days to weeks (Icoz and Stotzky, 2008). Also, it was demonstrated that these proteins do not accumulate in soil (Sims and Ream, 1997), which was one of the arguments for their biosafety.

Irrespective of its origin, RNA shares the same two main receiving compartments in the environment: soil and water. Early experiments on dsDNA stability in soil revealed a half-life of under 2 h (Greaves and Wilson, 1970; Keown et al., 2004). Recent experiments with $^{32}$P-labelled dsRNA applied to ‘active’ soil samples demonstrated similar half-life times (Parker et al., 2019). The authors identified two main degrading factors: bacterial uptake and extracellular RNases. On the other hand, quantitative evaluation of dsRNA persistence in water reservoirs by qPCR revealed a half-life of approximately 3 days (Fischer et al., 2017). The apparent discrepancy might reflect differences in bacteria as well as extracellular RNase abundance in these two environmental compartments. Also, biotic degradation in the environment appeared to dominate over abiotic factors, especially in soils (Dubelmann et al., 2014).

It cannot be wrong to assume that the same factors, determining dsRNA stability in soil and water, also play a role in dsRNA persistence on leaf surfaces.

Leaf surface can be considered as an arid zone with extensive solar irradiation, inconsistent temperature variations and low organic content. Cuticle surface wax renders it water-repellent and does not allow significant water accumulation. Together, these factors lead to changing microenvironments and do not allow extensive bacterial growth. The reduced biotic degradation results in dsRNA half-life of 36 h (Bachman, 2019) and persistence for up to 3 days with sufficient activity.

### 10.3 Challenges to dsRNA Stability During Insect Feeding

The next major event is the transit of dsRNA in the insect gastrointestinal tract. Here, the main degrading factors are chemical composition (i.e. pH, ions, compounds), secreted RNases and gut microflora.

RNases comprise a large family of enzymes that play different functions in the cells and organisms. They differ by structure, activity, specificity, localization and environmental stability, to name a few. These differences are observed not only among taxonomic groups but also among the enzymes encoded by a particular genome and reflect the many functions that RNases play in the cells. Insect species are no exception and also demonstrate significant differences in RNase (and, more importantly, dsRNase) composition (Singh et al., 2017; Peng et al., 2018, Peng et al., 2020). The involvement of insect dsRNases in RNA interference efficiency through feeding application was demonstrated by knockout of dsRNA genes. Two dsRNA genes named dsRNase1 and dsRNase2 were identified in Queensland fruit fly, Bactrocera tryoni. Their knockout demonstrated significant improvement of the insecticidal effect of externally applied dsRNA (Tayler et al., 2019). These data can lead to the suggestion to consider dsRNase genes as co-targets in complex RNAi insecticide formulations.

Secreted RNases are the main degrading factor that dsRNA encounters during insect feeding. The very first contact occurs in the upper gastrointestinal tract (Lomate and Bonning, 2016; Song et al., 2017). Experiments have revealed that naked dsRNA suffered extensive degradation within 5 min when incubated with saliva of the southern green stink bug, Nezara viridula (Lomate and Bonning, 2016).
In the midgut, dsRNA encounters additional challenges like changes in pH, ionic content and organic compounds that can increase degradation either directly or by destabilizing dsRNA structure, making it more susceptible to dsRNases. However, dsRNase activity along the gastrointestinal tract appeared to have species-specific variances. In *B. tryoni*, dsRNases appeared to be the most important factor determining dsRNA degradation in the midgut (Tayler *et al*., 2019). On the other hand, in *N. viridula*, dsRNase activity in the midgut is negligible compared with the saliva (Lomate and Bonning, 2016). These seemingly discrepant results suggest that a good knowledge of the biochemistry of the targeted insect is a prerequisite to achieve maximal insecticide activity by RNAi approach.

Since the dsRNase source in these experiments was not clear (insect, bacterial, or both), gut microflora can also be considered as an important degrading factor in the midgut. Although direct evidence is not yet available, experiments on RNA persistence in soil (Parker *et al*., 2019) might offer a glimpse of its significance.

### 10.4 Reaching Inside Cells

What is next for the dsRNA molecules that remained intact during their passage through the insect’s gastrointestinal tract? In order to express their activity, dsRNA must enter the epithelium cells and, eventually, reach the haemolymph (Garbutt *et al*., 2013).

First of all, dsRNA must enter the gut epithelium cells. This seems to be carried out by clathrin or caveolin-mediated endocytosis of molecules, adsorbed to the cell surface (Denecke *et al*., 2018).

There are at least two possible entry mechanisms. The first one might involve formation of complexes between dsRNA and dsRNA-binding proteins in a non-specific manner. Proteins, bearing dsRNA-binding motifs, apparently exist in both prokaryotes and eukaryotes. It might be expected that some proteins might be presented in the midgut, where they form complexes with dsRNA, which might adsorb to the epithelial cell surface and enter via endocytosis.

It can easily be assumed that such adsorption is non-specific but there might be some indication of other more specific mechanisms. Researchers have identified cell membrane-associated DNA protein in human HeLa cells (Siess *et al*., 2000). Further, an RNA/DNA-binding protein has been demonstrated to relocate to the cell membrane (Ren *et al*., 2014). Recently, quite interesting data were published that Argonaute proteins can be secreted from the cells (Weaver and Patton, 2020). Together, these results suggest the possibility that dsRNA can be actively imported into the cells via some specific pathway (e.g. receptor-mediated endocytosis).

However, all these data were obtained on human cell lines. Not much data is available for insect (ds)RNA-binding proteins, exposed to cell surfaces. In *Caenorhabditis elegans*, two membrane proteins SID-1 and SID-2 were identified, which are responsible for RNAi uptake and spreading in an endocytosis-independent manner. In insects, SID-2 has no homologues but SID-1 is conserved among almost all species except Diptera. There is no direct evidence for dsRNA binding by SID-1, which makes any conclusions about its role too preliminary (Denecke *et al*., 2018).

Several possible pathways of dsRNA entry have been suggested (Vélez and Fishilevich, 2018). One proposed pathway might depend on SID-1-like proteins. Another pathway might depend on endocytosis in several aspects. One is related to cholesterol uptake, while the other is related to formation of clathrin vesicles. In the latest case, involvement of yet unidentified dsRNA-binding proteins is suggested (Vélez and Fishilevich, 2018).

Studies on endocytosis in different insect species revealed differences in dsRNA localization and cytoplasm entry routes, which might explain the observed species-specific differences in RNAi efficiency (Vélez and Fishilevich, 2018). Unfortunately, all available data are controversial, which makes it difficult to identify the exact mechanisms.

Once engulfed, dsRNA must escape from the endocytosis vesicles into the cytoplasm. The efficiency of vesicle escape and subsequent intracellular transport are important for triggering the RNAi path (Shukla *et al*., 2016). This is really terra incognita, since very limited data are available. One can speculate that escape occurs in a manner similar to one exploited by viruses. Since several groups are reporting that work is
in progress, one might expect that the first data will appear soon.

The number of dsRNA molecules that eventually reach into the cytoplasm of epithelial cells might be as low as a few molecules per cell. Here, the only viable way to reach effective levels appears to be through an RNA amplification pathway (Zhang and Ruvkun, 2012). Unfortunately, no RNA-dependent RNA polymerase genes were identified in insects (Gordon and Waterhouse, 2007), which puts RNA amplification mechanism beyond consideration. Therefore, in insects, the RNAi effect seems to rely only on molecules, passing from the gut (Ivashuta et al., 2015).

10.5 How dsRNA Appears to Work as an Insecticide and What Improvements Are Needed

In Fig. 10.1 a model of dsRNA delivery from plant surface to insect body is depicted.

Stage 1 refers to the dsRNA application process and its stability on the leaf surface. Here, the most critical factors are environmental conditions like UV, ions, pH and, to some extent, RNases. Since dsRNA in the environment has a half-life of 2–3 days, formulations are necessary to achieve sufficient efficiency.

Stage 2 reflects dsRNA uptake by insects during feeding. At this stage, the main obstacles are dsRNases of the gastrointestinal tract. Since dsRNase patterns differ, the particular set of secreted enzymes might be the first reason for species-specific differences in RNAi efficiency.

During Stage 3, dsRNA must pass through the gastrointestinal tract and reach the epithelial cells in the midgut. Again, dsRNases, either insect-secreted or of bacterial origin, are the main degrading factor. Proper formulations (e.g. nanoparticles) might significantly increase dsRNA stability and, thus, RNAi efficiency.

At Stage 4, dsRNA must either enter epithelial cells or pass into haemolymph. The molecular bases of these processes are not very well understood in insects (Cooper et al., 2019). Obviously, these are basic natural processes like endocytosis and other trans-barrier and transmembrane trafficking mechanisms, but their exact nature is unrevealed. While most mechanisms of RNAi pathways appear to be conservative (Yoon et al., 2018), it is unclear how dsRNA might express its activity without RNA amplification process (Vélez and Fishilevich, 2018).

Fig. 10.1. Pathways for dsRNA from plant surface to within insect body.
10.6 Possible Improvements of the RNAi Design

The outlined pathway demonstrates that both biotic and abiotic factors cannot be controlled under field conditions. One possible solution is to design formulations that can improve dsRNA stability for a substantial period of time (for at least 5–7 days). There is an excellent review of delivery systems by Whitten (2019). Briefly, almost all known approaches like chemical condensation, peptide or protein complex formation are providing sufficient increase of RNA stability. Maybe the most promising direction is towards development of protein–RNA complexes with predefined properties. Such complexes have the potential to implement most, if not all, required features for efficient insecticidal effect.

An approach demonstrated by Ghosh et al. (2017) employs a specially formulated diet as an RNA protecting factor. It has been identified that formulations targeted for increased dsRNA stability are an absolute prerequisite and may be the only way to deliver sustainable effect. One could expect that novel solutions, some of them not in the mainstream, will also find their market niche.

Another parameter to be considered for efficient external application is the length of the applied dsRNA molecule. Foliar-applied actin-dsRNA against Colorado potato beetle remained active for 4 weeks under greenhouse conditions but its efficiency depended on the length (San Miguel and Scott, 2016). In similarly designed experiments with Diabrotica undecimpunctata howardi, a precursor 27 bases long did not demonstrate toxicity. The efficiency became significant when the length of the precursor was increased to 60 bases and reached a plateau when the length exceeded 70 bases to at least 240 bases (Bolognesi et al., 2012). Another important result was the discovery that a precursor 240 bases long with 100% identity to the target was significantly more efficient than one with the same length but containing the absolute minimum of identical bases (i.e. 21 or 27 base long RNAi target and non-specific carrier). These results clearly demonstrate that using long target-specific precursors is a more effective strategy than short pre-determined analogues of siRNA/miRNA (Wang et al., 2019).

A site cleavage preference during insect dsRNA processing (probably by Dicer) has been described (Guan et al., 2018). The preference appears to be species-specific, thus further explaining differences in RNAi efficiency. Preliminary analysis of such site specificity in the targeted insect might be considered in RNAi design for further efficiency improvement.

10.7 Concluding Remarks

The exact nature of processes that underline dsRNA efficiency as insecticide is largely unclear. Most of them are investigated in great detail, while others are not yet fully revealed even in model organisms. Moreover, it is unclear how these processes interact in order to provide a single pathway of dsRNA from the environment to the insect cells. At the moment, the only possibility is to extrapolate available scientific data in an attempt to generate a hypothetical picture of how dsRNA acts.

References


11 Boosting dsRNA Delivery in Plant and Insect Cells with Peptide- and Polymer-based Carriers: Case-based Current Status and Future Perspectives

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Abstract

Since the discovery of this naturally occurring endogenous regulatory and defence mechanism, RNA interference (RNAi) has been exploited as a powerful tool for functional genomic research. In addition, it has evolved as a promising candidate for a sustainable, specific and eco-friendly strategy for pest management and plant improvement. A key element in this technology is the efficient delivery of dsRNAs into the pest or plant tissues. While several examples using transgenic plants expressing the dsRNAs have proved the potential of this technology, non-transgenic approaches are investigated as alternatives, allowing flexibility and circumventing technical limitations of the transgenic approach. However, the efficacy of environmental RNAi is affected by several barriers, such as extracellular degradation of the dsRNA, inefficient internalization of the dsRNA in the cell and low endosomal escape into the cytoplasm, resulting in variable or low RNAi responses. In the medical field, carrier systems are commonly used to enhance RNA delivery and these systems are being rapidly adopted by the agricultural industry.

Using four case studies, this chapter demonstrates the potential of carriers to improve the RNAi response in pest control for aquatic-living mosquito larvae and RNAi-resilient Lepidoptera and to cross the plant cell wall, allowing efficient environmental RNAi in plants.

11.1 Introduction

Plants are crucial for the planet and all organisms living on it. Most essentially for humans, they provide a source of oxygen and food. However, the changing climate poses enormous challenges to the agricultural sector to provide sufficient food for our growing population. Next to the obvious effects on abiotic stress (drought, heat, flooding, etc.), climate change has introduced novel or increased biotic stresses (pests, diseases, etc.) (Peters et al., 2014). To achieve the food demands of our ever-growing world population, agriculture has often practised an unsustainable upscaling of production, leading to reduction of the biodiversity of the terrestrial ecosystems (Pegler et al., 2019). This has included the excessive use of synthetic pesticides
to protect crops from biological stresses, which has had a serious detrimental effect on the environment and led to the emergence of resistance to most classes of conventional pesticides. Therefore, there is a dire need for more sustainable and eco-friendly solutions for crop improvement and pest control.

Exploiting RNA interference (RNAi), a natural regulatory and defence mechanism present in most eukaryotic organisms, has emerged as one of the most promising strategies for crop improvement and pest control. This is due to the biodegradability of the active molecule (RNA) and the possibility of designing this natural molecule to be species-selective (Huvenne and Smagghe, 2010; Younis et al., 2014; Cagliari et al., 2019; Taning et al., 2020). In RNAi, the presence of free double-stranded RNA (dsRNA) in the cell triggers and directs the sequence-specific translational repression or degradation of homologous messenger RNA (mRNA) targets, resulting in downregulation or knockdown of protein expression. In the past decade, applications have been developed in the form of genetically modified plants (host-induced gene silencing (HIGS)) expressing specific dsRNAs that silence the expression of essential genes that are required for the survival of the pests (insects, viruses and bacteria), thereby exploiting the RNAi mechanism as a species-selective pest control strategy (Huang et al., 2006; Mansoor et al., 2006; Baum et al., 2007; Mao et al., 2007; Qu et al., 2007). Similarly, the RNAi mechanism can also be exploited as a strategy for crop improvement through the silencing of specific plant genes to provide desired phenotypes or resistance to (a) biotic stress (Li et al., 2009; Younis et al., 2014; Joshi et al., 2018).

Despite the successful development of interesting and promising crop varieties through the HIGS approach, public acceptance of genetically modified crops is very poor (Shew et al., 2017). Moreover, the technical difficulties arising from the lack of established transformation protocols for some cultivated plants, the high cost of production and the long time required from the laboratory to the market have further impeded the adoption of the HIGS approach (Mitter et al., 2017a). These drawbacks have motivated the search for (Scozza et al., 2013) and development of alternative non-GMO (genetically modified organism) strategies for the delivery of dsRNA molecules. Non-GMO strategies could circumvent the technical limitation of plant transformation and the negative public perception of GMOs and provide an easy-to-use, environmentally friendly and flexible tool to improve plant performance and crop protection (Shew et al., 2017; Cagliari et al., 2018). The non-GMO approach of environmental application of dsRNAs offers an easy design and flexibility to apply relevant dsRNAs when and where needed. This approach has already been shown to offer protection against several pests, such as the Colorado potato beetle (San Miguel and Scott, 2016) and the fungal pathogens Fusarium (Koch et al., 2016) and Botrytis (Wang et al., 2016). However, a drawback in RNAi-based methods for pest control and plant improvement is the high variability in the RNAi response. Two important factors affecting RNAi efficiency are differences in dsRNA uptake into cells and differences in the stability of the dsRNAs against, for example, dsRNA-degrading enzymes (nucleases).

11.2 Barriers to dsRNA Delivery

Owing to their large size and highly negative charge, dsRNAs cannot easily enter the cells (Whitehead et al., 2009; Scott et al., 2013), making cellular uptake a key factor in explaining the variability in RNAi efficacy in non-GMO applications. Although some core components are known, many questions still remain unanswered concerning the dsRNA uptake pathways (Cappelle et al., 2016; Cooper et al., 2019). In insects, two different uptake mechanisms have been described so far: a pathway mediated by specific dsRNA channels, as also described in nematodes (Winston et al., 2002); and an alternative pathway based on endocytosis-mediated uptake mechanisms (Saleh et al., 2006; Miyata et al., 2014; Cappelle et al., 2016) (Fig. 11.1). A genetic screen in the nematode Caenorhabditis elegans identified several genes with a crucial role in the local and systemic RNAi response: the systemic RNA interference deficiency (SID) genes (Winston et al., 2002). In C. elegans, the cellular uptake of environmental dsRNA is mediated by the intestinal membrane protein SID-2 (Winston et al., 2007), while the dsRNA-selective dsRNA-gated channel SID-1 is required.
for systemic RNAi (Winston et al., 2002). An absence of orthologues of SID-2 in insects suggests that the mediators of dsRNA might be different across metazoa (Cappelle et al., 2016; Vélez and Fishilevich, 2018). Although orthologues of SID-1 are present in some insect species, data suggests that these SID-1 orthologues are not essential for systemic dsRNA uptake (Tomoyasu et al., 2008). In the closely related coleopteran species Diabrotica virgifera and Tribolium castaneum, the former has two SID-1 orthologues which are both involved in dsRNA uptake (Miyata et al., 2014), while the three SID-1 orthologues in the latter seem not to be necessary, suggesting an alternative uptake mechanism (Tomoyasu et al., 2008). In Drosophila melanogaster, no SID-1 orthologues have been identified; however, uptake of dsRNA has been demonstrated by receptor-mediated endocytosis (Saleh et al., 2006; Ulvila et al., 2006). This endocytosis-mediated uptake mechanism makes use of (pattern recognition) scavenger receptors and clathrin-dependent endocytosis (Saleh et al., 2006; Cappelle et al., 2016) (Fig. 11.1). In humans, a clathrin-independent (caveolae) endocytic pathway contributes to the cellular uptake mechanisms (Kasai et al., 2019), but a similar pathway is not involved in dsRNA uptake in D. melanogaster or T. castaneum (Saleh et al., 2006; Xiao et al., 2015). In D. melanogaster S2 cells, two scavenger receptors, SR-CI and Eater, account for 90% of the dsRNA uptake (Ulvila et al., 2006) (Fig. 11.1). Analysis of the components in this alternative uptake mechanism in C. elegans suggested this mechanism might be evolutionarily conserved (Saleh et al., 2006). In plants, the cell wall poses an additional barrier for the internalization of the dsRNAs. While it was shown that exogenously applied RNAs can spread locally and systemically through the plant and induce RNAi-mediated plant pathogen resistance, the understanding of the mechanisms for uptake of extracellular nucleic acids is limited and data are scarce and inconsistent (Bhat and Ryu, 2016; Mermigka et al., 2016; Dubrovina et al., 2019). Similar to the endocytosis-mediated uptake mechanisms present in animals, pattern recognition receptors are shown to be involved, but further research is needed to shed light on the mechanisms of extracellular dsRNA uptake (Dubrovina et al., 2019).

Fig. 11.1. Cellular internalization mechanisms in insects. While SID-1 orthologues are identified in several insect species, they do not play an essential role in dsRNA internalization. In insects, the primary dsRNA uptake mechanism depends on endocytosis. After binding of the dsRNA to membrane-bound scavenger receptors, the complexes are internalized through clathrin-mediated endocytosis. After acidification of the endosomes, the dsRNA is released into the cytoplasm where it is processed by the core RNAi machinery.
With endocytosis established as the major cellular internalization mechanism in plants and insects, the next barrier is the release of the dsRNA from the endosomes into the cytoplasm, where they are processed by the RNAi machinery (Dicer and RISC) (Saleh et al., 2006) (Fig. 11.2). Endosomal release occurs after acidification of the endosomes. A vacuolar H^+^-ATPase was suggested to play a role in this endosomal escape (Saleh et al., 2006). However, this escape from the endosomes is not always efficient and impaired endosomal release was demonstrated as a cause of low sensitivity to RNAi (Shukla et al., 2016; Yoon et al., 2017) (Fig. 11.2).

Besides cellular uptake and endosomal release, stability of the dsRNA is also an important factor undermining RNAi efficacy (Fig. 11.2). Despite being considered as an unstable molecule, dsRNA can persist on leaves for up to 20 days in greenhouse conditions (Mitter et al., 2017a, b). Experiments with photochambers and in field conditions showed that UV radiation is not a major contributor to instability of the dsRNA (Bachman et al., 2020). In contrast, wash-off by rain or dew is an important factor in foliar application (Bachman et al., 2020). In an aquatic environment, dsRNA can persist up to 4–7 days (Fischer et al., 2017); however, in soil the dsRNA is only stable up to 24–36 h (Dubelman et al., 2014). The instability of dsRNA is mainly attributed to the presence of microbial nucleases (Dubelman et al., 2014). Next to the microbial nucleases, damage to the plant (during dsRNA application) can result in the release of nucleases and subsequent degradation of the exogenously applied dsRNA. Especially in insects, extracellular degradation of dsRNA by nucleases in the gut has been identified as a key factor explaining reduced RNAi efficacy (Christiaens et al., 2014, 2016, 2018; Prentice et al., 2017; Guan et al., 2018; Ghodke et al., 2019; Castellanos et al., 2019). Next to the gut nucleases, also the extracts from saliva exhibit nuclease activity that can cause the rapid degradation of the dsRNA (Allen and Walker, 2012; Christiaens et al., 2014). Although the characterization of these nucleases requires further study, several candidates have been identified in the insect gut (Arimatsu et al., 2007; Liu et al., 2012; Wynant et al., 2012; Almeida Garcia et al., 2017; Spit et al., 2017; Prentice et al., 2019).

Increasing RNAi efficacy can be achieved by the use of dsRNA carrier systems. These systems are designed to efficiently deliver their dsRNA cargo into the cells by avoiding RNAi barriers such as an inefficient cellular uptake, a low endosomal release and extracellular

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**Fig. 11.2.** Barriers of environmental RNAi. External and internal barriers can affect the efficiency of the RNAi response. External factors include the degradation of the dsRNA by microbial nucleases (1) and UV radiation (2) and the wash-off of the applied dsRNA by rain or dew into the soil, where it is rapidly degraded by nucleases (3). Internal factors include the inefficient cellular uptake of the dsRNA (4), low endosomal release (5) and the presence of nucleases in the salivary glands, midgut and haemolymph of insects (6).
degradation of the dsRNA (Akinc et al., 2008; Vogel et al., 2019). Complexation of the dsRNA with the carriers increases the environmental stability of the dsRNA molecules, protects them against degradation by nucleases, improves cellular internalization and/or stimulates endosomal release, and this without affecting the ability to silence the target genes. These carriers can be based on naturally occurring or synthetic molecules and may include viral particles, lipids, metals, sugars, peptides, proteins and polymers. The peptide- and polymer-based carriers are the best studied (Vogel et al., 2019; Christiaens et al., 2020a, b). Polymers are macromolecules of variable sizes, composed of many repeating subunits, and can be naturally occurring or synthetically designed. The use of polymeric carrier systems has a long history to enhance RNA delivery in medical applications but applications in the agricultural industry are growing rapidly (Christiaens et al., 2018). Similarly, the use of naturally occurring peptides or proteins to direct the delivery of dsRNA provides a promising prospect and has already been developed in the biomedical and pharmaceutical fields (Milletti, 2012). Peptide-based carriers mainly make use of cell membrane penetrating peptides (CMPPs), which are small polycationic or amphipathic peptides that can facilitate cellular uptake of various molecular cargo, including nucleotides (Wang et al., 2014; Gillet et al., 2017).

While different synthetic and natural carrier systems have been investigated in relation to RNAi efficacy, this chapter presents a selection of four case studies to demonstrate the potential of the carrier systems to overcome specific barriers and improve RNAi efficacy in plants and insects: (1) a natural polymer for the control of aquatic-living mosquito larvae; (2) a synthetic polymer for the protection of dsRNA in the strong alkaline environment of lepidopteran guts; and (3) a polymer- and peptide-based carrier system to improve environmental RNAi in plants by assisting the RNAs to cross the cell wall. In the fourth case, we focus on the design of a peptide-based carrier, showing the potential of adding additional domains to improve its functionality. For a more comprehensive review on the barriers and the use of carrier systems to improve RNAi responses in plants and insects, refer to the recent reviews by Vogel et al. (2019) and Christiaens et al. (2020a, b).

### 11.3 Case 1: Delivery of dsRNA in Insects in Aquatic Environments

Blood-feeding mosquitoes serve as vectors for disease-causing agents responsible for the death of more than one million people each year (Zhang et al., 2015) and also act as vectors of infectious diseases that affect animal production (Bartlow et al., 2019). While the direct injection of dsRNA in adult mosquitoes has been shown to effectively trigger RNAi, microinjection delivery is not feasible as an application method for vector control in the field (Zhang et al., 2010, 2015). A viable strategy for the RNAi-based control of mosquitoes would be through the delivery of the interfering RNA with the ingested food at larval stage. However, the aquatic lifestyle of the mosquito larvae poses several technical challenges, such as the instability of the dsRNA and the dispersion of the dsRNA from the food, causing a low dose of dsRNA in the organism and subsequently an inadequate RNAi response (Zhang et al., 2010) (Fig. 11.3). To overcome these challenges, Zhang et al. (2010) developed a delivery system based on a natural polymer, chitosan. Chitosan is a non-toxic and biodegradable polymer prepared by deacetylation of chitin, the most abundant natural polymer after cellulose (Dass and Choong, 2008). The chitosan/dsRNA nanoparticles are formed by self-assembly through electrostatic forces between the polycationic chitosan and the negatively charged dsRNA (Zhang et al., 2015). Using the chitosan/dsRNA nanoparticles in feeding experiments with Anopheles gambiae and Aedes aegypti larvae significantly improved the RNAi efficacy (Zhang et al., 2010, 2015; Mysore et al., 2013; Kumar et al., 2016). The application of these nanoparticles improved the retention of the dsRNA in the food gel, an important element in feeding-based RNAi in aquatic environments; in addition, the nanoparticles significantly stabilized the dsRNA and enhanced delivery into the gut epithelial cells (Zhang et al., 2010) (Fig. 11.3). Although the mechanisms by which the cellular internalization is achieved were not completely elucidated, it is suggested that the nanoparticle carriers may facilitate dsRNA uptake by the endocytosis pathway in the gut (Zhang et al., 2010).
11.4 Case 2: Overcoming the High pH in the Lepidoptera Midgut

Not all carrier systems are appropriate for all applications. This implies that carriers must be optimized or sometimes even tailor-designed to the biology of the target organism in question (Christiaens et al., 2020a). The synthetic character of certain polymer-based carriers allows the design of carrier systems adapted for specific conditions. Lepidoptera can be considered as a worst-case scenario for RNAi-mediated pest control. Due to their slow cellular uptake and the strong dsRNA-degrading capacity of nucleases in the very alkaline (pH > 9) gut environment, these insects are generally very resilient to RNAi, especially upon oral delivery (Terenius et al., 2010; Garbutt et al., 2013; Christiaens and Smagghe, 2014; Christiaens et al., 2018) (Fig. 11.4). To overcome these barriers, a formulation was needed to protect the dsRNA against nucleolytic degradation to allow uptake into the cells and that was stable at high pH (Christiaens et al., 2018). Despite their proven efficiency in Diptera, the natural polymers were found to be unsuitable for use in Lepidoptera as the complexation was not stable in the strong alkaline environment in the gut of most Lepidoptera (Christiaens et al., 2018). Therefore, a series of nanoparticles based on cationic polymethacrylate derivates were designed to specifically shield the dsRNA from the degrading effects at high pH. The stability of the nanoparticles at high pH was enhanced by the modification of the polymer with protective guanidine side groups. Complexation of the dsRNA with these guanylated polymers resulted in an increased RNAi efficacy in in vivo feeding experiments with Spodoptera exigua larvae (Christiaens et al., 2018). This increased RNAi efficacy was shown to be due to an improved protection of the dsRNA against nucleolytic degradation, protecting the dsRNA for up to 30 h against S. exigua gut juice, and an enhanced cellular uptake of the dsRNA (Christiaens et al., 2018) (Fig. 11.4).

In addition to the protection in the high-pH environment by polymers, uptake in the midgut epithelium is needed for an RNAi response. Parsons et al. (2018) suggested that the synthetic polymer mimics cell-penetrating peptides to allow efficient internalization into Spodoptera frugiperda midgut epithelial cells. Similarly, synthetically modified cationic polymers have been shown to facilitate dsRNA uptake in feeding experiments with larvae of the Asian corn borer, Ostrinia furnacalis, and significantly improve the
RNAi efficacy in this insect (He et al., 2013). Next to the improved internalization and protection against extracellular endonucleases, polymer-based carrier systems have been suggested to improve RNAi efficacy by promoting endosomal escape. Especially in Lepidoptera, the endosomal release can be inefficient and contribute to the low RNAi response (Shukla et al., 2016; Yoon et al., 2017). According to the protein sponge theory, the buffering capacity of the nanoparticles could lead to osmotic swelling and rupture of the endosomes (Akinc et al., 2005) (Fig. 11.4).

11.5 Case 3: Crossing the Plant Cell Wall

As well as the challenge posed by the difficulties for the dsRNA to cross the cell membrane in animal cells, delivery of interfering RNAs in plant cells is faced with another barrier: the cell wall. In several studies, polymer-based carriers have been shown to deliver plasmid DNA and proteins into intact plant cells (Chang et al., 2013; Hussain et al., 2013; Martin-Ortigosa et al., 2012; Demirer et al., 2019), suggesting the potential of these systems to deliver interfering RNAs. However, the use of these carriers to improve non-GMO RNAi in plants remains understudied, with only a few papers reporting the delivery of RNAi molecules into the plant cell using nanoparticles (Demirer and Landry, 2017). The study by Mitter et al. (2017b) showed delivery of dsRNA into Nicotiana tabacum using layered double hydroxide clay nanosheets (BioClay). When loaded with dsRNA, this nanoparticle led to the sustained release of the dsRNA as the BioClay degraded (Mitter et al., 2017b). The slow release allowed the detection of dsRNA for up to 30 days after being sprayed on the plant and led to successful antiviral effects for at least 20 days (Mitter et al., 2017b). In another study, single-walled carbon nanotubes were used to improve the cellular delivery of small interfering RNAs in Nicotiana benthamiana plants (Demirer et al., 2019). Infiltration of complexed sense and antisense siRNA leads to efficient uptake of the complexes and subsequent desorption and hybridizing of the complementary siRNA strands activating an RNAi response (Demirer et al., 2019). Similar to the protection against
nucleolytic degradation observed in insects, the polymeric carrier protects the siRNA against degradation by RNaseA (Demirer et al., 2019).

In addition to the polymer-based carriers, peptide-based carriers have been shown to be able to deliver dsRNA cargo into plant cells. Peptide-based carrier systems using cell membrane penetrating peptide (CMPP) domains have been successfully used to initiate rapid and efficient RNAi-mediated silencing of exogenous and endogenous genes in leaves of diverse plant species, such as Arabidopsis thaliana, Nicotiana benthamiana, Solanum lycopersicum and popular (Numata et al., 2014, 2018), N. tabacum suspension-cell cultures (Unnamalai et al., 2004; Numata et al., 2018) and rice callus tissue (Numata et al., 2018). While these results showed the potential of these delivery systems, it is likely that the delivery of dsRNA can be improved by altering the lengths and/or amino acid composition of the peptides (Unnamalai et al., 2004). It is suggested that longer polypeptides with many positive charges might form complexes too tight to dissociate inside the cell, leading to a lower RNAi efficacy (Bettinger et al., 2001). The influence of the amino acid composition was shown in a comparative study of 55 CMPP-based carriers, revealing that the cell penetrating efficiency of Lys-containing CMPP-based carriers is relatively higher in plant cells than in animal cells (Numata et al., 2018). In addition, several CMPPs were found to function with specific plants or tissues. The inability to identify one peptide carrier with high cell-penetration efficiency for all plant species and cell types suggests that optimization of the CMPP domain will be essential for each application (Numata et al., 2018).

11.6 Case 4: Modifying carriers to improve functionality, uptake and endosomal release

Optimization of the CMPP-based carrier systems can significantly improve their ability to provoke an RNAi response. Within the CMPPs, the short cationic arginine-rich transactivating transcriptional activator (Tat) peptide of the human immunodeficiency virus 1 (HIV-1) has been specifically studied and engineered to improve its uptake efficiency and endosomal escape (Vivès et al., 1997; Wadia et al., 2004; Gillet et al., 2017) (Fig. 11.5). To improve the oral delivery of dsRNA in the cotton boll weevil, Anthonomus grandis, the Tat peptide was enhanced with the inclusion of a haemagglutinin peptide to destabilize the membrane of the endocytic vesicle and promote endosomal escape (Wadia et al., 2004; Erazo-Oliveras et al., 2012; Gillet et al., 2017). Direct conjugation of the cationic CMPP domains to anionic RNAs results in charge neutralization, which renders the carrier system inactive and limits delivery into the cells. In addition, this causes the aggregation/precipitation of the complex and leads to cytotoxicity (Turner et al., 2007; Meade and Dowdy, 2008; Eguchi et al., 2009). To circumvent the charge neutralization, the engineered peptide transduction domain was fused to the dsRNA-binding domain of the human protein kinase R (Eguchi et al., 2009). These modifications allowed a swift internalization of the complexes into the cell through endocytosis, an efficient endosomal escape and protection against nucleolytic degradation in the insect gut, leading to an enhanced RNAi response (Gillet et al., 2017).

11.7 Perspectives

RNAi-mediated pest control and improvement of plant performance have emerged as one of the most promising strategies, combining specificity and sustainability. Although exogenous application of RNA molecules is known to trigger RNAi responses in plants and insects, several barriers impede the use of non-GMO-based RNAi. Among these barriers, stability of the dsRNA and efficiency of the cellular internalization are the major challenges. The conjugation of RNA to different types of carriers is reported to improve the stability of the dsRNA, protect the dsRNA against nucleolytic degradation and facilitate an efficient internalization into the plant or insect cells, resulting in an improved RNAi response. The exploitation of chemical creativity to design carriers with specific properties, and the large biological diversity in which novel interesting proteins to direct dsRNA delivery can be identified, provides us with a wide diversity of
untested candidates that will allow the discovery of many potentially interesting carriers.

An interesting class of proteins for the design of protein-based carriers is the carbohydrate-binding proteins or lectins, allowing carbohydrate-targeted delivery of dsRNAs. Many lectins are shown to be efficiently internalized by insect cells and can even be transported across the epithelium into the underlying tissues (Powell et al., 1998; Caccia et al., 2012; Shen et al., 2017). In addition, many lectins were found to be stable in a large range of pH and temperatures and are resilient to proteolytic degradation (Chan et al., 2012; Walski et al., 2014), suggesting these proteins could offer protection to the dsRNA. These properties could make lectin-based carrier systems powerful tools for oral delivery of dsRNA. One example is the development of a lectin-based carrier using the mannose-specific *Galanthus nivalis* agglutinin (GNA) (Van Damme et al., 1987; Shibuya et al., 1988). Previously this lectin was used for the delivery of peptides and proteins in various insect cells through the generation of fusion proteins (Raemaekers et al., 1999; Raemaekers, 2000; Fitches et al., 2002, 2004; Down et al., 2006). Similarly, fusion to a dsRNA-binding domain would enable the cellular delivery of dsRNA (Bogaert et al., 2005; Cao, 2016), although further research is needed to investigate the potential of this dsRNA carrier.

As the functionality of dsRNA carrier systems has been shown, some caution must be taken when working with these carrier systems (Vogel et al., 2019). Many of the carrier systems are inspired by those used in the medical field, which implies that these could be capable of entering mammalian cells as well as arthropod or plant cells (Vogel et al., 2019). These aspects need to be taken into consideration in the development of potential applications.

**Fig. 11.5.** Modification of the TAT-based peptide carrier enhances its ability to provoke an RNAi response. Direct conjugation of the positive-charged TAT domain to the negative-charged dsRNA results in charge neutralization, causing a limited delivery into the cells. Fusion of a dsRNA-binding domain (dRBD) circumvents the charge neutralization and allows efficient cellular internalization. To promote endosomal escape, the carrier was modified with a haemagglutinin domain to destabilize the membrane of the endocytic vesicle.
Several studies have already confirmed the potential of carrier systems to improve RNAi for applications in pest control and plant improvement after environmental application of dsRNA. However, improving our knowledge on the factors affecting dsRNA stability and uptake mechanisms of carriers and dsRNA, in both plants and insects, will allow the generation of improved carrier systems, consequently improving this technology for future applications.

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Environmental Safety Assessment of Plants Expressing RNAi for Pest Control

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12.1 Introduction

Problem formulation (PF) is normally considered the first part of the environmental risk assessment (ERA) process and involves the identification of the possible hazards associated with a stressor (e.g. genetically modified (GM) RNA interference (RNAi)-expressing plants or RNAi-based pesticides). This initially requires an examination of all existing information to determine which hazards are identified by current scientific literature or experiences with the stressor and similar organisms or products. It also requires an element of brainstorming in order to envisage new potential hazards that might arise, particularly considering how the new stressor will be used and managed. The hazards identified in the PF are characterized in order to determine whether they have the potential to cause adverse environmental impacts and the potentially harmful characteristics become the main focus for the risk assessment. The PF also examines information on the potential receiving environments for the new stressor in order to determine what other biota might be exposed and which ecosystem functions might be affected. In addition, the PF identifies where there is lack of knowledge or experience with a new stressor and/or its receiving environments and therefore what studies are required to determine its environmental impacts. The risk hypotheses developed from the PF are used to hypothesize pathways to risk and to support the design of experimental studies to determine environmental impacts.

Any environmental risk assessment needs to provide quantitative information on two main components of the pathway to risk: exposure and hazard. Each of the two components can be determined based on the evaluation of several factors to estimate the exposure function \( f(\text{exp}) \) and the hazard function \( f(\text{haz}) \).

12.2 Exposure to dsRNA Expressed in Genetically Modified Plants

12.2.1 Environmental exposure and fate of dsRNA, siRNA and miRNA

Environmental risk assessment of RNAi-based pesticides (i.e. double-stranded RNA (dsRNA),
small interfering RNA (siRNA), microRNA (miRNA) involves a characterization of the potential ecological effects of exposure to these pesticides combined with a characterization of the anticipated concentrations of RNAi-based pesticides in environmental systems to which organisms will be exposed (Auer and Frederick, 2009; Lundgren and Duan, 2013). Developing estimates of environmental concentrations of RNAi-based pesticides requires specific knowledge on the entry and fate of RNAi-based pesticides in these environmental systems, which primarily are expected to be agricultural soils and adjacent surface water (Parker and Sander, 2017).

The release of RNAi-based pesticides to receiving environments from GM plant tissue differs greatly from the environmental release of sprayable RNAi-based pesticides or conventional synthetic pesticides. Whereas the amount of an exogenously applied pesticide entering the receiving environment depends primarily on its application rate, the amount of an RNAi-based pesticide produced in the tissue of a GM plant is determined by production and processing of the dsRNA within the plant tissue and the route(s) of entry from the plant tissue into the environment.

The amount of RNAi-based pesticides entering receiving environments has not yet been quantified from either spray application or GM crops. In the case of the latter, release rates from certain GM crops for which data are available have been estimated using release rates of Cry proteins from GM plant tissue to receiving environments (Clark et al., 2005) and reported concentrations of both RNAi-based pesticides and Cry proteins in GM plant tissue (US Environmental Protection Agency, 2015). From this available information, release rates of RNAi-based pesticides from GM crops to agricultural soil are estimated to occur at levels of micrograms per hectare (3–4 orders of magnitude lower than Cry protein release rates), resulting in nanogram or lower concentrations of RNAi-based pesticides per gram of soil (Parker and Sander, 2017; Parker et al., 2019).

One validated method that uses quantitative reverse transcription-polymerase chain reaction (RT-qPCR) is able to quantify RNAi-based pesticides at low levels applicable to release rates of RNAi-based pesticides from GM crops in receiving environments (Zhang et al., 2020). Alternative methods to measure RNAi-based pesticides in receiving environments require them to be present at relatively high concentrations (Fischer et al., 2016) or to be radio-isotopically labelled (Parker et al., 2019) and therefore may be unable to quantify RNAi-based pesticides in the field.

After entry into receiving environments, the fate of RNAi-based pesticides is determined by the relative rates and extents of multiple processes, including abiotic, enzymatic or microbial degradation and adsorption to solid–water interfaces (Parker and Sander, 2017; Parker et al., 2019). A few studies using the aforementioned hybridization assay have reported dissipation of detectable dsRNA pesticides or model dsRNA analogues applied at relatively high concentrations (i.e. μg/ml or μg/g levels) to soil or sediment–water microcosms (Dubelman et al., 2014; Albright et al., 2017; Fischer et al., 2017). These studies estimated half-lives for dsRNA dissipation ranging from hours to days. One study conducted using 32-phosphorus (32P) labelled dsRNA investigated the fate of dsRNA at lower concentrations (ng/g) in soil microcosms (Parker et al., 2019). In addition to enabling experiments to be conducted at lower dsRNA concentrations that are closer to expected concentrations in receiving environments, the use of 32P-labelled dsRNA enabled delineation of specific fate processes, including dsRNA degradation and adsorption to solid–water interfaces (Parker et al., 2019). These experiments revealed that both processes affecting dsRNA fate occur simultaneously in soils and therefore must be further evaluated to determine expected concentrations of RNAi-based pesticides in receiving environments.

Degradation of RNAi-based pesticides may occur by abiotic, enzymatic or microbial pathways. Abiotic degradation pathways include denaturation of dsRNA to single-stranded RNA and acid- or base-catalysed hydrolysis of the ribose-phosphodiester bonds comprising the RNA backbone (Parker and Sander, 2017). Microorganisms in receiving environments may accelerate the degradation of RNAi-based pesticides either through the production of extracellular hydrolases competent towards the pesticides, or through direct uptake and utilization of the pesticides. While reducing the abundance of viable microorganisms through either
X-ray sterilization or filtration of solutions extracted from soils only slightly decreased the degradation of 32P-labelled dsRNA in microcosm experiments, it dramatically reduced the formation of specific 32P-labelled degradation products indicative of microbial utilization (Parker et al., 2019). Together, these results provide a preliminary indication that both extracellular enzyme activity and microorganism viability lead to biological degradation of RNAi-based pesticides in receiving environments.

RNAi-based pesticides are also expected to adsorb to solid–water interfaces on particles in soil or sediment. In soil microcosms, adsorption of 32P-labelled dsRNA to particles was found to be rapid and extensive, particularly in soils with finer texture (Parker et al., 2019). Adsorption of RNAi-based pesticides in environmental media is expected to result primarily from electrostatic interactions between negatively charged phosphodiester groups along the pesticide backbone and positively charged soil constituents (e.g. iron and aluminium (oxyhydr-)oxides), as previously observed for DNA (Cai et al., 2006). Adsorption sites may be limited in abundance, particularly in the presence of competing adsorbates including other nucleic acids, phosphate, and organic acids co-occurring with RNAi-based pesticides in environmental media (Cai et al., 2007). Saturation of adsorption sites may explain the absence of significant adsorption observed in microcosm experiments conducted at high RNAi-based pesticide concentrations (Albright et al., 2017). Once adsorbed to an interface, longer RNAi-based pesticides (i.e. dsRNA molecules) are hypothesized to form train-and-loop structures common among linear polyelectrolytes, resulting in kinetically slow desorption which requires simultaneous detachment of the pesticide from all points of attachment to the interface (Parker and Sander, 2017). Relative to dissolved RNAi-based pesticides, RNAi-based pesticides adsorbed to sediment and soil particles appear to undergo slower degradation (Fischer et al., 2017; Parker et al., 2019); slower degradation of adsorbed nucleic acids relative to dissolved molecules has been widely attributed to protection of the adsorbed molecules from hydrolyses (Aardema et al., 1983; Lorenz and Wackernagel, 1987; Romanowski et al., 1991; Paget et al., 1992; Blum et al., 1997; Crecchio and Stotzky, 1998).

Taken together, current results suggest an important role for both degradation and adsorption to solid–water interfaces in determining the fate of RNAi-based pesticides in receiving environments. To constrain estimates of anticipated concentrations of RNAi-based pesticides in environmental systems, the rates and extents of these processes must be determined as a function of physicochemical and biological properties of the soil or other receiving environment (i.e. soil pH, texture, biological activity), as well as the properties of the RNAi-based pesticide (i.e. length, sequence) and the concentration at which it occurs. Furthermore, the use of delivery formulations to increase the stability and/or cellular uptake of the RNAi-based pesticide may impact these processes, for example by reducing degradation rates or inhibiting adsorption to sediment and soil particles. In addition, the link between environmental concentrations and organism exposure must be established by characterizing: (i) the bioavailability of RNAi-based pesticides adsorbed to solid–water interfaces; and (ii) the bioactivity of RNAi-based pesticides after partial degradation in the receiving environment.

12.2.2 Environmental exposure routes from plants and plant products to invertebrates

The principal pathway of exposure from plants to invertebrates involves herbivore organisms feeding on plants that, upon ingestion, introduce a number of compounds that are channelled to the digestive system. Herbivore organisms in any ecosystem, including agro-ecosystems, are numerous and normally linked to a few plant species (oligophagy) as their food source. Herbivores are active both on the aerial parts of the plant (initiating grazing food chains) and in the rhizosphere (detritus food chains). Trophic chains can be rather complex and it is not surprising to find, in many agro-ecosystems, organisms active at the fourth trophic level (e.g. Gillespie and Wratten, 2017).

The three main channels through which environmental exposure for invertebrates to plant-expressed components can occur are air, plants and soil. Exposure through the air
is initiated when pollen or seeds are dispersed from the plants into the wider environment and may involve organisms living in sites outside the cropped area. This type of exposure route is expected to implicate mainly herbivores, e.g. pollen feeders like bees, ladybirds, etc. or seed feeders like many beetles. An indirect exposure to other herbivores can occur when wind-dispersed pollen grains dust leaves of wild or cultivated plants where more herbivore organisms could be affected (Pleasants et al., 2001; Perry et al., 2010).

The exposure to plant-expressed compounds through trophic chains can initiate in any moment of the cropping season when a herbivorous species starts feeding on the plant. However, it does not stop at harvest, since plant residues may remain on soil for some time and can be moved incidentally by mechanical operations or naturally dispersed in nearby environments, including water bodies (Palm et al., 1996; Zwahlen et al., 2003; Rosi-Marshall et al., 2007; Tank et al., 2010). Herbivores and higher-order consumers can then become exposed off-site. Finally, exposure through the soil is also expected due to the emission of root exudates and litter to which soil-dwelling organisms at different trophic levels can then be exposed.

In the framework of ERA of genetically modified plants, different types of data need to be collected to provide estimates on the likelihood of exposure through the above-mentioned channels. First and foremost, data on the expression of dsRNA in various plant parts along the cropping season need to be collected. Scientific literature is rather poor in quantitative data referring to dsRNA expression in planta, which is normally derived only by comparison with the expression of housekeeping genes. The benchmark study in this respect was conducted by Bachman et al. (2016) during the characterization of the MON 87411 maize event that expresses dsRNA targeting the DwSnf7 gene, which was developed to provide an additional mode of action to confer protection against corn rootworm species. In planta studies were also conducted on the same maize event by Ahmad et al. (2016).

As stated above, the exposure is not limited to herbivore arthropods but can involve indirectly organisms at higher trophic levels (e.g. predators, parasitoids, hyperparasitoids) along the food chains based on the host plants expressing new compounds. In the specific case of dsRNA expressed in genetically modified organisms, information on the actual exposure along the food chain is very limited. The most compelling evidence of movement of dsRNA along the food chain comes from tritrophic studies conducted by Garbian et al. (2012), who investigated bidirectional transfer of RNAi between the honey bee Apis mellifera and its parasite spider mite, Varroa destructor. A dsRNA targeting V. destructor was supplied to a bee colony which was successively infested with Varroa mites. dsRNA was detected in Varroa individuals and, over time, the population of the parasite was sensibly reduced, demonstrating that movement of dsRNA along the food chain did not impair its biological activity. These individuals were also able to induce reverse movement when put in contact with a new honey bee colony. This particular example indicates a possible profitable use of dsRNA in the beekeeping sector. However, opposite scenarios could occur if a similar movement of dsRNA would affect predators of insect pests and jeopardize the contribution of natural pest control in the field. While several studies are ongoing to estimate the hazardous characteristics of dsRNA on some natural enemies (see below), studies aimed at identifying their possible indirect exposure in natural conditions are still scarce.

### 12.2.3 RNAi efficiency and (cellular) uptake of dsRNA in invertebrates

Several steps are necessary before exposure of an organism to the noxious substance present in the environment actually occurs. First of all, the compound needs to enter the target organism to exert its effect, then the substance (e.g. dsRNA) needs to undergo metabolic processes inside the body (e.g. cellular uptake, cleavage to siRNA) for the physiological exposure to occur.

RNAi efficiency is known to be very variable among invertebrate species, especially when dsRNA is taken up through the oral route. In nematodes, Caenorhabditis elegans is considered a model species for RNAi studies for different reasons, one of them being a very high sensitivity to RNAi. However, many other nematode species, including animal parasites and even other closely related Caenorhabditis soil-living species, show a much less robust RNAi response. In
arthropods, coleopteran insect species (beetles) contain some of the most RNAi-sensitive species, while insects belonging to other orders are often recalcitrant (e.g. Lepidoptera) or display a variable efficiency at best (e.g. Diptera, Hemiptera). Many studies have investigated potential factors explaining this variability, which include differences in RNAi core machinery gene repertoire, the stability of dsRNA in the insect body, the efficiency of cellular uptake of dsRNA from the gut lumen, the endosomal release inside the cell and the influence of viruses on the RNAi core machinery. A great amount of research is also conducted to improve RNAi efficiency in these less sensitive species, for example by using different formulations to increase the dsRNA stability and cellular uptake. Here, an overview is given on the variability of RNAi efficiency, focusing mainly on nematodes and arthropods since data on molluscs and annelids are very scarce at this moment.

*C. elegans* is the model species for RNAi research. This is partly because RNAi was first described in this species (Fire et al., 1998) and because *C. elegans* had already been a model species for biological, genetic and molecular research for several decades (Kaletta and Hengartner, 2006). Undoubtedly, it is also facilitated by the fact that *C. elegans* is highly sensitive to dsRNA taken up from the environment. Efficient RNAi gene silencing can be achieved by injecting the worms with dsRNA but also by oral or transdermal uptake (Timmons, 2006). Although unknown during the early years of RNAi, many other nematodes show a much less robust response to dietary uptake of dsRNA. A meta-study looking into the RNAi response and RNAi machinery in a wide range of nematodes, including plant and animal parasites, found that *C. elegans* is a rather special case, having an expanded RNAi machinery gene repertoire (Dalzell et al., 2011). The study found that many non-*Caenorhabditis* species possess less than half the number of RNAi-related genes considered to be involved in *C. elegans*. Particularly, genes that are known to be involved in cellular uptake of dsRNA were found to be absent in many parasitic nematodes. The study also showed that *C. elegans* has a highly evolved cellular uptake mechanism for dsRNA, involving different pathways and specific channel proteins encoded by *sid* genes. Uptake from the gut happens via Sid-2-mediated endocytosis and the dsRNA is then released in the cytoplasm from the internalized vesicles via Sid-1 channel proteins (McEwan et al., 2012). The same Sid-1 is also responsible for cellular export of dsRNA and uptake in cells that are not lining the gut. Therefore, Sid-1 is a major component of the successful systemic RNAi that is observed in *C. elegans*. Dalzell et al. (2011) found that not all nematodes, particularly parasitic species, possess these genes in their genome.

In arthropods, the cellular uptake pathways have not been completely characterized yet. While most insects do have one or more *sid-1-like* (*sil*) genes in their genome, their role in cellular dsRNA uptake is not clear. In all species where the involvement of (clathrin-mediated) endocytosis in cellular dsRNA uptake was investigated, this pathway turned out to be heavily involved. However, silencing of *sil* genes only affected RNAi efficacy in some of the species. An overview of this was presented in a cellular uptake study in the Colorado potato beetle, which is known to be highly sensitive to RNAi (Cappelle et al., 2016). In dipteran insects, there are clear indications that cellular uptake of dsRNA, at least from the midgut, is inefficient in some species. Studies in *Drosophila melanogaster* and *Drosophila suzukii* have shown that liposome encapsulation of dsRNA, aimed to improve cellular uptake, greatly increases RNAi efficacy (Whyard et al., 2009; Taning et al., 2016). Also in some other dipteran insects, such as mosquitoes, studies have shown that delivery formulations are necessary for efficient oral RNAi (Zhang et al., 2010).

A study by Shukla et al. (2016) showed that cytoplasmic release of dsRNA from internalized vesicles also plays a role in some lepidopteran insects. These researchers could see, through confocal microscopy, that fluorescently labelled dsRNA was taken up by the lepidopteran cells into endosomal vesicles but then not released into the cytoplasm. This was further confirmed when no siRNAs could be detected in these lepidopteran cells, indicating that no processing of the long dsRNA happened (Shukla et al., 2016; Yoon et al., 2017).

Another factor explaining the higher RNAi sensitivity in *C. elegans* and possibly other nematodes compared with arthropods is the presence of an RNA-dependent RNA polymerase-dependent amplification system,
whereby secondary siRNAs are created (Sijen et al., 2001). In this way, the silencing signal can be amplified and prolonged. In fact, most of the siRNAs in C. elegans are such secondary siRNAs, highlighting the importance of this amplification pathway (Pak and Fire, 2007). In arthropods, the presence of these RNA-dependent RNA polymerases (RdRPs) has only been reported in some ticks and mites (Kurscheid et al., 2009; Gribić et al., 2011). No homologues for this particular RdRP have been identified in insects so far. However, given the sensitivity of some insects to RNAi, it cannot be excluded that other, different amplification systems might be present in these species.

While variable cellular uptake efficiency and the lack of an amplification mechanism clearly play a role in some groups of insects, the most important factor affecting RNAi efficacy in insects might be the stability of dsRNA in the insect body. Many studies have shown that nucleases that are present in haemolymph, saliva and especially the midgut of a wide range of insect species are capable of causing rapid nucleolytic degradation of dsRNA that is taken up in the insect body. Nucleolytic degradation in saliva has been demonstrated in the saliva and haemolymph of Hemiptera and in the midgut of Coleoptera, Orthoptera and Lepidoptera. A study investigating RNAi efficacy and dsRNA persistence in the insect gut of three Coleoptera revealed a clear positive correlation between the two (Prentice et al., 2017). In their in vitro gut juice incubation assays, the most sensitive of the three showed a very long persistence (more than 10 h) while the dsRNA in the least sensitive of these three insects was degraded within 30 min. Several studies have discovered several nucleases in the genome of insect species, for example in Bombyx mori, Schistocerca gregaria, Locusta migratoria, Cylas punctifolius and Anthonomus grandis (Liu et al., 2012; Wynant et al., 2014; Song et al., 2017; Prentice et al., 2017; Almeida Garcia et al., 2017, respectively). Finally, in the RNAi-insensitive lepidopteran Ostrinia furnacalis, a nuclease was discovered and characterized which is specific for lepidopteran insects and which negatively affects RNAi efficacy in this species (Guan et al., 2018).

More information on these cellular and physiological barriers in insects can be found in a review by Cooper et al. (2019). These types of physiological barriers will prove a challenge for scientists and industry to apply this technology against a wide range of insect species. Advances in dsRNA delivery, for example by using nanoparticles or other delivery vehicles, might help us to overcome these barriers (Joga et al., 2016; Christiaens et al., 2018b). Of course, these new delivery methods will have an impact on the risk assessment of these RNAi products. For example, these delivery vehicles will prolong the persistence in the environment, they might expose the dsRNA to non-target organisms (NTOs) which would otherwise not be exposed and they can also overcome barriers in NTOs that might otherwise prevent dsRNA from being taken up by its cells. These considerations will have to be taken into account during the development and regulation of future RNAi-based products.

While RNAi efficiency and its variability among invertebrates is obviously of great importance for product developers, it can also have implications for risk assessment. In the current pesticide as well as genetically modified organism (GMO) regulatory frameworks, toxicity testing on NTOs is an important stage. Knowledge on the efficiency of dsRNA uptake in invertebrates could guide us in the choice of NTOs to perform these tests on. For example, it could be questioned whether it is useful to test a novel product on a phylogenetically distant species that is known to be insensitive to environmental RNAi, while a more closely related NTO known to be highly sensitive could be chosen. Of course, potential formulations and delivery methods will impact these choices, as they could lead to exposure in species that would not be exposed to naked dsRNA.

12.3 Hazards of dsRNA Expressed in Genetically Modified Plants

12.3.1 Off-target, non-target and unintended effects of RNAi-based GM plants

Due to the mode of action, RNA interference is a potentially very specific means of silencing genes, e.g. in pest insects, mites or pathogens (Mysore et al., 2018; Niu et al., 2018; Zotti et al., 2018). Within the body of a sensitive species,
long dsRNA is cleaved by the enzyme Dicer into siRNAs, which are the effective molecules involved in silencing genes that produce RNA with a complementary sequence. siRNAs are about 20–22 nt in length and can therefore be effectively designed to attack specific sequences within genes of interest, usually involving lethal effects on target species or drastically reducing reproductive performance (e.g. Whyard, 2018). However, several possibilities of harm to non-target organisms have been hypothesized (Lundgren and Duan, 2013). These unwanted effects might be related to sequence-dependent mechanisms if the same target sequence is found in non-target organisms. Also, sequence-dependent mechanisms might be the cause of harm to target or non-target organisms if the same sequence targeted by the siRNA is found in other parts of the genome (off-target effects).

A sequence-dependent mechanism was the cause of a silencing effect on the vATPase A gene in two ladybird beetles when fed dsRNA designed to target the same gene in the western corn rootworm (WCR), Diabrotica virgifera virgifera (Haller et al., 2019). The extent of the silencing and its biological impact were different in the two predatory species, being higher in Coccinella septempunctata, in which a significantly reduced survival rate in the bioassays was recorded. In Adalia bipunctata, during laboratory bioassays the authors only noted a prolonged developmental time. When the genome of the two species was studied in bioinformatics analyses, there was a difference in the number of 21 nt matches of the dsRNA with the vATPase A of C. septempunctata (34 matches) and that of A. bipunctata (six matches). This indicates that the degree of the negative effective could be attributed to the different presence of target sites in the genome. Further studies including additional species of Coccinellidae (Pan et al., 2020) confirmed that taxonomic similarities are a good proxy to estimate the possibility of non-target effects, since taxonomically related species share a higher percentage of genomes. However, gene silencing has been noted in some cases also on quite distant species, i.e. belonging to completely different insect orders. For example, Chen et al. (2015) studied the effects of dsRNA targeting rpl19 gene from Bactrocera dorsalis on a number of non-target species by measuring silencing with RT-PCR. The maximum effect was obtained on the co-specific B. minax, but significant effects were also obtained on the hymenopteran Diachasmimorpha longicaudata, which shared 72% sequence homology with B. dorsalis. The available studies clearly indicate the necessity of characterizing the possible sensitivity of non-target species to the dsRNA in an early phase of the development of a new RNAi-based product.

For the development of the MON 87411 maize expressing Cry3Bb1, Cry34Ab1/Cry35Ab1 and DvSnf7 dsRNA to induce multiple insect resistance, Bachman et al. (2016) characterized the spectrum of insecticidal activity of a 240 nt dsRNA targeting the Snf7 gene in D. virgifera virgifera. Insects belonging to ten different families and four different orders were tested in continuous feeding diet bioassays with DvSnf7 dsRNA. The results demonstrated that the spectrum of activity for DvSnf7 was narrow and activity was only evident in a group of beetles within the Galerucinae subfamily of Chrysomelidae, which show > 90% identity with WCR Snf7. A shared sequence length of ≥ 20 nt seemed to be required for efficacy against D. virgifera virgifera and all orthologues susceptible for gene silencing by DvSnf7 contained at least three 21 nt matches with the DvSnf7 sequence. However, these sequence identity requirements could be different between insect species, as research has shown that the length of siRNAs which are the result of Dicer-2 processing of long dsRNA is variable (20–22 nt) between species of different orders (Santos et al., 2019). Therefore, further research is needed to elucidate the sequence identity requirements for efficient RNAi. Taning et al. (2020) investigated potential effects of feeding dsRNA specific to pollen beetle (Brassicogethes aeneus) target genes by the bumblebee Bombus terrestris. Besides observing that this dsRNA had no effect on lethal and sublethal endpoints, the authors also investigated expression changes of 24 B. terrestris genes, which had the highest sequence identity with the non-target dsRNA. They found no changes in expression for any of these genes, despite siRNA matches of up to 20 nt.

Off-target effects are commonly related to the siRNA sequence and may occur when a partial complementarity of the siRNA to an unintended target within an organism is found (Jackson et al., 2006). It is not uncommon that off-target binding sites exist in several different
organisms, given the small sizes of siRNAs and the large genome of even quite simple organisms (Qiu et al., 2005). As shown above, sequence complementarity is needed to trigger off target effects; however, siRNAs containing some mismatches may still effectively trigger silencing (Christiaens et al., 2018a). The most striking example of off-target effects was shown in experiments with honey bees that were fed diet containing dsRNA targeting gfp, a gene that does not exist in the bee’s genome (Nunes et al., 2013). Although dsGFP is not expected to induce a response in honey bees, the authors reported phenotypical effects on specimens of Apis mellifera (i.e. altered pupal pigmentation and larval development). Examples are not limited to insects: Zhou et al. (2014) conducted a study on C. elegans and showed that nuclear Ago NRDE-3 protein associates with off-target silencing effects following administration of exogenous RNAi.

Unintended effects or RNA interference might sometimes occur, due to non-sequence-dependent mechanisms. A saturation of the RNAi machinery (e.g. on the protein RISC complex) is possible when a large number of dsRNAs enters the body of an organism, with consequent temporary inhibition of cellular use of RNA and compromise of some of its natural functions. However, to our knowledge this mechanism has never been proved in invertebrates.

RNA in invertebrate species is involved in the functioning of the immune system, especially against virus infections. The presence of exogenous RNA is known to trigger this response in mammals, and due to the similarities between the mammalian and arthropod immune system (Lundgren and Jurat-Fuentes, 2012) a possible alteration of the immune system functioning has been hypothesized (Lundgren and Duan, 2013), though rarely experimentally proven in arthropods.

### 12.3.2 Activity spectrum on soil- and plant-dwelling organisms

Due to the very general mechanism involved in RNA interference, theoretically speaking any gene can be silenced in species of interest (e.g. plant pests or pathogens) with the use of dsRNA. Highly conserved genes could therefore represent a common target among a high number of species, which are not meant to be affected if exposed non-target organisms. The extensive review by Christiaens et al. (2018a) was based on a thorough literature search in July 2016 regarding possible silencing effects on invertebrates due to RNA interference. As of June 2019, no new studies had addressed soil invertebrates as revealed by doing a literature search on Web of Science (WoS) using the search terms of Christiaens et al. (2018a), but restricted to the main soil invertebrate taxa in agricultural soils: collembolans, mites, enchytraeids and lumbricids.

The open literature is still void of testing results involving RNAi and soil invertebrates. Ecotoxicological testing of RNAi with soil invertebrates has been reported only twice in the literature, i.e. for DvSnf7 (Bachman et al., 2016) and for v-ATPase A dsRNAs (Pan et al., 2016). In these studies, collembolans were exposed to the dsRNA active ingredient through food and the earthworm was exposed through soil. Worst-case scenarios were explored by manipulating the dsRNA similar to application of sprayable RNAi pesticides. None of the studies employed an increasing dose approach enabling an LC (lethal concentration) or EC (effect concentration) estimation, but this is not warranted if range-finding indicates no effects and a high often unrealistic dose is an option in a limit test (OECD Test Guideline 232; see OECD, 2016). So, ten times the expected maximum environmental concentration was tested for DvSnf7. In planta exposure was not addressed as recommended by Arpaia et al. (2017) and in this case the choice of test species should be litter feeders and litter decomposers. Both Bachman et al. (2016) and Pan et al. (2016) concluded that there were no effects on the soil invertebrates. However, a typical dilemma of the assessment occurred for the collembolan exposed to v-ATPase A dsRNAs: the developmental time was decreased. This would be interpreted as a case of hormesis if it was observed for a chemical, but for an RNAi it was not considered to indicate adversity and the RNAi was deemed harmless. It remains to be elucidated if such an effect is due to unintended effects or a stress response leading to hormesis.

A bioinformatics approach aiming for in silico screening of potential risks is possible
for the two most tested soil invertebrates, the earthworm *Eisenia fetida* and the collembolan *Folsomia candida*, as their genomes and transcriptomes are available (Faddeeva et al., 2015; Bhambri et al., 2018, respectively). However, available genomic or transcriptomic information for a broad range of soil NTOs is still needed. Currently the database of the US National Center for Biotechnology Information (NCBI) includes the genome of three annelids, 17 collembolans and 30 mites, and the Transcriptome Shotgun Assembly (TSA) database of NCBI includes eight collembolans, 25 mites and two earthworms (available at www.ncbi.nlm.nih.gov, accessed 12 November 2020), but these transcriptomes are not evenly distributed across the taxonomic and functional diversity of soil invertebrates.

For soil invertebrates, we still need candidate dsRNAs with a reproducible effect to include as a positive control. Positive controls are available for chemical testing in OECD Guidelines for the Testing of Chemicals programme (OECD, 1994), but not for exposure through food or soil of dsRNA. Protection of soil ecosystem services has received increasing attention at the European Union (EU) level (Krogh, 2021), but hitherto no assessment protocols are available, and the ERA is stuck with assessment of life history parameters and biodiversity.

The paragraph above on the cellular uptake of dsRNA in invertebrates gives a good overview of the species that might constitute possible effective targets for dsRNA-based pesticides or prolactin-induced proteins (PIPs). Obviously, most of the knowledge in this area comes from insects and nematodes, due to their relevant role as pests in agriculture. Particular attention has been given to the use of dsRNA as a control for noxious organisms that are quite recalcitrant to other forms of pest control (for example, *D. virgifera virgifera* could not be satisfactorily controlled with the use of *Bacillus thuringiensis* (Bt)-expressing genetically modified maize). This is reflected in the results of the review by Christiaens et al. (2018a), who indicated that the great majority of studies had been conducted with the aim of silencing genes in insects (2862 studies), though there was a bias towards the model species *Drosophila melanogaster* (Diptera) which, alone, was the subject of 1243 publications. The current research trend is in line with the existing literature, and emphasis is being given to several insect pests, such as sap feeders (e.g. Castellanos et al., 2019; Sun et al., 2019; Tian et al., 2019) or invasive species (e.g. Christiaens et al., 2018b; Bento et al., 2019) that are still known to represent difficult pests to manage in many agro-ecosystems in different areas worldwide. Yet very little is being published regarding non-target species not taxonomically related to targets and for which there is not much information about their genome.

### 12.4 Conclusions and Knowledge Gaps

RNAi-based mechanisms are a promising new means of pest control which could couple high effectiveness, due to completely new modes of action, to an extreme selectivity as a consequence of carefully selected target sequences in the genome of pests. However, even an accurate design of the dsRNA to induce interference does not exclude the possibility of off-target or non-target effects. Bioinformatics can give important support in designing RNA sequences that target genes expressed specifically in target pests. Nevertheless, the limited availability of genomic sequences of arthropod pests, the possibility of gene silencing if mismatches between the target and the siRNA sequences exist, and the likelihood of sequence-independent silencing suggest that laboratory, or higher-tier, bioassays remain fundamental in assessing possible environmental risks for non-target organisms due to the use of dsRNA. Current environmental risk assessment frameworks regarding possible effects on non-target organisms are expected to be effective to estimate RNAi-based products (Arpaia et al., 2020; Papadopoulos et al., 2020), though for some soil invertebrates more realistic exposure scenarios need to be developed. Arpaia et al. (2017) indicated some features of the dsRNA mode of action that need also to be considered during environmental risk assessment. For example, since mRNAs are transcribed only when needed by the organism, it is important to specifically consider the environmental conditions under which tests are being conducted. This is true for assessing silencing in both non-target genes and potentially existing off-target sequences.
Moreover, there is uncertainty about the possible modes of action, and consequent effects, in case of off-target silencing. Due to the various modes of action that can be activated while silencing genes with dsRNA, it is very unlikely that one single set of test species will serve as an adequate proxy of non-target species for all products using RNAi technology.

Several knowledge gaps need to be filled in order to have a thorough understanding of the possible environmental impacts of this new means of pest control. It must be noted that most scientific publications describing RNAi-expressing GM plants did not explicitly investigate the potential exposure of invertebrates to dsRNA expressed in such plants. For example, the actual presence of dsRNA in different plant parts over time has only occasionally been reported. As indicated above, only a few soil-dwelling species have been specifically tested for sensitivity to dsRNA, therefore studies encompassing more species, especially those known to be involved in providing ecological services to agriculture, are certainly needed.

The movement of dsRNA along food chains has been studied to only a limited extent. Indirect exposure was demonstrated for Varroa mites when feeding on honey bee colonies to which dsRNA was added to the diet (Garbian et al., 2012). In this study, not only was transfer of the nucleic acid ascertained, but it was also found that dsRNA remained biologically active and the transfer could be reversed from mite individuals to new honey bee families. Data on other pests and their natural enemies are urgently needed. Likewise, some recent reports of potential interference of exogenous dsRNA with the immune system in bees (e.g. Niu et al., 2016; Brutscher et al., 2017) need confirmation of the mechanism and its consequences.

Finally, we note that the molecular mechanisms for uptake have mostly been studied in C. elegans and therefore we still need to fill relevant gaps for other arthropod systems of relevance.

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13 Food and Feed Safety Assessment of RNAi Plants and Products

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13.1 Introduction: Steps in the Risk Assessment

The risk assessment of genetically modified (GM) plants for food and feed use is based on a comparative approach (EFSA GMO Panel, 2011) where the composition as well as phenotypic and agronomic characteristics of the GM plant are compared with those of a conventional counterpart with a close genetic background and to additional non-GM comparator lines, which are assumed to have a history of safe use. Comparative risk assessment identifies effects intended by the genetic modification as well as possible unintended effects arising from transgene insertion into functional genome regions or from inadvertent impacts of the transgene product(s) on plant metabolic pathways. If differences and/or lack of equivalence between the GM plant and its comparator(s) above natural variation are identified, possible adverse effects on human and animal health have to be considered.

This type of hazard identification is the first step in the risk assessment of a GM plant. Intended and unintended differences in contrast to comparator(s) are then evaluated with respect to adverse health effects. This involves in the first place toxicological and allergenicity assessment of newly expressed proteins (NEPs). In the case of RNA interference (RNAi) plants not expressing any new protein, these assessments are inapplicable. Instead, as the introduction of a gene silencing construct may cause silencing of ‘off-target’ genes, bioinformatics searches for ‘off-target’ sequences in the plant genome should be part of hazard characterization. If plant metabolic genes are silenced, unintended interferences with endogenous metabolic pathways are also possible and may cause alterations in metabolites and precursors of suppressed metabolic routes, justifying – on a case-by-case basis – analysis of specific RNAs or metabolites (EC, 2013).

An important aspect of risk assessment is the determination of exposure to the food and feed derived from GM plants, which involves identification of the population groups and animal species exposed as well as the extent of exposure. A starting point for the extent of exposure is the expression product of the introduced genetic modification, which is double-stranded RNA (dsRNA) in the case of RNAi plants. Its level, as well as levels of constituents altered as a result of the genetic modification, should be...
determined in plant parts used for food or feed. For estimating exposure from dietary intake or feed consumption, the stability of the dsRNA and derived small interfering RNAs (siRNAs) during storage and processing of plant material as well as during oral consumption need to be taken into account.

The final risk characterization of food and feed derived from GM RNAi plants is based on the results from the evaluation of potential adverse effects on human and animal health and from exposure assessment.

### 13.2 Potential Hazards of Food and Feed Derived from RNAi Plants

#### 13.2.1 Adverse changes of plant metabolism

The principle of RNAi is used to modulate agricultural, phenotypic or compositional characteristics of plants by promoting gene silencing (Fire et al., 1998; Dykxhoorn et al., 2003; Frizzi and Huang, 2010). This strategy does not pose an inherent hazard to consumers or the environment, because it exploits gene regulation mechanisms that occur naturally in plants and animals. There are already manifold examples of spontaneously occurring RNAi-mediated genetic traits that were selected by conventional plant breeding. Such ‘natural’ gene silencing traits involve, for example, changes in the coat color of soybean seed (Tuteja et al., 2004) and maize stalk (Della Vedova et al., 2005), or mediate a low glutelin level in rice (Kusaba et al., 2003). So far, the RNAi strategy has been adopted in biotechnology-derived food crops to generate virus-resistant varieties (Sherman et al., 2015), optimize their agronomic performance (Ogita et al., 2003), provide pest and pathogen protection (Baum et al., 2007; Gordon and Waterhouse, 2007; Mao et al., 2007; Koch and Kogel, 2014), facilitate industrial processes like starch production (EFSA GMO Panel, 2006), improve the nutritional profile (Andersson et al., 2006; Regina et al., 2006) and reduce allergen levels (Le et al., 2006). Some prominently discussed RNAi-mediated products that have achieved market approval include the Flavr Savr™ tomato with reduced polygalacturonase expression for delayed fruit softening (Redenbaugh et al., 1992), Plenish™ soybean with reduced omega-6 desaturase for high oleic acid content (EFSA GMO Panel, 2013) and Arctic™ apple with reduced polyphenol oxidase expression for delayed browning (Sherman et al., 2015; Waltz, 2015).

The intended decrease in the expression of a target gene may require safety considerations on a case-by-case basis. For example, the purpose of soybean with reduced omega-6 desaturase activity (also known as soybean 305423) is to obtain oil for frying and bakery with an increased content of heat-stable oleic acid (C18:1) at the expense of heat-labile polyunsaturated fatty acids (PUFAs) (C18:2 and C18:3). The consequences of this intended change in plant metabolism and composition need to be assessed to ascertain that the altered fatty acid profile does not impact on human and animal health in an exposure scenario where conventional vegetable oils are replaced with oil from soybean 305423 (EFSA GMO Panel, 2013). This assessment is focused on soybean oil and does not extend, for example, to soy milk and tofu for human consumption or defatted toasted meal for animal consumption, as such products are not expected to differ in composition between conventional soybean and soybean 305423, except for their altered fatty acid profile. However, the low contribution of fatty acids from these other soybean products to overall exposure is not anticipated to modify their nutritional impact. There is a detailed discussion of the risk assessment of an RNAi crop with altered metabolic composition in section 13.4.1, below.

Unintended effects caused by silencing genes in plant metabolic pathways

The engineering of plants with RNAi-mediated traits is achieved using the same transgenic techniques employed in the production of other GM crops grown and consumed widely today. In particular, RNAi plants are generated by inserting DNA sequences that lead to the expression of dsRNA or short hairpin RNA (shRNA), which are processed into siRNAs and microRNA (miRNA), respectively. These processed RNA molecules of 20–30 nt in length, collectively termed small RNA (sRNA), suppress gene expression at the transcriptional or post-transcriptional level,
but are themselves designed to lack translation initiation signals and open reading frames necessary for protein biosynthesis (reviewed by Casacuberta et al., 2015; Petrick et al., 2013). Because the sRNA effectors are not translated to heterologous proteins, the risk assessment is focused on the direct and indirect consequences of the gene silencing machinery. The standard comparative analysis is well suited to detect possible unintended effects of RNAi-mediated silencing that may occur in addition to the intended gene expression changes.

An example of a potential indirect effect of RNAi-mediated silencing became apparent with the compositional analysis of the aforementioned high-oleic acid soybean 305423. In fact, the comparison between soybean 305423 and its non-GM (conventional) counterpart ‘Jack’ confirmed the expected change in fatty acid composition (increased levels of oleic acid at the expense of PUFAs), but also revealed an unexpected increase in the level of odd-chain fatty acids heptadecanoic acid (C17:0), heptadecenoic acid (C17:1) and nonadecenoic acid (C19:1). It is not known whether this effect results from off-target gene silencing (see below), from the manipulation of fatty acid synthesis pathways, from another unidentified response to the genetic modification, or as a consequence of either the simultaneous expression of a transgenic acetolactate synthase (ALS) enzyme (conferring herbicide tolerance) or the genetic background of the recipient soybean variety. In any case, a nutritional assessment came to the conclusion that the slight changes observed in the concentration of odd-chain fatty acids would not constitute a health hazard for humans and animals (EFSA GMO Panel, 2013).

Unintended effects caused by off-target gene suppression

In addition to the intended effects induced by expression of the non-coding RNA, unintentional changes may occur in the plant by suppression of genes that were not foreseen as RNAi targets. RNAi-mediated silencing is hybridization-dependent and therefore takes place in a sequence-specific manner. Nevertheless, suppression of genes with less than perfect sequence complementarity is possible (Senthil-Kumar and Mysore, 2011). In some cases, indications for such off-target effects may come from the screen for agronomic performance and phenotypic characteristics or from the compositional analysis (see section 13.2.5, below), as changes of gene expression may impact on one or more of these routinely measured parameters and analytes. Genome-wide bioinformatics studies retrieving transcripts that match the newly expressed sRNA sequences would potentially indicate possible off-target effects (see section 13.2.3, below, for appropriate bioinformatics tools). However, the genomes of typical crops are at best only partially sequenced and known reference genomes do not take into account the sequence variability occurring between varieties (Ramon et al., 2014; Casacuberta et al., 2015).

Despite these limitations of bioinformatics-based predictions, whole-genome homology searches may nonetheless reveal unintended silencing targets.

The EFSA GMO Panel acknowledged the limitations of bioinformatics searches for possible off-targets of sRNA produced by GM plants. A predictive strategy is nevertheless possible, due to the fact that plant miRNAs are usually perfectly or nearly perfectly complementary to their target transcripts (Paçes et al., 2017). Thus, a set of parameters may allow for the prediction of RNAi off-targets in plants, whereas in humans and animals the extent of complementarity between the sRNA molecules and their targets is more flexible, thus preventing sufficiently reliable predictions (Pinzón et al., 2017). Besides the abundance of each sRNA produced, the degree and position of base pairing between the sRNA and the target mRNA are the primary factors determining the efficiency of silencing (Rhoades et al., 2002; Allen et al., 2005; Pasquinelli, 2012; Liu et al., 2014). Based on the current knowledge gained from the target specificity of natural miRNAs, the EFSA GMO Panel described in Annex II of the minutes of its 118th Plenary meeting (EFSA GMO Panel, 2017) a practical approach to identify sequences with potential off-target silencing. This procedure considers all 21 nt sRNA sequences that derive from a given dsRNA precursor and comply with the following rules:

- No more than 4 base mismatches with no gap or 3 mismatches and one gap in
the alignment between the 21-mer sRNA sequence and a potential target mRNA transcript, whereby each G:U base mispair counts as half a mismatch.

- Only one gap can be present in the sequence alignment between the 21-mer sRNA sequence and a potential target transcript, and this single gap cannot be longer than one nucleotide.
- The sequence alignment should not reveal any mismatches or gap at position 10/11 of the sRNA sequence.
- The sequence alignment should also not reveal more than two mismatches (or no more than one mismatch and one gap) in the first 12 nucleotides from the 5′ end of the sRNA sequence.
- The minimum free energy of the imperfect duplex of the sRNA sequence with a potential target, divided by the minimum free energy of the perfect complement, should be > 0.75.

The ensuing risk assessment of potential off-target silencing in the plant should consider the abundance and the number of different sRNAs showing relevant similarity to the same transcript, as the potential for gene repression increases with multiple sRNA sequences being able to bind to the same mRNA molecule (Hannus et al., 2014). Depending on the nature and function of the potential off-targets, the safety assessment may require extra studies in addition to the standardized agronomic/phenotypic characterization and compositional analysis.

13.2.2 Mechanisms and potential for non-target gene silencing in humans and livestock, including gut microbiome

Mammals have an RNA silencing machinery, which is distinct from that of plants and other animal orders. While in plants there is a complex RNAi system with different types of siRNAs and Dicer proteins and a distinct miRNA pathway, mammals have a single set of Dicer and Argonaute (AGO) proteins for both miRNA and siRNA pathways (Pačes et al., 2017). This implies that in mammals siRNAs can function in the same way as miRNA, i.e. bind to mRNAs depending on homologies to the ‘seed region’ which comprises nucleotides 2–8 from the 5′-end of the miRNA (Brennecke et al., 2005) and therefore have less strict target specificity than siRNAs in plants. In fact, in mammals sRNAs that are perfectly complementary to a target mRNA sequence are loaded into an AGO2 RNA-induced silencing complex (RISC) guiding target RNA cleavage, while siRNAs and miRNAs with minimum seed region homology are loaded on all four mammalian AGO proteins, resulting in translational inhibition (Meister et al., 2004; Gebert and MacRae, 2019). Lower requirements for sequence complementarities between miRNAs and mammalian mRNA make predictions of putative target sequences more difficult. As there is no distinct siRNA pathway in mammals, efficient induction of RNAi by long dsRNA, which has to be processed first into active siRNAs, is limited by poor Dicer activity in most mammalian cells (Nejepinska et al., 2012; Flemr et al., 2013; Pačes et al., 2017).

Another specific feature of plant siRNAs and miRNAs which distinguishes them from siRNAs and miRNAs in mammals is their 3′-terminal methylation at the 2′-hydroxyl group (Li et al., 2005; Yu et al., 2005). 3′-terminal methylation probably protects small RNAs from degradation (Li et al., 2005; Ren et al., 2014) and may promote recognition by plant Argonaute proteins in RISC (Yu et al., 2005). In contrast, mammalian AGO proteins preferably bind to non-methylated miRNAs (Tian et al., 2011). On the other hand, it was shown by Ma et al. (2004) that 2′-OH methylation only moderately decreased the binding affinity of siRNA for the PAZ domain of a human AGO protein, while binding was heavily reduced by most other 2′-OH modifications at the 3′-terminal nucleotide. In line with this, Chau and Lee (2007) found no obvious effect of 2′-OH methylation on the efficiency of silencing in mammalian cells. However, these authors also showed that siRNAs derived from a plant hairpin transgene and extracted from transgenic plants were not effective for gene silencing in mammalian cells. Among other things, they attributed this lack of cross-species function to a putative plant-specific siRNA modification.

These molecular mechanisms indicate that there is no evidence that plant-produced dsRNA
and siRNAs are functional in mammalian cells. Another limiting factor is the high number of miRNAs required to exert an effect on gene expression (Brown et al., 2007). The expected unfavorable stoichiometry between exogenous small RNAs and mammalian mRNA targets will therefore further restrict gene silencing effects of dietary siRNAs in humans and livestock. In this context it also has to be mentioned that, in contrast to plants, fungi and nematodes, mammalian genomes do not possess an RNA-dependent polymerase (RdRP) homologue (Stein et al., 2003; Maida and Masutomi, 2011), implying that there is no amplification of ingested siRNAs and that each exogenous siRNA effector molecule would have to be delivered with the diet. Nevertheless, there is still some controversy about the bioavailability of relevant amounts of functional exogenous, plant-derived miRNAs in mammalian plasma and tissues and their possible effects on endogenous gene expression (Zhang et al., 2012b; Dickinson et al., 2013; Witwer et al., 2013; Pacès et al., 2017) (see sections 13.3.2 and 13.3.3, below).

If siRNAs from ingested food or feed stay intact after entering the digestive tract of humans and livestock, they may have effects on gut microbiota. Although RNA taken up by microorganisms is generally degraded and used for bacterial nutrition, there is some evidence that faecal miRNAs derived from mammalian gut epithelial cells penetrate gut bacteria and co-localize with bacterial nucleic acids (Liu et al., 2016). These authors showed that some of these miRNAs can regulate bacterial gene expression and thereby affect growth of certain bacterial species. Effects on gene expression in microorganisms encompassed decreases, as well as enhancements of transcripts, and were obviously distinct from RNA interference in eukaryotic organisms. Prokaryotes do not have an intrinsic RNAi machinery, but they produce non-coding sRNAs of around 100 nt that can up- or down-regulate mRNA stability and translation by base pairing to target mRNAs (Mayoral et al., 2014; Wagner and Romby, 2015). Although stem-loop structures similar to eukaryotic precursor miRNAs have been detected for sRNAs from Wolbachia-infected insect cells and although sRNAs from these bacteria were shown to regulate expression of Wolbachia genes as well as expression of host genes (Mayoral et al., 2014), so far there is no evidence that plant-derived dietary miRNAs have an effect on the gut microbiome.

13.2.3 Bioinformatics tools for prediction of off-target sequences of interfering RNA

Bioinformatics tools are available that may help identify potential ‘off-target’ binding sites in the transcriptome of the recipient plant. However, these have not been specifically developed for the purpose of safety assessment of GM crops. Algorithms searching for off-target effects as part of the optimization of design of siRNA/miRNA are offered as a single tool or, frequently, as part of a package. These include both accessible online web applications and open source, stand-alone software. Several of these tools predict which genes’ mRNA transcripts will be targeted by sRNAs. The sequences of the latter can be entered by the user as such or as part of a larger cDNA sequence, often in FASTA format or with reference to a database accession. Such predictions assist in the selection and design of artificial siRNA or miRNA molecules that effectively bind a target with low off-target effects binding to mRNA transcripts of other genes (Lukasik and Zielenkiewicz, 2019). This also applies to the retrospective identification of targets of small RNAs that have been added to cells in massive functional screening experiments, i.e. ‘miRNA screening’, and have shown an effect (Lemons et al., 2013). The predicted targets can then be compared and confirmed with parallel data on downregulated genes from, for example, transcriptomics. Target-identifying tools can also be used for annotation, namely for genes encoding sRNA precursors in genomics data, or for sRNAs that have been identified in transcriptomics studies. Another common purpose includes, amongst others, investigation of isoforms (e.g. single-nucleotide polymorphisms (SNPs)) in naturally occurring sRNAs (Lukasik and Zielenkiewicz, 2019). A large number of such applications have been brought together in portals such as Tools4Mirs (https://tools4mirs.org/, accessed 30 March 2020), which harbours 170 tools, including 59 software items and ten websites that can be used for target prediction. The user could, for example, use multiple tools
for target prediction to reach a ‘consensus’ outcome on the likeliest targets.

A basic approach to identify possible ‘off-target’ genes for the siRNA/miRNA would be to search for sequence homologies between the cDNA sequence of interest and its counterparts from RNA transcriptome databases. Using BLASTn, for instance, the query sequence could be aligned with sequences from NCBI’s RefSeq collections of mRNA transcripts from various organisms. The latter could contain data for the recipient plant species that has been genetically modified, or for humans, animals and other species representing environmental non-target organisms. The alignments in the BLAST outputs should then be judged for compliance with certain criteria that are known to affect the in vivo alignment and binding of mi/siRNA to the target mRNA within the RISC complex. For example, in animals a perfect match of the seed sequence of 6–8 bp at the miRNA molecule’s 5’ end is required. Some ‘wobbly’ mismatches are tolerated in this seed sequence but they decrease efficacy. Mismatches are also tolerated to a limited extent in the guide part of the miRNA molecule. These reportedly prevent degradation by Slicer but still block translation of the bound mRNA. Other factors include conserved residues and guanine-cytosine (GC) contents, amongst others, based on experience gained with miRNAs for certain species. Such factors affecting the efficacy of target binding and inhibition of gene expression are commonly automated as part of the specialized algorithms. These other factors are also relevant for the purpose of risk assessment, given that the seed sequence alone is relatively small (starting at 6 nt), which would easily render hundreds of genomic sequences that could be recognized but still remain without any major impact on gene expression.

Mainstream target prediction tool websites that specifically also focus on RNA targeting in plants include, for example, psRNATarget (Dai and Zhao, 2011) and TAPIR (Bonnet et al., 2010). On the psRNATarget website (http://plantgrn.noble.org/psRNATarget/analysis#, accessed 30 March 2020) users can enter the sequences of either the sRNA or target RNA and select various variables, such as the seed region (i.e. by default nt 2–13 being recognized as critical in plants), the penalty for mismatches and opening gaps in the seed and other regions, and whether or not bulges or caps should be allowed in the structure of the miRNA–mRNA complex. The outputs thus list the various sRNAs or target genes, show the alignments with the matching parts of the sRNA and mRNA molecules compliant with the criteria and indicate whether the complex probably will be cleaved or inhibit translation. The algorithm underlying psRNATarget not only takes into account Crick–Watson base pairing using scoring matrices for matches, mismatches and gaps, but also features optionally an energy calculation for the unwinding of the adjacent RNA parts upon binding, which correlates with accessibility (Dai and Zhao, 2011).

Similarly, the TAPIR website offers a comparable search feature, yet both the miRNA and target sequences have to be entered at the same time. Variables that can be modified by the user include score and free energy, i.e. the binding energy of the mismatched sequences as compared with that of a perfectly matching pair.

Another tool that more specifically focuses on potential off-targets of small RNA in a wide array of organisms is pssRNAit (http://plantgrn.noble.org/pssRNAit/, accessed 30 March 2020). WMD3, a program for designing artificial miRNAs, also has a feature to BLAST a query sequence against DNA data sets from a large collection of plants (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Blast;project=stdwmd, accessed 30 March 2020).

13.2.4 Possible non-specific effects of dsRNA and siRNA in mammals

Molecules of dsRNA as well as the derived siRNAs, which constitute the molecular effectors of gene silencing in RNAi plants, occur naturally in food or feed and, therefore, constitute a ubiquitous component of the diet for both humans and animals. Systemic exposure following consumption of plants containing dsRNA or siRNA is limited in higher organisms by extensive denaturation and degradation of ingested RNA and by biological barriers preventing their cellular uptake. Inflammatory responses have been observed following systemic administration of siRNA in animal models (Judge and MacLachlan, 2008; Robbins et al., 2009). Such responses of the innate immune system are mediated by receptors that
recognize nucleic acids such as Toll-like receptors (TLRs) or the RNA-binding protein kinase PKR. However, the observed inflammatory response might also be due to the delivery system or to chemical modifications introduced into the nucleic acid backbone to increase stability, rather than being elicited by the presence of native siRNA molecules (Heidel et al., 2004; Ma et al., 2005; Petrick et al., 2013). In any case, inflammatory reactions upon oral exposure to siRNA or other nucleic acids are not expected.

13.2.5 Comparison of data requirements for safety assessment of food and feed from RNAi plants and from plants expressing recombinant proteins

A universally accepted strategy is in place for the evaluation of the safety of GM crops and their products used as food or feed. This general approach, described in relevant documents issued by international organizations (Codex, 2003; ILSI, 2004; EFSA GMO Panel, 2011), analyses both the safety of intended effects introduced by the genetic modification and possible unintended effects resulting inadvertently from the new trait or from the genetic transformation process. A common cornerstone of all GM plant safety evaluation guidelines is the comparative assessment. This entails an extensive analysis comparing the GM crop with a genetically close, conventional counterpart with a history of safe use. Common, recurrent features of this analysis include the following.

- A molecular characterization of the inserted DNA including sequences introduced, their copy number, orientation, possible rearrangements, etc., as well as their expression (e.g. mRNA or NEPs) in different plant tissues and in different developmental stages, and stability of inheritance. For RNAi-modified crops, particularly relevant is the expression of the RNA encoded by the inserted genes, as well as the mRNA of the genes targeted for silencing, whilst no new proteins are expected to be expressed. Horizontal gene transfer, which also has to be assessed, may only be relevant if genes are introduced that convey a selective advantage to the recipient.
- An extensive comparative compositional analysis of the GM crop versus a conventional counterpart, grown in various locations representative of the conditions under which the crop is intended to be produced commercially. This quantitative analysis entails a wide range of macronutrients, micronutrients (e.g. vitamins, minerals), antinutrients and toxins, which are characteristic and relevant for the crop species and which are listed in consensus documents developed under the international frame of the Organization for Economic Co-operation and Development (for example, for maize composition see OECD, 2002). Despite considerable natural variability in nutrient/antinutrient content, the compositional analysis provides an indicator of possible unintended effects resulting from the genetic modification and, therefore, is also applicable to the risk assessment of RNAi plants. The outcome of this analysis should reflect the intended changes if the introduced trait is intended to affect plant composition, for example its fatty acid content (see section 13.4.1, below, for a specific case study).
- Phenotypic and agronomic characteristics of the crop are analysed in a similar way and this may reveal possible unintended changes caused by the genetic modification, as well as providing important data for the environmental risk assessment. The determination of agronomic/phenotypic characteristics may also help to confirm specific traits that are intended to improve plant growth, grain yield or protection from biotic and abiotic stresses (see section 13.4.2, below). Thus, this part of the comparative evaluation is also pertinent to the risk assessment of RNAi plants.

Based on these comparative tests, it can be decided if there is sufficient information to conclude the risk assessment or to proceed with assessment of additional data. Usually the items that are also addressed during risk assessment of GM crops include: (1) potential toxicity and allergenicity; and (ii) nutritional impact (Codex, 2003; EFSA GMO Panel, 2011; EC, 2013).
For **potential toxicity and allergenicity** of NEPs and other compounds introduced or whose levels have been altered by the genetic modification, common items of the ‘weight of evidence’ approach include a bioinformatics-based comparison of the amino acid sequence of NEPs with those of known toxic and allergenic proteins. This is because all known food allergens are proteins, which raises the question as to whether any NEP could indeed become an allergen (or a toxin). The query sequences also include those that are hypothetically formed from open reading frames (ORFs) present in the insert and crossing its borders with the host’s genomic DNA. This latter comparison would also still be applicable for hypothetical peptides encoded by the ORFs within the inserted construct encoding silencing RNA.

Other commonly assessed factors that are specific to proteins and not to non-coding RNA include:

- Information on the gene donor: is there a known history of toxicity or allergenicity, i.e. the propensity to cause toxic or allergic reactions? Is it known if these properties are linked with the product of the gene used?
- Resistance of NEPs to *in vitro* degradation by the digestive proteolytic enzyme pepsin, which indicates a greater likelihood of *in vivo* passage of the protein through the gastrointestinal tract, and possibility to cause toxicity or interact with the immune system in the consumer or animal.
- *In vivo* toxicity trials in laboratory animals with the NEPs or any other compound altered or introduced by the genetic modification and administered to the animals in purified form.

Toxicity testing with whole GM food and feed products in experimental animals should only be performed as a last resort given the inherent insensitivities and other practical and ethical limitations, and with a clear hypothesis of potential adverse effects. Nevertheless, a unique feature of the European legislation is the mandatory requirement for 90-day feeding studies, which need to be provided even in the absence of any hazard or risk hypothesis (Devos *et al.*, 2016). For food allergenicity testing, there are no validated animal models yet.

The **nutritional impact** of any intended and unintended changes in the nutrient profile of the host crop caused by the genetic modification may be particularly relevant if there are substantive changes in nutrient levels (e.g., beyond background variability) and if the particular crop is known to be a relevant source of the specific nutrient. In such cases, it should be estimated to what extent these changes will affect the intake of the particular nutrient by consumers and domestic animals. To estimate the intake, the quantitative data on the altered nutrient levels from the compositional analyses need to be combined with data on the intake of the crop and derived product, such as from the EFSA Food Consumption Database. In some rare cases, it may be necessary to extend the data with new studies in representative animal models.

In summary, the paradigm of comparative assessment is well suited for the safety evaluation of RNAi plants. Unlike the vast majority of GM crops currently on the market, which have been designed to express heterologous proteins (so-called NEPs) that confer a desired phenotype like herbicide tolerance, pest protection or increased yield, RNAi-mediated traits involve the expression of non-coding RNA without NEP biosynthesis. Therefore, NEP-related aspects of the safety assessment process are not applicable. This includes the search for homology of NEPs with known protein toxins and allergens, their digestibility (as allergenic or toxic proteins may be refractory to degradation by digestive enzymes) and, in the absence of a proven history of safe use, rodent studies to test the potential oral toxicity of NEPs. However, in order to predict unintended effects from off-target gene silencing, EU Regulation No. 503/2013 requests that for the authorization of RNAi plants in the EU an *in silico* bioinformatics analysis is carried out to identify potential off-target genes in the plant genome (EC, 2013).

Any additional studies considering intended or unintended effects of sRNA should be considered as needed on a case-by-case basis. For example, the use of RNAi as an insecticidal tool raises the question of whether sRNAs that are lethal to insects could also harm humans and farm or companion animals. There is a detailed discussion of the risk assessment of an RNAi crop conferring insecticidal properties in section 13.4.2, below.
13.3 Exposure Assessment

13.3.1 Expression level of dsRNA and siRNAs in plants

The first determinant for exposure of humans and farm animals to siRNAs from consumption of plant-derived food or feed is the level of dsRNA expression in the respective GM RNAi crop which provides the basis for a maximal estimate for exposure, assuming a worst-case scenario with no barriers to bioavailability. The expression level of transgenes in different plant tissues is dependent on the regulatory sequences, but may also be affected by environmental changes and the age of the plant (Meyer et al., 1992; van der Hoeven et al., 1994). Transgenes including dsRNA constructs introduced into GM plants are often under control of a strong constitutive promoter like the cauliflower mosaic virus 35S promoter (P35S), accounting for a high constitutive expression in all plant tissues. Nuclear expressed dsRNA, however, is to a large part processed in plant cells into siRNAs by Dicer-like (DCL) proteins from the plant RNAi machinery (Chau and Lee, 2007; Frizzi and Huang, 2010; Zhang et al., 2015). Thus, when quantifying specific RNA levels relevant for dietary intake, both dsRNA and siRNAs have to be taken into account. Chau and Lee (2007) found that in transgenic tobacco plants expressing a hairpin construct under P35S control, the hairpin-specific siRNA level was about 50 ng/g leaf tissue. Petrick et al. (2013) calculated a daily dietary exposure to transgene-derived siRNA from a putative RNAi soybean product of 45 µg/kg for adults, assuming a transgene-derived siRNA rate of 1.5% of the total RNA and a maximum amount of total RNA in grain tissue of 986.6 µg RNA/g. However, this transgene-specific siRNA percentage and the resulting exposure estimate seems to be too high. Ivashuta et al. (2009) reported endogenous small RNAs (21–24 nt) as a whole to be present at levels of maximally 1.61 µg/g soybean grain, which corresponds to clearly less than 1.5% of total plant RNA, with similar levels found in conventional maize and rice grain. Using a validated quantification assay based on the QuantiGene Plex 2.0 (Affymetrix) technology (Armstrong et al., 2013), Bachman et al. (2016) detected DvSnf7 dsRNA in transgenic insecticidal maize MON 87411 at levels up to 0.17 ng/g dry weight in grain, while the mean level in leaf was 14.4 ng/g fresh weight. There was no information, though, on the proportions of long dsRNA originating from the transcript versus small siRNAs resulting from DCL processing.

For RNAi plants conferring resistance against certain insects via host-induced gene silencing (HIGS), for example MON 87411 expressing dsRNA targeting an essential insect transcript, it has to be considered that dsRNAs require a certain minimal length in order to be taken up efficiently and become biologically active in insects (Bolognesi et al., 2012). This implies that processing of dsRNA into siRNAs, which has been shown to occur readily for nuclear expressed dsRNAs, needs to be kept at a minimum. This is especially important for certain insect groups like Lepidoptera, which are less susceptible to RNAi (Tereniuis et al., 2011) and therefore require delivery of a very large amount of dsRNA to be efficiently targeted. One way to prevent the rapid turnover of dsRNAs in plants is the construction of transplastomic plants where dsRNA accumulates in the chloroplasts, thereby being protected from Dicer (Zhang et al., 2017). In contrast to nuclear transformants, no detectable levels of siRNAs were found in transplastomic Nicotiana benthamiana plants, but only large amounts of the unspliced hairpin RNA (Bally et al., 2016). Similar results were obtained by Zhang et al. (2015) for tobacco and potato lines expressing insect gene-specific dsRNAs from the plastid genome. Moreover, differences between plant tissues were reported. While in transplastomic potatoes specific dsRNA transcripts were below the detection limit in tubers, levels of insect gene-specific dsRNAs up to 0.4 % of the total cellular RNA accumulated in leaves (Zhang et al., 2015). Assuming a total RNA amount of around 500 µg/g leaf tissue, this corresponds to 2 µg of dsRNA, which is about 150-fold higher compared with the amount of DvSnf7 dsRNA reported by Bachman et al. (2016). Transplastomic crop plants are thus distinct from nuclear transformants due to the restriction of substantial dsRNA production.
to chloroplast-containing photosynthetic tissues and due to deviant amounts of dsRNA and siRNAs.

13.3.2 Oral exposure from dietary intake

All foods and feeds contain naturally occurring coding and non-coding RNA, but animal tissues generally have a higher RNA concentration than plants (Jonas et al., 2001). The overall content of RNA in plant-derived food and feed is in the order of 1 mg/g tissue, but up to 95% by weight of this total amount consists of highly abundant transfer RNA (tRNA), ribosomal RNA (rRNA) and mRNA. As outlined above, siRNA and miRNA make up less than 5% of the RNA content of plant tissues. Although present at such minor levels, siRNA and miRNA sequences found in plant tissues (for example, cereal or soybean seeds) display a high similarity or even identity to genomic regions of humans and livestock animals (Lassek and Montag, 1990; Heisel et al., 2008; Ivashuta et al., 2009). This observation opens the possibility that dietary sRNA, of natural occurrence or inserted into RNAi plants, may elicit biological responses in humans or animals. It should be noted, however, that even with the usually intended overexpression of the transgene-derived sRNA in RNAi plants, this additional sRNA represents only a very minute fraction of the total dietary RNA occurring in food and feed.

Many lines of evidence demonstrate that the mammalian digestive tract provides an extremely effective barrier to the local or systemic uptake of exogenous RNA molecules, which are inherently unstable in their natural form. First, the degradation of ingested RNA begins in saliva, which is a rich source of ribonucleases (Bardoni and Shugar, 1980; Park et al., 2006). Secondly, the harsh milieu in the stomach with low pH promotes further RNA degradation as well as depurination (Loretz et al., 2006; O’Neill et al., 2011). The dominant gastric enzyme pepsin, which was thought to be protein-specific, has been shown to digest effectively nucleic acids including RNA (Liu et al., 2015). Thirdly, pancreatic nucleases, phosphodiesterases and nucleoside phosphorylases, secreted into the intestinal lumen, degrade ingested RNA into oligonucleotides, nucleotides and free bases (Jain, 2008; O’Neill et al., 2011). Also, the intestinal epithelium, like any other cellular membrane, presents a physical barrier to hydrophilic compounds like nucleic acids (Khatsenko et al., 2000). Any RNA that may bypass cellular membranes by transport into intestinal cells through endocytosis will be targeted to endosomal vesicles, sequestered into lysosomes and thereby degraded by lysosomal nucleases (Gilmore et al., 2004). Thus, the systemic absorption of orally ingested RNA is negligible. For a 20mer DNA oligonucleotide, constructed with stabilizing phosphorothionate linkages to prevent degradation in the gastrointestinal tract, oral bioavailability in rats was at best 0.3% after gavage administration (Nicklin et al., 1998). Kendal D. Hirschi and colleagues reported on an unusual sRNA from herbs, flowers and vegetables with a comparably high stability in gastrointestinal fluids (Yang et al., 2016, 2018). This atypical sRNA of 20 nt, denoted as MIR2911 although it is a product of 26S rRNA breakdown, arises abundantly from ribosome degradation in macerated plant tissues. When tested in cabbage extracts, around 0.1% of input MIR2911 survived a 60 min in vitro incubation in gastrointestinal fluid, whereas for comparison MIR168 (see below) was digested under identical conditions around 1000 times more efficiently. MIR2911 was reported to occur at femtomolar concentrations in the plasma of rodents fed vegetable-enriched diets and was found in human plasma (Yang et al., 2015a, Yang et al., 2015b), where it appears to be stabilized against degradation and elimination by some host factors (Yang et al., 2016, 2017, 2018). However, a follow-up report considered that MIR2911 was probably misidentified in human plasma as a plant-derived sRNA, but instead matches human genome sequences (Witwer, 2018).

Other authors found only trace levels at best of exogenous dietary miRNA molecules in the plasma of mice or humans (Chen et al., 2013; Snow et al., 2013; Dickinson et al., 2013; Witwer et al., 2013; Huang et al., 2018), confirming the generally ineffective uptake and transfer of miRNA from food or feed to recipient organisms. A major problem is the use of exceptionally sensitive methods allowing for the detection of few nucleic acid molecules, which gives rise to false positive results due to non-specific amplification or sample
contamination (Zhang et al., 2012a; Tosar et al., 2014). A survey of publicly available sequencing data sets found foreign miRNA in human body fluids and tissues, although at low abundance. Intriguingly, there is no enrichment of foreign RNA in human tissues that are most directly exposed to dietary intake, like liver, and there is no depletion of foreign sequences in compartments that are comparably well separated from the bloodstream, like for example the brain. The majority of foreign miRNA detected in this survey originates from rodents, which are common laboratory animals but do not contribute to human nutrition. It was, therefore, concluded that the apparent detection of foreign sRNA sequences in mammalian/human body fluids or tissue results from technical artifacts or misidentification (Kang et al., 2017; Witwer, 2018).

The detection of plant-derived or other dietary sRNA entering the bloodstream of animals or humans (see for example Zhang et al., 2012b; Yuan et al., 2016) is prone to artifacts and fails independent experimental reproduction, and the general consensus is that only a very small fraction, if any, of ingested sRNA will be absorbed into the circulation. Additionally, minor traces of sRNA that might be absorbed into the blood are not spared from degradation and excretion. For siRNA molecules, a rapid breakdown in human plasma has been described with nearly 75% degradation within 2 min of incubation (Layzer et al., 2004). Samples of siRNA injected intravenously into mice exhibited a short half-life in the range of only a few minutes and were subject to rapid hepatic and renal clearance (Vaishnaw et al., 2010; Christensen et al., 2013). It can be concluded from the above findings that plant sRNA molecules never reach sufficiently high concentrations and stability to exert biologically relevant effects in mammals and humans. In the unlikely event that traces of ingested RNA molecules are absorbed from the gastrointestinal tract, not degraded within the cardiovascular system and not readily eliminated through the liver or kidneys, these remaining RNA molecules, in the absence of any delivery vehicle and amplification mechanism, would not be able to cross lipid membranes, escape lysosomal degradation and, hence, reach the cytoplasm of cells (Sioud, 2005; Manjunath and Dykxhoorn, 2010). Taken together, the instability in biological fluids and matrices, in combination with biological barriers, reduces the likelihood that ingested sRNA will display local or systemic biological activities in mammals and there is currently no reason to believe that this conclusion may not also apply to other vertebrates, including birds and fish (EFSA GMO Panel, 2018). There is also no basis for the hypothesis that engineered sRNA in GM feed and foods may develop different nutritional properties than the background of natural sRNAs already present in all GM and conventional crop.

### 13.3.3 Likelihood of transfer of dsRNA or siRNA from plant to mammalian cells

Mammalian cells do not efficiently take up dsRNA or sRNA. The genetic basis for RNA uptake mechanisms has been investigated in detail in the nematode *Caenorhabditis elegans*. RNAi-mediated gene silencing is induced by soaking worms in siRNA-containing solutions (Tabara et al., 1998; Maeda et al., 2001) or by feeding them with bacteria that express dsRNA (Timmons et al., 2001; Newmark et al., 2003). A straightforward RNA uptake in *C. elegans* is mediated by transmembrane protein channels like SID-1, SID-2 and SID-5 (SID, systemic RNA interference-deficient) that promote RNA endocytosis, transfer of RNA into the cytoplasm of cells and RNA spread from cell to cell (Winston et al., 2002, 2007; Feinberg and Hunter, 2003; Jose and Hunter, 2007; McEwan et al., 2012). Two mammalian proteins, referred to as SIDT1 and SIDT2 (SID transmembrane family member 1 and 2), have been annotated as homologues of the RNA transporter SID-1. However, these mammalian proteins have more similarity with the *C. elegans* cholesterol uptake protein CHUP-1 than with SID-1. Also, SIDT1 and SIDT2 contain putative cholesterol-binding motifs known as cholesterol recognition/interaction amino acid consensus (CRAC) domains. Accordingly, the expression of SIDT1 and SIDT2 in human cells in culture demonstrated that they function as transmembrane cholesterol transporters, but are unable to mediate the intracellular uptake of dsRNA or miRNA (Méndez-Acevedo et al., 2017). Another aspect of RNAi observed in *C. elegans* is the amplification of sRNA, a mechanism that is restricted to plants, fungi, some nematodes and some other invertebrates.
Amplification takes place when sRNA molecules hybridize to target RNA sequences and prime these targets to be copied by RNA-dependent RNA polymerase (RdRP) (Hunter et al., 2006; Mittelbrunn and Sánchez-Madrid, 2012). Such an amplification of sRNA is absent in insects and vertebrates, including mammals and humans (Tomari and Zamore, 2005; Miller et al., 2012).

Nonetheless, reports from the Nanjing University in China suggested that natural plant miRNA, once ingested by animals or humans, may exert local activities in the intestinal mucosa and even systemic effects. The authors switched the regular feed of mice to a diet consisting entirely of unprocessed rice and, already 3–6 h later, detected several rice miRNAs at femtomolar concentrations in the bloodstream and liver (Zhang et al., 2012b). One of these detected miRNAs (miR168a) displays sequence identity with a region of the mouse gene coding for LDLRAP1 (low-density lipoprotein receptor adapter protein 1). Although the respective mRNA was not affected following the consumption of rice, the authors reported that the level of LDLRAP1 protein was lower in the liver of mice fed rice than in controls receiving a standard rodent diet. The authors also reported elevated LDL-associated cholesterol levels in plasma and attributed this effect to the suppressed LDLRAP1 expression resulting from miR168a uptake. This finding is contradicted by a 90-day feeding study as well as by a three-generation study, both with a 70% inclusion of rice into the diet of rats. In these studies, no LDL changes were detected relative to control groups (Zhou et al., 2011, 2012). Another study, published by Dickinson et al. (2013), attempted to replicate the findings of Zhang and colleagues. After feeding mice with rice-containing diets at an inclusion rate of up to 75%, these authors detected only trace levels, if any, of plant-derived miRNA molecules in plasma. Zhang et al. (2012a) also described the presence of miR168a in the serum of Chinese persons with high dietary intake of rice. However, traces of plant-derived siRNA or miRNA taken up into the cells of the gastrointestinal epithelium, or systemically absorbed, would not be sufficient in terms of their concentration to trigger the gene silencing machinery or exert any other biologically relevant effect. In summary, humans and farm or companion animals do not have the mechanisms to take up and amplify dietary RNA in a way that their genes would be subjected to foreign sRNA-mediated regulation. Given the history of safe consumption of nucleic acids including RNA, oral toxicity studies of dsRNA and derived sRNA are currently not warranted (EFSA GMO Panel, 2018).

### 13.4 RNAi-specific Risk Assessment

In view of the above considerations, there is no reason to expect that GM plants tested in depth by the usual comparative and, if necessary, nutritional assessments are any less safe than conventional comparators just because a particular trait is generated by the RNAi pathway. This conclusion is supported by two specific examples outlined below.

#### 13.4.1 Case study of an RNAi crop with altered metabolite composition

RNAi-based GM crops that have previously been filed for regulatory approval, particularly in North America but also elsewhere, include papaya, potato and cucurbits with transgenes aimed at silencing genes of invading viruses, and maize with resistance against an infesting insect (corn rootworm). In addition to these crops, GM soybean and potato that had been modified with silencing constructs targeting endogenous genes involved in biosynthesis of fatty acids and starch as well as apple and tomato for the suppression of plant genes involved in enzymatic browning (polyphenol oxidase) and ripening (polygalacturonidase), respectively, have been submitted for approval. A case in point for such compositionally altered crops is high-oleic soybean, which will be explored in further detail in this section. Whilst the risk assessment of this crop included the regular, recurrent items summarized in section 13.2.5 above, we will discuss here some features that were specific to this type of compositionally altered RNAi crop.

The modification in soybean 305423 targeted the biosynthesis of PUFAs. PUFAs biosynthesis includes several subsequent steps of enzymatic dehydrogenation, which introduces double bonds in the carbon chain of the fatty acid, hence the ‘unsaturation’. Oleic acid
(C18:1), for example, is the mono-unsaturated form of stearic acid (C18:0) with a backbone chain of 18 carbon atoms length, and a double bond between the 9th and 10th carbon atoms (C9-C10). It is a substrate for the fatty acid dehydrogenase enzyme FAD2 (oleoyl phosphatidylcholine dehydrogenase). Its FAD2-2 and FAD2-3 isomers are constitutively expressed throughout the plant, whilst FAD2-1 is strongly expressed during embryogenesis in seeds. FAD2 converts oleic acid to the PUFAs linoleic acid (C18:2), by introducing a second double bond between the 12th and 13th carbon atoms. Linoleic acid, in turn, can be further enzymatically transformed to the PUFAs linolenic acid (C18:3) with three double bonds.

For industrial purposes, such as for frying and bakery, it is desirable to increase the oxidative stability of PUFAs-rich vegetable oils. This can be done by decreasing the content of PUFAs, which are particularly prone to oxidation. This way, the oils will tend to become rancid less quickly. Whilst catalytic hydrogenation was historically used for this purpose, there are potential consumer health issues with the trans-fatty acids that may be formed during this process. As an alternative, the use of high-oleic acid mutants of oilseed crops with decreased levels of PUFAs would help to avoid these issues.

Around the globe, for example, there is widespread cultivation of high-oleic sunflower varieties, which originate from a mutant created through chemical mutagenesis, in which the FAD2-1 gene has been partially duplicated, causing gene silencing (Schuppert et al., 2006). Also, transgenesis exploiting RNA interference has been applied to introduce constructs silencing expression of FAD2-1 in experimental and commercial oilseed crop lines. Examples include soybean, cotton, Indian mustard, carinata, flax and camelina (Kinney and Knowlton, 1998; Chapman et al., 2001; Sivaraman et al., 2004; Du et al., 2018). A more recent, pre-commercial example is super high-oleic safflower, which has been modified with transgenes encoding hPrNAs that target the FAD2-2 and FATB genes (Wood et al., 2018). Genome editing using TALENs or CRISPR-Cas9 has also been applied to achieve similar results, introducing mutations in FAD2 genes to create high-oleic variants of oilseed rape, rice and soybean, for example (Haun et al., 2014; Abe et al., 2018; Okuzaki et al., 2018; Al Amin et al., 2019). A genome-edited soybean line with deletions created with TALENs in the FAD2-1 gene was recently introduced into the US market, and its oil is offered to the food industry under the trade name ‘Calyno®’ for frying and dressing, and use as a sauce (FDA, 2019).

As an example of a risk assessment of high-oleic soybean, transgenic GM soybean line 305423 (tradename ‘Plenish®’) assessed by the EFSA GMO Panel contained two types of modifications, namely: (i) a gene-silencing construct targeting the FAD2-1 gene imparting the high-oleic phenotype; and (ii) the ALS gene encoding a mutant acetolactate synthase (ALS) gene mediating resistance to ALS-inhibiting herbicide active substances.

With regard to safety, soybean 305423 had to be assessed as a GM crop, in line with the internationally harmonized principles of comparative safety assessment. Soybean 305423 had been transformed with a DNA construct containing a partial sequence of the soybean fad2-I gene aimed at silencing the expression of the host’s endogenous counterpart. This gene was under the control of the promoter and terminator sequences from the Kunitz trypsin inhibitor gene 3 (Kti3) coding for the antinutrient and allergenic trypsin inhibitor protein, thus allowing for seed-specific expression of the inserted genes. The inserted DNA also carried the gm-hra gene encoding an ALS enzyme conferring herbicide resistance (EFSA GMO Panel, 2013).

Molecular characterization of soybean 305423 showed that DNA had been inserted at four distinct sites, with a relatively complex pattern comprising, for example, seven copies of the fad2-I fragment and five of the Kti3 terminator. Expression data (Northern blotting) indeed showed inhibited expression of fad2-I in seeds of soybean 305423 as well as of Kti3 as a corollary effect. The latter can be attributed to silencing caused by the presence of Kti3-related sequences (promoter, terminator) in the introduced DNA. Bioinformatics-supported comparisons of the amino acid sequences hypothetically formed from the ORFs of the inserts and flanks with known toxic and allergenic proteins did not reveal any relevant similarities. An experiment testing the stability of inheritance indicated recombination between the Kti3 promoter elements at one of the integration sites in a single
progeny plant, accounting for loss of the \textit{gm-hra} gene cassette, but this was not considered as relevant for safety assessment (EFSA GMO Panel, 2013).

Compositional analysis of seeds from experimental field sites showed that, whilst non-transgenic soybeans contained 19\% oleic acid, 55\% linoleic acid and 8\% linolenic acid, these figures had markedly changed in 305423 soybeans to 73\% (+54\%), 4\% (−5\%), and 4\% (−4\%), respectively. This confirmed that the fatty acid composition had indeed changed from a preponderance of the PUFA linoleic and linolenic acids to that of the MUFA oleic acid, as intended (EFSA GMO Panel, 2013).

The potential nutritional impact of these changes was assessed, taking into account that consumers need to attain adequate intakes of linoleic and linolenic acids. It was assumed that the new oil from soybean 305423 would totally replace conventional vegetable oils in targeted foods. Based on consumption data, the intakes of the various types of fatty acid by different consumer groups, ranging from toddlers to elderly people, were estimated. The reductions in PUFA intakes thus obtained under these conservative scenarios did not raise health concerns, though post-market monitoring for verification of these consumption data was also recommended (EFSA GMO Panel, 2013).

In conclusion, the risk assessment of soybean 305423 as an example of a crop with silenced endogenous genes shows that various generic factors come in play. This could also be translated to other traits achieved through RNAi, such as disease resistance based on suppression of the plant host’s intrinsic vulnerability factors. For example, Northern blotting or other means of RNA expression analysis will be important to confirm the targeted suppression of the endogenous gene of interest. Moreover, it should be verified if endogenous genes bearing similarity with inserted elements (such as observed for \textit{Kti3} on soybean 305423) are also affected, though not the aim of the modification, as a corollary effect. Bioinformatics-supported comparisons of the amino acid sequences of peptides that could be hypothetically formed from ORFs with sequences of allergenic and toxic proteins will help to identify potential safety issues should the inserted DNA sequences unexpectedly be translated into peptides. Extensive comparative analysis of compositional, phenotypic and agronomic characteristics will help to identify any potential intended and unintended effects of the modification. For the changes identified, the impact on toxicity, allergenicity and nutritional value of the modified RNAi crop should be assessed.

13.4.2 The case of insecticidal RNAi maize

RNAi-mediated silencing, first observed in nematodes and plants (Dougherty \textit{et al.}, 1994; Fire \textit{et al.}, 1998; Brodersen and Voinnet, 2006; Jones-Rhoades \textit{et al.}, 2006; Vazquez, 2006), was later demonstrated also in some insects (Ghildiyal \textit{et al.}, 2008). Several reports demonstrated the proof-of-principle that it is possible to induce an RNAi-mediated suppression of essential genes by feeding parasitic nematodes (Huang \textit{et al.}, 2006; Yadav \textit{et al.}, 2006; Fairbairn \textit{et al.}, 2007) or the larvae of insect pests with GM plants engineered to express specific dsRNA precursors (Baum \textit{et al.}, 2007; Mao \textit{et al.}, 2007) or the larvae of insect pests with GM plants engineered to express specific dsRNA precursors (Baum \textit{et al.}, 2007; Mao \textit{et al.}, 2007). In maize MON 87411, this principle is employed for the management of western corn rootworm (WCR). \textit{Diabrotica virgifera virgifera}, the most important maize pest in the US ‘corn belt’. A single insert has been introduced to express a modified version of \textit{Bacillus thuringiensis} Cry3Bb1 protein, a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme and an expression cassette containing two sequences of the \textit{D. virgifera} (Dv)Snf7 gene coding for an essential vacuolar sorting protein. The reader is referred to the relevant scientific opinion of the EFSA GMO Panel for the risk assessment of the Cry3Bb1 and EPSPS proteins (EFSA GMO Panel, 2018). The Snf7 dsRNA expression cassette consists of two fragments of the coding sequence of the \textit{DvSnf7} gene in an inverted repeat configuration flanked by the e35S promoter from cauliflower mosaic virus, the heat shock protein 70 intron from \textit{Zea mays} and the 3′ untranslated sequence of the \textit{E9} gene from \textit{Pisum sativum}. The \textit{DvSnf7} inverted repeat sequence generates a 240 bp precursor with hairpin structure that is processed to generate siRNA molecules. When the WCR larvae feed on MON 87411 maize, silencing of the \textit{DvSnf7} gene leads to insect lethality, thus protecting the plant from root damage.
The mechanism of RNA uptake and systemic spread in the WCR is poorly understood but may involve SID-like (SIL) proteins and also clathrin-mediated endocytosis. An RdRP-mediated amplification is absent in the WCR (Huvenne and Smagghe, 2010; Fishilevich et al., 2016).

As outlined in section 13.2.1 above, in the EU a bioinformatics analysis is required according to Regulation (EU) No. 503/2013 to identify potential off-target genes that may be influenced in their expression by the siRNA approach. Following the recommendations of the EFSA GMO Panel for an RNAi off-target search in plants, it was found that none of the maize transcripts in the available databases showed perfect match to any of the siRNAs possibly produced. A few maize transcripts have sequences matching the siRNAs with one to four mismatches. Some of these sequences presented matches for more than one (up to five) possible siRNA. However, a scrutiny of the anticipated function of the proteins encoded by these mRNAs matching the siRNA sequences indicated that off-target effects, if they took place, would not raise safety concerns, because the possible depletion of these potential targets is not expected to affect agronomic, phenotypic, compositional and nutritional characteristics of the GM maize. This conclusion is confirmed by the comparative analysis of maize MON 87411 and non-GM comparators. A field trial for the assessment of agronomic and phenotypic characteristics did not reveal any statistically significant differences between maize MON 87411 and this conventional counterpart. Also, no changes in the composition of grains were detected, i.e. concentrations of none of the 78 tested maize constituents were significantly different in maize MON 87411 compared with its conventional counterpart and also present at levels outside the equivalence range defined by non-GM reference varieties grown in the same field trial. Of course, an off-target gene silencing may also theoretically occur in organisms exposed to the RNAi plant, for example upon food and feed consumption. As described in sections 13.3.2 and 13.3.3 above, dietary dsRNA and sRNA are, however, rapidly denatured, depurinated and degraded after ingestion, due to the particular milieu of the gastrointestinal tract and the presence of multiple digestive enzymes in humans, mammals and other vertebrates. Further biological barriers like cellular membranes or lysosomes limit the uptake of dsRNA and sRNA. Therefore, it is not expected that sRNAs with DvSnf7 sequences are able to exert any biological effects once maize MON 87411 is ingested by humans, or by farm or companion animals (EFSA GMO Panel, 2018). A 28-day oral repeated-dose study in mice with DvSnf7 dsRNA, conducted in accordance with the principles laid down in the OECD Test Guideline 407, lends further support to the above conclusion. In this study, the DvSnf7 dsRNA was administered by daily oral gavage at doses of 1, 10 and 100 mg/kg body weight. No treatment-related effects were observed in the animal body weights, food intake, clinical parameters, clinical chemistry values, haematology, gross pathology and histopathology (Petrick et al., 2016). Considering the possible Snf7 dsRNA and sRNA content of maize MON 87411, which is difficult to assess quantitatively, the authors of this toxicity study calculated that a human would need to eat 60 million kilograms of maize MON 87411 per day to reach the dose of 100 mg/kg body weight that in the mouse study remained without any effects. The lack of biological activity of ingested dsRNA or sRNA is also documented by a previous 28-day toxicity study in mice using dsRNA of 218 bp, or a pool of four 21-mer siRNA molecules, targeting a mouse vacuolar ATPase transcript. The daily dose administered by gavage was 64 mg/kg for the dsRNA and 48 mg/kg for the siRNA. This 28-day toxicity study revealed no adverse effects and, importantly, no changes of vacuolar ATPase expression in any tissue, including the gastric mucosa (Petrick et al., 2015), thus supporting the notion that no consequences are expected from the dietary uptake of dsRNA or siRNA present in food or feed. Taking into account all of the above, maize MON 87411 is considered equivalent, with respect to its food and feed safety and its nutritional profile, to non-GM maize counterparts.

13.5 Conclusion

The concept of gene silencing in GM plants based on the principles of RNAi has been exploited from the early days of commercial crop biotechnology. Applications straddle traits of both agronomic importance, such as disease and pest resistance, and of consumer and producer
benefit, such as oilseeds with altered fatty acid composition. The internationally harmonized risk assessment approach for the food safety of GM crops can also be applied well to the subcategory with RNAi-based gene-silencing traits, notwithstanding some special features, such as the lack of newly expressed proteins. Moreover, the issues of off-target effects of the silencing RNAs within the plant, as well as the hypothetical uptake by consumers after ingestion of foods derived from RNAi-based GM crops, has been at the focus of scientific discourse. The current state of knowledge indicates that cross-kingdom interactions of consumed plant sRNA with the intrinsic RNAi machinery of humans and farm animals is a highly remote possibility at best, with unlikely impacts of any potential health concern. The featured case studies both underscore the applicability of current guidelines of the EFSA GMO Panel, enshrined in Implementing Regulation No. 503/2013, and more generally, those of the international Codex Alimentarius of the FAO/WHO Food Standards Programme.

Acknowledgement

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14 Regulatory Aspects of RNAi in Plant Production

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14.1 Introduction

Technologies based on RNA interference (RNAi) may be used in plant production in different contexts. With respect to applicable regulations, a major distinction is to be made between plants producing small RNA molecules due to modifications of the genome and topically applied plant protection products (PPPs) based on double-stranded RNA (dsRNA).

The first group may be further divided into those using RNAi technology to achieve changes in the plant’s metabolism and those where plant-produced RNA molecules are intended to impact other organisms that interact with the plant.

For PPPs, relevant aspects are whether the product contains living organisms or only purified molecules. The intended use of the product is another relevant aspect with respect to regulation. It is expected that PPPs will be among the first products utilizing the RNAi mechanism in the European Union (EU).

Based on these considerations, it is clear that the main relevant regulatory frameworks are in the areas of genetically modified organisms and plant protection products.

14.2 Regulation of Modified RNAi Plants

A meaningful utilization of RNAi effects in plants is generally only possible by modifying the plant’s genome in a way that does not occur naturally by mating and/or natural recombination. Based on this premise, these plants fall within the scope of Directive (EC) 2001/18 in the EU and are therefore regulated as genetically modified organisms (GMOs) (EC, 2001). Any person intending to place such products on the market or to carry out a deliberate release into the environment of a GMO for any other purposes than placing on the market within the Community requires authorization to do so. It is important to note that Directive (EC) 2001/18 covers the authorization of the deliberate release of living organisms but does not cover any product produced from a GMO if it no longer contains living organisms. Most agricultural crops are not authorized under directive (EC) 2001/18, since food and feed products are covered by Regulation (EC) 1829/2003 (EC, 2003). For this reason, there are few genetically modified plants conceivable where developers would seek authorization under Directive (EC) 2001/18 only.

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Crops for fibre or energy production might be such cases if they cannot be used for food or feed too, but a more relevant group of products is ornamentals. There has been an application for carnations with altered flower colour due to silencing of a gene in the anthocyan pathway. The carnation with unique identifier IFD-25958-3 was authorized for use as a cut flower in 2015 (EC, 2015). Although there are a few instances where RNAi plants have been assessed directly under Directive (EC) 2001/18, Annex II of the Directive is of high importance, as it lays down the principles of the environmental risk assessment that are also followed for the assessment of applications under Regulation (EC) 1829/2003, especially if cultivation is within the scope of the application.

Under Regulation (EC) 1829/2003, authorization may be granted for: (a) genetically modified plants for food or feed uses; (b) food or feed containing or consisting of genetically modified plants; and (c) food produced from or containing ingredients produced from genetically modified plants or feed produced from such plants (EC, 2003). It should be noted that, in contrast to Directive (EC) 2001/18, products that no longer contain a living GMO are covered by Regulation (EC) 1829/2003.

Detailed rules for the implementation of Regulation (EC) 1829/2003 are laid down in Commission Regulation (EC) No. 641/2004 and Commission Implementing Regulation (EU) No 503/2013 (EC, 2004, 2013c). These regulations provide rules concerning applications for authorizations. Commission Implementing Regulation (EU) No. 503/2013 especially details procedures on the preparation and presentation of data for applications and is therefore of high relevance for applicants and risk assessors. In this implementing regulation the only direct reference to RNAi can be found within the European legislation on genetically engineered organisms. Under the section on toxicology, RNAi is covered indirectly by the mention of gene silencing as a genetic modification with potential toxicological impact. Annex I describes specifically all the information that an application shall contain. Within the section on molecular characterization, information on the expression of the insert is requested. Under point 1.2.2.3.(e), data requirements for gene silencing and RNAi approaches are specified as follows:

When justified by the nature of the insert (such as silencing approaches or where biochemical pathways have been intentionally modified), specific RNA(s) or metabolite(s) shall be analysed.

For silencing approaches by RNAi expression, potential ‘off target’ genes should be searched by in silico analysis to assess if the genetic modification could affect the expression of other genes which raise safety concerns ... Under Regulation (EC) 1829/2003, two genetically modified soybeans (MON87705 and DP305423) and one maize (MON87411) producing small RNA molecules due to modifications of the genome have been authorized for placing on the market. In these cases, all uses with the exception of cultivation have been approved. In both soybean events the composition of fatty acids and oils has been changed. The change in composition has been achieved by a silencing approach targeting genes of the fatty acid metabolism of the modified plant itself. In contrast to this internal silencing effect, the construct in maize MON87411 results in the expression of dsRNA that targets an essential gene in a different species, namely *Diabrotica virgifera*, the corn rootworm, thus conferring resistance to this coleopteran pest.

As delineated above, products containing dsRNA that are not to be used in the food or feed sector and do not contain living organisms are not covered by EU legislation on GMOs. Such products, however, may be subject to other regulations, depending on the intended activity and use. Within the area of plant production, the most relevant examples of such products will be PPPs.

### 14.3 Regulation of PPPs Utilizing RNAi Mechanisms

Double-stranded RNA might be a new class of active substances in externally applied PPPs. From the scientific literature, such products may be used to control a range of different pathogens and pests.

In general, each active substance and any product placed on the market to protect plants needs an authorization. In the EU, the legal basis for this is provided by Regulation (EC) No.
1107/2009 (EC. 2009). The regulation foresees a two-step approach. In the first step the active substance must be assessed in an EU-wide process led by the European Food Safety Authority (EFSA) and approved by the EU Commission. In the second step the PPP containing the active substance is assessed by the Member States (MS). With Regulation (EC) No. 1107/2009 a zonal approach was introduced to streamline the authorization process (EC, 2009). The EU has been divided into three zones. The northern zone comprises the Scandinavian and Baltic countries, the central zone the countries of central and Eastern Europe and the southern zone the countries contiguous to the Mediterranean Sea plus Bulgaria. In a zone, one Member State (zonal rapporteur Member State) (zRMS) assesses the risk of a PPP for its whole zone. MS of this zone are obliged to follow the conclusion of the assessment of the zRMS. However, MS can claim national specificities and decide on specific risk management options for their country.

Data requirements for assessing active substances are laid down in Regulation (EC) No. 283/2013 (EC, 2013a) and for PPP in Regulation (EC) No. 284/2013 (EC, 2013b), respectively. Furthermore, the implementing Regulation (EU) No. 546/2011 (EC, 2011a) defines uniform principles for evaluation and authorization of PPPs. The aim is to ensure a high level of protection of human and animal health and the environment in all Member States. Additionally, guidance documents produced by the Organisation for Economic Co-operation and Development (OECD), the European and Mediterranean Plant Protection Organization (EPPO) and EFSA give detailed guidance on the methodological requirements for the risk assessment active substances and PPPs.

The above-mentioned documents define the data requirements and the decision criteria in detail. In consequence, a required data set is more or less fixed. It can be expected that RNA-based PPPs will have different properties than the chemicals mostly used as active substances in PPPs until now. Therefore, adaptations of the data requirement for the risk assessment might be reasonable for different reasons. For example, non-target arthropods are exposed to an active substance by contact, but for dsRNA-based PPPs usually oral exposure is needed to cause effects. A further aspect might be that new kinds of risks have to be addressed, such as off-target effects. Additionally, the models used for the prediction of environmental fate are designed to assess specific chemicals, but are probably not applicable for assessing dsRNA-based products. Therefore, new tools might be introduced into the risk assessment of PPPs. One of these new methods might be bioinformatics supporting risk assessment.

Although the initial focus of the regulations was set on chemicals as active substances, the legislature has had other substances already in mind to protect plants. Therefore, article 77 of the Regulation (EC) No. 1107/2009 (EC, 2009) emphasizes the possibility that

... the Commission may ... adopt or amend technical and other guidance documents such as explanatory notes or guidance documents on the content of the application concerning micro-organisms, pheromones and biological products, for the implementation of this regulation. The Commission may ask the Authority to prepare or to contribute to such guidance documents.

The reason might be that other data requirements for these product classes are needed, compared with chemicals. In fact, for microorganisms, pheromones and botanicals, specific data requirements were developed and implemented (EC, 2011b, 2014a, b, 2016). However, discussions on adaptation of the data requirements are at an early stage (OECD, 2020) so that no specific guidance documents for dsRNA as active substance or dsRNA-based PPPs are available in the EU.

However, the effectiveness of dsRNA-based PPPs will, inter alia, depend on the stability of the dsRNA in the environment and transport into the cells of target organisms. Producers are likely to develop formulations containing synergists or co-formulants that stabilize dsRNA in the environment or to enhance transport into the target cells. Such targeted formulations of PPPs will also require consideration in the risk assessment.

The properties of dsRNA-based PPPs raise several additional questions related to procedural issues, which have to be clarified. For example: how much difference in the nucleotide sequences is acceptable to be considered as one active substance? Furthermore, would two dsRNAs
differing in one nucleotide be considered as two active substances?

For economic reasons dsRNA would probably be produced in microbial production systems using genetically modified microorganisms. Questions regarding the variability in the product and the purity regarding the nucleotide sequence are as yet unanswered. Additionally, it must be guaranteed that the genetically modified microorganisms are completely inactivated. Otherwise an additional authorization for placing GMOs on the market is needed. It is important to note that products which do not contain a living organism and are not to be used as food or feed, like topically applied PPPs, are not regulated under GMO regulations within the EU.

Answers to these questions are urgently required in order to develop clear criteria to characterize the active substances and PPPs based on dsRNA.

The authorities responsible for placing PPPs on the market are confronted with a new class of products. The thoughts we highlighted in this chapter are a preliminary list of open questions and it is time to discuss these regulatory and biosafety issues intensively in order to define an adequate framework and design appropriate risk assessment procedures. Active ingredients and PPPs containing dsRNA are in the pipeline and the first applications can be expected within a few years.

References


15 The Economics of RNAi-based Innovation: from the Innovation Landscape to Consumer Acceptance

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15.1 Introduction

RNA interference (RNAi) is an innovative technology of gene silencing which offers great opportunities for the development of sustainable solutions for crop protection (Palmgren et al., 2015; Borel, 2017; Limera et al., 2017; Zotti et al., 2018). The most original aspect related to the economics of RNAi is the opening of a completely new innovation scenario consisting of new formulations of RNAi-based products for topical use, which are considered to be able to meet the need to find safer and more effective strategies for pest control and combat agricultural losses (Mitter et al., 2017; Wang and Jin, 2017; Niu et al., 2018). The possibility of substituting agrochemicals with more natural molecules is seen as the major advantage of these new technologies, which provide contributions towards a more sustainable agriculture (Collinge, 2018). In this context, academic interest in the economic aspects of this new technology is growing rapidly, suggesting that this innovative set of technologies is going to reshape the state of the art of the agricultural biotechnology (agbiotech) sector under multiple aspects, including the market structure (Bonny, 2017) and, most probably, public acceptance.

15.2 Market Potential of RNAi Innovation

After decades of debate on genetically modified organisms (GMOS), one of the most controversial ‘science and society’ issues able to divide scientific community and public opinion, a new wave of techniques has replaced the previous transgenic approach to plant breeding, introducing the possibility of imitating natural genetic recombination and thus avoiding the introduction of foreign genetic material. Among them, the economic landscape of RNAi-based innovation has been analysed. Frisio and Ventura (2019) investigated the structure of the global patent landscape of RNAi agricultural applications, identifying significant differences in the role of private and public research and evidencing the specialization of some universities and the rising power of Chinese research. Results revealed that China’s pattern of innovation is able to stay at the forefront in most modern
agricultural biotechnologies, in stark contrast to the European scenario, where the regulatory landscape continues to impede the exploitation of agbiotech inventions. Mat Jalaluddin et al. (2019) provided an analysis of the global trend of RNAi-based product commercialization, using both bibliometric and patent data. They outlined that resistance against viruses, fungi and insect pests are the priorities for research activity and that the global market is rapidly moving toward huge investments in this field, with potential positive impacts on the development of RNAi technologies. These technologies could have very promising opportunities for being developed and applied in a broad range of agrifood products as well as in the formulation of innovative methods for biocontrol.

15.3 The Frontiers of Innovation: RNAi for Biocontrol

A new wave of RNA-based commercial products is ready to reach the market, with the first plant protectant product (to control rootworm) approved in the USA (EPA, 2017). Thus, the identification of the global scenario for RNAi technology innovation applied to biotic control, using patent data as indicators of innovation output, can provide some useful insights about this specific innovation scenario and its future applications (Chi-Ham et al., 2010; Frisio et al., 2010; Lundin, 2011; Egelie et al., 2016).

The analysis has been carried out by mining the Questel-Orbit patent database through specific keywords for the identification of those inventions regarding the use of RNAi technology for plant biotic resistance. For this purpose, a set of keywords related to the term ‘RNAi’ have been searched in the ‘title and abstract’ field. The search has been limited to a number of International Patent Classification (IPC) and Cooperative Patent Classification (CPC) classes associated with biopesticides (IPC code A01N and CPC code Y02A-040). Time coverage of data is limited to the past 10 years (2010–2019). The original data set contains information about worldwide innovation in agricultural RNAi-based inventions, amounting to a total of 641 patent families. Then, with the aim of extracting from the data set only those inventions specifically developed for plant protection, a text-mining analysis has been performed through double check in the patent title, abstract, claims and technical concepts, to identify those inventions referring to biotic control for agricultural application. The final data set is composed of 223 patent families, corresponding to 1224 single patents. In some cases, data elaboration has been performed making the distinction between inventions and patents. The term invention relates to the first filing of a patent application, anywhere in the world (usually in the applicant’s domestic patent office). The statistics are based on the count of single inventions that provide information on the origin of the invention itself. Conversely, the term patent also refers to the set of patents filed in several countries that are related to the same invention, thus representing the so-called patent family. This variable is more indicative of the spread of innovation and its market, as the size of patent family is considered a proxy for the value of the invention.

Time trends outlined that RNAi technology applied to plant resistance is a field of innovation that has witnessed a good development globally in recent years, with an annual average number of new inventions equivalent to 22, corresponding to 122 patent applications. Nevertheless, Fig. 15.1 shows a peak in the numbers of patent filings in 2014 and a subsequent decline starting from 2015. Since patent applications are normally published after 18 months, data can be considered complete until 2017. The data set is composed of 223 inventions, whose legal status is ‘alive’ for 96% of cases, while the only nine inventions classified as ‘dead’ have been at some stage revoked, or lapsed. The analysis of the evolution over time of patent trends based on the nationality of the assignee indicates that, on a global level, the three main countries involved in this innovation sector are China (41.7%), the USA (26%) and the European Union (EU) (20%). The European data are quite surprising, since previous studies focusing on the analysis of the more global patent landscape of RNAi technology for plant improvement (Frisio and Ventura, 2019) revealed the marginal role of European players in producing innovations in this sector. This probably means that, amongst the different applications of RNAi technology, European research and development (R&D) activity shows greater competitiveness in the implementation
of RNAi-based solutions for biotic resistance. The major contribution to the European innovation capacity derives from Germany, accounting for 26% of EU patents, principally applied by the agbiotech firms Bayer and BASF and, for public research, by the Max Planck and the Fraunhofer research institutes. The relevant role of Chinese applicants is most probably due to the massive investments in public research made by a government that considered agbiotech innovation a national priority. Nevertheless, the importance of Chinese applications dramatically decreases when considering the diffusion of inventions, represented by the number of patents filed in foreign patent systems, for which China accounts for only 8% of the total patenting activity.

The analysis of the type of assignee (Table 15.1) reveals that almost 47% of inventions are produced by public research, a value ten points greater than the private sector (35%). Moreover, nearly 18% of inventions derive from collaboration between public and private assignees. However, statistics related to the share of patents show that private players are more capable of exploiting inventions through their protection in different patent systems, as the value of the private sector’s contribution moves from 35% of inventions to 55% of patents. It can be deduced that public sector R&D is competitive in producing innovative ideas and products for the application of RNAi technology in agriculture, but misses the opportunity to implement innovations in the form of more

Table 15.1 Analysis of the type of assignee. (Source: own elaboration on Questel-Orbit data.)

<table>
<thead>
<tr>
<th>Assignee Type</th>
<th>% Share of Inventions</th>
<th>% Share of Patents</th>
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<td>Single Assignee</td>
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<td></td>
</tr>
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<tr>
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<td>Public–Public</td>
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Fig. 15.1. Time trend for plant-RNAi inventions. (Source: own elaboration on Questel-Orbit data.)
market-oriented solutions. A more detailed classification indicates that the public sector is principally composed of academic institutions, while the private sector is composed of the ‘Big Four’ agbiotech companies for 35% of the total data set, with an additional 25% represented by other biotech companies.

The top player is Dow Agrosciences (merged with Du Pont in 2017), the seed company most interested in investing in the development of this technology. Notably, this firm shares several patents with three public research institutions, showing a great level of public–private collaboration activity. Apart from the former ‘Big Six’ agbiotech companies, top assignees (Table 15.2) are small–medium firms specializing in very specific innovation sectors. For example, FuturaGene Ltd focuses on sustainable wood production, Forrest Innovation Ltd aims at providing eco-friendly solutions for mosquito vector control, RNAgri was born as a start-up specifically focused on RNAi-based products for modern agriculture. Considering the content of inventions, the innovative nature of this specific use of RNAi technology emerges from the fact that 65% of patents do not have a single plant as target (30% plant not specified, 25% multiple applications and 10% multiple major crops). The remaining patents have maize as the major target plant (14%), followed by wheat and rice.

As for the analysis of the type of plant resistance, Fig. 15.2 shows that the main trait is insect resistance (79% of inventions), which is an impressive share indicating that this technology is considered to be more effective or even more easily applicable for insect control. Fungal control is included in 6% of patent application and relates to resistance to Magnaporthe grisea, Botrytis, Verticillium and Zymoseptoria species. Considering the minor categories, virus resistance accounts for 5% of patents, while nematode resistance (principally to the Heteroderidae family) represents 4% of the applications.

Finally, with regards to the subset of insect resistance, the analysis of the target species (Fig. 15.3) reveals that 32% of inventions relate to Hemiptera. The great majority of these patents derive from China and are intended to confer

<table>
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<tr>
<th>Applicant</th>
<th>No. of inventions</th>
<th>No. of patents</th>
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<td>Dow Agrosciences llc</td>
<td>24</td>
<td>274</td>
</tr>
<tr>
<td>with Fraunhofer Institute</td>
<td>18</td>
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Fig. 15.2. Trait analysis. (Source: own elaboration on Questel-Orbit data.)

Fig. 15.3. Main targets for insect resistance.
resistance to the Aphididae family. The second type of insect resistance targets Coleoptera, almost entirely represented by the resistance to *Diabrotica* in maize. An additional 10% of patents aim at conferring resistance to both the Hemiptera and Coleoptera, while 14.2% are aimed at resistance to Lepidoptera. With regard to the type of application, the analysis showed that 24% of inventions contain a specific mention of the spray/topical application of the RNAi-based product.

### 15.4 RNAi: Stakeholder and Consumer Perceptions

Despite the fact that technological innovation plays a crucial role in enhancing the global sustainability of food chains and meeting changing consumers’ needs and choices, growing evidence suggests that consumers tend to appreciate technology applications in many fields of their everyday life but tend to reject innovation when applied to the food domain. For this reason, academic research is focusing on the identification of the drivers of consumers’ acceptance of innovative products, in order to find the most appropriate tools to mitigate consumer scepticism and resistance to these new technologies. In relation to new breeding techniques for crop improvement, public opinion has always shown one of the highest levels of rejection, principally based on the perceived unnaturalness of crop genetic modification (Mielby *et al.*, 2013; Kronberger *et al.*, 2014). Nevertheless, the literature suggests that not all the biotechnology solutions are perceived as being the same by consumers. Shew *et al.* (2017) showed that respondents valued CRISPR and GM food similarly and substantially less than conventional food, which could be detrimental for meeting future food demand. They also concluded that RNAi may be a better market alternative to more traditional biotechnologies such as GM crops expressing *Bacillus thuringiensis* (Bt) insect resistance. Topical application on plants avoids the need for genetic modification of plants, which could decrease consumer scepticism. Britton and Tonsor (2019) investigated the acceptance of a hypothetical RNAi beef product, concluding that consumers require a discount for buying the innovative product compared with conventional ones. Nevertheless, they also stated that the way RNAi technology is framed in food labels could have an influence on its acceptance. Results could support policy makers in understanding the current determinants of consumer attitudes toward RNAi technologies, in particular the role played by communication. If the information gap represents one of the main barriers to consumer acceptance, policies including information campaigns or educational programmes could be recommended to make consumers more aware and informed during food choice. This aspect has been confirmed by the outcome of a meeting with stakeholders (seed companies, farmer associations, producers) organized by iPlanta in October 2018 in Brussels. The meeting offered the opportunity to exchange knowledge on RNAi technology, biosafety and socio-economic impacts. All stakeholders attending the meeting showed a high interest towards this innovative technology, especially as a potential solution for farmers’ needs, but also expressed concerns mostly related to consumer acceptance of RNAi-based products. The meeting outlined the importance of defining common ground to discuss solutions with scientists and stakeholders and for engaging with consumers to reduce the knowledge gaps.

### 15.5 Conclusions

RNAi for plant biotic resistance is a field of innovation that has been receiving increasing interest in recent years, showing promising future applications and developments. Innovation is being produced by both public and private players. As for the latter category, some emerging small—medium firms are gaining market share by developing tailored solutions for specific problems. In this initial stage of development, insect management is the trait that is receiving the greatest attention in relation to RNAi technology, but new solutions for pest control reveal broad opportunities for the creation of new products for the agbiotech industry. A more comprehensive analysis of the economic costs and benefits for their production in the European Union will have to take into account certain aspects of the innovation supply chain.
Specifically, one of the major issues is how these new highly specific molecules will be classified in the existing EU regulation system (chemicals, bioregulators, biostimulants or biopesticides) (Taning et al., 2019). If properly communicated to consumers, and inserted in the correct legal framework, the economic perspective of RNAi technology in the EU will lead to a growing market, rich in opportunities for all the actors of the agri-food chain.

References


Abstract

Communication is an increasing prerequisite to justify academic existence and value, and for project funding of all kinds to show relevance and value, including the future of European networks like COST Actions. Academia is slowly adapting to this expectation and learning the profession of communication. Language and vocabulary are key issues in communication, and particularly to reach the many important non-scientific audiences. Therefore, this chapter starts with a description of some new plant breeding technologies relevant for communicating, in general terms, the science behind plant improvement. This is followed by selected examples of the application of these techniques to improve current and future crop varieties. Finally, key messages gathered from the European iPLANTA project for policy makers, non-specialists and specially interested citizens are communicated. This is to show a wider audience how RNAi can contribute to sustainable food solutions and food security with minimal environmental impacts.

16.1 Plant Breeding Tools to Meet Future Sustainable Food Security

Plant breeding uses a range of techniques to develop new varieties of plants that cope with current and future environmental stresses, including climate challenges, pests and diseases, in order to produce optimal feed and food products. These plant varieties typically have improved yield and quality, combined with improved use of natural resources. They are therefore more sustainable and contribute towards feeding the growing global population. Plants need to meet increasing challenges from pests and diseases, changing climates and demands to reduce inputs of water and fertilizer. However, to meet all these challenges we need to substantially increase plant breeding efforts and use a range of new scientific tools. These new tools include new breeding technologies that allow greater levels of genetic modification of plants, to introduce desired characteristics not achieved before (Madre and Agostino, 2017). These plant breeding technologies are discussed below, including the introduction of RNA interference (RNAi) into plants, which allow genes to be turned up or down.
16.1.1 Conventional plant breeding

All plant varieties grown and eaten today have been genetically changed (modified) by plant breeding. Most current varieties have been changed by what are known as conventional methods like selection, crossing selected beneficial parents, laboratory techniques like doubling the chromosomes from gamete cells like pollen to get pure-bred offspring with two identical alleles of each gene from one parent (the pollen donor), or mutating cells to increase genetic variation and get new characteristics, like pink grapefruit.

16.1.2 Genetically modified plants

Genetically modified organisms (GMOs) can be defined biologically or legally, and not necessarily be classified the same in both ‘worlds’. If the genetic change has been done by the extraction of DNA from an organism and adding it to another organism by laboratory techniques (termed transgenesis), they are defined as GMO under international United Nations (UN) protocols and hence by national and European Union (EU) legislation. When defined according to law there are different legal interpretations in different countries, sometimes embracing a range of non-transgenic technologies as well as the biological GMO. In the EU, GM regulations were interpreted by the EU Court of Justice in 2018 to include plants that have been mutated by certain gene editing techniques that change plant gene arrangements, but not those that have been mutated by other means such as irradiation (Heitz, 2020). Other countries, such as the USA, China, Canada and Australia, do not consider that gene edited mutants should be legally classified as GMOs. Discussions are ongoing to achieve international consensus on these definitions. The regulation and assessment of GMOs in the EU, particularly GM RNAi plants, are discussed in Chapters 13, 14 and 15.

16.1.3 Mutation breeding techniques

Mutations spontaneously occur in nature and many have been used to produce new plant types and varieties. Conspicuous examples are variegated ornamental plants and contorted or dwarf types. Mutation frequencies can be increased by using chemicals to disrupt cell division so that uneven numbers of genes occur in cells, or by irradiation, which damages genes. The mutated plants are examined for desirable types which are then selected, tested and propagated to produce new varieties. Examples include most dwarf and semi-dwarf wheat varieties currently grown, which put more resources into grain production than vegetative parts as in taller varieties. Genetic technologies have enabled the genetic components of most crops to be characterized so that genes can be precisely modified and edited to change their expression. For example, genes producing plant toxins and allergens can be inhibited or removed to improve plant quality using these gene editing techniques.

16.1.4 RNA interference

Since the discovery of RNAi (Baulcombe, 2019 and references therein), the mechanism has gained increasing recognition due to its important applications in medicine and feed/food production. RNAi is a central tool in functional genomics, since it allows basic studies of all genes, which is important to understand gene function and genomic interaction between genes/DNA and RNA sequences. The results and uses are from low to full downregulation of single genes or gene families, in order to change plant characteristics and improve plant varieties while protecting natural resources (Christiaens et al., 2018). RNAi can lead to improved plant protection against pests, diseases and environmental stresses. Food and feed quality can be improved to reduce losses along the food chain and provide better nutritional value for consumers. In the USA a corn (maize) variety has been commercialized with resistance to the root worm pest and a papaya has been bred with virus resistance. Oilseed crops such as soybean have had their oils modified to contain improved fatty acid profiles; and spoilage of fruits such as apple during storage has been reduced. Also, allergens are being removed from some crops, such as wheat.
16.2 Applications of RNAi for Gene Regulation and Public Acceptance of New Plant Foods

Some RNAi products have been approved for marketing globally, but in Europe most have only been approved for animal feed use. The major challenge in Europe is the extensive regulatory demands to get a GMO marketed in most countries. This means that only large companies can afford the extra costs of producing large amounts of information to demonstrate safety and stability of the products, reducing the opportunities for small and medium-sized actors. In addition, the EU has the problem that a number of countries are blocking the cultivation of approved GM crops and there is political and social opposition to the consumption of GM foods that have been assessed for safety.

Globally there are marked differences in the public and political acceptance of GMOs between continents, such as America and Europe. This is not based on scientific evidence, but on perceptions of the different consumers often driven by non-governmental organizations (NGOs) that oppose the use of GM technology in foods (though not in medicine). Many of the new products of GM plants have nutritional and quality benefits of direct value to consumers and yet public acceptance is problematic. Recent examples are the products of the companies Impossible Foods (IFs) and Beyond Meat, which illustrate how different perceptions, public acceptance and regulations affect consumer availability, and how beneficial products can affect and change consumer choices. Recently, we have seen consumers shifting towards more environmentally friendly food choices and an increasing awareness of the environmental impacts of livestock farming in particular.

The goal for IFs is to develop a green alternative to the 95% human population that prefers eating meat due to its flavour. The company found that haemoglobin causes the meatiness characteristic of beef, and that it could be mimicked by adding haeme from soybean roots. However, it would require harvesting and extraction from large amounts of roots. By contrast, transferring the haeme-producing gene to laboratory cell cultures allows efficient production of large amounts of haeme. This is added to the pure vegetable components of the impossible burger and, in tests, people can not tell the difference between a regular meat burger and an IFs burger. Consumers welcomed this new choice and it has become a number one seller at Burger King and the stores selling it. IFs has been upfront about why it depends on a GM product to add the meat flavor to its 100% veggie-burger, and this has been accepted by US consumers. In Europe the approval of this GM product might be blocked by political and activist groups unless public perceptions and approval are changed. Europe so far only has the Beyond Meat burger, since this is not using GM. However, it does not have the meaty flavor caused by haeme, so appeals mostly to vegetarians.

IFs is already hitting the global food market in the USA beyond all expectations (Gravier, 2019; Fontanazza, 2020). When looking ahead to completely new food production solutions that possibly increase sustainability beyond any previous food alternatives, Solar Foods is a powerful example. The company uses microbes to produce proteins directly using atmospheric conditions, water and solar energy. Such solutions depend on advanced understanding of functions and availability of genes and precise regulation of the selected genes. In addition, we see emerging companies like Solar Foods from Finland making food from air and water, possibly 20,000 times more sustainable than current food production (Southey, 2019; Solar Foods, 2020). Headlines like ‘New food solutions will save the planet but kill farming’ have been produced by journalist George Monbiot in The Guardian newspaper (Monbiot, 2020). IFs achieved ‘Generally Regarded As Safe’ (GRAS) approval for restaurant provision from mid-2018, retail sales in 2019 and started home-deliveries in 2020.

16.3 How RNAi Communication Can Hit the Goals of Relevance, Surprise, Solution, Challenge and Obstacle

Successful communication is achieved when relevance is combined with a selected message being received and understood by the target audience. This takes clear wording and messages. Additionally, communication must fit into attention spans and compete with many platforms in a rapidly changing media world.
From updated professional media courses given by the European Co-operation in Science and Technology (COST, created in 1971) academy, the messages are that, for social media, Facebook is still the platform reaching most people with 2.3 billion users, followed by YouTube with 1.9, WhatsApp 1.5 and FB Messenger with 1.3 billion users (ABS CBN News, 2019). YouTube's rapid rise is due to videos being more efficient at reaching target audiences than still photos and written texts. LinkedIn is of special value to professionals; despite having a lower number of users, it provides a platform for a well-educated and influential audience. It also publishes short stories and papers in addition to job market information. Twitter and Instagram are widely used but are mainly picture driven and less text oriented, with correlated limitations.

16.3.1 Relevance

There is growing global consumer demand for meeting climate change goals while feeding our growing population. This will require increased production of high-quality foods with reduced levels of inputs on existing land surfaces. Such sustainable production demands some plant production and food systems to replace animal sources. This additionally meets the increased market trend for plant-based food. Greta Thunberg is a strong advocate for this drive, being selected as one of the most influential leaders in 2019 by *Time* and *Nature* magazines (Alter et al., 2019; Nature, 2019). Her message that her generation's future is lost unless meat consumption is dramatically reduced is helping to drive food shifts towards meat replacements. Applying RNAi technology to plant production will contribute to meeting these new food demands. RNAi products improving plants and protecting them from external damaging factors like pests and climate change will be very relevant for achieving these sustainability goals.

16.3.2 Surprise

Our brain is designed to save power and run on default unless surprised (Luna and Renninger, 2015). Therefore, to successfully gain the attention of an audience, surprise and telling the story while attention is held is an important factor. This can be done with videos providing a few key messages to increase impact, or by piggybacking on popular podcast hosts. Podcasts have longer airtime and professionals can adapt the messages and wording to deliver more complicated messages.

A good example of a successful plant food message success is how the IFs burger has achieved a market share beyond any expectations (Gravier, 2019; Fontanazza, 2020). In addition, messages from emerging companies like Solar Foods stating they can make food from air and water (Southey, 2019; Solar Foods, 2020) have prompted headlines like ‘New food solutions will save the planet but kill farming’ (Monbiot, 2020), making a large impact.

16.3.3 Solutions

While achieving attention, it is important to provide solutions to challenges arising in the minds of audiences. For example, nutritionally enhanced foods provide solutions to allergies, vitamin deficiencies and alternatives to meat. RNAi plants provide solutions to controlling pests and diseases without the need for pesticides. Thus, the new crop varieties provide clear advantages for production and quality. Covid-19 vaccines based on mRNA demanding -70 °C storage can be avoided if replacing the unstable mRNA with short RNAi, showing how RNAi can contribute with extremely high impact solutions.

16.3.4 Challenges

To meet future food requirements, we require improved productivity and quality of crops and protection against pests, diseases and environmental stresses using minimal inputs and on limited land areas. Plant breeding can meet this challenge if it is permitted to use the wide range of new technologies. RNAi is an important technology to meet this challenge, either activated through plant breeding or in developing new biological plant protection treatments (see Chapters 9 and 11).
16.3.5 Obstacles

The main obstacles to meeting the challenges and providing solutions should be presented clearly, together with strategies for overcoming them. For GM plants, and RNAi in particular, the main challenges are public perception and regulations. Regulatory frames should be soundly science based and determine whether a new variety is safe for consumption and its environmental impacts are the same as or less than those for similar varieties. However, a major additional obstacle in some countries is that there is political interference in the regulatory process and non-acceptance of the scientific findings. This is often driven by lobbying organizations who are opposed to many aspects of new scientific development and have a powerful influence on public perception, politicians and decision makers. In addition, regulations are often lagging behind scientific progress so that they are not fit for assessing new technologies. Good science communication is thus required to demonstrate clearly the present and future roles that new varieties can play in meeting sustainable food solutions. In addition, we need updated regulatory processes that permit improved plant solutions and we need to assess expected new plant breeding technologies and their products (Hartung and Schiemann, 2014; Zetterberg and Edvardsson Björnberg, 2017).

16.3.6 Consumer choices

Future food solutions will depend on available tools, including plant breeding technology, and an understanding of the choices paramount to keep working democratic principles for anchoring decisions for the common good. Consumer trends show rapid shifts demanding real changes to increase sustainable food production, while protecting the environment and meeting future climate changes. IFs, for example, is clear on the need for gene technology and gene transfer to make true ‘meat-like’ alternatives to satisfy current meat consumers. Other plant breeding solutions such as RNAi will be required to develop sustainable production of nutritious foods to provide choice to growing populations.

16.3.7 The wording best used in communicating RNAi

In order to improve communication with non-scientific audiences and the general public it is important that the language used is clear and succinct and does not use words that have double meanings or emotive effects. Therefore, in relation to RNAi, ‘genetic interference’ must be clearly explained to avoid negative reactions and should be described as on/off switches for genes to prevent the expression of undesirable characteristics. Scientists also typically say RNAi causes ‘knockdown’ of gene expression which results in increasing/reducing protein production. The analogy with on/off switches could be taken further to describe the system as being like a dimmer switch which can increase or decrease the expression of a gene. Gene silencing is also used to describe RNAi activity and this could be described as analogous to the volume switch on a radio where the sound level can be regulated. Also ‘mutant’ is considered a negative word that people may associate with, say, Zombie films. It should be explained that mutation is simply a change, which can be for the good and produce many desirable variants, such as those described above.

16.4 RNAi: Key Messages

The selected messages below are collected from the COST Action iPlanta working groups. Some of the messages overlap as the working groups themes do to secure full coverage of RNAi thematics, and the messages are formed and worded to reach a wide audience.

16.4.1 Exploiting RNAi to improve plant production

- RNAi is a biological phenomenon exploited by scientists to develop molecular tools for controlling pests and diseases, by changing the expression of desirable or undesirable genes, to secure future plant production. The plant’s RNA can be guided to target genes in pests and diseases on the plant by inhibiting essential gene(s) in the pest or pathogen. This process is very selective and
generally considered much safer than alternative methods of control, providing promising new solutions for crop protection.

- In addition, RNAi is being developed as externally applied treatments (e.g. sprays) which are highly selective to target pest species, have little or no effect on non-target and beneficial organisms and low environmental persistence. Hence biological pesticides provide a very efficient alternative technical solution for sustainable pest and disease control.

16.4.2 Applications

- The understanding of RNAi is now adequate to allow safe use for commercial applications as a tool to treat human and plant diseases and to develop improved crop varieties in agriculture and horticulture.
- In crops, some applications of RNAi are by genetically modified plants, others are through non-GM methods that are regulated differently.
- Robust pre-market regulatory procedures exist for the risk assessment and authorization of GMO RNAi plants (see below).
- Several crops have been modified by RNAi to change their quality characteristics and some have been commercialized in Europe. The amylogen potato with modified starch for industrial production has been approved for cultivation in the EU and imports of food/feed commodity crops such as soy and maize with changed nutritional characteristics have been approved.
- For pest control, the main commercial crop application is for control of root worm in maize in the USA. The main commercialized application in trees has been in papaya for control of ringspot virus. Plum with resistance to sharka virus has also been approved in North America but not elsewhere. Also, RNAi has been developed in trees such as poplar, prunus and citrus with great promise but not yet commercialized.
- Despite the promising applications of RNAi, in Europe the number of field trials has decreased, likely due to the cost and strict regulation of GMOs. This is blocking the potential of RNAi plants to aid adaptation of future crop and reforestation material to meet future requirements and climate change challenges.
- For external RNAi treatments of pests and diseases that does not involve GM plants, appropriate regulatory frameworks, including science-based risk assessment procedures, have not been developed and therefore are needed (see Chapters 9 and 11).

16.4.3 Biosafety of RNAi

- RNA interference is widely present in plants and animals, meeting criteria for a history of safe use. In addition, dsRNA activity in higher animals is limited by existing biological barriers, meaning RNAi is safe in feed and food.
- RNAi can selectively target pest organisms, including viruses, and is a promising alternative to chemical control and increasing agriculture's sustainability.
- Biosafety assessments using bioinformatic tools allow design of specific dsRNA, avoiding adverse effects on known non-target organisms (NTOs). However, this depends on information about the NTO’s genome sequence, so that until we have genome sequences for higher numbers of NTOs, initial bioinformatic analysis needs further confirmation with tests of NTO sensitivity.

16.4.4 Socio-economics of RNAi

- Consumer preferences are rapidly changing towards more sustainable production, affecting previous consumption models. Increasing demands for environmentally friendly food products like plant-based solutions can be delivered by RNAi technology.
- RNAi solutions can additionally have positive economic impacts for agriculture,
by both reducing costs and increasing productivity.

- Current European regulation and its operation is a major barrier for the introduction of RNAi-based products, hampering innovation capacity and competitiveness of EU firms and negatively affecting consumers and the environment.

**Websites**

- Beyond Meat: https://www.beyondmeat.com
- Impossible Foods (IFs): https://impossible-foods.com
- Solar Foods: https://solarfoods.fi

**References**


Glossary

**ABC**: ATP-binding cassette

**agbiotech**: agriculture biotechnology

**AGO, Ago**: Argonaute

**AM**: assisted migration

**amplification of RNAi**: amplification might be required for efficient RNA-mediated silencing. In *Caenorhabditis elegans* and in plants, primary siRNAs can act as primers for the synthesis of additional dsRNA, using the target mRNA as a template, in a reaction catalysed by a RNA-dependent RNA polymerase (RdRP). The newly synthesized dsRNA is then cleaved by Dicer to generate secondary siRNAs, thereby amplifying RNA silencing.

**Argonaute** (AGO, Ago): a family of evolutionarily conserved genes. Their protein products are involved in various RNA interference processes because of being part of the RNA-induced silencing complex (RISC).

**BLAST**: Basic Local Alignment Search Tool

**Bt**: *Bacillus thuringiensis*

**CAS**: CRISPR-associated

**cDNA**: complementary DNA

**CDS**: coding sequence

**COST**: European Cooperation in Science and Technology programme

**CP**: coat protein

**CPB**: Colorado potato beetle

**CQD**: carbon quantum dot

**CRAC**: cholesterol recognition/interaction amino acid consensus

**CRISPR**: clustered regularly interspaced short palindromic repeats

**CMPP**: cell membrane penetrating peptide

**DCL**: Dicer-like proteins in plants

**DdRP**: DNA-dependent RNA polymerase

**Dicer (Dcr)**: a ribonuclease III enzyme; a double-stranded RNA-specific endonuclease that processes dsRNAs to 20–25 nt siRNAs during RNA interference and excises miRNAs from precursor miRNA-hairpins.

**diRNA**: defective interfering RNA derived from RNA viruses

**dsRNA**: double-stranded RNA, i.e. RNA with two ribonucleic acid strands. dsRNAs longer than 30 nucleotides are the precursors of the siRNA that can trigger RNAi.
**easiRNA**: epigenetically activated 21 nt small interfering RNA, a type of siRNA

**EFSA**: European Food Safety Authority

**EMBRAPA**: Empresa Brasileira de Pesquisa Agropecuaria (Brazilian Agricultural Research Corporation)

**endo-siRNA**: endogenous siRNA, produced from endogenous dsRNA; involved in genome protection and gene regulation

**EPPO**: European and Mediterranean Plant Protection Organization

**ERA**: environmental risk assessment

**ERF**: ethylene responsive factor

**EU**: European Union

**exo-siRNAs**: exogenous siRNA derived from exogenous dsRNA; involved in antiviral defence

**FAD**: fatty acid dehydrogenase

**FAO**: The Food and Agriculture Organization of the United Nations

**GA**: gibberellic acid

**GE**: genetically modified

**GMO**: genetically modified organism. Organism in which in vitro prepared DNA is incorporated into its genome early in development. The newly inserted DNA is present in both somatic and germ cells, is expressed in one or more tissues and is inherited in a Mendelian fashion.

**HC-Pro**: helper-component proteinase

**HDR**: homology-directed repair

**HIGS**: host-induced gene silencing

**hpRNA**: hairpin RNA; a structure in which adjacent segments of RNA fold together and are stabilized by base pairing, creating a loop of single-strand RNA. Short hairpin RNAs can be engineered to suppress the expression of desired genes in cells. hpRNAs can be transcribed from RNA polymerase II promoters in vivo, thus permitting the construction of continuous cell lines.

**HTS**: high-throughput sequencing

**ihpRNA**: intron-spliced hairpin RNA

**indels**: insertions and deletions

**IPC**: International Patent Classification

**iPlanta**: COST action CA15223 to study RNAi genetic improvement methods, funded by the European Union

**IPM**: integrated pest management

**LA**: linoleic acid

**LDH**: layered double hydroxide

**LDLRAP1**: low-density lipoprotein receptor adapter protein 1

**IncRNA**: long non-coding RNA

**MEEC**: maximum expected environmental concentration

**MIGS**: miRNA-induced gene silencing

**miPDC**: miRNA precursor deposit complex

**miPEPS**: miRNA encoded peptides

**miRNA**: microRNA. Small RNAs that interact with components shared by the RNA-induced silencing complex (RISC). miRNAs play a central role in the regulation of gene expression in cells.

**miRBase**: miRNA database

**miRISC**: miRNA-induced silencing complex

**miRLC**: miRISC loading complex

**miRNPs**: microRNA ribonucleoproteins

**MN**: meganuclease
**mRNA**: messenger RNA, the RNA template for protein synthesis. mRNA is formed by transcription of the template DNA strand, followed by the excision of introns and the joining of exons to form mature mRNA. The mRNA is next translated to polypeptides making up proteins.

**MS**: Member States

**NBS**: nucleotide binding site

**NBT**: new breeding technique

**ncRNA**: non-coding RNA

**NEP**: newly expressed protein

**NGS**: next-generation sequencing

**NEHJ**: non-homologous end joining

**nt**: nucleotide

**NTO**: non-target organism

**ODM**: oligonucleotide-directed mutagenesis

**OECD**: Organisation for Economic Co-operation and Development

**Off-target gene silencing effects**: Suppression of genes other than the target gene. Off-target effects have been correlated with the concentration of siRNAs, as well as similarities between the off-target transcripts and the 5′ ends of siRNAs.

**ORF**: open reading frame

**PAMP**: pathogen-associated molecular pattern

**PAZ**: Piwi/Argonaute/Zwille

**PDR**: pathogen-derived resistance

**PF**: problem formulation

**phasiRNA**: phased siRNA

**PIP**: prolactin-induced protein

**piRNA**: PIWI-interacting RNA

**PIWI**: P-element induced wimpy testis, a domain in particular AGO proteins

**PPO**: polyphenol oxidase

**PPP**: plant protection product

**pre-miRNA**: miRNA precursor; a small hairpin precursor before Dicer cleavage

**pre-siRNA**: siRNA precursor

**pri-miRNA**: primary miRNA; a primary miRNA transcript before processing to pre-miRNA

**PRINT**: particle replication in non-wetting templates

**PTD–DRBD**: peptide transduction domain–dsRNA-binding domain

**PTGS**: post-transcriptional gene silencing. The transcription of the gene is unaffected; however, gene expression is suppressed because mRNA is degraded and/or not translated. PTGS is involved in regulation of gene expression and provides nucleotide sequence-specific protection against a variety of foreign genetic elements, including viruses.

**PUFA**: polyunsaturated fatty acid

**qRT-PCR**: quantitative reverse transcriptase polymerase chain reaction

**R&D**: research and development

**RC**: RNA control

**RdDM**: RNA-directed DNA methylation

**rDNA**: ribosomal DNA – a part of the genome encoding ribosomal RNA

**RdR6**: RNA-dependent RNA polymerase VI, involved in the amplification of RNAs in some organisms such as *C. elegans* and plants.

**RenSeq**: resistance gene enrichment sequencing

**RISC**: RNA-induced silencing complex. An enzyme complex containing aspecific Argonaute protein that uses the sequence encoded by one of the siRNA strands or the miRNA guide strand to target mRNA of complementary sequence for degradation and/or translational repression.

**RLC**: RISC loading complex
**RNAi:** RNA interference. The RNAi pathway is based on two steps, each involving ribonuclease enzymes. In the first step, the trigger RNA (either dsRNA or pre-miRNA) is processed into short RNAs (sRNAs) by the RNase III enzyme Dicer. In the second step, siRNAs are loaded into the effector complex RISC. The siRNA is unwound during RISC assembly and the single-stranded RNA hybridizes with mRNA target. Gene silencing is a result of nucleolytic degradation or translational repression of the targeted mRNA by the slicing enzymatic activity of a specific Argonaute protein (Slicer).

**rRNA:** ribosomal RNA
**rsd:** RNAi spreading defective
**SAGO:** siRNA-specific Argonaute
**SCR:** southern corn rootworm
**shRNA:** short hairpin RNA
**sid:** spreading RNA interference defective
**SID:** systemic interference deficiency
**SIGS:** spray-induced gene silencing
**siRNA:** small (or short) interfering RNA. Short pieces of dsRNA, approximately 21–25 base pairs long, involved in RNAi. In the majority of cases, siRNA duplexes composed of equal length sense and antisense strands, are paired in a manner to have a 2 nt 3′ overhang.

**Slicer:** enzyme that cleaves mRNA in the RNAi RISC complex; action of an Argonaute protein involved in cleaving mRNA
**SNP:** single nucleotide polymorphism
**SPc:** star polycation
**sRNA:** small RNA, encompassing both siRNA and miRNA
**ssRNA:** single-stranded RNA
**TALEN:** transcription activator-like effector nuclease
**tasiRNA:** trans-acting siRNA

**TGS:** transcriptional gene silencing. Gene expression is reduced by a block at the transcriptional level. Transcriptional repression is caused by epigenetic modifications like DNA methylation and chromatin modification. The major role of TGS is the suppression of transposon activity. In transgenic plants TGS is mediated by dsRNA with homology to promoter sequences.

**TGN:** Trans-Golgi network
**TILLING:** targeting induced local lesion in genomes
**TLR:** Toll-like receptor
**TO:** target organism
**tRNA:** transfer RNA
**transgene:** a gene transferred into another organism using genetic engineering (genetic modification) techniques.

**transgenic organism:** an organism in which a DNA from another organism (a transgene) is stably incorporated into its genome. The transgene is present in both somatic and germ cells, is expressed in one or more tissues, and is inherited in a Mendelian fashion.

**USDA-APHIS:** US Department of Agriculture Animal and Plant Health Inspection Service

**UTR:** untranslated region

**VIGS:** virus-induced gene silencing: silencing that is induced by the presence of viral genomic RNA. Only replication-competent viruses cause silencing, indicating that dsRNA molecules might be the inducing agents. Restriction of virus growth in plants is mediated by PTGS, which can be initiated by production of dsRNA replicative intermediates.

**vRNA:** viral RNA
**VLP:** virus-like particle, virion-like particle
**VNP:** virus-based nanoparticle
**vsiRNA:** virus-derived small interfering RNA
**VSR:** viral suppressor of RNAi
**WAGO**: worm-specific Argonaute  
**WCR**: western corn rootworm  
**ZFN**: zinc-finger nuclease  
**ZRMS**: zonal rapporteur MS

**Abbreviations for virus names**

- **ALSV**: apple latent spherical virus  
- **AMV**: alfalfa mosaic virus  
- **BBTV**: banana bunchy top virus  
- **BGMV**: bean golden mosaic virus  
- **BSMV**: barley stripe mosaic virus  
- **CaMV**: cauliflower mosaic virus  
- **CMV**: cucumber mosaic virus  
- **CPV**: cypovirus (cytoplasmic polyhedrosis virus)  
- **CTV**: citrus tristeza virus  
- **CymMV**: cymbidium mosaic virus  
- **PMMoV**: pepper mild mottled virus  
- **PNRSV**: prunus necrotic ringspot virus  
- **PPV**: plum pox virus  
- **PRSV**: papaya ringspot virus  
- **PSBMV**: pea seedborne mosaic virus  
- **PVY**: potato virus Y  
- **SCMV**: sugarcane mosaic virus  
- **TEV**: tobacco etch virus  
- **TMLCV**: tomato yellow leaf curl virus  
- **TMV**: tobacco mosaic virus  
- **TSWV**: tomato spotted wilt virus  
- **TuMV**: turnip mosaic virus  
- **TYLCV**: tomato yellow leaf curl  
- **TYMV**: turnip yellow mosaic virus  
- **WMV**: watermelon mosaic virus  
- **ZYMV**: zucchini yellow mosaic virus
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RNAi for Plant Improvement and Protection

Edited by Bruno Mezzetti, Jeremy Sweet and Lorenzo Burgos

RNA interference (RNAi) has the potential to make major contributions towards sustainable crop production and protection with minimal environmental impacts compared to other technologies. RNAi is being developed and exploited both within plants (i.e. host-induced gene silencing, HIGS) and/or as topical applications (e.g. spray-induced gene silencing, SIGS) for targeting pest and pathogen genes and for manipulating endogenous gene expression in plants. Chapters by international experts review current knowledge on RNAi, methods for developing RNAi systems in GM plants and applications for crop improvement, crop production and crop protection. Chapters examine both endogenous systems in GM plants and exogenous systems where interfering RNAs are applied to target plants, pests and pathogens. The biosafety of these different systems is examined and methods for risk assessment for food, feed and environmental safety are discussed. Finally, aspects of the regulation of technologies exploiting RNAi and the socio-economic impacts of RNAi technologies are discussed.