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DNA Sequencing and Crop Protection

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1. Introduction

Many plant pathogenic organisms (e.g. viruses, fungi, bacteria and phytoplasmas) as well as plant pests (e.g. insects and mites) cause serious and widespread diseases and injuries to different cultivated plants with heavy economic consequences for the growers.

Crop management to reduce damage by diseases and pests is based on integrated control strategies involving exclusion, eradication, and protection. In the recent years the choice among the different options is driven not only by the overall costs versus the increased yield and/or quality, but also by the energy consumption, time taken, environmental impact and overall sustainability.

One of the crucial step in crop protection is the rapid, accurate and reliable plant pathogen/pest detection, identification and quantification. It allows to control the spread of the diseases/pests by screening vegetal propagative material and to implement quarantine regulations. Moreover pathogen/pest detection and identification are fundamental for epidemiological studies and for the design of new control strategies.

Traditionally, the most used approach to identify plant pathogens relied with visual inspection of symptoms usually followed by laboratory analyses based on morphological identification using microscopy and isolation and culturing of the organisms. In some cases these methods are still used, but actually these conventional approaches require skilled and specialized expertise which often takes many years to acquire, are laborious, time consuming and not always sensitive and specific enough. Moreover closely related organisms may be difficult to discriminate on morphological characters alone, symptoms are not always specific and not all the microorganisms are culturable in vitro. For all these reasons in the last decades much effort has been devoted to the development of novel methods, in particular nucleic acid based molecular approaches, for detecting and identifying plant pathogens and pests (Lopez et al., 2003; McCartney et al., 2003; Alvarez, 2004; Lievens & Thomma, 2005). The use of DNA-based methods derives from the premise that each species of pathogen carries unique DNA or RNA signature that differentiates it from other organisms. Knowing the pathogen/pest nucleic acid sequence enables scientists to construct oligos to detect them. These oligos are at the basis of many highly specific analytical tests now on the market (Louws et al., 1999).

The present chapter will describe the routine applications of DNA-based technology in different aspects of crop protection. It will highlight the perspectives of innovative

approaches, based on nucleotide sequences, in providing new sustainable and environmentally-friend strategies for disease and pest management. Moreover the role of next-generation sequencing (NGS) technology in supplying these innovative approaches will be outlined.

2. Molecular tools for detection and identification of plant pathogens and pests

Many molecular techniques based on the hybridization or amplification of target sequences, have been developed for many plant pathogenic organisms and pests as well. A great and indisputable improvement in molecular diagnostic was achieved with the introduction of the polymerase chain reaction (PCR) in the mid 1980s (Henson & French, 1993). PCR technology is now widely used for plant pathogen detection because it is rapid, specific and highly sensitive. Even RNA-based viruses can be detected by PCR, by first reverse-transcribing the RNA into DNA. The specificity, that is the ability to distinguish closely related organisms, depends on the designing of proper primers and improvements in sequencing technologies are making the selection of reliable PCR primers routine (Schaad & Frederick, 2002; Schaad et al., 2003). Highly conserved gene regions are often the target for designing primers. Often, to select the target sequences to be used in nucleic acid diagnostic, the same known gene is isolated and sequenced from target and non-target pathogens. Then regions of the sequence that are different are used for primer design. Alternatively, randomly selected DNA fragments are used. With the advancements in high-throughput DNA sequencing, more and more genomes of plant pathogens are available (http://cpgr.plantbiology.msu.edu) and a huge number of nucleotide sequences has been deposited in databases, i.e. GeneBank. Searching in these databases the sequences of a particular organism and screening them for their feasibility as potential targets in a PCR-based assay of a particular organism makes the primer design more and more rapid and precise.

In viruses the most common target is the coat protein gene. In bacteria, phytoplasmas and fungi very often the DNA encoding the ribosomal RNA (rDNA) is used, that occurs as a repeated, structured unit consisting of relatively conserved ribosomal RNA (rRNA) subunit genes (16S, 23S and 5S in the case of prokaryotes and 8S, 5.8S and 28S in the case of fungi) which are separated by internal transcribed spacers (ITS) with relatively high variability. This allows to design primers binding to conserved regions and amplify variable regions on sequences that are conserved between species. These variable regions can be used for species identification (White et al., 1990). Usually the most used techniques are conventional specific PCR, nested-PCR to reach high sensitivity or PCR followed by restriction fragment length polymorphism (PCR+RFLP) to reach high specificity. However, ribosomal sequences do not always reflect sufficient sequence variation to discriminate between particular species (Tooley et al., 1996). Therefore, but also to corroborate discrimination based on ITS sequences, other housekeeping genes are becoming more intensively studied, including beta-tubulin (Fraaije et al., 1999; Hirsch et al., 2000), actin (Weiland & Sundsbak, 2000), elongation factor 1-alpha (O'Donnell et al., 1998; Jiménez-Gasco et al., 2002), and mating type genes (Wallace & Covert, 2000; Foster et al., 2002).

Besides detection and identification, quantification of a pathogen is fundamental in crop protection because it is related to the risk of disease development and economic loss and

thus directly linked to disease management decision. For this reason real-time PCR, allowing amplified DNA quantification using fluorescent dyes, and guaranteeing reduced risk of sample contamination avoiding post-PCR sample processing, is one of the most rapid species-specific detection techniques currently available.

We should also consider that plants can be infected by different pathogens at the same time, thus technologies able to detect multiple pathogens simultaneously are required. Multiplex PCR, using several PCR primers in the same reaction, allows these kind of analyses saving time and reducing costs. However multiplex PCR can be difficult to develop because the products from different targets need to be different sized to ensure that they can be distinguished from one another and they must all be amplified efficiently using the same PCR conditions (Henegariu et al., 1997). Also real-time PCR allows the simultaneous detection of different targets by using probes with different fluorescent reporter dye (Weller et al. 2000, Boonham et al., 2000). Microarray technology is an emerging tool in crop protection for unlimited multiplexing analyses (Fessehaie et al., 2003; Lèvesque et al., 1998; Lievens et al., 2003; Uehara et al., 1999; Mumford et al. 2006; Boonham et al. 2007), but nowadays existing microarray methods are still complex and relatively insensitive, and a widely accepted diagnostic format has yet to be adopted.

PCR-based techniques are also being used in an insect pest management context to identify insect pests and insect biotypes, to understand population structure, tritrophic interactions and insect-plant relationships (Caterino et al., 2000; Heckel, 2003; Gariepy et al., 2007). For this purpose the ribosomal and mitochondrial regions have been proven to be highly informative. In particular the cytochrome c oxidase I and II (COI and COII) and the 16S and 12S subunits of mitochondrial DNA as well as the internal transcribed spacer regions (ITS1 and ITS2), the 18S and the 28S subunits of the rDNA are used (Gariepy et al., 2007).

However DNA-based identification of species should not be considered as a replacement for the traditional methods, but it is a great help in all the situations in which morphological characters are not helpful such as in the case of young stages or cryptic species. In particular it can help in the study of known or putative vectors of plant pathogens giving innovative contributions to faunal studies and insect vector monitoring and allowing more rational control strategies (Bertin et al., 2010a; Bertin et al., 2010b; Cavalieri et al., 2008; Saccaggi et al., 2008; Tedeschi & Nardi, 2010).

Molecular techniques have also been used to facilitate ecological studies on parasitoids and predators in biological control programs (reviewed by Gariepy et al., 2007; Greenstone, 2006), while microsatellite analysis can be used to separate strains and evaluate genetic diversity of natural enemy populations. All these information may be able to predict natural enemy host range, climatic adaptability and other important biological traits that can be used in the selection of efficient candidate biological control agents (Unruh & Woolley, 1999).

Anyhow all these molecular detection methods such as PCR and real-time PCR, rely on previous knowledge of the pathogen/pest for design of sequence-specific primers.

The advent of the second generation sequencing affords unique opportunity to directly detect, identify and discover pathogens without requiring prior knowledge of the pathogen

macromolecular sequence. This technology is unbiased and allows to consider, on the whole, bacteria, viruses, fungi and parasites (Quan et al., 2008).

This approach, already applied in medical diagnostic, starts to be regarded in phytopathology. As correctly stated by Studholme et al. (2011), efficient sample preparation methods, bioinformatics pipelines to efficiently discriminate pathogens and host sequences and functionally associating candidate sequences with disease causation should be developed before using high-throughput sequences as a routine diagnostic tool. Moreover plant samples always contain a complex microbiota that alone cannot reveal the causal agents of a disease and biological knowledge are fundamental for a good interpretation of the results.

The total nucleic acid from the sample of interest has the advantage that it avoids introducing bias and is suitable for a variety of substrate, but the elimination of host nucleic acid is very critical to increase the pathogen signal towards the threshold for detection.

The huge number of sequences obtained should be trimmed and filtered to remove low-quality sequences and reads containing any primer sequences. Then the sequences are compared against databases of known sequences using a pair-wise sequence similarity method. If no similar sequences are found, other methods can be used, such as the ones that separate sequences into clusters based on their physical characteristics (Abe et al., 2003; Chan et al., 2008).

If a new pathogen is identified on the basis of sequences alone, problems could be occur for its naming and taxonomy, because Koch's postulate are rarely completed, thus maybe a prefix or suffix can highlight this status (Studholme et al., 2011).

3. Application of DNA barcoding in crop protection

DNA barcoding is a universal typing system to ensure rapid and accurate identification of a broad range of biological specimens. This technique allows the species characterization of organisms using a short DNA sequence from a standard and agreed-upon position in the genome. This concept was proposed by Hebert et al. in 2003 with the description of the first marker as a "barcode", the mithocondrial COI gene, for species identification in the animal kingdom. The DNA target sequence should be identical among the individuals of the same species, but different between species, extremely robust with highly conserved priming sites and highly reliable DNA amplifications and sequencing, phylogenetically informative and short enough to have lower processing costs and to allow amplification of degraded DNA (Valentini et al., 2008). However the perfect DNA target region does not exist and more than one marker have been proposed. The COI region has been almost widely accepted for barcoding animals because of its generally conserved priming sites and third-position nucleotides with a greater incidence of base substitutions than other mitochondrial genes. Moreover the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox & Herbert, 2001; Wares & Cunningham, 2001). On the contrary in plants the mtDNA has low substitution rates and a rapidly changing gene content and structure, which makes COI unsuitable for barcoding in plants (Wolfe et al., 1987). For this reason two regions of chloroplast DNA, ribulose-bisphosphate carboxylase (rbcL) and maturase K (matK) have been recommended for initiating the barcoding process of plant species (CBOL Plant

Working group, 2009; Consortium for the barcode of life, 2009). Due to the only 70% species discriminatory power, additional loci need to be used in this field (Vijayan & Tsou, 2010). On the contrary in the case of fungi, the ITS of nuclear DNA (nrDNA) has recently been proposed as the official primary barcoding marker (Seifert, 2009), while no standard regions have been found yet for viruses, bacteria and phytoplasma, but studies are ongoing (Contaldo et al., 2011).

The primary intent of DNA barcoding was to use large-scale screening of one or a few reference genes in order to assign unknown individuals to species, and to enhance discovery of new species (Hebert et al. 2003; Stoeckle, 2003), so this approach was extensively used by taxonomists and ecologists in particular for biodiversity studies in spite of limitations and pitfalls (Valentini et al., 2009; Moritz & Cicero, 2004). Today new applications are emerging and the use of DNA barcoding has been proposed for a new approach in plant protection, that is for the rapid and precise identification of invasive alien species (IAS). Non-indigenous pests, and the plant pathogens they harbor are a serious threat for agriculture and forestry with huge ecological and economical consequences. IAS occur in all major taxonomic groups, including viruses, fungi, bacteria, nematodes and insects. All these organisms are able to reproduce and spread, causing massive environmental damage also because in the new environment often no natural specific predators or parasitoids are present. Early detection and rapid response are most cost-effective and more likely to succeed than action after a species has become established. For this reason inspections and quarantines are key factors of prevention. As previously stated the development of accurate identification tools for plant pathogens and pests is fundamental for plant health in agriculture but at the same time the taxonomic knowledge available to identify harmful quarantine organisms via their visual characteristics is gradually decreasing. DNA barcoding, using standardized protocols, will easily allow to identify specimens comparing their sequences against a database of known sequences from identified specimens. Based on DNA, this system is applicable to all life stages even those that cannot be identified by conventional means such as eggs and larvae of insects. This approach does not require extensive knowledge of traditional morphological taxonomy and a minimum of technical expertise is sufficient (Floyd et al., 2010).

Several major research initiatives have already started to assemble reference libraries of DNA barcodes of pest species in quarantine. The International Plant Protection Convention (IPPC) agreed that barcoding currently has a clear potential as a diagnostic protocol.

In New Zealand, since 2005, DNA barcoding using COI sequence has been employed routinely for the highest risk insect species, fruit flies and lymantriid moths. In particular, case studies with the pink gypsy moth *Lymantria mathura* Moore, the yellow peach moth *Canocethes punctiferalis* (Guenée) and the fall web worm *Hyphantria cunea* (Drury) demonstrated the effectiveness of DNA barcoding for border diagnostics. Moreover barcoding improved the chances of finding new organisms, even those that may not have been identified already as high risk to New Zealand (Armstrong, 2010).

In Europe, a project financed by the 7th Framework Program of the European Union and called QBOL (http://www.qbol.org) aims for the development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health. QBOL is a project that makes collections harboring plant pathogenic quarantine organisms (arthropods, bacteria, fungi, nematodes, phytoplasma and viruses) available (Bonants et al.,

2010). Informative genes from selected species on the EU Directive and EPPO lists are DNA barcoded from vouchered specimens and the sequences, together with taxonomic, phylogenetical and phytosanitary features, will be included in an internet-based Q-bank database (http://www.q-bank.eu) (Bertaccini et al., 2011). Therefore DNA barcoding will supply not only a strengthen link between traditional and molecular taxonomy as a sustainable diagnostic resource, but also a diagnostic tool for plant health.

Moreover, considering its application in identification and interspecific discrimination of phylogeographic groups within a single species, important advices can be achieved by IAS. Indeed, it will be possible to assess from where exotic specimens originated and get data on the natural host range, climatic range, and potential biocontrol agents, all crucial information for the development of successful control programs (Valentini et al., 2009).

Another practical application of DNA barcoding could support crop protection strategies: DNA barcoding is also a useful tool for searching for candidates of biological control agents and evaluating their potential risks. Normally, searching and screening of control agents require long-term feeding experiments, while DNA barcoding allows to identify and select control agents based on their gut contents (Jinbo et al., 2011; Neumann et al., 2010), detecting prey DNA from the gut content or feces of the predators (Symondson, 2002; Valentini et al., 2008).

Anyhow whatever purpose the DNA barcoding is used for, very good sequence reference datasets for groups with well worked out taxonomy are important key factors.

Due to the evolution of DNA sequencing technology and to the possibility to have nowadays an easy access to sequencing via companies, the DNA-barcoding had a general great impulse and spreading. When using the classical sequencing approach via capillary electrophoresis, environmental samples require an additional step of cloning the different amplified DNA fragments into bacteria, followed by sequencing hundreds or thousands of clones to reveal the full complexity of those samples. Such a cloning step is both expensive and time consuming, thus limiting large-scale applications. New DNA sequencing technologies bypassing the cloning step have recently been developed, opening the way to applying large-scale DNA barcoding studies to environmental samples.

4. Molecular crop protection using recombinant DNA techniques

Conventional breeding techniques which are based on processes of crossing, back crossing and selection have been used since the beginning of agriculture. With these methods, that have been used for hundreds of year, farmers could obtain plants with faster growth, higher yields, pest and disease resistance, larger seeds, or sweeter fruits. In spite of the undoubted good results reached, conventional breeding proved to be time consuming and could not stand the pace with the rapid co-evolution of pathogenic micro-organisms and pests.

A great improvement was achieved in the last 30 years by tissue cultures and micropropagation for the production of disease-free, high quality planting material and for rapid production of many uniform plants. Moreover the rapid advances in molecular biology technologies led to new knowledge for cloning and sequence analysis of the genomic components of plants and the relative pathogens and pests. Comparative mapping across species with genetic markers, and objective assisted breeding after identifying

candidate genes or chromosome regions for further manipulations brought to the development and application of recombinant DNA techniques in crop pest management.

The introduction in plants (transgenic plants) of new genes from other organisms (native or modified forms) allows to develop resistant plants against insects or pathogens (mainly viruses) or more tolerant plants towards herbicides. In contrast to conventional breeding, which involves the random mixing of tens of thousands of genes present in both the resistant and susceptible plant, recombinant DNA technology allows the transfer of only the resistance gene to the susceptible plant and the preservation of valuable economic traits.

The transfer of new genes in plants have been obtained mainly using *Agrobacterium*-mediated DNA transfer or by particle bombardment, electroporation and polyethylenglycol permeabilisation (Fisk & Dandekar, 1993). Strategies using recombinant DNA allowed to obtain different degrees of resistance against viruses, fungi, bacteria and insects.

The engineering of virus resistance into plants is considered one of the most important way to control these pathogens, due to the fact that no direct chemical control is available and the control of the insect vectors or the use of certified virus free material are not overly successful. The first approach, based on the pathogen-derived resistance (PDR) (Sanford & Johnson, 1985) was applied to many plant species. It entails the introduction of a virus-derived gene sequence into the genome of the host plant, then the products of these sequences (mRNA and proteins) interfere with the specific stages in the viral infection cycle such as viral replication or spread. The use of the viral coat protein (CP) as a transgene gave the most important results, with delay and attenuation of symptoms or complete immunity. Powell et al. (1986) first introduced, in this way, resistance against Tobacco mosaic virus (TMV) in tobacco plants then many other researchers obtained several engineered crops for commercial cultivation such as tomato resistant to TMV, Tomato mosaic virus (ToMV) (Sanders et al., 1992), Cucumber mosaic virus (CMV) (Fuchs et al., 1996), potato resistant to Potato virus X (PVX) (Jongedijk et al. 1992), Potato virus Y (PVY) (Malnoe et al., 1994) and Potato leafroll virus (PLRV) (Presting et al., 1995). Moreover tobacco containing the CP genes for three viruses, Tomato spotted wilt virus (TSWV), Tomato chlorotic spot virus (TCSV), and Groundnut ringspot virus (GRSV) developed resistance to all of them (Prins et al., 1995). Alternatives to CP-mediate protection are the introduction of sequences coding for non-structural viral proteins, the so called replicase-mediated protection, the movement-protein-mediated resistance and the REP protein-mediated resistance to single stranded DNA that proved to be effective for controlling virus diseases in a wide range of crops (Prins et al., 2008). Moreover new and important opportunities came from the RNA-mediated resistance, founded on the post-transcriptional gene silencing process called also RNA interference (RNAi) or RNA silencing. It is based on a plant natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner that will be deepened in the following paragraph.

Strategies proposed to control fungal and bacteria diseases by transgenic plants are similar, because of their similarity in modes of pathogenesis and plant response. As a defense strategy against the invading pathogens (fungi and bacteria) the plants accumulate low molecular weight proteins which are collectively known as pathogenesis-related (PR) proteins. Several transgenic crop plants with increased resistance to fungal pathogens are being raised with genes coding for the different compounds such as the glucanase or

chitinase enzymes that degrade the cell wall of many fungi (Grover & Gowthaman, 2003), or lysozyme and cercopsins that cause the hydrolytic cleavage of cell wall murein (Strittmatter & Wegener, 1993) and the formation of ion channels in the bacterial membrane with the leakage of cell components respectively (Florack et al., 1995). For both fungi and bacteria also the use of phytoalexins gave good results together with many other strategies well resumed by Grover & Gowthaman (2003).

Insect resistant crops are one of the major success of recombinant DNA technology in agriculture. Cotton resistant to lepidopteran larvae and maize resistant to both lepidopteran and coleopteran larvae are nowadays widely used and allowed to reduce the use of pesticides and the overall production costs (Gatehouse, 2008).

The soil bacterium *Bacillus thuringiensis* (Bt) is one of the most important source of genes encoding insecticidal proteins, which are accumulated in the crystalline inclusion bodies produced by the bacterium on sporulation (Cry proteins, Cyt proteins) or expressed during bacterial growth (Vip proteins). These are specific to particular groups of insect pests (mainly Lepidoptera and Coleoptera), and are not harmful to other useful insects. Moreover different Bt strains show different specificity against different insect pests. For this reasons induced resistance using Bt genes is one of the first application of crop protection with recombinant DNA technology. Expression of Bt genes in tobacco and tomato provided the first example of genetically engineered plants for insect resistance (Barton et al., 1987; Vaeck et al., 1987). Then after, hundreds of field trials with different transgenic crops with Cry genes (e.g. cotton, rice, maize, potato, tomato, cauliflower, cabbage, etc.) have proven the efficacy of this approach in controlling important insect pests with a consequent impressive reduction in pesticide use (Gatehouse, 2008).

The possibility of insects to evolve resistance to transgenic Bt crops has been expected by many researchers due to the widespread of Bt transgenic crops and to the prolonged exposure to Bt toxins (Tabashnik et al., 2008), but despite a few documented cases (Bates et al., 2005), most insect pest populations are still susceptible. To preserve this susceptibility, some agronomical devices are crucial, such as the refuge strategy, that is the presence near Bt crops of host plants without Bt to promote survival of susceptible pests (Tabashnik et al., 2008). At the same time, to reduce the risk of insect resistance, new strategies are proposed such as the expression of multiple Cry genes in transgenic crops (Zhao et al., 2003) or the use of completely different genes.

Certain genes from higher plants were also found to result in the synthesis of products possessing insecticidal activity. For instance many plants produce protease inhibitors that are small proteins which inhibit the proteases present in insect guts or secreted by microorganism, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo et al., 2002). One of the examples is the Cowpea trypsin inhibitor gene (CpTi) which was introduced into tobacco, potato, and oilseed rape for developing transgenic plants (Boulter et al., 1989). It was observed that the insecticidal protein was a trypsin inhibitor that was capable of destroying insects belonging to the orders Lepidoptera, Orthoptera, etc. while it has no effect on mammalian trypsin, hence it is non-toxic to mammals.

Another approach that maybe will have more chances in the future is the production of transgenic plants expressing genes encoding lectins (Rao et al., 1998) considering that many

plant lectins have strong insecticidal properties causing a deterrent activity towards feeding and oviposition behavior (Murdock & Shade, 2002; Michiels et al., 2010).

The increasing availability of DNA sequence information allow to discover genes and molecular markers associated with different agronomic traits creating new opportunities for crop improvement.

The complete genome sequencing can be considered the highest level of genetic markers, providing the opportunity to explore genetic diversity in crops and their wild relatives on a much larger scale than with the earlier technologies. Nowadays the complete genome sequences are becoming available for many plants opening new important opportunities for the creation of transgenic plants resistant or tolerant to pathogens and pests.

5. Crop protection using RNA interference

RNA interference (RNAi) is a naturally occurring regulatory mechanism which causes sequence specific gene silencing. Actually it refers to diverse RNA-based processes that all result in sequence-specific inhibition of gene expression either at the transcriptional, post-transcriptional or translation levels.

This phenomenon was first observed in plants as a unexpected result of attempt to make the color of petunia blooms more purple. Trying to overexpress the production of anthocyanin pigments introducing exogenous transgenes, flowers showed, instead of deepen flower colour, variegated pigmentation, with some lacking pigment altogether (Napoli et al., 1990). This mechanism, with apparent silencing of both endogenous and exogenous genes, was indicated as post-transcriptional gene silencing (PTGS) and termed "co-supression". Some years later RNAi was described in the nematode *Caenorhabditis elegans* (Maupas) as a response to double-stranded RNA (dsRNA) injected into the body cavity, which resulted in sequence-specific gene silencing expressed in distal tissues and in the progeny (Fire et al., 1998). RNAi could be induced also by feeding the worms on the engineered *Escherichia coli* producing dsRNA against the target gene (Fire et al., 1998; Timmons & Fire, 1998; Timmons et al., 2001), by in vitro transcription of transgene promoters (Tavernarakis et al., 2000), or simply by soaking the worms in a solution containing the dsRNA (Tabara et al., 1998). Then Ketting & Plasterk (2000) demonstrated that the co-suppression recorded in plants and the phenomena observed in nematodes are based on the same mechanism and the term RNAi was adopted.

It is now commonly accepted that it has evolved as a mechanism for cells to eliminate foreign genes; indeed it is considered to be an evolutionary ancient mechanism for protecting organisms from viruses (Waterhouse et al., 2001). Many viruses have RNA as their genetic material and it is widely assumed that ds-RNA is generated by viral RNA polymerases either as an intermediate in genome replication (RNA viruses) or as an erroneous product due to converging bidirectional transcription (DNA viruses) (Kumar & Carmichael, 1998; Ketting et al., 2000, Jacobs & Langland, 1996). All the pluricellular organism hosts are able to recognize dsRNA and then activate the RNAi defensive mechanism. An endoribonuclease called Dicer cleaves this dsRNA into short dsRNA fragments called small interfering RNA (siRNA) about 20-25 nucleotides long. Then a multiprotein complex, the RNA-Induced Silencing Complex (RISC), uses the siRNA as a template for recognizing and

destroying single stranded RNA with the same sequence, such as mRNA copies used by the virus to direct synthesis of viral protein. It was also demonstrated that some animal and plant viruses are able to produce proteins to suppress host-mediated RNA silencing allowing viral spread within the host (Li et al., 2002; Ding et al., 2004).

This kind of homology-dependent gene silencing appears to be evolutionarily conserved in all eukaryotic taxa and it was recorded in plants, fungi, invertebrate, including insects, trypanosomes, planaria and hydra, and vertebrates (Bosher & Labouesse, 2000). Even if some specifics of the silencing mechanism may differ between kingdoms, dsRNA seems to be the universal initiator of RNAi.

Since its discovery, RNAi has become a remarkably potent technology to knockdown gene functions in a wide range of organisms representing a valuable tool for functional genomics; moreover it has also found a great potential application in agriculture and in particular it offers a new approach to crop protection. Due to its high specificity, it can be considered a species-specific tool against pests. This phenomenon has been studied in various host-pest systems and efficiently used to silence the action of the pest. New RNAi based strategies have been proposed in last years for conferring plant resistance to phytopathogenic viruses, bacteria, fungi and nematodes and some have been used in commercial agriculture (Escobar et al., 2001; Huang et al., 2006; Gonsalves, 1998). Moreover particular attention was devoted to RNAi efforts targeting insects.

Considering its ancestral role of conferring protection against viral infection, the possibility to manipulate genetically the plants in order to obtain transgenic resistance was supported. Different plant-virus systems have been investigated for inducing immunity by means of RNAi technology and they are well resumed by Wani et al. (2010) and Wani & Sanghera (2010). Very recently Zhang et al. (2011) were able to achieved robust resistance to multiple viruses (Alfalfa mosaic virus, Bean pod mottle virus, and Soybean mosaic virus) with a single dsRNA-expressing transgene in soybean plants opening new interesting perspectives in practical viruses control. It has also been assessed that plant viruses have evolved mechanism to overcome the host antiviral silencing by encoding diverse viral suppressors of RNA silencing (VSRs). These suppressors enhance virus accumulation in the inoculated protoplasts, promote cell-to-cell virus movement in the inoculated leaves, facilitate the phloem-dependent long-distance virus spread, often cause more severe disease (Díaz-Pendón & Ding, 2008; Li & Ding, 2006).

In the case of bacteria very interesting efforts were carried out to generate resistance to crown gall disease caused by the soil bacterium *Agrobacterium tumefaciens* (Smith & Townsend) that causes serious economic losses in perennial crops worldwide. Escobar et al. (2001) obtained transgenic *Arabidopsis thaliana* L. (Heyn) and *Lycopersicon esculentum* L. plants expressing two self-complementary RNA constructions designed to initiate RNAi of tryptophan monooxygenase (iaaM) and isopentenyl transferase (ipt) genes. Expression of these two oncogenes causes the overproduction of the plant hormones auxin and cytokinin resulting in the formation of wild type tumors. The resultant transgenic lines retained susceptibility to *Agrobacterium* transformation but were in some cases highly refractory to tumorigenesis, providing functional resistance to crown gall disease. This procedure could be exploited to develop broad spectrum resistance in ornamental and horticultural plants which are susceptible to crown gall tumorigenesis.

RNAi mechanism has been proposed also to improve plant resistance to fungi. The first gene silencing phenomena in a fungus was described in 1992 by Romano and Macino in *Neurospora crassa* Shear & Dodge. Later on many other fungal species were studied and suppression of gene expression by dsRNA-expressing plasmid or related-systems has been shown in various Ascomycota such as *Venturia inaequalis* (Cooke) Winter (Fitzgerald et al., 2004), *Aspergillus nidulans* (Eidam) Winter (Hammond & Keller, 2005), *Magnaporthe oryzae* Couch (Kadotani et al., 2003), *Aspegillus fumigatus* Fresenius (Mouyna et al., 2004), Basidiomycota such as *Cryptococcus neoformans* (Sanfelice) Vuillemin (Liu et al., 2002) and Zygomicota.

After the first demonstration of RNAi mechanism in the free-living nematode *C. elegans*, the possibility that dsRNA mediates gene silencing also in other worm species was mentioned by Fire et al. (1998). Important advances have been made in the biotechnological application of RNAi towards plant parasitic nematode control. RNAi silencing of a gene that plays a key role in the development of the nematode, either directly or indirectly, can adversely affect the progression of pathogenesis. Genes that are good targets for this technology are likely to be nematode specific and have sequence conservation with orthologues from related species to maximize the spectrum of resistance (Karakaş, 2010). However RNAi in plant parasitic nematodes by injection or soaking is not feasible, because they are too small to be microinjected with dsRNA and do not normally ingest fluid until they have infected a host plant (Bakhetia et al., 2005). But in 2002 Urwin et al. induced the nonfeeding preparasitic second stage (J2) of two cyst nematodes *Globodera pallida* (Stone) and *Heterodera glycines* Ichinohe (which parasitize potato and soybean respectively) to take up dsRNA from a solution containing the neuroactive compound octopamine. Subsequently they observed a shift from the normal female/male ratio of 3:1 to 1:1 by 14 days postinfection (dpi) targeting cysteine proteinases and a reduction of the number of parasites targeting C-type lectine gene. Later, other two stimulation reagents, resorcinol and serotonin, were also used to induce dsRNA uptake by J2s much more effectively (Rosso et al., 2005). In the last years RNAi assays on sedentary forms followed one another and included root knot nematodes (e.g. Meloidogyne), cyst nematodes (e.g. Heterodera and Globodera), and the migratory pine nematodes *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle. All these studies, that are well summarized by Lilley et al. (2007) and Rosso et al. (2009), referred to the successful targeting of a number of genes, expressed in a range of different tissues and cell types, for silencing in various plant parasitic nematodes. Plants that express dsRNA or hairpin RNA in the nematode feeding cells provide a new option for creation of resistant varieties. Plant parasitic nematodes can be found deep enough in the soil to escape the action of pesticides. The development of trapping plants that attract nematodes and block their development through the expression of dsRNA that target essential nematode genes, may be an efficient way of reducing nematode populations in the field (Rosso et al., 2009).

In the field of entomology, the RNAi technique has already widely applied for different kind of researches, to improve the knowledge of RNAi mechanisms *in primis*, but also to study gene function as well as expression and regulation of gene cascades (Price & Gatehouse, 2008; Whyard et al., 2009; Bellés, 2010; Mito et al., 2011). Successful results were obtained in preventing bee mortality by Israeli Acute Paralysis Virus Disease (IAPV) infection under natural beekeeping conditions (Hunter et al., 2010), but many efforts focused on the possibility to control insect pests by means of RNAi. For these studies different insect

models were used, such as *Drosophila melanongaster* Meigen (Misquitta & Paterson, 1999; Dzitoyeva et al., 2001; Schmid et al., 2002), *Tribolium castaneum* (Herbst) (Bucher et al., 2002; Tomoyasu & Denell, 2004; Tomayasu et al., 2008), honeybees (Amdam et al., 2003), *Acyrthosiphon pisum* (Harris) (Mutti et al., 2006; Jaubert-Possamai et al., 2007), the green peach aphid *Myzus persicae* Sulzer (Pitino et al., 2011), the western corn rootworm *Diabrotica virginifera virginifera* (LeCompte) (Baum et al., 2007; Alves et al., 2010). In almost all the experiments carried out, dsRNA was injected directly in the organism, but this kind of dsRNA delivery is not feasible in the protection of crops against phytophagous insects.

The big obstacles to make RNAi approach extensively feasible for crop protection is related to the selection of target sequences, to the production of big quantities of dsRNA to be released, and to the delivery systems of dsRNAs.

Concerning the selection of target sequences, up to now RNAi application tooks advantages from already known genes while cDNA libraries allowed to identify novel genes (Turner et al., 2006; Baum et al., 2007).

The recent availability for many organisms of the full genome sequences makes possible, at least theoretically, the silencing of every single gene to evaluate how the organism reacts. A great improvement in the selection of target genes can be given by the next-generation sequencing (NGS) technologies, with the direct sequence of the cDNA generated from messenger RNA (RNA-seq) (Wang et al., 2009; Haas & Zody, 2010). This new method, called also "Whole Transcriptome Shotgun Sequencing", uses recently developed high-throughput sequencing technologies to convert long RNAs into a cDNA fragments with adaptors attached to one or both ends. Then these fragments are sequenced in a high-throughput manner to obtain short sequences. The resulting reads are aligned to a reference genome or a reference transcript, or assembled ex-novo without the genomic sequence to produce a genome-scale transcription map (Wang et al., 2009). This new method results very useful in the case of non-model organisms for which any genome or transcriptome sequence information is available. Wang et al. (2011) used Illumina's RNA-seq and digital gene expression tag profile (DGE-tag) technologies to screen optimal RNAi targets from *Ostrinia furnalalis* (Guenée) demonstrating that the combination of these two technologies is a rapid, high-throughput, cost less and easy way to select the candidate target genes for RNAi.

One of the biggest limitations to a cost-effective commercial use of this technology is the production of mass quantities of dsRNAs. Tenllado et al. (2004) proved the efficacy of crude preparations of dsRNA in silencing genes of plant viruses using bacterial crude preparations obtained by lysing cell pellets with a French Press avoiding the use of time-consuming protocols to purify nucleic acids. So they anticipated that inducible expression of dsRNA in bacteria could easily be scaled-up as a rapid and cost effective expression system for a large variety of viral dsRNAs. They suggested the use of large fermenters normally used for the commercial production of entomopathogenic nematodes for the mass production of interfering products in agriculture. Moreover they stated that bacterial cell cultures provide a practicable and reproducible approach for mass production of dsRNA because they are easily pelleted, stored and distributed, without losing their interfering properties with plant virus infection.

Concerning the delivery system of dsRNA, the feeding approach instead of the haemocoel injection is more attractive because more feasible in crop protection, that is the target

organism should be able to take up the dsRNA autonomously, especially in the case of insects , e.g. by feeding and digestion (Huvenne & Smagghe, 2010). Several studies have proved the possibility of introducing dsRNA in insect through feeding as resumed by Huvenne & Smagghe (2010) and recently confirmed by Pitino *et al.* (2011) who demonstrated that siRNAs can travel from the plant phloem through the aphid *M. persicae* stylet and reach the intestinal tissues triggering the silencing of aphid target genes. Thus the solution for a practical crop protection application is the introduction of dsRNA in the plants that are ingested by insect herbivores. That is possible by *Agrobacterium* infection, by particle bombardment or by virus infection. The first method refers to transgenic RNAi plants that are created, as other genetically modified plants, by means of isolation, amplification and cloning into a plasmid of the target gene. Then the plasmid is allowed to undergo recombination into an *A. tumefaciens* binary vector. Then plants are transformed with *A. tumefaciens* and the vectors replicate to produce ds-RNA for RNAi induction (Johansen & Carrington, 2001). Alternatively bombarding cells with dsRNA can produce transient silencing. On the contrary with Virus Induced Gene Silencing (VIGS) RNA is introduced in the plants by means of modified viruses as RNA silencing trigger (Watson et al., 2005; Wani et al., 2010).

Tenllado et al. (2004) found that a spray made of crude dsRNA was efficient at gene silencing of plant viruses. But even if the dsRNAs were able to stable remain on the leaves for several days conferring resistance to viral infection, this method was recognized not to be cost-effective. Likewise a crude extract of *E. coli* HT115 containing large amounts of dsRNA and applied to maize plants as a spray was able to inhibit Sugarcane mosaic virus (SCMV) infection (Gan et al., 2010). More recently Wang et al. (2011) demonstrated that a direct spray of dsRNA can produce a down-regulation of gene and finally a development delay and death in the Asian corn borer *O. furnalalis* larvae by penetration through the exoskeleton before reaching the haemolimph and producing RNAi effects. This finding opens new perspectives in dsRNA delivery and provides new insight that RNAi targets can be selected from the whole insect instead of gut-specific target after feeding with dsRNA.

Concerning the future of RNAi in crop protection there are good chances for its application in the field as well as interesting commercial implications in particular against viruses and insects. Probably the future of crop protection against insect pests by means of RNAi technology is more related to sap-sucking pests such as aphids, whiteflies, psyllids and leafhoppers. In fact the current expression of Bt insecticidal proteins in plant offers a better degree of protection in field condition against Lepidoptera and Coleoptera, while none of Cry toxins of *B. thuringiensis* is effective on sup-sucking pests (Virla et al., 2010). The good new results obtained with the whitefly *Bemisia tabaci* (Gennadius) (Upadhyay et al., 2011), the aphid *M. persicae* (Pitino et al., 2011) and the brown planthopper *Nilaparvata lugens* Stål (Zha et al., 2011) are promising. Anyhow, recent reports of resistance to Bt toxins expressed in the plants (Tabashnik et al., 2008) will provide more interest for alternative strategies also for Lepidoptera and Coleoptera.

There is also another application of RNAi that could open new opportunities in crop protection. Allowing the specific down-regulation of genes, this technology will permit the identification of new targets for crop protection compounds such as fungicides and insecticides (Busch et al., 2005) for an ever more specific action. With this approach vulnerable targets for new pest-specific insecticides with novel mode of action can be assessed.

Anyhow for a wide practical application of RNAi in crop protection some challenges remain. For instance the long-term effectiveness of the approach will need to be established, in particular it is necessary to investigate how the internal defense mechanism of the insects will respond to long-term exposure to dsRNA.

6. Conclusion

The general trend in crop protection is the progressive reduction of chemicals because of a more consciousness towards human health and environment. Nowadays biotechnology and genomics offer new important possibilities for this purpose. The possibility to transfer genetic information between different organisms allowed to create transgenic plants resistant to different pathogens and pests. The application of this technology to the most economically important crops and the dissemination throughout the world provided a considerable decrease in pesticide use. In parallel genomics help us to understand the precise role of simple sequences and the proteins they encode within the organism opening new perspectives for the production of pesticides with innovative mode of action and for the development of new specific control strategies against plant pathogens and pests such as the RNAi approach. The improvement of the sequencing technology and in particular the advent of the NGS together with the evolution of bioinformatics enable large-scale sequencing projects permitting a new comparative genomics approach and thus it will facilitate the identification of candidate genes to be used in crop protection. The possibility to down-regulate or over express genes in plants or in plant pathogenic organisms is a very powerful tool, within pest management and agriculture in general, to meet farmer and consumer demands.

7. References

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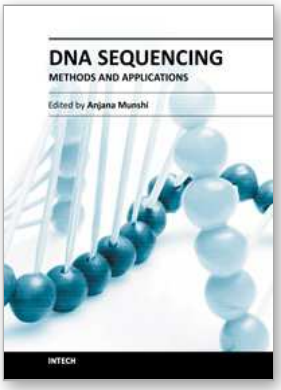

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This book illustrates methods of DNA sequencing and its application in plant, animal and medical sciences. It has two distinct sections. The one includes 2 chapters devoted to the DNA sequencing methods and the second includes 6 chapters focusing on various applications of this technology. The content of the articles presented in the book is guided by the knowledge and experience of the contributing authors. This book is intended to serve as an important resource and review to the researchers in the field of DNA sequencing.

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