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Review Article

Plant gene co-suppression; basis of the molecular machinery of interfering RNA

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Abstract

RNA interference (RNAi), also known as post-transcriptional gene silencing (PTGS) co-suppression, is considered one of the most significant discoveries in molecular biology during the last several years. First recognized in plants, the starting point for its historical overview begins in the late 1980s and early 1990s when researchers used genetic engineering to alter flower color. RNAi is considered a gene down-regulation mechanism demonstrated to exist in all eukaryotes, where small RNAs (of approximately 21-24 nucleotides in size) function to guide specific effector proteins (Argonaute protein family) to a target nucleotide sequence by complementary base pairing. Subsequently, the effector protein complex down-regulates the expression of a RNA or DNA target. Although the small RNAi-directed gene regulation system was independently discovered in plants, fungi, worms and mammalian cells, scientific attention has been focused mainly on the regulation of development, biotic and abiotic stress responses and genome stability through controlling plant gene expression. On the other hand, the small interfering (si) RNA-mediated RNA silencing also functions as a neutral antiviral defense mechanism. The purpose of this review is to provide an overview of the discovery and molecular characterization of RNAi in plants.

Keywords: Double stranded RNA; Plant gene silencing; Post-transcriptional gene silencing; rasiRNA; RNA-directed DNA methylation; RNA interference; tasiRNA.
Abbreviations: AGO_argonaute-like protein; DCL_DICER-like protein; PTGS_post transcriptional gene silencing; rasiRNA_repeat associated small interfering RNA; RNAi_RNA interference; siRNA_small interfering RNA; tasiRNA_trans-acting siRNA

Introduction

Gene silencing referred to as gene quelling in plants and fungi, and interfering RNA (RNAi) in animals, is considered a conserved regulatory mechanism of gene expression and it has been mostly characterized in eukaryotic cells (Duan et al., 2012). According to some authors (Hunter, 2000; Duan et al., 2012), RNAi was formally characterized when it was found that injecting antisense-stranded RNA was an effective way to inhibit gene expression. This was the first attempt to use an antisense RNA technique to inactivate a *Caenorhabditis elegans* gene (Fire et al., 1998), and both Andrew Z. Fire and Craig C. Mello shared a physiology Nobel Prize for this magnificent work. As far that is known, RNA silencing leads to a nucleotide sequence-specific process that induces mRNA degradation or translation inhibition at the post-transcriptional level in plants (PTGS). On the other hand, in plants, it sometimes can cause epigenetic modifications at the transcriptional level, which depend on a process called RNA directed DNA methylation (RdDM) (Prins et al., 2008). In addition, siRNA-mediated RNA silencing also serves as natural antiviral defense mechanism (*e.g.*, virus-induced gene silencing [VIGS]) (Ding, 2010). Recent discoveries have shown that RNA silencing pathway is composed of a series of different important components. Among others, gene suppression starts with a double stranded RNA (dsRNA) trigger, followed by the action of an intermediary processor called DICER or a DICER-like protein (DCL). The processor product, which consists of small RNAs (siRNAs or miRNAs) of about 21 to 24 nt in size activates an effector complex called RISC (RNA-Induced Silencing Complex), where the Argonaute protein (AGO) works as a key player to initiate the regulation of gene expression. The siRNAs-guided AGO cut the target RNA or serve as priming tool for the RNA dependent RNA polymerase (RDR) and the SGS3 protein to amplify the dsRNA target RNA by reverse transcription. These molecular interactions stabilize the dsRNA substrate for DCLs to produce secondary siRNAs and maximize the silencing process (Peragine et al., 2004).

Because of its effectiveness and relative ease of use, gene silencing technique has become a potential tool in both basic and applied research. In either case, for the effective silencing of a target gene by the use of RNAi it is necessary to generate dsRNA trigger molecules. Potential applications are: (i) Plant functional genomics, (ii) Metabolic engineering of transgenic plants, (iii) Engineering of crops that would be resistant to pests (insects, nematodes) and diseases (bacteria, fungi, viruses) (Ricaño et al., 2014).

Interfering RNAs; first antisense RNA approaches

In the early 1990s, a few research groups attempted to increase the pigment content in petunia flowers (*Petunia sp.*), through the addition of copies of selected petunia genes involved in pigment biosynthesis pathways that were joined to very strong promoters and inserted into the petunia genome. Although respective results showed a decrease in floral color, those expected should be just the opposite. This means that some transgenic plant lines used in the experiments exhibited a suppression or co-suppression (gene silencing) that may be coordinated, of both the transgene and the homologous endogenous plant gene. In light of the above considerations, it was concluded that plant tissues exhibiting gene suppression (co-suppression), showed strong evidence of the reduction of steady-state levels of transgene and homologous messenger RNA (mRNA) (Napoli et al., 1990; van der Krol et al., 1990).

Although the molecular mechanism behind this phenomenon was unrecognized, shortly before these first experiments was published, a co-suppression work concerning the production of transgenic plants resistant to the RNA virus Tobacco Etch Virus (TEV) by artificial expression of the TEV coat protein (Lindbo and Dougherty, 1992a; 1992b; Lindbo et al., 1993).

According to some researchers (Hunter, 2000; Duan et al., 2012), almost simultaneously RNAi was discovered when it was found that the injection of antisense-strand RNA was an effective way to inhibit gene function. This was the first attempt to use the antisense RNA approach to inactivate a *Caenorhabditis elegans* gene (Fire et al., 1998). Due to the above results and thanks to further investigations, it was concluded that the active molecules that triggered this phenomenon could be considerable amounts of dsRNA that interfere *in vitro* RNA preparations. Whereas introns and promoter sequences did not appear to compromise RNAi, exon sequences were highly required, which determine the "rules" and interaction of these types of organic structures (Fire, 1999). It is known that RNAi acts systemically when is injected into the animal´s tissue that inhibit gene function in cells through the organism. Through a variety of experiments that involve *C.elegans* and other organisms, it has been indicated that RNAi acts to destabilize cellular RNA after this has been processed (Alvarado and Newmark, 1999).

Timmons and Fire (1998) conducted a simple experiment that showed amazing results related to the potency of RNAi. Using bacteria that had been genetically engineered to express dsRNA, they fed nematodes with molecules corresponding to a *C. elegans* gene called *unc-22* and the result was the development of a similar *unc-22* phenotype mutants that were dependent on their nourishment requirements. The conditional expose of nematodes to dsRNA established the basis for an effective way to select for *C. elegans* mutants and posteriorly identify their corresponding genes (Tabara et al., 1999).

Although there is little scientific background related to RNAi potential against various types of viruses capable of infecting animal cells (*e.g.,* dengue virus and *Drosophila*) (Kakumani et al., 2013; Karlikow et al., 2014), some studies suggest RNAi involvement in plant pathogenicity.

dsRNA is rapidly processed into short RNA duplexes of about 21 to 28 nucleotides in length. A clear example of the natural function of these small RNA molecules is the fact that mRNAs or viral genomic/antigenomic RNAs are recognized by them and subsequently cleaved or translationally repressed. In addition, we can mention that short RNAs are implicated in guiding chromatin modification (Peragine et al., 2004).

It is important to mention that some authors consider that RNAi was first discovered in plants (not in worms) as PTGS (Meister and Tuschl, 2004). Through the use of tools for creating transgenic plants, several attempts have been made to engineer organisms with more desirable characteristics, which is the case of Jorgensen and colleagues (1996) who tried to decrease the purple color of petunias. This is how the concept "co-suppression" was used to explain the ability of exogenous elements to modify gene expression.

To date, we know that there are also a few more types of molecules related to siRNA that suppress gene expression through PTGS in land plants (*i.e.* Trans-acting siRNA; abbreviated "TAS", "ta-siRNA" or sometimes "tasiRNA" and "rasiRNA", which are a class of small RNAs involved in the RNAi pathway) (Hamilton and Baulcombe, 1999; Vaucheret, 2006). Fig 1 shows representative endogenous plant siRNAs in *Arabidopsis.*

RNAi processing; DICER and argonaute proteins, the orchestra directors

RNAi originates from three different metabolic pathways that share a common molecular mechanism. These are currently known as: miRNA, siRNA and Piwi-associated RNA (RNAi that prevents the mobility of transposons in the genome), although the last one has been only found in animals (Shabalina and Koonin, 2008). It is considered that gene silencing is triggered by a miRNA or siRNA complex that works as a template and pattern recognition to identify a nucleotide sequence ready for degradation (Saini et al., 2007).

Since the whole mechanism involves both endogenous miRNA and exogenous siRNA and larger precursors are produced by small dsRNA molecules of appropriate size in order to be linked to an effector protein, this phenomenon is mediated by an endoribonuclease enzyme class III called DICER. These enzymes are important intermediaries of siRNA and miRNA pathways and generate small double stranded RNA molecules that are imperative substrates for an Argonaute protein, which is considered a common effector that constitutes a ribonucleoproteinic complex that conducts, at the same time, the mRNA degradation besides that it is linked to a single RNA sequence of about 20-30 nucleotides that is complementary to the target gene (Wilson and Doudna, 2013).

DICER has different structural domains, although the most important are those called PAZ (Piwi-Argonaute-Zwille) and helicase *(i.e.* specific amino acid sequence responsible for unpacking genes). Argonaute proteins contain four domains: terminally N, PAZ, middle (MID) and Piwi-C terminal, which is characteristic of this type of complexes (Tolia and Joshua-Tor, 2007). Likewise, PAZ domains have similarities to the oligonucleotide-oligosaccharide structures and theoretically; they recognize the 3'end of the RNA substrate (Doyle et al., 2012). Recent studies have also shown that PAZ domains not only link the 3'substrate but also their 5'-phosphorylated, which cleavage position is recognized at a distance of 22 nucleotides (Park et al., 2011).

DICER enzymes also contain a helicase domain and a couple of dimerized RNase domains, although variability between organisms can be observed (Shabalina and Koonin, 2008). The helicase domain of DICER is perfectly aligned with the long dsRNA precursor. It has been proposed this uses ATP to unpack the dsRNA precursor and thus to generate a large number of siRNAs from a single molecule of dsRNA (Cenik et al., 2011).

After an intensive search for the enzymatic mechanisms of gene silencing, DICER enzymes were first identified as

Table 1. Representative enzymes involved in PTGS or RNAi in different species.

Peptide	Motifs	Species	Annotation	Reference
DICER	RNase III, DEAD*, PAZ, dsRBD	*Homo sapiens*	miRNA precursor processing	Hutvágner et al. (2001)
DCR-1	RNase III, DEAD, PAZ, dsRBD	*Caenorhabditis elegans*	miRNA precursor processing	Grishok et al. (2001)
DCL1	RNase III, DEAD, PAZ, dsRBD	*Arabidopsis thaliana*	miRNA precursor processing	Xie et al. (2004)
AGO1	PAZ, PIWI	*Drosophila melanogaster*, *Homo sapiens*	Short RNA binding	Okamura et al. (2004)
AGO2	PAZ, PIWI	*Drosophila melanogaster*, *Homo sapiens*	Short RNA binding	Mourelatos et al. (2002)
AGO3	PAZ, PIWI	*Homo sapiens*	Short RNA binding	Meister et al. (2004)
AGO1	PAZ, PIWI	*Arabidopsis thaliana*		Kidner and Martienssen (2004)
Gemin3	DEAD	*Homo sapiens*	RNA helicase	Mourelatos et al. (2002)
Drosha	RNase III	*Homo sapiens*	Processing of primary miRNA transcripts	Lee et al. (2003)
Exportin-5		*Homo sapiens*	Nuclear export of miRNA precursors	Bohnsack et al. (2004)
HYL1	dsRBD	*Arabidopsis thaliana*		Vazquez et al. (2004)
HEN1		*Arabidopsis thaliana*		Boutet et al. (2003)

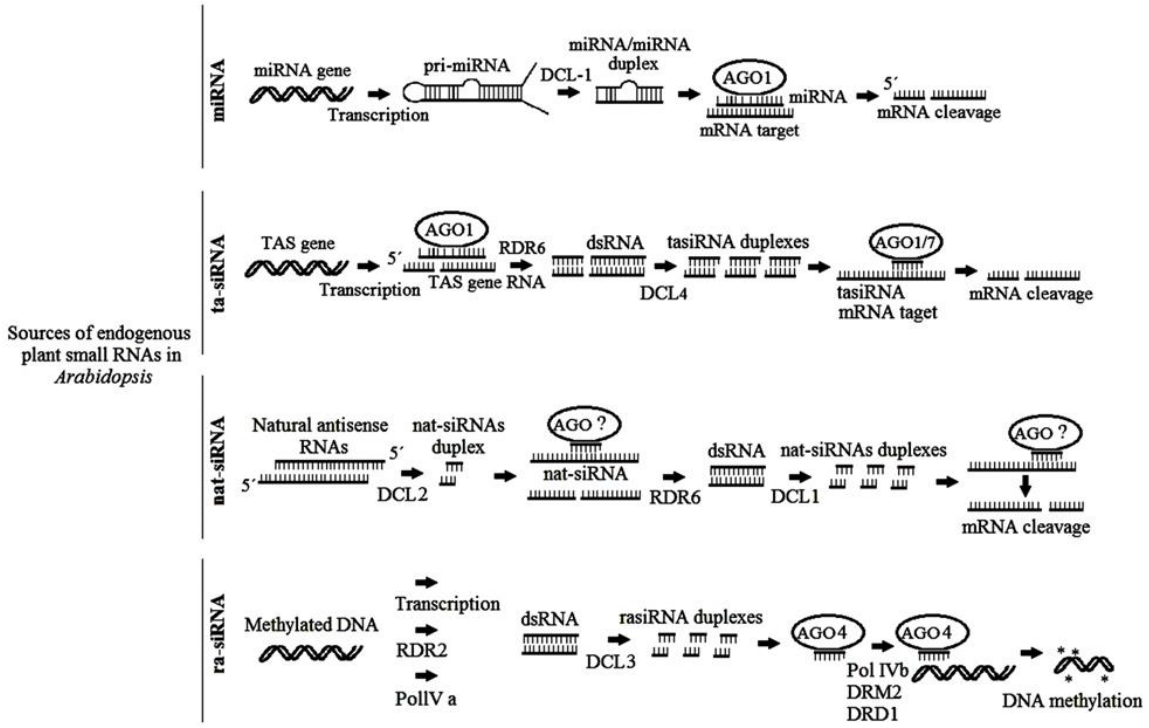

Fig 1. Endogenous plant siRNAs in *Arabidopsis*. Overview of the biogenesis of miRNAs, ta-siRNAs and rasiRNAs in plants (Adapted from Hamilton and Baulcombe, 1999; Vaucheret, 2006).

responsible for processing dsRNA to siRNA in *Drosophila* (Bernstein et al., 2001). In genera such as *Arabidopsis*, DICER DCL1 (DICER-like1) proteins act sequentially on pri-miRNAs for synthesizing loops and posteriorly, small double stranded RNA molecules of about 21 nucleotides in length (Pattanayak et al., 2013). Through partial sequence alterations of RNA helicase domains caused by point mutations, it has been observed a reduction of the amount of mature miRNA sequences (Kasschau et al., 2003). Finally, in the conventional RNAi model, DICER enzymes interact in the cytoplasm to degrade their substrates before coupling to the RISC complex through which gene silencing occurs.

Many organisms express multiple members of this superfamily of proteins. For example: *Homo sapiens*, *Drosophila melanogaster* and *Arabidopsis thaliana* express up to 8, 5 and 10 peptides respectively. In general, individual members of each family are highly specialized in carrying out the process of gene silencing (Doyle et al., 2012). One of the most prominent roles in this class is its relationship with pre-ribosomal RNA synthesis (pre-rRNA) (Nicholson, 1999). Table 1 shows some representative DICER enzymes in different species.

During the miRNA formation, HASTY proteins (exporter miRNA proteins) translocate their precursor into the cytoplasm. Subsequently, the double-stranded precursor is dissociated and the guide miRNA sequence is incorporated into a complex of several proteins containing AUG, usually to form a specific RISC complex (miRISC) (Voinnet, 2009). The PAZ domain of AGO1 complex binds to the miRNA and helps to incorporate the miRISC. The miRISC-miRNA complex prevents the expression of target genes, either by mRNA cleavage or inhibiting its translation (Meng et al., 2011). In the miRNA processing, introns that are among pre-miRNA sequences are removed through RNA splicing, whose process performs a post-transcriptionally RNA maturation. Table 2 shows some of the most important stem-loop miRNA sequences discovered to date.

It has been recently discovered, that there are ribonucleotide structures at the intermediate stage of the metabolic complex that allow the synthesis of specific molecules known as non-coding RNAs (ncRNAs), which are considered regulatory RNA molecules (of about 200 nucleotides) not translated into proteins (Perkel, 2013).

Is short hairpin RNA-induced silencing related to RNA-directed RNA degradation?

It is known that gene silencing occurs at both transcriptional and post-transcriptional levels. Cytosine methylation of promoter sequences is thought to be the principal mechanism of transcriptional gene silencing, although the exact role of this phenomenon remains unclear. It seems that methylation deactivates the promoter sequence through blocking its transcription factors, and also, by the heterochromatinization of the promoter sequence (attracting-chromatin-remodeling proteins) (Wang and Waterhouse, 2000).

As mentioned above, PTGS was first observed in the early 90s through experiments related to transformation of petunia (referred to as cosuppression) (van der Krol et al., 1990). Since then, a considerable number of theories have been proposed to understand the PTGS mechanism as well as its induction (Meins, 2000; Waterhouse et al., 1998).

The presence of a micro sequence of about 79 nt length in an ACC oxidase transgene (*i.e.* 1-aminocyclopropane-1-carboxylate) is a further evidence of dsRNA gene silencing, since this involvement is strongly implicated in petunia species by a correlation between the gene suppression of a chalcone synthase and the presence of transgene copies of the same DNA sequence. Therefore, through transcription it is produced dsRNA that could be potentially induced by transgene insertions (Waterhouse et al., 1998; Wang and Waterhouse, 2000).

Some transgenes such as chalcone synthase and nitrite reductase contain dsRNA that are related to coding regions that may induce gene silencing when RNA is highly abundant. On the other hand, using single stranded RNA could be effective when sense co-suppression is activated by plant-encoded RNA-dependent RNA polymerase. Silencing is enhanced by fusing identical short sequences with long non-target sequences in artificial constructs that possess short hairpin RNA (hsRNA; artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNAi) (Miller et al., 2001).

Through selective inhibition of sequences involved in multiple metabolic pathways, it is possible to improve agronomic traits in crop plants (*e. g*. modification of desaturase genes in cotton which modifies the fatty acids content of seed oil) (Liu et al., 2000) and, although it is unknown whether plant silencing is subject to saturation, it may be possible to perform a simultaneous inhibition of genes by cloning complementary sequences into a hsRNA construct or by plant transformation. In addition, the specific inhibition of these genes could be achieved using less conserved regions on sequences such as the 5´ and 3´ un-translated, where a variable region of an appropriate size must be preset to allow a specific inhibition. In the above case, a clear example of a desirable gene silencing event may be the alteration of seed´s oil, proteins and carbohydrate composition without affecting their metabolism in the stem and leaves of the plant (Wang and Waterhouse, 2000).

hsRNAs transgenes have considerable advantages over conventional protein-mediated resistance transgenes, for example: (i) Transgenes encoding virus-derived hpRNAs appear to be more efficient than conventional sense or anti-sense viral transgenes in conferring virus resistance upon plants, (ii) A single copy of hpRNA transgene is enough to induce immunity to viral infection, (iii) They minimize the risks associated with recombination between transgene RNA and viral RNA (Wesley et al., 2001). Virus-induced gene silencing could provide an efficient tool for high-throughput functional analysis in species that are not readily transformable with hsRNA transgenes, such as *Arabidopsis* and *Japonica* rice that are readily transformable using *Agrobacterium tumefaciens*. It is noteworthy that recent studies suggest that RNA silencing may play an important role in but is not a determining factor for the multiplicity of infection (Donaire et al., 2016).

It is worth mentioning that miRNAs are being exploited as new platforms for developing solid knowledge in different science fields like medicine, nanotechnology and integrated pest management. In that sense, the production of RNAi in plant-base biofactories could be effective in several disciplines, for example: biomaterial synthesis has allowed developing sensing treatments using nanovehicules capable of conducting miRNAs and antisense RNA against liver, spleen and kidney cancer (Conde et al., 2015). RNAi technology is also applied to bone research and orthopedics, since bone formation needs to be enhanced providing new options for the treatment of several conditions such as osteoporosis, bone tumors, nonunion and critical size effects (Ferreira et al., 2015). Likewise plant-made Chikungunya virus vaccines may be an option to fight the epidemic that is caused by different insect vectors (Ou et al., 2014). RNAi potential to control insect pests should be encouraged and

Table 2. Some of the most important stem-loop miRNA sequences discovered to date.

Description	Annotation	Mature sequence	Reference
Arabidopsis thaliana miR156a stem-loop	Regulatory roles through complementary to mRNA	ath-miR156a-5´ (21-40 nt) ath-miR156a-3´ (83-104 nt)	Rhoades et al. (2002)
Arabidopsis thaliana miR167a stem-loop	Target of mRNAs coding for auxin response factors, DNA binding proteins related to control transcription in response to the phytohormone auxin	ath-miR167a-5´ (19-39 nt) ath-miR167a-3´ (101-121 nt)	Reinhart et al. (2002)
Arabidopsis thaliana miR168a stem-loop	Target of mRNAs coding for Argonaute (AGO1) proteins	ath-miR168a-5´ (18-38 nt) ath-miR168a-3´ (103-123 nt)	Rhoades et al. (2002)
Arabidopsis thaliana miR169a stem-loop	Target of mRNA coding for CCAAT binding factor (CBF)-HAP2-like proteins	ath-miR169a-5´ (18-38 nt) ath-miR169a-3´ (190-209 nt)	Xie et al. (2005)
Arabidopsis thaliana miR170a stem-loop	Target of mRNAs coding for GRAS domain (family of transcription factors whose members have been implicated in radial patterning in roots, signaling by gibberellin and light signaling	ath-miR170a-5´ (18-38 nt) ath-miR170a-3´ (190-209 nt)	Rajagopalan et al. (2006)
Arabidopsis thaliana miR172a stem-loop	Target of mRNAs coding for APETALA2-like transcription factors	ath-miR172a (78-98 nt)	Xie et al. (2005)
Nicotiana tabacum miR6020b stem-loop	Regulatory roles through complementary to mRNA	nta-miR6020b (21-41 nt)	Li et al. (2012)
Description	Annotation	Mature sequence	Reference
Physcomitrella patens miR1049 stem-loop	Regulatory roles through complementary to mRNA	ppt-miR1049 (89-109 nt)	Axtell et al. (2007)
Oryza sativa miR156a stem-loop	Regulatory roles through complementary to mRNA	osa-miR172a (7-26 nt)	Reinhart et al. (2002)
Populus trichocarpa miR156d stem-loop	Family of plant non-coding RNA	ptc-miR156d (11-30 nt)	Lu et al. (2005)
Ricinus communis miR156a stem-loop	Target of mRNAs coding for Argonaute (AGO1) proteins	rco-miR156a (6-26 nt)	Zeng et al. (2010)
Saccharum officinarum miR408c stem-loop	Regulatory roles through complementary to mRNA	sof-miR408c (247-267nt)	Dezulian et al. (2005)
Selaginella moellendorffii miR156 stem-loop	Regulatory roles through complementary to mRNA	smo-miR156c (11-31 nt)	Axtell et al. (2007)
Solanum tuberosum miR6022-stem-loop	Regulatory roles through complementary to mRNA	stu-miR6022 (197-217 nt)	Li et al. (2012)
Zea mays miR156b stem-loop	Regulatory roles through complementary to mRNA	zma-miR156b-5´ (21-40 nt) zma-miR156b-3´ (86-106 nt)	Zhang et al. (2009)

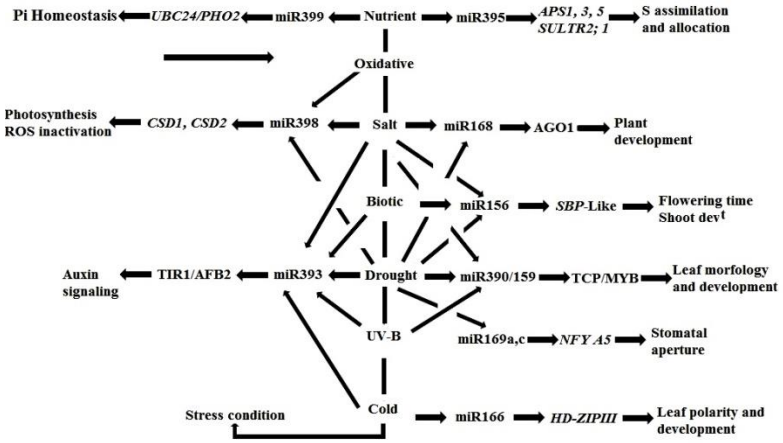

Fig 2. Summary of miRNA involvement in response to stresses. AGO-like and SBP-like proteins carry out plant development including flowering time. RAD gene encodes small MYB-like protein that is specifically expressed in the dorsal region of developing flowers. NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. HD-ZIPIII (homeodomain-leucine zipper class III) protein plays overlapping, distinct and antagonistic roles in key aspects of development. UBC24/PHO2 control inorganic phosphate homeostasis. CSD1/2 (superoxide dismutase). TIR1/AFB2 auxin receptors that mediate Aux/IAA proteins [Adapted from Kruszka et al. (2012)].

more research must be necessary to understand the barriers for an efficient application.

Gene silencing; methylation patterns are maintained through inverted repeats

Methylation is considered a complex mechanism that triggers cells division in order to stably gene activity through inherent states. In many organisms, DNA methylation is thought to have involved in defense against a foreign DNA, since methylation systems consist of several methylase enzymes that overlap short palindromic sequences, as well as peptides that cleave the same nucleotide structure (*i.e.* restriction enzymes) (Kuhlmann et al., 2014).

In the late 1990s it was hypothesized to involve DNA–DNA pairing (Waterhouse et al., 1998). Now we know that there is a post-transcriptional gene inactivation mechanism (*i.e.*co-suppression) which is not usually equal to that meiotically heritable, and may not be autonomous. Therefore, the silencing signals could travel systemically throughout the plant, showing a probable co-suppression to involve unusual RNA structures, especially inverted repeat-containing RNAs. This can autocatalytically destroy RNA products of homologous genes causing silencing. According to Wassenegger and Pélissier (1998) there is also evidence for interaction between DNA methylation and RNA which can promote post-transcriptional gene silencing and direct methylation of homologous DNA sequences, respectively.

Arabidopsis thaliana has provided insights into how plants do show DNA methylation in order to regulate the synthesis of micro-sequences that regulate gene expression. Due to the above, we may know how some organisms develop proper methylation patterns in their genome, since multiple copy gene silencing is required to duplicate regions of eukaryotic genomes that are recognized and stably silenced (Bender and Fink, 1995). *de novo* DNA methylation as well as transcriptional gene silencing is followed by transition to efficient maintenance of cytosine methylation in a symmetric sequence context by persistence of gene silencing (Kuhlmann et al., 2014). It is considered that epigenetic gene silencing is important to maintain genome integrity and is mediated by histone posttranslational modifications, chromatin remodeling complexes and DNA methylation through repressive histone marks usually correlated with transcriptionally silent heterochromatin, although mutation of *Arabidopsis Morpheus Molecule 1 (MOM1)* causes transcriptional de-repression of heterochromatin independently of changes in DNA methylation (Moissiard et al., 2014). Likewise, Arabidopsis *HIT4* gene (regulator involved in heat-triggered reorganization of chromatin and release of transcriptional gene silencing) relocates from chromocenters to the nucleus in response to stress (Wang et al., 2015).

Eukaryotic cells are capable of modulating the stability of their miRNAs in response to environmental and endogenous stimuli and/or to regulate mRNA transcription levels (regulating mRNA transcript level). Such alterations in reducing mRNA levels are mediated by RNAi *cis* regulator and by RNA-binding proteins. miRNA sequences are often related to the regulation of various biological processes such as stress mitigation (Staiger et al., 2013; Streitner et al., 2013; Sunkar et al., 2012).

RdDM is also an epigenetic process in plants that involves both short and long non-coding RNAs and the generation of theses microsequences is related to different transcriptional mechanisms comprising two plant specific RNA polymerases (*i.e.* Pol IV and V) (Matzke et al., 2015).

Concluding remarks and future perspectives

Knowledge of molecular basis that are implemented in plant defense mechanisms against diseases caused by biological agents or extreme abiotic conditions, is vital for sustainable agriculture. In past decades, these have achieved important advances in the field of plant-microbe interactions, which demonstrated the role they play on the recognition of receptor

patterns in disease resistance. Furthermore, it has been found the existence of several virulence factors caused by phytopathogens that are related to blocking patterns recognition, and signaling in immune responses. However, despite knowing the outcome of these physiological processes, it was not entirely clear which could be the molecular mechanisms that trigger these processes. Just a few years ago, the phenomenon was discovered and now we know that gene silencing is caused by RNAi, whereby it may regulate gene expression in eukaryote organisms.

Although understanding of RNAi has been developed in a very short period of time, many questions about its molecular mechanism should be answered in order to have a better comprehension of its process. While it is true that plant metabolic pathways regulate their gene expression through a silencing phenomenon are expressed in various ways throughout siRNA, miRNA and tasiRNA, all these molecules share common elements in their biogenesis and structural characteristics, as well as action mechanisms involved in common cellular components. However, despite miRNA and tasiRNA have certain conservation degree; they are adjusted in several ways among different species.

There are similarities between RNA and siRNA that mediate post-transcriptionally gene silencing, and it is known that both are expressed in different ways across species. This phenomenon could be due to functional triggers of RNAi silencing diversification, which participate in adaptive processes that change throughout evolution. To date, it is known that there are only five eukaryotic clades that have miRNAs, although each one has its own repertoire of sequences. When observing that every clade has its own unique complement of miRNAs, we could infer the existence of a clear example of molecular expansion (Tarver et al., 2012).

Although miRNAs discovery has delved into the role they play in plant gene regulation, more questions arise about their function, for example: Why multiplicity is observed in these molecules? Since different miRNAs can cooperatively regulate individual target DNA sequences although their expression differ between different cell types as well as conditions (Voinnet, 2009). Some other questions that arise from the understanding of miRNA functionality are the following: What is the reason why some times target DNA interactions show a protective effect on the stability of molecules, and in others, their purpose is the degradation? Or well, how random interactions between reactivated transposons and endogenous microRNAs might initiate easiRNA (epigenetically activated small interfering RNA) biogenesis? Since plant silent transposons are reactivated during stress and development (Sarazin and Voinnet, 2014).

Besides that biotechnological tools are based on complex theoretical basis, they should also have an industrial approach which allows their implementation outside the laboratory and provide a benefit to humanity. To mention some important examples; maize, that is highly susceptible to water stress, could take advantage if we scrutinize some of its *MIR* genes (microRNA genes) (Mittal et al., 2016). Likewise, it would be possible to increase the results in multi-environment tests that help optimizing crops under drought conditions (Campos et al., 2004). For more details about some stress responses in plants see Fig 2.

The recent discovery of some of the main RNAi molecular mechanisms, can discuss their future applications in agricultural biotechnology. It is important to mention that the resulting food security that comes from the application of such tool must be imperative. As a result of few studies on the human effects of the consumption of plant foods with high levels of micro sequences interfering their genome, considerable uncertainties arise that should be unveiled in the future, for example; the effect of these microarrays on the metabolism of those who directly consume them.

Artificial microRNAs (amiRNAs) were originally reported in 2006 by Schwab and collages, and thereby it is currently possible to have a better approach on the exact location of target genes in organisms of agricultural interest (*i.e.* crops and insects) through the generation of these types of molecules (Li et al., 2013). Such projections could improve research in crop plants and engineering through the development of a better predictable and genetically artificial manipulable sequences. Likewise, recent research in insects has shown the in vitro micro injection effect of synthetic double-stranded sequences in embryos (Gu and Knipple 2013).

Plants require at least 14 essential minerals coming from the soil for proper development; therefore, RNAi is involved in both regulation and homeostasis of nutrients (Kruszka et al., 2012). It is worth mentioning that constructions of genomic libraries have proved to be very valuable for studies of miRNAs associated with these metabolic processes (Wang et al., 2013). Thereby, biotechnological applications of miRNAs might require microarray studies helping to discover important miRNA associated metabolic responses to water, heat, salt, biotic stress, and UV radiation, as well as stress-mediated hormonal regulation and nutrient homeostasis, and resulting in future creations of "biotech" lines resistant to adverse environmental conditions. In addition, if plant-implemented glyco-engineering techniques based on RNAi silencing could reduce target glycosyltransferases transcripts, virus-like particles (VLPs) production in transgenic plants may be a reliable path to develop CHIKV (chikungunya) vaccines (Salazar-Gonzalez et al., 2015), for example.

Finally, application of RNAi in plants could also increase substantially sophisticated solutions involving the regulation of transduction signals in defense mechanisms, for example: against viruses, fungi and bacteria (Collinge et al., 2010), as well as the implementation of scalable tools to screen gene functions that participate in adaptation to drought (Gavin et al., 2015).

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Conflict of interests

The authors declare that there are no conflicts of interests

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